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Volume 24
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Preface

We have the great pleasure to welcoming you to Zurich for the first joint conference of the European Grassland Federation (EGF) and of the EUCARPIA section 'Fodder Crops and Amenity Grasses' with the key focus being on improving sown grasslands through breeding and management. Conditions for the sustainable production of sufficient food and feed have become increasingly complex and demanding. Population growth, changing consumer behaviour and the continuous loss of land surface suitable for agricultural production call for drastic increases in productivity. In addition, increasing pressure to reduce greenhouse gas emissions and pesticide usage in agriculture calls for environmentally sound production systems. These challenges are reinforced by increasing economic and environmental (climate change and loss of biodiversity) uncertainties. Grassland-based ruminant livestock production allows for the efficient, local and sustainable production of meat and dairy products, often relying on sown grasslands, either as regularly re-sown permanent pastures and meadows or as leys in a crop rotation.

Sown grasslands are not only a valuable element of arable crop rotations allowing the soil to recover and preventing the transmission of pests and diseases, but also provide a number of key ecosystem services. They are an important source of high quality roughage, reducing the need to import high-energy or high-protein feed supplements, and they furthermore play a substantial role in storing carbon, regulating water flows, providing habitats for many species, preventing soil erosion and enhancing landscape aesthetics. Grasslands are grown either as pure stands of high yielding forage grass or legume species such as perennial ryegrass or red clover, or as multi-species mixtures consisting of several grass, legume and sometimes forb species. Mixtures may be particularly attractive in terms of optimising yield (due to over-yielding effects in multi-species mixtures) and minimising nitrogen fertiliser inputs (due to transfer of fixed atmospheric nitrogen from legume to grass species). In addition they yield balanced roughage rations (where the high energy content of grasses is complemented by the high protein content of legumes) and can supply secondary plant metabolites with positive effects. In order to fulfil the high demands modern day livestock production has on sown grasslands with respect to forage quality and yield, management schemes need continuously to be optimised, seed mixtures to be adapted to specific needs and competitive cultivars of different forage crops species to be improved by breeding. All this is only possible by continuous research efforts and interdisciplinary collaboration among scientists in various research topics.

The 20th Symposium of the European Grassland Federation and the 33rd Meeting of the EUCARPIA Section 'Fodder Crops and Amenity Grasses' are, for the first time, held as a Joint Symposium in order to facilitate exchange between experts in the fields of agronomy, grassland ecology and breeding, to encourage discussions and to facilitate new collaborations for the advancement of sown grasslands for sustainable agricultural production. We are highly pleased to be able to present the research of a large number of scientists from all over Europe and overseas, which allows the exchange ideas on a broad range of topics including grassland multifunctionality, improving grasslands through management and breeding, the development of mixtures, mitigating future challenges and improving collaboration between practice and research.

We would like to thank all the authors for submitting such a broad range of interesting papers. We are grateful to all the reviewers and editors for providing critical feedback, which greatly helped to improve the quality of the manuscripts. We would like to acknowledge the great support of the presenting partners Agroscope, ETH Zurich, the Federal Office for Agriculture, Bern University of Applied Sciences and the Swiss Grassland Society (AGFF). We are grateful for the generous contributions of all sponsors and supporters. Finally, we would like to express our gratitude to all members of the local organising committee and the scientific committee and to all the innumerable helpers for making this exciting symposium possible.

We wish you an interesting scientific meeting with many new insights and contacts and hope that, apart from the science, you will find the time to enjoy the sights of Zurich and the landscapes and specialities Switzerland has to offer.

Andreas Lüscher & Roland Kölliker
Co-chairs of the organising committee

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Opening session

Grassland systems in Switzerland with a main focus on sown grasslands

Lüscher A.[1], Grieder C.[2], Huguenin-Elie O.[1], Klaus V.H.[3], Reidy B.[4], Schneider M.K.[1], Schubiger F.[2], Suter D.[1], Suter M.[1] and Kölliker R.[5]

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Abstract

Grasslands dominate Swiss agriculture and cover nearly 80% of the country's agriculturally utilised area. They form the basis for ruminant livestock production through the provision of high-quality roughage which accounts for three quarters of the total dry matter of dairy cow rations, as averaged over the whole country. While permanent grasslands dominate in mountainous and less favourable regions, sown grasslands form an important part of the crop rotation (1/3) in the lowlands. Sown grasslands for intensive forage production typically consist of mixtures of 3 to 7 grass and legume species, taking advantage of increased dry matter yield through overyielding or transgressive overyielding, complementary forage quality of the different species, weed suppression and symbiotic N_2 fixation. The Swiss-Standard-Mixtures System involves development and testing of species mixtures adapted to a broad range of purposes and environmental conditions. These mixtures rely on the availability of high yielding cultivars with appropriate competitive ability and optimal forage quality. Switzerland's forage crop breeding programme targets the improvement of the twelve prevalent forage grass and legume species including ryegrasses, fescues and clovers. Breeding research focusing on elucidating the genetic control of important traits and the development of genomics-assisted breeding tools ensures efficient breeding of improved cultivars. Continuation of the intense collaboration in research for forage plant breeding and grassland management, with involvement of all the actors, will be key to adapt sown grasslands to future challenges and demands.

Keywords: species-mixtures, multifunctionality, forage crop breeding, overyielding

Natural growth conditions

In Switzerland grasslands cover nearly 80% of the agriculturally utilised area (including alpine summer pastures). According to the 2017 agricultural census (FOAG, 2018), grasslands comprise 124,000 ha of temporary sown grasslands (8% of the agriculturally used area) and 609,000 ha of permanent grasslands (40%). In addition, approximately 465,000 ha of alpine pastures (31%) are used for temporary grazing during summer. The remainder of agricultural land is mostly arable land (18%) or covered by permanent crops (3%).

This dominance of grasslands can be largely explained by the environmental conditions – slope, altitude, rainfall – that often do not allow efficient arable farming. As a consequence, grasslands in Switzerland occur in diverse environmental conditions (Figure 1). For example, 95% of the permanent grasslands on agricultural land are located between 408 and 1,508 m a.s.l., on slopes between 1.3 and 62% and with an annual precipitation between 850 and 1,913 mm. Even more diverse are conditions of the alpine summer pastures: 95% of them are located between 941 and 2,557 m a.s.l., 6 and 92% slope, and 888 and 2,162 mm of precipitation. In contrast, arable crops and temporary grasslands occupy a much narrower environmental niche. Their 95% limits are between 366 and 853 m of elevation, 0 and 22% of slope and

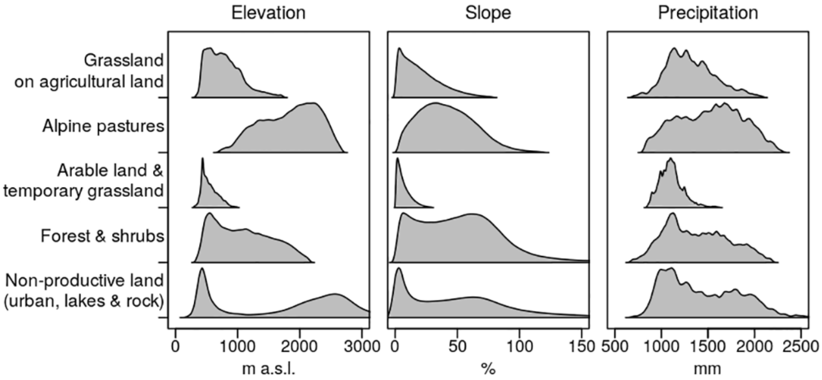

Figure 1. Density distribution of the environmental variables of elevation, slope and precipitation in the five major land use types in Switzerland. Data were derived from 4.1 Mio observation points of the Swiss Land Use statistics (FSO, 2018), elevation and slope of SwissAlti3D (Swisstopo, 2017) and gridded precipitation data (MeteoSwiss, 2016).

890 and 1424 mm of precipitation. Soil properties vary as much as these main drivers of soil formation (Amelung *et al.*, 2018).

General principles of Swiss grassland systems

In Switzerland, the overarching goal is sustainably productive, multifunctional and environmentally sound grassland systems. Three specific objectives are pursued to achieve this. The first is the production of high-quality forages from all types of grasslands described above that enable livestock to be fed on a grass-based ration. This requires harvesting forage at early development stage with high nutrient content and an appropriate fertilisation, and thus an intensive management. As a large proportion of Swiss farms cannot produce cereals or protein crops efficiently, due to topographic and climatic constraints, this intensive grassland-based forage production also contributes to feed-self-sufficiency, to closed nutrient cycles and to the re-coupling of the C and the N cycles. Temporary grasslands are the focus of the second objective. For these types of grasslands, the full benefits of modern agricultural development should be made available to optimise their production. This involves forage plant breeding, growing high performing elite cultivars, as well as the development of forage plant mixtures adapted to a wide range of conditions and types of utilisation. The third objective targets an enhanced multifunctionality of grassland systems, by means of differentiated and site-adapted management intensity at the farm and regional scale. The fields with the highest production potential should be managed intensively to produce high quality forage, necessary for a grass-based ration for productive livestock. This intensity should not exceed the carrying capacity of the site and be adapted to the growth requirements of the forage plant species to guarantee stable forage plant communities on the large areas of permanent grasslands. In return, the fields with a low production potential should be managed at low intensity to promote habitat diversity at the farm scale and to contribute to biodiversity conservation (Huguenin-Elie *et al.*, 2018).

Grass-based rations for dairy cows

Grassland-based milk production is of major importance in Switzerland. Traditionally, herbage from temporary and permanent grasslands provides the largest proportion of feed for ruminants. Due to climatic and topographic restrictions, intensive maize-concentrate based dairy systems are usually limited to the lowland area. In a survey conducted in 2013, the feeding strategy and ration composition of 157 dairy farms (0.7% of milk-producing farms) located in different climatic zones of Switzerland was investigated (Ineichen *et al.*, 2016). On average, of the examined farms, the proportion of fresh or conserved herbage in the year-ration was about 76% on a dry matter basis. This proportion increased with increasing altitude from 68% for farms in the lowland, to 78% in the hill region and 84% in the mountain zone. Feeding of maize silage showed the opposite trend. Its proportion of the feed ration decreased from 18% in the lowland, 9% in the hill region to 3% in the mountain zone. Substantial differences in herbage proportions were observed between feeding systems. Silage-free milk used for hard cheese production was produced with a higher proportion of herbage (83%) than milk produced from silage feeding (72%). On average of all farms, the ration contained 11% concentrates (DM). With an average milk yield of 6846 kg ECM cow^{-1} and 647 kg DM cow^{-1} year^{-1}, the concentrate utilisation was relatively low. Concentrate utilisation increased with increasing milk yield. However, variation in concentrate feeding between individual farms with similar milk yields was substantial, indicating a potential for optimisation in some farms (Figure 2).

During the last decades, and especially since the abolition of the milk quota system in 2009, the Swiss dairy production has undergone rapid structural changes. Since 2009 the number of dairy farms has shown a substantial decrease of -25%, with a shift towards bigger units and more intensive feeding systems. Grassland-based feeding, including grazing, is therefore actively supported with direct payments in the framework of the Swiss agricultural policy. The introduction of the 'RAUS'-programme, aiming to promote grazing, resulted in 85% of dairy cows currently having access to pastures for a minimum of 26 days per month from May 1 to October 31 (FOAG, 2018). In 2014 an additional programme supporting herbage feeding and limiting concentrate utilisation was introduced. In order to participate in this so-called 'GMF'-programme, farms are obliged to use a minimal proportion of herbage in the ration (75% DM, valley zone) and a maximum limit on the amounts of concentrates (10%). In 2018 the average participation at the programme was at 78% of the grassland area or 66% of the farms. Meeting the energy demand of high performing ruminant livestock through a largely grassland-based forage ration remains a major challenge for farmers. Increasing the starch content in forage legumes such as red clover may offer a valuable alternative to cereal based energy-complements (Ruckle *et al.*, 2017).

Advantages of multispecies mixtures

There is important evidence from experiments in intensively managed productive grasslands that mixtures perform better than the average of all monocultures (overyielding) or even better than the best performing monoculture (transgressive overyielding) (reviewed by Sanderson *et al.*, 2004, 2007; Lüscher *et al.*, 2014; Phenlan *et al.*, 2015). Compared to the average of monocultures, mixtures of legumes and non-legume species (mostly grasses) had about 30% higher biomass yield (Kirwan *et al.*, 2007; Nyfeler

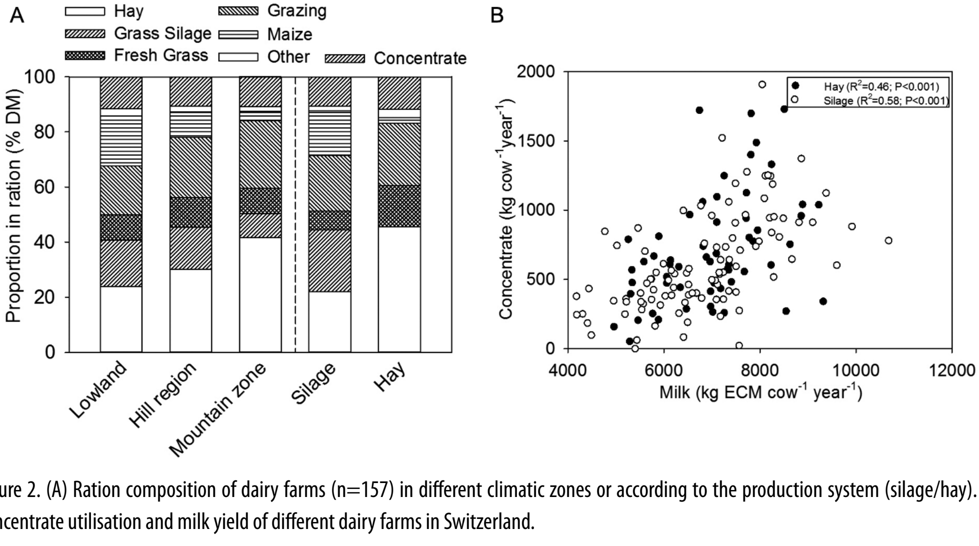

Figure 2. (A) Ration composition of dairy farms (n=157) in different climatic zones or according to the production system (silage/hay). (B) Concentrate utilisation and milk yield of different dairy farms in Switzerland.

et al., 2009; Küchenmeister *et al.*, 2012; Finn *et al.*, 2013; 2018; Malisch *et al.*, 2017; Cong *et al.*, 2018), improved weed suppression by up to 80% (Frankow-Lindberg, 2012; Suter *et al.*, 2017; Connolly *et al.*, 2018), and increased temporal stability of the yield (Haughey *et al.*, 2018). Significant N input through symbiotic N_2 fixation (Nyfeler *et al.*, 2011) led to enhanced total N yield (Suter *et al.*, 2015) compared to grass only swards. Forage quality of swards containing legumes was increased compared to grass swards (Gierus *et al.*, 2012; Lüscher *et al.*, 2014; Küchenmeister *et al.*, 2014). Legumes can increase the forage intake of mixed swards compared to pure grass stands due to a faster rate of digestion and particle breakdown and due to greater palatability (Dewhurst *et al.*, 2009; Niderkorn *et al.*, 2017) and some legumes can have positive effects due to their secondary plant metabolites as condensed tannins (Mueller-Harvey *et al.*, 2019).

These beneficial effects of mixtures were remarkably robust: they persisted over a wide pedo-climatic gradient. In the Agrodiversity Experiment (Finn *et al.*, 2013; Kirwan *et al.*, 2014; Connolly *et al.*, 2018), which has been conducted from Atlantic to continental and from temperate to arctic boreal conditions, both biomass yield and weed suppression were greater in mixtures than in the best performing monocultures at the great majority of sites. In this experiment, N yield has also been proven to be significantly greater in grass-legume mixtures than in grass monocultures at about 75% of tested sites (Suter *et al.*, 2015). Moreover, recent drought stress experiments have revealed beneficial mixture effects over the average for monocultures, in terms of persistence under severe drought (Hofer *et al.*, 2016; 2017; Finn *et al.*, 2018). Robustness of mixture benefits was also evident over a wide range of species proportions. In a three-year experiment, Nyfeler *et al.* (2009) demonstrated transgressive overyielding of mixtures' biomass yield at legume proportions between 20 and 80%. Persistent mixture benefits at varying species proportions were also evident in the Agrodiversity Experiment at all three experimental years, with proportions ranging between about 15 and 65%, irrespective of the species in mixtures (Finn *et al.*, 2013). Despite a strong decline in legume abundance over the three years, beneficial mixture effects on yield were still significant in the third year at 18 out of 24 sites, although the magnitude of the mixture effect was smaller at sites with lower legume proportion (Brophy *et al.*, 2017). Robustness of mixture benefits is further underlined by many experiments in extensively managed grasslands that have demonstrated overyielding (although not transgressive) and higher temporal stability (Tilman *et al.*, 2006; van Ruijven and Berendse, 2007; Hector *et al.*, 2010; Craven *et al.*, 2018) in species-rich swards compared with the average of the monocultures (Spehn *et al.*, 2002; Hille Ris Lambers *et al.*, 2004; Hooper and Dukes, 2004; Hooper *et al.*, 2005; Roscher *et al.*, 2005; Marquard *et al.*, 2009; Mommer *et al.*, 2010). Extensively managed, low-productive grasslands often have greater relevance for functions other than production (e.g. biodiversity conservation, maintaining food webs across trophic levels, touristic and recreational values) and experimental evidence suggests that species diversity is an important driver of ecosystem functioning and multifunctionality (Isbell *et al.*, 2011; Allan *et al.*, 2013; Weisser *et al.*, 2017).

The mechanism behind such mixture benefits is a better utilisation of the available growth resources due to species niche complementarity and positive interspecific interactions. The major driver was the legumes' ability for symbiotic N_2 fixation, which allows the production of more biomass with less N fertilisation. Due to symbiotic N_2 fixation and its growth stimulation of the legume's partner species (Høgh-Jensen and Schjoerring, 1997; Nyfeler *et al.*, 2011) total nutrient uptake from soil and fertiliser (N and P) are enhanced in grass-legume mixtures (Hoekstra *et al.*, 2015; Husse *et al.*, 2017). At the same time, nitrate leaching has not been considered as a problem in mown swards based on grass-legume mixtures, even if fertilised with up to 150 kg N ha^{-1} $year^{-1}$. This is in contrast to pure legume swards and highly N-fertilised pure grass swards, where leaching can be problematic (Loiseau *et al.*, 2001; Nyfeler, 2009). Low amounts of nitrate leaching in mixtures, despite their substantial N input from symbiosis, can be explained by (1) the efficient N uptake of the highly productive grass species, (2) the symbiosis taking place inside the plant, (3) being a steadily flowing N source, (4) which is tuned according to the sward's N demand (Hartwig, 1998). All four factors prevent periods with high concentrations of reactive N forms in the soil. In line with this, nitrous oxide emissions have also been found to be lower in grass-legume mixtures compared with N-fertilised pure grass swards (Schmeer *et al.*, 2014) or swards with very low legume proportions, which were in turn intensively fertilised because of missing N_2 fixation (Fuchs *et al.*, 2018). Besides complementarity in N_2 fixing ability, there is also evidence that complementarities in seasonal and annual growth patterns and also in rooting depths significantly contribute to mixture benefits (Nyfeler *et al.*, 2009; Mommer *et al.*, 2010; Hofer *et al.*, 2016; Husse *et al.*, 2016, 2017).

Designing optimal mixtures

Important distinctions of the results of agricultural experiments in intensively managed grasslands, compared with biodiversity experiments in extensively managed grasslands, are that (1) beneficial mixture effects occurred at low levels of species richness, and that (2) transgressive overyielding was often achieved. In contrast, a meta-analysis of low productive grasslands revealed that, averaged over all experiments assessed, the most species-rich mixtures had 12% less yield than the highest yielding monocultures (Cardinale *et al.*, 2007), though the mixtures were clearly more species rich. This raises the question of how mixtures should be designed to be most suitable for forage production.

From the flattening of the slope of the plant species richness-productivity relationship observed towards high species richness in many biodiversity experiments (e.g. Weisser *et al.*, 2017), it can be expected that the effect of adding supplementary species to productive four-species mixtures will be much weaker than the effect between monocultures and the four-species mixtures. In addition, we suggest that transgressive overyielding in intensively managed grassland experiments was related to a targeted selection of the species based on prior knowledge. This selection firstly focused on the best-performing forage plant species (forage yield and quality) under the given growth conditions and management intensity. Secondly, the complementarity of traits among species was maximised by combining species with differences in their symbiotic N_2 fixation ability, rooting depth, and seasonal and annual growth pattern. Such a strategy seems most promising to enhance yield and other ecosystem services (Lüscher *et al.*, 2011; Storkey *et al.*, 2015) and differs from the random species assembling design of many biodiversity experiments in low productive systems. The number of species necessary to optimize the benefits of mixtures becomes increasingly uncertain with an increase in the number of functions expected from the mixture. In theory, each supplementary species could bring benefits for an ecological function poorly supported by the other species, and thus, it could be expected that a large number of species would promote multifunctionality. For grassland communities it is still contentious whether this holds true (Gamfeldt and Roger, 2017; Meyer *et al.*, 2018). Although the diversity-multifunctionality relation in intensively managed grasslands has not yet been evaluated with more than four species, two- and four-species mixtures supported multiple functions and increased multifunctionality (Suter *et al.*, 2019).

The Swiss-Standard-Mixtures System

Growth conditions as well as management objectives vary widely among farms. Consequently, for practical use, a variety of distinct mixtures that cover this broad range of needs are required. The product range of the system of Swiss-Standard-Mixtures consists of forty-six seed mixtures, which offer mixtures with a high versatility of use as well as mixtures especially designed for mowing or grazing.

The duration of the ley is an important factor for the seed mixture composition and the classification of the mixtures in the system (Table 1). With increasing duration, the number of species in the seed mixture is increased. Essentially, this is done to optimise the components' time-dependent performance (temporal niche over the years). The suitability for distinct types of utilisation depends mainly on the main legumes in the seed mixture. The wealth of possible species combinations allows for adaptation

Table 1. Most important standard mixtures with their main species, duration, expected mean clover proportion, environmental requirements, range of utilisation and estimated market share.[1]

Mixture	Main legumes	Main grasses	Number of species	Duration (a)	Mean clover %	Water availability	Altitude	Utilisation				Seed market share %
								Green	Silage	Hay	Grazing	
102	VS, PS	LM	3	1	35	+	▼	●	○			9
106	TA, TU	LM	3	1	40	+	▼	●	●			
155	MS	LM	4	1	40	–	▼	●	●			
200	TP	LM	2	2	40	+	▼	●	●	○		17
230	TP	LM, DG	4	2	35	0	▼	●	●	○		
300	TP	DG, LH	5	3	60	–	▼	●	●	○		1
301	TP	DG, AE	4	3	60	––	▼	●	●	○		
320	MS	DG, LH	5	3	60	––	▼	●	●	○		3
323	MS	DG, FP	5	3	70	–––	▼	●	●	○		
326	OV	AE, FP	4	3	50	–––	▼	○	○	●		
330	TR, (TP)	LP, DG	6	3	35	0	▼	●	●	●	●	22
340	TR, (TP)	LP	6	3	50	+	▼	●	●	●	●	
362	TP	FA, PP	4	3	30	––	▼	●	●	○	●	
430	TR, (TP)	LP, DG, PP	7	4+	30	0	▼	●	●	●	●	28
440	TR, (TP)	LP, PP	6	4+	45	+	▼	●	●	●	●	
431	TR, (TP)	FP, DG, PP	9	4+	35	–	▼▲	●	●	●	○	
442	TR, (TP)	AP, FA, PP	8	4+	35	+/–	▼	●	●	●	○	
444	TR	AP, FP, PP	6	4+	35	++	▼▲	●	●	●	○	
462	TR	FA, PP	4	4+	30	–	▼	●	○	●	●	
480	TR	LP, CC, PP	7	4+	30	+	▼	●	○	●	●	
481	TR, (LC)	CC, PP, AG	9	4+	30	+	▼▲	●	○	●	●	
Salvia	diverse	diverse	38	4+	20	–/0	▼			●		1
Humida	diverse	diverse	35	4+	20	++	▼			●		
Montagna	diverse	diverse	30	4+	20	0	▲			●		
Broma	diverse	diverse	47	4+	20	––	▼			●		

[1] Species names: AE (*Arrhenatherum elatius*), AG (*Agrostis gigantea*), AP (*Alopecurus pratensis*), CC (*Cynosurus cristatus*), DG (*Dactylis glomerata*), FA (*Festuca arundinacea*), FP (*Festuca pratensis*), FR (*Festuca rubra*), LC (*Lotus corniculatus*), LH (*Lolium × hybridum*), LM (*Lolium multiflorum*), LP (*Lolium perenne*), MS (*Medicago sativa*), OV (*Onobrychis viciifolia*), PP (*Poa pratensis*), PS (*Pisum sativum*), TA (*Trifolium alexandrinum*), TF (*Trisetum flavescens*), TP (*Trifolium pratense*), TR (*Trifolium repens*), TU (*Trifolium resupinatum*), VS (*Vicia sativa*); water availability: ++ wet, + humid, 0 balanced, – occasionally dry, –– often dry, ––– regularly dry; altitude: ▼ below, ▲ above 900 m a.s.l.; utilisation: ● suitable, ○ medium.

to various environmental conditions, the most important of which are temperature – determined by altitude and exposition – and water availability.

The constant change in agricultural practice and the progress in plant breeding are considered by frequent adaptation of the system and the composition of the seed mixtures. Since the establishment of the standard mixtures in 1955 (Frey, 1955), 15 revisions have been conducted, including 120 changes in the product range (removal or addition of mixtures) and the same number of adjustments of existing seed mixtures. Since the establishment of the standard mixture system, one of the driving factors has been the intensification of agricultural production systems. This is clearly manifested by an increase in the percentage of mixtures containing white clover from 27 to 48% and of mixtures containing perennial ryegrass from 32 to 48% between 1955 and 2017 (Suter *et al.*, 2017). Nevertheless, more mixtures for less intensive, and even extensive utilisation, were part of the system in 2017 than in 1988 (Lehmann *et al.*, 1988), reflecting the introduction of environmental schemes and the impact of a paradigm shift in the agricultural policy in Switzerland in the early 1990s. This development could also be observed in the number of species of the most species-rich mixture in the system: in 1955 and 1988, it contained 8 and 9 species, respectively, whereas in 2017 it contained 47 species. Also, the increase in the total number of species included in the system, and, thus being traded, is impressive: it rose from 27 in 1988 to 99 in 2017. The success of seed mixtures depends heavily on the availability of high performing cultivars of the different species adapted to the respective environments. Although the importance of competition and coexistence for successful breeding of forage crops for mixtures has long been recognised (Hill, 1990; Lüscher and Jacquard, 1991; Lüscher *et al.*, 1992), selection of forage grasses and legumes still depends largely on population-based improvement of individual species (Posselt, 2010). However, targeted breeding efforts have resulted in a broad set of cultivars suitable for being cultivated in multispecies mixtures (Suter *et al.*, 2019).

Breeding forage crops adapted to a broad range of environments

The climatic conditions and cultivation systems outlined above call for forage crop cultivars adapted to local conditions and a broad range of applications. Therefore, Agroscope, the Swiss competence centre for agricultural research, maintains a large breeding programme covering the twelve prevalent forage crop species: perennial ryegrass (*Lolium perenne*), Italian ryegrass (*L. multiflorum*), hybrid ryegrass (*Lolium* × *hybridum*), meadow fescue (*Festuca pratensis*), tall fescue (*F. arundinacea*), red fescue (*F. rubra*), Kentucky bluegrass (*Poa pratensis*), cocksfoot (*Dactylis glomerata*), red clover (*Trifolium pratense*), white clover (*T. repens*), sainfoin (*Onobrychis viciifolia*) and birdsfoot trefoil (*Lotus corniculatus*). The programme results in a broad number of diploid and tetraploid (for ryegrasses, meadow fescue and red clover) cultivars regularly listed in Swiss and international lists of recommended cultivars (Suter *et al.*, 2019).

Breeding efforts mainly focus on the improvement of dry matter yield under frequent cutting, the seasonal distribution of yield, persistence over several growing seasons, forage quality, resistance to biotic and abiotic stresses and improvement of seed yield. For perennial ryegrass, specific breeding targets are tolerance to dry and hot summers, winter survival, and the ability to persist at higher altitudes. In addition, very early heading cultivars that combine a high yield in spring with a rapid regrowth and a long vegetation period are developed. Italian ryegrass is primarily selected for the ability to survive two winters as well as dry and hot summers, while in meadow fescue the focus lies with a high level of endophyte (*Epichloë uncinatum*) infection for better fitness and improved competitive ability during summer (Malinowski *et al.*, 1997). In order to exploit the high drought tolerance of tall fescue, genotypes are selected with fine, flexible leaves, which indicate good palatability, in combination with a high digestibility. In red clover, the breeding programme focuses on persistence breeding based on old Swiss 'Mattenklee' germplasm, which was developed over decades of on-farm seed production (Kölliker *et al.*, 2003). In addition, red clover types with low phyto-estrogen content or types that are suitable for grazing are selected. In white clover, breeding focuses on dual-purpose cultivars (i.e. alternating mowing or grazing use) and a limited proportion of cyanogenic genotypes.

Although red and white clover with their high protein content complement the dietary composition of forage grasses in mixtures, pure grassland-based diets are often energy limited. Although so far not exploited in forage crop breeding, structural carbohydrates such as starch have the potential to increase feed value by improving the energy content of grassland-based diets. The first studies in red clover have shown significant differences in starch content in different red clover genotypes accounting for up to one third of their dry matter biomass (Ruckle *et al.*, 2017). Although harvest-losses and post-harvest degradation limit a full exploitation of starch in the ruminant diet, the genotypic differences indicate suitability of improvement of this promising trait through targeted breeding. This may even be more promising in white clover, which is used predominantly for grazing. Starch content has been shown to be significantly higher when plant material is harvested by the end of the day as compared to the end of the night when a large proportions of starch have been degraded (Ruckle *et al.*, 2017, 2018). Therefore, evening grazing of white clover stands offers a valuable option to exploit white clover genotypes with high starch content and offer an attractive alternative to cereal-based feed supplements. However, a more detailed understanding of starch metabolism in red clover and the development of genomics-based breeding tools are needed before starch can be incorporated as a trait in clover breeding programmes.

Sainfoin presents another forage legume with attractive forage qualities (Kölliker *et al.*, 2017). The species has been shown to contain considerable amounts of condensed tannins which have been shown to reduce bloat, diminish gastro-intestinal parasites and may also have the potential to lower methane emissions (Mueller-Harvey *et al.*, 2019). An extensive evaluation of 27 sainfoin accessions from a worldwide collection showed large variability in the content of condensed tannins which could be exploited through breeding (Malisch *et al.*, 2015). Although a predominantly outcrossing species, high rates of self-fertilisation have been observed for sainfoin under artificial directed pollination (Kempf *et al.*, 2015). This could enable the development of homogenous inbred lines facilitating the improvement of tannin content through molecular marker assisted breeding approaches.

For all species, the Agroscope breeding programme is mainly based on recurrent phenotypic selection to improve the breeding populations. In spaced-plant nurseries, individual genotypes are selected for desired traits such as disease resistance, forage quality or persistence. The seed of the first generation of a new variety is produced via open pollination among selected elite plants after cloning in a polycross (grasses) or directly in the nursery (clover). In a half sib progeny test the best progenies are selected and allowed to pollinate to create seed of the new candidate variety. The performance of the new candidate is tested in three-year plot trials on several sites. Although this breeding process has proven very efficient and yielded a large number of successful cultivars over the years, constant challenges such as evolving pathogen populations, changing environmental conditions and novel consumer demands ask for constant refinement of the breeding process. A detailed understanding of the genetic control of key target traits and the development of genomics-assisted breeding methods offer additional means for the targeted improvement of forage crop cultivars.

For example, in Italian ryegrass, a major quantitative trait locus (QTL) explaining more than 60% of the phenotypic variance of resistance to bacterial wilt (caused by *Xanthomonas translucens* pv. *graminis*) was identified (Studer *et al.*, 2006). Further characterisation of the QTL region using a pooled sequencing approach allowed to identify sequenced characterised markers and candidate genes potentially involved in *Xtg* resistance (Knorst *et al.*, 2018). Simulation of marker-assisted selection using these markers resulted in a decrease in the average marker score of up to 28%, indicating the suitability of these markers for marker-assisted improvement of *Xtg* resistance in Italian ryegrass. In addition, comparative genomic analysis of several *Xtg* isolates and comparison to other *X. translucens* pvs. identified a set of unique genetic features potentially involved in pathogenicity of the pathogen (Hersemann *et al.*, 2017). This detailed information on host resistance and pathogen virulence will not only further advance our knowledge of this complex host-pathogen interaction, but also allow for the development of refined breeding methods.

Proof of concept for marker-assisted introgression of disease resistance in Italian ryegrass was given for the crown rust-causing fungal pathogen *Puccinia coronata* f. sp. *lolii*. A single marker linked to a crown rust QTL on linkage group 2 of Italian ryegrass (Studer *et al.*, 2007) was used to introgress resistance from the progeny of a bi-parental mapping population into different breeding populations. While phenotypic selection alone resulted in an improvement of 2.5 disease scores (using a scale from 1 to 9), single-marker-assisted selection resulted in an improvement of 5.52 disease scores (Kölliker *et al.*, 2016).

Genetic resources and their value for breeding

In contrast to many field crops like maize, where the wild form of the cultivated species no longer exists, wild and semi-natural forms of common forage species still exist alongside current cultivars (Boller and Greene, 2010). Since forages are less domesticated than field crops (Harlan, 1983), the closeness of wild and cultivated forms eases the use of natural plant genetic resources (PGR) for breeding. Natural or semi-natural permanent grasslands harbour forage ecotype populations highly adapted to their habitats, showing variation for a range of adaptive traits like ear emergence and growth habit (e.g. Peter-Schmid *et al.*, 2008). With an area larger than 500,000 ha, corresponding to 34% of total agricultural area (FSO, 2018), Swiss permanent grasslands, which are spread over a large range of altitude and climatic conditions (Figure 1), therefore make a wealth of natural genetic variation. Following the proposition of Hertzsch (1959), the source of a breeding programme should be based on plants found on old permanent grasslands with an association similar for the respective situation. Accordingly, ecotypes from permanent grasslands have been used as the starting point for selection in Switzerland at Agroscope, as in many other forage breeding programmes in Europe since the beginning of the 20th century (e.g. Humphreys, 2005). Although breeders nowadays rely more on crosses between cultivars, new collections of adapted ecotype populations still bear the potential to improve the breeding pool. For the example of Italian ryegrass, introduced to northern Switzerland in the early 19th century (Stebler and Schröter, 1883), Boller *et al.* (2009) have shown that the average performance in terms of yield, vigour, and resistance to snow mould of 20 ecotypes collected across Switzerland in 2003 was superior to currently recommended cultivars. In this study, resistance to crown rust and leaf spot were the only traits where cultivars outperformed ecotypes. For Italian ryegrass, in contrast to meadow fescue, no clear distinction between ecotypes and cultivars could be made based on genetic markers, indicating continuous exchange between these two groups (Peter-Schmid *et al.*, 2008). Unlike other forage species where newly collected ecotypes are often crossed with existing breeding materials, new Italian ryegrass ecotype collections were directly used in the development of new varieties at Agroscope. For example, the variety Oryx (first seed produced in 1993, listed in 2000) was created from ecotypes collected in 1987 after just one cycle of selection among spaced plants in the nursery followed by one cycle of selection among half-sib families. The new variety Rabiosa (first seed produced in 2005, listed in 2015), which is currently ranking best in the Swiss list of recommended forage varieties (Suter *et al.,* 2015), was created from ecotypes collected in 1996 after two cycles of selection among spaced plants followed by one cycle of selection among half-sib families.

This direct use of ecotypes for the creation of high performing varieties clearly demonstrates the high value of adapted (semi-) natural populations in permanent grasslands, and this has meanwhile also been recognised by the public. Since 1999, the Federal Office for Agriculture has been running a national action plan for conservation and use of plant genetic resources. Within this programme, Agroscope has collected accessions of different forage species for *ex-situ* conservation. This collection comprises accessions of forage grasses mainly derived from ecotypes, but also accessions of forage legumes that were derived mainly from landraces, as in the case of red clover. Most accessions have been described morphologically and agronomically in several projects, of which the data are now available in a national database (www.bdn.ch). Description of these genetic resources is ongoing, as for example in the EU-funded EUCLEG project (www.eucleg.eu), where red clover accessions are phenotypically and genotypically analysed. To protect the continuous adaptation of ecotypes in their place of origin at a national level, special support measures are currently tested by the Swiss federation in a pilot project since 2018 for effective *in-situ* conservation of forage ecotypes together with the farmers. All these measures will help to protect and valorise these genetic resources for a successful plant breeding in the future.

Conclusions

Roughage from grasslands provides the basis for ruminant livestock production, thereby making a valuable and sustainable use of local natural resources in many countries of alpine regions and central Europe. Management practices, the breeding of adapted cultivars, and optimised seed mixtures for various purposes and site conditions have allowed a very high level to be reached in terms of productivity and sustainability. However, challenges such as changing climatic conditions, new evolving pathogen populations, increasing nutrient requirements of cows due to increasing milk yields, changing farm structures and consumer demands, all call for constant refinement of grassland management and utilisation. This can only be achieved by combining progress on all the different scales of grassland systems. Developments in the area of molecular genetics and genomics, but also high throughput and high precision phenotyping, offer promising tools to breed the best genotypes. Exploitation of positive interactions in the plant-soil-microbiome system will allow the production of more forage on an individual field with fewer inputs, and site-adapted and diversified management intensities among fields of a farm or region will allow increased multifunctionality of our grassland systems.

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Theme 1.
Efficient and multi-functional forage production on sown grasslands

Multifunctionality of permanent grasslands: ecosystem services and resilience to climate change

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Abstract

Permanent grasslands are highly relevant for the provision of many ecosystem services in Switzerland. This includes not only forage production for livestock, but also climate regulation, erosion control and cultural services (among others). How these services are affected by climate change, but also to what extent Swiss grasslands contribute to climate change or can actually mitigate it, are important research questions. Within the Swiss FluxNet, a network of ecosystem flux measurement sites in Switzerland, greenhouse gas (GHG) fluxes and C sequestration have been measured at three grassland sites of different management intensities for more than a decade. At the intensively managed site on the Swiss Plateau, legumes are currently tested as a N_2O mitigation option. Overall, environmental conditions as well as management events have clear impacts on net ecosystem fluxes. Particularly during a restoration year (which included ploughing, harrowing, resowing, fertilisation), the grassland turned into a large GHG source, driven by extraordinarily high N_2O and CO_2 losses. On the other hand, increasing the legume fraction in the sward, thereby substituting organic nitrogen fertiliser, reduced N_2O emissions while still maintaining yield and forage quality levels. Thus, management strongly affected the climate regulation service provided by the grassland. Climate impact studies, but also agroeconomic assessments complemented these long-term GHG measurements. All three grasslands were surprisingly resilient to severe droughts, both simulated and naturally occurring. Feed production, but also sward composition did not show any legacy effects of preceding droughts. Thus, multifunctionality, i.e. the provision of multiple services, seemed resilient as well, and offers promising options with respect to anthropogenic climate change.

Keywords: forage production and quality, climate regulation, legume fraction, restoration, drought impact, economic assessment

Introduction

Grasslands in Europe occur under highly variable environmental and socio-economic conditions. Thus, a wide variety of management intensities and vegetation compositions exist. This also applies to Switzerland where about 70% of the Swiss agricultural area consists of grasslands, i.e. about 737,000 ha (FOAG, 2017). These grasslands range from the Swiss Plateau up to the alpine areas, with temporary grasslands in crop rotations but also permanent grasslands (with regular restoration) at lower elevations.

Permanent grasslands, used as meadows and pastures, are highly relevant for the provision of many ecosystem services in Switzerland. According to MA (2005), four types of ecosystem services are differentiated: supporting, provisioning, regulating and cultural ecosystem services. Among others, forage production for livestock (provisioning service), but also climate regulation and erosion control (both regulating services) as well as cultural services (e.g. for tourism) are in the focus of current attention. For example, climate regulation is assessed from different perspectives, since on the one hand, grasslands contribute to the greenhouse gas (GHG) emissions of Swiss agriculture (which contributes 12.6% to overall Swiss emissions; FOAG, 2017), while grasslands, on the other hand, are also strongly impacted by more frequent droughts and heatwaves (e.g. Reichstein *et al.*, 2013). About 55% of agricultural GHG emissions in Switzerland originate from livestock, 19% from manure management, while 25% can be traced to soils and <1% to limestone and urine applications (FOAG, 2017). How these services are affected by climate change, but also to what extent Swiss grasslands contribute to climate change or can actually mitigate it, are thus important research questions.

Materials and methods

Within the Swiss FluxNet (http://www.gl.ethz.ch/research/bage/fluxnet-ch.html), a network of ecosystem flux measurement sites in Switzerland, GHG fluxes and C sequestration have been measured at three permanent grassland sites of different management intensities for more than a decade (Figure 1; Table 1).

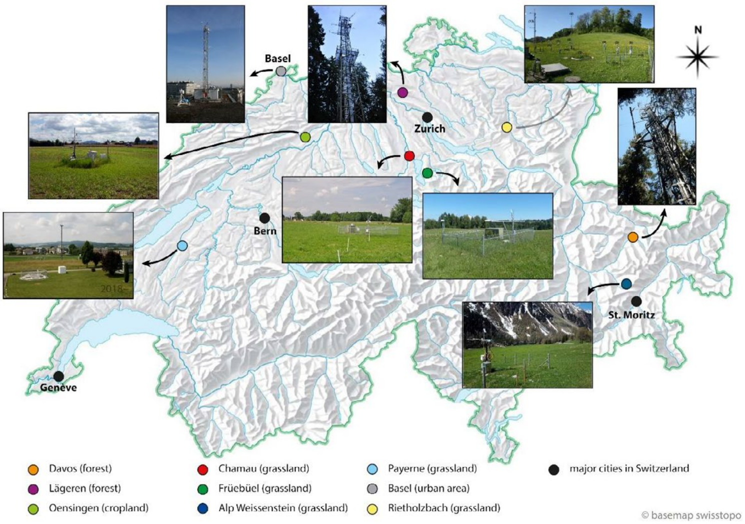

Figure 1. The Swiss FluxNet, a network of flux sites for measuring biospheric-atmospheric gas exchange. It currently encompasses seven long-term ecosystem sites, six run by the Grassland Sciences (ETH Zurich), covering the major land-use types in Switzerland: forest (coniferous: Davos; mixed deciduous: Lägeren), cropland (Oensingen), grassland (Chamau, Früebüel, Alp Weissenstein, Payerne – run by MeteoSwiss). Flux measurements in an urban setting (Basel) as well as within a watershed (Rietholzbach) complement the Swiss FluxNet. Map: Courtesy of W. Eugster.

Table 1. Site characteristics of the three grassland sites within the Swiss FluxNet.

Site characteristics	**Chamau (CHA)**	**Früebüel (FRU)**	**Alp Weissenstein (AWS)**
Latitude, longitude	47° 12′ 37″ N, 8° 24′ 38″ E	47° 6′ 57″ N, 8° 32′ 16″ E	46° 34′ 60″ N, 9° 47′ 26″ E
Elevation in m ASL	393	982	1,978
Annual precipitation in mm	1,150 to 1,200	about 1,600	about 900
Average annual temperature in °C	9 to 10	8	2
Soil type	Cambisol	Gleysol	Humous sandy loam
Management	intensive (4-6 cuts/yr, manure/slurry applicat-ions, regularly restored	intermediate (1-2 cuts/yr, some fall grazing, manure/slurry applications)	extensive (grazing)
Sward composition	about 25 species, incl. *T. repens*, *L. perenne*	about 35 species, incl. *D. glomerata*, *A. pratense*	about 20 species, incl. *F. rubra*, *T. repens*
Continuous flux measurements since	July 2005	August 2005	Summer campaigns from 2006 to 2014, continuously since winter 2014/2015
Drought experiment	2005 to 2007, 2009 to 2011	2005 to 2007, 2009 to 2011	2006 to 2007, 2009 to 2011
Legume experiment	Since 2015	-	-

Fluxes are measured with the eddy covariance (EC) method, which is based on high frequency measurements (10-20 Hz) of the vertical wind velocity and the mixing ratio of a trace gas, e.g. CO_2, CH_4, N_2O or water vapour. The turbulent flux is calculated from the covariance between these two measurements using time averaging of typically 30 minutes (for details, see Eugster and Merbold, 2015). Thus, these measurements run continuously, 24 hours a day, for 365 days per year, preferentially for many years. Flux measurements at Chamau (CHA), Früebüel (FRU) and Alp Weissenstein (AWS) started between 2005 and 2006 (Table 1), have been complemented by dedicated measurements campaigns or long-term experiments, such as drought simulations using portable rain shelters or manipulations of the vegetation composition of the sward (legume experiment at CHA). All data are available as open access.

At CHA, the intensively managed, permanent grassland site on the Swiss Plateau, legumes are currently tested as a N_2O mitigation option (Table 1, Legume experiment). The footprint of the flux tower, i.e. the area where the fluxes come from, has been divided into two parcels in 2015. The legume fraction of one of these parcels has been increased to 20 to 40% and no fertiliser has been applied, while the legume fraction of the other parcel stayed at 10 to 20% and fertiliser application continued. N_2O fluxes are measured continuously with the EC method described above (for further details, see Fuchs *et al.*, 2018).

In addition, research on drought impacts has been carried out at all three permanent grassland sites (Table 1, Drought experiment) to test the response of Swiss grasslands, located along an elevation gradient, to extreme drought as projected for the future (NCCS, 2018). Portable rain shelters were used to simulate consecutive, severe summer droughts during two three-year periods (2005-2007, 2009-2011), complemented by two years without drought simulations but with a similar measurement portfolio (2008, 2012). Rain shelters were typically set up for six to twelve weeks, reducing precipitation input by up to 50% (for details, see Gilgen and Buchmann, 2009; Prechsl *et al.*, 2015). In addition, agroeconomic assessments were carried out to determine the economic consequences of summer drought for Swiss farmers, based on yield responses scaled up to the farm level (for details, see Finger *et al.*, 2013).

Results and discussion

Net ecosystem CO_2 exchange, measured with the EC method, clearly shows CO_2 losses and gains at a very high temporal resolution (Figure 2), here for an intensively managed grassland on the Swiss Plateau (CHA).

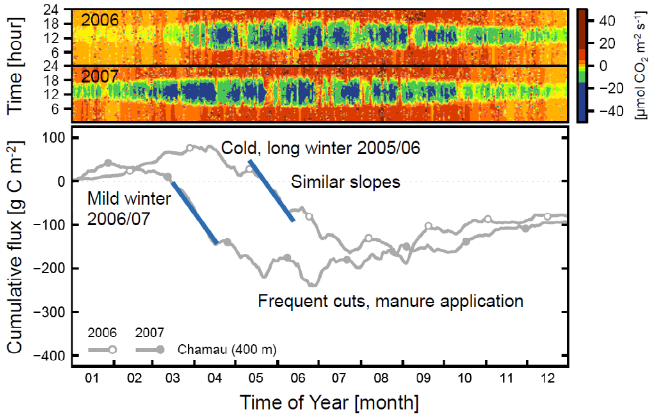

Figure 2. Net ecosystem CO_2 fluxes from a permanent grassland on the Swiss Plateau (CHA) for two years (2006, 2007). Top panels: Temporal variability of CO_2 fluxes is shown on diel as well as on annual scales. Colour codes depict CO_2 losses from the grassland (red/dark colours, positive sign) and CO_2 gains by the grassland (green-blue/light colours, negative sign). Bottom panel: Cumulative net ecosystem CO_2 fluxes are shown for two consecutive years. The same sign convention applies (losses: positive; gains: negative). Data based on Zeeman *et al.* (2010).

During the night and during cold winter months (as in 2006; Figure 2 top panels), the grassland was a strong CO_2 source because respiration from plants and soils dominated the ecosystem fluxes and sward photosynthesis was not large enough to (over-)compensate these CO_2 losses (Zeeman *et al.*, 2010). However, during the day and particularly during the summer months, the grassland was a strong carbon sink due to high photosynthesis rates. Mild winters, such as in 2007, can lead to carbon sink behaviour as early as in January, indicating that CO_2 uptake via photosynthesis overcompensated CO_2 losses via ecosystem respiration. Frequent cuts with subsequent applications of organic fertilisers during the growing season in both years led to immediate responses of the net ecosystem CO_2 fluxes, such that the grassland was a carbon source until photosynthesis of the regrowing sward again became larger than respiration. Adding up all fluxes cumulatively nicely illustrated the delayed start of sink activities in 2006 compared to 2007 (Figure 2 bottom panel), while uptake rates during both summers were very similar, only interrupted by management events (i.e. zigzag patterns). At the end of both years, regrowth decreased, and respiration again dominated the overall CO_2 fluxes. The total CO_2 flux budget summed up to a sink of about 90 g C m^{-2} $year^{-1}$. Accounting for all harvests (C exports) as well as organic fertiliser applications (C imports), the grassland was a small carbon sink of about 70 g C m^{-2} $year^{-1}$ during these two years (not shown) and thus contributed to the ecosystem service of climate regulation.

Since both environmental conditions as well as management events have clear impacts on the net ecosystem CO_2 fluxes and thus on the sink/source behaviour of this grassland, long-term measurements gain particular importance, namely to understand the underlying mechanisms but also to differentiate among drivers. During 2012, the grassland at CHA was restored, i.e. the grassland was ploughed under and re-sown to restore sward composition and thus yield and forage quality. However, during this year, the grassland turned into a large GHG source, driven by extraordinarily high N_2O and CO_2 losses (Table 2; Merbold *et al.*, 2014). The N_2O fluxes in 2012 were a magnitude higher than those in two benchmark years (2013 and 2014), representing about 50% of the total GHG flux budget compared to on average 5%. Although the vegetation re-established during the growing season 2012, the CO_2 budget was dominated by respiratory losses. This means, at a 10-year return time of such a restoration, about 50% of the long-term grassland carbon sink can be lost by one management event during these 10 years.

Strategies to reduce GHG losses from managed grasslands are therefore urgently needed. Options to reduce N_2O losses include reduction of nitrogen (N) fertiliser or increasing the legume fraction in the sward, thereby substituting (at our site, organic) nitrogen fertiliser application with N_2 fixation by legumes and their symbionts. Since 2015, a legume experiment at CHA helps to test this option on two neighbouring parcels (Fuchs *et al.*, 2018). While N_2O losses were similar for both parcels during the two years prior to the experiment (2013, 2014), increasing the legume fraction and simultaneously decreasing N fertilisation during 2015 and 2016 resulted in strongly reduced N_2O emissions (Figure

Table 2. Greenhouse gas (GHG) flux budget of the site Chamau in the restoration year 2012 compared to a benchmark year (average 2013 and 2014). Data from Merbold *et al.* (2014).

GHG fluxes	**CO_2-C**	**CH_4-C**	**N_2O-N**	**Total GHG flux budget**
Restoration year				
g m^{-2}	339	2.65	2.91	
g CO_2-eq. m^{-2}	1,245	88	1,363	2,696
%	46.2	3.3	50.6	100
Benchmark year				
g m^{-2}	-655	1.44	0.28	
g CO_2-eq. m^{-2}	-2,398	65	131	-2,202
%	92.3	2.6	5.1	100

3A). This reduction was between 40 and 50% (Figure 3B), accompanied by lower soil mineral N concentrations (not shown). Yields were about 10% lower on the unfertilised parcel but forage quality in terms of raw protein content tended to be higher due to the higher legume fraction (not shown). Thus again, management strongly affected the climate regulation service provided by the grassland. Moreover, increasing the legume fraction is a valid option to reduce N_2O emissions to mitigate climate change.

As shown above, Swiss grasslands can drive climate change and can help to mitigate it, but they are also driven by climate change. During an eight-year climate impact study (see Table 1, Drought experiment), the response of Swiss grasslands to severe summer drought was investigated along an elevational gradient (Gilgen and Buchmann, 2009; Prechsl *et al.*, 2015). Community above-ground biomass production of the three permanent grasslands responded quite differently to the simulated summer drought (Figure 4). While the grasslands at 400 m (CHA) and 2,000 m (AWS) showed significantly reduced biomass production in response to drought during most of the experimental treatment periods, the grassland at 1000 m (FRU) showed no reduction at all (or rather an increase for some periods). These site-specific responses scaled with annual precipitation: more pronounced reductions in biomass production occurred at lower annual precipitation (not shown). Thus, direction and magnitude of any effect of severe summer drought on feed production, a supporting ecosystem service in grassland, was highly variable and management adaptation strategies cannot be easily transferred across space.

On the other hand, all three grasslands were surprisingly resilient to severe droughts, one of the most prominent extreme weather events in global climate scenarios (Figure 4). Typically, already during the first, but certainly by the second regrowth after the removal of the rain shelters, biomass production did not show any drought effect any longer but recovered rather quickly. This was supported by fast recovery of leaf gas exchange and chlorophyll fluorescence (not shown; Signarbieux and Feller, 2011). Moreover, during 2008 and 2012, two years when no drought was simulated, no legacy effects were detectable, although grasslands had experienced three (2008) and six (2012) years of preceding severe summer droughts (Figure 4). Thus, performance of grassland plants as well as feed production, i.e. one of the most important ecosystem services in grassland, were surprisingly resilient, and offer promising options with respect to anthropogenic climate change.

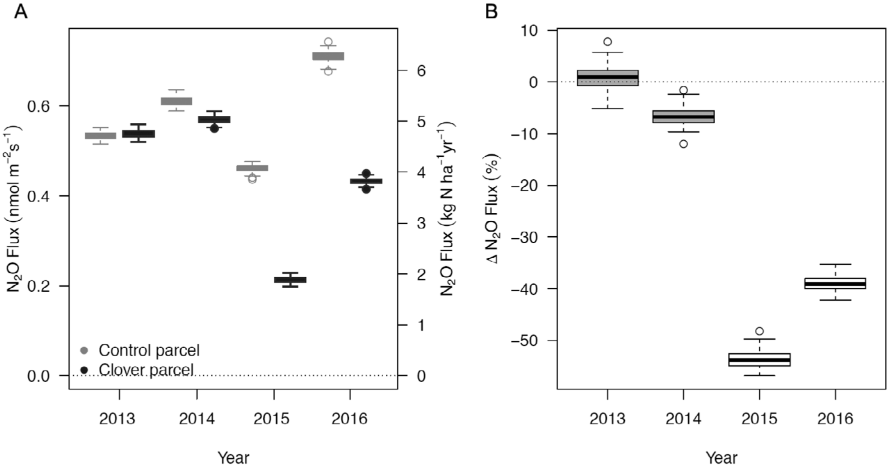

Figure 3. N_2O fluxes from a permanent grassland site in Switzerland (CHA). (A) Annual N_2O fluxes from the control (light grey) and the clover parcels (dark grey) for 2013 and 2014 (before the experiment) as well as 2015 and 2016 (during the experiment). (B) Relative differences between N_2O fluxes from the control and the clover parcels for all four years. Interquartile ranges are plotted, bold black lines represent respective medians (Figure from Fuchs *et al.*, 2018).

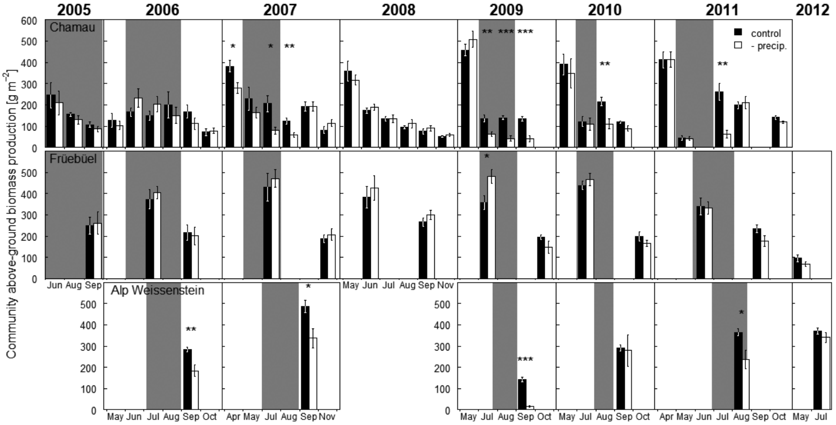

Figure 4. Effect of summer drought on community above-ground biomass production (feed production) at the three Swiss grassland sites Chamau, Früebüel and Alp Weissenstein. Periods of drought treatment are shaded in grey. No drought was simulated in 2008 and 2012. * 0.05≥*P*>0.01, ** 0.01≥*P*>0.001, *** *P*≤0.001. Data from Gilgen and Buchmann (2009), Prechsl (2013) and Prechsl *et al.* (2015).

In order to understand this fast recovery of plants in permanent grassland under drought conditions, the water uptake and uptake depths were studied in more detail using stable oxygen and hydrogen isotopes of soil and plant xylem waters (Prechsl *et al.*, 2015). Analysing the stable isotopic signatures with a Bayesian calibrated mixing model revealed that during pre-treatment periods in spring, plants took up water mainly (i.e. 70-90%) from the topsoil (except 2011; Figure 5). However, during the summer treatment period, drought-subjected plants relied more strongly (i.e. 40-70%) on water in the topsoil (top 10 cm) than control plants (only <40%). Instead, control plants shifted to deeper soil depths (20-35 cm) for water uptake (30-50%) in summer, while drought-subjected plants did not. After rain shelter removal and first rain events, in particular the drought-subjected plants took up their water in the topsoil (>90%,

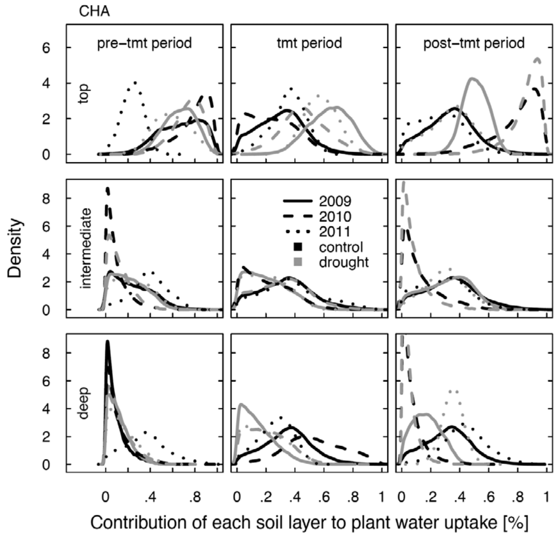

Figure 5. Effect of drought on water uptake depths of plants in a permanent grassland site (CHA). Probability density distributions for the contribution of three different soil layers (top, 0-10 cm deep; intermediate 10-20 cm; deep, 20-35 cm) to total plant water uptake are plotted for 2011. The density distribution was calculated with a Bayesian calibrated mixing model (SIAR) based on the measured water isotope data of soil and plant xylem waters (Figure from Prechsl, 2013).

except 2011), while control plants used the entire soil profile. This unexpected behaviour of drought-subjected plants was supported by root growth which mainly occurred in the top soil during the drought experiment (not shown; Prechsl *et al.*, 2015). Thus, location of meristems (close to the root collar) as well as abiotic conditions (most likely high oxygen as well as nutrient concentrations) triggered root growth and thus water uptake by root tips in the topsoil under drought. This in turn allowed very fast recovery of all performance and growth variables as soon as the first rain event occurred and water availability increased after shelter removal.

Complementing these grassland performance and ecosystem services assessments, an agroeconomic assessment was carried out as well (Finger *et al.*, 2013). Using the yield data from the three grassland sites and their responses to drought, loss of farmers' income due to drought was calculated. It turned out that the farmers on the Swiss Plateau lost the most income (about 30%), while the farmers in the alpine area lost the least (about 10%), despite yield losses being rather similar (about 30%: 2.4 t ha^{-1} vs 0.9 t ha^{-1}, respectively). These counter-intuitive results had their origin in the Swiss ecological direct payment scheme: while farmers in the lowlands relied heavily on hay prices and thus on hay production for their incomes, the income at alpine elevations strongly depended on direct payments which were independent of production levels. Thus, considering the increasing risk of such extreme weather events in the future, new economic instruments might be needed for farmers to deal successfully with drought risks.

Conclusion

Grasslands are both drivers of, as well as driven by, climate change. We provide ample evidence that grassland management is highly influential in achieving either positive or negative impacts of grasslands on climate change. This in turn allows the development of climate-smart agricultural practices to reduce or avoid GHG emissions. Moreover, our studies suggested a high resilience of grasslands against severe summer droughts, despite immediate, potentially also strong negative responses to these extreme events. Thus, multifunctionality, i.e. the provisioning of multiple ecosystem services, is high in permanent grasslands, even under drought.

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Strategy avenues for breeding plants for multispecies grasslands

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Abstract

Increasing the species diversity of agricultural crops and vegetation cover, as in multispecies sown grasslands, will enable them to cope better with the effects of climate change, including more frequent and more extreme weather events. It will also help agriculture to meet the environmental challenges posed by the need for low-input and more sustainable production systems. However, the use of species mixtures has significant implications for the plant breeder. Traditionally, grassland cultivars have been selected for increased agronomic value when grown as pure stands. However, the use of grassland species in mixtures requires cultivar selections to be revisited for their performance under conditions which include inter-species interactions. A number of approaches have been used to improve species for multispecies use. These may be classified broadly as either 'black box' or 'functional'. Each offers advantages and disadvantages, but no one approach is really fully adopted. The appropriate approach depends on plant characteristics, research objectives and state of knowledge. This paper does not attempt to establish an ideal methodology, but instead it presents a number of approaches that could be mobilised, and it explains how these may be used in combination to improve multispecies grasslands.

Keywords: multispecies grassland, breeding schemes, mixing ability, ecological mechanisms, genetic diversity

Increasing diversity in agroecosystems

A major challenge for agriculture is to develop more sustainable cropping systems able to produce well with minimal inputs, which can adapt to/cope with ongoing climate change (especially to high within- and inter-annual variability) and that also provide ecosystem services. A number of ecological studies have demonstrated the key role of species diversity in (1) ensuring stability of biomass production (Finckh *et al.*, 2000; Gross *et al.*, 2014; Hector *et al.*, 1999; Hoffmann and Sgrò, 2011; Nyfeler *et al.*, 2009; Weigelt *et al.*, 2008), particularly (2) with regard to the effects of environmental change (Lesica and Allendorf, 1999; Tilahun, 1995; Tilman *et al.*, 2001) and (3) in the context of reduced inputs (Frame and Newbould, 1986).

The introduction of more multispecies sown grasslands (now standard in many countries) into agroecosystems should foster more sustainable production. Multispecies sown grasslands can play positive ecological roles in cropping systems, particularly in their nitrogen economies (Gross *et al.*, 2010; Roscher *et al.*, 2004) and also in respect to their weed populations (Meiss *et al.*, 2010; Sebillotte, 1980; Viaux *et al.*, 1999). Particularly since 1960 (and earlier in some countries), the selection of cultivars for use as grassland species has been carried out to increase their agronomic value when grown in pure stands (Sampoux *et al.*, 2011). However, the use of grassland species in mixtures has significant implications for their breeding. Where these are grown in mixtures, the complex inter- and intra-species interactions should also be taken into account.

The behaviour of a species in a pure stand is unlikely to be the same as that under multispecies conditions (Allard and Adams, 1969; Clay and Allard, 1969; Hill, 1996). Indeed, the performance of a cultivar in isolation compared with that in a mixture may be quite different as a result of complex interactions between neighbouring plants in the mixture. This is the result of local selection pressures in the mixture generated by both intra- and inter-species neighbour interactions (Harper, 1978; Turkington and

Harper, 1979a,b; Burdon, 1980). The genetic composition of a cultivar influences its performance and sustainability in a species mixture (Prieto *et al.*, 2015). For example, compatibility differences between different combinations of white clover (*Trifolium repens*) and perennial ryegrass cultivars have been demonstrated (Evans *et al.*, 1989). In a sown grassland containing seven species (grasses and legumes) it has been shown that the choice of species cultivars influences both the yield and the abundance of the species recorded over a five-year period (Meilhac *et al.*, 2019). Thus, the genetic composition of the species employed in a mixture is of great importance (Aarssen and Turkington, 1985a,b).

Because of interactions between genotypes within and among species, the performance of a cultivar in a pure stand could be a very poor indicator its performance in a species mixture. Many studies (Atwood and Garber, 1942; Dijkstra and De Vos, 1972; Caradus *et al.*, 1989; Rowe and Brink, 1993) have found relatively low correlations of yield for clover cultivars in pure stands and in species mixtures. The lack of correlation also occurs among other species mixtures; for example, Finckh and Mundt (1992) showed that in the case of the wheat (*Triticum aestivum*)/stripe rust (*Puccinia striiformis*) system, there was a strong influence of plant-plant interactions on seed yield. Maamouri *et al* (2017) found only a partial correlation between mixture performance and pure performances for lucerne (alfalfa; *Medicago sativa*). In this study, which tested the correlation between the biomass and quality of alfalfa (genotypes in a pure stand and in an alfalfa/tall fescue (*Festuca arundinacea*) mixture) a general positive correlation was observed. But the study showed significant differences in the performance of some genotypes in pure stands *vs* their performance in mixtures. This result indicates that the interaction of alfalfa with its fescue neighbours modified the relative performance of some genotypes, but this modification depended on the genotype. Faced with this difficult prediction, we can use two useful approaches to improve species mixtures: the 'black-box' approach and the 'functional' approach.

The black-box approach

Determination of the genetic composition of species used in multispecies stands has commonly been conducted in binary mixtures containing one legume and one grass species (Allard and Adams, 1969; Garnier *et al*, 1997; Guy, 1982; Mousset, 1982; Trenbath, 1974). The black-box approach defines a mean value of the ability of a cultivar in association (see review by Finckh and Wolfe, 2006). The method for estimating this is derived from the diallele-tests used in maize (*Zea mays*), which determine the ability of lines to hybridise (Gallais, 1970). In the context of cultivars, the term 'general mixing ability' (GMA) is used to describe the average performance of a cultivar in a mixture. The GMA is determined on the basis of the performance of various binary mixtures which contain the cultivar of interest. Similarly, the term 'specific mixing ability' (SMA) is also used to describe the difference between the observed performance value of a mixture and the predicted value of this mixture from the GMA cultivars of which the mixture is composed. A cultivar is deemed to have a good ability for use in a mixture if its GMA value is high and its SMA value is low. There is a considerable literature concerning the analysis of genotypes/populations for their mixing ability (Finckh and Mundt, 1992). This approach, initially used for improving multi-varietal blends of cereals, including multi-lines among crop species (Gallandt *et al.*, 2001; Gizlice *et al.*, 1989; Jensen and Federer, 1965; Knott and Mundt, 1990; Lopez and Mundt, 2000; Mille *et al.*, 2006; Phillips *et al.*, 2005), can be used also with species mixtures (Finckh and Wolfe, 2006; Finck *et al.*, 2000; Gallais, 1990; Gallais and Bannerot, 1992; Zannone, 1985). In the context of species mixtures, the term 'ecological combining ability' can be used (Allard and Adams, 1969; Harper, 1967, 1977). Some studies, mainly on cereals, suggest that tests of binary mixtures can provide useful indications of species and general mixing ability for predicting performance of more complex mixtures of wheat (Knott and Mundt, 1990; Lopez and Mundt, 2000; Mille *et al.*, 2006), barley (*Hordeum vulgare*) (Gacek *et al.*, 1996) and even potatoes (*Solanum tuberosum*) (Phillips *et al.*, 2005) in terms of both yield and disease restriction. This approach works well if the plant material has a high 'producer effect' (high production under competitive pressures) and a high 'beneficial effect' on the neighbours (Dawson and Goldringer, 2012). But GMA estimation could not be transferred beyond the specific test material used in the context of the study. Even in the context of binary associations, the large number of possible combinations (from a large number of candidate cultivars) to be tested, soon becomes limiting. The latter limitation can be reduced if the tested plant material is exposed to a selected subset of populations of species (that will be in the mixture), called 'testers' which are representative of the species variability. On the other hand, if we can identify cultivar traits that are correlated or which explain the GMA, it should be possible to select the cultivars from these traits. Hill (1996) suggested that the difference between the clover response in pure stands and in mixtures could be explained by a variation in competitive ability of the clover. The traits involved in this competitive ability should then be regarded as good candidates. Annicchiarico and Piano (1994) showed that the competitive ability of white clover correlated most strongly with architectural traits, such as a petiole length and leaflet size. However, selection based on these traits in a pure stand could be efficient if the heritability of these traits does not differ in pure and in mixed stands. In the review of Annicchiarico *et al.* (2019) the heritabilities of legume-based mixtures, evaluated in pure stands vs in mixtures, did not differ significantly.

The efficiency of this approach assumes (1) that the SMA effects are small and (2) that the improvement of one target species will improve the performance of the whole plant community. The results of the few studies (Holland and Brummer, 1999; Maamouri *et al.*, 2017) that have compared SMA and GMA values in grass-legume mixtures support these assumptions. In spite of the conclusion from these results that seems to reinforce the trait-GMA approach in pure stands, we do need to be prudent. Direct selection in a mixture of Italian ryegrass (*Lolium multiflorum* Lam.) and tall fescue was 23% more efficient than selection in a pure stand based on a range of indices, including three traits associated with competitive ability (Annicchiarico, 2003). Other studies (Annicchiarico and Piano, 1994; Hill and Michaelson-Yeates, 1987; Pederson and Brink, 1988; Rowe and Brink, 1993;) showed the performance of a specific species in a mixture depends on the characteristics of the companion species. Annicchiarico and Piano (1994) showed that a significant rank inversion of white clover cultivars occurs between pure stands and mixtures in cases where the clover is associated with more aggressive grasses. Consistent clover responses between pure stands and mixtures were observed only for mixtures with low-yielding grass cultivars (Hill and Michaelson-Yeates, 1987). In the same way, no differences in clover yield were observed between mixtures with grasses having similar aggressiveness (Pederson and Brink, 1988; Rowe and Brink, 1993). The interactions between cultivars belonging to different species depend on the characteristics of each. The assessment of GMA for one cultivar needs to be tested for this cultivar with contrasting cultivars of associated species. Therefore, as mentioned above, it is necessary to have the good 'tester' to optimise the GMA approach in selection.

The functional approach

If the SMA values are close to, or higher than, the GMA values, and if the mixture contains more than two or three species having different characteristics, it is important to understand the ecological mechanisms behind SMA to optimise breeding for the species mixtures. A functional approach may create a more general framework for selection of plants to be used in complex mixtures.

Zuppinger-Dingley *et al.* (2014) showed contrasting trait values between grassland species which evolved in mixtures, compared with grassland species grown in pure stands. Based on the concepts and theories of community and functional ecology, it is expected that species having different resource requirements will optimise complementarity of resource use (Loreau *et al.*, 2001) and will thus achieve a better yield mix (Aarssen, 1997; Loreau *et al.*, 2001). The 'limiting similarity hypothesis' (Abrams, 1983; MacArtur and Levins, 1967) states that two species which possess contrasting ecological niches, thus adopting contrasting resource-use strategies, are more likely to coexist easily. Given the hypothesis that trait and resource (niche) axes are correlated (Violle and Jiang, 2009), species with contrasting trait values are more likely to favour contrasting niches and, thus, contrasting resource-use strategies. Resource-use complementarity is being assessed more commonly in plant ecology (McGill *et al.*, 2006; Weiher and Keddy, 1999; Weiher *et al.*, 2011) via measurements of particular traits (e.g. height or rooting depth). Some studies of grassland forage species (Caputa, 1967; Charles and Lehmann, 1989; Frick *et al.*, 2008; Mosimann and Charles, 1996; Mosimann *et al.*, 2004) aimed to establish rules, based on cultivar traits contained in each species, for assembling cultivars in multispecies mixtures. These rules are based on empirical studies (particularly those in Switzerland) and on increasing numbers of modelling studies. The underlying mechanisms in these involve the process of species niche complementarity. They provide essential information on traits of interest in the interactions between grassland species in mixtures (traits that allow greater complementarity between species or greater system resilience to the vagaries of the weather). However, (1) they have been carried out mainly from pools of cultivars selected under conventional pure-stand conditions, which may limit the range of functional variability tested (Prieto *et al.*, 2017), and (2) they do not incorporate this knowledge in the context of improving genetics and plant breeding for the mixtures. Litrico and Violle (2015) suggested a selection based on 'interaction traits', i.e. the major traits involved in the interactions between individuals in the mixture. These interaction traits are related to resource foraging, such as (1) spatial resource-use complementarity (e.g. rooting depth), (2) temporal resource-use complementarity phenology (e.g. the period of vegetative production), and (3) light partitioning and aboveground architecture (e.g. stem branching). In this approach, in addition to the identification of 'interaction traits' and their mean values for each species in a mixture, the variance values of these traits should be defined. Intra-species diversity determines the adaptive capacity of a species to confront selection pressures that are dynamic within mixtures and depend on environmental factors. Hence, the intra-species variance of interaction traits is required when the grassland is sown, to optimise mixture performance over several years. A number of experiments, especially on grassland species, have manipulated the intra-species diversity (i.e. genetic diversity) and have revealed changes in yield and/or species coexistence (Fridley and Grime, 2010). Meilhac *et al.* (2019) showed the benefit of diversity within each species of a multispecies grassland. In such a context, if the levels are known for the mean and the variance of the interaction traits maximising the value of the species mixture, the usual scheme of recurrent selection can be applied for each species separately. In this way, it will be sufficient to add new selection criteria (interaction traits). The variance of the interaction traits can be obtained in two ways: (1) by selecting a number of cultivars, each with low variance of interaction traits but with contrasting means of these traits, and pooling them, or (2) by selecting the cultivar with the largest variance in the interaction traits. However, in this case we need to change the distinction-uniformity-stability (DUS) rule for registration. Obviously, the correlations between interaction traits and agronomic traits should be considered and the selection indices methods can be improved to simultaneously control the changes in means and variances of the interaction traits and the means of the agronomic traits. The assembly of cultivars from different species would then be carried out at the end of the selection process for each species. The selection process can be efficient if just a few interaction traits lead to a major effect. But if the interaction traits are numerous, and with low individual effects, this functional approach is scarcely feasible. It is likely it will be difficult to determine all the interaction traits and the precise values of the trade-offs between agronomic and interaction traits under a range of conditions. In such cases, it is important to consider the direct interactions between species within mixtures during the selection process.

Is a 'mixed' approach best?

An alternative approach for selecting species for mixtures is to expose the mixture components to direct selection pressures resulting from the intra- and inter-species interactions (Allard and Adams, 1969; Donald, 1978; Evans *et al.*, 1989; Hill and Michaelson-Yeates, 1987). This approach (also a black-box approach) takes direct inter-species interactions into account in the selection process. This is the 'evolutionary plant breeding' method (Suneson, 1956). It has mainly been applied to composite multiline populations (Blijenberg and Sneep, 1975; Danquah and Barrett, 2002; Ghaouti and Link, 2007; Ghaouti *et al.*, 2005; Hockett *et al.*, 1983; Qualset, 1968; Soliman and Allard, 1991; Suneson, 1956; Thomas *et al.*, 1991). The first step is to create variability across pairwise multiple inbred lines of interest. Different hybrids from these crosses are then pooled into a 'composite cross population'. Within this population, the hybrids will reproduce (natural inbreeding) for several generations under the influence of local selection pressures due to the heterogeneous individuals in the neighbourhood. In this way, natural selection will continue over maybe 10 to 20 generations. Pure lines are then selected naturally and adapted in terms of survival and reproduction in an environment composed of a mixture of other lines. The efficiency of this selection in a multi-line mixture depends on the correlation between fitness and the agronomic value of the selected individuals in coexistence (Allard and Hansche, 1964). To mitigate the consequences of a negative correlation or a lack of correlation between fitness and agronomic value, artificial selection manipulations can also be used within these mixtures in parallel with natural selection (Patel *et al.*, 1987; Raggi *et al.*, 2017). The 'evolutionary plant breeding' method for selection in multi-line mixtures can be seen in the context of multispecies mixtures (Eagles, 1983; Wright, 1985) and can allow the simultaneous selection of multiple species.

Very often, an objective for a farmer who sows a multispecies grassland is to retain, over time, all the species that were sown in the mixture. Improvement of multispecies grasslands requires examination of the relationship between the productivity of the mixture (community-scale response) and the individual species' competitive and productive capacities within the mixture (Denison, 2010; Denison *et al.*, 2010). Efficient selection schemes to improve complex mixtures should contain a stage which improves simultaneously the different species. Examples of this type of selection scheme are rare in the literature, and in most cases they consider only two species under selection in a mixture (Wright, 1985). In this type of selection scheme, the black-box approach and the functional approach should be combined. Such a mixed approach could use the variation of interactions that are not predictable from ecological studies of community dynamics, to provide a more accurate adjustment of the optimal functional range of variation. In the first stages of this type of selection scheme, there is a need to select a pool of candidate genotypes from each species on the basis of (1) the mean and variance values of the interaction traits and (2) the mean value of agronomic traits (such as disease resistance, components of quality and biomass production). This stage should consider and optimise the trade-off between traits through selection indices. The second stage could include two options in accordance with the correlation between the fitness and performance values of the genotypes. If the fitness and performance values are correlated, this stage could lead directly into an evolutionary plant breeding process in the species mixture. If the fitness and performance value are uncorrelated, the second stage could be the evaluation of the progeny of the candidates directly in a mixture with other candidates of other species (J.P. Sampoux, unpublished results).

In spite of (1) the diversity (and non-exclusives) of possible approaches to improve multispecies grasslands and (2) the choice of the method which depends on the objectives, more particularly on the level of performance expected; i.e. if the objectives are at the mixture level (total yield) and/or at the species level (comparable or non-comparable performance of each species in the mixture), the mixed approach could well be the best way to improve the agronomic values of a multispecies grasslands. However, there is a need to increase our knowledge with respect to all species used in grasslands and their interactions within a multispecies composition to improve the mixed approach and to develop proofs of concept. In addition, it will be useful to screen the current genetic resources of grassland species (cultivars and ecotypes) to estimate the variability of interaction traits and the genetic correlations between the interaction traits and the agronomic traits.

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The influence of nitrogen fertilisation on the productivity of multi-species swards in three production years

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Abstract

Field trials were carried out with the aim of studying the forage yield and quality of grass and grass-legume swards fertilized with three nitrogen rates (N0, N60, and N120) in three production years. Mixtures were grouped into four sward types: mixtures of grasses only (G); mixtures of white clover (*Trifolium repens*) and grass (Tr+G); white clover, red clover (*Trifolium pratense*) and grass (Tr+Tp+G) mixtures; bird's-foot trefoil (*Lotus corniculatus*) and grass (Lc+G) mixtures. On average, the N fertilizer rate increase from 0 to 120 kg ha^{-1} contributed to a significant increase in crude protein (CP) content for all mixtures (on average 18 g kg^{-1}), and a significant dry matter (DM) yield increases: by 2.14 Mg ha^{-1} in the first year, by 2.01 Mg ha^{-1} in the second, and by 1.91 Mg ha^{-1} in the third year of yielding. Legume-containing mixtures achieved a higher CP content in comparison with grass-only swards at all fertilisation rates. The results show a substantial decrease in DM yields over successive years of yielding. The average yield decrease between the first and third year of yielding (by 8.81 Mg ha^{-1} or 57%) was more expressed for mixtures containing red clover (Tr+Tp+G).

Keywords: grass-legume mixture, nitrogen fertilization, production year, crude protein

Introduction

Nitrogen (N) fertilization is a management factor that greatly influences the perennial grass and legume herbage yield and crude protein (CP) content. The use of N fertilisation contributes to increases in dry matter (DM) yield, but, at the same time, has a negative effect on the content of legumes in the sward (Adamovics and Gutmane, 2016). Crude protein content is closely connected to the proportion of legumes present in the sward. Using different grass-legume containing mixtures, the protein content could be more affected by the type of seed mixture than by the N level applied (Meripold *et al.*, 2016). The objective of our study was to determine the influence of nitrogen fertilisation on the yield and quality of multicomponent swards in three production years.

Materials and methods

Field trials were conducted at three experimental sites in Latvia. At each site, the same mixtures were sown in June 2014, without a cover crop, in three replications, with a 10 m^2 plot size. The multicomponent swards were composed of *Phleum pratense, Dactylis glomerata, Lolium perenne, Lolium boucheanum, Festulolium, Festuca pratensis, Festuca arundinacea, Festuca rubra, Poa pratensis, Trifolium pratense, Trifolium repens*, and *Lotus corniculatus*, and grouped in four types: mixtures composed only of grasses (G); white clover (*Trifolium repens*) and grass (Tr+G) mixtures; white clover, red clover (*Trifolium pratense*) and grass (Tr+Tp+G) mixtures; bird's-foot trefoil (*Lotus corniculatus*) and grass (Lc+G) mixtures. The following P-K fertilisation treatments were used for all mixture types: P78, and K90. There were three N-fertilisation levels: N0, $N60_{(30+30)}$, and $N120_{(60+60)}$ kg ha^{-1}. Swards were cut four times during the vegetation season. The CP content of plants was determined by modified Kjeldahl. The data were statistically analysed using the three-way analysis of variance with 'mixture type', 'fertiliser', and 'year of sward use' as factors, and the difference among means was detected by LSD at the $P<0.05$ probability level (Excel for Windows, 2003).

Results and discussion

The N rate increase from 0 to 120 kg ha^{-1} contributed to a significant DM yield increase in all production years, for all mixture types (Table 1). The highest DM yield increase was found for grass-only swards (increase of 3.72 Mg ha^{-1} on average for the three production years). For all legume-containing mixture types (MT), the increase in DM yield was lower, but still significant. The DM yield increase (by 1.06 Mg ha^{-1}) was lowest for the MT including red clover (Tr+Tp+G).

There was a significant (P<0.05) MT-by-year interaction. Stable productivity was demonstrated by grass-only swards, where no significant differences between the yields of the first and second production years were found. For all legume-containing MT, DM yields in the second production year decreased on average by 3.70 t ha^{-1} or 25%. The decline in productivity between the second and third production years was detected for all MT – on average by 1.42 t ha^{-1} or 17% for grass swards, and by 4.37 t ha^{-1} or 40% for all legume-containing MT.

The CP content in the DM yield of swards was significantly (P<0.05) affected by the MT, production year, and N rate. The increase in N fertilizer rate from 0 to 120 kg ha^{-1} contributed to a significant CP content increase in the DM yield of all MT (Table 2). The highest CP content increase, by 22.3 g kg^{-1} (on average for three production years), was found for grass-only swards. The CP content increase (by 15.0 g kg^{-1}) was lowest for the Tr+G swards.

On average for three production years, grass-only MT had a significantly lower CP content in comparison with all legume-containing MT. There was a significant (P<0.05) interaction between year and MT. In the first production year, there were no significant differences between the grass-only and Lc+G swards. The increase in the proportion of perennial legumes (Tr and Lc) in swards contributed to a significant CP content increase in the second production year for all legume-containing MT. In the second and third production year, all legume-containing MT had a higher CP content in comparison with grass-only MT.

Table 1. Effect of N rate and production year on the average dry matter yield (Mg ha^{-1}) of the four mixture types.[1]

Production year (Year)	Mixture type (MT)	N rate, kg ha^{-1} (N)			
		N0	N60	N120	Mean
First	G	5.91	8.71	10.35	8.32
	Tr+G	14.07	14.09	15.02	14.39
	Tr+Tp+G	15.22	15.00	16.21	15.48
	Lc+G	13.15	13.94	15.32	14.14
	Mean	12.09	12.93	14.23	13.08
Second	G	6.04	8.37	9.95	8.12
	Tr+G	9.56	10.55	11.52	10.54
	Tr+Tp+G	11.01	11.09	11.86	11.32
	Lc+G	10.57	10.69	11.89	11.05
	Mean	9.30	10.17	11.30	10.26
Third	G	5.20	6.89	8.01	6.70
	Tr+G	5.73	6.92	7.16	6.60
	Tr+Tp+G	5.90	6.79	7.33	6.67
	Lc+G	5.40	6.86	7.35	6.54
	Mean	5.56	6.86	7.46	6.63

$LSD_{0.05}$ MT = 0.70; N = 0.61; Year = 0.61; MT×N = n.s.; MT×Year = 1.21; N×Year = n.s.; MT×N×Year = n.s.

[1] LSD = least significant difference; n.s. = not significant.

Table 2. Average crude protein content in DM yield for three years of sward use, g kg^{-1}.

Year of sward use (Year)	Mixture type (MT)	N rate, kg ha^{-1} (N)		
		N0	N120	Mean
First	G	102.1	144.2	123.1
	Tr+G	138.7	151.2	144.9
	Tr+Tp+G	139.2	150.1	144.6
	Lc+G	113.2	131.8	122.5
	Mean	123.3	144.3	133.8
Second	G	104.6	126.5	115.5
	Tr+G	164.4	184.1	174.2
	Tr+Tp+G	166.8	185.9	176.3
	Lc+G	166.3	189.7	178.0
	Mean	150.5	171.5	161.0
Third	G	138.2	140.9	139.5
	Tr+G	175.5	188.3	181.9
	Tr+Tp+G	165.0	181.0	173.0
	Lc+G	157.5	175.4	166.4
	Mean	159.0	171.4	165.2

$LSD_{0.05}$ MT = 11.44; N = 8.09; Year = 9.91; MT×N = n.s.; MT×Year = 19.81; N×Year = n.s.; MT×N×Year = n.s.

Conclusion

Increasing the N fertilizer rate from 0 to 120 kg ha^{-1} contributed to a significant increase in DM yield and CP content for all mixtures. The highest DM yield and CP content increase was found for grass-only swards. Significant differences in DM yields were found among the sward production years, with the highest yield in the first harvest year.

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Properties of red clover in monoculture and in mixtures under the influence of nitrogen fertilization

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Abstract

The aim of this research was to determine the production properties of red clover cultivated in monoculture and in mixtures with perennial ryegrass during two years with application of fertilizer in different rates of N. The experimental factors were two mixtures of perennial ryegrass and red clover arranged in different proportions of 70:30 (30CP), 50:50 (50CP) and monoculture of red clover (100CP). N was applied as ammonium nitrate (34% N) in three different levels: 0, 50 and 100 kg N ha^{-1}. After each cut, the botanical composition was studied, and the herbage production parameters and crude protein content were analysed. The share of red clover in the crop had a significant effect on all of the studied properties except for the leaf:stem ratio and leaf surface. Higher proportion of red clover increased yield, plant height, specific leaf area (SLA) and leaf area index (LAI). The height of red clover plants and LAI were higher in monoculture than in mixtures by 12% and 46%, respectively. The crude protein content was 8% higher in mixtures than in pure crop. N fertilization reduced the yield of red clover, and increased other production parameters as well as the crude protein content.

Keywords: red clover, perennial ryegrass, morphology, mixture, yield

Introduction

Red clover is one of the most important perennial forage plants used in livestock nutrition. It has high nutritional value and gives fodder of very good quality suitable for all categories of domestic farm animals. It is a very valuable component of grass-leguminous mixtures. Due to its nitrogen-fixing properties, it reduces the need for the use of N mineral fertilizers, ensures higher content of proteins in the mixture as well as their better utilization, and it also ensures more uniform and higher production. According to Iepema *et al.* (2006), red clover in combination with perennial ryegrass and white clover can realize high yields with low amounts of N. The use of red clover in animal nutrition is limited by its short lifespan of 2-3 years. Factors influencing its persistence are improper management, disease and impaired competition. The cultivation of red clover with high-quality grasses and the application of N mineral fertilizers reduce its persistence (Oram *et al.,* 2014). So, there is a need for breeders to create varieties of red clover that will have greater competitiveness and persistence and enable its successful cultivation in mixtures and widespread use in meadows and pastures.

The aim of this research was to assess the effect of the share of red clover in perennial ryegrass-red clover mixtures and the effect of fertiliser N application rate on the yield of red clover, its morphological properties and the crude protein content.

Material and methods

A field study was conducted at the Institute for Animal Husbandry, Belgrade, Serbia (44° 49' 10" N, 20°, 18', 45" E; altitude 110 m.a.s.l.), in spring 2014. At the site, the mean annual precipitation was 714 mm and mean annual temperature 16.7 °C. The soil is a low carbonate chernozem with pH of 7.26, content of $CaCO_3$ of 3.30 g kg^{-1}, humus 43.50 g kg^{-1}, total N of 1.97 g kg^{-1}, P of 0.40 g kg^{-1} and K of 0.13 g kg^{-1}. The design of the experiment was split-plot with three replications and a plot size of 2×5 m. The red clover (*Trifolium pratense*; local variety K-39) was sown in monoculture (100%) and in mixtures with perennial ryegrass (*Lolium perenne* cv. Calibra) to provide clover proportions of 30 and 50%. A seeding rate for red clover of 20 kg ha^{-1} was used and 25 kg ha^{-1} for perennial ryegrass. The amount of seed for sowing in mixtures was determined based on the planned sown proportions. N fertilization was supplied as ammonium nitrate with 0, 50 and 100 kg N ha^{-1}. Dry matter yield was recorded for three cuts in 2014 and 2015. For each cut the share of red clover was assessed by separation of species from 1 m^2 subplots. Morphological traits were evaluated on five randomly selected plants per plot. After measuring plant height, leaves were separated from the stem, the leaf area (cm^2) was recorded with ImageJ and the LAI was specified. Thereafter leaf and stem were oven dried, measured and used for calculation of leaf/stem ratio and SLA. Data were statistically processed using analysis of variance including the factors – mixture with different shares of red clover (30CP, 50CP, 100CP) and nitrogen fertilization (0N, 50N, 100N). Statistical analysis was performed in program STATISTICA 8.0 (StatSoft, Inc., 2007).

Results and discussion

The proportion of sown clover and N fertilisation level showed significant impact on DMY of red clover. The red clover DMY increased with increasing sown proportion in the mixtures, and was on average 6.0, 8.5 and 9.5 t ha^{-1} for the 30, 50 and 100% red clover treatment (Table 1). However, the yield of the 30CP mixture represents 63% of the yield of pure red clover and 89% for the 50CP mixtures, indicating higher productivity of red clover plants in mixtures with perennial ryegrass than in pure red clover crop as well as lower interspecific competition. The fertilization significantly reduced the yield of red clover by 24% (50N) and 29% (100N). Plant height is one of the most important traits of yield and persistence of red clover. There was a significant effect of mixture on plant height, which was highest in the pure red clover crop (69.80 cm) and lowest in red clover plants in the 30CP mixture (57.5 cm). According to Hoekstra *et al.* (2018), plant height is an indicator of persistence and competitive ability of plants. In monoculture there is high competition between plants for light, so the plant height is higher than in the mixtures. Measurement of leaf area is in direct connection with competition for light. The SLA value was the highest in plants of a pure crop and was lowest for plants in the 30CP mixture. The difference was significant only between plants in the 30CP mixture and plants in pure crop or in 50CP mixture. N fertilization increased values of SLA. The LAI was the highest in pure crop (4.11 cm^2) and lowest in the 30CP mixture (2.42 cm^2). N fertilization increased the LAI value from 2.74 to 3.57 cm^2. Knops

Table 1. Effect of mixture and N fertilization on red clover dry matter yield (DMY), plant height, specific leaf area (SLA), leaf area index (LAI), leaf/stem ratio, leaf area (LA), and content of crude protein (average over two years ± standard error of means).[1]

	DMY (t ha^{-1})	Plant height (cm)	SLA (cm^2 g DM^{-1})	LAI (m^2 m^2)	Leaf/stem ratio (g g^{-1})	LA (cm^2)	CP (g kg^{-1})
Effect of mixture							
30CP	6.0 ± 0.3^{b}	57.5 ± 1.6^{c}	265.82 ± 7.81^{b}	2.42 ± 0.16^{c}	0.39±0.01	141.27±5.97	16.27 ± 0.24^{a}
50CP	8.5 ± 0.7^{a}	62.4 ± 1.8^{b}	287.47 ± 9.49^{a}	3.35 ± 0.38^{b}	0.40±0.02	159.35±5.91	16.61 ± 0.13^{a}
100CP	9.5 ± 0.9^{a}	67.0 ± 0.9^{a}	291.42 ± 6.27^{a}	4.11 ± 0.43^{a}	0.38±0.01	154.24±9.78	15.21 ± 0.29^{b}
Level of significance							
mixture	**	**	**	**	ns	ns	**
Effect of N fertilization							
0	9.7 ± 0.9^{a}	62.8±2.6	287.02 ± 10.73^{a}	2.74 ± 0.10^{b}	0.36 ± 0.01^{c}	137.89 ± 5.51^{b}	16.03±0.39
50	7.4 ± 0.7^{b}	61.1±1.3	264.65 ± 4.73^{b}	3.57 ± 0.47^{a}	0.39 ± 0.01^{b}	165.98 ± 8.94^{a}	15.71±0.25
100	6.8 ± 0.5^{b}	63.0±1.8	293.06 ± 6.65^{a}	3.56 ± 0.50^{a}	0.41 ± 0.01^{a}	150.98 ± 7.00^{b}	16.35±0.23
Level of significance							
N fertilization	**	ns	**	*	**	**	ns
Interaction	ns	ns	**	**	ns	**	**

[1] ns = not significant, * significant at the 0.05 level, ** significant at the 0.01 level; Values with different letters are significantly different.

and Reinhart (2000) also confirm that nitrogen fertilization increases values of SLA and LAI by 82 and 202%, respectively. Fertilization increased leaf area with 9 and 20%. The N treatments had significant effect on leaf/stem ratio. It ranged from 0.36 g g^{-1} in 0N to 0.41 g g^{-1} in 100 N treatment. The CP content was higher in mixtures than in pure crop by 8%.

Proportion of red clover showed statistical differences between mixtures that were in line with the sowing density. N fertilization also had significant impact on red clover proportion (Figure 1). There were significant differences between treatment with 100 kg ha^{-1} and the other two treatments.

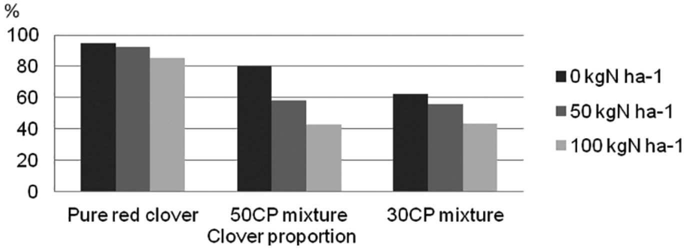

Figure 1. Mean red clover content (%) for three mixtures and three levels of N fertilization.

Conclusion

This study shows that perennial ryegrass is compatible for growing in mixtures with red clover. In pure red clover crop, there is greater competition for light than in red clover-perennial ryegrass mixtures. Red clover plants are more productive in the mixtures than in the pure crop. The N fertilization decreases the DMY of red clover but increases the parameters of leaf area and leaf/stem ratio. The content of CP in red clover increases when cultivated in mixture with perennial ryegrass.

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Functional groups drive positive diversity effects on yields across multiple Norwegian sites

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Abstract

A multi-site grassland biodiversity experiment was established at three sites across Norway in 2016. The sites were Holt (north), Kvithamar (lowland, central Norway), and Særheim (southwest). Monocultures and multi-species mixtures varying in richness and relative abundances of up to seven agronomic species were sown at low and high N fertiliser. The species varied in functional traits and were categorised according to (1) grasses or N-fixing legumes and (2) tall-rapid or low-slow establishment. Annual yield results from the first growing season in the year after establishment are reported. While productivity varied across the experimental sites (higher yields the further south), diversity effects driven by the grass-legume functional groupings were identified consistently across sites. There were strong positive grass-legume interactions, and of lower magnitude but also strongly positive grass-grass interactions. There were positive effects of N fertiliser on yield that decreased as the percentage of legume in the sward increased. At equi-proportional communities, benefits of increased fertiliser were still evident at the higher richness levels.

Keywords: multispecies mixtures, N fertiliser, diversity effects, percentage of legume, intensive management

Introduction

Positive effects on annual yield of mixing four agronomic grass and legume species have been shown across multiple sites spanning a wide climatic gradient (Finn *et al.*, 2013). In addition, mixing up to four agronomic species and fertilising at a low rate of N can produce comparable yields to heavily fertilised grass monocultures (Nyfeler *et al.*, 2009). However, it is not known how varying fertiliser management intensity will impact on the yields of mixtures of up to seven species. Here we present results from a first-year ley multi-site Norwegian experiment that manipulates sward species composition, richness and relative abundances from a pool of seven (five grass and two legume) species, at two fertiliser rates.

Materials and methods

A multi-site biodiversity experiment was established at three sites across Norway in 2016. The experimental sites were located to span the varying Norwegian climatic gradient: Holt (69 °N, 14 m.a.s.l. – north), Kvithamar (63 °N, 28 m.a.s.l. – mid-Norway) and Særheim (58°N, 14 m.a.s.l. – southwest). The grass species were perennial ryegrass (*Lolium perenne*), timothy (*Phleum pratense*), meadow fescue (*Festuca pratensis*), smooth meadowgrass (*Poa pratensis*) and colonial bentgrass (*Agrostis capillaris*), and the legume species were red clover (*Trifolium pratense*) and white clover (*Trifolium repens*). The first three grasses and red clover are rapid establishing and tall-growing species, while the last two grasses and white clover are relatively slow establishing and low-growing species. At each site, monocultures and multispecies mixtures of up to seven species were sown, and the richness and relative abundances of species in mixtures were varied according to a simplex design (Cornell, 2002); there were 30 plots, comprising richness levels 1 (7 plots), 2 (9 plots), 3 (2 plots), 4 (3 plots), 5 (7 plots), 6 (1 plot) and 7 (1 plot). The 30 plots were repeated at low and high N fertiliser at rates determined locally (Holt: 85 and 170 kg N ha^{-1}, Kvithamar: 120 and 240 kg N ha^{-1}, Særheim: 130 and 260 kg N ha^{-1}), giving 60 plots in total.

We analysed the plot-level annual dry matter (DM) yield collected at multiple harvests across the 2017 growing season using Diversity-Interactions modelling (Kirwan *et al.*, 2009). This regression-based modelling approach estimates the contributions of each species (identity effects) and species interactions (diversity effects) on ecosystem functions. A random site effect was included to adjust for variability from site to site. We tested a range of models to identify how the species in our seven-species pool interacted with each other and to test what impact varying N fertiliser had on identity and diversity effects for annual DM yield.

Results and discussion

While productivity varied across the sites in the 2017 growing season, with the most northerly site (Holt) being the least productive (Figure 1), we found consistent and positive effects of mixing species on annual DM yield across the sites. Functional group membership dictated interactions between the species: the interaction between any grass and any legume was strong and positive, interactions between any two grasses were also positive, but weaker, while the interaction between the two legumes was not significant (Table 1). There was no evidence of differences in interaction strengths for grasses that differed in establishment rate and growing height. There was a positive effect of high versus low N fertilisation on annual DM yield, but the strength of this effect declined as the percentage of legumes in the sward increased (Table 1). Predictions from the model showed that there were still benefits of using high over low N fertiliser at both the 6- and 7-species communities ($P<0.001$ in both tests).

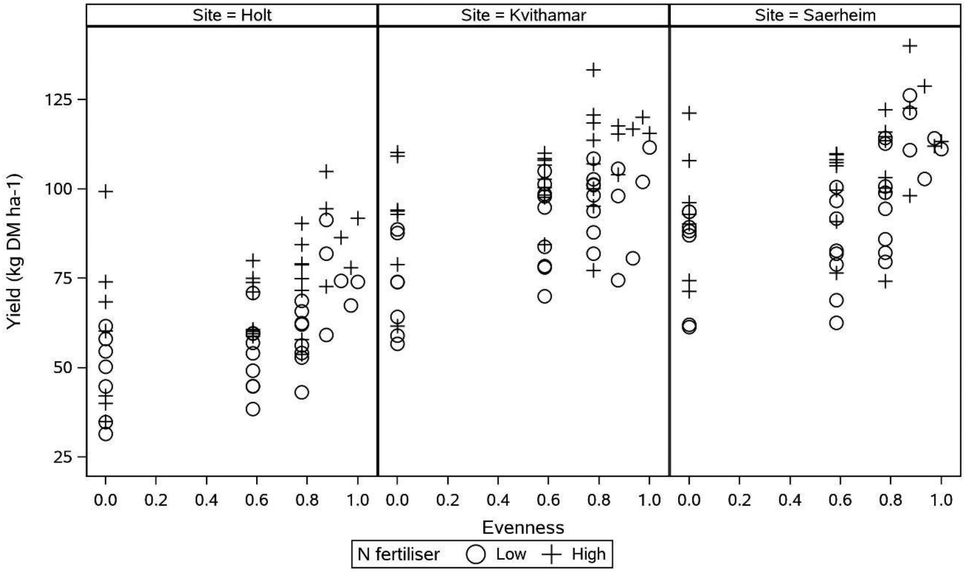

Figure 1. Annual DM yield (kg ha^{-1}) versus evenness (E) of each community for each site. Evenness is scaled to lie between 0 (monoculture) and 1 (seven-species mixture with equal relative abundances) and is computed as

$E = \frac{2s}{(s-1)} \sum_{i<j} P_i P_j$, where P_i is the proportion of species *i*, and there are $s=7$ species in the species pool.

Table 1. Estimates of the Diversity-Interactions model coefficients are shown.[1]

Effect	**Estimate**	**Standard error**	***P*-value**
Lolium perenne	89.4	11.71	
Phleum pratense	70.6	11.49	
Festuca pratensis	66.0	11.50	
Poa pratensis	55.0	11.49	
Agrostis capillaris	50.8	11.50	
Trifolium pratense	76.1	11.71	
Trifolium repens	58.8	11.71	
N Fertiliser treatment	18.7	2.12	<0.001
N Fertiliser × Legume %	-17.6	4.97	<0.001
Grass-grass	51.7	7.37	<0.001
Legume-legume	12.7	19.92	0.524
Grass-legume	80.2	7.46	<0.001

[1] The first seven rows give the estimated annual DM yield (kg ha^{-1}) of each species in monoculture, on average across sites. The N fertiliser treatment is coded 0 for low and 1 for high N fertiliser. Legume % was the percentage of legume (red clover plus white clover) sown in the sward and ranged from 0 to 100% across the plots. The last three rows provide estimates of functional group level interactions.

Conclusion

This work highlights the particularly important role of legumes in highly diversity agronomic grassland swards. While there are positive effects of N fertiliser on both monoculture and mixture swards, the yield benefits of the fertiliser declined as the legume percentage in the sward increased. However, at equi-proportional mixtures at richness levels 6 (legume=33%) and 7 (legume=29%), there were still yield benefits to the increased fertiliser rates.

Acknowledgements

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Development of multi-species grasslands in low input dairy goat farming systems: multiple services and benefits, limits

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Abstract

French dairy goat farming systems have been gradually intensified, resulting in an increase of purchased inputs. Using multi-species grasslands can be a solution to develop low-input farming systems and therefore improve dairy goat farms sustainability. In 2012, a system-experiment in Western France was designed to evaluate if this low-input solution leads to high-performance farming systems. The system-experiment aimed at evaluating three independent farmlets differing in herbage utilisation method (grazed or mown) and kidding period (February or September). Grasslands were composed of 52% DM grasses, 37% legumes and 11% unsown species, in average. Between 2014-2017, the average forage yield was 6.2±1.6 tons DM ha^{-1} $year^{-1}$. Although fresh forage allowed high forage intake, average milk yield was low for all farmlets (646±63 l $goat^{-1}$ $year^{-1}$). However, the large herbage proportion in the ration was associated with a low quantity of concentrates and dehydrates (282±38 kg $goat^{-1}$ $year^{-1}$) and low feeding costs (157±39 € per 1000 l), in particular for the farmlet with grazing and kidding in February. These results show that the development of multi-species grasslands for low-inputs dairy goat systems presents multiple benefits.

Keywords: multi-species grasslands, dairy goat, low inputs, feed self-sufficiency

Introduction

France is the largest producer of goat's milk in Europe with about 550 million litres collected. Feeding systems in goat farms are very diverse, ranging from pastoralism to above-ground systems. Since 2000, goat farms have moved towards intensive farming, largely increasing their need in purchased inputs, particularly feeds. To increase productivity, grazing has been progressively abandoned and indoor breeding developed. As a result, feed self-sufficiency of goat farms in western France is only 61%, compared to 82% in dairy cattle systems (Broccard *et al.*, 2016). Larger use of multi-species grasslands can be a solution for improving goat farms sustainability with a better control of feeding costs and a greater security of systems (Lüscher *et al.*, 2014). The objective of this study is to evaluate the multiple services, benefits and limits of multi-species grasslands in low input dairy goat farming systems, based on the results obtained at Patuchev, an INRA system experiment. The objective of the Patuchev system experiment is to design low-input goat farming systems and evaluate their performance based on (1) biological regulations and interactions between the components of the farm and (2) an increase of self-sufficiency with the use of local feed resources and inputs (Caillat and Jost, 2015).

Materials and methods

Patuchev was located at Lusignan, Western France, and since 2013, evaluated three farmlets with 60 Alpine goats and 10 hectares of pastures and crops. The three farmlets shared the same challenges and objectives (e.g. 75% of feed self-sufficiency, 800 kg $year^{-1}$ of milk per goat with less than 300 kg $year^{-1}$ of concentrates and dehydrates) achieved with different managements. Two farmlets mainly use grazing pastures, one with kidding in February (GF) and the other in September (GS). For the third farmlet (IS), goats were bred indoor during all the year, with a ration based on ventilated hay, and kidding in September. Each farmlet area is divided in 6 or 7 pastures paddocks of one hectare, and 4 or 3 hectares of annual crops, respectively for IS and for GF and GS systems. Pastures were sown with multi-species seed mixtures (timothy, fescue, red and white clover, lucerne). Every year, for each farmlet, two plots of grasslands were sown and grown for three or four years in a 10-year rotation. In IS system, plots were mown (EM). In GF and GS systems, plots were grazed and mown (GM). For plots, forage yield, botanical diversity and forage quality were evaluated every week (grazing) or before harvest (mowing) between 2014 and 2017. For animals, feed intake and milk yield were measured weekly and body weight monthly.

Results and discussion

Multi-species grasslands to stimulate intake and provide a balanced diet

The average composition of multi-species grasslands was 52% grasses, 37% legumes (% of DM before harvest) including 14 to 17% lucerne and 6 to 13% red clover (according to the type of harvest) and 11% weeds. EM plots had more unsown species (+5.9%) mainly Italian ryegrass and *Rumex*, that were preferentially consumed by grazing goats and tended to disappear with time on GM plots. Average herbage yield (± standard deviation) was 6.2±1.6 Mg ha^{-1} dry matter (DM), with manure as the only fertilizer (2 to 3 Mg ha^{-1}). Over the studied period, we observed a decrease in forage yield leading to a decrease in farm self-sufficiency (from 100% in 2014 down to 75% in 2017 for the IS farmlet). Average herbage intake was 698 kg DM goat^{-1} year^{-1} and represented about 66% of the total ration.

Sowing is a crucial moment

In the three farmlets, no herbicides were used. The results of the botanical diversity assessment (17 plots with 229 samples) showed a high development of weeds during the first year after sowing (16.4±15.5% DM) for all harvest types (Figure 1). The proportion of weed decreased faster in the second year when plots were grazed and cut (17.7±18.3% DM versus 8.1±9.2% DM).

This high proportion of weeds reduced the development of the sown legumes and grass species and contributed to reduce forage yield during the first year. Checking the sowing is an even more important aspect where grasslands are grown for only 3 or 4 years. This short duration is considered as a solution to limit well-known gastro-intestinal parasitism problem for grazing goats. The choice of species in the mixture is also very important. Species such as Italian ryegrass or red clover may be very aggressive for species with slower development (lucerne, fescue) and may limit the durability of grassland (Jost and Caillat, 2017).

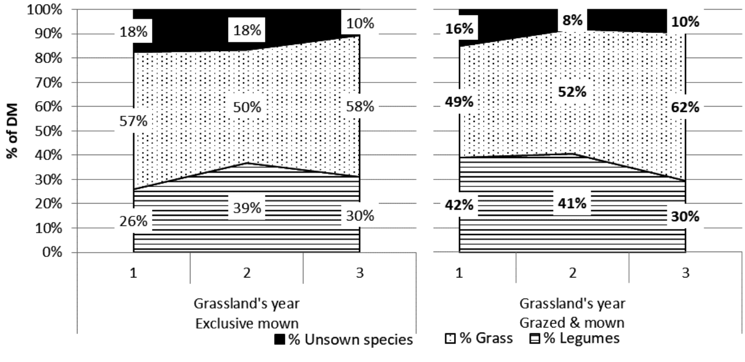

Figure 1. Changes in the botanical diversity according to grassland age and harvest method.

Positive balance with low feeding costs

Average milk yield was similar between goats of the three farmlets (620 to 695 l goat^{-1} year^{-1}). This result was 27% lower than the average local milk yield (obtained from a network of 22 specialized dairy goat farms Western France French Livestock Institute). However, the three farmlets achieved a high proportion of forage in the diet (67%) compared to the local farm result (50%). They also achieved a low quantity of concentrates and dehydrated (282±38 kg goat^{-1} year^{-1}) compared to local farm results (479±48 kg goat^{-1} year^{-1}). As a result, the three farmlets achieved lower feeding cost (157±39 € per 1000 l) than the local network (218±8 € per 1000 l), in particular for the GF farmlet (123±13 € per 1000 l) compared to GS and IS systems (174±37 € per 1000 l).

Conclusion

The results showed that the development of multi-species grasslands is an interesting option in low-inputs dairy goat systems, mainly by enhancing feeding self-sufficiency and grazed forage. However, these benefits imply productive grasslands with high feeding value. The sowing operation is crucial: choice of sown species and soil management are important to limit weeds development, not to forget organic fertilisation management. Despite a lower milk yield, diet with high proportion of forages remains cost-effective when the purchase of inputs is controlled. In addition to economic benefits, multi-species grasslands seem to be the forage resource most naturally adapted to the diversity of French soil and climatic conditions and allow to improve the sustainability. Finally, increasing the use of multi-species grasslands in French dairy goat farms improves the image of goat cheeses, as products which contribute to the maintenance of biodiversity. This aspect will be an asset for the development of cheeses with label of quality like Protected Designation of Origin or Organic Agriculture.

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Effect of intra-specific diversity on the performance of subterranean clover across contrasting Mediterranean environments

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Abstract

Breeding multiline mixtures rather than pure lines of inbred species should provide wider adaptation and more resilience. Subterranean clover (*Trifolium subterraneum* L.) is an inbred pasture species for Mediterranean-climate environments, encompassing the major subspecies *subterraneum* (S) and *brachycalycinum* (B). The former features higher variation for the key adaptive trait flowering time. This study assessed the effect on the clover performance of increasing intra-specific variation, by comparing pure lines, 2- and 4-line mixtures within subspecies, and 4- and 8-line mixtures across subspecies. Mixtures included lines selected for contrasting phenology within or across two sites of origin. Forage dry-matter (DM) and seed yield and seedling regeneration were recorded across four managed environments of Sardinia obtained by the factorial combination of two soil types by two drought stress levels in a one-year assessment. On average, mixtures resulted in higher forage DM and seed yield than pure lines, as well as higher forage yield stability across environments as revealed by AMMI analysis, for the subspecies S featuring greater phenological variation. In contrast, mixtures had no effect in the subspecies B. The increase of intra-specific diversity provided by mixtures of the two subspecies did not produce any significant advantage over mixtures of the separate subspecies.

Keywords: adaptation, breeding, drought stress, multiline mixture, *Trifolium subterraneum*, yield stability

Introduction

Although modern agriculture has favoured the adoption of pure lines of inbred crops, there is large evidence that biodiversity provides crop varieties with a buffering effect towards biotic and abiotic stress pressures, thereby increasing crop resilience, performance stability and system sustainability (Marshall and Brown, 1973; Kiær *et al.*, 2012; Barot *et al.*, 2017). Diversity of key traits may be beneficial when assembling a mixture. Flowering time is recognised as the single character mostly affecting germplasm adaptation to the environment. Information is needed on the optimal level of intra-specific diversity in terms of number of genotypes within a mixture and range of expression for adaptive traits.

Subterranean clover is a major inbred pasture species for Mediterranean-climate environments, where two main subspecies, namely subsp. *subterraneum* (S) and *brachycalycinum* (B), are bred as pure lines for cropping, largely exploiting native genetic resources (Nichols *et al.*, 2013). Both subspecies show composite natural populations formed by several genotypes, where S constantly features higher within-population variation than B for flowering time (Piano, 1984; Piano *et al.*, 1993).

By mimicking the natural populations to some extent, we assembled genotype mixtures of different complexity both within and across subspecies, aiming at assessing the effect of increasing intra-specific variation on the clover performance across contrasting Mediterranean environments.

Materials and methods

Genotypes of both S and B subspecies were chosen for contrasting phenology from germplasm collected in each of two Sicilian sites (Maletto, Ma; Vizzini, Va) where the two subspecies grew sympatrically. Flowering time ranged four and six days for B lines, and 25 and 21 days for S lines in Ma and Vi, respectively. Pure-stand genotypes were compared to 2- and 4-line mixtures of same subspecies assembled within or across sites of origin, and to 4- and 8-line mixtures across subspecies (the former by mixing lines within each site, the latter by mixing lines across sites). The experiment was sown in November in Sanluri, Sardinia, and was carried out across four managed environments obtained by the factorial combination of two soil types (clay loam and sandy loam) by two drought-stress levels (rain-fed, with 248 mm of rain from November to June, and irrigated cropping with a total of 573 mm of water). Forage DM yield was cumulated over three harvests in the rain-fed treatment and four in the irrigated one. Seed yield, assessed as the number of seeds per m^2, and seedling regeneration, which are two pillars for a self-regenerating crop as subterranean clover, were recorded in July and early November, respectively. The data were subjected to ANOVA with two factors: plant material and environment. Contrasts compared pure lines *versus* mixtures in all possible combinations. Interactions for forage DM yield of pure lines and mixtures across the four environments were modelled by AMMI analysis (Gauch *et al.*, 2008).

Results and discussion

The environments significantly ($P<0.05$) differed for all traits. As expected, the irrigated environments outyielded the rain-fed ones for forage DM yield. Seed yield tended to be lower in the clay loam soil, especially without irrigation, than in the sandy loam soil, and the rain-fed clay loam soil exhibited lowest autumn seedling regeneration (data not shown). The variation for plant material and the interaction of plant material with environment were significant ($P<0.001$).

Few contrasts were significant at $P<0.05$, and all of them regarded subsp. *subterraneum* (Table 1). Binary and 4-component mixtures in S tended to produce more forage DM and had higher seed yield across environments than the average of pure lines (Table 1). Four-component mixtures had no significant advantage over the average of 2-component mixtures. Mixing the two subspecies, either in 4- or 8-component mixtures, did not improve the performance compared to pure line means or single-species mixtures (data not shown). No significant contrasts were observed for seedling regeneration in autumn. Although certainly affected by seed production, the dynamics of self-regeneration are also likely influenced by the proportion of hard seeds, which was not considered in this study.

Table 1. Significant contrasts for forage and seed yield between pure stands (PS) and 2- or 4-component mixtures (Mix-2 and Mix-4, respectively) of subterranean clover (subsp. *subterraneum*) genotypes from two Sicilian collection sites (Maletto, Ma; Vizzini, Vi) grown across four managed environments in Sardinia.[1]

Subspecies	**Site**	**Contrast**	**DM yield (Mg ha^{-1})**	**No. seeds m^{-2}**
S	Ma	PS vs Mix-2	4.21 vs 4.31 NS	10,521 vs 14,444 *
S	Vi	PS vs Mix-2	3.17 vs 3.76 *	11,543 vs 19,899 ***
S	Ma+Vi	PS vs Mix-2	3.69 vs 4.03 *	11,032 vs 17,171 ***
S	Ma+Vi	PS vs Mix-4	3.69 *vs* 4.32 *	11,032 vs 15,325 **

[1] S: *subterraneum*; NS: not significant; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Nominal forage DM yield (i.e. AMMI-modelled yields from which the site main effect was subtracted) of pure lines and mixtures as a function of the first GE interaction principal component (PC 1) score of the four environments revealed that plant material × environment interaction affected mostly S lines. Binary and 4-component mixtures in this subspecies tended to be more stable-yielding across environments than the constituent lines (Figure 1).

In conclusion, greater intra-specific variation resulted in higher forage DM and seed yield along with better forage yield stability across environments only for subsp. *subterraneum*, which featured greater phenological variation than subsp. *brachycalycinum*, thus possibly resulting in greater adaptive variation.

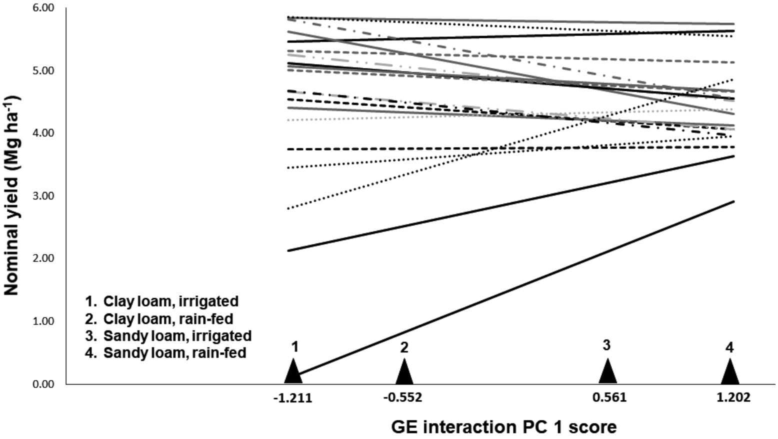

Figure 1. AMMI-1 nominal dry-matter yield (Mg ha^{-1}) of individual subterranean clover genotypes (solid lines; black: S, grey: B), 2- and 4-component mixtures of same species (dashed and dashed-double dotted lines; black: S, grey: B), 4- and 8-component mixtures across species (dashed-dotted and dotted lines; grey), and three check cultivars (dotted lines, black) across four managed growing environments.

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Optimizing yield and flower resources for pollinators in intensively managed multi-species grasslands

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Abstract

Increasing crop diversification in intensively managed grasslands can promote herbage yield and multiple ecosystem services. In this study, we address if composition of multi-species mixtures (grasses, legumes and non-leguminous forbs), and cutting frequency can be optimized to produce high yield and support insect pollinators. Four perennial grassland mixtures (3-, 5-, 11- and 13-species mixtures) were established at three sites with three cutting strategies (four cuts per year, two cuts per year, and no cut). Flower abundance, richness and flower-visiting insects were monitored every 2 to 4 weeks throughout the flowering season. The 11- and 13-species mixtures, which were designed to enhance pollinators, produced similar or even higher annual herbage yield than the 3- and 5-species mixtures under certain cutting regimes. The 3- and 5-species mixtures had a high total flower abundance due to excessive flowering of lucerne under the two-cut strategy and white clover under the four-cut strategy. Pollinator profiles were plant-species specific. Legume species mainly attracted large bees (honey bees and bumblebees), while the dominant forbs attracted mainly syrphids and other flies. Our results suggest that multi-species mixtures, in particular grass-legume-forb mixtures, under a two-cut strategy may produce high herbage yield and flower resources, supporting insect pollinators.

Keywords: cutting strategy, flower abundance and richness, forb, grassland mixtures, herbage yield, pollinator profile

Introduction

Perennial grass-legume mixtures characterized by high productivity, efficient nitrogen (N) use and strong weed suppression were proposed as a means for increasing sustainability of grassland production (Lüscher *et al.*, 2014). Recent studies have shown that adding certain forbs such as chicory (*Cichorium intybus* L.), caraway (*Carum carvi* L.) or plantain (*Plantago lanceolata* L.) to grass-clover mixtures can further enhance or largely maintain the high productivity of grass-clover mixtures (Cong *et al.*, 2017, 2018). Agri-environmental schemes such as planting of wild flower strips are commonly practised by farmers and land managers to improve biodiversity of pollinators (Whittingham, 2007). It remains unclear whether adding certain flowering forbs to productive grassland mixtures could be managed to provide flowers for pollinators, without compromising yield. We specifically investigated if yield (biomass production) and floral resources for pollinators could be optimized simultaneously by varying botanical composition of mixtures and cutting frequency of the grasslands. We tested four different perennial seed mixtures (3-, 5-, 11- and 13-species mixtures) at three sites varying in surrounding environment using three cutting strategies (no cutting, two cuts per year, four cuts per year). In addition, we also examined how different functional groups of insect pollinators responded to plant species and study sites.

Materials and methods

Experimental plots were established at three sites in mid Jutland, Denmark: Foulum, Venning and Aalum. The three sites differed in soil type and other properties and landscape composition. At each study site, the experiment was set up as a split-plot randomized block design with four replicates. Within each block, three cutting strategies (no cut, two cuts and four cuts) were assigned to the main plots. Within the plot, different seed mixtures were assigned to sub-plots. The 3-species mixture consisted of three productive legumes (white clover, *Trifolium repens* L.; red clover, *Trifolium pratense* L.; lucerne, *Medicago sativa* L.), designed for maximizing protein content in animal fodder. The 5-species mixture was a grass-forb mixture (perennial ryegrass, *Lolium perenne* L.; lucerne, chicory, caraway and plantain) designed for maximizing energy output in biogas production. The 11-species mixture comprised six competitively inferior forb species added to the 5-species mixture, while the 13-species mixture was based on the 11-species mixture by adding perennial ryegrass and white clover. The latter two mixtures were designed for enhancing diverse flowering as a resource for wild pollinators. The experimental plots did not receive fertilizers, pesticides or irrigation. Biomass production was measured as annual herbage yield in both 2015 and 2016. Plots were visited once every 2-4 weeks throughout the flowering season in 2016. On every observation date, flowering was monitored by counting flower units of each of the sown plants in three evenly spaced 50 × 50 cm sub-plots within each plot. Flower-visiting insects were observed during three five-minute observation trials for each flowering plant.

Results and discussion

Annual herbage yield significantly differed between mixtures ($P<0.001$), but the effect depended on study site and cutting strategy. Generally, the 13-species and 11-species mixtures in general produced similar or sometimes higher yield than the 3-species mixture at the two-cut strategy or the 5-species mixture at the four-cut strategy (Table 1). Increasing cutting frequency from two to four significantly enhanced biomass proportion of ryegrass, white clover and plantain, but markedly decreased that of lucerne. The 3- and 5-species mixtures had a high accumulated flower abundance due to excessive flowering of lucerne under the two-cut strategy and white clover under the four-cut strategy, which was consistent across three sites (Table 1). The 11- and 13-species mixtures presented a higher diversity of flowers during the flowering season. Interestingly, accumulated flower abundance was not significantly reduced under the two-cut strategy compared to no cut (data not shown).

Pollinator profiles (visits by different functional groups of insects) were plant-species specific; i.e. at all sites, plant species attracted similar types of insects (Figure 1). Three of the legume species (white clover, red clover and birdfoot's trefoil) were visited mainly by large bees (honeybees and bumblebees) while lucerne attracted many butterflies, in addition to large bees. The three most attractive forbs mostly supported non-bees. Field scabious was the single most attractive plant species, receiving visits by a range of different functional groups of insects, most notably a range of syrphid flies. Plantain received many visits by a single syrphid fly, *Melanostoma mellinum,* while caraway attracted a high diversity of flies (including syrphids), wasps, solitary bees and beetles. These results could be used to strategically enhance specific pollinators by growing certain plant species.

Table 1. Annual herbage yield (t DM ha^{-1}) averaged across two years and flower abundance (total flower units m^{-2}) averaged across all monitoring dates in 2016 of four mixtures under two cutting strategies at three sites.[1]

Variables	Mixtures	Foulum		Venning		Aalum	
		2 cuts	4 cuts	2 cuts	4 cuts	2 cuts	4 cuts
Yield	3-species	9.6±0.6	11.0±0.6	9.7±0.5^{b}	11.0±0.1	9.0±0.4^{b}	9.4±0.4^{a}
	5-species	11.0±0.6	10.5±0.2	12.7±0.4^{a}	11.4±0.6	10.1±0.5	7.5±0.4^{b}
	11-species	11.6±0.2	11.0±0.2	11.2±0.5^{ab}	11.0±0.5	9.2±0.2^{b}	8.9±0.6^{ab}
	13-species	11.7±0.5	11.5±0.2	11.2±0.5^{ab}	12.2±0.2	9.3±0.4^{a}	10.5±0.2^{a}
Flower abundance	3-species	68±6	31±7	148±17^{a}	50±7^{a}	85±4^{a}	50±9^{a}
	5-species	66±6	17±2	128±6^{a}	14±2^{b}	97±6^{a}	6±2^{b}
	11-species	70±7	21±3	73±4^{b}	27±5^{ab}	38±4^{b}	26±5^{b}
	13-species	56±2	29±2	41±7^{b}	44±6^{a}	29±7^{b}	22±4^{b}

[1] The lowercase letters indicate significant differences ($P<0.05$) between mixtures within each cutting strategy at a given study site using one-way ANOVA with Tukey's *post hoc* test.

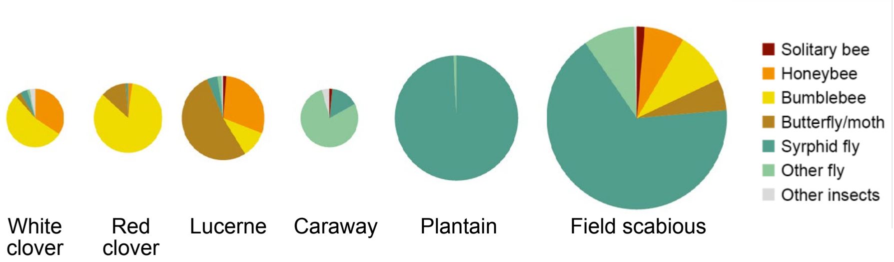

Figure 1. Pollinator profiles of legumes (white clover, red clover and lucerne) and forbs (caraway, plantain and field scabious) averaged across three sites in 2016.

Conclusion

Our results showed that multi-species grassland mixtures can be designed to support a high diversity of pollinators without compromising herbage yield. In particular, adding forbs to the grass-legume mixtures and using a two-cut strategy rather than four cuts per year, may increase flower resources available for a larger range of wild pollinators.

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Effects of plant diversity on yield in intensively managed grasslands

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Abstract

Multi-species grassland mixtures offer an opportunity to increase sustainable production from intensively managed European grasslands. We investigated the effects of mixing species and functional groups (FGs) with the aim of improving forage yield and yield stability. A simplex design including six species monocultures and 13 mixture communities was sown in 2017 in a field experiment in the south-east of Ireland. Three FGs were represented by two grass, two legume and two herb species. We sowed monocultures of all six species, as well as 2-, 4-, 5- and 6-species mixtures with FG richness varying from 1 to 3. All experimental plots received 150 kg ha^{-1} of nitrogen (N) fertiliser *per annum* and were compared against a *Lolium perenne* monoculture with 300 kg ha^{-1} of N fertiliser. Aboveground biomass was harvested and measured on seven occasions. We modelled the effects of species identities and FG interactions on total annual aboveground yield. We found that interactions from cross-FG complementarity were stronger than intra-FG interactions and caused positive diversity effects on yield. Yield of communities with an equal proportion of each of the six species fertilised with 150 kg ha^{-1} of N outperformed the *Lolium perenne* monoculture fertilised with 300 kg ha^{-1} (12,219 kg ha^{-1} yr^{-1} and 10,732 kg ha^{-1} yr^{-1} respectively, $P<0.05$). This adds to the evidence base indicating multispecies mixtures as an agronomic option to reduce inorganic fertiliser use and associated greenhouse gas emissions.

Keywords: intensive grassland, plant diversity, grass, legume, herb

Introduction

Ruminant systems in oceanic regions of Europe are mostly specialized in intensively managed grasslands for feeding livestock. Plant diversity in four-species mixtures has been shown to have a strong effect on productivity of intensively managed grasslands throughout Europe (Finn *et al.*, 2013). The positive yield effects of multispecies mixtures could be sufficient to compensate for a substantial reduction in nitrogen fertilizer and its associated greenhouse gas emissions. In this experiment, we investigated the effect of functional group (FG) diversity on yield in communities with up to three functional groups (FGs). We specifically tested the relative effects of within-FG versus between FG interaction effects.

Materials and methods

Forty-three plots with replicates of 20 different forage plant communities were established in 2017 at Johnstown Castle, Wexford, in the south-east of Ireland (52°17'57.8'N, 6°30'23.3'W). Plots of 35 m^2 (7×5 m) were sown with combinations of six different species across three FGs: grasses (*Lolium perenne, Phleum pratense*), legumes (*Trifolium pratense, Trifolium repens*) and herbs (*Cichorium intybus, Plantago lanceolata*). The sown communities comprised six monocultures and 13 mixtures, containing two to six species. Following a simplex design, species proportions were 100% of the advised monoculture seeding weight for monoculture (3 replicates each); 50% of each species from the same FG for the 2-species mixtures (2 replicates each); 25% of each species from two FGs for the 4-species mixtures (2 replicates each), and; 16.7% of each species for the 6-species mixtures (3 replicates). The 5-species mixtures were dominated by each species in turn, such that there was 60% of one species and 10% of the other four species from the other two FGs (1 replicate each). Communities received 150 kg ha^{-1} of nitrogen fertiliser *per annum*. A *L. perenne* monoculture with 300 kg ha^{-1} of nitrogen was included (4 replicates). Plots were harvested on seven occasions during the 2018 growing season, at 4.5 cm height with a Haldrup harvester. Total annual aboveground yield was analysed using general linear models using proc GLM in SAS (SAS Institute Inc. 2013), with species proportions and interactions among proportions as predictors, as in Kirwan *et al* (2009). Here, we present results from the model based on the six individual species' yields in monoculture (identity effects), the pairwise interactions within the three functional groups, and the pairwise interactions across the functional groups.

Results and discussion

Total annual yield of the monocultures varied from 8,610 kg ha^{-1} yr^{-1} (*C. intybus*) to 11,672 kg ha^{-1} yr^{-1} (*T. pratense*) (Table 1). There was a synergetic pairwise interaction among pairs of species that were from two different FGs. This positive effect was marginally non-significant for the grass × herb interaction (P=0.080) and significantly positive for both the legume × herb and legume × grass interaction effects (P=0.007 and 0.012 respectively). However, within-FG interactions were not significant (P=0.90, 0.52 and 0.14 for grass × grass, legume × legume and herb × herb respectively) (Table 1).

These results demonstrate three aspects of diversity effects. Firstly, differences in yield among species could be quite variable (Figure 1). Secondly, the interaction effect was derived from cross-FG complementarity more than intra-FG interactions as suggested by the review from Díaz and Cabido (2001). Thirdly, the strength of the cross-FG interaction effects on yield was also dependent on the identity of the FG. The equi-proportional 6-species mixture community fertilised with 150 kg N had a significantly higher estimated yield (12,219 kg ha^{-1} yr^{-1}) than the *Lolium perenne* monoculture with either 150 kg N (9,260 kg ha^{-1} yr^{-1}, P<0.001) or 300 kg N (10,732 kg ha^{-1} yr^{-1}, P<0.05).

Table 1. Model estimates (±SE) of species identity effects (performance in monoculture), and the within- and across-functional group interaction effects.

Parameter	**Estimate (kg ha^{-1} yr^{-1})**	**SE (kg ha^{-1} yr^{-1})**	**t-value**	***P*-value**
Lolium perenne 300N	10,732	468	22.93	<0.0001
Lolium perenne	9,260	523	17.70	<0.0001
Phleum pratense	11,198	539	20.76	<0.0001
Trifolium pratense	11,672	521	22.39	<0.0001
Trifolium repens	10,303	521	19.77	<0.0001
Cichorium intybus	8,610	521	16.52	<0.0001
Plantago lanceolata	10,695	521	20.52	<0.0001
Grass×grass	-382	2,997	-0.13	0.8993
Legume×legume	1,946	2,962	0.66	0.5163
Herb×herb	-4,499	2,962	-1.52	0.1397
Grass×legume	6,582	2,458	2.68	0.0121
Grass×herb	4,458	2,458	1.81	0.0801
Legume×herb	7,055	2,422	2.91	0.0068

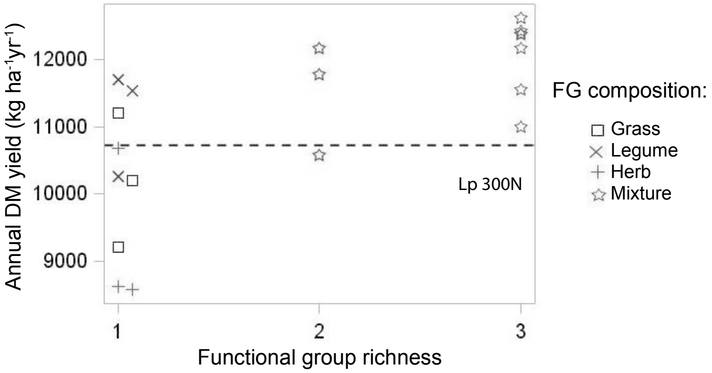

Figure 1. Harvested dry matter yield of the different communities categorised by functional group richness in 2018 (first full year of harvesting after sowing). Lp 300N = *Lolium perenne* monoculture with 300 kg ha^{-1} of nitrogen fertilizer per year. Note that the communities with FG = 1 were jittered to separate monocultures (left) and two-species communities from the same FG (right).

Conclusion

Plant diversity showed a strong positive effect on annual dry matter yield. This effect was explained by a combination of species identity, FG identity and FG interactions. The mixing effect of FGs was dependent on FG identity. Combining legumes with other FGs (with 150 kg ha^{-1} yr^{-1} of nitrogen) achieved a fertiliser replacement value sufficient to out-yield a *L. perenne* monoculture with 300 kg ha^{-1} yr^{-1} of nitrogen.

Acknowledgements

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Changes in sward botanical composition in a ley cutting system over four years

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Abstract

A field trial with two levels of *Trifolium pratense* L. (CLR) (10% and 22% seed weight) in four ley mixtures was conducted at Tenhult, Sweden, and changes in botanical composition were monitored over four harvesting years. Besides CLR, all mixtures contained 4-5% *Trifolium repens* L. (CLW). Two mixtures (A, B) contained *Phleum pratense* L. (TIM), *Festuca pratensis* L. (MFE) and *Lolium perenne* L. (RGE). The other two (C, D) contained *Phleum pratense* L. (TIM) and festucoid Festulolium (*Festuca arundinacea* Schreb. × *Lolium multiflorum* Lam.) (FEH). Three cuts per year were taken for four years. The botanical composition (% of harvestable biomass) was scored visually before each cut. A significant interaction was found between seed mixture, cut and year. Increased percentage of CLR in the seed mixture gave a significantly higher CLR content in all cuts, but by year 4 the CLR content was low in all swards. CLW content was low, but remained fairly constant over time, for all mixtures. With mixtures A and B, MFE increased from about 10 to 40%, but TIM had the highest content in the first cut in all years and increased to about 50% in year 4. RGE had the highest content (up to 40%) in the first two years but decreased to low levels (2-3%) in year 4. With mixtures C and D, FEH increased from about 30 to 94% from year 1 to year 4, whereas TIM was totally outcompeted after year 2.

Keywords: clover, ley, grasses, botanical composition

Introduction

The ability of different grassland species to compete in a cutting system determines the sward botanical composition after some years. The tall fescue hybrid festucoid Festulolium cultivar Hykor is a very high-yielding variety with a high leaf to stem ratio (Cernoch and Groenbaek, 2015; Ostrem *et al.*, 2013) and can therefore compete strongly against clover in mixed swards. Vliegher and D'Hose (2015) found that, on average over four years, the content of red and white clover in a cutting system was lower in a seed mixture with cv. Hykor than with perennial ryegrass. The aim of the research reported in this paper was to examine changes in botanical composition in a cutting system over four harvesting years and different seed mixtures to explore long term effects of competition. The hypothesis was that large changes in botanical composition will occur for all species over four years.

Materials and methods

A ley trial with two levels of red clover (CLR) (*Trifolium pratense* L. cv. Vicky), 10% (mixture A and C) and 22% (mixture B and D) seed weight in the mixture with four replicates, was conducted at a field site in Tenhult, Sweden (57°44'01.6'N, 14°16'41.5'E; ~230 m.a.s.l.). Four seed mixtures were tested. Besides CLR, all mixtures contained 4-5% white clover (CLW) (*Trifolium repens* L. cv. SW Ares). Two mixtures (A, B) contained timothy (TIM) (*Phleum pratense* L. cv. Switch), meadow fescue (MFE) (*Festuca pratensis* L. cv. Tored) and perennial ryegrass (RGE) (*Lolium perenne* L. cv. Kentaur). The other two mixtures (C, D) contained *Phleum pratense* L. cv. Switch) and festucoid Festulolium (FEH) (*Festuca arundinacea* Schreb. × *Lolium multiflorum* Lam. cv. Hykor). Three cuts per year were taken for four years (2014-2017). The first cut was in early June, at heading of MFE and RGE (when about half of the panicle was visible on 50% of shoots). Botanical composition (% of harvestable biomass) for different species was scored visually just before each harvest. The nitrogen application rate was 80, 50 and 40 kg ha^{-1} to the first, second and third cut, respectively. The potassium application rate was 100 and 60 kg ha^{-1} to the first and second cut, respectively. Sulphur was applied to all cuts (in total 100 kg ha^{-1}). The soil at the site is sandy loam with and organic matter content of 2.5%. The 'Mixed' procedure in SAS Version 9.3 was used for statistical analyses (Littell *et al.*, 2006).

Results and discussion

There was a significant ($P \leq 0.001$) interaction between seed mixture, cut and year for the botanical composition of CLR, CLW and TIM, but FEH had a P-value of <0.05. The species MFE and RGE, had for botanical composition a significant ($P \leq 0.05$) interaction between seed mixture and year. Two methods of repeated measures (ar(1) and cs in SAS) were tested, but did not describe the correlation structure better between cuts and years than the random effect treatment×replicate, which describe an even correlation over time. Increased content of CLR in the seed mixture gave a significantly ($P \leq 0.05$) higher content of CLR in first cut the first and second ley year when comparing mixture A with B and in first and third year when comparing mixture C with D. After year 3 there was a strong drop in sward CLR content in all mixtures. The CLW level was low in the first cut but remained fairly constant over time for all mixtures. For mixtures A and B, MFE in the first cut increased over four years from about 10 to 40%, but TIM had the highest content in the first cut in all years and increased to about 50% in year 4 (Table 1).

Table 1. Changes in botanical composition (% of harvestable biomass) of the first cut in years 1-4 for seed mixtures A-D.[1]

Seed mixture	**Harvest year**			
species	**1**	**2**	**3**	**4**
Mixture A				
CLR, 10%	15	17	18	8
CLW, 5%	2	1	2	2
MFE, 20%	11	10	26	34
RGE, 15%	38	34	21	3
TIM, 50%	35	38	34	53
Mixture B				
CLR, 22%	26	23	21	10
CLW, 5%	2	1	2	2
MFE, 17%	9	10	21	38
RGE, 13%	27	29	20	2
TIM, 43%	36	38	36	49
Mixture C				
CLR, 10%	18	18	16	2
CLW, 5%	2	1	3	2
FEH, 35%	29	41	70	94
TIM, 50%	51	40	11	2
Mixture D				
CLR, 22%	29	23	25	4
CLW, 4%	2	1	2	1
FEH, 30%	28	48	65	94
TIM, 44%	41	29	8	2

[1] CLR = red clover, CLW = white clover, MFE = meadow fescue, FEH = hybrid tall fescue, TIM = timothy and RGE = perennial ryegrass; SE of CLR = 1.9, CLW = 0.5, MFE = 1.6, RGE = 1.3, TIM = 1.8 and FEH = 2.0.

RGE had the highest content (up to 40%) in the first two years but decreased to low levels (2-3%) in the first cut in year 4. Thus RGE could not persist for more than three years under the conditions at the trial site. With mixtures C and D, FEH in the first cut increased from about 30% in year 1 to 94% in year 4, indicating that FEH is very competitive and persistent. In those mixtures, TIM was totally outcompeted after year 2, and in the first cut in year 4 its content was only 2%.

Increasing CLR proportion from 10 to 22% in seed mixtures with high-/medium-competing grasses substantially increased ($P \leq 0.05$) sward CLR proportion in the first cut the first ley year for both pairs of mixtures compared, but although high levels of CLR content year two and three, the differences was not always significant. This effect is probably positively correlated with the moderate nitrogen fertilisation rate applied in the trial. At the first cut of the 4th year, there is a larger difference in content of CLR between mixtures A and C or mixtures B and D, than between mixtures A and B or mixtures C and D.

Conclusion

Festucoid Festulolium is clearly a very high-competing and persistent species, outcompeting all other species by year 4 in mixed leys. This long-term effect is difficult to avoid. Possible solutions could be to apply very low levels of nitrogen or keep the ley for a maximum of three years. Perennial ryegrass is persistent only up to year 3 in mixed leys.

Acknowledgements

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The use of *Plantago lanceolata* to reduce nitrate leaching from the urine patch

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Abstract

In pastoral livestock production systems nitrate leaching occurs predominantly from urine patches where nitrogen concentrations are typically too high for pasture plants to take up all available nitrogen. Reducing nitrate leaching is increasingly important in New Zealand to improve both surface and ground water quality. Plantain (*Plantago lanceolata*) has the potential to reduce nitrate leaching from the urine patch through four mechanisms which reduce the nitrogen load of urine patches and reduce the rate of nitrification in soil.

Keywords: *Plantago lanceolata,* plantain, nitrate leaching, nitrification, urine patch

Introduction

Regional councils in New Zealand are placing restrictions on nitrate leaching losses from agricultural land with the aim of improving surface water quality. The predominant source of nitrate leaching in grazing systems is the urine patch, with up to 90% of the nitrogen (N) leached to ground water coming from this area of high N load (Ledgard *et al.*, 2009).

Plantain (largely cv Tonic), commercialised in New Zealand over 20 years ago (Stewart 1996), has become a successful forage plant for livestock particularly sheep, beef cattle and deer. Recently, 'diverse' pastures containing ryegrass, clovers and forage herbs (including plantain), have been shown to reduce urinary nitrogen concentration in dairy cows (Totty *et al.*, 2013). Initially, the focus was on species diversity per se rather than specific pasture species; however, further work has shown a specific role for plantain in reducing N leaching (Box *et al.*, 2016).

Plantain has the potential to reduce nitrate leaching using four mechanisms. There is increasing evidence that plantain provides some benefits to reduce urinary N concentration (Box *et al.*, 2016) and total N output (Cheng *et al.*, 2017) and to slowing nitrification (Judson *et al.*, 2018) and thus loss of nitrogen from the urine patch (Woods, 2017; Carlton *et al.*, 2018; Navarrete *et al.*, 2018). Plantain is largely a winter-dormant flat weed while the domesticated and cultivated forms range from late-heading winter-dormant types to upright winter-active forage plants. For clarity 'plantain' in this paper refers to winter-active, upright and productive forage plantains (almost exclusively the cultivars Tonic and Agritonic).

The aim of this paper is to review the evidence for the mechanisms through which plantain reduces nitrate leaching from the urine patch.

Urinary N output

The amount of N excreted in urine is proportional to the amount of N consumed in the diet although typically 50% of dietary N is excreted in urine (Luo and Kelliher, 2010). This relationship between N intake and urine excretion has been generated largely from ryegrass (*Lolium perenne*)-white clover (*Trifolium repens*) diets (Luo and Kelliher, 2010). There is increasing evidence that plantain diets partition

N away from urine (presumably to faecal material or retained by the animal) in cattle (Navarrete *et al.*, 2016, Cheng *et al.*, 2017). For example, Cheng *et al.*, 2017 reported less dietary N excreted in urine from young dairy cattle fed plantain diets (41 g N day^{-1} – 33% of dietary N) compared with ryegrass diets (65 g N day^{-1} – 54% of dietary N) despite similar daily N intake (approximately 120 g N day^{-1}). The *in vitro* fermentation experiments of Navarrete *et al.* (2016) offer some possible explanation, with significantly lower ammonia production from diets enriched with two secondary plant compounds (aucubin and verbascoside) that are commonly found in plantain.

Urinary N concentration

Livestock grazing plantain typically have a much lower concentration of urinary nitrogen than those grazing ryegrass-white clover pastures, despite similar N intakes (Box *et al.*, 2016, Cheng *et al.*, 2017). For example, Cheng *et al.*, (2017) reported urinary N concentrations less than half (0.35 vs 0.14%) for young dairy cattle consuming plantain compared with those on ryegrass. Lower urinary N concentration is an obvious consequence of a reduced dietary N partitioned to urine (as outlined above) but is also a function of increased urine volume from the presence of a diuretic (O'Connell *et al.*, 2016) and increased water intake associated with plantain being typically low in dry matter content (Cheng *et al.*, 2017). Urine-patch N loading rate is an important factor in determining nitrate leaching (Selbie *et al.*, 2015). There is, however, considerable variation amongst plantain cultivars for the potency of diuresis (Judson *et al.*, 2018).

Biological nitrification inhibitors in urine

Nitrification refers to the conversion of soil ammonium through to nitrate, with ammonia oxidising bacteria the main drivers of nitrification in soils (Di *et al.*, 2009). Reducing the rate at which this process happens has the potential to reduce nitrate leaching by allowing pasture plants a longer period to take up nitrogen before soil ammonium is converted to the readily leachable nitrate form and potentially lost to groundwater.

Urine from animals fed plantain behaves differently when applied to soil (Judson *et al.*, 2018). Urine from ryegrass-fed lambs was quickly converted to nitrate, such that at 21 days post-application almost 50% of available urinary N had been converted to nitrate compared with a much slower rate (>25% of available urinary N) for urine from lambs grazing plantain.

Biological nitrification inhibitors in the sward

Soil incubation experiments demonstrated inhibition of nitrification when aucubin was incorporated into soil (Dietz *et al.*, 2012). Further, nitrification activities were lower and nitrifying bacteria reduced 200-fold in the presence of plantain plants when given access to ammonium fertiliser (not urine) (Verhagen *et al.*, 1995). Carlton *et al.*, (2018) also recorded reductions in the abundance of ammonia oxidizing bacteria in soils containing plantain.

Systems effects

Lysimeter studies allow the effect of these four mechanisms working together to be assessed. Woods (2017) recorded a 45% reduction in urine-patch N leaching when urine from dairy cows fed perennial ryegrass-white clover was applied to Italian ryegrass (*Lolium multiflorum*) swards containing 42% plantain on a DM basis. Further, when urine derived from dairy cows grazing swards containing plantain (42%) was applied to the same swards, the reduction was 89%, compared to normal urine on ryegrass swards. In similar work, Carlton *et al.*, (2018) demonstrated a 74% reduction in nitrate leaching using swards containing 20-30% plantain.

Farm system work has begun assessing the leaching losses from hydrologically isolated paddocks (Navarrete *et al.*, 2018). This facility allows the collection of leachate through a drainage system from large areas of either ryegrass, plantain or plantain-clover mixtures under grazing by dairy cows and is a useful 'proof of concept' measurement. Early indications (Navarrete *et al.*, 2018) suggest reductions in nitrate leaching of around 50% from pure plantain on a paddock scale are possible, but continued measurement is required.

Conclusion

Regulations restricting the amount of nitrate leached from farmland are increasing in New Zealand. Up to 90% of nitrate leached from farms comes from the urine patch. There is a growing body of evidence that plantain (of the appropriate genetic background) is capable of reducing nitrate leaching by reduce N-loading of the urine patch and slowing the nitrification rate in the soil, but further farms systems work is required to confirm the effect on farm.

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Can we deduce general management recommendations from biodiversity-ecosystem functioning research in grasslands?

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Abstract

Experiments using grassland model systems have increased our theoretical understanding of linkages between biodiversity and ecosystem functioning. Nowadays, there is strong interest in using beneficial biodiversity effects for more sustainable land use. Therefore, we performed a restricted review on published papers to evaluate whether existing biodiversity-ecosystem functioning literature enables us to make recommendations on how to use plant diversity to improve grassland management for forage production. Among the screened studies, many reported positive effects of 'higher plant diversity' of mixtures on, e.g. productivity, weed suppression and further production-related functions. However, it was difficult to extract clear recommendations, as many experiments were of purely scientific design and data analysis did not directly address agronomic questions. Still, we also found studies with clear implications for grassland management, indicating that biodiversity is still an underestimated production factor. Besides intensive outreach activities to communicate these results to farmers, novel experiments should directly address agronomic questions such as the practical use and long-term maintenance of biodiversity effects for forage production.

Keywords: biodiversity, ecosystem services, productivity, mixtures, ecosystem functioning

Introduction

Many experiments have progressed our fundamental understanding of the linkage between biodiversity and ecosystem functioning, most of them in grassland-like model systems (e.g. Finn *et al.*, 2013; Weisser *et al.*, 2017). Recently, there is strong interest in utilising beneficial biodiversity effects on ecosystem functioning for more sustainable land use. Furthermore, governments and institutions have integrated the idea of mutual benefits for ecosystem services and biodiversity into their planning and decision-making.

Materials and methods

We reviewed published papers and datasets to assess whether current biodiversity-ecosystem functioning research enables us to deduce recommendations on how to use plant diversity to improve forage production. In this summary paper, we refer to a selection of papers with the aim of covering both extensive and intensive grasslands, including the two largest European biodiversity experiments in extensive grasslands (the *Jena Experiment*; Weisser *et al.*, 2017) and in intensive (temporary) grasslands (the *Agrodiversity Experiment*; Finn *et al.*, 2013).

Results

We found a number of interesting and relevant research results reporting positive effects of species mixtures on many functions and services such as productivity, weed suppression and other ecosystem services such as reduced risk of nitrate leaching (Table 1).

Despite 25 years of biodiversity-ecosystem functioning research in grasslands, it was surprisingly difficult to extract clear recommendations from the many published studies on how to improve grassland management. The reasons for this were manifold. Study results were either not directly meaningful to

Table 1. Effects of plant diversity on grassland functioning and their practical relevance in extensive and intensive grasslands. Note that all results come from either temporary or long-term sown experimental grasslands.

Biodiversity effect	**Management intensity**	**Direct practical relevance**	**Design of experiment/study**	**Comments**
Plant species richness in mixtures increases yield compared to average monoculture yield (simple overyielding)	intensive	*low*	Ten different experiments with grassland model communities with one to 64 species; management with up to 200 kg N ha^{-1} a^{-1} (Craven *et al.*, 2016; Weigelt *et al.*, 2009)	Simple overyielding of minor agronomic relevance; effect often saturates at low diversity; partly unrealistic species combinations, treatments and management
	extensive	*low*	Different experiments with grassland model communities with one to 64 species; no fertiliser addition (Cardinale *et al.*, 2012; Weigelt *et al.*, 2009)	Simple overyielding of minor agronomic relevance; partly unrealistic species combinations and treatments
Plant species richness in mixtures increases yield compared to yield of best performing cultures (transgressive overyielding)	intensive	*high*	31 replicated experiments; temporary swards with one to four species; different clover proportions; up to 450 kg N ha^{-1} a^{-1} (Finn *et al.*, 2013; Nyfeler *et al.*, 2009)	Wide agro-environmental gradient; directly applicable to high yielding temporary grasslands; biodiversity effect exceeds fertilisation effect; only four species involved
	extensive	*high*	Field trial of establishing grasslands on ex-arable sites using seed mixtures of 25-42 plant species; no fertiliser addition (Bullock *et al.*, 2007)	Results close to practice with economic assessment of the biodiversity effect; implications also for grassland restoration
Effects of plant species richness on grassland yield persist under drought conditions	intensive	*high*	Two experiments with temporary swards with one to four species; different clover proportions; up to 200 kg N ha^{-1} a^{-1}; rainout shelters (Hofer *et al.*, 2016; Finn *et al.*, 2018)	Implications meaningful for practice; importance under future climatic conditions; biodiversity benefits were larger than drought impairment; only four species involved
	extensive	*medium*	Six experiments with grassland model communities with one to 64 species; drought induced by different methods such as shelters (Craven *et al.*, 2016)	Effects on mean yield of minor agronomic relevance (simple overyielding); importance under future climatic conditions
Plant species richness reduces the abundance or biomass of unsown species (weed suppression)	intensive	*high*	31 replicated experiments; fertilised temporary swards with one to four species, different clover proportions (Connolly *et al.* 2018)	Results directly applicable to temporary grasslands; only four species; specification of weed species unclear (includes unsown but valuable species)
	extensive	*medium*	Experiment with grassland-like model communities ranging from one to 64 species; no fertiliser addition (Roscher *et al.* 2009)	Partly unrealistic composition of species; effect saturates at relatively low diversity; weed species unclear (may include unsown but valuable species)

farmers, as they have been analysed in a purely scientific way and do not report relevant information such as the effect of a mixture compared to the best monoculture (transgressive overyielding). Another problem with application to farm conditions is the sometimes quite artificial treatment of the plots (weeding) to keep the planted diversity levels. Nevertheless, a number of studies could be found where clear practice-oriented recommendations on how to utilise beneficial effects of plant diversity for grassland management could be extracted (Table 1).

Conclusion

Besides a gap between purely scientific research projects and the requirements for practical implementation, this review found consistent evidence of positive effects of functional plant diversity on forage production in both intensive and extensive grasslands. Thus, the number of functionally relevant plant species has to be acknowledged as an important pillar for sustainable forage production. On the contrary, it remains doubtful if the use of biodiversity effects can also safeguard high diversity of native plants in today's grasslands. To achieve a broad adoption of plant diversity effects in grassland production systems, we identified several requirements that should be considered such as the need for intensive outreach activities, the development of ready-to-be-used seed mixtures (e.g. Suter *et al.*, 2019) and the exploration of how the desired plant diversity can be maintained under intensive management.

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Carbon sink activity of managed grasslands: case study of three multi-treatment field sites

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Abstract

Here, we analysed changes in soil organic carbon stocks for three long-term sites (>15 years) of a national observatory in France (SOERE-ACBB). These sites each comprise a number of tested managements practices (e.g. temporary *vs* permanent grasslands, grazing, mowing, and fertilization) offering an opportunity to study effects of grassland properties (i.e. plant functional types, rooting, litter, ...) in relation to C sequestration potential, climatic-management interactions and trade-offs. C sequestration was evaluated by repeated soil sampling campaigns (3 dates, 2005-2017) to 0-60 cm depth. Furthermore, to assess the development of sequestrated C, soils were analysed in detail (1) for their soil organic matter pools (i.e. labile, passive, inert; using the Zimmermann *et al.*, 2007 method) and (2) spatial distribution of soil C stocks at field scale.

Keywords: soil organic carbon pools, soil inventory, grazing, mowing, duration of grassland management, crop rotation

Introduction

Temperate grasslands have been recognized to contribute significantly to the terrestrial C sink by sequestering C in soil organic matter. Grasslands may thus have a great potential to sequester an important amount of carbon, contributing to slow down the current rise in 'greenhouse' gases and associated effects. However, the quantification of this C sink activity has been greatly questioned, due to the uncertainty associated with those values being as important as the sink itself. Average values of 0.7 ± 0.1 t C ha^{-1} yr^{-1} have been cited in different studies (eddy covariance-technique, EC) while soil inventory measurements reported only 0.05 ± 0.3 t C ha^{-1} yr^{-1} (Soussana *et al.*, 2010). So far, soil inventories are the most direct approach to investigate C sequestration via changes in SOC, even though this method requires a large number of soil profiles, sampled over long-time scales (>5 years). Several studies have reported only small changes in SOC in the top and medium soil layers (0-30 cm) under long term management practices, suggesting that most grasslands are in equilibrium (Smith *et al.*, 2014). However, even small changes in management practices are likely to modify equilibrium state affecting both top and deeper (>30 cm) soil layers C (Weismeier *et al.* 2012). Here, we analysed C sequestration (i.e. SOC stock changes over time) for a number of grassland fields and managements options (e.g. temporary vs permanent grasslands, grazing, mowing, and fertilization) in order to assess management interactions and trade-offs.

Materials and methods

The study is based on data of the national observatory on Agro-ecosystem, Biogeochemical Cycles and Biodiversity (SOERE-ACBB) in France. The observatory comprises 3 sites, located on (1) two semi-natural permanent grassland sites (SOERE-ACBB Theix and Laqueuille, Auvergne, Central France) and (2) temporary sown grasslands sites under long and short-term crop rotation cycles (SOERE-ACBB Lusignan, Poitou-Charentes, Central France) (Figure 1). According to duration of applied management, intensity of use and fertilisation, management treatments could be classified along a gradient of disturbance-fertilisation-age. The permanent grasslands comprised 7 treatments (grazing under high/

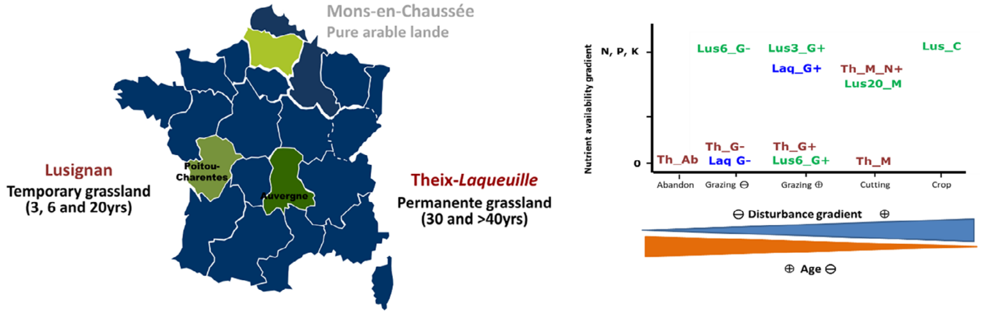

Figure 1. Map of French study sites and scheme of treatments following a gradient of nutrient availability and disturbance (duration of applied management practices) for the semi-natural permanent grassland sites Theix (Th) and Laqueuille (Laq) with abandonment, high grazing (G+), low grazing (G-), mowing (M) treatments, and the temporary sown grasslands sites Lusignan (Lus) under short-term (3 and crop), long crop rotation cycles (6 years) and permanent grassland (20 years).

low animal stocking rate, mowing with and without fertilization NPK, and abandoned). The Lusignan field experiment comprises 6 treatments (sown grasslands with 3- and 6-year life time and permanent grasslands, under mowing and grazing with and without fertilization). At each experimental site, fields were sampled three times since the setup of the management treatments, according to a spatial sampling design. To assess in more details the changes in soil C stocks, soil cores were separated into different layers and soil organic matter fractions were analysed according to the soil fractionation method (Zimmermann *et al.*, 2007).

Results and discussion

According to soil inventories, grasslands are a neutral to moderate sink of C, depending on grassland age and management practices (grazing, mowing, fertiliser supply) (Figure 2). Established grassland had a higher C sequestration potential than young grasslands, which is contradictory to the equilibrium assumptions. Likewise, at permanent long-term grasslands, management practices (intensity of biomass use) have an important role on C sequestration potential.

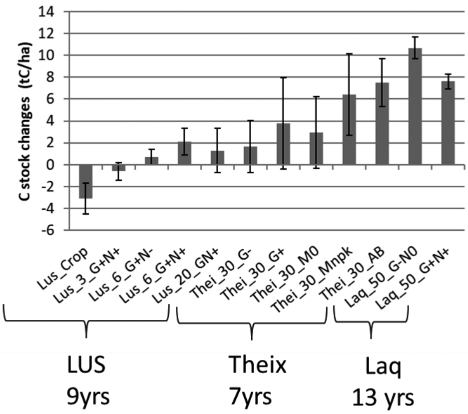

Figure 2. SOC stock changes (0-60 cm) over time (after 9 years at Lusignan, after 7 years at Theix, after 13 years at Laqueuille) for treatments following a gradient of nutrient availability and disturbance (duration of applied management practices); for the semi-natural permanent grassland sites Theix (Th) and Laqueuille (Laq) with abandonment, high grazing (G+), low grazing (G-), mowing (M) treatments; fertiliser application (zero: N0; low: N-; high: N+) for the temporary sown grasslands sites Lusignan (Lus) under long term treatments (20, 30, 50 years) and short-term crop rotation cycles (crop, 3 yrs, 6 yrs).

The spatial analyses of soil C stocks showed that grazing intensity (high/low), grazing vs mowing, and also grassland age (length grasslands in crop rotation) had a significant effect on the spatial distribution of soil carbon stocks. Higher C sequestration was shown in long-term grasslands under low grazing intensity.

In terms of vertical distribution in the soil, analyses provided evidence that C was mostly stored in deeper soil layers (20-60 cm) whereas topsoil C stocks were more vulnerable to management practices. Fractionation of soil organic matter revealed temporal changes between soil C pools and soil layers, i.e. humidified C pool declined, and inert, stable soil C increased between two inventories (5 years). Concerning the effect of grassland use and age, grazed fields had higher C sequestration rate than mown fields, which underlined the trade-off between intensity of biomass removal (i.e. removal of photosynthetic tissue, litter and reduction in root production) and C inputs for C sequestration.

Conclusion

Age and intensity of biomass use are of great importance when considering C sequestration. To draw better conclusions more long-term experiments, differing in pedo-climatic conditions and management practices, are needed to draw sound conclusions on C sequestration in managed grasslands.

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Harvesting techniques for legumes (especially leaves) as protein feed for monogastric animals

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Abstract

Leaves from legumes like alfalfa or red clover have a big potential as protein source due to their high protein and essential amino acid content. Especially for monogastric animals like pigs or poultry in organic farming, there are limited sources of essential amino acids. Hence, the aim of this study was to evaluate technical approaches for harvesting legume leaves with regard to achieving a high protein content and high yield per hectare using three different harvesting methods. The methods were applied in a field trial: (1) stripping leaves directly from the plants and harvesting the stems thereafter, or (2) and (3) harvesting the complete plant at two different dry matter (DM) contents. Results from three cuts showed that harvesting the whole plant with a DM content of about 30% gave the highest DM yields of alfalfa and red clover, with 3,803.7 kg ha^{-1} (± standard deviation 515.7) resp. 3,885.2 kg ha^{-1} (±958.8) compared with harvesting the whole plant with about 60% DM content with a DM yield of 3,086.6 kg ha^{-1} (±840.4), respectively 3,366.6 kg ha^{-1} (±969.2). The lowest DM yields with 2,519.6 kg ha^{-1} (±692.9) and 2,495.2 kg ha^{-1} (±920.2) were achieved, respectively, by harvesting leaves and stems of alfalfa and red clover separately.

Keywords: alfalfa, red clover, harvesting leaves

Introduction

Several studies have shown legumes have a large potential for supplying proteins and (essential) amino acids for monogastric animals (Sommer and Sundrum, 2015; Weltin *et al.*, 2014). Sommer and Sundrum (2015) advise a separation of stems and leaves in order to achieve a higher concentration of protein and amino acids. Dried leaves of legumes could be easily mixed into the concentrate for monogastric animals. According to Weltin *et al.* (2015), alfalfa leaves have a crude protein content up to 30% of leaf DM. Therefore, techniques for harvesting the leaves and stems separately are compared to common harvesting techniques for the whole plant. The objective of this study was to investigate the DM yield of three harvesting techniques. One technique stripped the leaves directly from the plants and harvested the stems thereafter. The other techniques harvested the complete plant at a DM content of 30 or 60% with post-harvest separation of the leaves and stems.

Materials and methods

The alfalfa (*Medicago sativa* L.) variety Plato and the red clover (*Trifolium pratense*) variety Titus were sown in August 2016 on an organically managed plot near Freising (Bavaria, Germany). One cut was taken in autumn 2016, and in spring and summer 2017 two cuts were taken before the field trial started. Therefore, the third cut of 2017 was used for the field trial at the growth stage flowering (BBCH code 65 of Federal Biological Research Centre, Federal Plant Variety Office and Chemical Industry). In 2018, the first and third cuts were harvested at the growth stage late bud (BBCH 55) which was the target growth stage for cutting. Three different harvesting techniques were applied: variant V1 harvested the leaves and stems separately. Therefore, a special prototype machine with a roller traverse to the direction of travel, with a multiplicity of metal tines actuated by a tractor, was used to strip the leaves from the plant stems. The leaves were then collected on a conveyer belt and thrown on a loader wagon by an accelerator. The harvested leaves were transported to a dumpsite for weighing. The prototype was not configured to cut the stems while stripping. Therefore, the stems were cut afterwards, windrowed and harvested in a further step. The DM yields of leaves and stems were then added together. In contrast to variant V1, the second and third harvesting techniques (V1, V2) harvested the whole plants. All variants were cut at the same time. After cutting the whole plants in variant V2, the plants were windrowed after one day to a target DM content of between 30 and 35%, harvested with a loader wagon and transported to a hot-air-drying facility. The objective for variant V3 was a DM content of 60% at harvest; therefore, completion of several steps like tedding, turning and windrowing of the material were needed within three days before the material was ready for harvesting with a loader wagon. The harvested material from V3 was then transported to a warm-air barn-drying facility. The separation of leaves and stems for the harvest material of V2 and V3 need to be done with sieving after drying and chopping. Each variant was replicated four times using a complete randomised block design. The average DM yield was calculated for each technique per hectare (kg ha^{-1} per cut). In variant V2 at the third cut in 2017 the DM content of the windrow was used to determine DM content at harvest, because data for the DM content of the harvested material in this variant were not available. Statistical analysis was conducted using R with an analysis of variance followed by a Tukey test.

Results and discussion

The DM yield of alfalfa in variant V2 was significantly higher than in V1 and V3 (Table 1). The lowest DM yields for alfalfa were achieved in V1, at 2,519.6 kg ha^{-1} (±692.9) by harvesting stems and leaves in two steps. Compared to V2 the yield of V1 was 33% lower. Shinners *et al.* (2007) also harvested leaves and stems separately and achieved a DM yield for alfalfa of 3,510 kg ha^{-1} which was about 40% higher than our results. According to Shinners *et al.* (2007) the standing fraction was cut immediately after stripping, whereas in variant V1 leaves and stems were harvested separately in two steps. The prototype stripped the leaves from the plants, and the stems were cut in a second step. During the stripping, the leaves were thrown onto a loader wagon driven next to the prototype, so stems were knocked over by the tyres of two machines, thus causing higher losses. To improve V1, and reduce DM yield losses, the stripper and mower should be used in one working step as done by Shinners *et al.* (2007). The lowest DM yields of red clover in all variants was obtained in V1, with 2,495.2 kg ha^{-1} (±920.2) which was significantly lower than the other variants which had higher yields. For alfalfa, variant V3 had lower DM yields than V2, as a result of the shatter losses that occurred in V3 because the whole plants were tedded several times compared with V2. The study of Manns *et al.* (2009) which investigated the effect of an increasing number of working steps, in addition to higher DM content, also lead to increased shatter losses in alfalfa.

Table 1. DM yield per cut for alfalfa and red clover harvested with variants V1, V2 and V3.[1]

Variant	**Alfalfa**		**Red clover**	
	DM yield per cut [kg ha^{-1}]	**SD**	**DM yield per cut [kg ha^{-1}]**	**SD**
V1	2,519.6^{a}	692.9	2,495.2^{a}	920.2
V2	3,803.7^{b}	515.7	3,885.2^{b}	958.8
V3	3,086.6^{c}	840.4	3,366.6^{b}	969.2
P-value	<0.05		<0.05	

[1] Data shown are average values of 3 cuts in 2 years. SD: standard deviation. Means followed by the same letter showed no significant difference at $P<0.05$.

Conclusion

The highest DM yields for alfalfa and red clover were obtained for variant V2. The stripping technique for leaves had a higher potential because harvesting of more stems is possible. For alfalfa, variant 3 (whole plant) resulted in lower DM yields because of the higher shatter losses relative to variant V2.

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Water use efficiency in forage grassland systems is driven by species diversity and identity

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Abstract

In the context of climate change, improving water use efficiency (WUE) is an important objective for forage grassland systems. It is still controversial whether enhancing diversity of forage plant species could be an appropriate method and there is only limited information available on the effect of identity of forage plant species. Therefore, a two-site field experiment was carried out with *Lolium perenne* grown in mixture with *Trifolium repens* or *Cichorium intybus* and with monocultures of all three species. We measured soil water content, precipitation amount and dry matter yield to calculate WUE for two harvests in two years. WUE of the corresponding mixtures did not exceed WUE of the *C. intybus* monocultures in either year, or of the *T. repens* monocultures in the first year. These findings provide evidence that not only species diversity, but also identity drive WUE in forage grassland systems.

Keywords: forage grassland system, species diversity, species identity, water use efficiency

Introduction

Climate change will be a challenge for current forage grassland systems (Olesen *et al.*, 2011). Hence, increasing the biomass production while maintaining or reducing the water amount used, i.e. enhancing water use efficiency (WUE), is of major interest. Combining forage plant species of different functional groups and with varying functional traits could play a key role in this regard (Hofer *et al.*, 2016). To investigate the impact of species diversity and identity on WUE, a field experiment was conducted with a shallow-rooted non-legume (*Lolium perenne*) grown in mixture with a shallow-rooted legume (*Trifolium repens*) or a deep-rooted non-legume (*Cichorium intybus*) and with the corresponding monocultures. Due to supposed complementarity in resource use we expected WUE of both mixed stands to exceed WUE of corresponding pure stands. We further assumed that WUE differs between species of different functional groups and with varying functional traits, both in pure and mixed stands.

Materials and methods

The ongoing experiment, established in 2014, is located at two contrasting sites near Goettingen in Lower Saxony, Germany. The mixtures of *L. perenne* with *T. repens* or *C. intybus* (600:400 seeds m^{-2}) and the monocultures of all species (1000 seeds m^{-2}) were grown in plots (3×5 m) randomized in blocks with four replications. No fertilizer was applied. In 2016 and 2017, aboveground biomass was harvested four times per year. Subsamples were dried and weighed to determine dry matter yield (DMY). Once a week, volumetric soil water content was measured to a depth of 60 cm by means of a permanently installed access tube and a portable probe (Diviner 2000, Sentek Pty Ltd, Stepney, Australia) and interpolated to the day of harvest. Differences of soil water content between second (June) and first (May) and between third (August) and second harvest (ΔSWS) were assessed. Precipitation amounts (P) were also recorded at both sites to determine water consumption (WC) according to a simplified soil water balance equation (Zhang *et al.*, 2004) as P – ΔSWS. Thus, WC includes both transpiration of the vegetation and evaporation of the soil depending on the vegetation. Finally, we calculated WUE for the second and third harvest as DMY/WC. The statistical analyses of WUE, WC and DMY were performed separately for pure and mixed stands of *L. perenne* and *T. repens* or *C. intybus*. Linear mixed effects models were fit using the R 3.5.1 (R

Foundation for Statistical Computing, Vienna) package 'nlme' (Pinheiro *et al.*, 2018). Due to the nested experimental design, stand, harvest, site, year and their interactions were included as fixed effects, and plot nested in block as random effects. To meet assumptions of homoscedasticity and normality of residuals, square-root transformations and variance structure functions were used. Minimum adequate models were determined based on the second order Akaike Information Criterion. Testing whether the effects of the final model were significant was done with Wald chi-square tests. Where significant effects were found, Tukey post-hoc tests followed to identify significant differences between means.

Results and discussion

For pure and mixed stands of *L. perenne* and *C. intybus*, WUE depended on stand type consistently across years ($P<0.001$). By contrast, for pure and mixed stands of *L. perenne* and *T. repens*, WUE of the different stand types varied between the two years (stand × year interaction: $P<0.001$, Table 1).

Contrary to our expectation, in either year, WUE of *L. perenne* and *C. intybus* mixtures only exceeded that of *L. perenne* monocultures, but equalled that of *C. intybus* monocultures (Figure 1). Regarding *L. perenne* and *T. repens*, the mixtures used water more efficiently than *L. perenne* monocultures and as efficiently as *T. repens* monocultures in 2016, while in 2017 mixtures outperformed both corresponding monocultures. The results mainly reflect the DMY, as WC showed no (*L. perenne* and *C. intybus*) or only minor (*L. perenne* and *T. repens*) variation between stand types (Figure 1, Table 1). We hypothesise that the differences in DMY and thus WUE were derived from differences in plant-available nitrogen (N). In contrast to *L. perenne*, both partner species had access to additional N sources: *T. repens* as a legume to atmospheric N_2 via symbiotic fixation and *C. intybus* to N from deeper soil layers via deep roots. These hypotheses are largely in line with findings of Hofer *et al.* (2017).

Table 1. *P*-values for main and interaction effects on water use efficiency (WUE), water consumption (WC) and dry matter yield (DMY) for pure and mixed stands of *Lolium perenne* and (a) *Trifolium repens* or (b) *Cichorium intybus*.

Effect	***P*-value**					
	WUE		**WC**		**DMY**	
	a	**b**	**a**	**b**	**a**	**b**
stand	<0.001	<0.001	0.038	-	<0.001	<0.001
harvest	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
site	0.024	0.003	0.471	0.034	-	0.039
year	0.089	-	0.002	<0.001	0.943	<0.001
stand × harvest	-	-	-	-	-	<0.001
stand × year	<0.001	-	-	-	-	-
harvest × site	-	0.018	<0.001	<0.001	-	-
harvest × year	<0.001	-	0.001	0.007	<0.001	-
site × year	-	-	-	-	-	0.009

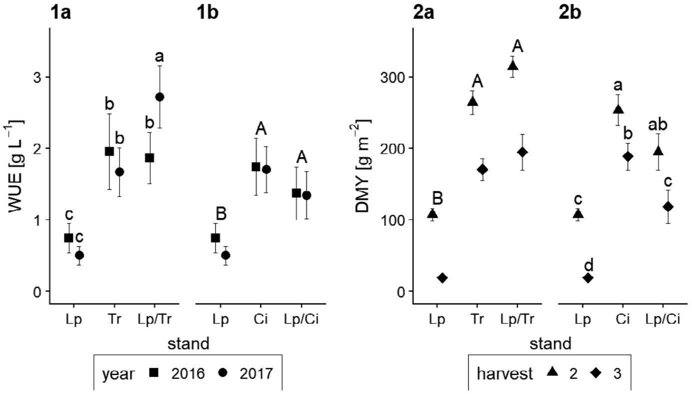

Figure 1. Means and standard errors of (1) water use efficiency (WUE) and (2) dry matter yield (DMY) for pure and mixed stands of *Lolium perenne* (Lp) and (a) *Trifolium repens* (Tr) or (b) *Cichorium intybus* (Ci). Different lowercase letters signify significant differences (P<0.05) between means per year or harvest, different uppercase letters between means across years or harvests.

Conclusion

The results indicate that WUE in forage grassland systems is not only driven by species diversity, but also by species identity. In addition, in our experiment it is possible that nitrogen and not water was the primary limiting resource for DMY and thus WUE.

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Antioxidant activity, phenol and flavonoid concentrations of different red clover extracts

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Abstract

The paper presents the results of examination of 10 populations of red clover (*Trifolium pratense* L.) originating from natural and semi-natural grasslands of Serbia. The field experiment was conducted at the Institute for forage crops, Kruševac, over three years (2009-2011). Plants from the third year of the experiment were used for analyses of the composition of bioactive components and antioxidant activities. Methanol extracts were extracted from the above-ground parts of full-grown plants. Antioxidative activity, total phenolic content and flavonoid concentrations were determined by spectrophotometric method. Antioxidant activity, expressed as IC_{50} values, ranged from 152 μg ml^{-1} (CS118) to 656 μg ml^{-1} (TA055). Results showed that total concentration of phenols ranged from 31.4 mg GA g^{-1} (TA055) to 104.8 mg GA g^{-1} (RA100), while flavonoids concentrations ranged from 50.3 mg Ru g^{-1} (RA123) to 112.4 mg Ru g^{-1} (CS108). Further work would lead to the determination of the optimal concentration of bioactive components in the red clover breeding material. The use of such genotypes would have a significant impact on the animal health and diet, and indirectly, on humans.

Keywords: *Trifolium pratense*, phenols, flavonoids, natural populations, animal health

Introduction

Red clover is a widespread perennial forage legume and a very important component of grasslands in temperate regions (Herrmann *et al.*, 2008). Red clover fodder has a high nutritional value and is rich in minerals, vitamins and secondary metabolites (Tucak *et al.*, 2018). The presence of secondary metabolites such as polyphenols or tannins has a significant impact on sustainable livestock production (Mueller-Harvey, 2013) and animal health. High quality food is a key factor in the prevention of various diseases. In the food industry today an effort is being made to improve the healthiness of food (Decker and Park, 2010). Products obtained from healthy animals are a good source of bioactive compounds with positive effect on human health (Mortensen *et al.,* 2009; Pogorzelska-Nowicka *et al.*, 2018).

In order to obtain high quality varieties of red clover with an improved profile of bioactive components, 10 populations were examined in field and laboratory conditions. The paper presents results of the antioxidant activity of isolated methanol extracts, the concentrations of total phenols and flavonoids.

Materials and methods

The examined material is part of the seed collection of the Institute of Forage Crops, Kruševac. Population code, latitude, longitude, elevation as well as a detailed design of a more comprehensive experiment are already published (Petrović *et al.,* 2014). In order to break the dormancy, seeds were scarified with sand paper. After that the seeds were sown in plant pots to germinate. When plants reached the growth stage of 3-4 mature leaves, they were transferred to the nursery on the property of the Institute for Forage Crops, Kruševac (Globoder) (43°34’55’ N, 21°34’8’ E; altitude 150 m). For the purposes of these analyses, plants in the third year of the experiment were used. Three bulk samples were formed from each population. Samples consisted of above ground parts of 3-5 fully developed single plants. The plants were air-dried in the shade for 7 days, at a temperature of 18-22 °C and humidity of 55-65%. The dried material was then ground and placed in dark stained glass jars. Extraction of bioactive compounds was performed with methanol. The concentrations of total phenols and flavonoids were determined by spectrophotometric methods (Singleton *et al.,*1999; Quettier *et al.,* 2000). The ability of the plant extract and reference substance to scavenge DPPH+ free radicals was assessed by using the method adopted with suitable modifications of Kumarasamy *et al.* (2007). One-way analysis of variance (ANOVA) was used to examine differences in mean values among populations for each chemical trait and antioxidant activity. Tests of significance were calculated with post-hoc tests of Bonferroni at $P<0.05$.

Results and discussion

There was a statistically significant difference between the populations for all observed traits. The results showed that the level of antioxidant activity ranged from 152 mg ml^{-1} (RA100, CS118) to 656 mg ml^{-1} (TA055). Populations originating from the locations with an altitude exceeding 1000 m a.s.l. (CS118, CS108, RA100) had the most pronounced ability to scavenge DPPH+ free radicals. The concentration of total phenols, expressed as gallic acid equivalent in the examined extracts of red clover populations, ranged from 31.4 mg GA g^{-1} (TA055) to 104.8 mg GA g^{-1} (RA100). The lowest flavonoid concentration, expressed as the equivalent of rutin, was measured in the RA123 population (50.3 mg Ru g^{-1}) while the highest value was measured in the CS108 population (112.4 mg Ru g^{-1}). Heightened concentrations of secondary metabolites and antioxidant response in plants occur most frequently in stressful environmental conditions (Vaseva *et al.*, 2012; Sivesin and Seguin, 2005). Populations collected in the area above 1000 m a.s.l. (with typical features of the mountain climate) had higher concentrations of secondary metabolites and increased antioxidant activity. However, green matter yield of these populations was low (Petrović *et al.,* 2014).

On the other hand, populations from eastern Serbia (ES042, ES043, ES077) with an average concentration of secondary metabolites and average antioxidant activity (Table 1) were characterized by sufficiently high yield and quality of dry matter (Petrović *et al.,* 2014).

Populations from eastern Serbia represent potentially valuable breeding material. However, in order to obtain varieties that may be of use for improving animal health and thereby potentially reducing the need for drug use, it is necessary to conduct further analyses on the examined material of the red clover.

Table 1. Concentration of phenols, flavonoides, antioxidant activity and standard deviation of ten *Trifolium pratense* populations.[1]

Population	**Total phenolic content (mg GA g^{-1} of extract)**	**Flavonoid concentration (mg Ru g^{-1} of extract)**	**Antioxidant activity[2] (µg ml^{-1})**
ES042	74.8±3.58 bc	79.5±5.52 cd	157±0.83 ab
ES043	68.0±3.60 c	93.8±4.85 b	213±7.72 f
ES077	95.2±4.86 a	80.7±5.07 bc	184±6.62 cd
RA089	81.5±0.94 b	88.2±4.65 bc	208±7.37 ef
RA100	104.8±4.26 a	75.1±4.54 cd	152±3.01 a
RA123	51.8±3.35 e	50.3±2.90 f	260±0.89 g
TA055	31.4±4.70 f	51.7±5.33 ef	656±8.07 h
TA097	62.3±4.43 de	65.5±4.56 de	195±4.14 de
CS108	96.1±3.05 a	112.4±4.81 a	172±3.17 bc
CS118	78.7±3.45 bc	59.1±1.50 ef	152±1.63 a
average	74.5	75.6	235

[1] Means followed by a common letter are not significantly different according to Bonferroni test at $P<0.05$ level.

[2] Antioxidant activity is higher as values are lower.

Conclusion

Analysis of secondary metabolites and antioxidant activity of ten red clover populations showed variability of the examined traits: total phenolic content, flavonoid concentration and antioxidant activity. Based on the obtained results we conclude that the red clover populations from eastern Serbia are suitable for further breeding processes. It is necessary to conduct a detailed screening of secondary metabolites and breading procedures in order to increase the content of antioxidants.

Acknowledgements

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Changes in root traits of forage mixtures through successive defoliations and with different N sources

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Abstract

Few studies have monitored the *in situ* seasonal rooting patterns of forage mixtures. In a field experiment, we investigated the changes in root traits of perennial forage mixtures through successive defoliations and with different N sources. A second-year mixture of timothy (*Phleum pratense* L.) and tall fescue (*Festuca arundinacea* Schreb.) was fertilized with either calcium ammonium nitrate or cattle slurry at a rate of 150 kg available N ha^{-1}. As a third treatment, lucerne (*Medicago sativa* L.) was grown with these two grasses, without N fertilization. Root length and diameter were monitored weekly with a minirhizotron camera to a 60-cm depth. Root diameter decreased from first to third defoliation (-22%) in all treatments, while root length increased from first to third defoliation for the grass-only mixture and between the second and third defoliation for the grass-lucerne mixture. The net root elongation rate was 83% greater in spring than after any defoliation and it became negative in autumn as root disappearance exceeded root growth. The N source did not affect root traits in the grass-only mixture. Lucerne in the grass mixture resulted in lower values of net root elongation rate early in the season but greater values later on.

Keywords: rooting patterns, minirhizotron, forage mixture, root length, defoliation, N source

Introduction

Few studies have characterized *in situ* the rooting patterns of crops, particularly those of multi-species forage mixtures. Root traits of perennial forage species are known to express seasonal patterns (Reid *et al.*, 2015; Chen *et al.*, 2016) and to be affected by N fertilization (Skinner and Comas, 2010). Defoliation may also alter root growth dynamics (Skinner and Comas, 2010; Reid *et al.*, 2015; Chen *et al.*, 2016). However, the influence of fertilization and defoliation is still poorly understood, especially in forage mixtures. We investigated the *in situ* changes in root traits of perennial forage mixtures during the growing season through successive defoliations and with different N sources.

Materials and methods

An experiment was established in 2016 at Saint-Augustin-de-Desmaures (Quebec, Canada, Lat. 46°44'N; Long. 71°31'W) on a silty clay soil. In a randomized complete block design with three replications, a mixture of timothy (*Phleum pratense* L.) and tall fescue (*Festuca arundinacea* Schreb.) was fertilized in 2017 with either calcic ammonium nitrate or dairy cattle slurry at rates of 90 and 60 kg available N ha^{-1} in spring and after the first defoliation, respectively. As a third treatment, lucerne (*Medicago sativa* L.) was grown with these two grasses, without N fertilization. All mixtures were defoliated three times in 2017 when tall fescue reached the late boot/early heading stage at the first defoliation and with an interval of approximately 35 days between the following defoliations. Root traits were monitored with a minirhizotron camera (BTC-100X video-camera, Bartz Technology Corporation, CA, USA) in three transparent tubes per plot inserted into the soil at a 30°-angle to the vertical. Root images were taken from the soil surface to a 60-cm depth every week from May to September, and every other week in October and November. Images were analysed using the WinRhizoTRON MF 2018a software (Regent Instruments, QC, Canada). The growing season was split into five periods: spring, post defoliations 1, 2 and 3, and autumn. The length of live roots (hereafter root length) and root diameter at the time of each defoliation were subjected to an ANOVA using the MIXED procedure in SAS (SAS 9.4, 2013) and the daily net root elongation rate was calculated for each period from a piecewise regression analysis in SAS. Differences were considered significant at $P \leq 0.05$.

Results and discussion

Root length increased from first to third defoliation for the grass-only mixture but only between second and third defoliation for the grass mixture with lucerne (Figure 1A). Root length was not affected by the N source except at the second defoliation when the grass-only mixture had a greater root length than the grass mixture with lucerne (Figure 1A). This generally greater root length of the grass-only mixture at second and third defoliation compared to the grass mixture with lucerne could be due to greater N stress (N nutrition index of 0.7 and 0.3 at second and third defoliation, respectively) that favoured a larger resource investment in roots than in shoots at the end of the growing season (Skinner and Comas, 2010). Root diameter was not affected by the N source and it decreased by 22% from first to third defoliation as a result of the growth of finer roots as the growing season progressed (Figure 1B).

The net root elongation rate was 83% greater in spring than after any defoliation in all treatments (Figure 2). Defoliation is known to reduce root growth in favour of vegetative regrowth (Reid *et al.*, 2015). The specific effect of defoliation, however, is difficult to isolate from that of development stages or seasonal variation (Chen *et al.*, 2016). The net root elongation rate became negative in autumn as root disappearance exceeded root growth (Figure 2). In the grass-only mixture, the N source did not affect the net root elongation rate in any period. However, lucerne in the grass mixture affected the root growth dynamics during the growing season as indicated by generally lower values of net root elongation rate than with the grass-only mixture in the first two periods and greater values in the last three periods. This could be due to the intrinsic morphological differences between the grass fibrous root system and tap-rooted lucerne, with lucerne investing more in a taproot at the expense of lateral finer roots at the beginning of the growing season. Moreover, because thick roots are known to have a longer longevity (Reid *et al.*, 2015), this could contribute to explain the smallest declining rate of root elongation for the grass-lucerne mixture in autumn.

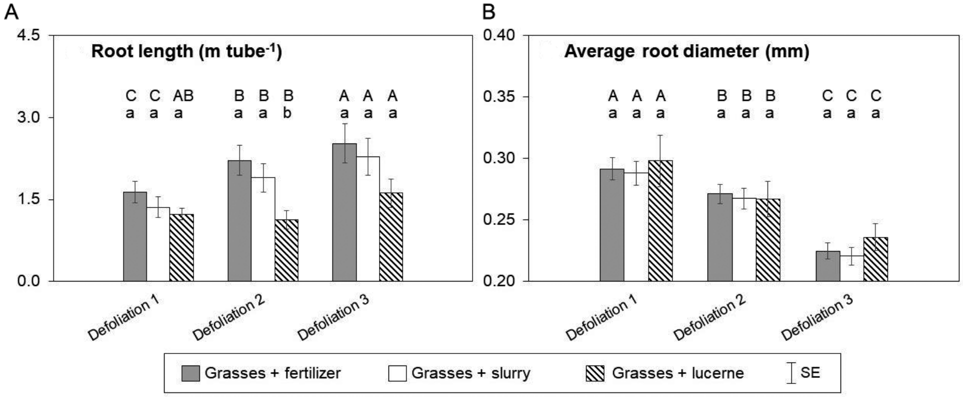

Figure 1. Length of live roots (A) and average root diameter (B) at defoliations 1, 2, and 3 in 2017 for the grass mixture fertilized with calcic ammonium nitrate (grasses + fertilizer) or with dairy cattle slurry (grasses + slurry) and for the grass mixture with lucerne without N fertilization (grasses + lucerne). Different letters indicate a significant difference at the 5% level between defoliations for the same treatment (uppercase letters) and among treatments within each defoliation (lowercase letters).

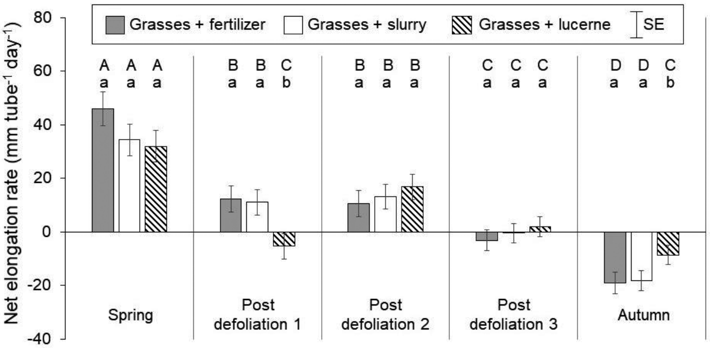

Figure 2. Daily net root elongation rate for five periods of the 2017 growing season for the grass mixture fertilized with calcic ammonium nitrate (grasses + fertilizer) or with dairy cattle slurry (grasses + slurry) and for the grass mixture with lucerne without N fertilization (grasses + lucerne). Different letters indicate a significant difference at the 5% level between periods for the same treatment (uppercase letters) and among treatments within each period (lowercase letters).

Conclusion

Root length generally increased through successive defoliations during a growing season, while root diameter decreased. The source of N fertilizer applied on the grass-only mixture did not affect any of the root traits, while the presence of lucerne in the grass mixture affected the root growth dynamics during the growing season with lower values of net elongation rate than the grass-only mixture early in the season but greater values later in the season. Research is ongoing to use our data to estimate root turnover in an effort to obtain better insights into root contribution to soil organic carbon.

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Temporal synchrony of white clover populations with perennial ryegrass affects yield and yield stability of grass-clover mixtures

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Abstract

Forage plant mixtures are known to increase dry matter yields over monocultures but may also improve yield stability within years. Populations whose seasonal growth pattern differs from that of their mixture partner can be expected to show complementary resource use which may increase overyielding and reduce temporal yield variability. We grew *L. perenne* pure stands and eight populations of *Trifolium repens* either in pure stands or in mixture with *Lolium perenne* over three years at three sites. Seasonal yield synchrony between *T. repens* and *L. perenne* pure stand yields did not differ between *T. repens* populations. Increasing pure stand yield synchrony decreased overyielding and increased yield variability of mixed stands. While synchrony was not affected by *T. repens* population, our findings indicate that pure stand seasonal yield distribution can be a criterion for selecting compatible species populations for forage crop mixtures.

Keywords: asynchrony, genetic variation, clover, grass, mixed cropping

Introduction

Forage plants frequently show higher yields when grown in mixed rather than in pure stands, which can at least partly be attributed to complementarity in resource use between partner species. Specifically, asynchronous seasonal biomass production has been related to increased yield (Husse *et al.*, 2016) and yield stability (Prieto *et al.*, 2015) of forage plant mixtures. Temporal synchrony could be decreased by selecting populations that maximise differences in phenology between the partner species (Litrico and Violle, 2015).

To evaluate this option for increasing mixture performance, we grew eight populations of *Trifolium repens* in pure stands and in mixed stands with *Lolium perenne* over three years at three sites. We expected synchrony in biomass production with *L. perenne* to differ between *T. repens* populations, and to be unrelated to these populations' pure stand yield. We further expected that increasing synchrony would decrease overyielding and increase yield variability of mixed stands.

Materials and methods

Eight populations of *T. repens* from DSV (Deutsche Saatveredelung) and one population of *L. perenne* were sown in pure stands and in two-species mixed stands at three sites in Lower Saxony, Germany, with four replicate blocks at each site. Stands were established on plots of 3×5 m in summer 2014 (sites 1 and 2) or in spring 2015 (site 3). From 2015 to 2017, they received four annual cuts (five cuts in 2015 at site 3). Dry matter yield was measured for each cut. For mixed stand plots, we determined transgressive overyielding as difference between the yield of the mixed stand and that of the best corresponding pure stand (*T. repens* for all sites and years), relative to that pure stand's yield. Additionally, we quantified within-year yield variability of mixed stand plots as the coefficient of variation of each year's four (five) harvest yields. To determine within-year synchrony between *T. repens* and *L. perenne*, we calculated a synchrony index (Φ) adapted from Loreau and de Mazancourt (2008) to pure stand yield data as: $\Phi = \mathrm{var}(y_{Tr} + y_{Lp}) / (\mathrm{sd}(y_{Tr}) + \mathrm{sd}(y_{Lp}))^2$, where y_{Tr} and y_{Lp} are the dry matter yields of single annual cuts of *T. repens* and *L. perenne* in pure stands, respectively. Φ varies between zero (no synchrony) and one (maximum synchrony).

We used linear mixed effects models as implemented in the *nlme* package of the R software environment (The R Foundation for Statistical Computing, Vienna) for two data analysis steps. First, we modelled *T. repens* pure stand yield, logit(Φ), mixed stand transgressive overyielding and mixed stand yield stability as a function of *T. repens* population, year, site and their interactions, with experimental block as a random effect. Next, we modelled *T. repens* pure stand yield, mixed stand transgressive overyielding and mixed stand yield stability as a function of Φ, site, year and site × year. Both intercept and slope of Φ were permitted to vary randomly for each combination of year, site and block. In all cases, the minimum adequate model was identified based on the second-order Akaike Information Criterion. A marginal pseudo-R^2 (R_m^2), incorporating fixed, but not random effects, was calculated for each final model.

Results and discussion

Population, in interaction with site, affected *T. repens* pure stand yield as well as within-year yield variability of mixed stands, but was unrelated to Φ or to mixed-stand transgressive overyielding (Table 1). Transgressive overyielding was highest at site 3, which also had the highest *T. repens* monoculture yields, and increased with experimental duration across the three sites (Figures 1A, B). Site and year interactively affected Φ, but together explained only a small part of the observed variability in Φ (Table 1). No significant relationship between Φ and pure stand yield of *T. repens* could be found (P=0.79, model R_m^2=0.79; Figure 1A). By contrast, transgressive overyielding decreased with increasing Φ (P=0.003, model R_m^2=0.31; Figure 1B). Finally, mixed-stand within-year yield variability was significantly (P<0.0001) and positively related to Φ (R_m^2= 0.60; Figure 1C).

Within-year synchrony between *T. repens* and *L. perenne* varied considerably between sites and years, and showed pronounced variability that could not be explained by the experimental design, as shown by the small R_m^2 of the fitted model. This presents a challenge for breeding efforts to realise the potential increases in mixture performance through decreased temporal synchrony that our results indicate.

Table 1. *P* values and pseudo-R^2 (R_m^2) of single and interactive effects of *Trifolium repens* (Tr) population, year and site on Tr dry matter yield in pure stands, on within-year synchrony between dry matter yields of Tr and *Lolium perenne* (Lp) grown in monoculture (Φ), and on transgressive overyielding and within-year yield variability of a Tr-Lp mixture.

Effect	Pure-stand yield Tr (kg ha^{-1} a^{-1})	Φ	Mixture transgressive overyielding	Mixed-stand within-year yield variability
Population	0.0003	not in final model	not in final model	0.0045
Year	<0.0001	<0.0001	<0.0001	<0.0001
Site	<0.0001	0.0007	0.0004	0.0001
Population × site	<0.0001	not in final model	not in final model	0.0001
Year × site	<0.0001	<0.0001	not in final model	<0.0001
R_m^2	0.81	0.21	0.23	0.60

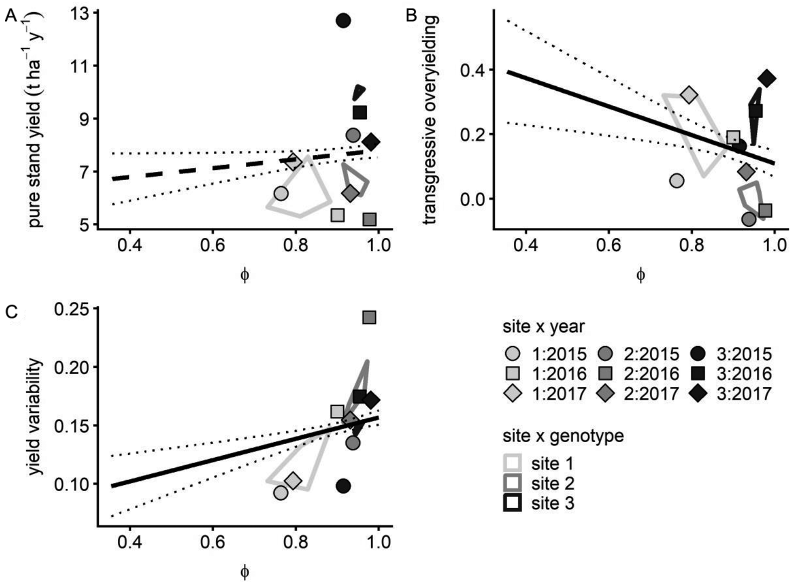

Figure 1. Relationship between within-year synchrony (Φ) of pure stand yields of *Trifolium repens* (Tr) and *Lolium perenne* and (A) Tr pure stand yield, (B) mixed stand transgressive overyielding and (C) mixed stand within-year yield variability. Symbols: means per site and year across Tr populations; convex hulls: range of population means per site across years; black lines: model prediction (solid: significant, dashed: non-significant effect) with 0.95 confidence intervals (dotted).

Conclusion

Both mixed stand yield and yield stability increased with decreasing temporal synchrony of *T. repens* with its partner species *L. perenne*. Seasonal compatibility, however, was highly dependent on environment and did not differ between the investigated populations of *T. repens*. It is therefore doubtful if genetic variability of seasonal growth patterns within *T. repens* can be used to increase the performance of *T. repens-L. perenne* mixtures.

Acknowledgements

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Lucerne (*Medicago sativa* L.): persistence in intensive mowing systems

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Abstract

Lucerne (alfalfa) is being reintroduced on several Belgian dairy farms. However, lucerne can be rapidly exhausted under the intensive Belgian mowing system. We investigated (A) mixing lucerne with white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.), and (B) an extensive cutting regime in a lucerne monoculture. The importance of seed inoculation with *Rhizobium meliloti* was also investigated. In trial A, lucerne monoculture was compared with lucerne+perennial ryegrass, lucerne+white clover and non-inoculated lucerne on small field trial plots for botanical composition and crop yield. Trial A was established in spring 2015 and again in spring 2016 (both 2-year trials). Trial B compared regimes of 3 cuts/year and 4 cuts/year in a stand-alone stand of lucerne under practical field conditions with conventional machinery for crop yield, nutritive value and persistence. Trial B was established in spring 2015 and lasted 3 years. The field trials revealed that perennial ryegrass or white clover are good as companion species with lucerne in wet weather conditions during and after sowing the crop. The 4 cuts/year regime is preferable, as higher crop yield, nutritive value of the fresh matter and persistence were achieved compared with a 3 cuts/year regime. Seed inoculation is absolutely necessary on sites where there have been decades of absence of lucerne. Without inoculation, crop yield losses of up to 50% were observed in the first year.

Keywords: cutting intensity, botanical composition, crop yield, seed inoculation

Introduction

Lucerne is becoming more popular on Belgian dairy farms as a highly palatable crop for cattle. It has considerable drought resistance and potentially high yields in intensively mown grassland, but its crop persistence is relatively low. Lucerne can be exhausted by intensive mowing and frequent passage of heavy machinery. The farmer advisory board usually advises 4 cuts per year. However, most farmers have several parcels of land with perennial ryegrass under a 5 (-6) cuts regime and, for practical reasons, the mowing regime coincides for every parcel in the 5-cut system. This leads to rapid exhaustion of the lucerne. Mixing the lucerne sward with perennial ryegrass and/or white clover is a possible solution to extend the lifetime of the sward. Both species are more resistant to frequent cuts and passage of machinery and can fill gaps in the sward where lucerne disappears. On more extensive farms with 4 cuts per year, the cutting regime is sometimes further reduced to 3 cuts due to the requirements of agro-environmental programmes. The effect of number of cuts per year on nutritive value and persistence of the lucerne is a point of intensive discussion. Lucerne seed inoculation with *Rhizobium meliloti* is advised because the crop is usually introduced after having been absent for decades. Nevertheless, farmers question whether this is necessary. The objectives of this study were to determine: (1) if mixtures with white clover or perennial ryegrass can improve the crop yield in an intensive mowing system with 5 cuts, (2) if extensification (3 instead of 4 cuts) of stand-alone lucerne improves the persistence, and (3) if inoculation of lucerne seeds is profitable.

Materials and methods

Two field trials (A and B) with lucerne (var. Arpege) were carried out in Merelbeke (Belgium) next to each other on the same field (sandy loam; $pH_{KCl} = 6.0$; 1.4% C). Trial A.1 was sown in April 2015, following a randomized complete block design with 4 treatments in 3 blocks: LU (lucerne 25 kg ha^{-1}), LU+WCL (lucerne 25 kg ha^{-1} + white clover 3 kg ha^{-1}), LU+PR (lucerne 12.5 kg ha^{-1} + perennial ryegrass 20 kg ha^{-1}) and LU- (non-inoculated lucerne 25 kg ha^{-1}). Except for LU-, all lucerne seeds were inoculated with *Rhizobium meliloti*. K fertilization was 66 kg K ha^{-1} and 200 kg K ha^{-1} in the sowing year and the second year, respectively. LU+PR received 120 kg N ha^{-1} in the second year while the other treatments received no N fertilizer. Field plots of 6×1.5 m were harvested with a Haldrup forage harvester at a cutting height of 5 cm. Dry matter (DM) crop yields of each replicate were calculated after drying the subsample in a forced-draft oven at 80 °C for 24 hours. To determine the botanical composition, a grab subsample per plot was separated into the individual sown species. Trial A.2 was an exact copy of trial A.1 but started in April 2016. Both A.1 and A.2 were cut three times in the year of sowing and five times the year after. Trial B (1 ha) was sown in April 2015 with 25 kg lucerne ha^{-1} and cut three times. In 2016 and 2017, the field was split into strips with a 3-cut regime and a 4-cut regime. Conventional machinery was used for all tasks. Crop yield was determined in 3-fold for every cut and samples from every cut were ensiled in small containers for 12 weeks. Both samples from the fresh matter and the silage product were analysed by NIRS to determine nutritional parameters. Fodder Unit milk was calculated following De Boever *et al.* (1988). In March 2018, the persistence of lucerne was assessed by determining the surface coverage with plants (lucerne + unsown species) using image analyses and counting of the number of plants and shoots per m^2 in 8 replications per strip.

Results and discussion

In trial A.1, the highest crop yield in the year of sowing was achieved in the stand-alone lucerne (LU; Table 1). The combination with white clover or perennial ryegrass had small negative effects of crop yield. In trial A.2 it was the opposite. Mixing lucerne with white clover led to significantly higher crop yields in the year of sowing. This can be explained by the detrimental conditions when sowing lucerne in the very wet conditions during May and June 2016 (200 to 250% of normal rainfall in both months). This indicates that white clover and perennial ryegrass can compensate for lucerne under wet establishment conditions. Poor establishment of lucerne in 2016 was also revealed by its low proportion in the swards of LU+PR and LU+WC. Regardless of the very wet spring of 2016, LU, LU+WC and LU+PR achieved very high crop yields in the second year of trial A.1. Although the establishment of lucerne was difficult in A.2, the proportion of lucerne gradually increased in the following year. LU and LU+WC showed significantly higher crop yields than LU+PR. No inoculation of lucerne (LU-) resulted in lower crop yields, in both trials and both years. In the year of establishment of A.1, there was a crop yield loss of more than 50%. In both trials A.1 and A.2 the difference in crop yields between LU and LU- became smaller in the second year, and in A.2 there was no longer any significant difference.

Table 1. Field trial A, annual mean crop yield (n=3) and botanical composition per cut of the sward.[1]

		Year of establishment				2nd Year					
		Crop yield	% lucerne in sward – cut no.			Crop yield	% lucerne in sward – cut no.				
		(Mg ha^{-1} yr^{-1})	1	2	3	(Mg ha^{-1} yr^{-1})	1	2	3	4	5
Trial A.1 2015	LU	11.1^{d}	96	100	100	19.7^{b}	100	100	100	ND	ND
	LU+PR	10.1^{c}	73	86	65	21.5^{c}	75	65	87	ND	ND
	LU+WC	9.5^{b}	94	100	99	20.6^{bc}	98	96	98	ND	ND
	LU-	5.4^{a}	85	99	100	19.0^{a}	100	100	100	ND	ND
Trial A.2 2016	LU	7.3^{b}	90	100	100	18.8^{b}	100	100	100	100	100
	LU+PR	12.1^{c}	35	50	91	16.8^{a}	71	81	99	98	83
	LU+WC	12.2^{c}	22	49	78	18.3^{b}	73	82	97	99	97
	LU-	6.3^{a}	85	98	100	17.9^{b}	100	100	100	100	100

[1] Statistically significant differences in crop yield (Tukey $P<0.05$) are indicated with different letters. ND = not determined.

The 4-cut regime lead to significantly higher crop yield and to a significantly higher nutritive (energetic) value (calculated in Fodder Unit of milk) in cuts 1 and 3 of 2016, in comparison with the 3-cut regime (Table 2). Likewise, amounts of true protein digested in the small intestine were higher in the fresh material of the 4-cut regime in 2016 (data not shown). This means that a larger interval between cuts leads to lower digestibility due to senescing of plant matter, without any advantage for crop yield. In the silage product however, we observed no significant differences in nutritive value. The results from 2017 confirmed these results (data not shown). In March 2018, there were no differences in either the plant cover or the number of plants per m^2. In the 4-cut regime, however, the number of shoots was significantly higher compared to the 3-cut regime (Table 2). This indicates that more of the soil surface in the 3-cut regime was covered with unsown species like *Poa annua* L., *Capsella bursa-pastoris* L. and *Taraxacum officinale* L., which was also observed visually. Lucerne persistence was therefore higher in the 4-cuts regime than in the 3-cut regime.

Table 2. Field trial B, mean crop yield (n=3), the mean nutritive (energetic) value (n=3) of the fresh harvested matter for every cut and the mean number of shoots per m^2 (n=8).[1]

Treatment	**Crop yield (Mg ha^{-1} yr^{-1})**		**Fodder Unit Milk 2016 (g kg^{-1} dry matter) – cut no.**				**Shoots per m^2**
	2016	**2017**	**1**	**2**	**3**	**4**	**April 2018**
3 cuts/year	12.7^a	16.0^a	697^a	682	698^a	-	689^a
4 cuts/year	15.2^b	18.2^b	790^b	736	736^b	795	831^b

[1] The year of establishment was 2015 (3 cuts in both treatments). Statistically significant difference (Tukey $P<0.05$) are indicated with different letters.

Conclusion

Under good weather conditions in the months after sowing lucerne, adding seeds of perennial ryegrass or white clover had no added value. Under wet conditions after installing the lucerne mixture, perennial ryegrass and white clover compensated for slow growth of lucerne. Adding perennial ryegrass or white clover can thus be seen as an insurance against detrimental environmental conditions when using lucerne as forage crop. A 4-cut regime is preferred to a 3-cut regime, as the 3-cut can lead to less crop yield, less nutritive value of the fresh matter and even reduces lucerne persistence. Seed inoculation can lead to an approximately two-fold yield in the year of installation of stand-alone lucerne.

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The influence of red clover-based organic manure on spring wheat productivity

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Introduction: In order to utilise a larger amount of symbiotically fixed N, the aboveground mass of *Fabaceae* family plants is used as green manure. A major part of nitrogen is found in readily degradable organic compounds. Modern technologies of green manure management must be based on N fixation in the biomass or immobilization in the soil, and synchronisation of N release with plant N requirements (Dannehl *et al.*, 2017). The aim of the present study was to assess the impact of red clover preparation as green manure, namely ensiling and composting, on the productivity of the following spring wheat.

Materials and methods: Two field trials were carried out in 2015-2016 at Dotnuva (loamy *Cambisol*) and at Joniskelis (clay loam *Cambisol*) sites to test the effect of two types of green manure on the yield of spring wheat. Green manure was produced from red clover herbage mass by ensiling (EnsRC) and aerobic composting with winter wheat straw (ComRC+S). Fertilization treatments were: (1) control with no fertilizers (Control); (2) fresh red clover mass incorporated in the autumn (RC); (3) ensiled red clover mass (EnsRC); (4) composted red clover and straw mass (ComRC+S); (5) pelleted cattle manure (PCM). Organic fertilizer rates are presented in Table 1.

Results and discussion: At the Dotnuva site, the highest yields were achieved in the RC and PCM treatments, where the grain yield was 28 and 17% higher compared with the control (Table 1). At the Joniskelis site, no significant effects were determined. Among all amendments, yield of spring wheat tended to be the lowest in EnsRC at both experimental sites. The higher C:N ratio might be responsible for this as it probably stimulated N immobilisation and reduced the available N.

Table 1. Red clover-based organic manures and their effects on spring wheat grain yield.[1]

Organic manure	Characteristics of incorporated manure			Spring wheat grain yield, kg ha^{-1}	
	DM kg ha^{-1}	N kg ha^{-1}	C:N	Site: Dotnuva	Site: Joniskelis
Control	–	–	–	2,182 b	4,015 ab
RC	2,269	75	15:1	2,792 e	4,237 ab
EnsRC	2,632	50	21:1	2,286 bc	3,774 ab
ComRC+S	1,592	50	16:1	2,404 cd	3,818 ab
PCM	18.1	50	17:1	2,553 d	4,297 b
Mean	2,076	56		2,371	3,985

[1] Means not sharing the same letter are significantly different at $P<0.05$ (Duncan MRT).

Conclusion: Green manure produced from red clover herbage mass by ensiling (EnsRC) had the highest C:N ratio and most probably reduced plant available N. Hence there was a trend for EnsRC to give the lowest grain yield of all tested amendments.

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Early emergence of autumn sown seed mixtures of different species composition to improve insect diversity at various sites

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Introduction and methods: During the last decades, insect biomass and diversity in our landscape has been declining (Hallmann *et al.* 2017). Few measures to improve biodiversity have yet been tested in intensively used grassland (Fritch *et al.,* 2017). Extensification in combination with reseeding can help alleviate the negative effects on biodiversity and insect biomass (Sheridan *et al.,* 2008). In 2018, a seed mixture of two legume species (red clover and birdsfoot trefoil), a seed mixture of different forb species (29) and a seed mixture of forbs, legumes and grasses (42 species) were sown at the rotavated edges of intensively used meadows (>4 cuts/year). The aim is to compare 4-5 cut systems and reduced cutting frequency in sown conservation strips and to check the effects on biodiversity. In this study we test how different seed mixtures sown at the edges of intensively used meadows have established. Conservation strips were sown in autumn 2018 at 6 sites. The sites (540-680 m a.s.l.) are more than 6 km from each other and are scattered over the region 'Oberschwaben' in south-west Germany. The region 'Oberschwaben' is a hilly landscape, where dairy farming with intensive grassland management predominates. After sowing the different mixtures, a rating of the germination success was carried out in October 2018.

Results and discussion: At sites without rain until 24 October 2018 there were no seedlings visible, except for the mixture 'legumes'. The fact that at some sites there were more seedlings in the legumes' mixture, even at sites with virtually no rain, can be due to subterranean vegetative tillers of previously occurring *Trifolium pratense* plants. The rating showed that the establishment up to 24 October 2018 depends on the timing, and mainly on the sum, of precipitation after the sowing process. In a study by Woodcock *et al.* (2014), a mixture with forb and legume species yielded more flowers over 4 years than the legume mixture. These mixtures consisted of only 12 or 18 species, whereas in our experiment we use a higher number of perennial grassland species in order to increase the chance of establishment and supply of flowers.

Conclusion: More research on biodiversity of sown margin strips in intensively used grassland is needed. The first ratings showed that the time of sowing the seeds is crucial for establishment because germination and establishment of young seedlings depends on water availability.

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Breeding forb species for intensively managed multi-species mixtures

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Introduction: Adding certain forbs such as chicory (*Cichorium intybus* L.), caraway (*Carum carvi* L.) or plantain (*Plantago lanceolata* L.) to grass-clover mixtures can further enhance or largely maintain the high productivity of grass-clover mixtures (Cong *et al.*, 2018). This study aimed to screen varieties of three competitive forbs – chicory, caraway and plantain with high yield and quality performance – and to examine whether these varieties perform well when grown with traditional grass-clover mixtures.

Materials and methods: The experiment was established in Stevns, Denmark in 2014. Four varieties of chicory, plantain and two varieties of caraway were grown in pure stands or with a mixture of ryegrass (GR) and red clover (RC) in two seed proportions (20% or 60%). All plots received 60 kg year^{-1} of plant-available N in cattle slurry. Herbage yield was determined in three annual cuts from 2015 to 2017. Herbage quality parameters (NDF, protein content and soluble sugar) were measured in the spring-cut herbage in pooled samples.

Results: We found that chicory (cv. Balance) produced the highest yields in pure stands. And adding this variety to grass-clover mixture further enhanced yield. The cv. Balance-containing mixtures also had higher protein content due to higher red clover proportion in mixtures. Compared to the grass-clover mixture, the mixtures with 60% plantain (cv. Wildtype) produced higher yield, as well as protein, whereas the mixtures with 20% plantain (cv. Ranger) produced higher yield and protein content. The mixture with 60% caraway (cv. Volhouden) produced higher yield and protein than the mixture with caraway (cv. Prochan), whereas the opposite performance was observed in 20% seed mixtures.

Table 1. Annual herbage yield (averaged over three years) and quality of sward composition.[1]

Sward composition	Forb variety	Yield (kg ha^{-1} yr^{-1})	%NDF	%Protein	%Sugar
100CI (Chicory)	Spadona	7,809^{b}	48.7	13.3	16.5
	Amateurras	8,072^{b}	48.3	13.5	16.4
	CCR9AA	8,223^{b}	48.9	12.5	17.7
	Balance	8,946^{a}	48.7	12.7	17.0
100PL (Plantain)	Wildtype	7,767^{ab}	50.5	11.2	18.2
	01961	7,101^{b}	50.5	11.9	17.1
	Ranger	7,203^{b}	48.9	12.8	17.3
	Tangoaa	8,482^{a}	51.0	11.8	15.6
100CA (Caraway)	Volhouden	6,753^{a}	49.4	11.3	19.1
	Prochan	6,451^{a}	50.1	11.9	18.0
20RC80GR		7,152^{b}	50.2	9.6	22.0
20RC20GR60CI	Balance	11,661^{a}	50.2	12.0	16.6
20RC20GR60PL	Wildtype	10,515^{a}	50.4	11.4	18.1
20RC20GR60CA	Volhouden	7,645^{b}	49.2	10.3	20.1
40RC40GR20CI	Balance	9,400^{a}	50.6	10.5	19.6
40RC40GR20PL	Ranger	10,367^{a}	50.9	11.6	16.3
40RC40GR20CA	Prochan	9,758^{a}	51.2	10.5	18.9

[1] 20RC means 20% red clover; 80GR means 80% perennial ryegrass. Herbage yield with different letters indicated significant differences ($P<0.05$) between varieties of each species, or between the grass-clover-forb mixture and grass-clover mixture.

Conclusion: These findings suggest that including new forb varieties in grass-clover mixtures holds potential for enhanced productivity and protein yield of intensively managed grasslands.

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Grass-clover mixtures: benefits for arable and livestock farms and biodiversity

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Introduction: Grass-clover mixtures show many benefits for sustainable agriculture. In the Netherlands, organic arable and livestock farmers often work together in a so-called partner farm concept: the arable farms grow one-year grass-clover leys to widen their crop rotation and as fodder for a livestock farm in exchange for manure. The aim of this research was to investigate the effect of different grass-clover mixtures and monocultures in a one-year ley on both aboveground and belowground parameters in light of the benefits of the ley for livestock farms, arable farms and biodiversity.

Materials and methods: Monocultures and selected 2-, 3- and 4- species mixtures of a range of grass (*Lolium perenne*: Lp, *Lolium multiflorum*: Lm) and clover (*Trifolium pratense*: Tp and *Trifolium repens*: Tr) species were assessed in a one-year organic ley, without fertilisation. Aboveground measurements included yield, digestibility, nitrogen (N) content and the proportion of weeds and clover. Belowground parameters were soil penetration resistance, soil structure, rooting density and soil mineral N content. Earthworms were counted, weighed and assigned to functional groups. Normally distributed data were analysed with ANOVA, all other data were analysed with Kruskal-Wallis tests.

Results: Grass monocultures showed good weed suppression, high root density, and especially Lp had a positive effect on soil structure. Clover, conversely, showed high herbage DM yield (particularly Tp; Figure 1) and N yield, and Tr showed high digestibility. Moreover, clover had a positive effect on the soil mineral N, and earthworm abundance tended to be higher in clover monocultures. When (some of) the four species were combined in grass-clover mixtures they combined the positive effects of the species and often outperformed the (best) monocultures (Figure 1). Mixtures including Lm showed lower clover content (due to fast Lm growth in spring, slowing clover development), resulting in lower N content and N yield.

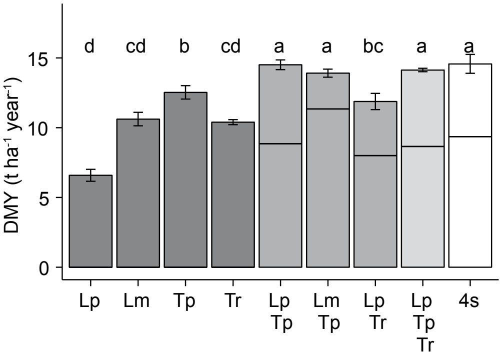

Figure 1. Mean cumulative herbage dry matter yield (t DM ha^{-1} year^{-1}) ± 1 SE for the different sward types. Lp: *Lolium perenne*, Lm: *Lolium multiflorum*, Tp: *Trifolium pratense*, Tr: *Trifolium repens*, 4s: four species mixture. The horizontal lines across bars show the predicted yield based on monoculture performance. Means with the same letters are not significantly different (P>0.05) (n=3).

Conclusion: For the livestock farm, the Lp:Tp mixture would appear to be the most beneficial, as it showed the highest herbage DMY, N yield and DOM yield. In contrast, for the arable farm both the Lp:Tr and the four-species mixtures performed best, due to the combination of high weed suppression, good soil structure, high root score and intermediate soil mineral N levels. For biodiversity, the clover monocultures, Lp:Tr and three-species mixtures scored best due to higher earthworm abundance and potentially positive effects on insects. Therefore, if all results are combined, the three-species mixtures (Lp:Tp:Tr) performed best on average.

Grass-clover yield and composition responses to mineral fertilizer N versus cattle slurry

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Introduction: Nitrogen (N) fertilization of grass-clover leys is common practice in dairy production systems. When fertilized with N, grass will be better than clover at exploiting the N. Previous studies have shown that in terms of botanical composition, the advantage to grass is less pronounced when N is applied as manure as opposed to mineral fertilizer (Søegaard, 1998; Nesheim *et al.*, 1990). The aim of this experiment was to investigate how different types and amounts of N fertilization affect the dry matter production and clover content.

Materials and methods: An experiment was set up in spring 2018 in a second year grass-clover field (undersown in spring barley (*Hordeum vulgare* L.) in 2016) with white clover (*Trifolium repens* L.), red clover (*Trifolium pratense* L.) and perennial ryegrass (*Lolium perenne* L.). Plots (1 m^2 including borders), laid out in a randomized block design with four replicates, received three types of N fertilizer (mineral NO_3^- and NH_4^+, and NH_4^+ as cattle slurry) in three amounts (0, 180 and 360 kg plant-available N ha^{-1} yr^{-1}). Fertilizer was divided in three equal portions and given prior to spring growth, after first cut and after second cut. Herbage was harvested four times in subplots (0.25 m^2) during the growing season and the yield and botanical composition was determined by manual sorting.

Results: Total DM yield increased with increasing N from 0 to 180 kg N ha^{-1} yr^{-1} ($P<0.004$). No significant differences were found between 180 and 360 kg N ha^{-1} yr^{-1}. Average yield increase in response to N was 326 g m^{-2}. The clover fraction was negatively affected by increasing amount of N ($P<0.001$). At 360 kg N ha^{-1} yr^{-1}, the clover fraction, determined following slurry application, was significantly lower than that following the two mineral N treatments ($P<0.05$). The experiment did not confirm earlier findings that clover fractions are significantly higher when the same amount of plant available N was added as slurry rather than mineral N. Mechanisms responsible could include ammonia volatilization.

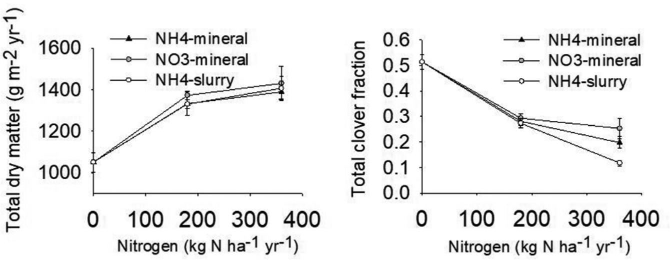

Figure 1. Annual yield and clover fraction N response for the three types of N fertilizer. Mean ± SE (n=4).

Conclusion: DM production increased from no N to medium N. Further N fertilization did not increase DM yield significantly. The clover fraction showed a negative response to increasing N-amount, surprisingly the clover fraction was supressed by the slurry compared to the mineral fertilizer at the highest N level.

Nesheim L., Boller B.C., Lehmann J. and Walther U. (1990) The effect of nitrogen in cattle slurry and mineral fertilizers on nitrogen fixation by white clover. *Grass and Forage Science* 45(1), 91-97.

Søegaard, K. (1998) Timing of N-application after defoliation of clover-grass. *Grassland Science in Europe* 3, 979-982.

Extending forage production with lucerne-grass mixtures in a Mediterranean rainfed environment

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Introduction: Growing forage mixtures based on perennial grasses and legumes is of considerable interest for farmers in Mediterranean rainfed livestock systems, especially if they produce more dry matter (DM) during late spring, summer and early autumn. The objective of our study was to assess the DM yield of perennial lucerne-grass mixtures based on local germplasm and adapted varieties and their performance within and across years.

Materials and methods: Mixtures based on grazing- and drought-tolerant *Medicago sativa* L. accessions [‘Bulk’ (L1), semi-erect type; elite line ‘Surigheddu’ (L2), erect type] and grasses [*Dactylis glomerata* L. cv ‘Kasbah’ (C), true summer-dormant; *Festuca arundinacea* Schreb. cv ‘Flecha’ (F), summer but not true type dormant] were established in 2014 in an experimental field in Sassari (Sardinia, Italy) following a randomized block design. Binary (L1C, L1F, L2C, L2F) and four species (L1L2CF) mixtures were compared with their respective monocultures (L1, L2, C, F) for DM yield. Cuts were taken seasonally in 2014-15, 2015-16, and 2016-17. Multifactor ANOVA and Tukey test were used to analyse data.

Results: Annual DM yield ranged from 6.9 to 12.7 t ha^{-1} year^{-1} in mixtures, from 7.3 to 11.5 t ha^{-1} year^{-1} in lucerne monocultures, and from 3.5 to 7.1 t ha^{-1} year in grasses. No interactions were found between year and sward type. Among pure stands, the semi-erect type L1 performed better than the erect L2. F performed better than C. This latter showed the lowest DM yield. Mixtures based on L1 showed the best performances. Moreover, L1C showed the best forage seasonal distribution among swards but similar to L1.

Table 1. Sward type, three-year average annual DM yield of sown species, and DM yield distribution in each season.[1]

Sward type	Annual DM yield (t ha^{-1} yr^{-1})	DM yield distribution (% of annual DM yield)			
		Autumn	Winter	Spring	Summer
L1	10.43 abc	12-16	9-29	42-55	29-58
L2	7.38 d	3-13	0-7	26-54	36-61
C	3.83 e	49-65	29-50	0-22	0
F	6.19 d	26-53	32-38	8-62	0
L1C	10.77 ab	14-25	14-26	42-45	30-50
L1F	12.20 a	13-28	0-17	29-43	27-44
L2C	7.46 d	22-33	14-23	0-48	26-44
L2F	8.13 cd	18-36	0-25	28-48	13-36
L1L2CF	9.80 bc	14-29	18-30	0-43	26-44

[1] Means with same letters are not different at 5% level.

Conclusion: The use of mixtures based on drought-tolerant *M. sativa* improved the seasonal distribution of forage production under rainfed conditions. They could be profitably adopted to extend green forage season during late spring, summer and early autumn.

Lüscher A., Mueller-Harvey I., Soussana J.F., Rees R.M. and Peyraud J.L. (2014) Potential of legume-based grassland-livestock systems in Europe: a review. *Grass Forage Science* 69, 206-228.

Grassland mixtures for Norwegian agriculture in a longer growing season

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Introduction: Seed mixtures for Norwegian grassland generally contain few species, with the small-seeded *Phleum pratense* the major species. An expected longer growing season demands species with high regrowth capacity, and with species complementing each other both above- and below ground. The objective of our study was to determine the production capacity (dry matter yield (DMY), t $ha^{-1}yr^{-1}$) of six mixtures differing in species number and proportion of species.

Materials and methods: Triplicated field trials with six grassland mixtures were established at three climatically different sites (Fureneset, 61°N coast, 10 m a.s.l.; Løken, 61°N inland, 450 m a.s.l.; Tjøtta, 65°N coast, 11 m a.s.l.) in 2016 and were cut two or three times during 2017 and 2018. Four mixtures contained *P. pratense*. Red (*Trifolium pratense*) and white (*T. pratense*) clover were included in five of six mixtures. Two trials were conventionally and one (Tjøtta) organically managed with fertilization; in total 180 kg $ha^{-1}yr^{-1}$ at Løken and Tjøtta, and 300 kg $ha^{-1}yr^{-1}$ at Fureneset. The species included in different mixtures were *Agrostis capillaris* (Ac), *Bromus inermis* (Bi), *Dactylis glomerata* (Dg), *Festuca arundinacea* (Fa), *F. pratensis* (Fp), *F. rubra* (Fr), *Festulolium* (FpxLp, 4×) (FL), *Lolium perenne* (4×) (Lp), *L. hybridum* (Lh), *P. pratense* (Pp), *Poa pratensis* (Ppr), *T. pratense* (2×) (Tp), *T. repens* (Tr) (Table 1). The data were analysed by ANOVA.

Results: The preliminary results for two years show differences in DMY between the three locations ($P<0.0001$, Table 1); however, with a site × mixture interaction (*0.0246*). The mixtures containing *P. pratense* produced more DMY than the mixtures without *P. pratense* at both southern sites. This was in contrast to the organically treated trial in which *P. pratense*-mixtures yielded less than the other mixtures, possibly due to limited availability of nitrogen needed for optimal production.

Table 1. Dry matter yield expressed as mean of two ley years for the three trial sites.

Proportion of species[1] (seed weight, %)	Dry matter yield (t ha^{-1} yr^{-1})					
	Fureneset		Løken		Tjøtta	
1: Pp-45, Fp-25, Ppr-20, Tp=Tr-5	13.31	*a*	10.98	*a*	6.72	*b*
2: Pp-45, Fa-25, Ppr-20, Tp=Tr-5	13.82	*a*	10.34	*a*	7.19	*b*
4: Pp-40, Fp-20, Fr-10, Ppr-10, Ac-10, Tp=Tr-5	13.07	*ab*	11.06	*a*	6.38	*b*
3: Pp-30, Fr-10, Ppr-20, Bi-30, Tp=Tr-5	12.96	*ab*	10.49	*a*	7.42	*b*
5: Fp-20, Fr-10, Ppr-20, Dg-20, Lp-20, Tp=Tr-5	11.45	*b*	9.64	*a*	8.03	*ab*
6: Fa-20, Lp-40, Lh-20, FL-20	11.68	*b*			9.18	*a*
Mean of two ley years	12.72	*A*	10.50	*B*	7.49	*C*
Standard error of mean	0.26		0.21		0.39	

[1] See materials and methods for species description. Means with a common letter are not different at 5% level (REGWQ), a-b in columns and A-C in row.

Conclusion: Both number and choice of species, and species proportion in the seed mixtures seem to influence the total DMY of the tested mixtures. However, more experimental ley years combined with nutritional forage quality and species proportion in the plant stands are needed to test for the persistency of, and to make recommendations for, species mixtures for Norwegian grassland.

Trade-offs and synergies between sown grassland ecosystem services and disservices under different nitrogen fertilization strategies

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Introduction: Mineral nitrogen (N) fertilizers are commonly used to maximize agronomic performance of grasslands. However, increasing the agronomic benefits of fertilization for ecosystem services while limiting N losses is challenging. Moreover, how fertilization practices modify the synergies and trade-offs between different (dis)services and ultimately the multi-functionality of grasslands is still unclear. Here, we quantified the effects of N fertilization timing (N-timing) and rate (N-rate) on proxies of two grassland services (forage and seed yields) and three disservices (time-integrated nitrate leaching, NH_3 and N_2O emissions) in *Leymus chinensis* grasslands. We assumed that synergies/trade-offs between these ecosystem (dis)services could be substantially modulated by N-timing, because the fate of added N would depend on the plant phenological status and uptake capacity.

Materials and methods: The experiment was conducted in the *Songnen* Grassland of China, using in situ mesocosms with 4 replicates. We used a complete factorial design with 2 factors: N-timing (in autumn of the previous year or in spring) and N-rate (0, 56, and 112 kg N-urea ha^{-1}). N leaching was monitored by lysimetry, and NH_3 and N_2O emissions by the static chamber method during 2.5 months after fertilization. Data were analysed by two-way ANOVA.

Results: At a given N-rate, seed and forage yields were higher under autumn than spring fertilization, except forage yield under low N-rate (Table 1). Total NH_3 emissions were lower for spring than autumn fertilization with high N-rate. N_2O emissions were lower for spring than autumn fertilization whatever the N-rate (Table 1). No N leaching was detected.

Table 1. Effects of N-timing and N-rate on the different (dis)services (yield, kg ha^{-1}; gas emissions, µg-N m^{-2} h^{-1}).[1]

Variables	Aut-N-0	Aut-N-56	Aut-N-112	Spring-N-0	Spring-N-56	Spring-N-112
Seed yield	47.13±1.41 e	94.36±0.67 c	138.63±1.19 a	47.13±1.41 e	65.91±1.04 d	99.43±0.60 b
Forage yield	2,489±114 d	4,266±142 c	6,873±99 a	2,489±114 d	4,667±66 c	5,402±82 b
NH_3 emission	21.87±0.27 d	49.65±0.42 c	107.24±1.74 a	25.92±0.29 d	48.18±1.56 c	89.12±0.43 b
N_2O emission	3.30±0.07 d	6.28±0.19 c	13.10±0.27 a	1.96±0.10 e	3.49±0.21 d	11.46±0.23 b

[1] Values are means ± standard error (n=4). For each variable, values with different letters significantly differed ($P<0.05$).

Conclusion: Interaction between N-timing and N-rate modifies the multi-functionality of semi-arid, sown grassland, and this knowledge can be used for N risk: benefit analyses when evaluating fertilization strategies. High N fertilization in autumn is preferable for maximizing forage and seed yields, whereas spring N fertilization is a better choice for limiting disservices associated with emissions from N compounds.

Protein production potential of lucerne grown in monoculture or in mixture with clover, forb or grass species

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Introduction: Forage legumes play an essential role for the protein supply of ruminants. High crude protein contents can be achieved with clover monocultures, but yields are usually lower compared to grass-legume mixtures (Nyfeler *et al.,* 2009). The objective of this study was to test if combinations of lucerne (*M. sativa* (Ms)) with either red (*T. pratense* (Tp)) or white clover (*T. repens* (Tr)) or selected forbs (*P. lanceolata* (Pl)*, C. intybus* (Ci)) may both maximize dry matter and protein yields.

Materials and methods: A field experiment with four replicates for each mixture was conducted in 2016-17 at two sites in Switzerland. As references, two grass-legume mixtures (SM 320 and 325 (Suter *et al.,* 2012)) and Ms varieties (Catera and Fleetwood) in pure stands were included. All plots were harvested five times, with no data available from the first cut in 2016 due to incomplete sward establishment. Fertilizer, as cattle slurry, was applied only after the first cut in 2017 (to supply 20 kg N ha^{-1}). Forage nitrogen content was measured with a C/N-analyser (Dumas). Data were analysed with a linear mixed-effects model in R.

Results: No differences in total dry matter (DM) yield could be observed in either year, except for the mixture Ms + Tr in the second year, with a significantly lower yield than the other mixtures and MS pure stands (Table 1). In 2016, crude protein (CP) yields were significantly higher for Ms pure stands and Ms + Tp, as compared to Ms + Ci and Ms + Pl. This was due to the lower CP contents of the mixtures with forbs (data not shown). Almost no differences in the CP contents occurred in 2017 between the mixtures, most likely related to the increasing dominance of Ms in the mixtures (especially in combination with forbs, data not shown). Consequently, CP yields of the mixtures in 2017 behaved similarly to the DM yields.

Table 1. Effect of partner species on dry matter (DM) and crude protein (CP) yield (means of two sites).[1]

Partner species	DM yield (dt ha^{-1} yr^{-1})		CP yield (kg ha^{-1} yr^{-1})	
	2016	2017	2016	2017
Monocultures Ms	115.1 a	185.7 a	2,103 a	3,594 a
Mixture Ms + Tp	117.5 a	177.8 a	2,107 a	3,348 a
Mixture Ms + Tr	107.0 a	144.6 b	1,978 ab	2,819 b
Mixture Ms + Ci	110.0 a	186.0 a	1,521 c	3,436 a
Mixture Ms + Pl	114.8 a	186.4 a	1,578 bc	3,561 a
Grass-legume mixtures	119.1 a	182.8 a	1,845 abc	3,485 a

[1] A common letter implies no difference at 5%.

Conclusion: Total DM yield was not determined by the mixture except for MS + Tr. Provided a balanced botanical composition, as it was the case only in the first year, protein production potential could be maximized with the mixture of Ms + Tp and with Ms pure stands.

Nyfeler D., Huguenin-Elie O., Suter M., Frossard E., Connolly J. and Lüscher A. (2009) Strong mixture effects among four species in fertilized agricultural grassland led to persistent and consistent transgressive overyielding. *Journal of Applied Ecology* 46 (3), 683-691.

Suter D., Rosenberg E., Mosimann E. and Frick R. (2012) Standardmischungen für den Futterbau Revision 2013-2016. *Agrarforschung Schweiz* 3 (10), Beilage/encart.

Species diversity enhances multifunctionality in sown grass-legume mixtures

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Introduction: Legume-based grassland systems provide several key functions for the provision of high quantity and quality forage at low to moderate nitrogen (N) fertilizer input (Lüscher *et al.* 2014). Despite their high agronomic and economic importance, there is no up-to-date evaluation of the diversity-multifunctionality relationship in these systems.

Materials and methods: A diversity experiment was set up with sown monocultures, 2- and 4-species mixtures comprising *Lolium perenne* L., *Dactylis glomerata* L., *Trifolium pratense* L., and *Trifolium repens* L., and was maintained for three years at 150 kg N fertilization ha^{-1} yr^{-1}. Nine functions were measured that represented (1) production: aboveground biomass yield, between-year temporal yield stability (mean yield/standard deviation), aboveground weed biomass; (2) N cycling: symbiotic N_2 fixation (N_{sym}), N efficiency (N yield/N applied), NO_3 in soil solution; and (3) forage quality: crude protein content (CP), organic matter digestibility (OMD), metabolizable energy content (ME). All functions were jointly regressed on sown species proportions using multivariate linear mixed-effects regression (all stands used).

Results: Across the three years, all functions regarding production and N cycling revealed significantly enhanced performance in the 4-species equi-proportional mixture (used as a reference) compared to averaged monocultures ('overyielding'). Thus, the reference mixture had 62% more biomass yield, a 73% increase in temporal stability, 80% less weed biomass, 99% and 50% higher N_{sym} and N efficiency, respectively, and 85% less NO_3 in the soil solution (Table 1). The reference mixture and averaged monocultures did not significantly differ in CP, OMD (g kg^{-1} yield), and ME (MJ kg^{-1} yield) (Table 1). This, however, resulted in significant overyielding between 49% and 68% in all three forage quality functions on a per-hectare basis (kg or MJ ha^{-1} year^{-1}) ($P<0.01$). Thus, the use of grass-legume mixtures enhanced the performance of all measured functions, without trade-off between them, indicating enhanced multifunctionality in mixtures.

Table 1. Percent overyielding of nine functions representing forage production, N cycling, and forage quality, measured over three experimental years.[1]

Service	Function	Unit *(Ratio)*	Targeted direction	Overyielding
Production	Aboveground biomass yield	Mg ha^{-1} year^{-1}	positive	62% ***
	Temporal stability of yield	μ / σ	positive	73% **
	Weed biomass	Mg ha^{-1} year^{-1}	negative	-80% ***
N cycling	Symbiotic N_2 fixation	kg ha^{-1} year^{-1}	positive	99% ***
	N efficiency	N yield/N applied	positive	50% ***
	NO_3 in soil solution	mg liter^{-1}	negative	-85% *
Forage quality	Crude protein content	g kg^{-1} yield	positive	-8% ns
	Organic matter digestibility	g kg^{-1} yield	positive	-2% ns
	Metabolizable energy	MJ kg^{-1} yield	positive	-3% ns

[1] μ: mean, σ: standard deviation; *** $P\leq0.001$, ** $P\leq0.01$, * $P\leq0.05$, ns: $P>0.05$.

Conclusions: Sown grass-legume mixtures at moderate N fertilization sustain high multifunctionality and, compared to monocultures, enhance levels of individual functions that jointly promote sustainable forage production.

Lüscher A., Mueller-Harvey I., Soussana J.F., Rees R.M. and Peyraud J.L. (2014) Potential of legume-based grassland-livestock systems in Europe: a review. *Grass and Forage Science* 69, 206-228.

Temporal changes in the genetic diversity of perennial ryegrass swards during three years of cultivation

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Introduction: Perennial ryegrass (*Lolium perenne*) cultivars are genetically diverse populations. The genetic diversity of ryegrass sown in swards may change because of environmental factors, management practices, and companion cultivars or species in the mixed sward (Verwimp *et al.,* 2018). These genetic changes may affect the sward performance. To investigate whether cultivar-specific responses exist, we monitored the genetic diversity of plots of seven cultivars included in the GrassLandscape field trial (Blanco-Pastor *et al.,* 2018) at Merelbeke (BE) during three growing seasons.

Materials and methods: The cultivars Expo, Mara, Clerpin, Barutti, Merks, Meloni, and Fennema were established in October 2015 in field plots of 2 m^2 with 3 replicates. The field plots (n=21) were sampled in the spring of 2016, 2017 and 2018 (n=3), by collecting 72 leaf samples evenly distributed within each plot. We used pool-GBS (Verwimp *et al.,* 2018) to estimate the allele frequencies for 45k robust SNPs. The genetic composition of the 63 pools was described with PCA. The genetic diversity was characterized by calculating mean Expected Heterozygosity (H_e), and temporal changes were identified with ANOVA.

Results: The PCA confirmed that the seven cultivars are clearly distinct from each other (Figure 1A) and that their genetic composition was maintained during three years of cultivation, except for Barutti (Figure 1B). The ANOVA of mean H_e values revealed a significant interaction between cultivar and year (P<0.01) and significant differences between cultivars (P<0.01), but not between years. The three plots of Barutti displayed a sharp decline in H_e from the first to the second year (Figure 1B).

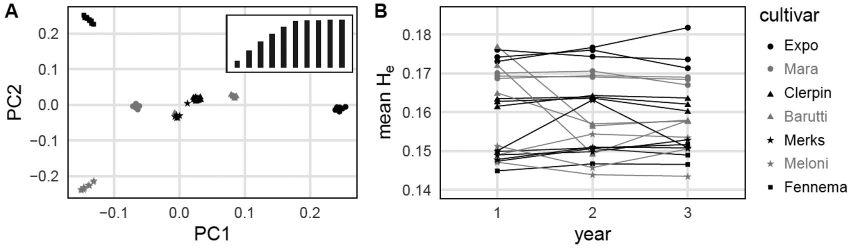

Figure 1. (A) PCA scores of 63 sample pools based on the allele frequencies of 45k SNPs. The screeplot (inset) shows the cumulative eigenvalues of the first 10 components. (B) Mean H_e of 21 field plots (represented by lines) over the three consecutive years of cultivation.

Conclusion: The genetic diversity of perennial ryegrass monocultures can change during cultivation and these changes depend on the sown cultivar.

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Verwimp C., Ruttink T., Muylle H., Van Glabeke S., Cnops G., Quataert P., ... Roldán-Ruiz I. (2018) Temporal changes in genetic diversity and forage yield of perennial ryegrass in monoculture and in combination with red clover in swards. *PloS one* 13, e0206571.

Increased inherent energy in grasses acts complementarily with birdsfoot trefoil to improve pasture-based dairy heifer performance

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Introduction: The dairy industry has experienced a dynamic increase in pasture-based milk production and heifer replacement systems. However, reduced dry matter intake (DMI) by grazing cattle is a major factor limiting growth and milk production. Thus, grass-legume pastures that require fewer inputs, have high forage mass and nutritive value, and have high DMI are critical to the viability of pasture-based dairies. The objective of this study was to determine if increased inherent energy in grasses and condensed tannins in birdsfoot trefoil have a complementary effect on dairy heifer DMI and growth performance.

Materials and methods: Eight pasture treatments were evaluated consisting of tall fescue (*Schedonorus arundinaceus*; TF), meadow bromegrass (*Bromus riparius*; MB), high-sugar orchardgrass (*Dactylis glomerata*; OG), and high-sugar perennial ryegrass (*Lolium perenne*; PR) in monocultures and binary mixtures with birdsfoot trefoil (*Lotus corniculatus*; BFT). For each of three grazing seasons, post-pubertal Jersey dairy heifers were allocated to the pasture treatments. Rotational stocking was used by moving heifers to new paddocks every 7 days, resulting in three 35-day rotation cycles. Forage mass and nutritive value were determined before and after each 7-day grazing period, and forage disappearance (apparent DMI) was calculated by comparing pre-grazing forage mass to post-grazing forage mass. Heifer body weight was determined at the end of each 35-day rotation.

Results: Grass-BFT mixtures had greater mass, crude protein (CP), and heifer average daily gain (ADG), but lesser digestible fibre (DNDF) and water-soluble carbohydrates (WSC) than their respective grass monocultures (Table 1), whereas, grass monocultures differed in energy (NEg) with high-sugar grasses having greater NEg. Furthermore, the high-sugar grass-BFT mixtures ranked the highest for ADG. However, grass-BFT mixtures did not have greater NEg, and only exhibited greater DMI for PR+BFT, compared with monocultures (Table 1).

Table 1. Rotation-cycle mean herbage mass, nutritive value, and heifer DMI and ADG.[1]

TRMT	Herbage (kg ha^{-1})		CP (%)		DNDF (%)		WSC (%)		NEg (Mcal kg^{-1})		DMI (kg AU^{-1} day^{-1}*)		ADG (kg day^{-1})	
PR+BFT	3,681	b	18.3	a	24.0	g	9.4	c	0.68	b	11.1	bc	0.70	a
OG+BFT	3,587	b	15.3	b	31.5	d	7.0	e	0.61	c	11.7	ab	0.66	ab
MB+BFT	4,142	a	17.2	a	28.5	f	6.0	f	0.60	cd	12.8	a	0.64	bc
TF+BFT	3,610	b	14.5	b	29.8	e	8.4	d	0.50	e	9.6	cd	0.58	cd
OG	3,118	c	10.9	c	39.2	a	8.5	d	0.65	b	11.6	ab	0.58	d
PR	2,328	d	10.4	cd	32.8	c	16.7	a	0.73	a	9.0	d	0.54	d
MB	3,548	b	10.9	c	35.9	b	7.9	d	0.61	c	11.8	ab	0.53	d
TF	3,066	c	9.4	d	34.7	b	11.2	b	0.56	d	9.4	d	0.42	e

[1] Means with a common letter are not different at 5% level (Fisher's protected LSD). Sorted by ADG.

Conclusions: We conclude that when grown in mixtures, increasing the inherent energy in grasses will have a complementary effect with BFT resulting in greater dairy heifer growth performance. However, it does not appear to improve overall DMI.

Production capacity of forage legumes and persistence to root rot in organic mixed swards

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Introduction: Red clover (RC; *Trifolium pratense* L.) is an important forage legume in Sweden cultivated in mixtures with grass. One main constraint is the poor persistence of red clover plants, originating in root rot caused by infection of several soil-borne pathogens. The objective of our study was to investigate production capacity, persistence and disease development of white clover (WC; *Trifolium repens* L.), lucerne (LU; *Medicago sativa* L.), birdsfoot trefoil (BFT; *Lotus corniculatus* L.) and RC grown in mixed swards with timothy (TI; *Phelum pratense* L.) in a 3-year set up. Results from the second harvest year are reported.

Materials and methods: A field experiment with four replicates per plot was established in 2015 at Nibble, Järna (N 59° 19', E 18°4'). RC, WC, BFT and LU respectively were sown in a mixture with TI. All plots were harvested with a forage harvester at a height of 5-7 cm. The prevalence of root rot was determined and fungal pathogens were quantified by real-time PCR. The data were analysed by ANOVA.

Results: Dry matter (DM) yields of RC-TI and BL-TI were much larger than those of WC-TI and BFT-TI (Table 1). DM yields were lower overall compared to the first harvest year. WC showed the highest disease severity index (DSI) of 63, not significantly different from RC 46, while the DSI of BFT and LU were 31 and 23, respectively. DSI was significantly higher for WC whereas the DSI of the other legumes did not differ significantly compared to harvest year 1. The target pathogens were detected with PCR in all of the legume species.

Table 1. Dry matter yield (kg ha^{-1}), dry matter content (%DM) of red clover, white clover, birdsfoot trefoil and lucerne with companion grass, and composition of species at Nibble, Järna in the second harvest year (2017).[a]

Treatments	Forage yield (kg DM ha^{-1})					Composition of species, average of 3 cuts			
	1st cut	2nd cut	3rd cut	Total	DM (%)	Sown legume	Other legume	Timothy	Weed
RC-TI	3,140	1,218^{b}	2,755	7,133^{a}	20.7^{c}	59.2^{b}	3.8^{b}	24.7^{b}	10.4^{b}
WC-TI	2,676	825^{b}	1,343	4,844^{b}	26.2^{a}	37.2^{c}	0.0^{c}	45.3^{a}	16.1^{a}
BFT-TI	2,862	1,014^{b}		3,876^{b}	26.4^{a}	40.4^{c}	6.2^{a}	24.3^{b}	7.8^{d}
LU-TI	3,214	3,085^{a}	2,228	8,517^{a}	23.6^{b}	74.6^{a}	2.2^{b}	16.4^{c}	6.4^{c}
CV[1]	13.9	14.0	14.8	9.3	5.0	11.0	12.8	14.2	9.2

[a] 1st cut in all treatments: 16 June, 2nd cut in: RCTI, WCTI and LUTI: 26 July, 2nd cut in BTFTI: 15 Aug, 3rd cut in: RCTI, WCTI and LUTI: 16 October. Different letters indicate significant differences according to Tukey's HSD-test ($P<0.05$). [1]Coefficient of variation.

Conclusion: Swards containing LU and RC showed high yield and high persistence, as legume contents in the second harvest year were significantly higher than for WC and BFT. WC suffered severely from root rot whereas LU was significantly healthier than RC and WC. Molecular detection showed prevalence of the target pathogens in all of the legume species; hence, BFT and LU show a tolerance to developing severe disease symptoms.

Biodiversity and forage quality of semi-natural and cultural grasslands in Lithuania

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Introduction: Conservation of semi natural grasslands is highly important for biodiversity protection as these grasslands provide habitats for numerous plant and animal species. Semi natural grasslands make up only 5% of the total agricultural grassland area in Lithuania; therefore, efforts need to be taken to encourage farmers to preserve them. The objective of this study was to evaluate biodiversity and forage quality of cultural and semi natural grasslands as an initial step of the project aimed at investigating social, environmental and economic value of natural and cultural grasslands in Lithuania.

Materials and methods: Plant species composition and forage quality were evaluated in cultural grasslands and two types of semi natural grasslands (*Molinia* meadow and lowland hay meadow) in the central – western part of Lithuania. Grassland fields were located on four private farms (one field per farm), two fields were cultural grasslands, one field was *Molinia* meadow and one was lowland hay meadow. Species composition was evaluated in three test plots in each cultural grassland field, 9 test plots were chosen in *Molinia* meadow and 13 test plots were chosen in lowland hay meadow. The size of the test plot was 1 m^2, plots were chosen randomly in the boundaries of the field, the least distance to the field margins and between the plots was 10 m. Each test plot was harvested for forage quality evaluation. Crude protein, dry matter digestibility (DMD), neutral detergent fibre (NDF) and water-soluble carbohydrates (WSC) were evaluated using near-infrared spectrometer NIRS-6500. The structure of fatty acids was determined using the gas chromatography method by analysing methyl esters of the fatty acids (Shimadzu GC – 2010 PLUS, Shimadzu Corporation, Japan).

Results: As expected, number of plant species was 2-3 times higher in semi-natural grasslands compared to cultural grassland. Cultural grassland plant biomass accumulated more WSCs and omega-3 fatty acids; however, it also contained higher percentage of NDF and lower percentage of proteins. Semi natural grasslands differed significantly among each other in several forage quality parameters as well as number of plant species. The lowest NDF, highest DMD and omega-6 content was assessed in *Molinia* meadow plant biomass samples (Table 1).

Table 1. Plant species richness and forage quality of semi natural and cultural grasslands.[1]

Trait (Mean ± SE)	Cultural grassland	*Molinia* meadow	Lowland hey meadow
Crude protein, %	7.7±0.69 a	12.8±0.74 b	14.4±0.57 b
Neutral detergent fibre, %	59.2±0.97 a	49.0±1.67 b	54.6±1.58 a
Water soluble carbohydrates, %	19.2±1.26 a	15.1±0.56 b	12.7±1.11 b
Dry matter digestibility, %	56.7±0.88 b	58.4±1.40 a	52.6±2.29 b
Omega-3, % of total fatty acid content	0.98 ±0.05 a	0 b	0.12±0.06 b
Omega-6, % of total fatty acid content	23.0±2.32 a	35.8±1.68 b	17.7±1.46 a
Number of species	7.5±0.34 a	22.7±2.03 b	15.8±1.16 c

[1] Means with a common letter are not different at 5% level (Fisher LSD).

Conclusion: Comparison of quality parameters of plant biomass, harvested in cultural grassland, *Molinia* meadow and lowland hay meadow demonstrated that semi natural grasslands can produce cattle feed of similar or even higher quality to that from cultural grasslands.

Theme 2.

Improving sown grasslands and their management for future challenges

Nitrogen fertilization of grass-clover leys

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Abstract

Grass-clover leys have advantages in terms of yield stability, feed quality and milk production if fertilizer application is optimized. Economically optimal N application, taking into account yield, protein content and clover value for milk production, vary from no N at high clover content (~50%) to more than 240 kg N ha^{-1} at low clover proportion (~20%). Thus, there is a need to differentiate fertilizer N application according to clover proportion, which may be affected by establishment, weather and sward deterioration over the years. Automated image analysis has opened the possibility for identifying clover content which, linked to decision support systems, can be used for differentiated fertilizer application. In-field variability in clover content needs further investigation, but an example of clover content variation from 0 to 70% across an 11 ha field is shown. Furthermore, the technology may assist other management decisions such as reseeding time. Compared to annual crops and grazed systems, under cutting-only management, nitrate leaching from fertilizer inputs is much reduced. In an experiment with increasing levels of N in cattle slurry, applied for up to 4 years, the nitrate concentrations were very low and no increase in leaching was found when applying cattle slurry at up to 300 kg total-N ha^{-1}yr^{-1}. However, there is a need to investigate consequences at the crop rotation level of fertilizer strategies for both nitrate leaching and N_2O emissions.

Keywords: grassland mixtures, nitrogen response, clover fraction, image analysis, precision agriculture

Introduction

Inclusion of forage legumes in grasslands reduces dependence on industrial fertilizer, lowers production costs and increases protein self-sufficiency (Lüscher *et al.*, 2014). Grass-clover leys have been demonstrated to increases yield stability (Frankow-Lindberg *et al.*, 2009) compared to fertilized grass-only leys, due to niche complementarity (Nyfeler *et al.*, 2009). Increasing clover proportions generally improves feed quality (Phelan *et al.*, 2015) due to the greater protein content of clover (Søegaard, 2009; Suter *et al.*, 2015) and enhances milk production (Johansen *et al.*, 2017) due to greater motivation to eat, even though digestibility may not be affected (Sturladóttir *et al.*, 2014). For sheep, the optimal proportion of white clover fed with ryegrass to optimise intake was 25-50% (Niderkorn *et al.*, 2017). Furthermore, grass-clover leys potentially have low levels of N leaching per hectare compared to annual crops – even under fertilized conditions – due to high N uptake and removal (Eriksen *et al.*, 2015).

Future scenarios for dairy production predict that milk yield per cow will increase considerably, which is only possible if feed quality is optimized and this is to some extent related to clover proportion (Johansen *et al.*, 2017). At the same time, farm size in terms of land and cows per farm, is increasing, which for high-yielding milking cows limits the proportion of the diet covered by grazing. In Denmark the majority of dairy cows are fed indoors – only 25% of the milking cows were grazed in 2015 (Kristensen and Søndergaard, 2017) – meaning that the cattle manure is collected and is available as fertilizer on the farm, including also for use on arable crops in mixed crop rotations. In order to optimize the fertilization of grass-clover leys it would be ideal to know the clover proportion in the sward, as the fertilizer response is greater at low clover proportions. Visual inspection of the clover proportion is associated with great uncertainty and may be impossible when large fields need to be covered. Therefore, automated and precise methods for estimating clover proportion in mixtures are needed. Over the last decade studies have shown that clover proportions can be estimated by analysing digital images (e.g. Himstedt *et al.*, 2009; Rayburn, 2014; McRoberts *et al.*, 2016), but in order to implement digital image analysis in practice, suitable camera equipment and decision support tools need to be in place, so that information on clover proportions can be used in 'close to real-time' to adjust fertilization levels.

This paper is on optimization of fertilizer N use in intensive grassland-livestock systems with short-term leys lasting typically 2-4 years and embedded in crop rotations on dairy farms, focusing on management practices and automated clover recognition that optimize yield and clover proportion for optimal silage quality, and potentially ensure a low environmental impact.

Fertilizer response

White clover (*Trifolium repens* L.) and red clover (*Trifolium pratense* L.), which togther with lucerne (*Medicago sativa* L.) are the most important forage legumes of the temperate zone (Halling *et al.*, 2004), are not dependent on fertilizer-N, as they can perform symbiotic fixation of atmospheric N, at times greater than 300 kg N ha^{-1} yr^{-1} (Carlsson and Huss-Danell, 2003; Rasmussen *et al.*, 2012). When grown with a companion grass, typically perennial ryegrass (*Lolium perenne* L.) that shows a yield response to N application up to 500 kg N ha^{-1} (Whitehead, 1995), the N fertilizer demand of the mixture depends on the clover content.

An example of the response of grass-clover mixtures to fertilizer N application is shown in Figure 1. Especially in the 1-yr-old swards there are considerable increases in DM production with fertilization, but with decreasing clover content as a result. Similarly, the crude protein content initially decreased followed by an increase at very high N levels. Clearly, optimal fertilization is a balance between yield, quality and N efficiency, and when calculating the economic optimum for N application it is necessary to use a model that includes yield, protein content and clover content.

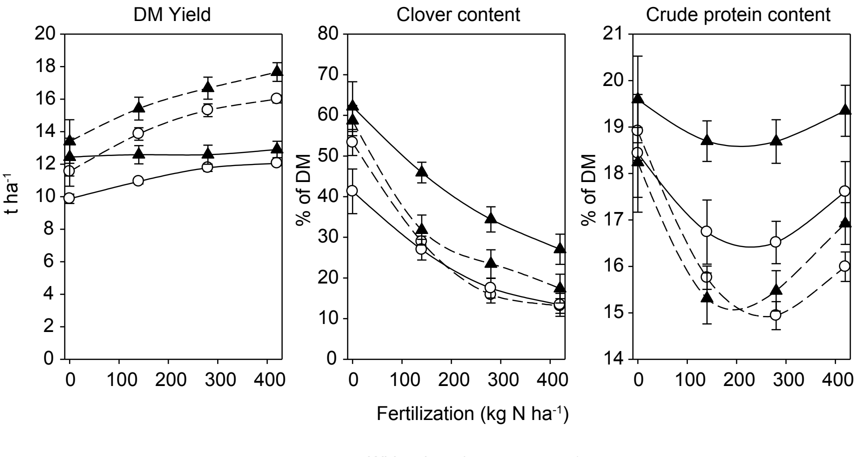

Figure 1. Yield, clover and crude protein content at increasing N fertilizer rates in 1st and 2nd production years of two grass-clover mixtures (from Birkmose and Frandsen, 2018). Average of three sites on sandy soil with cereals as previous crop. Fertilizer application in spring and before 1st and 2nd regrowth. Four and five cuts per year in 2- and 3-species mixtures, respectively.

Frandsen *et al.* (2019) estimated the net profit of forage production by valuing legume proportion and protein content for feeding of lactating cows and subtracting the cost of N fertilizer. In the case of a high clover content (~50%), N fertilizer application was uneconomic, but with a low clover content (~20%) the economic optimum was more than 240 kg N ha^{-1}. Thus, there is a very clear incentive to differentiate N fertilizer application according to actual or expected clover content.

Interaction with ley age

The common motivation for grassland cultivation is yield loss due to sward deterioration caused by, e.g. compaction from wheel traffic and invasion of less productive unsown species (Hoving and Boer, 2004). With the use of perennial ryegrass and clovers with low persistency to fulfil the requirement for high herbage quality in a diet with a high proportion of roughage, it is difficult to maintain leys for longer than 2-3 years. However, there has been little focus on the interactions between grassland mixtures, ley age and fertilization. In one experiment, fertilizer responses were similar across years and mixtures (Figure 2). Yields were maintained over a 4-year period in a mixture with white clover because increasing ryegrass yields compensated for the decreasing clover yield. In similar plots with ryegrass, white and red clover there was a considerable yield decrease over the years because ryegrass was not able to compensate for the clover yield decrease. However, this yield decrease may have been less in the three-species mixture in year 3 and 4 if the fertilizer application had been adjusted; for example, if based on actual clover proportions in the field.

Fertilization according to clover proportion

Skovsen *et al.* (2017) successfully used machine learning to analyse red, green and blue images of grass-clover mixtures, training a deep convolutional neural network to specify plant species in every pixel of the image. The relationship between the convolutional neural network-driven analysis and the actual dry matter-based clover content in photographed patches is shown in Figure 3. Across a wide spread in

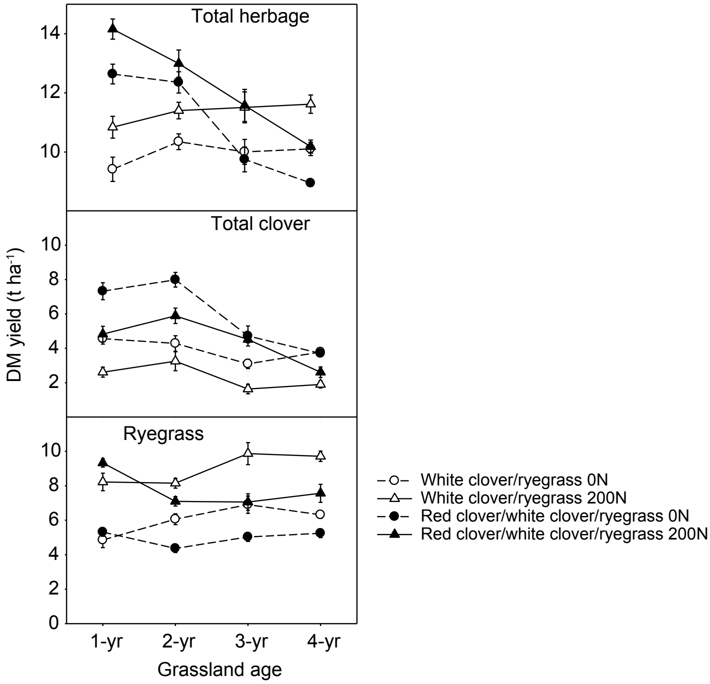

Figure 2. Annual yields (total herbage, clover and ryegrass) in successive years of two- and three-species swards (from Eriksen *et al.,* 2014).

yield, clover content, weeds and time in the season, the clover dry matter content was predicted with a standard deviation of 8 percentage points across all sample pairs. The digital image analysis has been commercialized in the CloverCam system by Agrointelli for mounting on a mower (Figure 3), from which GPS coordinates and clover content is continuously uploaded to a decision support tool (SEGES Crop Manager, Frandsen *et al.*, 2019) where the farmer can monitor the variations in clover content for use in fertilizer decisions. Although, the technique has been shown to work, there is still a need to enhance the stability across different mixtures of species and varieties, and improve the understanding of how these potentially respond differently to fertilization. Initial work on distinguishing between white and red clover using this methodology demonstrates this is possible, but also there are limitations due to different vertical distribution of these species in the sward (Skovsen *et al.*, 2018). In the spring cut, where the sward was high, white clover was typically not visible in the canopy from above. Furthermore, the potential application of the technique depends on the in-field variability in clover content. This is largely unknown and needs investigation, but the expectation is that on real farms there is considerable variation. An example of real-farm variability based on 122 images per ha is shown in Figure 4, where the clover proportion in autumn varied from 0 to 70% across a field of 11 ha. The automated image analysis also opens the possibility for electronic mapping of weeds. Within five years after establishment of a grass-

Figure 3. The Clovercam System by Agrointelli. A high-resolution RGB-camera and a processing terminal, displaying the current legume proportion.

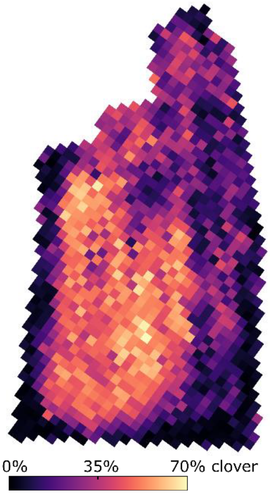

Figure 4. Interpolated map of the clover content in an 11-ha grass-clover field, based on 1380 images corresponding to 122 images per hectare (from Skovsen *et al.*, 2019).

clover sward, increasing proportions of weed species such as dandelion and couch grass are often found (Trott *et al.*, 2004) causing severe sward deterioration. The possibility of taking this into account may influence fertilizer application rates as well as decisions on when to reseed the sward. Thus, for optimal fertilizer application, there is a need to develop models where estimation of clover proportion at one point in time can be used for predicting the response of fertilizer application.

Environmental consequences

In most annual crops increasing N inputs results in increased N leaching losses. In grazed systems a close link between inputs of N fertilizer or via N_2-fixations and nitrate leaching has also been shown; i.e. increasing total N input gave an exponential increase in nitrate leaching associated with increased dry matter production, N uptake and N recycling in animal excreta (Ledgard *et al.*, 2009). However, in cutting-only systems, this link appears to be much weaker, where N leaching was demonstrated to be independent of application level for inputs up to 350 kg N ha^{-1} (Wachendorf *et al.*, 2004). The effect of fertilizer N application should be evaluated not only in the short term though, since continued application of N, especially animal manure, may give rise to long-term effects increasing N leaching. In an experiment with increasing levels of N in cattle slurry applied for up to 4 years (Figure 5), volume-weighted nitrate concentrations were all far below the EU limit for drinking water (11.3 mg NO_3-N l^{-1}) and with no increase in leaching when applying up to 300 kg total-N $ha^{-1}yr^{-1}$ in cattle slurry. Yield response to slurry application of up to 300 kg total-N ha^{-1} was found in the 1-yr-old sward, but only to 200 kg total-N in the 2-4-yr-old swards. However, this application above yield maximum did not result in increased nitrate leaching, only in decreased clover clover content (not shown). Similarly, others have found that nitrate leaching stays low for fertilization levels clearly higher than for grazed systems, up to a threshold value after which it increases exponentially (Delaby *et al.*, 2014).

In addition to N leaching, fertilizer application may also increase the risk of fertilizer-specific losses of N_2O. It has been found that fertilizer N, compared to biological N fixation, increases N_2O emissions (Klumpp *et al.*, 2011; Li *et al.*, 2011; Schmeer *et al.*, 2014). Furthermore, differences in fertilization may affect soil organic C and N build-up and the well-known residual effect in subsequent crops (Rasmussen *et al.*, 2012; Eriksen *et al.*, 2008; Johnston *et al.*, 1994), thus mitigating the fertilizer GHG-effect. The effect of fertilization in this context is, however, not known, as fertilization will increase the grass component of grass-clover swards changing the composition of crop residues added to soil (Eriksen, 2001; Fox *et al.*, 2018).

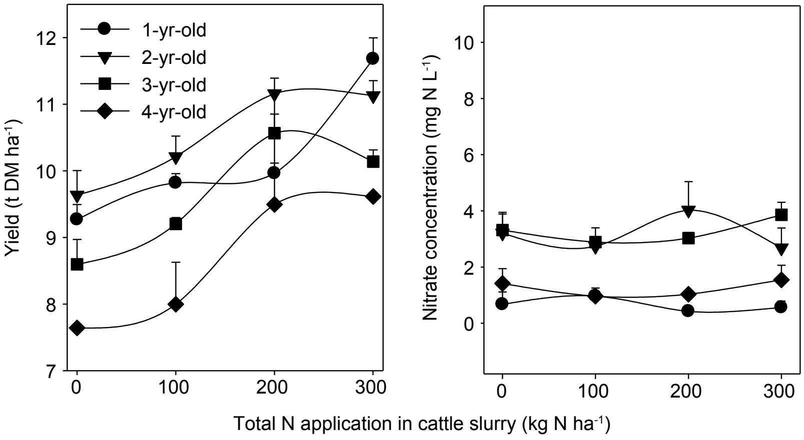

Figure 5. Annual yield of grass-clover mixtures and associated volume-weighted nitrate concentrations in leachate from 1 to 4-yr-old grass-clover leys with annual cattle slurry application

Conclusion

There is a huge potential for improving fertilizer N application in intensive grassland-livestock systems with leys by avoiding uneconomical applications of N in clover-rich swards and applying optimal levels in swards with little clover content. Automated image analysis is a possibility for identifying clover content for differentiated fertilizer application that may be especially attractive on farms with a huge ley area or where contractors carry out the fertilisation. This can improve yield, quality and potentially the persistence of the swards. Furthermore, the technology may assist other management decisions such as reseeding time. Compared to annual crops and grazed systems, under cutting-only management, nitrate leaching from fertilizer inputs is much reduced. However, there is a need to investigate consequences at the crop rotation level of fertilizer strategies for both nitrate leaching and N_2O emissions.

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Does nature protection status affect grassland utilisation and farming? – A German case study

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Abstract

Grassland area and management are affected by various national strategies, agricultural funds and regulations, especially grassland in conservation areas. To depict changes, we analysed annual data of the Integrated Administration and Control System for the period 2010 to 2015 from nine federal states of Germany. We see a positive trend of grassland area, as a whole and in conservation areas. In nature conservation areas the grassland is dominantly managed by large farms specialised on grazing. This implies that policies focussing on biodiversity protection in grassland must especially tackle these farms. Furthermore, we conclude that the conservation status of an area has effect on grassland development, farm size and farm type.

Keywords: grassland area, nature conservation, farm type, management

Introduction

Recently national strategies, agricultural funds and regulations aim to protect grassland and enhance its biodiversity, thereby probably affecting grassland area and management. Both are important triggers on biomass yield, carbon storage, climate protection (Jones and Donnelly, 2004) and biodiversity (Isselstein *et al.*, 2005). To assess trends and changes a variety of statistical and geographic data on grassland area are available, but these often lack in timeliness, spatial coverage and information on management. We aim to depict recent trends in grassland area and management in Germany based on data of the Integrated Administration and Control System (IACS). Thereby we take a closer look on nature conservation status (NCS) as a possible trigger on grassland management.

Materials and methods

We analysed grassland in the period 2010 to 2015 based on data of Integrated Administration and Control System (IACS) data of nine federal states (Brandenburg/Berlin, Baden-Wuerttemberg, Rhinland-Palatine, Schleswig-Holstein/Hamburg, Lower Saxony/Bremen, North Rhine Westphalia). The data set covers 58% of the agricultural land in Germany. IACS is part of the Common Agricultural Policy of the European Union (CAP) managing the area-related payments to farmers by establishing an annual database on land use and animal stocks. The advantage of IACS is its high temporal and spatial accuracy related to land use and the additional information on farm structure and animal stocks. To enable time series and spatial analysis we created a national 10×10 m point matrix. To each point of the matrix, we assigned relevant information on IACS land use and NCS, based on geographic location and spatial shares within the 10×10 m cell (Nitsch *et al.*, 2017). Until 2014 an IACS land parcel could contain arable and grassland plots, due to the fact that the location of the plots within the parcel are not mapped, we calculated the grassland area with NCS of each parcel based on the spatial shares of grassland and land under conservation. A high NCS was assigned to Natura 2000 sites, Biosphere Reserves, and nature conservation areas.

Results and discussion

In our study area about 2.48 mio. ha of grassland were cultivated in 2010. A high share of this grassland (approx. 68%) was located in areas with an NCS, 22% even in areas with a high NCS. Grassland area has increased from 2010 to 2015, the increase was smallest in areas with a high NCS after 2014 (Figure 1).

The grassland within areas of high NCS was generally managed by larger farms. Especially the share of grassland managed by farms with more than 100 ha of grassland was more than three times higher in areas with a high NCS (Figure 2).

The dominant farm types differ markedly. In areas without NCS, specialised dairy farms prevail in grassland utilisation (Figure 3). In areas with high NCS dairy farms manage less than 20% of the respective grassland, whereas farms specialised in grazing prevail.

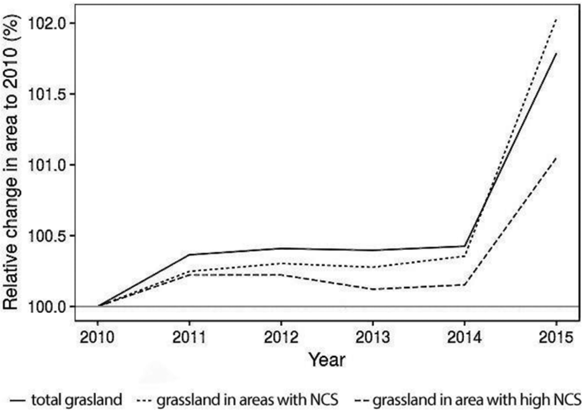

Figure 1. Growth rate of grassland area from 2010 to 2015 in relation to 2010.

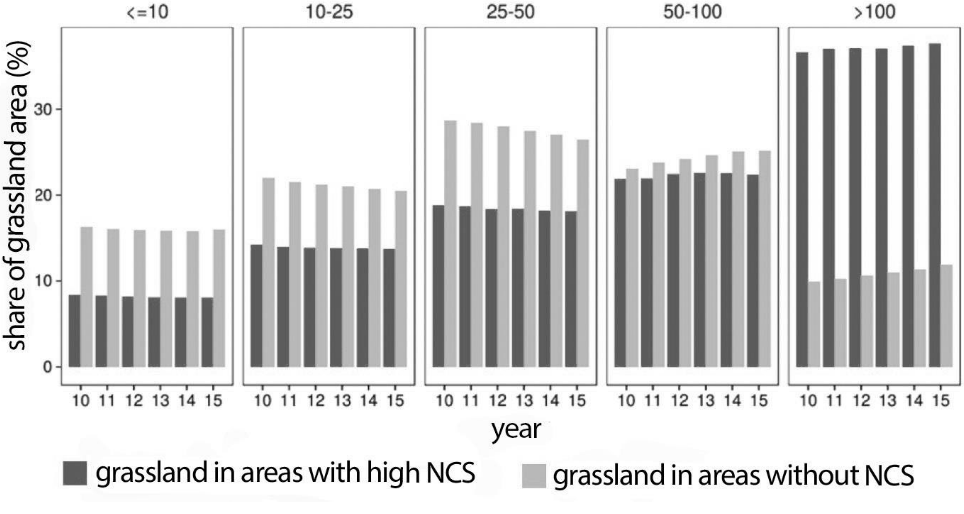

Figure 2. Share of grassland cultivated by farms of different size classes (boxes), in relation to the total grassland in areas with or without NCS (colours).

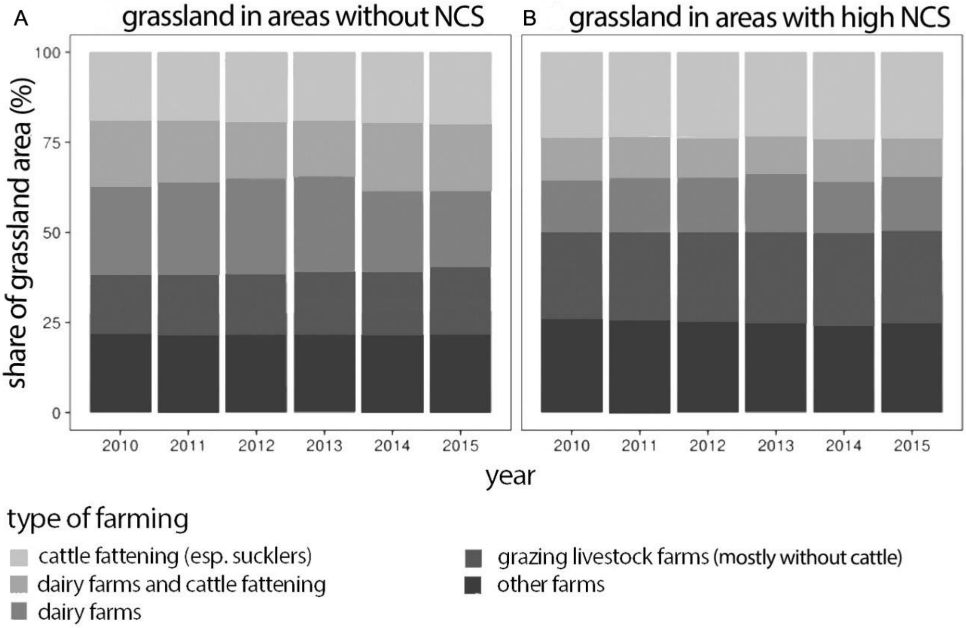

Figure 3. Types of farming (A) outside of and (B) inside of nature conservation areas.

Conclusion

The overall trend of grassland area in IACS for Germany is positive, especially in areas with high NCS. Dairy farms prevail in the utilization of grassland in areas without NCS. The grassland in areas with high NCS is predominantly managed by large farms keeping suckler cows, or grazing sheep, horses and so on. This implies that with respect to issues of biodiversity protection in grassland policies must especially tackle these farms, while questions related to the dairy farms are of secondary importance.

Acknowledgements

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The effect of fertilizers on grass dry matter yield and biomass quality

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Abstract

In a grassland experiment at the Research and Study farm 'Peterlauki' (56°53'N, 23°71'E) of the Latvia University of Life Sciences and Technologies (LLU), it was found that fertilizer is an essential factor in increasing the dry matter yield of grasses. Combined application of phosphorus and potassium (P80K120) increased the dry matter yield of five grass species (*Phalaris arundinacea*, *Festulolium*, *Phleum pratense*, *Festuca pratensis,* and *Festuca arundinacea*) by 21% on average (from 9% for tall fescue to 31% for timothy and 32% for meadow fescue). When ≥90 kg ha^{-1} of nitrogen was used, biomass production of all grass species increased considerably. No significant differences in phosphorus concentration were observed for the fertilizer variants, whereas potassium concentration increased under the influence of both fertilizer systems. In unfertilized variants, 49.7 kg ha^{-1} of N, 27.8 kg ha^{-1} of P_2O_5, and 117.2 kg ha^{-1} of K_2O were removed by plant biomass from the soil. When grasses were fertilized with phosphorus and potassium (P80K120), the removal of plant nutrients increased by 17% of nitrogen, 16% of phosphorus, and 23% of potassium.

Keywords: grasses, dry matter yield, nutrient concentration

Introduction

In order to establish perennial grass swards from which high-quality yields can be obtained, proper fertilization is required. Fertilizer efficiency directly depends on the soil, agroclimatic conditions of a region, cultivation technologies applied, and the species and varieties of grasses. Trials with perennial grasses have shown that regular application of mineral fertilizers leads to at least a 30-50% increase in dry matter yield (Adamovics and Gutmane, 2016). It has also been found that phosphorus and potassium fertilizers have a positive effect on perennial grass productivity (Christian *et al.*, 2006).

In the long-term use of only two-component fertilizers, such as nitrogen and phosphorus without potassium, or only phosphorus and potassium-free nitrogen, the productivity of grass sward decreases, its botanical composition changes, and the soil nutrient content depletes. The purpose of the research was to find out how grass dry matter yield and its chemical composition change.

Materials and methods

A field trial was conducted at the 'Peterlauki' Study and Research Farm (56°53' N, 23°71' E) of the Latvia University of Life Sciences and Technologies from 2012 to 2016. Soil characteristics: sod calcareous soil Luvisols (according to FAO classification); granulometric composition: heavy dusty sand clay. Soil agrochemical parameters: pH KCL 6.7 (LVS ISO 10390: 2006); organic matter content: 21 g kg^{-1} (by Tyurin method, LV ST ZM 80-91), phosphorus content: 52 mg kg^{-1} P_2O_5, and potassium content: 128 mg kg^{-1} K_2O (according to Egner-Rhym method, LV ST ZM 82-97). The following grass species were included in the study to be used for fuel (pellet) production: reed canary grass (*Phalaris arundinacea*), festulolium (*Festulolium*), meadow fescue (*Festuca pratensis* Huds.), timothy (*Phleum pratense* L.), and tall fescue (*Festuca arundinacea* Schreb.). Summer barley was grown for two years before establishing the energy grasses. The experimental plots were sown with a seed-drill 'Hege 80' on May 10, 2011, and the seed rate was 1000 germinating seeds per square meter. The fertilizer variants were: N0P0K0 (control), P_2O_5 – 80 kg ha^{-1}, K_2O – 120 kg ha^{-1} (=PK – background), PK+N30, PK+N60, PK+N90, PK+N120, PK+N150, and PK+N180 kg ha^{-1}. The application of mineral fertilizers started in the year after sowing. The plot size was 10 m^2; the variants were arranged randomly, each in three replicates. Chemical composition of plants was determined for the first cut using the following methods: dry matter content (DM) – dried grass biomass was ground and dried in the oven at a temperature of 105° C for four hours; N – by modified Kjeldahl. The mineral elements P and K were analyzed by atomic adsorption spectrometry. The data were statistically analyzed using a two-way analysis of variance with grass species and fertilizer as factors, and the difference among means was detected by LSD at the $P<0.05$ probability level (Excel for Windows, 2003).

Results and discussion

Dry matter yield of perennial grasses was dependent on the type and level of fertilizer applied (Table 1). PK-fertilizer significantly ($P<0.05$) increased the DM yield of grasses – on average by 0.85 t ha^{-1} or 21% compared to the unfertilized control variant (without fertilizer application). Regarding the species of grasses, PK-fertilizer increased the DM yield of grasses by 0.44 t ha^{-1} for tall fescue (+9% compared to control) and by 1.16 t ha^{-1} for timothy (+31% compared to control). The second highest increase in DM yield was for meadow fescue – by 1.10 t ha^{-1} or 32% compared to control. Nitrogen fertilizer, compared to the background variant (PK), significantly ($P<0.05$) increased grass dry matter yield by 1.98 t ha^{-1} with 60 kg ha^{-1} N respectively by 2.91 t ha^{-1} with 120 kg ha^{-1} N.

For all grass species included in the study (reed canary grass, festulolium, meadow fescue, timothy, and tall fescue), a significant increase in DM yield was observed with the application of nitrogen. Dry matter yields were 1.81, 2.02, 1.83, 1.70, and 1.60 t ha^{-1} higher with 60 kg ha^{-1} N compared to PK-fertilizer), whereas the increase in nitrogen rates of up to 90 and 120 kg ha^{-1} N, produced no further significant increase ($P>0.05$) in DM yield.

In the PK-variant, the nitrogen and phosphorus content in grass biomass decreased on average by 1 and 7%, respectively (Table 2), whereas potassium content increased by 2% compared to the control variant. The application of 60 kg ha^{-1} N caused a lower content of nitrogen and phosphorus in grass DM yield – by 3 and 10%, respectively, compared to the control variant, and by 2 and 4%, respectively, compared to the PK-variant. The application of 90 and 120 kg ha^{-1} N increased the content of both nitrogen and potassium in grass biomass, as well as also slightly increased the phosphorus content, which, however, was lower compared to the control and PK-variant.

Out of all grass species studied, the highest nitrogen content in DM was detected for meadow fescue: from 1.53% (control variant) to 1.77% (N90P80K120). The highest increase in nitrogen content of the biomass of all grass species was observed when ≥90 kg ha^{-1} N were applied. From the studied grass

Table 1. Effect of nitrogen fertilizer on the dry matter yield of grasses (average of 2012-2016).[1]

Fertilizer applied,	Grass species (F_B) – dry matter yield, t ha^{-1}					
NPK, kg ha^{-1} (F_A)	Reed canary grass	Festulolium	Timothy	Meadow fescue	Tall fescue	Mean
0-0-0	4.64	3.93	3.80	3.42	4.67	4.28
0-80-120	5.20	4.89	4.96	4.52	5.11	5.13
60-80-120	7.01	6.91	6.79	6.22	6.71	7.11
90-80-120	6.95	7.09	7.52	6.57	6.52	7.50
120-80-120	7.28	7.32	8.06	7.15	7.13	8.04
Mean	6.22	6.16	6.23	5.58	6.25	6.41
$LSD_{0.05}$ F_A= 1.14; F_B= 0.37; F_{AB}= 1.84						

[1] LSD = least significant difference.

Table 2. Impact of fertilization on the chemical composition of grasses (average of 2012-2016).

NPK, kg ha^{-1}	Species of grasses – content of NPK in % of DM					NPK content (on average)	
	Reed canary grass	Festulolium	Timothy	Meadow fescue	Tall fescue	%	g kg^{-1}
Nitrogen (N)							
0-0-0	1.34	0.95	1.14	1.53	1.01	1.17	11.7
0-80-120	1.04	0.95	1.14	1.73	1.00	1.16	11.6
60-80-120	1.08	0.93	1.09	1.62	0.99	1.14	11.4
90-80-120	1.17	1.32	1.44	1.77	1.17	1.37	13.7
120-80-120	1.12	1.33	1.43	1.61	1.08	1.32	13.2
Phosphorus (P_2O_5)							
0-0-0	0.31	0.28	0.29	0.30	0.30	0.30	3.0
0-80-120	0.29	0.24	0.28	0.32	0.29	0.28	2.8
60-80-120	0.27	0.24	0.25	0.34	0.26	0.27	2.7
90-80-120	0.29	0.29	0.26	0.35	0.28	0.29	2.9
120-80-120	0.28	0.29	0.26	0.35	0.26	0.29	2.9
Potassium (K_2O)							
0-0-0	2.25	2.50	2.26	2.56	2.30	2.37	23.7
0-80-120	2.33	2.25	2.36	2.66	2.50	2.42	24.2
60-80-120	2.29	2.34	2.57	2.65	2.50	2.47	24.7
90-80-120	2.12	2.70	2.60	2.76	2.25	2.49	24.9
120-80-120	2.19	2.77	2.57	2.74	2.51	2.56	25.6

species, also the highest phosphorus content in DM was found in meadow fescue. Changes in phosphorus content of the various grass species were similar for all fertilizer variants, but the slight differences were not significant. Potassium content in the biomass of grasses increased mostly under the influence of both PK fertilizer and NPK fertilizer.

Conclusion

1. For all grass species studied, a significant ($P<0.05$) increase in dry matter yield was achieved at a level of 60 kg ha^{-1} N, whereas the increase in dry matter yields for a level of 90 and 120 kg ha^{-1} N was not significant.
2. The highest nitrogen content in DM was found for meadow fescue. The nitrogen content in the biomass of all grass species increased when ≥90 kg ha^{-1} N were applied. Potassium content in the biomass of grasses increased under the influence of both PK fertilizer and NPK fertilizer.

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The unknown 25% – What kind of grassland is not reported to IACS?

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Abstract

Grassland is of great importance for biodiversity and climate protection. Against this background it is important to know where grassland is located and how much grassland exists. However, in Germany, the area of grassland stated by the land registry offices is 25% higher than the grassland area according to IACS data (Integrated Administration and Control System). The question is what kind of grassland is not covered by IACS. To answer this question we analysed the data of six federal states accounting for 53% of Germany's total grassland area. We analysed the distribution of IACS and non-IACS grassland with the help of a 10×10 m point raster. Using Logit-models we determine the influence of various natural and legal factors on the likelihood that grassland is registered in IACS. However, our results show that the chosen variables have only a minor explanatory power.

Keywords: grassland, agriculture, land use, IACS, ATKIS Base DLM, logit models

Introduction

Regarding the extent of grassland in Germany, there are relevant differences between the different data sources. For instance, the statistical offices state that 2.5 million ha of grassland are agriculturally used in the federal states of Brandenburg (BB), Baden-Wuerttemberg (BW), Lower Saxony (NI), North Rhine-Westphalia (NW), Rhineland-Palatinate (RP) and Schleswig-Holstein (SH) in 2015 (Statistisches Bundesamt, 2016). This figure is primarily based on information derived from the Integrated Administration and Control System (IACS). The IACS is part of the Common Agricultural Policy of the European Union (CAP) managing the area-related payments to farmer. In contrast according to the digital landscape model (ATKIS Base DLM) which is operated by the surveyor's offices grassland covers for the same federal states and year are 3.2 million ha or 26% more than stated by the IACS (own calculation). Due to the general climatic conditions grassland as land cover in Germany is dependent on regular utilization.

Against the background of the great importance especially of extensively managed grassland for biodiversity (Isselstein *et al.*, 2005) and grassland in general for climate protection (Jones and Donnelly, 2004) it is interesting to know what kind of grassland is not reported by IACS. Thus, our research question is: Do certain abiotic or legal factors influence the likelihood that grassland is registered in the IACS?

Materials and methods

The study covers the German federal states of BB, BW, NI, NW, RP and SH covering more than 50% of Germany's grassland area. A German wide 10×10 m point raster was created where each point contains information on local characteristics like ATKIS land cover, protection status, slope, and elevation (Nitsch *et al.*, 2017). Part of the IACS is the Land Parcel Identification System (LPIS) covering all agricultural areas receiving EU funds. An LPIS parcel may consist of several fields with different agricultural land uses. In these cases we have to use probabilities based on field size as we do not know the exact location of all agricultural land uses (cf. data selection for logit models below).

Our multivariate logit model has a binary dependent variable indicating whether the grassland is registered in ATKIS only (0) or in IACS (1). We consider the ATKIS code (obaa/attr) 43001/1020 as grassland, excluding for instance heath, swamp or mire. We include the following independent variables in the model: soil climate region (nominal), area flooded every 100 years (HQ100, binary), area designated according to nature protection laws (Natura 2000 areas, biosphere reserve and/or nature reserve; binary), peatland (binary), slope, elevation and soil quality index. For the last three variables we included also the squared transformed variable to increase the flexibility of the function form (e.g. depicting saturation effects).

The initial data set (used for Figure 1) contains 444 million raster points that are either recorded in ATKIS as grassland and/or in IACS. For the logit models (Table 1) we excluded the following data: (1) ATKIS grassland within IACS but not registered as grassland in IACS (but e.g. as arable land), (2) points registered in IACS with a probability of being grassland below 90%. From the remaining 277 million points we draw five random samples comprising each for 0.5% of the data set whereat the sampling method Bernoulli was chosen. We present the model with the median model accuracy (described by Nagelkerke's pseudo R^2) for result description. In order to depict the absolute and relative importance of the variables we run the model with and without z transformation. The analyses were conducted by PostgreSQL (version 9.5) and R (version 3.2.3).

Results and discussion

Especially in arable farming regions with intensive livestock farming in northwestern (Muensterland, Vechta-Cloppenburg) and northeastern Germany (Oderbruch) as well as in the arable farming regions in BW and RP the grassland area in ATKIS is by relatively larger than the one registered in IACS (Figure 1).

We calculated three logit models to compare the explanatory power of the different variable types: (1) soil climate regions only, (2) site specific variables only and (3) both types of variables together. This analysis shows that 47% of the explained variation can be exclusively attributed to interregional differences (soil climate regions), while 26% are explained by the various site-specific variables (see pseudo R^2 of the different models in Table 1). Being located on peaty soils or in an area flooded once per century increases the odd of being registered in IACS by 58% and 26%, respectively. For every 100m increase in elevation the odds of being registered in IACS increases by 31%. Our results show that the location of a grassland plot within a designated area for nature protection is only marginally influencing the likelihood of being registered in IACS (Table 1).

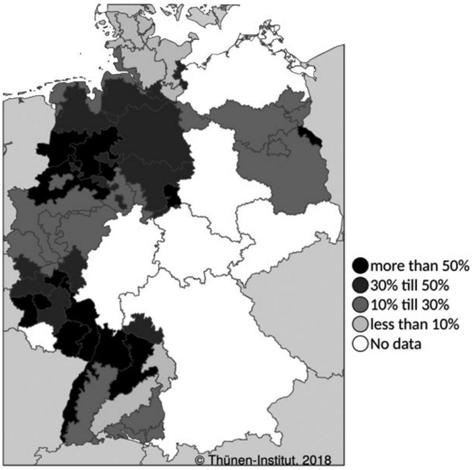

Figure 1. Proportion of ATKIS grassland outside of IACS in relation to IACS grassland in the soil climate regions of BB, BW, NI, NW, RP and SH in 2015.

Table 1. Results of the logit models: all variables, all variables z transformed, only site-specific variables, only soil climate regions.

All variables:	**Coefficients**			**All var., z transformed**		
	Estimate	**Std. Error**	**Effect (%)**	**Estimate**	**Std. Error**	**Effect (%)**
Dummy var. (soil climate regions, intercept)						
Site specific factors						
Nature protection	4.0 E-02	5.7 E-03	4.1	1.7 E-02	2.4 E-03	1.7
Peatland	4.6 E-01	6.8 E-03	58.0	1.9 E-01	2.8 E-03	20.6
HQ100	2.3 E-01	9.0 E-03	25.7	6.3 E-02	2.5 E-03	6.5
Soil quality index	1.8 E-02	7.2 E-04	1.8	3.2 E-01	1.3 E-02	37.4
(Soil quality index)^2	-1.8 E-04	7.5 E-06		-3.0 E-01	1.3 E-02	
Elevation	2.7 E-01	7.4 E-03	31.0	5.9 E-01	1.6 E-02	84.4
(Elevation)^2	1.7 E-03	8.2 E-04		2.5 E-02	1.2 E-02	
Slope	-2.9 E-02	8.8 E-04	-2.9	-2.1 E-01	6.3 E-03	-18.0
(Slope)^2	5.3 E-05	2.6 E-05		1.1 E-02	5.1 E-03	
Pseudo R^2: Nagelkerke	0.084			0.084		
	Pseudo R^2**: Nagelkerke**					
Soil climate region (only):	0.062					
Site specific factors (only):	0.045					

The regional analysis as well as the Logit models indicate that the relative difference between the grassland area recorded in ATKIS and the one in IACS is negatively correlated to overall grassland share in a region. This could be due to a base effect: i.e. in all areas a more or less fixed share of grassland (cover) on the total agricultural area exists that is not agriculturally used for various reasons. However, the share of the agricultural land used as grassland declines as the conditions for arable cropping are getting better.

Conclusion

The various natural and legal factors explain only a small share of the likelihood that an ATKIS grassland is registered in IACS. Therefore, we assume that other factors like farming structure and intensity as well as general demographic factors are more important to understand the distribution of IACS grassland in dependence of ATKIS grassland.

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The effect of soil variability and nutrient management within the farm on soil fertility status

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Abstract

Farms dominated by soils with high proportions of fine soil particles (silt and clay) offer many challenges in improving soil fertility (pH, Phosphorus (P) and Potassium (K) status) due to their inherent chemical properties. They require intensive and specific fertiliser application strategies to achieve their full agronomic potential. It is one of the main factors inhibiting the output of these heavy soils. Soil tests carried out over four years, across six heavy soil farms shows that 0-55% of paddocks are in the optimum range for pH, P and K in any given year. Despite regular application of significant quantities of nutrients, nutrient levels, particularly P, remain low. The objective of this study was to examine paddock scale historical data on farm to assess soil fertility dynamics across a range of heavy soil types. Farm outputs were related to nutrient status in order to understand the fate of nutrients; taken up by the grass sward, stored in the soil or lost through leaching/ runoff. Results indicate that nutrient build-up on heavy soils requires significant investment of resources and extended time periods. In this study it was found that across soils a surplus of 78.7 kg ha^{-1} P is required to increase soil test phosphorus (STP) by 1 mg l^{-1} and 30.5 kg ha^{-1} of K to increase soil test potassium (STK) by 1 mg l^{-1}.

Keywords: heavy soils, phosphorus, potassium, farm requirement

Introduction

An adequate supply of P is essential in agricultural soils to meet the demands of grazing animals and crop growth. (Daly *et al.,* 2015) The restricted nature of rock phosphates and the dependence on imports from China, North Africa and Asia into Europe is coming under pressure as reserves diminish (Cordell *et al.,* 2009). 75% of P imported is used to manufacture fertiliser for agriculture and 11% is used in animal feeds and food production (Johnston *et al.,* 2000). In many European countries, recommended rates of P and K fertiliser are based on the index system where there are targets for soil test P and K which help guide application rates. In Ireland, soil test phosphorus (STP) is measured through Morgan's extractable P and is categorized in 4 indices (1-4), index 1 (deficient), index 2 (low), index 3 (optimum) and index 4 (excessive) (Lalor *et al.,* 2008) and similarly with K. For optimal grassland production, the desired soil pH is 6.3 on mineral soils while (P) should range from 5.1-8.0 mg l^{-1} (Morgan's extractable P) and (K) should range from 100-150 mg l^{-1} (Morgan's extractable K). Where lacking, P and K are necessary in surplus amounts in order to increase soil P concentration and consequently achieve the optimum agronomic potential in the soil. On mineral soils, a recommended application rate of 34 kg ha^{-1} P and 90 kg ha^{-1} K is required to increase soil test from index 1 to index 3 (Wall and Plunkett, 2016). As P accumulates in the soil (Gourley *et al.,* 2010), it builds fertility, or may be absorbed and locked up particularly in high clay content soils with significant proportions of iron and aluminium. (Kurz *et al.,* 2005). It can be lost in eroded material or lost through leaching when in soluble form (Heathwaite 1997). The maximum annual allowance of P fertiliser at a stocking rate of 2 LU ha^{-1} is between 39 kg ha^{-1} on index 1 soils and 0 kg ha^{-1} on index 4 soils (Communities, 2010). The objective of this study was to examine farm and paddock scale historical data to assess soil fertility dynamics and determine nutrient use efficiency, particularly in terms of P, across a range of heavy soil types.

Materials and methods

This was a longitudinal study on six Irish grassland dairy farms across a range of agroclimatic regions between January 2014 and December 2017. Nutrient balances at paddock scale (per hectare basis) were used to assess the relationship between nutrient application and soil nutrient levels. Nutrient balance was calculated per paddock based on nutrient inputs (chemical/organic fertiliser, concentrates) and off-takes (milk, meat, silage, grass). Changes in soil test pH, P and K were correlated with the application of lime, P and K. The grass based, spring calving dairy farms based in the Republic of Ireland were chosen through the Teagasc Heavy Soils Programme, which was established to demonstrate methods to improve grassland productivity and utilisation, decrease volatility and sustain viable farm enterprises on poorly drained soil. A prerequisite for farmer selection was that farms had to have a proven record of managing grassland, measuring grass, and recording fertiliser and feed inputs and farm production. The farm locations are as follows: Kishkeam, Cork (1), Athea, Limerick (2), Castleisland, Kerry (3), Rossmore, Tipperary (4), Doonbeg, Clare (5), Ballinagree, Cork (6). These farms are situated in high rainfall areas (long-term annual averages of 982-1757 mm) with poorly drained soils and high proportions of clay and organic matter (OM). Historic soil fertility records along with fertiliser, meal and slurry inputs and grass, silage, milk and meat outputs were recorded by farmers and formulated to derive a nutrient balance at paddock scale each year. A total of 162 paddocks were examined in the study and relationships between various parameters were analysed by correlating soil tests against surplus nutrients applied to establish key factors in the soil fertility status over a four-year period. In year 1, soil test records showed an average soil pH of 5.8 (range: 5.0-6.4), an average P value of 3.5 mg l^{-1} (range: 0.2-11.8) and an average K value of 103.8 mg l^{-1} (range: 28.0-280.0). In year 4, soil test results showed an average soil pH of 6.4 (range: 5.2-7.4), an average P value of 5.61 mg l^{-1} (range: 1.0-21.4) and an average K value of 101.7 mg l^{-1} (range: 30.4-275.0) (Table 1). The data was analysed using SAS version 9.4.

Results and discussion

Grass growth was significantly impacted by STP, STK and soil pH ($P<0.05$). On the milking platform, P surplus and change in soil test P had a significant impact on grass growth ($P<0.05$). STP and STK was more strongly influenced by soil test pH ($P<0.05$) than the application of surplus P and K fertiliser.

Farm 2 (59% (of farm area) Stagnic Brown Earth) had a significantly higher P requirement in comparison to Farm 6 (52% Brown Earth) (Figure 1A). Farm 5 (51% Gley) had a negative requirement for P (the average surplus P applied to Farm 5 decreased STP) due to the high organic matter content in the soil. Farm 1 (55% Stagnic Luvisol) had a surplus K requirement of 38 kg ha^{-1} and Farm 6 had a K surplus requirement of 108 kg ha^{-1} to increase STK by 1 mg l^{-1} (Figure 1B).

Table 1. Proportion of paddocks within each phosphorous and potassium index (Lalor *et al.*, 2008), in year 1 (2014) and year 4 (2017) of the study.

Index	Phosphorus (P) (mg l^{-1})	Potassium (K) (mg l^{-1})	Proportion of paddocks/index (year 1)		Proportion of paddocks/index (year 4)	
			P	K	P	K
1	0.0-3.0	0-50	0.39	0.11	0.30	0.11
2	3.1-5.0	51-100	0.30	0.44	0.27	0.45
3	5.1-8.0	101-150	0.24	0.28	0.28	0.27
4	>8	>150	0.07	0.16	0.15	0.17

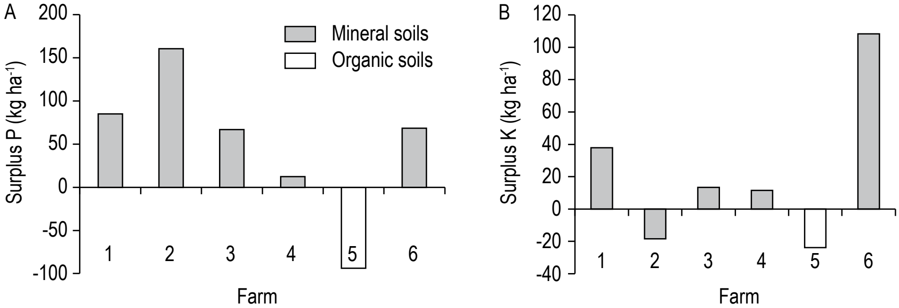

Figure 1. (A) Surplus Phosphorus (kg ha^{-1}) and (B) surplus Potassium (kg ha^{-1}) required to increase STP, respectively STK, by 1 mg l^{-1} on each mineral soil farm.

Nutrient requirement varied between soil types. Surplus P requirement varied from 218 kg surplus P ha^{-1} to increase 1 mg l^{-1} on a Stagnic Brown earth to 20 kg surplus P ha^{-1} on surface water gleys. Alluvial, Humic Ground Water Gleys, Iron Pan Podzolics and Peats had a negative change in STP in spite of the addition of significant quantities of P.

Conclusion

There is a substantial difference in nutrient requirement to increase STP across each farm. These variations are largely due to varying soil particles physical and chemical properties. The inability of soils with high proportions of soil organic matter (>20%) to build reserves is clearly evident from farm 5. There is a problem facing these heavy soils farms as regards achieving optimum soil fertility levels.

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Polyphenolic variability and quantification within leaf tissues of the commercial grass species *Dactylis glomerata*

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Abstract

Phenolic compounds are of growing interest within the biorefining industry, with their presence in *Dactylis glomerata* having previously been highlighted. Differences in the profile and accumulation of phenols between seven accessions and three sequential cuts were investigated. Methanolic extracts of these phenols were analysed and quantified by LC-PDA-MS^n, with compounds grouped into hydroxycinnamates, flavonoids and hydroxcinnamate-flavonoid conjugates. Total phenols per accession were also quantified for cuts one and two. Quantities of grouped compounds were significantly different for all cuts ($P<0.001$). Significant differences between total phenols per accession were seen within the initial cut ($P=0.008$) but not the second. Data suggested that accumulation of phenols was more greatly affected by cut than accession, with flavonoids consistently showing the highest content. Plants of a later cut were concluded to be optimal for biorefining and therefore any differences seen between accessions can be nullified and ruled out as a means of selective breeding.

Keywords: phenols, cocksfoot, bio-refining, hydroxycinnamate, flavonoid

Introduction

The prospect of co-extracting phenolic compounds from plants in a biorefinery is of growing interest to pharmaceutical and health food industries (Shahidi and Ambigaipalan, 2015). Due to anti-oxidising, anti-inflammatory and anti-carcinogenic properties, phenolic extracts can be deemed a high commodity product, with extracts from the Chinese herb *Salvia miltiorrhiza* comprising a substantial portion of the US$60 billion current global market for botanical extracts (Persistence Market Research, 2018). Hauck *et al.* (2013) reported on the composition of polyphenolic compounds in both fresh and ensiled *Dactylis glomerata* L. (Cocksfoot) variety AberTop following on from previous research identifying the hydroxycinnamate esters in this variety (Parveen *et al.,* 2008). The work presented analyses the change in phenolic composition between seven *D. glomerata* accessions over a period of summer growth.

Materials and methods

Replicate leaf tissue samples (4×) of multiple plants existing from seven F1 half sib progeny accessions were cut at three tri-weekly intervals throughout June-July 2018, from trays kept on an outdoor hardstanding. Samples were placed on dry ice and frozen at -20 °C before being freeze dried and milled. Phenols were extracted from 30 mg samples in 70% methanol and the resulting supernatant dried by vacuum centrifugation. Solid phase extraction was undertaken on samples according to methods described in Hauck *et al.* (2013). Samples were analysed via LC-PDA-MS^n on a Thermo Finnegan LTQ system with an injection volume of 10 μl. Recorded data were analysed using 'Qual Browser' in Thermo Xcalibur 2013. Peaks detected were identified by UV absorbance and MS^n spectra based on data reported by Hauck *et al.* (2013). Compounds were quantified based on UV absorbance, given in milli-area units per gram (mAU/g). Statistical analysis of data was undertaken using GenStat 18th Ed (VSN International Limited), via an ANOVA.

Results

Compounds were grouped into hydroxycinnamates, flavonoids and hydroxycinnamate-flavonoid conjugates; significant differences in the quantity of grouped compounds were found both within and across cuts (Figure 1, $P<0.001$ and $P<0.001$, respectively). Accumulation of conjugate increased with cut; total relative content of flavanoid compounds was consistently greater than both hydroxycinnamates and hydroxycinnamate-flavonoid conjugates. Significant differences in phenolic content between accessions were seen in cut 1 ($P=0.008$), but not in cut 2 (Figure 2*).* Mean quantity of all phenolic compounds was significantly different between cuts *(*$P=0.002$*).*

Discussion

Significant differences in phenol quantity across all accessions throughout the three cuts were indicative of time of cut having an impact on phenol production. Time of cut also had an effect on the ratios of hydroxycinnamate:flavonoid:conjugate compounds, as shown in Figure 1. Whilst flavonoid content was always the greatest, hydroxycinnamate compounds generally decreased with increasing conjugate production over time. As a biorefinery crop, these data indicate an optimum time for cutting the grass, dependent on

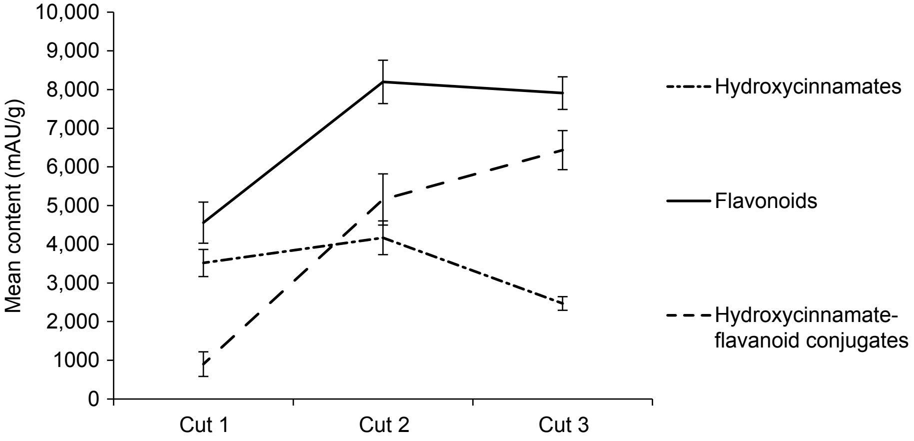

Figure 1. Changes in the mean content (mAU/g) of hydroxycinnamate, flavonoid and hydroxycinnamate-flavanoid conjugate compounds across all accessions over three cuts, taken on 18th June, 09th July and 30th July. Standard error of the mean bars are shown.

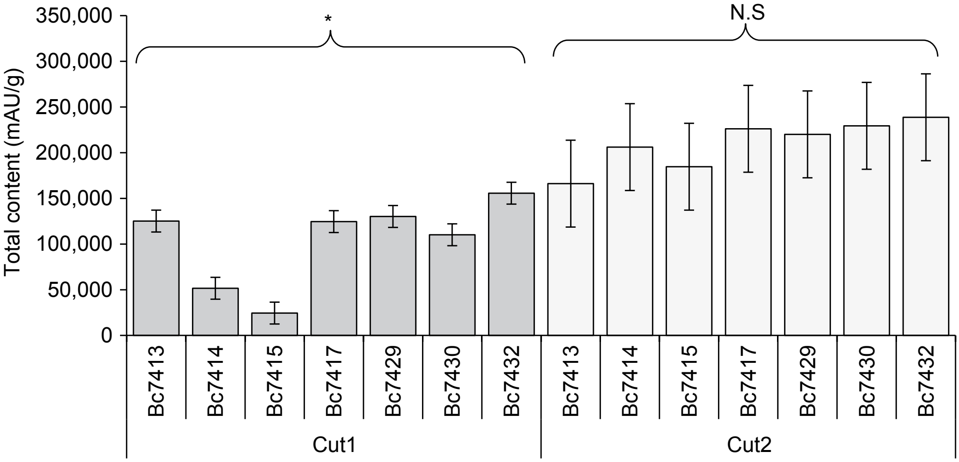

Figure 2. Total phenol content within the seven accessions per cut, taken from mean content of the four replications. Standard errors of the mean bars indicate overlapping data. Significant differences are denoted by '*', none significant data by 'N.S.'

which group of compounds are considered most favourable. Data also showed that flavanoid compounds were accumulated to the greatest extent, regardless of cut, suggesting that this would the most consistent and plentiful biorefinery product.

An insight into the influence of accession on phenol production can be seen in Figure 2; quantities of phenolic compounds were only significantly different between accessions in the earliest growth stage – cut 1. As cut 1 produced the lowest quantity of phenols across accessions on average (89770 mAU/g), harvesting of leaf tissues for optimum biorefinery potential would – on the basis of this study – occur in mid-late summer. This additionally suggests that selective breeding for accessions with the highest quantity of phenols would have little influence on the biorefining potential of cocksfoot. Yet, it must be iterated that replications were from trays of plants, instead of individuals; sampling individuals would give greater insight.

Conclusion

Differences in the ratio of grouped compounds were seen between cuts, although flavonoid production was consistently greatest. Significant differences were only seen between accessions at the earliest cut; selection for accessions with the greatest quantity of phenols would likely not benefit any breeding efforts, given optimum time of cut would be later in the season when differences were not significant.

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Sward lifting in compacted grassland: effects on soil structure, grass rooting and productivity

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Abstract

One way to ameliorate compaction in the topsoil (0-30 cm), without destroying the sward, is soil loosening by sward lifting. To explore the potential of this form of non-inversion tillage, we applied this treatment once, either in spring or autumn, to a moderately compacted grassland on a sandy soil and measured the effects on soil structure, grass rooting and productivity for up to two growing seasons. We also explored whether complementary overseeding with *Lolium multiflorum* Lam. would extend the duration of soil loosening effects. Our results show that sward lifting improved soil structure and rooting for at least 10-12 months, but did not result in a consistent or lasting increase in herbage yield or nitrogen (N) uptake. Loosening in spring decreased herbage yield (-27%) and N uptake (-16%) in the following growth period, but these decreases were largely compensated for (herbage yield) or more than compensated for (N uptake) by increases in the next three growth periods. The increase in N uptake in the first growing season (+13 kg N ha^{-1}) was reversed in the second season (-14 kg N ha^{-1}). Loosening in autumn increased herbage yield (+8%) and N uptake (+15%) in the first growth period (after winter), but not in the four growth periods thereafter. Cumulative yield tended to be higher (+4%), supporting the view that soil loosening should be carried out in autumn rather than in spring. The positive effects of loosening on herbage yield and N uptake were explained by a temporary increased soil N mineralization; initial negative effects by mechanical damage to sward and roots. Finally, complementary overseeding did not extend the duration of soil loosening effects.

Keywords: soil compaction, grassland, sward lifting, soil loosening, soil structure

Introduction

Soil compaction is a common problem in permanent grassland. Soil compaction may affect root growth and activity, e.g. through physical impedance and oxygen deprivation, and hence impair crop growth. Compaction of the topsoil layer (0-30 cm) can be ameliorated by sward lifting, a form of non-inversion tillage. Although soil compaction is likely to have negative effects on the production of grassland, little research has been done to assess the effects of amelioration methods. A field experiment by Van Eekeren and Ter Berg (2008) showed (short-lived) positive effects of sward lifting on soil structure, but not on herbage yield. These authors hypothesized that the effectiveness of soil loosening could be enhanced by overseeding (i.e. adding grass seeds to the existing sward), assuming that the rapidly growing roots of seedlings would be more likely to quickly occupy and stabilize the macropores created by soil loosening than the older roots of the existing sward. The objective of the present study was to explore the potential of sward lifting, with and without overseeding, as a method to ameliorate soil compaction.

Materials and methods

The experiment was conducted in permanent grassland on a compacted (based on a high penetration resistance), undrained, water-retaining fine sandy soil in the southern part of the Netherlands (51°61'N, 5°80'E). Treatments were applied either in the late spring (after the first harvest) or early autumn of 2014, and effects were measured in 2014 (2nd, 3rd, 4th and 5th harvests) and 2015 (1st, 2nd, 3rd, 4th and 5th harvests) growing seasons (spring treatments) or the 2015 (1st, 2nd, 3rd, 4th and 5th harvests) growing season (autumn treatments). The field experiment was set up as a randomized complete block design, with time of treatment (spring or autumn) assigned to two main plots located adjacent to each other. Within each main plot, all treatments (control, soil loosening, overseeding, and soil loosening combined with overseeding) were replicated on five plots (10×2.7 m) in five randomized blocks. Overseeding was carried with a Vredo Agri (Vredo Dodewaard BV, Dodewaard, the Netherlands), sowing Italian ryegrass (*Lolium multiflorum* Lam. cv. Mont Blanc) at a rate of 25 kg ha^{-1}. Soil loosening was carried out with an Evers Agro sward lifter with five shanks, spaced 60 cm apart and fitted with hardened, 20 cm wide winged tines which operate to a depth of 25 cm (Evers Agro BV, Almelo, the Netherlands).

Soil measurements consisted of penetration resistance, root biomass, and a visual assessment of soil structure and rooting. Sward measurements consisted of herbage yield and herbage N uptake. Herbage yield was determined by cutting the grass with a Haldrup grass harvester (J. Haldrup a/s, Løgstør, Denmark). The harvested material was weighed, and samples were oven-dried at 70 °C for 48 hours to determine dry weight and calculate herbage biomass and N uptake. Total N content in the dried samples was determined by a Dumas-based method (NEN 16634-1, 2008). Treatment effects were statistically analysed in pairwise comparison between treatment and control for each of the late spring and early autumn treatments separately, using the ANOVA-procedure in the Genstat statistical package (17th edition) (De Boer *et al.*, 2018).

Results

Soil loosening in spring and autumn reduced soil penetration resistance for at least 10 months and 12 moths respectively, compared to the controls. Soil loosening in spring had no effect on total root biomass (0-40 cm depth) or root biomass per 10 cm soil layer, measured after 1 and 4 months. When applied in autumn, soil loosening also had no effect on total root biomass, as measured after 6 and 12 months. However, after more than 12 months, root biomass was higher in the 10-20 cm and 20-30 cm soil layers of the loosened plots, compared to the controls. Soil loosening in spring had a positive effect on soil structure in the 0-25 cm soil layer for at least 10 months, with the strongest effect observed after 1 month. When applied in autumn, soil loosening also had a positive effect on soil structure in the 0-25 cm soil layer for at least 10 months.

Soil loosening in spring had a significant effect on herbage yield of each of the four growth periods in the first growing season (2014): while yield of the first growth period was lower, the yields of the second, third and fourth growth periods were higher, compared to the controls. Cumulative yield of the first year was not influenced (Table 1). In the second growing season (2015), no effect of loosening was observed on the first, second and third growth periods, but yields of the fourth and fifth growth periods were lower, and cumulative yield tended to be lower in the loosened plots, compared to the controls. When applied in autumn, soil loosening significantly increased the yield of the first following growth period (2015 growing season), but had no significant effect on subsequent growth periods (Table 1). The cumulative yield tended to be higher in the loosened plots, compared to the controls. Soil loosening in spring decreased N uptake during the first following growth period (although this effect was not significant, P=0.19), but tended to increase or increased N uptake during the next three periods, compared to the controls (P=0.07, P=0.01 and P=0.07, respectively). Cumulative N uptake during this first year (2014) was not significantly influenced (Table 2). In the second year (2015), N uptake tended to be higher (P=0.08) during the first growth period, but was lower for the fourth growth period and tended to be lower (P=0.06) for the fifth growth period. Cumulative N uptake in the second year was not influenced. When applied in autumn, soil loosening increased N uptake in the first following growth period (in 2015), compared to the controls. However, N uptake in the next four growth periods was not different from the controls, and no effect was observed on cumulative N uptake.

Table 1. Cumulative herbage yield (kg DM ha^{-1}) of a compacted grassland, as influenced by sward lifting and/or overseeding applied in the spring or autumn of 2014 (n=5).

Time of treatment	**Year**	**Treatment**[1]				***P*-value**[2]		
		C	**L**	**OS**	**L + OS**	**L**	**OS**	**L + OS**
Spring 2014	2014	8,944	8,603	8,417	8,426	0.40	0.09	0.37
	2015	11,451	11,078	11,836	11,673	0.10	<0.01	0.49
Autumn 2014	2015	11,232	11,523	11,294	11,962	0.09	0.36	0.48

[1] C = control; L = lifting; OS = overseeding.

[2] *P*-values of the main effect for L and OS and of the interaction for L + OS. A difference is significant where $P<0.05$.

Table 2. Cumulative nitrogen uptake (kg N ha^{-1}) in a compacted grassland, as influenced by sward lifting and/or overseeding applied in the spring or autumn of 2014 (n=5).

Time of treatment	**Year**	**Treatment**[1]				***P*-value**[2]		
		C	**L**	**OS**	**L + OS**	**L**	**OS**	**L + OS**
Spring 2014	2014	217	230	–[3]	–	0.16	–	–
	2015	275	261	277	280	0.23	0.04	0.09
Autumn 2014	2015	265	272	277	292	0.14	0.03	0.56

[1] C = control; L = lifting; OS = overseeding.

[2] *P*-values of the main effect for L and OS and of the interaction for L + OS. A difference is significant where $P<0.05$.

[3] Not determined.

Discussion and conclusion

The positive effects of sward lifting on soil structure and rooting in the compacted grassland of our study persisted over two growing seasons, but did not result in consistent or lasting herbage yield increases. Our results indicate that soil-loosening experiments should measure not only herbage yield but also herbage N uptake. Longer-term N uptake patterns enable to distinguish whether yield increases are caused by temporary increased N mineralization, or by other factors, such as improved root growth. Furthermore, our results indicate that grassland experiments in which soil N dynamics are influenced should run for at least two growing seasons, to account for potential compensatory effects or reversal of effects after the first growing season. Lastly, our study showed that complementary overseeding did not extend the duration of soil loosening effects. Apparently, new root growth from the existing sward was effective enough to stabilize these effects (De Boer *et al.*, 2018).

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Allelopathic effects in species mixtures of legumes

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Abstract

Allelopathic root exudates affect natural and managed grasslands in various contexts such as weed control, reseeding, overseeding and crop rotation by causing autotoxicity and/or heterotoxicity. In this study, we investigated the allelopathic effect of two species of legumes, Alsike clover (*Trifolium hybridum*; AC) and Black medic (*Medicago lupulina*; BM), and a mixture (1:1) of the two species. Leachates of four donors (bare soil, AC, BM, and Mix) were used for daily irrigation of the two-legume species, and two weeds: fat-hen (*Chenopodium album*; FH) and barnyard grass (*Echinochloa crus galli*; BYG). PCA analysis showed a clear distinction between autotoxicity and heterotoxicity of AC and BM. The mixture exerted an exacerbated allelopathic effect on AC compared to all the other receivers. Root leachates of AC, BM, and the Mix suppressed the overall growth of FH significantly ($P<0.001$) by 74, 91, and 85%, whereas BYG was inhibited by 11, 47, and 26%, respectively. We conclude that the allelopathic suppression of root leachates of AC and BM and their mixture on weed reduction is selective. In addition, their autotoxicity and heterotoxicity may affect legume growth itself, and thereby their contribution to sustainable agricultural system performance.

Keywords: allelopathy, intercropping, Alsike clover, Black medic, weeds

Introduction

Numerous studies have demonstrated that including legumes in crop mixtures may increase productivity, owing to the N fixation by legumes (Döring *et al.*, 2013) and may also have a potential to reduce weed growth (Baraibar *et al.*, 2018). These functions in legume-based systems are known to be mediated by root exudates (Li *et al.*, 2016). Accumulation of root exudates in the soil may influence the growth dynamics of crop and weed plants by causing autotoxicity and/or heterotoxicity. Alsike clover and Black medic are two legume species from different genera and have several contrasting characteristics regarding growth habit, growth rate, and response to water. Little is known about their root phytochemical allelopathy. The aim of this study was to investigate the effect of their root leachates in mixture vs monocultures on the same legume species and on two weeds from different families. We asked: (1) do root leachates of Alsike clover and Black medic and their mixture have an allelopathic effect on crop growth and weed growth of barnyard grass and fat-hen? and (2) Do the emitted allelopathic compounds in the root leachates alter plant growth?

Materials and methods

A pot experiment was conducted in a greenhouse from 1 February 2018 in Berlin-Dahlem (Albrecht Daniel Thaer-Institute of Agricultural and Horticultural Sciences) and was run for 120 days (average daily temperature: 20.8 °C; average daily relative humidity: 42.4%). Pots with a size of 1.5 l (Ø15 × H12 cm) were filled with substrate collected from the top-soil of a non-cropped bare field in the experimental field station of Humboldt University of Berlin in Dahlem (52° 28' N, 13° 18' E; 51 m a.s.l.). The soil type was sandy loam with a pH-value of 5.6, and nutrient contents per 100 g soil of 12.1 mg P, 8.3 mg K, 3.7 mg Mg, 124.2 mg Ca, and 504.4 mg Fe^{3+}. The experimental design was a two-factorial randomized complete block design with 4 donor treatments (D), 4 receiver plants (R; target plant) and 4 replicates

(Figure 1). Irrigation by donor leachates started from the first day of sowing and increased gradually and kept at ± 90-95% of water holding capacity (WHC). The donors received 300, 198, 150, and 137% WHC to irrigate AC, BM, BYG, and FH, respectively according to the needs of the receivers. Harvesting occurred once, at the end of the experiment. Ten variables were measured: shoot (fresh weight (FW), dry weight (DW), and height (cm), root (FW, DW, and length (cm)), total FW, total DW, growth stage (vegetative or flowering) and root:shoot ratio (based on DW). PCA analysis was conducted using R environment (version 3.5.1) on the data sets of the receivers to visualize change in plant growth as a response to different root leachates.

Results and discussion

The PCA analysis for the 4 receivers showed that the first two axes (PC1 and PC2) accounted for 68.4%, 74.7%, 81.2%, and 82.4% of the variation of AC, BM, BYG, and FH, respectively (Figure 2). The dimensions that most influenced the 2 axes above the expected average contribution (1/number of variables = 10%) were similar for all the receivers and included shoot (FW & DW), root (FW & DW), total (FW & DW), and R:S ratio. The autotoxicity of AC (Figure 2A; the centroid of the cluster is located on the negative side of PC1 and the positive side of PC2) was less strong than its heterotoxicity on BM (Figure 2B; the centroid of the cluster is located on both negative sides of PC1 and PC2). In contrast, BM showed stronger autotoxicity than heterotoxicity, suggesting that AC and BM have a different metabolic profile and different associate microbes that may exacerbate or alleviate their allelopathic effect in the mixture via degradation or alteration of root leachates. While heterotoxicity of only BM on legume species was reported by Sheaffer *et al.*, (2002), our bioassay is the first to demonstrate autotoxicity of AC and heterotoxicity of both legume species on each other. The significant reduction in FH by all plant leachates (AC, BM, and Mix), but not of BYG (Figure 2C,D), is consistent with findings from Hiltbrunner *et al.*, (2007) who reported that living mulches of legumes suppress dicotyledonous weeds more strongly than monocotyledonous weeds. The improved plant growth in the donors owing to gradual leaching proportional to plant age shows a potential strategy to counteract the allelopathy of AC and BM (data not shown).

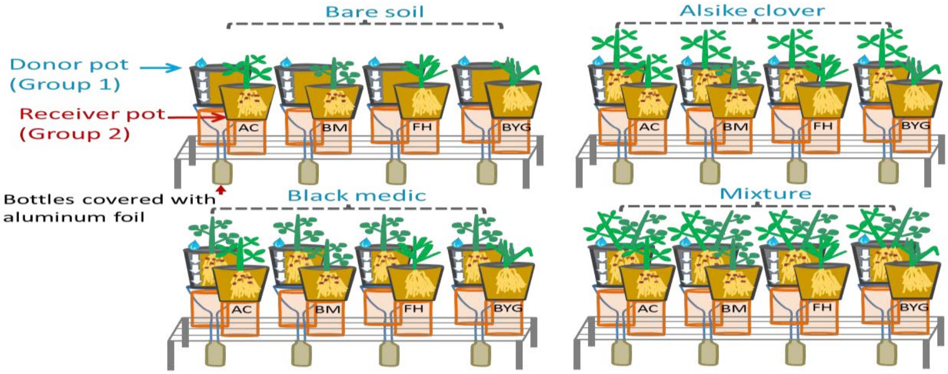

Figure 1. Design of the pot experiment. Four types of donor pots: bare soil, Alsike clover (AC), Black medic (BM), and mixture (1:1) and 4 types of receiver pots: Alsike clover (AC), Black medic (BM), fat hen (*Chenopodium album*; FH), and Barnyard grass (*Echinochloa crus gali*; BYG). One block is 32 pots (treatments); 4×4 donor (=16) and 4×4 receivers (=16). Total number of pots for 4 blocks (represent 4 replicates) was 128 pots. 24 plants of each species were in each pot; but in the mixture 12 AC:12 BM.

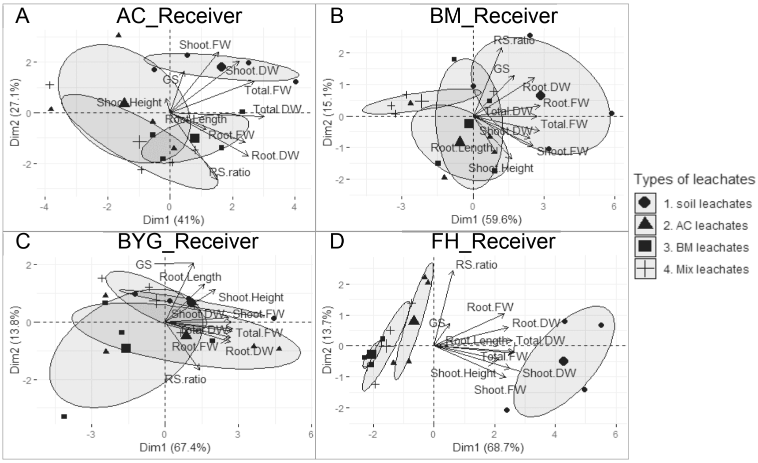

Figure 2. The PCA score biplot for the four receiver species: (A) Alsike clover (AC), (B) Black medic (BM), (C) Barnyard grass (BYG) and (D) fat-hen (FH) in response to four types of leachates: soil leachates (circle symbols), AC leachates (triangle symbols), BM leachates (square symbols), and the mixture leachates (cross symbols). The biplot was obtained by using 10 variables. Number of replicates per each treatment = 4. Arrows indicate the variable loadings of the PCA, whereas the angels between them (cosine) approximate their correlations. Dim = dimension.

Conclusion

Root leachates of AC and BM and their mixture exhibit autotoxicity and heterotoxicity that may affect their growth, and thereby their contribution to cropping systems, and their allelopathic suppression to weeds is significant on dicotyledonous species. These effects may be useful in weed control in sown grassland.

Acknowledgements

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Species richness increased yield stability in intensively managed grasslands subjected to experimental drought

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Abstract

Climate change is expected to cause an increase in the frequency and intensity of drought events. Over two years we investigated the effects of experimentally imposed drought on intensively managed grassland communities (5×6 m plots) of varying richness (1, 2 and 4 species), and comprising four species (*Lolium perenne* L., *Cichorium intybus* L., *Trifolium repens* L., *Trifolium pratense* L.). In each year a summer drought period of nine weeks with complete exclusion of precipitation was simulated, inducing severe drought stress at Reckenholz (Zürich, Switzerland), and extreme drought stress at Wexford (Ireland). Mean yield and plot-to-plot variance of yield were measured across harvests during drought and after a subsequent post-drought recovery period. At both sites, there was a positive relationship between species richness and yield under both the rainfed control conditions and under drought. At both sites, four-species communities had lower plot-to-plot variance of yield compared to monoculture or two-species communities under both rainfed (-49% smaller standard deviation) and drought conditions (-24%), which demonstrates higher yield stability in four-species communities. At the Swiss but not the Irish site, a high degree of species asynchrony could be identified as a mechanism underlying increased temporal stability in four-species communities.

Keywords: multispecies swards, mixture effect, stability, drought, extreme weather event

Introduction

Climate change is expected to cause an increase in the frequency and intensity of drought events, which have strong negative effects on the aboveground biomass (yield) of grassland ecosystems. We investigated the use of multi-species mixtures in intensively managed grasslands as a practical adaptation strategy for increasing yield stability under drought.

Materials and methods

A field experiment was established at two sites (1) Wexford, Ireland and (2) Zürich, Switzerland. Four agricultural grassland species were selected based on the factorial combination of nitrogen-fixing (N_2-fixing) and root-depth traits; two non-fixing species, *Lolium perenne* L. (shallow-rooted grass) and *Cichorium intybus* L. (deep-rooted forb), and two N_2-fixing species, *Trifolium repens* L. (shallow-rooted legume) and *Trifolium pratense* L. (deep-rooted legume). Main-plots (5×6 m) were sown following a simplex design (Hofer *et al.* 2016), such that there were: monocultures of each of the four species, six binary combinations (50% of each of two species), an equi-proportional mixture (25% of each of the four species), and four-species mixtures dominated by each species in turn (79% of one species, 7% of the other three). At each site, a total of 35 main-plots were arranged according to a randomised incomplete block design.

A summer drought of nine to ten weeks was simulated at each site over two years (see Hofer *et al.*, 2016). Precipitation was completely excluded from one randomly selected half (split-plot) of the main-plot by using rain-out shelters. Aboveground biomass was harvested five times annually at Wexford and six times at Zürich. Plots received mineral nitrogen (N) fertiliser at a rate of 130 kg N ha^{-1} y^{-1} (year 1) and 150 kg N ha^{-1} y^{-1} (year 2) at Wexford, and 200 kg N ha^{-1} year^{-1} in both years at Zürich. At each harvest, dry matter content of each split-plot yield was determined by drying a subsample of the harvested fresh biomass. Analysis of the yield responses to experimental drought only included data from the control and drought treatment for the three harvests: mid-drought, end-of-drought and post-drought harvests in each year (the post-drought comprised the first harvest after the removal of rain-out shelters). Mean yield across all harvests, plot-to-plot variance of yield (per level of species richness and drought treatment), and the stability index S (yield mean/standard deviation across harvests) were measured across harvests during drought and after a subsequent post-drought recovery period. To investigate a potential mechanism underlying the observed levels of yield stability, an analysis of species asynchrony was conducted for the mixtures (see Haughey *et al.*, 2018 for details). The smaller the value, the more asynchronous the temporal pattern of growth across species in the community.

Results and discussion

At both sites, there was a positive relationship between species richness and yield. Under rainfed control conditions, mean yields of four-species communities were 32% (Wexford, Ireland) and 51% (Zürich, Switzerland) higher than the average of the four monocultures ($P<0.001$ both sites). This positive relationship was also evident under drought, despite significant average yield reductions due to drought (-27% at Wexford; -21% at Zürich). At both sites, four-species communities had lower plot-to-plot variance of yield compared to monoculture or two-species communities under both rainfed (-49% smaller standard deviation) and drought conditions (-24%), which demonstrates higher yield stability in four-species communities.

A significant positive correlation between the stability index S and species asynchrony was identified at Zürich under both control (*rho* = 0.649) and drought conditions (*rho* = 0.745, Figure 2), but not in Wexford. At Wexford, species asynchrony was strongly reduced by drought ($P<0.001$), resulting in a distinctly reduced stability under drought at this site. Overall, while the asynchrony-stability correlation was driven by drought at Wexford (overall *rho* = 0.304, $P=0.051$), the asynchrony-stability relationship was more affected by species richness at Zürich.

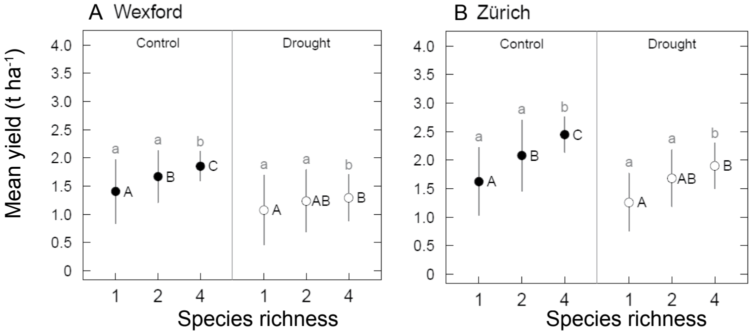

Figure 1. Effects of species richness and drought on yield mean across harvests and plot-to-plot standard deviation under rainfed control and drought conditions at Wexford (A) and Zürich (B). Within each site and treatment, different letters indicate a difference at $P<0.05$ based on regression analysis, except SD under drought at Zürich, which is at $P<0.1$ (means: inference in black upper-case letters; SD: inference in grey lower-case letters). Reproduced from Haughey *et al.* (2018), and refer therein for details.

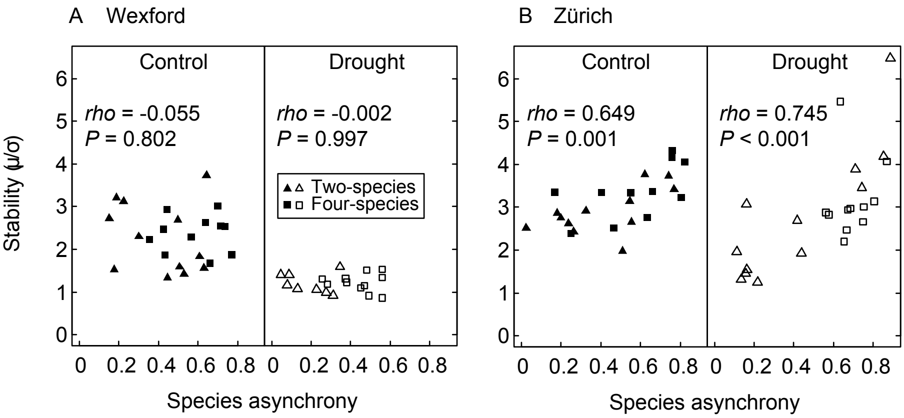

Figure 2. Relationship between species asynchrony and yield stability at Wexford (A) and Zürich (B), under rainfed control and drought conditions. The stability index $S = \mu/\sigma$ was computed with μ and σ being the mean and the standard deviation across all harvests. *Rho*: Spearman rank correlation.

Conclusion

There was a positive relationship between species richness (1, 2 and 4 species) and yield, and a negative relationship between species richness and yield variation. This demonstrates higher yield stability in four-species grassland communities under both rainfed control and drought treatments. These results indicate the high potential of multi-species grasslands as an adaptation strategy against drought events and that mixtures can help achieve sustainable intensification under both unperturbed and perturbed environmental conditions.

Acknowledgements

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Legacy effect of legume proportion in the preceding ley on the bacterial community composition under a *Lolium multiflorum* following crop

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Abstract

Sustained legume-induced legacy effects of sown grassland mixtures on the performance of a following crop have been previously demonstrated, though it is currently unclear if any such effects are observed in the soil microbiome. In March 2015, following the completion of the AnimalChange mixtures experiment (2012-2014), the first soil samples were taken. These swards were then completely removed and all plots were reseeded with a *Lolium multiflorum* monoculture. Soil samples were subsequently taken in June, August and September. The bacterial 16S rRNA gene was PCR amplified from all samples and an amplicon-based Illumina Miseq sequence analysis conducted. In March, when legumes were still present, a highly significant effect of legume proportion was observed on the composition of the bacterial community ($P<0.01$). Interestingly, even after re-seeding with *Lolium multiflorum*, a significant legacy effect between the former legume and non-legume monocultures was seen in the bacterial community in the following June and August ($P<0.05$), with the effect disappearing at the September sampling ($P>0.05$). Such legume-induced legacy effects on the microbiome may represent an important functional component of the increased performance of following crops in rotations, which utilize a legume component in the preceding ley.

Keywords: legacy effect, legumes, following crop, bacterial community composition

Introduction

It is known that sustained legacy effects on the composition of the soil microbiome can be induced by agricultural management (e.g. manure application, Zhang *et al.,* 2018) and also by environmental perturbations (e.g. Banerjee *et al.,* 2016). What remains unclear, however, is how the influence imparted by a plant functional group on the structure and functionality of the soil microbiome can endure even after that group's complete removal from a system. A recent study described a positive legume-induced, soil transferred, legacy effect on the sward performance of a following crop of *Lolium multiflorum* (Fox *et al.,* 2018). The present study aimed to gain additional insights into the persistence of this legume-induced legacy effect by investigating its impact on the bacterial community composition from the same experiment.

Materials and methods

Under the auspices of the EU-project AnimalChange, a field mixtures experiment was established at Zürich-Reckenholz (47°26'12' N, 8°31'51' E), conducted on a cambisol (top soil 42% silt, 26% clay, pH = 7.1) over a period of three years (2012-2014). The experiment utilized four model plant species, one grass (*Lolium perenne* L., cultivar (cv.) Alligator) and one forb (*Cichorium intybus* L., cv. Puna II) and two legumes (*Trifolium repens* L., cv. Hebe and *Trifolium pratense* L., cv. Dafila). The experiment contained plots with a sown legume proportion of 0, 50 and 100% grown on plots 3×5 m in size (for more details see Hofer *et al.,* 2016). Following the completion of the AnimalChange experiment, soil samples were taken for microbial analysis on 16 March 2015. In the spring of 2015 (21 April), these preceding swards were completely removed using a glyphosate herbicide and all plots were re-seeded with a *Lolium multiflorum* L. monoculture. This sward was allowed to become established and soil samples were subsequently taken three times during 2015 (10 June, 25 August and 29 September). Soil DNA was extracted from all collected samples and the bacterial 16S rRNA gene (V3-V4 region) was PCR amplified and an amplicon-based Illumina Miseq sequence analysis conducted (Frey *et al.,* 2016). Raw sequence reads ran through a customized bioinformatic pipeline and a bacterial operational taxonomic unit (OTU) table was constructed. The raw data were imported into the R statistical software package where they were subjected to a square root transformation, converted to relative abundance and a Bray-Curtis dissimilarity matrix was constructed. To take into account the differing replicate number between the sown legume proportions, the dissimilarity matrix was sub-sampled to the lowest number of replicates 1000 times, and a PERMANOVA analysis (9999 permutations) was conducted on each, with the mean *P* value taken. Group centroids were calculated via PERMDISP analysis.

Results and discussion

At the March sampling, when the legumes were still present, there was a highly significant effect of sown legume proportion on bacterial community composition ($P<0.01$). The 100% legume proportion harboured a significantly distinct bacterial community compared to the non-legume control ($P<0.02$, distance between centroids = 0.056), while the 50% legume proportion was not significantly distinct from either the 0 (distance between centroids = 0.038) or 100% (distance between centroids = 0.019) legume proportions (both $P>0.05$). This would suggest a gradual shift in the composition of the microbiome as the composition of the plant community changes from non-legumes to legumes (Figure 1).

Interestingly, at the first sampling after the removal of the previous experimental swards in June, there was still a significantly distinct bacterial community associated with the former 100% legume proportion when compared with the former non-legume treatment ($P<0.05$, distance between centroids = 0.038). This effect again persisted into the next sampling in August ($P<0.05$, distance between centroids = 0.033). At the final sampling event in September, six months after the destruction of the previous AnimalChange sward, the bacterial community composition between the previous legume and non-legume monocultures was no longer significant ($P>0.05$, distance between centroids = 0.027).

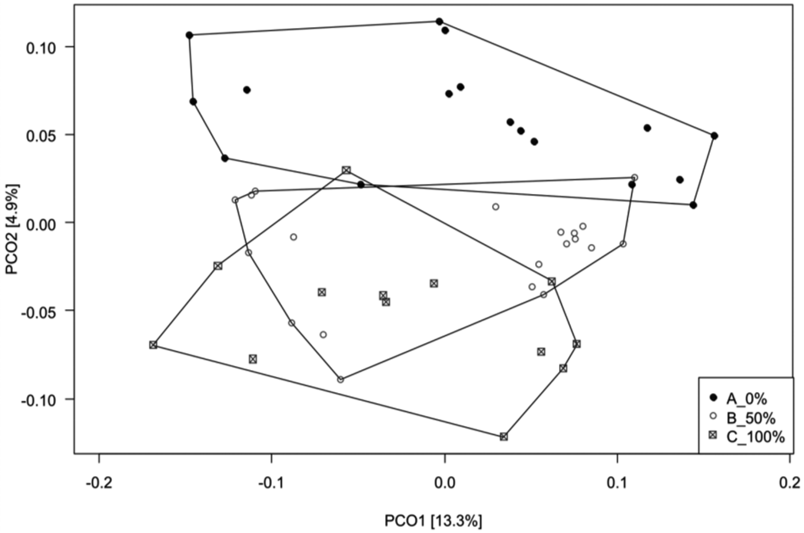

Figure 1. PCoA plot of the bacterial community composition among the different legume proportions in the seed mix; 0 (black dot), 50 (white dot) and 100% (crossed square), when the swards of the AnimalChange experiment were still present in March. Convex hulls indicate the different groupings of the bacterial community from sown legume proportions.

There are a few possible mechanisms behind this observation. Firstly, rhizobacteria may have remained attached to legume roots which persisted in the soil matrix. The composition of the bacterial community may also have adapted to utilize the changing quality of the soil organic matter (SOM and litter) due to legume presence, as these plants are known to have a high N content and a low C/N ratio (Nyfeler *et al.,* 2011). Hammelehle *et al.,* 2019 found that after a two years cultivation of a clover-grass mixture, the residual N potential of clover derived symbiotically fixed nitrogen in the soil was 10 g m^{-2}. Thus, the legacy effect observed may be a result of the soil bacteria continuing to utilize these legume-derived residues as a nutrient source. Intriguingly, these results would also suggest a role played by soil bacteria in the previously reported positive legume legacy effect on following crop performance (Fox *et al.,* 2018), though further research into the functionality of the bacterial community is necessary to establish any cause and effect mechanism.

Conclusion

These results demonstrate a legume-induced legacy effect on the composition of the soil bacterial community. This observation builds on (and may well be an explanatory mechanism for) the previous observation of the legume-induced legacy effects on following crop performance.

Acknowledgements

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Comparison of sowing methods for restoration of Natura 2000 protected hay meadows

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Abstract

Species-rich hay meadows are protected by the European Habitat Directive, part of Natura 2000. Of late, more and more hay meadows are losing their favourable conservation status. Thus, their typical plant species composition has to be re-established, in some cases, by re-seeding. This study presents a comparison of different sowing methods on their effectiveness for re-establishing the typical species composition of protected hay meadows. Four methods were examined: (1) sowing a seed mixture of local provenance; (2) transfer of fresh plant material; (3) transfer of fresh plant material with deep soil preparation; and (4) hay transfer. Establishment rate and fraction of total biomass for sown, low-nutrient indicator species, and additional species valuable for this habitat type were measured. Highest establishment rate and fraction of total biomass were found for method 2 ('transfer of fresh plant material') in the first year after sowing. However, establishment rate and biomass within plots sown by a seed mixture increased with time. In the second year after sowing, plants from the seed mixture treatment showed higher establishment rates than those freshly transferred. Both methods, transfer of fresh plant material and seed mixture, are effective in the short-term restoration of a species-rich hay meadow with regard to a favourable conservation status.

Keywords: species-rich grassland, sowing method, restoration, seed mixture, transfer of plant material

Introduction

Species-rich hay meadows have a high ecological value. Thus, the European Habitat Directive, part of Natura 2000, protects these natural habitats which are of Community interest. These protected hay meadows have to be maintained at a favourable conservation status. If changes of grassland management or other disturbances lead to negative transformation of the typical species composition, the favourable conservation status then has to be restored. In some cases it is not enough to return to extensive management with low fertilizer inputs (recommendation: 20 m^3 ha^{-1} slurry or 100 dt ha^{-1} manure only every second year) to restore plant species diversity. Thus, the typical species have to be re-established via seeding using an appropriate method. The objective of this study was to compare different sowing methods in terms of effectiveness for establishing the typical species composition of protected hay meadows. Typical species composition of the protected vegetation type 'Lowland hay meadows' are mainly low-nutrient indicator species. Four methods to re-introduce seeds within the sward were examined, including sowing with a seed mixture of local provenances and transfer of plant material (fresh or dried). Furthermore, to suppress competition by weeds and species of the old grassland sward on the introduced seeds, different seed bed preparations – shallow (rototiller) or deep (plough) – were compared.

Materials and methods

Three field experiments were established on grassland sites in southern Germany. Two of the meadows, Buehlertann and Dornhan, had lost their favourable conservation status due to an intensive grassland management (3-4 cuts, fertilization with slurry). The meadow in Friedingen had never been mapped as a protected lowland hay meadow within the framework of Natura 2000 and was also intensively managed in the past. At the sites in Friedingen and Dornhan an extensive grassland management (2 cuts, no fertilizer) was re-established two years prior to the start of the experiment. In Buehlertann, the site was intensively managed until the start of the experiment. The experiment was carried out in a two-factor strip design, testing four methods to re-establish seeds and two kinds of seedbed preparations. The sowing methods were as follows: sowing a seed mixture (50% grasses, 47% herbs, 3% legumes) of local provenances (SM), transfer of fresh plant material (FT), transfer of fresh plant material with deep soil preparation (FT + P) and hay transfer (DT). The seedbeds in SM, FT and DT were shallow cultivation using a rototiller twice. In FT + P, a deep seedbed preparation was conducted first by ploughing, and then by cultivation with a rototiller. Every sowing method was applied in randomly distributed plots of 60 m^2 within three blocks at each site. The experiment in Friedingen was established in 2014. Buehlertann and Dornhan followed in 2015 and 2016, respectively. Plant material for the transfer methods was gathered mid-June on protected hay meadows with an excellent conservation status A (except Buehlertann: B) situated within 1 to 2 km distance to the experimental site. For FT and FT + P, each donor site was mown early with the morning dew on the grass. The required amount of plant material was transferred immediately to the experimental site and spread onto the corresponding plots. After two days, the transferred material was turned by hand to improve the drop out of seeds. For DT, the rest of the sward material of the donor site was dried on-site. The hay was transferred in mid-September, at the same time when SM was sown using the seed mixture (4 g m^{-2}). The species-rich grassland seed mixtures used consisted of seeds of local provenances and were obtained from a commercial seed vendor. For optimal connection of seeds with soil, they were compacted by rolling. Vegetation surveys were done every year, end of May/ beginning of June, including estimations of fraction of total biomass for every species according to Klapp and Stählin (1936). For the 1st and 2nd years after sowing, vegetation data were analyzed with regard to low-nutrient indicator species which are ecologically valuable for lowland hay meadows and listed in LUBW 2017. For these valuable species, establishment rate (ER) was calculated according to the equation:

Establishment rate (%) = number of established species/ number of sown species × 100

This formula considered varying numbers of sown species at different sites (numbers of low-nutrient indicator species within seed mixtures: Friedingen and Buehlertann 28, Dornhan 29; numbers of valuable species in transferred plant material: Friedingen 22, Buehlertann 9, Dornhan 16). We performed linear mixed effect models in combination with analyses of variance (ANOVA) to test for differences in establishment rate and fraction of total biomass for valuable species. The models used sowing method and site as fixed factors. To account for influences by experimental site or conditions of the year of vegetation survey, number of block and year of observation were added as random factors. In all tests, the level of significance was set to P=0.05. All statistical analyses were performed using the statistical software R 3.4.3. For linear mixed effect models and multiple post hoc comparisons, the software package 'nlme' version 3.1-131 was used.

Results and discussion

Establishment rate (ER) and fraction of biomass of sown, low-nutrient indicator species significantly differed with applied sowing methods in both years after sowing (all P<0.0001, Figure 1). Only in the second year after sowing were there differences between sites for both response parameters (ER: F=22.06, P=0.007; biomass: F=15.99, P=0.012). However, effects of sowing method on ER and biomass varied slightly across sites in all years (all interactions 'sowing method:site' P<0.01).

Highest establishment rate (32.1±4.2%) and fraction of total biomass of valuable sown species (22.0±3.1%) were found in the methods 'transfer of fresh plant material' after one year. However, ER and biomass increased with time within plots sown by a seed mixture (Figure 1). In the second year after sowing, species sown by seed mixtures showed higher ER (43.5±2.0%) than those transferred with fresh plant material (37.0±3.2%). This delayed establishment might be caused by seed dormancy. Furthermore, within the transferred material from the first cut in June, not all species of the sward might have developed ripe seeds. Transfer of dried plant material showed worst performance in terms of species number and biomass. This is in agreement with Kirmer *et al.* (2012), which predicted higher seed losses

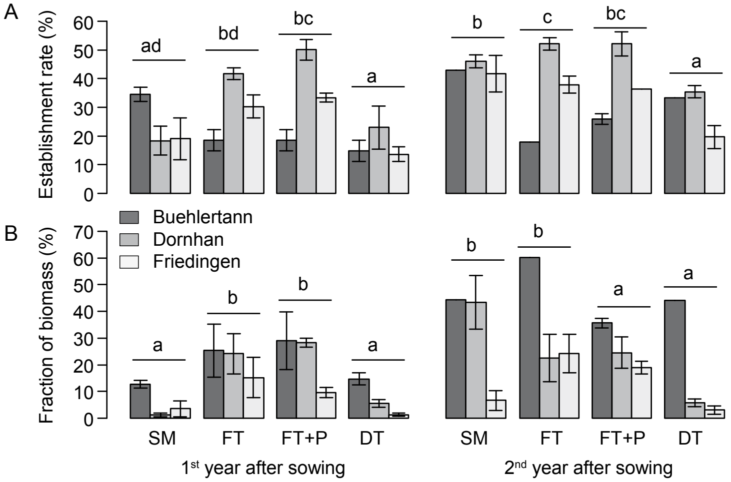

Figure 1. (A) Establishment rate of sown, low-nutrient indicator species (%) and (B) their corresponding fraction of total biomass (%) for the 1st and 2nd year after sowing within the different methods (SM = seed mixture, FT = transfer of fresh material, FT = transfer of fresh material with previous ploughing, DT = transfer of dry material, mean ± SE); small letters indicate significant differences within methods (average over the 3 sites) and year according to post-hoc tests ($P<0.05$).

during the hay preparation process and transportation. Deep soil preparation did not lead to a sustained advantage for species establishment.

Conclusion

Both methods, transfer of fresh plant material and seed mixtures, are effective for a short-term establishment of a species-rich hay meadow. If the re-established species composition is maintaining itself on a long-term basis, the favourable conservation status might be restored. With sowing by seed mixtures, more species might be re-established. A combination of both methods could be considered for even higher restoration success.

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Strategies for a sustainable management of intensive sown grassland: effects of the farmers' choices

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Abstract

To motivate Swiss farmers to evaluate promising strategies of the use of sown grassland the Strickhof Sown Grassland Cup was founded with the aim of comparing sustainable grassland production systems. Participating teams could choose from a range of clover-grass mixtures, set the date of cutting and fertilizer application. The results show that it is most important to take the right decision regarding the optimal cutting date in spring in order to avoid low energy forage. Legume mixtures have a high potential to produce high yield even with a low input in nitrogen. Farmers seem to sometimes overestimate the nitrogen fertiliser needs of grass-clover mixtures, which strongly reduces nitrogen efficiency.

Keywords: sown grassland, competition, sustainable intensification, nitrogen efficiency

Introduction

In view of future population trends and changing consumer habits, it is essential to secure and expand agricultural production. From that perspective, the aim is to develop production systems that have a lower demand in fossil energy and thus enable greater yields with lower environmental impact (Lehmann, 2011).

Due to the ability of legumes to fix atmospheric nitrogen, it is possible to reduce the nitrogen fertilizer rates without negatively affecting the yield and quality of the forage by using legumes in grassland-based forage production systems. Therefore grass-clover mixtures play an important role in Swiss forage production. A targeted low-input strategy using legumes can lower the environmental footprint of forage production without impeding productivity (Peoples *et al.*, 2019).

To show the potential that lies in grass-clover mixtures in combination with a good strategy of grassland use, the Strickhof Sown Grassland Cup (SGC) was started. For the organisers, the aim was to promote reflection and debate about optimal management of sown grasslands. For the participating teams, the aim was to achieve the best possible yield, forage quality and financial efficiency through appropriate mixture choice and optimal strategy of cutting and fertilization. The participating teams could choose their mixture, cutting and fertilizer strategy. The winner had to combine high yields with high nutrient contents and low costs. The SGC serves as a lighthouse project for Swiss farmers to evaluate optimal strategies to maximize milk production per hectare.

Materials and methods

The SGC was conducted four times during 8 years (2006-2013). Each competition lasted 1.5 years. Each competition took place at a new location, and started with new sown grassland and a possible new strategy. The locations were located between 450 and 550 metres above sea level, in regions with 900-1100 mm yearly rainfall. The teams chose among the following three mixtures: (1) Italian ryegrass-clover mixture (*Lolium multiflorum, Lolium* × *hybridum, Lolium perenne, Poa pratensis, Trifolium repens, Trifolium pratense*; SM240), (2) Red clover-grass mixture (*T. pratense, L.* × *hybridum, Dactylis glomerata, Festuca pratensis, Phleum pratense*; SM300), and (3) alfalfa-grass mixture (*Medicago sativa, T. pratense, L.* × *hybridum, D. glomerata, Ph. pratense*; SM320). The field trials were carried out in a randomized block design consisting of 5 to 7 treatments depending on the number of participating teams

(1 treatment = 1 team). Plot size was 18 m^2 and each treatment had three replicates. The mixtures were sown following the harvest of the preceding cereal crop, mostly in mid-August. Yields were recorded from the first cut in autumn until the last cut in October the following year. The swards were cut at 6 cm height, individually weighed and samples taken for dry matter (DM) analysis and nutrient content analysis using near-infrared reflection spectroscopy. Fertiliser was applied by the organizers according to instructions given by the teams. The fertilizer used was either mineral fertilizer or cattle slurry (with 1 kg available nitrogen per m^3).

For the calculation of the winning strategy, there was a calculation model used, calculating the potential milk production per hectare from the yield, energy content and protein content of the produced forage. To compare the treatments for their use of nitrogen and the efficiency of the nitrogen, the total yield was divided by the amount of available nitrogen applied in each treatment. The statistical analysis was done by xlstat using year, team and mixture as qualitative factors and nitrogen as quantitative factor.

Results and discussion

For the following results only the yield from spring to autumn was compared without the first cut in autumn after sowing. The average yield of the mixtures over all four competitions was very high under Swiss conditions, with 18.6 t DM ha^{-1} per year (Figure 1).

The amount of applied nitrogen was in average only 60 kg ha^{-1} $year^{-1}$ but two teams applied more than 200 kg N ha^{-1} (Figure 2). For the SM240, the results indicate that many teams decided to apply more nitrogen fertiliser than the amounts necessary for high yields (Figure 2A). Nitrogen efficiency in term of biomass yield per unit of applied nitrogen was strongly correlated to the amount of applied nitrogen, without any clear effect of the mixture type (Figure 2B). On average, the teams nevertheless applied less fertiliser to the red clover-grass and the alfalfa-grass mixture than to the Italian ryegrass-clover mixture, and some reached very high nitrogen efficiencies with these types of mixtures (Figure 2B).

The teams followed strategies mostly focused on high biomass yields resulting in comparably low nutrient contents (mean: 5.32 MJ NEL kg^{-1} DM, standard deviation: 0.27). Most teams (59%) followed a 4-cut-scheme. The others either chose a 3 or 5-cut scheme. The first two cuts accounted for 72% of the annual

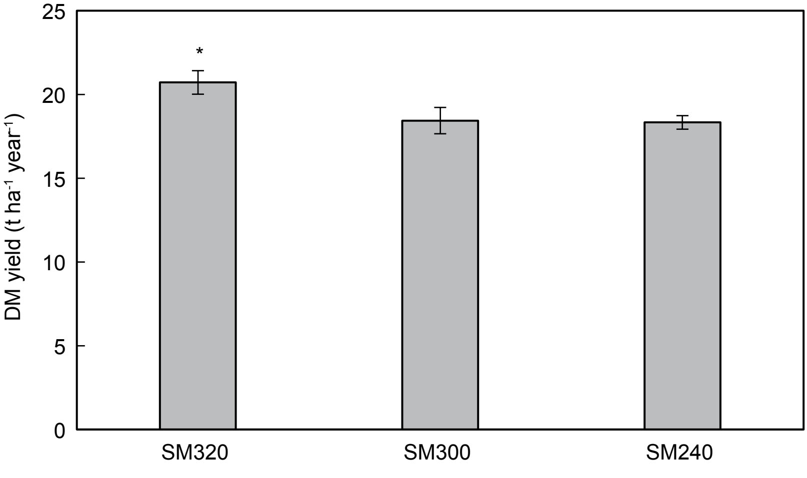

Figure 1. Annual dry matter (DM) yield (± 1 standard error) of the three mixtures. The alfalfa-grass mixture (SM320) had significantly higher yield (P<0.05) than the Italian ryegrass-clover (SM240) or red clover-grass mixtures (SM300). SM240 and SM300 did not differ in biomass yield or in nutrient content.

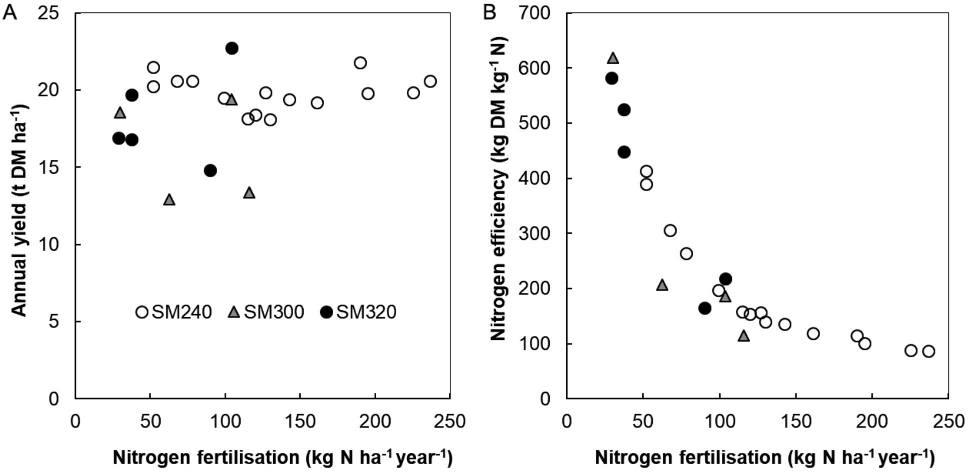

Figure 2. Effect of nitrogen fertilisation on (A) the biomass yield and (B) the nitrogen efficiency in term of biomass yield per kg of applied N, for the Italian ryegrass-clover mixture (SM240), the red clover-grass mixture (SM300) and the alfalfa-grass mixture (SM320). Data are mean values per participating team for four editions of the SGC.

yield. Strategies that missed the optimal cutting date faced very low nutrient contents that could not be compensated with the remaining cuts.

Conclusion

It is crucial to take the right decision regarding the optimal cutting date in spring to avoid low-energy forage. Legume-grass mixtures have a high potential to produce high yield even with low inputs of nitrogen. Farmers seem to sometimes overestimate the nitrogen fertiliser needs of grass-clover mixtures, which strongly reduces nitrogen efficiency.

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What is the effect of incorporating white clover (*Trifolium repens* L.) into grazed grassland on the farm-gate nitrogen balance?

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Abstract

Pasture-based milk production systems are the most cost-efficient feed systems in regions where grass grows for most of the year. Incorporating white clover into pasture-based systems can reduce the requirement for nitrogen (N) fertiliser application and can increase herbage dry matter intake and milk production. This study examined the effect of grass-clover swards receiving 150 or 250 kg N ha^{-1} yr^{-1} on milk production and farm-gate N balance compared to grass-only swards receiving 250 kg N ha^{-1} year^{-1} over a four-year period. Annual herbage production was similar in all three systems. Milk solids (MS) production was greater on the grass-clover treatments (1,337 and 1,373 kg MS ha^{-1} yr^{-1} on grass-clover swards receiving 150 and 250 kg N ha^{-1}, respectively) compared to the grass-only treatment (1,261 kg MS ha^{-1} yr^{-1}). The N-use efficiency was similar on the two treatments receiving 250 kg N ha^{-1} yr^{-1} and was greatest on the grass-clover treatment receiving 150 kg N ha^{-1} yr^{-1}.

Keywords: white clover, grazing, nitrogen balance, N use efficiency

Introduction

The objective of intensive dairy production systems is to maximize milk production from grazed grass (Dillon *et al.*, 2008). Irish dairy farms have the potential to produce up to 15 t herbage dry matter (DM) hectare^{-1} (ha) over a long growing season (O'Donovan *et al.*, 2011) and this, combined with a predominantly spring-calving, pasture-based system, puts Ireland at a competitive advantage in terms of low-cost feed production compared to most EU countries. Perennial ryegrass (*Lolium perenne* L.) is the predominant forage species used in grazing systems in temperate regions of the world (Fulkerson *et al.*, 2007). It has long been recognised that white clover (*Trifolium repens* L.; clover) provides a forage nutritionally superior to perennial ryegrass and offers an alternative to N fertiliser application or an additional source of N for the sward for herbage production (Phillips and James, 1998). Intensive pasture-based systems usually rely on N fertiliser to ensure adequate supply of good quality herbage for grazing animals. In grass-clover swards increasing the rate of N fertiliser application can reduce sward clover content (Enriquez-Hidalgo *et al.*, 2018). Recently Egan *et al.* (2018) has succeeded in maintaining a high clover content in swards receiving >200 kg N ha^{-1}. Despite the positive benefits of N in pasture-based milk production systems, losses of N occur as it moves through the system. Increasing N-use efficiency is a key objective in pasture-based milk production systems. The objective of this study was to investigate the farm-gate N balance of pasture-based milk production systems – grass-only receiving 250 kg N ha^{-1}; grass-clover receiving 250 or 150 kg N ha^{-1}.

Materials and methods

The experiment was undertaken at the Animal & Grassland Research and Innovation Centre, Teagasc, Moorepark, Fermoy, Co. Cork, Ireland (52°16'N; 8°25'W; 49 m a.s.l.) from 2013 to 2016. The soil type was a free draining acid brown earth of sandy-loam-to-loam texture. Soils had a pH of 6.4 and had Index values of 3 and 4 (adequate or luxury supply) for phosphorus and potassium, respectively. The grazing experiment is described in detail by Egan *et al.* (2018). Briefly, a farm systems experiment was established in February 2013 and conducted over four grazing seasons (2013-2016). There were three treatments:

grass-only receiving 250 kg N ha^{-1} (Gr250); and grass-clover receiving 250 or 150 kg N ha^{-1} (Cl250 and Cl150, respectively). A separate farmlet was established and permanently fenced for each of the three sward types (treatments). Nitrogen was applied to all treatments in each rotation as urea (46% N) until the end of April and as calcium ammonium nitrate (27.5% N) from early May to mid-September. All treatments were stocked at 2.6 cows ha^{-1}. In February each year spring-calving dairy cows were selected from the Moorepark herd and blocked according to calving date, lactation number, pre-experimental daily milk yield and daily milk solids yield, and then randomly assigned to one of the three treatments. Cows remained on their respective treatment for the full lactation each year. Concentrate supplementation to each group was the same, and was fed predominantly in spring and late autumn. Cows rotationally grazed swards, achieving approximately 9 rotations per year. Measurements undertaken include annual herbage production, sward clover content and milk solids (MS) yield. The Moorepark Dairy Systems Model (Shalloo *et al.,* 2005) used grazing management data, sources and quantities of N inputs and types and quantities of N outputs from each treatment to simulate performance of the dairy production systems. As stocking rate was the same in all systems it was assumed that culling rate and calves sold from the farm were the same. Production data were then used in an N-balance model (Ryan *et al.*, 2011) to calculate N-use efficiency and N surplus.

Results and discussion

Herbage production was 13.6, 14.0 and 13.6 t DM ha^{-1} yr^{-1} on Gr250, Cl250 and Cl150, respectively. Average sward clover content for the four years was 23% on Cl250 and 27% on Cl150. Milk solids yield was greatest on Cl150 and Cl250, compared to Gr250 (Table 1). The N fertiliser application rate on the clover treatments did not affect MS production. Total N input was greatest on Gr250 and Cl250 due to the greater quantity of fertiliser N applied to these two treatments compared to Cl150. Milk was the greatest source of N output and was similar and greater on the two clover treatments compared to Gr250 (Table 1). The N surplus was least on Cl150 and greatest on the treatments receiving 250 kg N ha^{-1}. Previous studies have reported N use efficiency for grass-only systems with 250 kg N ha^{-1} of 44% (McCarthy *et al.*, 2015), similar to the current study. When N application was reduced from 250 to 150 kg N ha^{-1}, N use efficiency increased from 40 to 59%, respectively. This study shows that incorporating

Table 1. Milk solids (MS) production per hectare and farm gate N balance for three grazing treatments – Gr250 (grass-only receiving 250 kg N ha^{-1}), Cl250 (grass-clover receiving 250 kg N ha^{-1}) and Cl150 (grass-clover receiving 150 kg N ha^{-1}).

	Gr250	**Cl250**	**Cl150**	**s.e.**	***P*-value**
MS yield (kg MS ha^{-1})	$1,261^a$	$1,373^b$	$1,337^b$	18.8	<0.05
Nitrogen inputs					
Fertiliser applied (kg N ha^{-1})	229	232	156	–	–
Concentrate (kg N ha^{-1})	8	8	8	–	–
Atmospheric deposition (kg N ha^{-1})	9	9	9	–	–
Replacement heifers (kg N ha^{-1})	2.2	2.2	2.2	–	–
Total N input^1 (kg N ha^{-1})	249^a	250^a	175^b	3.8	<0.001
Nitrogen Outputs					
Milk (kg N ha^{-1})	87^a	94^b	92^b	0.9	<0.001
Cull cow (kg N ha^{-1})	8.2	8.2	8.2	–	–
Calf (kg N ha^{-1})	3	3	3	–	–
Total N outputs (kg N ha^{-1})*	98^a	105^b	103^b	1.3	<0.01
N surplus (kg N ha^{-1})	150^a	145^a	72^b	3.8	<0.001
N use efficiency (%)	40^a	42^a	59^b	0.7	<0.001
N surplus per kg MS produced (kg N kg MS^{-1})	0.12^a	0.11^a	0.05^b	0.004	<0.05

* Inputs and outputs may not sum exactly to total figures due to rounding.

clover in to grazing swards can increase MS production. Additionally, reducing N application rate to grass-clover swards did not reduce herbage production but increased N use efficiency. The N fixed by clover was not accounted for in this N balance. It is likely that N available through N fixation will increase the quantity of N available within the system and therefore the surplus N (Cuttle *et al.* 1992), and reduce the NUE. Based on calculating the N fixed by difference, the N fixation on Cl250 was 26 kg N ha^{-1} yr^{-1} and on Cl150 it was 80 kg N ha^{-1} yr^{-1}. Including those estimates in the inputs reduces the NUE of Cl250 to 32% and Cl150 to 51%.

Conclusion

Incorporating clover into intensive pasture-based systems at reduced N fertiliser input offers potential to increase NUE, without negatively impacting on MS yield or herbage production.

Acknowledgements

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White clover population affects mixture forage quality

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Abstract

Grass-legume mixtures improve forage yield and nutritive value compared to grass monocultures. Adding further species to grass-clover mixtures may further enhance forage yield, but will also influence the quality of the forage. Little is known about the role of genetic variation within mixture partners on the forage quality of mixtures. Therefore, we established a field experiment to investigate how and to what extent different populations of white clover (*Trifolium repens* L.) affect the forage nutritive value of different mixtures. Eight novel populations of white clover were grown as monocultures, as binary and as three-species mixtures with *Lolium perenne* L. and *Cichorium intybus* L. at two sites differing in resource availability. Aboveground herbage was cut four times annually over three years and analysed for crude protein, acid detergent fibre, and water-soluble carbohydrate concentrations. The results showed that forage nutritive value was affected by the interaction of white clover population × crop stand. The range of variation among white clover populations regarding all nutritive parameters was higher in white clover monocultures than in mixtures.

Keywords: multi-species swards, white clover, perennial ryegrass, forbs, nutritive value

Introduction

Plant species vary in their nutritional composition; legumes contain comparatively higher crude protein (CP) and lower fibre and water-soluble carbohydrates (WSC) than grasses (Brink *et al.*, 2015). For legume species such as white clover there is also variation in the nutritive value within the species, i.e. varieties differ from each other (Cupina *et al.,* 2013). Forage from grass-legume mixtures often has a better balance of nutrient composition compared with the respective monocultures. The choice of suitable components and, in particular, the proportion of legumes in mixtures is important for their forage nutritive value (Brink *et al.*, 2015). Breeding new plant germplasm with improved traits can therefore enhance the nutritional composition of mixtures (Evans *et al.*, 1995; Casler, 1999). So far, no studies have assessed whether differences in white clover populations affect the nutritive value of clover either in pure stands or in simple and multi-species mixtures including forbs. We established a field experiment with eight novel populations of white clover (*Trifolium repens* L.), which were grown as monocultures, as binary and three-species mixtures with perennial ryegrass (*Lolium perenne* L.) and chicory (*Cichorium intybus* L.). We hypothesized that white clover populations vary in forage nutritive value and also affect the forage nutritive value of mixtures with different non-legume companion species.

Materials and methods

The experiment was established at two sites on research stations of the University of Goettingen, Germany, from 2014 to 2017. The two sites are noticeably different in terms of soil depth, fertility and temperature. The fertile site (51.29° N, 9.55° E) has a low elevation (157 m a.s.l.) with a Gleyic Fluvisol; the marginal site (51.34° N, 9.58° E) has an elevation of 342 m a.s.l., the soil is a Calcaric Leptosol. Eight novel populations of white clover (*Trifolium repens* L.) were grown in monoculture (1000 seeds m^{-2}), in binary mixtures with either perennial ryegrass (cv. ELP 060687) or chicory (cv. Puna II) (400:600 seeds $m^{-2)}$, and in three-species mixtures of white clover with ryegrass and chicory (400:300:300 seeds m^{-2}). The white clover populations differed in phenology, leaf size and yield potential. On a scale of 1 to

9 for phenology, leaf size and yield potential for white clover varieties included in the German National List of Varieties (Bundessortenamt, 2018), white clover populations in this study were rated 3 to 9 for phenology and 3 to 7 for both leaf size and yield potential. There was no application of nitrogen fertilizer. The experiment followed a split-plot design with clover population as the main-plot and crop stand as the split-plot factor, and four replicate blocks. Plots measured 5×3 m and were cut four times per year (2015 to 2017). Herbage was cut at a height of 5 cm with a combine harvester. Sub-samples were subjected to near infrared spectrometry to determine CP, acid detergent fibre (ADF) and WSC concentrations. Mean annual values of these forage nutritive values were calculated as weighted averages of each harvest's value, with that harvest's dry matter yield as weighting factor. Linear mixed effects models with white clover population, stand, site and year and their interactions as fixed effects and block, main plot and split-plot and as nested random effects, were fitted using the R software environment (The R Foundation for Statistical Computing, Vienna). All models were visually checked for homogeneity of variance and appropriate variance structures were included where necessary. Minimal adequate models were identified based on the second order Akaike Information Criterion.

Results and discussion

Concentrations of CP, ADF, and WSC varied significantly with clover population interacting with crop stand (Table 1). Variation in CP among white clover populations was 2.1% while it was 1.9% for the binary and 1.7% for the complex mixtures. The respective results for ADF were 2.4, 1.1 and 0.7%, and for WSC 1.3, 1.1 and 0.7% (Figure 1). The presence of chicory in mixtures increased the concentration of ADF, showing the substantial contribution of stem material in altering the nutritional composition of forage. The binary mixture of white clover and perennial ryegrass had the highest concentration of WSC (Figure 1). Our results showed that the identity of companion species had a substantial effect on the forage nutritive value.

Table 1. *F* and *P*-values to test the effects of white clover population, stand, site and year and their interactions on the mean annual concentration of crude protein (CP), acid detergent fibre (ADF) and water-soluble carbohydrates (WSC).

Factor	CP		ADF		WSC	
	F-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Population	18.1	<0.0001***	12.6	<0.0001***	4.6	0.0001***
Stand	1,710.0	<0.0001***	465.5	<0.0001***	1,563.8	<0.0001***
Site	0.2	0.6666^{ns}	12.1	0.0130*	165.8	<0.0001***
Year	716.6	<0.0001***	205.7	<0.0001***	4.5	<0.0001***
Population × stand	2.5	0.0007***	2.6	0.0002***	3.1	0.0001***
Stand × site	130.1	<0.0001***	71.7	<0.0001***	60.0	<0.0001***
Stand × year	77.9	<0.0001***	93.4	<0.0001***	77.2	<0.0001***
Site × year	105.8	<0.0001***	37.8	<0.0001***	27.7	<0.0001***
Stand × site × year	19.2	<0.0001***	8.6	0.0001***	38.9	<0.0001***

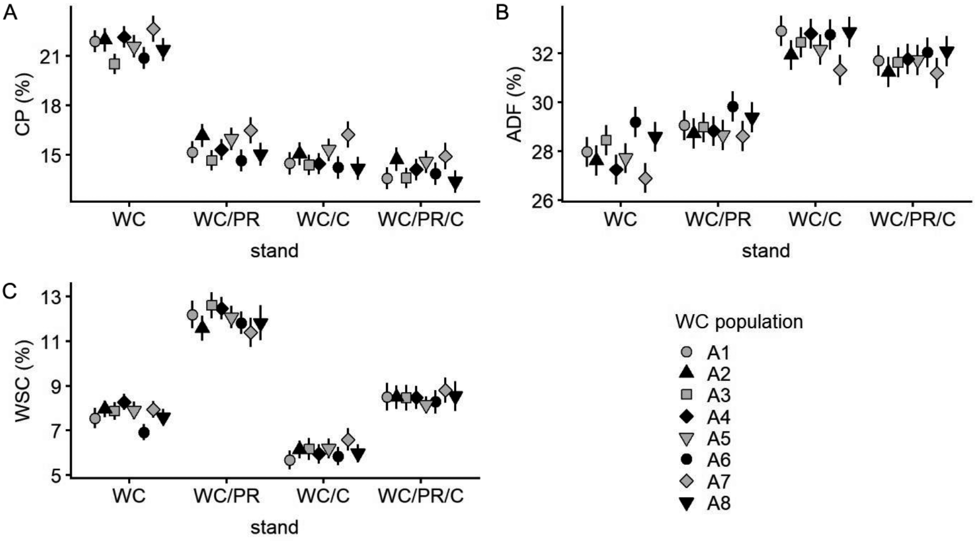

Figure 1. Effect of white clover (WC) population on the nutritive value of WC monocultures and mixtures with perennial ryegrass (PR), chicory (C) or both. CP: crude protein; ADF: acid detergent fibre, WSC: water soluble carbohydrates. Model estimates with 0.95 confidence intervals.

Conclusion

White clover population influences the forage nutritive value of monocultures as well as of mixtures with larger differences among the monocultures. However, the effect of the botanical composition is generally stronger than the clover population effect.

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Comparative evaluation of energy efficiency of state-of-the-art barn hay drying systems

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Abstract

High quality roughage is crucial for milk production. Driven i.a. by good marketing opportunities for 'hay milk products' and current improvements in hay drying technology, barn dried hay becomes an alternative to silage again. The aim of this study was to analyse the energy efficiency of state-of-the-art barn hay drying systems at an experimental plant. The harvested material was filled in equal shares into two identical boxes (c.p.) each with 30 m^2, an identical centrifugal fan and comparable air distribution systems. In one box a dehumidifier in recirculation mode was used for drying. In the second box a heat exchanger with a heat recovery unit in the exhaust air was used to warm up the atmospheric air in two steps for drying. Collected data were i.a. energy consumption using adequate sensors. The measured energy requirement for drying hay with the dehumidifier was 0.65 kWh per kg water loss in summer or respectively 0.51 kWh per kg water loss in autumn compared to 1.18 kWh per kg water loss for the heat exchanger and heat recovery unit in summer.

Keywords: barn hay drying system, dehumidifier, energy demand

Introduction

Various preservation methods are available for the production of high-quality forage crops. High quality roughage is crucial for milk production and therefore most farms rely on silage. However, milk produced with silage is not suitable for certain hard cheese products. Driven i.a. by good marketing opportunities for 'hay milk products' and current improvements in hay drying technology, barn dried hay becomes an alternative to silage again. New, powerful technology is available today, with the help of which wilted grass with about 60% dry matter (DM) content can be dried in short time using warm air or a dehumidifier to storable hay. For the efficiency (energy consumption, drying time, etc.) of these new technologies, only few data from companies are currently available, except one study from Pöllinger (2014) or older date for cold air-drying systems (e.g. Baumgartner, 1991). Therefore, the objective of our study was to analyse energy efficiency of state-of-the-art barn hay drying systems at an experimental plant.

Materials and methods

In Hübschenried, north of Lake Ammersee in Bavaria (Germany), an experimental plant with two identical boxes (c.p.) each with 30 m^2 and comparable air distribution was built. Furthermore, two identical centrifugal fans (type RVN 630-35/10, 7.5 kW, manufacturer GB Birk) were used. Due to the structural conditions on site, on fair weather days only the air under a south-facing canopy can be used for drying. In one box a dehumidifier in recirculation mode was used for drying (Table 1). In the second box a heat exchanger with a heat recovery unit in the exhaust air was used to warm up the atmospheric air in two steps for drying (Table 1). The heat exchanger was heated by a mobile oil heater (300 kW, manufacturer Mobiheat GmbH), simulating e.g. waste heat utilisation.

Hereinafter referred to as system S1 and system S2. System S1 was used in two experiments in July and October 2018 for drying the third and fifth cut from permanent grassland (harvested on 13.07.2018 and 10.11.2018). System S2could so far only be tested in July 2018. The harvested material was filled in equal shares into the boxes (c.p.). In all experiments the boxes were ventilated with atmospheric air

Table 1. Barn-hay drying systems at the experimental plant.

System	S1	S2	
Drying system	Air dehumidification	Waste heat utilisation with heat recovery	
Technique	Dehumidifier	Heat exchanger	Heat recovery unit
Type	Agrifrigor HT 60		ERC-T 30/56
Manufacturer	Frigortec GmbH	Heribert Waltinger GmbH	Arwego Armin Schneider e.K.

only during filling. Both boxes were then covered with a dome to avoid air flowing from one system to the other and to ensure an efficient continuous air recirculation through the dehumidifier and the heat recovery unit. Collected data were i.a. energy consumption, weight of the boxes, air volume, temperature and humidity, using adequate sensors. The DM content of the forage filled into S1 was 71.3 in July and 70.7% in October; whereas for S2 DM content of harvested material was on average 73.5%. For all filled boxes, data about the drying process and energy consumption up to 86% DM content of the hay were logged. The energy consumption for the post-ventilation phase required in practice was not taken into account in all experiments, since in practice generally solar heated air would have been used. In system S1 in July about 3,550 kg DM and in October 2,250 kg DM were dried in total. In system S2 3,500 kg DM were dried in July. Consequently, the filled amount of wilted grass corresponded to a 'water cap' of 47.7 kg water m^{-2} resp. 31.2 kg water m^{-2} in system S1 and 42.1 kg water m^{-2} in system S2. All values were at a very low level, since in practice, depending on the material to be dried, a 'water cap' of 70 to 100 kg water m^{-2} is recommended (Pöllinger, 2014).

Results and discussion

The measured energy requirement for drying hay in July 2018 was 0.65 kWh per kg water loss or 0.51 kWh per kg water loss in October 2018 for system S1. In system S2, energy consumption for drying with nearly 100% simulated waste heat utilisation was 1.18 kWh per kg water loss in July 2018. Compared to values of the energy efficiency promotion program set by the Federal Office for Agriculture and Food (BLE) the energy consumption was slightly higher (BLE, 2017) (Table 2). BLE based the reference value on the technology used in the past five years. For comparing the measured energy consumption with the values set by BLE, the values have to be calculated according to 50% technical drying during night. Only system S1 reached its target value at the October trial. When comparing the measured values with a dehumidifier from Pöllinger (2014) with the specifications of BLE, the energy consumption was on average, despite the energy-efficient technology, even at the expected value for a hay drying system e.g. with waste heat utilisation.

Table 2. Comparison of the energy demand of the experimental plant to the energy efficiency promotion program (BMEL, 2016) und a barn hay drying system with dehumidifier and the possibility of roof suction at AREC Raumberg-Gumpenstein (Pöllinger, 2014, values calculated to 50% roof suction with 50% technical drying based on day lengths).

Energy consumption in kWh kg^{-1} water loss	Energy efficient promotion program	Hübschenried waste heat utilisation July 2018	Hübschenried dehumidifier July 2018	Hübschenried dehumidifier October 2018	AREC dehumidifier all cuts 2011/12 (converted)
100% technical drying	1.04 kWh kg^{-1}	1.18 kWh kg^{-1}	0.65 kWh kg^{-1}	0.51 kWh kg^{-1}	–
50% heat by means of roof suction + 50% technical drying	0.52 kWh kg^{-1}	0.63 kWh kg^{-1}	–	–	–
50% heat by means of roof suction + 50% energy-efficient technology	0.31 kWh kg^{-1}	–	0.41 kWh kg^{-1}	0.32 kWh kg^{-1}	Min-Max: 0.17-0.79 kWh kg^{-1} average: 0.51 kWh kg^{-1}

However, the range from minimum to maximum energy consumption showed that very large differences were possible depending on the operating conditions (Table 2). Therefore, the results showed that the actual energy consumption with state-of-the-art barn hay drying systems depends on many parameters. In contrast to the fix properties of the wilted grass for drying and the weather on the days following harvest, the plant control by the farmer, e.g. an efficient exhaust air or recirculation mode is crucial for good drying results (Thurner *et al.,* 2018).

Conclusion

The use of state-of-the-art barn hay drying systems requires a lot of know-how of the plant operators, but equally important is the exact control of temperature and humidity using sensors to produce barn hay with as little energy demand as possible. If the technology is used efficiently and outside air conditions are adequate, the BLE target values can be reached.

Acknowledgements

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Effects of including perennial ryegrass (*Lolium perenne* L.) in different species mixtures on yield, feed quality and botanical composition in first year of ley

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Abstract

Perennial ryegrass (*Lolium perenne* L.) is not widely used in forage production in Norway; until recently only in regions with very mild winter climate. Due to its high digestibility and yield potential, and trends towards milder winters, the interest for using this species in silage production has increased. However, variable winter weather with frost and ice can damage perennial ryegrass extensively, and it is therefore regarded as a rather short-lived species under these conditions. In this paper, we report results from field experiments for first-year leys established in 2016 at three different locations from south to north in Norway. Different seed mixtures of grass and clover species were sown with and without the addition of perennial ryegrass. In 2017, plots were fertilised with either medium level of nitrogen (N) or low N-level (half of the medium level). Dry matter yields, botanical composition and feed quality (determined by NIRS) from each cut were recorded. Perennial ryegrass dominated in all mixtures and reduced weed invasion, regardless of location. Inclusion of ryegrass led to higher yield production compared to mixtures without ryegrass; it increased digestibility but the content of crude protein tended to be lower, probably due to a dilution effect caused by the higher yield production.

Keywords: species composition, weed invasion, northern latitudes, crude protein, NDF, digestibility

Introduction

Extended growing seasons and milder winters open up possibilities for using grass species formerly not used under northern latitudes such as perennial ryegrass *(Lolium perenne)*. Grass mixtures containing perennial ryegrass are available on the market, and farmers use these mixtures increasingly for silage production. Perennial ryegrass is productive and competitive and has a high feed quality, but it is less winter hardy than the more commonly used species of timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) (Höglind *et al.* 2010). If ryegrass dominates totally in the mixtures, losses of this species by winter damage could result in open gaps where weeds could potentially invade. Therefore, the effect of adding perennial ryegrass to the mixture is most likely dependent on the companion species, and their ability to balance the dominance of ryegrass during the first year. We report here on results from first year ley of multispecies field experiments at three climatically contrasting locations from south to north in Norway. In this paper, we compare seed mixtures with and without perennial ryegrass in terms of dry matter (DM) yields, botanical composition and feed quality.

Materials and methods

Field experiments were sown at three sites across Norway in 2016: Holt (69°N, 14 m a.s.l.-north), Kvithamar (63°N, 28 m a.s.l.-mid-Norway), Særheim (58°N, 14 m a.s.l.-southwest), sown 9 June, 24 June or 5 July, respectively. The species used were perennial ryegrass, timothy, meadow fescue, smooth meadow-grass (*Poa pratensis*), common bent (*Agrostis capillaris*), red clover (*Trifolium pratense*) and white clover (*T. repens*). At each site, monocultures and species mixtures of up to seven species making 30 communities were sown in a simplex design (Cornell, 2002). The communities were fertilized with compound fertilizer; in kg ha^{-1} per site: 170N – 36P – 148K (Holt); 240N – 40P – 174K (Kvithamar);

260N – 44P – 188K (Særheim) and replicated with low N-level (half of this amount, same amount of P and K) making 60 plots of 10.5 m^2 per site. The mixtures used in this study are shown in Table 1. Plots were harvested with a Haldrup experimental harvester twice at Holt (5 Jul, 22 Aug), three times at Kvithamar (15 Jun, 26 Jul, 11 Sep) and Særheim (7 Jun, 24 Jul, 11 Sep), with first cut at early heading of timothy. Subsamples of ca 300 g were separated for first and last cut for botanical composition and DM yields were determined for all harvests after drying subsamples at 60 °C for 48 h. Subsamples for NIRS analyses were ground to 1 mm (Cyclotec™ Sample Mill 293) and analysed (NIR systems 6500, Silver Spring MD, USA). Data were analysed using analyses of variance with the glimmix procedure (SAS Institute Inc., 2012) with site, mixtures, N-level and inclusion or absence of ryegrass as factors.

Results and discussion

The ryegrass content of the DM yield was affected by mixture type ($P<0.025$) with the highest content in mixture D (87%) and lowest in mixture A (48%) where all species were present (Figure 1). Ryegrass was sown in lower amount in mixture A (14%) than in the other mixtures and was also competing with other rapidly establishing species such as red clover, timothy and meadow fescue. The number of weeds was significantly ($P<0.0001$) reduced when ryegrass was included in the mixtures, particularly in mixture D. The DM yields were lowest at the northern site Holt (7.66 t ha^{-1}) and highest at Særheim (11.03 t ha^{-1}) ($P<0.0001$), reflecting the shorter growing season at the northern site. Inclusion of ryegrass gave higher yields, but this was significant only in mixture D, where the plots with ryegrass had highest yields of all mixtures, while the same mixture without ryegrass had the lowest yield of all (Table 2). Mixture D was composed of the slow establishing species smooth meadow-grass, common bent and white clover, and the plots without ryegrass had large amounts of weeds (Figure 1). In these mixtures, perennial ryegrass dominated completely when it was included. The crude protein (CP) and NDF contents were lowest, and DM digestibility (DMD) was highest at the northern site ($P<0.0001$). This is in accordance with results of Nordheim-Viken *et al.* (2009) who found the same tendencies in timothy treated with lower temperatures and long day compared to higher temperatures and shorter day.

Inclusion of ryegrass gave lower CP, but this was significant only in mixture D, which had the highest content of CP of all mixtures when ryegrass was not included. This probably reflected both the high content of legumes and weeds in this mixture, and a low yield level (Figure 1, Table 2). Ryegrass did not affect the NDF content per se, but it was significantly lower in mixture D without ryegrass than in all other mixtures, also reflecting the botanical composition. DMD was significantly enhanced by the inclusion of ryegrass ($P<0.0001$), but there were no interactions between inclusion of ryegrass and mixture type.

Table 1. Species mixtures used with and without inclusion of perennial ryegrass. The percentage of each species in the seed mixture was calculated based on seed weights used when sown in monoculture.[1]

Mixture	Ryegrass	Total seed rate	Species						
		kg ha^{-1}	Lol	Phl	Fes	Poa	Agr	Trp	Trr
A	Abs	19.3	0	0.17	0.17	0.17	0.17	0.17	0.17
	Incl	21.6	0.14	0.14	0.14	0.14	0.14	0.14	0.14
B	Abs	22.5	0	0.25	0.25	0.25	0.25	0	0
	Incl	25.0	0.2	0.2	0.2	0.2	0.2	0	0
C	Abs	22.7	0	0.33	0.33	0	0	0.33	0
	Incl	25.8	0.25	0.25	0.25	0	0	0.25	0
D	Abs	16.0	0	0	0	0.33	0.33	0	0.33
	Incl	20.8	0.25	0	0	0.25	0.25	0	0.25

[1] Lol = perennial ryegrass, Phl = timothy, Fes = meadow fescue, Poa = smooth meadow-grass, Agr = common bent, Trp = red clover, Trr = white clover.

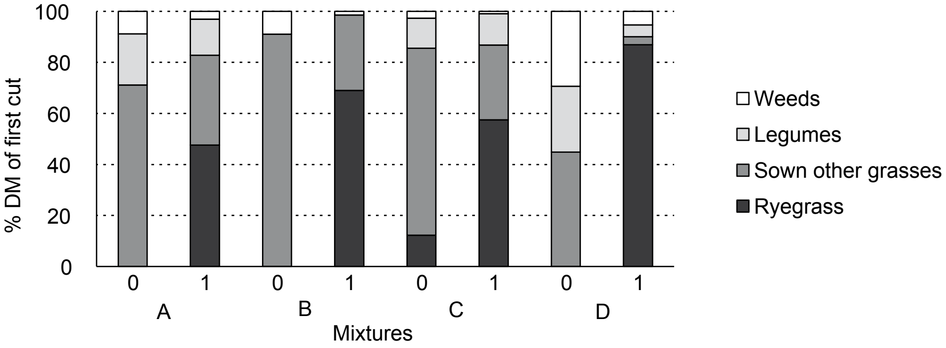

Figure 1. Percentage of perennial ryegrass, other sown grasses, legumes and weeds of DM yield in first cut averaged for the three sites and two N-levels for mixtures A-D without sown ryegrass (0), and mixtures with ryegrass (1).

Table 2. DM yield, crude protein, NDF and digestibility of total yield in 2017 averaged over the three sites in mixtures with (Incl) and without (Abs) perennial ryegrass.[1]

	Mixtures								**Averaged over mixtures**	
	A		**B**		**C**		**D**			
	Abs	**Incl**	**Abs**	**Incl**	**Abs**	**Incl**	**Abs**	**Incl**	**Abs**	**Incl**
DM yield t ha^{-1}	9.6 abc	10.3 abc	8.7 c	9.8 abc	9.5 bc	10.9 ab	6.9 d	11.1 a	8.8 B	10.5 A
Crude protein g kg^{-1}	125 b	113 bc	106 bcd	90 d	120 bc	102 cd	163 a	101 cd	128 B	101 A
NDF g kg^{-1}	573 b	548 b	598 a	558 ab	569 ab	562 ab	484 c	540 b	556 A	552 A
Digestible DM g kg^{-1}	691 c	716 ab	708 abc	729 a	696 bc	715 abc	719 ab	721 a	703 B	720 A

[1] Different letters behind mean values within rows indicate significant difference at $P<0.05$ (Tukey).

Conclusion

Inclusion of perennial ryegrass improved yield production, digestibility of the yields and also reduced weed invasion in the first year of ley. Mixtures containing rapidly establishing species balanced the ryegrass dominance better than slowly establishing species.

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Non-woven fabrics supporting the greening of newly formed ski slopes

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Abstract

The use of protective non-woven fabrics for greening ski slopes and its impact on the development of a grass-legume mixture was investigated. The research was carried out on a ski slope located on Jaworzyna Krynicka in the Beskid Sądecki at an altitude of 830 m a.s.l. A seed mixture of the following composition was sown on a newly shaped ski slope in the early spring: *Poa pratensis* 30%, *Festuca rubra* 30%, *Festuca arundinacea* 10%, *Festuca ovina* 10%, and *Trifolium repens* 20%. A part of the sown surface was covered with partially biodegradable protective non-woven fabrics with various basis weights of 64, 105 and 124 g m^{-2}. The uncovered area was used as reference. The non-woven fabrics used positively influenced the development of monocotyledonous plants and white clover. Plants under non-woven fabrics were distinguished by a faster development compared to the reference. On the uncovered surface 30% of the seeds did not germinate, the plants developed from the seeds were much smaller and the level of vegetation cover was lower. In addition, there was a positive relationship between the non-woven fabrics basis weight and the dynamics of plant development.

Keywords: ski routes, non-woven fabrics, grass-legume mixture, vegetation cover

Introduction

Newly shaped ski slopes do not have a dense turf, thus erosion of soil, in particular of organic matter may occur. It is a challenge to establish stable vegetation on such initial surfaces as rapid as possible. During the formation of the surface, large amounts of soil are moved and the parent rock is often brought out (Gliński and Przesmycki, 2011). The remaining surface substrate is poor in nutrients and biological activity, which are necessary for the development of protecting vegetation. In addition, this medium usually contains low amounts of organic matter that would increase soil water capacity and improve conditions for plant growth and development. The aim of the work was to examine the use of protective non-woven fabrics for greening ski slopes and its impact on the rate of grass-legume mixture development.

Materials and methods

The research was carried out on a ski slope located on Jaworzyna Krynicka in the Beskid Sądecki at an altitude of 830 m (49°25'08.3'N; 20° 55'01.0'E). In the early spring, a seed mixture with the following composition: *Poa pratensis* 30%, *Festuca rubra* 30%, *Festuca arundinacea* 10%, *Festuca ovina* 10% and *Trifolium repens* 20% was sown on a newly shaped ski slope (18° gradient, eastern exposition). A randomized block design with four replications was used. The four treatments were: uncovered (reference) plot, and covered with non-woven fabrics of 64, 105 and 124 g m^{-2}. The plot size was 50 m^2. Directly before sowing, mineral fertilization was applied at the following quantities: 60 kg N ha^{-1} in form of ammonium nitrate, 30 kg P ha^{-1} in form of superphosphate and 60 kg K ha^{-1} in form of potassium salt. Immediately after sowing, a part of the sown surface was covered with partially biodegradable protective non-woven fabrics with various basis weights of 64, 105 and 124 g m^{-2}. The uncovered area was used as the reference treatment. After 2 months, the coverage rate of vegetation was evaluated using the Weber squares technique (Clarke, 1986). Analysis of variance was performed (ANOVA) using Statistica 6.0. Significance of differences was verified by means of Tukey's test on the confidence level of 0.05.

Results and discussion

The role of the non-woven fabric on a ski slope is different in relation to its use in the production of fruit and vegetables (Ambroszczyk, 2009; Krzysztofik, 2013). The main threat to the initial development of seedlings and plants on the slope is the excessive exposure to direct solar radiation as long as the shallow root system is not able to provide the plant with sufficient amount of water. A large number of seedlings die off under such extreme conditions. Therefore, covering the slope with a non-woven fabric seem to be an indispensable support for seedlings against unfavourable climatic and soil conditions (Sowiński and Wołoszyn, 2013).

The uncovered area showed the lowest coverage of vegetation among all variants (Table 1). On this surface, mainly some grass and a few legume plants appeared. The height of seedlings was up to 8 cm, and the soil coverage by plants only reached 15%. On the reference plots a proportion of 30% of non-germinated seeds were visible. Some of the seedlings, especially grasses, showed clear indications of dryness, because of the weak root system and direct exposition to sunlight. Uncovered seeds and fertilizer were also exposed to runoff during intense precipitation – up to 70 mm per day with an annual rainfall about 1000 mm.

Under the lightest non-woven fabrics (weight of 64 g m^{-2}) the coverage of the soil surface with plants was 60%, and the average vegetation height was 15.3 cm. The plants were well developed, dark green and the grasses were predominant in the sward with *Festuca arundinacea* as the most represented species.

Variants covered with non-woven fabrics with a weight of 105 and 124 g m^{-2} showed the best soil coverage with vegetation ranging between 85-93%. There was a statistically significant difference of these two variants compared to the reference and the variant covered with the lightest non-woven fabric. The average height of the sward of variant with heaviest non-woven fabrics was 22.1 cm. The positive results obtained from using non-woven fabrics with the highest basis weight were probably caused by pressing the seeds to the soil and protecting seeds, soil and fertilizer components against surface runoff (Grześkiewicz, 2002). In addition, the white non-woven fabric increased the albedo surface area that is comparable to the albedo of fresh snow. This is a very beneficial phenomenon resulting in smaller temperature amplitudes during the day (Sowiński and Wołoszyn, 2013).

Table 1. Average height and coverage rate of vegetation after 2 months.[1]

Treatment	Height (cm)	Coverage (%)
Uncovered (reference) plots	7.5 a	12.7 a
Non-woven fabrics 64 g m^{-2}	15.3 b	60.0 b
Non-woven fabrics 105 g m^{-2}	21.5 c	86.3 c
Non-woven fabrics 124 g m^{-2}	22.1 c	91.2 c

[1] Different letters within the same parameter mean the significant difference ($P<0.05$).

Conclusion

1. The non-woven fabrics used for the greening of ski slopes ensure a rapid initial development of sown plants, which favours the rapid establishment of vegetation.
2. A positive relationship was found between the basis weight of non-woven fabrics and its suitability for greening measures of newly shaped ski slopes.

Acknowledgements

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The root growth of forage grasses at different soil P levels

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Abstract

The size of forage grass roots has a significant impact on nutrient use efficiency. Our aim was to examine how initial soil P fertility level (Fair P: 6.2 mg P l^{-1} soil, Good P: 17.5 mg P l^{-1} soil) and annual P application (20 kg P ha^{-1}) contribute to the above- and belowground growth of silage grass. The field trial was located at Kotkaniemi experimental farm (Yara Suomi Ltd., Finland) where the two soil P levels were created in a 40-year fertilization experiment. Plant and root samples (cylinder 20 cm depth, ø 10 cm) were taken at the time of the first harvest and three times after the harvest, and root and shoot dry mass (DM) was measured and shoot:root-ratio (S:R) was calculated. Significantly higher root DM was measured when the soil P level was good and P was applied (Good: P20). Root decay was observed in all treatments after harvest. In general, low S:R was measured due to a long period of water deficit and temperatures above the long-term average. Our results indicate that good P supply, either from soil or by additional fertilization, has a positive impact on grass root growth.

Keywords: grass root, soil P level, shoot:root ratio, P fertilization

Introduction

Most of the living grass biomass, over 60%, is beneath the surface (Gibson, 2009) and 70-90% of roots are in the 0-20 cm depth (Bolinder *et al.*, 2002). Silage grasses are harvested multiple times in a growing season, and it has been shown that root growth is reduced for 6-18 days after harvests. The growth cessation depends on the extent of above ground biomass removal (Crider, 1955). Simultaneously with the root growth cessation, nutrient uptake, such as P is also decreased (e.g. Oswalt *et al.*, 1959). Among other nutrients, the availability of N and P affects root growth. In Italian ryegrass (*Lolium multiflorum* L.) N fertilization is shown to increase shoot:root ratio (S:R), root dry mass (DM) and root area, but decrease the specific length (Henry *et al.*, 2005). The results of P fertilization effects on root DM and length are somewhat contradictory as some studies show no effect of P fertilization on root growth (Ros *et al.*, 2018) and in other studies the results depended on the species (Waddell *et al.*, 2017). The objective of our study was to examine how soil P fertility level and P application affected the above- and belowground growth of silage grass.

Materials and methods

The effects of two soil fertility P levels (Fair: 6.2 mg P l^{-1} soil; Good: 17.5 mg P l^{-1} soil) and annual P fertilization (P0: 0 P kg ha^{-1} and P20: 20 P kg ha^{-1}) on the root growth of silage grass ((timothy (*Phleum pratense* L.), meadow fescue (*Schedonorus pratensis* Huds.), tall fescue (*Schedonorus arundinaceus* Schreb.)) were evaluated in a field trial in a third-year grass ley located at Kotkaniemi experimental farm (Yara Suomi Ltd., Finland). Soil P levels ranging from low to high were created in a previous, static 40-year long-term fertilization experiment with increasing NPK levels. The soil type of the experimental site was clay loam. The plots, with three replicates, were harvested three times during the growing period and fertilized with N-P-K-S fertilizer (250/250 – 0/20 – 200/200 – 0/1.2). In 2018, the plots were irrigated (20 mm) one week after the first harvest due to a long drought period. One plant and root sample (cylinder height 20 cm, ø 10 cm) was taken from each plot (three replicates) at one week before the first harvest (May 24) and three times after the first harvest (June 5, 19 and 26). After sampling, the soil samples were soaked in water for 2 h and the roots were washed using 3.55 mm and 1.0 mm sieves to collect all roots. Four individual timothy plants with roots were separated and photographed. Separated plants were added back to sample taken with cylinder from ley mixture, shoots were separated from roots from the root neck. Roots and shoots were dried at 60 °C to constant weight. Root and shoot dry mass (DM) was measured and converted to g m^{-2} form and S:R was calculated. Root area (m^2 m^{-2}) and diameter (mm) were analysed from the images by using Image J program. Analysis of variance (ANOVA) was performed to compare differences between all treatments and sampling dates. Means were compared using Fisher's Least Significant Difference (LSD) when the F-value in the ANOVA was statistically significant ($P<0.05$).

Results and discussion

Soil P level and P application had a positive effect on root DM at the time of harvest and immediately after harvest (Figure 1). The root DM was measured significantly higher one week before harvest than 20 or 27 days after harvest when P was applied (Fair: P20 and Good: P20). Root decay was observed in all treatments after harvest and a full recovery of the root DM was not observed within a month.

In the aboveground biomass no differences were observed at harvest. Differences in regrowth between the treatments in the aboveground growth also levelled off during the monitoring period, even though the regrowth was slightly faster in the Good P20 treatment. The long period of water deficit and temperatures above the long-term average during the spring and summer explains the low above ground biomass. Root area (m^2 m^{-2}) varied between 0.005 to 0.008 being the lowest in the Good P20 treatment, whereas the mean root thickness was approximately 0.2 mm and it was unaffected by the treatment.

In the first harvest S:R was in the range 0.13-0.35 which is comparable to the results of Bolinder *et al.* (2002) who found the S:R was between 0.16 and 0.75 for grasses. Low S:R may be caused by a long period of water deficit and temperatures above long-term average in spring and summer, as significant amounts of the biomass accumulated in roots rather than shoots.

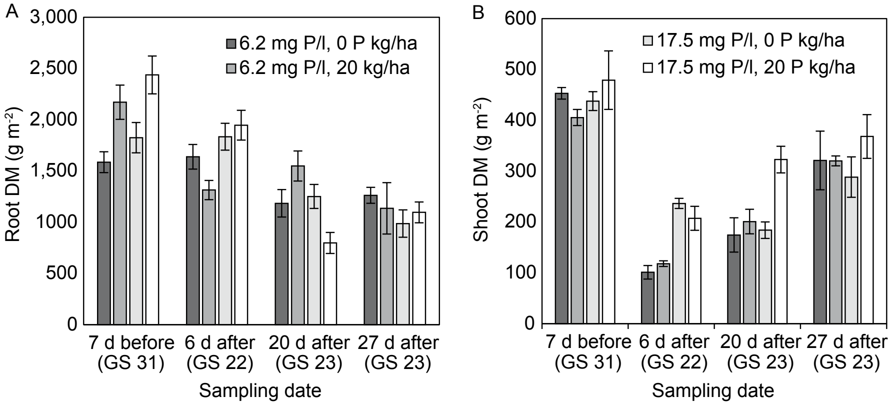

Figure 1. (A) Root dry mass (DM, in the 0-20 cm soil layer) and (B) shoot DM (g m^{-2}) means of four treatments (low soil P fertility level 6.2 mg P l^{-1} soil with 0 and 20 P kg ha^{-1} fertilization and high soil P fertility level 17.5 mg P l^{-1} with 0 and 20 P kg ha^{-1} fertilization) one week before harvest and three times (6, 20 and 27 days) after harvest and the growth stages (GS). The data shown are means ± standard error (SE), n=3.

Conclusion

Root DM was greatest in the 'Good P20' treatment at the time of harvest and immediately after it. This indicates that good P availability, either from soil or by additional fertilization had a positive impact on grass root growth. However, more study of the effects of soil P availability and P fertilization on grass root growth is needed.

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Importance of functional traits of the *Lolium perenne* variety on forage quality decreases when grown in mixtures

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Abstract

Grasslands intended for dairy production are managed intensively with respect to fertilisation and defoliation frequency by growing varieties of *Lolium perenne* as the dominant component. Plant breeding has brought about improvements in the yield, forage quality and functional traits of these varieties. The majority of functional trait variability is represented by phenology (early vs late) and growth form (prostrate vs upright). Whether variety-specific functional traits of *L. perenne* affect forage quality in mixtures has not yet been analysed over extended durations. This study quantified the effects on forage quality of four varieties of *L. perenne*, using a factorial combination of phenology and growth form, grown in binary or four-species mixtures compared with a monoculture.

Keywords: multispecies swards, forage quality, functional traits, extensive farming

Introduction

Research has emphasised benefits with respect to nutrient capture, yield and livestock performance of cultivating mixtures of grasses, legumes and forbs, e.g. *Plantago lanceolata* (Hofer *et al.*, 2016; Pembleton *et al.*, 2016). However, *Lolium perenne* represents the principle component of temperate grasslands for dairy production and farmers frequently cultivate selected varieties differing in their yield potential, growth form or phenology and supply the sward with nitrogen (N) to maintain productivity (Wachendorf and Taube, 2001; BSA, 2016). In less-intensively managed grassland swards, the N intensity is lower and usually higher abundances of forbs and legumes accompanied by grasses are found (Trott *et al.*, 2004). Whether variety-specific functional traits of *L. perenne* affect forage quality in such diversified systems is not yet clear. Therefore, this study quantified the effects of variety-specific traits of *L. perenne* grown in mixtures compared with their respective monocultures in a multi-year field experiment. We hypothesized that growing mixtures results in higher crude protein and energy levels compared to *L. perenne* monocultures.

Materials and methods

The field experiment was conducted at the research farm 'Reinshof' near Göttingen (mean annual temperature 9.3 °C, average annual rainfall 645 mm, silty-clay soil), Germany over five full harvest years. The experimental setup was a 4-factorial randomised block design with four replications and a plot size of 15 m^2 established during August of the preceding year. Four varieties of *L. perenne* were chosen to provide a factorial combination of phenology and growth form. Two early and two late-heading varieties with either a prostrate or upright growth form were sown as monocultures ('G'), in binary mixtures with *T. repens* ('GC' with a proportion of 75:25% germinable seeds of grass:clover) or in a four-species mixture ('GCF') together with *T. repens*, *P. lanceolata* and *Taraxacum officinale* (40, 20, 20 and 20%, respectively). Only G was supplied with 200 kg N ha^{-1} annually. On each harvest date, the botanical composition was quantified by separating a subsample (500 g FM) into grass, clover, plantain, dandelion and unsown species. Mean harvest dates for cut 1 to cut 4 were: 15.05, 20.06, 28.07 and 30.09, respectively (only 3 harvests during year 3 and 5 were possible). Prior to separation, a subsample was dried in a forced air oven (48 h, 60 °C) for dry-matter determination. Then the crude protein (CP) content was determined by near infrared reflectance spectroscopy (NIRS) and the energy contents (MJ ME kg^{-1} DM) were calculated according to Losand *et al.* (2007). Data were checked for normality and weighted means of CP and ME for each cut were derived, averaged over years. The effects of the factors on forage quality were calculated by linear mixed effects models using 'R' software by assuming cut, mixture, phenology, growth form and their interactions as fixed and plot nested in block as random factors. Minimum adequate models were determined by the Akaike's Information Criterion. For significant fixed effects, multiple contrasts were performed using 'emmeans' ($P \leq 0.05$). The factor 'mixture' identified G, GC and GCF.

Results and discussion

We found a significant interaction of mixture × phenology × cut ($P \leq 0.001$) and of growth form × mixture ($P \leq 0.05$) for the CP content, which ranged between 10.8% (first cut G early) and 20% (third cut G late). The ME contents were affected significantly by the interaction of growth form × mixture × cut ($P \leq 0.01$) and the interactions of phenology × growth form ($P \leq 0.001$), phenology × cut ($P \leq 0.05$) and phenology × mixture ($P \leq 0.01$) ranging between 11.2 and 12.4 MJ ME kg^{-1} DM. Therefore, Table 1 represents mean values for the interaction of mixture × phenology × cut.

For the CP contents, the effect of phenology remained stable over G, GC and GCF in cuts one and two, where late ranged above early and early above late, respectively. Thereafter no differences were found for GCF. In cut three, however, late ranged above early in G, and in cut four early ranged higher than late in GC ($P \leq 0.05$). Differences between mixtures in relation to cut × phenology were observed. When early varieties were grown, mixtures clearly ranged above G in cuts one and four. For late varieties, GC ranged above G and GCF in cuts one, two and four, while GCF ranged above G in cuts two and four. Cultivation of prostrate varieties resulted in higher CP contents compared to upright varieties irrespective of mixture (15.7 vs 14.9%, respectively).

The effect of phenology on the energy contents remained stable in G for the first three cuts, where late ranged above early treatments (Table 1). In cut two this could also be confirmed for GC. During cuts one to three, G ranged above GC and GCF irrespective of phenology, whereas the opposite was found in cut 4. For G, prostrate treatments ranged above upright in cuts one and two, with the opposite in cut three (not shown). Irrespective of growth form, however, G ranged above GC and GCF in cuts one, two and three (mean difference of 0.3 MJ ME kg^{-1} DM), while GC ranged intermediate. In cut four,

Table 1. Effect of mixture, phenology and cut on the CP [% DM] and energy contents [MJ ME kg^{-1} DM].[1]

	Mixture	Phenology	Cut 1	Cut 2	Cut 3	Cut 4
CP content [% DM]	G	early	10.8±0.7 Aa	13.9±0.3 Bab	16.7±0.5 Ab	13.7±0.6 Aa
		late	15.2±0.7 Ba	11.2±0.3 Aa	20.0±0.5 Bc	13.7±0.6 Aa
	GC	early	13.5±0.3 Ab	14.4±0.2 Bb	17.2±0.2 Ab	19.8±0.3 Bc
		late	18.5±0.3 Bb	12.9±0.2 Ac	17.7±0.2 Ab	19.0±0.3 Ac
	GCF	early	13.8±0.3 Ab	13.4±0.2 Ba	13.9±0.2 Aa	17.5±0.3 Ab
		late	16.2±0.3 Ba	12.5±0.2 Ab	13.7±0.2 Aa	17.5±0.3 Ab
ME [MJ kg^{-1} DM]	G	early	12.3±0.02 Ac	12.0±0.02 Ac	11.8±0.02 Ac	11.2±0.03 Aa
		late	12.4±0.02 Bc	12.2±0.02 Bc	12.0±0.02 Bc	11.3±0.03 Aa
	GC	early	12.2±0.02 Ab	11.8±0.02 Ab	11.6±0.02 Ab	11.5±0.03 Ab
		late	12.2±0.02 Ab	11.9±0.02 Bb	11.7±0.02 Ab	11.5±0.03 Ab
	GCF	early	12.0±0.02 Aa	11.7±0.02 Aa	11.4±0.02 Aa	11.6±0.03 Ab
		late	12.0±0.02 Aa	11.7±0.02 Aa	11.4±0.02 Aa	11.5±0.03 Ab

[1] Presented are means and numbers following ± represent standard error of the mean. Means with the same uppercase letters within in interaction of cut × mixture are not significantly different between phenology, while means with same lowercase letters are not significantly different between mixtures for every interaction of cut × phenology ($P \leq 0.05$).

G showed lower energy contents than GC and GCF (11.3 vs 11.5 MJ ME kg^{-1} DM). Consequently, mixtures showed some potential to increase the forage quality late in the season. The growth form had no influence when grown in GC and GCF. Generally, forage quality contents have to be regarded in view of the accumulated biomass. With respect to this, upright varieties of the *L. perenne* component resulted in higher yields when grown in G, while no effect was found in either mixture (S. Heshmati, unpublished data). However, GCF showed higher total yields compared with monocultures. Higher yields were accompanied by increased between- and within-years variability in GC and GCF compared with G, without effect of the *L. perenne* variety. The component yields of *T. repens*, *P. lanceolata* and *L. perenne* were affected by the growth form. While the total yield and the yield of the grass component was increased by growing upright varieties, irrespective of mixture (G, GC and GCF), prostrate varieties in GC and GCF increased *P. lanceolata* and *T. repens* component yields.

Conclusion

Generally, the forage quality differed between cuts due to environmental conditions during the experimental years. The functional traits of *L. perenne* matter primarily when grown as monoculture. Mixtures showed potential to increase the energy contents late in the season (cut 4) when compared to a pure grass sward.

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Characterising permanent grassland-based farming systems in Europe

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Abstract

Permanent grasslands (PG) provide a wide range of ecosystem services (ES) and are also the basis for many highly valued landscapes and support rural vitality and recreational potential in many regions; and yet in many parts of Europe, PG systems are threatened by abandonment and afforestation, intensification, or conversion to arable land. To support the maintenance and sustainable management of PG, the Horizon 2020 SUPER-*G* project (SUstainable PERmanent Grassland Systems and Policies) has set out to (1) increase understanding of the importance and functioning of PG within a range of important European biogeographic regions; (2) benchmark PG performance across Europe; (3) develop integrated approaches for sustainable PG management; and (4) develop tools and policy mechanisms inclusive of stakeholder and citizen priorities. A PG-based farming systems typology will provide one of the strata for the implementation of these activities. This paper presents first outcomes of an inventory of PG-based farming systems in five biogeographical regions of Europe.

Keywords: permanent grassland, grassland typology, farming systems, ecosystem services

Introduction

According to Eurostat data for 2013, PG covers almost 60 million hectares across the 28 EU-countries and accounts for 34% of the total Utilised Agricultural Area (UAA), although there are large differences between countries and biogeographical regions (BGRs) in terms of proportion of UAA, spatial fragmentation and distribution (Huyghe *et al.*, 2014). This results in contrasting priorities for the specific roles played by PG depending on local conditions. PG provide a wide range of ecosystem services (ES) and can support social infrastructure and high levels of biodiversity that in turn enhance ecosystem function and value to society (Cardinale *et al.*, 2012). In various parts of Europe, PG systems are threatened by abandonment and afforestation, intensification, or conversion to arable land. To curb these trends, the Common Agricultural Policy reform in 2013 introduced protective measures, including the obligation for national governments to maintain the ratio of PG to the total agricultural area. The ability of farmers and land owners/managers to maintain and manage grasslands with a view to ES delivery depends on local conditions (including soil type, slope, ground water level and climate), farming system (FS) (e.g. dairy, beef, sheep, goat, and their intensification level), the profitability of the farming business and any financial support/incentive provided by rural development programmes.

PG typologies and FS typologies are closely intertwined, and both are needed as frames to enable the assessment of technological innovations and policy interventions geared towards the tailored optimisation of economic performance and ES delivery by PG-based systems. Farming systems and management regimes, such as grazing, cutting, fertilization and irrigation or drainage, have an impact on plant species composition and productivity. Grassland composition, farming system and production intensity are also key factors governing the delivery of ES such as pollination, biodiversity, water regulation and erosion control, carbon sequestration, and landscape value.

Methods

We made a first and still tentative inventory of PG-based farming systems, their geographical distribution, their characteristics and the role PG plays in them, as the starting point for a FS classification. The latter will serve to quantify how farm and PG management options affect economic performance and ES delivery by PG-based systems. Subsequently, the classification aims to help in ranking FS types in terms of ES delivery, to define the main threats they face, and to design dedicated policies to support them and mitigate the threats.

The inventory and analysis of FS characteristics was based on (1) available databases at European scale: Eurostat database where farms are classified using FADN classes and PG surface is clearly separated from other land uses; and (2) literature review and the collection of expert knowledge within the project partnership, i.e. the main farm types as identified by each of the partner countries' formal or informal classifications.

Features that discriminate between PG-based farm types were identified using Multi Correspondence Analysis (MCA), performed in R (R Core Team, 2018) and FactomineR package (Le *et al.*, 2008). Variables included BGR (Alpine, Atlantic, Boreal, Continental, Mediterranean); farm products (crops, bioenergy, live animals, meat, dairy, wool); PG exploitation (haymaking, silage, grazing); PG agronomic management (overseeding, mineral fertilisation, manure fertilisation, irrigation); animal species (cattle, goats, sheep, horses, pigs, other); and the proportion of total farm UAA that is PG.

Results and conclusion

Preliminary outcomes are presented in Figure 1 and 2. Ellipses representing confidence intervals of the distributions show a good cohesion of FS around centroids when plotted by BGR, but also a high degree of overlapping (Figure 1). Overlaps between BGRs and between countries suggest the need for a harmonised farm classification across Europe. The position of FS along axes in Figure 1 depends mainly

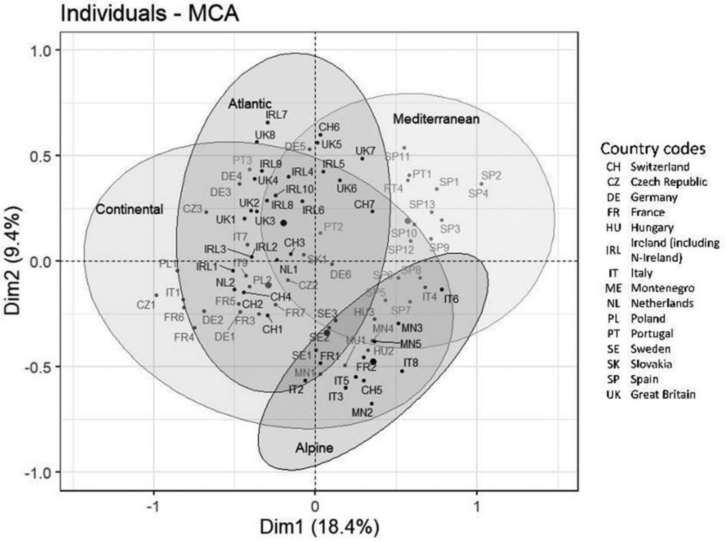

Figure 1. Plot of the described farms types in the first two MCA dimensions.

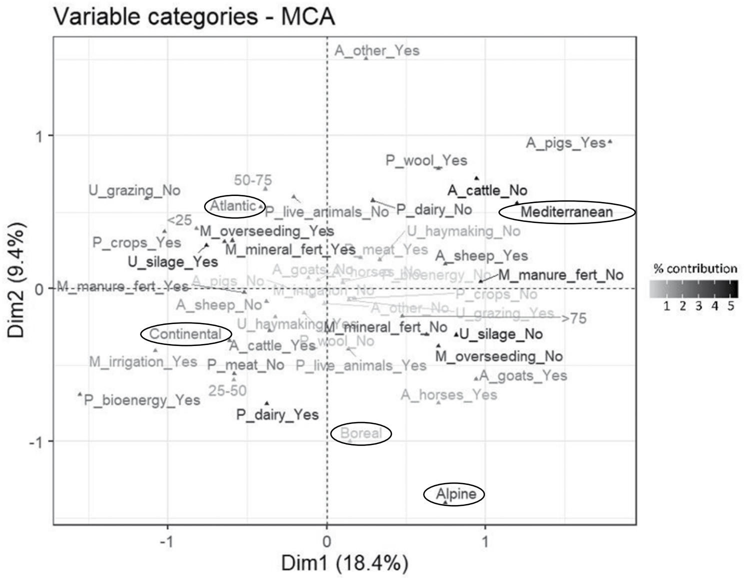

Figure 2. Plot of the variables, including BGRs outlined in black, in the first two MCA-dimensions. Grey font shades indicate the different percentages each variable contributes to Dimension 1.

on variables related to farm products, PG exploitation and PG management (Figure 2), as highlighted by the percentage contribution of these variables on axis one. In particular, the main drivers affecting the position of BGR ellipses was the absence of cattle, and consequently the absence of dairy products, most agronomic management options and cutting for silage. Conversely, the proportion of the farm area occupied by PG had limited power to separate FS types. These preliminary results will be used to address the next step in the farm system classification, to validate the information on the most important variables and rationalise farming systems between biogeographic regions and countries.

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The effect of altering autumn closing management on the water-soluble carbohydrate composition of perennial ryegrass (*Lolium perenne* L) the following spring

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Abstract

Water soluble carbohydrates (WSC) in perennial ryegrass (*Lolium perenne*: PRG) stubble are used post-grazing by plants to stimulate regrowth of the remnant leaf and the emergence of the first leaf. As the plant begins to photosynthesize again, WSC are accumulated and stored until the plant reaches the third-leaf stage. If the plant is not given the required regrowth interval to replenish WSC reserves, subsequent regrowth may be supressed, and persistency reduced. The objective of this study was to determine if altering autumn closing date or the number of days a paddock was closed for affected the PRG plants' WSC concentration prior to grazing the following spring. This study, established in autumn 2017, had 3 autumn closing managements: Early (25 September – 9 November), Normal (10 October – 24 November) and Late (25 October – 9 December) and each paddock assigned had a different number of days closed over winter. Prior to grazing the following spring, 45 tillers were cut to ground level and the WSC concentrations per tiller were determined. The treatments analysed were autumn closing management and number of days closed. Autumn management had no significant effect (P>0.05) on WSC concentration in the stubble (20.5 mg tiller^{-1} ± 0.61). The number of days closed had a significant effect (P<0.001) on WSC: paddocks closed for less than 100 days had a higher mean WSC (21.7 mg tiller^{-1} ± 1.73) than paddocks closed for over 150 days (18.6 mg tiller^{-1} ± 1.45), indicating a fall-off in WSC concentration after plants had reached the 3-leaf stage.

Keywords: water soluble carbohydrates, autumn management, closing, post-grazing

Introduction

Pasture-based dairy production systems in Ireland based on swards of PRG experience a herbage deficit at the commencement of spring calving (Egan *et al.*, 2017). Altered autumn grazing management, by closing paddocks earlier, has been proposed as one way to increase the availability of herbage in the following spring. Lawrence *et al.* (2016) reported that for each one-day delay in closing between 1 October and 14 November spring herbage was reduced by 10 kg DM per ha. Depending on autumn closing management and spring grazing date, the number of days an individual paddock is closed for can vary, resulting in a large variation in growth over winter. PRG grows by continuously producing new leaves; only three leaves are actively photosynthesising and as a fourth leaf emerges the oldest leaf begins to senesce and there is a continuous cycle of tissue turnover in the sward. The number of days it takes for a new leaf to appear depends on the time of the year (9-14 days in September and up to 35 days in January) (Davies 1977). When three new leaves have fully emerged ('3-leaf stage'), post-grazing WSC levels are completely restored; WSC stores are responsible for regrowth of the remnant leaf and the emergence of the first leaf post-grazing. Only after the first leaf starts photosynthesizing can WSC be produced for storage. It is important that WSC levels in the stem are allowed to adequately replenish in order to stimulate regrowth, as there is a positive correlation between regrowth capacity and WSC concentration (Fulkerson and Donaghy, 2001). Grazing at the 3-leaf stage ensures the WSC are completely restored and also that the ceiling yield is not reached at a regrowth of about 3.5-4 leaves post-grazing (Fulkerson and Slack, 1994). An earlier study by Alberda (1957) showed management practices such as defoliation interval strongly influence the WSC content in grasses. The objective of this study was to determine whether the WSC content in PRG swards closed over winter is linked to autumn management or the number of days closed.

Materials and methods

A farmlet systems study was set up at the Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark, Ireland in 2017. The soil was free-draining acid brown earth of sandy to loam texture. The trial was carried out on 30.6 ha, randomized into 6 farmlets with 5.1 ha in each. Each farmlet was split into 8 paddocks with a stocking rate of 2.9 cows ha^{-1}. There were 3 autumn closing managements: Early (25 September – 9 November), Normal (10 October – 24 November) and Late (25 October – 9 December). WSC measurements and leaf stage were taken on all paddocks prior to defoliation in spring 2018. Forty-five mature, vegetative, individual tillers were cut to ground level from each paddock within three hours of sunrise to avoid any diurnal changes that occur from sunrise to mid-afternoon, and washed to remove excess soil. Each tiller was cut from ground level to 3.5 cm (the targeted post-grazing residual stubble). The stubble, >3.5 cm, was weighed and dried at 60 °C in a pre-heated oven for 48 hours; dried samples were weighed to determine dry matter and then milled using a 1 mm sieve. WSC (mg tiller^{-1}) were determined using the anthrone method (Thomas, 1977). Data were subdivided into number of days closed (n=16), (paddocks closed for <100 days, 100-150 days and >150 days) to determine the effect of the closed period on WSC concentration. WSC data were analysed using PROC mixed in SAS 9.4. Terms included in the model were: fixed terms, i.e. treatment (autumn closing management) and number of days closed; and the random term was paddock.

Results and discussion

Autumn closing management (early, normal and late) had no significant effect (P>0.05) on WSC concentration in the stubble (20.5 mg tiller^{-1} ± 0.61); as a consequence the results discussed will be that of the number of days closed. The WSC concentrations in the current study were similar to those reported by Fulkerson and Slack (1994), who found that the WSC content at the 4-leaf stage was 22% of the DM. Few, if any, studies have reported on the WSC content past the 4-leaf stage. All paddocks closed in this experiment were past the 3-leaf stage and the leaf stage increased with the number of days closed (>4 leaves). A significant (P<0.01) decline in the WSC concentration was observed with number of days closed. The WSC concentration was lower in the >150 days treatment compared to the <100 days (18.6 vs 21.6±0.476 mg tiller^{-1}, respectively). Thus, the concentration of WSC available pre-grazing to sustain the growth of PRG was significantly lower in paddocks closed for a greater number of days. The reduction in WSC (Table 1) intensified as the number of days a paddock was closed over winter increased, which may be a result of increased tissue turnover in the sward (Lawrence *et al.*, 2017). This could show that continual leaf appearance and senescence without grazing may lead to the plant using stored WSC. Previous studies have shown that once WSC levels are restored at the 3-leaf stage there is no further build-up of WSC levels in the plant (Donaghy, 1998). As high WSC levels are linked to potential regrowth, a reduction in WSC in the stubble may impact on PRG regrowth capacity when the plant is grazed in the spring, leading to reduced persistency in the long-term (Fulkerson and Donaghy, 2001). Although in the current study, the decline in WSC did not result in an immediate decline in regrowth,

Table 1. Average WSC (mg tiller^{-1}) for the number of days a paddock was closed over winter.[1]

	Number of days closed			SE	Significance
	<100 days	100-150 days	>150 days		
WSC (mg/tiller)	21.6 ^{a}	20.2 ^{b}	18.6 ^{c}	0.476	0.01

[1] Values with different superscript letters are significantly different (P<0.01).

soil temperatures were low (<4 °C) prior to grazing which may have reduced the impact of WSC, as perennial ryegrass requires a soil temperature (>5 °C) for growth. The results of the current study show that if swards are closed for more than 150 days WSC concentration is negatively impacted. Grassland research in New Zealand and Australia has proposed grazing at the '3-leaf' stage to ensure the WSC in the stem has been fully restored and that there is minimal feed wastage as a result of senescence.

Conclusion

The benefit of grazing PRG at the 3-leaf stage is evident when looking at the WSC content in the stubble over winter. Increasing the number of days closed over winter leads to a reduction in WSC; however, ensuring that sufficient herbage is amassed on farm in spring to meet herd demands is vital. Paddocks closed earlier in autumn should be targeted for grazing earliest in spring to reduce the negative effect on WSC, which may result in a decline in regrowth potential, although not observed in the current study.

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Analysis of options to increase the sustainability in the Swiss dairy sector – a literature review

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Abstract

The dairy sector makes great efforts to improve its sustainability. The impacts of 15 options for improving sustainability were evaluated by means of a literature review. These options cover the aspects of productivity (milk yield per day of life), composition of the feeding ration (e.g. proportion of grassland and pasture forage), specific feed components (e.g. use of certified soya), animal husbandry (e.g. regular outdoor exercise), animal health (herd healthcare measures), biodiversity improvements (participation in a biodiversity improvement programme), and socio-economic aspects (e.g. multi-year contracts). Environmental (11 indicators), social (3 indicators) and economic (3 indicators) impacts were assessed. For some combinations of improvement options and indicators, a univocal assessment was achieved. Examples for an overall favourable effect were feeding of certified soya or herd healthcare measures. For several combinations, the literature provided controversial conclusions, where the effect depends on the context of the production system. Trade-offs between indicators occurred frequently, such as animal-friendly housing improving animal welfare, but also increasing ammonia emissions. Recommendations to improve some options are provided. Furthermore, tools for a quantitative sustainability assessment are suggested (e.g. life cycle assessment (LCA), product cost calculation, work budget calculation).

Keywords: dairy production, sustainability assessment, literature review, environmental indicators, social indicators, economic indicators

Introduction

The dairy sector is the most important agricultural sector in Switzerland and its sustainability is an increasing concern of the society. The question therefore arises as to whether certain measures lead to the safeguarding or improvement of sustainability in milk production. Based on a literature review, the impact of 15 selected measures implemented in the Swiss dairy sector on selected sustainability indicators was evaluated (Haupt *et al.*, 2018).

Materials and methods

Experts in two workshops defined various measures intended to safeguard or increase the sustainability of the Swiss dairy sector. These measures were evaluated with the aid of literature sources, based on the indicator set developed by Roesch *et al.* (2017) for the comprehensive assessment of the environmental, economic and societal pillars of sustainability of Swiss farms. The indicators analysed in the environment dimension of sustainability were non-renewable energy demand, global warming potential, P and K resource demand, land occupation, food-conversion efficiency, deforestation, water demand, eutrophication, acidification and ecotoxicity potentials, and biodiversity. In the societal dimension of sustainability, the effects on landscape aesthetics, milk characteristics, and animal welfare were evaluated. The following economic indicators were included: labour performance indicators, costs and investment as well as revenues, gains and income. The literature review took 190 sources into account (Haupt *et al.*, 2018).

Results and discussion

The effect of the measure *yield per day of life* (kg milk produced per day of life) is debated in the literature. Determining factors are the feeding intensity and the type of feed, the number of lactations, the genetic potential of the dairy cow, and the milk-to-meat-output ratio. Up to a system-specific yield limit, higher yields tend to result in lower environmental impacts per kg of energy-corrected milk (ECM). An increased useful (i.e. productive) life of the cows, a balanced milk-to-meat-output ratio, and a feeding strategy adapted to the individual system (e.g. to the energy requirements of the dairy cow) are favourable. More intensive production systems tend to have a lower global warming potential per kg ECM, mainly due to better feed conversion efficiency. A longer useful life can demonstrably lead to higher profits.

The impact of *concentrate use intensity* (g concentrate feed per kg milk) on the environment is a matter of debate. Producing concentrate feed tends to have higher environmental impacts than grassland forage, particularly for aquatic eutrophication, ecotoxicity, P and K resources, and deforestation. The effect on milk production depends on current milk yield, the concentrate efficiency, the feeding ration, and the genetic potential of the dairy cow. Low concentrate intensity and a high proportion of grassland forage increase earned income.

The influence of the *proportion of grassland and pasture forage* in the ration (kg dry matter in the annual ration) is also debated; the findings are somewhat similar to the concentrate use intensity. Grassland forage tends to give lower milk yields per cow than feedstuffs from arable land, with negative impacts on feed conversion. Competition for land for food production decreases when grassland forage is produced on land unsuitable for arable farming. Milk characteristics – in particular the fatty acid composition – are favourably affected. A higher proportion of grassland forage has positive effects on the economic dimension of the farm, and leads in particular to a higher labour utilisation.

The feeding of *certified soya* according to the Soya Network Switzerland, has a favourable effect on the environment, chiefly through the avoidance of deforestation and the associated greenhouse-gas (GHG) emissions. Uncertified soya is cheaper than certified soya, but since 99% of the soya used in Switzerland is already certified, the economic benefits are minor.

The impact of the measure *ban on the feeding of urea* in the environmental dimension is unclear. In some environmental impact categories, the production of urea is less favourable than soybean cultivation; in others, it is more favourable. A comprehensive analysis is not yet available, however. Refraining from feeding urea to livestock tends to have a positive effect on animal welfare. The effect on the properties of the milk is a matter of debate. Using urea would have positive effects on economic indicators. A price comparison with soybean meal shows that urea offers major potential savings.

Fatty-acid-optimised feed tends to have a favourable effect on the environment. Numerous studies point to reduced methane emissions from digestive processes after the feeding of fats, but comprehensive life cycle assessment studies are largely lacking. The influence on milk composition is also favourable, and at the proper dosage no negative effects on animal welfare are to be expected. Fatty-acid-optimised feed, however, entails additional costs.

The impacts of *sperm sexing* were little investigated, but the sharply rising demand for sexed sperm points to a positive profitability. *Calf management* encompassing the adequate supply of colostrum, demand-based feeding with solid feed, an adequate supply of fresh air, and housing first of all in individual cubicles, and rearing in group housing organised according to age, has a positive impact on animal welfare and animal health. The reduced transport of animals has a positive influence on animal welfare and the environment. Compared to agricultural production, however, transport plays a minor role in the total of environmental impacts. The benefits of *dual-use breeds* are debated; dual-use breeds produce more meat per kg of milk produced, but their milk yields are lower. The higher output of meat can replace beef from beef cow systems, which may reduce GHG emissions of the whole system.

Animal-friendly stabling (PAS) impacts positively on animal welfare, but leads to a higher eutrophication potential. Loose housing has positive effects on working-time requirement and earned income. Moreover, the investment costs for loose housing are lower than for tied housing.

The *Regular Outdoor Exercise for Livestock* (ROEL) programme makes it possible for the cows to get plenty of movement through access to pasture, or in an outdoor exercise area or an outside climate area. The land requirement and nitrous oxide emissions are higher than for indoor-reared herds, whilst ammonia emissions are lower. The ROEL programme is beneficial for animal welfare and tends to have a positive effect on milk composition, since pasture fodder and grassland forage both have a positive influence, nutritionally speaking, on the fatty-acid profile of the milk. For the most part, the ROEL programme has a positive effect on profitability. Higher work efficiency and additional direct payments can lead to higher overall productivity.

Herd healthcare measures have a positive effect on the environment, if they lead to an improvement in the state of health of the animals. Healthier animals live longer, perform better, and require fewer resources. An absence of disease has a positive effect on animal welfare, and possibly also on cell count, and hence milk quality. Based on the available literature, the effects of herd-health programmes on the financial aspects (farm expenses and revenue) are contradictory.

Participation in the IP-Suisse *biodiversity point system* is beneficial for biodiversity. Because the costs of providing ecological and landscape-conservation services are highly heterogeneous, no generally valid economic impacts can be determined.

Conclusion

The literature review allowed to show favourable and unfavourable impacts of the investigated measures on the environmental, social and economic dimensions of sustainability. Options with a single mode of action (like feeding certified soya) and clearly targeted effects tend to have consistent outcomes in different systems. For options influencing the system productivity and intensity, the feeding ration (roughage, concentrates) or the animal husbandry, the effective outcome strongly depends on the production system and its context. Furthermore, trade-offs often appeared within as well as between the three sustainability dimensions. A quantitative evaluation of the measures, by using existing tools such as LCA, product cost calculation, work budget calculation is therefore recommended.

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Effects of trampling-resistant seed mixtures on persistence, botanical composition, forage nutritive value and herbage yield

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Abstract

Increasing herd size is causing problems with trampling damage to pasture, causing dirty conditions for dairy cattle. This four-year study compared rotationally grazed swards with four seed mixtures in order to evaluate (1) persistence, botanical composition, forage nutritive value and herbage yield (results presented here) and (2) trampling resistance with respect to vegetation cover (results presented in a companion paper by Eva Salomon *et al.*). The mixtures were sown in a completely randomised block design (three replicates) and grazed on 17 occasions. All mixtures contained forage or amenity varieties of smooth meadow-grass (*Poa pratensis*) and red fescue (*Festuca rubra*), with/without white clover (*Trifolium repens*), plus perennial ryegrass (*Lolium perenne*) or tall fescue (*Festuca arundinacea*). Botanical composition was analysed in all years, herbage yield and forage nutritive value only in years 3-4. At the end of the experiment, the seed mixture with tall fescue had significantly ($P<0.001$) more smooth meadow-grass (70%) than that with perennial ryegrass (23%). There was no significant difference in annual herbage yield (4,300-5,400 kg DM ha^{-1}). The mixture with clover and ryegrass had significantly higher metabolizable energy and crude protein content (mid- to late grazing) and less fibre (NDF) than corresponding mixture without clover.

Keywords: dairy, grazing, botanical composition, forage, amenity grasses, trampling

Introduction

Trampling-resistant swards are crucial for grazing management, animal welfare and environmental protection. During four grazing seasons, four seed mixtures were compared regarding their ability to withstand trampling, measured as botanical composition, forage nutritive value and herbage yield.

Materials and methods

The experiment was conducted on a clay soil (Uppsala, Sweden). The grazing season in 2014 and 2015 had normal total precipitation, that in 2016 was warmer than normal and that in 2017 was dry in summer with heavy rain in September. Four seed mixtures designed to give a trampling-resistant sward were sown randomly in triplicate blocks, giving 12 field plots (each 12×36 m), in July 2012 (Nilsdotter-Linde *et al.*, 2015). All mixtures contained smooth meadow-grass and red fescue of forage or amenity type, with (vs without) white clover, plus perennial ryegrass of forage or amenity type or tall fescue of amenity type (summarized in Table 1). The main comparisons were: perennial ryegrass vs tall fescue (A-B), with (vs without) white clover (A-C), and forage varieties vs amenity varieties (C-D). Mixtures without clover were fertilised with 30 kg N ha^{-1} in spring (before grazing) and at midsummer (just after grazing). The plots were grazed in 2014-17 by dry cows/heifers for a total of 17 rotations (4-5 year^{-1}), lasting on average 3.9 days (range 1-7) and with 44 animals ha^{-1}. Cows got access to all plots at each grazing event when the plots had plenty of herbage and the cows were able to freely choose between treatment plots. In 2016-17, herbage yield was sampled plot-wise before each grazing event using a lawn mower-collector (Viking MB 448 VC). Four new areas (0.46×10 m) were cut to 0.05 m height in each case. Total herbage yield was weighed, mixed and sampled for analysis of dry matter (DM) and nutrient contents. Dried, milled samples per grazing occasion were mixed (in proportion to yield) within year to give samples

Table 1. Composition of seed mixtures A-D used in the field experiment.

Species		Variety (type)	Content, %			
			A	B	C	D
Trifolium repens	White clover	Undrom (small leaves)	20	20		
Poa pratensis	Smooth meadow-grass	Kupol (forage)	35	35	44	
		Julius (amenity)				44
Festuca rubra	Red fescue	Gondolin (forage)	10	10	12	
		Cezanne (amenity)				12
Lolium perenne	Perennial ryegrass	Foxtrot (late, diploid, forage)	35		44	
		Bizet 1 (late, diploid, amenity)				44
Festuca arundinacea	Tall fescue	Borneo (amenity)		35		

representing early, mid- and late grazing, which were analysed for DM, ash, crude protein, ash-free neutral detergent fibre (NDF), cellulose and lignin and 96-h *in vitro* digestibility. Metabolizable energy was then determined (Eriksson *et al.*, 2012). Sampling for analysis of sward botanical composition was performed in early season (May) 2014-2018 and late season (Aug/Sept) 2015-17, with the May 2014 sample as covariate in the analysis. Small grab samples were taken from each corner and in the centre of a quadrat (0.3×0.3 m) thrown randomly 10 times per plot. Dry weight of different species was determined plot-wise. Pre- and post-grazing sward height was measured randomly 10 times per plot and grazing event (Jenn Quip rising plate meter, NZ Agriworks Ltd.). Data on herbage yield, botanical composition and forage nutritive value were analysed by the procedure 'Mixed' in SAS ver. 9.4 (SAS Institute Inc., USA), with plot (1-12) as repeated subject, in a model with the following independent variables: seed mixture (A-D), year and sampling time. For data on herbage yield, nutritive values and botanical components year was 2016-17, 2016-17 and 2015-18, respectively, while sampling time for the three datasets was rotation number, early-, mid- and late grazing and spring and autumn, respectively. Covariate, block, plot position and interactions were tested in the model and included when significant (mainly seed mixture × year for botanical components). Results shown are least square means (LSM) for main effects or interactions.

Results and discussion

In spring after four grazing seasons, there was significantly ($P<0.001$) more smooth meadow-grass with mixture B (tall fescue) than A (perennial ryegrass), and with C (forage grasses) than D (amenity grasses) (Table 2). Smooth meadow-grass content tended ($P<0.1$) to increase with sward age for A and C and increased significantly ($P<0.001$) for B by 2018. In 2015, red fescue content was significantly larger in mixes without than with clover, while mixtures with white clover did not differ. White clover content was 24% in spring and 42% in autumn ($P<0.01$). Clover content decreased significantly ($P<0.001$) from the first two to the last two grazing years, from 50% to 16%. After four years of grazing, there was significantly ($P<0.05$) more perennial ryegrass in A (37%) than tall fescue in B (2%) (Table 2). In seed mixtures A, C and D, perennial ryegrass content was maintained at 25-30% in 2015-17 and thereafter increased significantly ($P<0.05$) in spring 2018 (40%). There were fewer herb weeds in A (7%) and B (5%) than in C (15%) and D (17%). White clover was considered a herb weed in C and D and increased over time. The other main weeds were dandelion (*Taraxacum* spp.) and couchgrass (*Elytrigia repens*, 2%). There were no significant differences in DM herbage production between seed mixtures for single grazing occasions (900-1,100 kg DM ha^{-1}) or seasons (4,300-5,400 kg DM ha^{-1}). There were no significant differences between mixtures in sward pre- or post-grazing height. Thus, it seems unlikely that grazing preference affected the results. Mixture A had a significantly higher energy content than C (Table 3), and higher content of crude protein in mid- and late grazing than C (without white clover) (Table 4). Mixture C (forage grasses, no clover) had a significantly lower content of crude protein at late grazing than D (amenity grasses).

Table 2. DM proportion (10 g kg^{-1}) of sown grass species in mixtures A-D, 2015-18.

Year	Smooth meadow-grass				Red fescue				Per. ryegr. vs tall fescue	
	A	B	C	D	A	B	C	D	A	B
2015	12^{aAB}	16^{aA}	19^{aA}	21^{aA}	11^{aA}	7^{aA}	29^{bA}	25^{bA}	22^{aAB}	18^{aA}
2016	10^{aA}	20^{aAB}	19^{aA}	14^{aA}	12^{aA}	14^{aA}	15^{aB}	9^{aB}	16^{aA}	10^{aAB}
2017	22^{aB}	27^{aB}	24^{aA}	23^{aA}	20^{aA}	30^{aB}	23^{aAB}	26^{aA}	27^{aBC}	17^{bA}
2018	23^{acAB}	70^{bC}	33^{aA}	10^{cA}	21^{aA}	14^{aA}	16^{aB}	24^{aA}	37^{aC}	2^{bB}

$^{a-c}$ Values within rows and species with different letters differ significantly between mixtures; $^{A-C}$ values within columns with different letters differ significantly between years ($P<0.05$).

Table 3. Dry matter (DM), energy and nutrient content in mixtures A-D, 2016-17.

Characteristics	Seed mixture			
	A	B	C	D
Dry matter, g kg^{-1} DM	231^{a}	252^{ab}	267^{bc}	290^{c}
Energy, MJ kg^{-1} DM	11.2^{a}	10.8^{ab}	10.4^{bc}	10.1^{c}
Crude protein, g kg^{-1} DM	210^{a}	213^{a}	172^{bc}	187^{c}
NDF, g kg^{-1} DM	358^{a}	366^{a}	436^{bc}	440^{c}
Cellulose, g kg^{-1} DM	176^{a}	188^{ac}	206^{b}	203^{bc}
Lignin, g kg^{-1} DM	30.5^{a}	31.5^{a}	28.5^{a}	30.1^{a}

$^{a-c}$ Values within rows with different letters are significantly different ($P<0.05$).

Table 4. Crude protein content (g kg^{-1} DM) in mixtures A-D in early, mid- and late grazing.

Grazing period	Seed mixture			
	A	B	C	D
Early summer	209^{aA}	209^{aA}	193^{aA}	196^{aA}
Mid-summer	196^{abA}	202^{bA}	155^{cB}	170^{acB}
Late summer	232^{abB}	229^{bdB}	170^{cC}	200^{dA}

$^{a-d}$ Values within rows with different letters differ significantly between mixtures, $^{A-C}$ values within columns with different letters differ significantly between grazing periods ($P<0.05$).

Conclusion

There were no significant differences in yield between the four mixtures tested. Proportion of white clover and tall fescue decreased and smooth meadow-grass increased over time, mainly in mixture B with tall fescue. Perennial ryegrass persisted well over the six years after establishment. Mixture with ryegrass and white clover (A) had less fibre, more energy and more protein in mid- and late grazing periods than corresponding mixture without clover (C).

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Effect of water stress on grass species in sown permanent swards

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Abstract

Grass species are expected to show a species-specific response to drought. Two seed mixtures, containing grass species in different proportions, were investigated in a four-year field experiment at two drought-endangered sites: 835 m a.s.l. (low altitude) and 1,205 m a.s.l. (high altitude). Three cutting frequencies (2, 3 or 4 cuts year^{-1}) coupled with increasing fertilization rates (equivalent to 1, 1.4 or 2 livestock units ha^{-1}) were applied. Just before the first cut, the yield proportion of each species was assessed. Drought indices, based on cumulated precipitation and evapotranspiration, were computed for each regrowth based on meteorological data and the effect of drought was explored. The effect of drought on the yield proportion of the sown grasses was quantified by means of mixed models accounting for design effects, the abovementioned factors and the cumulated water stress (CWS), for both the growth period until the first cut and the growth period corresponding to the last regrowth of the previous growing season. All species were affected by CWS. As expected, *Arrhenatherum elatius* and *Festuca arundinacea* increased their yield proportion under high water stress. The opposite was observed for *Lolium perenne*, whilst a less clear response was obtained for the remaining grass species.

Keywords: grasses, water stress, sown swards, yield proportion

Introduction

Extreme weather events, including drought, are expected to increase in the Alps in the future because of climate change (Gobiet *et al.*, 2014). Tall fescue (*Festuca arundinacea*), tall oat grass (*Arrhenatherum elatius*) and cocksfoot (*Dactylis glomerata*) are considered drought-tolerant grasses. For this reason, they are included in grassland seed mixtures for areas endangered by recurrent drought. Perennial ryegrass (*Lolium perenne*), on the other hand, is considered to be a grass species requiring a good water supply to thrive. Several components are pivotal to provide yield stability under perturbations, like resistance (the ability to withstand perturbations) and resilience (the extent to which biomass production recovers to pre-drought level) (Van Ruijven and Berendse, 2009). In mixed swards, resistance and resilience of single species to drought result in an alteration of competition effects between species, which can be described in terms of yield proportion of the species. This paper explores therefore the relationship between the yield proportion and the intensity of the drought events.

Materials and methods

In a four-year field trial, conducted at two drought-endangered sites (low altitude: San Genesio/Jenesien 835 m a.s.l., 46°31'25"N 11°20'22"E; high altitude: Falzes/Pfalzen, 1,205 m a.s.l., 46°49'18"N 11°53'42"E), two seed mixtures containing *Festuca arundinacea* in different proportions (Fa40 and Fa60, see Table 1 for details) were sown at a sowing rate of 30 kg ha^{-1} and managed at different intensities (low: 2 cuts year^{-1} with a fertilization level equal to 1 livestock units ha^{-1}; medium: 3 cuts year^{-1} with a fertilization level equal to 1.4 livestock units ha^{-1}; high: 4 cuts year^{-1} with a fertilization level equal to 2 livestock units ha^{-1}). The trials were fertilised with the farm's own manure. The experimental design was a Latin rectangle with 3 replications and a plot size of 4×4 m. Just before the first cut, the yield proportion of species was assessed visually. Two meteorological stations located in the surroundings of the field trials were used to compute precipitation sums (PS) and potential evapotranspiration (PET) according to Penman and Monteith (Allen *et al.*, 1998) for each regrowth. Cumulated water stress (CWS = Σ PS/ Σ PET) was used as a drought index and computed for both the growth period until the first

Table 1. Composition of the investigated seed mixtures in percent seed weight.[1]

Seed mixture	Ae	Dg	Fa	Fr	Lp	Php	Pop	Tr
Fa40	13	14	40	5	4	3	15	6
Fa60	9	8	60	4	3	2	10	4

[1] Ae = *Arrhenatherum elatius*, Dg = *Dactylis glomerata*, Fa = *Festuca arundinacea*, Fp = *Festuca pratensis*, Fr = *Festuca rubra*, Lp = *Lolium perenne*, Php = *Phleum pratense*, Pop = *Poa pratensis*, Tr = *Trifolium repens*.

cut (CWS_{cor}), to which the botanical assessments refer, and for the growth period corresponding to the last regrowth (between the penultimate and the ultimate cut) of the previous growing season (CWS_{pre}). Decreasing values indicate an increasing water stress. Botanical data collected in the first year, which were those most affected by the competitive ability of grass species during the establishment phase rather than by water stress, were not included in the analysis. The yield proportion of the above-mentioned species, expressing the competitive relationship between a given species and all other species comprising the sward, was analysed by mixed models accounting for the design effects (rows and columns of the Latin rectangle), the factors altitude, seed mixture, management intensity and their interactions, and for the serial correlation between observations in different years in the same plot, which were treated as repeated measurements. Drought indices were treated as a covariate and stepwise forward added to the model, determining also the degree of the polynomial using the Akaike's Information Criterion. All tests were performed at a significance level of P=0.05.

Results and discussion

CWS_{cor} and CWS_{pre} in particular showed a relatively broad range within and between investigation years and no apparent systematic trend over time (CWS_{cor}: 0.99-1.22, 0.97-1.41, 0.83-0.95; CWS_{pre}: 0.97-1.30, 0.81-1.67, 0.60-1.19 for 2012, 2013 and 2014, respectively). This allows to tentatively interpret the effect of CWS despite its entanglement with the effect of the competitive capacity of the single grass species, which plays a relevant role in the first years after establishment. CWS affected all grass species. Depending on the species, CWS_{cor} or CWS_{pre}, or both, were found to affect the yield proportion (Table 2). *Festuca arundinacea*, *Phleum pratense* and *Poa pratensis* were only affected by CWS_{cor}, whilst *Dactylis glomerata* and *Festuca rubra* responded to CWS_{pre} only. *Arrhenatherum elatius* and *Lolium perenne* were found to be affected by the water stress of both periods. *Festuca arundinacea* was least negatively affected by CWS_{cor} than all other species (Figure 1). The yield proportion of *Arrhenatherum elatius* increased with decreasing water stress according to CWS_{cor}, but it responded in an opposite way to CWS_{pre}. On the whole, however, considering both CWS_{cor} and CWS_{pre} simultaneously, increasing yield proportion

Table 2. *P*-values of the effects of CWS_{cor} and CWS_{pre} on the yield proportion (%) of the investigated grass species and parameter estimates for intercept, CWS_{pre} and CWS_{cor} only.

Species	*P*-value of effects					Function
	CWS_{cor}	CWS_{cor}^2	CWS_{pre}	CWS_{pre}^2	$CWS_{cor}*CWS_{pre}$	
Ae[#]	<0.001		<0.001		<0.001	$= 13.24 - 5.57*CWS_{cor} - 12.30*CWS_{pre} + 8.77*CWS_{cor}*CWS_{pre}$
Dg			<0.001	0.004		$= -6.61 + 42.74*CWS_{pre} - 15.91*CWS_{pre}^2$
Fa[#]	<0.001					$= 6.61 - 2.66*CWS_{cor}$
Fr[§]			0.085	<0.001		$= 3.62 + 29.13*CWS_{pre} - 12.92*CWS_{pre}^2$
Lp[§]	0.040		<0.001			$= -1.17 - 4.85*CWS_{cor} + 17.59*CWS_{pre}$
Php	0.081	<0.001				$= -40.25 + 82.42*CWS_{cor} - 36.95*CWS_{cor}^2$
Pop[#]	<0.001	<0.001				$= 18.45 - 27.30* CWS_{cor} + 11.61*CWS_{cor}^2$

Data transformation: [#]square root, [§]angular transformation.

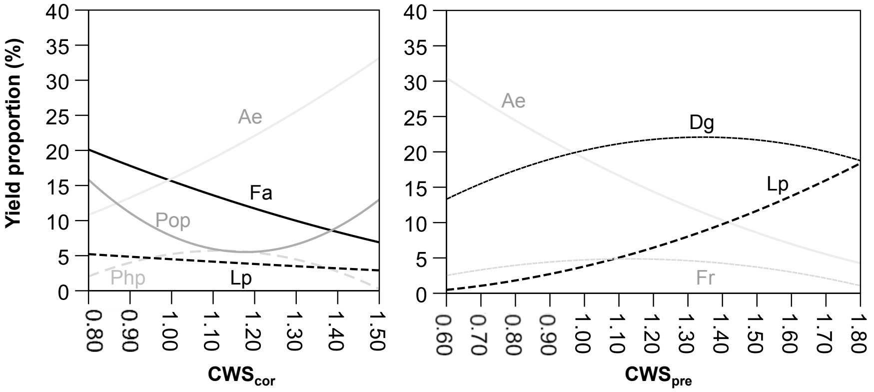

Figure 1. Effect of CWS$_{cor}$ and CWS$_{pre}$ on the yield proportion (%) of the investigated grass species (back-transformed predicted values).

was observed with increasing water stress (data not shown). *Lolium perenne* was found to be susceptible to dry conditions, as its yield proportion increased very slightly with water stress during the corresponding growth period, whilst it was clearly negatively affected by drought occurred during the previous growth period (CWS$_{pre}$).

Less expected was the positive effect of both high and low water stress on the yield proportion of *Poa pratensis*, as well as the detrimental effect of high water stress on the yield proportion of *Dactylis glomerata*. The effect of water stress on *Phleum pratense* and *Festuca rubra* was less pronounced.

Conclusion

Depending on the sown grass species, water stress occurring during the same growing season or during the previous one, or in both (*Arrhenatherum elatius* and *Lolium perenne*), affected the yield proportion. In particular, *Arrhenatherum elatius* and *Festuca arundinacea* increased their yield proportion under high water stress, whilst the opposite was observed for *Lolium perenne*. These effects may be related to the different resistance and resilience of the species sown and therefore to changes in the interspecific competition within the sward.

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Effect of light intensity on growth and development of different grass species

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Abstract

Intensive shading can be a reason for degradation of grass swards, with thinning of the turf and grasses becoming more delicate and elongated. This situation can also occur on the pitches of modern football stadiums, where stands and/or roofs exert a strong shading effect on the ground. To understand the effect of light intensity on grass growth and development an experiment was established during the year 2015 in Warsaw, Poland. Three different grass species were sown in pots located in three places with various sunshine levels, in 3 replications. During the observation period, three growth parameters were measured: plant height, number of leaves per tiller and, at the end of observation, the dry mass of roots. Light intensity was measured by quantity of photosynthetically active radiation (PAR). The biggest differences in plant height were shown by *Lolium perenne,* while *Festuca rubra* showed the least. All grass species were characterized by a low number of leaves on stems in full shade (only 2-3). Root mass decreased in the middle and full shade, compared with full sunlight.

Keywords: grass growth, shading, *Lolium perenne, Poa pratensis, Festuca rubra*

Introduction

Shade stress is one of the main problems limiting growth and development of grasses (Bell and Denneberger,1999; Dudeck and Peacock, 1992; Prończuk and Prończuk 2006). Turfgrasses growing in shade are exposed to many factors which limit their growth and development. Grasses in gardens are often exposed to different types of shade caused by buildings, roofs, trees or bushes. The shaded environment is characterized by specific microclimate with lower light intensity, fluctuating temperatures, limited air movement, increased CO_2 concentration and significantly higher humidity in comparison with non-shaded areas (Dudeck and Peacock, 1992). These environments promote development and expansion of fungi on the turfgrass. In addition, turfgrasses growing in the shade of trees are exposed to competition for water and nutrients and to allelopathic effects. The aim of this work is to compare growth and development of three grass species (*Lolium perenne* Taya, *Poa pratensis* Coventry, *Festuca rubra* Musica) under three light intensities: full sunlight, intermediate shade and full shade.

Materials and methods

The study was established in May 2015 in Warsaw, Poland. Three grass species (*Lolium perenne* Taya, *Poa pratensis* Coventry, *Festuca rubra* Musica) were sown in pots (diameter 15 cm) with sowing density: *Lolium perenne* 20 g m^{-2}, *Poa pratensis* 12 g m^{-2}, *Festuca rubra* 15 g m^{-2}, in three replications, and grown under three different light intensities. The first treatment was full sunlight. The second treatment was middle shade. The third position was in full shade, near the wall of a house from south side and high trees from west and north side. In this option the sunlight reached only from east side, from 7 to 9 am. During a sunny day (07.07.2015), light intensity (photosynthetically active radiation-PAR) measured by LightScout Quantum Meters (Spectrum Technologies Inc., Plainfield, USA) at midday, in first treatment (full sunlight) amounted 1450, second (intermediate shade) 330 and third (full shade) 130 μmol photon m^{-2} s^{-1}, while during a cloudy day (08.07.2015) (the light intensities was 580, 247 and 87 μmol photon m^{-2} s^{-1}, respectively.

The plants were irrigated in the evening, maintaining constant soil humidity. Mineral fertilizer (Yara International ASA, Oslo, Norway) was applied twice per season (0.5 g fertilizer per each pot). The fertilizer had the following composition: 12% nitrogen, 11% phosphorus, 18% potassium. Measurements of plant height and number of leaves on tillers were taken every two weeks. The observations were finished on 8 September 2015 when the mass of roots was determined.

Results and discussion

Our observations showed that the chosen three grass species differ between each other in the reaction to light intensity. All grass species responded by increase in plant height in the treatment with shade, but the effect on tillering was significantly higher in the full sunlight treatment, especially for *Poa pratensis* (Figure 1).

The first differences were noticed in the seedling phase. The fastest germination was noted for *Lolium perenne* grown under full shade, 5 days after sowing, followed by *Festuca rubra* (8 days) and *Poa pratensis* after 13 days, in full shade also. In the middle shade treatment seeds grew with two days delay. Seeds in strong sunlight the last to germinate, with 5 days delay, in comparison with full shade.

Clear differences were observed in plant height between grass species and light intensity (Figure 1). The lowest height characterized grasses in full sunlight. The most dynamic growth characterized *Lolium perenne,* in every variant, the highest measure was in full shade (242% compared with full sunlight). *Poa pratensis* showed the highest measure in middle shade, which in the last observation achieved 283% in comparison with the full sunlight. *Festuca rubra* in both shade positions showed similar results (233% in middle shade and 223% in full shade, compared strong sunlight).

In strong sunlight, the first tillers were noticed two weeks after sowing, for *Lolium perene* and *Festuca rubra*. In variants with shade, the tillering was weak for all tested species (Figure 2). Root dry mass was also dependent on light and shade. The largest root mass was observed for *Lolium perenne* and *Poa pratensis* in strong light exposition. The dry mass of roots in full shade treatment was reduced to about 1 g per pot (Figure 3).

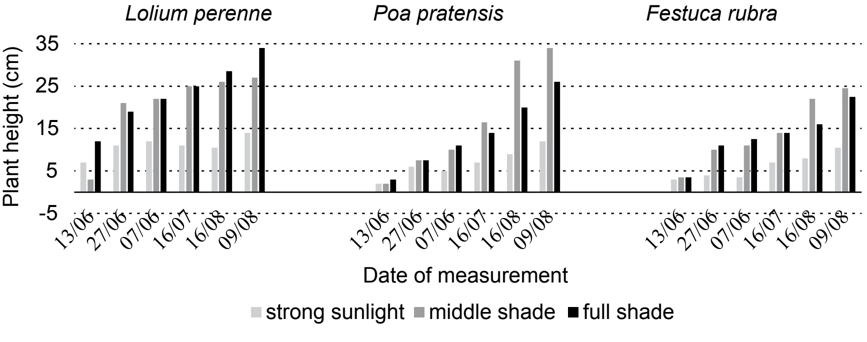

Figure 1. Plant height in different light intensity during the observation period [cm].

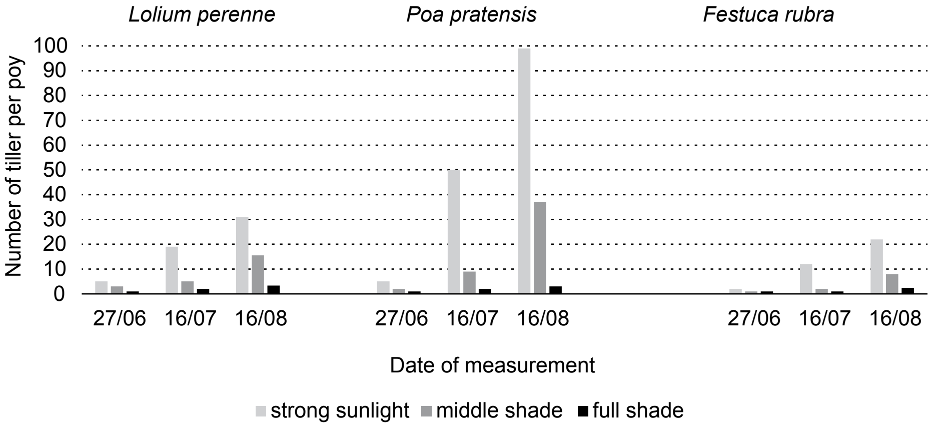

Figure 2. Effect of shade on tillering of three grass species [number of tiller per pot].

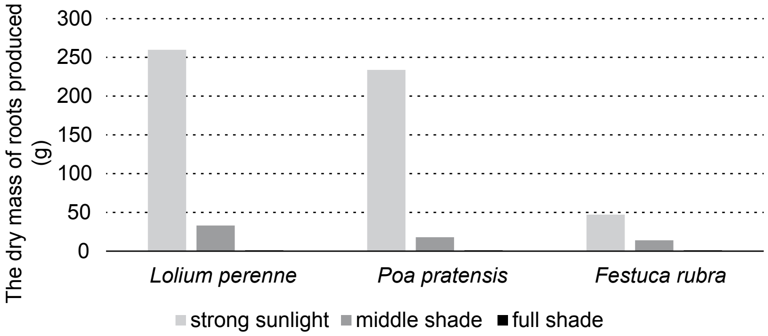

Figure 3. The dry mass of roots produced [g].

Conclusion

The growth and development of grass species depends on light intensity. Both full and middle levels of shade result in elongation of the plants. Grass tillering was limited in favour of plants increasing in height. The dry mass of roots is strongly dependent on the influence of shade stress. *Festuca rubra* seems to be more tolerant of shade than the other grass species tested in this experiment; the degradation of root mass and number of tillers of fescue in shade, compared with full light, was smaller than in other species.

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The effect of digestate fertiliser on biomass of *Phalaris arundinacea* L. grown for energy production

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Abstract

Growing and using perennial grasses for bioenergy can provide a number of benefits, especially in marginal areas. One of them is the inclusion of neglected lands into circular bioeconomy using waste products, such as fermentation residue (FR) or digestate from biogas plants for increasing soil fertility and plant productivity. In a three years field experiment different rates of FR, based on N content (from N 30 kg ha^{-1} to N 150 kg ha^{-1}), were used in different concentrations and fertilisation regimes (once, two-fold, three-fold use) for fertilisation of reed canary grass (*Phalaris arundinacea* L.). FR provided a significant (P<0.05) dry matter yield (DMY) increase in all treatments: annual DMY ranged from 3.93 to 11.44 t ha^{-1} in two-cut regime and from 5.89 to 13.94 t ha^{-1} in a single-cut regime. The highest and economically most effective biomass yields were produced when using the entire amount (60 kg ha^{-1} N) in a single application at the beginning of the vegetation, and when 120 and 150 kg ha^{-1} N were split into three applications.

Keywords: dry matter yield, fermentation residue, fertilisation, *Phalaris arundinacea* L.

Introduction

Fermentation residue (FR) or digestate from biogas plants contains a relatively high percentage of available plant nutrients, including nitrogen (Risberg *et al.*, 2017). Directly applied in liquid form to plants both for basal and top-dressing it could be an excellent organic fertiliser especially for energy grasses, thus providing yield increase and plant nutrient recycling (Moller *et al.*, 2008; Arthurson, 2009). In addition, it ensures an effective way to utilize residues from biogas plants and contributes to the reduction of artificial fertiliser use (Holm-Nielsen *et al.*, 2009; Rancane *et al.*, 2015). However, if liquid fertilisers, including FR, are used inappropriately to certain requirements, it could be a source of pollution for groundwater, surface waters and the atmosphere (Nyord *et al.*, 2008). High levels of N-NH4 in fermentation residue may pose a risk to N losses of ammonia (Riva *et al.*, 2016) and potential of nitrate leaching, especially in light soils (Chu *et al.*, 2007). Therefore, studies are needed to determine the optimal dosage and fertilisation regimes. The aim of this study was to evaluate the differences in the dry matter yield of *Phalaris arundinacea* under two harvest regimes using different rates of fermentation residue in different regimes.

Materials and methods

The field experiment was conducted from 2012 to 2015 in the central part of Latvia (56°37' N, 25°07' E). Small plots (1 m^2) of *P. arundinacea* were established in the sod-podzolic sandy loam soil (Eutric Retisol – WRB 2015) with pH KCl 6.4, organic carbon – 25.1 g kg^{-1}, plant available phosphorus (P) 35.7 mg kg^{-1}, plant available potassium (K) 76.1 mg kg^{-1}. In August 2012 *P. arundinacea* was sown (15 kg ha^{-1}) in small plots of 1 m^2, bordered with plastic plates up to the depth of 20 cm. Before sowing the liquid fraction of separated FR were evenly dispersed and incorporated into the soil. The following years FR was applied on the surface. The DM content of FR ranged from 4.4 to 5.4%; the content of main plant nutrients ranged within: 2.7 to 5.1 g l^{-1} N; 0.17 to 0.34 g l^{-1} P; 2.74 to 3.07 g l^{-1} K. Six FR treatments were compared with each other and with control (no fertilisation). FR rates were calculated based on the N content (kg N ha^{-1}): 1. 30 N; 2. 60 N; 3. 60 N (30+30); 4. 90 N (30+30+30); 5. 120 N (40+40+40); 6. 150 N (50+50+50). The experiment was established as a randomised block design with four replicates. *P. arundinacea* was harvested using two-harvest (the 1st cut at the full heading; the 2nd cut in early September) and single-harvest (in the late September or early October) regimes. Data were processed by ANOVA.

Results

The dry matter yield (DMY) of *Phalaris arundinacea* during three ley years fluctuated within a wide range depending on fermentation residue rate, ley year and harvest regime. Using two-cut regime DMY ranged within (t ha^{-1}): 7.07 to 11.44; 3.93 to 8.99; and 3.98 to 7.68 t ha^{-1} in the 1st, 2nd and 3rd ley year, respectively. Using single-cut regime DMY ranged within (t ha^{-1}): 5.82 to 10.69; 6.48 to 13.94; and 5.89 to 12.06 in the 1st, 2nd and 3rd ley year, respectively (Table 1).

Although the DMY differed between treatments, in general, higher productivity was provided by single-cut regime. Harvesting *P. arundinacea* once at senescence allows it to remove plant nutrients from above-ground parts to the rhizomes (Lindvall *et al.*, 2015), ensuring more rapid re-growth in spring and prolonging period of growth (Partala *et al.*, 2001). It generally resulted in higher dry matter yield.

Significantly higher biomass yield in the two-cut regime was harvested during the 1st ley year; in the single-cut regime DMY in all 3 years was more similar; however in some treatments (N 60; N 30×3; N 50×3) significantly higher DMY was in the 2nd ley year. All treatments provided a significant ($P<0.05$) increase of DMY, which correlated with the rate of fertiliser. The highest DMY in both harvest regimes

Table 1. Dry matter yield (DMY) during three ley years in two cutting regimes, using different rates of fermentation residue. Comparisons made within each cutting regime per each year and for average over three years.[1]

Treatment	**Total DMY in two-harvest regime, t ha^{-1}**			
	1st ley year	**2nd ley year**	**3rd ley year**	**An average in 3 years**
N0	7.07±0.43	3.93±0.38	3.98±0.42	4.99±0.25
N30	9.11±0.41	5.3±0.76	5.17±0.83	6.53±0.44
N60	11.44±0.37	7.62±0.86	6.29±0.63	8.45±0.48
N30 ×2	8.69±0.62	6.56±1.05	4.95±0.37	6.73±0.37
N30 ×3	9.88±0.17	7.31±0.78	6.82±1.03	8.01±0.54
N40 ×3	10.98±0.48	8.42±1.38	6.85±0.87	8.75±0.73
N50 ×3	10.29±0.43	8.99±1.28	7.68±0.65	8.99±0.62
LSD0.05	0.79	2.00	1.37	0.92
Treatment	**DMY in one-harvest regime, t ha^{-1}**			
	1st ley year	**2nd ley year**	**3rd ley year**	**An average in 3 years**
N0	5.82±0.51	6.48±0.94	5.89±0.66	6.06±0.42
N30	9.05±0.32	9.68±1.03	9.03±1.69	9.25±0.86
N60	9.81±0.80	11.83±1.62	8.99±0.73	10.21±0.61
N30 ×2	8.56±0.54	11.45±1.60	10.78±1.31	10.26±1.10
N30 ×3	9.56±0.50	13.28±1.29	11.43±1.65	11.42±1.09
N40 ×3	10.69±0.18	11.42±1.24	10.24±1.29	10.78±0.75
N50 ×3	12.00±0.71	13.94±1.86	12.06±1.37	12.67±0.86
LSD0.05	1.18	2.14	2.26	1.12

[1] Values are means ± standard error of the mean.

were provided by: FR with N 60 kg ha-1 using the entire amount at the beginning of vegetation; and FR with N 120 and 150 kg ha^{-1} split for three applications.

Conclusion

Fermentation residue (FR) provided a significant ($P<0.05$) dry matter yield (DMY) increase in all treatments. Higher efficiency was achieved by higher rates of FR, used in the early spring immediately after resumption of vegetation. Using of FR at lower air temperatures on wet soil contributed in higher biomass yield formation in general.

The highest and economically most effective biomass yields were produced when using the entire amount (60 kg ha^{-1} N) in a single application at the beginning of the vegetation, and when 120 and 150 kg ha^{-1} N were split into three applications.

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The best seeding density and N fertilization level for red clover and Italian ryegrass in pure stands and in mixture

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Abstract

Climatic conditions could have high influence on the productivity of forage plants. The aim of this study was to investigate the behaviour of two forage plants of major importance for the forage culture base worldwide. Research were made in Chinteni commune, Cluj County, Romania. The biological material consists in *Trifolium pratense* and *Lolium multiflorum* in pure stands and in mixture. Both forage species were sown on 2 different distances between rows (12.5 and 25 cm) and were fertilized with nitrogen (N) in 3 doses (N0: control, N50: fertilized with 50 kg N ha^{-1}, and N100: fertilized with 100 kg N ha^{-1}). The studies were made in 2016 and 2017, years very different from each other in matter of rainfalls. The results highlighted variations among dry matter and protein productivity on both species and mixture and showed that even when adverse climatic conditions are recorded high harvest could be obtained. The best technology for Italian ryegrass and mixture showed to be sowing on 12.5 cm and fertilization with 100 kg N ha^{-1}, unlike red clover which recorded the maximum yield on 12.5 cm and fertilization with 50 kg N ha^{-1}.

Keywords: monoculture, forage mixture, nitrogen fertilization, crude protein

Introduction

Agriculture is extremely vulnerable to climate change (Smith *et al.*, 2011). Higher temperature could eventually reduce yields of desirable crops while encouraging weed and pest proliferation. Changes in precipitation patterns increase the likelihood of short-run crop failures and long-run production declines (Nelson *et al.*, 2009). Forage mixtures formed of red clover and Italian ryegrass are generally very successful and are capable of generating very high yields, even on soils less supplied in mineral elements. Italian ryegrass is a species with elevated nitrogen requirements, so in association with red clover it will use almost all the nitrogen fixed by the leguminous species (Rotar *et al.*, 2013; Sobkowicz *et al.*, 2016). The aim of this study is to investigate DM and protein yield of red clover and Italian ryegrass in pure stands and in mixture in order to identify the best seeding density and N fertilization level of these two species in the context of different climatic conditions.

Materials and methods

The experiment was installed in Chinteni commune, Cluj County, Romania and carried out in 2016 and 2017. The sum of precipitation was 816 mm in 2016 and 560 mm in 2017. The experimental plots were sown on 2 seeding density (12.5 or 25 cm between rows), fertilized each year with 3 doses of nitrogen (0 kg N ha^{-1} year^{-1}, 50 kg N ha^{-1} year^{-1} and 100 kg N ha^{-1} year^{-1}), and mowed 3 times per year, in 3 replications. The biological material consisted of red clover and Italian ryegrass in pure stand and a mixture resulting in the sward types presented in Table 1. Experimental plots were analysed in terms of dry matter yield (DM) and crude protein (CP) content (g kg^{-1} DM). Data presented in this paper are the sum of these 3 cuts. The data were analysed by Statistica vs 10, t-test for single means and Partial Correlations.

Table 1. Experimental plots.

Species	Experimental plots	Sward type
Trifolium pratense	Tp_mono1, Tp_mono2, Tp_mono3	monoculture sown on 12.5 cm: unfertilized and fertilised with 50 kg N ha^{-1} $year^{-1}$ and 100 kg N ha^{-1} $year^{-1}$
	Tp_mono4, Tp_mono5, Tp_mono6	monoculture sown on 25 cm: unfertilized and fertilised with 50 kg N ha^{-1} $year^{-1}$ and 100 kg N ha^{-1} $year^{-1}$
Lolium multiflorum	Lm_mono1, Lm_mono2, Lm_mono3	monoculture sown on 12.5 cm: unfertilized and fertilised with 50 kg N ha^{-1} $year^{-1}$ and 100 kg N ha^{-1} $year^{-1}$
	Lm_mono4, Lm_mono5, Lm_mono6	monoculture sown on 25 cm: unfertilized and fertilised with 50 kg N ha^{-1} $year^{-1}$ and 100 kg N ha^{-1} $year^{-1}$
Mixture of Tp and Lm	TpLm_mixt1, TpLm_mixt2, TpLm_mixt3	mixture of Tp and Lm sown on 12.5 cm: unfertilized and fertilised with 50 kg N ha^{-1} $year^{-1}$ and 100 kg N ha^{-1} $year^{-1}$
	TpLm_mixt4, TpLm_mixt5, TpLm_mixt6	mixture of Tp and Lm sown on 25 cm: unfertilized and fertilised with 50 kg N ha^{-1} $year^{-1}$ and 100 kg N ha^{-1} $year^{-1}$

Results and discussion

Results show significantly higher DM yields on mixture compared to monoculture with the maximum value of 13.81 t ha^{-1} $year^{-1}$ for TpLm_mixt in the year 2016 (Table 2). All the sward types achieved the highest yields using 12.5 cm sowing-distance between rows. DM production increased proportionally with nitrogen fertilization in monoculture with Italian ryegrass and in mixture. In which concerns dispose of productions among the 2 experimental years results showed higher yields in 2016 for all the sward types analysed. Results showed very significant interaction between Sward type × Seeding density, while interactions between Sward type × N fertilization and N fertilization × Seeding density are not significant from statistical point of view.

The experimental plots had the same reactions also when CP content was considered, except for fertilisation factor. Fertilization determined an increase in CP content proportional with the N doses for all the sward types (Table 3). The highest average of 164 g kg^{-1} DM was achieved on monoculture with Tp, followed by mixture. The lowest protein content was obtained on monoculture with Lm. Results showed that interaction between Sward type × Seeding density, between Sward type × N fertilization and N fertilization × Seeding density are not significant from statistical point of view.

Table 2. Effect of nitrogen fertilization, sowing-distance between rows and the sward type on the dry matter yield in 2016 and 2017.

Factor	Factor level	DM yield in 2016 (t ha^{-1} $year^{-1}$)	DM yield in 2017 (t ha^{-1} $year^{-1}$)	*P*-value (from means 2016-2017)
Sward type	Tp_mono	11.72	11.06	<0.001
	Lm_mono	8.86	8.50	
	TpLm_mixt	13.81	13.67	
N fertilization	0	9.24	8.89	<0.001
	50	11.92	11.70	
	100	13.13	12.66	
Seeding density	12.5	11.93	11.45	<0.001
	25	10.98	10.72	
Interaction Sward type × N fertilization				0.019
Interaction Sward type × Seeding density				<0.001
Interaction N fertilization × Seeding density				0.023

Table 3. Crude protein content in monoculture and mixture.

Factor	Factor level	Crude protein content in 2016 (g kg^{-1} DM)	Crude protein content in 2017 (g kg^{-1} DM)	*P*-value (from means 2016-2017)
Sward type	Tp_mono	164	163	<0.001
	Lm_mono	122	112	
	TpLm_mixt	132	122	
N fertilization	0	135	129	<0.001
	50	140	131	
	100	143	137	
Seeding density	12.5	142	134	<0.001
	25	137	131	
Interaction Sward type × N fertilization				0.346
Interaction Sward type × Seeding density				0.078
Interaction N fertilization × Seeding density				0.130

Conclusion

Results pointed out significantly higher DM yields on mixture compared to monoculture. The best seeding density is 12.5 cm where all the sward types achieved the highest yields. Red clover holds high potential in which concerns the crude protein content, followed by mixture and in smallest percentages on Italian ryegrass. Also, CP is highly influenced by N fertilisation and climate condition.

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Effects of mixture types and organic fertilisation on the yield of sown grasslands

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Abstract

Seed meadows are becoming an important topic in today's European Programs and finding a nutritional and protein balance is 'a must' on all farms. Sowing meadows are among the most important crops in at least two points of view: first they are extremely productive, delivering high quality feed, and then there are excellent crops because they store and enrich the soil in nitrogen. Application of fertilizers (slurry and manure) has a favourable effect both on the productivity of the mixtures and on the quality of the feed obtained. For mixtures which are based on alfalfa the application of high doses of manure is not economically justified, but are justified when mixtures are based on red clover. The same things happened when high quantities of slurry are applied on the same type of mixtures.

Keywords: manure effect, slurry, multi-species mixture, Transylvania

Introduction

Many researchers have studied seeded grasslands, elaborating suggestions for growing mixtures of perennial fodder plants. Frame (2000) underlines the role of seeded grasslands in Eastern European agriculture, assessing that grass mixtures and corn for silage are the basic fodders for many EU countries. Sleugh *et al.* (2000) and Zemenchik *et al.* (2002) shows that *Fabaceae* have a higher nutritional value than grass species, thus mixtures of *Poaceae* and *Fabaceae* can improve fodder quality compared to a pure *Poaceae* field. Grassland productivity is a complex attribute, whose determination is based on a series of interacting elements. Rotar and Carlier (2010) states that seeded grasslands have valuable features, unequalled by other fodder crops. Among these, it first stands out the high productions that can be 3 to 5 times larger than those of permanent grasslands but with low number of species. In Romania, thanks to a subsidy programs, small farms with 'high genetic merit livestock' valuable animals begin to develop so that the establishment of sown meadows becomes a necessity. Our objectives was to determine the influence of differentiated fertilization with manure and slurry on mixtures of *Poaceae* and perennial *Fabaceae* on the evolution of sward and determining the productive potential of simple and complex mixtures of perennial *Poaceae* and *Fabaceae* in order to ensure economic use for the studied area.

Materials and methods

The field experiment was established in 2013 in the sub-Carpathian Depression in Transylvania and carried out over a three-year period (2013-2015). The experimental setup was a randomized block design with 4 replicates, 9 different seed mixtures and 8 different fertilizer treatments, either as a slurry or solid manure. The species mixtures were as follows: 7 simple mixtures based on alfalfa or red clover, and 2 complex mixtures. The simple mixtures were composed of two species in which the ratio of *Fabaceae* was 70% and *Poaceae* 30%. The complex mixtures were composed of 5 species, each species with a 20% participation ratio. The experimental mixtures are: V1 = *Trifolium pratense* + *Lolium multiflorum* 30 kg ha^{-1}, V2 = *Trifolium pratense* + *Phleum pratense* 25 kg ha^{-1}, V3 = *Trifolium pratense* + *Poa pratensis* 25 kg ha^{-1}, V4 = *Dactylis glomerata* + *Festucaa pratensis* + *Lolium perenne* + *Trifolium pratense* + *Trifolium repens* 35 kg ha^{-1}, V5 = *Dactylis glomerata+Festuca pratensis* + *Lolium perenne* + *Trifolium pratense* + *Trifolium repens* 35 kg ha^{-1}, V6= *Medicago sativa* + *Festuca pratensis* 30 kg ha^{-1}, V7 = *Medicago sativa* + *Dactylis glomerata* 35 kg ha^{-1}, V8 = *Medicago sativa* + *Festuca arundinacea* 35 kg ha^{-1},V9 = *Medicago* *sativa* + *Bromus inermis* 35 kg ha^{-1}. Each of the two type of fertilizer was tested in four different doses: slurry F1 = 0 slurry, F2 = 5 t ha^{-1}, F3 = 10 t ha^{-1}, F4 = 20 t ha^{-1} and manure: F5 = 0 manure, F6= 10 t ha^{-1}, F7 = 30 t ha^{-1}, F8 = 50 t ha^{-1}. Determination of species composition were made with the help of the gravimetric or weighting method, a method that is mostly used for the study of grassland vegetation, especially to record the changes occurring in grassland vegetation under the action of certain improvement measures or methods (Pacurar and Rotar, 2014).

Results and discussion

Here, we only present the data for the last experimental year. In V1, both manure and slurry have statistically significant increases compared to the control. Dry matter (DM) production of the mixture of *Trifolium pratense* and *Phleum pratense* (V$_2$) as a result of fertilization with liquid fertilizers compared to the control, from 1.08 at the application of 5 t ha^{-1} of slurry to 2.46 t ha^{-1} DM at application maximum dose of 20 t ha^{-1} of gulle (Table 1). In this case the difference between the harvest obtained at the maximum dose of 20 t ha^{-1} gulle and the harvest obtained at the dose of 10 t ha^{-1} gulle is 0.03 t ha^{-1} DM, what means that this difference does not justify the maximum dose of fertilizer. Applying manure to the same mixture of *Trifolium pratense* + *Phleum pratense* results in a reaction somewhat different from the recorded application of liquid fertilizers. Thus, at the first 2 gradients DM production increases, while at the application of 50 t ha^{-1} manure the difference in harvest versus application of 30 t ha^{-1} of manure is less than 1.52 t ha^{-1} DM. This determines that the application of 50 t ha^{-1} is not justified. In the mixture of *Trifolium pratense* and *Poa pratensis* (V3) the production is largely based on red clover, which in last year reaches 75% in the floristic composition in the fertilized variant with 20 t ha^{-1}of slurry, 80% in the variant fertilized with 50 t ha^{-1} of manure. The maximum harvest at this mixture is 15.4 t ha^{-1} DM and is obtained in the fertilized variant with 20 t ha^{-1}of slurry. The complex mixture consisting of *Festuca pratensis* + *Phleum pratense* + *Dactylis glomerata* + *Medicago sativa* + *Lotus corniculatus* (V4) behaves differently from simple clover-based mixtures. The application of 50 t ha^{-1}of manure does not lead to any production increase. In the mixture of *Trifolium pratense* + *Trifolium repens* + *Dactylis glomerata* + *Festuca pratensis* + *Lolium perenne* (V5) the production in two experimental years are similar, this is explained by the large share of *Dactylis glomerata* species of 89% of participation in last year, a species that responds well to fertilization. Slurry and manure increase in coverage of *Festuca pratensis* in the mixture

Table 1. Effect of the sward mixture in different doses of fertilization on DM production (t ha^{-1}).[1]

Sward mixed	F1	F2	F3	F4	F5	F6	F7	F8	Mean
V1	5.6	8.01	10.92	10.94	5.13	7.15	8.23	11.43	8.43
V2	8.53	9.61	10.96	10.99	8.06	9.1	11.78	10.2	9.90
V3	8.46	8.47	8.95	12.98	6.99	7.94	10.78	9.92	9.31
V4	10.01	10.95	11.63	11.57	10.19	11.41	11.44	10.55	10.97
V5	7.79	11.93	10.57	9.33	9.27	11.74	10.85	10.23	10.21
V6	11.14	12.94	10.72	7.44	12.88	11.56	11.15	7.86	10.71
V7	14.82	14.23	12.9	9.03	14.06	11.77	9.43	8.75	11.87
V8	15.21	14.27	13.15	11.12	14.33	12.66	12.46	11.52	13.09
V9	14.85	14.26	13.19	12.05	12.47	12.55	12.59	11.28	12.91
Mean Tp mix	7.53	8.70	10.28	11.64	6.73	8.06	10.26	10.52	9.21
Mean Cx mix	8.9	11.44	11.1	10.45	9.73	11.575	11.145	10.39	10.59
Mean Ms mix	14.01	13.93	12.49	9.91	13.44	12.14	11.41	9.85	12.15
Mean	10.15	11.35	11.29	10.67	9.96	10.59	10.94	10.25	
SEM	3.41	2.62	1.12	0.88	3.36	2.21	0.60	0.35	

[1] Tp mix = simple mixtures with *Trifolium pratense*; mean of V1, V2 and V3; Cx mix = complex mixtures; means of V4 and V5; Ms mix = simple mixtures with *Medicago sativa*; mean of V6, V7, V8 and V9; SEM = standard error of the mean.

of *Medicago sativa* and *Festuca pratensis* (V6). The application of 20 t ha^{-1} of manure significantly reduces the yield compared to the control, compared with the application of 5 t ha^{-1} slurry. In the mixture of *Medicago sativa* + *Dactylis glomerata* (V7) both manure and slurry slightly decrease harvest yields. The alfalfa dominates the mixture with a coverage between 63 and 73%. In the last year the share of the alfalfa falls greatly between 20 and 22%, and the production of the dry matter decreases from 14.82 t ha^{-1} DM (unfertilized variant) at 9.03 t ha^{-1} DM to fertilize with 50 t ha^{-1} DM of manure. Large doses of fertilizers favour the installation of *Dactylis glomerata.*

The mixture of V8 made up of *Medicago sativa* + *Festuca arundinacea* reacts with the application of liquid fertilizers by reducing the yield of -0.95 t ha^{-1} DM at the application of 5 t ha^{-1} of slurry and -4.09 t ha^{-1} DM at the application of 20 t ha^{-1} of slurry. The manure applied to this mixture determines differences in harvests that are negative but lower than those obtained when applying liquid fertilizers, ranging from -1.67 t ha^{-1} DM at the application of 10 t ha^{-1} of manure and -2.82 t ha^{-1} DM to the application of 50 t ha^{-1} of manure.

The yields of the mixture of *Medicago sativa* and *Bromus inermis* (V9) are lower than those obtained at the same intensification in *Medicago sativa* mixtures with *Dactylis glomerata* or *Festuca pratensis*. The harvest reaches 9.91 t ha^{-1} DM at the application of 20 t ha^{-1} of slurry or 12.05 t ha^{-1} DM to fertilization by 50 t ha^{-1} of manure.

Conclusion

Sown meadows are an extremely advantageous systems for a necessary and stable hay production in Transylvania. In general both fertilizer (manure and slurry) favours the installation of the *Poaceae* rather than *Fabaceae*. For Transylvania we recommend a mixture consisting of *Trifolium pratense* and *Lolium multiflorum*, or a mixture of *Medicago sativa* and *Dactylis* glomerata but without high doses of fertilization at the last mixtures.

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Effects of trampling-resistant seed mixtures on pasture vegetation cover

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Abstract

Increasing herd size is causing problems with trampling damage to pasture, causing dirty conditions for dairy cattle. This study compared four seed mixtures in four grazing seasons, in order to evaluate (1) trampling resistance with respect to vegetation cover (results presented here) and (2) effects on botanical composition, biomass production, and nutritional content (results presented in a companion paper by Nilsdotter-Linde *et al.;* these proceedings). The seed mixtures were established in a completely randomized block design with three replicates. In total, the experiment had 17 grazing events. Degree of vegetation cover/bare soil was evaluated by spatial analysis using Unmanned Aircraft System photographs in spring and after each grazing event. The degree of vegetation cover was high overall (>88%). The fourth grazing event (in October) with heavy rain caused trampling damage (23-34% vegetation cover). The following spring the vegetation had a cover of 70-85%. The fifth grazing event (summer) with heavy rain caused trampling damage (25-60%). After 29 days' rest the sward recovered and was grazed. Overall, there were minor differences between the seed mixtures in terms of withstanding trampling. Grazing management, with sufficient rest time after serious sward damage, was a successful strategy to withstand trampling and allow sward recovery.

Keywords: dairy cow, rotational grazing, spatial analysis, sward recovery, trampling

Introduction

Increasing herd size is causing problems with trampling damage to pasture, causing dirty conditions for dairy cattle. Trampling-resistant swards are crucial for grazing management, animal welfare, and environmental protection. The objective of this study was to compare four seed mixtures during four grazing seasons regarding their ability to withstand trampling, including their ability to recover after damage.

Materials and methods

The experiment was conducted on a clay soil east of Uppsala, Sweden. In the first and second grazing season total precipitation was close to the 30-year mean value (The Swedish Meteorological and Hydrological Institute). The third grazing season was warmer than average, while the fourth grazing season was drier throughout summer with a rainy autumn. No season had wet conditions. Four seed mixtures, formulated to give a persistent sward resistant to trampling, were sown randomly in three replicate blocks, giving a total of 12 field plots (each 12×36 m) in July 2012, and differences in sward establishment between the seed mixtures were evaluated (Nilsdotter-Linde *et al.*, 2015). The experimental area was surrounded by pasture and one short side of the experimental area had two buffer plots where gate entrance and water were placed at the far side. All mixtures contained varieties of smooth meadow-grass and red fescue, either forage or amenity types, with/without white clover as a base, plus perennial ryegrass, also either forage or amenity type, or tall fescue of amenity type (Table 1). Pre- and post-grazing sward height was measured randomly10 times per plot and grazing event (Jenn Quip rising plate meter, NZ Agriworks Ltd.). The experimental area was grazed by dry cows and/or heifers for a total of 17 rotations (4-5 year^{-1}), with each grazing event lasting on average 3.9 days (range 1-7) and with 44 animals ha^{-1}. Cows got access to all

Table 1. Seed mixtures used in treatments A-D in the field experiment.

Species		Variety (type)	Seed mixture, %			
			A	B	C	D
Trifolium repens	White clover	Undrom (small leaves)	20	20		
Poa pratensis	Sm. meadow-grass	Kupol (forage)	35	35	44	
		Julius (amenity)				44
Festuca rubra	Red fescue	Gondolin (forage)	10	10	12	
		Cezanne (amenity)				12
Lolium perenne	Perennial ryegrass	Foxtrot (late, diploid, forage)	35		44	
		Bizet 1 (late, diploid, amenity)				44
Festuca arundinacea Schreb. Schreb.	Tall fescue	Borneo (amenity)		35		

plots at each grazing event when the plots had plenty of herbage and the cows were able to freely choose between treatment plots.

RGB images of the experimental area were taken at 125 m height by an Unmanned Aircraft System in spring before grazing started and directly after each grazing event (in total on 21 occasions), to assess sward damage. The images were evaluated by spatial analysis, pixel size 0.05×0.05 m, using Isocluster Unsupervised classification in ArcGIS toolbox (2014). Among 18 colour classes created, those representing bare soil and living vegetation were determined. Close-up images were used to confirm the classification. Generally, 1-3 classes were determined as bare soil and the rest as living vegetation. The calculated vegetation cover was used in statistical analysis, with a mixed model (SAS ver. 9.4, SAS Institute Inc., Cary, NC, USA) with handling time of images as repeated measurements, seed mixture as fixed factor and block as random factor.

Results and discussion

No significant differences were found between treatments A-D in pre- or post-grazing sward heights. Thus, it seems unlikely that grazing preference affected the results regarding sward damage. Statistical analysis of average vegetation cover revealed few significant differences between grazing events, with the degree of vegetation cover being high overall (>88%) over time (Figure 1A). After the fourth and fifth grazing events, there was, on average, a significantly lower ratio of vegetation cover than after the other grazing events, due to severe trampling damage. The fourth grazing event was in mid-October, when plant growth rate was declining due to reducing daylight hours; 30 mm rain fell when the cows were grazing, and the trampling damage resulted in 23-34% vegetation cover (Figure 1A). The effects of this single, severe trampling event on wet soil with plants preparing for winter dormancy were still visible the following spring, confirming findings by Pande *et al.* (2000). The second spring had a vegetation cover of 70-85%, which was lower than the first and fourth spring (Figure 1B). The fifth grazing event was in early summer, but 44 mm of heavy rain fell when the cows were grazing, causing further trampling damage (25-60% vegetation cover) (Figure 1A). The sward recovery period was extended until enough herbage was available, which required 29 rest days (normal rest period 2-5 weeks). After the sixth grazing event, the vegetation cover was 93-95%, with high recovery observed for all four seed mixtures (Figure 1A). During the growing season, crop growth is optimal and soil hydrological conditions are more stable than during crop dormancy, factors which enable faster sward recovery after trampling damage (Warren *et al.,* 1986).

Overall, there were minor differences between the four seed mixtures in vegetation cover after grazing events causing trampling damage (Figure 1A). Seed mixture D, with amenity grass types, gave on average

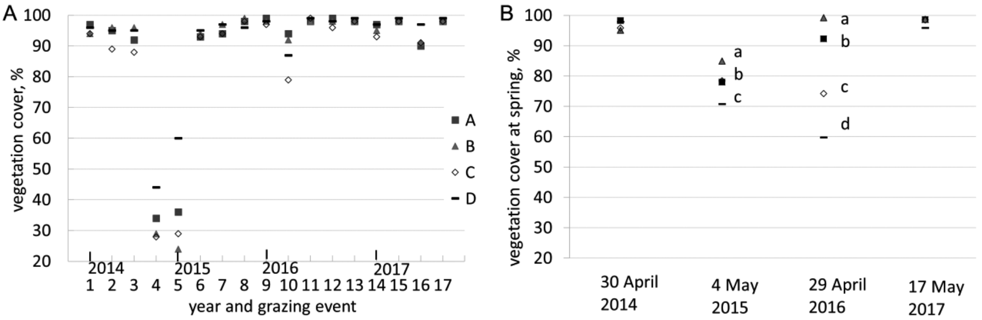

Figure 1. (A) Average ratio of vegetation cover (LSMeans with a standard error of 1.64%) for seed mixtures A, B, C, and D after 17 grazing events ($P<0.05$). (B) Average ratio of vegetation cover (LSMeans with a standard error of 2.1%) for seed mixtures A, B, C, and D in spring. Points marked with different letters within years are significantly different ($P<0.05$). See Table 1 for details.

a significantly higher degree of vegetation cover after the fourth and fifth grazing events than seed mixture C with forage grasses. Differences between treatments at the start of the grazing season during the four years were mainly between the treatments with and without clover (Figure 1B).

Conclusion

The four seed mixtures had significant trampling damage, though to different extents, at two grazing events, and seed mixture D, with amenity-type grasses, had the highest vegetation cover. All seed mixtures had high ability to recover. Grazing management with enough rest time after serious sward damage proved to be a successful strategy to withstand trampling and allow sward recovery.

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The application of plant growth regulator in Italian ryegrass seed crop

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Abstract

In seed crops of many temperate grass species stem lodging occurs during reproductive development. Nitrogen (N) spring application maximizes grass seed production, but influence the incidence and severity of lodging. Therefore, we analyzed the effect of different N rates (0, 50 and 100 kg ha^{-1}) in spring and growth regulator trinexapac-ethyl (TE) doses (0, 0.5 l ha^{-1} and 1 l ha^{-1}; Moddus 250-EC) on morphological parameters of two commercial Italian ryegrass (*Lolium multiflorum* Lam) cultivars, K-13 diploid and K-29 tetraploid type. Before swathing, internodes length, stem length, spike length and the number of spikelet from 10 randomly picked tillers per plot were measured. The increase of N rate significantly increased the fifth and sixth internodes length of cv. K-29. Also, significant decrease of internodes length was achieved in cv. K-29 with 1 l ha^{-1} TE, while stem length in cv. K-13 was unchanged by TE and N spring application. Cv. 29 had higher spike length than cv. K-13 and TE decreases length in both cultivars, in K-13 with 1 l ha^{-1} TE, while in K-29 with both doses of TE. The use of higher N fertilizer rates together with TE in Italian ryegrass seed crop can be more valuable practice for tetraploid cultivars.

Keywords: growth regulator, Italian ryegrass, nitrogen application, seed production

Introduction

Italian ryegrass is one of the best forage grasses in Serbia, producing high-quality forage from early spring to late summer. The Serbian production of forage and grass seed is often in areas characterized by seasonally very variable conditions (Simić *et al.*, 2009, 2012). Italian ryegrass seed crops are often routinely fertilized with a predetermined amount of nitrogen (N) fertilizer in spring (Vleugels *et al.*, 2017) which influences N uptake and consequently chlorophyll concentration (Rowarth *et al.*, 1999). The recommended N doses for ryegrass varies considerably, but especially based on soil texture, length of growing season, ploidity of ryegrass, as well as weather conditions (Kusvuran, 2011). If the level or source and timing of N application are incorrectly managed, a reduction of grass seed yields might occur. High ryegrass plant density could place leaf laminae at different heights within the canopy, affecting their ability to compete for light and, consequently, leading to lower effectiveness of photosynthesis, affecting plants susceptibility to wind and possibly lodging (Griffith, 2000). Lodging is ubiquitous in Italian ryegrass grown for seed, especially under high N fertilization. However, lodging could be prevented by using plant growth regulators (PGR), such as trinexapac-ethyl at appropriate growth stage and in adequate dose (Trethewey *et al.*, 2016). The objectives of this study were to determine the impact of N early spring application on one hand, and impact of plant growth regulator trinexapac-ethyl (TE) application on the other hand on crop agronomic performance in the first-year Italian ryegrass seed production.

Materials and methods

Field experiment was conducted under no irrigation conditions during the 2018 harvest season, near Belgrade, Serbia (N: 44° 50' 18.9'', E: 20° 17' 0.6'', and altitude: 66 m a.s.l.), located in a semi-humid region, on degraded chernozem soil. Soil properties were: pH (KCl) 6.64; 30.36% sand, 35.28% silt, 34.36% clay, 2.70% organic matter, 0.42% of $CaCO_3$, 0.0014 μg N g^{-1} of N-NO_3, 0.0035 μg N g^{-1} of N-NH_4, 103.43 mg kg^{-1} available P and 178.48 mg kg^{-1} available K (Van Reewijk, 2002). Italian ryegrass was sown in third decade of October with seeding rate of 20 kg ha^{-1} and 20 cm inter-row spacing. The experimental design was a randomized complete block with four replications and individual plot size 10 m^2. One factor was N rate (0, 50, and 100 kg ha^{-1}), and the other factor was PGR (TE) rate (0, 0.5 and 1 l ha^{-1}). Nitrogen was applied in early spring. TE applications were made with a hand-held CO_2-presurized sprayer equipped with flat fan nozzles (XR Tee Jet 11002) which delivered a spray volume of 240 l ha^{-1}. Those treatments were applied when ryegrass crop was at 2nd internode detectable growth stage (BBCH 32). Prior to the seed harvest, biometrical traits such as number of generative tillers, internodes' length, stem length, spike length and the number of spikelets per spike were measured on 10 randomly sampled tillers from each plot. The data were analysed by ANOVA and LSD test indicated differences between treatments.

Results and discussion

Overall response expressed through morphological parameters varied among cultivars, doses of N and TE applied (Table 1). In general, diploid cv. K-13 showed less sensitivity to both different N and TE application treatments.

Table 1. The influence of nitrogen and trinexapac-ethyl application on ryegrass morphological parameters. Means followed by a common letter are not different at the 5% level (LSD test).[1]

Treatments	Diploid cv. K13									
	NT	1st IN	2nd IN	3rd IN	4th IN	5th IN	6th IN	SL	SPL	NS
N (kg ha^{-1})										
0	12.9	4.79	8.52	9.85	13.9	24.3	14.6	75.9	27.7	26.4
50	14.1	5.26	8.83	9.80	13.4	22.3	12.6	72.1	27.1	27.2
100	12.4	5.24	9.24	9.64	12.6	21.2	14.6	72.5	26.4	27.3
F test N	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
PGR (L ha^{-1})										
0	13.1	6.03a	9.91	9.76	12.2	24.1	12.4	74.4	27.3b	26.2b
0.5	12.5	5.17a	8.82	10.15	13.6	19.2	15.3	72.3	28.4a	28.2a
1	13.7	4.09b	7.86	9.38	14.1	24.4	14.0	73.9	25.6c	26.6ab
F test PGR	ns	**	ns	ns	ns	ns	ns	ns	**	*
Average	13.1	5.10	8.86	9.76	13.3	22.6	13.9	73.5	27.1	27.0
Treatments	**Tetraploid cv. K29**									
	NT	1st IN	2nd IN	3rd IN	4th IN	5th IN	6th IN	SL	SPL	NS
N (kg ha^{-1})										
0	15.1	4.75	9.10	11.1	11.3	8.20b	6.81b	52.5b	22.0b	24.6
50	14.5	5.98	10.4	11.0	13.3	18.4a	13.5a	74.4a	23.7a	24.3
100	14.6	5.76	10.6	11.7	12.6	17.0a	13.8a	73.6a	22.3b	24.1
F test N	ns	ns	ns	ns	ns	**	**	**	*	ns
PGR (L ha^{-1})										
0	14.6b	6.44a	12.2a	13.0a	11.5b	15.5	15.1a	76.7a	24.1a	24.7
0.5	17.7a	6.17a	10.2b	11.3b	13.8a	13.7	9.35b	64.8b	22.9b	23.9
1	11.9c	3.88b	7.77c	9.43c	11.9b	14.4	9.59b	59.0b	21.1c	24.4
F test PGR	**	**	**	**	*	ns	*	**	**	ns
Average	14.7	5.50	10.0	11.2	12.4	14.5	11.4	66.8	22.7	24.3

[1] NT = number of tillers; 1st IN =first internode length (cm); 2nd IN = second internode length (cm); 3rd IN = third internode length (cm); 4th IN = fourth internode length (cm); 5th IN = fifth internode length (cm); 6th IN = sixth internode length (cm); SL = stem length (cm); SPL = spike length (cm); NS = number of spikelet; PGR = plant growth regulator.

There were no statistically significant differences for all measured parameters in regard to different N rates concerning diploid cv. K-13. The recommended TE rate (0.5 l ha^{-1}) induced unexpected effects manifested through increased spike length and number of spikelet, while TE rate of 1 l ha^{-1} resulted in reduction of the first internodes' length (unexpected due to the time of TE application). Tetraploid cv. K-29 expressed clear influence of N application rates to all internodes length, with statistically significant differences for the 5th and 6th internodes, as well as for the stem and spikelet lengths. On the other hand, very obvious influence of TE application rates on all parameters measured was recorded with exception of the number of spikelets. Both recommended and twice the recommended doses induced length reduction of all internodes except the 4th (where reverse effect was recorded, probably due to stronger N rate influence). The length reduction was more pronounced for treatment with higher TE rate. The spike length reduction was also established for both PGR doses, but with no effect on spikelet number. The 2018 vegetation season was characterized by adequate precipitation evenly distributed throughout the period of ryegrass vegetative development resulting in positive influence on N uptake and consequent crop growth, also stated by Chastain (2000).

Conclusion

The results of this research showed that applications of N and PGR in Italian ryegrass seed crop could be an effective strategy for tetraploid but not for diploid type of ryegrass. The results of the present study indicated that in order to improve tetraploid Italian ryegrass features for seed production in the first production year, a medium N rate is preferable (50 kg ha^{-1}) with trinexapac-ethyl application at recommended dose (0.5 l ha^{-1}).

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Investigation of seed maturity and harvesting time of sainfoin and birdsfoot trefoil

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Abstract

The objectives of this study were to investigate the course of the seed maturation, to identify a straightforward technique for the determination of harvesting time, and to validate the optimum harvesting time in the case of desiccant application. During five years seed maturation dynamics of sainfoin (*Onobrychis viciifolia* Scop.) and birdsfoot trefoil (*Lotus corniculatus* L.) was started with testing at 28-42 days after the start flowering of the stand and was continued once a week for 2-3 months. It was determined that in order the earliest time of harvesting a composite sample collected from different places in the stand must be analysed. Harvesting time may be established more precisely, using the sum of the degree-days, than the number of days from the start of the stand flowering, when 10% of clusters are in bloom. The earliest time for sainfoin harvesting is when the quantity of brown and semi-brown pods in the stand reaches 75% or about 42-49 days after the start of flowering. At this time the sum of degree days is 805 °C. Due to high seed shattering the duration of optimum harvesting time is very short, up to one week. The earliest time for birdsfoot trefoil harvesting comes when the quantity of brown and light-yellow pods in the stand reaches 67%. Then the sum of degree-days from the start of flowering is 954 °C. In dry and warm weather, the pods open themselves and then harvesting duration is short – from 3 to 7 days.

Keywords: *Onobrychis viciifolia, Lotus corniculatus,* seed maturity, harvesting time

Introduction

In Lithuania, birdsfoot trefoil and sainfoin are less widespread than red clover. However, the area under these perennial legumes tends to increase, as these species are very suitable for use in ecologically clean agricultural production. The stands are able to remain in flower for a long time in rainy weather. The seed maturation is non-uniform. If seed harvesting is delayed or done prematurely the seed losses are high (Lazauskas, 1991; Frame *et al.*,1998). There is limited experimental evidence on seed maturation and harvesting time of these legumes. In all cases it is rather difficult to determine the harvesting time of birdsfoot trefoil as the stand matures unevenly and mature pods tend to dehisce (Hare and Lucas,1984). Thus, maturation and harvesting of seed of sainfoin and birdsfoot trefoil is dependent on the weather conditions and the peculiarity of the species. The objectives of this research were: (1) to identify a straightforward technique for the determination of harvesting time; (2) to investigate the process of seed maturation; iii) to validate the optimum harvesting time in the case of desiccation.

Materials and methods

The maturation dynamics of sainfoin and birdsfoot trefoil seed was tested over 5 years at 4-6 weeks from the start of flowering, the point when 10% of clusters are in bloom. The tests lasted for 2-3 months on 1 m^2 plots with four replications. Once a week (during dry weather – twice) the seed stands were desiccated with the diquat Reglone® and after 3-7 days were threshed with a laboratory thresher. In a parallel area of the crop (subplot) at each time prior to desiccation, 3-4 samples were collected from a 0.25 m^2 area for estimating the stand maturity. The pods were detached and divided into separate groups. Sainfoin: (a) green; (b) yellowish green; (c) semi-brown; (d) completely brown, mature. Birdsfoot trefoil: (a) green, underdeveloped; (b) yellowish green, long and thick, transparent; (c) light yellow; (d) dark brown; (e) with shattered seeds. The pod numbers of each group are expressed as percent. In order to establish the course of seed maturation, in a parallel area of the crop 100-150 newly flowering inflorescences (bunches) were marked with red thread throughout the period 14 days after the start of the stand flowering and then every week until the end of flowering. 10-15 inflorescences were detached at 21, 28, 35, 42, 49, 56 days after marking. The seeds present in the pods were dried, hulled, and tested for quality. With a view to establishing the effect of desiccation the above mentioned groups of inflorescences were marked with thread. 10-20 marked desiccated inflorescences, as well as the same number of non-desiccated inflorescences from each group, were left for maturation. The marked inflorescences were detached after 3-7 days, threshed and later tested for quality.

Results and discussion

Seed growth and development from the stage of setting to maturity is best demonstrated by the 1000 seed weight and variation of germination. The most valuable clusters of sainfoin flowered in June or the first part of July. One can expect a high-quality seed yield of birdsfoot trefoil only from the clusters which start flowering no later than in the second 10-day period of July. The 1000 seed weight of the clusters flowering later than the second 10-day period of July was 0.54-0.96 g and the seed content in clusters was 2.4-times lower; furthermore, the seed germination did not meet the standard requirements. Clusters of sainfoin and birdsfoot trefoil which flowered at the beginning of the stand flowering and during the mass flowering ripened the seeds after 6-7 weeks. For full maturity sainfoin needs 749 °C degree days, birdsfoot trefoil needs 780 °C degree days (Table 1).

When desiccation is carried out in each week during the experimental period, clusters with various degrees of ripeness are found in the stand, ranging from flowering to shattered seeds. Desiccation of the youngest clusters of all legume species (at 3-4 weeks from the start of flowering) was detrimental. The 1000 seed weight of the non-desiccated plants grown and ripened naturally was significantly higher and the seed germination was also higher. The seeds from semi-brown (sainfoin) respectively, and light yellowish (birdsfoot trefoil) desiccated pods did not differ in terms of quality from the seeds of the non-desiccated pods of the same maturity. Therefore, when determining the harvesting time, the clusters of this group may be attributed to the group of ripened ones. It is important to know the earliest feasible harvesting time of legumes, as in this case it is possible to make better adjustment to rainy weather. The earliest harvesting (desiccation) time was established taking into account the following conditions: (1) the seed yield must be the highest or increasing within the limits of error; (2) seed germination must meet the standard requirements. The earliest sainfoin harvesting time can be better established according to the number of brown (mature) and semi-mature (part of pods are yellowish green) clusters rather than only by the number of brown clusters. Variation coefficients are 23% and 48% respectively (Table 2). For the determination of harvesting time, the sum of degree days (V 5%) from the start of flowering is a more accurate indicator than the number of days (V 13%). The mean data show that the earliest desiccation time for sainfoin stands is when the amount of brown and semi-brown clusters is 75%, i.e. approximately

Table 1. The number of days and the sum of degree days from start of flowering to the seed maturity of separate clusters (non-dessicated plants, average five years).

	Legumes	Days from the beginning of flowering	Sum of degree days from start of flowering (°C)	1000 seed weight (g)	Germination (%)
Clusters that flowered at the mass flowering time	Sainfoin	46±1.4	749±14	20.7±3.4	78±5
	Bird's foot trefoil	47±2.3	780±16	1.20±0.1	90±11
Clusters that flowered at the end of flowering time	Sainfoin	53±1.7	779±18	20.3±3.8	67±11
	Bird's foot trefoil	58±2.0	807±16	1.09±0.2	75±7

Table 2. Mean data for the earliest possible harvesting time of legume seed crops (Dessicated plants, average five years).

Indices	**Sainfoin**		**Birdsfoot trefoil**	
	Mean	**Variation, %**	**Mean**	**Variation, %**
Sum of days from start of flowering	50±2.8	13	58±4.2	19
Sum of degree days from start of flowering °C	805±18	5	954±33	9
Mature pods, %	53±10.5	48	40±7.1	46
Mature and semi mature pods, %	75±7.1	23	67±4.1	16
Seed germination, %	84±6		89±5	
1000 seed wt., g	20.1±2.4		1.2±0.1	
Seed yield, g/m^2	91±11		34±8	
Harvest time, weeks from start of flowering	07-aug		08-okt	

in the 7th-8th week from the start of flowering. The earliest desiccation time for birdsfoot trefoil is when the amount of brown and light-yellow pods in the stand achieves 67%.

Conclusion

The earliest time for sainfoin harvesting is when 75% of the clusters in the stand are brown and semi-brown; i.e. approximately in the 6th-8th week from the start of flowering, when the sum of the degree days is 805 °C. The optimum harvesting time is short – one week, due to high levels of seed shattering. The earliest time for birdsfoot is when 67% of the pods in the stand are brown and light-yellow. In a drier and warmer summer this is at approximately the 7th-8th week, and in cooler and rainier summers in the 9th-10th weeks from the start of flowering, when the sum of the degree days is 954 °C. The optimum desiccation time when dry and warm weather prevails (pods burst) is short, about 3-7 days.

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Plant species covers in sown grass fields on Icelandic dairy farms

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Abstract

Plant species cover was inspected in two consecutive years in 152 cultivated perennial grass fields on 8 Icelandic dairy farms. Factors analysed were the effect of farms, field age from last sowing, and time of inspection within the growing season. Forty-one species or genera were identified on the fields, with 9 grass species comprising 80-99% of the total vegetation cover, depending on the farm. *Phleum pratense* was the species with the most cover, on average 39%, and was registered on 89% of grass fields. *Poa pratensis* was recorded on 93% of grass fields, with average cover of 24%. Field age and farm practice (rate of field renovation) explained most of the variation in species cover, and time of inspection to a lesser extent.

Keywords: perennial forages, species covers, field age

Introduction

The backbone of Icelandic agriculture is cultivated semi-permanent grass fields intended for livestock. Crop rotation or frequent grass renovation is, however, common practised on dairy farms to secure better forage quality and yield. *Phleum pratense* is by far the most important introduced forage species in cultivated grass fields. It is normally either seeded in pure stands or in mixture with *Poa pratensis* or earlier also with *Festuca rubra*. However, forage species like *Lolium perenne*, *F. pratensis* and *Trifolium pratense* are becoming more relevant for dairy farmers in the light of warmer climate. These species have recently often been mixed with *Phleum pratense* instead of *Poa pratensis* even though they are less persistent. The objective of this study was to evaluate plant species cover in sown grass fields in relation to age of cultivation and time of inspection within season.

Materials and methods

Plant species cover was inspected for two consecutive years in early summer (primary growth) and again in mid-summer before second cut (secondary growth) in 152 cultivated perennial grass fields totalling 432 ha on 8 high-output dairy farms. The fields supported swards that were at least one-year old, 95% of them seeded initially with *Phleum pratense* in pure stand or as a main species in mixtures with one or two other forage species, and 5% of the grass fields were seeded with *Lolium perenne* as a main species. With the help of the farmers, the fields were grouped into 5 field-age classes; 1-5, 6-10, 11-20, 21-30, 31+ years from sowing. A single, trained examiner estimated species cover subjectively by crossing diagonally all fields four times. Species cover was registered in percentages with 5% intervals except 1-5% and 95-100% where the interval was 1%. The main effects of three factors on species cover were finally analysed with this linear model; $Y = \mu + a + b_{(a)} + c_{(a)} + e$, where Y = mean cover (%) of species or group of species in question, a = farm effect (8), $b_{(a)}$ = field age effect nested within each farm (5), $c_{(a)}$ = time of inspection effect nested within each farm (2) and e = error. The average field cover was weighted (corrected for field size) within each farm. The analyses were made with the JMP statistical software from SAS using standard least square procedures. The F statistic was used for testing that the effect was zero. If the factor has $P<0.05$ the effect is considered statistically significant. Species were divided into three groups for clarification: introduced forages, indigenous forages and weeds. Indigenous forages are edible species but with moderate or low agronomic value (low yield and/or palatability). Weeds are considered to be more or less non-edible species.

Results and discussion

Forty-one species or genera were identified in this study, 5 introduced (seeded) species, 11 indigenous forage species and 25 weed species. The most frequent species are listed in Table 1 and showing their mean cover, model fit and the effects of factors studied.

The model with these three factors explained most of the variation in species cover with few exceptions. The most dominant species was *Poa pratensis* which was found in 93% of the fields, and second was *Phleum pratense* which was found in 89% of the fields. The two most frequent indigenous forages were *Deschampsia cespitosa and Taraxacum officinale* which comprised on average 12% of the field cover. The most frequent weed species were *Rumex longifolius* and *Ranunculus acris* but they comprised only about 1% of the field cover on average. The inspection time had a significant effect on field cover in some species but not in others. The cover of *Phleum pratense* was significantly less in the secondary growth than the primary growth. On the other hand, the cover of *Agrostis* spp., *Poa annua* and *Achillea millefolium* was significantly more in the secondary growth than in the primary growth. Field age had a profound effect

Table 1. Mean cover of the most frequent species in 152 sown grass fields with standard deviations (st. dev.), adjusted model fit (R^2) and its three main effects (farm, age class and inspection (insp.) time within season.

Latin name	Frequency	Cover		Model fit	Effects, *Prob* > *F*[1]		
	%	%	st.dev.	adj. R^2	farm	age class	insp.time[2]
Introduced forages							
Poa pratensis	92.8	24.3	16.2	0.78	<0.0001	<0.0001	0.3614
Phleum pratense	88.7	38.5	24.1	0.84	0.0001	<0.0001	0.0275
Festuca spp.	30.9	2.6	7.3	0.07	0.5777	0.2834	0.4795
Lolium perenne	4.6	1.9	2.3	0.98	<0.0001	<0.0001	0.4693
Trifolium pratense	4.3	0.1	0.2	0.67	0.0015	<0.0001	0.4482
Indigenous forages							
Deschampsia cespitosa	78.3	10.5	15.2	0.87	<0.0001	<0.0001	0.5632
Taraxacum officinale	64.0	1.6	2.9	0.78	<0.0001	0.0003	0.4910
Poa annua	51.8	2.1	2.6	0.71	<0.0001	0.0115	0.0010
Alopecurus pratensis	45.7	10.0	22.4	0.77	<0.0001	<0.0001	0.6733
Agrostis spp.	33.6	3.4	5.9	0.59	<0.0001	0.1143	<0.0001
Stellaria media	33.4	0.4	1.5	0.06	0.2269	0.5482	0.2589
Alopecurus geniculatus	25.7	2.0	4.6	0.89	<0.0001	<0.0001	0.5017
Trifolium repens	20.4	0.3	1.8	0.00	0.7179	0.4883	0.2917
Achillea millefolium	19.9	0.3	2.8	0.37	0.0062	0.4986	0.0026
Weed species							
Rumex longifolius	43.8	0.7	3.0	0.90	<0.0001	<0.0001	0.3672
Ranunculus acris	24.3	0.5	3.8	0.63	0.0001	0.0004	0.0909
Capsella bursa-pastoris	19.9	0.0	0.0	0.45	0.0067	0.0433	0.0153
Lenotodon autumnalis	19.2	0.1	0.4	0.02	0.4877	0.8763	0.0050
Cerastium fontanum	15.3	0.0	0.0	0.30	0.5034	0.0309	0.2869
Rumex acetosa	13.8	0.2	1.5	0.06	0.3711	0.3938	0.3836
Ranunculus repens	11.0	0.4	2.1	0.89	<0.0001	<0.0001	0.3194
Polygonum aviculare	12.2	0.0	0.1	0.33	0.0451	0.0356	0.7023
Chamomilla suaveolens	10.2	0.0	0.1	0.02	0.9114	0.1145	0.2781

[1] The *P*-value for the effect test. If $P<0.05$ the effect is statistically significant.

[2] Primary or secondary growth.

on the species composition. The field cover of introduced forages declined and indigenous forages increased significantly with age (Figure 1). The average weed cover was not significantly different between age groups. However, there was a huge variation in weed cover between single fields, both in young and old fields. The cover of introduced *Phleum pratensis* declined rapidly with age and was replaced somewhat with introduced *Poa pratensis* in the beginning before it also loses cover (Figure 2). This study supports the findings of Thorvaldsson (1996) who concluded that one of the most important factors affecting the cover of plant species in grass fields were the age of the sward and that *Poa pratensis* partly replaces *Phleum pratense* with time before it also loses cover.

Figure 1. The effect of age on the cover of three groups of species in 152 grass fields. The bars are standard deviations of the means.

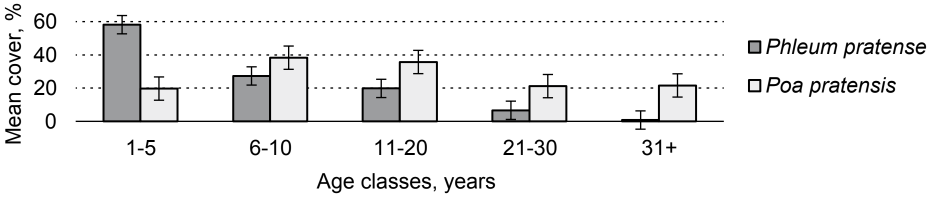

Figure 2. The effect of age on the cover of the two most dominating grass species in 152 grass fields. The bars are standard deviations of the means.

Conclusion

Introduced forage species retreat along with age at different rates and are replaced by indigenous forage species. The mean cover of weed species does not correlate with field age.

Acknowledgements

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Effect of different management measures on the occurrence of *Rumex obtusifolius* in mountain grasslands

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Abstract

Rumex obtusifolius infestation is a serious problem in gathering places of livestock in mountain grasslands. In order to control this weed a long term experiment was established in the Nízké Tatry Mts (Slovakia) in 2004. The treatments were: (1) unmanaged control, (2) cutting twice per year, (3) herbicide application (glyphosate) and grass-forbs mixture reseeding in the first experimental year followed by cutting twice a year in all subsequent experimental years. Cover of plant species (%) were visually estimated in the experimental plots every year (2004-2011) in late June. The results showed that a single herbicide application with a subsequent reseeding with a grass-forbs mixture reduced the cover of *R. obtusifolius* to 3% after the first experimental year. In subsequent years this cover was further decreased by cutting twice a year, and this even led to disappearance *R. obtusifolius* in 2008, which remained until the end of the experiment. Cutting twice a year led to a gradual reduction of *R. obtusifolius* from 77% to 3% during the experimental time, but it did not lead to its complete eradication. In contrast, unmanaged plots supported *R. obtusifolius* and its high cover of about 75% was maintained during all experimental years. This long-term experiment showed that single-use herbicide application and subsequent reseeding with a grass-forb mixture, and cutting twice a year in the following years can completely suppress *R. obtusifolius* and establish grasslands with desirable species composition. However, long-term cutting twice per year also seems to be an effective method to control *R. obtusifolius*, especially on sites where herbicide application is prohibited.

Keywords: dock weed, gathering places of livestock, reseeding, glyphosate, botanical composition

Introduction

Rumex obtusifolius is a problematic perennial weed in many parts of the world. In grasslands, *R. obtusifolius* frequently grows on highly productive, nutrient-rich soils independent of the cutting frequency (Strnad *et al.*, 2012). It is also a dominant plant species in the abandoned former resting places of sheep or cattle in the mountains. For its control, biological, mechanical, cultural or combined methods are usually used, but its total eradication is rarely successful (Zaller, 2004). The success of control management depends on *R. obtusifolius* dominancy, soil nutrient concentrations, source of seeds and previous management. In order to find applicable control methods for *R. obtusifolius* and to support species rich mountain grasslands suitable for forage production, a long-term experiment was established in the Nízké Tatry Mts (Slovakia).

Materials and methods

An experiment was established in 2004 in the National Park Nízké Tatry (Slovakia, 48° 51' 22' N, 19° 14' 57' E). The experimental site was 1,140 m a.s.l. Mean annual precipitation is 800 mm and the average temperature during vegetation season (IV.-IX.) was 8 °C. The terrain of the experiment showed an easterly exposure with a slope of 8-10°. Geological subsoil was created by porphyric granodiorite, granite and quartzite. Soil is classified as cambisol, subtype modal and variety acidic (pH approximately 5.0). In 2004, the mean nutrient concentrations in the soil were 6.44 g kg^{-1} Ntot, 260 mg kg^{-1} plant available P and 890 mg kg^{-1} plant available K. *Rumex obtusifolius* and *Urtica dioica* were the dominant vascular plant species in the experimental area. The experiment was arranged in three randomised blocks with three treatments and a single plot size of 15 m^2. The applied treatments were: U-unmanaged control; 2C-cutting twice per year only; H-herbicide application (Roundup Biaktiv-glyphosate) and subsequent grass-forbs mixture reseeding (after 3 weeks) with 18 autochthonous plants (*Dactylis glomerata* L., *Festuca pratensis* Huds., *Phleum pratense* L., *Poa pratensis* L., *Festuca rubra* L., *Trisetum flavescens* (L.) P. Beauv, *Trifolium repens* L., *Trifolium pratense* L., *Lotus corniculatus* L., *Plantago lanceolata* L., *Achillea millefolium* L., *Carum carvi* L, *Taraxacum officinale* Weber, *Alchemilla vulgaris* L., *Daucus carota* L., *Acetosa pratensis* Mill., *Leucanthemum vulgare* Lam., *Prunella vulgaris* L.) in the first experimental year, with cutting twice per year in all following years. The percentage covers of all vascular plant species were visually estimated in each experimental plot every year (2004-2011) at the end of June. Repeated measures ANOVA was used to analyse effect of time, treatment and time × treatment on *R. obtusifolius* cover. Redundancy analysis (RDA) in the CANOCO 5.0 program (Ter Braak and Šmilauer, 2012) was used to evaluate multivariate vegetation data (2004-2011).

Results and discussion

The cover of *R. obtusifolius* was significantly ($P \leq 0.001$) affected by treatment, year and treatment × year interaction according to the repeated measures ANOVA. The highest cover of *R. obtusifolius* was observed in the U treatment during all experimental years (Figure 1A) and remained relatively stable (about 75%) during the total duration of the experiment. This was caused by nutrient enrichment (N, P, K) in former cattle resting places. A gradual decrease of *R. obtusifolius* from 77% at the beginning of the experiment to 3% in the last year of the experiment was recorded in the 2C treatment. This finding contrasts with the study of Hann *et al.* (2012), which indicated that *R. obtusifolius* is well adapted to cutting management. But other studies (Hann *et al.*, 2012; Hejcman *et al.*, 2012) showed that *R. obtusifolius* also thrives on unmanaged grasslands with higher nutrient availability, as occurred in our experiment. In the H treatment the cover of *R. obtusifolius* also sharply decreased from 76 to 3% after the first year of the experiment. Cutting twice per year in the following experimental years resulted in a further reduction of *R. obtusifolius*. From the year 2008, a total eradication of *R. obtusifolius* was revealed and diverse grassland was established (Figure 1B). Therefore, treatment H (using herbicide) seems to be a reliable method for *R. obtusifolius* eradication, but the use of herbicides is limited in protected areas.

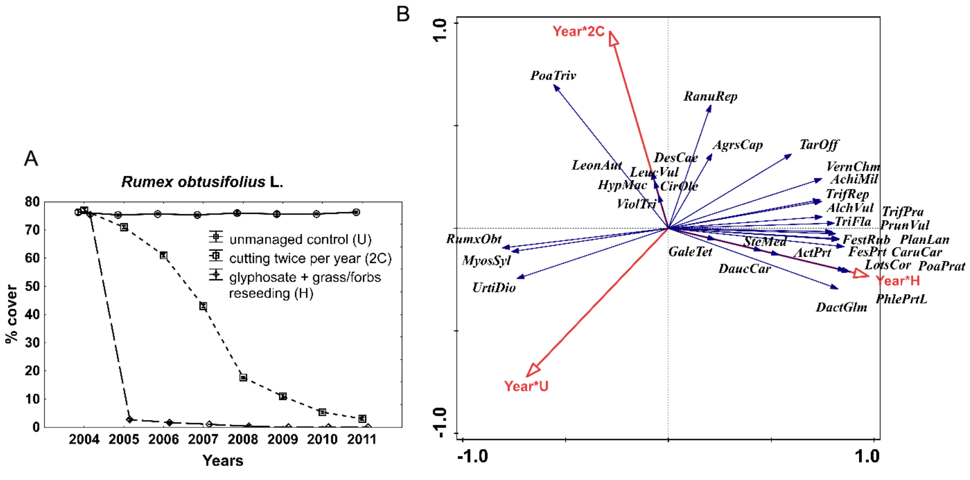

Figure 1. (A) Changes in the cover (%) of *R. obtusifolius* during 2004-2011; (B) Ordination diagram shows the result of RDA analysis of vegetation data. For the treatment abbreviations see Materials and methods section. Species abbreviations are based on the first three or four letters for genus and species name. RDA analysis explained 63.96% variability on the first ordination axis (*F-value* = 60.3, $P \leq 0.001$).

RDA analysis revealed that the species-rich grassland was more effectively established by means of the H treatment, where the number of plant species was the highest (Figure 1B). Results of this long-term experiment showed that both treatments 2C and H could, over a long-term perspective, be sufficient for the control of *R. obtusifolius* in mountain grasslands.

Conclusion

Rumex obtusifolius is very well established in unmanaged, nutrient-rich mountain grasslands, especially in abandoned former cattle resting places. The herbicide application in combination with reseeding of grass-forb mixtures can completely suppress *R. obtusifolius* and lead to establishment of species rich-grasslands with desirable species composition suitable for forage production. However, due to potential environmental risks the use of glyphosate is highly debatable. Therefore, long-term cutting twice per year seems to offer a more suitable and environmentally friendly management for *R. obtusifolius* control in mountain areas.

Acknowledgements

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Revitalisation of agricultural land endangered by drought

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Abstract

Global climatic change and increased aridity of landscape influence also the Central European landscape. Both factors can significantly complicate revitalisation processes of degraded landscape. The main aim of our research was to test the auxiliary soil substances in order to improve the parameters of damaged soils in arid areas of the Czech Republic. A model plot of a degraded farmland in the Southeast of the country was used for testing the application of top-quality lignite from local sources combined with hydro-absorbents, which improve the physical and chemical properties of the soil and conditions for vegetation growth. Within the project different legume and grass species were evaluated. After two years of evaluation it can be concluded, that there is a significant positive effect of auxiliary soil substances both on the plant emergence and stand development. The observed results significantly differ among the species and depend on climatic conditions in different years.

Keywords: arid areas, lignite, hydro-absorbent, legumes, grasses, field emergence, vegetation cover

Introduction

Climatic change and increased aridity of landscape influence the agricultural systems and may decrease the biodiversity. Especially summer heat waves affect significantly Central European landscape (Ballester *et al.*, 2010). The most limiting stress factor for plants during these heat waves is water shortage. Auxiliary soil substances (ASS) are a possibility how to reduce the drought influence, improve the soil quality and environment for plant growth. Together with growing of drought resistant species ASS can significantly increase the effectiveness of re-vegetation (Harris and Zuberer, 1993). Hydro-absorbents exist in both natural (lignite) and synthetic form. Lignite modifies soil organic matter quality and mineralogy, improves some soil properties such as clay content, C sub(org) content, CEC and porosity. On the contrary, lignite has a higher C/N ratio and higher recalcitrant C content compared to recent soil organic matter. This results in a decreasing expression of microbial soil functions involved in the turn-over of the main bio-elements, like C, N, P and S, due to lignite acting as a diluting factor, especially when lignite is used as soil amendment (Clouard *et al.*, 2014). Synthetic hydro-absorbent application improves soil water management and enables even action of lignite in the soil. Both substances could significantly improve stand characteristics (Šarapatka *et al.*, 2006).

The objective of our study was to evaluate the influence of two forms of ASS applied together (natural lignite and synthetic hydro-absorbent) on stand characteristics and stand establishment of selected legume and grass species.

Materials and methods

At the experimental site near Hodonín (SE of the Czech Republic), two trials were carried out in 2017 and 2018 to measure the influence of ASS on stand development of legume and grass species. The experimental site is located in an arid continental climatic region and shows a degraded sandy soil with a low pH, between 4.5 and 5. The one hectare site was divided in two parts. In the first part, ASS was applied – lignite (30 t ha^{-1}) and hydro-absorbent (0.5 t ha^{-1}) together, in two size fractions (fine fraction 0.2-0.8 mm and coarser fraction 0.8-2.0 mm). The second part of the site was used as a control plot.

ASS was applied after ploughing and harrowing in the spring of the first experimental year. After ASS application, the experimental area was disked again, and small experimental plots, each of 10 m^2, were set up. Each species was replicated three times, using randomized block design. The trials were sown on 4th of May 2017 and 25th of August 2018. Tested species and seeding rates (calculated according to the real germination rate and thousand seed weight) are presented in Table 1.

The first evaluation of field emergence was made on May 16th 2017 and May 5th 2018. Four evaluations of stand cover were made in 2017: June 14th, July 26th, August 31st and October 20th. A follow up was also conducted in 2018 (May 30th, July 19th, September 18th and November 5th). Cover was evaluated using a wooden square frame of 50×50 cm, containing 25 grids of 10×10 cm. Presence/absence of the species was counted by means of a pin point method three times in each plot. ANOVA and Tukey post-hoc tests were used for the statistical analysis.

Results and discussion

Average stand cover values in the years 2017 and 2018 are presented in Table 2. Plant cover differed significantly among the species. *Lolium perenne* showed the highest cover among grasses (44.1%), whereas *Onobrychis viciifolia* performed best among legumes (49.7%). *Onobrychis viciifolia* is considered to be drought resistant (Carbonero *et al.*, 2011); but *Lolium perenne* not. The results of *Lolium perenne* are surprise for us, especially compared to *Festuca* species, generally considered as drought tolerant (Hofer *et al.*, 2016). The lowest cover among legumes and grasses was observed for *Medicago lupulina* (15.4%) and *Poa pratensis* (2.5%), respectively. This fact can be explained by very dry climatic conditions that occurred during spring of both vegetation seasons, and the fact that *Poa pratensis* is known to have a long

Table 1. Grass and legume species used in the experiment.

Grass species	Variety	Seeding rate (kg ha^{-1})	Legume species	Variety	Seeding rate (kg ha^{-1})
Festuca pratensis	Rožnovská	33	*Anthyllis vulneraria*	Pamir	18
Festuca rubra	Zulu	19	*Medicago lupulina*	Ekola	18
Lolium perenne	Ahoj	24	*Melilotus albus*	Meba	24
Phleum pratense	Levočský	14	*Onobrychis viciifolia*	Višňovský	216
Poa pratensis	Lipoa	22	*Trifolium repens*	Jura	12

Table 2. Average stand cover values for all evaluation terms in 2017 and 2018.[1]

Species	Stand cover (%)		
	Control variant (without ASS)	Variant with applied ASS	Average per species
Festuca pratensis	22.8 ^{d}	27.8 ^{e}	25.3 ^{de}
Festuca rubra	17.9 ^{c}	36.1 ^{f}	27.0 ^{e}
Lolium perenne	36.9 ^{f}	51.2 ^{h}	44.1 ^{g}
Phleum pratense	9.9 ^{b}	15.7 ^{bc}	12.8 ^{b}
Poa pratensis	3.0 ^{a}	2.0 ^{a}	2.5 ^{a}
Anthyllis vulneraria	27.6 ^{e}	32.7 ^{ef}	30.1 ^{e}
Medicago lupulina	20.2 ^{d}	10.6 ^{b}	15.4 ^{bc}
Melilotus albus	37.9 ^{f}	21.9 ^{d}	29.9 ^{e}
Onobrychis viciifolia	41.3 ^{g}	58.1 ^{h}	49.7 ^{h}
Trifolium repens	24.3 ^{d}	40.6 ^{g}	32.4 ^{ef}

[1] Upper index numbers mean statistically different stand cover values tested by Tukey post-hoc test. Table presents both post-hoc test on the interaction of species and ASS application, and post hoc test on the average cover differences among species.

period of emergence. ASS lead to higher cover by following grass species: *Festuca rubra* (cover increase by 18.2%), *Lolium perenne* (14.3%), *Phleum pratense* (5.8%, n s) and *Festuca pratensis* (5.0%, n s). In case of *Poa pratensis,* applied ASS had no effect.

Our results were influenced by climatic conditions, which were similar in 2017 and 2018. Long lasting severe drought and high temperatures during the summer of both years had significant effect on plant survival. Experimental stands were strongly damaged, because plants did not develop enough their root system after the emergence period. In both years, we found positive effect of applied ASS on stand cover in evaluated species.

Conclusion

Positive effect of auxiliary soil substances both on plant emergence and vegetation development was observed in both years. The results significantly differ among the species. Subsequent influence of long-lasting severe drought and high temperatures during the summer both years had significant effect on plant survival. The most drought resistant species were *Onobrychis viciifolia* and *Lolium perenne.* On contrary *Medicago lupulina* and *Poa pratensis* were the most drought sensitive species.

Acknowledgements

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The ensilability of different varieties of *Festulolium*

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Abstract

Festulolium varieties are hybrids of fescue (*Festuca*) and ryegrass (*Lolium*). This hybrid (Schedonorus) was developed to utilise the positive characteristics of the two species, namely, yield and persistence. In this study, *Festulolium* varieties were cultivated in small plots at Agroscope in Changins, Switzerland, from 2015 to 2017. For the ensilability test conducted in 2016, the forage of the first and second cuts of six *Festulolium* varieties, two fescue varieties (Préval and Paradisia) and two hybrid ryegrass varieties (Daboya and Dorcas) were harvested from three plots. The forage was wilted on the field to 28% dry matter (DM) for the first cut and 57% DM for the second cut and ensiled in laboratory silos. After storage for 90 days, the silages were opened, and the silage quality was analysed. The forage of the first cut had a higher crude protein and sugar content as well as a lower ADF/NDF content compared to that of the second cut. The *Festulolium* varieties had nearly the same chemical composition as the ryegrass varieties, but differences concerning the fibre, crude protein and sugar contents were detected relative to the fescue varieties. All the silages had only traces of butyric acid and showed good fermentation qualities. Because of the higher DM content of the forage of the second cut, lactic acid fermentation was limited, resulting in a smaller decrease in pH in all varieties.

Keywords: *Festulolium*, ryegrass, fescue, variety trials, silage quality

Introduction

Festulolium varieties are hybrids of fescue (*Festuca*) and ryegrass (*Lolium*). Ryegrass varieties have wide adaptability, rapid establishment, a high response to high soil fertility and give high yields of highly digestible forage. However, ryegrasses can suffer from poor persistency and climatic stress in drought conditions. In comparison, fescues contribute more mid-summer growth and have greater drought tolerance. Nevertheless, fescues have relatively poor palatability and digestibility (Grogan *et al.*, 2018). The *Festulolium* hybrids were developed to combine the positive characteristics of the two species, namely, yield and persistence. Between 2015 and 2017, Agroscope in Changins, Switzerland, tested different varieties of *Festulolium*, fescue and ryegrass for their agronomic properties (Frick *et al.*, 2018). No silage additive was added. The aim of the investigation presented here was to compare the ensilability and silage qualities of the *Festulolium* varieties with those of the fescue and ryegrass varieties.

Materials and methods

The forage of six *Festulolium* varieties (Felimare, Helus, Hopej, AberNiche, Bb 2540 and Fedoro), the fescue varieties Préval and Paradisia and the hybrid ryegrass varieties Daboya and Dorcas were sown in small plots in Changins, Switzerland, in 2015. The experimental design was a randomized complete block design with four blocks (replicates), but only the forage of three blocks was used for the silage trial. In 2016, the forage of the first cut, cutting date 9 May, and the second cut, cutting date 27 June, were wilted and ensiled in laboratory silos with a capacity of 1.5 L. The forage of the different plots' repetitions were ensiled separately. Three laboratory silos were used for each treatment. No silage additive was added. Before ensiling and after 90 days of storage, samples were taken, and the dry matter (DM) and nutrient content were analysed using near-infrared spectroscopy (NIRS) (Ampuero and Wyss, 2014). Additionally, the fermentation parameters (pH, acids and ethanol) in the silages were analysed. The data were analysed by one-way ANOVA with the three species *Festulolium*, ryegrass and fescue as variables.

Results and discussion

In this study, the forage was wilted on the field to an average DM content of 28% for the first cut and 57% for the second cut (Table 1). There was a dry period from the first to the second cut, so the plants on the field already had a high DM content. The forage of the first cut had a higher crude protein and sugar content and a lower NDF and ADF content compared to that of the second cut. The *Festulolium* varieties had a similar chemical composition to the two ryegrass varieties. Compared to the varieties of *Festulolium* and ryegrass, the fescue varieties had a higher crude protein and NDF/ADF content and lower sugar content for the first cut and a higher crude protein and sugar content for the second cut. Unlike the first cut, the second cut of the fescue varieties had a lower NDF/ADF content compared to the *Festulolium* and ryegrass varieties.

The different fermentation parameters are indicated in Table 2. All the silages had only traces of butyric acid and showed good fermentation qualities. The higher DM content of the forage of the second cut, which was due to the weather conditions, probably meant that lactic acid fermentation was limited, resulting in a smaller decrease in pH. The *Festulolium* varieties showed similar fermentation qualities to those of the ryegrass varieties. The lactic acid fermentation in the two fescue varieties was more intense compared to that of the *Festulolium* and ryegrass varieties.

Table 1. The DM and nutrient content of the first and second cuts of the different varieties at ensiling (values in g kg^{-1} DM).[1]

	First cut					Second cut				
	DM	Protein	ADF	NDF	Sugar	DM	Protein	ADF	NDF	Sugar
	%	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}	%	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}
Felimare	28.8	87	220	407	177	57.8	84	336	568	102
Helus	28.0	90	232	418	175	59.3	73	367	620	93
Hopej	28.0	84	240	452	171	55.5	67	346	598	105
AberNiche	31.1	86	213	403	182	58.0	71	336	576	113
Bb 2540	31.0	91	252	454	157	58.8	71	352	598	101
Fedoro	29.3	92	244	443	169	56.1	70	349	594	107
Ø Festulolium	29.4	88	233	430	172	57.6	73	348	592	103
SE	0.36	1.7	3.3	5.1	1.7	0.61	2.8	3.0	6.6	1.4
Daboya	29.7	90	241	441	172	56.1	80	326	552	106
Dorcas	28.9	82	233	440	176	56.1	81	342	584	100
Ø Ryegrass	29.3	86	237	440	174	56.1	80	334	568	104
SE	0.63	3.0	5.7	8.9	2.9	1.06	4.8	5.3	11.4	2.4
Préval	25.4	115	303	531	112	58.2	119	331	527	89
Paradisia	25.1	131	296	523	107	54.4	132	315	502	89
Ø Fescue	25.2	123	299	527	109	56.3	126	323	514	89
SE	0.63	3.0	5.7	8.9	2.9	1.06	4.8	5.3	11.4	2.4
P-value	***	***	***	***	***	ns	***	**	***	***

[1] DM = dry matter; Protein: crude protein; ADF = acid detergent fibre; NDF = neutral detergent fibre; Sugar = ethanol water soluble carbohydrates; SE = standard error; ns = not significant; ** $P<0.01$; *** $P<0.001$ refers to the effect of species.

Table 2. The fermentation parameters of the silages of the first and second cuts of the different varieties (values in g kg^{-1} DM).[1]

	First cut					**Second cut**				
	pH	**LA g kg^{-1}**	**AA g kg^{-1}**	**BA g kg^{-1}**	**Et g kg^{-1}**	**pH**	**LA g kg^{-1}**	**AA g kg^{-1}**	**BA g kg^{-1}**	**Et g kg^{-1}**
Felimare	4.4	67	17	1	7	5.9	3	1	<1	22
Helus	4.4	65	18	1	9	6.0	2	1	<1	15
Hopej	4.6	57	20	1	11	5.7	8	2	<1	18
AberNiche	4.8	54	13	1	13	5.8	4	1	<1	7
Bb 2540	4.4	50	15	1	9	5.8	3	1	<1	15
Fedoro	4.5	58	12	2	16	5.8	7	1	<1	17
Ø Festulolium	4.5	58	16	1	11	5.8	4	1	<1	16
SE	0.04	2.1	1.0	0.5	1.1	0.04	0.7	0.1	0.03	1.8
Daboya	4.4	48	14	1	8	5.6	6	2	<1	10
Dorcas	4.4	52	17	1	9	5.7	6	2	1	14
Ø Ryegrass	4.4	50	16	1	9	5.7	6	2	<1	12
SE	0.06	3.6	1.7	0.9	1.8	0.06	1.2	0.3	0.05	3.1
Préval	4.5	65	10	2	13	5.7	6	2	1	1
Paradisia	4.6	65	11	6	13	5.6	10	3	1	2
Ø Fescue	4.5	65	10	4	13	5.7	8	2	1	1
SE	0.06	3.6	1.7	0.9	1.8	0.06	1.2	0.3	0.05	3.1
P-value	ns	*	*	*	*	*	*	*	***	**

[1] LA = lactic acid; AA = acetic acid; BA = butyric acid; Et: ethanol; SE = standard error; ns = not significant; * $P<0.05$; ** $P<0.01$; *** $P<0.001$ refers to the effect of species.

Conclusion

The six investigated *Festulolium* varieties showed similar chemical compositions to those of the hybrid ryegrass varieties. Compared to these two species, differences concerning the fibre, crude protein and sugar contents were found in the fescue varieties. In general, the *Festulolium* varieties were easy to ensile and the silages were of good quality.

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Effect of irrigation on forage yield of grass-legume mixtures in a summer-dry mountain region of Switzerland

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Introduction: Climate scenarios for Switzerland predict decreased summer precipitation, potentially affecting grassland productivity and management. Knowledge about the effects of irrigation is crucial in order to address the expected changes for grassland management. The objective of this field study was to determine the influence of irrigation on different official grass-legume mixtures for leys in a mountain region with summer dryness in Switzerland.

Materials and methods: The two-year field experiment was conducted in Domleschg (GR) at 840 m a.s.l. on an organic farm. Official grass-legume mixtures (SM 301, 323, 330, 442 and 450; Suter *et al.* 2017), varying in grass- and legume species composition, were established in a randomized complete block design with four repetitions. Irrigation treatments consisted of either 'non-irrigated' plots which received water by precipitation only or 'irrigated' plots with additional water supplied by drip irrigation. Fertilization was applied in early spring as cattle slurry in amounts that supplied N at a rate of 65 kg N ha^{-1}. Plots were cut four times per year. Data were analysed using a linear-mixed-model in R.

Results: With precipitation of 741 mm in 2015, the annual dry matter (DM) yield in non-irrigated plots was on average 122 dt ha^{-1}. Irrigation (+511 mm) increased the DM yield on average by 32.4 dt ha^{-1} (P<0.05). Significant differences in DM yield between the irrigation treatments were mainly observed for cuts 2 and 3 during the dry summer months from June to August (Figure 1). The highest yield increase due to irrigation (+48%) was found for mixture 442 based on tall fescue. Yield differences were least pronounced for mixture 323 based on lucerne. Irrigation had a strong impact on the grass-legume composition of individual mixtures (data not shown).

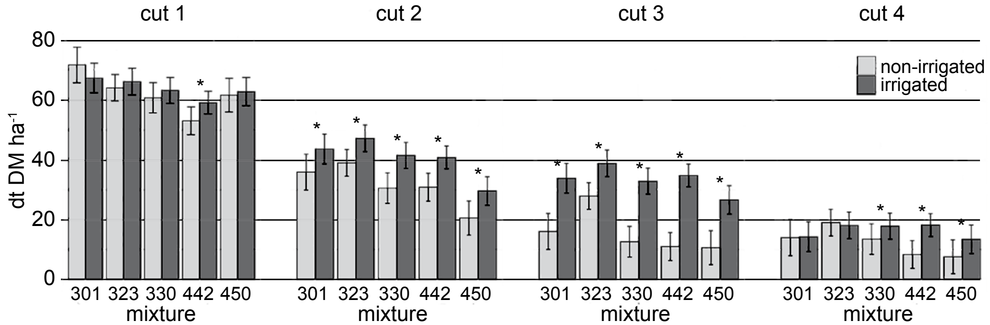

Figure 1. Average dry matter yield of individual cuts of official grass-legume mixtures, irrigated or non-irrigated (mean ± SEM). Asterisks indicate a significant difference at P<0.05.

Conclusion: The results indicate that irrigation increased the DM yield significantly, due mainly to higher dry matter yields in cuts 2 and 3 during the dry summer months. Individual mixtures show a species-dependent DM yield response to irrigation.

Suter D., Rosenberg E., Mosimann E. and Frick R. (2017) Standardmischungen für den Futterbau Revision 2017-2020. *Agrarforschung* (8), Supplement.

Ryegrass-red clover mixtures: impact of harvest stage on yield and nutritional value

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Introduction: Dairy farms can improve their protein self-sufficiency by producing more home-grown proteins. This solution can decrease the feeding cost, secure the production system, improve product traceability and reduce the potential negative impacts on the environment (Rouillé, 2014). Ryegrass and red clover mixtures can meet these requirements. Thus, the aim of our study was to produce grass silage with a higher protein content by harvesting grass for silage at an earlier stage and more frequently than is usually practised by dairy farmers.

Materials and methods: During four years at Trevarez experimental farm (Brittany, France), three grass fields of mixtures of hybrid ryegrass and red clover of 4 to 5 hectares each were split into two parts (Early and Control). On the 'Early' plots, grass was harvested to produce silage with a higher content in proteins by harvesting the first cut at an earlier stage (head at 15 cm, BBCH scale 31) than in the Control plots (first cut at early heading stage of the grass, BBCH scale 51). On the 'Early' plots, the next cuts occurred after a shorter interval than on the 'Control' plots: 5 weeks of regrowth versus 7 weeks. Yields, botanical composition, nutritional values, working time and costs were recorded for each cut of each treatment.

Results: The Early harvest system led to 5 cuts per year and resulted in reduced yield per ha in two of four experimental years (average -1.5 t DM ha^{-1}), compared to the Control system with 3 cuts per year. However, the early harvested forage always offered higher nutritive values both in energy and proteins (Table 1). Thus, the yields of net energy and proteins per ha were improved, reaching 9,392 UFL and 1.64 t CP ha^{-1} (respectively +18% and +32%). However, an increase in working time by 2.5 hours per ha was necessary to realise these early cuts. On average the cost of 1 ton of DM increased by 20 € for the early silage system: for instance, the cost of the two first early cuts reached 55 €/t DM compared to 35 €/t DM for the first control cut representing 50% of the total yield per year (Lefrançois, 2018).

Table 1. Net energy (INRA UFL kg^{-1} DM) and crude protein (g kg^{-1}) content in the forage, and difference (Δ) in energy (UFL ha^{-1}) and protein yield (t CP ha^{-1}) between the Early and the Control harvesting system.

Harvesting system	2014		2015		2016		2017	
	UFL	CP	UFL	CP	UFL	CP	UFL	CP
Control	0.75	99	0.84	126	0.88	121	0.84	105
Early	0.92	162	0.92	148	0.95	168	0.88	165
Δ Early-Control per ha	+1,102	+0.85	+1,037	+0.21	+5,796	+1.2	-1,139	+0.41

Conclusion: Early cuts of protein-rich mixtures can improve farm protein self-sufficiency, but their cost must be compared to the potential increase in dairy sales. An early harvesting system leads to a slightly reduced yield per hectare and not all farms can afford such a consequence in terms of forage stocks. Farmers may be advised to harvest early cuts till June and keep longer intervals when grass growth slows down to limit costs.

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The influence of lime on the fertility and physical composition of heavy soil

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Introduction: Poor soil pH (optimum 6.3) is a major limiting factor as regards to output potential on grassland farms; playing a key role in overall soil fertility. Optimum soil pH increases soil microbiological activity, it promotes the release of nutrients and increases the efficiency of organic and chemical nitrogen (N), phosphorus (P) & potassium (K) fertilisers. The objective of our study is to determine the effects of contrasting rates of lime on the physical and chemical properties of heavy soils.

Materials and methods: A randomised complete block design was established in a single paddock on three commercial dairy farms with contrasting heavy soil types: (1) Brown Podzolic, (2) Stagnic Luvisol, and (3) Humic Surface Water Gley. Seven treatments were imposed; namely 7.5, 5.0 and 2.5 tonnes ha^{-1} of ground limestone (AG) and 7.5, 2.5 and 1.5 tonnes ha^{-1} of granulated limestone (GRAN), in a single application at the beginning of the experiment and an untreated control throughout each of the 4 blocks. The plot study ran for 3 years from March 2015 to March 2018 and was assessed at 6-month intervals. The change in soil chemistry (pH) and nutrients using Morgan's, Mehlich III and EDTA test for P, K, Magnesium (Mg), and soil physical properties (bulk density, shear strength and penetration resistance) were analysed at each interval. The data was analysed using SAS version 9.4.

Results: There was a significant effect ($P<0.001$) of treatment on soil test pH (Table 1). Granulated lime increased pH faster over time. Morgan's soil test phosphorus (STP) was significantly affected by treatment on Farm 3 ($P<0.001$) but not Farm 1 and 2. Granulated lime only seemed to affect P index at high rates of application. Both products appeared to have positive effects in increasing Morgan's STP but a negative effect in increasing soil test potassium (STK). The change in (Mg) was mostly negative, however it started at the highest possible index 4 on each site (>100 mg l^{-1}). There was no evidence of soil softening between treatment rates or products.

Table 1. Mean soil pH over three-year period on each farm at each treatment rate with standard error of the mean (SEM).[1]

Treatment	Farm 1	Farm 2	Farm 3
Control	6.13^{e}	5.76^{c}	5.90^{b}
AG 7.5	6.53^{b}	6.03^{ab}	6.18^{a}
AG 5.0	6.35^{cd}	5.93^{bc}	5.99^{b}
AG 2.5	6.29^{d}	5.79^{c}	5.97^{b}
GRAN 7.5	6.68^{a}	6.18^{a}	6.24^{a}
GRAN 2.5	6.42^{bc}	5.91^{bc}	5.97^{b}
GRAN 1.5	6.26^{de}	5.83^{c}	6.00^{b}
SEM	0.023	0.027	0.027

[1] Within each farm, means with a common letter are not different at 5% level.

Conclusion: Due to the initial high pH level on farm 1 (mean pH 6.05), STP did not increase significantly, especially at high rates of product. Excess Calcium (Ca) in the soil reacts with P to form an insoluble compound. The naturally high K index of the soil and addition of Ca limestone may be the reason for the reduction of Mg levels in the soil.

Lignin – digestibility relations in various grassland species

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Introduction: The relationship between chemical composition and digestibility in forbs differs from that in grass, which is often used as a research standard. Therefore, predictions of the digestibility of forb-rich forage can be inaccurate. Relations between *in vitro* organic matter digestibility (IVOMD) and neutral detergent fibre (NDF) compounds such as acid detergent lignin (ADL) might increase insight into IVOMD differences among species. The objective of this study was to quantify such relations in four forbs.

Materials and methods: Pure stands were established with salad burnet (*Sanguisorba minor*), caraway (*Carum carvi*), chicory (*Cichorium intybus*), and ribwort plantain (*Plantago lanceolata*) in 2008. Swards were cut four times during 2009 and 2010 to get a wide range in digestibility within each species. Lignin, cellulose and hemicellulose contents were published previously (Elgersma *et al.*, 2014). This paper reports further analyses of IVOMD data in relation to the lignin content of DM and to the lignin proportion of cell walls. IVOMD, NDF and ADL were measured during four harvests in two consecutive years. Relationships among cell wall compounds and IVOMD were determined. The data were analysed by ANOVA.

Results: The IVOMD and ADL content were negatively related (Figure 1A); slopes of regression lines were similar among forbs, while the intercept was lower for salad burnet than for caraway and plantain ($P<0.05$). The ADL content *in planta*in was higher ($P<0.05$) than in other forbs, as was the degree of cell wall lignification ($P<0.05$; Figure 1B). Plantain had on average a lower IVOMD than caraway and chicory ($P<0.05$), but a similar level to salad burnet (636, 743, 715 and 641 g kg^{-1} OM, respectively) (Elgersma *et al.*, 2014). Thus, in salad burnet, factors other than lignification must be associated with IVOMD. The higher IVOMD of caraway than of plantain and salad burnet could not be explained by the degree of cell wall lignification as there were no relations, either among or within species (Figure 1B); in contrast, IVOMD declined with lignification (R^2=0.67) in mixed perennial ryegrass-white clover.

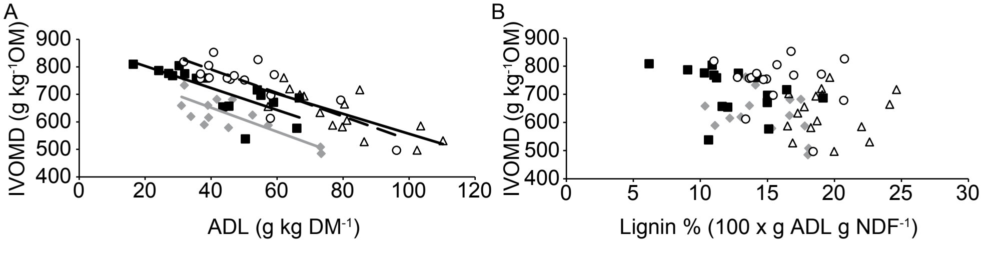

Figure 1. Relations between IVOMD and lignin content (A) and lignin proportion in cell walls (B) for four species (♦ salad burnet, ■ chicory, ○ caraway, Δ ribwort plantain); R^2 of regression lines: 0.64, 0.59, 0.67 and 0.58, respectively ($P<0.05$).

Conclusion: More research is needed to understand the lignification and composition of cell walls in various grassland species as relations between IVOMD and lignin content differed among species. Grass-based model predictions would underestimate caraway digestibility.

Elgersma A., Søegaard K. and Jensen S.K. (2014) Herbage dry-matter production and forage quality of three legumes and four non-leguminous forbs grown in single-species stands. *Grass and Forage Science* 69, 705-716.

Long-term CO_2 fluxes of permanent grassland: Impacts of weather and management

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Introduction: The contribution of permanent grasslands to mitigate climate change is still unresolved. Grasslands can act as source or sink of carbon dioxide (CO_2), depending on the environmental conditions and management. Considering the full greenhouse gas budget of managed grasslands, the CO_2 uptake often exceeds the loss of N_2O and CH_4 (in CO_2 equivalents), turning grasslands into net greenhouse-gas sinks (see Hörtnagl *et al.*, 2018). Taking into account the potential that grasslands have to sequester CO_2, our research objective was to investigate long-term CO_2 fluxes and to identify how management and extreme weather (e.g. dry spells; spring and summer droughts) influence these fluxes.

Materials and methods: Based on a 14-year data set, we assessed the impacts of weather conditions and management on the net ecosystem CO_2 fluxes of an intensively managed grassland on the Swiss Plateau, at Chamau (canton Zug). CO_2 fluxes were measured at the site from 2005 onwards, using the eddy covariance method. We assessed annual C budgets using CO_2 fluxes as well as C exports at harvest and C inputs via organic fertilizer applications. The calculated net C exchange over the whole period was then validated with measured changes in soil C stocks from soil sampling.

Results: Our results clearly show how the seasonal cycles of CO_2 fluxes were influenced by meteorological conditions and management measures over this period, and how resilient the grassland was in reacting to extreme weather events (e.g. drought). Regrowth after frequent cuts (4-6 per year) showed large seasonal variability but little inter-annual variability due to environmental impacts on growth. Management events strongly affected net ecosystem CO_2 fluxes, with the CO_2 losses typically increasing after harvests, until photosynthetic CO_2 uptake compensated and again exceeded respiratory losses. In particular, grassland restoration (i.e. ploughing and subsequent reseeding) in 2012 turned the grassland into a temporary large CO_2 source, although vegetation re-established during this year. We compared topsoil (0-10 cm) C stocks in 2005 with those in 2018, but this did not reveal significant differences between sampling dates, indicating a carbon neutral behaviour over the 14 years of permanent grassland management.

Conclusion: Flux measurements combined with management information (i.e. C exports and imports) allowed assessment of the resilience of this permanent grassland to extreme weather events and to calculate its full C budgets over more than a decade. Such data provide valuable information on C sequestration potential (i.e. changes in the C stock) for intensively managed grassland, suggesting potential management options to increase its C sink strength.

Hörtnagl L., Barthel M., Buchmann N., Eugster W., Butterbach-Bahl K., Díaz-Pinés E., Zeeman M., Klumpp K., Kiese R., Bahn M., Hammerle A., Lu H., Ladreiter-Knauss T., Burri S. and Merbold L. (2018) Greenhouse gas fluxes over managed grasslands in Central Europe. *Global Change Biology* 24, 1843-1872.

Below ground nitrogen of clover-grass mixtures and its residual nitrogen potential for subsequent crops

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Introduction: Estimates of symbiotic nitrogen fixation (SNF) in clover-grass mixtures usually take into account only the above ground clover N. Thus, this study aims at quantifying below ground N inputs from SNF by roots and N derived from rhizodeposition, their transfer to associated grass and their protection within soil organic matter pools as well as the residual N potentially available for subsequent crops.

Materials and methods: A microplot study with a *Trifolium pratense – Lolium perenne* mixture was performed within organic and conventional field plots of the DOK long-term experiment (Hammelehle *et al.* 2018). Clover was multiple ^{15}N urea leaf labelled during two cultivation years. Quantification of SNF and fluxes of symbiotically fixed N in the soil plant system were done with a combination of ^{15}N enrichment and natural abundance approaches. Results of the microplot study were scaled up using data from the DOK field plots.

Results: The mean SNF of clover across organic and conventional treatments was 37 g m^{-2} in two years (Figure 1). At the end of the second cultivation year about 5% of this amount of total SNF was found in clover roots. Over the two years 50% of total SNF went into rhizodeposition from where it continued its flux to soil organic matter pools, microbial and soluble N (15% of total SNF) and the grass (35% of total SNF). At the end of the two years a residual N potential of SNF of 10 g m^{-2} was built up. It consisted of clover roots (5% of total SNF), clover stubble (1% of total SNF), grass roots (6% of total SNF), and soil N pools (15% of total SNF).

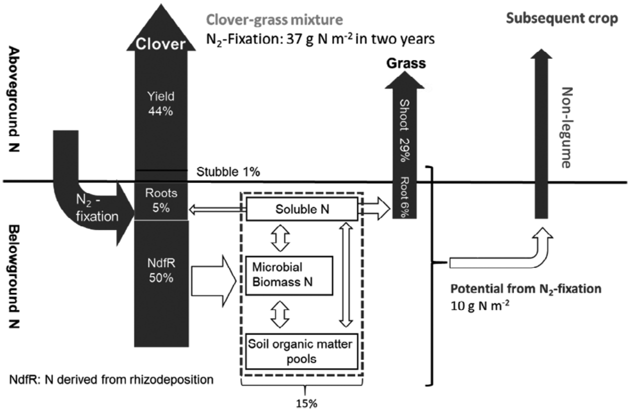

Figure 1. Partitioning of fixed clover N after two consecutive years of clover-grass mixture and its residual N potential for subsequent crops. Mean of organic and conventional (mineral N fertilised) DOK treatments.

Conclusion: A realistic estimation of N$_2$-fixation in clover-grass mixtures needs to take into account below ground N pools and fluxes.

Hammelehle A., Oberson A., Lüscher A., Mäder P. and Mayer J. (2018) Above- and belowground nitrogen distribution of a red clover-perennial ryegrass sward along a soil nutrient availability gradient established by organic and conventional cropping systems. *Plant and Soil* 425, 507-525.

Implications of forage response to flooding for ruminant production

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Introduction: Continual improvement of forage feed germplasm has achieved increases in ruminant production (Kingston-Smith *et al.,* 2013). Climate change models predict rises in temperature, CO_2 and weather system changes. Understanding forage stress response to this and its effect on ruminant production systems will allow optimisation of plant breeding traits for the future. This study has focussed on the effect of climate change-related flooding on the plant-mediated metabolism of protein in ingested perennial ryegrass (*Lolium perenne).*

Materials and methods: In a flooding experiment, *Lolium perenne* var AberWolf was grown from seed under standard glasshouse conditions for 8 weeks before dividing into two treatments; half were maintained in current conditions (Control treatment) and the remainder were completely submerged in water for 1 week (Flood treatment). Incubation (digestion) of 0.5 g FW leaf sections (1 cm) in *in vitro* fermentations in 15 ml Hungate tubes containing 10 ml of anaerobic phosphate/bicarbonate buffer, backfilled with CO_2 was conducted to assess the impact of treatment on foliar protein stability. Samples from both treatment groups were incubated at 39 °C to simulate anaerobic rumen conditions for 0, 2, 4, 6 and 24 hours. Grass samples were retrieved from the tubes at each time point. Protein was extracted, quantified and digested for analysis by nano-flow LC MS/MS 6550 Q-TOF analysis (Agilent) and data were processed using Mascot Daemon (Matrix Science) against the NCBInr database.

Results: Protein degradation during fermentation occurred regardless of pre-treatment. However, kinetics of total protein loss after 24 h incubation resulted in a degradation rate 2.7 times faster in flooded samples compared to the control (Figure 1). Peptide composition between samples showed significantly fewer unique peptides from flooded plants compared to the control (data not shown).

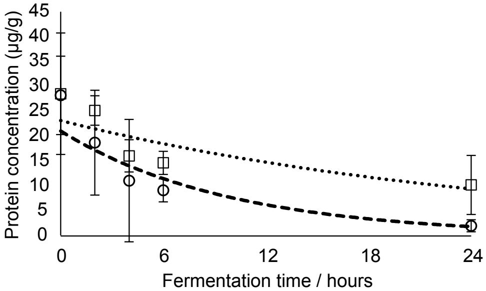

Figure 1. The effect of anaerobic fermentation time (hours) on the concentration of protein (mg gFW^{-1}) between control (square points) and flood (round points) samples. Error bars represent ± 1 SD.

Conclusion: Plants alter their metabolism in response to stress, hence changes in climate will likely impact on metabolism of forage protein by ruminants. Changes in such stress responses under flood conditions highlight the loss of potentially important essential amino acids. This alongside increased protein degradation impacts on the overall nutritive value of forage feeds and may have significant practical implications for the future of ruminant nutrition.

Kingston-Smith A.H., Marshall A.H. and Moorby J.M. (2013) Breeding for genetic improvement of forage plants in relation to increasing animal production with reduced environmental footprint. *Animal* 7, s1, 79-88.

Grassland management options to extend the lifespan of sown grasslands

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Introduction: Long-lasting sown grasslands would be advantageous for grassland-based production systems and the environment. However, the botanical composition of sown grasslands typically deteriorates after three to five years of utilisation. The aim of this experiment was to test management options to extend the lifespan of sown grasslands.

Materials and methods: The experiment began in spring 2014 on a mown and grazed grassland sown in 2011. At the experiment's onset in 2014, the sward had 20% *Lolium perenne*, 10% *Poa pratensis*, 10% other forage grasses, 15% clover, 30% forbs and 15% poor quality grasses. Two grazing levels (no grazing, cattle grazing in spring) and three seed addition levels were compared in a factorial design arranged as a strip plot with six replicates. The seed addition treatments were (1) no seeds, (2) overseeding with a mixture of *L. perenne*, *P. pratensis*, *Dactylis glomerata*, *Festuca rubra* and *Trifolium repens*, and (3) allowing the sward to shed seeds (self-reseeding) at the second harvest following an early harvest in April.

Results: Cattle grazing in spring enhanced the proportion of forage grasses in the sward (P=0.031), which translated into higher yields (P=0.005; Figure 1A). The effect of the seed-addition treatments was statistically not significant for the total proportion of forage grasses but almost significant for the yield (P=0.062, interaction with grazing P=0.005). Overseeding improved the proportion of the oversown grass species in the plots without grazing but not with grazing ($P_{seed\ addition}$=0.018, interaction with grazing P=0.087; Figure 1B). The proportion of forage grasses decreased between 2016 and 2018 in all treatments (data not shown).

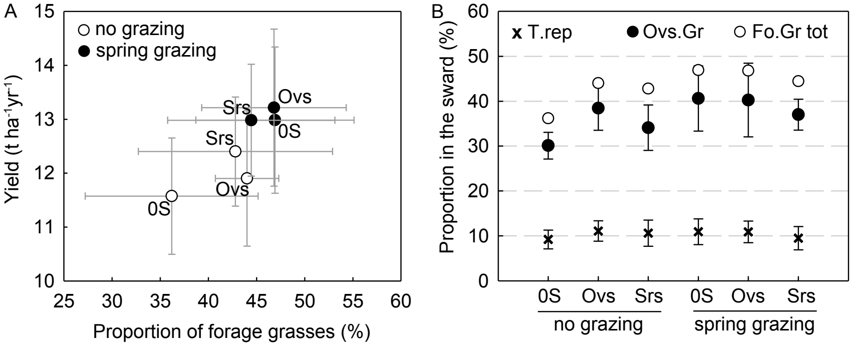

Figure 1. (A) Dry matter yield of the six management treatments as a function of the proportion of forage grasses in the swards and (B) proportions of *Trifolium repens* (T.rep), oversown grass species (Ovs.Gr) and total forage grasses (Fo.Gr tot) in swards managed with or without spring grazing, and without seed addition (0S), with yearly overseeding (Ovs) or with yearly self-reseeding (Srs). Data are the means of six replicates over the years 2015 to 2018 ± SD.

Conclusion: The mowing-only management without seed addition was clearly the least appropriate to sustain yields and a satisfactory botanical composition. Spring grazing seems more promising than overseeding, and especially compared with self-reseeding, to extend the lifespan of sown grasslands with *L. perenne* and *P. pratensis* as dominant grass species.

Changes in grassland management in the Middle Wieprz River Valley, eastern Poland

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Introduction: The Middle Wieprz River Valley PLH060005 is an area of special habitat protection located in the Lublin province, eastern Poland. Site covers an area of 1,523.34 ha including the well-preserved Wieprz valley, a wild, meandering river, with old river beds, wet meadows and xerothermic grasslands, located on steep slopes. The dominant grasslands are wet, rush and hay meadows. Recently, major changes in land management have been observed, due to the lower demand for feed from grasslands. The study objective was to assess changes in the use of grasslands and, consequently, changes in plant communities.

Materials and methods: The research was carried out in 2005-2017 on five grassland areas of total 176 ha. An inventory of the vegetation, including hay, wet and rush meadows, using the Braun-Blanquet method was carried out, defining the range of patches of plant communities. Land use in this area was verified by means of site inspections using a GIS receiver and mapping the range of cut or uncut plots in two periods: 2005-2007 and 2016-2017. In the second period, hyperspectral imaging data obtained from a gyrocopter were also used for this purpose. QGIS software was used to assess the surface changes in the use of grasslands.

Results: In 2005-2007 most meadows were cut twice a year (56 ha), while 1-cut meadows were also grazed by cattle in the end of summer. Typical pastures occupied an area of 20 ha with 150-200 grazing cattle. When analysing the frequency of cutting in 2016-2017, it was found that most meadows are not utilised (77 ha) or cut once in the growing season (76 ha), while 23 ha are covered by meadows cut twice a year. On the uncut wet meadows of the *Molinietalia* order, changes in vegetation and transformations towards high sedge-beds of the *Magnocaricion* alliance were observed. Hay meadows from *Arrhenatheretalia* order were the most stable communities. It should be stressed that most abandoned meadows are rush communities. During the period analysed (2005-2017), the greatest changes occurred in pastures (Ass. *Lolio-Cynosuretum*); only some of them (12 ha) are still used for grazing with 25-30 cattle in the growing season.

Conclusion: The greatest changes occurred in pastures, where the livestock density decreased and an associated disappearance of *Lolio-Cynosuretum* was observed. Moreover, the area of abandoned meadows increased, while the 2-cut meadows decreased. Most abandoned meadows developed into rush communities during the last decade. The suspension of agri-environmental subsidies to these communities in Poland contributed to their abandonment.

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Impact of nitrogen fertilization on dry matter yield of fen grassland – results of a 56-years field experiment

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Introduction: A long-term N fertilization plot experiment has been continuously conducted on drained fen grassland since 1961 in order to study the impact of different nitrogen (N) rates on the yield of drained fen grassland. Long-term field trials on drained fen grassland particularly depend on annually varying water conditions and on internally changing physical soil characteristics due to drainage. Therefore, it could be expected, that dry matter (DM) yields are characterized by high annual variation. For the prediction of DM yield in relation to nitrogen (N) rates, the incorporation of further information about typical characteristics of the drained fen soil at plot scale is necessary.

Materials and methods: In the plot experiment, the annual N rate was varied at five levels: 0, 60, 120, 240 and 480 kg N ha^{-1}. All plots were cut three times annually, and one third of the N fertilizer amount was applied to every cut. Potassium and phosphorus were given each year in early spring at the rate of 31.8 and 139.4 kg ha^{-1}, respectively. The trial was established in a randomized complete block design with four replications. Dry matter yields of all cuts were measured during the entire experimental period. Peat layer thickness and dry bulk density were recorded in 2015, as well as elevation of the single plots, describing the different distances between sward and ground water table beneath the trial area. As a first approach, DM yield was regressed on N rate (linear, quadratic), elevation, peat layer thickness and dry bulk density with models per each year using multiple linear regression (SAS 9.4 software, proc REG and MIXED) for the period from 1971 to 2016. We fitted the full model and all possible sub-models, and model fits were evaluated by Akaike Information Criterion and adjusted R^2. The blocking structure was not considered in the model because of a rarely existing variance component due to the blocks when DM yield was analyzed by ANOVA.

Results: The N rate affected DM yield variously between the years as well as between the cuts. We could not find a similar pattern for all years (we found no N effect as well as a linear or a nonlinear N effect). A significant relationship between DM yield and N rate was found in 84% (cut 1), 60% (cut 2), 44% (cut 3), and 73% (total annual yield) of the years. Including the soil characteristics in models, these percentages increased to 89% (cut 1), 68% (cut 2), 65% (cut 3), and 81% (total annual yield) of years. The impact of the soil characteristics on the DM yield differed too. Especially, elevation enhanced the model fit. Peat layer thickness and dry bulk density were also often incorporated in the best model, either separately or in common. In case of significant relationship between DM yield and N rate, the quadratic function was mostly best. In any case, the model fit improved (e.g. for total DM yield and 2008 at adjusted R^2=0.641 and AIC=112.8 for quadratic model, adjusted R^2=0.738 and AIC=107.3 for quadratic model + peat layer thickness as additional explanatory variable).

Conclusion: Based on first results, more sophisticated models incorporating the years are intended as basis for deriving practical conclusions for fen grassland management.

Individual-based modelling as a tool to identify combinations of key traits to enhance overyielding in legume-based mixtures

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Introduction: The role of many plant traits involved in carbon (C) and nitrogen (N) economy on species balance and total aboveground biomass production of grass-legume mixtures is still to be determined. A modelling approach using the Virtual Grassland model (Louarn and Faverjon, 2018) was developed to explore key trait combinations favourable to overyielding and mixture persistence.

Materials and methods: The model was previously calibrated for alfalfa-based mixtures (Faverjon *et al.*, 2018). Taking this calibration as a departure point, a first step consisted in running a sensitivity analysis. This allowed us to identify the model parameter (i.e. plant traits) most sensitive to inter-specific competition among 30 candidate traits. A second step then consisted in testing the impact of sensitive parameter combinations (selected to account for distinct physiological functions: light acquisition, mineral N acquisition, kinetics of growth and resource use efficiency) on virtual mixture performance. Each trait combination was assessed using principles of de Wit's replacement design (Figure 1A) to assess overyielding (Figure 1B). Three pedo-climatic conditions were analysed that differed in soil N fertility (0N, 120N, 300N).

Results: Based on our simulations (Figure 1B,C), maximal overyielding was achieved in cases where trait values were divergent for N acquisition (i.e. allowed complementarity in the use of different N pools) but convergent for light interception (i.e. limiting the asymmetric competition for light). The best combination of traits was not the same in all the pedo-climatic conditions tested and depended on the availability of mineral N in the soil. Random trait combinations could frequently lead to sub-optimal yields (i.e. less than a neutral situation of competition, Figure 1B) and even to under-yielding situations (i.e. less than the average of monocultures). Such situations were frequent when legumes displayed late growth kinetics.

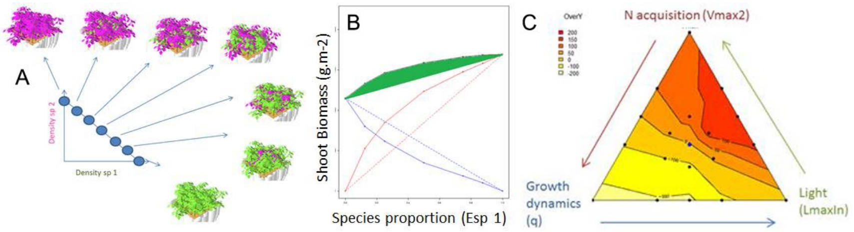

Figure 1. Virtual experiment design (A), overyielding estimate used to asses a binary mixture performance (B, coloured area) for a neutral situation of competition and (C) example of overyielding map for the 120N level.

Conclusion: Our study demonstrates how heuristic modelling can help to identify favourable combination of traits targeting a given pedo-climatic condition.

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Yield and chemical composition of grass-legume swards containing different proportions of alfalfa (*Medicago sativa*)

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Introduction: Mixtures of legumes and grasses may provide higher and more stable yield, positively affect the soil environment, and provide more varied forage than from grass or legume monocultures (Nyfeler *et al.,* 2011). Legumes enrich the feed with proteins, fats, macro- and microelements and reduce the content of crude fibre. Grass-legume mixtures provide excellent feed for milk production and allow farmers to reduce concentrates and N fertilization. Alfalfa (*Medicago sativa*) is one of the most important fodder plants used for feeding dairy cows. The aim of the study was to assess the effects of variation in the percentage of alfalfa in the mixture of forage grasses on chemical composition and nutritional value of the resulting yield.

Materials and methods: The experiment was conducted in 2016-18 (March-October) at an Experimental Station of the Agricultural University of Cracow (village of Prusy: 50°7'1'N 20°5'14'E). The experiment included a grass pure stand (control) and three grass-legume mixtures with variable weight share (10, 20, and 30%) of alfalfa cv. Kometa. The variants were planted on 1.65×6.00 m plots, with four replicates of each variant. The soil at the experimental field was loess-based degraded chernozem of bonitation class I (very good soil). The results were subjected to an analysis of variance (ANOVA), and the significance of differences between means was determined with Duncan test, using Statistica 12 PL software.

Results and discussion: The treatments with the medium and higher proportion of alfalfa showed significantly ($P \leq 0.05$) improved yield and dry matter. Plant samples obtained from the treatments with 20 and 30% share of alfalfa contained more ($P \leq 0.05$) total protein in absolute dry matter than the control. A small increase of crude fibre content found in the analysed plants (variant 30%) was not significant. We noticed a greater uptake of nutrients as phosphorus, potassium, magnesium and calcium, with sodium as the only exception. The addition of alfalfa also improved plant uptake of zinc, copper, manganese and iron (variants 20% and 30%).

Conclusion: Addition of alfalfa to grass mixtures resulted in a significant increase in the desired components and nutritional value of the fodder plants. The recommendation for agricultural practice is to use mixtures with the variant containing a 20% share of alfalfa.

Acknowledgements: The work has been financed with subsidies for maintaining the research potential granted by the Polish Ministry of Science and Higher Education.

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Yield potential and chemical composition of selected grass-legume forage mixtures in Poland

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Introduction: Taking into consideration regions of similar pedo-climatic conditions, it may be stated that production potential and nutritional value of plant biomass derived from grasslands depends mainly on the plant mixture and proportions of individual species. Grasses cultivated with legumes should grow and develop at a similar rate and have comparable habitat requirements (Terlikowski 2014). The aim of this study was to determine yield and chemical composition of selected new forage mixtures commercially available in Poland.

Materials and methods: Field experiments were conducted on a farm in the Małopolska region, southern Poland, using large plots (6.4×60 m). The soil was loess-derived degraded chernozem. We evaluated four types of mixtures: meadow mixture for dairy cows, universal meadow mixture with legumes, haylage mixture and mixture for cattle fattening. The first contained 5% red clover, 5% white clover, 5% cocksfoot, 10% timothy, 20% red fescue, 12% meadow fescue, 25% perennial ryegrass, and 18% Italian ryegrass. The second contained 5% red clover, 6% Swedish clover, 8% timothy, 8% red fescue, 10% meadow fescue, 8% hybrid ryegrass, 22% peren. ryegrass, 24% Ital. ryegrass and 7% westerwolds ryegrass. The third contained 10% lucerne, 5% crimson clover, 20% hybrid ryegrass, 25% Ital. ryegrass, 30% peren. ryegrass and 10% festulolium. The fourth contained 5% Swedish clover, 5% smooth-stalked meadowgrass, 10% red clover, 15% timothy, 30% peren. ryegrass, 15% Ital. ryegrass and 20% hybrid ryegrass. Control was permanent meadow with dominant grasses peren. ryegrass, smooth-stalked meadowgrass and cocksfoot. ANOVA was used and significance of differences between means was determined with Duncan test, using Statistica 12 PL software.

Results and discussion: Significant differences *($P \leq 0.05$)* in dry matter yield between the mixtures were found, ranging from 9.90 to 11.50 t ha^{-1} (6.01 t ha^{-1} for control). The haylage mixture produced the highest yield, while the mixture for cattle fattening was markedly less productive. Total protein content was highest in the haylage mixture (159.2 g kg^{-1}) and the universal meadow mixture the poorest (148.1 g kg^{-1}). The mixtures differed considerably in their content of raw fibre (271.8-285.3 g kg^{-1}), the haylage mixture had the lowest amount of this component. Plant samples from the variants with higher share of legumes contained more macronutrients (phosphorus 2.34-3.24 g, potassium 16.31-23.53 g, magnesium 3.51-5.53 g, calcium 1.03-1.06 g kg^{-1} DM; difference between variants was 10%) and micronutrients (zinc 77.81-89.04 mg, copper 4.19-4.49 mg, manganese 43.00-46.89 mg and iron 95.96-102.07 mg kg^{-1} DM; difference between variants was 8%.

Conclusion: Results demonstrated a high yield potential of the examined mixtures and large concentrations of organic and mineral components.

Acknowledgements: The work has been financed with subsidies for maintaining the research potential granted by the Ministry of Science and Higher Education.

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The economic value of species diversity in intensively managed grasslands

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Introduction: Species diversity (D) in grasslands can affect biomass yield and its quality in terms of both expected outcomes and their variability, i.e. production risk. For farmers, all these components are economically relevant. However, they have not been broadly addressed in previous agricultural economic studies. We contribute to filling this gap and quantify the D effects on grassland yields and quality using field trial data from intensive grassland production throughout Europe. We economically evaluate the D effects on grassland production and value it in terms of farmers' utility.

Data and methods: We used biomass yield, quality and D (Simpson Index) data from 16 intensively managed grassland experiments across Europe, each with 30 plots of varying D (richness and evenness; Kirwan *et al.,* 2014). First, employing a stochastic production function approach (Finger and Buchmann 2015), we estimated the D effects on three expected outcomes: biomass yield (kg forage yield ha^{-1}), milk production potential (MPP; kg milk kg^{-1} forage) and MPP yield (kg milk ha^{-1}) and their variances. Second, we used the expected utility framework to economically value farmers' utility gain from D. For this, we monetized the outcomes using potential milk sale revenues (π = MPP yield milk price) and computed the certainty equivalent for risk averse farmers.

Results: Species diversity of the reference mixture (the equiproportional four species mixture) increased biomass yield by ~30% and decreased its variance by ~-50%, while it had no effect on MPP (Table 1). Thus, D increased MPP yield (~30%) and decreased its variance (~-45%). The certainty equivalent increased with D, e.g. by ~35% for the reference mixture compared to the monocultures.

Table 1. D effects on expected outcomes and their variances for the reference mixture.[1]

	Biomass yield (kg ha^{-1})	**MPP (kg kg^{-1})**	**MPP yield (kg ha^{-1})**
Expected outcome			
Species diversity	+30%***	-1%^{s}	+29%***
R^2_{adj}	0.77	0.47	0.79
Variance of outcome			
Species diversity	-51%**	-76%^{ns}	-47%*
R^2_{adj}	0.16	0.03	0.16

[1] ns = not significant; * $P<0.1$; ** $P<0.05$; *** $P<0.01$.

Conclusion: Our results show that farmers can attain positive economic benefits from D in intensively managed grasslands. Consequently, D can contribute to sustainable intensification of grassland-based production.

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Theme 3.
Molecular genetics and genomics for improved breeding of forages

Advances and perspectives in the application of genomic tools for research and breeding of forage crops

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Abstract

Next-generation sequencing technologies have offered the possibility to sequence the genome of a few forage legume and grass species such as red clover or perennial ryegrass for which reference genome sequences and annotated gene sets are now publicly available. Nevertheless, due to the complexity of the forage crop genomes and the funding allocated to them, the quality of these reference genomes still needs improvement to obtain contiguous chromosome-scale assemblies. The latest sequencing technologies will facilitate the assembly process, and implementation of novel bioinformatics methods can improve the quality of the genome annotation. With or without the aid of a reference genome sequence, it is now possible to genotype a large number of individuals or pools of individuals with several thousands of markers, bringing routine implementation of marker-assisted selection within reach for a large number of forage crop breeding programmes. Exemplary illustrations are provided in the context of parentage testing, genome wide association studies, genomic selection and population genetics to identify adaptive traits.

Keywords: parentage analysis, association mapping, genomic selection, population adaptation

Introduction

It was ten years ago that the first author of this paper was invited to prepare a presentation with the title 'Marker-assisted Selection in Forage Crops and Turf: A Review' (Roldán-Ruiz and Kölliker 2010) for the Conference of the Eucarpia Fodder and Amenity Species Section in La Rochelle, France. The attempts that had been undertaken at that time to implement marker-assisted selection (MAS) in forage species were reviewed. While the value of QTL (quantitative trait locus) and association mapping as tools to dissect the genetic control of agronomic traits and to identify relevant genomic regions was acknowledged, the promise of genomic selection (GS) in forage crops was highlighted (Roldán-Ruiz and Kölliker 2010). Though at that time no single reference genome sequence of a forage crop had yet been published, the enormous potential of ongoing developments in DNA sequencing and SNP genotyping, both instrumental to accelerate the development and deployment of molecular tools in plant breeding, was anticipated.

Looking back to Roldán-Ruiz and Kölliker (2010) and thinking about the content of the present paper, the statement 'Most people overestimate what they can do in one year and underestimate what they can do in ten years' (attributed to Bill Gates) came to our mind. It is not the purpose of this paper to evaluate whether or not the elements handled in the future perspectives presented ten years ago have become a reality. However, we see that the path that was drawn at that moment is now being walked, and that advancements that we did not dare to consider ten years ago have been achieved. Next-generation sequencing (NGS) technologies have enormously impacted our knowledge of crop genomes and gene regulation. NGS technologies have also been pivotal in the development of cost-efficient methods to genotype very large numbers of single nucleotide polymorphisms (SNPs) in a large number of individuals or populations, enabling the application of GS approaches in forage crops. In some cases, this implies a rethinking of the breeding and selection schemes applied, so as to make them more suitable to the implementation of genomic selection.

The endeavour of developing and implementing genomics tools in forage crops is not simple. Most forage species are outcrossing and both breeding populations and varieties are genetically heterogeneous and characterised by high levels of heterozygosity. A further complication in some species is their polyploid nature. All these characteristics also complicate genetic improvement, slowing down the selection gain and leading to varieties, which are typically synthetic populations generated by random mating of elite parent plants.

In this paper we first summarize the state of the art of forage crop genome sequencing and advances in marker system development. We then illustrate current directions in the development and implementation of genomics tools with a few examples. However, as not all breeding programmes will necessarily implement these kinds of tools in the (near) future, one of the examples discussed concerns the use of a relatively simple approach to use markers for parentage analysis, which can benefit a large number of breeding programmes that currently mainly consider the maternal contribution to trait performance through a focus on half-sib family phenotype evaluation. Finally, we present some personal views of how genomics approaches can benefit both breeding and grassland management and bring them closer to each other. The paper is centred on four forage crops which are among the most frequently cultivated and studied worldwide, *Lolium perenne* (perennial ryegrass), *Trifolium pratense* (red clover), *T. repens* (white clover) and *Medicago sativa* (alfalfa), with references to other species where relevant.

Reference genome sequencing of forage crops

A reference genome sequence and a complete structurally and functionally annotated gene set are important resources to explore the structure and function of a crop's genome, while further resequencing and/or genotyping elucidate genetic variability among individuals. As such, efficient implementation of genomics-based breeding strategies relies on the availability of an annotated reference genome sequence. In this section we first summarize the current status of forage crop reference genome sequencing and then discuss future steps.

Current status of reference genome sequencing in forage crops

Two perennial ryegrass ($2n=2\times=14$; genome size estimation: $1\times = 2{,}068\text{-}2{,}695$ Mbp) reference genome sequences are currently publicly available: a first annotated draft genome sequence was published in 2015 (Byrne *et al.,* 2015), and a shotgun assembly of an independent genotype in 2016 (Velmurugan *et al.,* 2016). Nevertheless, both assemblies are fragmented and not anchored on the seven chromosomes as expected. Byrne *et al.* (2015) generated a draft genome assembly of *L. perenne* using a mixture of Illumina paired-end and mate-pair reads and PacBio long sequence reads. A predominantly homozygous genotype that had been inbred for six generations was used to avoid assembly problems related to heterozygosity. A *de novo* assembly generated 48,415 scaffolds with a total length of 1,128 Mbp covering about half of the genome. Velmurugan *et al.* (2016) also used a predominantly homozygous genotype and generated an assembly comprising 424,750 scaffolds with a total length of 1,110 Mbp. A genetic linkage map was used to anchor just over 200 Mbp of the sequence assembly.

CEGMA and transcript mapping scores of 96% have shown that the expected gene space was almost completely covered in the sequence assembly of Byrne *et al.* (2015), but because only half of the expected genome size is assembled into scaffolds, this indicates that the missing fraction of the genome sequence mainly concerns intergenic or repetitive sequences. K-mer analysis indicated that the *L. perenne* genome contains about 76% repetitive sequences. They annotated 28,455 gene models, using *de novo* assembled transcripts as experimental evidence, and used the GenomeZipper approach to construct a synteny-based gene order for 11,311 annotated genes. While these gene structure models are of good quality (87.4% accurate at the nucleotide level when compared to 503 manually curated reference genes (Veeckman *et al.,* 2018)), BUSCO and the PLAZA core Gene Family (coreGF) gene annotation completeness measures indicate that these gene sets are incomplete (BUSCO 81.6% ([S:61.6%, D:20.0%], F:2.5%, M:15.9%, n=1440); coreGF: 76.9% complete, n=7,076, 1,709 missing gene families), most likely because they did not incorporate *ab initio* gene prediction (Veeckman *et al.,* 2016). Further analyses of the genome sequence have improved the structural and functional gene annotation (Veeckman *et al.,* 2019). First, the EVidence Modeler (Haas *et al.,* 2008) tool was used for automated gene structure annotation and was combined with evidence from secondary sources, such as a complementary set of *ab initio* gene predictions, orthology guided transcript assemblies (Ruttink *et al.,* 2013), and aligned proteomes of closely related species (*Brachypodium distachyon*, rice, maize, sorghum). The resulting set of 39,967 annotated genes was more accurate (97.0% accuracy compared to 503 gold standard gene models) and more complete (BUSCO 92.6% ([S:89.0%, D:3.6%], F:2.5%, M:4.9%, n=1,440); coreGF:89.4% complete, n=7,076, 538 missing gene families). Next, functional annotations were generated by adding the 39,967 *L. perenne* gene set to a private build based on the PLAZA v4.0 Monocots with 29 comparator species (Van Bel *et al.,* 2018). In total 160,545 homology groups and 236,721 orthogroups were created, establishing orthology relationships between *L. perenne* genes and closely related species. Functional annotation was expressed using the Gene Ontology (GO) syntax, and added using InterproScan and projection of functions to orthologous genes (Burge *et al.,* 2012). Taking both Interpro and GO (20,224 genes), and AnnoMine (19,301 genes) annotations into account, a functional description is now available for 23,879 *L. perenne* genes.

In red clover (2n=2×=14; genome size estimation: 1×=420 Mbp), De Vega *et al.* (2015) generated a chromosome-scale reference genome sequence for one genotype from the variety 'Milvus' by integrating whole genome shotgun (WGS) sequencing of Illumina short reads, Sanger-based bacterial artificial chromosome (BAC)-end sequences, and a physical map and two linkage maps. Illumina WGS short reads were *de novo* assembled and marker information from two genetic maps was used to anchor and orient scaffolds into a pseudo-molecule scale assembly. Further scaffolding was based on BAC-end sequences and a physical map comprising 29,730 BACs. The final version of the genome assembly consisted of 309 Mbp in 39,904 scaffolds, of which 164.2 Mbp were anchored onto seven pseudo-molecules, and 75.2 Mbp were contained in 542 unanchored scaffolds longer than 50 Kbp. De Vega *et al.* (2015) also annotated 40,868 genes and 42,223 transcripts. Of those, 22,042 genes were anchored onto the seven pseudo-chromosomes. For most of the transcripts (93.6%) a homologous protein was found in the UniprotKB database, and 1,580 of the remaining transcripts (3.7%) had a novel ORF. A total of 31,576 transcripts (74.8%) was annotated with at least one GO term.

In white clover (2n=4×=32; genome size estimation: 1× = 430 Mbp) no genome sequence is publicly available yet, but work is in progress in New Zealand (Griffiths *et al.,* 2013, 2019; Martinez *et al.,* 2015). Sequencing libraries with different insert lengths (0.5-20 Kbp on Illumina short read platforms to reach 200× coverage and 1.5-14 Kbp by Illumina Truseq Synthetic Long-Read sequencing) were performed on an inbred genotype. To assign sequences to homeologous sets, sequences (0.5-8 Kbp) were also generated on an inbred genotype of the diploid species *T. occidentale,* progenitor of the modern white clover. Genome and transcriptome sequencing of this species complex has provided insight into the origin and evolution of white clover, and knowledge of subgenome interactions (Griffiths *et al.,* 2019). It is worthy to note that a reference genome assembly of the closely related species *T. subterraneum* (530 Mbp) could also be used as a model for white clover (Hirakawa *et al.,* 2016; Kaur *et al.,* 2017).

In alfalfa (2n=4×=32; genome size estimation: 1×= 800 Mbp) a genome sequence of a diploid genotype is accessible through queries in the Alfalfa Breeder's Toolbox (https://www.alfalfatoolbox.org; Monteros *et al., 2*018) and further work is in progress on tetraploid genotypes of alfalfa in the United States and in France (Debellé *et al., 2*012; B. Julier, INRA, pers. comm). The American project makes use of a diploid and a tetraploid genotype. For the diploid genotype, a 2× genome was obtained with PacBio sequencing and further analysed for gene annotation. For the tetraploid genotype, it was necessary to add Hi-C to Illumina and PacBio sequencing to generate a *de novo* assembly of scaffolds corresponding to the eight alfalfa chromosomes (unpublished data). In the French project, sequencing is based on paired-end and mate-pair Illumina WGS read data of a tetraploid genotype. As *de novo* assembly of these data is hampered by the autotetraploid nature of the species, current sequence assembly relies on the *M. truncatula* genome sequence, thus approaching alfalfa sequencing as a complex resequencing of the model species. With available alfalfa data, orthologs of about 47% of known *M. truncatula* genes were recovered (Julier *et al., 2*014). Currently, in the EU project EUCLEG coordinated by B. Julier (INRA), the same tetraploid genotype is being further analysed with NRGene (Ness-Ziona, Israel) to obtain a more complete genome sequence.

The next challenge/step of genome assembly

The main goal of reference genome sequence assembly is to create a representation of the actual DNA sequence of the chromosomes as contiguous sequences. Despite the progress in the last decade, no high-quality, complete genome sequence is currently available for any forage species, as discussed in the previous section. Main reasons are the fact that the assembly of large and complex plant genomes is still a big challenge, and the lower investment intensity compared to crops in which financial revenues for the breeding industry are larger. Indeed, the genomes of forage crops display features that typically hamper *de novo* assembly: a high proportion of repetitive sequences dispersed along the genome, high levels of heterozygosity in outbreeding species, and fairly large genome size in some species. Repetitive sequences are typically collapsed during *de novo* assembly thus shrinking total assembly length. In addition, if long-range sequence linkage information cannot be used to chain unique sequences across flanking repetitive regions, the assembly is truncated at the repeat region borders, causing fragmentation of the assembly. Conversely, highly divergent alleles in heterozygous genotypes will be reconstructed as independent sequences, leading to redundant sequences for a given chromosomal locus. Furthermore, in polyploid species such as alfalfa and white clover, homeologous chromosomes may or may not be collapsed depending on sequence divergence, thus leading to varying levels of redundancy along the chromosomal sequence and creating ambiguity in gene order and gene content per sub-genome.

The next major step of chromosome-scale genome assembly, therefore, is to fully exploit the latest NGS technologies for complex genome assembly, including long range sequencing such as PacBio, Oxford Nanopore or other third generation sequencing methods, optical mapping, chromosomal conformation capture coupled with NGS (Hi-C), 10X Genomics, and other emerging technologies. These technologies will allow the incorporation of repetitive sequences into the assembly and reconstruct the pseudo-chromosomes independent from assumptions underlying projected synteny, so that genome evolution can be studied in an unbiased fashion. Furthermore, an accurate and complete gene annotation set for such a chromosome-scale assembly will contribute to the interpretation of trait-associated SNPs. Integrated comparative genomics platforms will provide the tools for functional annotation, pathway analysis of differentially expressed gene sets, trait-SNP-gene linkage, orthology and synteny analyses, to delineate species-specific genes, lineage-specific gene loss or gene family expansion, and chromosomal rearrangements; hence, provide the background information required to understand the underlying biological processes driving phenotypic traits.

However, the genomic sequence of a single individual does not reflect the diversity in genome content and organization that exists in a crop species. It has been shown that copy number variation and presence/absence variation greatly contribute to intra-species genetic diversity (Bai *et al., 2*016). Therefore, the 'pan-genome' concept, originally introduced to describe the variation among closely related bacterial strains of the same species, has recently been extended to plant species. A pan-genome consists of two sets of sequences: those present in every individual (the 'core' genome), and those present in only a subset of individuals (the 'distributed' or 'dispensable' genome). Pan-genome analyses in *Arabidopsis thaliana* (Cao *et al., 2*011), rice (Yao *et al., 2*015), and maize (Hirsch *et al., 2*014) have highlighted extensive differences in genome constitution at the within-species level. Interestingly, pan-genome analyses in rice have revealed that gene families of the dispensable genome showed enrichment in regulation of immune and defence responses (Wang *et al., 2*018) or played a role in adaptation to stress (Hardigan *et al.,* 2016) and the development of novel gene functions. Pan-genome analyses are therefore a logical next step to understand genome evolution and mechanisms of adaptation of (forage) crops.

High-throughput, flexible, SNP genotyping tools

A reference genome sequence is typically a representation of the genome of a single individual. Further steps are necessary to discover DNA-sequence polymorphism in the genepool of the species that can be used for further research and for the development of breeding applications. In addition, the price per marker should be sufficiently low in comparison to their benefits in order to be used effectively. In some species such as rice, *B. distachyon* and soybean, complete genome resequencing was performed for a set of genotypes expected to represent relevant genome sequence variation (Lam *et al., 2*010; Yao *et al., 2*015; Gordon *et al., 2*017; Sedivy *et al., 2*017). In contrast, for forage species with relatively large genomes, the most common option consists of resequencing specific genomic regions using various forms of reduced representation libraries. These regions can be randomly spread across the genome and/or targeted on interesting genes.

Protocols involving restriction enzymes (e.g. Genotyping By Sequencing (GBS), RADseq, DArTseq) are commonly applied (Davey *et al., 2*011; Elshire *et al., 2*011). These approaches allow simultaneous discovery and screening of a large number of polymorphisms (from several thousands to more than a million SNPs) for a reasonable price (<50-100 € per sample) and are particularly helpful in forage species at both plant and population levels (Byrne *et al., 2*013; Annicchiarico *et al., 2*015; Fé *et al., 2*015). Most interestingly, they allow a quantitative estimation of allele dosage at the level of the individual, which is useful for polyploid species, such as alfalfa (Julier *et al., 2*018), or at the level of the pool (Byrne *et al., 2*013; Schlotterer *et al., 2*018, Rode *et al., 2*018; Verwimp *et al., 2*018), which is a cost-efficient approach when population-level allele frequency data are required. These methods benefit from comparing reads to a reference genome sequence but reference-free bioinformatics pipelines are also available (Garsmeur *et al., 2*018). Points of attention for these techniques are (1) the sequencing effort must be properly tuned to the number of DNA-fragments in the genomic library sequenced to avoid missing data, (2) different restriction enzyme(s) target different loci which could hamper comparison between studies, (3) bioinformatics analysis can be complicated for non-experts and require large computational resources, and (4) some of these methods are protected by IP rights which could increase their cost. Nevertheless, given the large number of polymorphisms that can be cost-efficiently screened, these approaches are highly efficient for Genome Wide Association Studies (GWAS) in forage species (Fé *et al., 2*015; Biazzi *et al., 2*017).

Targeted resequencing of regions of interest, including candidate genes, may rely on several techniques such as amplicon sequencing (e.g. NextSeq Series Amplicon Sequencing by Illumina; GT-Seq, or similar techniques, Floodlight Genomics LLC) and sequencing of probe-capture enriched WGS libraries (myBaits®, SureSelect). These techniques can be used on both individuals and pooled samples, allow the detection of novel polymorphisms, and consistently target the same loci across independent studies. Their drawbacks are: (1) the required *a priori* knowledge of the targeted sequences, (2) only a moderate number of loci (several hundreds to thousands) are targeted, (3) the required bioinformatics skills for experimental design and downstream data processing. Yet, once established, these approaches are very efficient to study candidate genes (e.g. Veeckman *et al.,* 2018) or to genotype individuals of a mapping population (Holtz *et al.,* 2016).

Finally, to genotype known SNPs, depending on the number of loci and the number of individuals to be surveyed, single locus methods such as KASP™ (Kompetitive allel specific PCR, LGC), or methods that allow the simultaneous screening of thousands of SNP in tens to hundreds of individuals, such as micro-arrays are used (Li *et al.,* 2014; Blackmore *et al.,* 2015). In these cases, no new polymorphism can be identified but the scoring of the genotypic classes can be more straightforward than the bioinformatics needed for sequence-based approaches. Nevertheless, it is difficult to estimate allele dosage in polyploid individuals and impossible to estimate allele frequencies for pooled samples. In addition, the development of an assay to screen a specific SNP polymorphism can be hampered by high density of sequence polymorphisms in the flanking regions, which is a non-negligible issue in highly heterogeneous, outcrossing species.

Summarizing, a plethora of methods is available for research and breeding of forage crops, which might overwhelm the less-trained geneticists or breeders. Some guidelines, depending on the goal, can be summarized as follows:

- Analysis of genetic relationships and population structure; requires intermediate number of markers (1000-5,000) spread across the genome: GBS with an infrequent cutting restriction enzyme or multiplex amplicon sequencing are appropriate if populations are surveyed; KASP™ assays or micro-arrays for individuals.
- Linkage and QTL mapping; requires low to intermediate numbers of markers (500-1000) spread across the genome: KASP™ assays or targeted resequencing to screen target genes; microarrays or sequencing of reduced representation libraries (e.g. GBS or RADseq) to saturate the map.
- GWAS; often requires a large number of markers (>5,000) in forage species due to a very fast decrease of linkage disequilibrium with distance: Microarrays or sequencing of reduced representation libraries (e.g. GBS or RADseq); possibly in combination with KASP™ assays or targeted resequencing approaches to screen specific candidate genes.
- GS; requires less markers than GWAS because of the use of kinship but often a GWAS analysis is performed first to identify genes with strong effects, which effects are incorporated in prediction models: A strategy could be to perform GWAS with a large number of markers and then implement further cycles of prediction and selection using only markers with significant effects identified by GWAS.

Application of genomics tools to forage crop breeding

The increasing availability of genomics resources for some forage crops and of high-throughput, cost-effective NGS-based genotyping approaches that allow screening thousands of SNPs in individual plants and populations, as discussed above, have opened new possibilities for forage crop breeders. We discuss four examples in this section.

Parentage analysis as a tool to increase selection gains

Forage crops are usually bred through family selection or polycross selection. Seeds are commonly harvested per plant (half-sibs), which implies that throughout the breeding process, the maternal origin of the offspring is known but the paternal origin is not. Selection on the mother genotype works for traits that can be assessed before flowering, e.g. plant vigour and some disease resistances, because poor performing genotypes can be removed from the field before flowering thus avoiding the pollination of other genotypes in the trial, but this is less efficient for traits that become apparent after flowering, e.g. seed yield (Boller *et al., 2*010). For these traits, knowing also the paternal identity of offspring can dramatically increase selection gains (more than two times; Riday, 2011), because offspring resulting from pollination by genotypes with a poor agronomic performance can be discarded. As a second application, parentage analysis allows the elimination of progeny from self-fertilization, e.g. in alfalfa or red clover, which, in turn, counters inbreeding and associated inbreeding depression (Busbice and Wilsie, 1966) and increases selection gains.

In practice, DNA is extracted from the seed plant and all possible pollinator plants upon seed harvest. DNA is then extracted per offspring seedling, and all parents and offspring samples are genotyped with a suitable set of marker loci. Finally, the parentage of the offspring is assessed through software, such as FAMOZ (Gerber *et al., 2*003) or Cervus (Kalinowski *et al., 2*007) for diploid species, or the exclusion-based paternity testing SAS code (Riday *et al., 2*013) or the R-package PolyPatEx (Zwart *et al., 2*016) for tetraploid species. The implementation of this approach does not imply any adaptation of the breeding process, and it only requires the availability of efficient and cost-effective management and laboratory protocols for timely delivery of paternity data to enable selection (Riday, 2010).

The first application of marker-assisted parentage analysis in forage crops was described by Riday (2011), who applied it in an open-pollinated red clover trial. Two multiplex PCR sets covering 11 SSR loci (Sato *et al., 2*015) allowed unequivocal determination of the parentage of most progeny plants. In the last years we see that marker-assisted parentage analysis is finding its way into regular forage breeding programmes and several works have highlighted the potential of this approach, or illustrated practical applications in red clover (Vleugels *et al., 2*014, 2018), white clover (George *et al., 2*018), alfalfa (Riday, 2013, 2015, 2019), perennial ryegrass (Van der Weijde, unpublished results), switch grass (Liu and Wu, 2012) and timothy (Tanaka *et al., 2*018).

Vleugels *et al.* (2014) applied it in a diploid red clover breeding trial to identify offspring derived from two high seed-yielding genotypes. A set of 18 SSR loci allowed parentage assignment for 96.6% of the progeny plants. Further, it was demonstrated that marker-assisted parentage analysis speeded up breeding progress for seed yield in diploid red clover. Similarly, Vleugels *et al.* (2018) used marker-assisted parentage analysis (based on a set of 9 SSR loci) in tetraploid red clover, with great success. In this case, SSR markers were used to verify if progeny from pair crosses were the result of self- or cross-pollination. A study on the application of parentage analysis in breeding for seed yield of tetraploid red clover has been performed by the same authors and will be submitted for publication soon. Parentage analysis was fairly successful also in this case, as 53.6% of progeny plants that were not the result of pollination by a genotype with the desired properties could be discarded.

In alfalfa, Riday *et al.* (2013) developed a method to assess the parentage as a means to study the pollen contribution of genotypes in a polycross. By genotyping 19 SSR loci, the correct pollen-donor could be determined for over 90% of progeny plants and the author concluded that parentage analysis can be used as a tool to select against inbreeding in alfalfa. Later studies in alfalfa have used parentage analysis in seed production fields to study selfing rates. Riday *et al.* (2015) found average selfing rates of 11.8% in an alfalfa seed production field. Riday *et al.* (this volume) investigated 32 alfalfa seed production fields, and found self-pollination rates between 5.3% and 29.7%, with an average of 12.2%.

Parentage analysis is currently being tested also in the perennial ryegrass breeding programme of the company Barenbrug, in collaboration with ILVO. The performance of SSR markers and that of SNP markers screened using a highly multiplexed amplicon sequencing approach are being compared.

Preliminary results indicate that the resolution achieved using SNP markers is higher than that of SSRs (Van der Weijde, unpublished results). This is due to the higher number of polymorphisms revealed by amplicon sequencing, as complete sequence stretches are screened. The implementation of high throughput customised SNP screening methods such as highly multiplexed amplicon sequencing (method validated in *L. perenne* in Veeckman *et al.,* 2018) can be envisioned as a means to combine parentage analysis and MAS for genes of interest in a cost-efficient manner.

Association mapping

With the emergence of GWAS, the dissection of the genetic architecture of complex traits moved beyond QTL mapping using bi-parental populations. In forage species, because of their outcrossing nature and associated high levels of heterozygosity and diversity, QTL mapping studies are case-specific and biased towards the choice of the parental genotypes and their phenotypes (i.e. only a few alleles per gene, and only the phenotypic range available in the offspring can be assessed while a much larger genetic variation is present in a typical forage breeding programme). With GWAS, broad-based and diverse panels are evaluated for the association between each genotyped marker and a phenotype of interest. The panels used for GWAS can be purpose-developed assemblies of genotypes, collections of domesticated and undomesticated germplasm, breeding populations, etc, reflecting better the variability available in the crop.

In the last decade, multiple GWAS studies have been published in *Lolium* spp. (Skot *et al.,* 2005; Skot *et al.,* 2011; Fé *et al.,* 2015; Van Parijs, 2016; Byrne *et al.,* 2017; Thorogood *et al.,* 2017), *Medicago* spp. (Annicchiarico *et al.,* 2015) and *Trifolium* spp. (Inostroza *et al.,* 2018). Recent advances in NGS technologies, as discussed above, enabled association studies on genome-wide sets of genetic variants. In some case, the genotyping focused on random loci using methodologies such as GBS (Fé *et al.,* 2015; Byrne *et al.,* 2017), while in other cases gene-targeted methodologies (Van Parijs, 2016; Veeckman *et al.,* 2018) were used. Further, as in forage crop breeding the phenotype of a population grown in a sward (e.g. half- or full-sib families, varieties, accessions, etc.) is the unit on which agronomic performance for most traits is evaluated, GWAS can be envisaged at population level rather than at individual genotype level. In this case, genotyping can be carried out using pool-sequencing approaches as discussed above. For example, Fé *et al.* (2015) applied GWAFF (Genome Wide Allele Frequency Fingerprints) in a GWAS and GS approach for heading date in perennial ryegrass. Similarly, gene-targeted markers in combination with GBS markers spread across the genome are used in the EU project GrassLandscape (coordinated by J.P. Sampoux, INRA), in which a GWAS is performed on agronomic and potentially adaptive traits phenotyped at population level on 500 perennial ryegrass accessions (Keep *et al.,* this volume).

The aim of GWAS is to reveal the global landscape of a trait or its genetic architecture. This involves the number of causative genes or alleles, their interactions, and the distribution and patterns of their effects (Hansen, 2006). During GWAS they become catalogues of allelic variants, and favourable alleles and their corresponding genotype–phenotype associations are identified. This information constitutes an unprecedented resource for breeders and scientists to understand crop functional genomics. For example, Skot *et al.* (2011) described a wide range of haplotypes in Lp*FT3*, an ortholog of the FT gene of *Oryza sativa*, and demonstrated significant associations between this variation and flowering time. But GWAS is also a powerful tool to reveal not yet described pathways. For example, alleles with a large beneficial effect on cell wall digestibility in perennial ryegrass were discovered in 6 genes by Van Parijs (2016), including genes that were not previously defined as candidate genes for this trait. Similarly, Thorogood *et al.* (2017) identified loci associated with self-incompatibility (SI) in *L. perenne* on linkage groups that had not previously been reported to be involved in SI.

Looking into the future, and considering current and foreseen advances in phenotyping (Tardieu *et al.,* 2017; Singh *et al.,* 2018) we can state that the concept of 'trait' is being redefined, and the analysis of conventional developmental variables will be complemented with high-resolution descriptions of plant characteristics and responses to environmental signals and biotic interactions, even at metabolic or molecular level, summarized in a variable vector depicting dynamic developmental processes (Liu and Hai, 2018).

From a methodological point of view, current limitations of GWAS, such as issues related to population structure and low frequency of causal alleles leading to false negative results, require further attention in the future. These issues can be solved by developing novel statistical models to explore rare functional alleles (e.g. Kaakinen *et al.,* 2017 developed a method that collapses rare variants within a genomic region and models the proportion of minor alleles in the rare variants on a linear combination of multiple phenotypes) or employing artificially designed populations such as MAGIC (Multi-parent Advanced Generation Inter-Cross) to balance allelic frequencies and control population structure. Nevertheless, although GWAS studies have been highly efficient to reveal the genetic architecture of quantitative traits, in such studies only a relatively small part of the genetic variability is readily explained, which could be due to the difficulty to detect genes with low effects or to the interaction between genes (Maher, 2008; Verma and Ritchie, 2018).

Genomic selection

Genomic selection, introduced by Meuwissen *et al.* (2001), describes breeding strategies in which the effects of many DNA markers throughout the genome are used to predict the breeding value of selection units (genotypes, families or populations). Theoretical analyses have shown that GS can be very useful for forage species breeding when phenotypic evaluation of individual plants poorly predicts their performance under sward conditions, if selection within families is based on imprecise genetic values, or if phenotypic evaluation takes a long time (Resende *et al.,* 2014).

Several studies have evaluated the value of GS for different traits (heading date, yield and quality) in perennial ryegrass (Fé *et al.,* 2015, 2016; Grinberg *et al.,* 2016; Faville *et al.,* 2018; Guo *et al.,* 2018; Pembleton *et al.,* 2018) and in alfalfa (Li *et al.,* 2011; Annicchiarico *et al.,* 2015; Biazzi *et al.,* 2017; a recent review for alfalfa can be found in Hawkins and Yu, 2018). Studies are in progress in red clover on a MAGIC population in order to use a moderate set of markers (Isobe *et al.,* 2013) and on a set of improved and wild populations (B. Julier, EUCLEG project). The phenotyping is performed mainly at the sward-plot level on different population types: (1) full-sib families (Fé *et al.,* 2015, 2016), (2) half-sib families (Grinberg *et al.,* 2016; Biazzi *et al.,* 2017; Faville *et al.,* 2018), (3) synthetics derived from 4-6 parents (Pembleton *et al.,* 2018). Exceptions are two alfalfa studies (Li *et al.,* 2011; Annicchiarico *et al.,* 2015) based on spaced-plant phenotyping. Correspondingly, the genotyping is performed either at the plant level when spaced-plants are used, on the mother plant of the phenotyped population (Grinberg *et al.,* 2016; Biazzi *et al.,* 2017; Faville *et al.,* 2018) or at the population level (Fé *et al.,* 2015, 2016; Pembleton *et al.,* 2018). In the latter case, allele frequencies are estimated based on the number of reads obtained through GBS or RNA-seq. The use of allele frequencies to build genomic prediction models is something relatively unique to forage species.

All these studies have shown that the quality of the genomic prediction depends mainly on the heritability of the trait, the number of plants or populations used to build the calibration (training population), the genetic link between the training population and the breeding population, and on the number of good quality markers (without too many missing data or with the possibility to impute them accurately), which has to be optimized depending on the LD in the studied population. From the studies available up to now, it emerges that GS should be more efficient than phenotypic selection in particular for traits such as yield or forage quality which are better estimated at the sward-plot level for at least two years. But the size of the training population should be large enough (>500) as well as the number of markers (>5,000 well spread across the genome with the possibility to add QTL-markers identified by GWAS). Additionally, possible loss of genetic diversity by GS in comparison to traditional schemes using phenotypic selection should be avoided (Lin *et al., 2*017).

In conclusion, we have no doubt that GS will aid forage species breeding in the future. But there are still some steps to be taken: (1) the cost and speed of genotyping have to decrease further (<20 € per plant or population), (2) the statistical analyses need to be automated for use by non-experienced operators, (3) the choice of the training population has to be evaluated (one population per recurrent population, or per breeder, or per genetic group). To start GS is difficult because it cannot be done half way and the cost of genotyping a large number of plant materials could be huge so gathering efforts is essential for species with low market value.

Contribution of genomics to our understanding of adaptation mechanisms in grasslands

Forage crops are mostly cultivated as mixtures of two or more species, and abundant literature has dealt with interspecific interactions and how these relate to the agronomic performance and ecosystem services delivered by grasslands (Suter *et al., 2*015; Barot *et al., 2*017; Brophy *et al., 2*017 and references therein). However, recent progress in community ecology has stressed the importance of intraspecific diversity to explain ecosystem functioning (Hart *et al., 2*016). Given the high genetic diversity harbored by any single variety of a typical forage crop, in which each individual carries a unique genotype, it can be anticipated that these 'diversity effects' will also play a significant role at the within species level. This implies that diversity effects might be essential to understand plant-to-plant interactions and adaptation not only in mixed species stands, but also in monocultures (a single variety or several varieties of the same species).

To date, few publications have reported on the relationship between intraspecific diversity and grassland functioning. Examples available try to establish a relationship between the number of varieties of a given species included in a grassland mixture and its agronomic performance (Meilhac *et al., 2*019). How the genetic composition and diversity of the grassland stand changes and eventually adapts to the environment, and whether possible genetic changes at the within-species level are related to interactions with other species present in the stand has obtained little attention in the past. While this used to be a costly endeavor, implying genotyping of a very large number of samples, pool genotyping approaches as the ones discussed above, make this feasible.

As an illustration, Verwimp *et al.* (2018) investigated these effects in a 4-year field trial established with perennial ryegrass and red clover, in monospecific or bispecific settings combining up to four varieties (the perennial ryegrass varieties Merks and Meloni and the red clover varieties Lemmon and Crossway). They determined the temporal changes in the genetic composition and genetic diversity of the perennial ryegrass component at the field plot level using GBS genotyping of pooled samples. Only subtle temporal changes in genetic diversity were observed when one perennial ryegrass variety, either Merks or Meloni, was cultivated. In contrast, large temporal changes in the genetic composition of the ryegrass populations were found in field plots in which both ryegrass varieties had been sown. The relative proportion of the two varieties in these plots changed throughout the cultivation period, revealing a dynamic behavior of the ryegrass component in the field plots in which both varieties were combined. Consistent patterns were observed whether or not perennial ryegrass was cultivated as monoculture or in mixture with red clover. These changes in the relative representation of the perennial ryegrass variety might be related to the functional characteristics of the varieties, similar to changes in species abundance (Barot *et al., 2*017), and might equally contribute to grassland resilience.

Though further research involving a larger number of varieties and variety combinations is required, these first results show that the incorporation of genomics approaches in studies dealing with the optimization of mixtures can be of great interest. A similar approach can be used to investigate the differential performance of a single variety in different environments. For example, Verwimp *et al.* (this volume) is currently investigating variety- and location-specific responses of seven perennial ryegrass varieties when cultivated in Merelbeke (BE), Lusignan (F) or Poel (D). While the same seed lots of each variety were used to sow the plots at the different locations, environmental conditions during the establishment phase in subsequent seasons might have resulted in slightly differentiated populations. Understanding these dynamic changes will provide information on the response of the different varieties to the three contrasting environments. Further, by identifying genomic loci and marker alleles displaying differential abundances in the different environments might pinpoint relevant adaptive genetic diversity in perennial ryegrass, which in turn can be exploited by breeders. Indeed, differential allele abundances in different environments are possibly the result of adaptation of the population to the prevailing conditions, and could be identified using approaches to detect outlier loci, which are indicative of signatures of selection (see Ahrens *et al., 2*018 and references herein for a recent methodological review and examples). Exploring this possibility will contribute to our understanding of the plasticity of varieties of forage crops under different environmental conditions, and will facilitate the selection of climate change robust varieties.

Concluding remarks

We have summarized the current status of genomic research in the most broadly used forage crop species and highlighted possible directions of future research. Developments in the field of DNA sequencing and genotyping are instrumental for the implementation and deployment of molecular approaches in this group of crops. However, the high diversity of technologies and methods available, the pace at which new options are published, and the high level of specialisation in specific disciplines that is required, might overwhelm the users. Taking full advantage of recent technological and conceptual advances in practical breeding applications will benefit highly from the setting up of multidisciplinary collaborative teams.

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Quantitative trait loci mapping of rust resistance in tetraploid alfalfa

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Abstract

Leaf rust caused by *Uromyces striata* compromises yield and forage quality of alfalfa (*Medicago sativa* L.). The objective of this research is to investigate the genetic basis of leaf rust resistance in alfalfa and identify quantitative trait loci (QTL) associated with rust resistance using genotyping-by-sequencing (GBS) SNP markers in a bi-parental pseudo testcross F1 population. The population consisted of 184 progenies generated by crossing a rust-susceptible fall-dormant cultivar, 3010 (female) to a resistant non-dormant cultivar CW1010 (male). The parents and progenies were screened under field conditions using a visual rating for severity on the scale of 1-5. Single dose allele SNPs (SDA) were used for constructing linkage maps and QTL mapping. The results showed a moderate negative correlation (r=-0.26, P<0.05) between fall-dormancy and rust severity, suggesting that the more dormant genotypes were more susceptible to rust. Five QTLs were detected in CW1010 and three in 3010. The most important QTL mapped on chromosome 1B of CW1010 explained 13% of the variation. Altogether, the five QTLs detected in the resistant parent CW1010 explained 38% of the phenotypic variation. The SNP markers identified can be a valuable genomic resource to build upon for marker-assisted selection of rust-resistant alfalfa.

Keywords: alfalfa, GBS, rust, *Uromyces*, QTL mapping, SNP

Introduction

Leaf rust caused by *Uromyces striatus*, a fungus species of the *Pucciniaceae* family, can cause substantial yield loss (Webb and Nutter Jr., 1997). Leaves, petioles and sometimes stems of rust-infected alfalfa display masses of reddish-brown spores from circular pustules (Frates and Davis, 2008). Rust also reduces the persistence of established alfalfa and results in plant death and thinning of newly planted seedlings. Even though alfalfa rust can be controlled by agronomic practices and application of fungicides, rust-resistant cultivars would be the most sustainable way. There is no information available on the genetic and genomic basis of rust resistance in alfalfa. Therefore, the aim of this study is to map QTL associated with alfalfa leaf rust resistance using genotyping-by-sequencing (GBS) SNPs.

Materials and methods

Rust was scored in the field on an F1 population of 184 single plant progenies developed by crossing single plants of two alfalfa cultivars, 3010 and CW 1010. Each progeny was replicated via stem cuttings and planted in Watkinsville, GA in a RCBD design with 3 replications with four clones of each progeny per replication. Rust severity (RS) rating was assigned visually on a scale of 1-5 according to the North American Alfalfa Improvement Conference (NAAIC) protocol with slight modification. Phenotypic data of rust scores were analysed using PROC GLM in SAS 9.4 (SAS Institute, 2004) and the least square (LS) means of the genotypes were used as the trait value in QTL analysis.

The parents and 184 progenies (bulk of 4 clones) were genotyped by sequencing (GBS) for SNP discovery. The genetic linkage map for each parent was constructed using JoinMap 5.0. Linkage groups were assigned based on *Medicago truncatula* reference genome. Rust QTLs were detected using composite interval mapping (CIM) in Windows QTL Cartographer version 2.5 (Statistical Genetics, NC State University). A QTL was declared significant when the peak LOD value is >3.0. The QTLs detected from the resistant parent CW 1010 were named Us-RustR1, Us-RustR2 etc. to represent *U. striatus* resistant QTLs. The QTLs mapped on the linkage map of the susceptible parent 3010 were referred to as Us-RustS1, Us-RustS2 and so on. Since the two parents also differed in dormancy with the resistant parent being non-dormant (FDR = 10) and the susceptible parent being very dormant (FDR = 2), the relationship between fall-dormancy ratings (FDR) and RS as well as winter hardiness (WH) and RS of the F1 individuals were determined using Pearson correlation coefficient (r). Dormancy and WH data were taken over two consecutive years 2015/2016 and 2016/2017 (Adhikari *et al.,* 2008). Sequences of the SNPs under QTLs peak and flanking regions were compared against *Medicago* A17 reference assembly (Mt4.0) using BLASTn search.

Results and discussion

The female parent 3010 had an RS score of 4.0 (susceptible) while the male parent CW 1010 showed resistance with a RS score of 2.3 (Figure 1). The F1 mapping population segregated for RS and exhibited a near normal distribution (Kolmogorov-Smirnov D=0.08, $P<0.01$), suggesting that the trait is quantitative (Figure 1). The mean RS score for the population was 3.2 and the LS means of the 184 F1 progenies ranged from 1.5 to 4.9. Transgressive segregants were present on both tails of the distribution (Figure 1). Rust resistance showed a negative correlation ($r=-0.26$, $P<0.05$) with FDR and a negative correlation (r=-0.27, $P<0.05$) with WH, a possible indication that the more dormant and winter hardy alfalfa genotypes are more susceptible to rust.

Five significant ($P\leq 0.05$) QTLs associated with rust resistance were mapped on homologs 1B, 7A, 8A, 7C and 2C of the CW 1010 linkage map, suggesting that the inheritance of rust resistance in alfalfa is most likely polygenic (Table 1). The most important QTL identified on homolog 1B was detected with a $LOD=8.1$ and had an $R^2=0.13$. This QTL shares the same genomic location with a stable dormancy QTL ndorm8 reported previously (Adhikari *et al.,* 2008), supporting the possible correlation between rust resistance and non-dormancy in this population. All rust QTLs mapped on the maternal (3010) genetic map showed negative effects on rust resistance. The 3010 QTL Us-RustS1 located on chromosome 1A (91.7-94.2 cM) also co-localized with a stable dormancy QTL, dorm1 (Adhikari *et al.,* 2008). Some of the QTLs for rust resistance also shared genomic regions with WH QTLs (Adhikari *et al.,* 2008). The five QTL mapped in the CW1010 parent accounted for 38% of the total phenotypic variation and the three QTLs detected in the 3010 parent accounted for 21% of the phenotypic variation. The moderate negative correlations between the phenotypic values of FD, WH, and rust resistance/susceptibility observed in this study are a possible indication that dormant and winter hardy germplasm might be

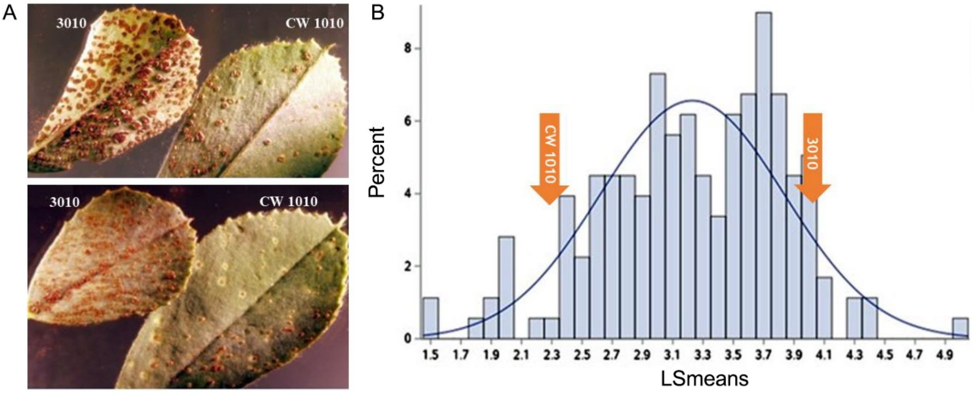

Figure 1. Magnified images showing rust pustules on the abaxial and adaxial surfaces of leaflets from the susceptible (3010) and resistant (CW 1010) parents (A). Distribution of rust infection scores on alfalfa F1 individuals (B).

Table 1. Alfalfa rust QTLs and positions on the resistant parent (CW 1010) and the susceptible parent (3010) maps. Peak and flanking markers along with their LOD and R^2 are indicated.

Parent	QTL code	Chr.	Peak Marker	Peak LOD	R^2	Allele effect	1-LSI (cM)	Flanking markers
CW 1010	Us-RustR1	1B	TP41786	8.1	0.13	(+)	21.4-23.6	TP83000, TP7086
CW1010	Us-RustR2	7A	TP27424	7.5	0.11	(–)[1]	29.3-30.7	TP87998, TP51544
CW1010	Us-RustR3	8A	TP63006	5.6	0.05	(+)	19.3-19.7	TP68653, TP46925
CW1010	Us-RustR4	7C	TP38417	3.2	0.05	(+)	43.6-49.3	TP4972, TP54614
CW1010	Us-RustR5	2C	TP40466	3.0	0.04	(+)	29.8-38.7	TP40466, TP70925
3010	Us-RustS1	1A	TP78651	4.5	0.08	(–)	91.7-94.2	TP995, TP15998
3010	Us-RustS2	8B	TP75477	3.6	0.07	(–)	21.5-22.5	TP42683, TP7327
3010	Us-RustS3	3D	TP74002	3.4	0.06	(–)	37.8-40.5	MRG_16533948, TP1280

[1] Indicates a QTL with negative effect but detected in the rust resistant parent, Chr. = Chromosome, 1-LSI (cM) = one-LOD support interval.

more vulnerable to rust. However, to draw a solid conclusion, a more extensive investigation in alfalfa germplasm with diverse genetic backgrounds including dormant and non-dormant with different levels of winter hardiness is needed.

Conclusion

This study suggests a polygenic inheritance and incomplete resistance to *U. striata* leaf rust in alfalfa. Since this is the first QTL work on alfalfa rust and the mapping was done in an F1 pseudo test-cross population, more investigations are required to explore the entire set of genetic factors involved in alfalfa rust resistance. SNP markers in QTL regions can be a valuable genomic resource to develop rust resistant alfalfa via marker assisted selection.

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Genomic selection for lucerne biomass yield in different stress-prone environments

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Abstract

Climate change and reduced irrigation water emphasize the need for lucerne (*Medicago sativa* L.) varieties with improved yield under drought and saline conditions. Marker-based selection may offer opportunities, owing to low yield gains displayed by phenotypic selection. This study generated preliminary results on the ability of genomic selection to predict lucerne breeding values and the extent of genotype × environment (GE) interaction for several stress-prone environments. Some 128 genotypes issued from an elite broadly-based reference population were genotyped via Genotyping-by-Sequencing, and phenotyped according to biomass yield of their half-sib progenies over a period ranging from five months to two years in four agricultural environments of Algeria, Argentina, Morocco and Tunisia featuring drought or salinity stress, and three environments of Italy with contrasting managed drought stress. AMMI analysis and estimates of genetic correlation revealed large GE interaction even across drought-prone environments. Genome-enabled predictive ability was mostly low albeit not quite unfavourable when compared with phenotypic selection opportunities. It tended to be higher under favourable conditions than under stress and in the last harvest, suggesting that it may increase for yield collected over a longer time span. Genomic selection was particularly convenient for selecting varieties with wide adaptation across stress conditions.

Keywords: adaptation, alfalfa, breeding, drought tolerance, yield, genomics, salinity

Introduction

Reduced rainfall due to climate change, and reduced quantity and quality of irrigation water due to water demand for non-agricultural uses, emphasize the need for lucerne varieties with improved yield under drought and saline conditions. However, plant breeding is challenged by low rates of genetic yield gain for phenotypic selection (Annicchiarico *et al.*, 2015a) and large genotype × environment (GE) interaction that emerged, *inter alia*, from results of lucerne varieties and landraces across sites of the western Mediterranean basin (Annicchiarico *et al.*, 2011). Specific-adaptation responses proved to be associated with different adaptive traits that are difficult to combine into novel varieties with moderately wide adaptation (Annicchiarico *et al.*, 2013). This adaptation target could be useful, however, to cope with the wide year-to-year climatic variation that features in many Mediterranean-climate locations.

Genotyping-by-Sequencing (GBS; Elshire *et al.* 2011) and other high-throughput techniques have facilitated the application of genomic selection to plants. For species bred as synthetic varieties, the prediction of breeding values could conveniently rely on genomic models developed from genotyping data of candidate parent plants and phenotyping data of their half-sib progenies (Annicchiarico *et al.*, 2015a). A pioneering study for lucerne biomass yield of two reference populations in the absence of marked drought or salinity stress revealed the distinct advantage of GBS-based genomic selection over phenotypic selection according to predicted yield gains per unit time (Annicchiarico *et al.*, 2015b). This study aimed to verify the ability of genomic selection to predict lucerne breeding values for a range of environments mostly subjected to severe drought or salinity stress, and to assess concurrently the extent of GE interaction across stress environments, for biomass yield data that were preliminarily available from a multi-environment phenotyping assessment.

Materials and methods

Some 128 genotypes from a broadly-based Mediterranean reference population underwent GBS using the Elshire *et al.* (2011) protocol with modifications as described in Annicchiarico *et al.* (2015b). Their half-sib progenies were phenotyped in densely planted micro-plot trials including three to five replicates performed in three managed environments (MS) of northern Italy with contrasting drought-stress level, two drought-prone rain-fed sites of Algeria (Alger) and Argentina (Santiago del Estero), one drought-prone Moroccan site with supplemental irrigation (Marrakech), and a Tunisian site (Médenine) irrigated with moderately saline (9.37 dS/m) water. Dry matter yield was recorded over five months in moisture-favourable MS, and over a period of eleven months to two years elsewhere. GE interactions for yield of the half-sib progenies across stress environments were modelled by Additive Main effects and Multiplicative Interaction (AMMI) analysis (Gauch *et al.*, 2008), analysing $\log_{10}$-transformed data to compensate for heterogeneity of genotypic variance across sites. SNP calling held two homozygote classes *AAAA* and *aaaa*, and one heterozygote class pooling *AAAa*, *AAaa* and *Aaaa* variants. We retained for analyses 9,269 SNP markers with less than 20% missing data across genotypes, imputing missing data by the KNNI method. Genomic predictions were assessed by Ridge Regression BLUP and Support Vector Regression with linear kernel.

Results and discussion

Nominal yields (i.e. AMMI-modelled yields from which the site main effect was subtracted) of nine top-yielding half-sib progenies as a function of the first GE interaction principal component (PC 1) score of the stress-prone environments revealed large GE interaction of cross-over type (Figure 1). PC 1 indicated distinct half-sib progeny responses in Marrakech, as confirmed by genetic correlations (where this site showed nil or negative correlation with the other environments). GE interaction PC 2 was also significant ($P<0.001$). The genetic correlations of the agricultural environments among themselves or with MS environments never exceeded 0.30, except for Médenine with moisture-favourable MS ($r_g=0.59$).

The two genomic selection models exhibited similar predictive ability. Genomic predictions were fairly high for moisture-favourable MS, and poor for the stress-prone sites of Alger, Medénine and Santiago del Estero (Table 1). The possibility to reach better predictions using future data for a longer time span was supported by better predictions obtained for the last harvest in Alger and Medénine. One possible reason for less accurate predictions in stress environments was their trend towards higher experiment

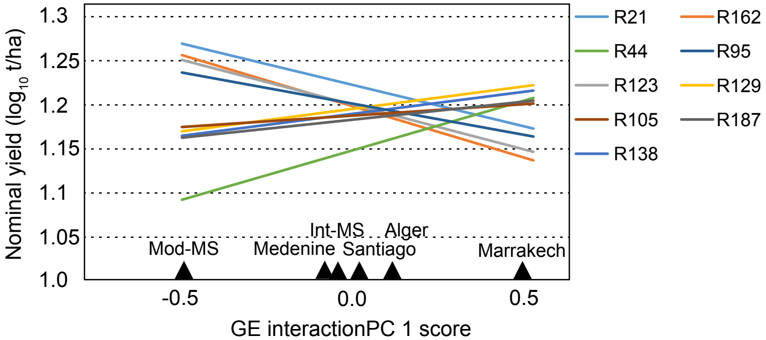

Figure 1. AMMI-1 nominal dry-matter yield of nine top-yielding lucerne half-sib progenies across moderate-drought or intense-drought managed stress environments (MS) and four agricultural environments.

Table 1. Assessment period and mean total biomass yield of managed-stress environments (MS) of Italy and agricultural environments, and predictive ability of genomic selection for lucerne breeding values (as correlation of modelled and observed data over 100 ten-fold stratified cross-validations for one top-predictive model).

Test environment	Period (month/year)	Mean yield (t ha^{-1})	Predictive ability	
			Total yield	Last harvest yield[1]
MS, intense drought	4/2014 – 4/2016	14.11	0.12	0.22
MS, moderate drought	10/2012 – 5/2014	10.11	0.18	0.10
MS, moisture-favourable	6/2012 – 10/2012	7.62	0.36	–
Alger (Algeria)	12/2015 – 12/2016	8.53	0.05	0.12
Marrakech (Morocco)	10/2015 – 10/2016	12.56	0.19	0.23
Médenine (Tunisia)	9/2015 – 11/2016	38.64	0.05	0.13
Santiago del Estero (Argentina)	12/2015 – 11/2016	13.43	0.10	–

[1] Excluding environments with relatively short experiment duration.

error than favourable environments. However, the value of genomic selection ought to be assessed in relation to opportunities offered by phenotypic selection as determined by narrow-sense heritability, selection cycle duration, and selection intensity (which is affected in turn by evaluation costs). In this respect, even a prediction ability around 0.15 would be sufficient to grant some advantage to genomic selection for lucerne yield relative to phenotypic selection (Annicchiarico *et al.*, 2017).

The genomic prediction ability for progeny mean yield over stress environments (computed from relative yields in each environment) was 0.14, which is promising for selecting widely-adapted varieties. Phenotypic selection would be less convenient in this prospect, owing to high evaluation costs and low heritability caused by high GE interaction.

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An assessment of genomic selection in perennial ryegrass

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Abstract

Perennial ryegrass (*Lolium perenne* L.) pasture is a major source of nutrition for livestock in temperate environments. Genomic selection (GS), a strategy that uses genomic markers to predict trait performance, is an option for improving the rate of genetic gain in this species. To assess genomic prediction models for heading date (HD) and herbage accumulation (HA), divergent selection was undertaken independently for these traits in five selection populations. Two selection populations (C1) were the generation directly subsequent to the training set while three populations (C3) had advanced through two further cycles of recombination before GS was applied. Genomic-estimated breeding values (GEBV) were derived using genotyping-by-sequencing data. GS for HD resulted in a significant (P<0.05) change of up to 7.2 days in a single divergent GS cycle. GS was most effective in C1 populations, with a mean difference of 6.5 days between high and low GEBV progenies compared with 4.3 days in C3 populations. Interim results from a three-year HA trial indicate high GEBV progenies outperforming low GEBV progenies in one of two environments, although with statistical significance (P<0.05) for only one population. These findings show GS can be effective in perennial ryegrass, as well as highlighting potential challenges.

Keywords: genomic prediction, SNP, perennial ryegrass, GEBV, flowering time, dry matter yield

Introduction

Genomic selection (GS), a strategy that uses genomic marker data to predict breeding values for selection candidates (Meuwissen *et al.*, 2001), is a promising methodology for improving the rate of genetic gain in forages. Selection decisions may be made earlier than with conventional breeding, reducing the generation interval, and GS provides breeders with a tool to exploit within-family genetic variation (Casler and Brummer, 2008). Recently, genomic prediction models have been developed and described for ryegrass (Faville *et al.*, 2018; Fè *et al.*, 2015, 2016; Grinberg *et al.*, 2016; Pembleton *et al.*, 2018) and these studies have highlighted the potential of GS as a tool to accelerate improvement for a range of agronomic traits. The objective of the current study was to empirically evaluate GS in ryegrass by applying genomic prediction models developed previously for heading date (HD) and herbage accumulation (HA; a proxy for dry matter yield), for divergent selection in populations with varying degrees of relatedness to the original training set.

Methods

HD and HA genomic prediction models (Faville *et al.*, 2018) were used for divergent selection in five breeding populations (Grasslands Innovation Ltd). These were related to two populations of the training set (Pop I and Pop III; Faville *et al.,* 2018) and were either the generation subsequent to the training set (C1) or a generation two cycles of recombination advanced from the training set (C3). The HD model was trained using phenotypic data from two environments (Faville *et al.* 2018) while the HA model was trained with phenotypic data from three environments, predominantly Waikato (60%). Genomic-estimated breeding values (GEBVs) were computed for 139-259 seedlings per population. DNA extraction, genotyping-by-sequencing (GBS) library development, sequencing of GBS libraries and single nucleotide polymorphism (SNP) genotype-calling were as described by Faville *et al.* (2018). Raw GBS sequence data from both the training set and selection candidates were combined for SNP-calling and a genomic relationship matrix was estimated by KGD methodology (Dodds *et al.*, 2015).

GEBVs for HD and HA of the selection candidates were derived by Genomic Best Linear Unbiased Prediction (GBLUP). Independent selections were made in each breeding population for HD and HA, with the top 10% (high GEBV group) and bottom 10% (low GEBV group) polycrossed to create divergent synthetic populations. Seed was harvested in 2017. For HD, 25 random seedlings per synthetic were established in spaced plant field trials (40 cm spacing, row-column design with 45 repeated clonal checks) in Waikato (37.78°S, 175.32°E) and Manawatu (40.21°S, 175.37°E), New Zealand in April 2017. HD was the number of days after 22/10/17 that five spikes emerged. For HA, balanced bulks of seed from each synthetic were direct-drilled as 2 m rows (0.6 g seed per row) at the same locations, in a row-column design with n=4 replicates and two check cultivars. Four seasonal cuts were completed as per Faville *et al.* (2018) and HA was mean dry weight per row over all cuts. For both HD and HA trials phenotypic data were analysed by linear mixed model, using residual maximum likelihood. Final means for each synthetic were calculated as BLUP's.

Results and discussion

After one divergent selection cycle, low and high-GEBV synthetics differed significantly ($P<0.05$) for HD in all but one population (Table 1), with high GEBV synthetics heading later than low GEBV synthetics. On average GS impact was highest in C1 populations (mean +6.5 days between high and low GEBV synthetics) compared with C3 (+4.3 days), suggesting predictive ability of the genomic prediction model declined with diminishing relatedness of the selection population to the training set. After 15 months of a 3-year trial, performance differences were indicated for mean HA between high and low GEBV synthetics. At Waikato, HA of high GEBV synthetics exceeded that of low GEBV synthetics from all breeding populations (mean +13.3 g dry weight) albeit with statistical significance ($P<0.05$) for only one population (Table 1). By contrast, HA selections performed erratically in Manawatu (mean -4.2 g dry weight), with low GEBV synthetics out-yielding high GEBV in three populations (Table 1). Although an interim result, this may reflect the predominance of Waikato phenotype data in the dataset used to train the prediction model. The Waikato site is characterised by higher annual and summer air temperature, as well as contrasting soil type, higher annual rainfall (Faville *et al.* 2018) and greater biotic pressure from insect herbivory. The trends observed will be confirmed through further measurement, the HA and HD

Table 1. Performance of synthetic ryegrass populations generated by divergent GS from five breeding populations, for either heading date (HD; mean days after 22/10/2017 until five spikes observed) or herbage accumulation (HA; mean dry weight, in g).

Trait	Population	High GEBV	Low GEBV	Difference (high-low GEBV)	LSD$_{0.05}$	Significance^1
HD	Pop I C1	26.5	19.3	+7.2 days (37%)	3.2	*
(Both sites)	Pop I C3a	26.7	24.2	+2.5 days (10%)	3.3	ns
	Pop I C3b	24	17	+7.0 days (41%)	3.4	*
	Pop III C1	29.5	23.7	+5.8 days (24%)	3.3	*
	Pop III C3	35.7	32.2	+3.5 days (11%)	3.3	*
HA	Pop I C1	85.9	64.8	+21.1 g (32%)	19.9	*
(Waikato)	Pop I C3a	88.7	73.3	+15.4 g (21%	19.2	ns
	Pop I C3b	77.2	65.7	+11.4 g (18%)	19.5	ns
	Pop III C1	90.3	86.6	+3.7 g (4%)	19.7	ns
	Pop III C3	100.2	85.1	+15.1 g (18%)	19.5	ns
HA	Pop I C1	68.2	61.4	+6.9 g (11%)	11.3	ns
(Manawatu)	Pop I C3a	70.4	85.6	−15.2 g (21%)	11.0	*
	Pop I C3b	57.3	68.2	−10.9 g (19%)	11.4	ns
	Pop III C1	77.0	81.9	−4.9 g (6%)	11.3	ns
	Pop III C3	83.2	79.9	+3.2 g (4%)	11.1	ns

[1] ns = not significant; * $P<0.05$.

trials continuing for 21 and 12 months, respectively. Other experiments underway will evaluate response to a second cycle of GS and assess GS against a range of conventional selection strategies.

Conclusion

Our results indicate that GS can be effective for both low and high heritability traits in perennial ryegrass. They also highlight challenges for successful implementation of GS, including the importance of target environment data used to build prediction models and the influence of relatedness between training and selection populations on predictive ability.

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Regeneration of suspension cultures for genome editing in perennial ryegrass

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Abstract

Perennial ryegrass (*Lolium perenne* L.) is the most important cool-season forage grass in temperate regions around the world. With an increasing understanding of the genetic basis underlying important traits and the availability of genome sequence resources, genome editing (GE) will allow us to target and fully exploit specific genetic mechanisms for crop improvement. However, GE techniques have not been implemented in perennial ryegrass yet, and generation of mutations in target genes still relies on random mutagenesis. Here, we report on the development of the prerequisite tissue culture steps to provide adequate material suitable for GE. Suspension cultures (SCs) were initiated from various explants of several genotypes and their regeneration potential was monitored over four months. Independent of the genotypes and the type of explants, all the SCs lost their regeneration potential within three months following their initiation.

Keywords: tissue culture, suspension cultures, plant regeneration, protoplasts

Introduction

Currently, mutation induction in perennial ryegrass relies on random mutagenesis only, as programmable nuclease-mediated GE has not been reported yet. A GE platform, based on Cas9 sequence-specific nuclease derivatives, would allow inducing mutations of interest through the induced DNA double-strand break and imperfect DNA repairs. GE reagents, either as ribonucleoprotein (RNP) complexes or DNA constructs encoding for RNP components, can be delivered using several methods. RNPs have advantages over other DNA-based techniques in that no exogenous DNA sequences are potentially integrated into the host genome, thus restricting programmable nuclease activity over time and reducing off-target effects (Woo *et al.*, 2015). However, the plant cell wall represents a barrier hampering the delivery of pre-assembled RNPs and, thus, protoplasts are the most amenable cell forms for this purpose. However, regeneration of protoplasts into plants has been found to be extremely challenging in several plant species. Highly regenerative SCs are thus the preferred substrate for protoplast isolation and plant regeneration in *Lolium* (Folling and Olesen, 1999). Indeed, because of their low aggregation, SCs should be excellent substrate for protoplast isolation as larger surface is exposed to the cell wall degrading enzymes. Here, we report the regeneration potential of SCs initiated from different explants including anther- and seed-derived calli as well as seed devoid of prior callus induction phase. The SCs showing the highest regeneration potential should then be used as substrates for protoplast production to be used for GE and subsequent regeneration of mutant plants.

Materials and methods

SCs were initiated from various genotypes and their regeneration potential assessed over a four-month period by counting regenerated plantlets. SCs were initiated from seeds of registered cultivars according to Dalton (1988) as well as from previously induced friable embryogenic calli. Callus induction from seeds was performed according to Wang *et al.* (1993) and Bajaj *et al.* (2006), respectively. Anther-derived calli were obtained from highly androgenic genotypes according to Begheyn *et al.* (2017). Weekly maintenance of the SCs and monthly evaluation of their regeneration potential was realized following the methods of Folling and Olesen (1999). Briefly, 2-3 ml of small dense colonies growing in the SCs were plated onto regeneration media and emerging shoots larger than 1cm were counted 4-6 weeks later. DAPI and Hoechst staining were applied to confirm the presence of nucleated cells in SCs and calli regenerated therefrom.

Results and discussion

Callus induction from seeds and anthers all yielded calli within 6 to 8 weeks. Calli displayed distinct morphological differences that were observed amongst all genotypes and explants. An appropriate subculture regime allowed for the isolation and propagation of friable embryogenic calli. SCs initiated directly from seeds (Dalton, 1988) required approximately two months to be established and displayed the highest regeneration rate compared to the SCs initiated with other explants.

A total of 45 green plants were regenerated from the same SC during two regeneration tests conducted within three months following its initiation (Table 1). SCs initiated from seed-derived calli were able to regenerate green plants although more albino were observed in comparison to SCs initiated directly from seeds. Adequate renewal of the SCs was able to maintain their regeneration potential as shown with culture #9.1 renewed from #9 (Table 1).

SCs initiated from anther-derived calli could only regenerate albino shoots, on the other hand. According to the regeneration test conducted, all SCs, independent of the explants and genotypes used, had lost their regeneration potential within three months following their initiation. Independent of the explants and genotypes, different growth rates and aggregation degrees were observed between the SCs.

Table 1. Regeneration potential of suspension cultures (SCs). Regeneration tests were conducted monthly by plating two to three ml of small dense colonies onto PCP media (Folling an Olesen, 1999).[a]

SC	Initiation	Genotype	Explant	Regeneration test	Outcome	Green plants in the soil
#1	March 2018	Arolus	Sd	May	+++	42 [b]
#1	March 2018	Arolus	Sd	June	+++	3
#1.1	May 2018	Arolus	Sd		-	–
#1.2	May 2018	Arolus	Sd		-	–
#1.3	May 2018	Arolus	Sd		-	–
#9	May 2018	Arara	S		++	8
#1	March 2018	Arolus	Sd	July	-	–
#1.1	May 2018	Arolus	Sd		-	–
#1.3	May 2018	Arolus	Sd		-	–
#9	May 2018	Arara	S		+	–
#1	March 2018	Arolus	Sd	August	-	–
#1.3	May 2018	Arolus	Sd		-	–
#9	May 2018	Arara	S		+	–
#9.1	June 2018	Arara	S		++	1
#18	July 2018	6-10	AC		++	–
#19	July 2018	6-10	AC		+	–
#20	July 2018	3-12	AC		+	–
#21	July 2018	3-12	AC		+	–
#27A	July 2018	6-47	AC		++	–
#27B	July 2018	6-47	AC		++	–

[a] – indicates no callus growth or regeneration; + indicates callus growth only; ++ indicates callus growth & shoot regeneration (only albino or albino > green); +++ indicates callus growth & shoot regeneration (only green or green > albino); AC, anther-derived calli put in SC; S, seed-derived calli put in SC; Sd, SC directly induced from seeds (Dalton, 1988). SCs #1.1, #1.2 and #1.3 were derived from #1, #9.1 was derived from #9.

[b] Only a subset of the observed shoots was regenerated into plants.

Concerning aggregation, pectolyase treatment appeared to have less effect than using an appropriate subculture and renewal regime. However, there is a lack of quantitative methods for measuring SCs aggregation and regeneration potential and currently they are evaluated by simple visual observations and picture comparisons respectively. Preliminary experiments aiming at the isolation of protoplasts from SCs following the methods of Folling and Olesen (1999) yielded a very low number of protoplasts (<100 protoplasts/g SC) when compared to leaf mesophyll-derived protoplasts ($>7\times10^6$ protoplasts/g fresh leaves).

Conclusion

The progressive loss of regeneration potential of the SCs and albinism reported above are typical issues in tissue culture experiments, as observed by Dalton (1988) and Folling and Olesen (1999) for instance. SCs initiated from seeds without prior callus induction were the most regenerative material in our hands in the three months following SC initiation. SCs initiated from seed-derived calli were also able to regenerate green plants in the three months following SC initiation, although at lower frequency than the SCs initiated from seeds. Anther-derived calli displayed high regeneration on solid media but did not regenerate any green plants from SCs. Protoplast isolation from SCs requires further optimization to yield higher protoplast density in order to be used to regenerate plants as reported by Dalton (1988) and Folling and Olesen (1999). Once a working protocol for protoplast production from SCs is developed in our lab, we suggest performing GE and regeneration within three months from SC initiation.

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Genomic polymorphisms involved in phenotypic variation in the natural diversity of perennial ryegrass (*Lolium perenne* L.) across Europe

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Abstract

In the next decades, grasslands are likely to experience damage due to the foreseen anthropogenic climate change. Most grassland species show large natural ecotype diversity that could be used to recombine natural climatic adaptations and agronomic value to create improved populations of grassland species adapted to future regional climates. The FACCE-JPI ERA-NET+ *GrassLandscape* project intended to investigate these issues for perennial ryegrass, using a set of 385 accessions from the natural diversity of this species collected right across Europe and provided by genebanks of different countries. For each of these populations, high-throughput genotyping provided the allele frequencies of 189,781 SNPs. GWAS were implemented on the average value of each population for 40 agronomic and/or potentially adaptive traits recorded in three climatically contrasted locations (France, Belgium, Germany). Hundreds of markers were detected as being linked to at least one trait. Most markers were detected for phenology traits. Genomic prediction models calibrated on natural populations showed high accuracy for many traits (between 0.44 and 0.99) when predicting other natural populations, but the ability to predict cultivars seemed highly dependent on the genetic architecture of the trait. These results will certainly be valuable contributions to the objective of improving the climatic adaptation of perennial ryegrass but also to improve the management of genetic resources of this species.

Keywords: genebank, association study, genomic prediction, perennial ryegrass, genotyping by sequencing, natural adaptation

Introduction

The impacts of climate change are already becoming visible notably with an increase in the frequency, length and intensity of summer droughts in many regions of Europe. Breeders' elite genetic pools do not necessarily contain the genetic diversity required to respond to the challenges of adaptation to climate change. Untapped diversity could be valorised with this aim but should be first described and assessed. Natural populations of perennial ryegrass (*Lolium perenne*) originating from a large part of the distribution range of this species were phenotyped for agronomic and potentially adaptive traits and genotyped using a high-throughput technology. The objectives were to identify major effect loci that affect the natural phenotypic differentiation of the species and to evaluate the potential of genomic prediction models to accurately characterise wild populations. Such models have already been successfully used to predict the genetic value of perennial ryegrass plants or populations in breeding programmes (Pembleton *et al.*, 2018; Cericola *et al.*, 2018). Furthermore, using high throughput molecular markers has already proved efficient to help mine gene banks in other major species like wheat (Crossa *et al.*, 2016).

Materials and methods

This study used 385 natural perennial ryegrass populations originating from all across Europe that were provided by various genebanks, as well as 14 cultivars (synthetic populations). Phenotyping was carried out over three years (2015 to 2018) in 3-replicates field trials sown in 2015 in three sites (IPK in Poel Island Germany, ILVO in Melle Belgium and INRA in Lusignan France). The populations were evaluated for a number of traits including sowing-year heading, heading earliness, aftermath heading, disease resistances, forage quality indicators, year-round growth dynamics through regular canopy height measurements, responses to drought and cold, persistency, spike length, spikelet count, flowering stem density and isotopic discrimination of ^{13}C. Phenotypic variation was analysed using the '*lmer*' function of the '*lme4*' R library. Genotyping-by-sequencing provided the allele frequencies per population (DNA extracted from at least 300 individuals by population) of 189,781 nuclear genome SNPs. A Genome-Wide-Association-Study (GWAS) was undertaken for each trait implementing the '*GWAS*' function of the '*rrBLUP*' R library, with a correction for kinship using a genomic relationship matrix calculated as described by Cericola *et al.* (2018). SNP markers were considered significant allowing a 10% maximum false discovery rate. Genomic prediction models were implemented for all traits using the '*mixed.solve*' function of the '*rrBLUP*' R library. The predictive ability was evaluated using 100 iterations of random sub-sampling of a calibration set of 335 populations leaving 50 populations to be predicted. At each iteration, the breeding values of the cultivars were also predicted but these were never included in the calibration set.

Results and discussion

A total of 385 SNP markers were detected as linked to at least one trait. For a given trait, the number of independent detected markers varied from none for many traits such as autumn growth rate to 65 for heading earliness that explained 80% of the phenotypic variance. The twelve most significant markers for heading earliness (including one explaining up to 48% of the phenotypic variance) were all within 1000 base pairs of a SNP marker discovered by Fè *et al.* (2015) as being linked to the same trait. This suggests the nearby presence of a major gene with stable effect in different genetic backgrounds. The predictive abilities of genomic models varied from 0.33 for regularity after sowing (h^2=0.75) to 0.89 for the autumn growth rate in 2017 at IPK (h^2=0.9). Such accuracies were relatively high when compared to previous studies (Pembleton *et al.*, 2018). Heading earliness and autumn growth were both accurately predicted for natural populations but only heading earliness was well predicted for the cultivars (Figure 1). The GWAS implemented to a large range of natural populations is not expected to be efficient if the polymorphism of causal loci is confounded with neutral structure patterns. This appears to be the case

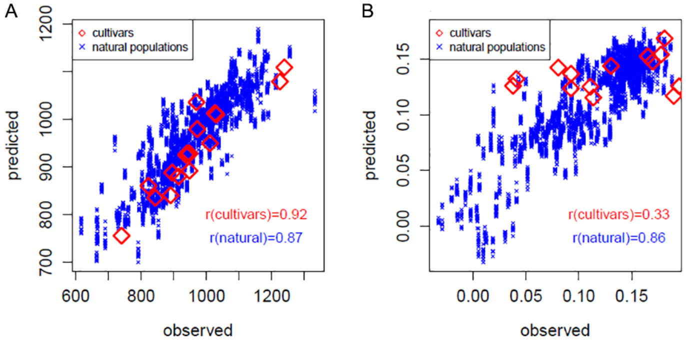

Figure 1. Predicted breeding values using genomic prediction models plotted against the observed phenotypic values for heading earliness expressed in degree days (A) and autumn growth at IPK in 2017 expressed in mm/degree-day (B). The Pearson correlations between predicted and observed values are indicated.

for a number of the traits we investigated, leading to many false negatives with GWAS. However, the contrasted ability of genomic models to predict the genetic value of cultivars for heading earliness and autumn growth can likely be imputed to the contrasted complexities of their polygenic determinism. The effect of major genes involved in heading earliness appears accurately predicted independently of the genetic background, even after the recombination cycles that have led to elite cultivars. On the other hand, the genomic prediction of more polygenic traits based on training data from natural populations probably mainly relies on kinship which becomes inefficient after reshuffling of the genome.

Conclusion

This study on natural populations identified major genes whose effects seem stable in different genetic backgrounds even after reshuffling. The complexity of genetic architectures was however difficult to apprehend due to the confounding effect of the neutral genetic structure. For many traits, there are cases of phenotypically similar populations that are distant spatially and genetically. As such, original genotypes recombining valuable alleles at different loci could be designed. Furthermore, the spatial distribution of trait values and allele frequencies could give insight into how natural selection has acted.

Acknowledgements

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Genome organization and (in)stability of wide hybrids

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Abstract

Interspecific hybridization offers a unique opportunity to combine traits of agricultural interest from two species, or even genera, into a single organism or, alternatively, to introgress one specific gene/trait/chromosome segment from one species into another. As such, it has become a popular tool in plant breeding and several wide hybrids have been developed and are now widely used by farmers. Here, genome organization and stability in two hybrids – *Triticale* (hybrid of wheat and rye) and its derivatives and *Festulolium* (hybrid of ryegrasses and fescues) is investigated. In both hybrids genome dominance was observed (the wheat genome in Triticale and the ryegrass genome in *Festulolium* were dominant). The stability of the hybrid genome composition in *Triticale* was disrupted by elimination of rye chromosomes, both in amphiploids and in introgression lines. This was caused by the high frequency of univalents of rye chromosomes that were eliminated during meiotic divisions. It seems that the formation of univalents was already predetermined in somatic nuclei as revealed by 3D-FISH. In *Festulolium*, the chromosomes of fescue were slowly, but constantly, replaced by those of ryegrass. Similarly, the *Lolium* genome dominance in *Festulolium* was also observed at the transcriptomic level.

Keywords: genome dominance, genome instability, chromosome pairing, hybridization, allopolyploidy, gene expression

Introduction

Allopolyploidization represents the merge of genomes of two or more species via interspecific hybridization preceding or following genome doubling. Besides its potential in breeding for combining agriculturally important traits from two species, genomic analyses of allopolyploids allow us to study fundamental aspects of plant speciation and evolution. It has been evidenced in several cases that one of the parental genomes in interspecific hybrids becomes dominant. This 'genome dominance' can be observed in various forms and in all cases leads to the partial elimination of the 'weaker' parental genome from the hybrid progeny. In particular, Zwierzykowski *et al.* (2006) observed slow, but gradual replacement of *Festuca* chromosomes by those of *Lolium* between F1 and F6. However, it seems that the proportions of parental chromatin stabilize in later generations (Zwierzykowski *et al.*, 2006; Kopecký *et al.,* 2017). In this study, we took a multidisciplinary approach, including flow cytometry, genomic *in situ* hybridization (GISH), transcriptomic analysis using RNAseq and confocal microscopy, to analyse genome dominance and genome instability in two hybrids: *Triticale* and its derivatives and two *Festulolium* forms (amphiploids and introgression lines).

Materials and methods

For *Triticale*, we used various introgression lines: either centric chromosome arm substitutions, whole-chromosome (complete rye chromosome or with deletion of proximal part of short or long arm) substitutions or telosomic additions. For the analysis, we used flow cytometry sorting of G1 nuclei combined with fluorescent *in situ* hybridization (FISH) to label rye chromatin, centromeres and telomeres and confocal microscopy accompanied with 3D imaging (Pernickova *et al.*, 2019).

For *Festuloliums*, we used various cultivars (either amphiploid or introgression types) and tetraploid F1 and F2 hybrids of *F. pratensis* × *L. multiflorum*. We performed GISH analysis to estimate the stability of various cultivars over the generations (Kopecký *et al.*, 2017). For analysis of allele-specific gene expression, we used RNAseq of F1 and F2 hybrids (Stočes *et al.*, 2016).

Results and discussion

Triticale *hybrids*

Results obtained from 3D FISH experiments performed on substitution and addition wheat-rye introgression lines showed that almost every hybrid line contained a portion of nuclei with non-attached rye telomeres with frequencies up to 12.5% in 5RS.5BL (disomic centric translocation of short arm of rye chromosome 5R with long arm of wheat 5B chromosome) and 54% in $1RS_{del}$.1RL lines. Similarly, improper positioning of rye telomeres was observed, with the highest frequencies for 5RS.5BL (6.25%) and $1RS_{del}$.1RL (18%). Interestingly, similar frequencies of non-attached telomeres were also observed in meiocytes (Pernickova *et al.*, 2019) and this corresponds with the reduced pairing frequencies of introgressed rye chromosomes in a wheat background and to the lower transmission rates. As such, our study indicates that non-attached or mis-positioned rye telomeres may improperly migrate to the telomere bouquet at the beginning of meiosis leading to reduced synapsis and chromosome pairing and lower transmission rates of the chromosome to the progeny.

Festulolium *hybrids*

The transmission rate of Festuca chromosomes in one introgression-type cultivar was influenced by selection (Figure 1). In fact, the genome composition changed from 26.9L+0.53T+0.05F (where L refers to complete *Lolium* chromosome, T for chromosome with translocation and F for complete *Festuca* chromosome) to 27.0L+0.48T. Selection of the plants having introgression prior to mating or after intercross, by collecting the seeds only from these plants can maintain the introgressions and even increase their numbers (Figure 1).

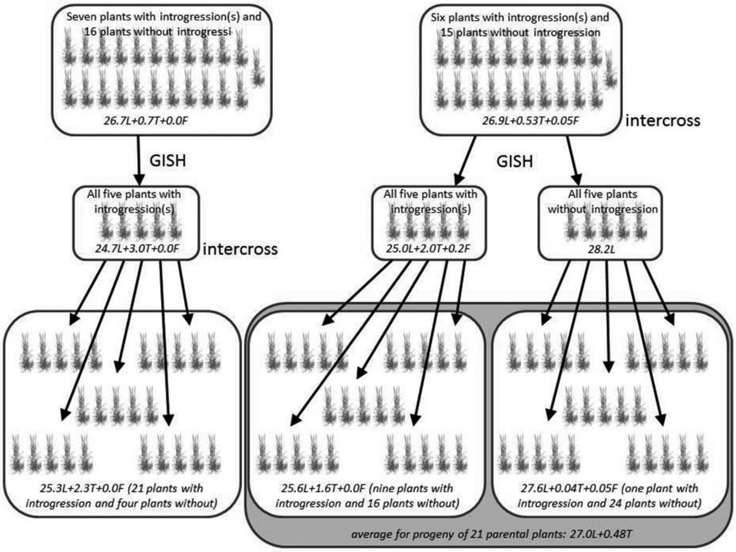

Figure 1. Shift in genome constitution between two successive generations of introgression-type *Festulolium* cultivar revealed by GISH using two strategies (L refers to the complete *Lolium* chromosome, T to chromosome with translocation and F to complete *Festuca* chromosome).

Transcriptomic analyses

Interestingly, genome dominance was also found at the level of RNA expression. Using a set of orthologous genes identified in our previous study (Stočes *et al.*, 2016), we analysed gene expression in F1 and F2 generations of tetraploid *F. pratensis* × *L. multiflorum* hybrids. Out of all differentially expressed genes (DEGs) 38% showed *Lolium* expression level dominance (ELD; this refers to the overall expression level of a particular gene in the hybrid in comparison to the expression levels in the parental plants), while 18% displayed *Festuca* ELD. The difference in ELD was diminished in the F2 generation, where almost equal percentages of DEGs displayed either *Lolium* (21%) or *Festuca* (22%) ELD. Allele specific expression analysis (ASE; quantifying the contribution of the *Lolium* allele and the *Festuca* allele to the expression levels of a particular gene) showed that 20 and 20% of genes were expressed preferentially from the *Lolium* allele, while only 6 and 8% of genes were expressed preferentially from the *Festuca* allele in the F1 and F2 generations, respectively. The rest of the subset of genes, 74% for F1 and 72% for F2 did not display significant differences in ASE.

Conclusion

The mechanism(s) underlying the phenomenon of genome dominance are still unknown. However, their discovery may help to maintain higher transmission rates of introgressed alien chromatin to successive generations.

Acknowledgements

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Implementation of genomic predictions in timothy (*Phleum pratense* L.)

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Abstract

Timothy (*Phleum pratense L.*) is a perennial, cross-pollinating hexaploid (2n=6×=42) grass species distributed naturally throughout Europe and parts of North Africa and Asia. It is one of the most important input factors in the Norwegian agriculture. New improved cultivars with high yield capacity and forage quality, which are well adapted to the future climate, are very important for an economically sustainable Norwegian milk and meat production. In view of the recent developments in sequencing, molecular marker technologies and theoretical foundations, it is feasible to start the development of genomic selection (GS) based breeding schemes in timothy. The main objective is to develop GS methods for breeding of the forage grass timothy by implementing the following strategies: (1) Develop single nucleotide polymorphism (SNP) marker allele frequencies (GWAFF) for 1000 full-sib families of timothy using genotyping-by-sequencing technologies. (2) Assess the relative efficiency of GS for important traits like yield and forage quality and trait stability across environments using different GS models. (3) Apply genome-wide association studies in conjunction with GS to unravel the genetic architecture of yield and forage quality. (4) Validate the GS predictions for yield and forage quality in 242 unrelated full-sib families. A total of 874,163 SNPs markers were identified in all the full sib families. These markers were used to test different genomic selection models for yield and quality traits. Genomic predictions results will be discussed in this presentation.

Keywords: genomic selection, genotyping by sequencing, forage grass, genomic predictions

Introduction

Forages are economically the most important crop in Norway, and timothy seeds constitute more than 50% of the seed sold for establishing leys for herbage production. A combined effort of improved agronomy and breeding of high-yielding and persistent cultivars is needed in order to secure stable supplies of high-quality forages for meat and milk production. Yield (which is highly dependent on climate adaptation, winter survival and persistency) and herbage quality, are the two main traits determining the economic value of herbage from perennial grasses in livestock production. Improving these traits will speed up the development of superior cultivars, contributing to a more efficient, sustainable, and economically viable livestock farming. However, these key traits are laborious and requiring multi-environment trials over several years. Therefore, there is a great need for developing and implementing new breeding technologies in timothy breeding. Genomic selection (GS) is a method that combines molecular markers with phenotypic and pedigree data for prediction of breeding values based on all markers distributed genome-wide (Meuwissen *et al.,* 2001). With recent development in sequencing and theoretical foundations, it is feasible to start the development of GS based breeding schemes also for timothy.

Materials and methods

847 second generation full-sib families (FS-2) available as remnant seeds stored at the breeding station of Graminor AS. These FS-2 families were progeny tested (2003-2013) in field trails at two locations in Southern Norway, at Bjørke, Hamar – a lowland location, and at Løken, Valdres – a highland location. Yield data are available for 3 harvest years with 2 or 3 cuts per year. Forage quality data, i.e. crude

protein, *in vitro* digestibility, NDF, ADL, water-soluble carbohydrates, and various estimates of energy concentration, are available for each of 3 cuts of 630 families, sampled at Bjørke in a single year. 216 new FS-2 families were sown at Bjørke and Løken, and complete yield and forage quality data will be available in the fall of 2018. These families will be genotyped and utilized for cross-validating GS models to ensure the prediction accuracy is high enough to rely upon. All the FS-2 families were sequenced using a robust next generation sequencing technology 'Genotyping-by-sequencing' (GBS) (Elshire *et al.* 2011) and the SNP calling were performed by Universal Network Enabled Analysis Kit (UNEAK) pipeline from TASSEL (Lu *et al.* 2013).

Results and discussion

Due to the lack of timothy genome sequence, we have performed *de novo* SNP calling using UNEAK pipeline. In total, we obtained 3,194,960 SNPs with minimum minor allele frequency of 0.05. Further, we used filtering methods such as; a SNP must be genotyped at least 10% of 918 samples, and we retained 874,163 SNPs. These SNPs will be used to perform the genome wide association studies for heading date, dry matter yield and forage quality. The traditional GBLUP and Bayesian methods are implementing to estimate the GEBV for dry matter yield and quality data. Our results show that genomic prediction have worked quite well in timothy for yield related traits. The predictive ability plot (Figure 1; Table 1) for dry matter yield (DYM) for three cuts in three years shows good correlation between the GEBV and the observed phenotype. The accuracy (correlation between the genomic estimated breeding values and the observed phenotypes) is quite high ranging from 0.959 to 0.994 for DYM (Table 1). Further, advanced machine learning models will be implemented in GS models for timothy yield and quality and these results will be presented and discussed.

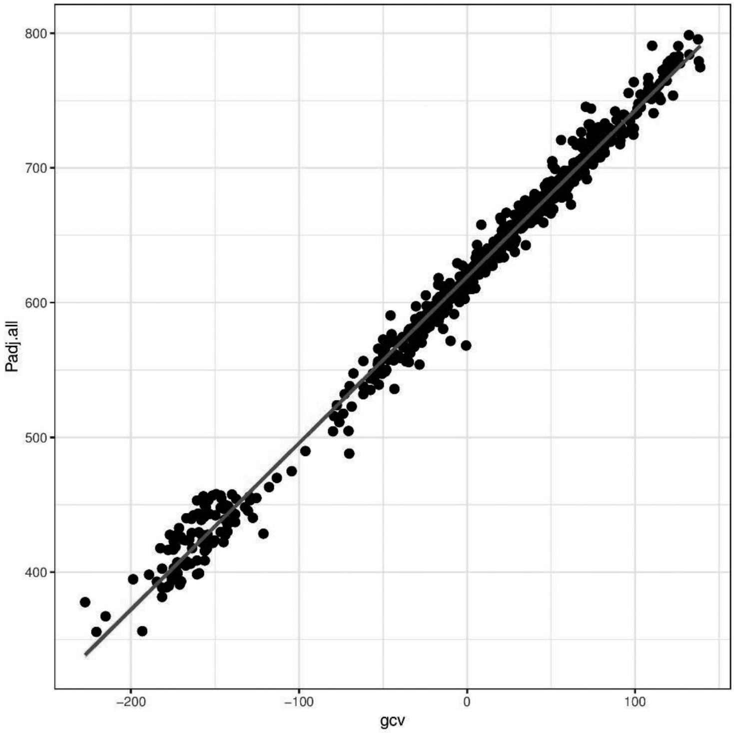

Figure 1. Correlation between observed phenotypes and genomic estimated breeding values for dry matter yield (DMY) in timothy. X-axis represents gcv; genomic estimated breeding values calculated based on cross validation. Y-axis represents the actual phenotype observed.

Table 1. Accuracy and heritability for dry matter yield trait.[1]

Trait	Bias	Accuracy	h^2
KGDM101	1.235	0.994	0.318
KGDM102	1.448	0.983	0.240
KGDM103	1.162	0.993	0.366
KGDM201	1.187	0.995	0.348
KGDM202	1.217	0.990	0.321
KGDM203	1.185	0.973	0.277
KGDM301	1.384	0.981	0.221
KGDM302	1.716	0.972	0.167
KGDM303	1.251	0.959	0.266

[1] KGDM101, KGDM102 and KGDM103: dry matter yield for 1st cut, 2nd cut and 3rd cut in 1st year. KGDM201, KGDM202 and KGDM203: dry matter yield for 1st cut, 2nd cut and 3rd cut in 2nd year. KGDM301, KGDM302 and KGDM303: dry matter yield for 1st cut, 2nd cut and 3rd cut in 3rd year. Heritability is calculated as narrow sense heritability.

Conclusion

GS is being used in many animal breeding programmes, and increasingly also in plant species. Progress in breeding of timothy by classical methods has been rather slow, and there is great need for implementing new breeding technologies. In view of the recent development in sequencing, molecular marker technologies and theoretical foundations, it is feasible to start the development of GS based breeding schemes also for timothy. Our genomic prediction results showed that it is feasible to implement genomic selection in timothy.

Acknowledgements

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Association study for traits related to growth in alfalfa

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Abstract

A breeding population of alfalfa (*Medicago sativa*) was phenotypically characterised over the course of a growing season. Individual plants were genotyped using genotyping by sequencing (GBS), followed by a genome wide association study (GWAS) to identify single nucleotide polymorphisms (SNPs) associated with growth traits. One SNP associating with plant regrowth height following harvest was identified and matched with a candidate gene from the red clover (*Trifolium pratense*) genome assembly.

Keywords: alfalfa, GBS, GWAS, SNP, plant competition

Introduction

GWAS is a powerful method of detecting genetic markers for agronomic traits (Şakiroğlu and Brummer, 2017). The method is particularly useful as it does not require prior detailed knowledge of genome structure, or generation of a specific mapping family. We used GBS (Elshire *et al*, 2011) followed by GWAS in an effort to identify SNP molecular markers putatively associated with the traits plant spread, height and biomass regrowth after cutting, using germplasm of a breeding population produced from a polycross of commercial alfalfa cultivars. These architectural and vigour traits are important in influencing plant competition for light, which determines energy availability and resource acquisition. The traits were measured on individuals growing in a spaced-plant nursery, an environment that promotes the development of plant canopy traits under favourable conditions.

The study formed part of a phenotypic and genotypic assessment of potential germplasm for breeding alfalfa suitable for use in UK conditions. Controls used included Sabilt, an old variety bred at the Welsh Plant Breeding Station, and Timbale, one of the few alfalfa varieties grown presently in the UK. These were included for comparison with the breeding population as they are known to be reasonably well adapted for the UK. Mesa Sirsa, a variety known to perform poorly in the UK, was included for cross reference in other studies.

Materials and methods

Experimental design and site

The experiment was established in August 2016 in IBERS, Aberystwyth (52°43'N, 4°02'W) at a lowland site (20 m a.s.l.). Plants were grown in a glasshouse from seed of (1) Syn2 progeny of polycrosses carried out using the alfalfa cultivars 'Geminii', 'Timbale', 'Diane', 'Daisy', 'Marshall' and 'Sabilt'; and (2) control cultivars 'Mesa Sirsa', 'Sabilt' and 'Timbale'. When the seedlings were four months old, they were transplanted into a spaced-plant nursery, laid out in rows 1m apart with a distance of 1m between rows. A total of 624 plants of the Syn2 population were planted, together with 30 plants each of the control cultivars using a randomised complete block design with three replications.

Procedure

A single leaf sample (100 mg fresh weight) was collected from each genotype in the Syn2 population and DNA extracted using QIAGEN's DNEasy kit. After extraction and normalization to 10 ng μl^{-1} these were sent to The Elshire Group Ltd (Palmerston North, New Zealand) for the production of sequence data using GBS. In summer 2017 (between March and August), phenotypic data on plant height, spread and stem number were collected weekly on nine occasions, numbered 1-9. All plants were cut to a height of 7 cm on 7 June and 17 July. In the first cut, herbage fresh weight was measured per plant.

Data interpretation

SNPs were identified from the raw sequence data using the Tassel 3.0 unique pipeline for *de novo* SNP discovery without a reference genome (Şakiroğlu and Brummer, 2017). Any SNP that had fewer than 10 reads, and those present in fewer than 20% of genotypes were omitted from the analysis. The minimum and maximum allele frequencies were set at 0.05 and 0.95 respectively and only biallelic SNPs were included in the analysis. This resulted in 17,730 SNPs. GWAS was performed with the statistical package R, using rrBLUP (Endelman, 2011). The sequences of potentially significant SNPs were searched in the Phytozome v11 database with the BLASTn algorithim, using the *Fabidae* clade as reference.

Results and discussion

One trait in the Syn2 population was found to be significantly associated with a SNP marker. Values of this trait, 'plant height of regrowth after second cut' (PH_9), are presented in Table 1 for all four populations. The cultivar 'Timbale' had the tallest plants, followed by 'Sabilt' and 'Mesa Sirsa'. The Syn2 population produced the least regrowth after cutting.

The result of the GWAS for the trait PH_9 is presented in Figure 1. BLAST analysis showed that the SNP associated with this trait was gene 9617 in the red clover genome assembly. This gene codes for sucrose-phosphate synthase, an enzyme involved in sucrose biosynthesis. It has previously been associated with QTL controlling plant growth and yield (e.g. Castleden *et al.*, 2004).

Where forage legumes are commonly grown in mixtures with grasses, their yielding ability (yield in pure stands) and competitive ability (enhancement or suppression of yielding ability in mixtures) are both relevant, but may be unrelated, or even negatively related. For forage legumes frequently grown in pure stands e.g. red clover and alfalfa, yielding ability is of primary interest. Work on alfalfa by Zannone *et al.*, (1986) highlighted (1) the impact of plant height on the competitive ability of alfalfa populations, as expected for an upright, dominant species in favourable environments, and (2) the consistency of alfalfa genotype yields across pure stand and mixture conditions, which also results mainly from the crucial importance of plant height for high yielding ability.

Most forage breeding programmes have used spaced plant evaluations despite the questionable link between these and, for example, sward yield (e.g. Waldron *et al.*, 2008). Ease of planting and maintenance in spaced plant nurseries facilitate selection, but on a plant-for plant basis, spaced plants have greater access to water, light and nutrients than those in sward conditions. Differences in resource competition within the two environments may consequently result in separate genetic responses occurring, which potentially reduces the relevance of selection in spaced plants for mixed sward yield. Even in species primarily intended for use in pure stands, such as alfalfa, individuals in spaced-plant nurseries will inevitably experience less intraspecific competition than those in more densely planted plots. GWAS may prove useful in this context by comparing germplasm separately in spaced plant and pure or mixed stand conditions from the same genetic base, in order to identify genomic regions associated with specific or wide adaptation to all conditions.

Table 1. Mean plant height (regrowth following cutting, PH_9) ± SD for four populations of alfalfa grown as spaced plants.

	Syn2 population	Mesa Sirsa	Sabilt	Timbale
Plant height (cm)	89.7±21.6	105.8±53.1	138±43.5	151.7±41.3

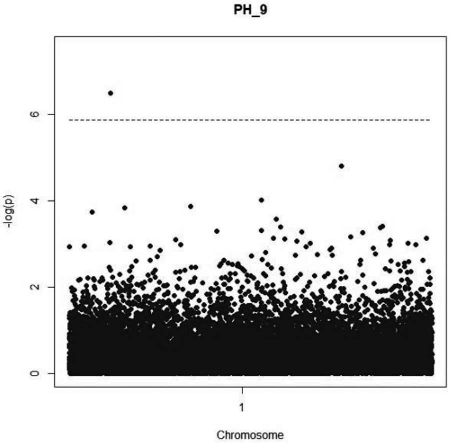

Figure 1. Manhattan plot of SNP loci for the phenotypic trait PH_9 in spaced plants of a Syn2 alfalfa population. The dotted line represents the FDR (false discovery rate) of 0.05. An arbitrary chromosome number was used due to the absence of an alfalfa reference genome.

Conclusion

Here we have identified one SNP marker associated with the height of regrowth after cutting in alfalfa. If validated, this marker could be used to increase the efficiency of selection programmes in alfalfa.

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Using molecular markers to select early and late flowering plants of perennial ryegrass

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Abstract

We crossed early and late heading plants of perennial ryegrass (*Lolium perenne* L.) and generated molecular markers in *LpCO1* and *LpFT3* to investigate if combined phenotypic and genotypic selection could be complementary criteria to generate homogeneous and contrasting groups with early or late heading date in the progeny.

Keywords: molecular markers, heading date, *Lolium perenne*, *LpCONSTANS1*, *LpFT3*

Introduction

In the breeding programme of perennial ryegrass (*Lolium perenne* L.), we cross early and late heading plants to create heterosis. While the progeny of these pair-crosses shows a wide variation in heading date (HD), the HD needs to be fixed in the next steps of breeding and selection to create a homogeneous variety. We developed molecular markers for two candidate genes (*LpCO1* and *LpFT3*; Skot *et al.*, 2005, 2007, 2011) and tested if combined phenotypic and genotypic selection can be used to create early or late heading F_2 populations.

Materials and methods

In spring 2011, we crossed an early (P_1) and a late (P_2) heading plant in a crossing cell. The F_1 progeny was planted in a nursery in autumn 2011 and scored for HD in spring 2012. Next, we created seven groups of F_1 plants based on combinations of phenotypes (early, intermediate and late) and/or the presence of the *LpCO1-a* or *LpCO1-b* allele (Figure 1). Each group of F_1 plants was multiplied in isolation to produce an F_2 progeny. We planted about 100 plants per F_2 progeny together with the two grandparents and 144 F_1 parents in a nursery in autumn 2013. The parental alleles of *LpCO1* and *LpFT3* were determined via Sanger sequencing of several PCR amplicons (800-1,100 bp) in promoter and transcribed regions. Together, these amplicons differentiated alleles a, b, c, and d of *LpCO1*, and alleles a, b, and c of *LpFT3* (Figure 1). KASP assays (KBioscience, UK) were developed for four SNPs for *LpCO1*, and three SNPs for *LpFT3* to differentiate the respective alleles per gene in the two grandparents, 144 F_1 parents and 626 F_2 progeny. The HD of the F_1 plants was determined in 2012, 2014 and 2016, and of the grandparents and F_2 progeny in 2014 and 2016. Heading was recorded as the date at which 3 spikes were visible. HD was expressed as Growing Degree Days (GDD) counting from 1 January, with base temperature of 0 °C. Statistical analysis was carried out in R version 3.5.1 using RStudio 1.1.456 (RStudio Inc., USA), with the package Agricolae.

Results and discussion

Sequencing of *LpFT3* in the parental genotypes P_1 and P_2 revealed three alleles. P_1 was homozygous for *LpFT3-a*, which corresponds to the previously reported FT3-A allele (Skot *et al.*, 2011), except for one SNP and a 6 bp deletion in the second intron. P_2 was heterozygous and contained *LpFT3-b*, which resulted from a cross-over event in the first exon leading to a novel haplotype composed of the upstream region of the *LpFT3*-A allele and the downstream region of the previously reported rare allele *LpFT3*-D (Skot *et al.*, 2011); and *LpFT3-c* corresponds to *LpFT3*-D except for a 2 bp deletion at 169 bp upstream of the translational start codon in *LpFT3-c* compared to the sequence of *LpFT3*-D. Analysis of the

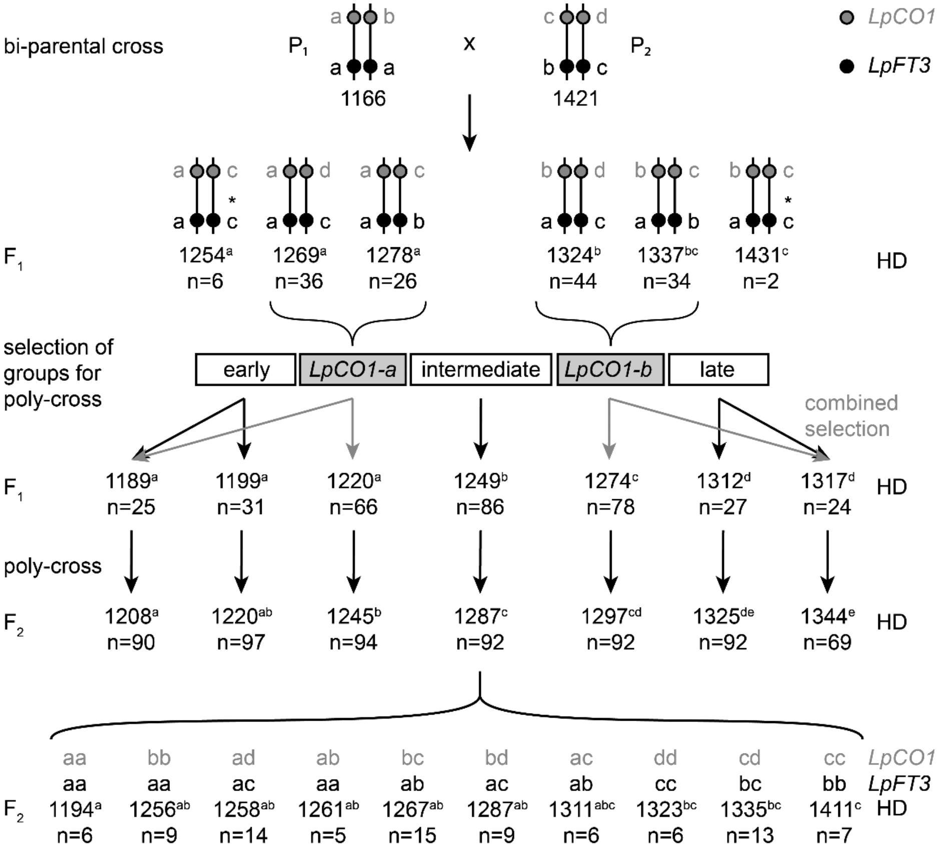

Figure 1. Genotypic constitution at *LpCO1* and *LpFT3* in parental lines (P_1 and P_2) and their F_1 and F_2 populations. F_1 plants are grouped by phenotype (white boxes) or genotype of *LpCO1* (grey boxes), or both, to generate F_2 populations via poly-crosses. Mean HD per genotypic class. Asterisks indicate cross-over events between *LpCO1* and *LpFT3* in the P_2 parent. The mean HD (expressed in GDD) is given for each group of parental F_1 genotypes as well as the F_2 progeny. n denotes the number of plants per group. Groups that are not statistically different are indicated by letters in superscript.

segregation of *LpFT3* alleles revealed no significant differences in mean HD between F_1 plants carrying *LpFT3-ab* or *LpFT3-ac* alleles. Analysis of the segregation of four *LpCO1* alleles revealed an association of the *LpCO1-a* allele with early heading (1271 GDD) and the *LpCO1-b* allele with late heading (1332 GDD). Although *LpCO1-c* tends to display slightly later HD compared to *LpCO1-d*, no significant difference was found between the *LpCO1-c* and *LpCO1-d* alleles.

Next, we created seven groups of F_1 plants (Figure 1): three groups based on phenotypic selection only (early, intermediate, and late); two groups based on genotypic selection only (*LpCO1-a/FT3-a* versus *LpCO1-b/FT3-a*); and two groups based on a combination of phenotypic and genotypic selection (early and *LpCO1-a/FT3-a* versus late and *LpCO1-b/FT3-a*). F_2 progeny of these seven poly-crosses were phenotyped together with the grandparents and F_1 parental plants. The mean HD of the F_2 progeny per group was consistent with that of the F_1 group used to generate them. Ranking of the mean HD across the seven groups revealed that the combination of phenotypic and genotypic selection resulted in the strongest contrast in HD of the F_2 (1208 GDD and 1344 GDD). These combined phenotypic and genotypic selected groups were significantly different from their respective genotypic selection groups, but not from the respective phenotypic selection groups. This suggests that genotypic selection on *LpCO1-a/LpFT3-a* and *LpCO1-b/LpFT3-a* in the F_1 is by itself not sufficient to improve the selection towards contrasting HD genotypes as compared to phenotypic selection alone, but that the combination leads to stronger HD contrasts. The four *LpCO1/LpFT3* alleles were segregating further in the F_2 progenies, thus creating additional genotypic classes. For example, in the group selected with intermediate HD in the F_1, all possible combinations of the four parental alleles were observed in the F_2, and genotypes carrying *LpCO1-cc/LpFT3-bb*, *LpCO1-cd/LpFT3-bc* and *LpCO1-dd/LpFT3-cc* alleles showed significantly later heading compared to genotypes carrying *LpCO1-aa*. Ranking of the homozygous genotypic classes showed the effect of the *LpCO1/LpFT3* allele combinations, ordered from early to late HD: *LpCO1-aa/LpFT3-aa* (1194 GDD); *LpCO1-bb/LpFT3-aa* (1256 GDD); *LpCO1-dd/LpFT3-cc* (1323 GDD); *LpCO1-cc/LpFT3-bb* (1411 GDD). Almost all heterozygous pairs of the *LpCO1/LpFT3* allele combinations displayed mean HD that was intermediate to the respective homozygous classes, except that the heterozygous combinations *LpCO1-ab/LpFT3-aa* and *LpCO1-bc/LpFT3-ab* had similar HD as homozygous *LpCO1-bb/LpFT3-aa* plants. This suggests a relatively strong effect of the *LpCO1-b/LpFT3-a* allele in counteracting very early (*LpCO1-a/LpFT3-a*) or very late heading (*LpCO1-c/LpFT3-b*).

Conclusion

Phenotypic selection combined with genotypic selection enhances the contrasts between early and late heading groups, but selection effectiveness depends on the allelic constitution of the parental lines. In order to estimate the effect of the various alleles of *LpCO1* and *LpFT3*, and their interaction, requires larger numbers of plants, first to generate sufficient numbers plants for all possible genotypic classes, and second to break linkage between current *LpCO1/LpFT3* allele combinations.

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Genome-wide association study for agricultural traits in perennial ryegrass

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Introduction: The genetic control of the majority of agronomic traits is polygenic in nature and individual genes usually have a relatively small effect; therefore, such traits have complex genetic architectures and are still poorly understood. A genome-wide association study (GWAS) was performed for several important perennial ryegrass traits to identify the markers with the largest effects on the examined traits.

Materials and methods: In total, 481 individual diploid perennial ryegrass plants belonging to 264 distinct populations were scored for various agricultural traits: autumn size, winter survival, spring growth, growth habit, heading date, plant height, regrowth in autumn, and prevalent diseases during 2012-2014 at Bjørke (Norway) trial site. Leaf material was sampled for genotyping by sequencing (GBS), and eight GBS libraries were prepared using the *Ape*KI restriction enzyme (Elshire *et al.*, 2011). DNA samples of 88 perennial ryegrass genotypes were barcoded and pooled into individual GBS libraries which were sequenced on the Illumina Hiseq 4000 platform (single-end 100 bp run). Sequence data for each genotype were then aligned against the perennial ryegrass genome assembly for biallelic SNP calling using Genome Analysis Toolkits (GATK) (McKenna *et al.*, 2010). GWAS analysis was performed using 'rrBLUP' package (Endelman, 2011) with the threshold limit of -log10(P) set to 5.

Results: In total, we identified 2,658,219 high-confidence polymorphic markers which were used in the genome-wide association studies (GWAS) of all phenotyped traits described above. Three of GWAS markers, all located on linkage group (LG) 2, were associated with plant size in autumn. The markers for heading date were more distributed with the majority of them located on LG2 and LG3. Also, seven markers were associated with regrowth in autumn (on LG4, LG5, and LG7), and five with rust resistance (on LG4 and LG5). The majority of markers for winter survival in the year 2014 were assigned to LG4 and LG5, whereas the significant markers for winter survival in 2013 were distributed across all LGs.

Conclusion: We conclude that identified marker-trait associations shed more light on the complex nature of important perennial ryegrass trait inheritance and some of the identified markers might be developed to be implemented in breeding programmes.

Acknowledgements: This study was carried out under the auspices of the Public Private Partnership for pre-breeding in perennial ryegrass project funded by the Nordic Council of Ministers and the Development of Molecular Markers for Genomic Selection of Adaptation in Perennial Ryegrass project funded by the Research Council of Lithuania.

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Studying physiological parameters contributing to cold acclimation mechanisms in *Lolium multiflorum/Festuca arundinacea* introgression forms

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Introduction: Abiotic stresses, including low temperature, can significantly reduce plant yield, especially in susceptible crop cultivars. Plant frost tolerance is an inducible trait and requires an earlier period of plant exposure to low but non-freezing temperature in order to be developed. This process, referred to as cold acclimation (CA), induces, e.g. alterations in the cellular proteome and metabolome, and increases in the stability of biological membranes. *Lolium* species are high yielding and have excellent forage quality under favourable environmental conditions, while *Festuca* species express stronger persistency and higher tolerance to abiotic and biotic stresses. The *L. multiflorum/F. arundinacea* introgression forms have already proved to be good models for recognizing some crucial components of molecular mechanisms involved in the cold acclimation process (Augustyniak *et al.*, 2018).

Materials and methods: Two tetraploid introgression forms of *L. multiflorum/F. arundinacea* with contrasting levels of frost tolerance, high frost tolerant (HFT) and low frost tolerant (LFT) genotypes, were selected. Plant frost tolerance and changes in physiological parameters (electrolyte leakage, lipid peroxidation and chlorophyll fluorescence) were evaluated (Augustyniak *et al.*, 2018). In addition, the level of superoxide anion generation, the expression profiles of the gene coding chloroplast fructose bisphosphate aldolase (pFBA) using real time qRT-PCR and the activity of pFBA, were assayed. All the experiments were performed at several time points of CA during three weeks and under control conditions before CA. The significance of the observed differences was estimated at a level of 0.05 (Fisher's LSD-test).

Results: The obtained results revealed that electrolyte leakage and the level of lipid peroxidation were excellent physiological parameters for evaluating the impact of CA on the stability of cellular membranes under frost conditions. The ability of photosynthetic acclimation to low temperature was demonstrated to be a crucial attribute of plant frost tolerance. The analysis of cellular antioxidant activities revealed that the HFT introgression form possessed a higher capacity antioxidant system, compared to the LFT form. All the physiological parameters showed clear and significant differences between the analysed introgression forms at the majority of the experimental time points. The expression level of pFBA was different between the analysed plants on the 1st and 14th day of CA, and was significantly higher in the HFT form.

Conclusion: The performance of both photosynthetic and antioxidant systems is postulated as being fundamental for the process of cold acclimation in forage grasses. Furthermore, the capacity to acclimate photosynthetic machinery and antioxidant systems to low temperature seems to be one of the most crucial components of plant metabolism in increasing levels of frost tolerance. However, further research to confirm this conclusion, is still required.

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Augustyniak A., Perlikowski D., Rapacz M., Kościelniak J. and Kosmala A. (2018) Insight into cellular proteome of *Lolium multiflorum/Festuca arundinacea* introgression forms to decipher crucial mechanisms of cold acclimation in forage grasses. *Plant Science* 272, 22-31.

Accumulation profiles of COR proteins during cold acclimation of *Lolium multiflorum/Festuca arundinacea* introgression forms

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Introduction: The cellular membrane systems are the primary sites of frost injury in plants and the freeze-induced membrane damage results primarily from the severe dehydration associated with freezing. Thus, a key function of cold acclimation is to stabilize membranes against freezing injury. The level of cellular membranes' integrity in frost conditions can be easily estimated on the basis of electrolyte leakage measurements (Kosmala *et al.*, 2009; Augustyniak *et al.*, 2018). The COR proteins, such as Wcor80 and Cor14b, have been proved to be involved in that aspect of acclimation process for numerous plant species. The Wcor80 is a dehydrin, whereas Cor14b protects thylacoid membranes from photoinhibition (e.g. Crosatti *et al.*, 1999).

Materials and methods: Two *Lolium multiflorum/Festuca arundinacea* introgression lines, a high frost tolerant (HFT) and a low frost tolerant (LFT) line, were used here as models for forage grasses to decipher some mechanisms of frost tolerance, associated with the integrity of biological membranes (Augustyniak *et al.*, 2018). The research involved the estimation of electrolyte leakage and lipid peroxidation to evaluate biological membranes' integrity and the analysis of accumulation profiles of two COR proteins (Cor14b and Wcor80) at several time-points of cold acclimation process.

Results: The two introgression lines differed significantly with respect to their membranes' integrity under low temperature treatment. The HFT line demonstrated lower levels of peroxidation and electrolyte leakage during cold acclimation progression, compared to the LFT line. The accumulation levels of both COR proteins were different between the analysed introgression lines. The Cor14b protein was shown to accumulate to higher levels in the HFT line during the experiment. However, this level was almost constant in both analysed introgression lines. The level of Wcor80 accumulation increased significantly during 2 weeks of cold acclimation, and after a further week it decreased significantly in the two lines. At most experimental time-points the accumulation level of Wcor80 was higher in the HFT line.

Conclusions: A clear relationship between the levels of frost tolerance and accumulation of both COR proteins, were revealed. Furthermore, the levels of Wcor80 and Cor14b abundance during cold acclimation could be useful molecular 'markers' to monitor an efficiency of cold acclimation process and to 'predict' levels of membranes' integrity and frost tolerance in *Lolium-Festuca* grasses. However, this aspect of our research will require further work performed on a larger plant population.

Acknowledgments: The research was performed within a project funded by the Polish Ministry of Agriculture and Rural Development (no. 35; 2014-2020).

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Environment-genotype-phenotype associations to detect candidate loci for climatic adaptation in perennial ryegrass

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Introduction: Perennial ryegrass (*Lolium perenne*) is a grass species prevalent in natural grasslands across Europe and also one of the main species bred for forage usage worldwide. In Europe, increased risk of heat waves and severe drought could lead to production losses and high mortality in natural populations. To address this problem, we need to develop breeding programmes delivering genotypes adapted to future conditions. A first step required to design such breeding programmes is the identification of genes associated with climatic adaptation.

Materials and methods: We used high-throughput genotyping (ca. 190,000 SNPs from genotyping-by-sequencing pool-Seq) and phenotyping (155 traits) to detect candidate loci under positive environmental-induced selection. We investigated associations with bioclimatic indices, ecophysiological indices and soil properties. Our approach combined redundancy analysis (RDA; Forester *et al.*, 2015) and mixed model predictions to detect candidate loci and identify environmental variables imposing selection. Additionally, we implemented a genome-wide association study (GWAS) to identify phenotypes linked to environmental adaptation in perennial ryegrass.

Results: The RDA analysis identified 2,819 candidate SNPs. 396 of these candidates were highly associated with one or more environmental variables (mixed model, $P<0.001$). A high proportion of these candidates (176 SNPs) showed highest association with environmental variables linked to heat/water stress. Eleven out of 176 SNPs were also highly associated with nine different traits in the GWAS analysis ($P<0.001$): acid detergent lignin content, acid detergent fibre content, spring canopy height, spring canopy height independent of heading earliness, autumn growth rate, growth vigour, autumn maximum height, water soluble carbohydrates content and soil coverage.

Conclusions: Our results reveal that adaptation to heat stress in perennial ryegrass is associated with multiple genes. Most adaptive loci did not show association with phenotypic traits with agronomic value. But eleven alleles associated with adaptation to heat stress were also negatively associated with agronomic value, and therefore, should be avoided in breeding programmes. These alleles were associated with higher lignin and fibre content, lower spring canopy height, lower autumn growth rate, lower water-soluble carbohydrates content and lower soil coverage. This study will provide opportunities for the development of new varieties of perennial ryegrass both adapted to future warmer climatic conditions and with high agronomic value.

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Gene expression differences correlated with higher forage quality in perennial ryegrass

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Introduction: Within perennial ryegrass (*Lolium perenne* L.) there exists significant variation to select for, and improve, forage quality. In this study we initiated a forage quality marker assisted breeding endeavour through the identification of differentially expressed genes related to cell wall biosynthesis in tetraploid perennial ryegrass genotypes.

Materials and methods: Leaf tissue at the third summer harvest was collected from eight plants from Maurizio, Trivos, or Aston Energy cultivars that varied in forage quality parameters. Eight total RNA samples were sequenced on an Illumina PE150 platform. *De novo* reference assembly used CLC Genomics Workbench (Qiagen, Aarhus, Denmark), and read mapping and determination of total counts per million used Kallisto (Bray *et al.*, 2016).

Results: The eight plants from three cultivars were phenotyped for organic matter digestibility (Goering and Van Soest, 1970) (OMD) over two years, with samples e102 and e346 having the highest OMD. Gene expression analysis was conducted for seven of these samples and gene expression counts (TPM) extracted for genes involved in cellulose synthase and lignin biosynthesis. A CAD-like transcript was correlated with OMD at r=0.47 and 0.61, for year 1 and 2 respectively. A CSL-like transcript was correlated with OMD at r=0.71 and 0.62, for year 1 and 2 respectively.

Table 1. Organic matter digestibility and total counts per million (TPM) of cinnamoyl alcohol dehydrogenase (CAD-like) and cellulose synthase (CSL-like) transcripts.

Entry	OMD year1	OMD year2	CAD-like (TPM)	CSL-like (TPM)
e102	87.0	87.1	–	–
e111	84.8	84.6	1.6	0.8
e119	80.7	80.6	2.2	0.8
e133	83.3	83.0	0.8	0.8
e209	84.1	84.1	2.4	0.6
e221	78.9	80.2	2.0	0.5
e338	82.7	81.6	1.8	1.1
e346	86.9	87.6	4.4	1.3

Conclusion: The quantitative nature of the forage quality trait along with heterozygosity within and among perennial ryegrass plants showed substantial variation for both digestibility and gene expression. However, significant correlations suggest further studies into gene expression and function to affect cell wall digestibility and other forge quality traits.

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Genomic prediction of forage yield in a tetraploid perennial ryegrass breeding programme

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Introduction: Studies have shown that perennial ryegrass breeders have succeeded in achieving significant breeding gain for forage yield. Indirect selection using genome-wide markers is one tool that offers breeders an opportunity to further accelerate genetic gain for forage yield. In perennial ryegrass this can be achieved by enabling multiple cycles of genomic selection (GS) to be completed in the same time it takes to perform a single cycle of conventional selection.

Material and methods: A small population of 150 half-sib families was established by intercrossing plants of an elite commercial tetraploid cultivar. Maternal plants were genotyped using a genotyping-by-sequencing strategy and half-sib progenies were phenotyped for forage yield in replicated sward plots over 2 years under two cutting managements (conservation management (CM) and simulated grazing management (SGM)). We used a simplified procedure for genotype calling that did not distinguish between the three heterozygote states (Li *et al.*, 2014), which is challenging in the absence of very high sequence read depth (>60×). Genomic-BLUP was carried out with the R package rrBLUP (Endelman, 2011), and we performed Monte-Carlo cross validation by assigning 80% of the population as training with the remainder used for testing and repeated this 1000 times. The mean predictive ability was determined as the average Pearson correlation coefficient between predicted and observed phenotype over all iterations.

Results and discussion: Predictive models were developed using a marker set of genotypes in each maternal plant at over 45,000 genomic positions. Predictive ability for forage yield ranged between 0.03 and 0.28 across different cutting periods and managements (Table 1).

Table 1. Predictive ability (Pa) for seasonal forage yield. Spring, summer, autumn simulate grazing managements, 1st cut silage (cut 1 under CM), and total yield after applying a seasonal correction factor (spring: €0.16/kg, summer: €0.04/kg, autumn: €0.11/kg).

Seasonal forage yield	Pa (mean of 1000)
Spring grazing	0.28
Summer grazing	0.03
Autumn grazing	0.16
Annual grazing	0.16
1st cut silage	0.22
Weighted	0.22

Conclusion: Pa for seasonal forage yield may encourage use of GS in routine selection. It is possible to go from seed-to-seed within a single year using GS. Therefore, despite Pa being low, our capacity to complete at least five cycles of GS in the time it takes to complete a single cycle of conventional selection ensures favourable relative selection efficiency for GS.

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A diploid phased genome assembly of the Italian ryegrass (*Lolium multiflorum* Lam.) cv. Rabiosa

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Introduction: Grasses of the genera *Lolium* and *Festuca* are the main feed sources for livestock production in temperate regions. Over many decades their importance has led to the development of new cultivars through traditional breeding programmes. However, newer improvement methods such as genomic selection could benefit greatly from a high-quality reference genome assembly. In the past, attempts in delivering such a dataset have been hampered by the complexity of the genome and the high heterozygosity of individual genotypes (Byrne *et al.*, 2015). We sequenced an individual of the *L. multiflorum* (Italian ryegrass) cv. Rabiosa, producing a highly contiguous and complete assembly.

Materials and methods: Paired-end, mate-pair and 10× Chromium libraries of a single *L. multiflorum* genotype (cv. Rabiosa) were sequenced on Illumina instruments and assembled de novo with NRGene's DeNovoMAGIC™ 3.0 pipeline. Bionano optical maps and Hi-C data were produced to validate the scaffolds and build pseudomolecules. Messenger RNA was extracted from four organs of young plants of the same genotype, sequenced following the standard Illumina protocol, and used to aid in gene model prediction. The assembly was also fully annotated for repeated sequences and transposable elements.

Results: The de novo assembly resulted to be about 4.5 Gb in size, and highly contiguous (N50 3.05 Mb, N90 0.28 Mb, 1.43% Ns). Interestingly, the size of the assembly close to the size of the diploid genome, the large number of genes (~70,000), the large proportion of duplicated single-copy genes, and the extensive collinearity between pairs of scaffolds support the finding that the two alleles were assembled separately, thus resulting in a diploid assembly. The addition of large-scale scaffolding methods (optical maps, Hi-C contact maps, 10× reads) was able to identify and correct mis-assemblies, resulting in fully-phased pseudochromosome sequences. Comparing allelic regions, there is very little conservation of the intergenic space, a likely consequence of high TE turnover and high differentiation among ryegrass populations.

Conclusions: The availability of a complete and highly contiguous genome assembly of Italian ryegrass paves the way to the exploitation of the forage crops genetic resources using a modern genomic platform. Compared to the available *Lolium* genome assemblies (Byrne *et al.*, 2015; Knorst *et al.*, 2018), the Rabiosa assembly improves contiguity by >40-fold, contains both haplotypes of the diploid parent, and assigns most of the sequence to chromosomes. Since the high intra- and interspecific genetic variation of ryegrass cannot be encompassed by a single reference, we aim at developing multiple genome assemblies of this quality, spanning the diversity of the whole *Festuca* and *Lolium* breeding material.

Byrne S.L., Nagy I., Pfeifer M., Armstead I., Swain S., Studer B., ... Asp T. (2015) A synteny-based draft genome sequence of the forage grass *Lolium perenne*. *Plant Journal* 84, 816-26.

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Mapping self-fertility for hybrid breeding in perennial ryegrass (*Lolium perenne* L.)

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Introduction: Self-incompatibility (SI) prevents the creation of homozygous parental lines by self-pollination for hybrid breeding in perennial ryegrass (*Lolium perenne* L.). Although SI is highly effective, its breakdown leading to self-fertility (SF) has been reported. Recent research has fine-mapped one major locus conferring SF in a perennial ryegrass population to a 1.6 cM region on linkage group (LG) 5 (Do Canto *et al.*, 2017). Our goal was to confirm, by marker-trait association using genome-wide markers, this previously described locus as being solely responsible for SF variation.

Materials and methods: A genotyping-by-sequencing (GBS) library was prepared using 75 individuals from a SF F_2 population described by Do Canto *et al.* (2017). SNPs were called as described in Begheyn *et al.* (2018) using a genome assembly of *Lolium multiflorum* L. (unpublished). SNPs passing a minor allele frequency threshold of 5% with a minimum of 30 genotypes were used for marker-trait association. Kruskal-Wallis was used to detect significant associations (Bonferroni corrected: $-\log_{10}(P/n) \geq 4.64$) between SNPs and the SF phenotypic traits from Do Canto *et al.* (2017). Synteny to *Hordeum vulgare* L. was used to confirm the position of scaffolds harbouring significant SNPs.

Results: After filtering, 2,215 SNPs were used for marker-trait association (Figure 1). A total of 44 SNPs were found to be significantly associated with SF. Of these, 37 SNPs could be allocated to genome positions and peaked in close proximity of the SF locus on LG 5.

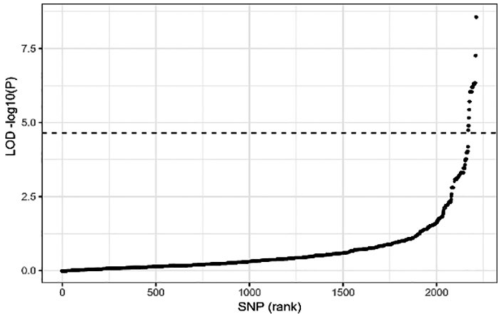

Figure 1. Marker trait association analysis between SF scores and SNPs. x-axis = ranked order of SNPs; y-axis = LOD score; dashed line = significance threshold (Bonferroni corrected: 4.64).

Conclusions: The marker-trait association confirmed the presence of one major SF locus on LG 5. Moreover, these data are currently exploited for marker development to further refine the SF locus and identify causal gene candidates.

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Do Canto J., Studer B., Frei U. and Lübberstedt T. (2017) Fine mapping of a self-fertility locus in perennial ryegrass. *Theoretical and Applied Genetics* 131(4), 817-827.

Investigating the effect of seeding rate on forage grass sward composition

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Introduction: Maintenance of sward density over time is a desirable trait in forage grasses as it allows for the preservation of yield and reduces the requirement for costly reseeding (O'Donovan *et al.*, 2016). The simulation of contrasting sward densities can be achieved by sowing at a range of seeding rates, and higher densities have been shown to have a significant positive impact on dry matter yield in perennial ryegrass (Creighton *et al.*, 2011). Monitoring changes in the genetic profiles of sward population structure can allow for the identification of genotypes with the ability to survive under different management conditions. Genotyping by sequencing (GBS) can be utilised to generate genome-wide allele frequency fingerprints in perennial ryegrass populations (Verwimp *et al.*, 2018). Our goal is to use this technique to detect population genetic changes in perennial ryegrass populations over years and investigate whether these changes can be attributed to seeding rate and sward density, in both perennial ryegrass and hybrid cultivars.

Materials and methods: This study was carried out at Teagasc, Ireland (50°09N; 8°16W). Two tetraploid perennial ryegrass cultivars (AberGain and Twymax) and a tetraploid and diploid hybrid cultivar (Amalgam and Ligunda, respectively) were sown in August 2013. Plots were sown at high (37 kg ha^{-1}), medium (22.2 kg ha^{-1}) and low (7.4 kg ha^{-1}) seeding rates. Varieties were sown as 1.5×5 m plots in a randomised complete block design with four replications. Plots were managed under simulated grazing, with 9 cuts per year. After 3 years, plots were sampled by collecting 10 hand-grab samples along a meter transect, 4 times per plot. Original seed from each variety was germinated and harvested after 2 weeks (4 replicate samples for each cultivar). DNA from plant material was isolated and GBS libraries were prepared after double digestion with PstI and MspI. We also prepared artificial mixtures of breeding families to confirm our ability to detect differences in allele frequencies. Single nucleotide polymorphisms were identified, and frequency of the variant allele determined in each sample.

Results: We were able to identify differences in allele frequencies among breeding families and generate reproducible genome-wide allele frequency fingerprints. We did not identify any clear differences in allele frequency fingerprints associated with seeding rate. Future work will expand the analysis to focus on treating GBS data as multi-allelic markers.

Conclusion: GBS is a useful tool to identify differences in genetic make-up of forage grass populations, and we envisage it being particularly useful for monitoring changes in cultivar mixtures that are typical of many grassland farming systems.

Creighton P., Kennedy E., Shalloo L., Boland T.M. and O'Donovan M. (2011) A survey analysis of grassland dairy farming in Ireland, investigating grassland management, technology adoption and sward renewal. *Grass and Forage Science* 66(2), 251-264.

O'Donovan M., McHugh N., McEvoy M., Grogan D. and Shalloo L. (2016) Combining seasonal yield, silage dry matter yield, quality and persistency in an economic index to assist perennial ryegrass variety selection. *Journal of Agricultural Science* 155(4), 556-568.

Verwimp C., Ruttink T., Muylle H., Van Glabeke S., Cnops G., Quataert P., ... Roldán-Ruiz I. (2018) Temporal changes in genetic diversity and forage yield of perennial ryegrass in monoculture and in combination with red clover in swards. *PloS one* 13(11), e0206571.

Shedding light on the genetic control of anthracnose resistance in red clover

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Introduction: Red clover (*Trifolium pratense* L.) is one of the most important forage legumes in temperate climates. It is an excellent feed for cattle due to its high protein content and digestibility, and its ability to fix atmospheric nitrogen also improves and maintains soil fertility (McKenna *et al.,* 2018). In recent years, an increase in red clover crop failure due to southern anthracnose, caused by *Colletotrichum trifolii*, has led to demand for resistant cultivars. This study aims to shed light on the genetic control of anthracnose resistance through genome sequencing, and genome-wide association studies.

Materials and methods: Plant material segregating for anthracnose resistance was developed at Agroscope (Zurich) by crossing individual resistant (r) or susceptible (s) plants selected from five cultivars. A total of 432 F_1 progeny of four reciprocal bi-parental crosses were tested in the greenhouse in a randomized complete block design with four replications. Eight weeks after sowing, plants were spray inoculated with a single-spore isolate as described (Schubiger *et al.,* 2003). Seven weeks after inoculation, the percentage of surviving plants was assessed. Surviving plants were spray inoculated again with a mixture of seven additional isolates and survivors of both inoculations were recorded. Before inoculation, plant material of six-week-old seedlings was taken for DNA extraction. Genotyping by sequencing (GBS) was performed on pooled leaf samples of susceptible and resistant plants, respectively and genomic regions associated with anthracnose resistance were identified.

Results and conclusion: Single-isolate inoculation yielded a segregation ratio of 1:1 in F_1 progenies of crosses between resistant and susceptible parents, and a ratio of 1:0 in F_1 progenies of two resistant parents. This indicates that one dominant gene governs resistance (Figure 1) in all the crosses investigated. Inoculation of the surviving resistant plants with the isolate mixture resulted in further segregation, suggesting the existence of at least one additional resistance gene and strain-specific resistance. GBS revealed several SNPs potentially associated with resistance, which provide the basis for further characterization of anthracnose resistance in red clover.

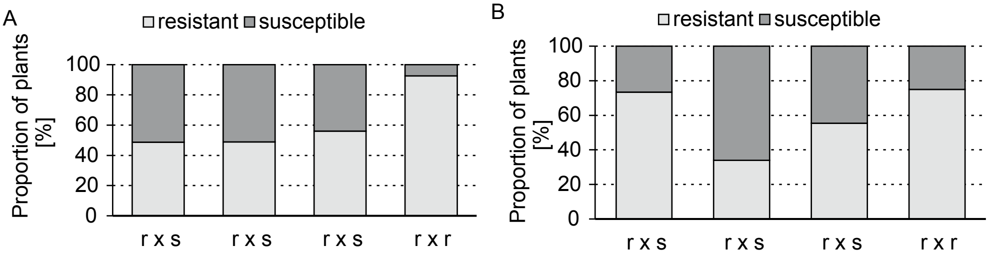

Figure 1. Percentage of F_1 plants resistant (r) or susceptible (s) after inoculating with (A) a single spore isolate, (B) an isolate mixture.

McKenna P., Cannon N., Conway J. and Dooley J. (2018) The use of red clover (*Trifolium pratense*) in soil fertility-building: A Review. *Field Crops Research* 221, 38-49.

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Effects of two CONSTANS-like mutations on alfalfa plant height and flowering date

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Introduction: Some genes have major impacts on quantitative trait variation. In an allele mining study in alfalfa (*Medicago sativa*), non-synonymous mutations have been described in five candidate genes (Gréard *et al.*, 2018). In a CONSTANS-like gene whose polymorphism is linked to flowering date but also to plant height in alfalfa (Herrmann *et al.*, 2010), two non-synonymous mutations were identified, one leading to a stop codon in the first exon, the other one, located in the fourth exon, being in linkage disequilibrium with a silent mutation already identified as related to plant height and flowering date (Herrmann *et al.*, 2010). The objective of this study was to evaluate the effect of these two mutations on the phenotype.

Materials and methods: Separately for each mutation, genotypes carrying one to three doses of the mutation (AAAB, AABB and ABBB) were polycrossed in order to obtain offsprings with zero to four doses (AAAA, AAAB, AABB, ABBB and BBBB). KASPar markers were defined to determine the mutation doses. Each allele dosage was represented by 17 to 50 progeny genotypes. However, no quadruplex (BBBB) was found for the mutation leading to a stop codon. The genotypes were planted in a spaced plant design. Flowering date was evaluated in August 2018 and plant height was measured three weeks after cut, in September 2018. The data were analysed by ANOVA (proc lm of R, Tukey test for mean comparison).

Results: The mutation leading to a stop codon, probably lethal as quadruplex (BBBB), had a significant impact on flowering date: triplex genotypes (ABBB) flowered three days earlier, on average, than the other dosages (Figure 1A). The other non-synonymous mutation had a significant additive effect on plant height: nulliplex genotypes (AAAA) were on average 12 cm shorter than triplex and quadruplex ones (ABBB and BBBB) (Figure 1B).

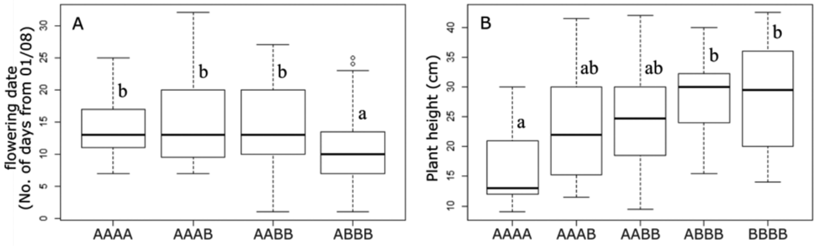

Figure 1. Flowering date (A) and plant height (B) depending on allele dosage.

Conclusion: The two mutations in CONSTANS-like had a strong impact on the phenotype. The KASPar markers could be used in breeding programmes to speed up genetic progress, especially for plant height, a component of forage yield. The allele mining strategy, going from the identification of allele polymorphism to the test of the effect of mutations on the phenotype, could be a method to exploit natural diversity for alfalfa breeding.

Gréard C., Barre P., Flajoulot S., Santoni S. and Julier B. (2018) Sequence diversity of five *Medicago sativa* genes involved in agronomic traits to set up allele mining in breeding. *Molecular Breeding* 38, 141.

Herrmann D., Barre P., Santoni S. and Julier B. (2010) Association of a CONSTANS-LIKE gene to flowering and height in autotetraploid alfalfa. *Theoretical and Applied Genetics* 121, 865-876.

The evaluation of genetic variation in *Lolium perenne* ecotypes by using molecular markers

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Introduction: *Lolium perenne* L. (perennial ryegrass) is a native plant that grows naturally in North and West Iran. Due to its rapid growth, it is one of the most important forage crops in Iran. Some species of *Lolium* spread in the most semi-arid, temperate zones and cold areas (Sharifan, 2008). This great adaptability makes *Lolium perenne* valuable in soil conservation, rangeland recovery and forage production. The aim of this study was an assessment of genetic diversity (genetic distance) among identified unknown accessions of *Lolium perenne* collected in 2015 from Iran by RAPD marker and its application in later breeding processes such as hybridization and synthetic varieties.

Materials and methods: In this study, RAPD markers were used to evaluate the genetic variation among 10 fresh samples of *Lolium perenne* from a population collected randomly from natural rangelands in different areas of Iran. DNA of the leaves of these ten ecotypes was extracted according to the method of Dellaporta (1983) and genetic variation was assessed with 15 primers.

Results: ten RAPD primers produced 135 bands of which 75 (55%) were polymorphic. The number of the bands for the primers varied from 12 to 29%. The percentage polymorphism for each primer varied from 75 to 85% and the size of the DNA fragments was from 200 to 2,800 bp. The dendrogram constructed using UPGMA method, distinguished 4 main groups (dendrogram cut off at a distance of 65) (Figure 1). The RAPD primers divided *L. perenne* on the basis of the geographical distribution and climate similarities. Azadshahr and Ramian ecotypes showed the highest similarity (81%). They belonged to North of Iran (Golestan province). The greatest genetic difference was observed between ecotypes Kordestan 1 and 2 and the other ecotypes.

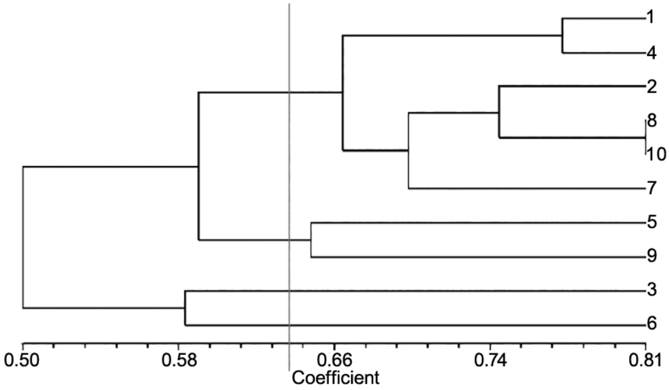

Figure 1. Dendogram by UPGMA.

Conclusion: This study revealed that studied *Lolium perenne* germplasm has a considerable genetic variation and RAPD marker is useful to study DNA polymorphism in *Lolium perenne*.

Dellaporta S.L., Wood J. and Hicks J.B. (1983) A plant DNA minipreparation. *Plant Molecular Biology Reporter* 1(14), 19-21.

Sharifan F. *et al.* (2008) Genetic diversity in agronomic and morphological traits in *Lolium* collection of national plant Gene Bank of Iran. *10th Iranian congress of Crop Science, 18-20 Aug, 2008*, karaj.IRAN.221-222.

FestucaBase: Molecular resource for forage grass *Festuca pratensis*

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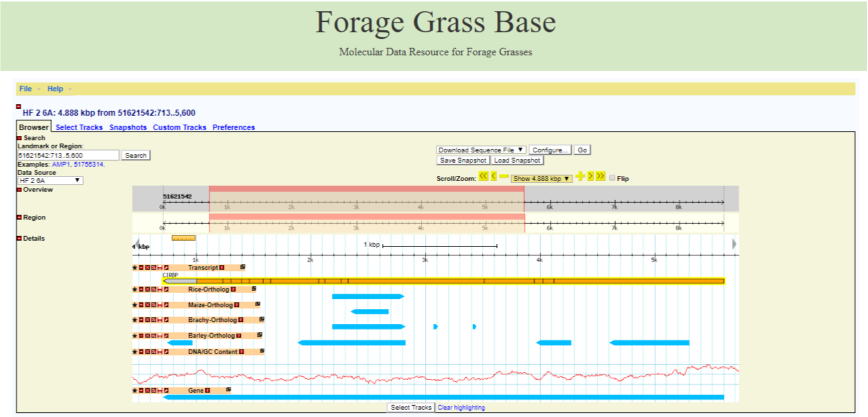
Introduction: Among several forage crops, meadow fescue (*Festuca pratensis* Huds.) is one of the most important forage grasses. *Festuca pratensis* is a diploid (2n=14), outbreeding species that belongs to the genus *Festuca*. Together with *Lolium*, it is the most important genera of forage grasses in temperate regions. *Festuca pratensis* has good winter survival, with high quality dry matter yields and persistency, and is suitable both for frequent-cutting conservation regimes and grazing. It is a significant component of species-rich permanent pastures in temperate regions, ensuring high forage yield under harsh climatic conditions where other productive forage grass species are unable to grow. The first draft genome sequences of two *F. pratensis* cultivars 'HF' and 'B14/16' are reported in this study.

Materials and methods: *De novo* sequencing of *F. pratensis* genome was performed by Illumina mate pair sequencing and assembly was performed by SOAPDenovo assembler. Further, gene annotation was performed by in-house developed python scripts. Bootstrap (HTML, CSS), Javascript, PHP and Python were used to develop ForagegrassBase. The Generic Genome Browser (GBrowse) and BLAST were also installed. R packages are used for BLAST results visualizations.

Results: Here, using the draft genome and functional annotation datasets of two *F. pratensis* cultivars, we have constructed the *F. pratensis* genome database http://foragegrass.org/, the first open-access platform to provide comprehensive genomic resources related to this forage grass species. The current version of this database provides the most up-to-date draft genome sequence along with the structural and functional annotations for genes. In addition, comparative genomic tracks developed by comparing the *F. pratensis* genomes to the barley, rice, *Brachypodium* and maize genomes are integrated in the genome browser (Figure 1). BLAST and Gbrowse tools are provided for visualization and access to genomic data.


Forage Grass Base
Molecular Data Resource for Forage Grasses

HF 2 6A: 4.888 kbp from 51621542:713..5,600

Landmark or Region: 51621542:713..5,600
Examples: AMP1, 51755314.
Data Source: HF 2 6A
Scroll/Zoom: Show 4.888 kbp

Figure 1. *Festuca pratensis* genome browser contains comparative genomic tracks developed by comparing the *F. pratensis* genomes to the barley, rice and maize genomes.

Conclusion: This database provides valuable resource for a range of research fields related to *F. pratensis* and other species belonging to the forage crops, as well as for plant research communities in general. The genome database can be accessed at http://foragegrass.org. In the near future, we will expand this database by adding genomic tools for other forage grass species, as soon as their genomes are available.

A cytogenetic map of *Festuca pratensis* and examples of genome reshuffling in *F. pratensis* × *Lolium perenne* hybrids

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Introduction: The genome composition and karyotype structure of *Festuca-Lolium* species is still not fully recognized. Seven chromosomes of *F. pratensis* and *L. perenne* are similar in shape and size, and thus their identification based on their morphology is not possible. Both species are closely related and the intercrossing of these species results in fertile intergeneric hybrids. The genomes of hybrids undergo changes after the hybridization and these changes can occur as intergenomic rearrangements leading to chromosome instability. The objective of our study was to identify chromosome specific sequences and to recognize all *F. pratensis* chromosomes, and to analyse processes that can contribute to genome instability in *F. pratensis* × *L. perenne* hybrids.

Materials and methods: The plant materials consisted of diploid (2n=2×=14), autotetraploid (2n=4×=28) *F. pratensis*, and allotetraploid hybrids (2n=4×=28) obtained by crossing *F. pratensis* (2n=4×=28) and *L. perenne* (2n=4×=28) (F_1-F_9 generations). The karyotype structure and genome composition of all the plant materials were examined by FISH and GISH, using BAC clones and sequences from a DNA library representing most frequently present in the genome of *F. pratensis*.

Results: The experiments comprised *in situ* hybridization of 192 DNA clones in diploid *F. pratensis*. These experiments revealed six types of FISH patterns: dispersed-, chromosome painting-, centromeric-, knob-, single locus-like sequences, and those without observed hybridization signals. The location of single locus sequences was also analysed in autotetraploid *F. pratensis*. Based on our FISH results, the first detailed cytogenetic map for this species was created. Analysis of processes leading to genome instability proved that the position of intergenomic rearrangements in analysed hybrids did not colocalize with fragile sites related with 35S rDNA; however, their position overlapped with internal telomeric sequences in several plants.

Conclusion: The development and exploiting of BAC library and DNA library representing sequences most frequently present in the *F. pratensis* genome enabled the construction of the first detailed cytogenetic map of this species. Furthermore, the analysis of genomic composition of *F. pratensis* × *L. perenne* hybrids indicated processes that can contribute to the instability of their genomes.

Acknowledgments: The study was partially supported by the NSC (no. 2018/28/T/NZ9/00074).

An allele frequency-dependent methodology for mapping and characterising a newly discovered major gene for self-compatibility in perennial ryegrass (*Lolium perenne* L.)

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Introduction: Self-incompatibility (SI) is a mechanism widespread in flowering plants that prevents fertilisation by self-pollen and, in doing so, favours outcrossing and maintenance of heterozygosity. In the grasses SI is primarily controlled by two multi-allelic loci; S, on linkage group (LG) 1 and Z, on LG 2. SI acts as a barrier to hybrid crop breeding programmes and prevents the production of inbred lines, thus hampering the synthesis of F1 hybrids, limiting breeders to develop cultivars from improved populations or synthetic varieties where a large proportion of potential heterosis is lost (Do Canto *et al.*, 2016). This has, in part, led to limited genetic gains in perennial ryegrass breeding and overcoming SI would lead to greater flexibility in breeding. In perennial ryegrass (*Lolium perenne* L) an additional locus, independent from S and Z, has been recently identified on LG 6 that completely overrides the SI system causing self-compatibility (SC) in plants. Here we report on current progress and prospects of our efforts to fine map the SC locus using whole genome next generation sequencing (NGS), subsequent single nucleotide polymorphism (SNP) allele frequencies of bulked segregants and sequence alignments with an annotated *Lolium* reference genome.

Materials and methods: A F_3 population segregating for SC was first phenotyped to produce pools for Bulked Segregant Analysis (BSA). This population was derived from selfing a single F_2 plant heterozygous for a SC mutation on LG 6. Due to the non-transmission of pollen gametes not in possession of an SC allele on LG 6, the F_3 population was either fully self-fertile, being homozygous for the mutant allele, or half self-fertile and heterozygous, segregating in a 1:1 ratio. Phenotyping was performed based upon pollen grain activity following self-pollination. Individuals with grains displaying both compatible and incompatible reactions were pooled as half self-compatible whereas those with only compatible grains were pooled as fully self-compatible. Individuals were further pooled depending upon whether their phenotype could be determined with a high degree of confidence or not. DNA was then extracted from pooled leaf samples and sent for NGS.

Results: 474 individuals were phenotyped, with 223 as fully self-compatible with high confidence and 44 with low confidence whereas 182 were pooled as half self-compatible with high confidence and 27 with low confidence.

Ongoing work: Following whole genome NGS of bulked samples, SNP markers potentially associated with the SC gene will be identified based on allele frequencies. Subsequently, these markers will be aligned to an annotated perennial ryegrass reference genome for candidate gene appraisal. In addition to identifying candidate genes, we will also locate genetic markers for efficient introgression of SC into a wide range of germplasm to explore novel breeding methodologies in forage grass breeding programmes.

Do Canto J., Studer B. and Lubberstedt T. (2016) Overcoming self-incompatibility in grasses: a pathway to hybrid breeding. *Theoretical and Applied Genetics* 129, 1815-1829.

Response of alfalfa populations to recurrent selection for superior freezing tolerance

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Introduction: Freezing tolerance (FT), a major limitation to alfalfa (*Medicago sativa* L.) persistence and productivity in northern climates, can be improved by recurrent selection (RS). Here, we used GBS to characterize response to selection in breeding populations of two alfalfa cultivars, and to identify genome regions affected by RS for improved FT.

Materials and methods: Two cultivars (Ap-FT0 and Ev-FT0), and populations obtained from four to five cycles of RS for FT within these cultivars (Ap-FT5 and Ev-FT4) were grown indoors. DNA was extracted from 384 genotypes (100 Ap-FT0, 100 Ap-FT5, 95 Ev-FT0, 89 Ev-TF5), and four ApeKI-digested DNA libraries were prepared and sequenced on 8 Illumina HiSeq2500 lanes. Each genetic background (Ap- and Ev-) was analysed separately. SNP were called with GBS-SNP-CROP (Melo *et al.*, 2016) using *M. truncatula* genome (Tang *et al.*, 2014) as a reference. R packages vcfR (Knaus *et al.*, 2017) and Radiator (Gosselin *et al.*, 2017) were used for data analysis, and F-statistics were calculated on filtered datasets.

Results: About 1.5G reads were obtained from sequencing. Analysis with GBS-SNP-CROP yielded 12703 SNP in Ap and 11201 SNP in Ev, including 8800 common SNP. After removing one outlier genotype, comparable profiles of missingness were observed in both populations. In both cases, PCA revealed significant differentiation between initial cultivar and RS populations (P<0.001) (Figure 1). About 100 SNP displaying frequency shifts in response to RS were identified (P<0.01). Interestingly, 13 large regions containing multiple SNP with significant allele frequency shifts were consistently identified in both genetic backgrounds.

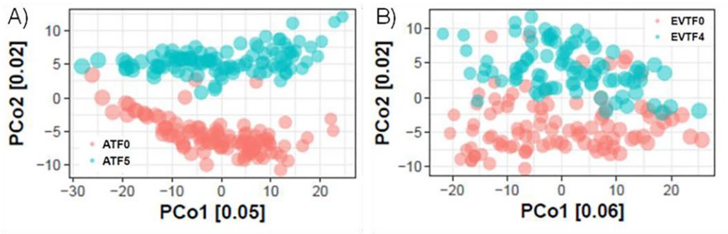

Figure 1. Projection of samples of (A) Ap-TF0 and Ap-TF5 (B) Ev-TF0 and Ev-TF5 populations over the first two PCA axis.

Conclusion: For both cultivars, improving FT by RS had no impact on overall genetic diversity, but affected allele frequency in several genome regions. Despite a moderate overlap between SNP datasets, common regions responded to RS in the two distinct cultivars. These regions are possibly associated with the increased freezing tolerance observed in recurrently selected populations. Several candidate genes were identified in these regions using homology with *M. truncatula* genome, and further characterisation of their response to selection is in progress.

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Differential gene expression and multi-omic analysis of drought stress in *Lolium perenne*

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Introduction: Perennial ryegrass (*Lolium perenne* L.) is the predominant forage grass in temperate climates, due to its high forage quality. Drought is a growth-limiting factor for such temperate grasses, which have to survive and recover after a period of prolonged drought. The aims of our study were to analyse whole genome RNAseq data in the context of differential expression (DE) and integration with Bayesian prediction models, to reveal the potential for drought adaptation in perennial ryegrass.

Materials and methods: 263 *L. perenne* F2 families and commercial cultivars were grown across a water deficiency stress gradient under semi field conditions in the RadiMax facility (Thorup-Kristensen *et al.,* 2018), with controlled subsoil irrigation at increasing soil depth. Leaf tissue for DNA and RNA extraction was sampled from plants on each side of this drought gradient. Aboveground quality traits related to yield, digestibility and carbohydrate contents were assessed. Root growth was monitored by cameras in minirhizotron tubes. InterProscan was used to assign gene ontology (GO) terms to the predicted protein sequences. Single nucleotide polymorphisms (SNPs) were called with freebayes. The phenotypes, SNPs and normalized gene count data were used as input for prediction with BGLR using Bayesian ridge regression with a Gaussian prior.

Results: Main results from DE analysis are summarized in Table 1. The majority of DE genes were involved in known stress-related pathways. Functional analysis also identified several DE regulative proteins, some of which may be key regulators of metabolic pathways associated with drought response. Prediction models integrating both gene count data and SNPs generally explained a larger portion of the phenotypic variance than models based on genomic information only, and performed better in terms of higher prediction accuracy assessed by cross validations.

Table 1. Differentially expressed genes, as identified with the Wald test in Sleuth at $\beta \geq 2$ and a FDR ≤ 0.0 cut off.

	# significant genes	Functional annotations – examples
Up	409	heat shock proteins, dehydrins, jacalin-like lectins, ring/zinc finger motifs
Down	412	chlorophyll A-B binding protein, biotic stress-associated lectins

Conclusions: Genetic variants in DE genes are promising targets for selection of more drought-tolerant lines. Gene expression reflects the plants' response to the environment and reflects processes taking place in the plants. Integrating transcriptome data in prediction models thus adds a high-dimensional layer of information not captured by genomic data alone (Westhues *et al.,* 2017).

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Genomic snapshots from the pre-breeding of perennial ryegrass (*Lolium perenne* L.)

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Introduction: In future, the cultivation area of perennial ryegrass (*Lolium perenne* L.) is expected to expand northwards in Scandinavia due to climate change leading to milder and wetter winters. In an effort to provide new material for breeding of adaptable cultivars we aim (a) to identify genome-wide markers that are associated with phenotypic traits and (b) to estimate their breeding values of synthetic populations by genomic prediction.

Material and methods: We selected 298 gene bank accessions of *L. perenne* and conducted field trials in Finland, Norway and Sweden. After scoring traits we produced 21 synthetic populations selected for one specific trait (see legend in Figure 1). Using genotyping-by-sequencing (GBS), we genotyped pooled individuals from synthetic populations and their individual parents after one growing season. Using genome-wide association studies (GWAS) and genomic prediction of pooled samples (Cericola *et al.*, 2018) we aimed to identify markers associated with heading date and high winter survival in the synthetic populations.

Results: Across several locations (and several years for winter survival), heading date, plant height and winter survival exhibited the highest heritability among all traits, their estimates being 0.455, 0.236 and 0.187, respectively. We identified 3,216,605 SNPs across all samples, which were reduced to 39,060 after removing variants with more than 25% missing data. GWAS analyses did not identify any significant markers for the three traits across locations and years. Genomic prediction, however, revealed that predicted trait scores for heading date and winter survival corresponded very well with the selected traits (Figure 1).

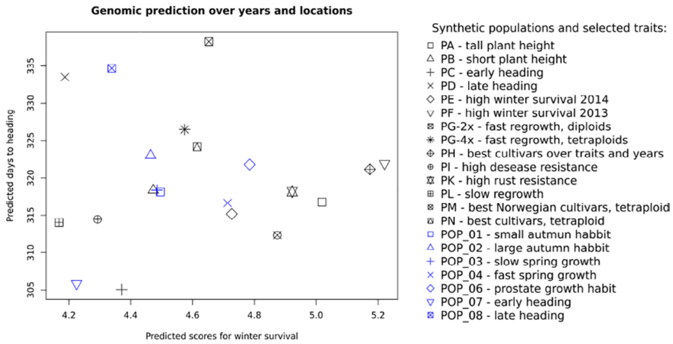

Figure 1. Genomic prediction using the R-package rrBLUP. Synthetic populations selected in Norway and Sweden are coloured black and blue, respectively.

Conclusion: Although genomic association was non-significant across locations (and years), we expect to find stronger associations within the locations. The correlation of predicted trait values and selected traits needs to be further tested and cross-validated.

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Expression of *LpIRI1* and *ADAGIO 1-like* genes in diploid and tetraploid *Lolium perenne* lines with contrasting freezing tolerance

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Introduction: Resistance to freezing temperatures is a crucial trait of perennial ryegrasses grown in the northern parts of Europe. Numerous studies indicate that tetraploid cultivars produce higher herbage yields, yet can be more susceptible to low temperatures compared with diploids (Helgadóttir *et al.*, 2018). The objective of this study was to evaluate candidate gene expression in induced tetraploid and parental diploid perennial ryegrass lines contrasting in their freezing resistance after two weeks of cold acclimation. The candidate genes *LpIRI1* and *ADAGIO 1-like* were chosen based on marker-trait association analysis result.

Materials and methods: Tetraploid plants were induced in 5 freezing-susceptible (LT_{50} from -6.8 to -6.1 °C, S group) and 5 freezing-resistant (LT_{50} from -9.7 to -9.8 °C, R group) diploid perennial ryegrass lines by treating the embryos with 10 mM colchicine solution. Ten plants in 4 repeats of each diploid and tetraploid line were grown in peat substrate in horticultural cell packs for 4 weeks in the greenhouse, transferred to the phytotron set at 20 °C, 300 µmol m-2 s-1 PAR, 16/8 h for one week, then the temperature was lowered to 2 °C and kept for 2 weeks. Plant stem bases were harvested for RNA extraction before and after cold acclimation at 2 °C. Total RNA was extracted using GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific, Lithuania), cDNA was synthesised using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania), qRT-PCR were performed with 7500 Fast Real Time PCR System (Applied Biosystems, USA) using Luminaris Color HiGreen qPCR Master Mix (Thermo Fisher Scientific, Lithuania). Expression of two candidate genes *LpIRI1* and *ADAGIO 1-like* was measured, *TBP-1* and *YT521-B* were used as reference genes, all reactions were performed in 4 biological and 3 technical replicates.

Results: The low temperature treatment increased the expression of both candidate genes. The mean *ADAGIO 1-like* $\Delta\Delta$CT was 0.97±0.21, and mean *LpIRI1* $\Delta\Delta$CT was 6.8±1.58. Slight gene expression differences were recorded in ploidy and resistance groups: *LpIRI1* $\Delta\Delta$CT in diploid and tetraploid S group was 6.8±0.73 and 8.5±0.95 respectively; the $\Delta\Delta$CT in R group was 6.2±1.49 for diploids and 5.6±1.26 for tetraploids. *ADAGIO 1-like* $\Delta\Delta$CT in S group diploids and tetraploids was estimated to be 1±0.11 and 0.7±0.15, and in the R group it was 1.2±0.21 and 1±0.12 respectively.

Conclusion: The differences in gene expression between the diploid and autotetraploid plants were too low to serve as a possible explanation for the higher diploid perennial ryegrass freezing resistance compared to tetraploid plants. Most likely, the larger cell size of tetraploids determines their poorer survival during exposure to freezing temperatures.

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Helgadóttir Á., Aavola R., Isolahti M., Marum P., Persson C., Aleliūnas A., Brazauskas G., Krisjánsdóttir T.A., Asp T. and Rognli O.A. (2018) Adaptability and phenotypic stability of *Lolium perenne* L. cultivars of diverse origin grown at the margin of the species distribution. *Journal of Agronomy and Crop Science* 204, 493-504.

Genetic distance study for improved hybrid breeding in perennial ryegrass (*Lolium perenne*)

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Introduction: Currently, the development of varieties in perennial ryegrass (*Lolium perenne* L.) relies heavily on synthetic breeding. This leads to breeding material not favourable for hybrid breeding as it is closely related and as heterotic groups within the breeding material are difficult to identify (Pembleton *et al.*, 2015). The objective of our study is to assist the exploitation of heterosis by using an amplicon-sequencing (AmpSeq) based approach to improve the initial selection of heterotic groups. AmpSeq allows the calculation of the pairwise genetic distance of parental components, which can be used to develop a yield prediction model. The method enables breeders to target specific genes or entire pathways to retrieve haplotype structures of parental components and to establish a genotyping platform.

Materials and methods: Parental components of cytoplasmic male sterility (CMS)-based tetraploid hybrid breeding programmes were selected for this analysis. Hybrid combinations of 20 pollinators and twelve CMS parents, resulting in 26 hybrids, were tested in yield trials in the years 2014 to 2017. Complete randomized yield trials were analysed using the Agrobase® software. Amplicons of genes linked to agronomically important traits were used for the analysis. AmpSeq was performed with an in-house protocol modified on pools of 60 individual tetraploid plants of each parental component per sample. Pairwise genetic distance was calculated using CLC Genomics Workbench (Version 11.0), Shapiro-Wilk test and Pearson correlations were calculated using R.

Results: Fresh yield of the 26 tested hybrids showed wide ranges in total yield performance ranging from 108.6 dt ha^{-1} to 154.6 dt ha^{-1}. Crown rust (scored in France) reached values from 4.0 to 8.3 (susceptible to resistant: 1-9). Sequence information used for calculation of genetic distance between parents of the hybrids showed a negative correlation for crown-rust resistance ($P<0.001$) and a positive correlation to relative yield ($P<0.05$). Diverse genes were more suitable for correlation than conserved genes.

Conclusion: AmpSeq, to describe the haplotype structure of tetraploid parental components for hybrid breeding in perennial ryegrass, can be used as a tool to identify heterotic groups within the breeding material. Correlation of sequence information to performance in yield and quality-related traits is possible. This will allow the implementation of a prediction model for the performance of hybrids in modern forage grass breeding.

Acknowledgements: This programme receives funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 722338 – PlantHUB.

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Determination of nuclear DNA content and ploidy of newly collected *Festuca ovina* populations from Turkey by using flow cytometer

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Introduction: The genus *Festuca* consists of approximately 400 species with similar morphology and variable ploidy levels. The taxonomy of the genus is difficult and contentious due to morphological similarity. Previous studies demonstrated the importance of ploidy analysis in systematics of the genus (Huff and Plazzo, 1998). The objective of the study was to determine ploidy of newly collected 400 *Festuca ovina* populations by flow cytometer.

Materials and methods: *Festuca ovina* populations have been collected in Turkey through a national research project to use as genetic resource in breeding forage and turf type *Festuca ovina* cultivars. The samples were prepared by using Partec commercial kits (CyStain PI absolute P). and analysed using a Partec CyFlow Space flow cytometer (Munster, Germany). *Vicia sativa* (cv nulifer) was used as internal standard. Five individual plants were analysed for each population. A simple statistical procedure using confidence intervals was used to compare mean DNA content of the populations.

Results: Based on the results flow cytometric analysis, the 2C nuclear DNA content of *Festuca ovina* populations varied between 4.60 pg and 14.92 pg. Differences among populations were statistically significant. The populations were separated clearly into 4 different groups (4.60-4.85, 9.18-9.64, 12.43-13.20, and 14.33-14.92 pg) based on their nuclear DNA contents. DNA contents of the groups were correlated to their ploidy level with root tip chromosome counts on selected plants. According to the results of the study, approximately 60% of the populations were determined as diploid. Ratio of the tetraploids, hexaploids and octaploids were 20, 11 and 5%, respectively. 4% of the populations included individuals with different ploidy levels. A few triploid plants were also determined in the study. Upon morphological examination of the collected *Festuca* plants and going through the Flora of Turkey (*Festuca* treatment by Markgraff-Dannenberg), it was found out that the specimens used in this study were belong to *Festuca valesiaca* Schleich. ex Gaudin, *Festuca chalcophaea* Krecz. & Bobrov *subsp. Chalcophaea*, and *Festuca heterophylla* Lam taxa which are commonly found in Anatolia.

Conclusion: The newly collected *Festuca* populations can have a mixture of different species and ploidy levels. Therefore, they need to be characterized before including them in breeding programmes, and flow cytometer is the one of the best methods for this type of study.

Acknowledgments: This study was funded by TUBITAK (TOVAG 214O077).

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Theme 4.
New methods and technologies to assess plant traits, swards and forages

Methodological advances, challenges and perspectives in field phenotyping and its application to forage crops

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Abstract

Novel phenotyping techniques related to remote and proximal sensing hold great promise for landscape phenotyping, variety trials and to increase genetic gain in plant breeding. For their successful implementation into plant breeding, a lot of measures need to be considered that are related to timely, high-throughput data acquisition and processing. While data acquisition includes the identification of the regions of interest, data processing includes a range of modelling steps to extract targeted traits from sensor data. Here, we will highlight these steps by providing examples from our work with sensors operated in a high-throughput manner from drones or from the field phenotyping platform (FIP) at ETH Zurich, respectively. We put these results into perspective with other ongoing research approaches and discuss how to use the developed concepts for monitoring of forage crops.

Keywords: remote sensing, phenotyping pipeline, data assembly, modelling, biomass estimation, stress resistance

Introduction

Phenotyping, or more precisely high-throughput phenotyping, has become a new bandwagon in crop breeding research. Expectations are that this bandwagon will close 'the genotype-phenotype gap'. Despite progress in genotyping, yields of many major crops, such as wheat, have stagnated in many countries (Brisson *et al.*, 2010), making it more urgent to invest into new breeding approaches. Today, DNA fingerprinting and sequencing has become routine in many breeding programmes and genomic selection holds great promise to increase the breeding progress. However, using measured yield or quality parameters to feed genomic prediction models suffers from the shortcoming that the processes contributing to the expression of these parameters are treated as a black box. Furthermore, yield particularly can only be assessed relatively late in the breeding process, when enough plant material is available. Thus, breeders measure traits or do eyeball ratings of traits positively correlated to yield and quality in early breeding cycles. These secondary traits are usually assessed throughout the growing season – starting with plant emergence and ending with yield components. This analytical approach follows an ideotype concept, i.e. the measurement of morphological and physiological traits conferring an adaptation to a particular environment, management or end use (Martre *et al.*, 2015).

The process of measuring secondary traits or plant features of interest can be summarized as 'phenotyping'. The terms genotype (the genetic composition of an individual) and phenotype (the measurable effect including both genetic and environmental influences) were coined as early as 1911 by the geneticist Wilhelm Johannsen (1911), but phenotyping has more recently become an almost separate field of research (Furbank and Tester, 2011; Walter *et al.*, 2015). The development of high-throughput phenotyping was driven by the availability of low-cost sensors, and sufficient computer power including software development to process and combine large images. It started off with large-scale, automated platforms in greenhouses and growth chambers aiming to handle the ever increasing sizes of genetic testing material (Granier and Vile, 2014; Hartmann *et al.*, 2011; Nagel *et al.*, 2012). Such material was and is generated in large-scale genetic transformation pipelines, mutation breeding approaches, or it consists of mapping populations, which were generated to map the genomic regions controlling quantitative traits. The use of modern phenotyping methods by means of imaging sensors started under controlled conditions and later the field (Hund *et al.*, 2019). Initial steps to establish field phenotyping approaches consisted of the development of different carrier systems like the so-called phenomobiles, followed by drones and large, fully automated installations (see reviews by Cendrero-Mateo *et al.*, 2017; Hund *et al.*, 2019; Luis Araus *et al.*, 2018). The field phenotyping platform at ETH represents one of these fully automated installations (Kirchgessner *et al.*, 2015). A large array of active and passive sensors covering almost the entire electromagnetic spectrum are mounted on lots of these carriers to assess all kinds of plant features. In contrast to their indoor counterparts, many field phenotyping approaches were developed with the clear vision to directly assist the breeding progress.

However, the most crucial question is which traits are to be monitored to support genetic gain for the targeted traits yield and quality. This brings us back to the ideotype concept. It might be tempting to use the whole hyperspectral reflectance signal of a canopy and feed it into a machine learning pipeline to train a yield prediction model. This approach will likely fail, if it does not consider the basic physiological mechanism contributing to yield. Thus, most scientists target the traits which are thought to affect yield. The aim is to (1) replace breeders' ratings with new, high-throughput methods or (2) to measure additional features a breeder cannot assess. Such features are, for example, the canopy temperature (Deery *et al.*, 2016) but also the response of growth and development to changing meteorological conditions during the growing season (Grieder *et al.*, 2015).

Phenotyping of elite germplasm in a breeding nursery is not a piece of cake. The observation process needs to be performed precisely during occurrence of the critical stages at which the material segregates best and at which the secondary proxy trait shows the highest correlation with the targeted primary trait. In many cases multiple observations in time are required to measure the targeted traits, for example when aiming to determine the onset of shooting throughout time (Kronenberg *et al.*, 2017) or the response to temperature (Grieder *et al.*, 2015). Moreover, sensor and data fusion may be required to increase the predictive value of remote sensing features to a level relevant for assessing the targeted breeding traits (Maimaitijiang *et al.*, 2017). If traits that are routinely assessed in a breeding programme should be replaced by a new proxy measure, the relative efficiency of this indirect selection should be either higher than that of the proxy trait, or it should contribute to a significant reduction in the overall phenotyping costs.

General considerations for setting up a phenotyping pipeline

Target traits and decisions on sensors and carriers

The targeted traits drive the decision on the carrier and sensor combination. The very first decision to make, when aiming to include high-throughput field phenotyping (HTFP) techniques into the breeding programme, is which traits should be measured and to which level of precision. It requires previous knowledge about the importance of the trait in the breeding process and the feasibility to measure it using HTFP techniques. This information greatly drives the choice of the carrier system and the selection of the appropriate sensor. For breeders, the simple and robust application of both carrier and sensor including a timely data processing is thereby of major importance. Despite research progress and intense collaboration between breeders and the phenotyping research community, there is still a lot of room for improvement and for establishing applicable solutions. There is a wide range of literature introducing carrier systems and sensors for crop phenotyping (Cendrero-Mateo *et al.*, 2017; Deery *et al.*, 2016; Hund *et al.*, 2019; Luis Araus *et al.*, 2018; Tattaris *et al.*, 2016). Here we refer only to some systems to highlight the diversity of these approaches that are important steps towards generating applicable solutions.

If only the senescence behaviour is targeted, the widely used normalized difference vegetation index (NDVI) may be the right choice of proxy trait. It can be measured using active point sensors, such as Yara N-Sensor, Green Seeker or Crop Circle, which work reliably under varying illumination conditions. If the number of pots is in the range of a few hundred, the best carriers are probably the breeder's own feet. On the other extreme, high-end, active Light Detection And Ranging sensors (LIDAR) or multiple hyperspectral image sensors could deliver 3D spectral information of the canopy, potentially allowing the measurement of photosynthesis-related traits or the spread of diseases. Due to their weight, these devices are usually mounted on phenomobiles or integrated in dedicated phenotyping platforms. The analysis of images derived from these platforms often requires sophisticated image processing techniques.

We believe that the typical breeding applications fall between the two extremes outlined above. While large phenotyping platforms and traditional breeder's rating will deliver the necessary tools and trait calibrations, flexible carrier systems will bring these tools into the breeding nursery. The most flexible and cost-efficient carrier system today are drones or simple phenomobiles. Drones may be of first choice, when large areas have to be covered in a short time period and mainly when general canopy characteristics are in focus. Phenomobiles come into play when more detailed information is required and the timing of the measurement is less critical. Furthermore, in canopies of tall or dense crops, such as maize, sorghum or rapeseed, phenomobiles may be restricted to early developmental stages. At minimum, the carriers should be equipped with a high-resolution consumer-grade RGB camera. In the example of cereals, RGB imaging can be used to measure a wide range of potential features. While this potential is huge, feature extraction is not yet widely used in routine breeding. The reasons for this are related to the complex computation process, which most of the time requires trait and crop-specific image-processing algorithms. Nevertheless, proof-of-concept studies show that RGB information can be used directly to assess a wide range of traits: to count seedlings (Jin *et al.*, 2017; Liu *et al.*, 2017, 2016), detect ears and determine the heading stage (Fernandez-Gallego *et al.*, 2018; Sadeghi-Tehran *et al.*, 2017; Zhu *et al.*, 2016), follow the early canopy cover (Kipp *et al.*, 2014; Liu *et al.*, 2017) or to measure leaf chlorophyll content (Baresel *et al.*, 2017). Yet, RGB imaging can be also used to measure height from multiple images of a scene, applying 'structure from motion' algorithms (Aasen *et al.*, 2015; Bareth *et al.*, 2016; Holman *et al.*, 2016; Madec *et al.*, 2017) or to measure the response of canopy growth to changes in temperature (Grieder *et al.*, 2015) and likely also responses to other changes in meteorological conditions. When only global characteristics of closed canopies are required, hyperspectral and thermal image sensors are suitable. Drones carrying lightweight thermal and multispectral sensors offer the opportunity to monitor hyperspectral and thermal characteristics of large areas in a short time. Compared to the somewhat slower observation with phenomobiles or fixed installations, this minimizes the risk of changes in the illumination conditions during imaging. Such changes influence the signal reflected by the canopy and considerably reduce the heritabiltiy of the trait.

In addition to the above-mentioned passive 2D sensors, active sensing devices can be used to scan the environment with potentially higher precision. The most relevant examples in this context are LIDAR sensors that use laser light. LIDAR systems are widely used for crop phenotyping and the lightweight versions can also be carried by unmanned aerial vehicles (UAVs). LIDARs can be used to generate surface models, to measure canopy height (Friedli *et al.*, 2016) or canopy structure (Jimenez-Berni *et al.*, 2018).

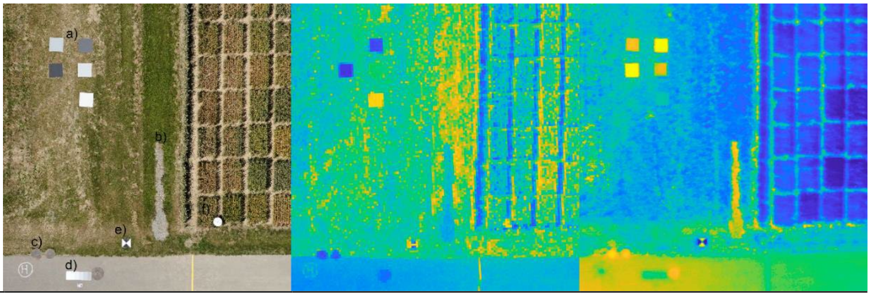
Yet, the appropriate choice of sensors, carrier systems and an appropriate experimental design (not discussed here) to generate relevant imaging data is only the prelude to a successful phenotyping process chain. Relevant field phenotyping approaches require a range of different steps that can be grouped into 'data acquisition' and 'data modelling'. Data acquisition comprises three steps: (1) mission planning; (2) the mission itself; and (3) data assembly and identification of the regions of interest. In the following, we elaborate aspects of the data acquisition pipeline for the case example of drone-based phenotyping, since this carrier system is currently of most relevance for applications of high throughput field phenotyping.

Mission planning

For data acquired with drones, photogrammetric software, such as Pix4D (Pix4D, 2016) and Agisoft PhotoScan (LLC, 2016) offer the first step to construct orthomosaic images and digital surface models. To ensure that orthomosaics and digital surface models are assembled at sufficient quality, ground control points (GCPs) are required. These GCPs are the implicit standard to obtain information of the orientation and position of each image to be located. Moreover, GCPs are needed to align images taken at different times during crop development. At least one visible GCP per image is required to achieve high vertical precision, while a lower number may be sufficient if only a high horizontal precision is required (Harwin *et al.*, 2015). The PhenoFly Planning Tool enables an appropriate placement of GCPs given the attained ground sampling distance as a function of flight height, sensor size of the camera and focal length of the lens (Roth *et al.*, 2018).

The ground sampling distance covered by a sensor pixel (GSD) determines which feature can be resolved. For a given sensor size and sensor resolution, the GSD depends on the distance of the camera to the object and on the focal length of the lens. The maximum GSD to detect a feature in an image should be smaller than 1/5 of the feature size (O'Connor *et al.*, 2017). This means that a feature, like a plot, plant, leaf or a flower head, should at least be covered by five pixels in its smallest object dimension. Due to the requirement of achieving a high GSD, a high sensor resolution is the first choice when aiming for high-quality images of 2D sensors. The number of recorded pixels of a sensor range from 1,024×768 for today's thermal cameras over 2,048×1,088 for multispectral sensors to 6,000×4,000 for visual (RGB) sensors (e.g. Aasen *et al.*, 2018). Thus, generally RGB imaging is at the higher end of sensor resolution while hyperspectral and thermal imaging mark the lower end. An example for the results of different sensors is given in Figure 1.

Given a certain sensor resolution, the GSD can be adjusted by the combination of the lens system and the carrier system determining distance between canopy and sensor. Phenomobiles can bring cameras



Sensor	RGB	NIR 800 nm band	Thermal
Model	Sony, alpha 9	Imec, SNm5x5model	FLIR, A65
Res. (px)	4,000×6,000	409×216 (per channel)	640×480
Alt. (m)	80	60	60
GSD (mm)	6.5	65	42

Figure 1. Field scene taken with three different types of sensors (Model, sensor resolution (Res.), flying altitude (Alt.) and ground sampling distance (GSD) are given). The central and right panels show the reflectance in false-colour. The following features are indexed: spectral calibration targets (a), field path with drainage strip of gravel (b), duct covers (c), grey-scale calibration target (d), thermal ground control point (e), and machine-readable ground control point (f).

close to the plant and allow for GSD below 1 mm that allow resolving of even small seedlings or fine details of the plant. An example where such resolutions are required would be the counting of wheat seedlings at the one-leaf stage where GSD should be below 0.2 mm (Jin *et al.*, 2017) or the detailed analysis of early canopy growth in dense time intervals to measure temperature response (Grieder *et al.*, 2015). For multirotor-UAVs, the distance to the ground usually needs to be above 15 m (due to security considerations and to the down-wash of air by the rotors), limiting attained GSD to values above 1 mm. This fact and the limited flight time (due to battery capacity) induce the requirement for a precise flight planning to capture images with sufficient quality (Roth *et al.*, 2018). Motion blur and the camera's maximal trigger frequency can become additional limiting factors when aiming to cover large areas with low GSD. Furthermore, overlapping of images, placement of ground control points, but also the required depth of field, in which the objects appear sharp, need to be considered when planning a flight mission. For example: a copter with a full-frame 24 MP camera and a 55 mm lens achieves a GSD of 3 mm at 28 m altitude. Using the flight parameters of a 92×75% image overlap and a target area of 36×40 m requires a flying time of 9 min to capture about 600 images (Figure 2 and 3). Exemplary data shown here were calculated using the PhenoFly planning tool (Roth *et al.*, 2018); the extracted 2D and 3D information is presented in Figure 3.

Data assembly and identification of the regions of interest

The orthomosaics (Figure 3A-C) can be used to georeference each image and identify the regions of interest which are usually the centres of the experimental plots of interest. After this step, multiple images taken from different positions above the plots can be used to retrieve 3D information (Figure 3D-F) while multiple images taken at different time points can be used to quantify temporal changes of image features, as discussed in the section about modelling.

Modelling of phenotypic data

Besides the timing of the measurement and the right choice of the carrier and sensor system, the data analysis pipeline is the major bottleneck for high-throughput phenotyping. Thus, after ensuring sufficient data quality during the phenotyping campaigns in the field, the extraction of the relevant information for selection is the major task. The sensor-derived raw data of a canopy can have up to five dimensions which can potentially be used for selection. The first three dimensions relate to the orientation of the available

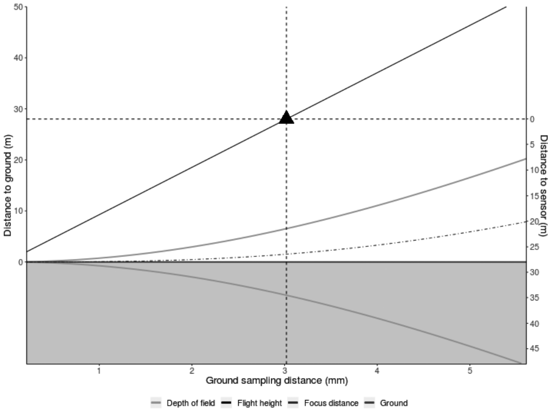


Figure 2. Ground sampling distance depending on the distance to the ground for a full-frame 24 MP sensor equipped with a 55 mm lens. Data were simulated for the case of a bright day (exposure value of 15) and a f-number of 8. The triangle marks an altitude of 28 m resulting in a ground field of view of 18.1 × 12.1 m, a GSD of 3.02 mm and an f-number dependent depth of field of ± 10.5 m (grey, solid lines between which the objects appear sharp). Data were generated using the PhenoFly Planning Tool https://shiny.usys.ethz.ch/PhenoFlyPlanningTool/.

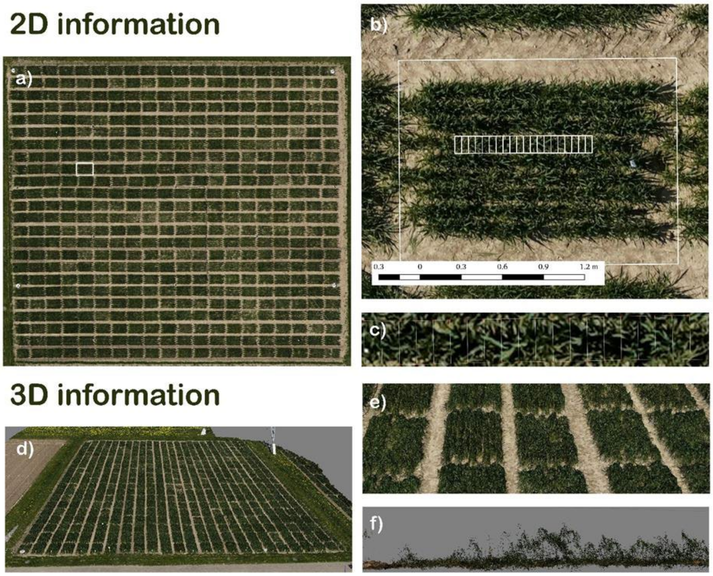
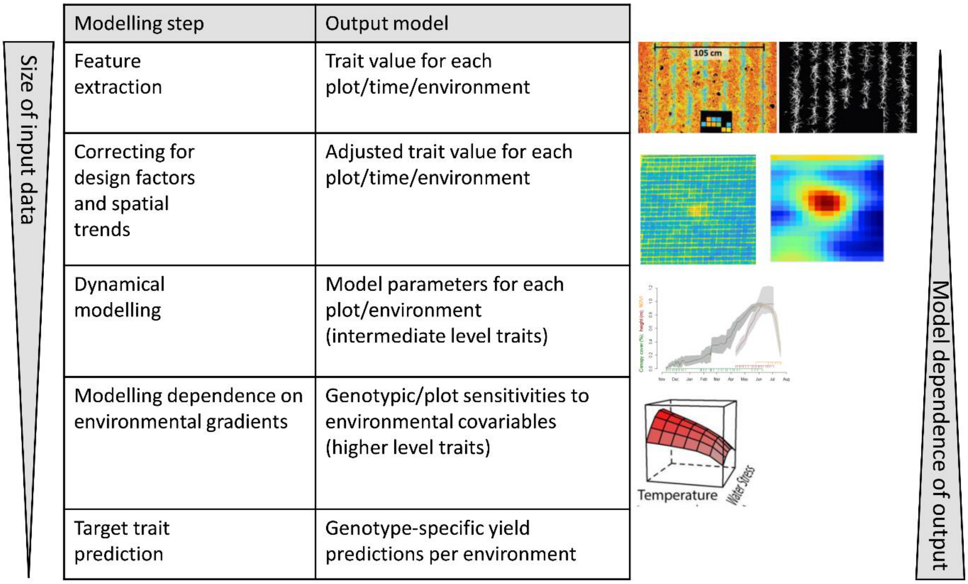


Figure 3. Two-dimensional othomosaic of wheat lot 3 of the field phenotyping platform FIP (Kirchgessner *et al.*, 2015) derived from 588 images of a copter flight at 28 m height with a ground sampling distance of 3 mm (A), individual plot (B), and subplot of one metre length dissected into 20 subsections (C). Three-dimensional image generated by structure from motion of the same lot (D); individual plots in 3D view (E); vertical cross section though the point cloud of the subplot in C showing the vertical position and colour of each respective point (F). Data were acquired in the framework of the TraitSpotting project.

information in the canopy. This information can be derived from point sensors (1D), images (2D; e.g. Figure 3D-F), or point clouds derived from laser scanners or photogrammetry (3D; e.g. Figure 3B). An additional dimension is the distinct spectral reflectance information for each point, pixel or point cloud (Figure 3F) and yet another dimensions is offered by following the change of this information through time. Meteorological data, derived from weather stations or soil sensors, delivers additional covariates to the time dimension. The most prominent use of these covariates is the expression of the temporal dimension as thermal rather than ordinal time.

Generally, the complexity of this multidimensional space is reduced stepwise: first by extracting features from the spatially distributed spectral information and second by summarizing the change of these features over time. There is an additional step in between, as suggested by Eeuwijk *et al.* (2018): correcting for the influence of nuisance factors in the field by means of design factors and spatial trends. Eeuwijk *et al.* (2018) classify the cascade of modelling steps from the original sensor-derived raw data to the prediction of the primary target traits yield and quality in five steps: feature extraction, correction of nuisance factors in the field, dynamical modelling, model dependence on environmental gradients and target trait prediction (Figure 4). Thereby, the size of the input data decreases from one step to the next, whereas the dependency on the previous model output increases. This means that there are many different ways to integrate the sensor-derived raw information into breeding decisions. At the far end of this pipeline, modelers develop crop models for yield and quality prediction. Today many of the steps described above are just in the exploration phase. Implementing them in robust and generic software solutions to generate the desired output within days rather than weeks will be necessary to deliver these tools to breeders.



Modelling step	Output model
Feature extraction	Trait value for each plot/time/environment
Correcting for design factors and spatial trends	Adjusted trait value for each plot/time/environment
Dynamical modelling	Model parameters for each plot/environment (intermediate level traits)
Modelling dependence on environmental gradients	Genotypic/plot sensitivities to environmental covariables (higher level traits)
Target trait prediction	Genotype-specific yield predictions per environment

Figure 4. Modelling process to convert sensor-derived raw data into yield and quality prediction. As this of the input data is decreasing at each modelling step, the model dependency in increasing. Modified according to Eeuwijk *et al.* (2018).

Implementing field phenotyping for forage crop breeding: short and long-term aims

Biomass estimation

Unlike grain crops, forage crops have the advantage that the harvest product can be more directly estimated by means of remote sensing techniques. Yet, given small differences in elite germplasm, a robust biomass estimate will likely involve different traits measured at the appropriate time. However, one-trait measurements may work well for single plants or in special cases. For example, image-derived canopy cover of clover (*Trifolium*) showed a high genetic correlation with dry matter production (rg=0.88, $P<0.001$) with a broad-sense heritability (H2) value of 0.56 (Inostroza *et al.*, 2016). Fresh matter yield may also be estimated using simple indices based on RGB images, such as the green/red index as shown in the example of alfalfa (*Medicago sativa*) (Figure 5). For robust estimates from year-to-year, additional information may be required.

Flowering time and number and size of flowers

There are many efforts to determine flowering time and to count the number of ears in grain crops (Fernandez-Gallego *et al.*, 2018; Sadeghi-Tehran *et al.*, 2017) which may be utilized for the same purpose in forage crops. At the moment the most promising approaches are machine-learning techniques to detect flowers under many contrasting conditions. These techniques will require large sets of images taken under a wide range of conditions for training in order be robust enough for routine application.

Species detection in mixtures

Forage crop breeding also involves the analysis of species mixtures. Phenotyping may be used to evaluate the change in the relative abundance of the different species. Some limited functionality is available in standard software using different colour spaces given clear colour contrasts among the species. However,

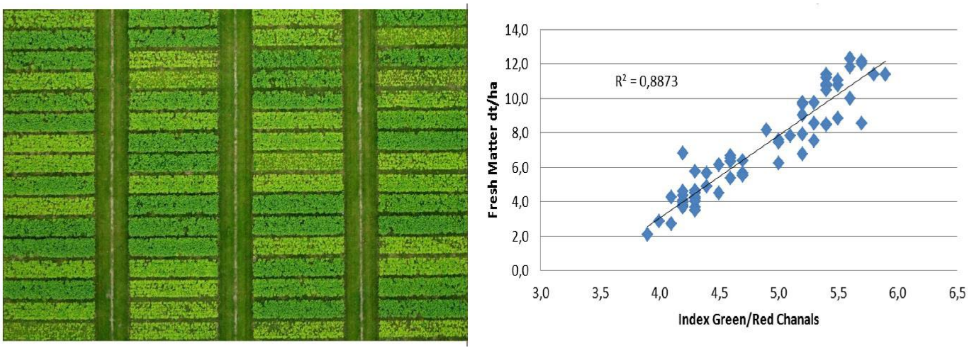


Figure 5. Experiment of 16 alfalfa entries planted in four replications at the test site of the Deutsche Saatveredelung (DSV), Asendorf, Germany (left); Fresh dry matter yield as related to the green/red index derived from RGB images using a multicopter as carrier.

often this requires manual thresholding for each measuring campaign. Again, artificial intelligence approaches are the first choice for classifying different crop species in images.

Canopy development after successive harvests

Not only the temporal dynamics of height growth after successive cuts, but also the response of this growth dynamics to changes in meteorological conditions could be evaluated. Height of experimental plots can be measured efficiently using either terrestrial laser scanners or structure from motion data derived from drone campaigns as explained above. For single plants, i.e. early in the breeding cycle, laser scans can be used to measure the increase in height and canopy volume as a proxy for biomass accumulation (Figure 6).

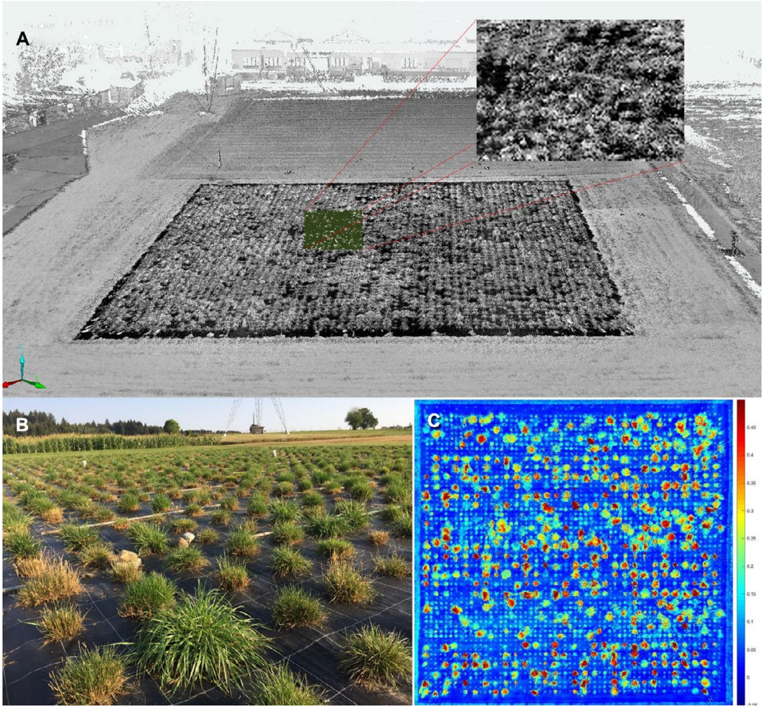


Figure 6. Terrestrial laser scan of a diverse panel of >1000 perennial ryegrass (*Lolium perenne* L.) genotypes comprising both turf and forage types (A), Faro Focus 3D, 4 scans registering, and visualization done with Faro Scene software) to retrieve traits like height and canopy volume (B). The false-colour height map shown in panel (C) was developed with a custom MatLab script. The experiment was planted as completely randomized block design, arranged in three replicates

Diseases and abiotic stresses

Diseases and resistances to diseases are at the top of the list in most breeding programmes. While early disease development is difficult, if not impossible, to assess by means of remote sensing, we may be able to quantify later phases. In cereal crops, the major challenge is to separate senescence-driven changes in canopy reflectance from disease-driven changes. In perennial forage crops this may be less of a problem, and thus disease detection may be more straightforward. Some diseases like crown rust (*Puccinia coronata*) in *Lolium* are easy to detect by eye due to the yellow colour of the diseased canopy of the susceptible genotypes. The disease is a good candidate for drone-based phenotyping, which may even be measured using simple indices derived from RGB images (Figure 7). For more complex situations, we expect that the assessment of disease development rather than a snapshot in time will be necessary to quantify the disease and distinguish among genotypes. Abiotic stress factors of major focus are overwintering ability, measurable by means of the greenness of the canopy after winter, or drought tolerance, measurable by means of thermography.

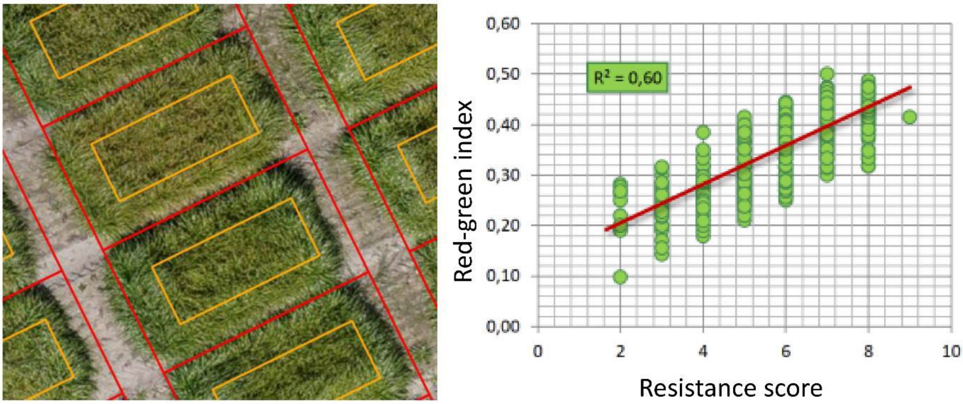


Figure 7. *Lolium perenne* plots at the test site of the Deutsche Saatveredelung (DSV), Asendorf, Germany (left) showing the plot (red) and measuring area (yellow with 30 cm distance to the plot border). Red-green index derived from RGB images with a ground sampling distance of 2 cm as related to the general rust resistance score (1 = susceptible to 9 = resistant) of the plots.

Conclusion

Intensive research and education in field phenotyping will deliver proof-of-concepts and know-how to implement and improve standard protocols for crop breeding. These protocols need to take into account a wide range of aspects from choice of sensors to the application of appropriate statistical models to extract relevant features. In order to improve their applicability, the phenotyping protocols need to be fine-tuned for each trait and each crop, thereby integrating with first-hand information of breeders. Importantly, it is not the technology but the targeted trait and its contribution to yield (and quality) that must be the focus of our efforts.

Acknowledgements

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A strategy for identifying phenotypic traits which favour cold acclimation and stress resistance in white clover

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Abstract

Temperatures in early spring and autumn are lower than required for active growth of white clover (*Trifolium repens* L.) and thus reduce its competitiveness with companion grass (*Lolium perenne* L.). The general objective of this work was to develop a strategy for identifying phenotypic traits that favour cold acclimation and cold stress resistance and determine the chromosomal regions (QTL) which control the expression of these traits, in germplasm naturalized in the southern cold regions of Argentina and Chile (Patagonia). The main specific objective was to select useful genotypes for breeding white clover cultivars with better earlier spring growth. Subsidiary objectives were: (1) selection of 192 genotypes from 28 populations and two cultivars to generate an association mapping population (AMP); (2) AMP phenotypic characterization in the field at three contrasting cold environments (the morphological, physiological and biochemical traits measured presented high variability associated to air temperature); (3) molecular characterization carried out through next generation sequencing technology (Capture Seq,) and tetraploid SNP-calling; and (4) performance of genome-wide association studies (GWAS). QTLs associated with white clover cold resistance were identified. Additionally, this strategy may facilitate faster progress in the breeding process, thereby increasing the genetic gain.

Keywords: genotype selection, phenotyping in field, cold-affected traits, QTLs associated with cold resistance

Introduction

White clover (*Trifolium repens* L.) is the most important forage legume in temperate regions of the world. It is used in association with grasses as N supplier and for improving forage quality. An important factor affecting grass-clover mixture performance is the lower growth rate of clover at low temperatures (5-15 °C). This affects the clover competitiveness in spring and autumn, reducing its contribution to the mixture DM yield. Common forage breeding methods are based on the estimation of genetic merit from phenotypes of progeny and related pedigree information, followed by the selection of superior individuals based on their estimated breeding values (Hayes *et al.*, 2013). The observed phenotype can be confounded with genotype × environment effects that can bias estimates of genetic merit. Molecular markers have revolutionized plant breeding through the ability to dissect genetically complex traits such as cold tolerance and select for favourable alleles through marker-assisted selection (MAS). However, MAS applications in white clover have been limited because of its complex genetic condition; white clover is allotetraploid, cross-pollinated and self-incompatible. The general objective of this work was to develop a strategy for identifying phenotypical traits that favour cold acclimation and stress resistance, and determine the chromosomal regions (QTLs) which control the expression of these traits in white clover.

Materials and methods

An association mapping population (AMP) for cold stress resistance comprised 192 genotypes selected from 28 white clover naturalized populations from Argentinean and Chilean Patagonia and from two cultivars, 'Weka' and 'Haifa', according to the procedure described by Acuña *et al.* (2014). The AMP phenotypical characterization was conducted at three sites: Santa Rosa (36°32' S, 71°55' W), Atacalco (36°53' S, 71°37' W), and Puente Marchant (36°54' S, 71°32'W). These sites are located on a transect rising to the Andes Mountains, ~36° S, and have a winter temperature gradient related to the altitude (140, 659, and 1,054 m.a.s.l., respectively); mean daily minimum air temperatures were 2.5, 1.6, and -1.4 °C during the coldest month. Plant morphology and growth characters, spectral indices, and water-soluble carbohydrates (WSC) were measured during the seasons 2013-14, 2014-15, and 2015-16, as described by Vásquez *et al.* (2018). An alpha-lattice experimental design, with 24 incomplete blocks and two replicates was used. A phenotypic linear mixed model was implemented to estimate the variance components using the Restricted Maximum Likelihood (REML) method within the ASReml-R package. The white clover AMP was genetically characterized using targeted hybridization followed by next-generation sequencing (Capture Seq Method, RAPiD Genomics, LLC, FL, USA). The population structure was studied by discriminant analysis of principal component (DAPC), the genome–wide association analyses were performed using a Q+K linear mixed model implemented in the GWASpoly R package, and BLAST search for putative candidate genes was done, as described by Inostroza *et al.* (2018).

Results and discussion

A large amount of genetic variability was found among genotypes for all phenotypic traits of the white clover population characterized for cold stress resistance. Principal component (PC) analysis captured 53.6% of total variance in the two first PC. The most important variables contributing to the PC1 were petiole length and leaf area, the normalized difference vegetation indices NDVI and MTCI, and shoot dry matter accumulation (DMA). On the other hand, the higher contributions to PC2 were made by stolon traits (SIL, SID, and SER) (Figure 1).

GWAS analyses performed with the diploidized genetic model (DA) did not detect any significant SNP-trait association. On the other hand, GWAS analyses performed with tetraploid genetic models and phenotypic values obtained across locations and years (combined model) identified 53 genomic regions underlying the variation of seven phenotypic traits. The BLAST search was performed for sequencing reads containing the most robust associations ($P<0.001$). For six SNP markers, eight putative candidate genes were found in the surrounding genomic region (Table 1). Based on their annotations in the

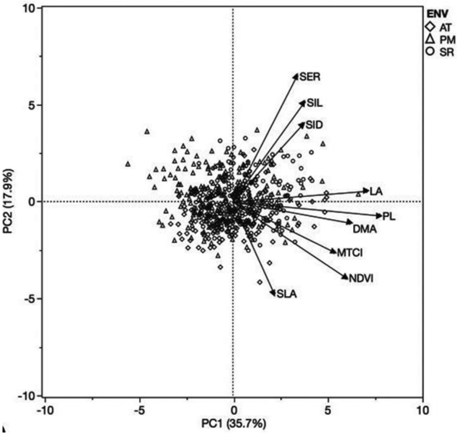

Figure 1. Biplot of the first two principal components (PC1 and PC2) for PCA of nine traits evaluated in 192 genotypes of a white clover population under three contrasting winter-cold conditions (ST, Santa Rosa; AT, Atacalco; PM, Puente Marchant) during the second growing season (2014-2015). The traits are stolon internode diameter (SID), stolon internode length (SIL), stolon elongation rate (SER), petiole length (PL), leaflet area (LA), specific leaf area (SLA), normalized difference vegetation index (NDVI), Merris terrestrial chlorophyll index (MTCI), and shoot dry matter accumulation (DMA).

Table 1. Single nucleotide polymorphism associated with different traits and candidate genes based on *Medicago truncatula* genome annotations.[1]

Marker	Chr	SNP Position (Kbp)	Gene	Annotation	Potential function	Phenotypic traits
cIWC709	2	1059	Medtr2g007640	plastid lipid-associated protein	tolerance of the photosynthetic apparatus to stress conditions	SprgDM, TotalDM, PL, LA
cIWC2377	3	53563	Medtr3g114670	prolyl 4-hydroxylase alpha-like protein	osmoprotection	WSCdr
cIWC2377	3	53563	Medtr3g114910	RING-finger E3 ubiquitin ligase, putative	cold tolerance	WSCdr
cIWC3539	5	3568557	Medtr5g012000	plant invertase/pectin methylesterase inhibitor	carbohydrate metabolism	WSCdr
cIWC3540	5	3568563	Medtr5g012270	peptide/nitrate transporter	nitrogen allocation	WSCdr
cIWC4819	7	8890	Medtr7g026890	serine/threonine-kinase ATM-like protein	plant signal under abiotic stress	SprgDM, TotalDM, PL, LA
cIWC4865	7	21084	Medtr7g058430	E3 ubiquitin-protein ligase PRT1-like protein	cold tolerance	SprgDM, TotalDM, PL, LA
cIWC4865	7	21084	Medtr7g058530	serine/threonine kinase, plant-type protein	plant signal under abiotic stress	SprgDM, TotalDM, PL, LA

[1] Phenotypic traits description: Spring dry matter accumulation (SprgDM); annual dry matter accumulation (Total DM); petiole length (PL); leaflet area (LA); water soluble carbohydrate degradation rate (WSCdr).

reference genome (*Medicago truncatula*), these genes are plausible candidates underpinning phenotypic variation. Genes were found that were associated with abiotic stress tolerance, osmoprotection, cold tolerance, carbohydrate metabolism and nitrogen allocation.

Conclusion

Some leaf and stolon morphology characters, stolon water soluble carbohydrates concentration, and DM accumulation are phenotypical traits highly related with cold acclimation and stress resistance, and chromosomal regions (QTLs) which control their expression were identified.

Acknowledgements

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Using drone imagery to estimate lodging in red fescue (*Festuca rubra*)

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Abstract

Red fescue (*Festuca rubra*) is a grass species used in many different seed mixtures for lawns and pastures. The climate in Denmark is well suited for producing seeds of red fescue. The species is exposed to lodging at flowering, and therefore plant growth regulators (PGRs) are used to prevent lodging at flowering, thereby ensuring sufficient pollination. The objective of this study was to investigate how colour images taken from a drone can be used to estimate the degree of lodging and performance of the crop. We established a field experiment in 2014 and studied the lodging in 2017. Four different crop densities and four different PGR applications were applied. The degree of lodging was scored from the ground on a 10-point scale on two dates before harvest. Based on the images from the drone, we found a high and positive correlation between the blue intensity of images and lodging. There was a negative correlation between lodging and crop seed yield.

Keywords: aerial images, lawns and pastures, image analysis, lodging, seed production

Introduction

Red fescue (*Festuca rubra* L.) is used in seed mixtures for lawns and pastures in temperate climates. Perennial grasses grown for seed production like red fescue are prone to lodging at high nitrogen levels, which are applied to maximise seed yield. Lodging at flowering reduces pollination and seed set and, consequently, the seed yield (Stanisavljevic *et al.*, 2010). Plant growth regulators (PGRs) can be applied to avoid lodging. Trinexapac-ethyl is a foliar-applied PGR resulting in shorter and more erect stands. It has to be applied when the first node is visible (late April or May) (Anonymous, 2018; Zapiola *et al.*, 2006). This study aimed to use aerial images to determine the level of lodging and to evaluate the relationship between seed yield and lodging of red fescue plots, which received different trinexapac-ethyl dosages and seeding rates. We compared the results with a visual ranking done on the ground. Furthermore, we wanted to relate the level of lodging to the seed yield.

Materials and methods

We established the red fescue field trial in 2014 at the research station of the University of Copenhagen in Taastrup (55°38′ N, 12°17′ E), Denmark. Red fescue cv. Maxima was undersown in spring barley cv. Odyssey on 15 April 2014. The experiment consisted of four seeding rates (2, 4, 6 and 8 kg ha^{-1}) and four Moddus M (250 g l^{-1} trinexapac-ethyl, Syngenta) doses (0, 0.3, 0.6 and 1.2 l ha^{-1}) were applied. Each plot was 12×1.5 m of which half was sprayed with the PGR on 9 May, and the other half was left unsprayed as a control. In 2017, lodging was assessed in sprayed plots by visual ranking done by two persons on the ground using a scale from one to ten. Score 1 indicated no lodging and 10 indicated that the whole stand was lying down on the ground (Figure 1). We ranked the plots on 27 June and 17 July. Images were captured of all plots with a RGB camera mounted on a DJI Phantom 4 Pro drone on 27 June. In the images, plots were cropped with in-house software called PlotCut and a variety of indices were calculated for each plot to quantify the colour differences recognized in the areal orthomosaic (Figure 1). The blue intensity (0–255) correlated slightly better with the visual rankings of lodging than the average intensity of all colours. Therefore, the blue intensity was used as an indicator of lodging. Pearson Correlation Coefficient Test was used to test the correlations between (1) the visual rankings of lodging and the blue

Figure 1. An aerial image of the experimental plots and their corresponding scores of lodging on 17 July. Score 1 indicates no lodging and 10 indicates that the whole stand was lying on the ground. Rows 1a and 2a show unsprayed plots and rows 1b and 2b show sprayed plots.

intensity of images and (2) the yield and the blue intensities for all plots. Statistical analysis was done using SAS® version 9.4 software.

Results

PGR significantly affected lodging as evaluated by visual ranking and blue intensity of images ($P \leq 0.001$), whereas the seeding rates had no impacts. The evaluation of treatment effects showed they were unaffected by ranking date. There was no interaction between PGR applications and seeding rates ($P=0.14$). Addition of 0.6 and 1.2 l ha^{-1} of PGR resulted in the same low ranking of lodging (≈2), whereas spraying with 0.3 l ha^{-1} of PGR resulted in severe lodging (≈7) compared to the other application rates.

There was a high correlation between lodging and the blue intensity of images ($P<0.001$; coefficient correlation: 0.92 for ranking on 27 June and 0.91 for ranking on 17 July, respectively) (Figure 2A and 2B). There existed a negative correlation between yield and lodging indicated by the blue intensity ($P \leq 0.01$; correlation coefficient: -0.39) (Figure 3).

Discussion and conclusion

Based on visual inspections of images, lodging and brightness were positively correlated and tests showed that the blue intensity correlated slightly better with ground truthing of lodging than the overall brightness. Rankings done on the ground were highly correlated with the blue intensity of the areal images of the plots. Drone imagery was fast, and assessments are objective. Estimation of lodging, however, requires calibration, which means that a suitable vegetation index (or colour intensity) has to be calibrated against ground truthing. A drone can easily cover one ha per minute. Correlation between

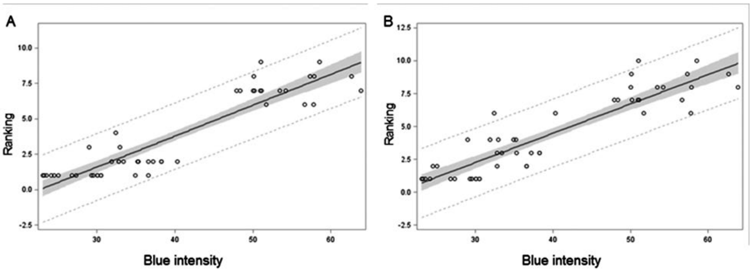

Figure 2. The relationships between lodging ranked from the ground (y-axis), and the ranking based on the blue intensity (0-255) (x-axis). The blue area around the regression line corresponds to the 95% confidence limits for the line, and the dotted lines correspond to the 95% prediction limits of single observations. (A) Ranked on 27 June and (B) ranked on 17 July.

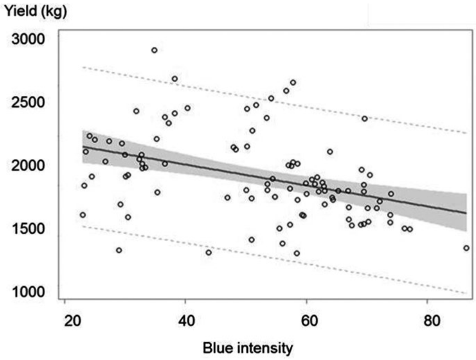

Figure 3. Relationship between blue intensity indicating lodging and the seed yield (kg ha^{-1}). See further explanations in the legend of Figure 2.

lodging estimated from drone images and yield was not large (R^2=-0.39) indicating that other factors than lodging affected the seed yield. Seeding rates did not affect yield or lodging. Red fescue produces rhizomes making it able to cover the ground after some time, even from low initial crop densities. This explains that lodging and seed yield in the fourth year of the crop were unaffected by seeding rate. In conclusion, drone imagery is considered a successful tool to estimate lodging and may be a useful tool for site-specific application of PGRs.

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Yield estimation of perennial ryegrass plots in breeding trials using drone images

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Abstract

Non-destructive yield estimations of perennial ryegrass plots in breeding trials can be valuable to determine growth rates or to estimate yield where an accurate weight determination is difficult. We have used a drone to capture visual images of 7 yield trials comprising 252 diploid and tetraploid perennial ryegrass populations in 2 replicates. Visual images were taken before mowing and just after mowing of the first cut and were used to estimate the difference between the surface height before mowing and the stubble height after mowing, resulting in a mean canopy height (MCH) per plot. For 5 out of 7 trials a satisfactory to high correlation was found between MCH and fresh (FY) and dry matter yield (DMY) per ha. For 2 trials, however, weak to non-existent correlation values were obtained. Further exploration of the images revealed that in these trials (and three other trials), the leaves of grass plants of several plots were 'bending', what disturbed the MCH-FY/DMY relationship. The combination of mean intensity (spectral information) of a plot (as a proxy for bending leaves) and MCH resulted in an improved yield estimation.

Keywords: dry matter yield, drone, breeding trials, canopy height, HIS-colour transformation

Introduction

The toolbox of the practical plant breeder can be extended with digital images using a conventional camera mounted on a drone, in combination with photogrammetric processing and open-source geographic information system (GIS) analysis software. These images do not only provide a global overview of the experimental field and its homogeneity, but they can also be used to extract accurate information to assist the selection process (Araus and Cairns, 2014). The perennial ryegrass breeding program of ILVO (Belgium) already relies on the use of drone images for the evaluation of persistency (Borra-Serrano *et al.*, 2018). In addition, estimations of biomass derived from visual images can be used to determine growth rates or yield of micro-plots (Borra-Serrano I., unpublished results). Traditionally, rising-plate meters or ruler measurements have been used in this respect, but these methods become less precise at higher yields (>2,700 kg DM per ha) and the determination of the number of measurements per plot is not straightforward (Baert, 1986; Holshof and Stienezen, 2016). We wanted to evaluate the possibility to incorporate drone-based yield estimations using canopy height models and other variables/indices that can be extracted from orthomosaics in a practical breeding program of perennial ryegrass.

Materials and methods

The experimental field was located in Merelbeke (Belgium) and consisted of 7 yield trials (Table 1). The trials were sown on April 20th 2016 and were mown 3 times in 2016 and 5 times in 2017 at a height of 5 cm. The plots were fertilized in 2016 with 92 kg N ha^{-1}, 10 kg P ha^{-1} and 76 kg K ha^{-1} and in 2017 with 311 kg N ha^{-1}, 25 kg P ha^{-1} and 315 kg K ha^{-1}. In 2018, they were fertilized on March 15th with 81 kg N ha^{-1} and 100 kg K ha^{-1}. Each plot measured 1.35×5.8 m (7.83 m^2) and each trial was bordered by plots of a reference variety. Within a trial, 2 replicates of a variety or breeding population were arranged in a randomized complete block design. The trials were mown on 2 and 3 May 2018 with a Haldrup Plot

Harvester. A sample of about 300 g fresh material was taken for dry matter content determination (72 h drying at 70 °C). Just before and just after mowing, a set of nadir overlapping images (60% forward-longitudinal-lap and 60% side-lap) was captured by a RGB (red-green-blue) sensor (model α6000, Sony Corporation, Japan) mounted on a 12-rotors UAV (model Onyxstar Hydra-12, Mikrokopter, Germany). These visual images were used to build orthomosaics and digital elevation models (DEM), based on the Structure from Motion (SfM) technique using Agisoft Photoscan v1.2.6 Professional Edition (Agisoft LLC, Russia). The DEM estimates the surface height before mowing and the stubble height after mowing. The difference between those two DEMs resulted in a canopy height model from which the mean canopy height (MCH) of individual plots was determined in QGIS 2.14.16-Essen (QGIS Geographic Information System; Open Source Geospatial Foundation Project), together with the mean intensity (MI) values per plot derived from the I-channel of the hue-intensity-saturation (HIS) colour transformation. The data were analysed in TrialWizard Version 3.12 (Beerepoot Consultancy BV, the Netherlands) to perform corrections based on 'moving mean' and 'nearest-neighbour' analysis, resulting in an average per variety/population within a trial. These averages were analysed further in R (R Core Team, 2018) for the determination of Pearson correlation coefficients and multiple linear regression analysis.

Results and discussion

The DMY of the first cut was in the range of 2,900 till 6,200 kg dry matter per ha (Table 1). The mean canopy height varied between 0.14 and 0.39 m (above stubble height). It is important to stress that all varieties and populations were in the vegetative stage at the moment of the first cut, so the influence of ears in the image analysis was not considered. Table 2 shows the correlations between FY, DMY and MCH. We found high and significant correlation coefficients for 5 out of 7 trials, but for trials Lp1602 and Lp1606 the correlation was low or close to zero and non-significant for FY-MCH and DMY-MCH (and even for FY-DMY in Lp1606). Visual exploration of the orthomosaic showed that the leaves of several varieties and populations were 'bending' in the trials Lp1601, Lp1602, Lp1604, Lp1605 and Lp1606. As the bottom-side of the leaves of perennial ryegrass is smooth and shiny, a fraction of the plots appeared as a white coloured, due to sunlight reflection. We used MI per plot as a proxy for 'whiteness' (equivalent to the fraction of bending leaves in a plot) and incorporated this in a multiple linear regression to predict yield.

The incorporation of MI resulted in a substantial improvement of the explained variance (R_a^2) and a decrease in the standard error of the estimates of FY and DMY (*SE*, Table 3), except for DMY in trial Lp1603, where no improvement was found (no significant difference between the two models). Table 1 shows that the MI range for this trial was lower than the other trials: the leaves were not bending yet in this trial with late heading material.

Table 1. Number of entries (= varieties or breeding populations), ploidy level, heading date group and ranges (=minimum-maximum value) of fresh yield (FY), dry matter yield (DMY), mean canopy height (MCH) and mean intensity (MI) of the first cut in 2018 per yield trial.

Trial	Number of entries	Ploidy level	Heading date	FY range (10^3 kg ha^{-1})	DMY range (10^3 kg ha^{-1})	MCH range (m)	MI range (-)
Lp1601	42	Diploid	Intermediate+late	23-34	3.9-5.8	0.19-0.33	76-106
Lp1602	24	Diploid	Intermediate	25-33	4.1-6.2	0.18-0.32	77-106
Lp1603	24	Diploid	Late	19-26	3.4-4.4	0.15-0.22	74-91
Lp1604	36	Tetraploid	Intermediate+late	26-39	3.8-5.5	0.16-0.39	82-108
Lp1605	42	Tetraploid	Intermediate	25-39	3.6-5.1	0.18-0.33	82-113
Lp1606	30	Tetraploid	Late	23-30	3.4-4.4	0.14-0.24	77-107
Lp1607	54	Diploid	Late	17-30	2.9-4.9	0.15-0.25	69-97

Table 2. Correlation between fresh yield (FY), dry matter yield (DMY) and mean canopy height (MCH).[1]

Trial	***rFY-DMY***	***rMCH-FY***	***rMCH-DMY***
Lp1601	0.90^{***}	0.86^{***}	0.82^{***}
Lp1602	0.89^{***}	0.53^{**}	0.34^{ns}
Lp1603	0.84^{***}	0.78^{***}	0.71^{***}
Lp1604	0.90^{***}	0.63^{***}	0.67^{***}
Lp1605	0.77^{***}	0.64^{***}	0.60^{***}
Lp1606	0.56^{**}	-0.01^{ns}	0.27^{ns}
Lp1607	0.91^{***}	0.77^{***}	0.62^{***}

[1] Correlation values were calculated with the mean of the two replicates within a trial. ns = not significant; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Table 3. Regression results with fresh yield (FY) and dry matter yield (DMY) as variables and mean canopy height (X_1) and intensity (X_2) as prediction variables.[1]

Trial	**Model 1: $Y=b_0+b_1X_1$**				**Model 2: $Y=b_0+b_1X_1+b_2X_2$**				**Comparison Model 1 – Model 2**	
	Y=FY		**Y=DMY**		**Y=FY**		**Y=DMY**		**ANOVA FY**	**ANOVA DMY**
	R_a^2	***SE***	R_a^2	***SE***	R_a^2	***SE***	R_a^2	***SE***	***P*-value**	***P*-value**
Lp1601	0.73	1.7	0.66	0.29	0.83	1.3	0.76	0.25	<0.001	<0.001
Lp1602	0.25	2.0	0.08	0.53	0.55	1.6	0.41	0.42	<0.001	0.0014
Lp1603	0.59	1.0	0.48	0.20	0.75	0.8	0.49	0.20	<0.001	0.2016
Lp1604	0.38	2.8	0.43	0.29	0.78	1.7	0.66	0.22	<0.001	<0.001
Lp1605	0.39	1.9	0.34	0.24	0.76	1.2	0.48	0.21	<0.001	0.0016
Lp1606	0.00	1.7	0.04	0.21	0.42	1.3	0.39	0.17	<0.001	<0.001
Lp1607	0.59	1.5	0.38	0.30	0.71	1.2	0.55	0.25	<0.001	<0.001

[1] Regression was performed with the average of the two replicates within a trial. R_a^2: adjusted determination coefficient; *SE*: standard error of estimate (10^3 kg ha^{-1})

Conclusion

The combination of mean canopy height and mean intensity per plot explained for 5 out of 7 yield trials with diploid and tetraploid perennial ryegrass more than 70% of the variation of the fresh yield of the first silage cut. Further research should also focus on developing a more robust calibration suitable for all trials as well as on the yield estimation of plots with generative plants (ear formation) in the second or third cut.

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Intraspecific variability of ^{13}C discrimination in response to water deficit in perennial ryegrass

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Abstract

The discrimination of isotope ^{13}C (Δ^{13}C) by plants is supposed to be a proxy of plant water use efficiency under strong genetic determinism. The aim of this study was to assess the intraspecific variability of Δ^{13}C response to contrasting water deficits in perennial ryegrass (*Lolium perenne*). The Δ^{13}C of above-ground biomass of twenty perennial ryegrass varieties grown in field conditions was measured throughout the growing season. Our results show that Δ^{13}C was negatively correlated with the intensity of water deficit. We observed a strong variability of Δ^{13}C among varieties including in well-watered conditions. In a second step, four varieties with contrasting Δ^{13}C were grown in a growth chamber under well-watered conditions. Stomatal functioning was characterized through stomatal dimensions and stomatal conductance response to irradiance variations. Our results revealed a high variability of the stomatal response to light. Some parameters related to stomatal closure and stomata length exhibited consistently a strong genetic determinism.

Keywords: forage grasses, ryegrass, *Lolium perenne*, water, drought, ^{13}C discrimination, stomata, water use efficiency, intraspecific variability

Introduction

Breeding new varieties with improved tolerance and resilience to drought is a major challenge for forage grasses used under temperate conditions with frequent summer droughts (Durand *et al.*, 2010). Water deficit is the main variable of climate change that will reduce productivity by decreasing plant growth and CO_2 assimilation due to stomatal closure. The discrimination of isotope ^{13}C (Δ^{13}C) by plants is supposed to be a proxy of plant water use efficiency (Farquhar and Sharkey, 1982). Water stress induces stomata closure and a decrease in Δ^{13}C, i.e. the organic matter is being enriched in ^{13}C compared to the atmosphere. This proxy is also under strong genetic determinism, and is therefore suitable for assessing the plant water status integrated over the growing period. In a first experiment (Exp 1), we aimed at assessing the intraspecific variability of Δ^{13}C response to contrasting water deficits in perennial ryegrass (*Lolium perenne*). Secondly (Exp 2), we investigated if the observed variability in Δ^{13}C could originate from genetic differences in stomatal conductance in well-watered conditions.

Materials and methods

Carbon isotope composition was measured using a continuous flow isotope ratio mass spectrometer. The relative abundance of ^{13}C ($\delta^{13}C$, ‰) was expressed as:

$$\delta^{13}C = (R_s - R_{ref}) / R_{ref} \times 1000$$

Where R_s and R_{ref} refer to the ^{13}C/^{12}C ratio in a sample and in caffeine (standard), respectively. Carbon isotope discrimination (Δ^{13}C) was calculated as in Roussel *et al.* (2009):

$$\Delta^{13}C \approx (\delta^{13}C_{air} - \delta^{13}C_{plant}) / (1 + \delta^{13}C_{plant} / 1000)$$

Where $\delta^{13}C_{air}$ is the isotopic composition of the air. Because $\delta^{13}C_{air}$ was not assessed in Exp 1, results are shown in terms of biomass isotopic composition ($\delta^{13}C_{plant}$). For Exp 2, $\delta^{13}C_{air}$ was assessed by growing maize plants and recording the $\delta^{13}C$ of leaves, results are therefore expressed as C isotope discrimination ($\Delta^{13}C$).

Exp 1: The $\delta^{13}C$ genetic variability was assessed in twenty varieties of perennial ryegrass (free of endophytes) from Europe and New Zealand, with similar heading dates. These varieties were tested in five locations in temperate regions of France (Beuvry-La-Forêt in the North, La Mézière and Montours in Brittany, Connantre in the East and Saint-Sauvant in the Centre West). Soil depth and texture were measured for each site and the soil water content at field capacity and permanent wilting points were determined. Meteorological data (potential evapotranspiration) were collected at the nearest weather station of the Météo France or INRA networks. Rainfalls were measured on the experimental sites. A simple soil water balance (adapted for one reservoir from Kunrath *et al.*, 2015) was used to determine the daily soil relative water content (SRWC), which is the ratio between the soil water content and soil water content at field capacity. Plots were sown in spring 2011 with high fertilization rates (300 kg N) replicated twice. A high frequency of cutting (up to seven cuts per year) was followed. The herbage was weighed fresh and the dry matter content was measured following 48 hours oven drying at 60 °C of an aliquot subsample. $\delta^{13}C$ of the leaf dry matter was measured on each herbage sample of spring, summer and autumn harvests.

Exp 2: Four varieties (Barutti, Clerpin, Delphin and Mara) with contrasted $\Delta^{13}C$ (defined from Exp 1) were grown in a growth chamber under well-watered conditions. Above ground $\Delta^{13}C$ was measured 36 days after germination in 100 plants of each variety. Stomatal conductance was monitored in response to irradiance variations in a subsample of 15 plants for each variety using a portable IRGA device (Li-6400). Kinetics of stomatal closure and opening were fitted numerically using a sigmoidal function (Schnute model). The statistical significance of the relation between each of the 11 parameters derived from the Schnute models and $\Delta^{13}C$ was assessed by Pearson correlation tests (α=0.05). Stomatal length and width were measured from leaf epidermal fingerprints obtained on 15 plants per variety.

Results and discussion

In Exp1, water deficit had a very clear impact on the average $\delta^{13}C$ of varieties (Figure 1), ranging between -31.5 and -29.5‰ under non-limiting water conditions and between -30 and -28‰ under drier conditions, i.e. under 70% of SRWC. In all sites, and mostly independently of SRWC, the data exhibited a similar genetic variability, the range between the heaviest and lightest isotopic signature being 1‰ approximately, i.e. half of the drought-induced variation.

Plants grown in well-watered conditions in Exp 2 showed a large variability in $\Delta^{13}C$ for each variety (Figure 2A). The ranking of average $\Delta^{13}C$, which ranged from ~24.2 (Clerpin, Delphin) to 24.8 (Mara), was consistent with the results of Exp 1. We also observed of strong genetic variability of the stomatal response to light variations. Some parameters related to stomatal closure exhibited a strong genetic determinism as well as a statistically significant relation with $\Delta^{13}C$ (P=0.023, Figure 2B). Stomatal length was mainly affected by ploidy (cv. Delphin is tetraploid), but no significant relation with $\Delta^{13}C$ was found.

Conclusion

$\Delta^{13}C$ in perennial ryegrass is a good proxy of the plant water status under strong genetic determinism which may be partly explained by stomatal functioning. Genetic variability of $\Delta^{13}C$ in *Lolium perenne* is conserved under moderate water deficits in the field and appeared as a relevant trait for the analysis of the genetic diversity of soil/plant/atmosphere water relations.

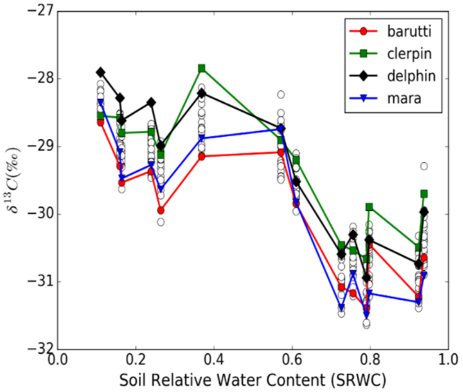

Figure 1. Relationship between the $\delta^{13}C$ of 20 perennial ryegrass varieties and the soil relative water content during regrowth. Each symbol represents the $\delta^{13}C$ of a cultivar averaged across years and locations for each SRWC. Solid lines represent the four varieties used in Exp 2 while all other varieties are shown in open circles.

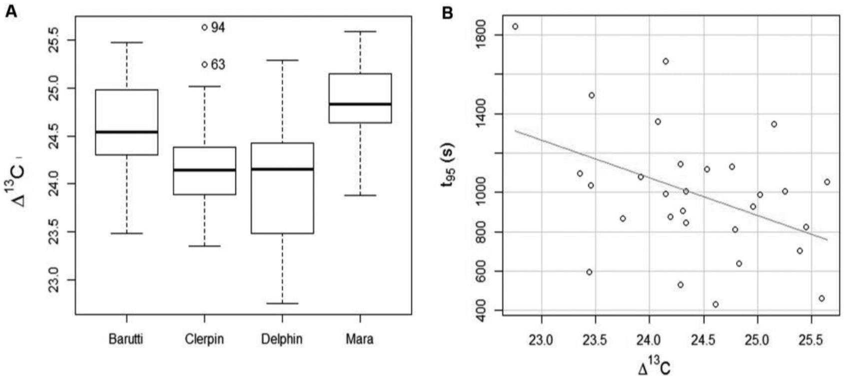

Figure 2. $\Delta^{13}C$ of the four ryegrass varieties measured in Exp2 (A) and its relation to a Schnute parameter representing the time of 95% stomatal closure (B): t95 = 5,653 – 190.9 × $\Delta^{13}C$ (R^2=0.1477).

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Evaluating LIDAR as a tool for yield measurement in perennial ryegrass and white clover monoculture sward plots

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Abstract

Tools for real-time, non-destructive and accurate measurement dry matter yield would be valuable for forage plant breeding. New LIDAR-based approaches for non-invasive assessment of forage yield may offer such a solution. A key specification for such tools is the ability to screen monoculture sward plots and develop an algorithm to measure yield with high accuracy, precision and repeatability. This would offer marked improvement over current practice, which is imprecise and/or costly, slow and destructive. Our study used LIDAR on a mobile platform for non-invasive biomass estimation in hybrid and diploid perennial ryegrass sward plots, and in white clover sward plots. This included data collection, algorithm development for volumetric estimation, and field validation. The LIDAR-based yield estimates (absolute and average) were compared against fresh weight (FW) and dry weight (DW) of mechanically harvested samples. Here we report that LIDAR is an effective tool for FW and DW measurement in perennial ryegrass and white clover monoculture sward plots, with a high level of accuracy of up to R^2=0.96). The implications of this research are that LIDAR offers a field-ready, precise, accurate and cost-effective way to measure yield in ryegrass and white clover.

Keywords: LIDAR, pasture plot, breeding, yield, fresh weight, ryegrass, white clover

Introduction

The ability to collect large volumes of reliable yield data is a longstanding constraint to forage plant breeding. Light Detection and Ranging (LIDAR) has been successfully developed for measuring yield in single rows of perennial ryegrass (Ghamkhar *et al.*, 2018), but is untested in sward plots. The two major forage species in New Zealand are perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens)* (Charlton and Stewart, 1999; McNally *et al.*, 2015), which together make up most of the country's mixed forage cultivation in order to benefit from the high nitrogen-fixation capacity of white clover and increase the productivity of perennial ryegrass (Clark and Harris, 1996; Chapman *et al.*, 2018). However, yield measurement of any mixed or individual forage species is a laborious and/or inaccurate task (Lopez-Guerrero, 2005). The aim of this study was to assess whether a ground-based LIDAR system can measure FW and DW yield in ryegrass and white clover monoculture sward plots with similar accuracy as demonstrated in ryegrass single rows.

Materials and methods

LIDAR absolute yield index or absolute biomass index and the harvested (FW) and dried (DW) herbage were compared on 60 randomly selected plots within 10 columns of ryegrass monoculture plots, and 44 randomly selected plots in one column of white clover monoculture plots. The clover and ryegrass experiments were at different sites in Canterbury New Zealand: the ryegrass experiment at a site near Darfield (43°27' S, 172°11' E) and the clover experiment at a site near Lincoln (43°37' S, 172°28' E). Plots were mechanically harvested to uniform height immediately after LIDAR scanning, weighed for FW, then dried and reweighed for DW and dry matter % calculation. The ryegrass experiment included diploid perennial (n=30) and hybrid (n=30) ryegrasses. Yield was also estimated by measuring the time of flight of laser beam between the LIDAR scanner and the herbage. An algorithm to convert LIDAR data to volumetric models used either (a) the top of the plant reflecting average height of the plots per unit of area (AVG = average biomass index) or (b) the sum of all spots of plants in the plot reflecting the total volume estimation of the plots (ABS = absolute biomass index). Seperate algorithms were developed and validated in Matlab® for grass and clover, then ported into C# to enable high-speed processing in the field. Diploid and hybrid ryegrass data were analysed separately and together, correlations calculated in Excel and data plotted using SigmaPlot.

Results and discussion

Correlation of FW and LIDAR estimates ranged from 0.87 to 0.96, and for DW from 0.84 to 0.94 (Table 1). Correlation between FW and ABS was higher for hybrid ryegrass (R^2=0.96) than diploid ryegrass (R^2=0.90). Correlations between FW and AVG for both mixed and hybrid ryegrass remained high (R^2=0.95 and 0.87, respectively), although it dropped more for the diploid material. The pattern of higher accuracy for FW than DW is consistent with results from single rows (Ghamkhar *et al.*, 2018). This result suggests that the accuracy of LIDAR measurements may be influenced by herbage mass, and the ABS model is more accurate than the AVG model.

In white clover plots, correlation between FW and ABS was R^2=0.92 (Figure 1). Although this was drawn from a sample size of 44 with limited morphological diversity, it provides initial assessment of LIDAR accuracy in white clover, which is in its very early days compared to ryegrass. The next step would be to develop models for mixed swards based on the results obtained in this study.

Table 1. Correlation of LIDAR and yield in ryegrass monoculture sward plots.

Germplasm	n	Algorithm	R^2		Mean (Std Dev)		
			FW	DW	FW (g)	DW (g)	DM (%)
Diploid	30	AVG	0.87	0.84	1,101 (174)	317 (49)	29 (1.5)
	30	ABS	0.90	0.88			
Hybrid	30	AVG	0.95	0.93	1,521 (349)	396 (78)	26 (0.8)
	30	ABS	0.96	0.94			
All	60	AVG	0.96	0.91	1,311 (347)	356 (76)	28 (1.7)
	60	ABS	0.96	0.92			

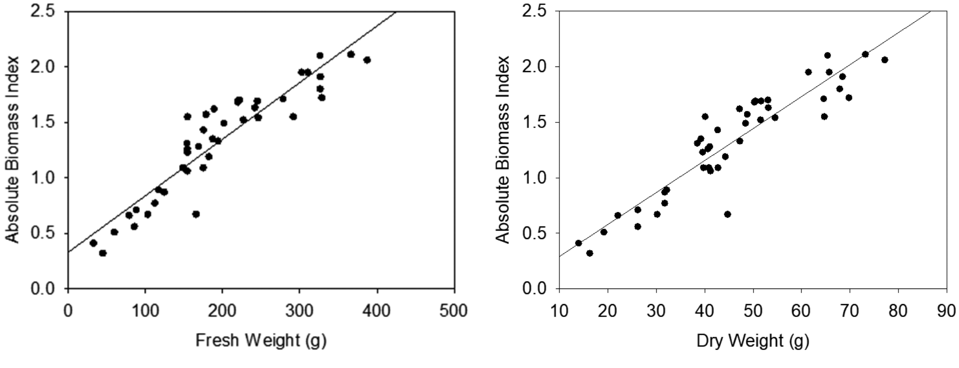

Figure 1. Correlation between LIDAR estimated volume and white clover fresh weight (left), dry weight (right). R^2=0.92 for both FW and DW correlation with LIDAR data.

Conclusion

Our data suggest LIDAR can provide an accurate tool for non-destructive measurement of fresh weight and dry matter yield in ryegrass and white clover monoculture plots. We hypothesise that grass-legume forage mixtures may also be measured non-destructively by developing models and algorithms based on the separate ryegrass and white clover algorithms developed and reported here.

Acknowledgements

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Differentiation of perennial ryegrass cultivars with regard to their leaf tensile strength

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Abstract

The objective of this work was to analyse the differentiation of selected cultivars of perennial ryegrass (*Lolium perenne*) with regard to leaf tensile strength (LTS). The investigations were carried out in 2013-2015 on plant material obtained from two cultivar-testing experiments, in which 15 diploid (2×) and 15 tetraploid (4×) perennial ryegrass cultivars (cvs) were analysed. LTS was estimated on fully developed leaf blades using a prototype testing-stand for measuring tensile strength of biological material designed on the basis of subassemblies of the Höttinger Baldwin Messtechnik Company. The fresh matter and dry matter weight, width and specific leaf area of the leaf blades were also determined. LTS of investigated cultivars differ significantly within ploidy levels and ranged from 3.16 N to 4.27 N (2× cvs) and from 4.57 N to 6.96 N (4× cvs). The LTS of the ploidy levels also differed significantly.

Keywords: cultivar, leaf tensile strength, perennial ryegrass, ploidy level

Introduction

Grass tensile strength may have implications for animal preference and on energy expenditure by animals during grazing and, consequently, on animal performance (Weinberg *et al.*, 2017). It influences bite mass, biting rate, sward preference and energy used by grazing animals (MacAdam and Mayland, 2003) and consequently affects feed intake, daily weight gain, and milk and meat production. The most important pasture grass species in temperate zones is perennial ryegrass. Information on leaf tensile strength (LTS) of breeding materials of this grass would help to select improved forage cultivars (Evans, 1964; Rogalski and Kozłowski, 1981) and to improve grassland management for better animal performance (Rae *et al.*, 1964). The objective of this work was to evaluate the differentiation of selected perennial ryegrass cultivars with regard to LTS.

Materials and methods

Leaf tensile strength of selected cultivars of perennial ryegrass was evaluated during the 2013-2015 growing seasons at the Brody Experimental Station of PULS (52° 26' N, 16° 18' E). Plant material originated from two cultivar-testing experiments established in late summer 2012. The soil was an Albic Luvisol (pH_{KCl} – 5.9, N_t – 6.80, P – 0.078, K – 0.116, Mg – 0.054 mg g^{-1}). Experimentation was done in randomized complete block designs (r=3) with plots of 10 m^2 (1×10 m, in which 15 diploid (2×) and 15 tetraploid (4×) perennial ryegrass (*Lolium perenne*) cultivars (Lp cvs) were examined. In each year the following rates of fertiliser were applied: N – 120, P – 26, K – 83 kg ha^{-1} and 4 regrowth periods were harvested. The annual mean temperature for 2013, 2014 and 2015 was 8.7, 10.1 and 10.3 °C, and total precipitation was 525, 633 and 488 mm, respectively. From each regrowth and each cultivar, 30 youngest fully developed grass leaf blades were randomly collected from plants in the vegetative growth stage (about 20 cm height – sward target for rotational grazing systems). LTS measured as force in Newtons (N) was estimated on 12 cm-long middle-section leaf blades on the day of sampling using a prototype testing-stand for measuring tensile strength of biological material designed on the basis of subassemblies (tensiometric sensors of appropriate nominal ranges, special measuring amplifiers with analogue/digital convertors) of the Höttinger Baldwin Messtechnik (HBM) Company (Goliński, 2009). The FM and DM weight, width and specific leaf area (SLA) of these 12 cm-long leaf blades were also determined. The data were analysed by ANOVA. Tests of the main effects of year, regrowth, cultivar and their ploidy level were performed by F-tests. Means were separated by the LSD and were declared at $P<0.05$.

Results and discussion

Results on the leaf tensile strength of all tested cultivars of perennial ryegrass are presented in relation to leaf blade width (Figure 1) and leaf blade weight (Figure 2). The mean values for the entire period of investigations show lower differentiation of diploid Lp cvs in LTS as well in leaf blade width and weight in comparison to tetraploid Lp cvs.

Perennial ryegrass cultivars were found to differ significantly within (Table 1) and between ploidy levels (Table 2). LTS of 2× and 4× Lp cvs were also significantly influenced by year and regrowth and their interaction. In the case of 2× Lp cvs the interaction of regrowth with cultivar was also significant. When mean values for the entire period of investigations are compared, LTS of 2× cvs of Lp was 34.2% lower

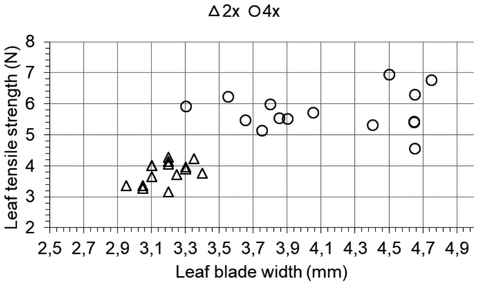

Figure 1. Leaf tensile strength of Lp cvs in relation to leaf blade width.

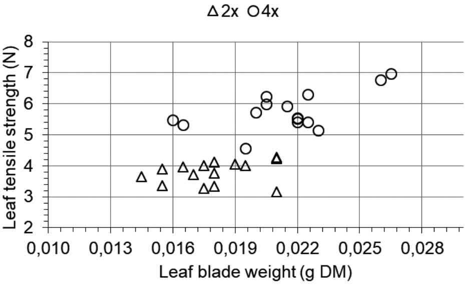

Figure 2. Leaf tensile strength of Lp cvs in relation to leaf blade weight.

Table 1. Testing of general hypotheses in three-factor analysis of variance for LTS.

Source of variation	Degree freedom for Lp 2× and 4× cvs	F-statistic value for	
		Lp 2× cvs	Lp 4× cvs
Year	2	281.8*	127.2*
Regrowth	3	348.6*	158.0*
Cultivar	14	238.8*	280.7*
Year × Regrowth	6	203.0*	81.3*
Year × Cultivar	28	0.7	1.3
Regrowth × Cultivar	42	1.5*	0.9
Year × Regrowth × Cultivar	84	1.1	0.7
Error	5,191		
Total	5,399		

[1] Significant at * $P<0.05$.

Table 2. Leaf tensile strength of Lp cvs during the total investigation period.[1]

Ploidy level of Lp cvs	Mean (N)	Coefficient of variation (%)	Cultivar means (N)		$LSD_{0.05}$ for cultivars based on plot error
			Minimum	Maximum	
2×	3.79	11.8	3.16	4.27	0.195
4×	5.76	12.8	4.57	6.96	0.318

[1] $LSD_{0.05}$ for ploidy level of Lp cvs = 0.069.

than 4× Lp cvs. The force required to break the leaf blades of diploid Lp cvs ranged from 3.16 N to 4.27 N. Thus, the difference between extreme cultivars was 1.11 N. In the tetraploid Lp cvs this difference was higher (2.39 N). It is also worth emphasising the smaller coefficient of variation recorded for 2× Lp cvs (11.8%) in contrast to a higher variation coefficient in the case of 4× Lp cvs (12.8%).

Cultivars of perennial ryegrass differ with regard to their morphological structure. For this reason it is of interest to investigate LTS with reference to selected biological features (Zhang *et al.*, 2004). Rogalski and Kozłowski (1981) reported a correlation between LTS and length and width of leaves. In our study the leaf blade weight was also considered because for the same length of leaf blade section this feature is positively correlated with leaf thickness and higher percentage of structural tissue. The negative correlation of ruminant preference with leaf strength may actually indicate a preference for grass cultivars with a higher proportion of mesophyll tissue (MacAdam and Mayland, 2003). Data shown in Table 3 confirm that 4× Lp cvs were characterised by a significantly higher LTS in comparison with 2× Lp cvs, when calculated per g fresh or dry leaf weight, per mm leaf blade width and per unit of SLA.

Table 3. Leaf tensile strength of Lp cvs in total investigation period with regard to leaf parameters.

Ploidy level of Lp cvs	Weight[1]		Width[1]	SLA	Leaf tensile strength per:			
	FM (g)	DM (g)	(mm)	(cm^2 g^{-1})	FM (N g^{-1})	DM (N g^{-1})	Width (N mm^{-1})	SLA (N (cm^2 g^{-1})$^{-1}$)
2×	0.068	0.018	3.19	219.0	56.7	216.6	1.200	0.018
4×	0.076	0.021	4.14	234.9	77.0	271.4	1.423	0.026
$LSD_{0.05}$	0.0064	0.0019	0.050	3.12	6.25	23.04	0.0222	0.0004

[1] Refers to the weight and width of a leaf blade section of 12 cm in length; FM = fresh matter; DM = dry matter; SLA = Specific Leaf Area = leaf area (width × 12 cm)/dry mass.

Conclusion

Leaf tensile strength of investigated perennial ryegrass cultivars differed significantly within ploidy levels and ranged from 3.16 N to 4.27 N (2× cvs) and from 4.57 N to 6.96 N (4× cvs). The LTS of the ploidy levels also differed significantly.

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UAV-based remote sensing used for yield prediction in temperate grasslands

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Abstract

Non-destructive remote sensing tools are useful for obtaining spatial information at larger scales. A new low-cost method in agriculture is the utilisation of RGB (red, green, blue) sensors attached to a small unmanned aerial vehicle (UAV), but little is known on the relation between RGB data and crop yield in grassland. In this study RGB imaging was used to predict yield in temperate grassland. Six types of crop were compared: two legume mixtures (clover-grass, lucerne-grass) as well as the pure stands of both legumes and grasses. All plots were harvested four times a year. UAV RGB sensor measurements were done in 2017 throughout the vegetation period every second week. In parallel to every UAV flight the biomass was assessed by manual harvesting, and crop height (CH) was also measured (50 measurements per plot). The spatial information and its derived CH were used as a predictor for dry matter yield by creating a linear regression model. The biomass yield in the experiment could be predicted well by CH. The results clearly show the advantages of remote sensing-based solutions in grassland management.

Keywords: grassland, yield prediction, canopy height, unmanned aerial vehicle, RGB imaging

Introduction

Remote sensing is a useful non-destructive tool in agriculture to reveal spatial information about plant canopy height (CH). Since biomass yield and CH are positively correlated with each other (Fricke and Wachendorf, 2013), a simple quantitative prediction of aboveground biomass can be carried out at relatively low cost. A new way to quickly cover large areas is red, blue, green (RGB) imaging through a high-resolution camera attached to a small, unmanned aerial vehicle (UAV). By photogrammetric structure from motion (SfM) processing of these digital images into point clouds, 3D spatial data can be generated. Previous studies showed that there is a correlation between the CH information from RGB images and the biomass in homogeneous cereal crops such as barley (Bendig *et al.*, 2014) and wheat (Schirrmann *et al.*, 2016). The aim of this study is to evaluate if biomass production can be predicted by UAV RGB imaging in temperate grassland, forage legumes, and grass-legume mixtures.

Materials and methods

The study was carried out at the organic experimental farm Neu-Eichenberg of the Universität Kassel (51°23' N, 9°54' E; 227 m above sea level) in northern Hesse, Germany. Field plots (1.5×10 m) of clover-grass (CG) and lucerne-grass (LG) in mixtures, as well as in pure stand of legumes (L_{CG}, L_{LG}) and grasses (G_{CG}, G_{LG}) were sown in the autumn of 2016 in four replicate blocks. CG contained 60% *Lolium multiflorum*, 30% *Trifolium pratense*, 5% *Trifolium hybridum* and 5% *Trifolium repens*, whereas LG included 40% *Medicago sativa*, 20% *Festuca pratensis*, 15% *Lolium perenne*, 10% *Lolium multiflorum*, 10% *Trifolium pratense* and 5% *Phleum pratense*. In the following year, biomass samples were taken every second week, including four harvests (17.05.17, 26.06.17, 08.08.17, 09.10.17) for the determination of dry matter yield (DMY; 105 °C). In parallel, manual height measurements were obtained (50 measurements per plot). RGB images were taken before destructive biomass sampling with a UAV (DJI Phantom 3 Professional; Shenzhen, Guangdong, China). UAV flights were conducted on days with no rain and low wind. During each flight, images (12 megapixels) were taken in a grid pattern with a spatial overlap of 80% at 10 m above the ground surface. Seven black and white targets as portable ground control points were evenly distributed and set up in the field, which were used for georeferencing the images after each flight by using a Leica DGPS. 3D point clouds from the RGB images were generated for each data set using a SfM-based imaging processing software (Agisoft PhotoScan) to calculate the digital surface model (DSM). In a further step, the software Quantum Geographical Information System (QGIS) was used to calculate the CH. To provide a digital elevation model (DEM), ground points in the path next to the plots were selected, which were interpolated to provide a continuous ground surface model over the whole field. CH was calculated by subtracting DEM from DSM. Subsequently, a mean CH value for each plot was calculated. The CH by manual height measurement (CH_R) and the UAV RGB images (CH_D) were used as an estimator for DMY using linear regression models. DMY had to be square root transformed to achieve normal distribution of data.

Results and discussion

The objective of the research was to examine the relationship between total biomass and RGB imaging in temperate heterogeneous grassland. DMY in the grass-legume mixtures varied substantially, as the crops were investigated at various growth stages through the vegetation period. Especially the sward growth in summer (2nd cut) was strongly affected by drought. CH_R and CH_D were used as an estimator for DMY (Table 1). Considering the total dataset and the individual treatments, the regression analysis for DMY and CH_R yielded in a coefficient of determination (R^2) between 0.58 and 0.78 with a root mean square error (RMSE) between 0.21 and 0.32 t ha^{-1}. CH_D correlated slightly better (R^2=0.59-0.81, RMSE=0.21-0.30 t ha^{-1}). The exclusion of the grass data from the 2nd cut resulted in higher R^2 and lower RMSE values. This effect was more evident at values of CH_R than of CH_D.

Overall the height values of CH_R varied between 9.42 and 89.54 cm and of CH_D between 1.01 and 71.06 cm (Figure 1A). On average over the entire dataset, CH_D was more than 4 cm lower than CH_R. Comparing the two measurement methods, the correlation showed an R^2 of 0.56 with an RMSE of 5.47 cm. Noticeable were the individual overestimated values on the part of CH_R. Again, after exclusion of the grass data of the 2nd cut, an improvement of R^2 with 0.70 and RMSE of 3.73 cm was observed (Figure 1B).

Malambo *et al.* (2018) showed in a field experiment with maize and sorghum that the growth stage affected the prediction accuracy of the SfM-based CH. In our study the grasses of the 2nd cut reached an earlier generative phase and, therefore, too many elongated shoots with visible ears and narrow leaves. In our study, manual height measurements were made only for a limited number of points per plot, which may be more sensitive to single protruding shoots. In addition, the results of this study indicated that

Table 1. Coefficients of determination (R^2) and root mean square errors (RMSE) of linear regression analysis between dry matter yield and manual height measurements (CH_R) as well as UAV-based RGB imaging (CH_D) for the whole dataset (All) and the different treatments: clover-grass (CG), lucerne-grass (LG) as well as the pure stands of legumes (L_{CG}, L_{LG}) and grass (G_{CG}, G_{LG}) of the mixtures, respectively. Values in brackets represent results for the dataset without pure grass swards of the second cut.

Crop type	CH_R		CH_D	
	R^2	RMSE (t ha^{-1})	R^2	RMSE (t ha^{-1})
All	0.64 (0.71)	0.32 (0.29)	0.70 (0.71)	0.28 (0.28)
CG	0.76	0.27	0.81	0.24
LG	0.69	0.27	0.63	0.29
L_{CG}	0.66	0.24	0.73	0.21
L_{LG}	0.78	0.21	0.59	0.29
G_{CG}	0.64 (0.78)	0.31 (0.24)	0.64 (0.68)	0.30 (0.30)
G_{LG}	0.58 (0.82)	0.31 (0.20)	0.65 (0.73)	0.27 (0.25)

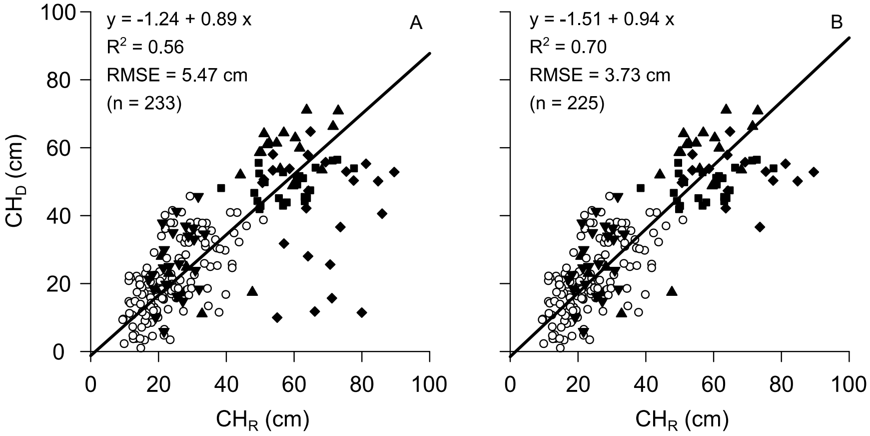

Figure 1. Linear relationship between canopy height from manual height measurements (CH_R) and UAV-based RGB imaging (CH_D) for the whole dataset (A) and for the whole dataset excluding pure grass stands of the second cut (B). The different symbols indicate sub-samples (○), which were taken between the first (■), second (◆), third (▲) and fourth (▼) harvest.

UAV RGB imaging was less susceptible to extreme values in the pure stand of grasses due to the surface cover and camera position.

Conclusion

UAV RGB imaging is a useful, resource-efficient method for biomass prediction in grassland. In this study, the advantage of SfM-based plant height measurement in heterogeneous swards became clear. In addition, UAV RGB imaging is a suitable alternative to the time-consuming manual height measurement or biomass sampling. The combination of height information with other remote sensing techniques, such as multi-spectral cameras, could further improve the prediction of biomass quantity and quality in a heterogeneous grassland.

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High-throughput phenotyping based on canopy reflectance and RGB-images for selecting drought tolerant alfalfa in rainfed Mediterranean environments

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Abstract

High-throughput phenotyping techniques can aid plant breeders to increase the efficiency of selection and are needed for implementing predictive models and genome wide association studies. The objective of this study was to evaluate the phenotypic relationship among multiple vegetation indices (VIs) and drought tolerance in an alfalfa diversity panel. Sixty-three alfalfa populations including landraces, cultivars and advanced genetics lines were stablished in two experiments that were managed under irrigated and rainfed conditions. Dry matter production was measured at two harvests during the first growing season. One day before each harvest, RGB-digital images and canopy reflectance were obtained and VIs were calculated. A phenotypic linear mixed model was implemented to estimate the best linear unbiased prediction (BLUP) using the restricted maximum likelihood method. Most VIs exhibited a significant association with DM production under drought conditions ($P<0.05$). The Normalized Difference Vegetation Index (NDVI) measured at the beginning of the growing season was the most effective selection criteria to select higher DM production population under rainfed Mediterranean environments.

Keywords: NDVI, broad sense heritability, image analyses, phenotypic correlation

Introduction

Perennial deep-rooted and drought tolerant forage legumes, such as alfalfa can improve pasture productivity in the Mediterranean region of Chile (del Pozo *et al.*, 2017). The alfalfa breeding program of INIA-Chile aims to develop drought tolerant populations for rainfed environments. Dry matter (DM) production is the most important agronomic trait in perennial forage species. Multiple trials and seasons are required by breeders to obtain reliable estimates of DM production. In early stages of a breeding program, visual scores are frequently used. In this study, DM production was considered as the key trait describing alfalfa drought tolerance in a rainfed Mediterranean environments. Today, remote sensing and digital image analyses offer high-throughput and reliable techniques for plant phenotyping. The objective of this work was to evaluate the phenotypic relationship among multiple vegetation indices (VIs) and drought tolerance in an alfalfa diversity panel.

Materials and methods

Seventy alfalfa populations provided by the Global Crop Diversity Trust Program (ID GS15014) were planted in 1×2.5 m plots. Seeds were germinated in germination trays and seedlings were established in 20 cm spaced rows every 10cm. Alfalfa populations are originally from 16 countries and they belonged to the subsp. *sativa* (62%), × *varia* (35%) and *coerulea* (3%). The alfalfa diversity panel included landrace, cultivars and advanced genetics lines. Two experiments were stablished in Cauquenes Research Station of INIA-Chile (35°57'S, 72°19'W). Experiments were managed under irrigated and rainfed conditions. The environmental conditions were characterized by the Mediterranean climate (523 mm annual rainfall) and the Alfisol soil (2.7% organic matter). Both experiments were arranged in an alpha lattice experimental design with five incomplete blocks, each with 14 populations, and with three replicates. In both experiments, dry matter (DM) production, digital images, and VIs were recorded. DM production was measured in two harvests during the first growing season (2017) and one harvest in the ongoing second growing season (2018-19). One day before the biomass harvest, digital images were taken with a conventional RGB-digital camera (Canon EOS Rebel t5i). Image analyses were performed with the software BreedPix (Casadesús *et al.*, 2007) and vegetation indices (VIs) were calculated. The VIs are based on either the average colour of the entire image, in diverse units related to its 'greenness' (HUE, Intensity, Lab and Luv), or on the fraction of pixels classified as green canopy relative to the total number of pixels of the image (GA and GGA; Table 1). During the second growing season, the Normalized Difference Vegetation Index (NDVI) was measured four times (8-14, 9-07, 11-07, 11-22-2018) with a GreenSeeker handheld sensor (Trimble, USA). The second and fourth NDVI measurements were performed one day before harvest. A phenotypic linear mixed model was implemented for estimating the variance components and predicting the Best Linear Unbiased Prediction (BLUP) of genotypic values (breeding values) using the restricted maximum likelihood method within the ASReml-R package in R software. The linear mixed model was based on a multi-location analysis (irrigated and rainfed). The variance components were used to estimate the broad sense heritability (H^2) on the population mean basis (Nyquist, 1991). Phenotypic correlation between DM production and VIs were calculated with the corrplot-R package.

Results and discussion

Most VIs derived from the RGB-images exhibited significant association with DM production ($P<0.05$; Table 1). The Lab-a index showed the highest association with DM production. Additionally, some greenness-related VIs exhibited a medium-high genetic control with H^2 values around 0.60.

The NDVI values determined at the beginning of the growing season (August and September) showed higher phenotypic variation among population than later measurements (Figure 1). Population growth and biomass accumulation at the beginning of the growing season represent the crops' ability to overcome and survive the dry season (4-5 months). In this period, NDVI values exhibited the highest genetic control with H^2 values of 0.85. Later NDVI measurements did not show phenotypic variation among alfalfa populations and they exhibited lower H^2 values (Figure 1).

Table 1. Correlation coefficient (DM-r) between dry matter production and some vegetation indices calculated in an alfalfa diversity panel grown in a Mediterranean environment, and broad sense heritability (H^2) of vegetation indices and DM production.

Vegetation indices	DM-r coefficient[1]	H^2
HUE	0.58***	0.63 ±0.09
Intensity	-0.40**	0.18 ±0.20
Lab-a	-0.62***	0.41 ±0.05
Lab-b	0.56***	0.67 ±0.06
Luv-u	-0.38**	0.29 ±0.17
Luv-v	0.55***	0.57 ±0.10
Green area (GA)	0.36**	0.54 ±0.11
Greenest area (GGA)	ns	0.26 ±0.18
$NDVI_{August}$	0.78***	0.85 ±0.03
DM production	-	0.29 ±0.06

[1] ns: not significant; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

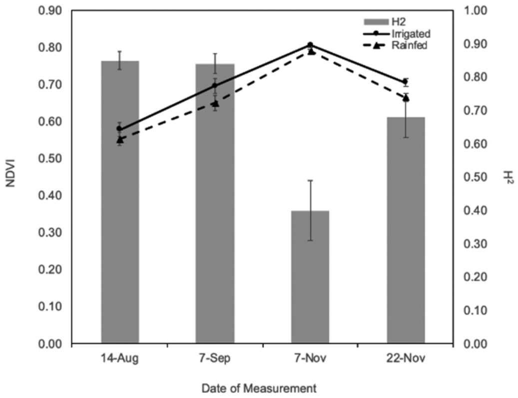

Figure 1. Normalized Difference Vegetation Index (NDVI; lines) and its respective broad sense heritability (H^2, bars) measured four times (8/14, 9/07, 11/07, 11/22/2018) in an alfalfa diversity panel grown under Mediterranean conditions.

Conclusion

Preliminary results allow us to conclude that VIs are phenotypically associated to the expression of DM production. Furthermore, some of them exhibited higher genetic control (expressed as H^2 values) than DM production. NDVI measured at the beginning of the growing season is more suitable as selection criteria to select higher DM production population under rainfed Mediterranean environments.

Acknowledgements

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DeltaGen: a freeware data analysis tool for forage plant breeders

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Abstract

Analysis supporting implementation and optimisation of breeding strategies is an ongoing challenge. The aim of this paper is to demonstrate key data analysis components within a new freeware plant breeding software tool 'DeltaGen'. We use two case studies: (1) comparing via simulation the genetic gain (ΔG) and costs per selection cycle of among half-sib (HS) family selection, versus (A_PWFgs-HS) among HS family phenotypic selection and within family genomic selection (GS), for seasonal clover content in white clover (*Trifolium repens* L.), (2) identification of breeding lines of perennial ryegrass (*Lolium perenne* L.) with broad adaptation. The analyses demonstrated quantitative genetic procedures within DeltaGen; variance component analysis, estimation of quantitative genetic parameters, genetic gain simulation and pattern analysis. The link: http://agrubuntu.cloudapp.net/PlantBreedingTool/

Keywords: plant breeding software, genetic gain, breeding methods, breeding simulation

Introduction

The efficiency of plant breeding methods can be assessed on ΔG and associated costs ($). Despite the widespread use of computation, there are few software packages that bring together the necessary functions to support such analyses by plant breeders. In this paper we demonstrate a new software tool 'DeltaGen', developed in R using the R/Shiny package. DeltaGen is an integrated tool for experimental trial design, data exploration, univariate and multivariate analysis, quantitative genetic analysis, pattern analysis, index selection, and breeding method simulation. In this paper, we will introduce this tool using two case studies: (1) genetic analysis and selection efficiency of 60 white clover HS families for mean seasonal clover content and (2) evaluation of perennial ryegrass breeding trials for seasonal growth across three years and five locations in New Zealand.

Materials and methods

Case Study 1. Estimation of genetic gain for seasonal clover content using contrasting breeding methods

A random sample of 60 HS, F_2 families were established in a transplanted field trial at Aorangi (40° 29' S, 175° 35' E), New Zealand, in early spring 2011. The experimental design was row-column with two replicates of 16 seedlings each. The HS families were planted into an established perennial ryegrass cultivar Samson (AR1) sward. After each assessment of seasonal growth (scores from 1 (poor) to 9 (high)), the trial was grazed with young beef cattle for three hours or less to ensure rapid and uniform defoliation. Data were collected for three years.

Case Study 2. Evaluation of perennial ryegrass breeding trials

A total of 118 breeding lines and 11 cultivars, were established in 2013 at five locations in New Zealand; Lincoln (43°38' S, 172°30' E), Palmerston North (40°19' S, 175°29' E), Ruakura (37°46 S, 175°18' E), Gordonton (37°40' S, 175°18' E), and Kerikeri (35°13' S, 173°56' E). Row-column designs with four replicates were used at all locations, with each plot sown as a 1m single row. The trials were grazed by sheep, using a protocol similar to Case Study 1. Seasonal growth was scored prior to grazing using a scale of 1 (poor) to 9 (high).

Data analysis

Analyses of variance of data were conducted using mixed models in the univariate option in DeltaGen (Jahufer and Luo 2018). In study 1: HS families (random effects), seasons and years (fixed effects). In study 2: entries (random effects), years, seasons and locations (fixed effects). Genetic gain in Case Study 1 was estimated using the 'Genetic Gain Simulation' option. In DeltaGen narrow sense heritability is estimated according to Nyquist (1991). For cluster and principal component analyses of the entry-by-location BLUP matrix in Case Study 2 the 'Pattern Analysis' option was used.

Results and discussion

Analysis of the data from Case Study 1 showed significant ($P<0.05$) additive genetic variation among the 60 HS families for mean clover content across seasons and years (Table 1). The interaction effects indicated relative performance of the HS families changed across seasons and years. Narrow sense heritability (h^2_n), on a family mean basis, was high.

Estimation of ΔG and costs for the HS and APWFgs–HS breeding methods (Table 2), using 'Genetic Gain Simulation' in DeltaGen, indicated that within family GS, under the set of assumptions, resulted in higher gains, and lower costs per unit gain.

In Case Study 2 there was significant ($P<0.05$) genotypic variation among the 129 entries within each location and also across seasons, years and locations (Table 3). The genotype-by-location interaction was also significant indicating a change in the relative performance of entries across locations. Pattern analysis (Figure 1) graphically summarized relative performance of the 82 common ryegrass entries across all locations. Of the four entry groups from cluster analysis, group 1 contained entries with broad adaptation to all locations.

The case studies provided a concise demonstration of key analysis procedures in DeltaGen; variance component analysis, estimation of quantitative genetic parameters, genetic gain simulation and pattern analysis. Once the field trial data were uploaded, all analyses were carried out within the single software platform. DeltaGen is available as freeware at http://agrubuntu.cloudapp.net/PlantBreedingTool/

Table 1. Case Study 1: Additive variance and interaction with seasons and years, and narrow sense heritability (h^2_n), for seasonal clover content among the 60 white clover HS families evaluated at Aorangi across three years.

Mean	Range	Additive	Additive-by-Year	Additive-by-Season	Additive-by-Season-by-Year	h^2_n
5.63	3.52-7.96	1.04±0.22	0.23±0.06	0.03±0.02	0.01±0.03	0.87±0.03

Table 2. Case Study 1: Predicted ΔG per selection cycle and associated costs for mean seasonal clover content of the 60 half-sib families evaluated across three years at Aorangi in two scenarios.[1]

Breeding method[2]	No. of replicates	No. of years	Among family selection pressure (%)	Within family selection pressure (%)	ΔG (%) per selection cycle	Cost[3] ($) per selection cycle	Cost[3] ($) per unit gain
HS	4	3	10	-	15.13	92,464	6,111
A_PWF_{gs}-HS	2	2	10	5	20.95	118,988	5,680

[1] Selection intensity = 1.76 (10%) among and 2.06 (5%) within, GS accuracy = 0.2, and parental control 0.5 for half-sib families. Costs ($) are in NZD.

[2] HS, half-sib family selection; APWFgs-HS, among-HS-family phenotypic selection and within-family genomic selection.

[3] Field trial costs ($): per single row score = 0.5, per replicate = 500 per year, per location = 25,000 per year, other (seedling establishment, polycrossing, etc.) = 10,000. Costs associated with GS: based on a cost of $90 per seedling and 100 seedlings genotyped from each of the six selected HS families.

Table 3. Case Study 2: Genotypic and genotype-by-location interaction variance components among 129 entries of perennial ryegrass evaluated across 5 locations, and 3 years.

Variance	Kerikeri across seasons and years	Kimihia across seasons and years	Palmerston North across seasons and years	Ruakura across seasons and years	Gordonton across seasons and years	Across seasons, years and locations
σ^2_A	0.39±0.06	0.17±0.03	0.24±0.04	1.5±0.2	1.2±0.2	0.45±0.07
σ^2_{AL}	–	–	–	–	–	0.26±0.02

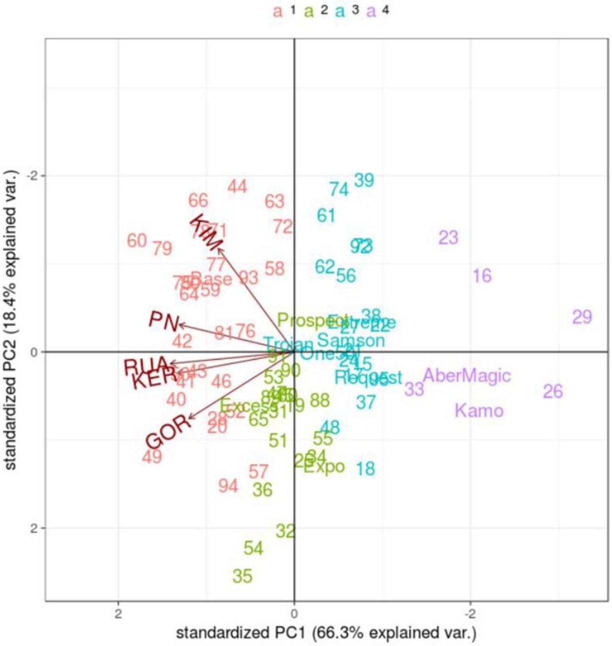

Figure 1. Study 2: Biplot from pattern analysis of the 82 common ryegrass entries-by-location BLUP matrix for seasonal growth, at KIM-Kimihia, PN-Palmerston North, RUA-Ruakura, KER-Kerikeri and GOR-Gordonton. Entry clusters indicated by different colours.

Acknowledgements

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Towards a high-throughput method to measure genetic diversity in grassland

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Abstract

Despite its economic and ecological relevance, the genetic diversity of grasses and legumes is rarely assessed in large-scale biodiversity surveys. This is due to the fact that morphology-based methods and low-throughput molecular markers are not well-suited for large-scale assessments. In addition, most grass and legume species lack the genomic information needed to develop high-throughput DNA-based methods. We hypothesize that a set of semi-conserved nuclear loci will provide enough sequence information to efficiently assess species richness and genetic diversity in mixed-species samples of grasses and legumes. We followed a targeted sequencing approach to enrich 611 nuclear loci from multiple genotypes of 16 economically relevant forage species. The target loci showed increasing within-species diversity as more genotypes were analysed, suggesting that they can be used in an amplicon-based method to measure genetic diversity on a large scale. Furthermore, some loci were also able to discriminate between species, which is a key feature for applications in mixed-species samples.

Keywords: probe capture, amplicon sequencing, grassland species, genetic diversity, high-throughput genotyping, species mixtures

Introduction

The genetic diversity within each plant species of a community is a valuable asset for grasslands. High levels of plant genetic diversity may stabilize and increase plant population yields (Abbott *et al.,* 2017). Furthermore, the genetic diversity of grasses and legumes, the most economically relevant plant families in grasslands, provides the basis for breeding superior forage crop cultivars.

Utilizing and protecting genetic diversity in grasslands requires detailed measurements on a large scale. However, biodiversity assessments of plants have so far focused largely on species richness, due mainly to technical limitations for measuring genetic diversity (Taberlet *et al.,* 2012). Developments in the area of high-throughput DNA sequencing and genotyping offer new possibilities to assess genetic diversity and species richness on a larger scale.

We hypothesize that a set of nuclear loci will allow us to measure genetic diversity in grass and legume populations and, at the same time, discriminate species in mixed samples. In this work, we assessed the within-species genetic diversity and the between-species discrimination potential of 611 nuclear loci. Those 611 loci are shared by a phylogenetically diverse set of plants, which comprises two grasses (*Lolium perenne* and *Brachypodium distachyon*), two legumes (*Trifolium pratense* and *Glycine max*), as well as *Arabidopsis thaliana*, *Theobroma cacao*, *Solanum lycopersicum*, and *Vitis vinifera* but occur only once in each genome. Our aim is to find the 10 to 20 most sequence-diverse loci, which we will use to produce an amplicon-based method to detect genetic diversity and species richness in grasslands on a large scale.

Materials and methods

Genomic DNA was extracted from plant individuals of 16 forage species (*Arrhenatherum elatius, Alopecurus pratensis, Cynosurus cristatus, Dactylis glomerata, Festuca pratensis, F. rubra, Lolium multiflorum, L. perenne, Phleum pratense, Poa pratensis, Trisetum flavescens, Lotus corniculatus, Medicago* *sativa, Onobrychis viciifolia, Trifolium pratense*, and *T. repens*). Each species was represented by 5 plants from three cultivars. 80 dual-indexed Illumina libraries were prepared for each plant, and 16 additional libraries were prepared with the pooled DNA of all plants from each species. The fragment size of the libraries was ~550 bp. Sequence capture was performed on the libraries with a custom MYbaits® v4 kit (Arbor Biosciences, MI, USA). The 100-nt long baits were designed to target 611 nuclear loci. The enriched libraries were sequenced (Illumina MiSeq) and separate *de novo* assemblies were made with one library of each species. Quality-controlled reads were mapped to their respective *de novo* assemblies. We used *k*-mer richness to measure sequence diversity at each locus in an increasing number of genotypes of the same species. *K*-mer calculations were performed with $k=25$ and a coverage cut-off of 10. Clustering based on k-*mer* composition was done with *kWIP* (Murray *et al.*, 2017).

Results and discussion

A total of 13,827,916 high-quality paired reads were obtained, of which 2,199,853 reads (15.91%) were on-target (Table 1), a rather low proportion. However, the mean depth at every on-target nucleotide position for all libraries was 33.39X, which is enough to capture the sequence diversity of the different alleles of the target loci in diploid plants. In contrast, the mean off-target sequencing depth was 2.38X, which indicates that off-target reads come from random pieces of the genome, while the on-target reads are concentrated on the loci for which the sequence capture baits were designed. In addition, legume samples had more on-target reads than grass samples (1,583,144 reads vs 616,709), even though grasses represented the majority of all the sequenced libraries. Thus, the overall low read mapping rate could have been due to hybridization issues when pooling samples from different species on the same sequence capture reaction.

A direct relationship between *k*-mer richness at each locus and number of plants (i.e. distinct genotypes) was observed (Figure 1A). As more plants are considered for analysis, the *k*-mer richness at each locus increases, indicating that these loci are suitable to detect within species diversity. Clustering of the reads mapping to one of the 611 loci (uce-11004050; Figure 1B) allowed to separate all individual legume plants according to the respective species. For grasses, the separation was less clear, although some species were almost completely resolved. Inconsistencies in species clustering can be due to differences in *k*-mer sequencing depth, since grasses had lower on-target mean sequencing depth than legumes did.

Table 1. Sequence capture summary results.

Group of libraries	Raw reads	QC reads	On-target reads	On-target mean depth	Off-target mean depth
Grasses	10,373,596	9,821,623	616,709 (6.28%)	15.78X	2.15X
Legumes	4,170,860	4,006,293	1,583,144 (39.52%)	59.92X	3.48X
Total	14,544,456	13,827,916	2,199,853 (15.91%)	33.39X	2.38X

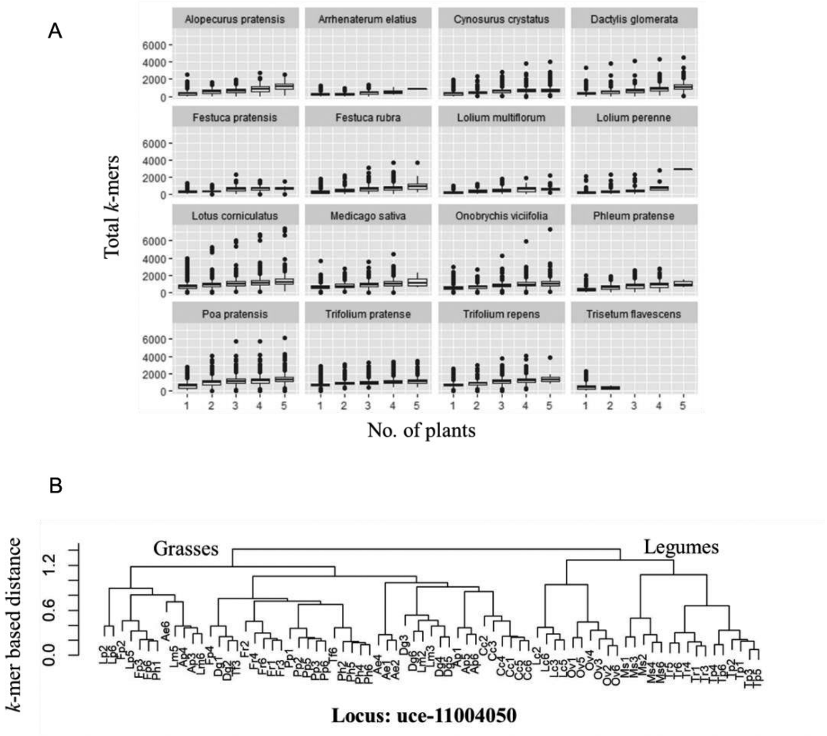

Figure 1. (A) The sequencing reads of one to five individual plants were categorized by locus and *k*-mer richness was calculated. Each data point corresponds to one locus. Each subplot corresponds to one species. Only *k*-mers with sequencing depth higher than 10× were considered. (B) Clustering of individual plants based on *k*-mer composition for locus uce-11004050.

Conclusion

Overall, the targeted 611 loci reflect the genetic diversity present in the different genotypes that were analysed for each species. Some loci, like the locus uce-11004050, are also able to distinguish species from each other. Our results constitute the first steps towards an amplicon-based method to measure genetic diversity in grassland plants. The loci displaying highest levels of between-species discrimination success and highest within-species diversity will be selected for further analysis.

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Inferring the date of floral transition from environmental data and heading date in perennial ryegrass

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Abstract

The present study describes a method for inferring the date of floral transition (FTD) from heading date (HD) observations, in order to analyse the interactions between genetic diversity and climate. The dataset included HD measurements made by the French Variety and Seed Study and Control Group (GEVES) on 20 cultivars of perennial ryegrass (*Lolium perenne*) for seventeen years and across seven French locations. The time between FTD and HD was computed using a temperature driven model based on a simplified beta response function. A high genetic variability of HD was observed. The method gave some good results for FTD but didn't succeed in some cases, driven the question of its generalisation.

Keywords: phenology, *Lolium perenne*, floral transition, heading date

Introduction

The impact of global warming on grassland productivity is a crucial issue for agriculture. Floral transition of temperate grasses is classically linked to the increase of biomass production of grasslands that occurs in spring (Parsons and Robson, 1980). Cultivars of grass species exhibit a large variation of phenology (Sampoux *et al.*, 2011), then a precise determination of the floral induction date (FTD) is needed to better understand and predict grassland production under the actual and future climates. For most cultivars in *Lolium perenne*, floral transition is achieved in two steps (Heide, 1994). First, the vegetative apex has to undergo a vernalisation period that is satisfied by its exposition to low temperatures. Only after this period, the apex becomes sensitive to photoperiod. Then, floral transition is completed only if the plant is exposed to long days. Critical values of temperature, daylength and induction phase durations (vernalisation and photoperiodic) are cultivar-specific (Aamlid *et al.*, 2000). After floral transition, production of leaf primordium stops and the reproductive structures (stem and spike) start to grow. The duration between FTD and the heading date (HD) is therefore mainly determined by the growth of the stem, which may be estimated using a simple temperature driven growth model, when water and mineral nutrition are non-limiting. Using a dataset of HD for multiple cultivars of *Lolium perenne* grown in seven locations in France for seventeen years, we developed a method to infer FTD taking into account the growing conditions preceding HD. We based our work on two main hypotheses. First, we assumed the FTD of each cultivar to be similar between years and locations because within our dataset, (1) the vernalisation was always completed and (2) the differences of latitude between locations result in negligible variations of photoperiod. Second, we assumed that the duration between FTD and HD was cultivar-specific and could be estimated from a simple temperature-driven growth model.

Materials and methods

The HD in twenty *Lolium perenne* cultivars were measured by the French Variety and Seed Study and Control Group (GEVES) from 2001 to 2017 on seven sites in temperate locations of France. Plants were implanted in rows in spring and fertilised to ensure optimal growth. HD is defined as the first date at which at least ten spikes per linear meter were visible. It was monitored three times a week in the second spring. Meteorological data were obtained from local INRA and Météo-France weather stations. Using the local meteorological data and soil depth, a water balance of the winter and the spring was computed in order to exclude from this analysis the years and sites where field capacity before HD was under 50%

(Allen *et al.*, 1998). The response of the growth rate of spike and stem to temperature was represented by a *beta* function (Yin *et al.*, 1995). Based on daily mean temperature, the model computes an 'efficient day' of plant growth, ranging from zero when daily temperature was lower or higher than minimum and maximum temperatures, respectively, to one for optimum temperature. These cardinal temperatures of *Lolium perenne* development being genetically invariant (Zaka *et al.*, 2017), the same *beta* function parameters were used for all cultivars. For each combination cultivar-year-site, we considered each date between 1st January and observed HD as a potential FTD (FTD_{pot}) (Figure 1A). For each FTD_{pot}, we calculated a duration, expressed in efficient days, to reach HD, using the model described above (Figure 1B). Then, for each cultivar, we evaluated the variance of the duration 'FTD_{pot} – HD' between years and sites for each potential FTD. The likeliest FTD of each cultivar was assumed to be that minimises the variance of the FTD_{pot}-HD duration (Figure 1C). The statistical unicity of the minimum variance was tested using the statistical Levene's test (R software).

Results and discussion

GEVES measurements revealed a high genetic variability with heading occurring between days 110 and 155 (Figure 2). In eleven significant cases, the FTD inferred using our methodology was realistic, *i.e.* consistent with minimum daylength reported in the literature (Heide, 1994). However, it was not possible to infer a significant FTD for nine other cultivars. Two main reasons could be suggested to explain these results. First, the mathematical approach used to calculated the effective growth days tends

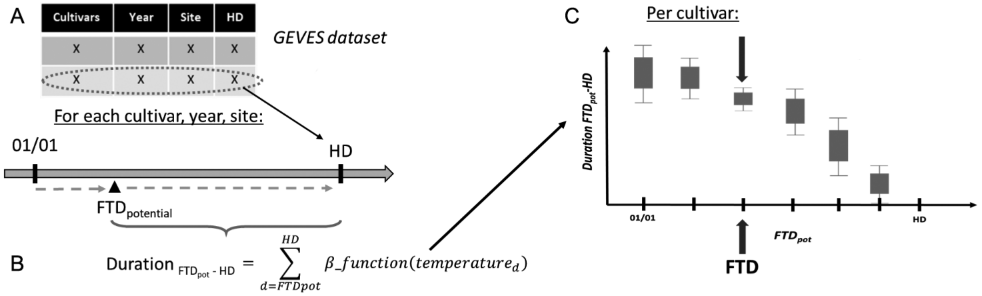

Figure 1. Description of the method used to infer FTD from HD and climatic context.

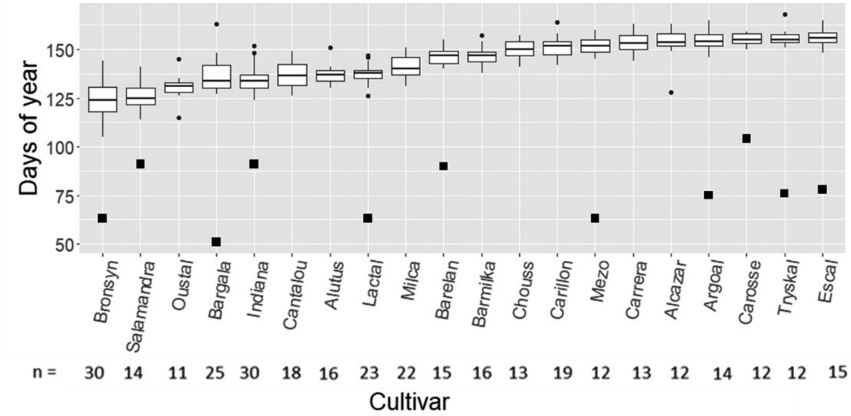

Figure 2. Observed HD of twenty cultivars of *Lolium perenne* (boxplot) and the eleven significant calculated FTD in day of year (black squares). Boxplots represent the variability of HD between year-location combinations for each cultivar (plot in the style of Tuckey). 'n' values under are the number of occurrences of each cultivar.

to minimise the 'weight' of cold days compared to the warm days, thus reducing the precision in the assessment of the duration between FTD and HD for cold years. Secondly, studies with cereals cultivars suggest that the number of leaves to grow before the first spikelet depends on the dual induction timing (He *et al.*, 2012). This variation in the final number of leaves may affect the duration of growth following FTD, which was not taken into account by our approach.

Conclusion

Lolium perenne cultivars present a high variability in HD, which is of interest for studying reproductive period in perennial forage grasses. This variability is explained at the same level by genetic and environmental factors. The method used gave realistic FTD with, however, no relation with HD. This result highlights that flowering in *Lolium perenne* cannot be represented with a simple method as it is currently done. Interaction between vernalisation and photoperiodic induction may take place in determining plant HD. Attention should be paid to relation between the induction phases and the plant morphogenesis to improve prediction of FTD.

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Capturing plant height from images taken with a drone to provide information for use in breeding forage grasses

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Abstract

In a nursery of isolated cocksfoot plants, the natural height of the plants was measured either with an electronic graduated ruler or estimated by 3D reconstruction from photos taken at several heights from a drone. This first experiment showed that 4.5-9 m was the optimal height for drone photography. At this height range, there was a correlation (r^2) of 0.77 between the two methods. Furthermore, the regression line is close to the y = x line. The drone method allows for comparable differentiation between varieties to that possible by manual measurement, while providing significant time saving and reducing the arduous work of manual measurement.

Keywords: cocksfoot, phenotyping, drone, Structure-From-Motion (SfM) photogrammetry

Introduction

Grass breeders are usually challenged by the phenotyping of numerous individual plants in nurseries for highly heritable traits, and for traits correlated with yield at the plot level. The best plants will then be polycrossed to produce the first generation of selection. In this approach, heading date and plant natural height are traits of particular interest. The kinetics of regrowth over cuts and the seasons is a further essential trait for selecting for specific management (Hazard and Ghesquière, 1997) as well as detecting the ability for growth when drought occurs or temperatures becomes limiting. However, capturing growth rate at individual plant level is often unaffordable by breeders. Measurement has to be done frequently and over a short time span. This study reports how 3D photogrammetry (Malambo *et al.*, 2018; Chang *et al.*, 2017), using a camera on a drone, could replace conventional plant height measurement that uses an electronic graduated ruler. The main aim was to evaluate how shooting height of the drone may affect the accuracy of plant height estimation.

Materials and methods

A nursery of 57 varieties of cocksfoot (*Dactylis glomerata*) under a 3-complete block design with single plot as a row of 10 plants spaced at 0.70×0.70 m apart was used to calibrate 3D-imaging. On 21 September 2017, natural plant height was measured using an electronic graduated ruler (SODALEC) by sampling 4 plants in each row in the whole design; i.e. a total of 684 plants. On the same day, a drone (DJI, Phantom 4 Advanced) photographed the whole design by shooting at heights of 4.5, 9, 14 and 19 m. From the 3-dimensional pixel data set, the shape of each plant was rebuilt by photogrammetry using the Agisoft Photoscan software (Agisoft LLC, St Petersburg, Russia). Plant height was estimated as the difference between the maximum height of each plant and the average height of surrounding ground. Individual plant heights were then compared with the height given by the electronic graduated ruler.

Results and discussion

The main characteristics of image-capturing and data process at the four heights of the drone are given in Table 1. When the shooting height was increased, the number of shootings required to encompass all the design decreased, while the pixel size is also increased. The highest accuracy (r^2) was found for shooting heights between 4.5 and 9 m. Pixel size was the smallest and so was distinctness of the image, by 1.1 mm at 4.5 m shooting height against 4.9 mm at 19 m shooting height. This was by the smallest pixel size that

Table 1. Mean and accuracy of cocksfoot plant height estimated by a drone photography and main characteristics of image-capture according to shooting height.

	Flight height (m)			
	4.5	**9.0**	**14.0**	**19.0**
Coefficient of correlation (r^2)	0.77	0.77	0.69	0.62
F variety test	2.15	3.14	2.71	2.36
Mean plant height (mm)	259	227	219	210
Flight time (h)	1.5	1	1	0.5
Number of images	1,249	729	688	622
Pixel size (mm)	1.11	2.25	3.65	4.89
Image memory size (Go)	8.3	4.9	4.6	4.1
Processing time (h)	79	34	40	39
3D-shape memory size (Go)	6.6	3.0	1.5	1.1

the plant height estimate was the closest to the plant height given by the ruler, 259 mm at 4.5 m shooting height against 273 mm manually, while it was 210 mm at 19 m of shooting height.

The accuracy of plant height estimated by drone photography is given by the squared coefficient of correlation between the two methods of measurement. With r^2 of 0.77, individual plant height in a nursery is estimated fairly accurately by a drone, given that r^2 between two successive measurements by an electronic graduated ruler is about 0.87. Only the height of the smallest plants tended to be underestimated by the drone (Figure 1). Regression value close to one indicates that the estimate can be directly used without any transformation.

From the breeder's perspective, it is essential to know how to correlate manual and drone measurements between variety means. This is shown in Figure 2. With a r^2 of 0.88, ranking among variety means did not change significantly across methods even though drone resulted in a bias about 13 mm relatively to ruler measurement. F-test values with variety as a factor remained close between shooting heights (Table 1) and with F-value from the graduated ruler (F=2.90).

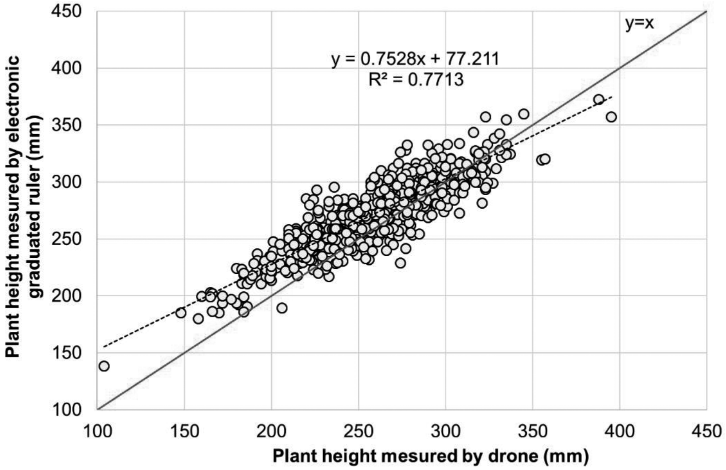

Figure 1. Correlation of individual plant height measured by electronic graduated ruler with an estimation from image-capturing by a drone in a 0.70×0.70 m spaced-plant nursery of 684 individuals of cocksfoot.

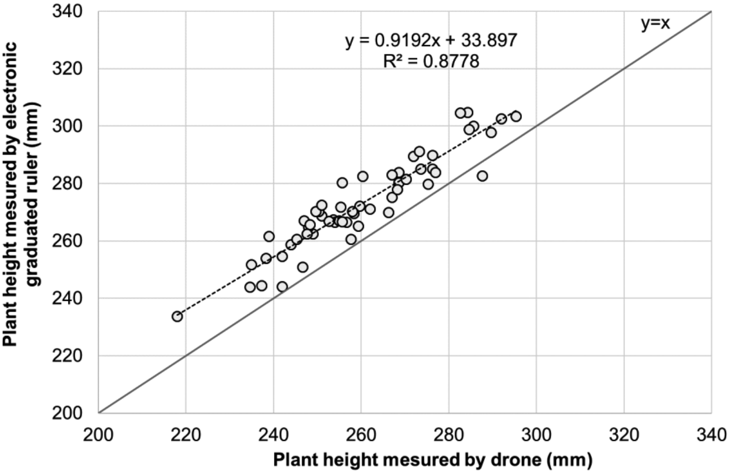

Figure 2. Correlation of mean plant height measured by electronic graduated ruler with an estimation from image-capturing by a drone at the scale of 57 cocksfoot varieties grown in a 0.70×0.70 m spaced-plant nursery (same plants as used in Figure 1).

As a main result, drone measurements enable considerable saving in time, with no loss in accuracy, to consider kinetics of growth by repeated measures on thousands individual plants. In the present study of 1,710 single plants, 1 h 30 was required to operate the photography of the whole design, plus 1 h needed for processing data and parametrizing the software. By contrast, it took 4 h 30 to measure manually plant height in all the design. Furthermore, promising data can be drawn from 3D-imaging for plant diameter and volume. On the other hand, it is obvious that new human expertise and computing resources are necessary for replacing of manual assessment by drone methods.

Conclusion

The use of photogrammetry from photos taken with a drone makes it possible to estimate the height of individual plants in a nursery. We will work on the use of this method to estimate the growth rate after cutting and, if possible, the biomass of forage species in micro-plots.

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Measuring height of red fescue (*Festuca rubra*) plots using drone imagery

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Introduction: The height of a grass crop can be an indicator of its seed production potential. If grasses become very tall, there is a risk of lodging and, if a grass crop is established with the purpose of producing seeds, lodging during the flowering period may result in inadequate pollination and low seed yield. Plant growth regulators (PGRs) can be applied to prevent lodging, and therefore information about crop height is relevant. In plant breeding, crop height is also a valuable trait. This study assesses a method for deriving red fescue (*Festuca rubra*) height from high spatial resolution (1.4 cm/pixel) 3D digital surface models of crop field trials produced via Structure from Motion (SfM) photogrammetry using aerial imagery.

Materials and methods: A field experiment with red fescue was established in 2014. Four different seeding rates (2, 4, 6, and 8 kg ha^{-1}) were sown, and four different dosages (0, 0.3, 0.6, and 1.2 l ha^{-1}) of a plant growth regulator (Moddus M (250 g l^{-1} trinexapac-ethyl, Syngenta)) were applied. In 2016 and 2017, aerial images were captured from a drone in two horizontal directions at 30 m altitude. Images were overlapping in order to generate 3D digital surface models from Structure from Motion (SfM) photogrammetry. The SfM algorithm was implemented in the Pix4D software (https://www.pix4d.com/). Crop height was not measured from the ground using other methods.

Results: The workflow was fast and reliable for crop height measurements. Two different methods for height measurements were demonstrated. A visualization of crop height derived from a 3D point cloud is shown in Figure 1.

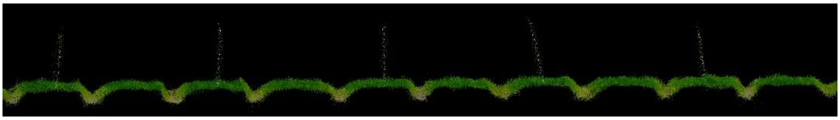

Figure1. 3D point cloud of a cross section of ten red fescue (*Festuca rubra*) plots based on a 3D model generated from drone images.

Conclusion: It is possible to achieve reliable height measurements of experimental plots using drone imagery. Height measurement using drone imagery has the advantage that the drone can capture the images within a short time (in our case about 10 minutes), and the data can then be stored and processed later when the researcher has time for analysis. The images can give very detailed site-specific information which is useful for interpreting field trials.

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Lucerne root traits can help in variety evaluation in lucerne-grass mixture

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Introduction: Growing lucerne (*Medicago sativa* L.) in mixture with grass is a common practice but less attention has been paid to development of lucerne below ground biomass under concurrence with the grass component. A recent study described a positive relationship between lucerne root morphology traits and stand forage productivity in a lucerne-grass mixture (Hakl *et al.*, 2018). Our objective was to investigate the relationships between root traits and lucerne variety performance in mixture with a companion grass.

Materials and methods: In 2008-2011 an experiment was conducted at the experimental field station Červený Újezd, Czech Republic. Treatments were a factorial combination of four lucerne varieties (Jarka, Oslava, Tereza, Zuzana) and three lucerne sowing ratios: lucerne monoculture (L100), 90% (L90) and 75% (L75) lucerne proportion sown with grass hybrid *Festuca pratensis* × *Lolium multiflorum* (variety Achilles). Each autumn, lucerne plants were removed by digging to a depth of about 20-25 cm in each plot and evaluated for root morphology traits. The root potential index (RPI) was calculated as the area of tap-root diameter cross section in cm^2 per m^2. In each cut lucerne forage dry matter (DM) yield, total DM yield of the mixture, and aboveground forage traits were measured. The measurement of all traits was described by Hakl *et al.* (2018). The data were analysed by two-way ANOVA.

Results: Within years, values of RPI between varieties corresponded to differences in their forage yield. In the last year of the experiment, variety Tereza provided the lowest RPI together with the significantly lowest lucerne yield, whereas Zuzana achieved the highest lucerne and total yield in line with the highest RPI value. Our results demonstrate that the higher RPI value could be a suitable indicator for lucerne variety productivity in the mixture because it supports higher lucerne stem density as the trait with the best correlation with lucerne yield (Hakl *et al.*, 2018).

Table 1. Root potential index (RPI, cm^2 m^{-2}), lucerne forage dry matter yield (L-DMY, t ha^{-1}) and total DMY of mixture (T-DMY, t ha^{-1}) averaged across sowing ratios of evaluated lucerne varieties.[1]

Variety	2009			2010			2011		
	RPI	L-DMY	T-DMY	RPI	L-DMY	T-DMY	RPI	L-DMY	T-DMY
Zuzana	94.51	14.40^a	16.77	108.64	15.36	17.45	85.71	14.61^c	15.81^b
Tereza	92.15	15.45^b	17.35	112.41	15.82	17.59	68.79	13.08^a	14.27^a
Jarka	83.26	14.76^a	16.99	85.04	15.13	16.88	72.63	13.91^b	14.81^a
Oslava	98.24	15.68^b	17.23	109.96	15.98	17.79	75.69	13.15^a	14.58^a
P	0.412	0.001	0.208	0.120	0.198	0.235	0.351	<0.001	<0.001
SEM	6.433	0.225	0.207	8.885	0.309	0.323	6.845	0.225	0.236

[1] Means with a same letter are not different at 5% level (Fisher LSD).

Conclusion: Root potential index can be considered an effective indicator for monitoring lucerne root development in lucerne-grass mixtures with regard to lucerne variety performance in the mixture. Understanding the relationships between root morphology and stand productivity can help in making effective evaluations of lucerne varieties.

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The ^{15}N natural abundance and enrichment techniques provide similar estimates of N transfer

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Introduction: Nitrogen (N) transfer from clover to grass could present a significant contribution of symbiotically fixed N to grass nutrition in clover-grass swards. However, depending on the method of estimation, the proportion of transferred N might differ. The aims of this study were (1) to compare the ^{15}N natural abundance (NA) and the ^{15}N enrichment technique to estimate the N transfer from clover to grass and (2) to test the applicability of the NA technique under different fertiliser strategies affecting the ^{15}N soil background value.

Materials and methods: The ^{15}N NA and enrichment (clover leaf labelling) technique were compared in sub plots of a long-term cropping-system field experiment (Hammelehle *et al.*, 2018) using treatments supplied with no fertiliser, animal manure (δ^{15}N of faeces: 6.7‰ and of slurry: 10‰), or mineral N fertiliser (δ^{15}N: 0.5‰). ^{15}N values and N contents of yields, stubbles, and roots were determined from *Trifolium pratense – Lolium perenne* mixed and *L. perenne* pure stands. Estimates of N transfer were determined using different surrogates of plant available soil N and clover and grass plant parts when using NA (Daudin and Sierra, 2008) and using different experimental setups when using ^{15}N enrichment (Giller *et al.*, 1991). The design of the study was a split-split plot. Data were fitted to a mixed effect model.

Results: During two consecutive cultivation years, the proportion of transferred N was considerable and was in the same range for both methods (^{15}N NA: 34%-42% of grass N; ^{15}N enrichment: 27%-46% of grass N). The ^{15}N enrichment technique suffered from a temporally non-uniform ^{15}N labelling of the clover root (results not shown). The NA δ^{15}N of pure stand grass as surrogate of plant available soil N tended to underestimate the proportion of N transfer, especially in the 1st year of cultivation (Table 1).

Table 1. Development of δ^{15}N values of clover and grass yields over two years from NA mixed and pure stands (mean of n=15; SEM=0.2).[1]

Species	Stand	δ^{15}N [‰] yield of harvest		
		2 MC	6 MC	17 MC
Clover	mixed	-0.5 e	-0.5 e	-0.2 e
Grass	mixed	3.9 ab	1.8 d	2.6 cd
Grass	pure	3.8 ab	3.3 bc	4.4 a

[1] Same letter indicates no difference between means at α<0.05 (post hoc *t*-test); MC: months of cultivation.

Conclusion: Independent of the ^{15}N method, about 1/3 of grass N derived from N transfer. Adequate NA procedures can be used even in the presence of a range of fertiliser types with differing δ^{15}N values, if soils have been under these fertilisation strategies for many years and fertilisation remains unchanged during the study.

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How accurate is the HarvestLab™ 3000 system in measuring dry matter concentration?

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Introduction: There is increasing interest in pasture-based dairy systems in Europe. These are often seen as cost-effective for providing feed, but their forage quality changes constantly. Hence, to be able to manage pasture precisely, we need to automate measurements of grass yield and quality in real-time, repeatable in the field. Near infrared spectroscopy (NIRS) is becoming more common as a measurement technique in feed quality analyses (e.g. Ampuero Kragten and Wyss, 2014) but there is little research on portable on-farm systems. Therefore, we evaluated the commercial system HarvestLab™ 3000 (Deere & Company, Moline, USA).

Materials and methods: The HarvestLab™ 3000 uses NIRS to measure a number of herbage quality indicators, such as dry matter content (DM, %). Thus, fresh herbage samples were collected on six locations in Central Switzerland with three replicates each at five observations throughout the vegetation period of 2018. Due to severe drought during the summer, four observations are missing, leading to a total of 78 samples. From each experimental plot, one square metre was cut in order to simulate a grazing situation. Thereof a mixed sample was analysed three times by the HarvestLab™ 3000 (calibration version 2017/21/03) and the mean value was recorded. Following this, the samples were oven-dried at 60 °C and the DM content calculated in the laboratory. To evaluate the accuracy of the HarvestLab™ 3000 measurements they were compared with the laboratory results.

Results: With a Pearson correlation coefficient of R=0.87 the HarvestLab™ 3000 measurements correlate well with laboratory results. According to Taylor (1990), the reached correlation can be interpreted as high. The standard error of prediction was 2.21% DM, whereas the laboratory instrument NIRFlex N-500 (Büchi Labortechnik AG, Flawil, Switzerland) reaches 0.29% DM (Ampuero Kragten and Wyss, 2014). Figure 1 shows a tendency that the system is overestimating the DM content when it is low and underestimating it when it is high.

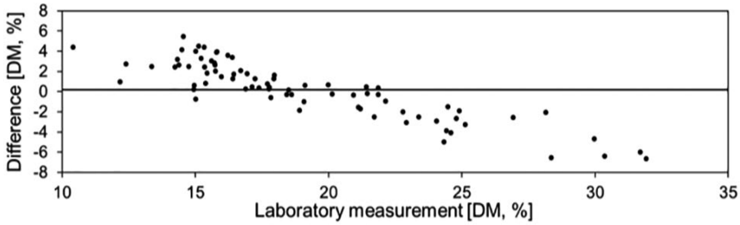

Figure 1. Laboratory results plotted against the difference to the HarvestLab™ 3000. The line indicates the mean difference between the two measurements (0.21% DM).

Conclusion: The HarvestLab™ 3000 is a rapid, user-friendly system and a good alternative to oven-dried samples. We conclude that the system is a suitable method to determine DM content on pastures, except under extremely dry or wet conditions. It can provide information to support pasture allocation and feed ration balancing.

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Comparison of different sensing technologies to estimate LAI of managed grassland

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Introduction: Climate change induced shifts of grassland growth dynamics require comprehensive monitoring to support optimal grassland management. Leaf Area Index (LAI) represents one of the most promising biophysical remote sensing parameters to monitor grassland (Darvishzadeh *et al.*, 2008). The objective of our study is to compare LAI time series of managed grassland derived from the earth observation satellite Sentinel-2 (S2), along with LAI from hyperspectral reflectance (HR) acquired by a field spectrometer (HandySpec/tec5) and in situ measurements from the AccuPAR LP-80.

Materials and methods: Sixteen HR measurements and twenty-four AccuPAR readings are obtained weekly at each of three subareas (20×20 m) within a cultivated grassland-field (4.6 ha) with four cuts per year. During the campaign, twenty-two cloud-free S2 images were acquired for the study site. The pre-processed images and products are downloaded from the 'Sentinel-2 value adder' (Vuolo *et al.*, 2016). LAI from HR is calculated by a neural net algorithm with fixed weights using an eight-band combination together with sun position information. This procedure is identical to the LAI calculation from S2 reflectance data.

Results: The Pearson correlation coefficient (r) between S2-LAI and HR-LAI is 0.97 ($P<0.001$), indicating a very strong positive linear relationship. Between S2-LAI and AccuPAR-LAI (r=0.88, $P<0.001$), as well as between HR-LAI and AccuPAR-LAI (r=0.84, $P<0.001$), a slightly lower but still high correlation can be observed. In advanced growth stages the AccuPAR-LAI tends to increase stronger and subsequently saturates due to consistent low photosynthetically active radiation below the canopy (Figure 1).

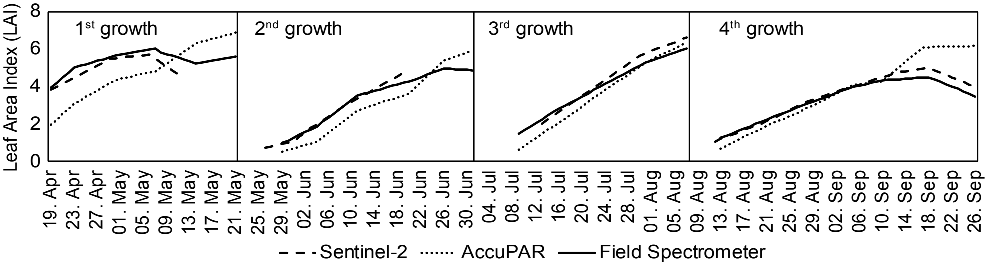

Figure 1. Development of LAI derived from different sensors for each cut in 2018.

Conclusion: We conclude that LAI time series of managed grassland from all three sensors are highly correlated, especially at early growth stages. Our findings are of increasing importance regarding the applicability of different LAI sources for grassland monitoring.

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Improving nitrogen use efficiency in forage bermudagrass (*Cynodon dactylon*)

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Introduction: Warm season perennial grasses are the foundation of pasture systems in the Southeast USA. Bermudagrass became the most important forage due its ability to adapt to a wide range of soils, aggressive growth, grazing tolerance, and ability to produce high yields of good quality forage when soil nutrients are not restricted. The forage varieties of bermudagrass require large amount of nitrogen (N) fertilizers for optimum growth and production. Only 50% of the N added onto cropland is converted into harvested products. Crops with higher nitrogen use efficiency (NUE) is critical to reduce costs and maintain agricultural production in a sustainable way. The overall goal of this study is to reduce N-fertilizer inputs without reducing yield and quality.

Materials and methods: A screening protocol was developed for evaluating NUE in bermudagrass (Schneider-Canny *et al.*, 2018). Single genotype of PI accessions (n=290) were screened for NUE using the developed protocol. Contrasting genotypes for NUE along with check cultivars (n=50) were further evaluated in greenhouse and hoop-house. A bucket (368×260 mm) contained 18 kg soil were used for evaluating three ramets of each accession in the hoop-house. Four N rates (0, 75, 150 and 300 mg N kg^{-1} soil) were divided into three equal amounts applied after each cut. Plants were harvested four times, about 30 days after each N applications. Data on biomass and crude protein (CP) were collected. Data were analysed for N-uptake efficiency (NUpE), N-utilization efficiency (NUtE) and NUE. Eighteen genotypes with contrasting NUE were selected and planted in field. Data collection on plot coverage, plant height, flowering, biomass production, frost tolerance, along with NUE related traits, are in progress.

Results: Genotypes showed significant influences ($P<0.001$) in all of the response variables. NUE had strong correlation with biomass production and NUpE, which even got stronger at higher N rates (Figure 1). In N-limiting conditions, bermudagrass showed a trade-off between biomass maintenance and CP content. Lower N applications increased biomass production over CP. However, when N is abundant the crop has the ability to improve CP. Several genotypes showed high NUE due their high NUpE and NUtE. The five bottom performing genotypes were 26%, 30% and 29% less N-use efficient than the top genotypes at three N rates, respectively. NUE of the accessions decreased significantly as more N was added to the plants. Superior genotypes for NUE will be used in the breeding programme to develop cultivar(s) with enhanced NUE in bermudagrass.

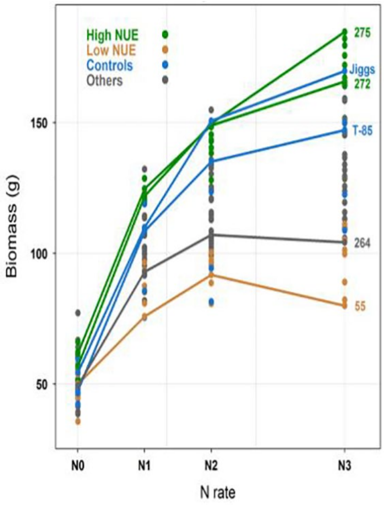

Figure 1. Accessions with high NUE produced high biomass at all N rates.

Conclusion: The bermudagrass germplasm presented high variability for NUE. There was no significant genotype × N rate interaction for NUE. Genotypes with high NUE tend to have the lowest CP content especially in the low N rates. NUE is highly correlated with biomass yield.

Schneider-Canny R., Chekhovskiy K., Muñoz P., Kwon S. and Saha M.C. (2019) Characterization of bermudagrass (*Cynodon dactylon* L.) germplasm for nitrogen use efficiency. *Euphytica*. DOI: https://doi.org/10.1007/s10681-019-2347-z.

Preliminary work on a national scale use of a grassland production index for insurance

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Introduction: In France, grasslands cover 40% of arable land. Climatic events can strongly impact forage stock management and farmers' financial situation. Since 2010, private insurers became gradually responsible for risk coverage. Index-based insurance for grassland production are being developed to offset high production losses in case of climatic disaster. From satellite images time series, annual grassland production is estimated at a small regional level and compared to the reference of the area made from the five previous years (Roumiguié *et al.*, 2016). The study aims to show the correlation between this method and on-farm yield measurement for assessing annual grassland production.

Materials and methods: A three-year protocol (2016-2018) has been set up on nine experimental farms across France. Between February and November, grassland production was monitored weekly either on the small regional area where are located the farms with the Grassland Production Index (GPI), or with a rising plate meter on the grassland paddocks (grazed and cut) of the farms. The GPI is calculated with a model integrating the daily fraction of green cover (fCover), a biophysical variable obtained from medium spatial resolution satellite images time series, a water stress index derived from spatialized meteorological data and phenological indicators. In-field production was calculated from the sum of weekly herbage growth. So far, the analysis has been carried out with both methods as grassland production in 2016 over 2017. Correlation between the two yield assessment methods is quantified by the coefficient of determination and the root mean square error.

Results: Grassland annual production variations in 2016 compared to 2017 ranged between -44% and +58% for on-farm data and -50% and +92% for GPI. GPI showed high correlation with biomass variations measured in field (R^2=0.74; RMSE=0.29) (Figure 1). One farm data had been removed because of unrepresentative practice within the monitored grassland area (pest damages combined with organic farming transition). Linear regression between the two datasets showed a deviation inferior to 20% in 7 cases out of 8. Only one farm had a negative correlation. The observed errors may be reduced by downscaling the calculation of GPI using high spatial resolution time series (like Sentinel-2) and improving ground data post-processing.

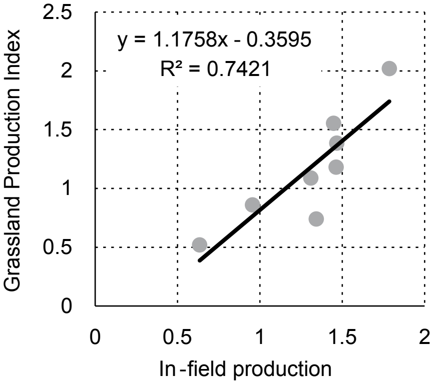

Figure 1. Correlation between GPI and in-field production for annual production ratio 2016 to 2017.

Conclusion: Assessment of grasslands production with GPI and in-field measurements showed a quite good correlation but needs a deeper analysis for some cases. This conclusion has to be confirmed with the addition of the 2018 data.

Roumiguié A., Sigel G., Poilvé H., Bouchard B., Vrieling A. and Jacquin A. (2016) Insuring forage through satellites: testing alternative indices against grassland production estimates for France. *International Journal of Remote Sensing* 38, 1912-1939.

Theme 5.
Breeding for future grassland challenges

Advances and perspectives in breeding for improved grass-endophyte associations

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Abstract

Endophytic *Epichloë* species form symbiotic associations with temperate grasses (*Poaceae* subfamily *Poöideae*). These symbionts are critical constituents of both natural grass ecosystems and cultivated agricultural grasslands providing improved persistence and production by protecting against biotic and abiotic stresses. The ecological significance of these endophytes is linked to a defensive mutualism attributable to secondary metabolites, particularly alkaloids, produced by the endophyte. These fungal-derived compounds can be effective in deterring insect pests, but some are also responsible for disorders in grazing animals. Asexual strains of *Epichloë* that retain desired bio-protective properties and improve animal productivity and health have been successfully developed and commercialised for use in pastoral farming systems in Australia, New Zealand, USA and some parts of South America. The development of novel animal-safe *Epichloë* endophytes for commercialisation can be hindered by poor compatibility between the host and endophyte. Breeding for improved grass-endophyte compatibility has helped overcome some of these obstacles. Emerging genetic tools, such as genome editing and genomic selection (GS) that improve manipulation and selection of both host and endophyte genomes are expected to improve breeding efficiency for endophyte compatibility traits.

Keywords: *Epichloë*, endophyte-grass interactions, alkaloids, animal health, molecular breeding

General introduction

Epichloë endophytes, which include the asexual species formerly known as *Neotyphodium* spp. (Leuchtmann *et al.*, 2014), form mutualistic associations with perennial ryegrass (*Lolium perenne*), an agriculturally important pasture grass to New Zealand (NZ) and Australia, as well as with tall fescue (*Festuca arundinacea*, synsyn. *Schedonorus arundinaceus* and *Lolium arundinaceum*) which, while grown in Australasia, is a more important grass in the USA and some parts of South America (Johnson and Caradus, 2019). Asexual *Epichloë* species systemically colonise aerial host tissues, vertically transmitting to the next generation of plants via colonising host seed. This process can be imperfect, leading to reduced rates of infected seed and loss of endophyte persistence in pastures. NZ pastures are heavily dependent on associations with asexual *Epichloë* species to protect against six major pasture insect pests, each of which can be affected by different secondary metabolites (alkaloids) produced by *Epichloë* strains (Popay and Rowan, 1994; Johnson *et al.*, 2013; Johnson and Caradus 2019). These endophytes also provide temperate grasses with tolerance to various abiotic stresses such as water and mineral deficiency (Arachevaleta *et al.*, 1989; Bacon, 1993; Malinowski and Belesky, 2000; Kane, 2011).

Epichloë strains exhibit a diversity of secondary metabolite chemotypes which have been categorised into four major groups – the ergot alkaloids, such as ergovaline and chanoclavine; the indole diterpenes, including lolitrem B and epoxy-janthitrem; pyrrolizidines, which includes lolines; and the pyrrolopyrazine metabolite, peramine (Spiering *et al.*, 2005; Tanaka *et al.*, 2005; Young *et al.,* 2006; Lane *et al.*, 2000; Schardl *et al.*, 2013). These are synthesised via fungal secondary metabolite pathways (reviewed in Lane *et* *al.*, 2000; Schardl *et al.*, 2013), with the quantity of alkaloids expressed affected by the host plant (Latch, 1994; Ball *et al.*, 1993; Easton *et al.*, 2002; Kaur *et al.*, 2015; Ekanayake *et al.*, 2017). Two of these alkaloid groups however negatively affect vertebrate behaviour, health and welfare. The indole diterpenes lolitrem B (Fletcher and Harvey, 1981; Gallagher *et al.*, 1981) and epoxy-janthitrems (Tapper and Lane, 2004), cause the neuromuscular disorder ryegrass staggers, with lolitrem B being the more potent of the two. While ergovaline acts as a grazing deterrent (Stuedemann *et al.*, 1989), consumption can cause heat stress and in extreme cases a syndrome known as fescue toxicosis (Bacon, 1995). A requirement for animal-safe endophyte strains in grazing systems – that are still capable of deterring insect pests while providing protection against abiotic stresses – has driven the demand for proprietary asexual strains with desirable alkaloid profiles. These are now used widely in many pastoral agriculture systems (Johnson *et al.*, 2013). Newly discovered strains can typically be isolated into axenic culture from surface-sterilised tissues of the original host, and thereafter manually inserted into the seedlings of commercial grass cultivars (Latch and Christensen, 1985). This combines the best characteristics of the host and the endophyte in novel symbiotic associations. *Epichloë festucae* var. *lolii* strain AR37 is a highly successful endophyte strain, estimated to contribute NZ$3.6 billion to the NZ economy through the life of its patent (Woodfield and Judson, 2018). It was commercialised to provide superior insect resistance and has been readily adopted by farmers (Caradus *et al.*, 2013). However, further strains with novel bioactivity continue to be sought to further improve pasture persistence and production.

Biology and lifecycle

Epichloë symbionts are most abundant in aerial host tissues but can also be found in the tips of the radicle and adventitious roots (but not in the tips of root branches) (Christensen and Voisey 2007). In above ground parts they are readily detectable in the shoot apex and true stem, leaf blade, ligule and sheath, and throughout the inflorescence, including the ovaries, but not in pollen (Tan *et al.*, 2001; Christensen and Voisey, 2007). Hyphae are found almost exclusively in the intercellular spaces of the host (Philipson and Christey, 1986). Since they do not produce obvious structures for feeding directly from host cells, they are presumed to obtain their nutrients from the apoplastic space (Schardl, 2001).

Asexual *Epichloë* transmission between plants occurs vertically through invasion of the embryo by hyphae. Within mature seeds (Figure 1A) hyphae are present in the embryo, scutellum, and a region between the embryo and the endosperm called the 'infection layer' (Philipson and Christey, 1986; Card *et al.*, 2011). While also numerous between the aleurone and seed coat, it is the hyphae in the embryo that colonise the seedling (Freeman, 1904; Liu *et al.,* 2017). Grass plants are comprised of collections of tillers, with each tiller containing a shoot apex at the base, from which arise a number of foliar units consisting of an internode, leaf lamina and sheath and an axillary bud (Evans and Grover, 1940). After seed germination (Figure 1B) hyphae between embryo cells establish a mycelial network in the seedling meristem (shoot apex) and then penetrate the intercellular spaces of organ primordia as they develop (Freeman, 1904; Philipson and Christey, 1986, Liu *et al.*, 2017; Figure 1C). Any vegetative or floral structures that have arisen from primordia that escaped hyphal colonisation appear to remain endophyte-free (Christensen and Voisey, 2007). After colonisation of the primordia, hyphal extension in leaves occurs primarily through intercalary growth, and stops when the leaves mature (Christensen *et al.*, 2008). Each leaf subtends an axillary bud, which has the potential to form a new daughter tiller, complete with its own shoot apex (Evans and Grover, 1940; Figure 1C). Endophyte colonisation of the axillary bud occurs prior to emergence of the new tiller, enabling the fungus to colonise the shoot apex of the daughter tiller, and from there, any nascent aerial structures (Philipson and Christey, 1986, Liu *et al.*, 2017). Upon vernalisation, the vegetative shoot meristem within each tiller has the potential to become a reproductive inflorescence primordium (Evans and Grover, 1940; Figure 1D). *Epichloë* mycelia, already *in situ*, extend into the reproductive tissues as they arise, eventually colonising the ovaries in the florets (Liu *et al.*, 2017 Figure 1E). Hyphae can be identified among the cells of most floral tissues; however, those in the ovary

Figure 1. Lifecycle of asexual *Epichloë* species. The grass reproductive cycle is presented in the outer ring, and the fungal transmission cycle is shown in the inner ring (figure reproduced from Gagic *et al.*, 2018). (A) Hyphae (in the lower part of the seed) are in the embryo, the scutellum, the nucellus and the 'infection layer' between the embryo and the endosperm (Philipson and Christey, 1986; Card *et al.*, 2011). (B) Hyphae (base of seed and arrow) colonise the shoot apex of the developing seedling. (C) Hyphal tips in the shoot apex move into leaf primordia (arrows), and extend primarily through intercalary compartment growth (Christensen *et al.*, 2008). Hyphal growth is synchronised with leaf growth. Axillary buds are colonised enabling hyphae to invade daughter tillers. (D) Hyphae colonise infloresence primordia early in development. (E) Hyphae penetrate the floral tissues, ovaries, and thereafter, the embryo and other seed structures (Sampson, 1933; Liu *et al.*, 2017; Zhang *et al.*, 2017; Gagic *et al.*, 2018).

penetrate the ovule and then the embryo upon fertilisation (Philipson and Christey, 1986; Zhang *et al.*, 2017; Liu *et al.*, 2017).

Epichloë species that are able to undertake the sexual cycle may also transmit horizontally between plants through ascospores produced on ectopic hyphal stromata that overwhelm or 'choke' host inflorescences (Schardl, 2001; Christensen *et al.*, 2002). These strains are regarded as pathogenic as they reduce, or can eliminate, host seed production. Some strains undertake both vertical and horizontal transmission on different inflorescences of the same plant. The sexual *Epichloë* species are not used to protect forage grasses due to their negative impact on seed production and also because they often lack effective levels of alkaloids.

Selected novel *Epichloë* strains require efficient vertical transmission through commercial seed production processes to ensure they are present in seed sown into pastoral farming systems. However, this process is often imperfect in both the native (original) and novel endophyte-grass associations (Afkhami and Rudgers, 2008; Gundel *et al.,* 2011). There are several stages at which vertical transmission of *Epichloë* may fail (Gagic *et al.*, 2018; Figure 1). Hyphae in infected seeds may not colonise the shoot meristem such that seedlings become endophyte-free after germination; hyphae in the shoot apex may not colonise the vegetative axillary meristems that will form the new tillers (resulting in endophyte-free tillers, inflorescences and seeds); and hyphae in the shoot apex may not colonise inflorescence primordia (resulting in endophyte-free florets, ovaries and seeds). Inadequate seed storage can also lower endophyte viability in a seed line resulting in further reductions in endophyte transmission into seedlings (Hume *et al.*, 2013).

Characterisation of novel associations across differing host genetic distances

In naturally occurring grass-endophyte symbioses with *Epichloë* species, there is strong evidence for co-evolution (Schardl *et al.*, 1997) and host specialisation (Schirrmann *et al.*, 2018). Efficient tiller colonisation by *Epichloë* strains, their transmission into most seeds, and ability to remain viable in seed for extended periods are all hallmarks of compatible associations. The establishment of an artificial symbiotic association by inoculation of seedling meristems (Latch and Christensen, 1985) with fungal mycelium can result in associations with compromised compatibility attributes that may delay or prevent commercialisation.

The genetic distance between the natural host of the endophyte (with which it co-evolved), and the new recipient host, is considered to play a large role in the fitness and seed transmission outcomes of the novel association. A model for naturally infected, obligate outcrossing hosts has been proposed that considers both the genetic distance between parent plants and likelihood of the expression of mutualism, and endophyte transmission efficiency on overall fitness of the symbiosis (Gundel *et al.*, 2010). This model predicts that transmission is maximised at moderate (e.g. inter-population) genetic distances between parent plants, in contrast to smaller (e.g. intra-population) and larger (e.g. interspecies) genetic distances, whereby in-breeding and out-breeding depression respectively are more likely to outweigh endophyte mutualistic effects, and hence reduce overall fitness of the association (Gundel *et al.*, 2010). Moreover, the probability of mismatching between endophyte genotype and seed genotype becomes greater as the genetic distance between parents increases, which will reduce endophyte compatibility and transmission (Gundel *et al.*, 2010).

The efficiency of vertical transmission in artificial associations may be considered under a similar framework. However, in contrast to the above, endophyte compatibility (and transmission) would be predicted to be high in recipients that are more closely related to their natural hosts than those that they are more distantly related to. Moreover, the reproductive behaviour of the natural host may impact the range of host genotypes that allow efficient endophyte transmission in new associations. For example, if the endophyte originated from, (and therefore had co-evolved with) an obligate outcrossing host, it may be more of a generalist that is compatible with a wider range of host genotypes in artificial associations than one that originated from a highly self-fertile host, which may be specialised to a narrower host range. Studies that have systematically examined the impact of host genetic distance on compatibility and vertical transmission for both natural and artificial associations are rare. However, support for these models can currently best be determined from independent sets of observations (e.g. Gagic *et al.*, 2018; Gundel *et al.*, 2010, 2012; Saikkonen *et al.*, 2010; Noh and Ju, 2012) and from experience in the development of commercially available endophyte strains. Outcomes observed for novel host-endophyte associations using hosts of various genetic distances to those of the natural host are summarised in Table 1.

In general, when forming novel associations within a species, such as when *E. festucae* var. *lolii* is isolated from a wild species of perennial ryegrass and inoculated into a selected cultivar of perennial ryegrass, the phenotypic features observed in the original symbiosis are largely retained, although this can vary widely depending on the genotype of both the fungus and the grass (Table 1). These types of novel symbioses formed from 'within species' partnerships, typically form stable associations with sufficient strength and stability to allow their deployment in pastoral agriculture. When forming novel associations across species, such as when *Epichloë* is isolated from a wild species of tall fescue and inoculated into a cultivar of perennial ryegrass, a reduction in the rate of transmission to seed along with significant changes in alkaloid levels has been observed (Easton, 2007; Easton *et al.*, 2007, 2009).

Table 1. Outcomes of novel associations between endophytes with their natural and the new recipient hosts.

Natural host	Recipient host	Genetic distance	Symbiotic phenotype	Co-selective breeding	Reference
Lolium perenne	*Lolium perenne*	Within species	Host genotype dependent but often fully compatible symbiosis formed with expected alkaloid profiles (but alkaloid levels may vary). Seed transmission and storage may be sub-optimal (e.g. AR37)	Selected for transmission, longevity of viability in seed and alkaloid production	Easton, 2007; Simpson and Mace, 2012; Kaur *et al.*, 2015; Gagic *et al.*, 2018; Ekanayake *et al.*, 2017
Festuca arundinacea	*Festuca arundinacea*				
Festuca pratensis	*Festuca pratensis*				
Festuca arundinacea	*Festuca pratensis*	Between species	Little characterisation performed	None	Adcock *et al.*, 1997
Lolium perenne	*Lolium boucheanum Kunth/ Lolium multiflorum*		Extensively colonised vascular bundles than with native *E. occultans*	None	Christensen *et al.*, 2001
Festuca arundinacea/ Festuca pratensis	*Lolium perenne*	Between genera	Changes in alkaloid profiles such as reduction in lolines; transmission can be variable	Selected for transmission, longevity of viability in seed and loline production	Easton, 2007; Easton *et al.*, 2009; Simpson and Mace, 2012; Faville *et al.*, 2015; Ekanayake *et al.*, 2017
Other fescues	*Lolium perenne*	Between genera	Variable outcomes from fully compatible (with some strains of *E. festucae*), to incompatible (i.e. plant stunting increased mortality of inoculated seedlings, poor transmission)	None	Christensen *et al.*, 1997; Tanaka *et al.*, 2012
Lolium perenne	*Festuca arundinacea*	Between genera	Incompatibility with *Lp*TG-2 endophytes e.g. *Epichloë hybrida* Lp1	None	Christensen, 1995
Lolium perenne	*Festuca pratensis*	Between genera	Little characterisation performed	None	
Elymus sp.	*Secale cereale*	Between genera	Plant stunting	Selected for improved phenotype of infected plants	Simpson *et al.*, 2014
Lolium perenne / Festuca arundinacea/ Festuca pratensis	*Festuca/ Lolium* hybrids	Mixed	*Lp*TG-2 endophytes and *Epichloë siegelii* incompatibility	None	unpublished data
Elymus sp.	Perennial ryegrass	Between tribes	Infection possible but loss of endophyte persistence	None	unpublished data

Note: *Festuca arundinacea* = *Lolium arundinaceum* = *Schedonorus arundinaceus*

*Lp*TG-2 =*Lolium perenne* Taxonomic Group 2; Christensen *et al.*, 1993

Transfer of loline-producing endophytes from *Festuca* to *Lolium* to improve cultivar performance

One approach to improve agronomic performance of endophyte-infected cultivars while having no adverse effects on livestock is to infect ryegrass with endophytes from tall fescue or meadow fescue (*Festuca pratensis*). Endophytes from these species produce loline alkaloids which have strong insecticidal properties and no harmful effects on livestock (Bush *et al.*, 1993). These alkaloids can be produced in large quantities, particularly in meadow fescue (11,000 ppm on a dry weight basis (Kennedy and Bush, 1983)) being the most abundant of the alkaloids detected in *Epichloë*-infected grasses. However, as described above, fully compatible outcomes become more difficult to achieve when the new host species is genetically distinct from the original host of the endophyte (Table 1; Easton, 2007; Simpson and Mace, 2012).

Transfer of *Epichloë* endophytes from tall fescue to ryegrass has been of significant interest to research groups worldwide as the production of the broad host-range insecticidal loline alkaloids in ryegrass is a highly desirable trait. The rate of inoculation success of these novel endophytes into ryegrass is similar to that of ryegrass endophytes into ryegrass (Easton *et al.*, 2007; unpublished data). Expression of the loline alkaloids at biologically effective levels, however, may be problematic. For example, when four tall fescue endophytes were inoculated into perennial ryegrass, total loline alkaloids were 30% of those found in tall fescue (Easton, 2007; Easton *et al.*, 2007). The range of loline derivatives that accumulated were also reduced, and in some infected plants, lolines were undetectable. There was no indication of an overall reduction in alkaloid production as peramine concentrations by tall fescue strain AR501 were 3-5 fold higher in ryegrass than in tall fescue hosts, and equivalent to those made by ryegrass endophytes when inoculated back into ryegrass hosts (Easton, 2007; Simpson and Mace, 2012). Elevated peramine concentrations by other tall fescue endophyte strains in ryegrass hosts have been reported elsewhere, while data on changes in ergovaline concentration are equivocal (Siegel *et al.*, 1990; Bush *et al.*, 1997). Siegel *et al.* (1990) and Bush *et al.* (1997) however, have reported comparable loline concentrations in ryegrass and tall fescue both infected with tall fescue endophytes. Rates of tall fescue endophyte transmission into ryegrass seed are generally also suboptimal and highly variable. While very high rates of infection have been realised (close to 100%), these have not been reliably achieved through several generations of seed production (Easton *et al.*, 2007, 2009).

Breeding studies on ryegrass infected with tall fescue endophytes report large differences between half-sibling families for inoculation success, loline production and seed transmission (Easton *et al.*, 2007, 2009). This genetic variation offers the opportunity to select and breed for improved and stable trait expression. In contrast, transmission into seed, and seedlings is not under simple host genetic control, and is also influenced by undetermined environmental factors (Easton *et al.*, 2009). No data has yet been published for storage of endophyte in seed, but we have observed reduced viability of tall fescue endophytes in perennial ryegrass when seed is stored at ambient temperatures (unpublished data). Reliable transmission and endophyte viability in seed is critical to produce a commercial endophyte-infected cultivar. It must be noted that, to date, there has been no successful commercialisation of *Epichloë* endophytes when transferred from one host species to another. It is much harder to achieve reliable transmission on a commercial scale where growing and harvest conditions and seed drying and storage are not as well controlled or more variable than in research situations.

Overcoming compatibility by plant breeding

The natural *E. festucae* var. *lolii* wild-type endophyte of perennial ryegrass found in New Zealand transmits and stores in seed extremely well, as does the commercial *E. festucae* var. *lolii* strain AR1. However, some strains such as AR37 have lower seed transmission and storage rates despite having originally been isolated from the same host species. This has largely been remedied through cycles of selection for host germplasm that transmits AR37 to nearly 100% of the progeny (unpublished). Host selection for changes in endophyte metabolite expression has also been possible (Easton, 2007) and has been achieved at commercial scale with *E. festucae* var. *lolii* strain Endo5 (Mason *et al.,* 2013). In this case, a number of cycles of selection effectively reduced the toxic alkaloid ergovaline to levels unlikely to cause serious animal health issues.

To achieve a commercially acceptable level of endophyte in seed (70%) (Johnson and Caradus, 2019), the starting point in nucleus seed generation needs to be 95-98% transmission rate to accommodate for a loss of 5% per generation during seed bulk up for sale (unpublished data). The effects of host selection and breeding for vertical AR37 transmission has been demonstrated in five *L. perenne* breeding populations (Gagic *et al.*, 2018), three of which (Populations I-III) had undergone previous selection for high seed infection by AR37, and two (Populations IV and V) had not. Mean AR37 transmission was significantly higher in Populations I-III (88, 93 and 92%, respectively) compared to Populations IV and V (69 and 70%, respectively) confirming that host genotype contributed greatly to differences in AR37 seed transmission. The same study also identified environment, in terms of year and geographical location, as having an important influence on AR37 seed transmission, while there was a significant but relatively small host genotype-by-environment interaction effect (Gagic *et al.*, 2018). Endophyte testing at different stages of the life cycle showed that endophyte loss occurred at pre-zygotic stages, such as infection of axillary buds (leading to endophyte-free daughter tillers) and also during flowering and seed formation (infected tillers did not produce infected seeds) (Gagic *et al.*, 2018). Post zygotic steps, such as the infection of germinating seedlings, were not evaluated and remain to be confirmed. Selecting for improved transmission and storage in AR37 does not appear to have carried some host yield penalty as modern cultivars are higher yielding than earlier unselected cultivars (as recognised by their placement at the top of the DairyNZ Forage Value Index – https://www.dairynz.co.nz/feed/pasture-renewal/select-pasture-species/about-fvi/).

Development of new tools for endophyte technologies and future opportunities

As discussed above, breeding for improved host-endophyte compatibility can be achieved through recurrent selection (Adcock *et al.*, 1997; Easton *et al.*, 2002) but this can be costly, laborious and can prolong the time taken to produce a cultivar. Indirect selection methods for endophyte compatibility in host plants based on DNA marker information, such as marker-assisted selection (MAS) or genomic selection (GS), are of value because they speed up the breeding process by shortening the length of a breeding cycle (Heffner *et al.*, 2010; Resende *et al.*, 2014). Using either approach allows selection of endophyte-compatible plants early in the plant's lifecycle, and prior to endophyte inoculation, avoiding the cost of inoculating large numbers of plants to obtain sufficient numbers of inoculated plants.

MAS involves selecting plants based on their genotype at one or a few DNA markers that are either linked to quantitative trait loci (QTL; defined section within the genome that contributes to trait variation), or which occur within genes functionally-associated with the trait. QTL regulating endophyte hyphal biomass and alkaloid concentrations have been identified in ryegrass (Faville *et al.*, 2015), indicating genes of significant effect, and a subset of these was consistent across different ryegrass host-endophyte associations. The challenges of implementing MAS directly using QTL is documented Heffner *et al.*, 2009), and may be particularly onerous in outbreeding forage species (Barrett *et al.*, 2007). MAS based on markers that occur inside host genes may therefore be more practical but at this stage information on the identity of genes critical to successful associations is only just beginning to emerge (reviewed in Scott *et al.*, 2018).

GS is a DNA marker-based breeding strategy in which the effects of thousands or millions of genome-wide single nucleotide polymorphism (SNP) markers are integrated in a statistical model to predict the phenotypes of selection candidates (Meuwissen *et al.*, 2001). It solves the limitations of MAS because the prediction model can be trained and applied directly in breeding populations and has the potential to capture the effects of most of the genes governing a polygenic trait (Solberg *et al.*, 2008). GS is a promising prospect for perennial ryegrass (Hayes *et al.*, 2013) and genomic prediction models and GS strategies have been developed for a range of traits in ryegrass (Fe *et al.*, 2015, 2016; Grinberg *et al.,* 2016; Faville *et al.*, 2018; Pembleton *et al.* 2018). In the context of host plant-endophyte interactions, Gagic *et al.* (2018) used genotyping-by-sequencing (Elshire *et al.*, 2011) SNP data to generate a genomic prediction model for vertical endophyte transmission in ryegrass with relatively high predictive ability for the trait (r=0.54 between observed and predicted phenotypic values). Application of this model for genomic selection in breeding populations will demonstrate the true predictive power of the model but these results suggest that GS approaches have high potential value for improving breeding efficiency for endophyte compatibility traits in host grass populations.

Beyond the molecular breeding approaches described above, emerging novel breeding strategies, notably F1 hybrid breeding (Pembleton *et al.,* 2015), could provide an effective means for accelerating the improvement of host-endophyte compatibility in forage grasses but this has yet to be explored. To better understand the key biological processes and interactions that underlie the maintenance and efficient vertical transmission of novel grass-endophyte associations, the use of 'omics' technologies (e.g. genomics, transcriptomics, proteomics and metabolomics) is now fundamental. Underpinning these platforms are the widespread availability of high throughput DNA sequencing, protein and analytical chemistry methods, with the development of associated computational and statistical tools. The availability of host and endophyte reference genome sequences (*Epichloë* genomes: Winter *et al.*, 2018, http://www.endophyte.uky.edu/; *Lolium perenne:* Byrne *et al.*, 2015) provides a foundation for understanding interactions between the host and endophyte. Transcriptome analyses to examine gene expression in key tissues (such as vegetative tiller crowns, inflorescence primordia, and ovary tissues) within high and low transmitting associations have the potential to reveal processes that are important for efficient vertical transmission (Zhang, 2018). In comparison to the recent popularity of using RNA-Seq to analyse transcriptome data of grass-endophyte interactions (Dupont, 2015, Dinkins *et al.*, 2017; Schmid *et al.*, 2017, Nagabhyru *et al.*, 2018; Zhang, 2018), proteomics (Bassett *et al.*, 2009) and metabolomics-based (Cao *et al.*, 2008; Rasmussen *et al.*, 2012) investigations have so far been less frequently applied. However, these will be valuable tools to quantify the gene products and metabolite differences between high and low transmitting associations, and to identify biomarkers that are diagnostic of high and low-transmitting associations. Moreover, the integration of multiple 'omics data has the potential to provide a system view that will allow the refinement and testing of hypotheses regarding the molecular mechanisms that underpin high and low transmission phenotypes.

CRISPR-mediated gene editing to accelerate breeding of plant-endophyte associations

With the advance of revolutionary gene editing techniques, such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat), that allow for the insertion, removal or replacement of genes at predetermined sites in the genomes of all organisms (Cong *et al.*, 2013), it is now feasible to re-engineer and improve the processes for the development of novel endophytes. CRISPR is currently being used to precisely design crop plants (Wang *et al.,* 2014; Komor *et al.,* 2017) and the technique has been applied to model fungi, including filamentous fungi (Zheng *et al.,* 2017). Proof of concept, using CRISPR-Cas9 (CRISPR associated nuclease) with *E. festucae* strain Fl1 has resulted in to eliminating expression of the mammalian toxin ergovaline, through the deletion of lpsB (unpublished results), which encodes an enzyme late in the ergovaline biosynthetic pathway (Fleetwood *et al.*, 2007). Endophyte secondary metabolites and their pathway regulators are future targets for genetic manipulation. CRISPR may also enable less genetically tractable strains to be re-engineered, enhancing our ability to improve pasture productivity in the absence of animal toxicoses. Once the molecular mechanisms that underpin endophyte compatibility issues such as high and low transmission phenotypes are identified, the genetics responsible from either symbiotic partner could be favourably manipulated by gene editing approaches. Whether country-specific regulations will allow gene editing to be ruled as a non-GMO technology will affect the possibility and ease of commercialisation, but ultimately consumers who buy meat and milk, will decide on whether this technology will be widely adopted.

Final perspectives

Epichloë endophytes are renowned for their importance in grasslands and are critical components of temperate grasses in intensive pastoral grazing systems. The positive effects of these endophytes on host fitness and yield whilst enhancing animal productivity and health has been a driving force for the development of novel endophytes. The necessity for near perfect vertical transmission of the endophyte to subsequent seed generations and the expression of a desired suite of alkaloids continues to be a challenge that modern science is capable of tackling. With the success of breeding for improved plant-endophyte associations and the development of emerging tools to speed up the breeding process, opportunities for new and improved endophytic solutions for sustainable agriculture are becoming a reality.

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New collections of *Lolium* and *Festuca* spp. show promising variation for stress tolerance related traits

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Abstract

Lolium perenne and *Festuca pratensis* subsp. *apennina* ecotypes, recently collected in Europe, were characterized for various traits. After a three-year field trial, a group of nine out of 41 ryegrass populations combining moderate winter damage, strong vigour, a high amount of ear producing tillers, large plant height and tussock size was separated by hierarchical clustering. For *F. pratensis* subsp. *apennina*, flow cytometry indicated the presence of both triploid (3×) and tetraploid (4×) material in 11 out of 16 populations (8 to 95% 3× plants). Preliminary screening in the field indicated that 3× material can be distinguished from 4× plants at the flowering stage by its indehiscent anthers, shorter awns and higher plant biomass. This well-described material might support the generation of improved ryegrass cultivars and more stable *Festulolium* hybrids.

Keywords: fescue, ryegrass, germplasm, collection, ecotypes, performance

Introduction

As one of the most important forage and turf grass species, *Lolium perenne* L. (perennial ryegrass) has been the subject of intensive breeding and research. Still, it suffers from frost in winter and early spring, heat, drought and various diseases. The identification of useful *L. perenne* genebank accessions or inter-specific crossings with *Festuca pratensis* Huds. (meadow fescue) are opportunities to improve stress tolerance of ryegrass cultivars and to broaden the genetic base of ryegrass breeding. However, in amphidiploid *Festulolium*, a hybrid species created to combine desired traits from both parental species, frequent homoeologous chromosome pairing during meiosis and a shift towards the ryegrass genome occurs with advanced generations (Zwierzykowski *et al.*, 2006). A close relative of meadow fescue, the allopolyploid *F. pratensis* subsp. *apennina* (de Not.) Hegi (apennine fescue), might contribute to develop more stable *Festulolium* hybrids by its strict homologous chromosome pairing during meiosis (Kopecký *et al.*, 2016). This species is known for its strong biomass development under the extreme conditions of its habitat (1,100-2,200 m a.s.l.), but is rarely present in genebanks. This study evaluates recently collected *L. perenne* and *F. pratensis* subsp. *apennina* ecotypes in order to provide useful and well-described germplasm for plant breeding and research.

Materials and methods

In 2014, 41 ecotypic *L. perenne* populations were collected across Europe and 30 individuals per population were cultivated from September 2015 until April 2018 at the Malchow/Poel site of the IPK in northern Germany (mean annual precipitation 592 mm, mean annual air temperature 8.9 °C). Plant vigour (VIG), winter damage (WID), disease infestation (crown rust RUC, *Xanthomonas* XNT, mixed diseases DIS) and the amount of ear producing tillers (FEC) at heading date were evaluated on a scale from 1 (weak/low) to 9 (strong/high). Heading (HAE) was counted in days after first of April. Plant height before cut (HBC, cm) and regrowth height seven days after cutting (REG, cm) were determined using a herbometer. Tussock size (TUS, cm) was calculated from the average of two perpendicular measurements of the diameter using a ruler. A cluster analysis was conducted in R based on the population medians for ordinal traits and the population means for numerical traits using the 'daisy' function of the 'cluster' package (Maechler *et al.*, 2018). *Festuca pratensis* subsp. *apennina* ecotypes were collected in Switzerland in 2017 between 1,500 and 2,000 m a.s.l. The sixteen populations collected vegetatively (between 10 and 85 plants per population) were cultivated in isolated plots (distance of 30 m in both directions, unreplicated, non-randomized) for seed multiplication in 2018 and a preliminary screening of plant biomass and disease infestation was conducted as described above. The ploidy level was determined using flow cytometry. Based on these results, the 3× cytotypes cultivated per accession in the field were reduced to a maximum number of five (Table 2).

Results and discussion

The agronomic performance of the ryegrass populations in the years 2017 and 2018 is presented in Table 1 and showed variation for most of the traits evaluated. Hierarchical clustering separated nine ryegrass populations originating from northern Germany, Belgium and France, which combined moderate winter damage, strong vigour, large plant height and tussock size as well as a high number of ear-producing tillers while tending to late heading.

For *F. pratensis* subsp. *apennina*, flow cytometry indicated the presence of both triploid (3×) and tetraploid (4×) material in 11 out of 16 populations (8 to 95% 3× plants, Table 2). The 3× cytotypes can be distinguished from 4× cytotypes at the flowering stage based on their short awns (3× = 0.59±0.42 (s.d.) mm; 4× = 1.23±0.58 mm) and their indehiscent anthers, indicating infertility. The 3× plants are of low relevance for plant breeding and were reduced to maximal five plants per population but maintained for comparative evaluations. Here, the *F. pratensis* subsp. *apennina* 4× plants were characterized by moderate to high biomass development, but some of them showed substantial disease infestation. Plant biomass of the 3× cytotypes was higher and general disease infestation about two score units lower than that of the 4× cytotypes.

Table 1. Overall performance of the 41 ecotypic *L. perenne* populations tested in the second and third year of field cultivation compared to the performance of the nine populations separated by the cluster analysis (Cluster 1).[1,2]

Year	Trait	Overall mean	Cluster 1 mean	Year	Trait	Overall mean	Cluster 1 mean
2017	WID_1	6.2/0.7	5.3/1.1	2017	REG_2	18.2/1.2	19.4/0.5
	TUS_1	20.1/3.4	23.7/2.8		DIS_1	3.9/0.6	3.8/0.7
	VIG_1	4.7/1.0	6.2/0.5		VIG_2	4.2/0.7	5.2/0.4
	HAE_1	47.7/9.9	52.4/12.4		TUS_3	23.9/4.5	29.0/2.9
	FEC_1	4.8/0.7	5.8/0.4		VIG_3	4.8/1.0	6.3/0.4
	RUC_1	1.8/0.4	1.7/0.4		HBC_2	22.9/2.1	25.6/1.1
	XNT_1	3.4/0.9	3.0/1.1		VIG_4	4.7/0.9	5.8/0.4
	TUS_2	21.8/3.4	25.1/3.3	2018	WID_1	5.5/0.6	5.3/0.3
	REG_1	19.7/1.4	20.2/0.8		VIG_1	4.1/0.6	4.9/0.4
	HBC_1	28.6/2.4	31.5/1.6				

[1] WID: winter damage; VIG: vigour, general condition of the plant; FEC: number of ear producing tillers; RUC: crown rust; XNT: *Xanthomonas*; DIS: mixed pathogen infestation; REG: regrowth height (cm), TUS: tussock size (cm); HAE: heading; HBC: herbometer height (cm).

[2] Values given are the means and the standard deviation.

Table 2. Accessions of *F. pratensis* subsp. *apennina,* altitude and number of individual plants collected, percentage of 3× cytotypes and mean scores for biomass development and general disease susceptibility of living plants during seed multiplication in 2018.

Accession	Alt.[a]	Number coll. and ploidy			Survival (%)[b]		Score biomass[b]		Score diseases[b]	
		Total	No. 3×	% 3×	4×	3×	4×	3×	4×	3×
GR 13219	1,551	51	19	37.3	93.7	80.0	4.5	6.3	5.9	3.0
GR 13255	1,540	20	2	10.0	83.3	50.0	8.2	9.0	2.5	5.0
GR 13259	1,680	85	18	21.2	80.0	80.0	4.3	8.0	6.1	3.0
GR 13261	1,700	8	0	0.0	88.0	100.0	7.9	8.6	2.2	1.0
	1,650	28	11	60.7						
GR 13262	1,580	37	35	94.6	100.0	80.0	5.3	7.3	4.8	2.8
	1,540	21	2	9.5						
GR 13266	1,920	38	0	0.0	97.4	–	6.6	–	4.6	–
GR 13267	1,760	38	3	7.9	97.1	100.0	7.2	6.7	2.9	1.0
GR 13270	1,590	36	0	0.0	97.2	–	5.9	–	5.5	–
GR 13274	1,616	37	7	18.9	100.0	100.0	7.3	7.8	1.9	1.0
GR 13276	1,903	36	0	0.0	91.7	–	5.5	–	6.6	–
GR 13277	1,760	10	0	0.0	100.0	–	5.7	–	6.0	–
GR 13280	1,600	35	0	0.0	100.0	–	6.6	–	3.4	–
GR 13281	1,670	47	37	78.7	100.0	100.0	6.9	8.0	2.7	2.0
GR 13284	1,800	35	12	34.3	100.0	100.0	7.9	9.0	1.6	1.0
GR 13285	1,653	38	19	50.0	100.0	100.0	7.3	7.8	3.6	2.4
GR 13290	1,850	37	18	48.6	100.0	100.0	8.2	9.0	1.8	1.0

[a] Altitude in m a.s.l.

[b] All 4× cytotypes and maximal five 3× cytotypes were cultivated for evaluation.

Conclusion

Large variation for many agronomic traits was observed in *L. perenne* and *F. pratensis* subsp. *apennina* accessions recently collected by the IPK Genebank. Based on the above evaluations, these genetic resources are well described and available as high-quality seed stocks.

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Digestibility, forage and seed yield of two new *Festulolium* amphiploids

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Abstract

In order to introduce more abiotic stress tolerance in *Lolium* fodder grasses, we carried out interspecific crosses between tetraploid genotypes of *Lolium perenne* (Lp) or *L. multiflorum* (Lm) and tetraploid genotypes of *Festuca pratensis* (Fp) or hexaploid genotypes of *F. arundinacea* (Fa). F1 plants were evaluated for spring growth, crown rust resistance, drought tolerance and winter hardiness. Two polycrosses were made, one with 3 selected genotypes of Lm × Fa and one with 5 selected genotypes of Lp × Fp. The syn1 seeds of the polycrosses were tested in a plot trial to determine yield and quality. At the end of the third year of the trial, plants from the two populations were randomly sampled and polycrossed to produce syn2 seeds. The two syn1 *Festulolium* populations had a higher annual dry matter yield than their *Lolium* parental species. Although the water-soluble carbohydrate content of the *Festulolium* populations was lower compared to the *Lolium* parents, their organic matter digestibility was higher thanks to a higher NDF digestibility. All this proves the potential of *Festulolium* hybrids to combine the stress tolerance of *Festuca* with the fodder quality of *Lolium*. However, in general, the seed yield of the syn2 populations was low. Chromosome analysis revealed a substantial amount of aberrations in chromosome numbers, which can be the cause of low seed yield. In addition, GISH showed no clear shift to one of the genomes that comprised the Lp × Fp population, while genome composition in the Lm × Fa clearly shifted towards the *Lolium* genome. Therefore, to validate the potential of *Festulolium* hybrids, it will be important in future breeding to select for higher seed yields and/or chromosome pairing stability at meiosis.

Keywords: *Festulolium*, digestibility, forage yield, seed yield

Introduction

Due to global warming more dry spells during summer will occur in NW Europe. Drought periods cause severe reductions in dry matter yield of perennial ryegrass (*Lolium perenne* L.), the most common sown fodder grass in temperate maritime environments (Aper *et al.*, 2014). The higher root biomass of tall fescue (*Festuca arundinacea* Schreb.) (Fa) makes this species more drought tolerant compared to *Lolium* (Cougnon *et al.*, 2016). However, the digestibility of the organic matter of Fa is at least 5% points lower compared to Lp (Cougnon *et al.*, 2013). Therefore, we aimed to combine the beneficial traits of both genera by interspecific hybridization. Due to genomic instability, the seed yield of amphiploid *Festulolium* cultivars may be low (Ghesquière *et al.*, 2010). May *Festulolium* combine high forage and seed yield and good digestibility? This paper reports the digestibility, forage and seed yield and genomic composition of two new *Festulolium* synthetics from our breeding programme.

Materials and methods

In 2011 we carried out interspecific crosses between tetraploid genotypes of *Lolium perenne* (Lp) or *L. multiflorum* (Lm) and tetraploid genotypes of *Festuca pratensis* (Fp) or hexaploid genotypes of *Festuca arundinacea* (Fa). F1 plants were evaluated for spring growth, crown rust resistance, drought tolerance and winter hardiness in 2012. Two polycrosses were carried out in 2013, one with 3 selected genotypes of Lm × Fa and one with 5 selected genotypes of Lp × Fp. The syn1 seeds of the polycrosses were sown in a plot ($8\ m^2$) trial on a sandy loam soil in Belgium, Merelbeke (50°58'55' N, 3°46'18"E) in September 2014 together with reference varieties of Lp, Lm, Fp and Fa (Table 1). Three replicates of each entry

Table 1. Dry matter yield, organic matter digestibility, sugar content and NDF digestibility of two *Festulolium* synthetics compared to cultivars of their parental species.[1]

Species	Ploidy	Cultivar	DM yield (t ha^{-1} yr^{-1})	OMD (%)	WSC (%/DM)	NDFD (%)
Lp	2×	Abermagic	15.2 bcd	79.7 a	20.1 a	70.2 c
Lp	4×	Aberbite	14.3 de	81.0 a	19.8 a	71.9 bc
Fp	2×	Merifest	13.5 e	78.5 a	13.6 b	73.1 b
Lp × Fp	4×	6381	15.9 b	80.8 a	15.7 b	75.4 a
Lm	2×	Podium	15.6 bc	72.2 bc	15.2 b	66.2 d
Lm	4×	Caballo	14.8 cd	72.8 bc	15.6 b	65.7 d
Fa	6×	Kora	17.5 a	69.9 c	14.4 b	70.1 c
Lm × Fa	5×	6323	15.9 b	74.2 b	15.1 b	70.1 c

[1] Means followed by a common letter are not different at the 5% level (Duncan MRT).

were arranged in a randomized complete block design. The plots were mown and sampled at 4 cuts in 2015, and 5 cuts in 2016 to determine the DM yield and the quality parameters: organic matter digestibility (OMD), water soluble carbohydrate content (WSC) and neutral detergent fibre digestibility (NDFD) by near infrared reflectance spectroscopy (NIRS). The data were analysed by ANOVA. At the end of the third year of the plot trial, plants in the two populations were sampled and multiplied to syn2 seeds in 2017. Seed yield of the synthetics was determined on the isolation plots of the polycrosses and multiplications. The seed yield of tetraploid Lp and Lm synthetics was measured on adjacent isolation plots as a reference. In 2018 we performed GISH on 10 plants of both the syn1 and syn2 of the two *Festulolium* synthetics. The proportion of the *Lolium* and *Festuca* genome in the *Festulolium* synthetics was analysed using DrawID (Kirov *et al.,* 2017).

Results and discussion

The dry matter yield of the Lp × Fp amphiploid was superior to the cultivars of the parental species. Its OMD was as high as the average OMD of tetraploid perennial ryegrass varieties but the WSC content was significantly lower. The NDF digestibility was significantly higher than those of both parental species. The Lm × Fa *Festulolium* had an intermediate DM yield between the varieties of both parental species. Its digestibility was superior to the cultivars of the parental species because of the combination of a similar WSC content to the *Lolium* parent and a similar NDFD to the *Festuca* component.

The seed yield of both *Festulolium* synthetics was inferior to the seed yield of the *Lolium* species. The seed yield of the Lp × Fp amphiploid remained rather stable in the two consecutive generations in comparison with the *Lolium* reference. The seed yield of the Lm × Fa amphiploid dropped extremely from syn1 to syn2 (Figure 1). Most probably this can be related to the chromosome number and stability of the genome composition in the *Festulolium* hybrids (Table 2). The chromosome number remained the same in the successive generations of the Lp × Fp amphidiploid and GISH revealed no clear shift to one of the composing genomes. In contrast, the chromosome number in the successive generations of the Lm × Fa amphiploid decreased and the genome composition shows a clear shift towards the *Lolium* genome.

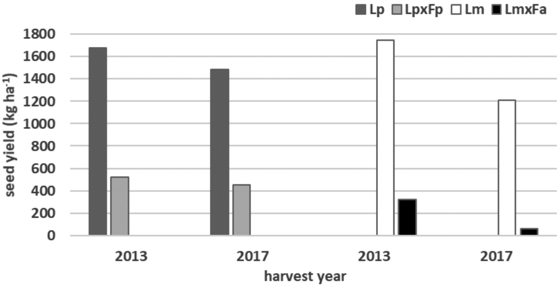

Figure 1. Seed yield of syn1 (2013) and syn2 (2017) of Lp × Fp and Lm × Fa amphiploid *Festulolium* compared to seed yield of tetraploid synthetics of Lp and Lm in the same years (unreplicated plots on the same fields).

Table 2. Chromosome number and proportion of *Lolium* genome in F1, syn1 and syn2 generations of Lp × Fp and Lm × Fa (mean and standard deviation).

Amphiploid	**Generation**	**Plants (n)**	**Chromosome number**		**% *Lolium* genome**	
			mean	**sd**	**mean**	**sd**
Lp × Fp	F1	1	28.0		49.0	
Lp × Fp	syn1	10	29.2	1.3	47.3	6.6
Lp × Fp	syn2	8	28.6	1.7	48.3	10.7
Lm × Fa	F1	2	35.0	0.0	40.6	0.6
Lm × Fa	syn1	10	34.8	3.3	49.7	4.5
Lm × Fa	syn2	10	30.6	3.2	59.4	7.2

Conclusion

Festulolium amphiploids may be superior in yield and fodder quality to their *Lolium* parental species. However, to validate their potential it is important to improve seed yield by looking for a stable chromosome composition.

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The imperative of improving yield of perennial forage crops: will genomic selection help?

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Abstract

Yield increases of forage crops are essential if they are to be profitably included in crop rotations. The many agronomic and environmental benefits of forage crops argue that their inclusion is necessary to develop truly sustainable cropping systems. One possible way to increase the genetic gain for biomass yield is to apply genomic selection as a way to greatly decrease cycle time. Here, we present initial results from a genomic selection experiment in which biomass yield was compared of populations developed using either phenotypic selection (clonal evaluation) or genomic selection for high or low yield. The results show that marker-only selection for high yield resulted in a population that yielded more than a population selected with markers for low yield. However, yields of marker-selected populations were not higher than the phenotypically selected population control and a second cycle of marker-only selection was ineffective. Reasons for this lack of progress are discussed. Despite these results, marker-based yield selection appears to have promise for forages.

Keywords: alfalfa, lucerne, genomic selection, biomass yield, cropping systems

Introduction

Designing and implementing stable, resilient, and environmentally benign cropping systems *requires* the incorporation of perennials into the landscape and into crop rotations. Herbaceous perennial forage crops are known to provide numerous environmental benefits to cropping systems (Olmstead and Brummer, 2008); for example, maize (*Zea mays* L.) yields are usually higher following alfalfa than following corn. If farmers are to include perennial forages in their cropping system, these crops need to show economic benefits on par with annual crops. Given that annual grain and oilseed crops are continually being improved for yield, increasing forage crop productivity is essential to keep these crops competitive in crop rotations.

Forage breeders must do more to increase yield. For most perennial forage crops, yield gains have been limited or non-existent, and those that have occurred often resulted from improvement in defensive traits, like disease resistance, rather than increased yield potential. Yield *per se* is not often not directly measured until late in the cultivar development process, but when it is evaluated under sward conditions, increases have followed (Brummer and Casler, 2014). Multiple harvests per year and multiple years of stand life make evaluating yield time and resource intensive. Two opportunities to increase the efficiency of selection are afforded by high throughput phenotyping using proximal or remote sensing and high throughput genotyping enabling genomic selection (Li *et al.*, 2015). In this paper, we present initial results from an empirical test of genomic selection to improve yield using only genetic marker selection.

Methods

Using a genomic selection model based on biomass yield measured in Ithaca, NY (Li *et al.*, 2015), we conducted genomic selection based solely on marker values. We grew and genotyped 400 individual seedlings from population NY0847 using genotyping-by-sequencing (GBS). This population was also used for phenotypic selection, resulting in population PSC1-H (NY1221). We conducted GBS as described previously (Li *et al.*, 2015), multiplexing 100 genotypes in a single lane of the HiSeq 2000. Following SNP scoring and imputation, we computed genomic enabled breeding values (GEBVs) for each individual plant and then selected the top 20 genotypes based on GEBVs. We restricted selections to no more than four individuals from any given maternal half-sib family to maintain variation in the population. These 20 individuals were intermated in the greenhouse by hand without emasculation to form the GSC1-H population. Analogous populations based on the lowest 20 GEBVs (GSC1-L) and a random selection of 20 plants were created simultaneously (GSC1-R). For the second cycle of selection, seeds of each maternal half-sib family derived from the intercross resulting in GSC1-H were germinated and DNA from 20 plants from each of the 20 families was isolated for marker analysis. We selected the top and bottom 20 individuals as done for Cycle 1 to form GSC2-HH and GSC2-HL, respectively. We then increased seed of all populations in a common environment in 2016.

To evaluate our progress from selection, we planted standard cultivar evaluation trials in spring 2017 at Ithaca, NY and Tulelake, CA. Plots were approximately 1 m wide and 3 m long; ten replications were included at each location. Seed was drilled at 17 kg ha^{-1}. In the first full production year (2018), yield was harvested with a mechanical harvester equipped with a computerized weigh system; dry weight samples were taken to adjust for moisture.

Results

First year yields show that the two locations gave largely similar results (Table 1). When comparing the first cycle genomic selection populations, GSC1-H had higher yield than GSC1-L. This indicated that our markers were able to discriminate between high and low yielding plants. However, GSC1-H was not higher than PSC1-H, the population selected phenotypically for higher yield and more disappointing, neither showed an improvement over the C0 base population. The GSC1-R population would be expected to fall between the high and low populations and the results show that it does. Performance of the C2GS populations was opposite of expectations (L was greater than H), suggesting that the transferability of our GS model from one cycle to the next was poor.

Discussion

There are several reasons why our GS model may not have been more successful. First, the model was based on spaced plant yield data and not on data generated from swards. Although many breeding programs are based on spaced plants, the correlation of spaced plant data with sward performance can be low, as shown in recent years in tall fescue (Waldron *et al.* 2008) and switchgrass (Sykes *et al.*, 2017). Thus, one way to improve GS modeling would be to develop models based on sward yields. Of course, the same argument can be used to improve traditional phenotypic selection.

Table 1. Total biomass yield (Mg ha^{-1}) for populations selected for high (H) or low (L) yield using phenotypic (PS) or genomic (GS) selection.

Entry	Ithaca	Tulelake	Overall
C0 (NY0847)	15.47	19.91	17.69
PSC1-H (NY1221)	15.36	19.77	17.55
GSC1-H	16.07	19.66	17.84
GSC1-R	15.29	19.28	17.28
GSC1-L	14.55	18.83	16.70
GSC2-HH	14.48	19.19	16.84
GSC2-HL	15.18	19.77	17.49
LSD (5%)	0.76	0.78	0.56

In addition, our GS model was a rather simple model that did not account for the full complexities of autotetraploid genetics – namely, it did not differentiate among the three heterozygote genotypic classes (for example, AAAT, AATT, and ATTT). A more refined model, possible using statistical frameworks developed since we did this selection, could result in a better prediction of yield.

We may be observing inbreeding depression in these results. Although we limited selections from individual families, we did not attempt to maximize genetic diversity otherwise. Finally, we have evidence from fall dormancy evaluation trials that the fall dormancy of the selected populations has increased, perhaps by genetic drift during our GS for higher yield. The inadvertent dormancy effect may counter the positive effect of yield selection using our GS model. In any case, the robustness of our GS model for more than one cycle was poor.

Conclusion

Genomic selection did not increase yield in this experiment. However, because we did see clear separation between high and low yield selections based on the marker model, we believe GS is a potential way to improve yield if other aspects of selection are improved. We need to base models on actual sward yield data, we need to control inbreeding depression, and we need to keep fall dormancy constant across cycles. We are conducting a second production year of data in 2019 to get a full picture of the productivity of these populations.

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Breeding energy grasses for increased biomass yield increases their nitrogen demand

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Abstract

Switchgrass (*Panicum virgatum* L.) and big bluestem (*Andropogon gerardii* Vitman) are undergoing development as perennial biomass crops in North America. Increasing biomass yield is the principal breeding goal, with the focus on creating more sustainable and profitable biomass crops. Since inception of the first breeding programmes in 1992, plant breeders have increased biomass yield and broadened the adaptation of numerous germplasm pools. The purpose of this study was to determine the impact of those breeding gains on nitrogen concentration and nitrogen removal rates in the harvested biomass. A total of 20 big bluestem trials and 23 switchgrass trials were established in 2012 or 2014 to evaluate advanced breeding populations developed by several breeding programmes. Each trial was conducted as a randomized complete block with up to four replicates and harvested for 2 or 3 years. Gains in biomass yield from 30 years of breeding ranged from 7 to 91%, depending on the species and source of germplasm. Because the genetic correlation coefficient between biomass yield and nitrogen concentration was near zero, changes in N were largely random and not generally significant. Thus, changes in nitrogen removal, computed as the product of biomass yield and nitrogen concentration, closely paralleled those of biomass yield per se. Increasing biomass yield increased nitrogen demand by an average of 2.2 kg per Mg biomass. Reducing nitrogen demand of these grasses will require combined selection for high biomass yield and reduced nitrogen concentration.

Keywords: switchgrass, big bluestem, *Panicum virgatum*, *Andropogon gerardii*

Introduction

Development of sustainable perennial grass production systems is a key element of many proposed cellulosic biomass systems (Robertson *et al.*, 2017). In the USA, switchgrass has been the model herbaceous species for these systems since 1992 (Sanderson *et al.*, 2007). Although it receives considerably less attention and funding, big bluestem is also a candidate for biomass production, owing to its high yield in monocultures and its candidacy as a component of high-diversity biomass polycultures (Mitchell *et al.*, 2016). Breeders have been extremely successful increasing biomass yield of switchgrass and, to a lesser extent, big bluestem. Genetic gains in biomass yield have been documented from several breeding programmes (Casler and Vogel, 2014; Casler *et al.*, 2018). Despite these gains and advancements, the role of nitrogen fertilizer in both commercial production systems and in breeding nurseries remains hotly debated and largely unresolved (Parrish and Fike, 2005; Robertson *et al.*, 2017). The purpose of this study was to determine the extent to which increases in biomass yield associated with breeding gains have led to increased nitrogen demand for biomass crops of switchgrass and big bluestem.

Materials and methods

Switchgrass and big bluestem field experiments were planted at 13 locations in April or May 2012 and 2014, representing a range of environments within USDA Hardiness Zones 3-6 (Casler *et al.*, 2018). Due to stand failures, there were a total of 19 big bluestem trials and 23 switchgrass trials. The experiments included 20 or 22 switchgrass populations and 11 or 12 big bluestem populations. Each experiment was designed as a randomized complete block with four replicates and plot sizes that varied with location. All plots were established with either 5-row or 7-row drill planters (0.9 m or 1.2 m width) at a seeding rate of 600 pure live seeds m^{-2}. Germination percentages and 1000-seed mass were used to adjust seeding rates to a pure-live-seed basis. Nitrogen fertilizer was applied to all plots at a rate of 100 kg N ha^{-1} in each harvest year. Plots were mechanically harvested about 7 to 20 days after killing frost for up to 4 years (2012 trials) or 2 years (2014 trials). Nitrogen concentration was predicted on dried and ground tissue samples, using existing near-infrared reflectance calibrations from a broad-based population of samples. Nitrogen removal (kg ha^{-1}) was computed as the product of biomass yield (Mg ha^{-1}) and nitrogen concentration (mg g^{-1} or kg Mg^{-1}). Data were analysed by mixed models ANOVA, contrasts, and linear regression.

Results and discussion

The cultivars in these field studies represented control cultivars plus those created during 30 years of breeding for increased biomass yield and broader adaptation. A previous paper documented significant gains in biomass yield, as high as 40% greater than the check cultivars, generated by one of four different mechanisms (Casler *et al.*, 2018). The correlation between biomass yield and nitrogen concentration was low in big bluestem (r = −0.18±0.07) and moderate in switchgrass (r = −0.49±0.07). Biomass yield had a 25-45% greater effect on nitrogen removal than nitrogen concentration, as indicated by standardized partial regression coefficients (data not shown).

Because of this result, nitrogen removal was strongly influenced by changes in biomass yield, mostly brought about by plant breeding efforts (Table 1). Linear regressions of nitrogen removal on biomass yield were significant for 18 of 19 big bluestem trials and 21 of 23 switchgrass trials. Extreme and mean values of these regressions are shown in Table 1 and Figure 1. On average, each Mg increase in biomass yield led to an increase in nitrogen removal of an additional 2 kg for big bluestem and 3 kg for switchgrass. Maximum values were 6 kg for big bluestem and 7 kg for switchgrass. Only 10 of the 42 trials showed no increase or a decrease in nitrogen removal associated with increased biomass yield. Trials with the highest nitrogen removal rates tended to be those located on soils with lower electrical conductivity and pH, or higher available water content and organic matter. Because the trials were harvested 2-4 weeks post-killing frost, it is not likely that these increases in nitrogen removal rates can be attenuated by any further delay in harvest time.

Table 1. Statistics for the linear regressions of nitrogen removal (kg ha^{-1}) on biomass yield (Mg ha^{-1}) for the two most extreme slopes (min and max) and the mean slopes for each of two species within the 2012 and 2014 plantings (all shown in Figure 1).

Species	Year	Location	Slope (kg N Mg DM^{-1})	R^2	*P*-value
Big bluestem	2012	State College, PA (min)	-0.05±0.34	0.00	0.87
Big bluestem	2014	Mead, NE (max)	5.71±1.62	0.58	<0.01
Big bluestem	2012	Mean over locations	1.57±0.40	0.61	<0.01
Big bluestem	2014	Mean over locations	2.10±0.58	0.60	<0.01
Switchgrass	2012	Chatham, MI (min)	-1.72±1.06	0.13	0.12
Switchgrass	2014	Grand Rapids, MN (max)	6.81±0.40	0.94	<0.01
Switchgrass	2012	Mean over locations	2.08±0.76	0.27	0.01
Switchgrass	2014	Mean over locations	3.00±0.37	0.79	<0.01

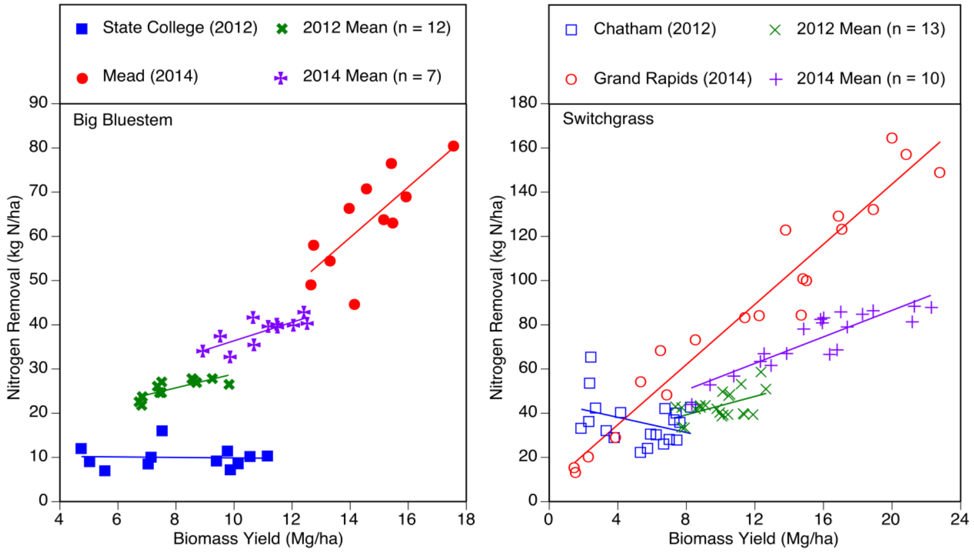

Figure 1. Scatterplots and linear regressions of nitrogen removal as a function of biomass yield for big bluestem and switchgrass. Each panel shows the regressions averaged across all locations within an establishment year (2012 or 2014) and the two individual trials with the highest and lowest slopes across all location-years (regression statistics shown in Table 1).

Conclusion

Energy grass breeders should alter their selection methods to simultaneously breed for increased biomass yield and reduced N concentration to attenuate increases in N removal.

Acknowledgements

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One hundred years of forage grass breeding at Aberystwyth

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Abstract

In 1919 the Welsh Plant Breeding Station was established through the visionary zeal of Sir George Stapledon who saw the urgent need to increase the productivity of grasslands in the UK following the 1914-18 war, when so much land had been ploughed up for cereals. There was a serious lack of reliable commercial grass seed, often having variable germination, contaminated with weeds and generally of a coarse type, with little persistency or leaf quality. In addition to improving grassland agriculture in general, Stapledon believed that the uplands could also provide a much greater contribution to animal production than previously achieved. Early breeding work focused on improving the yield and persistency of both grasses and clovers; notably through the 'S' strain varieties which provided a strong foundation for subsequent work. In the later part of the 20th Century breeding targets developed to include raising digestibility, and nutritional qualities such as water-soluble carbohydrate leading to improvements in ruminant efficiency; resulting in the commercially successful 'Aber' prefixed varieties. The current variety developments target both production and environmental goals, including nutrient use efficiency, improved root development and ecosystem services.

Keywords: Stapledon, forage breeding, history

Introduction

Having recognised the urgent need for improving the national grassland, Stapledon, along with T.J. Jenkin, R.D. Williams and with financial backing from a small group of wealthy benefactors, founded the Welsh Plant Breeding Station (WPBS). Based within Aberystwyth University they developed a strategy to not only create better quality forage types for re-sowing pastures lost to the war effort, but to improve the productivity of grassland nationally including better utilisation of the more marginal uplands.

For many years commercially available grass seed was known to be of inferior quality compared to established pasture grasses (Everitt, 1897; Wilkins, 1926). Many of the seeds available were essentially landraces (regional ecotypes) such as Pacey's Rye Grass, Devon Eaver, Ayrshire and Irish, which flowered profusely and produced large amounts of seed, but lacked quality or persistence. The situation of seed quality and availability came to a head following the 1914-18 war for three main reasons (Thomas, 1969). Firstly, many new leys were being established on land exhausted by years of cereals. Secondly, available seeds were poor quality, often with low germination and a wide variety of weeds. In 1914 Stapledon described this as the 'seeds scandal' in a report that ultimately led to legislation and official testing in 1917. Thirdly, the varieties used were stemmy and so lacking in persistency that after the first flush of growth they were often no better than allowing the land to 'tumble down' to grass with the eventual establishment of persistent indigenous plants.

1919-1939

In the early years of Stapledon's work a wide range of grass species were collected and assessed. Based on the observation that pastures managed over a long period for a particular purpose often contained the species and types suitable for the purpose they were being managed for, plants were collected from renowned fattening meadows in the English Midlands, Pembrokeshire, Kent and many other locations across the country. A handwritten note in one of the original ledgers reads 'Red Rodney Field, Leicestershire, considered the best pasture in England 'a bullock goes in lean, goes round the field once and comes out fat". Despite this poetic description the early breeding work was based on rigorous plant biology which included the understanding that most pasture grass species were self (or partially self) incompatible and being out-breeders suitable isolation procedures were necessary to ensure varieties were kept pure (Jenkin *et al.*, 1931). In April 1922, Stapledon set out his 'Book of Words' which described in minute detail the methodology and recording of trials, procedures and classification of plants and populations. Everything from noting parental lines to ensuring equipment was maintained correctly.

Throughout the history of the WPBS, (Breese and Davies 1969; Wilkins and Humphrys 2003) the breeding work has been driven by clearly defined agronomic objectives as a prerequisite to breeding operations regardless of the crop type. For herbage crops these objectives continue to be defined within the context of animal production with worthwhile economic returns. The requirements are therefore complex and have to be translated into physiological and biochemical criteria which can be assessed at the population and individual plant level. With this in mind two significant developments were reported in the WPBS Series H Bulletin no.3. The first was the use by Fagan (1924) of chemical analysis to assess the nutritive value of the herbage, the second was by Stapledon *et al.* (1924) in a paper entitled 'Grassland and the Grazing animal' which first presented the concept that agronomic work was moving away from a purely botanical outlook. The following year Davies (1925) published a paper on the relative palatability of pasture plants. By 1932 the 'S' varieties (or strains) were being developed alongside studies on chemical composition and palatability.

1939-1987

Through the 1930s the work of the WPBS was gaining recognition for its improved forage strains and was beginning to work more closely with an increasingly organised seed industry. However, of the strains produced, only relatively few were marketed as it had not been the policy of the station to add to the range of commercial varieties unless a particular deficiency could be specified, or significantly new and different aspect be offered (Breese and Davies, 1969). This meant that at the onset of war in 1939 there was still a shortage of seed of the new varieties (Lewis, 1942). Furthermore, many of the WPBS staff were seconded by the Ministry of Agriculture for advisory work to help increase agricultural output (Jenkin 1950), so breeding was virtually suspended. By 1943 physiological genetics had split away from forage breeding, (Jenkin, 1957) and a period of fundamental science embarked upon with regard to both genetics and agronomy. Leafiness was always high in breeding priorities, but its full significance was not fully established until Tilley and Terry (1963) developed the technique to measure *in vitro* digestibility thus quantifying nutritive value and delivering the means for highly targeted selection. The production of inter-specific and inter-generic hybrids and developments in cytology sparked interest in ploidy as a breeding tool. This led to the development of tetraploid hybrid ryegrasses (Sabrina) and the early *festulolium* varieties, Elmet and Prior.

1987-present

The late 1980s saw another shift in breeding perspective when the Grassland Research Institute (Hurley) combined with Aberystwyth to form the Institute of Grassland and Environmental Research. This prompted renewed scientific interest in ruminant nutritional requirements. A partnership was formed with Germinal Holdings which began a new era of plant breeding focused on increasing the efficiency of ruminant milk and meat production, with emphasis on water soluble carbohydrate. By then, *Lolium* was the key genus fulfilling a range of purposes. Amenity grasses had featured in the early years (a *Festuca rubra* and a *Phleum*) and an amenity programme was re-established in 1989. In 2008 the institute was re-united with the University to form the Institute of Biological, Environmental and Rural Sciences. The numbers of varieties produced and then commercialised has changed dramatically over time (Table 1). The initial interest in species such as cocksfoot, once the main focus for improvement, has been overtaken by the plasticity and quality of ryegrass.

Table 1. Numbers of varieties produced and in commerce 1930-2018 (Aberystwyth Strain Ledger).

	1930-1947		1968-1987		1987-2018	
	Produced	Commerce	Produced	Commerce	Produced	Commerce
Lolium perenne	8	3	25	1	56	25
L. multiflorum	4	1	7	52	5	3
Hybrid ryegrass	-	-	8	3	15	7
Dactylis glomerata	37	4	7	3	1	1
Phleum pratense	7	3	2	-	-	-
Festuca pratensis	2	2	1	-	-	-
Festuca arundinacea	2	3	3	1	-	-
Festuca rubra	6	2	2	1	4	3
Agrostis spp.	-	-	2	1	2	2
Festulolium	-	-	2	1	2	1

The current achievements at Aberystwyth are a result of applying sound scientific principles and novel genetics. Developments in gene sequencing open up great potential for targeting specific genes controlling desired traits rather than relying solely on chance recombination via conventional crossing. The application of genome-wide selection to forage species could deliver significant reductions in the time taken to breed varieties. Further developments in understanding the requirements of grazing animals has opened up new opportunities for breeders to modify the composition of forage plants. This is an exciting time with a massive resurgence of grass-based systems, where commercial and environmental factors combine to ensure that the development of high quality and yielding forage varieties is ever more important to meet the demands of modern agriculture.

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Comparison of plant persistency between and within *Festulolium* and *Dactylis* progenies under summer water deficit conditions

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Abstract

We investigated the effectiveness of individual plant selection for persistency in a dense nursery of spaced plants submitted to water deficit in the greenhouse. This was carried out using *Festulolium* backcross progenies and F1 and F2 *Dactylis* hybrids generated by crossing genotypes of temperate and Mediterranean origin. Results regarding individual plant biomass after eleven cuts over two years showed that leaf growth and earliness in spring are important drivers of persistency. The response to water deficit obviously included the effect of plant competition over time, but higher persistency was demonstrated in *Dactylis* of Mediterranean origin and heterotic-like response in hybrid progenies. By contrast, the beneficial effects of fescue-specific traits in *Festulolium* were partly masked due to the good ability of ryegrass for high recovery after water deficit. The results suggest that selection for persistency within *Festulolium* compared to *Dactylis* requires accounting for larger expression of plant-plant competition due to the interspecific level of variability and the predominant expression of the *Lolium* genome characteristics under high plant density.

Keywords: drought tolerance, competition, interspecific hybridization, breeding methods

Introduction

Under Mediterranean conditions, breeding for persistency in grass species is a challenge in terms of aiming to capture traits of plant tolerance to water deficit while not compromising productivity over years. Progeny selection can be carried out effectively in dense stands when seed availability is not limiting. However, progeny selection in field trials remains cost- and time expensive. In addition, the intensity of selection cannot be as high as when carried out at the individual plant level. Furthermore, the response to selection may be confusing after *Festuca* × *Lolium* hybridization. Indeed, genome composition of current *Festulolium* cvs has been found to have shifted to *Lolium* rather than to *Festuca* (Kopecky *et al.*, 2006; Zwierzykowski *et al.*, 2006), which is opposite to the expectation, given primary species-specific persistency and drought adaptation (Ghesquière *et al.*, 2015).

We investigated how inter-specific genetic variability can contribute differentially to express tolerance to water deficit among and within *Festulolium* accessions. *Dactylis* was used as a control of intraspecific genetic variability although it was enhanced by the use of hybrid progenies of Mediterranean origin. As water limitation cannot be easily reproduced in the field, we used glasshouse conditions in decreasing, more or less severely, watering from May to September. In addition, we used a plant density that was 10-times higher than is usual in a field nursery in order to increase water uptake competition.

Materials and methods

Twenty progenies of *Dactylis* and 13 progenies of *Festulolium* and related controls were compared as a split 4-complete-blocks design with rows of 10 individuals per progeny planted at an inter- and intra-row distance of 0.20 m. The experiment was established in October 2016 in a glasshouse with deep soil. In *Dactylis*, 18 F1 and F2 pair-crosses were produced by crossing individual plants of cv Ludac (of temperate origin) and cv Medly (of Mediterranean origin). Both parental cvs were included as controls. In *Festulolium*, 8 genotypes were HS families from a back-crossing generation (BC1) of a *L.*

multiflorum × *F. glaucescens* (2n=4×=28) hybrid into *L. multiflorum*. In addition to the hybrid (*Lm* × *Fg*, cv Lueur), further controls comprised Italian ryegrass, (*Lm*, cv Udine), hybrid ryegrass (*Lm* × *Lp*, cv Enduro and Kyrial) and a *Festulolium* cv similar to cv Lueur but derived from *F. pratensis* (*Lm* × *Fp*, cv Lifema). Individual biomass was harvested and dried through 6 cuts in 2017 and 5 cuts in 2018. The cutting regime was managed according to the large variability for heading date, especially among *Dactylis* genotypes. Summer water deficit was simulated by ceasing watering from May to September in year 1, and by applying 60 mm water only two times during the same period in year 2. As a consequence, cuts 4 and 5 in year 1, and cuts 8, 9 and 10 in year 2 were taken as being under water deficit conditions. Biomass at each successive cut was adjusted by individual mean biomass of all previous cuts as a covariate. In *Dactylis*, the impact of water deficit was profiled as the difference of adjusted biomass between the Mediterranean cv Medly and the temperate cv Ludac. In *Festulolium*, the contrast was similarly computed as between the drought-tolerant *Lm* × *Fg* hybrid and the susceptible *Lm* parent species.

Results and discussion

Individual biomass was found to highly correlate across cuts with minimum value at the peak of water deficit, r=0.53 in *Dactylis* at cut 5, or at heading time, r=0.56 in *Festulolium* at cut 7, but restoring each at 0.86 and 0.71 (respectively) at final cut. The profile of plant biomass over time varied largely depending on the plant population (Figure 1). In *Dactylis*, the contrast of biomass before drought in year 1 indicated that heading in the control cv of Mediterranean origin is earlier by about 3 weeks than in the control cv of temperate origin. Then, the biomass of the Mediterranean control continuously exceeded that of the temperate control to cumulate by 36% of the mean at final cut.

On the other hand, the contrast of biomass in *Festulolium* may widely reverse across cuts and years but end with advantage of the *Lm* × *Fg* hybrid by 60% of the mean. F-tests between *Dactylis* progenies increased similarly over time from the first water-limiting cut 4 up to $h^2 = 0.44$ at cut 11. Heritability was hardly significant among *Festulolium* BC1 progenies, with $h^2 = 0.05$ at final cutting. When adjusted by previous cuts, biomass dry weight at the final cut still correlated positively with unadjusted means in *Dactylis* (not shown) while the correlation was negative among *Festulolium* and related controls (Figure 2), emphasizing significantly higher mean tolerance of the *Lm* × *Fg* cv than of the *Lm* cv. However, in contrast to *Dactylis*, plant response in *Festulolium* was found to differ strongly from the persistency usually observed in field plots. Following adjustment, variability within *Festulolium* progenies highly decreased, but the variability within the BC1 progenies still overlapped widely with *Lm* and *Lm* × *Fg* controls. This suggests possible misleading selection if one aims to specifically improve persistency by using the traits and primary genetic control of the *Festuca* genome.

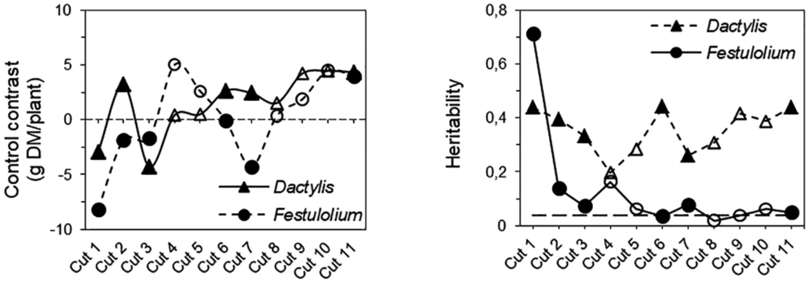

Figure 1. Contrasts in amounts of dry biomass between the tolerant cv and the susceptible control cv (left) and heritability s.s. between progenies (right) in *Dactylis* and in *Festulolium* across 11 cuts over 2 years. Cuts after limiting the watering shown as open symbols. The contrasts are T-test values and are significant ($P<0.05$) when below -2 or above 2; h^2 is significant when above 0.04 (dashed line) following F-test ($P<0.05$).

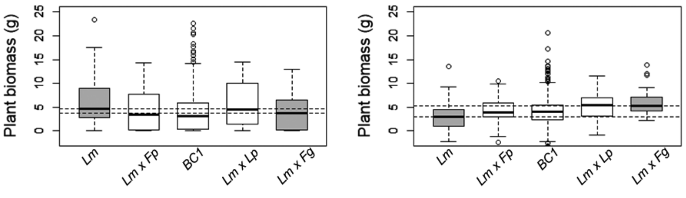

Figure 2. Range of individual dried plant biomass at final cut in year 2 among *Festulolium* progenies and *L. multiflorum* as parent species (*Lm*) when not adjusted (left) and adjusted (right) by plant individual mean value of biomass at all previous cuts as a covariate. The BC1 population comes from backcrossing *Lm* × *Fg* hybrid into *Lm*.

Conclusion

Persistency does not comprise just drought tolerance and survival traits but also traits of recovery linked to leaf growth and phenology. Thus, the response to water deficit in *Festulolium* is likely to differ from that of *Dactylis* because of interspecific recombination of contrasting traits according to genome sources. Our results underline that the effect of competition was amplified in glasshouse conditions and a dense spaced plant design with the risk of misleading selection. Adjusting biomass of recovery after drought by the profile of biomass production before drought allowed us to develop a better expression of true tolerance traits. Reducing the Mendelian sampling within *Festulolium* progeny and predicting the breeding value of each individual plant using large sets of molecular markers are further promising approaches to make selection more effective, as well as direct selection for species-specific trait.

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Combining abilities and heterosis for forage and quality traits of alfalfa (*Medicago sativa* L.) from second generation of selfing in a diallel design

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Abstract

Improvement of forage yield and quality is an important objective of alfalfa breeding. Development of free hybrids in alfalfa could be capitalized through selecting parents for achieving heterosis by crossing lines obtained from 2-3 generations of selfing. Experimental fields were established in two locations in 2014-2016 with clones of seven selected partly inbred (S_2) lines of ecotypes from different climatic regions of Iran and one Australian cultivar (as parents), and their 28 half diallel hybrids (F_1). Heterosis and combining abilities for total forage dry matter yield, plant height and quality traits including leaf to stem ratio (LSR), crude protein (CP), water soluble carbohydrate (WSC), ash, acid detergent fibre (ADF) and neutral detergent fibre (NDF) were estimated. The results of diallel analysis indicate high significant effects of hybrid, and general combining ability (GCA) and specific combining ability (SCA) effects. Positive significant SCA effects, mid-parent heterosis (MPH) and high-parent heterosis (HPH) were observed in some hybrids derived from eight partially inbred lines. The highest MPH and HPH observed for total DM yield were 56.02% and 52.99% respectively. Results indicate opportunities to exploit hybrid vigour by crossing partial inbreds (S_2) in order to improve forage yield in alfalfa.

Keywords: alfalfa, diallel, GCA, heterosis, hybrid vigour, SCA, selfing

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important forage crops in the world (Michaud *et al.*, 1988). Several studies have demonstrated the possibility of exploiting additive and non-additive genetic effects involving complementary gene interactions for alfalfa (Al Lawati *et al.*, 2010; Milić *et al.*, 2010). The identification of heterotic groups and utilization of heterosis in alfalfa semi-hybrids obtained by crossing genetically divergent germplasms has also be proposed (Brummer, 1999; Riday and Brummer, 2002). The effect of heterosis could be used for development of free-hybrids by crossing lines obtained from 2-3 generations of selfing (Rotili *et al.*, 1999). In general, both GCA and SCA increase with inbreeding and the best single crosses came from selected inbred parents (Rotili, 1976). The objective of this study was to conduct a diallel analysis for forage yield, plant height and quality traits and determine combining abilities effects and heterosis in a set of alfalfa lines derived from two generations of selfing of seven alfalfa ecotypes from Iran and one cultivar from Australia.

Materials and methods

Pair crosses following a half diallel scheme were performed with partial S_2 inbreds derived from seven alfalfa ecotypes from different climatic regions of Iran and one from Australia. The ecotypes Bami and Nikshahri were collected in a tropical area, Ghahavand, Kozare, Mohajeran and Silvana in cold regions, Rahnani originated from temperate regions and Sequel was from Australia. The selected partial inbreds were hand-crossed without emasculation in a half-diallel mating design. Selection of parents during selfing generations and crossings were performed as described by Rotili *et al.* (1999). The eight partial inbreds (parents) and their 28 F_1 progenies were grown in the experimental fields of the Seed and

Plant Improvement Institute, Karaj and Arak, Iran in early of autumn 2014 with three replications in a randomized complete block design. The experimental unit consisted of four rows of two meters in length with 50 cm spacing between rows and 10 cm between the plants (20 plants per row). Total forage dry matter (DM) yield (g plant^{-1}), plant height (cm), forage quality traits including leaf to stem ratio (LSR), Crude protein (CP), water soluble carbohydrate (WSC), ash, acid detergent fiber (ADF) and neutral detergent fiber (NDF) were determined in spring and summer of 2015 and 2016 for each location. The dried ground samples were analysed by near-infrared reflectance spectroscopy (NIRs) (DICKEY-john Instalab®). The average of three cuttings per year was calculated. The two years in two locations were regarded as a factor, i.e. environment. Seasonal data for traits were analysed with the MIXED procedure in SAS release 9.2 using entries, locations and years (described as environment) as fixed effects and blocks as random effects. General combining ability (GCA) and specific combining ability (SCA) were calculated using the theoretical framework developed by Griffing's (1956) for a diallel without reciprocal crosses and including all parents, as implemented in DIALLEL- SAS (Zhang *et al.*, 2005). Mid-parent heterosis (MPH) and high-parent heterosis (HPH) across environments were computed.

Results

Diallel analysis across two locations and two years indicated high significant GCA and SCA effects for all investigated traits. The results of the diallel analysis demonstrated that the traits are controlled by additive gene effects, as reflected in significant GCA values, as well as by non-additive gene effects, as reflected in significant SCA values (Table 1 and Table 2). Crossing Nikshahri and Ghahavand, which displayed large positive GCA effects (Table 1), resulted in hybrids with the highest positive significant SCA effect, and MPH and HPH (Table 2) for multiple traits. Significant SCA effects were detected in crosses between genotypes from different geographical areas such as Ghahavand × Nikshahri and Mohajeran × Nikshahri. However, the cross Ghahavand × Mohajeran (both collected in cold areas) also showed high positive SCA effects and significant positive MPH and HPH.

Conclusion

The existence of significant heterotic effects for forage dry matter yield and other interesting traits in this set of crosses demonstrates the possibility of using partial S_2 inbreds representing populations with a narrow genetic base in alfalfa breeding. However, in an autotetraploid species such as alfalfa, crossing partially inbred parents does not reach the maximum of heterozygosity recovery and hence the maximum heterosis in the first generation (F_1).

Table 1. Estimates of diallel effects for general combining ability (GCA) for investigated traits of eight selected partial inbreds of alfalfa ecotypes and cultivars over four environments (2 locations, 2 years).[1]

No		DM yield (g plant^{-1})	Plant height (cm)	LSR	CP	WSC	ASH	ADF	NDF
1	Bami	1.40	0.16	-0.03**	-0.39**	-0.44**	-0.16**	0.10	0.23
2	Ghahavand	5.91	1.77*	0.01	0.26*	0.27*	0.13*	0.52	0.55
3	Kozare	-10.91**	-2.84**	-0.07**	-0.68**	-0.74**	-0.24**	-0.85	-0.81*
4	Mohajeran	-3.05	1.48	0.01	0.16	0.40**	0.04	0.95*	0.75*
5	Nikshahri	12.98**	1.43	0.03**	0.41**	0.23	0.15**	0.53	0.47
6	Rahnani	-5.80	-1.12	-0.01	-0.14	-0.17	-0.06	-0.57	-0.53
7	Sequel	-5.41	-0.76	-0.01	-0.42**	-0.29*	-0.20**	-0.01	0.06
8	Silvana	4.88	-0.11	0.07**	0.80**	0.75**	0.33**	-0.67	-0.72
	SE(g)	3.68	0.81	0.01		0.09	0.09	0.42	0.35
	SE(gi-gj)	5.58	1.23	0.02		0.14	0.14	0.63	0.52

[1] LSR: leaf to stem ratio; CP: crude protein; WSC: water soluble carbohydrate; ADF: acid-detergent fibre; NDF: neutral-detergent fibre; SE(g): estimated the standard error of general combining ability gi; SE(gi-gj): estimated the standard error of the difference between the effects of general combining ability gi and gj; * significant at $P<0.05$ and ** at $P<0.01$.

Table 2. Estimates of positive and significant specific combining ability (SCA) values per cross (2nd line of each cell) and highest and significant MPH and HPH (3rd line of each cell) respectively, for all traits investigated.[1,2]

DM Yield (g plant^{-1})	Plant height (cm)	LSR	CP	WSC	ASH	ADF	NDF
2×4 (20.09**) (38.02, 32.53)	2×5 (6.05**) (21.26, 19.60)	1×2 (0.16**) (34.31, 33.01)	1×2 (1.84**) (27.23, 25.15)	1×2 (1.71**) (33.98, 29.24)	1×2 (0.65**) (23.02, 21.69)	2×4 (1.92*) (13.13, 10.82)	2×4 (1.65*) (11.15, 9.95)
2×5 (40.42**) (56.02, 52.99)	4×5 (5.51**) (19.62, 17.51)	1×4 (0.11**) (23.29, 17.49)	2×4 (1.40**) (21.94, 18.27)	2×4 (1.43**) (29.95, 25.90)	2×4 (0.64**) (22.08, 19.65)	2×5 (2.93**) (18.97, 17.30)	2×5 (2.95**) (16.31, 14.98)
4×5 (31.68**) (49.12, 40.52)	7×8 (4.79**) (15.36, 15.31)	2×4 (0.09**) (24.94, 20.18)	2×8 (1.45**) (26.42, 22.51)	2×8 (1.15**) (32.74, 30.67)	2×8 (0.49**) (24.38, 22.07)	4×5 (2.34*) (15.99, 12.06)	4×5 (2.06*) (13.28, 10.79)
7×8 (30.00**) (36.48, 27.04)		2×8 (0.10**) (29.03, 22.81)	3×5 (1.21**) (22.00, 18.11	5×6 (1.12**) (31.8, 27.12)	5×8 (0.47**) (21.11,19.41)	7×8 (2.42**) (13.04, 9.90)	
		3×5 (0.10**) (29.65, 20.56)	6×7 (1.43**)	6×8 (1.27**) (30.79, 27.98)	7×8 (0.72**) (23.80, 20.59)		
		6×8 (0.11**) (29.63, 22.81)	7×8 (1.44**) (22.00, 18.11)	7×8 (1.46**) (32.62, 28.58)			
SE (sij)							
7.71	1.97	0.03	0.39	1.41	0.90	0.16	0.82
LSD (0.05%) for MPH and HPH, respectively							
22.64, 26.64	5.51, 6.44	3.83, 4.49	0.07, 0.08	0.98, 1.14	1.04, 1.21	0.42, 0.49	2.31, 2.67

[1] LSR, CP, WSC, ADF, NDF: see T able 1. Code of parents of crosses including, 1: Bami, 2: Ghahavand, 3: Kozare, 4: Mohajeran, 5: Nikshahri, 6: rahnani, 7: Sequel and 8: Silvana.

[2] SE(sij): estimated the standard error of the difference between the effects of specific combining ability (sij); ** Significant at $P<0.01$.

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Selection progress in a commercial forage breeding programme

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Abstract

We analysed historic data from the Agroscope forage crop-breeding programme. A joint analysis of plot trials installed between 1991 and 2015 was performed, revealing the performance of variety candidates produced before or during this period. Linear regression analysis between trait performance and year of production of the variety candidates was then performed. Positive response to selection was found for most traits and species. In red clover (*Trifolium pratense*), average progress in dry matter yield (DMY, sum per year) was more than double in the second compared to the first main season, which may be attributed to strong selection for increased persistence and resistance to detrimental diseases like southern anthracnose (*Colletotrichum trifolii*). In tetraploid ryegrasses (*Lolium* ssp.), progress in DMY was similar for the second and first main season, resulting in a 2.9 to 4.1% increase per decade. In contrast, in diploid ryegrasses, progress in DMY was mostly lower or insignificant. Further results for different species are presented.

Keywords: breeding programme, selection progress, dry matter yield, quality, disease resistance

Introduction

Information on breeding progress in forage crops is available from different studies analysing historical varieties (e.g. Mc Donagh *et al.*, 2016; Sampoux *et al.*, 2011), but is still scarce in terms of progress within a practical breeding programme following a given selection scheme, or for species other than perennial ryegrass (*Lolium perenne*). Here we present data from 25 years of breeding activities at Agroscope, a publicly funded forage crop breeding programme.

Materials and methods

The Agroscope forage crop-breeding programme is mainly based on phenotypic selection among spaced plants in the nursery. After open pollination among selected elite plants (using single plants directly in the nursery for red clover, using cloned plants in the polycross for grasses), half-sib (hs) progeny are grown in trials with seeded rows (SR), which are evaluated for different traits. Before flowering, all rows of hs-families with bad performance within an SR trial are eliminated. Seeds are then harvested in bulk from all positively selected hs-families of a SR trial. These syn-2 seeds harvested in a SR trial are the basis of a new candidate. All candidates produced are tested in plot trials, usually in two series sown one year apart (2 years of installation) at 3 locations, resulting in 6 testing environments. Plot trials last for 3 years (installation year, first and second main season). Different traits related to yield, quality and disease resistance are assessed (Table 1).

A joint analysis of plot trials installed between 1991 and 2015, including candidates produced before or during this period, was performed using the model

$$y_{ijn} = g_i + e_j + ge_{ij} + b_{nj} + \varepsilon_{ijn} \qquad \text{Eq. (1)}$$

where y_{ijn} represents the observed phenotype on a plot basis, g_i the genotypic effect of candidate i, e_j the effect of environment j (combination of location and installation year), ge_{ij} the genotype-by-environment interaction, b_{nj} the block effect nested within environment and ε_{ijn} the residual. Comparability of the different trials (=installation years) was enabled via overlapping candidates and standard varieties. Least

Table 1. Results from linear regression of trait performance on year of candidate production. Presented regression parameters are number of candidates (n), regression coefficient (b) and coefficient of determination (R^2).[a]

Trait	*Trifolium pratense*						*Lolium multiflorum*					
	diploid			tetraploid			diploid			tetraploid		
	n	b	R^2	n	b	R^2	n	b	R^2	n	b	R^2
DMY_Y1 [b]	93	0.270	0.17 **	73	0.538	0.41 **	92	0.340	0.24 **	62	0.344	0.40 **
DMY_Y2 [b]	93	0.660	0.48 **	73	1.028	0.64 **	92	0.308	0.19 **	63	0.323	0.24 **
DOM_Y1 [c]	69	-0.010	0.02 ns	58	0.055	0.01 ns	71	0.436	0.13 **	49	0.184	0.03 ns
Vig_Y0 [d]	93	0.006	0.00 ns	73	-0.008	0.05 ns	92	-0.018	0.09 **	63	-0.017	0.13 **
Vig_Y1 [d]	93	-0.036	0.27 **	73	-0.051	0.58 **	92	-0.037	0.21 **	63	-0.034	0.33 **
Vig_Y2 [d]	93	-0.098	0.53 **	73	-0.131	0.71 **	92	-0.070	0.41 **	63	-0.063	0.43 **
Persistence [e]	91	-0.135	0.59 **	73	-0.212	0.77 **	92	-0.073	0.41 **	63	-0.083	0.46 **
Winter [f]	–	–	–	–	–	–	92	-0.039	0.39 **	63	-0.028	0.32 **
Rusts [g]	–	–	–	–	–	–	91	-0.047	0.16 **	63	-0.093	0.66 **
Leaf spots [g]	81	-0.075	0.30 **	59	-0.034	0.10 *	92	-0.015	0.07 *	63	-0.037	0.33 **
Anthracnose [g]	79	-0.154	0.72 **	64	-0.242	0.79 **	–	–	–	–	–	–

Trait	*Lolium perenne* (early maturing)						*Lolium hybridum*					
	diploid			tetraploid			diploid			tetraploid		
	n	b	R^2	n	b	R^2	n	b	R^2	n	b	R^2
DMY_Y1 [b]	106	-0.063	0.01 ns	75	0.351	0.32 **	38	0.144	0.03 ns	127	0.367	0.24 **
DMY_Y2 [b]	106	-0.037	0.01 ns	75	0.392	0.41 **	38	0.117	0.02 ns	127	0.333	0.21 **
DOM_Y1 [c]	80	0.155	0.02 ns	63	0.374	0.16 **	25	-0.045	0.00 ns	104	0.359	0.05 *
Vig_Y0 [d]	106	-0.007	0.02 ns	75	-0.016	0.09 **	38	-0.032	0.13 *	127	-0.005	0.00 ns
Vig_Y1 [d]	106	-0.018	0.08 **	75	-0.050	0.58 **	38	-0.032	0.12 *	127	-0.028	0.18 **
Vig_Y2 [d]	106	-0.027	0.14 **	75	-0.070	0.65 **	38	-0.026	0.14 *	127	-0.058	0.49 **
Persistence [e]	106	-0.036	0.16 **	75	-0.082	0.65 **	38	-0.010	0.01 ns	127	-0.084	0.43 **
Winter [f]	106	-0.014	0.05 *	75	-0.011	0.04 ns	38	-0.001	0.00 ns	127	-0.013	0.07 **
Rusts [g]	106	-0.100	0.48 **	75	-0.075	0.59 **	36	-0.067	0.32 **	127	-0.030	0.17 **
Leaf spots [g]	106	-0.028	0.17 **	75	-0.038	0.35 **	38	-0.011	0.04 ns	127	-0.048	0.26 **

[a] *, ** indicate significance at the 5% and 1% level, respectively. ns = not significant.

[b] DMY_Y1 and DMY_Y2 = sum of dry matter yield [dt ha^{-1}] of the first and second main season, respectively.

[c] DOM_Y1 = digestible organic matter [g kg^{-1}DM] from all cuts of first main season.

[d] Vig_Y0, Vig_Y1 and Vig_Y2 = vigour rating (1 = good, 9 = bad), average over all cuts in establishment year, first main season and second main season, respectively.

[e] Persistence = persistence rating (1 = high persistence, 9 = low persistence) at end of second main season.

[f] Winter = winter damage rating (1 = no damage, 9 = high damage), also including occurrence of snow mould;

[g] Rusts, Leaf spots and Anthracnose = rating of disease infestation (1 = no infestation, 9 = highly infested) of rusts (mainly caused by *Puccinia coronata*), leaf spots and southern anthracnose (*Colletotrichum trifolii*).

square means were predicted for every candidate. Linear regression analysis was then performed to assess the association between trait performance and production year (i.e. year of installation of SR trial) of the candidates.

Results and discussion

For diploid (2×) and tetraploid (4×) red clover (*Trifolium pratense*), average yearly progress in dry matter yield (regression coefficient b, measured in dt ha^{-1} y^{-1}), was approximately double for the second compared to the first main season (Table 1). The same pattern of increased progress for the second main season was also observed for plant vigour, a trait that was not significantly improved for the installation year. One reason for this observation is that the breeding material has been selected for resistance against detrimental diseases like southern anthracnose (*Colletotrichum trifolii*) that started occurring during this period (Schubiger *et al.*, 2003). This is highlighted by high R^2 values for persistence and southern anthracnose.

For 4× Italian (*Lolium multiflorum*), perennial (*L. perenne*) and hybrid ryegrass (*L. hybridum*), progress in DMY was similar among species and main seasons, ranging from 0.323 to 0.392 dt ha^{-1} y^{-1}. This corresponds to approximately 3% increase per decade (Table 2), which is similar (Sampoux *et al.*, 2011) or somewhat lower (McDonagh *et al.*, 2016) compared to earlier studies. No significant breeding progress was observed in the 2× forms of *L. perenne* and *L. hybridum*. For *L. hybridum* this can partly be explained by the low breeding activities (n=38). In 2× *L. perenne*, breeding activities are higher (n=106), so selection pressure for DMY was apparently too low. Reasons here for might be low correlation between vigour of spaced parental plants, a main selection criterion, and vigour or yield of offspring in swards (Kempf *et al.*, 2019). Progress in DMY of 2× *L. multiflorum*, a relatively short-lived species, was significant. By reducing susceptibility to winter damage (b=−0.039, R^2=0.39) and improving resistance to detrimental diseases like bacterial wilt (*Xanthomonas translucens pv. graminis,* data not shown), persistence of this material was improved (b=−0.073, R^2=0.41). Therefore, progress in DMY is probably an indirect consequence of improved plant survival.

Digestibility of organic matter (DOM) is not being used as a selection criterion in spaced plants in the nursery or in SR trials for the presented species, but selection is only performed among candidates. As consequence, no to very low selection progress could be observed for this trait in all species. In contrast, resistance to diseases like rusts undergoes a high selection pressure in the nursery and in SR trials, and a significant selection progress could therefore be observed for all grass species.

Table 2. Average selection progress in % per decade in dry matter yield for *Trifolium pratense* (Tp), *Lolium multiflorum* (Li), *L. perenne* (Lp) and *L. hybridum* (Lh).

	Species							
Trait	**Tp (2×)**	**Tp (4×)**	**Li (2×)**	**Li (4×)**	**Lp (2×)**	**Lp (4×)**	**Lh (2×)**	**Lh (4×)**
DMY_Y1	1.70%	3.20%	2.90%	2.90%	–	3.10%	–	3.20%
DMY_Y2	5.30%	8.00%	2.90%	3.00%	–	4.10%	–	3.30%

Conclusion

Significant breeding progress has been achieved during the past 25 years. Disease resistance and persistence show a higher response to selection on spaced plants in the nursery. This is why progress for these and related traits like yield in the second main season (DMY_Y2) showed more progress than yield *per se* and early development (e.g. Vig_Y0).

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Selecting vigorous plants in the nursery: useful or useless?

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Abstract

In breeding population varieties, selection based on *per se* performance of spaced plants is less effective than selection for performance of their offspring would be. This holds especially true for complex traits like biomass production. Nevertheless, many breeders use ratings of biomass from spaced plants as a selection criterion. Based on historical data from the Agroscope forage crop breeding programme, we analysed correlations between *per se* performance of spaced plants in a polycross and performance of their half-sib progeny as assessed in seeded rows. Correlations between plant *per se* and offspring vigour ($r_{P,O}$), showed high variation among the different polycrosses analysed. Values of $r_{P,O}$ ranged from positive to negative, with mean values being close to zero for all species. A significance at the 5% level could not be attested for most of the parent-offspring comparisons, partly attributed to a low number of plants per polycross. The inconsistent correlation patterns are likely caused by data character (scoring data), comparison levels (single plants vs swards), different plant materials and the nature of the trait itself (vigour as an omnium-gatherum of plant height and amount of leaves and stems). Breeders should not rely too much on selecting spaced plants based on their vigour.

Keywords: forage crops, spaced plant, *per se* performance, sward, offspring performance

Introduction

The efficiency of a forage crop breeding programme is described by the continuous and stable improvement of desired traits in the breeding pool. Measuring this success is often difficult, due partly to complex inheritance patterns, and biotic and abiotic factors, which mask a plant's potential performance (Resende *et al.*, 2013). Repeated scorings for different traits are an instrument to unravel a plant's actual fitness. Based on that, superior plants are selected to enhance parental plants for polycross installations. However, the selection of single plants is only reasonable if it improves breeding progress. Thereby, plant vigour is preferred as a selection criterion, because it supposedly summarizes a plant's excellence and is phenotypically correlated. However, it is a complex trait and the transmission of good vigour from selected plants to their progeny is difficult to predict. The assessment of the usefulness of selection could be complicated by the standard breeding method, e.g. parental plants were cultivated and evaluated as single plants, but the performance of their corresponding offspring will be evaluated in swards. It has been reported that correlations between performance of spaced plants and their offspring grown in swards are weak (Elgersma, 1990; Waldron *et al.*, 2008). Consequently, breeding progress is attenuated by two dilemmas: for complex traits, the parent-offspring correlation might be weak and the evaluation system with comparisons of spaced plants to plants grown in swards has proved to be of low validity in previous studies. Therefore, the present study aimed to scrutinize which parent-offspring correlations are present during one important step in the selection cycle. So far, to the best of our knowledge, this is the first report about such correlations in forage crops, based on long-term breeding data over a wide range of species.

Materials and methods

Phenotypic data of different forage grass species (Table 1) were analysed for *per se* performance of spaced plants in polycrosses (PC) installed between 2000 and 2015 and performance of their half-sib progenies grown in seeded rows (SR) in trials installed between 2002 and 2017. Experimental and observational units in PC consisted of plots of 2 to 4 plants of the same genotype. Each genotype was replicated onto 4 plots, arranged in a randomized complete block design. PC were planted in autumn of year 1 and screened

Table 1. Summary of correlations between plant *per se* and offspring performance ($r_{P,O}$) for plant vigour at heading of first growth ($V1_P$ and $V1_O$, respectively) in different forage grasses.[1]

Species	n_{Tot}	n_{Sig}	$n_{Sig, Pos}$	Points per correlation			$r_{P,O}$		
				min	max	mean	min	max	mean
Lolium perenne, 4n	20	4	2	6	15	10.2	-0.75	0.82	0.10
Lolium perenne, 2n	19	2	2	4	15	7.5	-0.63	0.80	0.14
Lolium multiflorum, 4n	11	0	0	10	23	14.3	-0.56	0.51	-0.02
Lolium multiflorum, 2n	13	0	0	7	22	12.9	-0.36	0.58	0.07
Lolium hybridum, 4n	24	2	2	5	25	12.1	-0.53	0.64	0.05
Lolium hybridum, 2n	5	0	0	6	10	8.4	-0.31	0.44	0.10
Festuca pratensis, 2n	18	2	1	7	25	10.6	-0.68	0.84	0.08
Festuca arundinacea	4	0	0	7	9	8.5	-0.45	-0.09	-0.27
Dactylis glomerata	6	1	1	7	13	9.3	-0.46	0.94	0.31
Festuca rubra	3	0	0	8	16	12.0	-0.34	0.43	-0.08

[1] Parameters n_{Tot}, n_{Sig} and $n_{Sig,Pos}$ give the number of total, significant and significantly positive correlations, respectively.

for vigour of the first growth in the subsequent season ($V1_P$, assessment at heading date in year 2). Seeds of PC were harvested separately per genotype in year 2 and were used for sowing SR trials in spring of year 3, whereby each PC resulted in one SR trial. SR trials were installed at one Swiss location, either at the site of Oensingen (47.284 °N, 7.733 °E), Zürich-Reckenholz (47.428 °N, 8.521 °E) or Ellighausen (47.609, 9.139) with 4 to 8 replicates per half-sib family. Experimental and observational units consisted of 2.5 m length rows. SR trials were screened for vigour in the establishment year ($V0_O$, assessed in autumn of year 3), early vigour in the first main season ($EV1_O$, assessment in April of year 4) and vigour of the first cut of the first main season ($V1_O$, assessment at heading date of year 4). For subsequent analysis, mean values of *per se* performance from PC trials and offspring performance from SR trials were calculated for each parental genotype. PC and their corresponding SR trials were only included if overlapping data were available for 4 or more genotypes. In total, the dataset comprised 123 pairs of PC and SR trials from 10 different species-ploidy combinations (Table 1). Spearman rank correlation coefficients between *per se* and offspring performance ($r_{P,O}$) and their significances were calculated for each pair of a PC and SR trial.

Results and discussion

Only low numbers of significant correlations were observed between *per se* performance of spaced plants in PC and their half-sib offspring grown in SR (Table 1). A broad range of values for $r_{P,O}$ was observed, of which the significant ones were mostly positive (Table 1, comparison of n_{Sig} and $n_{Sig,\ Pos}$). No general differences between species or ploidy-levels could be detected, whereby average $r_{P,O}$ per species was close to zero. Correlations of $V1_P$ with other traits assessed in rows (e.g. $V0_O$, $EV1_O$) were generally lower (data not shown) and are not presented. The low number of significant correlations between parent and offspring performance was rather unexpected, likely having a multifactorial background. The small numbers of plants per polycross is one aspect that has contributed to the low number of significant correlations, as the statistical power to detect these is limited. Statistical power is also impeded by the fact that PC and SR trials were performed in only one environment, giving a less precise estimate of the true genotypic *per se* and offspring value. Furthermore, the simplification of vigour to a scoring number from 1 to 9 could have biased the analysis.

Beside statistical issues, the comparison of spaced plants and row installations itself is precarious due to different environmental conditions. Spaced plants are at an advantage relative to row plants regarding competition for space, nutrients, water and light. It is expected that the performance of plants grown in swards exhibit half of the performance of spaced plants because genetic correlations between competitive and non-competitive impact is less than 1 (Casler and Van Santen, 2010). Reports from literature showed similar poor spaced plant-sward correlations to ours, with r=0.37 in *Festuca arundinacea* or r=0.6 in *Trifolium repens* (Resende *et al.*, 2013).

Furthermore, trait genetic architecture and heritability of a trait also give an impression about the success of selection. Vigour traits are highly complex and are determined by many genes, so variability in the breeding pool is essential to gain improvement. Heritability for such traits associated with fitness are considered to be low and the parent-offspring regression even lower (Wray and Visscher, 2008). In our data, spaced plants in PC already underwent strong selection pressure in the nursery and show less variability, which might have further reduced $r_{P,O}$. Furthermore, parental plants in PC were already of higher age (up to 5 years) and might have been influenced by different factors (e.g. viruses). Such age effects might have additionally reduced correlation with their relatively young (1 year) offspring grown in SR trials.

Conclusion

The present study illustrates that a direct comparison of spaced plants to their offspring in rows or swards is difficult, as their correlation is reduced by different factors (spaced plant – sward, parent – offspring). Whereas plant *per se* vigour might be inevitable for use as a selection criterion when starting with a large population in the nursery, its effectiveness is questionable for use in pre-selected plants that have been transplanted to clonal rows. If possible, such older plants in clonal rows should be selected based on their offspring performance. To do so, pre-selected plants could be polycrossed already in the nursery and their half-sib offspring could be grown in seeded rows parallel to the parental plants being in clonal rows. The inclusion of genomic selection procedures would simplify selection of such pre-selected plants based on their breeding value and improve the success of a breeding programme.

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Multi-species phenotyping of drought in the *Festuca-Lolium* complex

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Abstract

Climatic models predict that drought stress will be the most severe limitation for agricultural utilization of grass species. Thus, we investigated many species (basically all species of *Schedonorus* section of *Festuca* and Italian and perennial ryegrasses of both ploidy levels, and their hybrids) for summer drought and heat tolerance in 2017 and 2018 at the experimental field of IEB, Olomouc, Czech Republic. We found large difference among species and also between genotypes of wild species. The most drought-tolerant were Atlas fescue (*F. mairei*) and Mountain fescue (*F. glaucescens*). Large variation was found among the genotypes of Apennine fescue (*F. apennina*) with some of them suffering from the early onset of drought to some persisting as well as those of *F. glaucescens* and *F. mairei*. Similarly, interspecific hybrids of ryegrasses and fescues (× *Festulolium*) also varied between cultivars, mostly in correlation with their genome composition. This study allows estimating the potential of individual species and identification of specific genotypes for future breeding.

Keywords: drought tolerance, hybrids, ryegrass, fescue, climatic change, phenotyping

Introduction

Most climatic models predict climatic changes in the near future. This concerns not just rapid reduction of precipitation or temperature increase, but also more frequent occurrences of short- to mid-term periods of summer drought and heat. The last two summers in most regions of Europe provide excellent evidence for this. Thus, it is logical that drought tolerance should be one of the main aims of grass breeding and research. Together with gradual pressure on reduced water usage this calls for identification of new crops which can handle such conditions due to their highly improved abiotic stress tolerance. Merging two different genomes via interspecific hybridization allows introgression of specific traits from one species into another. This is a valuable approach in plant breeding and enables mitigation of the impact of future climatic changes. Italian and perennial ryegrasses are the key forage and turf grass species in Europe. However, they display low tolerance to summer drought and heat. On the other hand, some fescue species were identified as potential source of introgression of abiotic stress tolerance due to their crossability with ryegrasses. Close to one hundred *Festulolium* cultivars were released during the last 50 years and some of them became popular among farmers for their combination of yield and establishment inherited from ryegrasses and persistence and stress tolerance from fescues (Ghesquiere *et al.*, 2010). The aim of our study was to evaluate species from the *Festuca-Lolium* complex for their drought tolerance and to identify genotypes for future breeding.

Material and methods

A core collection of 48 genotypes of six species from genus *Festuca* sect. *Schedonorus* including one interspecific hybrid (*F. pratensis* × *F. apennina*), two *Lolium* species and seven *Festuca* × *Lolium* (× *Festulolium*) hybrids was established. Individual plants were cloned into twelve sections and planted in the experimental field of IEB, Olomouc, Czech Republic, in spring 2017. Irrigation was withheld between July 23 and August 31 (rain shelters were used). The phenotyping was done once a week based on scoring of plant condition (1 – weakest plant, 9 – the most vigorous plant; 0 – dead plant). The experiment was repeated in 2018, except that irrigation was withheld between June 09 and August 11. The phenotypic scores were transferred into percentages of change between the average initial score and the average score of the particular scoring date.

Results and discussion

The results were generally consistent between both years. In 2018, the drought stress applied to the plants was accompanied by summer heat (temperature surpassing 30 °C in 16 days of the drought stress). This caused more severe problems with recovery, especially in meadow fescue and Apennine fescue (and their triploid hybrids). We describe observations from both years for individual species (below) with examples of plant performance given in Table 1 and Figure 1.

- Italian ryegrass (*L. multiflorum*): The three cultivars tested suffered seriously during drought (2017) and drought and heat stress (2018). They did recover after re-watering in the first year, but not in the second year.
- Perennial ryegrass (*L. perenne*): All four cultivars suffered in both years, but recovered well to the level above the initial score.

Table 1. Phenotyping of plant impression of selected genotypes in 2017 and 2018.

Genotype	2017		2018	
	%ADS[a]	%AR[b]	%ADS[a]	%AR[b]
F. apennina 152	−65.38	−63.74	−15.07	−34.25
F. glaucescens 3	−40.25	−1.10	0.00	+55.10
F. mairei 312	+11.71	+33.33	−0.93	+53.27
F. arundinacea 1	−36.68	−9.55	−11.11	+44.44
Festulolium krasanii 1	−44.62	−4.62	−0.56	+17.51
Festulolium braunii	−48.44	−10.94	−49.69	−29.56
Festulolium krasanii 2	−31.82	−4.55	−2.35	+24.71
F. pratensis (2n) 1	−53.98	−47.16	−19.70	−34.85
F. pratensis (4n) 3	−14.29	+4.08	+4.95	+38.61
L. multiflorum 1	−33.61	−26.23	−40.00	−29.23

[a] Difference (in %) between initial score (at the initiation of drought stress) and the score at the end of drought stress (ADS=after drought stress).

[b] Difference (in %) between initial score (at the initiation of drought stress) and the score at the end of recovery period (two months after the end of drought stress period) (AR=after recovery).

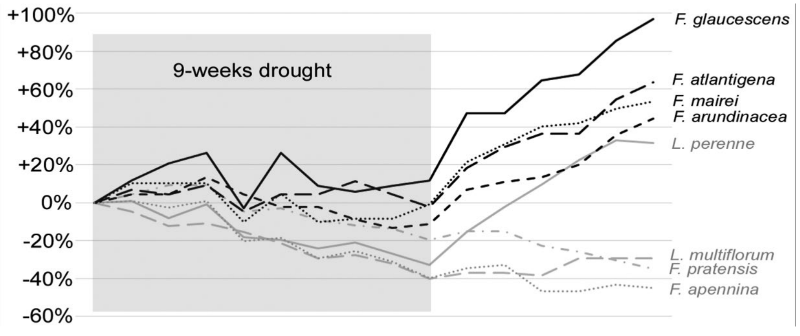

Figure 1. Performance of plant impression of selected genotypes of eight grass species during and after drought stress in summer 2018 (difference from initial score, in %).

- Meadow fescue (*F. pratensis*): Diploid meadow fescues suffered under the drought and heat. In the first year (drought only) they stagnated after re-watering, but plant scores remained low after re-watering in the second year (drought and heat). In contrast, three (out of four) tetraploid cultivars had relatively good regrowth after re-watering.
- Tall fescue (*F. arundinacea*): The growth of four genotypes was only slightly reduced by drought and heat, with good regrowth after re-watering.
- Atlas fescue (*F. mairei*) and Mountain fescue (*F. glaucescens*): All genotypes of both species kept growing during the first 4 weeks of drought stress with only minimal reduction in plant scores at the end of the drought stress and slow, but continual regrowth after re-watering.
- Apennine fescue (*F. apennina*) and triploid hybrid *F. pratensis* × *F. apennina*: Generally low tolerance to drought stress was observed. The accessions suffered during drought stress and did not recover well after re-watering (stagnated or showed reduced plant scores). However, two genotypes of *F. apennina* had relatively good drought tolerance with regrowth after initiation of re-watering (at the level of some *F. glaucescens* and *F. mairei* genotypes).
- Octoploid tall fescue (*F. arundinacea* subsp. *atlantigena*): Two genotypes stagnated at the initial level of plant scores and displayed rapid and immediate growth after re-watering.
- × *Festulolium* (*Festulolium braunii* = *L. multiflorum* × *F. pratensis* and *Festulolium loliaceum* = *L. perenne* × *F. pratensis*): High variability among cultivars, reflecting their genome composition, was observed. They generally followed the trend of their parents – meadow fescue and ryegrasses.
- × *Festulolium* (*Festulolium krasanii* = [*L. multiflorum* × *F. arundinacea*]× *F. arundinacea*): Three cultivars displayed the same or very similar trend as tall fescues.

Conclusion

The most promising sources of drought tolerance appear to be the Atlas and Mountain fescues (*F. mairei* and *F. glaucescens*) followed by tall fescues: octoploid *F. atlantigena* and hexaploid *F. arundinacea*. Valuable breeding material showing drought tolerance appears to be present also in *F. apennina*; however, precise selection for this trait has to take place in this species. Successful combining of drought tolerance from tall fescue and high yield from Italian ryegrass was already observed in cultivars of *Festulolium krasanii*.

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Effect of AR-1 endophyte on the agronomic performance of *Lolium perenne* in the UK

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Abstract

In New Zealand it is common to sow perennial ryegrass (*Lolium perenne* L.) infected with ryegrass endophyte (*Epichloë* spp.). This confers protection against attack by numerous insect pests and is considered as being essential in large parts of the country. Despite insect pests in the UK not being as common or as destructive as those in New Zealand, a trial was established to investigate the effects of AR-1 endophyte infection on the agronomic performance of the cultivars AberDart and AberMagic by sowing both cultivars with and without endophyte (E+, E-) in a plot trial. The presence of endophyte in the E+ cultivars and absence in the E- cultivars was confirmed before sowing. The trial was conducted over 3 harvest years at Aberystwyth with differing annual nitrogen fertiliser levels (100, 300, 600 kg N ha^{-1}). Dry matter yield (DMY) was measured at each of 7 harvests as was dry matter digestibility (DMD), water soluble carbohydrate (WSC) and crude protein (CP). At all harvests, except for the first spring harvest, fertiliser levels significantly increased DMY and CP (P<0.001) while WSC was decreased. No significant differences were observed between E+ and E- cultivars for DMY, DMD, WSC or CP.

Keywords: perennial ryegrass, dry matter yield, nitrogen, crude protein, water soluble carbohydrates

Introduction

In New Zealand it is common to sow perennial ryegrass (*Lolium perenne* L.) infected with ryegrass endophyte (*Epichloë* spp). This confers protection against attack by numerous insect pests and is considered as being essential in large parts of the country. However, the naturally occurring endophytes were also found to have negative effects on grazing stock known as 'ryegrass staggers' (Aasen *et al*, 1969). Livestock-safe endophytes have been produced, one of the earliest being AR-1 which controls Argentine Stem Weevil and Pasture Mealy Bug but does not produce the alkaloids lolitrem B or ergovaline, compounds known to cause ill-effects in livestock (http://www.grasslanz.com/UnderstandingtheScience/Ryegrassendophyteoptions.aspx). Despite insect pests in the UK not being as pervasive or as destructive as those in New Zealand, evidence exists that the cumulative effect of pests can affect the persistence of ryegrass in the UK (Clements *et al*, 1991). While it has been shown that endophyte gave no observable protection for European ryegrasses against frit fly (*Oscinella* spp.) and leatherjackets (*Tipula* spp.) (Lewis and Vaughan, 1997), the objective of this study was to determine whether AR-1 endophyte could benefit agronomic performance.

In addition, since there is evidence that endophyte concentration and alkaloid production was reduced under higher nitrogen levels (Rasmussen *et al.*, 2007) the study would also examine any effects of N fertilisation.

Materials and methods

Field plot performance was compared on four cultivars in a randomised complete block experiment. The cultivars used were the IBERS high-sugar varieties AberDart and AberMagic, with the same varieties which had been inoculated with AR-1 endophyte for the New Zealand seed market. Three N fertilizer treatments (23:4:13:7 $N:P_2O_5:K_2O:SO_3$, equivalent to 100, 300 and 600 kg N ha^{-1} year^{-1}) were applied to each population. Populations and fertilizer levels were fully randomized within each of four complete blocks (60 experimental plots in total). The experiment was carried out at the Gogerddan site (52°27'N, 4°01'W) of IBERS on Rheidol series soil, pH 6.8. Guard plots of AberDart, given 300 kg N ha^{-1} $year^{-1}$, were grown at both ends of each replicate block. Plots (2×1 m) were broadcast sown into a prepared seed bed at a rate of 22 kg of seed ha^{-1} in August 2008 followed by rolling. All plots were fully established by September 2008. In 2009, 2010 and 2011 the experimental plots were harvested at approximately 50 mm and weighed with a Haldrup plot harvester. Fresh samples of herbage (between 200 and 500 g) were weighed, oven-dried at 80 °C for at least 16 h, and re-weighed to determine DM content. Yields of DM were determined for 7 harvests in 2009, 6 in 2010 and 7 in 2011. Fertilizer was weighed and applied by hand to each plot in the first week of March and after each cut except the last, the dressings being split to provide an equal amount of fertilizer for growth during each harvest cycle. Over successive winters, all samples of oven-dried herbage were milled through a 1 mm sieve, and their DMD, WSC and N concentration was determined by the IBERS Analytical Services Unit (UKAS accredited to BS EN ISO 17025) using near infra-red spectroscopy. Visual estimates of the percentage ground cover of perennial ryegrass were made 5-10 days after the final harvest in each year.

All data were subjected to analysis of variance. Least significant differences (LSD) for comparing the E+ and E- populations were calculated using Genstat Fourteenth Edition (VSN International Ltd, www.vsni.co.uk, 2011).

Five plants of each cultivar were sown in pots in a glasshouse and were tested for the presence of endophyte prior to sowing the field experiment using Lactophenol Cotton Blue stain (Harvey *et al.*, 1982). Samples were also taken from the E+ field plots at the beginning of the second harvest year and tested for presence or absence of endophyte using the same method.

Results and discussion

Endophyte was detected in 90% of the AberDart AR-1 plants and 100% of the AberMagic AR-1 plants tested in the glasshouse prior to the trial. No endophyte was detected in the E- cultivars. Endophyte was also detected in 70% of the AberDart AR-1 plants and 60% of the AberMagic AR-1 plants in the field plots tested at the beginning of the second harvest year.

At all harvests, except for the first spring harvest, increased fertiliser levels significantly increased DMY and CP content ($P<0.001$) whereas WSC was significantly decreased by increasing N-levels. While significant differences were observed between cultivars at some cuts (Table 1) no significant differences were observed between E+ and E- plots for DMY, DMD, WSC or CP. No significant interactions were observed between cultivar and endophyte presence, cultivar and N-level, endophyte presence and N-level or between cultivar, endophyte presence and N-level. It is therefore clear that the presence of the endophyte does not give any additional benefits to either of the cultivars in the trial. Similarly, the presence of endophyte does not interact with any of the other factors.

Table 1. *P-value* ANOVA for agronomic traits across 3 harvest years.[1]

Dry matter yield in t ha^{-1}	**Cut 1**	**Cut 2**	**Cut 3**	**Cut 4**	**Cut 5**	**Cut 6**	**Cut 7**	**Total**	**% Grnd Cover**
Cultivars	NS	***	NS	NS	NS	*	NS	**	NS
N-levels	NS	***	***	***	***	NS	***	NS	***
%DMD	**Cut 1**	**Cut 2**	**Cut 3**	**Cut 4**	**Cut 5**	**Cut 6**	**Cut 7**	**Mean**	
Cultivars	NS	**	NS	NS	***	NS	NS	NS	
N-levels	NS	***	NS	NS	*	***	**	NS	
%N	**Cut 1**	**Cut 2**	**Cut 3**	**Cut 4**	**Cut 5**	**Cut 6**	**Cut 7**	**Total**	
Cultivars	NS	***	NS	**	***	NS	*	***	
N-levels	***	***	***	***	***	***	***	***	
%WSC	**Cut 1**	**Cut 2**	**Cut 3**	**Cut 4**	**Cut 5**	**Cut 6**	**Cut 7**	**Total**	
Cultivars	*	***	*	***	**	**	NS	***	
N-levels	**	***	***	***	***	**	**	***	

[1] No significant differences were observed for endophyte, cultivar × N-level, cultivar × endophyte, endophyte × N-level or cultivar × N-level × endophyte. *** $P<0.001$, ** $P<0.01$, * $P<0.05$.

Conclusion

From results of a 3-year trial under UK conditions it appears that the presence of the endophyte AR-1 does not confer any agronomic advantage, even under differing N levels.

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Agronomic evaluation of cocksfoot and tall fescue cultivars for adaptation to an Algerian drought-prone environment

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Abstract

A prime objective for improving perennial grasses for Mediterranean environments is the identification and selection of genotypes well adapted to semi-arid conditions. This study was conducted at an experimental site in Algeria over three consecutive seasons, with the aim of evaluating the agronomic performance and genetic diversity of 13 cultivars of two species of perennial grasses: six cocksfoot (*Dactylis glomerata* L.) and seven tall fescue (*Festuca arundinacea* Schreb.) cultivars. Variation was found in both species for dry matter yield, sward recovery and heading date. Tall fescue cultivars gave greater yield in spring with earlier heading date than cocksfoot, with no differences for the other traits. Among the genotypes, cv. Flecha was the most promising, together with Flecha E-542, Fraydo and Centurion, all of which are tall fescue cultivars. Principal component analysis indicated that persistence of grass cover is relatively independent from dry matter yield and water-use efficiency.

Keywords: cocksfoot, tall fescue, dry-matter yield, persistence, drought adaptation

Introduction

Poor persistence of sown perennial pasture grasses is a problem in regions of the World with Mediterranean climates, where the most stressful, life threatening season is a summer characterised by long and often severe droughts with high temperatures. This problem was well illustrated in a survey where 80% of farmer respondents in southern Australia expected the disappearance of the species which they had sown, within 10 years of establishment (Reeve *et al.*, 2000). Although there are multiple reasons for this, the on-going development of grass culivars with greater resilience in the face of summer drought is considered imperative (Oram and Lodge, 2003). Valuable attributes of North African perennial grass germplasm include summer dormancy (Shaimi *et al.,* 2009) and high winter growth (Reed *et al.,* 1980). As an example, Moroccan ecotypes of cocksfoot (*Dactylis glomerata* L.) showed 97% survival during summer, whereas European lines exhibited only 59% survival in the same trial (Knight, 1966).

Of the perennial grass species of common interest across the Mediterranean basin, tall fescue (*Festuca arundinacea* Schreb.) and cocksfoot (*Dactylis glomerata* L.) are of paramount importance. So far, these species have mostly been bred in and for more temperate climates, and attempt to simply introduce into the Mediterranean region cultivars selected under different conditions have repeatedly proven to be unsuccessful. Consequently, this study was undertaken to evaluate 14 *F. arundinacea* and *D. glomerata* cultivars, of Mediterranean origin, into an Algerian drought prone environment in order to identify the best-adapted plant material.

Materials and methods

The experiment was carried out at the Agricultural Research Station of the Field Crop Institute of Sétif (Algeria) which has a long-term average annual rainfall of 321.2 mm (varying from 170 to 470 mm). The climate is temperate continental, varying from arid to semi-arid. The soil on experimental location is a clay-loam with 44.7% clay, 41.3% silt, 13.9% sand, 1.3% organic matter and a pH of 8.2. The field, with chickpea as preceding crop, was prepared in the autumn of 2005. Superphosphate fertilizer (46% P_2O_5) was applied at 100 kg ha^{-1} just before the field was ploughed and nitrogen fertilizer was applied during the same months at a rate of 100 kg ha^{-1} as urea 35%. The sowing was done by hand in October 2005. Fourteen perennial forage grass varieties were sown, among which seven varieties of tall fescue (Tanit, Sisa, Flecha, Flecha Flecha E542, Centurion, Lutine, Fraydo) and seven varieties of cocksfoot (Jana, Medly, Kasbah, Delta1, Currie, Porto and Ottava). The plant material originated mainly from Portugal, Italy and France, and was obtained via the Permed project (http://www1.montpellier.inra.fr/permed/indexf.html).

The experiment was run for 3 years under rainfed conditions. It was established in plots (10 rows 2.5 m long and 0.20 m row spacing), in a simple lattice with four replications. Average sowing rate was 12 kg ha^{-1}. Harvesting was done on 6 inner rows, 2 m long, mechanically at a height of 7.0 cm. Fresh plant material samples were dried at 70 °C for 48 h at each harvest for dry matter determination (DMY, t ha^{-1}). Mowing was initiated in the autumn before the onset of frost kill, and in the spring and summer seasons when inflorescences emerged in at least 4 of the varieties. Recovery from each cut (REC) was expressed as the row cover percentage; i.e. the percentage of the row space occupied by living grown tissue. This was measured after each harvest, to determine the persistence of each of the cultivars. Heading day (HD) was recorded in spring on undisturbed rows in each plot and it was expressed as the number of days from 1 January to reach 50% heading. The data were analysed by ANOVA using (Systat Software, Chicago, IL, USA).

Results and discussion

Dry matter yields

The analysis of variance of the autumn dry matter yield accumulated during the three years indicated that the cocksfoot variety Kasbah and the tall fescue varieties Fraydo and Centurion were the top yielding with an average DM yield of 0.55 t ha^{-1}, while Porto recorded the lowest DM yield with an average of 0.24 t ha^{-1}. Concerning the spring DM yield, the ANOVA showed significant genotype and year effects (Table 1). The mean values ranged from 0.56 t ha^{-1} (year 3) to 2.21 t ha^{-1} (year 2). The average DM yields during the three years indicated that Flecha, Flecha E542, Fraydo and Centurion were the top yielding varieties with average DM yield of 1.61 t ha^{-1}, followed by Medly, Lutine, Sisa, Kasbah, Jana, Tanit, Delta1 and Currie with a mean yield ranging from 1.35 to 1.04 t ha^{-1}. The least yielding group was formed by Ottava (0.88 t ha^{-1}) and Porto (0.78 t ha^{-1}). Weak autumn production is explained by the variation of soil moisture available for vegetation. Autumn rains usually arrive very late, and rainfall in summer may

Table 1. Mean Square of the combined General Linear Model-ANOVA for dry matter, recovery and days to heading.[1]

Source of variation	DMa	DMs	RECa	RECs	HD
Species (S)	0.00 ns	3.08 ***	284.98 ns	382.49 ns	167.8 **
Year (Y)	0.82 *	29.60 ***	724.17 *	3,932.8 ***	10,793.2 ***
S × Y	0.01 ns	0.26 ns	492.72 ns	64.92 ns	31.58 ns
Genotype (species)	0.02 ns	0.38 *	591.58 ***	573.88 ***	212.3 ***
Block (year)	0.05 ns	0.43 ns	1,566.39 ***	1,760.8 ***	48.4 ns
Error	0.02	0.22	178.78	104.11	24.46
Multiple-R^2	0.36	0.77	0.52	0.72	0.91

[1] DMa,s = dry matter yield in autumn (a) and spring (s) (t/ha); RECa,s = recovery as row cover percentage (%) in autumn (a) and spring (s); HD = number of days from 1 January to heading. ns: not significant, * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

be completely absent which requires that the different varieties gradually enter dormancy, and thus this reduces their dry matter production.

Persistence and earliness

Variation among genotypes and years for row cover was significant, at least $P<0.05$, in both seasons during the three years, but there were no significant genotype × year interactions (Table 1). In fact, sward persistence expressed as final row cover averaged over cycles ranged from 37.16 (Porto) to 71.33% (Tanit) in autumn and from 40.80% (Porto) to 69.31% (Jana) in spring. Persistence is an important sward characteristic and traits that generally contribute to this characteristic are summer dormancy, grazing or defoliation tolerance, and earliness (Casler and Vogel, 1999). The swards took longer time to head during the first year of establishment compared to the second and third years, with a mean number of days to head, averaged over genotypes, of 134.7 in 2005/2006, against 114.5 and 102.9 days respectively in 2006/2007 and 2007/2008. The number of days to head showed significant differences between genotypes and years (Table 1). The tall fescues cvs. Faydo, Flecha and Flecha E542 exhibit the earliest flowering date with the cocksfoot Kasbah; whereas the cocksfoot Porto and the tall fescues Tanit and Lutine were in the latest group to head during the three seasons. Cultivars Currie and Sisa showed intermediate maturity during all three seasons. In the species cocksfoot and tall fescue, Volaire and Lelièvre (1997) note a strong linkage between geographic origin, earliness, and drought resistance. Earliness of heading can easily be selected for breeding programmes, and this might be useful as an indirect selection criterion, especially if the selection for drought-prone environments had to be performed for favourable sites or cropping years (Pecetti *et al.* 2011).

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Phenotyping diversity within a collection of sainfoin (*Onobrychis sativa* L.) populations

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Abstract

The aim of this study was to determine the productivity and morphological traits of natural populations collected from different locations in East Serbia in order to find ones with good agronomical traits. Nine collected populations and two control cultivars were evaluated in a field trial during three years in a spaced plant design. Morphological traits, dry matter yield and quality parameters (crude protein, ADF, NDF) were determined. Three populations (1, 7 and 11) showed better results than cultivars in their dry matter yield per plant and quality parameters. Cluster analysis divided the populations into three main clusters. The within population variability was high. Recorded differences among, as well as high variability of individual plants within populations, indicate that populations of sainfoin are a rich source of variability for the creation of new varieties of this species, for pure stand and for grass legumes mixtures.

Keywords: morphological traits, DMY, populations, variability

Introduction

Sainfoin (*Onobrychis sativa* L.) is a perennial forage legume with high potential as a forage source for ruminants in grassland-based livestock production systems (Kolliker *et al.,* 2017). In comparison with other forage species sainfoin is less demanding of the soil and produces high yields even in the poorest soils, because of its high resistance to cold, drought, and its high adaptive flexibility. Regrowth is slow and it is important to allow enough time to replenish root reserves to maintain its persistence and longevity (Hayot Carbonero *et al.,* 2011). High protein content, high palatability and its non-bloat characteristics make it a good choice for livestock or wildlife. Sainfoin blossoms produce copious amounts of nectar and are highly attractive to pollinating insects, particularly honey bees.

However, sainfoin cultivation is not widespread today, mainly because sainfoin cultivation relies mainly on old varieties or on populations whose characteristics are not clearly established. Further advances in sainfoin cultivation require the characterisation of existing genetic resources, both for its protection and its possible use. In the east and central part of Serbia, farmers have still kept old sainfoin populations that have good drought tolerance, which is characteristic for this area. Those populations, and the wild populations which are common in wild flora in this area, could be important genetic resources for these traits. Therefore, collecting and evaluating of those populations could be useful in the breeding process. The use of this information will be useful in further plant breeding programmes as it may help to develop improved varieties with a wide genetic base. The aim of the research in this paper was to determine the productivity and morphological traits of wild populations in order to find the populations with good agronomical traits, so that they can be included in the future breeding process.

Materials and methods

Nine sainfoin populations were collected from East and Central part of Serbia (Table 1). Their productivity, morphological traits and quality parameters of their dry matter was carried out on the demonstration field of the Institute for Forage Crops in Kruševac on degraded alluvial soil. Collected seeds were sown at distance 60×60 cm, in a spaced plant design and cultivars Makedonka and Norm were used as standards. Morphological analyses were made on 60 plants of each population in all three cuts in the second year of exploitation. The following parameters were monitored: plant height (cm), number

Table 1. Origin of collected sainfoin populations.

	Name		Latitude	Longitude	Altitude
1	p. Zajecar	landraces	N 43°91′35″	E 22°21′61″	250 m
2	cv Norm	cultivar			–
3	p. Beli potok	Wild populations	N 43°52′11″	E 22°08′98″	560 m
4	p. Bogovina	Wild populations	N 43°92′64″	E 21°87′62″	320 m
5	p. Josanica	Wild populations	N 43°73′87″	E 21°76′63″	520 m
6	p. Soko Banja	Wild populations	N 43°63′76″	E 21°82′28″	391 m
7	p. Luzinci	landraces	N 43°24′82″	E 20°22′20″	400 m
8	p. Sarbanovac	Wild populations	N 43°73′08″	E 21°88′99″	320 m
9	cv Makedonka	cultivar			–
10	p. Izvor	Wild populations	N 43°91′65″	E 22°34′38″	400 m
11	p. Novi Pazar	Wild populations	N 43°25′83″	E 20°24′20″	690 m

of stems per plant, number of nodes on five stems on each plant; the average value for all three cuts are presented in paper. Quality of dry matter was determined on samples from first cut in the second year of vegetation. In both years dry matter yield per plant was measured, and total values per year were presented. The data were analysed by ANOVA and variability among population for the chosen characteristics was expressed by the coefficient of variation (CV). Cluster analysis for all traits was calculated by the Ward method, using the Euclidean distance.

Results and discussion

Significant differences were observed between populations (Table 2). Considerable diversity was observed in most of the evaluated traits. Populations 1 and 11 reached the highest yield in both years of investigation. Plants from those populations had numerous stems per plant and achieved the highest dry matter yield per plant.

Table 2. Average values for plant height (cm), number of stems per plant, number of nodes per stem and total dry matter yield (g per plant) in second and third year of utilisation.[1]

		Plant height	Number of stems	Number of nodes	DMY 2nd year	DMY 3rd year	CP %	ADF	NDF
1	p. Zajecar	78.11	52.47	11.12	427.7	328.5	16.37	41.29	44.87
2	cv Norm	75.51	47.54	10.05	330.7	250.3	15.47	45.02	49.76
3	p. Beli potok	64.74	50.69	6.14	294.3	225.8	18.86	38.58	45.57
4	p. Bogovina	83.69	41.60	8.13	332.0	279.0	17.73	38.38	44.2
5	p. Josanica	66.60	34.98	8.30	230.1	211.2	16.32	43.37	51.65
6	p. Soko Banja	85.79	39.21	8.58	350.4	283.7	16.35	39.52	50.91
7	p. Luzinci	90.35	47.30	9.91	409.4	263.3	17.05	39.58	51.92
8	P. Sarbanovac	68.00	17.44	5.34	79.1	220.6	16.31	42.31	49.32
9	cv Makedonka	83.45	45.95	6.82	312.6	256.2	15.22	48.31	46.63
10	p. Izvor	52.36	32.63	6.48	116.7	146.3	16.49	39.50	47.99
11	p. Novi Pazar	88.13	46.06	7.99	428.2	296.6	17.24	51.55	50.13
	CV %	13.8	31.1	21.2	37.0	18.7	16.37	41.29	44.87
	LSD 0.05	12.1	16.4	2.3	83.4	78.2	6.9	8.1	5.7

[1] Parameters of quality (%) were determined in the second cut in second year of utilization.

Populations 8 and 10 had smaller plants, with reduced number of stems per plant and significant lower dry matter yield, and could be of interest for growing in mountain areas. Reduced organ size was recorded for mountainous accessions in a germplasm from central Italy, and this is a typical adaptation to mountainous conditions which has been observed for alpine plants (Negri and Cenci, 1988). Various studies demonstrate large variability in agronomic performance among and within sainfoin accessions, highlighting the big potential to improve this species by breeding (Bhattarai *et al.,* 2018, Mohajer *et al.*, 2013).

Hierarchical cluster analysis grouped populations into three main clusters (Figure 1). Cultivars were in the same cluster, but there were no matches of geographic distances of populations with hierarchical clustering of collection.

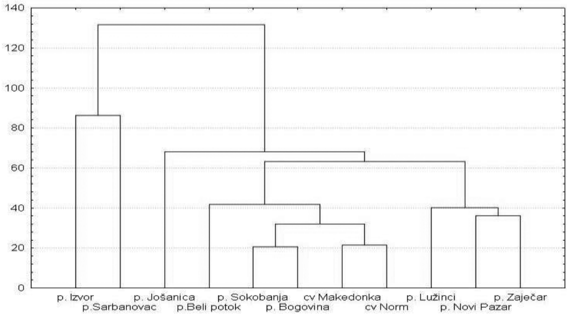

Figure 1. Grouping of populations based on all examined traits.

Conclusion

Recorded differences among populations, indicate that domestic populations of sainfoin in Serbia contain a rich source of variability for creation of new varieties of this species for different purposes (pure stands, grass-legumes mixture). It is necessary to continue investigation in order to determine their productivity in mixture with grasses and resistance to different agro-ecological conditions. Based on this study, the more promising plants from investigated populations will be selected to create improved populations.

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Self-pollination rates in Western U.S. alfalfa seed production fields

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Abstract

Previous research has shown that a proportion of alfalfa (*Medicago sativa* L.) seed produced in seed production fields is the result of self-pollination. In these studies, this rate has been historically measured around 30%. In this study, we surveyed alfalfa seed production self-pollination rates in three major seed production areas of the United States: Imperial Valley, California; San Joaquin Valley, California; and Washington State. In each of these regions, seed was collected from active commercial seed production fields. In each field, eight seed from forty individuals were sampled and using DNA marker comparisons between seed tissue and its maternal tissue, seed was classified as being the result of out-crossing or self-pollination. Based on this classification, a field-wide self-pollination rate was then estimated. Thirty-two fields were analysed. The average self-pollination rate was 12.2% with a standard deviation of 4.9% and with a maximum field self-pollination rate of 29.8% and a minimum field self-pollination rate of 5.3%. The median self-pollination rate was 11.6%. A log normal distribution provided the best distribution fit for the data. This study confirms that self-pollination occurs during alfalfa seed production at a lower rate than previously described. Future studies will determine if seed production field management practices influence alfalfa seed production self-pollination rates.

Keywords: *Medicago sativa*, lucerne, self-pollination, SSR markers

Introduction

Previous research has shown that a proportion of alfalfa (*Medicago sativa* L.) seed produced in seed production fields is the result of self-pollination (Riday *et al.*, 2015). In historical studies, the self-pollination rate was around 30%. In a more recent study using molecular markers to identify self-pollinated individuals, a 11.8% self-pollination rate was observed in an Idaho leafcutter bee (*Megachile rotundata* F.) pollinated alfalfa seed production field (Riday *et al.*, 2015). In the United States there are major seed production areas in the: Imperial Valley, California; San Joaquin Valley, California; and the Pacific Northwest which includes seed production areas in Washington State, Idaho, Montana, and Wyoming. Seed production management and pollinators utilized varied from region to region. The objective of this study was to broadly survey commercial alfalfa seed production field, alfalfa self-pollination rates in three major seed production areas of the United States: Imperial Valley, California; San Joaquin Valley, California; and Washington State.

Materials and methods

Seed from 32 commercial alfalfa seed production fields were sampled as part of this study. Twelve fields were sampled in the Imperial Valley; 9 fields were sampled in the San Joaquin Valley; and 11 fields were sampled in Washington State. At each field site, seed bearing racemes and leaf tissue from one alfalfa stem were collected from 40 plants each. Seed from each plant was threshed separately. DNA was extracted from tissue of each of the 40 plants collected from each of the 32 fields (1280 DNA samples). DNA was also extracted directly from individual seed from eight seeds from each of the 40 plants collect from each of the 32 seed production fields (10,240 DNA samples). Fifteen SSR markers were amplified on all

11520 DNA samples using PCR conditions and fragment visualization techniques described in Riday *et al.* (2015). The 15-alfalfa based SSR markers used in this study were: aw685684 (resized amplicon), aw690665, aw695035, bg222, bg234 (resized amplicon), bi68, bi86, bi131, M009, M014, M015, M026, M029 (resized amplicon), M048, and RCS5565 (red clover marker). Three of the SSR marker amplicons were enlarged by choosing a new reverse primer. DNA marker scores were evaluated by comparing 10240 progeny plant genotypes to their respective maternal genotype using SAS script described in Riday *et al.* (2013). Each of the 10,240 progeny were then classified as resulting from self-pollination or outcrossing. For each field, the frequency of progeny resulting from self-pollination was calculated. For each of the 1,280 maternal plants, the number of progeny that were the result of self-pollination events were recorded.

Table 1. Self-pollination rates (%) for commercial alfalfa (*Medicago sativa* L.) seed production fields in the western United States.

Field	Self-pollination rate	Parents with self-pollinated progeny					
		All progeny	1 progeny	2 progeny	3 progeny	4 progeny	5+ progeny
Imperial Valley, California							
Field 1	29.7	87.5	27.5	17.5	20.0	12.5	10.0
Field 2	13.4	57.5	27.5	10.0	20.0	0.0	0.0
Field 3	11.9	62.5	40.0	12.5	10.0	0.0	0.0
Field 4	16.9	72.5	27.5	32.5	10.0	0.0	2.5
Field 5	8.1	42.5	25.0	12.5	5.0	0.0	0.0
Field 6	12.5	60.0	32.5	17.5	7.5	2.5	0.0
Field 7	8.6	46.3	31.7	9.8	2.4	2.4	0.0
Field 8	8.1	47.5	30.0	17.5	0.0	0.0	0.0
Field 9	10.0	52.5	32.5	12.5	7.5	0.0	0.0
Field 10	20.4	68.4	31.6	10.5	5.3	10.5	10.5
Field 11	11.9	58.5	36.6	14.6	4.9	0.0	2.4
Field 12	12.1	57.5	35.0	15.0	2.5	2.5	2.5
San Joaquin Valley, California							
Field 1	17.2	67.5	25.0	25.0	10.0	5.0	2.5
Field 2	12.5	47.5	17.5	15.0	7.5	5.0	2.5
Field 3	9.1	42.5	27.5	7.5	2.5	2.5	2.5
Field 4	9.4	47.5	30.0	12.5	0.0	5.0	0.0
Field 5	14.1	57.5	22.5	22.5	10.0	0.0	2.5
Field 7	11.6	50.0	27.5	10.0	10.0	0.0	2.5
Field 8	10.1	46.3	24.4	12.2	7.3	2.4	0.0
Field 9	20.5	70.0	15.0	30.0	15.0	7.5	2.5
Field 10	8.8	47.5	30.0	12.5	5.0	0.0	0.0
Washington State							
Field 1	9.5	48.8	31.7	7.3	9.8	0.0	0.0
Field 2	5.3	32.5	25.0	5.0	2.5	0.0	0.0
Field 3	10.4	45.0	30.0	7.5	2.5	0.0	5.0
Field 4	11.5	56.4	35.9	12.8	2.6	2.6	2.6
Field 5	15.4	65.0	30.0	17.5	12.5	5.0	0.0
Field 6	17.3	69.2	33.3	15.4	10.3	7.7	2.6
Field 7	5.8	30.8	17.9	10.3	2.6	0.0	0.0
Field 8	8.7	38.9	22.2	11.1	0.0	2.8	2.8
Field 9	11.9	53.7	26.8	14.6	9.8	2.4	0.0
Field 10	7.5	32.5	15.0	10.0	5.0	2.5	0.0

Results

The average self-pollination rate among the 32 seed production fields was 12.2% with a standard deviation of 4.9% (Table 1). The lowest field-wide self-pollination rate was 5.3%, while the highest observed field-wide self-pollination rate was 29.7%. The distribution of field-wide self-pollination rates was right skewed with a median pollination rate of 11.6%. The field-wide self-pollination rate significantly deviated from a normal distribution with a lognormal distribution having the best fit. The average self-pollination rate in the Imperial Valley was 13.6±1.4%. The average self-pollination rate I the San Joaquin Valley was 12.6±1.6%, while the average self-pollination rate in Washington State was 10.3±1.5%. Of the 1,280 maternal plants tested, 46.9% had no progeny resulting from self-pollination; 28.0% had 1 out of 8 progeny resulting from self-pollination; 14.3% had 2 out of 8 progeny resulting from self-pollination; 7.1% had 3 out of 8 progeny resulting from self-pollination; 2.7% had 4 out of 8 progeny resulting from self-pollination; and 2.1% had 5 or more of 8 progeny resulting from self-pollination.

Conclusion

This study confirms that self-pollination occurs during alfalfa seed production at a low rate. The rate observed is lower than the approximately 30% self-pollination rate observed in older studies. There were no regional differences for self-pollination rates. Future studies will determine if seed production field management practices influence alfalfa seed production self-pollination rates. We hypothesize that there are likely management practices or environmental factors that contributed to the observed range of self-pollinations rates across the different seed production fields.

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Development of a high energy red clover

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Abstract

Red clover (*Trifolium pratense* L.) is one of the most important forage legumes in grassland agroecosystems worldwide. Although red clover has a high biomass potential and is particularly valued for its high protein content, its herbage lacks the high-energy carbohydrates required to meet the productivity potential of modern livestock breeds. Like most plants, red clover accumulates diurnal starch in its leaves during the day as a temporary carbon store of photosynthesis, but harvesting this starch is challenging. To develop a harvestable high-starch agronomic trait in red clover, a reverse genetic pipeline was established to identify beneficial alleles in the gene encoding the starch degradation enzyme GWD1 which naturally accumulate starch. Within the targeted region, we were able to identify several rare and common variants. Of these variants, one that was present at a rate of approximately 25% in the original population contributed to a starch increase of 20% in selected populations enriched for this allele. Implementation of such a high-starch trait in forage production is envisioned to maximize the energy content and protein absorption of forage crops in order to deliver a higher proportion of the feed intake from environmentally sustainable and locally produced roughage.

Keywords: red clover (*Trifolium pratense* L.), digestibility, glucan water dikinase (GWD1)

Introduction

Ruminant livestock diets are often supplemented with high energy corn, cereals, and soy derived from foreign supply chains. These supplemented feeds are a major cause local nutrient and carbon cycles to be broken, that leads to increased water pollution and greenhouse gas emissions, and aspect the public's poor perception of modern livestock production practices. Starch granules are produced in the chloroplasts of forage legume leaves during the day as a carbohydrate storage for night-time growth and respiration. Forage legumes, such as red clover, have the potential to accumulate more than 10 to 15% starch by dry weight (Ruckle *et al.,* 2017; Claessens *et al.,* 2016; Ruckle *et al.,* 2018). This amount of starch is sufficient to promote ruminant microbial activity, better protein absorption, and ultimately better productivity with fewer emissions. Reports that 15% starch supplementation from grain into a pasture diet can lead to 25% more daily animal productivity, 30% reduced methane emissions, and 20% reduced nitrate emissions (Reis and Combs, 2000; Benchaar *et al.,* 2001; Van Dorland *et al.,* 2007; Kingston-Smith *et al.,* 2013; Huntington, 1997). *In vitro* analysis of red clover leaves collected at the end of day (ED) versus the end of the night (EN) had 25% more digestible organic material and 15% less methane gas produced, which is consistent with more leaf starch at ED (Ruckle *et al.,* 2017).

Materials and methods

The red clover accession TP1245 from Agroscope (Reckenholz, Switzerland) was used in this study. Plants were grown in the glasshouse. Intense direct light was shaded to 50 to 100 μmol m^{-2} s^{-1}, depending on external conditions. The sunlight was supplemented with 150 to 200 μmol m^{-2} s^{-1} light from Clean Arc© metal halide lamps (EYE Lighting International, USA). A 14:10 light/dark photoperiod with a 19 to 23 °C day 14 to 16 °C night temperature regime was used. Plants were organized in a random design within the growth area. Leaves were collected and flash frozen in liquid nitrogen after 60 days of growth post-germination at the ED and EN. Starch was measured as described by Ruckle *et al.* (2017). Difference in starch content was determined between 45 genotypes of TP1245 (parental population) compared to 13 genotypes that were determined to be homozygous for the *TpGWD1-CBD1* allele. The difference was calculated by a one-way ANOVA per harvest time due to significant harvest time by genotype interaction. All statistical analyses were carried out in R statistical software version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria).

DNA was extracted using the Omega Mag-bind Plant DNA DS Kit 96 (Radnor, PA, USA). The 1 kb amplicon was generated by PCR with red clover gDNA as a template. PCR was carried out with Promega GoTaq Flexi (Promega; Madison, WI, USA) using the manufacturer's recommend protocol and 2.5 mM $MgCl_2$. Primers were designed to the published red clover genome (Phytozome v12.1; *Trifolium pretense* v2; De Vega *et al.,* 2015). Sanger sequencing (Microsynth, CH) was carried out to identify alleles by targeted resequencing of 380 genotypes of the TP1245 variety. The target amplicon specifically amplified a region with the primers 5'-CTTTAAATGCGGCATATTGTGTG-3' and 5'-TGACCTTCTCTTGCTCTGG-3'. A derived cleavage amplified polymorphic sequence (DCAPS) marker was designed to genotype plants for one of the identified alleles, *TpGWD1-CBD1.* DCAPS marker was amplified with 5'-TGATCCTTCTTCTGAGGTTTCTAC-3' and 5'-GTCCGTCTGATAGAATGACAAAAG-3' primers and digested with HPYCH4 (New England Biolabs, Ipswich, MA, USA).

Results and discussion

Different genotypes of red clover utilize their diel starch content differently. Some genotypes degrade less starch at night, and maintain relatively high starch contents in the morning (Ruckle *et al.* 2017). Such traits are interesting for increasing red clover energy content. One possible explanation for the difference in starch usage observed between genotypes is a deficiency in starch degradation. *TpGWD1* (Glucan Water Dikinase) encodes an important enzyme in the starch degradation pathway, and mutants in *GWD1* of several species are known to accumulate starch, because they do not degrade starch at night. To identify genotypes with higher starch content, a 1 kb fragment of *TpGWD1* was sequenced in a genetically diverse population of red clover. Targeted resequencing enabled the discovery of rare alleles in a genetically diverse population, allowing us to discover several allele variants in the coding space of a 1 kb region of *TpGWD1.*

One of the identified allele variants was in one of the carbohydrate binding domains (CBD) of *TpGWD1*, and was named *TpGWD1-CBD1.* Although this allele causes a non-synonymous change, there are possibly other alleles in TpGWD1 that are associated with the haplotype that contains this allele. To rapidly identify plants that were homozygous for this allele a molecular marker was developed that identified this allele within our genetically diverse population (Figure 1A). Plants that were homozygous for the *TpGWD1-CBD1* allele had 20% more mean leaf starch at ED (P=0.00686) and 60% more at EN (P=0.0205) compared to plants from the parental population that were not selected for the *TpGWD1-CBD1* allele (Figure 1B). The amount of dry matter produced by the plants homozygous for the *TpGWD1-CBD1* had a mean value of 2.00 g DW compared to 2.06 g DW of the parental population (P=0.674). Therefore, the increase in starch content does not appear to affect biomass production.

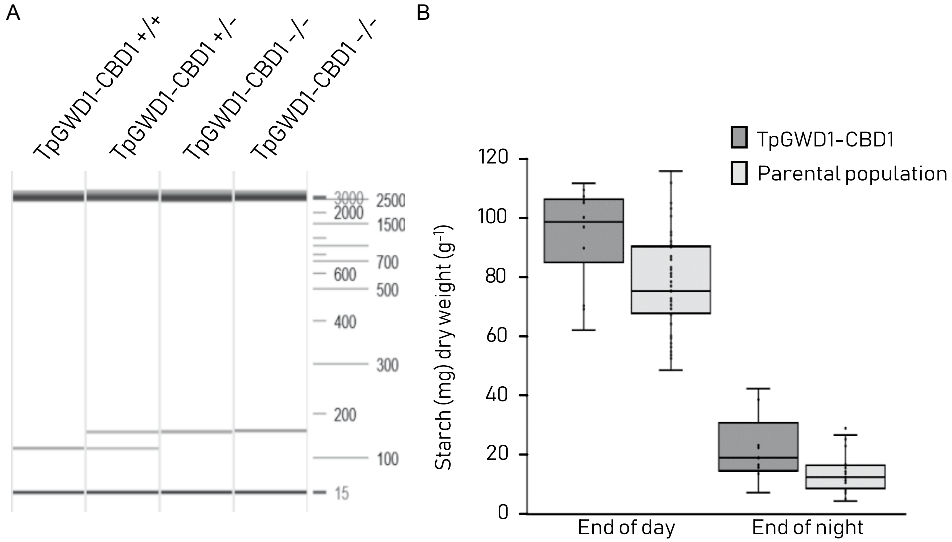

Figure 1. (A) Marker developed to identify the TpGWD1-CBD1 allele. (B) Starch content of the allelic variant *TpGWD1-CBD1* compared to the parental population, at ED and EN.

Conclusion

We have overcome preconceived restrictions based on the central dogma that starch content and growth are tightly linked to maximize biomass potential. There is likely a large degree of flexibility in carbohydrate metabolism that red clover utilizes to adapt to its environment or specified growth conditions. Genetic variation in the genes that encode for the enzymes in starch metabolism has the potential to increase starch content.

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Phenotypic selection improves the resistance of 'Mattenklee' red clover to Southern Anthracnose (*Colletotrichum trifolii*)

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Abstract

Southern Anthracnose, caused by *Colletotrichum trifolii*, is a serious disease of red clover (*Trifolium pratense*) and can lead to death of the plants. The degree of resistance is highly variable among cultivars. The objective of this study was to assess whether phenotypic selection within a cultivar improves resistance to Southern Anthracnose. From each of seven cultivars with moderate to low resistance level, we selected 10 resistant genotypes. Within each cultivar, the resistant genotypes were crossed by manual pollination. The resulting F_1 progeny of each cross was then evaluated in the greenhouse for resistance to *C. trifolii*, along with the original cultivars. Plants of the F_1 progeny survived at a rate of 53 to 77%, with a mean of 64%. The resistance of six out of seven cultivars was significantly improved after one cycle of selection. On average, the resistance level was increased by 28% (with a range of 18 to 52%) compared to the parental cultivar. The increase was highest in cultivars with a low initial resistance. The data suggest that few major genes are involved in Anthracnose resistance in the cultivars tested.

Keywords: *Trifolium pratense*, *Colletotrichum trifolii*, resistance, breeding

Introduction

Red clover (*Trifolium pratense* L.) is a high yielding, perennial forage legume, used mainly for cutting in pure stands or grass-clover mixtures and is often found in permanent grassland (Boller *et al.,* 2010). Due to its ability to fix atmospheric nitrogen, red clover has a high content of protein and is one of the most important forage legumes of temperate climates (Taylor and Quesenberry, 1996). Red clover is allogamous and has a strong gametophytic self-incompatibility system (Boller *et al.,* 2010). Swiss 'Mattenklee' (meadow clover) is an early flowering and persistent red clover type, which has been bred in Switzerland since the beginning of the 20th century based on locally adapted landraces.

In the early 1990s a fungal disease of red clover, Southern Anthracnose (*Colletotrichum trifolii*), was observed for the first time in Switzerland (Boller *et al.,* 1998). Plants of the Swiss 'Mattenklee' cultivars proved to be susceptible to this disease. *Colletotrichum trifolii* spreads rapidly with conidia under warm and humid weather conditions. The fungus causes light to dark brown lesions predominantly on stems and petioles. These injuries can girdle the whole stem very quickly so that leaves or whole shoots dry out and die, resulting in significant yield losses. On the diseased tissue, black fruiting bodies (acervuli) with conidia and bristles (setae) are formed. The fungus also attacks the upper part of the taproot and the plant base, which in most cases leads to premature death of the whole plant. In addition to red clover, Southern Anthracnose can infect other host plants like alfalfa (*Medicago sativa*) and other *Medicago* and *Trifolium* species.

The most economically efficient way to control Southern Anthracnose is through breeding of resistant varieties. In the United States, red clover was successfully bred for resistance to *C. trifolii* as early as the 1950s (Taylor, 2008). The objectives of this paper were (1) to show if resistance to *C. trifolii* is inherited in the Swiss 'Mattenklee' red clover and (2) to search for resistant genotypes in this plant material in order to develop new resistant and persistent red clover cultivars.

Materials and methods

Inoculum preparation

Cultures of eight single-spore isolates of *C. trifolii* were obtained from diseased red clover plants collected in Ellighausen (Switzerland). The cultures were grown on PDA for 14 days at 18 °C under 12 h ultraviolet light irradiation. The inoculum consisted of an equal part of each of the eight isolates and was prepared by scraping spores from the agar plates. The spores were diluted in distilled water containing two drops of Tween 20 l^{-1}. Spore concentration was adjusted to 3×10^6 spores ml^{-1}.

Evaluation of resistance

Red clover plants were grown in a greenhouse at a day/night temperature of 22/20 °C and a 16 hour photoperiod. Seedlings were planted in boxes (0.3×0.4 m) filled with cultivation substrate at a plant to plant distance of 4 cm. Each cultivar or progeny of a cross was grown in a randomized complete block design with three to four replicates (depending on the number of seeds available). Each replicate consisted of up to 40 plants. Five-week old plants were cut about 2-3 cm above ground and the number of plants was assessed. Two weeks after cutting, the plants were sprayed with the inoculum until the plants were dripping wet. Plants were then covered with a transparent polyethylene foil for five days. Two, six and ten weeks after inoculation plants were cut for the second, third and fourth time, respectively. Three days after the last cut the surviving (resistant) plants of each cultivar or progeny were counted. Resistance was expressed as percent of surviving plants to the number of plants prior to inoculation.

Plant material

Out of seven cultivars (with a moderate to low resistance level) 8 resistant plants per cultivar were selected. Within each cultivar 4 biparental crosses were made by manual pollination (subject to assumption that self-fertilization does not occur in our plant material). The resulting F_1 progeny of each of the 28 biparental crosses was then evaluated for resistance to *C. trifolii*, at the same time as the original cultivars.

Statistical analysis

Mann Whitney U-test was carried out for comparing the data of the cultivar and the F_1 progeny population. The observed ratios of resistant to susceptible plants in the F_1 populations were compared to theoretical ratio using Chi-square tests.

Results and discussion

Swiss 'Mattenklee' cultivars showed a low to moderate resistance to Swiss isolates of *C. trifolii*. Depending on the cultivar, they exhibited 15 to 50% resistance as determined by the percent of plant survival (Figure 1). The cultivars Merula and Pavo (both released in 2002) had the highest level of resistance. Surprisingly, the old varieties Renova and Rüttinova (released in 1964 and 1984, respectively) showed a comparable high percentage of surviving plants.

In the F_1 populations from crosses between resistant genotypes of identical 'Mattenklee' cultivars, plants survived at a rate of 53 to 77%, with a mean of 64%. The resistance of six out of seven cultivars was already significantly improved after just one cycle of selection. On average, the resistance level increased by 28% (with a range of 18 to 52%) compared to the parental cultivar. The increase was highest in Formica, a cultivar with a low initial resistance.

Only 9 out of 28 biparental crosses fitted a segregation ratio of 3:1 (resistant : susceptible) ($P>0.05$) and no progeny showed 100% resistant genotypes. The significant increase in the resistance level after one cycle of selection suggests that major effect resistance genes are involved in the cultivars tested.

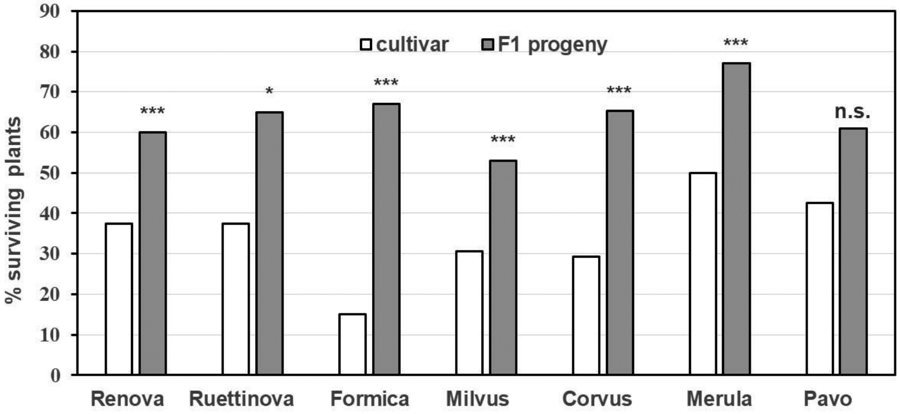

Figure 1. Resistance level, determined by percentage of surviving genotypes after an inoculation with *C. trifolii*, of seven Swiss 'Mattenklee' cultivars and the corresponding F_1 populations of crosses between resistant genotypes; * *P*<0.05, *** *P*<0.01.

Additionally, the segregation ratios in progeny populations from pair crosses between resistant genotypes indicate that more than one resistance gene is present.

Our data is in accordance with the observations of Jacob *et al.* (2015), where Pavo and Merula were among the most resistant cultivars tested, although at a much higher resistance level. Moreover, in both studies all tested cultivars showed improved resistance after just one cycle of recurrent selection.

Conclusion

The moderate resistance level of most Swiss 'Mattenklee' cultivars clearly indicates that at least one resistance gene, which confers resistance against *C. trifolii*, must be present in these cultivars. Moreover, the results show that the inoculation method described in this paper can be used to deploy resistance genes of old cultivars in breeding new resistant and persistent cultivars.

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Study of forage and seed yield of tall oatgrass populations from Serbia

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Abstract

Tall oatgrass (*Arrhenatherum elatius* (L.) P. Beauv. ex J. Presl & C. Presl.) is a perennial grass widespread in Serbia. It can be found from lowland to hilly-mountainous areas. It shows high, stable yield and good persistency, but its fodder quality is moderate, especially due to its early maturity nature. In some areas of Europe this species is highly neglected, while in Serbia it has maintained a place in herbage production. This experiment was carried out during two years on the experimental field of the Institute for Forage Crops, Kruševac, and comprised a collection of twenty-nine tall oatgrass populations collected earlier as landraces on farms in Central Serbia. The trial was set up in a spaced-plant nursery as a randomized block design. The aim was to determine values and variability for dry matter yield, seed yield and seed yield components. Dry matter yield was determined in three cuts in each of the experimental years. All results are shown as two-year mean values. Principal component analysis, cluster analysis and regression analysis were carried out.

Keywords: breeding, dry matter yield, principal component analysis, tall oatgrass

Introduction

Tall oatgrass (*Arrhenatherum elatius* (L.) P. Beauv. ex J. Presl & C. Presl.) is a perennial grass species naturally widespread in Serbia, from plains to hilly-mountainous regions. It is mostly present in the association *Arrhenatheretum elatioris,* considered productive natural meadows in Europe. It performs the best on fertile and well-drained soils, but it also shows acceptable growth on other soil types (Swedrzynski and Kozlowski, 1998). Its high and stable yield and good persistency qualify tall oatgrass for inclusion in mixtures especially suitable for dry and poor soils. Fodder quality of this species is moderate, especially due to its early maturity nature, but its total agronomic value is very high (Peeters, 2004). However, the disadvantages associated with early maturity can be avoided by adjusting the cutting frequency, which increases the proportion of leaves in the total dry matter. In some areas of Europe this species is highly neglected, while in Serbia it has maintained a place in herbage production. Generally, breeding of tall oatgrass is directed to improvement of dry matter (DM) yield, quality and digestibility, and towards late maturity.

However, new genotypes need to have good seed production, since seed multiplication has to be profitable for producers. Therefore, breeders should also not forget seed yield in breeding. High diversity in breeding germplasm is a prerequisite for the selection of superior genotypes, adaptability to different macro and micro habitats, and tolerance to abiotic stresses in the Serbian climate. Very often the initial breeding materials in grasses are ecotypes (Cagaš *et al.,* 2006; Sokolović *et al.,* 2004), while multivariate methods are of great help for the characterization of a breeding population (Ghariani *et al.,* 2006, Sokolović *et al.,* 2011). The aim of the work presented here was to identify within and among ecotypes diversity in a collection of tall oatgrass, describe complex trait correlation structure in the collection, and to define significant traits for population discrimination.

Materials and methods

The experiment was carried out during two years at the experimental field of the Institute for Forage Crops, Kruševac. We screened a collection composed of twenty-nine tall oatgrass populations (Sokolović *et al.,*

2004) that had been collected on farms in Central Serbia as landraces adapted to the local agro-ecological conditions. The trial was set up as a spaced plant nursery (60×60 cm), following a randomized block design with three replications and comprising 30 plants per population. In 2014 and 2015, components of forage yield (dry matter in the first, second and third cut [DMYI, DMYII and DMYIII], total dry matter yield [DMYT] and seed yield components (number of tillers per plant [NofT], 1000 seed weight [1000S] (g)) and seed yield per plant [SY] (g) were determined. All the results were expressed as two-year mean values and analysed by ANOVA. Cluster analysis was performed by the complete linkage method using Euclidean distances. A regression analysis of seed yield on DMYT was conducted. Principal component analysis was carried out based on population two-years means. All analyses were carried out using Statistica 10.0, Stat Soft Inc.

Results and discussion

Analysis of DMY in different cuts, as well as DMT and seed yield components, revealed very high within-population variability (Table 1), with some populations showing coefficients of variations above 70%. This was expected since the populations were collected from locations without intensive agricultural management and represent naturally adapted populations. Highly significant among-population differences were determined for all traits analysed. Coefficients of variation of population averages were between 17 and 19% except for 1000S, which was 10.7%.

Some populations showed high average values for important traits, meaning that they contain genotypes promising for breeding. Very interesting for breeding is knowledge about the interrelation between forage and seed yield. Regression analysis showed a positive relation between SY and DMYT in this collection of tall oatgrass populations (Figure 1).

The first two principal components represented a large amount of variability available among populations (79.06%). It is noticeable that most of the traits analysed were negatively correlated with the first PC,

Table 1. Range of mean values of traits and variability within the collection of tall oatgrass populations.[1]

Traits	DMYI (g plant^{-1})	DMYII (g plant^{-1})	DMYIII (g plant^{-1})	DMYT (g plant^{-1})	N of T	SY (g plant^{-1})	1000 S (g)
Pop. averages	82-174.4**	17.7-37.9**	13.1-31.1**	105.7-235**	91.2-180**	5.0-15.0**	2.4-3.9**
CV within pop. (%)	19.5-78.7	33.4-83.9	39.2-77.4	22.7-71.5	23.4-55.6	30.5-70.9	6.2-23.0
CV of pop. averages	17.70%	18.80%	17.20%	17.24%	17.11%	18.93%	10.76%

[1] ** ANOVA significant differences between populations at the 99%.

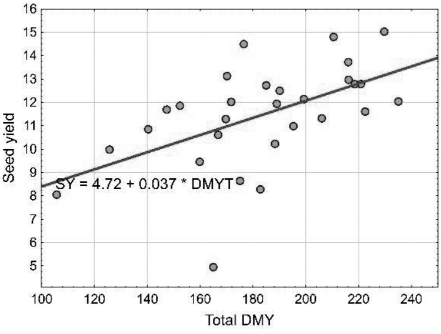

Figure 1. Regression analysis of DMYT and SY.

while 1000 seed weight was positively correlated with the second PC (Figure 2). Also, in this analysis, the vectors of DMY and SY were oriented in the same direction, confirming the tendency shown in Figure 1.

Hierarchical cluster analysis grouped the populations into three main clusters (Figure 3). There were no matches between geographic distances and the hierarchical clustering of the populations.

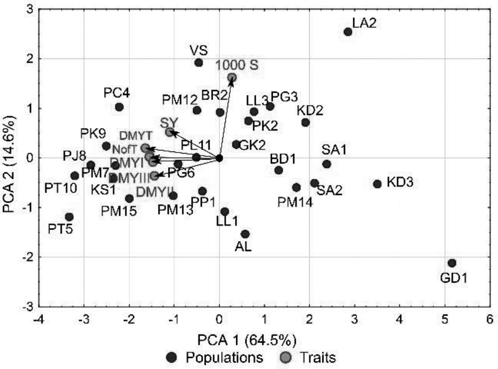

Figure 2. PCA biplot of populations between first two principal components with trait vectors.

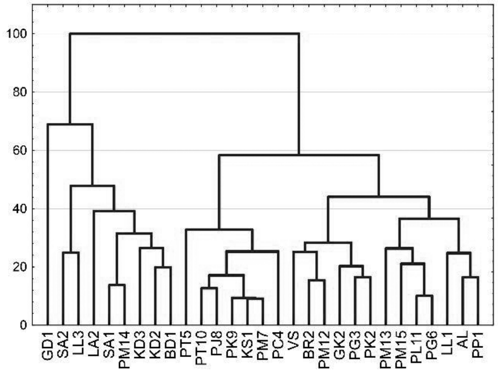

Figure 3. Grouping of populations based on all examined traits.

Conclusion

High within and among population variability were determined in this collection. Regression analysis revealed that forage yield and seed yield were related to dry matter yield, indicating that it might be possible to breed simultaneously for these two traits in tall oatgrass. The first two principal components described almost 80% of variability in the collection investigated and confirmed this result. This collection comprises many populations with desirable traits that are suitable for breeding.

Acknowledgements

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An experimental design to test tall fescue ecotype × endophyte combinations under diverse managements and environments

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Abstract

The success of tall fescue (*Festuca arundinacea*) is attributed in part to the presence of *Epichloë coenophiala,* a dominant symbiont endophyte that colonizes the above ground plant tissue and can enhance host tolerance to drought and provide protection from pests and pathogens. Mediterranean summer dormant (SD) tall fescue has a survival strategy by going dormant during summer thus offering multi-year persistence in stressed environments, unlike the widely planted summer active (SA) or continental tall fescue. We created isogenic clone pairs differing by presence or absence of different endophytes from genotypes of SA and SD ecotypes. Using these clone pairs, we then developed isogenic populations by intermating them for each genetic background. Our objective is to determine the relative merits of ecotype × endophyte combinations across multiple locations where hot and dry summers can deplete or eliminate stands of tall fescue. Clonal pair populations of SA and SD cultivars are currently being evaluated for yield and persistence under irrigated and non-irrigated, frequent and infrequent harvest management conditions in multiple, highly distinct environments across the Unites States.

Keywords: tall fescue, endophyte, summer dormant, summer active, isogenic populations

Introduction

Tall fescue is a cool-season forage grass noted for its high biomass production (e.g. in California; Orloff *et al.*, 2016) and its survival under diverse biotic and abiotic stresses. Tall fescue can be grouped into two primary ecotypes (Hopkins *et al.*, 2009). The summer active (SA) ecotype is the most widely grown and originates from Continental Europe. The summer dormant (SD) ecotype is native to regions around the Mediterranean Sea. The fungal endophyte, *Epichloë coenophiala* lives within many tall fescue plants, growing intercellularly in the stem and leaf blade and ultimately infecting seed, through which it is passed to the next generation (Christensen and Voisey, 2009). Generally speaking, plants infected with endophytes are more vigorous and persistent than isogenic uninfected plants (Arachevaleta *et al.*, 1989). Endophytes produce a range of alkaloids that protect the plant from herbivory, impart stress tolerance and insect resistance, and can be toxic to cattle (Bush and Fannin, 2009). Some endophyte strains do not produce toxic ergot alkaloids (Bush and Fannin, 2009). Non-toxic endophyte strains have been artificially inoculated in the laboratory into endophyte-free plants (Latch and Christensen, 1985). The resulting cultivars do not cause animal performance problems but have persistence attributes of a toxic infected tall fescue plant (Bouton *et al.*, 2002; Parish *et al.*, 2003). The endophyte effect of enhanced survival is most commonly observed in summer active ecotypes under heat and drought stress, with less benefit observed when environments are more conducive to grass production. The benefit to SD cultivars appears to be less, possibly because they already have a mechanism (dormancy) to deal with hot, dry environments (West *et al.*, 2007).

The environmental gradients from east to west in the southern Great Plains and north to south in California span adaptation zones for SA and SD tall fescue. We hypothesized that the benefit of summer dormancy and of endophyte infection will increase as environmental conditions become more severe. The objective of this experiment was to test that hypothesis across seven locations of contrasting severity, within each of which, multiple stress treatments are imposed.

Materials and methods

To evaluate the importance of endophyte, clone pairs were generated from 15 unique genotypes from each of six populations (two SA and four SD) with four different endophyte strains. Genetically identical clone pairs were established from unique endophyte-infected (E+) genotypes. Using these clone pairs, we then developed isogenic populations by intermating the E+ and E- clones for each genetic background. Seed was produced in 2015 and 2016 for each isogenic population and then tested for the presence or absence of endophyte as well as endophyte type, friendly or common toxic (CTE) using a PCR specific protocol (Young *et al.*, 2014).

Trials were established across seven locations in three states in the USA in order to evaluate persistence and yield of isogenic populations of SA and SD tall fescue with and without an endophyte symbiont across a range of stresses and environments. In autumn 2017, trials were seeded at Gene Autry, OK (34.17N; 96.58W), Vashti, TX (33.55N; 98.04W), Holtville, CA (32.80N; 115.45W), Davis, CA (38.52N; 121.77W), and Parlier, CA (36.60N; 119.51W); plots were established at Tulelake, CA (41.96N; 121.47W) in May 2018. The experimental design for each trial was split plot design with four replications. Plots consisted of two sown rows each 3.0 m long and spaced 0.2 m apart. Trials were sown using a small plot cone-drill into clean seed beds at all locations with a seeding rate of 22 kg ha^{-1}. In early summer, four treatments were imposed: continued full irrigation throughout summer or drought conditions (in CA, no water at all applied; in OK, no supplemental irrigation) combined within each irrigation treatment with either frequent or infrequent harvesting. Thus, within locations, treatments ranged from low stress (infrequent harvest and full irrigation) to high stress (no supplemental water and frequent harvest). Data from the forage yield trials were taken by cutting plots with a sickle bar plot harvester at a height of approximately 7 cm. Sub-samples were collected from each plot during the time of harvest and dried in a forced air oven at 60 °C to determine dry weight. All plot yields were adjusted to a dry weight basis and include sample weights. Data were collected from all locations in 2018 except Tulelake, CA.

Results and discussion

From initial harvest data collected during the autumn of 2018 at our Gene Autry, OK and Vashti, TX locations, yields under irrigation were higher on average for both SD and SA germplasms than germplasms under drought stress regardless of the presence or absence of fungal endophyte (Table 1). Yields of SD E+ germplasm were lower than that of E- SD germplasm across all treatments. However, yields of SA E+ germplasms were higher than that of SA E- germplasms regardless of treatment. We should caution against over interpretation of these data since this study is in its infancy and data collection will continue over the next several years at each site for multi-location analysis.

Table 1. Yields (Mg ha^{-1}) of isogenic SD and SA populations × endophyte combinations under four stress treatments averaged across two locations, Gene Autry, OK and Vashti, TX in the southern Great Plains, USA.[1]

Cultivar/germplasm	Ecotype	Endophyte	Drought				Irrigated			
			Infrequent harvest		Frequent harvest		Infrequent harvest		Frequent harvest	
			E+	E–	E+	E–	E+	E–	E+	E–
NFTF 1700	SD	AR502	4.3	4.9	4.2	4.4	5.1	6.4	5.9	6.0
NFTF 1700	SD	AR542	4.1	5.0	4.1	4.5	4.9	6.9	6.0	6.3
NFTF 1730	SD	AR584	4.4	5.2	4.2	4.7	5.9	6.9	5.8	6.3
NFTF 1810	SD	AR584	4.0	4.8	4.5	4.6	5.7	6.9	5.8	6.2
Texoma	SA	AR584	6.7	6.1	6.9	6.0	6.9	5.6	7.1	6.3
PDF	SA	CTE	6.0	6.0	6.0	5.7	9.0	5.6	8.6	6.1
Mean			5.0	5.3	5.0	5.1	6.3	6.4	6.5	6.2
LSD (0.05)			0.3	0.3	0.4	0.4	0.2	0.4	0.3	ns

[1] Plots were established in autumn of 2017 and all stands were uniformly excellent. Table represents preliminary data collection from 2018 only.

Conclusion

This experiment will continue for at least one more year, with yield and stand persistence monitored. The results of this trial will give us clear indications of the value of several different endophytes to yield and persistence of tall fescue under a wide diversity of environmental stresses. Information gathered from these trials will aid in recommending germplasm throughout both regions and help focus future breeding efforts.

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Benchmarking symbiotic performance of white clover cultivars: room for improvement?

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Abstract

To gain insight into variation in white clover-*Rhizobium* interactions, we assessed symbiotic performance of 48 individuals each from 17 cultivars from a range of leaf size classes, and included a wild ecotype population and a *T. repens* × *T. ambiguum* hybrid cultivar. Measurements of symbiotic response were based on plant shoot and root biomass, pink nodule number, and symbiotic potential. This was determined using an effective and a partially-effective *Rhizobium* strain separately. The plant biomass and symbiotic potential traits were strongly linked, and also inversely correlated with the root:shoot ratio. There were significant differences between cultivars for symbiotic interactions, with some performing equally well with both strains. Some cultivars exhibited extreme differences by performing very well with the effective strain, and very poorly with the partially-effective strain. Benchmarking symbiotic performance indicated clover population-specific genetic factors that may be explored as a breeding target for enhanced interaction with *Rhizobium* spp. to improve N-fixation.

Keywords: nitrogen-fixation, symbiotic potential, *Trifolium*, *Rhizobium*

Introduction

White clover (*Trifolium repens* L.) is an integral component of mixed pastures in temperate agriculture, providing forage and a sustainable source of fixed nitrogen (N) through its symbiosis with soil-dwelling *Rhizobium* bacteria (Abberton *et al.*, 2006). While there has been much focus on identifying and applying *Rhizobium* inoculants containing strains with high N-fixation capacity, there has been less attention on identifying and exploiting plant genetic factors influencing effective *Rhizobium* symbioses. Studies investigating symbiotic performance have focused on few individuals from a limited number of cultivars (Jones and Hardarson, 1979; Bonish, 1980). To assess potential untapped genetic variation in symbiotic interaction in white clover, we benchmarked a wide range of clover cultivars for symbiotic performance as part of programme to investigate clover-*Rhizobium* interaction as a breeding target.

Materials and methods

A selection of 17 white clover cultivars from a range of leaf-size classes and release dates, as described in Figure 1, were obtained from the Margot Forde Germplasm Centre (AgResearch, New Zealand). Seeds were surface-sterilised (Drew *et al.*, 2012), germinated, then transferred to 15×20×15 cm containers filled with sterile medium-grade vermiculite growth medium saturated with N-deficient McKnight's nutrient solution (Drew *et al.*, 2012). Containers were subdivided into four sections, each of which had a single cultivar with 16 seedlings, and each cultivar was replicated three times and nested within each treatment in a randomised block design. Treatments comprised: TA1 (effective *Rhizobium* strain); S12N10 (partially-effective strain); Positive control (mineral-N added, no rhizobia)); and Negative control (neither N nor rhizobia added). Plants were grown in a controlled environment (~22 °C) with a light intensity of 150-200 μmol m^{-2} s^{-1} and a 16 h photoperiod. *Rhizobium* strains suspended in water from agar were prepared (Drew *et al.*, 2012) and added to each seedling in the appropriate treatment the day after planting.

After seven days, each Positive control received mineral-N (200 ml of 150 mM NH_4NO_3) applied evenly across the container. After five weeks of growth, each plant was harvested and dried prior to measuring: root dry matter (DM); shoot DM; root:shoot DM ratio; shoot symbiotic potential (SP); root SP; and number of pink (N-fixing) nodules. SP $([DM_{\text{with } \textit{Rhizobium}} - DM_{\text{Negative control}}] / [(DM_{\text{Postive control}} - DM_{\text{Negative control}}])$ was calculated (Drew *et al.,* 2012) with an adjustment for the Negative control biomass. Data were analysed by regression analysis using a nested linear model within treatment, and canonical discriminant analysis (CDA) on the treatment:cultivar means was performed using 'candisc' (R Studio).

Results and discussion

The biplot of the seven measures based on the four treatments of the 17 clover populations showed a strong correlation among the plant biomass traits and symbiotic potentials, which were in turn inversely correlated with the root:shoot ratio (Figure 1). The number of Pink Nodules did not correlate with either biomass or root:shoot ratio. CDA analysis distributed the cultivar:treatment means into those treatments with or without *Rhizobium*, hence with or without nodules, whereas the vertical axis (CD2) separated the cultivar:treatments means by available nitrogen gradient (Figure 1). The Negative controls were tightly clustered and exhibited very high root:shoot ratios with low biomass, indicating the plants placed resources into root mass before growth slowed when seedling N reserves were exhausted. The Positive controls formed a tight cluster with the highest biomass metrics, as expected (Figure 1).

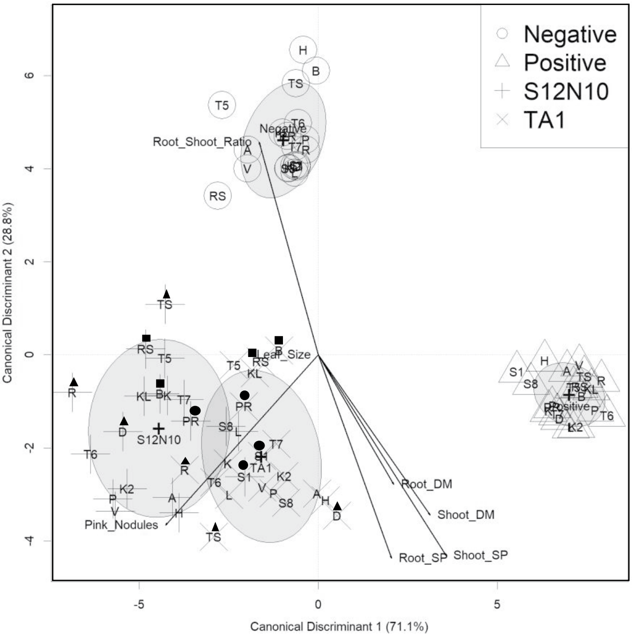

Figure 1. Trait biplot and canonical discriminant analysis (CDA) of standardised means of symbiotic responses from four treatments of 17 white clover populations [cvs Dutch (D ▲)); Kent (K); Louisiana (L); S100 (S1 ●); S184 (S8); Grasslands Huia (H); Regal (R ▲); Grasslands Pitau (P); Rivendel (V); Grasslands Kopu II (K2); Barblanca (B ■); Grasslands Prestige (PR ●); Klondike (KL); Riesling (RS ■); Grasslands Tribute (T5,6,7)), a wild ecotype (Tienshan (TS ▲)], and a *T. repens*×*T. ambiguum* hybrid (AberLasting (A). The % variation accounted for by the canonical discriminant axis is denoted in the axis legend. Exemplars of cultivar-strain performance (▲, ■, ●) are discussed in the main text.

The cultivar:TA1 (effective) strain treatment clustered in the direction of increased plant biomass traits compared with the partially-effective strain S12N10. Within these clusters, however, performance of individual cultivars varied greatly. In general, most cultivars, including the inter-specific hybrid A, were less productive with the partially-effective strain, but some cultivars had more extreme responses. The ecotype TS (▲) and cultivar D (▲) performed very well with the TA1 strain, but very poorly with the partially-effective S12N10 strain (Figure 1), indicating low compatibility with this strain. Cultivar R (▲) performed moderately well with TA1, but very poorly with S12N10 (Figure 1). In contrast, cultivars S1 (●) and PR (●), performed similarly well with both strains, indicating potential to form effective symbioses across a wider range of *Rhizobium* strains. At the other end of the spectrum, B (■) and RS (■) performed poorly with both strains relative to the Positive (+N) controls, suggesting an inability to form effective symbioses with these *Rhizobium* strains. Interestingly, the poor performing cultivar:strain combinations were distributed further along the root:shoot ratio biplot than most of the other cultivars, showing increased root growth when interacting with less effective strains. Further investigation with additional isolates is needed to establish if the symbiotic advantages shown by some cultivars extends to a wider variety of rhizobia strains.

Conclusion

These experiments benchmarked the symbiotic performance of a wide range of clover cultivars and revealed marked differences in cultivar interaction with *Rhizobium* strains. This indicated that their clover population-specific genetic factors may be explored as breeding targets for enhanced interaction with *Rhizobium* spp. to improve N-fixation for more sustainable agricultural production.

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Analysis of drought tolerance in perennial ryegrass (*Lolium perenne* L.) with methods of genomic prediction

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Abstract

Yield losses and reduced persistency are detrimental outcomes in growing perennial ryegrass (*Lolium perenne* L.) in areas affected by temporal drought. The aim of this study was to develop phenotyping and genomic prediction methods to assess drought tolerance in an efficient way. One of the most relevant traits for improving drought tolerance in perennial ryegrass is a good recovery of plants after water returns. Within 14 segregating biparental populations, a huge genetic variation for recovery was found with heritability for phenotypically scored biomass of up to 0.64. Using 2,140 genome wide distributed SNP markers, a genomic prediction of biomass scoring and biomass yield during recovery was set up. Prediction abilities of up to 0.510 were obtained for performance across two locations.

Keywords: recovery, rewatering, biomass production, SNP marker, prediction ability

Introduction

The year 2018 was characterised by extensive drought events in Northern and Central Europe with large impacts on grassland yields. Regarding Germany, grassland yields in Lower-Saxonia were up to 40% lower than long-term average, and average yield losses were reported between 0 and 30% from Southern to Northern Bavaria. It is possible that 2018 was a year that showed the impacts of global climate change, including increased occurrence of weather extremes. Perennial ryegrass (*Lolium perenne* L.), as one of the most important forage grass species in Europe, is likely to be particularly affected by global climate change. Current varieties have poor drought tolerance due to shallow rooting. Therefore, grassland yield is likely to decline in areas impacted by temporal drought periods. The aim of the project was to efficiently improve tolerance of perennial ryegrass to temporary drought through enhanced recovery after drought using innovative selection methods.

Materials and methods

The work was based on 14 diploid segregating biparental populations built from single plants differing in drought stress response. Each population comprised 140 individuals and was derived either from a cross of tolerant × tolerant (populations K01 to K06 and K11 to K14) or tolerant × susceptible clones (populations K07 to K10). Individuals were propagated clonally and phenotyped in a randomized two-location rain-out shelter experiment. Each family was divided into four blocks with 35 individuals complemented by five randomly distributed standard genotypes out of ten (28 copies each per location) in Freising/Pulling (Southern Germany) and Malchow/Poel (Northern Germany). During vegetation periods of 2017 and 2018, watering was withheld twice a year (in late spring and midsummer) and plants were kept below permanent wilting point for up to four weeks. After cutting of the plants a well-watered period of 4-6 weeks followed until the next cutting date. Biomass formation was scored visually (0 = dead plant, 1 = lowest to 9 = max. biomass) before cutting and single plant yield was recorded after recovery period. Four populations (K07, K10, K12 and K13; n=560 individuals) were genotyped with a 3.6 k *Lolium* SNP chip (Illumina Infinium; Blackmore *et al.,* 2015). Mean values adjusted for location and block effects were calculated using lmer function from R package 'lme4', heritabilities were derived from variance components. A genomic prediction approach was set up based on adjusted means (linear mixed model with best linear unbiased predictor). The predictive abilities were assessed with cross-validation schemes provided by the R package 'synbreed' (Wimmer *et al.,* 2012).

Results and discussion

Phenotypic data: Considering the number of dead plants between Freising and Malchow sites reflect the expectations derived from parents drought reaction. At the Freising site, out of susceptible families 81 (K07) and 110 (K10) individuals died, whereas from tolerant families 16 (K12) and 53 (K13) were dead after day 227 in the year 2018. At the Malchow site, the same ranking of populations was found with 20 (K07) and 58 (K10) dead plants as well as 3 (K12) and 32 (K13) dead plants at the end of the experiment. For comparing response to drought stress of different crosses in the rain-out shelter, adjusted means of biomass scoring data were analysed at family level. For the whole two-year observation period, heritabilities ranged between 0.30 and 0.64. For all 14 families, a decline of scores was observed after the first drought stress in 2017. The adjusted mean scores dropped from 5.8-7.7 to values between 3.6 and 5.7. Drought tolerant families always showed an increase in scoring at rewatering, while mean scores of susceptible families stayed reduced during recovery and even declined further in the following drought periods. These results complement the observations for the number of dead plants. Figure 1 shows trends for two populations: K10 (A, susceptible family) and K12 (B, drought tolerant family). For the last scoring (day 227; 2018), K10 and K12 ended up with a mean score of 2.24 and 6.26, respectively. Correlation between biomass score and single plant yield was high and equalled 0.83 for 2017 data and 0.82 for 2018 data.

SNP chip data: The latest version of Illumina iSelect SNP Chip for *Lolium* (Blackmore *et al.* 2015) contained 3,681 SNP markers of which 2,140 markers were informative within the four crossing populations. Figure 2 shows a principal component analysis of 560 F_1-individuals complemented by six parental clones. Parents were shown as genetically diverse and, as expected, genuine crossing products are located between their two parental clones. Self-pollinated genotypes are located at one of the parents and false pollinated genotypes appear in the middle of the chart. In sum 23, individuals were identified as self- or false-pollinated genotypes and were discarded from genomic prediction analysis.

Genomic prediction of drought tolerance: A genomic prediction was attempted for adjusted means of biomass scoring and single plant yield in 2017 and 2018, representing the performance of first of two recovery periods of each year. Table 1 shows the prediction abilities within and across families for the

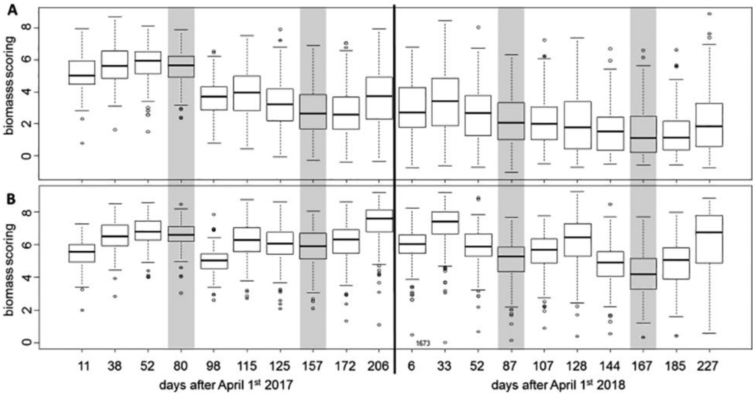

Figure 1. Boxplots of visual biomass scoring (adjusted means of two locations) within susceptible population K10 (A) and tolerant population K12 (B), n=140 individuals in each population; scoring dates during drought periods are marked with grey background.

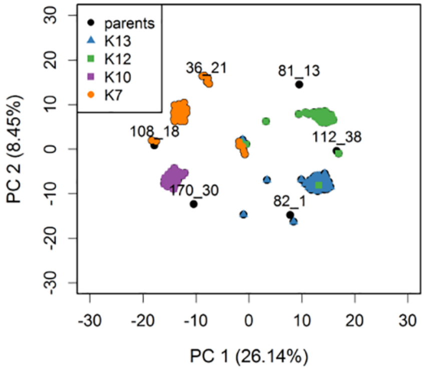

Figure 2. Principal component analysis of four crossing populations (coloured) and their population parents (black).

Table 1. Prediction abilities for four populations, two traits and two dates related to recovery after drought obtained for prediction within and across populations.

Population	Day 115, 2017				Day 128, 2018			
	Biomass scoring		Dry matter yield		Biomass scoring		Dry matter yield	
	within	across	within	across	within	across	within	across
K07	0.061	0.258	0.252	0.316	0.287	0.167	0.179	0.178
K10	0.480	0.510	0.346	0.382	0.492	0.463	0.144	0.195
K12	0.371	0.425	0.362	0.393	-0.043	0.267	0.189	0.253
K13	0.484	0.496	0.317	0.348	0.464	0.470	0.317	0.347

four populations under investigation ranging from -0.043 to 0.510. These values are comparable to others obtained for *L. perenne*.

Conclusion

Drought stress tolerance is a complex trait to select in plant breeding. A good recovery after drought is a relevant criterion for drought tolerance selection and we found that it can be assessed by methods of genomic prediction. One of the next steps will be to complement drought tolerance prediction with single plant NMR-based metabolome analysis.

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Salinity response of alfalfa populations selected for salinity tolerance using an indoor screening method

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Introduction: Root-zone salinity is an important abiotic stress limiting plant growth and productivity worldwide. Alfalfa has a low tolerance to salinity. Selection of salt-tolerant alfalfa genotypes through conventional breeding is difficult due to large within-fields variability in soil salinity. Our objective was to evaluate an indoor screening method of selection for salt tolerance in alfalfa.

Materials and methods: Recurrent phenotypic selection was applied for salinity tolerance using indoor-grown alfalfa plants in tanks filled with silica sand. A total of 520 genotypes of each population distributed in five large growing-tanks (1.0 m-high, 0.95 m-diam.) were surface irrigated with a modified Hoagland solution with an electrical conductivity of 12 dS m^{-1} representing a moderate salinity level. Plants were harvested twice and, after 90 days, the 10 larger plants per tank were selected for a total of 50 plants that were intercrossed. Using this indoor infrastructure, four cycles of recurrent selection (1, 2, 3 and 4) were performed within three cultivars (Apica, Halo, and Bridgeview). At each cycle, Salt Tolerant (ST) plants, with high yield when grown in saline conditions, were selected and intercrossed. Twelve populations (ST+1, ST+2, ST+3 and ST+4) within each cultivar were produced. To evaluate their responses to salinity, populations Halo, HaloST+3, Bridgeview and BridgeviewST+3 were inoculated with rhizobia and grown in Turface irrigated with Hoagland solution supplemented with one of five NaCl concentrations (0, 20, 40, 80, and 120 mM NaCl). The yield of each population was assessed under these conditions as an indicator of salinity tolerance. In addition, the concentrations of compatible solutes were measured in stems and nodules of each population/salinity combination to unravel salinity-tolerance mechanisms.

Results: Shoot biomass yields were significantly higher in ST+3 populations than in initial cultivars under a severe NaCl-stress of 120 mM. The ST+3 populations had higher sucrose concentrations in their nodules and similar sucrose concentrations in their shoots than Bridgeview and Halo cultivars (Table 1).

Table 1. Yield and sucrose concentrations in shoots and nodules of four populations of alfalfa grown in Hoagland solution supplemented with 120 mM NaCl (severe salinity stress).

Population	Yield (g/plant)	Shoot Sucrose (mg g^{-1} DW)	Nodule sucrose (mg g-1 DW)
Bridgeview	0.67 b	27 ns	61 b
Bridgeview ST+3	0.87 a	27 ns	75 a
Halo	0.76 b	27 ns	62 b
Halo ST+3	0.83 a	24 ns	80 a

Conclusion: Alfalfa populations recurrently selected for salt-tolerance seems to be more productive than unselected cultivars under a severe NaCl stress. Higher yield of ST+3 populations could be linked to higher nodule sucrose concentrations since this sugar has been shown to play a role of compatible solute involved in osmotic adjustment and could help maintain N_2 fixation under salinity stress.

Genetic diversity of *Epichloë* endophytes infecting *Schedonorus pratensis* and other forage grasses reveals complex correlation between haplotypes and loline alkaloid levels

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Introduction: Species belonging to the *Festuca-Lolium* complex are often naturally infected with *Epichloë*. These endophytic fungi grow asymptomatically in intercellular spaces of the aerial tissues of the host plants, and the asexual *Epichloë* species are exclusively seed-transmitted. The increasing knowledge on benefits provided by the endophytes and their secondary metabolites has led researchers and farmers to consider their potential use into commercial cultivars. Large-scale screenings are crucial to isolate and characterize animal-friendly strains for agriculture. Of interest, in this respect, is the asexual hybrid *Epichloë uncinata* isolated from meadow fescue, which can produce high levels of loline alkaloids without any known toxic alkaloids.

Materials and methods: In this study we collected approximately 15 to 20 plants of *Schedonorus pratensis,* and occasionally of *S. arundinaceus* (*Festuca arundinacea*), *S. giganteus* (*S. gigantea*) and *Lolium perenne* in 135 different locations across Europe, and seed lots of 255 meadow fescue accessions from the U.S. Department of Agriculture (USDA) and 96 from NordGenetic. Endophyte presence was initially tested with an *in planta* assay using two microsatellite markers, subsequently confirmed with an immunoblot essay. From these preliminary genetic data, 250 isolates were fingerprinted at five more microsatellite loci, which were uncharacterized for these endophyte species. Loline alkaloids were measured in all the infected *S. pratensis* plants, and endophytes were isolated from plants with the highest concentration and artificially inoculated in a perennial ryegrass elite cultivar.

Results: Of the 135 locations where plants were collected, 115 had *Epichloë* infected grasses. The number of infected locations varied between countries: for *S. pratensis* it spans from 70% in Denmark to 100% in Sweden, Norway and Austria. The screening on genebank seedlots confirmed *Epichloë* infection in 12 USDA and 53 NordGenetic accessions. The microsatellite-based fingerprinting essay identified 21 unique combinations of alleles (haplotypes) across 7 endophyte loci. Phylogenetic and principal components analysis showed a strong interspecific genetic differentiation among isolates. Loline alkaloid levels detected in native host plants will be presented at the conference. We observed very large variation in loline content among isolates, ranging from detection limits (<25 μg/g) up to 5,629 μg/g. Significant differences ($P<0.01$) in the average amount of total loline and in the proportion of the three loline alkaloids were found among haplotypes. When *E. uncinata* strains were inoculated in *L. perenne*, measured loline alkaloids were significantly lower than observed in the original host (<100 μg/g).

Conclusions: Scandinavian northern regions and the Alps are good sources of *Epichloë* infected meadow fescue. Germplasm repositories also represent a valuable resource for endophytes even though their viability in the seeds is reduced over time. Microsatellite markers provide a quick and reliable method to discriminate endophytes. The association between haplotypes and concentrations of loline alkaloids suggest a more complex genetic composition than expected in asexual, vertically transmitted species.

Breeding timothy (*Phleum pratense* L.) in the context of climate change

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Introduction: Timothy (*Phleum pratense* L.) is a winter-hardy perennial grass species largely used as forage in Canada. Timothy regrowth is hampered under dry and hot conditions (Mäkinen *et al.*, 2015), which are expected to worsen in a changing climate (Qian *et al.*, 2013). In a previous study on timothy response to drought, we already noticed that root biomass was tightly related to drought tolerance (Gagné-Bourque, 2016). This observation prompted us to assess root spatial distribution as a phenotypic feature potentially linked to timothy regrowth potential according to the experiment presented afterwards.

Materials and methods: To assess the genetic variation for this feature, a set of 30 timothy cultivars with contrasting shoot dry matter yield (DMY) was selected. Twenty-six plants per cultivar were grown in a growth chamber for 6 weeks at a day/night (D/N) temperature of 21/17 °C, a 16 h day length (dl) and without water limitation. The nine most vigorous plants were transplanted in tubes (95 cm deep, 16 cm diameter) and grown for 2 weeks in a growth chamber at a D/N temperature of 18/15 °C, a 16 h dl and without water limitation. We simulated the period from autumn to spring by gradually decreasing D/N temperatures and dl for ten weeks (min.: D/N temperature of 4 °C, 10 h dl) and then gradually increasing them for six weeks (max.: D/N temperature of 24/18 °C, 16 h dl). Plants were then harvested and a heat (D/N temperature of 30/20 °C) and drought (no watering) stress was applied for 7 days. After the stress, growing conditions were restored (D/N temperature of 24/18 °C, 16 h dl) were restored for 4 weeks. A second harvest was then carried out to measure shoot, total-root and sequential-root (20-cm increments from 0 to 100 cm deep) DMY.

Results: Phenotypic variation for shoot-DMY at the 1st and 2nd harvest, total-root-DMY, and the proportion of deep-root was observed between the 30 selected timothy cultivars. A significant positive relationship was observed between shoot-DMY and total-root-DMY at the second harvest (R^2=0.69) whereas no relationship was observed at the first harvest (R^2=0.11). The proportion of deep-root (40 to 100 cm deep) was positively related with the shoot-DMY (R^2=0.68), whereas the proportion of shallow-root (0-40 cm) was negatively related with that trait (R^2=-0.68). Additionally, the three populations showing the highest shoot-DMY and proportion of deep-root under controlled-conditions were among the highest yielding populations in the field (results not shown).

Conclusion: Results suggest that deeper root growth could be an adaptation strategy to tolerate heat and drought stress in timothy and should be considered when screening for new germplasm.

Gagné-Bourque F., Bertrand A., Claessens A., Aliferis K.A. and Jabaji S. (2016) Alleviation of drought stress and metabolic changes in timothy (*Phleum pratense* L.) colonized with Bacillus subtilis B26. *Frontiers in Plant Science* 7, 1-16.

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Qian B., De Jong R., Gameda S., Huffman T., Neilsen D., Desjardins R., Wang H. and McConkey B. (2013) Impact of climate change scenarios on Canadian agroclimatic indices. *Canadian Journal of Plant Science* 93, 243-259.

Broadening the genetic basis of cultivated tetraploid red clover (*Trifolium pratense* L. var. *sativum*) by polyploidization of diploid populations

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Introduction: Cultivated red clover (*Trifolium pratense* L. var. *sativum*) is one of the most important protein fodder crops in Germany. Tetraploid varieties are characterized by an increased biomass yield; therefore, they are often used in high quality seed mixtures. Tetraploid red clover does not occur naturally as breeding tetraploid varieties depends on colchicined progenies of a limited number of selected diploid plants or progenies of crosses between tetraploids. As the genetic basis of tetraploid varieties is limited, new tetraploid populations were developed by polyploidization of representative samples of diploid varieties.

Materials and methods: Seeds of 11 diploid varieties (Avisto, Global, Harmonie, Kontiki, Lucrum, Merula, Milvus, Nemaro, Odenwälder, Suez, Violetta) were germinated on filter paper for 5 days and the seedlings subsequently treated with colchicine (concentration of 0.2%, 0.4% and 0.6%, 2% DMSO, Tween80, pH 5.5) for 3 hours. After that, seedlings were rinsed with water for 30 min in a tea strainer. After a recovery period of 5 days on filter paper the seedlings were planted to cultivation substrate and cultivated until 5-6 leaf stage at which survival rate was estimated. Selection of tetraploid individuals was carried out by flow cytometry. Diploid and chimeric plants were discarded.

Results: In total 6,199 colchicine treated plants were tested for ploidy level resulting in 1,194 4×, 1,062 chimeric 2×/4× and 10 8× red clover plants. In general, a colchicine concentration of 0.2% resulted in 14.9% 4× plants (10 varieties tested), a concentration of 0.4% resulted in 21.7% 4× plants (11 varieties tested) and 0.6% colchicine resulted in 33.9% 4× plants (3 varieties tested). Chimeric plants occurred within a similar range (12.4, 19.8 and 32.8%, respectively). The survival rate of seedlings for 0.4% and 0.6% colchicine was about 30% (figures for 0.2% are not available). Average polyploidization rate depended on variety and ranged between 14.4% (Nemaro) and 34.9% (Lucrum; Figure1).

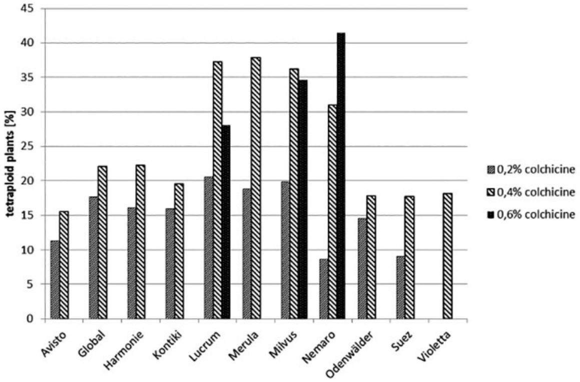

Figure 1. Proportion of tetraploid plants obtained by treatment with different colchicine concentrations (0.2%, 0.4% and 0.6%) in 11 varieties.

Conclusion: The increase of colchicine concentration from 0.2% to 0.4% showed an increase in polyploidization rate, whereas 0.6% showed an increase in only one of three varieties, so 0.4% colchicine and an incubation time of 3 hours can be recommended for diverse material.

Interaction of *Epichloë* infected forage grasses with fungal root endophytes

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Introduction: Fungal endophytes are known to have coevolved many symbiotic associations with grasses (Hodkinson, 2018; Hodkinson *et al.*, 2019). *Epichloë* endophytes are among the best known and produce a range of bioactive alkaloids that can effectively deter insect pests and are hence used in grassland farming systems in Australia, New Zealand, USA and some parts of South America (Johnson and Caradus, 2019). Very little is known about how *Epichloë* endophytes interact with other microorganisms especially other symbiotic endophytes. We therefore assessed the interaction of the above ground colonising *Epichloë* with a consortia of root fungal endophytes including *Cladosporium*, *Metarhizium* and *Penicillium* species. These root endophytes have been shown to improve the agronomic performance of grasses, especially barley (Murphy *et al.*, 2018). We tested for germination, days to flowering, shoot, root and seed yield. Endophyte presence was confirmed using culturing and microscopy. The ultimate aim was to discover whether there is potential to combine *Epichloë* endophyte infected grasses in agricultural forage systems with other known endophyte treatments such as the root endophyte consortium tested here.

Materials and methods: One growth experiment was set up for *Lolium perenne* and another for *Festuca arundinacea*. Experiment (1) For *Lolium*, treatments included germplasm containing four *Epichloë* strains (AR1, AR37, AR5 and wild type) and *Epichloë* free germplasm (GA66), with and without a consortium of fungal root endophytes (TCD04060502A, TCD04060502C, TCD04090103B, TCD04090604A). Experiment (2) For *Festuca*, *Epichloë* free and *Epichloë* infected AR584 germplasm was tested in combination with the same consortia of fungal root endophytes used for Experiment 1. In both experiments, plants were grown in pots with John Innes No.2 compost and eight seeds per pot in a fully randomised factorial design. *Epichloë* is vertically transmitted and present in the germinating seed. The fungal root endophytes were added as a spore solution (250 µl at 10^6 spores/ml = ~250,000 spores per seed).

Results: The experiment is ongoing for yield and flowering time determinations. Early results showed that germination rates of *Lolium* and *Festuca* seeds have been enhanced by the fungal root endophyte treatment. There was an overall significant increase of 21% in germination index (GI) associated with root endophyte treatment (P=0.03). Once biomass is harvested, the influence of the fungal endophytes on *Epichloë* alkaloid production will be assessed.

Conclusion: This is the first-time positive growth promoting interactions have been reported for a combination of above ground and below ground fungal grass symbionts. There is clearly significant potential to apply such combinations in agricultural forage grassland systems.

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Long-term crown rust survey in perennial ryegrass and *Festulolium* trials in Lithuania

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Introduction: Perennial ryegrass (*Lolium perenne* L., LP) is an important species valued for high yield and palatability; however, it is not tolerant of abiotic stress, whereas interspecific Festulolium (×F) hybrids are more tolerant to stress than LP and have similar nutritional value (Humphreys *et al.*, 2014). Crown rust (caused by *Puccinia coronata* f. sp. *lolli*) severely damages forage yield, quality and dry matter digestibility; thus, breeding for crown resistant cultivars is essential.

Materials and methods: Seven cultivars of LP (‘Alduva’, ‘Elena DS’, ‘Raminta’, ‘Sodrė’, ‘ Veja DS’, ‘Verseka’, ‘Žvilgė’) and 4 cultivars of ×F (‘Lina DS’, ‘Punia DS’, ‘Puga’ and ‘Vėtra’) were investigated. Experiments were carried out in field micro-plots using a randomised complete block design with 3 replications in Dotnuva, Lithuania during 2008-2018. One experimental cycle, comprising a sowing year and 2 years of use, was sown every year. The infection was scored as 1 = no rust damage, 5 = 25%, and 9 = more than 75%. The data were analysed using ANOVA with the factors: year of investigation, year of use and cultivar, using the statistical package Plabstat (Utz, 2011).

Results: Under Lithuanian conditions crown rust tends to occur in September; however, unexpectedly early infections were recorded in three years (2012, 2017, 2018) or did not occur at all in two years (2011 and 2015, Figure 1). The year of investigation was the major factor for rust incidence in both species (F-value 2,738; $P<0.01$ for LP, F-value 871; $P<0.01$ for ×F) whereas the influence of cultivar (LP F-value 3.19; $P<0.05$; ×F n.s.) or year of use (LP n.s.; ×F F-value 21.59; $P<0.01$) was much lower. Both species were similar in their rust resistance level.

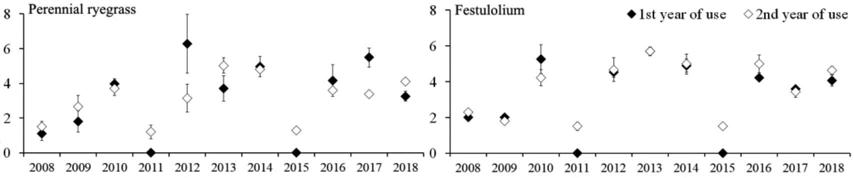

Figure 1. Crown rust infection of LP and ×F during the 1st and 2nd year of use over the period of 2008-2018. Error bars represent standard deviation.

Conclusions: though the onset and severity of crown rust infection was influenced by the year of investigation, the results suggest sufficient resistance of locally bred LP and ×F cultivars.

Acknowledgements: The study was funded by LAMMC long-term research program ‘Genetics and purposeful change of genotypes of agricultural and forest plants’ by Research Council of Lithuania, grant No. S-MIP-17-24.

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Analysis of seed shattering in a perennial ryegrass (*Lolium perenne* L.) backcross family derived from *Lolium temulentum*

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Introduction: Seed yield is critical for the commercial success of a grass variety. Whereas perennial ryegrass (*Lolium perenne* L., *L.p.*) exhibits a strong tendency towards premature seed shattering, darnel ryegrass (*Lolium temulentum* L., *L.t.*) is known to have non-shattering seeds. In our study the shattering behaviour of a *L.p.* backcross family (BC_2: [(*L.p.* × *L.t.*) × *L.p.*] × *L.p.*) was screened to get first insights into the differentiation and inheritance of the trait. With the obtained results, candidate genotypes with high shattering resistance and seed yield should be identified for breeding programmes.

Materials and methods: The shattering behaviour of the *L.p.* family was evaluated in a field trial (2017, 2018). The genotypes (n=64) were space-planted in rows in a nursery at Steinach (Germany) in autumn 2016. The inflorescences were covered with perforated bags (crispac bags, 305 × 450 mm) at the end of flowering and the edges of the bags were tied together with plano clips. Bagged tillers were cut when the awns had become fully ripe and shattering resistance was tested under controlled shattering provocation conditions in the laboratory. Natural (shattered seed in field) and technically provoked seed shattering (determined using a shaking device), and seed yield were determined for each genotype. The seed retention [%] (percentage of firm seeds of the total seed mass) was calculated for each genotype to determine the shattering resistance. Additionally, the seed yield per tiller [g] was calculated. The data were analysed by ANOVA and in cases of significance the Tukey' HSD test was used for pairwise comparisons of means. Pearson's correlation coefficient was calculated for the seed retention of the genotypes between the two years.

Results: A positive correlation between the seed retention estimated for two different years was observed (*r=0.506*, Figure 1), but at seed retention ≥60% the data points scattered in a wider band. In 2017, seed retention varied between 30% (low shattering resistance) and 82% (high shattering resistance) and in 2018 between 20 and 70%. The mean seed retention [%] and seed yield per tiller [g] were significantly lower in 2018 than 2017 (*post-hoc* Tukey HSD, $P<0.001$), indicating a strong annual effect (Figure 1).

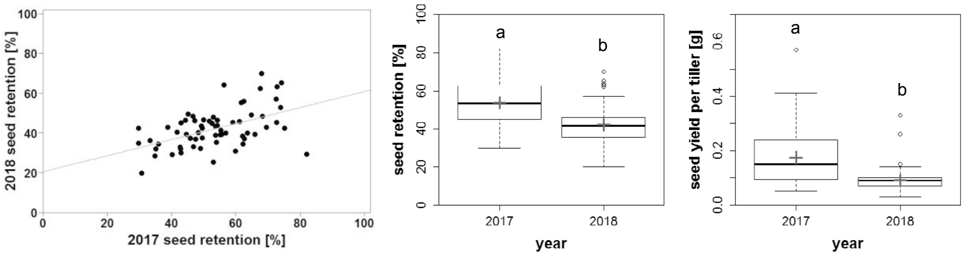

Figure 1. Between-year correlation of seed retention values (left), shattering resistance (middle) and seed yield (right) of a *L. perenne* family (BC_2: [(*L.p.* × *L.t.*) × *L.p.*] × *L.p.*, n=64). Seed retention [%] and seed yield per tiller [g] were investigated for each genotype in 2017 and 2018. Different letters: groups are significantly different (*post-hoc* Tukey HSD, $P<0.001$).

Conclusion: Within the *L.p.* backcross family investigated, genotypes with loose and firm seed set were observed. To select genotypes and to determine if the seed retention was improved by hybridization with *L.t.*, data of the recurrent *L.p.* parent will be generated (planned for 2019).

Physiological strategies to withstand soil water deficit in *Festuca arundinacea*, *Festuca glaucescens* and *Lolium multiflorum/F. arundinacea* introgression forms

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Introduction: Allohexaploid *Festuca arundinacea* (tall fescue) is a forage grass species with a relatively high capacity to avoid and/or to tolerate water deficit in soil. This is mainly due to its ability to develop a deep root system and/or to modify its crown tissue and leaf metabolism. On the other hand, *Festuca glaucescens* (often recognized as tetraploid *F. arundinacea* var. *glaucescens*) exhibits mainly a survival strategy by becoming quiescent under severe water deficit, which enables it to survive and to resume growth following irrigation. The introgression forms of *Lolium multiflorum* (Italian ryegrass) and *F. arundinacea* can represent a wide range of diversity with respect to the components of drought avoidance/tolerance mechanisms. These three groups of plants could be good models to go deeper in our understanding of different strategies to tolerate soil water deficit in forage grasses. Thus, the main objective of the presented research was to identify the key components of leaf metabolism associated with tolerance to water deficit and/or with the capacity to regenerate after stress cessation for the selected grasses.

Materials and methods: Within each group of plants, two genotypes distinct in their potential of tolerance/recovery, were selected. These genotypes, each one in three biological replicates, were transferred to five pots (7.50 dm^3), containing a sand : peat (1:3) mixture. A short-term drought treatment was applied, and measurements were performed at five time-points: before stress treatment (control), after 3, 6 and 11 days of watering cessation, and 10 days after subsequent re-watering. The experiment was performed in a growth chamber at a temperature of 22 °C (16 h day/8 h night, light of 400 µmol(quanta) m^{-2} s^{-1}. The effects of stress conditions on the accumulation of reactive oxygen species (ROS), integrity of biological membranes, functioning of photosynthetic apparatus (accumulation levels and activities of Calvin cycle enzymes) and functioning of enzymatic antioxidant system, were examined. A two-way analysis of variance (ANOVA), with genotype and time-point as classification factors, has been performed. Differences in parameters between the plants during experiment duration were evaluated using Tukey HSD test at $P=0.05$.

Results: A significant increase of the accumulation level of superoxide anion radical was observed under water deficit in both genotypes of *F. glaucescens*, whereas in *F. arundinacea* genotypes, as well as in the introgression forms its accumulation decreased. On the 11th day of stress treatment, the accumulation level of hydrogen peroxide (H_2O_2) increased in both introgression forms and *F. glaucescens* genotypes. Drought-induced lipid peroxidation was noted in both genotypes of *F. arundinacea*, as well as in the less stress-tolerant genotype of *F. glaucescens* on the 11th day of stress treatment. Interestingly, in the two analysed introgression forms, the conditions of water deficit lowered the level of lipid peroxidation.

Conclusion: We revealed that the balance between the photosynthetic and antioxidant activities of plant cells, together with the maintenance of high integrity levels of biological membranes could be crucial for grass metabolism to tolerate water deficit and to recover after stress cessation. However, furthermore detailed conclusions could derive from the ongoing research.

Acknowledgements: The research was performed within the project funded by Poland National Science Centre (project no. 2016/23/B/NZ9/00820).

Disentangling genetic and non-genetic yield trends of Dutch forage crops

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Introduction: High yields of grass and forage maize are important for enhancing profitability and circularity of dairy farming. The yield potential depends on the local climate, soil properties and crop genotype. Thus, genetic improvement is an important route for raising the yield potential of those forage crops. Actual on-farm yields and their increase are determined by the interaction between genetics, environment and management, while increases in potential yields are governed largely by genetics. The objective of our study was to disentangle genetic and non-genetic trends in grass and forage maize yields.

Materials and methods: We analysed yields of forage crops from VCU (value of cultivation and use) trials and from commercial dairy farms. The trials included 203 listed varieties of *Lolium perenne* L. (1975-2015), and 187 listed varieties of *Zea mays* L. (1991-2016). Genetic and non-genetic trends were estimated with the mixed model facilities of Genstat, following Laidig *et al.* (2014). On-farm yields (1990-2016) were collected from national statistics, and were analysed for year trends with linear regression.

Results, discussion and conclusions: In VCU trials, the average annual yield of perennial ryegrass was 12.8 t DM ha^{-1} (Figure 1). The annual genetic yield progress was +52 kg DM ha^{-1}, while there was a negative non-genetic trend of -14 kg DM ha^{-1}. For maize, the average yields in the trials were 19.0 t DM ha^{-1} with an annual genetic trend of +176 kg DM ha^{-1} and a non-genetic trend of +71 kg DM ha^{-1}. The non-genetic trends are a combined effect of changing climate and management. The average annual on-farm grass yields were 11.0 t DM ha^{-1}, without a trend. The average on-farm maize yields were 14.3 t DM ha^{-1} with an annual trend of +195 kg DM ha^{-1}. On-farm yields were 13% (grass) and 25% (maize) lower than trial yields. The lower annual trends and yields are partly caused by the time lag between testing and practical use of new varieties, but are also due to differences in soil quality and management.

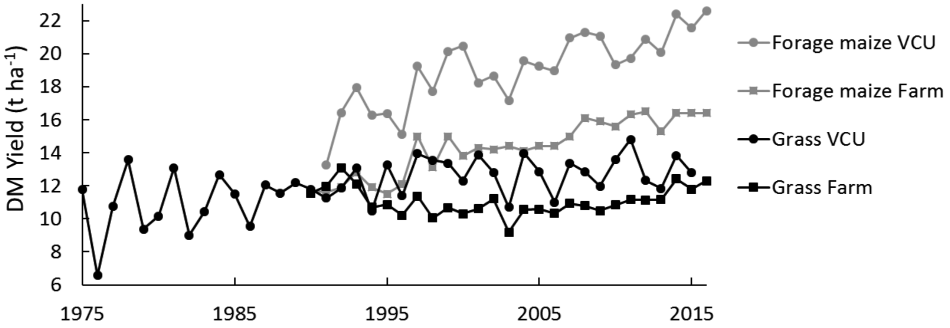

Figure 1. Annual DM yields (t ha^{-1}) of grass and forage maize in VCU trials and on farms.

Laidig F., Piepho H.P., Drobek T. and Meyer U. (2014) Genetic and non-genetic long-term trends of 12 different crops in German official variety performance trials and on-farm yield trends. *Theoretical and Applied Genetics* 127, 2599-2617.

Variations in polyphenol oxidase in red clover (*Trifolium pratense*) leaves and roots

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Introduction: Red clover contains relatively high concentrations of polyphenol oxidase (PPO). It has been suggested that these PPOs protect lipids and proteins from degradation through the generation of highly reactive quinones that covalently bind with nucleophilic sites, leading to cross-linking of protein polymers. This process could have a positive effect on the silage quality of red clover when fed to ruminants. If roots of red clover also contain these PPOs this could potentially affect the degradation of roots and influence the soil microbial community, and subsequent soil processes. The objective of this research was to determine if roots of red clover, like the leaves, also contain PPOs, and if this varies between cultivars.

Material and methods: The PPO concentration of roots and leaves of eight red clover cultivars (four Mattenklee and four Ackerklee types) was measured in November 2012 in an existing experiment (for more details see Hoekstra *et al.*, 2017). Swiss landraces of red clover are also called Mattenklee as opposed to the traditional Ackerklee or European field clover cultivars. PPO activity was analysed according to the protocol of Lee *et al.*, (2009).

Results and conclusions: The red clover variety Lemmon exhibited significantly higher PPO activity than the other varieties (Figure 1). There was also a significant effect of clover type, with Ackerklee varieties exhibiting significantly higher PPO activity than Mattenklee varieties. Roots of red clover also contain PPOs but there was no significant difference in PPO activity in the roots of the varieties examined. This could be due to a flaw in the method for extraction of PPO from roots, as this protocol has not been extensively researched.

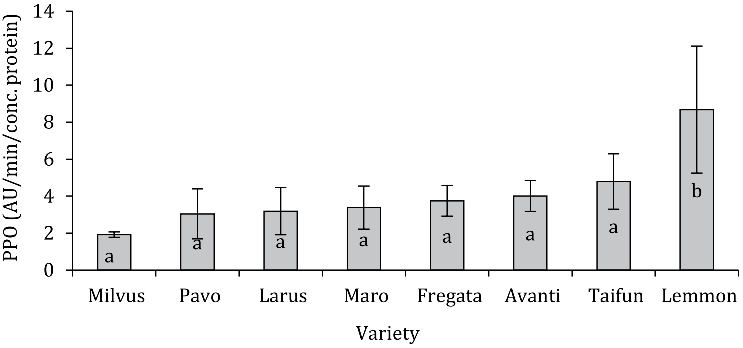

Figure 1. Active PPO in red clover leaves (P=0.042). Mattenklee types (cv. Fregata, Larus, Milvus and Pavo), Ackerklee types (cv. Avanti, Lemmon, Maro and Taifun).

Hoekstra N.J., De Deyn G.B., Xu Y., Prinsen R. and Van Eekeren N. (2018) Red clover varieties of Mattenklee type have higher production, protein yield and persistence than Ackerklee types in grass–clover mixtures. *Grass and Forage Science* 73(2), 297-308.

Lee M.R.F., Tweed J.K.S., Minchin F.R. and Winters A.L. (2009) Red clover polyphenol oxidase: Activation, activity and efficacy under grazing. *Animal Feed Science and Technology* 149(3-4), 250-264.

Traits underlying seed yield in red clover uncovered

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Introduction: Red clover (*Trifolium pratense* L.) is a valuable forage crop, but its seed yield is often unsatisfactory, especially in tetraploid varieties. Aiming to unravel the factors underlying seed yield in red clover, we investigated the relation between seed yield per plant, plant architecture, flower morphology, pollinator preference and fertility.

Materials and methods: In this study, 600 genotypes from 15 diploid and 15 tetraploid cultivars, representative of European breeding efforts, were used. Multiple linear regression was used to reveal relations between seed number per plant, number of ripe and unripe flower heads per plant, seed number per ripe flower head, flower number per head, corolla tube length and diameter, and flower anthocyanin content (flower colour) (Vleugels *et al.,* 2016). The relation between seed yield vs nectar production and composition, determined through HPLC, was studied among a subset of 54 genotypes from our first study with known differences in seed yield. In addition, pollinator preference was studied on 6 diploid and 6 tetraploid genotypes with contrasting seed yields, by evaluating pairs of genotypes for attractiveness to *Bombus pascuorum* bumblebees, the main pollinator of red clover in our region (Vanommeslaeghe *et al.,* 2018). Ovule development, the frequency of aberrations in male meiosis, and pollen quality were investigated among 19 diploid and 17 tetraploid genotypes with extreme seed yields, or subsets thereof (Vleugels *et al.,* 2019).

Results: Seed number per plant was well predicted by the number of flower heads per plant and the seed number per flower head. Tetraploid genotypes produced fewer flower heads (159.2 vs 209.7) and fewer seeds per flower head (8.9 vs 43.4) than diploids. Seed yield was not correlated to floral characteristics such as flower number per head, flower colour, corolla tube dimensions, nectar volume or nectar composition. Furthermore, no preference for diploids or tetraploids could be demonstrated in choice experiments with *B. pascuorum*. High degrees of ovule abortion occurred in genotypes from either ploidy level, but no clear link with seed yield appeared. However, tetraploid genotypes displayed more meiotic aberrations than diploids (17.3 and 7.0%, respectively), and lower pollen germination (39 and 63%, respectively).

Conclusion: Seed yield in tetraploid red clover is lower, predominantly because fewer seeds are produced per flower head, which is not explained by differences in flower morphology or pollinator attractiveness. Instead, although further research is needed to fully unravel the mechanisms underlying the lower fertility in tetraploids, meiotic aberrations, ovule abortion and pollen quality are likely to be part of the complex process of seed yield in red clover.

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Comparing drought tolerance between isogenic di- and tetraploid perennial ryegrass (*Lolium perenne* L.) genotypes

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Introduction: Limited availability of soil water is one of the main constraints for growing perennial ryegrass (*Lolium perenne* L.) as a fodder crop or its use in grassland mixtures for temporary drought prone areas. In an earlier study investigating the drought tolerance of 56 perennial ryegrass accessions (varieties and gene bank accessions), it was shown that on average, tetraploid varieties were more drought tolerant than diploid material (Westermeier *et al.*, 2015). From this study, it remained unclear whether this is an effect of the ploidy level or of the breeders' selection. Our experiment seeks to compare the drought tolerance of isogenic di- and tetraploid genotypes of perennial ryegrass.

Materials and methods: Eleven diploid perennial ryegrass clones were selected from diverse material with divergent drought response. Single tillers were treated with colchicine (0.2% + 2 %DMSO, Tween 80, pH 5.5) for 5 h and afterwards rinsed with water for 30 min. After formation of new tillers, selection of 4× tillers was done by flow cytometry. 2× and 4× plants complemented by a clone from cultivar Aubisque (4×), two *Festulolium* and one meadow fescue clones were propagated clonally and phenotyped in 15×15 cm pots for drought performance and recovery in the greenhouse with well-watered vs drought stress treatment in a randomized complete block design (three replications). Volumetric soil water content was continuously reduced in a time period of 31 days to 0% controlled by TDR measurements. Single plant biomass yield was recorded for the stress and recovery period.

Results: For dry matter yield under increasing drought stress no significant difference between diploid and tetraploid plants could be found. During the recovery period dry matter yield of tetraploid plants was significantly higher and exceeded the yield of diploid plants 4-fold (mean of 2× plants 0.20 g; mean of 4× plants 0.84 g). Figure 1 shows dry matter yield as the percentage of well-watered control during increasing drought stress and recovery.

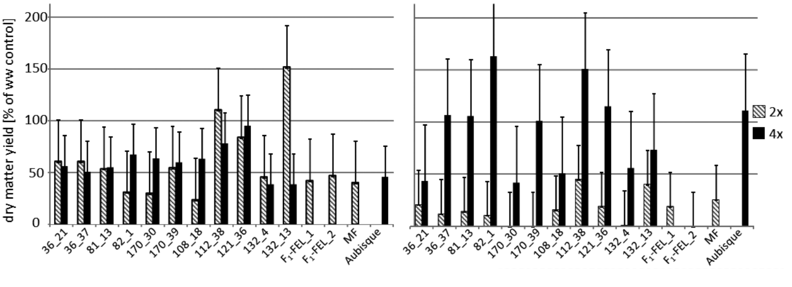

Figure 1. Relative dry matter yield of isogenic 2× and 4× clones in % of well-watered (ww) control. (Left) Increasing drought stress; (Right) recovery period. Error bars represent LSD 5%.

Conclusion: There is a clear effect of the ploidy level on recovery after drought stress. At comparable drought stress level 4× plants showed senescence one week later than 2× plants, one explanation for better recovery. Other possible reasons are stomata architecture of 4× plants and thus enhanced water use efficiency or accumulation of storage carbohydrates.

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Theme 6.
Plant-soil-microbe interactions in multi-species grasslands

Advances in understanding plant-soil-microbe interactions in multi-species grasslands

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Abstract

Grasslands are very important ecosystems across the globe because of the multiple ecosystem services they provide. Irrespective of its use as ley or permanent grassland, there is growing awareness that the functioning of grassland systems is highly dependent on the soil microbial communities. Here we reflect on the role of grassland diversity in delivering ecosystem services and its mechanistic underpinning via interactions with soil microbes. We do so by presenting a conceptual framework, inspired by current literature. From biodiversity studies in perennial grasslands we know that plant species richness promotes plant productivity. The underlying mechanisms are still being investigated, but it is clear that root-associated microbes that enable resource use complementarity between plant species, as well as suppression of soil-borne pathogens are at play. To understand and utilise these interactions to enhance productivity and nutrient retention, and to promote diversity, an integrative approach is needed. A key challenge is the understanding of how plant community traits steer the composition and functioning of the soil microbes, and how in turn the plant community responds to this. Furthermore, we need to recognise that these plant-soil feedbacks operate in the context of grassland management, resource availability, and land-use history. Recent work shows that also in more intensively used grassland species mixtures perform better, persist for longer and emit less N_2O than monocultures, indicating altered interactions with soil microbes in mixtures as compared to monocultures. However, the key importance to achieve these multiple benefits is which species (and thereby which traits) are mixed rather than increasing species richness *per se*.

Keywords: plant diversity, plant traits, plant-soil feedback, ecosystem functioning, multi-functionality, soil microbes

Introduction

In this paper we provide an overview of the relationships between plants and soil-microbes in multi-species grasslands. To put the current literature on this topic into context we present a conceptual framework of how plant-soil microbe interactions in grassland are governed, and how they link to ecosystem services (Figure 1). The pool of microbes plants can associate with depends on the soil microbial pool, which is affected by soil chemical and physical properties and soil management. Additional microbes can be introduced with seeding, such as plant endophytes or microbes included in seed coatings. The soil and rhizosphere microbial communities comprise plant growth suppressing (pathogens) and plant growth promoting (mutualists) organisms; these range from being specialists to more generalists. Saprotrophic organisms mineralise nutrients from organic matter and respond to organic matter input quantity and quality. From left to right (Figure 1) disease pressure is expected to decrease and mutualists and saprotrophs to increase. This shift in the soil microbial community composition increases resource-use efficiency and primary productivity. Increases in nutrient retention can be achieved by fine-tuning soil nutrient availability and uptake, so that losses via leaching or greenhouse gas emissions can be minimised. The impact of the soil biota on plant species co-existence depends on the promotion of subordinate plant species by soil microbes and on the grassland management that keeps the dominant plant species in check (cutting or grazing and fertilisation regime). Feedbacks to the soil microbial community occur via plant quantity and quality, indicated by the link with plant productivity, nutrient retention and plant diversity.

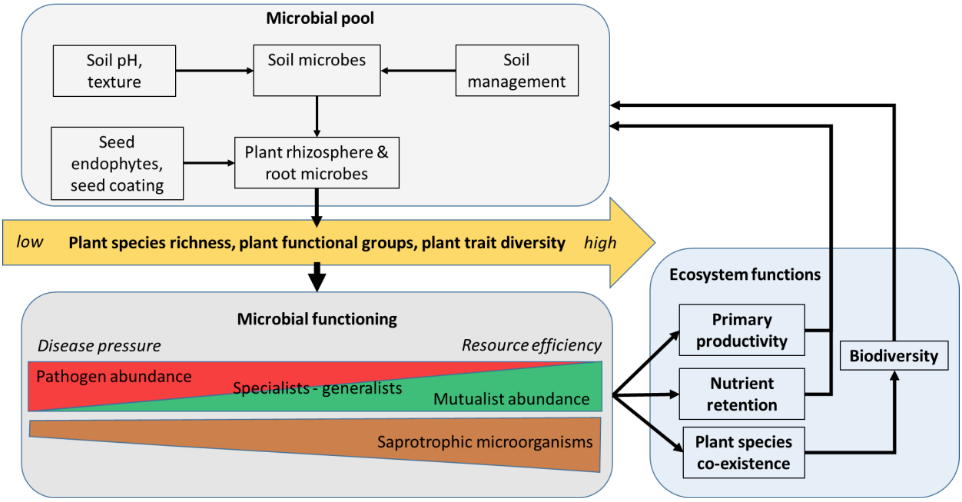

Figure 1. Conceptual scheme of plant diversity interactions with soil microbes and their feedback effects to ecosystem functions (plant productivity, nutrient retention, species co-existence and support of biodiversity). The arrow indicates increase in plant diversity from left to right.

Grassland diversity and ecosystem services

Grassland ecosystems are important ecosystems across the globe, not only for their economic value as feed for ruminants, but also for their role in supporting above- and belowground biodiversity, and for their capacity to serve as a greenhouse gas sink (De Deyn and Van der Putten, 2005; De Deyn *et al.*, 2008). The ability of grasslands to deliver these multiple ecosystem services is highly dependent on the grassland management intensity. Important management factors in sown grassland are the composition of the plant species mixture, the level of disturbance and the fertiliser regime (form, dosage and frequency). The impact of these factors is illustrated in a large overview study of grassland systems across five continents. That study showed a clear positive relation between plant diversity and plant productivity, and a negative impact of disturbance, such as overgrazing, on plant diversity (Grace *et al.*, 2016; Gross, 2016). On the other hand, this work also showed a negative relation between plant productivity on plant diversity, explained by highly productive species outcompeting less competitive species. These results indicate that maintaining productivity and plant diversity at the same time is not a trivial task. The authors also noted that soil fertility and soil suitability are important, but did not further integrate soil ecology into their model. Here we link plant and soil ecology, and put forward that plant interactions with soil microbes are of key importance to maintain productivity and diversity (Figure 1).

Soil ecology is essential to understand and steer the multi-functionality of grassland systems (Orgiazzi *et al.*, 2016). From grassland biodiversity studies we know that complementarity in resource use between plant species, for example by accessing nutrients from different sources, is important. However, these mechanisms cannot fully explain why plant species mixtures are more productive than plant monocultures (Mariotte *et al.*, 2018; Barry *et al.*, 2019). In the last decade, the role of soil borne pathogens and root mutualists in explaining increased productivity with plant diversity has gained attention (Schnitzer *et al.*, 2011; Kardol *et al.*, 2013; Van der Putten *et al.*, 2013). Recent work proposes how complementarity for resource use and the role of interactions with other organisms can be integrated to understand mechanistically the relation between plant diversity and productivity (Barry *et al.*, 2019).

Apart from plant productivity and maintenance of diversity, the retention of nutrients and sequestration of greenhouse gasses in grassland soils are also important ecosystem functions. The more intensively a grassland is used, the more important the counteraction of N_2O emissions next to CO_2 emissions becomes. The emissions of both these greenhouse gases from soil are mostly the result of microbial activity of interacting soil microbes. CO_2 emissions can be offset by enhanced plant uptake through photosynthesis and C-storage in organo-mineral complexes, which can be enhanced in plant species mixtures and associations between plants and their root symbionts (De Deyn *et al.,* 2008, 2011a, 2012). The production and consumption of N_2O by soil microbes is influenced by a number of interacting factors which make these emissions dynamic and hard to control. Nevertheless, there is also scope for using plant species mixtures for the control of these emissions in grassland systems (Abalos *et al.,* 2014, 2018). For a more extensive overview on the topic of greenhouse gas emissions from grassland (including the role of grass-legume mixtures) see the contribution of Buchmann *et al.* (2019) 'Multifunctionality of sown grasslands: ecosystem services and resilience to global change' in these Proceedings.

Plant-soil biota interactions in multi-species grassland

To understand the interactions between plants and soil microbes, we should first consider from where the microbes that associate with plant roots originate (Figure 1). From molecular studies in recent years it has become clear that root-associated and root-endophytic microbes originate primarily from the soil in which the plants are grown (Bulgarelli *et al.,* 2012). It is therefore not surprising that soil abiotic properties, such as pH and soil texture, that affect soil microbes also have a major impact on the microbial community composition in the plant rhizosphere (Paey *et al.,* 2016; Dassen *et al.,* 2017; Fierer 2017; Barel *et al.,* 2018). However, within a given soil different plant species and plant genotypes can harbour distinct microbial communities in their rhizosphere, due to selective promotion and suppression of subsets of the pool of microbes present in the soil (Bulgarelli *et al.,* 2012; Leff *et al.,* 2018; Barel *et al.,* 2018). This plant-induced differentiation in the community composition of bacteria and fungi in the root zone can be explained by differences in plant traits, for example by the different chemical compounds that are released in the rhizosphere (Bulgarelli *et al.,* 2012; Pieterse *et al.,* 2016). The strength of this effect can vary, and differences in microbial community composition in the rhizosphere are generally expected to be larger between plant functional groups (e.g. legumes vs non-legumes or grasses vs forbs) than between plant species (Dassen *et al.,* 2017; Mommer *et al.,* 2018). Apart from the more specialised root-associated microorganisms, such as pathogens and mutualists, decomposer microorganisms also respond to plant community composition, and to soil management practices via differences in quantity and quality of organic matter inputs to the soil (De Deyn *et al.,* 2011b,c; Lori *et al.,* 2017; Dassen *et al.,* 2017).

It is well-known that root symbiotic N_2-fixing bacteria of legume species play a central role in the complementarity between legume and non-legume species, whereby the bacteria bring extra nitrogen into the system by acquiring N from the air (Mariotte *et al.,* 2018; Barry *et al.,* 2019). As a result, legumes not only complement non-legumes, but can even facilitate them by leaving more mineral nitrogen in the soil. The benefits for non-legumes when grown with legumes have been recognized for a long time. More recently it was shown that legumes can also benefit from growing with non-legumes, a beneficial interaction that appears to be mediated by mycorrhizal fungi (De Deyn *et al.,* 2012). Another mechanism by which legumes could benefit from growing with non-legume species is slower population build-up of legume-specific pathogens in the soil. With respect to rhizosphere interactions that can promote nutrient-use efficiency the activity of nitrification inhibitors released by plant roots, and which block the microbial transformation from ammonium to nitrate, are noteworthy. These compounds have been reported for tropical *Brachiaria* pastures, and their potential occurrence and application in temperate grasslands is being explored (Subbarao *et al.,* 2009; Coskun *et al.,* 2017).

Plant-associated microbes may not only originate from the soil, but they can also be introduced via the seeds. For example, endophytic fungi in grass species tend to be transmitted vertically from mother plant to progeny, and these enhance plant defences against pathogens and herbivores (Clay, 1990). Similarly, specialised plant symbiotic microbes that are ascent in a given soil and not being transmitted vertically could be introduced with the plants via seed coating. One could think of specific species of legumes inoculated with effective specific strains of *Rhizobium*, in cases where the local inoculum density is too low (Yates *et al.,* 2005; Siefert *et al.,* 2018). This practice of seed coating with beneficial plant associated microbes is already common in several crop species. However, the design of an economic formulation of seed coating in which the microbial propagules remain viable for an extended period of time remains a bottleneck (O'Callaghan, 2016).

Plants not only affect the composition and activity of soil microbes, plants also in turn respond to the soil microbial community. This process is called plant-soil feedback and occurs both in natural and agricultural systems, and affects multiple ecosystem functions such as primary productivity and support of biodiversity (Mariotte *et al.,* 2018; Figure 1). The build-up of populations of soil-borne plant pathogens reduces plant productivity and plant persistence, and promotes or suppresses species co-occurrence depending on whether pathogens affect the dominant or subordinate plant species (Figure 1). It is generally thought that specialist plant pathogens build-up fast in plant monocultures, but much less so in plant species mixtures. The reduced abundance of diseases is attributed to host-dilution effects, as well as to disease suppression by the soil microbial community through competitive and antagonistic processes (Schnitzer *et al.,* 2016; Schlatter *et al.,* 2017; Mommer *et al.,* 2018). Apart from a reduction in pathogens, also an increased benefit of plant growth-promoting soil microbes can increase plant productivity and plant species persistence in species mixtures (Cortois *et al.,* 2016). In grasslands, generalist root-feeding nematodes may not decrease but increase with plant diversity when the plant community provides them with more resources. There is evidence for such an increase of root feeders. However, in plant species mixtures root biomass increased more strongly than plant-feeder abundance, suggesting reduced root-feeder impacts on individual plants (Cortois *et al.,* 2017). Similar to soil-borne pathogenic fungi and bacteria, root-feeding nematodes can also be controlled in soil via a suite of biological mechanisms, including the activity of nematophagous bacteria and fungi which live amongst the saprophagous microbes (Pereira da Silva *et al.,* 2018).

To date it has been hard to predict the outcome of plant-soil feedbacks in mixed grassland, but recent studies show that plant trait-based approaches are promising for enabling such predictions (Cortois *et al.,* 216; Semchenko *et al.,* 2018). These studies show that plants with acquisitive traits, such as high nitrogen concentration and thin roots and poor colonisation by mycorrhizal fungi, generate more negative plant-soil feedbacks than plant species with opposing traits (Cortois *et al.,* 216; Semchenko *et al.,* 2018). These studies comprised extensively used experimental grasslands, using plant species of moderately fertile soils. Plant species selected for fast growth on soils with high nutrient availability may be more sensitive to root pathogens and root feeders, and engage less in associations with beneficial root symbionts (Milla *et al.,* 2015; Martín-Robles *et al.,* 2018). It has to be noted that the sensitivity of plants to plant pathogens is also dependent on soil fertility, as stressed plants have less ability to employ adequate defence, especially when plants have been selected to grow under fertile soil conditions. Moreover, plant pathogens and mutualists in the soil do not operate in isolation, so their interaction with the saprotrophic micro-organisms should also be considered (Figure 1). It can be expected that nutrient retention and plant inputs to soil are larger in plant species mixtures than in monocultures, and thereby the role of root mutualists and saprotrophic soil microbes in nutrient cycling and disease suppression increases (Mariotte *et al.,* 2018). In addition to nutrient acquisition under normal conditions, root mutualists can also aid in counteracting leaching losses from soil during heavy rain events, thereby further contributing to resource-use efficiency of grasslands (Martinez-Garcia *et al.,* 2017).

Outlook

The increased awareness of the role of soil microbes in supporting the multi-functionality of grasslands is promising for further advancements in developing grassland ecosystems that optimise the different functions. The realisation that plant-soil feedbacks are mediated by plant traits offers practical handles, yet the underlying mechanisms warrant further study. The cross pollination between ecology and agronomy will facilitate the discovery of general principles from genotypes to ecosystems (Mariotte *et al.,* 2018). These developments will be further aided by new tools which enable the study of plant-soil feedbacks from the small to the large temporal and spatial scales (Van der Meij *et al.,* 2017; Mariotte *et al.,* 2018). For practical applications it will also be of key importance to develop tangible and affordable methods together with the end-users to evaluate the (belowground) functioning of their grasslands (Bünemann *et al.,* 2018).

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Total number of microorganisms in soils of natural grasslands in the Serbian Pešter plateau

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Abstract

Microorganisms as a biological component are good indicators of soil quality because they respond quickly to changes in the soil ecosystem. Microbiological abundance can therefore be used for the determination of soil health. Microbiological and basic agrochemical soil properties (0-30 cm) in natural grasslands were examined at ten locations on the Pešter plateau (southwestern Serbia) at altitudes between 1,015 and 1,283 m. Soils in the examined locations were acidic (pH ($_{KCl}$) of 3.87-4.31). The samples of soil from natural grasslands had different contents of mineral matter and humus and there were statistically significant differences among samples in presence of total microflora. The total number of microorganisms in soil samples varied from 6.810 to 7.567 (log of number) per one gram of absolutely dry soil.

Keywords: microorganisms, soil, natural grasslands

Introduction

The total number of microorganisms, where bacteria are the most abundant, can be used as a general indicator of biological abundance in the soil, and this is extremely important for creation and maintenance of soil fertility. The abundance and diversity of microorganisms in the soil is influenced by the physical and chemical properties of the soil, such as pH, quantity and type of organic matter, air-water regime and thermal regime, structure and mechanical composition of the soil, etc. Soil microbial communities are responsible for the cycling of carbon (C) and nutrients in grassland ecosystems including the maintenance of soil porosity, fertility, the decomposition of native organic matter, and mineralization of plant nutrient elements (van Eekeren *et al.,* 2007; Castro *et al.*, 2010). Any type of soil has its own characteristic microbiocenosis and the type of soil use may have positive or negative effects on microbiological activities, and this is directly reflected in soil fertility (Tintor *et al.,* 2009).

Rhizospheric microorganisms form a close community with the root and are directly influenced by root secretions. They are the source of carbon and energy for rhizospheric microorganisms, and their chemical composition and amount depend on the plant species. The aim of this investigation was to examine the total number of microbes in the soil of natural grasslands on the Pešter plateau in Serbia.

Materials and methods

In September 2018 soil samples from ten natural grassland localities were taken aseptically from a depth of 0-25 cm for microbiological analyses. All sites are located in the territory on the Pešter plateau (southwestern Serbia) at altitudes between 1,015 and 1,283 m. Soil sampling was carried out at three sites from different parts of each locality, then mixed and presented as one sample. The chemical properties of the soil were determined by standard methods in the chemical laboratory of the Institute for Forage Crops Kruševac. Biological activity of the soil (biogenicity) of grasslands was monitored on the basis of presence of the total number of microorganisms. In the laboratory, each of the samples was analysed in three repetitions. Total number of microorganisms was established with standard microbiological methods of introducing a certain specific quantity of soil suspensions agenized (Agar, Torlak, Belgrade) soil extract by using decimal dilutions (10^{-1}-10^{-6}), the method of agar panels (Pochon and Tardieux, 1962). The incubation lasted for five days on 28 °C. The number of grown colonies was calculated per

1g of absolutely dry soil. The results were processed by means of Statistics 8.0 computer program, using Fisher's LSD test.

Results and discussion

Based on the pH value, soils were acidic, between pH 3.97 and 4.31. There were differences among the analysed soil samples in terms of the content of humus, nitrogen, potassium and phosphorus (Table 1).

In our study the total microflora in soil samples varied from 6.810 to 7.567 (log of number) per one gram of absolutely dry soil (Table 1). Milošević *et al.* (2009) recorded high number of microbes in soil of grasslands on Pešter: 8.10-8.61 (log. number of microorganisms). According the results of Fisher test, statistically significant differences among soil samples were shown in terms of presence of total number of microorganisms. Results of this study showed that the presence of microorganisms in the tested soil samples is influenced by numerous factors and that each soil provides different conditions for the life of the microbe. The highest number of microorganisms was recorded in soil from location Boroštica (1,160 m) 7.567 (log of number). This location is provided with sufficient amounts of nitrogen and organic matter, pH ($_{KCl}$) 4.23, while in the soil (location Krstac, 1,015 m) with similar chemical characteristics determined the smallest number microorganisms (6.810). According to Lauber *et al.* (2009) pH of soil is considered to be one of the most important factors which affect abundance of microorganisms: this parameter may function as an integrating variable that provides an integrated index of soil conditions. These authors analysed soils collected from 88 sites across North and South America that represented a wide range of ecosystems. Their abundances were independent of the geographical location, but dependent on pH. The number of microorganisms is determined by the nutrients in the soil, and the indigenous microbial communities of each soil are unique for the particular soil as they have been shaped and evolved over time in accordance with the dynamics of the local habitat (Nazir *et al.,* 2013). Microorganisms do not exist in isolation; they interact and these interactions influence soil fertility as much or more than the organism's individual activities.

Table 1. The chemical composition and the total microflora (log of number) of studied soils.

Location	Altitude of location (m)	pH		Total nitrogen %	P_2O_5 mg/100 g	K_2O mg/100 g	Humus %	Total number of microorganisms[1] (/g soil)
		H_2O	KCl					
Krstac	1,015	5.30	4.14	0.336	16.00	15.34	5.37	6.810^{j}
Debelja	1,064	4.63	4.06	0.310	10.00	13.73	4.00	7.380^{c}
Vapa	1,080	4.99	4.15	0.254	11.20	33.24	3.34	7.204^{e}
Komarani	1,114	5.20	4.31	0.307	13.10	34.03	3.85	7.079^{g}
Cetanoviće	1,140	4.78	3.91	0.298	17.00	24.86	5.58	7.041^{h}
Boroštica	1,160	4.95	4.23	0.420	9.60	33.65	5.57	7.567^{a}
Leskova	1,184	5.42	4.27	0.332	9.00	22.44	3.22	7.415^{b}
Bukovik	1,190	5.20	4.16	0.387	12.20	16.30	4.09	7.146^{f}
Fijulj	1,200	4.86	3.87	0.389	18.40	20.42	5.00	6.903^{i}
Devreč	1,231	4.73	3.99	0.298	9.20	18.86	2.85	7.369^{d}

[1] Mean values with the same superscript(s) are not significantly different according to Fisher's LSD test (P<0.05). Values are log of number.

Conclusion

Physical and chemical characteristics of the soil, especially pH and organic matter content, are the most important properties that affect the number of microorganisms. Tested soil samples from locations at different altitudes have different chemical properties and differ in total number of microbes. These are preliminary studies, so it is necessary to continue research in this direction and to obtain complete information about presence of certain physiological and systematic groups of microorganisms in soil of grasslands of this area.

Acknowledgements

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Evaluation of N_2O emissions of multiple process-based models in intensively managed grassland

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Abstract

Process-based models are crucial for assessing greenhouse gas emissions of agricultural systems, such as grassland, but predicted N_2O emissions are often lacking reliability. We compared the performance of three frequently applied models (DayCent, PaSim and APSIM) using high-resolution N_2O flux measurements. We identified strengths and weaknesses in the prediction of N_2O fluxes for all models. Our findings therefore suggest the use of a multi-model ensemble approach to overcome shortcomings of individual models.

Keywords: model validation, nitrous oxide fluxes, process-based modelling, biogeochemical modelling, eddy covariance

Introduction

Grassland management can greatly affect the microbial production of nitrous oxide in soils. Process-based models are useful tools to assess the impact of changes in management practices and climate on yields and greenhouse gas (GHG) emissions of grasslands (Cowan *et al.*, 2015). Moreover, these models could be used for assessing national GHG inventories. However, accurate simulations of GHG exchange, in particular N_2O fluxes, are still a challenge for all existing models. Accurate predictions are impeded by our imperfect understanding of soil-plant-microbe interactions as well as difficulties in precisely measuring N_2O fluxes. These limitations result in considerable uncertainties in model predictions. In order to improve model performance, thorough evaluations against *in-situ* measurements are needed. Newly available high-resolution N_2O flux measurements promise to be especially helpful in this regard (Zimmermann *et al.*, 2018).

Materials and methods

Recently acquired eddy covariance measurements of N_2O fluxes at the ecosystem scale (several 100 m^2) provide an opportunity to test and evaluate state-of-the-art biogeochemical process models. We conducted a multi-model validation exercise with individual versions of three commonly used models (DayCent, PaSim and APSIM) comparing four years of modeled N_2O fluxes with *in-situ* observations from an intensively managed Swiss grassland site (Fuchs *et al.*, 2018). In detail, we addressed the inter- and intra-annual variability in N_2O fluxes and key driver variables of N_2O exchange.

Results and discussion

We found that DayCent predicted annual N_2O emissions more accurately than the IPCC (Tier 1) estimate, while APSIM and PaSim did not (Figure 1). Models performing best (minimized root mean square error RMSE) in the prediction of annual N_2O emission (DayCent in two variants) were outperformed by other models that performed best on the prediction of daily N_2O emissions (APSIM in two variants). The ensemble average achieved considerably better performance on the daily time-scale than individual models. The principal advantages of multi-model ensembles have already been well acknowledged for other applications (Hagedorn *et al.*, 2005).

Daily predictions were a challenge for all individual models, indicating that processes might not be represented adequately, and a better representation of grass and legume species and their effects on N_2O fluxes is needed.

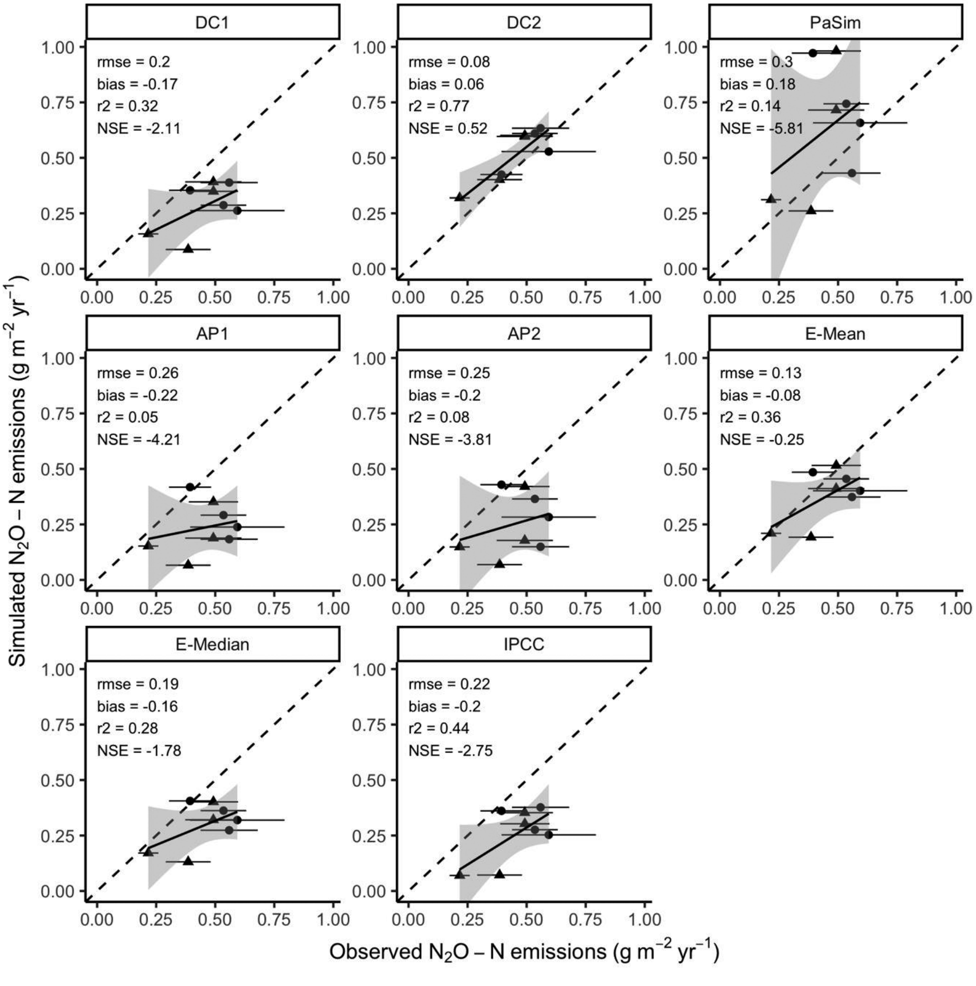

Figure 1. Annual values of observed (horizontal axis) versus simulated N_2O emissions (vertical axis) for models DayCent (DC1, DC2), PaSim, APSIM (AP1, AP2), the ensemble median (E-Median), the ensemble mean (E-Mean), and for the IPCC estimate in parcel A (circles) and parcel B (triangles). The dashed lines indicate the 1:1-lines, and the solid lines display the linear regression line between observed and simulated N_2O emissions.

Conclusion

According to our results, the use of multi-model ensembles leads to improved predictions of N_2O emissions. Being based on recently available high-resolution N_2O flux measurements a rigorous validation could be achieved. This approach could therefore also be suitable for upscaling of N_2O emissions for national inventories.

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The impact of mountain hay meadow management on litter decomposition and root colonization by arbuscular mycorrhizal fungi

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Abstract

Soil life and particularly arbuscular mycorrhizal fungi (AMF) play a fundamental role in ecosystem functioning and are crucial drivers of vegetation dynamics and efficient nutrient use. This study investigated the effect of liquid manure application and aerial irrigation on litter decomposition and AMF colonization in mountain meadowlands. Six intensity levels of irrigation and fertilization were experimentally tested in eleven meadows located in the Swiss Alps. For litter decomposition, a novel tea-bag approach was followed. AMF root colonization was assessed from field samples and a trap culture experiment. Decomposition measured with tea bag burials was higher under maximal management intensity levels. AMF colonization was 22% lower in high-input compared to low-input plots in the field while in the trap cultures colonization levels depended on the functional plant species group. Our findings shed light on the impact of management intensification in mountain hay meadows on belowground functioning. Ultimately, the findings of this study will help to develop pragmatic management recommendations to farmers that would allow acceptable yields while preserving functional diversity.

Keywords: subalpine meadows, arbuscular mycorrhiza, decomposition, fertilization, irrigation

Introduction

During recent decades, grassland management in montane and subalpine regions has been highly intensified (Tasser *et al.*, 2007). The application of slurry (i.e. liquid manure) instead of solid manure and the progressive replacement of traditional ground irrigation with water channels by aerial irrigation with sprinklers (Crook and Jones, 1999) are two examples of novel and common practices that were introduced to increase hay production. Their impact on plant and invertebrate communities in mountain grasslands has already been investigated (Andrey *et al.*, 2014). However, we still aim for a better understanding of belowground responses to human-induced land use changes (Wardle *et al.,* 2004). The objective of this study was therefore to gain knowledge about the influence of intensified irrigation and fertilization practices on litter decomposition and root colonization by arbuscular mycorrhizal fungi (AMF).

Materials and methods

Using a randomized block design, six management treatments were experimentally tested in mountain hay meadows (replicated at 11 study sites in SW Switzerland), namely: (1) management without either irrigation or fertilization, as control; (2) aerial irrigation only, by the means of sprinklers; (3) fertilization only, with slurry; (4-6) aerial irrigation combined with fertilization at, respectively, low, medium, and high input levels. For more information on the study design, see Andrey *et al.* (2014). Litter decomposition was measured by the relative residual mass (remaining after a given period of time) of buried green and rooibos tea bags and with two indices: the decomposition rate (k) that describes the speed of decomposition, and the litter stabilization factor (S) that indicates inhibiting environmental effects on the decomposition of labile material (Keuskamp *et al.*, 2013). Root colonization by AMF was analysed from field samples as well as from trap cultures where AMF were amplified in pots by growing them on *Plantago lanceolata*, *Trifolium pratense* and *Lolium perenne*.

Results and discussion

After four months, the relative residual mass of tea bags was lower in high-input than in control plots (41% and 44%, respectively, for green tea; 69% and 74%, respectively, for rooibos), which yielded higher k and lower S values (Figure 1). Furthermore, S was lower under irrigation alone. These results suggest accelerated litter decomposition under intensified meadow management. Intensification negatively affected root colonization by AMF in the field samples, which was 22% lower, on average, in high-input compared to low-input plots. In the trap cultures, lower AMF colonization was measured under medium and high management intensity for *Trifolium pratense* (minus 45% and 47%, respectively, compared to control plots). When irrigation and fertilization were applied alone, they did not affect root colonization by AMF (Figure 2).

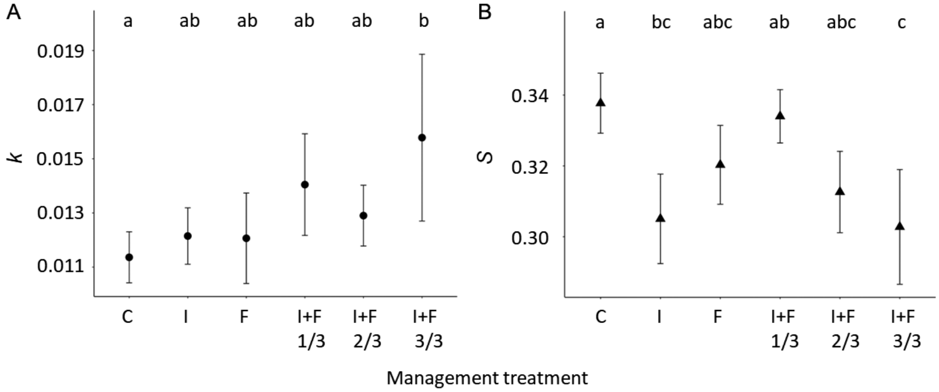

Figure 1. Decomposition rates k (a) and stabilization factors S (b) after four months of burial in response to the different management treatments. Management treatment abbreviations are: C = control; I = irrigated; F = fertilized; and I+F 1/3, I+F 2/3 and I+F 3/3 state for treatments fertilized and irrigated at respectively 1/3 (low), 2/3 (medium) and 3/3 (high) of the dose that would be necessary to achieve the maximum theoretical local hay yield. Different letters indicate significant differences among treatments at an alpha rejection value set to 0.05. Mean values ± standard errors (SE) are presented.

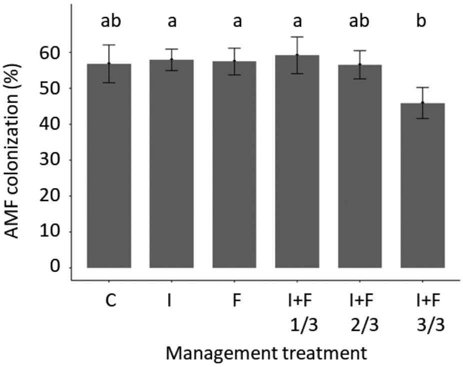

Figure 2. AMF root colonization in the field samples. For the management treatment abbreviations see Figure 1. Different letters indicate significant differences among treatments at an alpha rejection value set to 0.05. Mean values ± standard errors (SE) are presented.

Conclusion

Our findings indicate that the intensification of hay meadow management practices affects soil litter decomposition and root colonization by AMF. Possible consequences from faster decomposition are altered nutrient cycles with a lower carbon sequestration potential, while decreased root colonization by AMF may lead to restricted nutrient transfer, reducing plant growth and health. In line with former studies of the response norms of aboveground organisms to hay meadow management intensification, the present results suggest that moderate intensification may represent a good compromise in terms of biodiversity preservation, ecosystem functions and forage production.

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Tannin distribution across temperate forage species and their impact on root decomposition and soil respiration

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Abstract

Secondary plant metabolites such as proanthocyanidins (PA) are not well understood in terms of their impact on decomposition of SOM in seeded grassland swards. Hence, we designed a greenhouse experiment with five different forage species (sainfoin (*Onobrychis viciifolia*), big trefoil (*Lotus pedunculatus*), birdsfoot trefoil (*Lotus corniculatus*), salad burnet (*Sanguisorba minor*) and lucerne *(Medicago sativa*), to determine both the PA concentration in different plant compartments per species and the root decomposition rates. The experiment showed that sainfoin had the highest mean PA concentrations in the aboveground biomass, with 19 mg g^{-1} DM, yet contained little to no PAs in the root system (0.1 mg g^{-1} DM). Big trefoil had intermediate PA concentrations aboveground (9 mg g^{-1} DM), yet the highest belowground PA concentrations (5 mg g^{-1} DM) The CO_2 emissions resulting from root decomposition were largely in accordance with PA concentrations in the roots, the main exception to this rule being sainfoin, which resulted in the lowest CO_2 emissions, despite the absence of PAs.

Keywords: polyphenols, carbon immobilization, forage legumes, soil respiration

Introduction

Grassland systems are linked to higher soil carbon stocks compared to annual production systems, due to their large inputs of plant residues, above- (ABG) and belowground (BGB), and the absence of tillage. Accordingly, European grassland systems are estimated to increase their soil carbon stocks by 0.5 t C ha^{-1}, annually, while crop production results in annual losses of 0.8 t C ha^{-1} (Vleeshouwers and Verhagen, 2002). These numbers show the importance of grassland systems as a tool for climate change mitigation. Recent research suggests, however, that particularly diverse grasslands might possess an additional advantage, as it has been shown that plant secondary metabolites (PSM) affect ecosystem processes, such as litter decomposition or nutrient cycles. They can protect leaf litter from decomposition by inhibiting microbial activities and reducing enzymatic breakdown of SOM (Chomel *et al.*, 2016). However, previous research in this topic has focused on forests, where the pathways of tannins into the soil are clear, with leaching from litter being the main source. Contrary to that, in grasslands the aboveground biomass is frequently removed. One possibility is that tannins are obtained from the roots via exudates or fine root turnover. This hypothesis is feasible, given that polyphenols have been identified to be present in roots and also an impact of those polyphenols on nitrogen mineralization was identified for grassland species (Fox *et al.*, 1990). However, due to the limitations of the analysis of polyphenols the results are coarse and difficult to interpret and cannot be extrapolated to other grassland species.

Materials and methods

Five forage species were selected to be grown under controlled conditions in a greenhouse pot experiment: sainfoin (*Onobrychis viciifolia*), birdsfoot trefoil (*Lotus corniculatus*), big trefoil (*Lotus pedunculatus*), salad burnet (*Sanguisorba minor*) and, as a proanthocyanidin (PA)-free negative control, lucerne (*Medicago sativa*). Plants were grown in a sandy loam using pots of 40 cm height for minimal disturbance of root growth and arranged in a complete randomized block design. Nutrient levels and pH were adjusted to maximize growth rates. An automated irrigation system maintained a minimum water filled pore space of 50%. After harvesting the ABG, samples were frozen before being freeze-dried for further analysis, and the BGB was washed prior to freezing. PA analysis was performed using two analytical techniques: An HCl-butanol assay was performed according to Terrill *et al.* (1992) to determine the share of tannins that were either soluble in organic solvents, or present in either protein- or fibre bound form in the plant, using a standard obtained from plant material of sainfoin that was purified using a Sephadex LH-20 column. In addition, the solvent-soluble fraction was analysed using a UPLC-MS/MS system to determine the structural characteristics of the PA in the plants. The carbon concentration of all roots was analysed using a C/N analyser (Vario Max, Elementar) and their ash content by combustion in a muffle furnace. To determine the root turnover and the CO_2 emissions, a 2nd subset of roots was pooled, split into equal (DM-based) portions, rewetted and sown into mash begs with a mesh size of 37 μm to allow micro-organisms to pass through, yet prevent other losses. Over the entire experiment, soil moisture and temperature were measured and controlled. CO_2 fluxes were measured every second day, using a Licor 6400 connected with a soil respiration chamber (LI 6400-09).

Results and discussion

While concentrations of PAs varied substantially ($P<0.001$) among species both above- (range: 0-19.1 mg g^{-1} DM^{-1}) and belowground (range 0-4.6 mg g^{-1} DM^{-1}), there were differences among the above- and belowground biomass, with sainfoin having the highest PA concentration in AGB, yet almost no PA in BGB (Table 1). Lucerne was entirely free of tannins, as expected. Regarding the PA composition, salad burnet contained no prodelphindins (PDs), whereas sainfoin contained predominantly prodelphinidins (PD share: 81%), with birdsfoot trefoil (PD share: 13%) and big trefoil (PD share 66%) in between them (results not shown). Finally, while salad burnet showed comparably low concentrations in PA. However, it was very rich in hydrolysable tannins, with a concentration of 42 mg g^{-1} DM^{-1} above- and 57 mg g^{-1} DM^{-1} belowground (results not shown).

The cumulative CO_2 emissions resulting from the soil respiration also varied substantially among species ($P<0.001$; Figure 1). Considering the C-input by plant residues, birdsfoot trefoil showed the highest emissions at the end of the experiment with 543 g CO_2 m^{-2} over the 57 days of the experiment, compared to 283 g CO_2 m^{-2} in the bare soil (blank) that was used as negative control. Salad burnet and big trefoil generated the lowest CO_2 emissions, with 328 and 346 g CO_2 m^{-2}, respectively. While the low emissions of big trefoil are in accordance with its highest PA concentration in roots of all species, the low emissions of salad burnet can be explained by the presence of hydrolysable tannins, which have been identified previously to be efficient in reducing soil microbial activity (Adamczyk *et al.*, 2017). The reduced emissions of sainfoin can be explained by an elevated C/N ratio (results not shown) both in sainfoin (35:1) and salad burnet (55:1), compared to birdsfoot trefoil (17:1), big trefoil (18:1) and lucerne (22:1), as linear increments in the C/N ratio result in a exponential decrease in decomposition rates (Enríquez *et al.*, 1993). The increased emissions from birdsfoot trefoil compared to lucerne as the PA-free negative control might originate from the PA composition which, unlike in the other species, consists almost entirely of procyanidins (PCs). As previous research has identified that some tannin groups can also increase soil respiration rates (Adamczyk *et al.*, 2017), it might be that this is the case for PCs compared to either the hydrolysable tannins of salad burnet or the prodelphinidins mainly present in big trefoil.

Table 1. Proanthocyanidin (PA) concentration in mg g^{-1} DM for each species.[1,2]

	OV	LC	LP	SM	MS
AGB	19.1^{Ad}	1.2^{Ab}	9.1^{Ac}	2.3^{Ab}	0.0^{Aa}
BGB	0.1^{Bb}	1.0^{Ab}	4.6^{Bc}	1.0^{Ab}	0.0^{Aa}

[1] Upper case letters indicate differences between above and belowground biomass, while lower case letters indicate differences among species based on multiple comparisons in a linear mixed model.

[2] LC = *Lotus corniculatus*, LP = *Lotus pedunculatus*, MS = *Medicago sativa*, OV = *Onobrychis viciifolia*, SM = *Sanguisorba minor,* ABG = above ground, BGB = below ground.

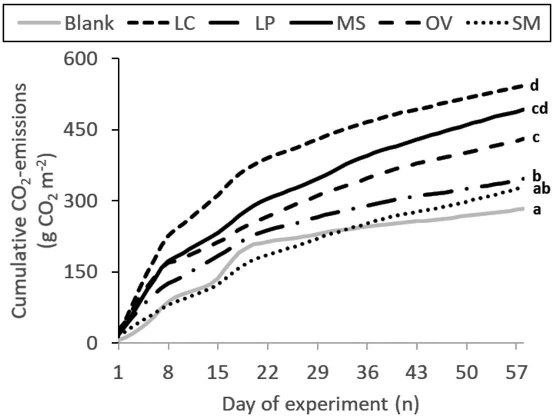

Figure 1. Cumulative CO_2 emissions resulting from root decomposition over the course of the experiment for each species (for abbreviations see Table 1) and compared to the baseline soil respiration ('blank') without any root input. CO_2 emissions from roots were corrected for C content in organic matter of the roots weighed in to compare CO_2 emissions per C-input.

Conclusion

For all species with the exception of sainfoin, the belowground proanthocyanidin (PA) concentrations were similar to the aboveground biomass. However, while plant secondary metabolites clearly do seem to have an effect on soil respiration and can result in substantially reduced CO_2 emissions, there is a strong dependency on both the type of tannin and how many tannins are included in the roots, and on other root properties such as the C/N ratio.

Acknowledgements

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Quantification of roots in soil, plant-fungal symbioses and in mixed plant populations with real-time PCR

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Abstract

Plant-soil and underground plant-plant interactions have direct implications for the conservation of biodiversity, plant productivity and understanding of local ecosystem responses to global environmental change. Study of associated organisms in the soil, symbiosis and in mixed plant populations, is limited by difficulties in the species quantification and sensitive detection. We have designed and tested new PCR primers and hydrolysis probes for TaqMan assays that target the nuclear ribosomal gene plant DNA. Species-specific probes and universal primers were developed for monocot and dicot plant species and evaluated for specificity and sensitivity in TaqMan assays on DNA extracted from soil and in mixed plant populations. The assays were sensitive and able to quantify rear targets in mixed plant populations. This approach appears to offer a quick and sensitive way to study roots in soil, symbiosis and in mixed plant populations composition and could provide insights into root function and responses not readily provided by other methods. These features identify the new primer sets developed in this study as useful complements to other universal primers for the study of plant diversity and community composition.

Keywords: grasslands, below ground species abundance, rooting distributions, TaqMan, ITS region, qPCR

Introduction

Roots are important in the functioning of ecosystems and are difficult to study in the field. This is mainly due to high spatial variability and difficulties in the possibility for selecting roots directly from the soil. The effects of soil microbial mutualists including mycorrhizae are poorly understood in natural communities despite the potentially large role they play in species coexistence and ecosystem function. High sampling intensity is necessary to account for the heterogeneous distribution of roots in the soil profiles. It is also difficult to identify the roots of individual species and accurately quantify the root mass using labour-intensive methods, such as root washing (Riley *et al.,* 2010; Jones *et al.,* 2011; Haling *et al.,* 2012; Frasier *et al.,* 2016). The main approach is to use DNA sequence data to identify roots to species either from single root fragments or from whole soil cores of roots composed of mixed samples of multiple species. Eukaryotic multi-copied 18S-23S ribosomal RNA (rRNA) genes that feature a wide coverage is critical in detecting the composition of eukaryotic organisms in ecosystems. Here, we designed universal primers for 18S, 5.8S and 23S rRNA based on consecutive conserved sites and evaluated their coverage efficiency and scope of application to different eukaryotic kingdoms (plant, fungi, animal). We investigated common evolutionally conserved regions in three kingdoms of eukaryotic 18S-23S rRNA sequences for the development 18S-5.8S-23S universal primers. After evaluation, several of them were considered as qualified universal primers based on coverage rate. This approach is quick and sensitive to the study of roots in the soil, symbiosis and mixed composition of plant populations and can give an idea about the function of the root and the responses that are not easily provided by other methods.

Materials and methods

Soil cores were taken from 50 locations in the area of Helsinki using a 20 cm diameter auger. Root-soil samples (about 50-100 g) were frozen at -80 °C and ground to a fine powder using a mortar and pestle in liquid nitrogen. DNA was extracted from soil (5-10 g) with a commercial E.Z.N.A.® Soil DNA Kit (Omega Bio-TEK) with a combination of an acid CTAB-based extraction method (Kalendar, 2018).

The 18S-23 rRNA plant gene sequences were taken from the NCBI database and a full set of rDNA sequences was downloaded from Repbase (https://www.girinst.org/repbase/). For each 18S, 5.8S and 23S rRNA genes, the sequence accessions were aligned and conservation assessed with the MULTALIN multiple alignment procedure (Corpet, 1988). The conserved segments of rRNA were used for the design of PCR primers, which was realised with the program FastPCR (Kalendar *et al.,* 2017b). We designed several primer pairs and hydrolysis probes for TaqMan assays for 18S, 5.8S and 23S rRNA genes of each plant species to compare amplification efficiency and reproducibility. None of the primers and probes chosen form dimers. All showed high PCR efficiency and were very effective for qPCR. qPCR amplification targeting the ITS region (ITS1-5.8S ribosomal gene-ITS2), using the universal plant 18S and 23S rRNA gene primers (Table 1). Root-soil samples were analysed for the quantity of target DNA of individual plant species. Real-time PCR was performed with a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad) equipped with a 384-well plate. qPCRs were conducted in a 15 µl mixture containing 1× DreamTaq (Thermo Fisher Scientific), 0.3 µM of each primer, 0.1 µM of each hydrolysis probe and about 20-50 ng of soil DNA template. The qPCR conditions were: initial denaturation of 95 °C for 2 min followed by 40 cycles of denaturing at 95 °C for 15 sec, annealing at 60 °C for 20 sec, extension at 72 °C for 30 sec and a final extension of 72 °C for 2 min.

Results and discussion

We tested the coverage efficiency of the universal primers designed for 5.8S and 23S plant rRNA genes and species-specific hydrolysis probe for each plant species studied. The specificity of given primers was evaluated by *in silico* PCR and DNA targets (Kalendar *et al.,* 2017a). We accumulated primary results on the analysis of soil samples (in total 136) and analysing the possibility to quantitatively measure rDNA genes of specific plant species (*Phleum pratense*, *Festuca* sp. (*F. arundinacea* and *F. pratensis*) and *Trifolium* sp. (*T. repens* and *T. pratense*) in soil providing our qPCR method to assess roots. These soil samples contained a mixture of the roots of the studied species, which had to be quantitatively and qualitatively determined. In addition, these soil samples were obtained for sections of the surface part of the soil (0-10 cm), medium (10-20 cm) and lower parts (below 20 cm). Field plots for which the analysis was carried out were subjected to repeated mowing of growing grasses. The objective of this study was to determine the depth in the soil where the roots of plants are detected, and the degree of survival of plant species after multiple mowing of these grasses. A series of experiments are being conducted to determine the sensitivity of the assays, the ability to detect DNA in mixed roots soil samples. We obtained total DNA from soil samples for all field samples and soil sections. The tendency of survival of cereals, compared with legumes, was identified. DNA roots are still detected in the lowest layers of the soil. However, the highest concentration of roots near the soil surface is revealed. The current result shows that this approach not only provides a new tool for studying the roots, but also that quantification of root DNA will provide information about root responses that could not be easily obtained otherwise. The multiple TaqMan assays developed are sensitive, detecting the fine roots plant DNA in the soil, and have a wide linear range of quantification. This DNA assay is likely to provide a better indicator of root function.

Table 1. Primer and probe sets used in TaqMan PCR for plant species detection.

ID	Sequence	Information	*Trifolium pratense* (KY860927)	*Phleum pratense* (MH628291)	*Festuca arundinacea* (KJ598991)	Tm (°C)
5447	gaagaacgtagcgaaatgcgatac	5.8S primer	1507→1530	2069→2092	271→294	56.9
5449	ggttcgctcgccgttacta	23S primer	1926←1944	2486←2504	688←703	56.4
5450	tcgagtctttgaacgcaagttgcgcc	Universal TaqMan (FAM-TQ1)	1561→1586	2123→2148	325→350	64.2
5451	ctcccaccccactaacctggggc	TaqMan *Festuca* sp. (HEX-Eclip)	-	-	401→423	64.8
5453	caagccgcatcccactcccgatga	TaqMan *Phleum* sp. (ROX-TQ3)	-	2211←2234	-	64.7
5454	tgaggcgatggtgctcacgggag	TaqMan *Trifolium* sp. (Cy5-TQ3)	1684←1706	-	-	64.6

Conclusion

We have developed a quick and sensitive DNA-based application for the quantitative determination of root growth of grasses directly in the soil. We were able to identify and quantify the plant species within single cores and number of roots soil sampled. In addition, this method also has potential to study underground interactions among plants in symbiosis and for the study of plant community composition.

Acknowledgements

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Variations induced by fertilizers on mycorrhizal status in natural grasslands

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Abstract

Grasslands are dynamic ecosystems, with a long duration of use and a complex assemblage of interactions. Over long periods of time, this induces the proliferation of symbionts within microbial communities with a highly beneficial impact on plant development. Due to constant removal of organic matter through mowing, nutrients derived from plant litter decreases. Most plants in grasslands are connected to a symbiotic network developed by mycorrhizas. Our study aimed to evaluate the response of mycorrhizas developed in dominant plants to the addition of several fertilizers in combination with mulching or mowing. A manure application of 10 t ha^{-1} combined with mineral nitrogen increased the value of colonization. A strong reaction was observed when wood ash was used as fertilizer, a quantity of 100 kg ha^{-1} acting toward an increase of fungal development in root depth. Management and fertilizers represent a mix for the amplification of mycorrhizal system, if it is desired to maintain the dominant species, or backward for changes in dominance.

Keywords: mycorrhizal colonization, management, fertilization, mowing vs mulching

Introduction

In the entire world, an increase of fertilization tends to produce more and more powerful imbalances on grasslands, amplifying the effects of management in these ecosystems. In Romania, due to socio-economic factors, the areas occupied by meadows are subjected either to abandonment or to a powerful process of intensification, which leads to the appearance of strong nutritional imbalances and ecosystem depreciation (Rotar *et al.*, 2014). Mycorrhizal fungi have the capability to interconnect the root systems with the soil particles, reacting rapidly to reduced levels of disturbance (Vidican *et al.*, 2014). The role of mycorrhizas in ecosystems is the judicious allocation of nutritional resources among plants (Lambers *et al.*, 2009), maintaining their overall balance. The aim of this paper is the identification of an optimal combination between fertilization and management in order to preserve the mycorrhizal status of dominant plants in mountain grasslands. Based on results we will be able to explore in the future different types of site-specific solutions for maintenance of mycorrhizal networks at a high level of efficiency.

Materials and methods

An experimental field with a randomized block design was installed in 2009 on a grassland in Gârda de Sus commune, Apuseni Mountains, on a *terra rosa* soil. Fertilization increases gradually from the control variant – unfertilized (F1) to 10 t ha^{-1} manure (F2), 10 t ha^{-1} manure + N50 kg ha^{-1} (F3), NPK – 50:25:25 kg ha^{-1} (F4), Eurofertil mezocalc 120 kg ha^{-1} + N50 kg ha^{-1} (F5), Eurofertil mezocalc 120 kg ha^{-1}(F6) and wood ash – 100 kg ha^{-1}(F7). The management of experimental parcels was done by mowing (C) or mulching (M). The analysed mycorrhizal parameter was the degree of colonization (the product between frequency and intensity of colonization) which shows the % of entire root volume explored by mycorrhizas in the roots of red fescue (*Festuca rubra* L.) and white clover (*Trifolium repens* L.), dominant (>50%) in the phytocoenosis. Root sampling was done in 2010 and 2011, in 2 vegetation sequences – at the maximum of vegetation growth (100 days after application of fertilizers) and at the end of vegetation period which corresponds to 150 days from fertilization. Roots were stained with a modified ink-vinegar method (Stoian and Florian, 2009) for the assessment of intraradicular mycorrhizas. In order to highlight the response of the mycorrhizas to the complex of factors, ANOVA test was preferred, and for assessing the oscillations between management and fertilization – the LSD type tests. All tests were performed with R Studio (R Core Team, 2018), package 'agricolae' (De Mendiburu, 2013).

Results and discussion

Tests conducted on the mycorrhizal mechanism indicate the high importance of fertilization and the vegetation sequence of the plants (Table 1). The climatic spectrum of each year is relevant only for *F. rubra*, dominant in the grassland. The combination of factors with greatest importance and synergistic effect for *F. rubra* is the sequence × fertilization × management. In the case of *T. repens*, the combination of management and the vegetation sequence is particularly important, especially due to lower coverage in phytocoenosis. Another aspect is the removal of upper vegetation through mowing, which is an opportunity to grow for these plants. Globally, the increase of fertilization levels will result in a higher production of above-ground biomass. This action will reduce the extension of root system, which will reduce the level of mycorrhization (Johnson *et al.*, 2008) in this context the management amplifying the effect of fertilization. The % root colonization in the dominant plant roots was evaluated according to their reaction at management × fertilization (Table 2). This type of interaction is most often encountered in the field and is the easiest to control. Under mowing conditions, the degree of colonization in the roots of *F. rubra* varies greatly from 1.20% (unfertilized) to 15.28% (ash) and over 21% in complex

Table 1. ANOVA test of interaction between factors on mycorrhizal colonization degree.[1]

Interaction	***Festuca rubra***		***Trifolium repens***	
	F value	***P*-value**	**F value**	***P*-value**
Year	11.16	0.001***	2.61	0.108 ^{ns}
Sequence	7.31	0.008***	0.43	0.515 ^{ns}
Fertilizer	13.22	0.000***	3.41	0.067 ^{ns}
Management	3.76	0.054 ^{ns}	0.90	0.345 ^{ns}

[1] ns: not significant; $P>0.05$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Table 2. Interaction of management × fertilizers on colonization degree.[1]

Management	**Fertilizer**	***Festuca rubra***		***Trifolium repens***	
		means	**Signif.**	**means**	**Signif.**
Cutting the plants (mowing)	Unfertilized	1.20	d	25.68	a
	Manure 10 t ha^{-1}	8.02	c	5.25	ef
	Manure 10 t ha^{-1} +$N_{50}P_{25}K_{25}$	7.22	cd	7.38	def
	N_{50} $P_{25}K_{25}$	4.81	cd	14.77	bcde
	Em 120 kg ha^{-1} + N_{50}	21.43	a	5.72	ef
	Em 120 kg ha^{-1}	6.84	cd	17.74	abc
	Ash 100 kg ha^{-1}	15.28	ab	8.27	cdef
Mulching	Unfertilized	3.73	cd	12.32	bcdef
	Manure 10 t ha^{-1}	9.55	bc	3.37	f
	Manure 10 t ha^{-1}+$N_{50}P_{25}K_{25}$	7.85	c	19.51	ab
	N_{50} $P_{25}K_{25}$	7.70	cd	15.91	bcd
	Eurofertil mezocalc 120 kg ha^{-1} + N_{50}	8.47	c	8.00	def
	Eurofertil mezocalc 120 kg ha^{-1}	4.19	cd	3.14	f
	Ash 100 kg ha^{-1}	8.09	c	11.23	bcdef

[1] Means followed by a common letter are not different at the 5% level.

mineral fertilization. The application of manure, with or without mineral nitrogen, stimulates the level of colonization.

The distribution of recorded data (Figure 1A) indicates large intervals of variation in mowing and fertilization with manure or ash. Mulching does not lead to spectacular increases in colonization and reduces the impact of fertilization. The disruptive effect of this type of management is diminished by fertilization (Figure 1), together acting to preserve the nutritional space of plants. Maximum colonization performance is observed in complex fertilization, manure or Em + N50. The clover's nitrogen symbiotic fixation capability is a solid basis for maintaining mycorrhizal colonization to over 25% in the absence of fertilization and mowing conditions, and only half of this value for mulching (Table 2). Complex mineral fertilization (NPK) stimulates colonization rates of 14-15% for both mowed and mulched plants. The highest colonization is observed at the application of manure + N50 and mulching, respectively Em + N50 under mowing conditions. Variation ranges are high in *T. repens*, indifferent to the applied fertilization system. The most stable combinations are mowing with ash fertilization, mulching with manure or Em fertilization.

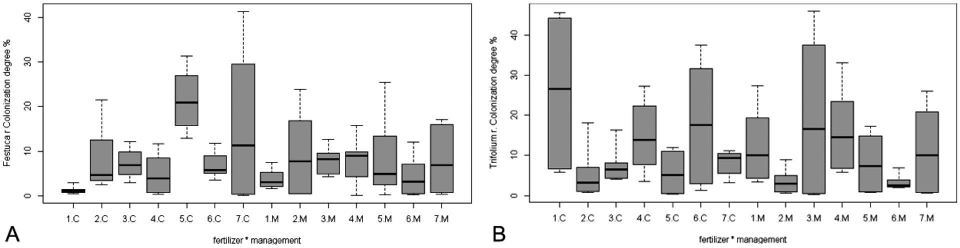

Figure 1. Stability of colonization degree induced by management × fertilizers. (A) *Festuca rubra*; (B) *Trifolium repens.* Numbers 1-7 – fertilization variants; C – mowing; M – mulching.

Conclusion

Fertilizers and a management based on mowing or mulching act to limit the level of colonization within a range of 5-20%. Wood ash can represent a valuable fertilizer due to its constancy in the case of mulching. Complex fertilizers increase the colonization degree.

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Soil microbial biomass and community structure in differently managed grasslands along a European gradient

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Introduction: Agro-ecological conditions and land use management are, among others, the key drivers of grassland soil microbial communities. In the BIOINVENT project, the soil microbiome of these systems was investigated along a pan-European transect.

Materials and methods: Soils were sampled in ten agro-ecological regions defined by favourable (F) and less favourable (LF) conditions, i.e. edapho-climatic conditions restricting plant growth, in each of five countries (Sweden, SE; Germany, DE; Switzerland, CH; Portugal mainland, PT and Azores, AZ). In each of these regions a gradient of management intensity was selected, from intensively managed grasslands with high nutrient inputs to grasslands with intermediate and extensive management intensity. The PLFA and NLFA (phospholipid- and neutral lipid fatty acid) analysis (Frostegård and Bååth, 1996) was used to estimate the microbial community structure as well as the biomass of the microbial groups.

Results: Canonical discriminant analysis (CDA) showed a clear separation of the PT samples from the others ($P<0.001$), among which the biggest difference came out between AZ and SE ($P<0.001$). The detailed study in each country showed that management affected soil microbial community structure, but that the growth conditions (F vs LF) had a larger effect in SE, DE and CH ($P<0.001$). The total microbial biomass was not affected by these factors; however, saprophytic fungal biomass was generally higher in extensive grasslands than under the other management intensities (Figure 1).

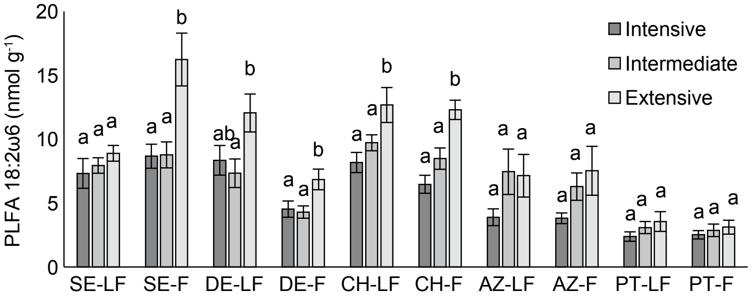

Figure 1. Saprophytic fungal biomass. F: favourable; LF: less favourable; SE: Sweden; DE: Germany; CH: Switzerland; AZ: Azores; PT: Portugal mainland. Different letters mean significant differences (ANOVA, $P<0.05$).

Conclusion: Agro-ecologic region had the greatest effect on soil microbial communities. Within agro-ecological regions, extensive grassland management provided the most prosperous habitat for fungal colonization.

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Effects of plant functional types on greenhouse gas fluxes in grasslands along a climatic gradient

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Introduction: Climate change is expected to induce strong shifts in biodiversity and vegetation composition of grasslands and thus on greenhouse gas (GHG) fluxes, because of interactions between the plant functional composition and nutrient inputs for soil processes involved in GHG exchange. The objective of our study was to investigate the effects of plant functional type (PFT) composition on GHG fluxes of CO_2, CH_4, and N_2O along a climatic gradient.

Materials and methods: Following a simplex design (Kirwan *et al.*, 2007), our study was carried out in 14 grassland sites in the North-Eastern Iberian Peninsula – traditionally extensively managed – along a climatic gradient, covering six climatic zones. We measured CO_2, CH_4, and N_2O fluxes under light conditions at the peak of the vegetation biomass using a photoacoustic field gas monitor and transparent static chambers. We then determined the proportion of each PFT. We tested with general linear models the effects of climatic (MAT, MAP, mean air temperature during sampling), soil conditions (pH, SOM and SON, C:N ratio), and PFT composition on GHG fluxes.

Results: Forb patches showed maximum CO_2 uptake in cold and humid sites, while legume patches exhibited the highest NEE in the warmer Mediterranean and montane sites. Woody patches showed the highest CH_4 emissions in semi-arid grasslands. However, when mixed with other PFTs we observed CH_4 uptake instead of emissions. The highest N_2O emissions in the Atlantic, subalpine and alpine grasslands occurred in legume patches. Legume-woody, forb-sedge and above all legume-grass interactions enhanced N_2O uptake (Figure 1).

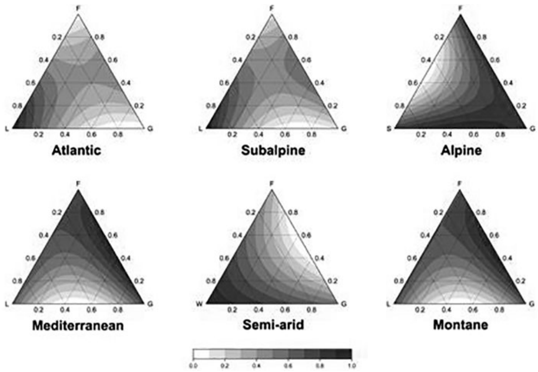

Figure 1. Ternary plots of N_2O fluxes (nmol m^{-2} s^{-1}) of PFT proportions (F: forb; L legume; G grass; S sedge; W woody), in grasslands of six climatic zones. Ternary plots are scaled to have a maximum of 1. Colour intensity shows a gradient from the maximum gas uptake/minimum emission (white) to the minimum gas uptake/maximum emission (dark).

Conclusion: PFT composition altered GHG exchange strongly mediated by climatic conditions. PFT interactions increased CH_4 and N_2O uptake compared to single PFTs.

Kirwan L. Luscher A., Sebastia M.T., Collins R.P. *et al.* (2007) Evenness drives consistent diversity effects in intensive grassland systems across 28 European sites. *Journal of Ecology* 95, 530-539.

The distribution of bacterial operational taxonomic units in European grasslands

Fox A.[1,2], Lüscher A.[1], Barreiro A.[3], Dimitrova Mårtensson L.-M.[3], Silva L.[4], Vieira Â.F.[4], Parelho C.[4], Cruz C.[5], Melo J.[5], Musyoki M.[6], Zimmermann J.[6], Rasche F.[6] and Widmer F.[2]
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Introduction: Under the auspices of the BiodivERsA project BIOINVENT (www.biodiversa.org/972), this study aimed to determine the distribution of bacterial operational taxonomic units (OTUs) in grasslands along a pan-European transect to help gain insights into the factors driving their colonization patterns.

Materials and methods: Soil samples were taken from three different permanent grassland management types (intensive, low intensive and extensive) in Sweden (SE), Germany (DE), Switzerland (CH), Portugal mainland (PT) and the Azores (AZ) (338 sites in total – 16 cores of 2.5 cm diameter were pooled per site). An amplicon-based Illumina Miseq sequence analysis was conducted on the bacterial 16S rRNA gene and an OTU table constructed. Sample reads were rarefied to the lowest sequencing number and a quintuple Venn diagram was made using the Venn package in R (R core team, 2018).

Results and discussion: A total of 28,174 unique bacterial OTUs were identified (Figure 1); of these 5,901 OTUs (20.9%) were shared among all countries. PT harboured the most unique OTUs (3,534, 12.5%), followed by AZ (2,142, 7.6%), while approx. 3-5% of the OTUs were unique to CH, DE and SE, respectively. The substantial number of shared bacterial OTUs would suggest a 'core' bacterial community among European grasslands. Country-specific variations in climate, grassland management and soil conditions may explain the relatively high proportion of unique OTUs present in each country.

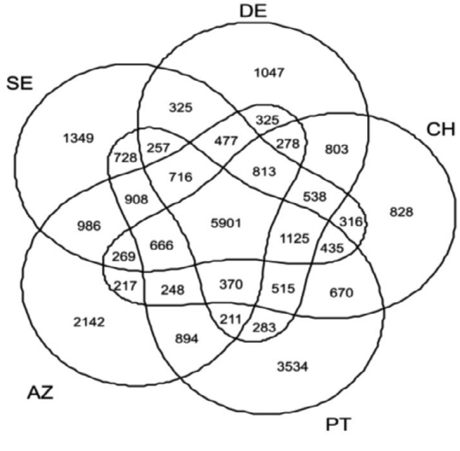

Figure 1. A quintuple Venn diagram showing the unique and shared bacterial OTUs from the countries of the BIOINVENT transect.

Conclusion: These results indicate a core grassland microbiome. The large number of country specific OTUs, in spite of the fact that the same management intensity spectrum was sampled in each country, would also suggest that a combination of country-specific climate, soil and management conditions are important determinants of the composition of the soil microbiome.

R Development Core Team (2018) R:A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Soil enzyme activities as driven by contrasting agro-ecologies and management practices in grasslands across Europe

Musyoki M.[1], Melo J.[2], Zimmermann J.[1], Guo Y.[1], Jongen M.[3], Cruz C.[2], Fox A.[4,5], Lüscher A.[4], Widmer F.[5], Barreiro A.[6], Dimitrova Mårtensson L.-M.[6], Silva L.[7], Vieira Â.[7], Parelho C.[7] and Rasche F.[1]

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Introduction: Interactions between soil enzyme activities involved in nitrogen (N) cycling, management and agro-ecological distinctions are vital for understanding the stability and sustainable productivity of grassland systems. What remains unclear, however, is if grassland management per se is a stronger regulator of soil N cycling enzymes than agro-ecological distinctions, as driven by the amount and type of nutrient resources applied. The objective of our study was to determine the extent to which management and agro-ecological distinctions regulate N-cycling enzymes in grassland systems across selected countries in Europe.

Materials and methods: Fluorometric quantification of Leucine-aminopeptidase (LEU) activity was analysed under contrasting managements (intensive, less intensive, extensive) across a Pan European agro-ecological gradient: Germany (DE), Switzerland (AZ), Sweden (SE), Portugal mainland (PT) and Azores (AZ). In each country, 12 sites were randomly selected from each management and from two agro-ecologies (favourable and less favourable growth conditions). Measurements were done in triplicate using 7-amino-4-methyl coumarin (AMC) substrate and the activities were expressed as nanomoles of AMC g^{-1} dry soil h^{-1}. The data were analysed using a linear mixed effect model and Spearman correlation in R (R core team, 2018).

Table 1. Country effects on LEU activity. Means with a common letter are not different at 5% level (Duncan MRT).[1]

Country	Leucine aminopeptidase activity	
	(nm g^{-1} dry soil h^{-1})	SEM
PT	135 ^{a}	74
AZ	410 ^{ab}	83
SE	647 ^{b}	112
DE	1,076 ^{c}	73
CH	1,420 ^{d}	73

[1] SEM= Standard error of means.

Results: Country showed significant effect on LEU activity ($P<0.001$) (Table 1), but not management and agro-ecological regions ($P>0.05$, results not shown). PT had the lowest LEU activity compared to all countries except AZ. In contrast, LEU activity revealed significant correlations with pH ($R^2=0.47$, $P<0.001=^{***}$), organic matter ($R^2=0.62^{***}$), total soil N ($R^2=0.58^{***}$) and total soil C ($R^2=0.67^{***}$).

Conclusion: Our results suggest that LEU activity responds strongly to site-specific soil chemical properties, probably masking effects of management intensity and agro-ecology within each country.

R Development Core Team (2018) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Linking above- and belowground diversity: plants and microbes as indicators of grassland management intensity

Silva L.[1], Fox A.[2,3], Lüscher A.[2], Barreiro A.[4], Dimitrova Mårtensson L.-M.[4], Vieira A.[1], Parelho C.[5], Cruz C.[6], Melo J.[6], Musyoki M.[7], Zimmermann J.[7], Rasche F.[7] and Widmer F.[3]

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Introduction: Within the framework of the BIOINVENT project (https://www. biodiversa.org/972), a Pan-European (Sweden, Germany, Switzerland and Portugal/Azores) survey of plant and microbial diversity in grasslands was undertaken. We tested the hypothesis that there was a high correlation between plant and fungal indicator species, depending on the management regime.

Materials and methods: Soil samples from grasslands under three management intensity levels were taken in summer 2017 from all regions. An amplicon-based Illumina Miseq sequence analysis was conducted on the fungal internal transcribed spacer (ITS) region and an OTU table built. Differences in floristic composition were sharp along the Azores gradient of management (semi-natural pasture – low management intensity; permanent pasture – medium management intensity, kept for 10 years; resown pasture – high management intensity, kept for 3 to 5 years). We used redundancy, co-correspondence, correlation and indicator species analyses to estimate the degree of association between plant and microbial communities.

Results: Plant and fungal indicator species were clearly found to be associated with the different management intensity levels (Figure 1), and high correlation values were found between the two types of indicators. That is, correlation was highest for plants and fungi that indicated the same type of grassland management. Similar results were obtained for bacteria.

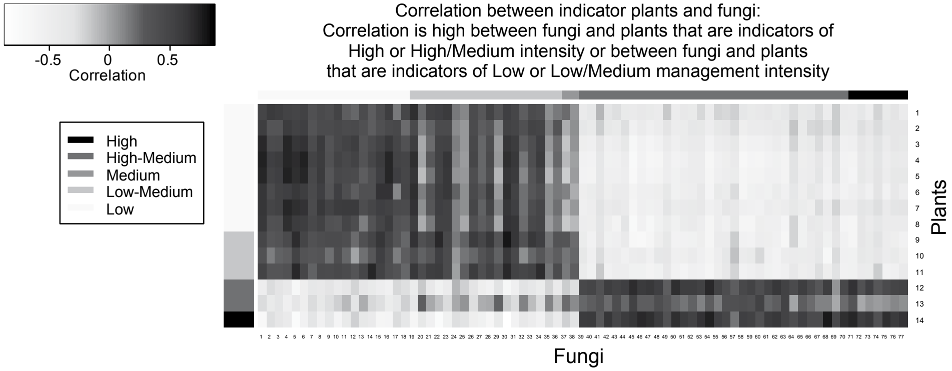

Figure 1. Results of an indicator species analysis based on 60 Azorean pastures. Only species with a significant indicator value ($P<0.05$), above 0.8, were included (14 plants, 77 fungi). Heatmap: Grey scale of the cells: darker – positive correlation between plants and fungi; Grey scales on sides: darker – higher management intensity.

Conclusion: A link between grassland management and indicator species was clearly revealed. Further, this was expressed as a high correlation between plant and microbial indicators, that is, between above and below-ground communities.

Legume belowground carbon, nitrogen and phosphorus input along an available phosphorus gradient in a Ferralsol

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Introduction: Limiting available P can affect plant belowground C, N and P input. The aim of this study was to investigate C, N and P derived from rhizodeposition during a defined growth phase and root decomposition, after shoot harvest, in response to levels of P fertilization.

Materials and methods: The legume, *Canavalia brasiliensis* was grown in a Ferralsol with limiting plant available P, which was fertilized with 0 or 50 mg of P kg soil^{-1}. At 5 weeks of growth the plants were labelled with the stable isotope ^{15}N and the radioisotope ^{33}P, using a cotton-wick-stem-feeding-method (Russell and Fillery, 1996), and pulse labelled with ^{13}C-enriched CO_2. Shoots were cut 4 weeks after labelling (w.a.l). Pots of a first set were destructively harvested the same day while pots of a second set were harvested at 8 w.a.l to measure δ ^{13}C, δ ^{15}N values and the ^{33}P activity in root free soil.

Results: ^{13}C, ^{15}N and ^{33}P recovered in root free soil reflect C, N and P derived from the plant. Mean δ ^{13}C values (Table 1) did not change along with P amendment. However, averaged over time they indicated increased belowground plant C input with P amendment. Mean δ ^{13}C decreased with time, because part of the belowground ^{13}C deposited was respired. Means of δ ^{15}N did not change with P amendment but increased across time. The mean ^{33}P activity was significantly higher for no P at 8 w.a.l, compared to high P at 4 w.a.l. Except the increase of plant derived P with time, this suggests greater input of plant derived P with lower P supply, due to greater belowground investment of the plant.

Table 1. δ ^{13}C δ ^{15}N and ^{33}P activity of the soil at 4 weeks after labelling (w.a.l) and 8 w.a.l.[1]

Time	P amendment	δ ^{13}C [‰]	δ ^{15}N [‰]	^{33}P [kBq]
4 w.a.l	no P	-9 ab	25 a	41 ab
	high P	-6 a	27 a	14 b
8 w.a.l	no P	-12 c	52 b	108 a
	high P	-10 bc	67 b	75 ab
ANOVA	Std. error	1	5	16
	P amendment	**	ns	ns
	Time	***	***	**

[1] A two-way analysis of variance was calculated to test the influence of treatment, time and P amendment:time (ns) on δ ^{13}C and δ ^{15}N values, as well as the ^{33}P activity in the soil. Values within a column followed by the same letter do not differ significantly (P=0.05) according to Tukey's test. All means are the average of four replicates. ** P<0.01, *** P<0.001.

Conclusion: δ ^{13}C values increased with P amendment and decreased with time. δ ^{15}N and ^{33}P values did not change with P amendment and in contrast to δ ^{13}C, increased with time. We are the first to demonstrate that simultaneous ^{13}C, ^{15}N and ^{33}P labelling is a feasible and sensitive way to follow plant derived C, N and P compounds into soil for a period of at least three months.

Russell C.A. and Fillery I.R.P. (1996) In situ ^{15}N labelling of lupin belowground biomass. *Australian Journal of Agricultural Research* 47, 1035-1046.

Bio-fertilizers may improve nutrient use efficiency in sown pastures

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Introduction: Sown and re-sown permanent pastures are the basis for sustainable meat production and many ecosystem services. The productivity of these ecosystems depends on the availability of high-quality forage crop cultivars, and on optimized management practices. Although it is generally accepted that symbiotic plant-microbe interactions may be used to increase sustainability of sown pastures, the advantages of using bio-fertilizers in these systems is still under discussion (Melo *et al.*, 2018).

Materials and methods: In the framework of the NovInoc project, we designed bacterial consortia (+BIO) formed by a 1:1 proportion of *Pseudomonas* sp and *Bacillus megaterium* identified based on their 16s rRNA which most promoted growth of black oat (*Avena strigosa* Schreb) under greenhouse conditions for 10 weeks. The consortium inducing the highest plant biomass production was used to perform a second cohort of greenhouse and field trials. Both bacterial isolates in the consortium were diazotrophic, P solubilizers and indole-acetic-acid producers. Non-inoculated seeds were used as controls. Distinct soil types were used in the pot and field trials. Fertilization was performed at 70% of the dose recommended by the seed suppliers. The pot assay had 10 replicates per treatment, and for each field assay (1 ha), 6 independent samples were analysed per treatment.

Results: The results showed that the microbial consortia +BIO, when provided with 70% of the recommended fertilizer doses, systematically improved plant productivity (Table 1) and plant N content (results not shown).

Table 1. Productivity of *A. strigosa* fertilized with 70% of the recommended dose of fertilizer (RFD) or with 70% RFD and Biofertilizer (+ BIO).[1]

	70% RFD	70% RFD + BIO	Increment (%)
Pot assay (n=10)	7,100±12%	8,400±11%	18
Field trial 1 (n=6)	8,400±7%	8,820±7%	5
Field trial 2 (n=6)	7,400±10%	8,510±10%	15

[1] Tests were performed in a pot and two field assays. Results represent the mean and the SD.

Conclusion: Although preliminary, these results highlight the increase in nutrient use efficiency that may be obtained in sown pastures by using biofertilizers formulated with diazotrophic and phosphate-solubilizing bacteria.

Melo J., Carvalho L., Correia P., Bastos de Souza S., Dias T., Santana M., Carolino M., Aguiar NO., Canellas LP., Cruz C. and Ramos A.C. (2018) Conventional farming disrupts cooperation among phosphate solubilising bacteria isolated from *Carica papaya*'s rhizosphere. *Applied Soil Ecology* 124, 284-288.

Changes in plant community composition along gradients of land management in São Miguel (Azores)

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Introduction: The natural vegetation in many areas of the world has been completely replaced by pastures and monocultures, leading to biodiversity and ecological losses (Tilman *et al.*, 2002). The Azores are ideal to model those changes, with habitats ranging from natural to completely artificial, at relatively small distances (i.e. Massot, 2015). We aim to test bioindicators providing an integrated insight of the changes in soil microbial community along two gradients of land management intensity (MI) in Azorean grasslands and forests.

Materials and methods: Samples were collected along two gradients of MI: High (grassland-corn rotation or production forest), Medium (permanent grassland or exotic forest) and Low (semi-natural grassland or natural forest). Soil analyses (bulk density, physicochemical parameters) followed standard protocols (e.g. Fernández-Ugald *et al.*, 2016). Plant community composition and cover were assessed for three replicate plots (grassland 2×2 m, forest 5×5 m) per site, and herbarium specimens collected. So far, two sampling seasons have been conducted, Winter (W) 2018, Spring (S) 2018; in total 72 samples [2 ecosystems, 1 gradient, 3 rep (W), 9 rep (S)]. Two more sampling campaigns will be performed in the same period of 2019. We applied cluster, ordination and indicator species analysis to the plant data.

Results and conclusion: Grasslands could be assembled into four groups (Figure 1), indicating a clear gradient from low to high MI. Some seasonal differentiation was found in the grassland-corn rotation (high MI), but not in the other two MI levels. Similar results were found for forests. These aboveground results will allow better understanding of possible differences in the respective belowground microbial communities that are presently being analysed.

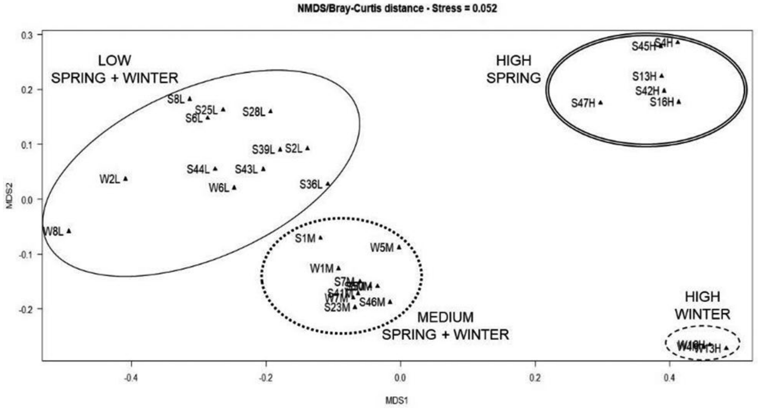

Figure 1. Ordination plot based on Bray-Curtis distance. Results for the grassland gradient. Management intensity: High, Medium, Low; Season: Spring, Winter.

Fernández-Ugald O., Jones A., Tóth G., Orgiazzi A., Panagos P. and Eiselt B. (2016) LUCAS Soil Component: proposal for analysing new physical, chemical and biological soil parameters; EUR 28038 EN.

Massot A. (2015) A agricultura do Arquipélago dos Açores (Delegação da COMAGRI). Departamento Temático.

Tilman D., Cassman K.G., Matson P.A., Naylor R. and Polasky S. (2002). Agricultural sustainability and intensive production practices. *Nature* 418, 671-677.

The interaction between soil biota and soil phosphorus fertilisation influences plant community biomass and composition

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Introduction: Both high residual soil fertility and lack of key soil organisms have been shown to be severe constraints to the restoration of semi-natural grasslands on ex-agricultural land (Reynolds and Haubensak, 2009). However, their effects have mostly been investigated in isolation, leaving their relative importance largely unexplored. Here we aimed to unravel the interactive effects of supplying soil nutrients and a wide range of soil inoculum sources on plant community biomass and composition.

Materials and methods: Nineteen plant species consisting of typical late-successional *Nardus* grassland species and mid- and early-successional species were grown in sterilised soil from reference nutrient-poor grassland, with or without soil inocula from oligotrophic, mesotrophic and eutrophic grasslands. The nutrient treatments consisted of a monthly nitrogen addition (3 levels) during the experiment and a one-time phosphorus addition (3 or 5 levels) at the beginning of the experiment. The data were analysed using mixed-effect models and redundancy analysis (RDA).

Results: We found an interactive effect between soil inoculation and soil phosphorus on plant community biomass and composition (Table 1). Soil phosphorus concentration had a positive effect on plant community biomass in the non-inoculated soils, whereas plant community biomass did not change with phosphorus concentration when soil biota were present. On the other hand, soil inoculation had positive effect on plant community biomass only in the unfertilised treatment. The biomass of late-successional target *Nardus* grassland species was negatively affected by soil phosphorus and inversely related to the biomass of mid-successional species, which benefited most from inoculation and high phosphorus availability. No effect of nitrogen was detected in this study, probably due to nitrogen leaching.

Table 1. Effect of soil phosphorus and soil inoculation and their interaction on plant community biomass and composition (ANOVA results).

Parameter	Community biomass			Community composition		
	numDF	F-value	*P*-value	DF	F-value	*P*-value
Intercept	1	90.6	<0.0001			
Soil phosphorus	1	9.4	0.003	1	8.6	0.001
Soil inoculation	3	12.1	<0.0001	3	3.4	0.001
Soil phosphorus × soil inoculation	3	4.5	0.005	3	2.1	0.002

Conclusion: The interactive effect between soil biota and soil phosphorus on plant-plant interaction plays a significant role in regulating plant performance in mixed grassland communities. Combining abiotic-biotic techniques (e.g. topsoil removal, soil inoculation from target areas) should be considered as necessary for restoration of plant species typical of *Nardus* grasslands.

Reynolds H.L. and Haubensak K.A. (2009) Soil fertility, heterogeneity, and microbes: towards an integrated understanding of grassland structure and dynamics. *Applied Vegetation Science* 12, 33-44.

Theme 7.

Collaboration between practice and research for practical advances

Revising official herbage cultivar evaluation to meet evolving EU stakeholder needs

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Abstract

All new herbage cultivars must pass official tests to prove they are novel entities (distinct, uniform and stable, DUS), with improved value for cultivation and use (VCU) before release into a member state (MS) and then EU-wide marketing. Herbage species present specific challenges as their allogamous nature adds complexity to the conduct and protection afforded in DUS tests. Also, current VCU systems measure production, disease/stress tolerances and nutritional value but do not have the resources to assess animal intake and far less the end product, ruminant performance from herbage cultivars. Also, where these cultivars are used in mixed stands, appraisal of their VCU under these competitive pressures is unassessed. The principles and constraints of the International Union for the Protection of New Varieties of Plants (UPOV) herbage DUS protocols and the resource inefficiency of standalone MS VCU systems are considered. An overview of current research shows that smarter means of testing is possible. This includes a proposal for using molecular markers as an 'mDUS' distinctness tool to solve problems where standard DUS characters cannot distinguish a higher VCU performing candidate from a lower performing registered cultivar, while still conforming to UPOV principles. Newer technologies for assessing VCU are considered but most currently do not provide the accuracy required by legally binding statutory VCU test schemes. Novel VCU characters are identified that can now be adopted to better assess the relative animal intake potential of new cultivars. An integrated pan-European (EU-VCU) test network is proposed, that places MS in data sharing collaborations while retaining their national listing authority. This could reallocate VCU test resources across the EU and make it possible to adopt new animal production indicators and potentially to study performance in mixed stands.

Keywords: cultivar, PBR, VCU, DUS, herbage testing, distinctness, registration

Introduction

Liberty Hyde Bailey (American botanist 1858-1954), created 'cultivar' as a portmanteau of *cultivated variety* to define any plant(s) selected or bred for decorative or useful characters. A cultivar was to be distinct from similar plants, retain its features when propagated and have a unique name. In his view 'variety' should be restricted to the botanical taxonomy of variants within a species, and although his nomenclature has not been strictly applied, the principles of value, distinctness and true-to-type multiplication remain unchanged.

In the early decades of the 20th century, plant breeding largely depended on voluntary levy schemes, which failed to support a profitable and sustainable breeding industry as seed of newly released cultivars moved freely between growers without adequate revenue returning to the breeder. The patchwork of protection schemes in Europe (Czechoslovakia 1921, France 1922, Austria 1938, Netherlands 1941, Federal Republic of Germany 1953) often only controlled cultivar naming, with little restriction on the release of plagiarised copies under other names (Laclavière 1965). Following World War II, political priorities around peace and food security began to break down nationally imposed protectionist measures on agricultural trade (Perren 1995). This involved the introduction of multilateral trade negotiations

(MTNs) reducing tariffs from around 40% to 5% by the late 1980s (Laird and Yeats 1990). In parallel to the General Agreement on Tariffs and Trade (GATT) and the Trade Related Aspects of Intellectual Property Rights (TRIPS), came international guidelines on the protection of cultivars (UPOV 1961, www.upov.int). Today, cultivar registration involves two parallel and independent processes: Proof that the new cultivar is sufficiently novel to be awarded Plant Breeder's Rights (PBR) by distinctness, uniformity and stability tests (DUS); Evidence of exceeding a Value for Cultivation and Use (VCU) threshold (most countries only require VCU if the intended use is for productivity, not for ornamental or amenity use). Successful completion of each process results in official government registration on a National List (NL) and access to that national market. Once listed in any EU member state (MS), the cultivar enters the Common Catalogue, so gaining right of sale across the EU. Typically, these tests take around four years from submission to decision, but DUS can be extended for extra tests and VCU milestones differ with species and MS protocol.

As the PBR and VCU tests are the gateway to the market place, first and foremost they must protect the intellectual property right (IPR) and commercial investment embedded in existing registered cultivars. These schemes must also stimulate and promote the release of 'unique', 'improved' candidates by revealing their novelty and quantifying their improved attributes. Candidates that are not 'unique' and 'improved' are correctly barred from the market. This places enormous responsibility on evaluators to protect breeders with existing profitable businesses while also promoting breeding progress to enhance growers' enterprises, support rural communities, lower environmental footprints and promote food security and quality.

The challenges faced when testing herbage species are made additionally complex as the marketable end-product is not the cultivar's productivity, but the secondary milk and meat produce of a ruminant animal. While this is also true when feeding grains to pigs or poultry, the inefficiency of rumen function compared to monogastric digestion (Table 1), requires an understanding of the adaptive responses to changes in feed composition by the rumen microflora. Responses in the rumen biome to mixed feeds of fresh or conserved herbage with or without concentrate supplementation adds complexity and variability to any nutritional study and can mask or demote the importance of cultivar differences in feed value. In addition, animals are often selective in what they graze, creating differences in the ingested quantity and quality of biomass compared to the machine-harvestable biomass. As applicants usually pay the test costs, financial limits exist on what parameters can be examined and this largely prevents testing of animal responses. Finally, as most herbage species are allogamous, DUS tests must account for the genetic diversity within a cultivar, unlike autogamous/clonal species where plants in a cultivar should be identical (within GxE tolerances).

The remit of this paper was to re-evaluate current EU statutory testing schemes, to assess their efficacy and point to opportunities to better harmonise performance assessments to the needs of ruminant farming and address current farm industry and farm policy challenges. The opportunities and practicalities to achieve this through novel genotyping, new technologies and adopting the latest livestock research is

Table 1. Average gross energy (GE), metabolisable energy (ME) and crude protein (CP) efficiencies (eff) and feed conversion ratios (FCR). Reproduced from Tolkamp *et al.*, 2010.

Species	Production	GE eff	ME eff	CP eff	FCR
Broiler	36-day slaughter, no rearing costs	0.28	0.41	0.52	1.69
Pigs	22-week slaughter, 4 litters/10 piglets	0.21	0.31	0.31	2.99
Sheep	1.72 lambs/ewe, 4 litters	0.06	0.10	0.09	10.4
Beef	Life time – 4 calves/cow	0.06	0.12	0.10	10.4

explored, including the potential for an EU testing network of data sharing alliances, in order to reallocate resources to meet these challenges.

European VCU evaluation strategies

Unlike PBR which is internationally harmonised through UPOV and within the EU by CPVO, VCU schemes differ between MS to reflect local farming practices and growing conditions. The main parameters measured by MS are nonetheless largely similar, though their relative importance can differ greatly (Table 2). The type and amount of data collected is often less if a species is designated as minor in a region. For example, ryegrass, cocksfoot, tall fescue and lucerne are major species in France, tested on at least six sites, with minor species restricted to three sites including the breeder's location. Similarly, the UK tests perennial ryegrass (PRG) at six sites and minor species such as Timothy and Italian/hybrid ryegrasses at three sites, whereas Italy tests PRG (considered as a minor species) at three sites.

Across all these varying schemes is a unifying objective to quantify the gross productivity and herbage quality that each cultivar can deliver by utilizing the incident conditions and tolerating the stresses. The baseline elements involve multisite, multiple years of testing to obtain an accurate mean comparison between each candidate and pass/fail standards that use exemplar national control cultivars and/or the performance range of all listed cultivars.

The performance characters examined typically include annual and seasonal DM production (by plot harvester), herbage quality and persistence. These assessments differ in levels of applied nitrogen, sward use (grazing-Gz, or cutting-Cn), cutting height/frequency and sample timing. So for PRG, the UK imposes an annually rotating 'Cn-Gz-Cn' three-year cycle, from two sowings on six sites; Ireland conducts two sowings each for two years, on separate sites for each management; France only has one sowing for three years of Cn management on up to eight sites; Italy (where PRG is a minor species) has a three-year trial sown at three sites. Disease resistance is also an important attribute, as there are the strong interlinks between high animal performance and high disease (and climate) tolerance in VCU

Table 2. Examples of national protocols for VCU testing of perennial ryegrass cultivars.[1]

Country		Sown trials × test years	No. of trial sites	No of cuts	Kg N ha^{-1}	Seasonality of yield data			Quality			Persistence	
						Sp	Sm	At	D	W	O	Density	Other
Denmark		1 × 2-3	4	4-5	320-360	•	•	•	•	•	•		•
France		1 × 3	5 (+3)	6	140-200	•	•	•			•		•
Germany		1 × 3	10	4-5		•	•	•	•				•
Italy		1 × 3	3	4-5	350	•	•	•					
Ireland	Gz	2 × 2	3	8-10	350	•		•	•	•		•	
	Cn/Gz	2 × 2	2	6									
Netherlands	Gz	2 × 3	3	5-6	350	•	•	•	•		•		•
	Cn/Gz	2 × 3		2+4-5	260								
Norway	Trials A	1 × 3	6	3	200-250[2]				•	•	•	•	•
	Trials B	1 × 2	4	3-4									
Spain		1 × 2		4-7	160-240				•				
Sweden		1 × 2	5-7	3	240				•	•	•	•	
Switzerland		1 × 3	11 (5)	5	250				•			•	•
United Kingdom[3]		2 × 3	6	5-10	260-395	•	•	•	•			•	

[1] All yield assessments by cutter bar harvesting (except where animals used as an overgrazing treatment, e.g. NL and DK). Gz = simulated grazing, Cn = conservation; Sp = spring, Sm = summer, At = autumn; D = digestibility, W = water soluble carbohydrate, O = organic matter.

[2] Determined after soil analysis.

[3] UK employs a Cn/Gz/Cn management for first, second and third year, respectively. Ireland/Netherlands Cn/Gz protocols have 2 Cn +4-5 Gz cuts.

trials. High tolerance protects green leaf driven intakes by preventing rapid leaf senescence in the grasses and leaflet losses in legumes. The frequency and degree of disease pressure and thus the cultivar resistance required differs even regionally within countries. Thus, authorities can in some climates rely on natural infection, while others must employ inoculation tests or field trials often sown with highly susceptible cultivars as infection sources and managed to induce infection of the candidates. All impose a minimum resistance threshold for listing and provide high to low scores for those that get registered.

Talbot (1984) and Conaghan *et al.* (2008) both report, for the UK and Ireland respectively, that the largest component of cultivar performance variation is location by year. These were interchangeable if all trials are within similar climatic and agronomic regions (agrizones). However, cultivar responses to acutely different edaphic classes or climatic conditions must be directly sampled. Nonetheless, within these constraints, the configuration of number of locations and number of trial years is of secondary importance if the comparisons are statistically valid. Hence, due to large locally specific adaptation effects, the lucerne VCU system in Italy grants national listing to cultivars if they demonstrate an improved performance through a high yielding ability in a specific cropping region (as represented by two test sites) or by a wide adaptation (based on mean yield over four contrasting sites) (Annicchiarico, 1992). Similarly, Talbot (1984) showed that production data from Scotland, England, Wales and N. Ireland could be directly combined to produce valid rankings of PRG cultivars for a combined UK national VCU scheme. This demonstrated that data sharing over a relatively wide but defined climatic range is achievable, albeit using a unified test protocol.

Comparing disease and climatic stress tolerance tests between MS authorities appears to show fundamentally different tolerances for the same cultivar. This is understandable, as Turesson (1922) showed that landraces or natural populations feature highly specific adaptation to the environmental conditions where they evolved. Clearly herbage breeding has only partially modified this legacy. Also, once the climatic tolerance of a cultivar is exceeded breakdown is normally rapid and acute. These G×E interactions are best expressed by the genetic correlation (r_g) for cultivar yield responses across environments and cropping conditions. Annicchiarico *et al.* (2015a) reported low and occasionally negative r_g for lucerne or red clover across drought-prone and moisture-favourable conditions (r_g ranging from -0.34 to 0.66; average r_g=0.10). This confirmed the need to evaluate cultivar under these distinct conditions. Also, disease strains can differ sufficiently to make a cultivar (or species) a total failure in one region but unaffected in a different climatic region. This is recognised in turf grasses, where, for example, the Deutsche Saatveredelung PRG cultivar Pascal was bred for the Mediterranean hot, dry climate, while Promotor was bred for winter-hardiness in north-west Europe (Bruins, 2016), where disease pressures will also differ substantially. Hence testing authorities set minimum acceptable standards for these attributes to reflect the regional levels of pressure that vary from critical to negligible. If necessary controlled condition tests are used for the most important diseases, to avoid dependence on getting sufficient natural infection levels every year. So in the Mediterranean climates of southern Europe, the hot dry summers and mild humid winters make drought tolerance and rust (*Puccinia* spp.) resistance essential characteristics for tall fescue and cocksfoot. In more continental climates in eastern Europe, drought tolerance, winter-hardiness and high temperature tolerances are vital and will favour early season growing grasses or annual types to optimise the use of transient moisture availability. In the oceanic climate of mild winters and adequate moisture of northern and western Europe where ryegrasses predominate, disease stresses vary annually and regionally requiring a baseline resistance to rust, mildews (*Erysiphales* spp.), *Drechslera* and leaf spot (*Helminthosporium*). In Scandinavia and mountainous regions of north-western Europe, winter-hardiness and snow mould (*Fusarium*) resistance are essential criteria, and a weakness in a Timothy candidate cultivar can result in a listing refusal despite a clear improvement in yield. Likewise, other authorities will impose minimal disease tolerance thresholds for listing.

Clearly evaluators place a different emphasis on different characters in different regions, and it would be hard to align test protocols across agrizones that have acutely differing growing conditions and farm practices. However, where the terrain of several MS overlap into similar agrizones, it would be feasible for MS to share data but so far, they do not. Moreover, if a MS has a specific growing area that is too small a region to justify a dedicated national VCU test site, data importing from a matching agrizone in another MS would be helpful but does not occur. Paradoxically, once one MS lists a cultivar it can be sold in every EU territory.

Improving herbage VCU evaluation relevance

As indicated, the overall critical challenge in forage VCU testing is to predict the animal performance each cultivar can potentially support, in order to inform herd feed management plans. The primary herbage VCU trait has always been biomass yield over the crop cycle and given its high G×E sensitivity (greater than forage quality, Annicchiarico *et al.*, 2015a), precise measurement is essential. As herbage allowance is a known key driver of animal performance (Dillon, 2006), this character will remain important also in any grazing value assessment. Notably, Aldrich and Elliott (1974) and McDonagh (2017) report similar yield ranking in mowed and grazed PRG trials, but Orr *et al.* (2000) found ranking to differ. Given the costs and production losses of reseeding, grazing persistence, especially at high stocking rates is also important (Frame, 2005), but longevity is not predicted accurately in short-term trials (1-3 yrs) where most evaluators base assessments on final sward densities in mowed plot trials. However, grazing persistency is associated with specific treading and excreting factors that a mowing regime does not simulate. Also, while defoliation frequency can be matched, pulling and preference/rejection impacts are not imposed. Hence conflicting results between mowed and grazed swards have been reported in major forage legumes such as white clover (Evans *et al.*, 1992), red clover (Taylor, 2008) and lucerne (Annicchiarico *et al.*, 2010). However, in Ireland changes in density recorded in on-farm grazing comparisons of PRG cultivars are used to realign the official plot VCU persistency results (O'Donovan, 2016).

To promote better on-farm performance, much progress has been made in chemically assessing cultivars for key nutritive value parameters, particularly with the development of NIRS calibrations for dried sample analysis. Research, such as Wilkins and Humphreys (2003), highlights the role of digestibility, crude protein (CP) and water-soluble carbohydrate (WSC) contents. Peyraud and Astigarraga (1998) have shown that a fall in herbage protein content below 12%, due to reduced N fertilizer use, was aligned with a 2 kg DM reduction in daily herbage intake and a 2.5 kg reduction in milk yield. Similarly, Emile *et al.* (1993) reported an increase of 1.4 kg of milk/day (7%) with a 4% increase in lucerne digestibility. Changes in grass WSC are correlated strongly with changes in net energy (Lila, 1977) and rapid fermentation to silage. They have also been shown to improve rumen efficiency when directly grazed (Miller *et al.*, 2001) or when fed ensiled (Merry *et al.*, 2006).

Relating digestibility to rumen function remains a complex task. It requires a balanced assessment of the value of the nutritionally poor fibre content (Van Soest and Wine, 1967) with the DM digestibility fractions (IVDMD, IVDOMD), while also accounting for factors such as rumen flow rate and microflora adaptation. The chemistry involves separation of fibre as either Neutral Detergent Fibre, (NDF), or the less variable Acid Detergent Fibre (ADF), comprising the cellulose and lignin fractions. Hence, quality assessment of lucerne in France uses ADF for more precise genetic evaluation and lower error. Nonetheless, *in-vivo* digestibility studies, mostly in sheep (Zhao *et al.*, 2017) but also in cattle (Hynes *et al.*, 2016), and for milk yield potential in dairy cows (INRA, 2018; Agnew *et al.*, 2004) have been used to calibrate these data into potential livestock responses. More recent claims by breeders of differences in fibre digestibility (dNDF) are linked to higher rumen passage rates and potentially higher DM intakes. This will be beneficial where performance is limited by intake (Downing and French, 2009; Harper and McNeill 2015). Flexibility of tall fescue leaves was used as an indirect criterion of 'appetibility' and 'ingestibility' in sheep in French VCU testing (Gillet and Jadas-Hécart, 1965), but had to be replaced by direct chemical analysis for better repeatability. Overall, VCU testing for nutritive value has raised digestibility annually by 0.5-1.0 g kg^{-1} DM (Wilkins and Humphreys, 2003; McDonagh *et al.*, 2016), but is less than for yield gains, typically 0.35-0.60% in grasses (Wilkins and Humphreys, 2003; Sampoux *et al.*, 2011; McDonagh *et al.*, 2016) or 0.10-0.60% for legumes (Annicchiarico *et al.*, 2015a).

This 'chemical prediction' approach remains important as direct animal responses in milk or meat output are highly variable and prohibitively expensive. Thus, routine screening of large candidate numbers on multiple sites to the accuracy required by evaluators is beyond current resources. For example, if the price of a mechanically harvested, chemically analysed small plot test is '1', then adding pre/post-grazing off-take and visual herbage response scores would be ×1.5-2, adding animal favouring sward morphology measurements could increase costs to ×5/×6 and measuring livestock productivity (milk/meat) could peak at ×10/×20 (AFBI cost estimates; Gilliland). These are very broad-brush estimates as costs depend on the animal numbers and species used, the complexity of animal characteristics measured and the number of study years. So direct measurement of livestock output is not an achievable objective and to date animal use has largely been curtailed to grazing stressors without measurement (Meehan, 2016), However, recent research is focusing on less expensive parameters linked with voluntary intake potential or grazability and grazing resilience. Grazing resilience is the ability of forage plants to withstand treading, pulling and fouling from the grazing animals and differs greatly between cattle that pull and sheep that bite and also between grasses and legumes. For lucerne, grazing tolerance also implies that the plants have to cope with frequent defoliation that depletes root reserves. There is, of course a long-established strong relationship between greater grazing persistence and smaller leaf size in white clover and a general trend for greater persistence with greater sward density in grasses (Gilliland *et al.*, 2009). While Evans *et al.* (1992) reported good alignment between frequently mowed and lax rotational cattle grazing of white clovers, alignment was poor under close intensive grazing. In grasses, no strong relationships exist and Byrne *et al.* (2017) has reported that simulating grazing by low and frequent cutting fails to replicate how grazing animals will perform.

A recent short review of research points to two avenues of opportunity for evaluations to assess intake estimates (Gilliland *et al.*, 2018). These are firstly to adopt indirect grazing performance predictors and secondly to make evaluations on the cultivars while under the grazing animal. Enhanced animal performance has been observed for PRG cultivars with greater fully expanded leaf content, defined as 'free leaf lamina' (FLL) (Cashman, 2014 and Wims *et al.*, 2013) and for those with higher 'sward leaf content' (Beecher *et al.*, 2015 and Flores-Lesama *et al.*, 2006). The measured differences have been reported to deliver gains of 1.6 kg milk cow^{-1} day^{-1}, (Cashman, 2014; Wims *et al.*, 2013). Similarly, McDonagh (2017) showed a correlation between intake and a high rate of leaf elongation linked to reduced pseudo-stem and true-stem contents. Importantly they found that flag-leaf length in DUS spaced plants is a strong predictor of FLL through the growing season, making it an easily captured indicator of animal intake potential for VCU tests. Furthermore, Tubritt *et al.* (2018) found significant differences in post-grazing heights of 3.7 to 4.8 cm among 30 PRG cultivars when grazed by cattle and proposed this approach as a predictor of grazability. Additionally, simple assessments for reduced secondary heading identified grasses with better grazability and utilisation (O'Donovan and Delaby, 2005). Likewise, Meehan and Gilliland (2019) have shown significant differences in DM content between PRG cultivars, of a magnitude expected to impact animal DM intakes and performance. This can be captured from existing data in standard VCU cut-plot trials. To put this in context, Vérité *et al.* (1970) reported that a reduction in internal DM content of 40 g per kg can reduce intake by 1 kg DM.

In lucerne, significant variation in quality traits was similarly evidenced among cultivars (Julier *et al.*, 2001) and within cultivars (Julier *et al.*, 2000) despite a negative physiological correlation with yield (Lemaire and Allirand, 1993; Julier *et al.*, 1997). However, as digestibility was not correlated with lodging, the possibility to breed for improved yield and feeding value is considered an achievable goal (Julier *et al.*, 2001). Variability of animal performance among all grass and lucerne cultivars presently registered in the French list is shown in Figure 1 as 'feed unit for milk'. The highest range of feeding value, 0.12 feed units, was found in PRG with no significant correlation with either yield in spring or earliness, which are negatively correlated to each other. So, the inclusion of very late heading cultivars of PRG in the French list seems to have impacted more on yield than quality.

All the parameters above are relatively easy and cheap to assess and would substantially enhance the intake/grazability evaluation for grass cultivar VCU. Some indirect methods have already been adopted, such as 'flexibility for management' of grasses in France. This criterion rewards cultivars for greater length of time between the start of growth in spring and heading. It indicates ease of control of grazing quality and a range of 5 days between the most and least 'flexible' cultivar is presently reported within a given PRG heading date group. Typical estimates of the gross potential value to farmers of higher utilization, range around €230-380 $ha^{-1}yr^{-1}$ (dairy) or €180-250 ha^{-1} (beef) per additional tonne ingested (DAERA, 2016) or an additional €181 net profit ha^{-1} (Hanrahan *et al.*, 2018).

Palatability is often heralded as an important intake characteristic, particularly by farmers and some anecdotal evidence does exist from sheep (Freer and Dove, 2002), but linked to levels of moisture and not cultivars. Typically, attempts in France to score PRG cultivar palatability and estimate voluntary intake in sheep was confounded by cultivar phenology and was abandoned due to poor repeatability over rotations and between years. Hence this parameter is unlikely to be adopted by evaluators unless a reliable link to voluntary intake is established.

VCU schemes generally assess cultivars in pure stands (Pu-S), despite many herbage species being grown on farm in mixed stands (Mx-S). GxE interactions for yield across Pu-S and Mx-S tend to increase where higher competitive stress is exerted on the test cultivar, as reported by Annicchiarico and Piano (2005).

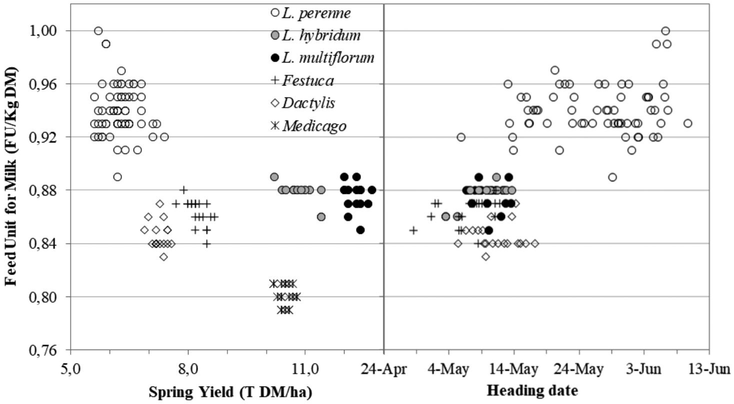

Figure 1. Feeding value of grass and legume species/cultivars as currently evaluated in the French NL since 2010 (data from http://www.herbe-book.org/). Spring yield is the total of all cuts before 10 July averaged for year 2 and 3 from 5 to 8 locations in France. Cutting is managed according to heading date of 1 to 3 control cultivars within each species, and further subdivided into early, intermediate and late heading for *L. perenne*. Feed Unit for milk is calculated from the weighed ADF content in the first three cuts of year 1. One feed unit refers to 1 kg of barley as 1,700 kJ kg^{-1} DM of net energy.

While a fairly high phenotypic correlation between Pu-S and Mx-S was found in lucerne (r^2=0.84), (Maamouri *et al.*, 2017), in the highly competitive white clover species, only moderate consistency has been found (r^2 of 0.53-0.67; Rowe and Brink, 1993; Annicchiarico, 2003). Hence MS such as the UK and France test white clover in mixture with perennial ryegrass. The corollary to this is to evaluate grass cultivars in mixture with a single, highly competitive clover species, as in Switzerland where red clover is used as the companion species. Grass-grass hierarchies also exist and Waldron *et al.* (2017) report a low Pu-S to Mx-S consistency for tall fescue (r^2=0.25) when mixed with a highly competitive companion. Currently competitiveness between grass species is not a VCU character, despite its agronomic importance, largely as separating grass/grass samples is a more skilful and costly task than grass/clover. However, testing cultivars in Mx-S could provide performance comparisons that are more farming relevant than using Pu-S alone.

New technologies and opportunities for improving evaluation in the future

Molecular biology

The emergence of high throughput genotyping may open new possibilities for DUS testing. Several thousand molecular markers can be obtained in forage species with Genotyping-by-Sequencing (GBS) (Elshire *et al.*, 2011), at a cost of €35-100/cultivar, depending on methodology and number of reads (Byrne *et al.*, 2013; Annicchiarico *et al.*, 2017). This has been claimed to offer new prospects for DUS testing (Annicchiarico *et al.*, 2016) and diversity analyses (Byrne *et al.*, 2013), for major genes identification and associated genetic study (Liu and Yu 2017), as well as breeders conducting genomic selection for complex quantitative phenotypic traits (Annicchiarico *et al.*, 2015b).

Satisfying UPOV distinctness requirements (www.upov.int/resource/en/dus_guidance.html) is becoming increasingly challenging in major perennial forages, e.g. lucerne and PRG, owing to the high and ever-increasing number of registered cultivars and their large within-cultivar morphophysiological variation. For example, the overall rejection rate for the grasses and lucerne in the French NL is about one third, with some candidates incurring 1 to 2 extra years of test following failure after a standard 3-year examination (Gensollen, personal communication, 2015). In the UK, candidate rejection rates for *Lolium* spp. and white clover (*Trifolium repens* L.) are around 20%, comprising 12% not distinct and 8% not uniform (Gilliland and Gensollen 2010; Gilliland, personal communication, 2018), though Italian ryegrass can be the more problematic species as the gene pool is more restricted. In general, non-distinctions are more frequent between cultivars from the same breeding programme or with contemporary market leaders. This does not indicate any malpractice but rather that breeders tend to, e.g. 'topcross' with a leading cultivar to import positive genes and select for a more elite type. However, the new synthetic may not adequately diverge from the protected 'topcross' cultivar, owned by another breeder.

Given published evidence, molecular markers would be expected to boost distinction rates in forage cultivars. The ability of such markers to structure a collection of cultivars was shown by Pembleton *et al.* (2016) for ryegrass and by Li *et al.* (2014) and Julier *et al.* (2018) for lucerne. Furthermore, the possibility to exploit GBS-generated markers derived from bulked plants for distinguishing herbage cultivars has been shown by Byrne *et al.* (2013) for PRG and Annicchiarico *et al.* (2016) for lucerne cultivars. Compared to the morphophysiological trait-based DUS criteria, a molecular distinction ('mDUS') was more efficient at discriminating lucerne cultivars (Annicchiarico *et al.*, 2016; Julier *et al.*, 2018). Indeed, they recorded a significant correlation between phenotypic distance and GBS genetic distances but, in contrast to the phenotypic distances, the genetic distances were always significant in a set of 20 cultivars (Julier *et al.*, 2018). It has been suggested that an mDUS approach might both establish distinctness and reduce the number of morphophysiological traits examined to those needed to confirm uniformity and stability and provide a certification description. This might reduce DUS test costs and provide rapid, definitive tests of cultivar identity, to resolve legal disputes on seed lot identity (and composition) and enforce PBR protection in commerce.

However, while molecular markers have been proposed for lucerne DUS testing within UPOV's Biochemical and Molecular Techniques working group (Julier *et al.*, 2017), UPOV has so far opposed marker-based distinctness as its primary concern is to protect the IPR of existing registered cultivars. Therefore, while some argue that mDUS distinction should routinely supplement or replace UPOV phenotypic DUS testing and thereby limit the rejection of non-distinguishable candidates of agronomic value, UPOV considers that the phenotypic-based IPR award given to existing registered cultivars takes precedence. So, if the candidate is indistinguishable using the current DUS tests and has an elite yet similar VCU to the protected cultivar, then even with an mDUS distinction, it is not a new entity as the registered cultivar IPR 'possess that phenotypic space', taxonomically and agronomically.

Even so, rejecting a candidate that is proven to be significantly better performing than the protected cultivar is counter intuitive to the objective of promoting breeding advances. Thus, it is arguable that where a candidate has a significantly different VCU to the registered cultivar and was bred from an independent source, then the DUS test system should not impede genetic gain or its release of into the marketplace. However, when a protected cultivar and candidate are indistinct in UPOV characters, this can be due to the candidate having been bred from that protected cultivar or the same genepool and not sufficiently diverged phenotypically or having been bred from an independent ancestry and phenotypically converged. These juxtaposing scenarios have differing implications for the IPR protection of existing cultivars:

If the candidate is indistinguishable using the current DUS tests:
- Because it has not sufficiently diverged morphologically from a shared genepool of the protected cultivar – Then even if the candidate has a higher agronomic value it would fall within the essentially derived (EDV) liability to the breeder of the original cultivar, regardless of any discriminating mDUS identity.
- Because it has converged morphologically with the protected cultivar, despite being from an independent genepool – Then if it has a 'proven' significantly different/better agronomy and an mDUS distinction, it is 'arguably a new entity' that merits registration.

Current phenotype based DUS tests cannot address this convergence/divergence problem as it requires a genotype examination. Hence, the International Seed Federation accepts a Jaccard coefficient 0.6 based on SSR markers on bulked plants to trigger a reversal of burden of proof to implement UPOV's EDV concept for ryegrass (www.worldseed.org/wp-content/uploads/2015/10/Guidelines_EDV_Ryegrass_Nov_2009.pdf).

So, this proposal introduces a fundamentally new approach in which a statistically significant VCU difference between protected and candidate cultivars could permit use of a statistically valid test of overall molecular distance (e.g. 100's of SNP markers) to confirm convergence (not absence of divergence) and permit an mDUS registration of the candidate. To implement this mode of mDUS supported distinction for herbage species, similar to maize distinction tests in France (UPOV, 2013), would require the prior genotyping of reference collections (essential in any marker based taxonomy) to set an agreed convergence threshold beyond the EDV threshold. It is contended that this new specified mDUS use need not infringe UPOV principles as markers are currently acceptable to UPOV (2013) within other restricted uses: A gene proven to be linked to a phenotypic trait can be used for distinction, though no such gene has yet been described for forage species; Markers can be used to structure a reference collection and assign a candidate cultivar to a subset of control cultivars for phenotypic comparison, thereby establishing distinctions by exclusion of controls. Given UPOV policy restrictions, even if highly-predictive genome-enabled models for the degree of diversity in allogamous species could be developed, it is uncertain if UPOV would sanction a VCU-DUS linked distinction and certainly only if protection of existing listed cultivars was maintained.

Another potential use of molecular technologies is in developing accurate genome-enabled predictive models for VCU production or quality traits. This could theoretically avoid large G×E interactions in field-based testing and reduce the resources needed. Recent genomic selection models for lucerne and ryegrass show predictive levels of 0.25-0.35 for yield (Annicchiarico *et al.*, 2015b; Faville *et al.*, 2018; Guo *et al.*, 2018; Jia *et al.*, 2018) and 0.10-0.60 for quality traits (Fé *et al.*, 2016; Biazzi *et al.*, 2017; Jia *et al.*, 2018) which is not yet accurate enough to replace, or even complement VCU testing, but remains a topic of research.

Digital and other novel technologies

The development of micro-plots as an evaluation platform for reproducing several distinct target environments, may represent a breakthrough in reducing VCU testing costs and improving the data available on responses to climatic variables that undergo large year-to-year variation under field conditions. Of particular value could be the automated 'rainout' shelters (e.g. Kant *et al.*, 2017) that have been used to study drought stress tolerances in terrestrial ecosystems and to impose soil moisture stress in PRG (Laidlaw, 2009). For example, Annicchiarico and Piano, (2005) developed a platform to simulate two drought stress levels by two soil types in 24.0 m × 1.6 m × 0.8 m deep containers equipped with micro-irrigators under a moving rain-out shelter. This has been used to compare lucerne cultivar yield responses to predict adaptation to contrasting sites in northern Italy and to compare both pure stand and mixed stand cropping capability.

Digital image analysis has been lauded as an economic means to estimate plot yields. However, the relationship between estimated and observed cultivar biomass yield (r^2=0.54-0.72; Inostroza *et al.*, 2016) is currently too low to be accepted for VCU trials, as inaccurate trait estimation may have greater negative consequences (rejecting an improved candidate) than in breeders' selection trials (discarding a promising parent). However, based on preliminary results (Himstedt *et al.*, 2010), this technology may be more useful for estimating proportions in legume/grass swards and could streamline VCU testing in mixed stands. Extending this use to grass-grass separations remains a greater challenge. Likewise, sward plate meters are becoming useful to estimate yields on farm and are an excellent grazing management tool. Recent WiFi linked plate meters can now send readings by mobile phone to the 'cloud' for real-time analysis. However, across a range of farm and test sites, the correlation accuracy to direct yield measures was only around r^2=0.7-0.85 (AFBI internal records) which is insufficient for VCU yield comparison of cultivars.

Near infrared reflectance spectroscopy (NIRS) has already proven to be faster and cheaper than chemical analyses and yet provides calibration equations at a high precision (R^2>0.95). It is currently used widely to measure fibre, protein and water-soluble sugar contents on dried samples in VCU trials. The innovation challenge is to omit the drying step and scan fresh tissue. Ultimately mobile NIRS scanners, either handheld or harvester mounted could provide rapid in-situ nutritional spectra for several parameters. Handheld NIRS is currently being used for on-farm silage analysis; however, fresh sampling presents difficulty in sample homogeny. Also, while lab based NIRS machines typically operate at 400-2,500 nm, hand held equipment typically has a reduced range (1,100-1,600 nm) and if full access to the software is restricted by the manufacturer it is not possible to perform the chemometrics to develop tailored calibrations. Furthermore, the precision validation checks conducted on laboratory NIRS equipment and the control of test conditions that ensures accuracy of prediction is less achievable for mobile units. So adoption of this technology into VCU tool boxes is not imminent as it requires improved functionality and procedures to curtail the random variation.

Technologies for non-destructive measurement of grass yield are also developing in capability, including remote sensing drones, WiFi linked digital weather stations, pasture optical/laser sensors, digital pedometers/rumination halters and GPS location systems. It is now possible to employ virtual fencing to precisely control where livestock graze, conduct in-field automatic water and animal weight recording and set up digital soil sensors to monitor temperature, moisture and nutrient flows. AFBI is currently building such a 'Precision Grassland Platform' at its Hillsborough Research Farm, with a network of over 25 data relay towers streaming real-time data from the platform to create a connected landscape of soil, plant and animal data. This is a high cost forefront research facility, likely beyond the commitment of most VCU schemes. While ideal for studying grazing behaviour, it has yet to be proven suitable for comparing large numbers of cultivars. Likewise, while some of these technologies are proving useful for monitoring sward growth and growing conditions, including on farms (e.g. www.agrisearch.org/grasscheck), precision levels are not yet sufficient to reliably rank cultivars in VCU trials. Given the speed of development in digital systems, it may become possible to make sufficiently precise measures of animal performance potential on large cultivar numbers and so these technologies need to be kept under constant review.

A vision for an 'EU-VCU' evaluation scheme

'EU-VCU' stands for a concept of an integrated pan-European herbage test network involving MS in data sharing collaborations. When examined in detail, differences between national VCU schemes seem substantial, but in a more general way follow similar processes and assess largely the same characters in a given species. So, these differences reflect regional best practices and while this diversity in procedures is justified, it need not preclude data sharing towards a common goal of ranking cultivar production potential under incident conditions. Hence, a single common test protocol is not obligatory in a data sharing scheme.

Given the need to limit breeders' fee charges, the number of VCU sites and years is typically set at the minimum necessary to obtain statistically valid, legally defensible MS pass/fail decisions. In addition, although animal value potential is the ultimate VCU goal, the parameters examined are predominantly the easy, relatively cheap, indirect criteria in the absence of animals. Sharing data within agrizones that cross political borders could allow MS to redistribute some of the resources currently only used for traditional characters, to assess informative animal performance characters. Some of these are easy to capture, such as FLL, DM% and pre-post grazing heights. MS sharing an 'agrizone' would have access to more data and from wider conditions, benefiting farmers in regions of their territory not serviced by a national test site. A MS could also be in more than one agrizone collaboration.

A partial model of this already exists, albeit for turf grasses, whereby Geves in France utilizes data from seven locations in France and five abroad (UK, Ireland, Germany, Netherlands and Spain), though mutual data sharing is not involved (Gilliland and Gensollen, 2009). While forage cultivar performance is arguably more regionally specific than turf grass quality, this network demonstrates the baseline principles for a forage data sharing scheme across MS.

Implementation challenges for an EU-VCU scheme

The practicality and wider consequences of an EU-VCU scheme need to be fully considered as there are both operational and organisational challenges to be addressed.

Establishing agrizones

The identification of agrizones should be readily achievable, as good examples already exist, such as the agro-climatic zone approach of van Wart (2013) to predict crop yield potential and the environmental classification of Europe produced by Jongman *et al.* (2006). The process would initially depend on the size and pattern of GxE interactions for herbage yield, with the distribution of disease and climatic stresses overlaid, but with quality characters likely to be least regionally variable. This demarcation in agrizones on a species by species basis could be achieved by a multisite examination of a relevant and broadly-based set of reference cultivars. According to statistical theory (Gauch, 1992), by prior modelling of cultivar yield responses via AMMI analysis or factorial regression analysis (Annicchiarico, 2002), future cultivar responses can be better predicted than by extrapolation from observed data. Modelling cultivar responses as a function of key environmental factors could be used to predict cultivar performance across the EU (and potentially adjoining countries), based on mean/long-term environmental data provided in a geographic information system. Therefore, an evaluation network across, e.g. MS, followed by G×E analysis would be the means of revealing redundant information and diverting resources to examine new animal intake and grazability predicting parameters.

From a breeders viewpoint rewarding cultivars for regional adaptation could better align with their selection criteria. For example, in northern Italy G×E interactions produce striking inversions of cultivar ranks between locations with contrasting summer drought stresses and soil type (Annicchiarico, 1992). Cultivars bred for highly heritable differences in root and aerial adaptive traits (Annicchiarico, 2007a) gave higher yields under specific conditions than widely adapted cultivars (Annicchiarico, 2007b). Furthermore, with the key climatic and agronomic features of each agrizone defined (e.g. different pathogens or moisture availability etc), this should identify the morphophysiological type of candidate cultivar required for that region. So categories of autumn dormancy in lucerne, summer dormancy in tall fescue and cocksfoot, leaf size class in white clover, reproductive phenology in grasses and growth habit in several species, could be determining factors. For example, lucerne cultivars with dormancy scores of ≤4.5 and ≥7 are known to be adapted to cold northern European and mild-winter southern European environments, respectively, because of the positive relationship between dormancy and winter hardiness (Annicchiarico *et al.*, 2015a). Likewise, small-leaved white clover or semi-prostrate lucerne are bred for grazed environments and would not suit agrizones where mowing predominated. In contrast, generally adapted morphophysiological types (e.g. erect lucerne with dormancy class >4.5 and <7 or highly winterhardy, disease resistant PRG) could be suitable for submitting into a wide target region encompassing several agrizones and MS.

In adopting new animal performance characteristics such as indicators of intake and grazability, it is not essential (though desirable) to know the precise magnitude of animal benefit. Evaluation is about achieving a progressive improvement in new candidate submissions over time and this can be achieved by expert panels agreeing indices to rank cultivars and inform listing decisions. So, there are no insurmountable impediments to immediately importing such parameters into standard VCU evaluation procedures.

Ensuring decision making is not compromised

There is a key risk that authorities and breeders might view an EU-VCU as an opportunity to reduce test sites and so registration costs. However, an overall reduction of VCU investment and testing sites from the current country-based system by using fewer sites scattered across Europe, would negatively impact on both breeders businesses and breeding support for national agri-food policies. This is because pass/fail standards are determined by the underlying accuracy of the tests. For example, in the UK the range of expression in the NL for each VCU character is used to calculate pass/fail standards. A candidate with a performance one LSD above the mean is scored +1/+2, but given -1/-2 if one LSD below the mean, for minor and major characters, respectively (Figure 2). Candidates with a positive total over all VCU characters are expected to be listed, if negative to be rejected and if zero require additional attributes to be listed.

As Figure 2 shows, if precision is reduced by having fewer (or more dispersed) sites, the confidence limits widen and more varieties are rejected for failing to meet clear improvement pass standards. This sets farmers' risk above breeders' risk, as the pass standard rises and so ensures no candidates that could damage farmers' enterprises are listed. Breeders lose good candidates that exceed existing cultivars, but not sufficiently to exceed the wider confidence limits. So, a statistical assessment is needed to design data sharing protocols within agrizones.

Winning stakeholder commitment

At the outset, it is vital to recognise, understand and address the negative reactions that stakeholders might initially have to this EU-VCU proposal. Breeders might at first view it as additional complexity overlaying an already multifarious process of individual national schemes and worry if test costs will increase. Evaluators may view it as additional work, a potential loss of control and a risk of test site closures and reduced funding. Governments will likely have concerns regarding legalities and liabilities for accepting candidates that will be partly tested outside of their control and fear loss of listing authority. Equally concerning, is that they may see an opportunity to cut back their testing schemes. Seed merchants might fear that more regionally specific listings will divide up seed markets and add costs through the need to carry additional varieties for specific regions within their sales area. Farmers are least likely to be resistant as they are only concerned in the end point recommendations and if these are more specific to their farming region, this will be an advantage. A similar positive view from EU policy makers is probable as an EU-VCU has more of a pan-EU functionality than the existing independent MS schemes. However, if the EU sought to use this proposal to implement centralised VCU registration, resistance from MS and breeders can be expected. There will likely also be hurdles due to decision makers in some MS only being government officials (e.g. Ireland, Germany – Federal and Bundesländer), whereas Wageningen University in the Netherlands oversees a Consultancy Group of industry, breeders, end-users and testers.

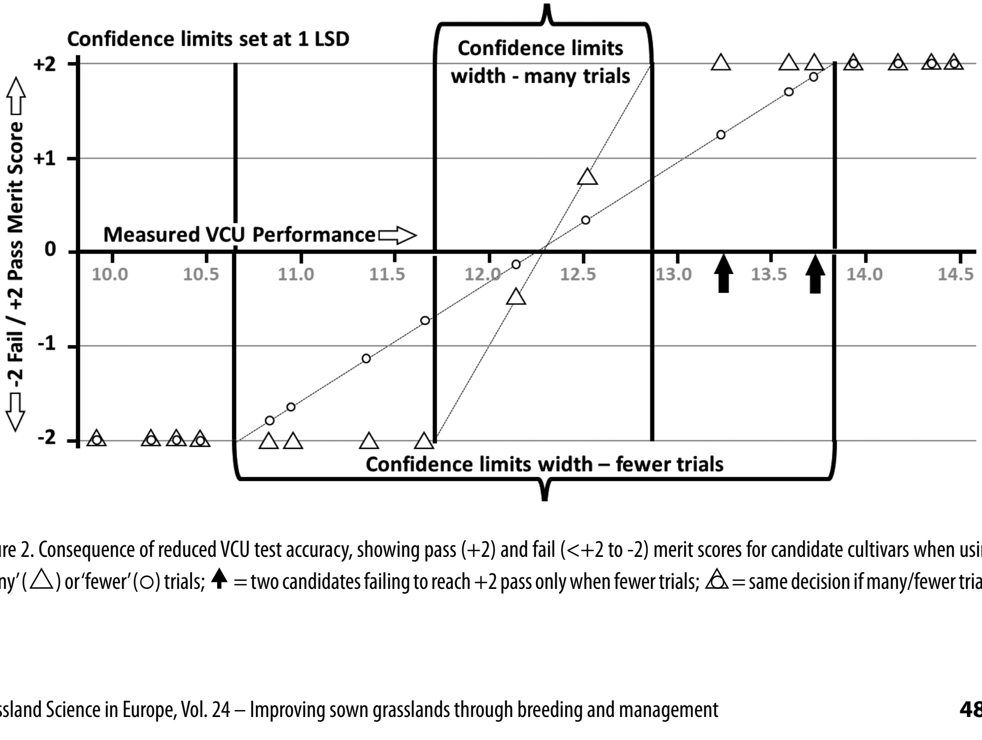

Figure 2. Consequence of reduced VCU test accuracy, showing pass (+2) and fail (<+2 to -2) merit scores for candidate cultivars when using 'many' (△) or 'fewer' (○) trials; ⬆ = two candidates failing to reach +2 pass only when fewer trials; ◬ = same decision if many/fewer trials.

In France the Ministry of Agriculture takes account of a consultative committee (Permanent Technical Committee of Selection) comprising breeders, public representatives and end-users. This overlays a diversity of funding streams (official, levy body, fees and industry support), that are often specifically justified to support only the markets within that territory.

So, despite clearly identified scientific benefits, allaying the concerns of stakeholders will be a substantive and delicate task. This process must begin with stakeholder engagement and this review paper can be the catalyst. Progress will require an independent body like the EGF or Eucarpia to champion the proposal and open discussions initially with EU policy makers and CPVO and then all interested parties. Imparting a balanced and complete understanding to all parties is vital. Most critically is that the EU-VCU model seeks collaboration between testing authorities, not the stricter rigor of the prescriptive CPVO or UPOV accredited DUS tests.

The key factors in the proposed model are:

- MS agree the cross-border agrizones for a species and the division of tasks for assessing 'standard' and 'animal-value' characters, ideally in a workload neutral arrangement.
- Sowing of common control cultivars to act as bridges across political borders within agrizones – some generally adapted types across multiple agrizones plus some specialist locally adapted types for specific agrizone standard setting.
- MS accept each other's candidates in their agrizone test sites on a full cost recovery basis.
- All test centres in an agrizone do not need to impose precisely the same test methodology to generate ranking for the same VCU parameter.
- Test centres to comply with agreed quality assurance standards and best practices, including management of the shared data base with each MS having 'real-time' full access.
- MS retain own listing authority, applying their own decision standards and indices.

So, assurances will be needed that MS authority will not be usurped, that an EU-VCU is better for the local agri-food economy, that there are no threats to the operations of the evaluators and that funds targeted for local jurisdictions are not being diverted elsewhere. Breeders already make decisions on which MS to seek listing, but instead of targeting a politically encapsulated market, they would target one or more agrizones, based on the selection criteria of their candidate cultivar. Some might be bred for broad adaptation and others to optimise performance under specific conditions and cultural practices. This would be attractive both to large corporate and small regional breeders as long as testing authorities reward cultivars equitably for improvement in either localised or generalist performances, which already occurs within most MS schemes. Nonetheless, as most breeding companies are internationally active, an EU-VCU could provide the means to focus variety distribution into targeted markets, where their genetic gain can impact most on sustaining grassland farming.

Conclusion

Stewart and Hayes (2011) reported that in countries where national lists strongly influence the market success of a cultivar, breeders' selection criteria are designed to target improvement in those test characters, placing performance on farm as a secondary priority. Current economic forecasts for the EU ruminant livestock sectors predict weakening product prices, rising labour, feed and infrastructure costs and further migration of subsidies away from productivity to public good priorities. Hence the common driver across the 150 million hectares of EU grassland is for an ever larger proportion of animal productivity to come from grazed swards and home grown forages. This is accentuated by global, public and political pressure to reduce GHG, ammonia and nutrient loss from ruminant farms (IPCC, UNFCCC) and for higher animal welfare. Added to this are increasing demands from leading farmers for more informative data on how new cultivars will benefit their herd's performance. Given the evidence of a dominant influence on breeding priorities and these external pressures on ruminant farming, evaluators must actively revise their test criteria to keep pace with the grassland practices of the most progressive farmers. Central to this is a major VCU challenge to better measure the ruminant intake and ingestion potential of grass and legume cultivars.

A key impediment is that the forage breeding sector generates weak incomes compared to the grain sectors. This places a downward pressure on test fees and often what funding governments are willing to invest. This compels evaluators to devise frugal test schemes and curtails their capacity to respond to increasing demands for livestock performance data that would increase testing costs. An EU-VCU scheme could release resources to conduct new evaluations while allowing MS to manage their NL and underpin the competitiveness of their agricultural industries, contribute to environmental protection, actively protect food quality and sustain their rural communities. Importantly, by data and task sharing, resources could be released to test cultivars for competitiveness in mixed stands for a more end-user relevant VCU evaluation. Moreover, sufficient scientific evidence exists to now devise effective testing protocols that incorporate grazability and intake indicators, as identified in this paper, to better rank cultivars for animal value, and meet the demands of leading farmers in grass-dominant EU regions. Relevant to this is an EU funded project (INVITE 2019) with 27 organisations in 9 MS that will be examining options for innovative evaluation of ryegrass, lucerne and 10 other forage species. Thus is now the time for the EU and MS to start work towards an EU-VCU strategy for herbage species, but on a data sharing rather than a prescriptive 'one size fits all' basis, which often underpins EU policy backed developments.

It is equally important that the DUS schemes do not impede the release of cultivars with improved VCU and particularly any with enhanced animal performance parameters. It will always be necessary to ensure that an allogamous cultivar is a stable entity with a definable VCU. This requires proof of uniformity in its inflorescence emergence and the morphological characters that contribute to full inter-pollination and competitive ability in swards. Therefore, DUS in herbage cannot be awarded without some phenotypical characterisation. Nonetheless, recent evidence on molecular techniques demonstrates that they are among the most powerful discriminating tools available, best able to distinguish between more and more phenotypically similar candidates. On-going molecular research priorities have sought to improve DUS test stringency, speed and cost base and define optimal kinship thresholds to ensure genetic distances between cultivars do not erode. Hence, an mDUS protocol can now be devised to resolve specific distinction problems between candidate-registered cultivar pairs. This would be specifically for the intolerable circumstance when a candidate with improved agronomy cannot be distinguished by current DUS protocols from a significantly poorer registered one. The critical factor will be to unequivocally retain the current IPR protection for the registered cultivar, with an EDV threshold as a final backstop.

Regarding other opportunities to improve VCU testing, innovations in digital technologies, machine vision, portable NIRS and major production gene identification currently function beyond the stringent requirements of VCU evaluation. Nonetheless, all need continual monitoring given the advancements achieved to date. Likewise devising short-term measures of long-term persistency, particularly under the grazing animal and resolving the dynamics of cultivar performance in mixtures are current challenges and an ongoing focus for research.

Overall, evaluators must stimulate and reward breeding innovation that better meets clearly defined local farming requirements and communicate those benefits in a modern way to promote their uptake. In essence none of the above is a departure from the principles set out by Liberty Hyde Bailey around 150 years ago, that a cultivar should be 'distinct from similar plants', 'retain its features when propagated' and 'have useful (farming) characteristics'.

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‘Grassland Compass’: a practical tool to improve grass production and grass utilization on Dutch dairy farms

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Abstract

On Dutch dairy farms there is an increasing need to improve grassland production and grass utilization while at the same time reducing the environmental impact because of sustainability goals, legislation on manure management and recent intended policies. The net dry matter yields from grasslands range from 8 to 19 t DM ha^{-1} y^{-1}. This range is caused by variation in farm management, which implies that there is room for improvement. However, for farmers it is often unclear which actions need to be taken, in which field, and in which part of the management (soil, sward, manuring, grazing, silage making, conservation). There are a number of farm decision support tools to improve the grassland management. However, a tool that provides a practical overview of all the strengths and weaknesses of grassland management on a particular farm has been lacking. The ‘Grassland Compass’ project developed a practical tool for both farmer and advisor with the aim of improving grass production and utilization at individual farm level. The project is an initiative of Dutch Universities of Applied Sciences in collaboration with feed industries, advisory services and dairy farmers. In spring 2018, during six weeks prior to the first cut, a prototype of ‘Grassland Compass’ was evaluated on 33 Dutch dairy farms. Results suggest that ‘Grassland Compass’ provides useful suggestions for improvements in grassland production and grass utilization.

Keywords: grassland production, grassland utilization, decision support tool, KPI

Introduction

For Dutch dairy farming, it becomes increasingly important to optimize the available farm resources. Sustainability goals in the dairy chain, legislation on manure management and new policies increase the need to optimize available resources. Dry matter yields from grasslands can differ by more than 10 t DM ha^{-1} y^{-1} in the Netherlands, with averages ranging from 8 to 19 t DM ha^{-1} y^{-1} (Aarts *et al.*, 2005; Van Eekeren *et al.*, 2010). These differences in yield can only partly be ascribed to differences in soil type and growth conditions. They are mainly attributed to differences in the dairy farmers’ management, such as differences in land use or in input of nutrients (Aarts *et al.*, 2005). This implies a potential for improvement of grassland production. Existing farm tools, e.g. ‘Kringloopwijzer’ (Annual Nutrient Cycling Assessment; Aarts *et al.*, 2015), provide quantitative performance data on grassland production, grass utilization and nutrient cycling at farm level but do not provide an overview of the strengths and weaknesses in the grassland production chain. A consortium of Dutch Universities of Applied Sciences, feed companies, advisory services and dairy farmers has therefore developed Graslandkompas (‘Grassland Compass’ (GC)). The aim of the GC-project was to develop a tool for farmer and advisor to get insight in the strengths and weaknesses of the grass production and utilization chain. The design of this tool should stimulate dairy farmers to carry out actual measures to improve the grassland production. This study aims to test the on-farm functionality of the GC (‘proof of principle’) by testing it on Dutch dairy farms.

Materials and methods

GC has a layered approach. Six ‘areas of influence’ (‘wind directions’) were identified that affect the process of grass production and grass utilization: soil, sward, manuring, grazing, silage making, conservation. Soil and sward relate to production factors, while the other wind directions relate to activities. Each wind direction consists of a number of key performance indicators (KPI). A KPI consists of a set of performance indicators (PI). PIs should meet three criteria: (1) they must be related to grassland production and/or utilization; (2) the outcome can be influenced by the farmer; and (3) they should be available or easy to determine during an on-farm test. Expert judgement was used to score the possible outcome of each PI. A PI was scored from 1 (poor) to 5 (good). KPIs were calculated as the weighted average of PIs (1-5 with intervals of 0.5). The wind directions were calculated as the weighted average of the KPIs. The arithmetic mean of the six wind directions led to the final GC score. Each wind direction consists of 3 to 6 KPIs. A KPI consists of 1 up to 15 PIs. GC was tested on 33 dairy farms in the Netherlands during a six-week period prior to the first cut in April-May 2018. Project partners nominated farmers who were interested in grassland management for testing GC on their farms. The common Dutch soil types of sand, clay and peat were represented with 14, 16, and 3 farms, respectively. Both the dairy farmer and advisor were asked to do a pre-test evaluation of the wind directions of GC, i.e. they were asked for their expectations of the score for each wind direction. Data collection was done through (1) retrieval of data from existing resources (e.g. soil or silage analyses results or results from Kringloopwijzer (Aarts *et al.*, 2015); (2) an interview with the farmer; and (3) field measurements (e.g. visual evaluation of soil structure and sward quality). The results of GC were discussed with the farmer and compared with the pre-test evaluation. A difference of 1 or more between the pre-test evaluation and the GC-result was considered to be relevant.

Results and discussion

The average milk production of the farms was registered (9,200 kg cow^{-1} y^{-1}) and is consistent with the Dutch average. 55% of the participating farmers had a BSc-education level. 52% of the farmers were aged between 20 and 39 years. Most farms had a GC above average (above '3') (Table 1). The KPI 'drainage' in the wind direction 'soil' often received the maximum score (28 out of 33 farms). Low scores in 'manuring' were mainly due to N surpluses and/or P_2O_5 surpluses. Low scores in 'silage making' were attributed to the PIs 'drying period', 'cutting height' and 'chop length'.

The wind directions 'soil' and 'grazing' have been assessed lower in the pre-test evaluation than the GC-result (Table 2). Farmers and advisors felt that they did not have enough knowledge regarding these two wind directions. 'Silage making' was assessed higher than the GC-result. Correlation between the GC result and farmer and advisor results was poor; $R^2<0.11$.

Some PIs were found to be subjective, so further validation is necessary. Some PIs showed seasonality, e.g. the PI rooting depth, which tends to be lower early in the growing season (Deru *et al.*, 2010). It is further recommended to always use GC in the same season when monitoring developments. Use of GC in spring is therefore recommended. The on-farm test of GC proved to be an excellent instrument to initiate

Table 1. Number of farms for each calculated mean score of GC and per wind direction (-).

Item	Calculated mean score								
	1	1.5	2	2.5	3	3.5	4	4.5	5
Grassland compass					3	16	14		
Wind direction									
Soil					1	4	7	19	2
Sward				1	10	10	11	1	
Manuring			1	5	10	11	4	2	
Grazing[1]					4	9	16	2	
Silage making			3	6	6	10	6	2	
Conservation			1	3	17	10	2		

[1] Two farms did not apply grazing.

Table 2. Difference in scores between GC and assessors per wind direction, both for GC higher than assessors and lower than assessors (number of farms per assessor or combination of both assessors).

Wind direction	GC higher than assessor			GC lower than assessor		
	Farmer	Advisor	Farmer and advisor	Farmer	Advisor	Farmer and advisor
Soil	10	5	4	0	0	0
Sward	2	0	0	1	1	0
Manuring	3	2	1	4	5	3
Grazing	5	2	2	0	0	0
Silage making	0	0	0	11	7	4
Conservation	1	3	1	4	4	3

discussions between farmer and advisor on improvements of grassland production and utilization, as the measures are farm specific.

Conclusion

The results of this study show that there are differences between the subjective evaluation of grassland production and grass utilization on individual farms as assessed by GC, farmers and advisors. Whether GC is able to provide useful insights into strengths and weaknesses in the grassland production and utilization chain depends on the reliability of the indicators and on the quality of the input data. Nevertheless, GC has shown that it can provide useful suggestions for improvements in grassland production and grass utilization.

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Estimating milk production potential of perennial ryegrass varieties

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Abstract

Substantial varietal differences exist among grass species in providing forage yield and quality that consequently affect milk production from pastures. Therefore, it is imperative to estimate the forage value of perennial ryegrass varieties in order to provide information to producers and breeders for more educated decision-making. The objective of this study is to quantify the relative value of different perennial ryegrass cultivars for milk production in coastal Oregon, USA. The current study investigated the dry matter (DM) yield and nutritive value of 20 perennial ryegrass varieties and estimated their milk production potentials in a replicated experiment in 2014-2016. In 2015, the total annual DM yields of the varieties ranged from 14,014 kg ha^{-1} (cv. 'Oroverde') to 16,908 kg ha^{-1} (cv. 'Trojan') but the difference was not significant (P=0.24). In 2016, cv 'Alto AR37' provided the highest DM yield of 19,494 kg ha^{-1}, while the lowest DM yield of 16,073 kg ha^{-1} was obtained from cv. 'Albion' (P<0.05). The varieties were ranked for relative feed value and estimated milk production using an index based on their net energy for lactation (NEl). Estimated milk production per hectare ranged from 30,789 to 26,506 liters with the cv. 'Aberzest' providing the highest estimated production (P<0.05).

Keywords: forage value index, milk potential, pasture quality, perennial ryegrasses

Introduction

Producers often renovate pastures based on seed availability and perceived adaptability with no real appreciation for the genetic selection, testing and breeding efforts that went into developing that cultivar. The grass seed industry in Oregon has had difficulty over time explaining the economic merit or value of an individual grass cultivar to the livestock industry. Chapman (2016) reported in New Zealand that environment × genotype interactions may change the way one cultivar performs in a specific environment compared to others. In Ireland, researchers developed a program to develop an economic ranking index for perennial ryegrasses (McEvoy, 2011). The economically important traits selected in their system were seasonal DM yield, forage quality, and sward persistency. Like all indexes, each parameter in the index had a different weight based on economic importance. In New Zealand, researchers have developed an economically based a forage value index (FVI) that ranks cultivars according to their overall value to a dairy farm business. Currently, there is not a (FVI) in place in the United States. There is paucity of the information on the expected performance differences between forage cultivars. Thus, the objective of this study is to develop a FVI for perennial ryegrass cultivars ranking in Oregon. Quality measurements weighted for energy are the driver of this index as energy is the single most limiting factor for milk production or growth in high quality perennial ryegrass pastures.

Materials and methods

This experiment was conducted on a silty loam soil in Tillamook, Oregon, USA in 2014-2016 growing seasons. Following cultivation and seedbed preparation, 20 perennial ryegrass varieties were broadcast, seeded on 1×3 m plots at the seeding rate of 18 kg/ha in the autumn of 2014. The experiment was a complete randomized block design with three replicates. All plots were fertilized with 300 kg ha of N as urea annually both years. Plots were irrigated and harvested six times with a Swift Current forage harvester (Swift Current, Saskatchewan) in both years. All yield measurements were recorded, and samples analysed using Near Infrared Reflectance Spectroscopy (NIR) at Dairy One Lab, Ithaca, NY.

A forage value index was developed to rank individual varieties and estimate milk production potential by using the forage analysis to determine to total net Mcal produced per ha for each variety. The total NEl was then used to calculate the milk production potential, using 0.89 Mcal (3.7 MJ) to produce one kg of milk (Weiss, 1999). With these values, we calculated how much total net energy per hectare was produced by a variety and estimated litres of milk potential per hectare equivalent. The data were analysed by analysis of variance (ANOVA) with three replicates as a randomized complete block design. Significant differences among treatment means were compared by Fisher's protected LSD at α=0.05.

Results and discussion

In the year of establishment (2014/2015), the perennial ryegrass varieties provided similar DM yields (*P*=0.24). In 2016, the top DM producer was cv. 'Alto AR37' at 19,494 kg ha^{-1}, while the lowest was cv. 'Albion' at 16,073 kg ha^{-1}. Energy from pasture is the result the digestion of soluble and structural carbohydrates, proteins, starch and fats. Net energy for lactation (NEl) is the estimated feed energy available for maintenance and milk production after digestive and metabolic losses and is calculated by analysing for the digestibility of all fats, carbohydrates and protein.

In addition to DM for years 1 and 2, Table 1 shows the FVI for both growing seasons combined, the relative value compared to the others in the study, and estimated litres of milk per hectare.

Table 1. Perennial ryegrasses ranked for milk production index, relative value, liters milk ha^{-1}.

Varieties	Total annual DM yield (kg ha^{-1})		Index value	Relative feed value	Estimated milk production[1]
	2015	2016			(litres ha^{-1})
Aberzest	16,178	19,318 de	27,938 f	108	30,789 f
Trojan	16,907	19,214 de	27,545 ef	106	30,356 fe
Aberstar	16,045	19,138 de	27,514 ef	106	30,322 ef
TrojanNEA2	16,607	18,731 bcde	26,983 def	104	29,736 def
Alto	16,092	19,181 de	26,794 def	103	29,528 def
BealeyNEA2	15,334	18,895 cde	26,685 def	103	29,409 def
Dromora	15,454	17,898 abcde	26,535 cdef	102	29,243 cdef
Remington	14,989	17,901 abcde	26,500 cdef	102	29,204 cdef
Elgon	15,777	17,932 abcde	26,497 cdef	102	29,201 cdef
Calibra	14,695	17,970 abcde	26,469 bcdef	102	29,170 bcdef
Alto AR37	14,323	19,494 e	25,789abcdef	99	28,421abcdef
Polim	14,823	17,259 abcd	25,541 abcde	98	28,147 abcde
Dunlace	15,209	16,789 ab	25,474 abcde	98	28,073 abcde
Bealey	14,912	17,560 abcde	25,280 abcde	97	27,860 abcde
Drumbo	14,867	16,863 abc	25,057 abcd	97	27,614 abcd
Kentaur	14,200	17,189 abcde	24,875 abcd	96	27,414 abcd
Albion	15,112	16,073 a	24,820 abcd	96	27,353 abcd
Oroverde	13,678	16,490 a	24,211 abc	93	26,682 abc
Tyrella	14,839	16,448 a	24,127 ab	93	26,589 ab
Tetragain	13,922	16,715ab	24,051 a	93	26,506 a
SEM	687.8	656.3	827.2		911.7
P-value	0.24	0.05	0.05		0.05

[1] Milk production estimates based on the assumption 0.89 Mcal (3.7 MJ) to produce1 kg of milk.

Conclusion

The DM yield results have indicated that the majority of the ryegrass cultivars from around the world performed well on the Oregon coast. However, substantial differences in performance were identified. Potentially, there were several different approaches that could have been used to develop an index for milk production, but energy is the most limiting factor in determining milk production in grazing dairies. Accounting for energy derived from the digestion of fats, proteins, soluble carbohydrates and structural fibre is a useful way to estimate performance and rank cultivars. The difference in the estimated milk production per ha was as high as 4,283 litre for the varieties tested. One can extrapolate this difference in milk production potential is worth between 1,500 and 1,800 US dollars per hectare. This clearly indicates the value of developing FVI for ryegrass varieties that provides science-based information to farmers and breeders to improve perennial ryegrass variety selection and development.

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A method to lead discussion groups for the analysis of grassland innovations

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Abstract

Capturing innovations from farmers and spreading them into practice requires a comprehensive analysis, combining the expertise of actors from science and practice, to identify requirements, strengths and weaknesses of innovations. Within the Inno4Grass H2020 thematic network, a framework for innovation analysis in participatory, multi-stakeholder discussion groups has been provided. A method comprising five elements was developed: (1) briefing, ensuring baseline knowledge sharing, (2) simplified, brainstorm-like SWOT-analysis, (3) PESTLE-analysis (Political, Economic, Social, Technological, Legal and Environmental factors), (4) exploration of predefined sub-topics and (5) standard questions to the innovator. The method was made available to the project partners, along with more general suggestions, in form of non-compulsory guidelines. A survey of the first 55 discussion groups revealed that the guidelines were used in 57% of the meetings. The frequency of application of the single elements of the method decreased with their increasing complexity (from element 2 to 4). With the progress of the project, the guidelines were used more frequently. On a 5-grade score system, the meetings were judged to be successful (4.4 on average) regardless of the method used. This suggests that a plurality of approaches does not negatively affect the effectiveness of discussion groups.

Keywords: grassland, innovation analysis, discussion groups, participatory approach, multi-stakeholder approach

Introduction

Inno4Grass (Shared Innovation Space for Sustainable Productivity of Grasslands in Europe, www.inno4grass.eu) is a European thematic network of 20 institutions from eight countries aiming to capture grassland innovations from practice, and analysing and establishing them through a better knowledge exchange between practice and science. Capturing innovations in grassland from innovative farmers requires the interaction of practitioners and scientists in discussion groups (practice and science meetings), which have been shown elsewhere to be an effective way for knowledge transfer and positively affect farm profit (Hennessy and Heanue, 2012). In order to provide meaningful information to potential adopters of the innovation, a comprehensive approach is needed, combining the expertise of actors along the grassland production chain representing both science and practice, and leading to the identification of requirements for a successful adoption of the innovation, as well as its strengths and weaknesses. Thanks to this outcome, farmers wanting to adopt the innovation can evaluate whether a certain innovation is at all feasible under the given conditions and whether the expected advantages and disadvantages fit their personal values and aspirations.

Materials and methods

Within Inno4Grass, a method to conduct practice and science meetings (P&SMs) with a participatory, multi-stakeholder approach has been developed. The method comprises five elements: (1) Briefing activities, ensuring that all participants have a common baseline knowledge about the innovation in order to be able to discuss it. For this purpose, information material is prepared and sent to the participants prior to the meeting. Then, in a 1-2 hour on-farm visit, the innovation is presented in more detail. In this phase, each participant can ask questions for a better understanding of the operational procedures. Afterwards, the discussion, led by a moderator (facilitator agent), starts. (2) First, a simplified, short-timed (15 minutes), brainstorm-like SWOT-analysis (Kreutzer, 2018), usually limited to strengths and weaknesses, is conducted. (3) In a further step, the results of the SWOT-analysis are more deeply examined by means of a PESTLE-analysis (Dimler, 2016), taking six different topics into consideration: Political, Economic, Social, Technological, Legal and Environmental factors. (4) Some relevant sub-topics are further explored (Table 1). They have been identified by the authors by means of a workshop as issues being presumably relevant to most innovations in grassland. (5) To conclude the P&SM, the moderator directs some final standard open questions to the innovative farmer concerning (a) the willingness of the innovator to implement the innovation again, if given this possibility; (b) the suggested modifications to the innovation and things that the innovator would do differently with today's knowledge; (c) an evaluation of the demand on the local market for the innovation. The guidelines for this method have been made available to all 20 project partners of Inno4Grass in form of non-compulsory guidelines, including also practical suggestions for the organisation. The use of the guidelines was recommended, but each project partner was free to decide whether to adopt them, and to which extent, as some partners were already applying other previously established methods. Structured feedback of the first 55 P&SMs held was provided by a survey, in order to gain insight about the methodology adopted and how successful the meetings were. This survey included the different elements of the guidelines and a self-evaluation of the organisers on a 5-scale score (from 1 = poor to 5 = excellent) of the success of the P&SMS for seven different issues (participation of the desired stakeholders; communication between moderator and participants; communication between participants; comfort of the innovator with the discussion; involvement of all participants in the discussion; suitability of information material prior to the discussion; keeping of the time schedule). An overall evaluation of the meetings was computed as a mean of these issues. Three project phases were distinguished: (1) common guidelines under discussion, (2) official introduction and preliminary test of the guidelines in a simulated P&SM, and (3) additional training on the discussion process by means of a real P&SM at an innovative farm. Descriptive statistics about the use of the guideline elements were computed within the single project phases and across them. The effect of the adoption of the guideline elements on the evaluation was tested by means of a t-test at a significance level of 5%. A t-test for unequal variances was used where appropriate according to a Levene's test (significance level of 1%).

Results and discussion

The survey of the first 55 discussion groups revealed that, across all P&SMs, the guidelines were used in 56.4% of the cases (Table 2). With the progress of the project (from phase 1 to phase 3), their use in general increased, as well as the use of the simplified SWOT-analysis and the final questions asked to the innovator (up to 70, 74 and 74%, respectively, in the last project phase). The frequency of application of the single elements of the method decreased with their increasing detail (from the simplified SWOT-

Table 1. Standard topics and sub-topics for a PESTLE-analysis considered to be relevant to most grassland innovations.

Topic	Sub-topic
Political	wishes/demands to the politics
Economic	labour; profitability (€ h^{-1}); marketing and logistics; investments
Social	family; free time; image (for himself/herself; for the vicinity, i.e. village; for the customers)
Technological	forage conservation; mechanisation (indoor and outdoor); processing
Legal	payments; cooperation between farms; home slaughtering
Environmental	animal welfare; nutrient cycle within the farm; grassland/fodder areas

Table 2. Use of the guideline elements depending on the project phase.

Project phase	N	Use of the guidelines in general (%)	Briefing activities (%)	Simplified SWOT-analysis (%)	PESTLE-analysis (%)	Use of the predefined sub-topics (%)	Asking the final questions (%)	Overall evaluation of the meetings (0-5 score)
1	10	30.0	100.0	10.0	0.0	0.0	50.0	4.63±0.13
2	21	57.1	100.0	52.4	28.6	23.8	52.4	4.36±0.08
3	24	69.6	100.0	73.9	26.1	17.4	73.9	4.32±0.08
Total	55	56.4	100.0	52.7	21.8	16.4	60.0	4.39±0.05

analysis to the use of the predefined sub-topics for the PESTLE-analysis). This suggests that the organisers of the P&SMs adapted the content of the guidelines to their specific situations.

In the perception of the organisers the P&SMs were found to be successful (4.4 on average across the project phases), with no apparent trend across the project phases. Based on the results of the t-tests, the score was found to be unaffected by the application of the five elements of the proposed method (all $P>0.05$).

Conclusion

The results suggest that the information provided by the guidelines was felt to be useful by the organisers of the P&SMs, but the single guideline elements were adopted less frequently with their increasing complexity. As no general effect of the use of the single elements of the guidelines was found on the evaluation success of the P&SMs, and a very positive evaluation was expressed in all project phases, these results suggest that a plurality of approaches does not negatively affect the effectiveness of the discussion groups. However, a certain bias of the evaluation can be assumed, as it was performed by the organisers themselves and not all project partners already organised P&SMs during the first project phase.

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Native diverse grasslands versus introduced pasture: the debate rages

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Abstract

Should we use native or introduced (exotic) forage species in our cultivated pastures and rangeland restoration? Around the world, that debate is far from over and those on either side are becoming entrenched. Both germplasm sources have their advantages and disadvantages. Native species are adapted to local edaphoclimatic conditions and are less likely to become invasive; however, they are less tolerant vis-à-vis pandemic (non-local) pathogens, pests, herbivores or (mis)management, such as overgrazing. In contrast, introduced grasses and forbs have been selected for their productivity, reproductive ease, nutritive value and management ease, but can overwhelm native, biodiverse plant germplasm and animals. Which will end up winning the debate? Native or introduced? The answer may be: both and neither. Both have advantages and disadvantages that favour their use in pastures but under different circumstances. Biological, economic and social aspects affect the debate as it unfolds around the world.

Keywords: cultivated, exotic, forages, grasslands

Introduction

The debate as to whether we should utilize native or introduced plant species in rangeland restoration or cultivated pasture is far from over as local economic and conservation interests and global food production and environmental sustainability concerns conflict (Low, 1997; Franklin *et al.*, 2006; Tscharntke *et al.*, 2012). Native grasslands in many parts of the world are being replaced by cultivated pastures or exotic forages as exemplified by the Argentinean Pampa (Herrera *et al.*, 2009) and the Great Plains of North America (Sanderson *et al.*, 2017). This trend may decrease vegetation structure and diversity (Sanderson *et al.*, 2017) along with greater global warming potential, depending on management intensity (Liebig *et al.*, 2010). Native grassland bird numbers and diversity increase with native grassland plant diversity (restored prairie or rangeland) compared to cultivated pasture, for example in the Great Plains and south-eastern regions of the USA (Monroe *et al.*, 2017). So why are introduced (exotic) monospecific or simple binary pastures supporting a few, introduced (exotic) domesticated ruminants so prevalent around the world? Historically, most managed grassland research and production has focused on high-input, high-output simplicity rather than low-input complexity. This approach has often favoured introduced forage species that produce more than natives.

Pros for introduced forages

Monoculture or binary cultivated pastures feeding a single ruminant species are usually easier to manage compared to sustainable and profitable biodiverse native grasslands for ruminant, wildlife and ecosystems services. Focus on such monocultures or simple grass/legume mixtures can be explained by the fact that their management is far less challenging (Franklin *et al.*, 2006; Muir *et al.*, 2018).

Large markets for widely adapted and/or studied introduced forage seed also favour greater commercialization vis-à-vis small-scale, localized native forage species (Muir *et al.*, 2018). Seed companies struggle to sell sufficient diverse native seed to turn a profit in small, local markets. Private and government subsidies to develop native germplasm are often the only way these locally-relevant forages end up on the market (Smith *et al.*, 2010). Similarly, in past decades in the USA, federally- or state-subsidized programmes preferentially supported using exotic forages in pasture, range, and grassland restoration because of their lower per-unit cost and achievable increases in forage production.

Domesticated forages introduced into environments where they are non-native sometimes perform better than their native counterparts. Klabi *et al.*, (2017) found this to be the case with introduced perennial legume *Medicago sativa* vis-à-vis native *Dalea purpurea* in a semi-arid Canadian native grassland (50°16'N 107°44'W); in much warmer conditions, Foster *et al.* (2017) found the same to be true for annual legumes in Texas (28°27'N, 97°42'W) where introduced species such as *Crotalaria juncea* or *Lablab purpureus* yielded as much as three times the dry matter and N as native *Strophostyles* spp. This superior performance from introduced germplasm is usually based on greater response to inputs and management, as well as ease of establishment; it can also reflect greater tolerance to mismanagement, as in the case of *Cynodon dactylon* or *Cenchrus ciliaris* that support heavy grazing (Franklin *et al.,* 2006) to the point of becoming invasive. More vigorous seedlings or greater seed production can also favour introduced species over natives (Badalamenti *et al.*, 2016), even resulting in introduced species occurring in areas where they were never intentionally established (Smith, 2010). Selection pressure by breeding programmes or, before them, centuries of farmer propagation have resulted in productive and grazing-tolerant domesticated forages compared to native species which have not received the same scrutiny.

Pros for diverse native grasslands

Biodiverse pastures and rangeland, whether native, exotic or mixtures, could support greater animal diversity and, as a consequence, likely greater animal production (Muir *et al.*, 2015). In portions of southern Texas, wildlife adapted to diverse habitats provide greater economic returns than the traditional livestock enterprises (Smith, 2010). In the south-eastern USA, conservation programmes support planting native grasses for grassland bird habitat (Monroe *et al.*, 2017). Grazing these native plant mixtures sustainably, however, increases management requirements vis-à-vis simplistic monospecific pastures supporting a single ruminant species.

Native germplasm will usually not be classified as a noxious invasive, and generally do not require seasonal inputs to maintain (modest) production potential (Smith *et al.*, 2010). Furthermore, native grasslands also provide for multiple-land uses underlying enhanced land value and greater rental rates. This is particularly apparent in areas of the southern USA where land management to facilitate hunting for game species such as northern bobwhites and white-tailed deer alongside cattle and sheep production are viewed as sustainable and complimentary land uses on native rangeland. Comparable examples exist in Australia, Argentina and southern Africa.

Compromise?

Diverse native grasslands have advantages where ecosystem services (such as C sequestration or edapho-hydrological stability) or animal biodiversity (wildlife and native pollinators) are important. Socio-cultural pressures from non-agricultural sectors increasingly favour these contributions and decry the invasiveness and environmental costs of introduced germplasm.

Cultivated exotic monocultures or simple mixture pastures, however, also have their place. They are more likely to respond positively to high inputs and produce greater outputs in monospecific ruminant systems on less land. These systems offer enticing short-term solutions for producing more food on less and less land for growing human populations.

Mixtures of native and introduced forages, whether on the same land or in independent patches, may be a feasible compromise in some conditions. However, aggressiveness and invasiveness of exotics, vis-à-vis natives, may preclude this approach. As agroecosystems become more efficient, and concomitantly, as competing land uses grow with human population, social values for varied grasslands will continue to evolve, though in many areas a clear trend away from exotic grasses and toward use of native grasses has emerged (Smith, 2010). We should be wary of being too singularly focused on native or introduced, and realize that optimal, or more importantly, sustainable pasturage for multiple species and services under uncertain future climate realistically lies somewhere in between.

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Diploid and tetraploid red clover varieties in different grass mixtures in Norway

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Abstract

Most red clover varieties used in Norway are diploids. However, according to practical experience tetraploid varieties may be more persistent and produce higher yields in mountain areas and in northern Norway. Nevertheless, due to problems with seed production, there are only one or two tetraploid varieties commercially available. In the present study, three diploids and three tetraploids were grown in two grass mixtures in a series of field trials. In both mixtures there were significant differences between varieties in the proportion of clover at the last harvest in the second and third year, but these differences could not be related to ploidity. In the second year the best two tetraploids had significantly higher yield than the diploid variety Lea, which is the most-used red clover variety in Norway. The ranking of the varieties was approximately the same as in the official variety trials with pure clover stands.

Keywords: dry matter yield, persistence, ploidity in red clover

Introduction

Most commercially available red clover varieties in Norway are diploids. However, according to Amdahl (2016) tetraploids are taller, have thicker stems and larger leaves resulting in higher forage yield in pure stands than for diploids. But the seed yield of tetraploids is significantly lower than that of diploids, which is also reported from Lithuania (Liatukas and Bukauskaite 2012), where the tetraploid average DM yield surpassed that of diploids by 6.5%, but the seed yield of tetraploids was 16% lower than that of diploids. Nesheim (2013) analysed results from 20 field trials at five NIBIO research stations from a 3-year-long variety testing, of which one test ended in 2009 and one test ended in 2011. In the first test the best tetraploid performed significantly better in northern Norway and in mountain areas than the diploid ones. In the second test no significant differences were found. Nesheim (2013) also analysed results from 14 field trials where four red clover varieties were seeded together with grasses, and found no indications of better performance of tetraploid varieties. The objective of the series of field trials presented here was to investigate further if there are any positive effects of newly approved tetraploid red clover varieties on yield and persistence in mixture with grasses. Data from the latest variety testing (Nesheim and Langerud, unpublished data) are included to check if the ranking of varieties was the same as in mixed stands.

Materials and methods

Field trials were established during two years (2013 and 2014) testing three diploid and three tetraploid varieties of red clover, seeded with either a grass mixture of timothy and meadow fescue, or a mixture of those species and 10% perennial ryegrass. Red clover comprised 10% of the total seed rate of 30 kg ha^{-1}. The objective of including perennial ryegrass was to investigate the effect of increased competition on the proportion of red clover. The mixtures were seeded on small plots (7×1.5 m) with two replicates. The trials were fertilised according to local recommendations, and the rate of nitrogen was approximately 100 kg per hectare. The trials were harvested two or three times each year in three years. The proportion of red clover at the last harvest each year was assessed visually as percentage of dry matter yield. The Norwegian Agricultural Extension Service was responsible for most of the field trials, located in eastern Norway (4 trials), central Norway (7 trials) and northern Norway (1 trial).

The official variety testing of fodder crops in Norway is accomplished on five NIBIO research stations. In this paper results from eight field trials (established in 2014 and 2015) with red clover are presented (Nesheim and Langerud, unpublished data). The trials were located at Løken (eastern Norway, mountain area), Apelsvoll (eastern Norway, lowland), Særheim (south-western Norway, lowland), Fureneset (western Norway, lowland) and Kvithamar (central Norway, lowland). Three diploid and three tetraploid varieties of red clover were seeded in pure stands on small plots with three replicates. No nitrogen fertiliser was applied; the application of phosphorus and potassium was according to local recommendations. The trials were harvested two-three times each year in three years.

For both series, statistical analyses were performed according to two different models, one with site/district (random) and variety (fixed) as factors and the other one with site/district and ploidity (fixed) as factors. For the variety trials, analyses of all data together revealed strong interactions between factors ploidity/variety and district, and data from each district were then analysed separately. The procedure GLM in SAS (release 9.4, 2002-2010, SAS Institute Inc., Cary, NC, USA) was used for all analyses. Means were separated according to Least Significant Difference in the LSMEAN statement.

Results and discussion

In the grass-clover trials there were significant differences between varieties in the proportion of clover at the last harvest in the second and third year, but these differences could not be related to ploidity (Table 1). In the second year the mixed stands with the best two tetraploids, Lars and Lasang, had significantly higher yield than the diploid variety Lea, which is the most used red clover variety in Norway.

Inclusion of 10% perennial ryegrass in the seed mixture did not affect either the proportion of clover at the last harvest (Table 1), or the yield level in the first two years. However, in the last year there was a significant negative effect of perennial ryegrass on the yield. This was probably due to a decrease in the proportion of perennial ryegrass from the second to the third year because of winter damage.

In the first year of the official variety testing, the group of tetraploids returned consistently higher yields than the group of diploids in three out of four districts, whereas single varieties were not separated in

Table 1. Percent clover at the last harvest, expressed as percentage of DM yield, and total dry matter yield for 2 or 3 harvests each year in 3 years.[1]

Variety/ploidity	Percent clover at last harvest					Dry matter yield, $t\ ha^{-1}$				
	1st year	2nd year		3rd year		1st year	2nd year		3rd year	
Gandalf 2×	23	21	a	19	ab	10.46	10.52	ab	9.12	
Lea 2×	22	18	b	19	abc	10.29	9.90	b	8.75	
Linus 2×	21	18	b	16	d	10.74	10.19	ab	8.80	
Lars 4×	23	20	ab	19	a	10.70	10.54	a	9.01	
Lasang 4×	24	20	ab	17	bdc	10.66	10.54	a	8.79	
Reipo 4×	22	18	b	17	dc	10.66	10.25	ab	8.79	
Mixture ryegr.	22	19		18		10.66	10.27		8.71	a
Mixture timothy	23	20		18		10.51	10.38		9.05	b
Diploids	22	19		18		10.50	10.20		8.89	
Tetraploids	23	20		18		10.67	10.44		8.86	
SEM	8	5		6		0.81	1.00		0.81	
P Variety	ns	0.01		0.01		ns	0.01		ns	
P Mixture	ns	ns		ns		ns	ns		<0.01	
P Ploidity	ns	ns		ns		ns	ns		ns	

[1] Results from 12 trials in different parts of Norway. Means followed by same letters are not different at the 5% level.

any of them (Table 2). In the third and last year, yield of single varieties differed significantly in central and eastern Norway. The tetraploid Lars yielded higher than the diploids Gandalf and Linus in the first district, whereas Gandalf outperformed all other varieties in the second. There were no consistent grouping according to ploidity (Table 2).

Table 2. Total dry matter yields (t ha^{-1}) in first and third year of ley in pure stands of red clover (Official Norwegian variety testing, two trials in each region).[1]

Variety/ploidity	Central, lowland		Mountain		East, lowland		West, lowland	
	1st year	3rd year	1st year	3rd year	1st year	3rd year	1st year	3rd year
Gandalf 2×	9.41	4.12 bc	8.54	6.80	9.64	3.76 a	7.31	4.82
Lea 2×	9.68	4.99 abc	8.39	5.53	9.09	3.06 b	7.07	5.31
Linus 2×	9.80	3.66 c	8.31	5.55	9.84	2.63 b	8.07	5.94
Lars 4×	10.56	5.91 a	9.30	6.86	10.10	2.97 b	7.60	5.05
Lasang 4×	10.64	4.78 abc	9.12	6.48	9.85	2.82 b	7.75	5.12
Reipo 4×	10.79	5.54 ab	9.24	6.53	10.33	2.71 b	7.97	5.17
SEM	0.35	0.45	0.42	0.43	0.19	0.14	0.42	0.35
P-value variety	ns	0.04	ns	ns	ns	0.02	ns	ns
Diploids	9.63	4.26	8.41	5.96	9.52	3.15	7.49	5.36
Tetraploids	10.66	5.41	9.22	6.62	10.09	2.83	7.77	5.11
SEM	0.17	0.32	0.19	0.28	0.15	0.17	0.24	0.22
P-value ploidity	<0.00	ns	0.01	ns	0.02	ns	ns	ns

[1] Means followed by same letters are not significantly different at the 5% level.

Conclusion

The vigour and higher yield potential of pure stands of tetra- versus diploids in the first year of ley were not expressed in trials with grass-clover mixtures, and there were no signs of tetraploids being more persistent than diploids. There were no clear-cut relationships between how varieties performed in pure stands and yields of mixtures where they were included.

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Effect of training and methodology development on the effectiveness of discussion groups on grassland innovation

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Abstract

Within the framework of the Horizon 2020 thematic network Inno4Grass (www.inno4grass.eu), 55 'practice & science meetings' have been carried out using a multi-stakeholder, participatory approach, in order to bridge the gap between science and practice and deliver a comprehensive analysis of grassland innovations across eight European countries. Three project phases can be distinguished: (1) common guidelines under discussion, (2) introduction and preliminary test of the guidelines, (3) additional training on the discussion process. A structured feedback on the meetings held was provided by the organisers by means of a questionnaire, including information about duration, preparatory material, participants, discussion methods and evaluation of the meetings by the organisers. This information allows computing of mean values and distribution of the most relevant variables. Only a few of them were affected by the project phase. In particular, there was an increase in the use of farm portraits and of scientific literature over the project phases. All aspects describing the success of meetings were positively assessed. This suggests that the methodology for the conduction of the practice & science meetings within Inno4Grass was already relatively well established at the beginning of the project.

Keywords: grassland, discussion groups, innovation analysis, participatory approach, multi-stakeholder approach

Introduction

Discussion groups are groups of farmers, facilitated by a moderator (agricultural extension agent or facilitator agent), that meet to discuss technical issues, share information and solve problems. Discussion groups offer an environment for members to share ideas and keep up-to-date with the latest innovations by means of peer-to-peer learning. They have been shown to positively affect the adoption of technical innovation and farm profit of dairy farms (Hennessy and Heanue, 2012). Within the Horizon 2020 thematic network Inno4Grass (www.inno4grass.eu), particular discussion groups, called 'practice & science meetings' (P&SMs), are conducted with a multi-stakeholder, participatory approach, to bridge the gap between science and practice, capture grassland innovations from practice and deliver a comprehensive analysis of them (Mairhofer *et al.*, 2019). This paper provides an overview of the methodological aspects adopted for the conduction of the meetings, their effectiveness, and the changes in methodology and effectiveness along the project development.

Materials and methods

Between January 2017 and September 2018, 55 P&SMs have been held in eight European countries (BE, DE, FR, IT, IE, NL, PL, SE). Common, non-mandatory guidelines consisting of general recommendations and five elements (briefing, simplified SWOT-analysis, PESTLE-analysis, predefined sub-topics for the PESTLE-analysis, and final standard questions to the innovator) were provided to the project partners (Mairhofer *et al.*, 2019). Over the duration of the project development, three phases can be distinguished, depending on the specific training and the state of development of the methodology, both provided to the participants during the project meetings: (1) common guidelines for the P&SMs being under discussion; (2) official introduction and preliminary test of the guidelines in a simulated practice & science meeting with project members only; (3) additional training provided by an external trainer on the discussion process by means of a real P&SM at an innovative farm with the involvement of the innovator and of external stakeholders. A survey of the meetings was held among the project partners who conducted the P&SMs by means of a questionnaire, including questions on duration, preparatory material, participants, discussion methods and a self-evaluation performed by the organisers of the success of the meetings for seven different issues (participation of the desired stakeholders; communication between moderator and participants; communication between participants; comfort of the innovator with the discussion; involvement of all participants in the discussion; suitability of information material prior to the discussion; keeping of the time schedule) on a 5-scale score (from 1 = poor to 5 = excellent). The effect of the project phase on the single variables was tested by a mixed model accounting for both the project phase (fixed factor) and the project partner (random factor) after checking the prerequisites for this analysis (normal distribution of residuals, homoscedasticity) by visual inspection of the residuals. The non-parametric Kruskal-Wallis-test was performed in case the prerequisites for ANOVA were not met. For binomial dependent variables, a binary logistic regression with the project phase as independent variable was performed with a cut off of 0.5 in order to test for significance. Multiple comparisons between project phases were obtained by repeating the analysis varying the reference category. The significance level was set at P=0.05.

Results and discussion

Training and methodology development, represented by the advancement state of project phases, did not significantly affect either the duration of the different elements of the P&SMs or the number of stakeholder categories taking part in the meeting (Table 1).

A duration of around two hours for the briefing activities seemed to be generally suitable for meetings, whilst slightly more time was apparently required for the discussion. On average, about seven stakeholder categories attended a single meeting.

Table 1. Mean ± standard error of mean of the duration of different elements of the practice & science meetings, of the number of stakeholder categories attending a meeting, and significance of the effect of the project phase.

Project phase	Duration (h)				Number of stakeholder categories
	On farm-visit[1]	Virtual farm tour[1]	Whole briefing[1]	Discussion[1]	
1	1.9±0.7	1.5±0.3	2.2±0.5	3.0±0.4	7.8±0.9
2	2.0±0.2	1.2±0.3	1.9±0.2	2.4±0.2	6.7±0.4
3	2.2±0.4	0.8±0.1	2.3±0.3	2.3±0.2	6.2±0.4
Average	2.1±0.2	1.1±0.1	2.1±0.2	2.5±0.3	6.7±0.3
Sig.	n.s.	n.s.	n.s.	n.s.	n.s.

[1] Kruskal-Wallis test, n.s. = not significant.

Table 2. Frequency (%) of use of information material during the briefing activities. The absence of superscript letters in common indicate significant differences between project phases according to the regression coefficients of the logistic regression ($P<0.05$).

Project phase	Scientific literature	Farm portraits[1]	Interview results	Presentation (slides) on the innovation topic
1	40.0^{ab}	20.0^{a}	50.0^{a}	40.0^{a}
2	19.0^{a}	52.4^{ab}	42.9^{a}	47.6^{a}
3	60.9^{b}	65.2^{b}	69.6^{a}	60.9^{a}

[1] Handout with a compact description of the innovation in the farm context.

Concerning the preparation of the meetings, an increase of the use of farm portraits (short, reader-friendly descriptions of the main farm characteristics) and increased provision of scientific literature, thereby ensuring a sound fact-checking and adherence to scientific standards (van den Pol-van Dasselaar *et al.*, 2019), became more and more established with the advancing project phases (Table 2).

None of the aspects that described the success of the meetings were affected by the project phase, as perceived by the organisers. High scores were obtained for all issues evaluated: participation of the desired stakeholders (mean ± standard error of mean 4.5±0.2), communication between moderator and participants (4.8±0.1), communication between participants (4.7±0.3), comfort of the innovator with the discussion (4.9±0.1), involvement of all participants in the discussion (4.3±0.3), and respecting the time schedule (4.8±0.1).

Conclusion

The methodology for the conduction of the P&SMs within Inno4Grass seemed to be well established from the beginning of the project, presumably as a result of skills and expertise already developed by the project partners in their previous experience. However, a certain evaluation bias can be assumed because of the organisers evaluating their own meetings. The results of Inno4Grass provide useful reference values for P&SMs in other contexts.

Acknowledgements

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Feed conversion efficiency in French dairy systems

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Abstract

Dairy production is facing social issues due to competition in the use of resources (feed, water and land). The French livestock Institute (Idele) has characterized feed conversion efficiency for the main dairy ruminants in France, i.e. cows, goats and sheep. The DIAPASON database from a reference farms network (INOSYS-Réseaux d'élevage) has been used to determine the efficiency of a large number of dairy systems in France between 2012 and 2016. Systems studied in this reference network are on average net producers of protein: 76% for dairy cows, 52% for dairy sheep and almost 50% for dairy goats. The ratio is lower if considering only energy conversion. Diet composition is of critical importance. Cereals are in direct competition with human nutrition, whereas grass as pasture or silage is not, giving a positive impact on efficiency. Thus, we can show that grass-based dairy systems, specifically those with pasture, are very efficient for turning grass proteins into high-quality proteins for human nutrition. A high variability within and between systems and species is shown and gives the opportunity to propose technical ways to improve this new approach on farm.

Keywords: dairy systems, feed conversion efficiency, goat, sheep, cow

Introduction

Dairy production is facing social issues due to competition in the use of resources (feed, water and land). Ruminants are often characterized as inefficient because they consume around 3 kg of plant protein to produce 1 kg of milk (Peyraud and Peeters, 2016). But the truth is more nuanced. This approach does not take into account that ruminants (1) have the ability to utilize the value of non-human edible resources, such as grass and by-products, and (2) supply high quality products with high nutritional values. In addition, animal husbandry has positive externalities beyond food production, like landscape and biodiversity conservation or carbon storage (Hoffmann *et al.*, 2014). A new approach has been proposed that deals with the evaluation of human-edible food from human non-edible feedstuffs in livestock feeding strategies. Some results using these new criteria are already available in the literature for different feeding systems in different countries (Wilkinson, 2011; Ertl *et al.*, 2015). The objective of our study is to determine the conversion efficiency – for both energy and proteins – of a large number of dairy systems in France for cow, goat and sheep production, especially for grass-based dairy systems.

Materials and methods

The DIAPASON database from a reference farms network (INOSYS-Réseaux d'élevage) has been used for a large number of dairy systems in France between 2012 and 2016. This network lasts since more than 30 years and allows the collection of various and numerous data routinely on more than 1000 farms. The total intake for both roughages and concentrates has been registered: inventory changes for all feeds except for pasture (estimation). The total meat and milk production has also been collected. Over the 5 years of the database, missing and incoherent data have been deleted leading to 1,384 data from 498 farms for dairy cows, 847 data from 274 farms for dairy goats, and 343 data from 108 farms for dairy ewes. The first step has been to use and complete an existing table of human-edible energy and protein fractions of the main feedstuffs used in France (Laisse *et al.*, 2016). For example, the human-edible protein fraction is 66% for wheat, whereas it is 0% for rapeseed or sunflower meals. For roughages, this fraction is estimated to 0%, except for maize silage (10%) to consider the grain fraction. Almost 100 feeds have been characterised. The second step is the calculation of two ratios to estimate the efficiency for energy (kcal/kg of feeds and animal products) and protein (kg of crude protein/kg of feeds and animal products). The 'total' efficiency considers the ratio between the total outputs (meat and milk production) and inputs (feedstuffs intake) while the 'net' efficiency considers only human-edible fractions of inputs. All calculations are made at the farm level, including both intake and production for all types of animals (young and adults for meat and milk). ANOVA using SAS software has been carried out, separately for each production (sheep, goat and cow).

Results and discussion

Grass-based systems studied in this reference network are on average net producers of proteins: 76% for dairy cows, 52% for dairy sheep and almost 50% for dairy goats. The ratio is lower if considering only energy conversion (Table 1).

For sheep milk production, feeding systems are very specific to the geographic area. In Corsica, feeding is based on pasture which represents 69.7% of the diet and only a small amount of concentrates is offered during the milking period. This system then has a high net protein efficiency (NPE) of 1.15. For the Pyrénées-Atlantiques, transhumant systems in mountain area, either only with dairy ewes or mixed with beef production, are both using the same amount of pasture (around 55%) and few cereals (1-2%) in the total diet. Differences in milk production (130 l/ewe vs 142 l/ewe respectively) explain the gap in NPE (0.91 vs 1.04 respectively). In the Roquefort sector, the pastoral system has more pasture in the diet than one based on hay (35.4% vs 26.2%) but is also less productive (238 l/ewe vs 279 l/ewe), which can explain a close NPE (1.05 vs 1.00).

For goat milk production, there is a large diversity of feeding systems, using different nature and quantity of roughage (fresh or dry grass, silage, maize). Total protein and energy efficiencies are quite low on average (about 0.16 and 0.1 respectively). Variations in net efficiency are mostly explained by the level of grass in the diet, the relation between milk production and the quantity of concentrate, and quality of roughages.

For dairy cow production, the main production areas in France are either on mountains or in lowlands. The grass-based systems are net energy and protein producers, except the lowland maize, and grass-based one in terms of energy. Systems using maize silage are dependent on milk production to improve the efficiency ratio. For example, this mixed system reaches 7,328 l/cow, whereas the others produce 5,434 l/cow in lowlands and 6,056 l/cow in mountain areas. Systems based on grass, either in mountain or

Table 1. Total and net efficiencies for protein and energy in dairy sheep, goat and cow systems (*P*-value ANOVA >0.01).

Dairy production	System	n	Total energy efficiency	Net energy efficiency	Total protein efficiency	Net protein efficiency
Sheep	Corsica, delivery only[1]	28	0.06^a	0.48^a	0.10^a	1.15^a
	Pyrénées-Atlantiques, delivery only[1], transhumant mountain area	17	0.06^a	0.61^a	0.10^a	1.04^a
	Roquefort sector, delivery only[1], pastoral	38	0.07^b	0.54^a	0.13^b	1.05^a
Goat	Fresh grass based	102	0.10^a	0.49^b	0.16^a	0.87^a
	Hay or grass-silage based	171	0.10^a	0.45^b	0.16^a	0.74^a
	Maize silage based	78	0.11^a	0.38^a	0.17^a	0.65^a
Cow	Mountain, grass-based	415	0.12^b	1.13^b	0.18^a	1.78^a
	Lowland, grass-based	133	0.11^a	1.53^c	0.17^a	2.42^b
	Lowland, maize and grass-based	222	0.14^c	0.79^a	0.21^b	1.45^a

[1] Delivery only: no cheese making on farm, all milk is delivered to industry.

lowland areas, are very efficient in protein supply because pasture and grass silages are not in competition with human nutrition, even though their milk productivity is lower.

A land use ratio has been proposed to estimate the land use efficiency of livestock systems (Van Zanten *et al.*, 2016). This ratio would allow the efficiency of mountain systems to be highlighted, as they use land that cannot be used for production of human-edible crops.

Conclusion

Feed conversion efficiency, for both energy and protein, is a critical issue for the future for ruminant production, as it is part of the competition between feed and food uses. First results for the three main French dairy production categories show the importance of grass share in the diet, use of by-products and concentrate efficiency. For the future, more issues have to be addressed on this database, like land use ratio and animal protein quality for human nutrition.

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High-elevation grassland re-establishment using adapted seed mixtures

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Abstract

The re-establishment of grassland vegetation at high elevations is challenging due to harsh climate, a short vegetation period and shallow, nutrient-poor soils. Moreover, environmental conditions differ greatly between sites, so site-adapted restoration measures are required. Seed mixtures allow for a flexible addition of adapted plant species at appropriate dates during the short vegetation period, either alone or in combination with transplanted turf or seedlings. We have reviewed seed mixtures proposed for the subalpine and alpine zones of the Alps. The 24 identified seed mixtures were often built around a common set of dominant species complemented by a large diversity of additional plant species (188 species). A subset of eight mixtures was tested in a randomised experiment at a high-elevation site. Despite considerable overlap in the core composition of the mixtures, large differences in yield of the first cut and the composition of the sward were found. This highlights the need to test seed mixtures for high-elevation restoration in the field.

Keywords: high elevation, restoration, multi-species mixture, sward establishment

Introduction

Growth conditions at high elevations differ tremendously from those in lowlands (Schneider *et al.*, 2017). Frost may occur even during the short vegetation period, soils are shallow and barely retain water and nutrients. Despite these limitations, land in most mountain areas in covered by vegetation, except for very steep, rocky places or areas under snow and ice. Natural events such as landslides and avalanches may destroy this protective vegetation cover, but also construction works, excessive grazing or ski runs may impair it, causing the loss of fine soil and humus. Disturbed sites may be repopulated by pioneer species only very slowly and it may take years until a stable, site-adapted vegetation re-establishes. Additional efforts to recreate a protective vegetation are therefore often necessary.

Sowing of multispecies seed mixtures is frequently used to re-establish vegetation at high elevations. It is crucial to sow species and genotypes adapted to the specific conditions of high-elevation sites. Our aim was therefore to review the available literature on seed mixtures for high-elevation restoration in the Alps and to test them in the field.

Materials and methods

The 13 mixtures identified by the literature review were complemented by two mixtures from commercial seed companies and nine mixtures specifically designed for restoration projects. Eight mixtures (six from the literature and two commercial mixtures) were further tested in a field experiment in the Eastern Swiss Alps at 1,850 m.a.s.l. (46.793°N, 9.687°E; cambisol on serpentinite; 3.6 °C mean temperature and 1,365 mm mean precipitation). Details about the mixtures can be found in Table 1. The trial was a randomised complete block design with plots of 2.5×7 m and three replicates. Mixtures were sown on 2 July 2018. A first cut was carried out on 27 September 2018 and cover proportions of functional groups were visually

Table 1. Species mixtures with their source, the number of species (Nsp) and the species with highest seed amounts.

	Akronym	Source	Nsp	Species with highest seed amounts
A	SM 491	Suter *et al.*, 2017	20	*Fes.nigre, Agr.gigan, Lot.alpin, Tri.badiu, Poa.prate, Tri.prate*
B	SM 492	Suter *et al.*, 2017	29	*Fes.nigre, Agr.gigan, Poa.prate, Ant.vulne, Fes.viola, Lot.alpin*
C	acidic	Krautzer, 2001	12	*Fes.nigre, Poa.alpin, Fes.ovina, Fes.pseud, Fes.viola, Phl.rhaet*
D	alkaline	Krautzer, 2001	8	*Fes.nigre, Poa.alpin, Lot.corni, Agr.capil, Ant.vulne, Fes.viola*
E	N5	TBA, 2013	25	*Fes.nigre, Lol.peren, Poa.alpin, Phl.rhaet, Agr.alpin, Ant.alpin*
F	N6	TBA, 2013	22	*Fes.nigre, Lol.peren, Ave.flexu, Phl.rhaet, Poa.alpin, Agr.rupes*
G	Terra verde 57	Schutz Corp.	13	*Poa.alpin, Fes.nigre, Phl.hirsu, Phl.rhaet, Poa.viola, Ach.mille*
H	OH-Pre alpin	OH seeds Corp.	15	*Fes.nigre, Fes.rubra, Lol.peren, Poa.prate, Fes.ovina, Dac.glome*

estimated. Differences in yield and cover proportions were analysed using one-way analysis of variance and subsequent pairwise least square difference tests with Holm correction.

Results and discussion

The 24 mixtures contained on average 26 plant species and 188 in total. The ordination of species composition showed that the mixtures evaluated in the trial differed in composition from the mixtures specifically designed for restoration projects (Figure 1). The mixtures in the trial were associated with warmer conditions and soils relatively rich in nutrients and humus. Since mixtures A and B, C and D, E and F originated each from the same authors, the analysis also indicated a pairing by author, likely because every author developed mixtures for a particular application or based on a limited set of available seeds.

Despite the fact that many of the dominant species in the mixtures were relatively similar, the tested mixtures differed greatly in yield and composition of the first cut (Table 2). The commercial mixture H had the highest yield and a high proportion of grasses and legumes. It was the only mixture that contained the lowland species *Lolium multiflorum* var. *westerwoldicum* and high yields may likely be transient (Suter *et al.*, 2013). The two mixtures A and B by Suter *et al.* (2017) also had high yields but were dominated by legumes and, to a lesser extent, by grasses. In contrast, mixtures C and D by Krautzer (2001) were

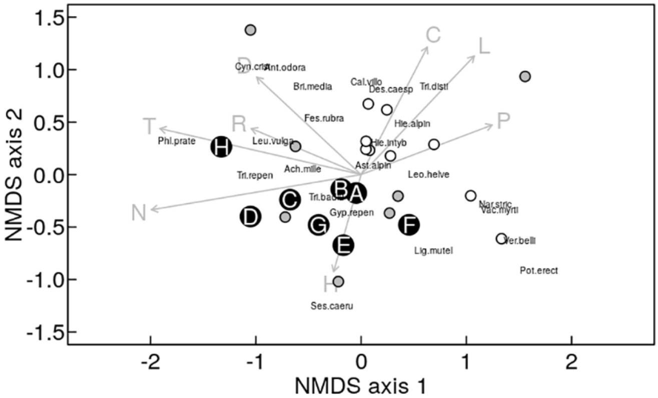

Figure 1. Non-metric multidimensional scaling of the species composition (presence-absence) of seed mixtures for high-elevation restoration. Black dots with capital letters indicate eight mixtures used in a field experiment, grey dots represent other mixtures from the literature, white dots mixtures of a restoration project. Text labels show the position of selected plant species in the ordination. Grey arrows are the fitted vectors of mean indicator values for nutrients N, temperature T, soil pH R, rooting depth D, continentalism C, light L, soil permeability P and humosity H taken from Landolt (2010).

Table 2. First assessment of yield (g DM m^{-2}), estimated total ground cover (%) and estimated cover % of sown grasses, legumes and forbs as well as unsown species in the first cut, two months after sowing.[1]

Nr	Yield	Total cover	Grasses	Legumes	Forbs	Unsown
A	329 ab	98 a	34 a	45 a	4 c	15 ab
B	272 ab	93 ab	27 ab	42 ab	5 c	20 a
C	264 ab	97 ab	6 b	48 a	36 a	7 b
D	312 ab	97 ab	6 b	50 a	33 ab	8 b
E	168 b	82 b	47 a	6 c	5 c	23 a
F	165 b	85 ab	32 a	23 bc	5 c	25 a
G	165 b	88 ab	33 a	12 c	22 b	22 a
H	386 a	100 a	40 a	47 a	7 c	6 b
LSD	215	16	25	22	13	13

[1] Different letters denote differences beyond the least significant difference (lsd) at the 5% level.

initially dominated by legumes and forbs, mainly *Achillea millefolium*. Mixtures E-G showed slower establishment, lower yields and a lower total coverage. As a consequence, they had the highest cover of unsown species.

Conclusion

The majority of recommended multispecies mixtures are representative of only a limited part of high-elevation grasslands. Mixtures by the same authors are often more similar among each other than to the mixtures of other authors. Despite a relatively similar core set of species in the eight tested mixtures, they strongly differed in yield of the first cut after establishment and the proportions of grass, legumes and forbs. This highlights the need for specific testing of mixtures for application in high-elevation restoration.

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Developments in the use of plantain (*Plantago lanceolata*) cultivars in New Zealand

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Abstract

This paper provides an overview of plantain (*Plantago lanceolata*) breeding and use in New Zealand since the release of the cultivar 'Ceres Tonic' in 1994. Plantain is now widely used in a range of farming systems and in a variety of pasture mixtures. These mixtures include those with perennial ryegrass, white clover, red clover, lucerne, chicory and forage brassicas. The agronomic features of this productive cultivar of plantain have enabled it to become a valuable component of pasture mixtures grazed by sheep, cattle and deer in New Zealand. Under common grazing practices plantain normally persists for 2 to 4 years. Some cultivars of plantain also offer a plant-based solution for significantly reducing nitrate leaching from the urine patch and nitrous oxide emissions by reducing the nitrogen load in the urine patch and by reducing the speed of nitrification in the soil.

Keywords: plantain, *Plantago lanceolata*, New Zealand, herbs

Plantain (*Plantago lanceolata* L.) is most commonly known as a ubiquitous flatweed but it has been used for grazing historically (Foster, 1988). The species has been transformed into a valuable short-lived pasture plant (2-4 years) in New Zealand through the breeding of the erect, winter-active, productive cultivar 'Ceres Tonic' (Stewart, 1996). This improved cultivar is rapid to establish, adapted to a wide range of soils and climatic conditions, tolerant to a wide range of insect pests and diseases, highly palatable to grazing animals, and capable of giving excellent animal performance results across many farming systems in New Zealand. Recently another erect winter-active cultivar 'Agritonic' has been released with improved recovery from selective herbicides which should allow improved weed control in many farming systems. Other cultivars with less winter activity such as 'Grasslands Lancelot' (Rumball *et al.,* 1997) have found a limited role in New Zealand.

Although it was clear 'Ceres Tonic' was productive (Minneé *et al.*, 2013) it was not clear how it could be best integrated into pastoral agriculture to benefit animal productivity. Initially seed was simply added to multi-species pasture seed mixtures where the benefits were less obvious; however, over time research has consistently shown that 'Ceres Tonic' plantain can increase productivity across many farming systems.

The activity of 'Ceres Tonic' plantain in the cooler months provides a valuable source of early spring growth (Moorhead and Piggot, 2009; Sinhadipathige *et al.*, 2012). This allows ewes lambed onto plantain pastures to produce lambs with a higher rate of liveweight gain than those on ryegrass based pasture (Judson *et al.*, 2009).

Given the broad natural distribution of plantain it is not surprising that plantain is adapted to a wide variety of climatic conditions and soil types. Compared to temperate grasses such as perennial ryegrass, it is more drought and heat tolerant and in warmer regions of New Zealand, such as Northland, where ryegrass is often invaded by C4 grasses including *Paspalum dilatatum* and *Pennisetum clandistinum* (Kikuyu), plantain provides valuable additional summer feed (Moorhead and Piggot, 2009).

Grazing management should aim to minimise seedhead frequency as leafy plantain is of high quality but old stemmy plantain pastures have reduced feed value (Fraser *et al.,* 1996; Robertson *et al.,* 1995).

Similarly, crops harvested for silage in the seedhead stage have poor quality (Bariroh *et al.,* 2018) so it is important to harvest these when leafy.

Plantain pasture mixtures provide benefits during lactation for ewes through improved intake, anthelmintic effects (Robertson *et al.,* 1995; Knight *et al.,* 1996; Rumball *et al.,* 1997) including reduced faecal egg output (Judson *et al.,* 2009), increased milk production (Kenyon *et al.,* 2010), and also for lamb finishing (Moorhead *et al.,* 2002; Somasiri *et al.,* 2013; Kemp *et al.,* 2013). In pastures where plantain is mixed with clovers, or clovers plus chicory, daily weight gains of weaned lambs grazed at high stocking rates have been in the range 250 to 350 g/day (Judson *et al.,* 2009; Kemp *et al*., 2010; Hutton *et al.,* 2011). Part of this improved performance will be due to faster rumen degradation rates (24.6%) compared to perennial ryegrass (11.7%) (Burke *et al.,* 2000). In dairy systems, Minneé *et al.* (2012) noted that plantain had the ability to improve milk solids production when included in the diet in summer/autumn with increases in both the nutritive value and dry matter intake being likely causes.

Recent studies have shown plantain herbage can have major effects on nitrogen in the urine from grazing animals through a combination of a higher moisture in the herbage, a diuretic effect and a reduction in the proportion of nitrogen excreted in urine relative to that in the dung (Totty *et al.,* 2013; O'Connell *et al.,* 2016). Furthermore, plantain produces nitrification inhibitors capable of reducing the rate of ammonium breakdown from urine to the leachable nitrate form (Dietz *et al.,* 2012) and, surprisingly, these inhibitors are present in the urine where it is most needed. Substantial reductions in nitrate leaching of up to 89% have been reported from lysimeter studies with mixed swards of ryegrass and plantain (Woods, 2017; Carlton *et al.,* 2018). This offers a valuable tool to reduce nitrogen leaching in intensive grazing systems by Judson *et al.* (2018).

Although plantain is considered a long-day plant, flowering appears to be driven largely by plant development stage and this has allowed thermal time equations to be fitted for each cultivar (Payne and Stewart, 2018). When first grown in New Zealand research seed-yields were a modest 200-350 kg ha^{-1} at optimal density plantings (Rowarth, 1990). However, with continued seed production research and close involvement of seed growers commercial seed yields have improved over the last 20 years by around 100 kg ha^{-1} per year to average over 2,000 kg ha^{-1} (Kelly, 2011).

In the 25 years since 'Ceres Tonic' was released, the features of erect winter active productive plantain cultivars have become a valuable component of pasture mixes in many New Zealand farming systems grazed by sheep, cattle and deer but especially in dryland systems and in warmer regions. These include its use in a wide range of mixes with grasses, clovers, chicory, lucerne or forage brassicas.

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Grass-clover mixtures in practice: key-factors of success

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Abstract

For more than six decades, Swiss grassland farming has been successfully relying on a system of elite grass-clover mixtures, the so called 'standard mixtures'. Today, leys are almost exclusively seeded with grass-legume mixtures and about 80% of these mixtures are traded as standard mixtures. The four key-factors for this outstanding success are: (1) an unambiguous system offers forty-six mixtures in a broad range from intensive forage production to improvement of biodiversity. Grouping of individual mixtures to main types enables an easy choice of the appropriate mixture. (2) Scientific development of seed mixtures in multi-site, multi-annual field experiments taking into consideration the breeding progress by the exclusive use of recommended varieties enables optimised recipes. (3) Extensive practical testing of the most promising candidate mixtures on-farm ensures robustness and feasibility. (4) Collaboration with extension services, the seed industry and the Swiss Grassland Society anchors the system in practice manifested by a quality label awarded by the Swiss Grassland Society.

Keywords: grass-clover mixtures, standard mixture system, extension, collaborative model

Introduction

Forage production in the Swiss Plateau has been relying on grass-clover mixtures from as early as the 19th century (Stebler, 1881). Numerous small businesses produced their own mixtures. This resulted in an unmanageable situation on the Swiss seed market, which made it difficult to choose the appropriate mixture for a particular farm's specific needs. Thus, official registration of grass-clover mixtures was made mandatory (Frey, 1955). At the same time, the idea of a system of elite mixtures called standard mixtures was developed and implemented by the Swiss agricultural research stations, now Agroscope. The success of the standard mixtures is impressive. In Switzerland 80% of the grass-clover mixtures are traded as standard mixtures, which are awarded by the Swiss Grassland Society with a quality label guaranteeing their excellence. Furthermore, nearly all sown grasslands are based on grass-clover mixtures rather than pure grass swards. This paper aims at presenting the key-factors of this success.

Key-factors

Key-factor 1. Mixtures adapted to a broad range of needs, but easy to recognise

The first published set of forty-one standard mixture recipes was classified according to (1) planned duration of utilisation, (2) growth conditions and (3) utilisation of the forage (Frey, 1955). This classification has proven successful ever since. It is also the basis for the current product line of forty-six standard mixtures (Suter *et al.*, 2017). The use of a three-digit code instead of names for mixtures made it possible to map the system onto its nomenclature. The first digit of the code designates the main group 'duration' of utilisation. This main criterion is also made visible by a distinct colour of the label on the seed bag (Figure 1). The second digit provides information related to the mixture's requirements regarding water supply. Mixtures that contain cocksfoot are considered to be more robust in case of an occasional water shortage than mixtures without cocksfoot and are labelled with a '3' for the second digit. The third digit refers to whether the mixture is designed for highly productive growth conditions (annual mean temperature 6.5-9.0 °C, sunny, annual precipitation 900-1,200 mm, well drained fertile soils) which is

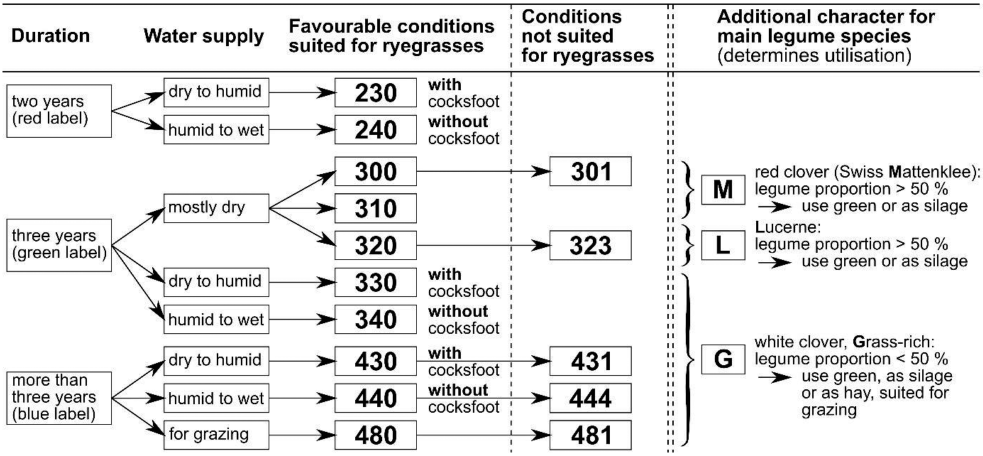

Figure 1. Main classification of important standard mixtures with their three-digit code and additional character.

indicated by a '0'. Other values for the third digit refer to mixtures for higher altitudes (e.g. 431 and 481) or for markedly wet (444) or dry (e.g. 323) conditions. An additional character adds information about the main legume species and the legume proportion of the forage. Mixtures with an 'M' (red clover of Swiss 'Mattenklee' type) or an 'L' (Lucerne) contain higher proportions of legumes (>50%) than mixtures with a 'G' (Grass rich, legume proportion <50%, main legume: white clover) and are restricted to the use as green forage or as silage. Mixtures with a 'G', however, are very versatile and can either be used green, as silage, as hay or be grazed. The three-digit code with an additional character enables the farmer to manoeuvre in the system, which makes it a lot easier to find the appropriate mixture compared to a scheme with long invented names.

Key-factor 2. Scientific development

Although some tools exist for at least a rough, conceptual design of a recipe (Caputa, 1948; Arens, 1973; Kirwan *et al.*, 2009), mixtures containing more than two species need to be developed, tested and optimised in a framework of multi-annual field experiments at several sites delivering detailed results on yield, botanical composition and forage quality. The exclusive use of recommended varieties makes it possible to benefit from the breeding progress already during mixture development. This also emphasises the importance of variety testing, which provides the basis for recommendations. For mixture development and variety testing eight experimental sites are available, located on the Swiss Plateau and at higher altitudes above 1000 m above sea level, covering a total of about 20 hectares.

Key-factor 3. Practical testing on farm

Controlled field experiments in small plots enable a valid evaluation of the agronomic potential of a mixture and a detailed comparison of different recipes. However, they provide rather limited information about how robust a seed mixture of a given recipe would be under very variable site conditions and utilisation. Thus, the recipes that have proven to be the most promising in small plots are subjected to on farm, upscaled strip-plot experiments under the respective farm's own utilisation practice. The different utilisation schemes, and even occasional mistakes regarding mowing, grazing or fertilising exert an important stress and contribute to the testing of the robustness and practical feasibility of a given seed mixture recipe. Only after passing these tests can a candidate recipe be selected and recommended as a new standard mixture.

Key-factor 4. Collaboration

The inclusion of extension services, the seed industry and the Swiss Grassland Society allows knowledge to be gained about trends in forage- and animal production and the required measures in grassland research and mixture development to be undertaken as a consequence. This collaboration further helps to anchor the system of standard mixtures in practice by supporting the on-farm testing programme and by a quality label awarded by the Swiss Grassland Society. The label emphasises the quality-based approach and guarantees for (1) scientifically developed seed mixture recipes, (2) composition of the seed mixture as published for the respective standard mixture, the use of (3) recommended varieties and (4) VESKOF® quality seed (Swiss-Seed, 2018) with a higher purity and a better germination ability than legally required. Regular examination of commercial seed samples ensures the label's acceptance. A small fee imposed on every seed bag carrying the quality label helps to finance the management and supervision of the label and to support research and development of mixtures.

Conclusion

The four factors – (1) a clear system of mixtures that accounts for duration of utilisation, growth conditions and utilisation of the forage, (2) scientific development of mixtures, (3) practical testing on farm and (4) tight collaboration among the stakeholders – all together form a package enabling the unparalleled success of the system of standard mixtures. This system undoubtedly is an important pillar of the broad use of multispecies mixtures in Swiss grasslands with their significant multifunctional advantages compared to pure grass swards.

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Predetermining factors for grazing efficiency differences in perennial ryegrass varieties

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Abstract

Perennial ryegrass (*Lolium perenne* L.; PRG) is the principal forage species sown in Ireland for ruminant production. This forage is predominantly fed as grazed grass and has been shown to be the most efficient feed for Irish ruminant production systems. Anecdotal evidence exists from farmers claiming that grazing livestock show preference for certain perennial ryegrass varieties. The aim of this study was to determine what varietal traits in perennial ryegrass influence grazing efficiency by examining differences in grazing intensity between varieties. Grazing efficiency was defined as the proportion of leaf tissue grazed, relative to that presented. Fifty-five PRG varieties were assessed. The plots were rotationally grazed by dairy cows for four grazing seasons from 2015 to 2018 inclusive. Varieties were shown to differ in their level of grazing utilisation (P<0.001). The average difference across the four years between the most and least grazed variety was 0.85 cm (3.72 and 4.54 cm respectively). Tetraploid varieties as a group were shown to have a greater utilisation performance than the diploid group (P<0.001) with mean Residual Grazed Surface Heights (ResGSH) of -0.15 and +0.11 respectively. Increased digestibility and a more open sward structure were associated with varieties found to have greater graze-out potential.

Keywords: cultivars, grazing, post-grazing, sward height, utilisation

Introduction

A key improvement to be made within Irish ruminant production systems is to employ PRG varieties that perform superiorly within grazing systems, as Irish farms depend hugely on grazed pasture (O'Donovan *et al.,* 2018). Increased feed intakes, improved pasture quality and even, tightly grazed swards are advantages of superior grazing varieties. Low post-grazing sward height (≤4 cm) is indicative of good grazing performance as this increases pasture intake and conditions the sward to increase leaf production which has a higher feeding value relative to stem and dead proportion (Stakelum and Dillon, 2007). PRG variety evaluation is conducted by the Irish Department of Agriculture, Food and the Marine (DAFM), which publishes a Recommended List. Plots are evaluated by mechanical defoliation on a rotational basis to mimic Irish rotational grazing systems. Mechanical harvesting protocols may be limited in the information they provide as varieties are employed on farm in a different system than they have been evaluated in. Creighton *et al.,* (2012) found no significant difference in herbage yield between varieties of PRG managed under cutting or animal grazing protocols. However, Cashman *et al.,* (2012) found a similar relationship between cut and animal grazed protocols for herbage yield but significant differences (P<0.001) in dry matter off-takes were observed between varieties under animal grazing. The aim of this study was to identify PRG varieties that perform superiorly in grazing systems. PostGSH was used as the measure of grazing efficiency and varietal traits likely to be responsible for differences in grazing efficiency were also investigated.

Materials and methods

In August 2014, 55 varieties of PRG of varying heading date and ploidy were sown in Teagasc Moorepark, Co. Cork in a randomised complete block design in 3 replicates. The varieties were both recommended and candidate varieties from the Irish DAFM Recommended List trials. The 7×3 m (21 m^2) plots were grazed once during their establishment phase in 2014 and data were collected from 2015 onwards. The plots were grazed on 32 occasions from 2015 to 2018. The 1-hectare paddock was rotationally grazed by lactating dairy cows when herbage mass was estimated to be 1,300 kg DM ha^{-1}. Prior to grazing the pre-grazing surface height (PreGSH) of each plot was measured using a Jenquip rising plate meter. The plate meter also measured the PostGSH once the cows left the paddock and this was used as the measure of grazing efficiency. Herbage samples were collected prior to grazing events 2-7 in 2015 and 3-7 in 2016 to calculate sward nutritive quality (Dry matter digestibility (DMD), crude protein (CP) and water-soluble carbohydrates (WSC)). These were harvested with Gardena hand shears at 4 cm above the base of the sward, dried at 60 °C for 48 hours, milled and scanned by near infra-red spectrometry (NIRS). Morphological traits were examined in 2015 and 2016, once in the vegetative stage and once in the reproductive stage; 20 tillers were used to determine extended tiller height (ETH) and extended sheath height (ESH). Free leaf lamina (FLL) was calculated by subtracting the ESH from the ETH. Each tiller was then dissected into its leaf, stem and dead (LSD) proportions and oven dried at 90 °C for 24 hours to express each fraction on a dry matter (DM) basis. Data were analysed in the statistical program SAS 9.3 (SAS Inst. 2011). PROC MIXED was used to analyse the effects of years and grazing events as fixed terms on PostGSH, DMD, CP, WSC, FLL and LSD proportions. Block was included as a random variable in the model. The relationship between PostGSH and the variables analysed in the previous mixed model were examined using PROC GLM. Residual grazed surface height (ResGSH) was calculated for each variety and averaged over ploidy group (Tubritt *et al.,* 2018).

Results and discussion

Pre and Post GSH differed significantly between the varieties. The mean PostGSH across all the varieties was 4.19 cm. Pearson rank correlation calculated between all years for PostGSH was 0.62. PostGSH differed significantly between ploidy. Tetraploids, on average as a group, had significantly lower PostGSH than diploids at 3.99 and 4.32 cm respectively. Figure 1 displays the difference in PostGSH between tetraploid and diploid varieties.

PreGSH was significantly and positively correlated with PostGSH (R^2=0.48) (Table 1). Diploids had higher PreGSH and therefore higher PostGSH compared to tetraploids. ResGSH accounts for the PreGSH effect on PostGSH, allowing for accurate comparison between varieties. Negative ResGSH values indicate that varieties have good grazing efficiency. Overall, the tetraploid group had superior grazing efficiency to diploids with in average a ResGSH values of -0.15 and +0.11 respectively, though the very best three diploids were grazed similarly or more tightly than the weakest tetraploids. Tetraploids were found to have significantly higher levels of DMD, CP, WSC and FLL than diploids ($P<0.01$). High values in these traits were allied with low ResGSH ($P<0.01$).

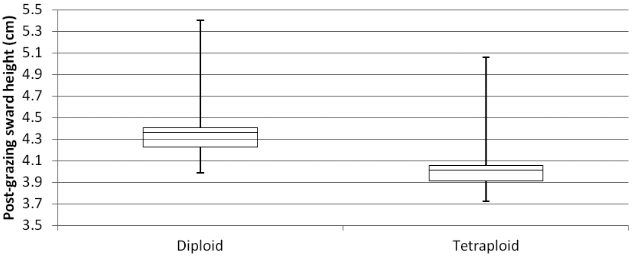

Figure 1. Difference in post-grazing sward height of diploids and tetraploids.

Table 1. Trait differences between diploid and tetraploid groups under animal grazing for key selected traits.

Trait	Diploid	Tetraploid	Significance[1]
PreGSH (cm)	10.00	9.60	***
PostGSH (cm)	4.32	3.99	***
ResGSH	+0.11	-0.15	***
Free leaf lamina (cm)	19.0	20.5	***
Dry matter digestibility (g kg^{-1} DM)	819.8	833.2	***
Crude protein (g kg^{-1} DM)	176.4	178.8	**

[1] $P>0.05$; * $P<0.05$; ** $P<0.01$ *** $P<0.001$.

Conclusion

The assessment of PRG varieties under animal grazing showed that varieties differed significantly in the level to which they are grazed by cows. Previous research has shown that grazing to low PostGSH increases the leaf proportion and nutritive value of the sward (McCarthy *et al.,* 2013). This study also provides evidence that tetraploid varieties were more conducive to grazing systems as they achieve lower ResGSH values. Genetic diversity existed within ploidy groups showing that further progress in grazing efficiency is possible. Assessment of PRG varieties using animals is costly and therefore identification of easily measured PRG traits for grazing efficiency is worthwhile. FLL, DMD and CP were three traits identified in this study that plant breeders could use to select for varieties with greater grazing efficiency. The study also suggests that farmers choosing grass varieties for grazing systems should select as high a proportion of tetraploid varieties within their seed mixtures as conditions and management permit.

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Comparison of perennial ryegrass varieties for differences in grazing efficiency

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Abstract

The aim of this study was to determine differences in grazing efficiency between varieties of perennial ryegrass (*Lolium perenne* L.) and to determine varietal traits influencing grazing efficiency. Grazing efficiency refers to the proportion of leaf tissue that is removed, relative to the amount presented to the animals prior to grazing. Low post-grazing sward heights are desirable as they condition the sward to increase leaf production and thus reduce the proportion of stem in the sward in subsequent rotations. Thirty varieties of perennial ryegrass from the Irish Recommended List were studied. The plots were grazed on nineteen occasions over two years, 2017 and 2018. Pre-grazing sward height, post-grazing sward height, herbage production, nutritive quality and morphology were measured throughout the trial. Varieties differed in their level of grazing efficiency (P<0.001) with post-grazing sward heights ranging from 3.54 to 4.60 cm. Residual Grazed Height (RGH) was derived as a measure of grazing efficiency calculated as the difference between achieved post-grazing height and predicted post-grazing height from a mixed model. Nutritive quality analysis was shown to have a significant effect on grazing efficiency. Organic matter digestibility (OMD) was shown to have a significant negative effect on post-grazing sward height (R^2=0.63). Tetraploids had greater OMD than diploids (787 g kg^{-1} DM vs 776 g kg^{-1} DM respectively).

Keywords: grazing efficiency, perennial ryegrass variety, residual grazed height

Introduction

Maximising the level of herbage utilisation is identified as a key factor influencing profitability on Irish ruminant farms as grazed grass is the cheapest source of ruminant feed relative to purchased feed (Hanrahan *et al.*, 2018). In Ireland grass swards are predominantly harvested by livestock in grazing systems. Identifying perennial ryegrass (PRG) varieties and traits responsible for superior performance within these systems is worthwhile so that varieties employed can perform optimally. Mechanically harvested (cutting) protocols used in variety evaluation trials such as the Irish Recommended List only provide a partial measure of variety performance and thus may be limited in the information they supply (Byrne *et al.*, 2018). The main disadvantage of 'simulated grazing' (cutting protocol) is that varieties that perform well within these evaluation trials may disappoint when they are used on farm which reduces confidence in the trials. Poor grazing efficiency is one such disappointment. Varieties exhibiting greater grazing efficiency are easier to manage and increase herbage utilisation. They are grazed to lower post-grazing sward heights (PGSH) which maintains the quality of the sward in subsequent rotations by conditioning the sward to increase leaf production. Poor grazing efficiency within swards results in discontent and unsettled animals that do not achieve graze-out targets. Farmers may choose to mechanically correct these poor graze-outs by 'topping' but this represents costs to the system, increasing the labour requirement and reducing grass utilisation.

Materials and methods

In 2016, the thirty leading varieties from the Irish Department of Agriculture, Food and the Marine Recommended List were sown and established in 8×4.5 m plots under a randomised block design in three replicates. Fifteen diploid (D) and tetraploid (T) varieties of intermediate and late heading types were used. The plots were rotationally grazed by dairy cows over two grazing seasons, 2017 and 2018. An average of 60 cows grazed the 0.6 hectare paddock on each occasion. Cows were allocated area based on the demand of the herd and the herbage cover in the paddock. Cows were moved on to the next allocation when the majority of plots were grazed to a height of 4 cm. Prior to grazing a 1.2×5 m section of each plot was harvested with an Etesia grass mower to a height of 3.5 cm. Mown herbage was weighed and 0.1 kg of the mown herbage from each plot was dried at 90 °C for 16 hours to determine dry matter (DM) content and herbage yield. A Jenquip rising plate meter was used to measure grass sward height prior to grazing and to measure the PGSH of each plot. A separate sub-sample was obtained from the mown herbage, freeze-dried at -50 °C for 72 hours, milled and scanned under Near Infra-Red Spectrometry (NIRS) for nutritive quality. Analysis of the morphological structure of the sward was undertaken by selecting 20 tillers from each plot and measuring the free leaf lamina (FLL, extended tiller length minus extended sheath length). The leaf, pseudostem, true stem and dead proportions of each plot were also measured on a DM basis. The data were analysed in the statistical program SAS 9.3 (SAS Inst. 2011) using a mixed model with block, grazing event and year used as variables. To account for pre-grazing height differences between varieties, the PGSH of each variety was predicted using pre-grazing height as an additional variable in the model. Residual grazed height (RGH) was used to evaluate grazing efficiency. It is calculated by subtracting predicted from actual PGSH. A negative RGH indicates a greater utilisation performance.

Results and discussion

Varieties were found to significantly differ in PGSH (P<0.001). The average PGSH across the two years was 4.1 cm which ranged from 3.54 cm (Astonenergy (T)) to 4.6 cm (Boyne (D)). Pre-grazing height was shown to have a significant positive relationship with PGSH (R^2=0.63). Boyne had the greatest pre-grazing height at 11.13 cm which was 2.07 cm higher than the lowest variety Astonenergy at 9.06cm. Astonenergy achieved the lowest RGH of -0.38; Clanrye (D) had the highest RGH of +0.34. Herbage yield is a major trait within variety evaluation and a key selection trait for farmers. Therefore, the superior varieties within this trait are those that achieve negative RGH values whilst also producing large herbage yields. Twymax (T) and Aspect (T) are examples of such varieties as they had RGH values of -0.2 and -0.18 and produced an average annual herbage yield of 13,400 and 12,500 kg DM ha^{-1} respectively. From Figure 1 a positive relationship can be observed between herbage yield and RGH (R^2=0.33). Not all varieties conform to this relationship though. For example, varieties such as Twymax and Rostta (D) produced similar levels of herbage (13,000 kg DM ha^{-1}) but had large differences in RGH; -0.2 and +0.14 respectively. Tetraploids were shown to have significantly better graze-out performance than diploids (P<0.001). This can be seen in Figure 1 with tetraploids dominating the left (negative) portion of the graph and diploids dominating the right (positive). Varieties identified for superior graze-out performance had increased levels of Organic Matter Digestibility (OMD) and a greater proportion of leaf within the sward. A 10 g kg^{-1} DM increase in OMD was associated with a 0.2 decrease in RGH (R^2=0.63). Tetraploid OMD (787 g kg^{-1} DM) was 115 g kg^{-1} DM greater than diploids (776 g kg^{-1} DM) which equates to a 0.23 difference in RGH. Varieties with increased leaf proportion had lower RGH values. A 50 g kg^{-1} DM increase in leaf proportion decreased RGH by 0.23 (R^2=0.54).

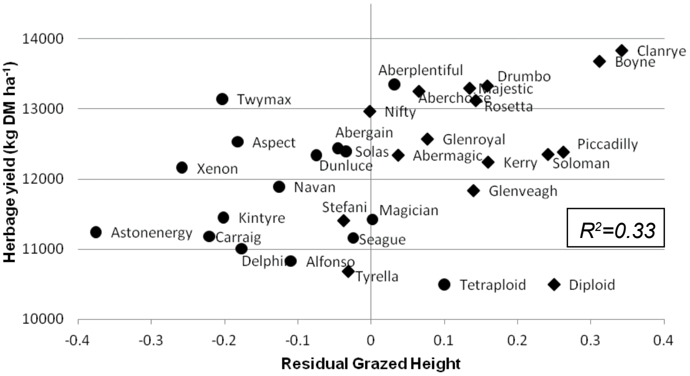

Figure 1. Relationship between varietal Residual Grazed Height and herbage yield.

Conclusion

Varieties were shown to differ in the level of efficiency that cows were able to graze them. Using RGH as a measure of grazing efficiency allowed varieties to be compared accurately by taking pre-grazing height differences into account. Herbage yield is a key trait of importance in variety selection and therefore high yielding varieties with negative RGH values are superior within grazing systems. Tetraploid varieties were identified as having a greater graze-out performance than diploids, although genetic variation between diploids shows room for improvement. As in other studies (such as O'Donovan and Delaby, 2005) increased OMD and increased leaf proportion were identified as factors responsible for improved grazing efficiency. Variety breeders can indirectly select for varieties with improved grazing efficiency by selecting for increased OMD and greater leaf proportion within the sward. The study also shows that farmers wishing to improve the grazability of their swards should incorporate increased proportions of tetraploids within their seed mix.

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Using the influencing factors for grassland farmers in bridging the gap between practitioners and scientists

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Abstract

In Inno4Grass, practical know-how on grassland-based farming systems is combined with results of scientific research and development in a participatory approach. The impact of the project will increase by the efficient spreading of the results to the larger farming community. This study therefore aimed to get insight into the main influencing factors for decisions of grassland farmers. An on-line questionnaire was issued among European grassland farmers in eight countries and yielded more than 1000 responses. The most important influencing factor for decisions with respect to grasslands was the farmers' own values and norms (>90%). Image of the farm / the sector, advisors, consumers, customers and family were also considered important. These results should be taken into account when trying to reach the larger farm community.

Keywords: Inno4Grass, mind-set, own values and norms, participatory approach, questionnaire

Introduction

In Inno4Grass, integration of current knowledge of farmers, advisors and researchers with new knowledge is achieved by active interaction between the different stakeholders. The aim is to create a flow of innovations on grassland management in Europe. Therefore, an overview of brokering systems was set up (https://www.encyclopediapratensis.eu/encyclopedia_praten-sis/about-grassland/the-actors/brokering-systems/), showing that they should be an important part of building a European innovation space for grassland-based farming (Goliński *et al.*, 2018). Furthermore, an on-line conference paper database (http://grassland.uni-goettingen.de/index.php) was set up and is now publicly available and easily accessible for all stakeholders (Paesel and Isselstein, 2018). Also, Inno4Grass is working with real-life meetings of practitioners and scientists (practice and science meetings) in eight countries to create a common learning and co-creating environment. Each meeting addresses a particular grassland-related topic. In these meetings, the points of view and the knowledge of scientists, practitioners and other actors are shared and combined. The evaluation of the meetings held thus far is positive (Peratoner *et al.*, 2019). The question arises, however, how to spread the results of Inno4Grass to the larger farming community in order to increase the impact of the project. A first step in answering this question is to identify factors that affect farmers' decisions with respect to grasslands. A study has thus been conducted aiming to get insight into these main influencing factors.

Materials and methods

An on-line questionnaire on innovations on grassland was developed in seven languages: Dutch, English, French, German, Italian, Polish and Swedish, using SurveyMonkey (www.surveymonkey.com). The questionnaire studied the attitude of grassland farmers. A list of ten potential influencing factors for decisions of grassland farmers was identified by the project consortium, i.e. by an array of people from practice (e.g. farmers unions, extension services) and science. Respondents were asked what influence these predefined people/items have on their decisions with respect to grasslands. Answers were scored on a Likert scale: very unimportant – unimportant – neutral – important – very important. Furthermore, some general questions were asked: country, available grassland area, main type of animal on grasslands (only one option possible). The partners of Inno4Grass actively spread the questionnaire to tens of thousands of grassland farmers in eight different countries (Belgium, France, Germany, Ireland, Italy, Poland, Sweden and the Netherlands), either via email or via social media. The questionnaire was available from winter 2017/2018 onwards and closed at the end of June 2018. Respondents that did not have grasslands were excluded. Results were analysed by analysis of variance (ANOVA) using SPSS version 23 to discern any effects of country, available grassland area and main animal type on influencing factors.

Results and discussion

The questionnaire yielded 1,091 valid responses from grassland farmers spread over the different countries (Table 1). Dairy cows were the most common animal type, followed by beef cattle. In Italy, most responses were related to sheep. The category 'other' included horses, pigs and buffalos (the latter especially in Italy), but also grasslands where the grass is harvested and sold and not used by any animals on the farm.

Country had a significant effect ($P<0.01$) on the influence of the majority of the predefined people/items on decisions of grassland farmers, while available grassland area and main animal type had not. The influence of customers had no country effect ($P=0.134$). Due to the large differences in number of respondents per country, weighted averages were calculated. Figure 1 shows that 'own values and norms' was the most important influencing factor. Image of the farm/the sector, advisors, consumers, customers and family were also considered to be important for decisions with respect to grasslands (>50%). It would be interesting to use in-depth interviews to study further the background of the answers, e.g. on what factors do grassland farmers build their own values and norms, is it family traditions, agricultural education or something else. In addition, people may adapt their opinion, e.g. as a result of education, training and examples from practice.

Table 1. Description of respondents per country: number of valid responses, average grassland area of the farm (ha) and main animal type (percentage of all respondents of the specific country).

Country	Number of valid responses	Average grassland area (ha)	Dairy cows (%)	Beef cattle (%)	Sheep (%)	Goats (%)	Other (%)
Belgium	24	36	50	34	8	4	4
France	80	78	42	25	23	1	9
Germany	40	87	77	8	0	0	15
Ireland	93	66	47	37	12	0	4
Italy	52	130	13	17	35	8	27
Poland	125	23	49	33	2	2	14
Sweden	112	128	49	29	4	0	18
the Netherlands	565	53	98	0	1	0	1

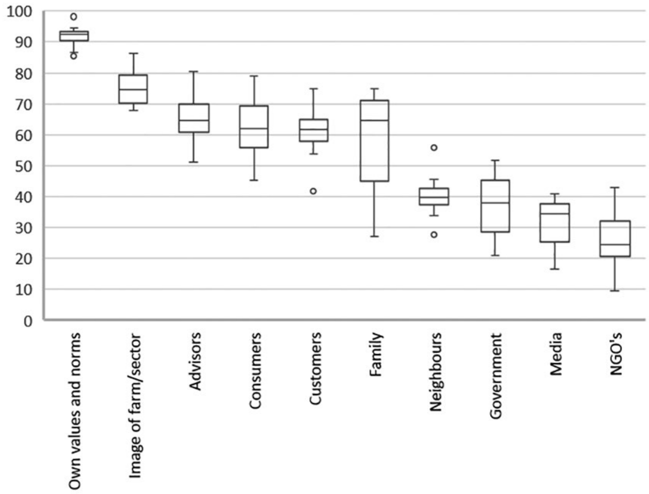

Figure 1. Influence of people/items on decisions with respect to grasslands (percentage of the respondents that scored either important or very important, weighted average per country).

Conclusion

The most important influencing factor for decisions of grassland farmers are their own values and norms, followed by image of the farm/the sector, advisors, consumers, customers and family. These results should be taken into account when trying to reach the larger farm community.

Acknowledgements

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Characteristics of *Lolium perenne* varieties for grazing

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Abstract

Grazing management in the Netherlands is characterised by alternating use of grassland for grazing and silage production. Therefore, the variety research for the Recommended List (VCU: Value for Cultivation and Use) of *Lolium perenne* L. (*Lp*) is based on cutting and grazing trials for late and intermediate varieties. The aim of this study was to examine whether data of the official Dutch variety testing are suitable to choose the best fitted varieties for grazing. It was shown that the choice of varieties depends on the type of grassland management. The varieties in the late heading group, especially diploid varieties, had advantages for grazing, like good summer growth and less stemmy regrowth, but did not realise an early growth in spring. The information on performance of individual varieties under grazing and mowing trials is relevant to support farm management.

Keywords: *Lolium perenne*, varieties, value for cultivation and use, grazing

Introduction

In the Netherlands, about 67% of the total area of grassland is grazed or partly grazed (Gies *et al.*, 2014). Common grassland management is characterised by alternating use of grass for grazing and silage production with *Lolium perenne* L. (*Lp*) being the main species. The first cut is often used for silage production. Then the grassland is grazed twice, followed by one cut for silage production and two for grazing. However, due to the increased attention for grazing in the Netherlands, more farmers start with a grazing cut instead of a silage cut and more grassland is grazed exclusively. These farmers require varieties of *Lp* with an early start in spring, a constant grass production during the growing season and limited effects of heading on grass intake. The aim of this study was to examine whether data of the official Dutch variety testing are suitable to choose the best fitted varieties for grazing.

Materials and methods

The variety research (VCU) for the Recommended List of Varieties of *Lp* in the Netherlands is carried out under practical conditions in both mowing and grazing trials (Anonymous, 2018) resulting in a yearly update of the Recommended List (Anonymous, 2019). New varieties – both diploid and tetraploid – are sown in a grazing and a cutting trial in two successive years on different locations and are compared with already listed varieties. Yield measurements are done in three consecutive years following the sowing year. In the mowing trials, a plot of 9 m^2 is harvested. The grazing plots, located at dairy farms, are 32-36 m^2 and split into five subplots. When grazing starts, a subplot is harvested with a Haldrup cutter, while the rest of the grass is grazed. Just before the following cut, another subplot is harvested with the Haldrup to measure the effect of grazing of the previous cut. This procedure is repeated till the end of the season. With a silage cut the same procedure of harvesting a subplot is used, while the rest of the grass is also mown and harvested.

Because varieties of *Lp* differ in heading date and growing pattern, the testing of varieties is split into two groups. There is one intermediate group with an average heading date from 20-31 May, and one late group with average heading dates from 1 June onwards. The variety means collected in the VCU trials in 2010-2017 were used to analyse the interaction between variety, ploidy and management. Growth per day was calculated by dividing the yields per cut by the number of growing days. The data were analysed using ANOVA.

Results and discussion

There was a strong correlation between ground cover (GC) under mowing and GC under grazing ($P<0.001$; Figure 1A). However, it was also clear that varieties with similar level of GC-mowing had a different GC-grazing. Therefore, the variety order depends on the management (grazing or mowing). On average, diploid varieties at the same level of dry matter yield under mowing (DMY-mowing) had a higher DMY-grazing compared to tetraploid varieties ($P<0.01$; Figure 1B upper line versus lower line). Next to this there was a strong correlation between DMY-mowing and DMY-grazing ($P<0.001$; Figure 1B). Again, varieties with similar levels of DMY-mowing showed a wide range of DMY-grazing, so the variety order strongly depends on management.

A negative correlation between heading date and spring growth was found (data not shown). As a result, late heading varieties that had less spring growth, but the same total annual DMY, differed in production later on in the season. Diploid varieties had a significantly higher production after the first cut compared to tetraploid varieties (Table 1, left). Late varieties had a higher production after the first cut compared to intermediate varieties.

A complicating factor is that the average yield of the first cut of the intermediate varieties was higher than that of the late varieties. It is thus not clear what the growth per day would have been if the yield of this first cut had have been the same for both groups. Stemmy regrowth in the early summer was the lowest for the diploid varieties in the late group (Table 1, right).

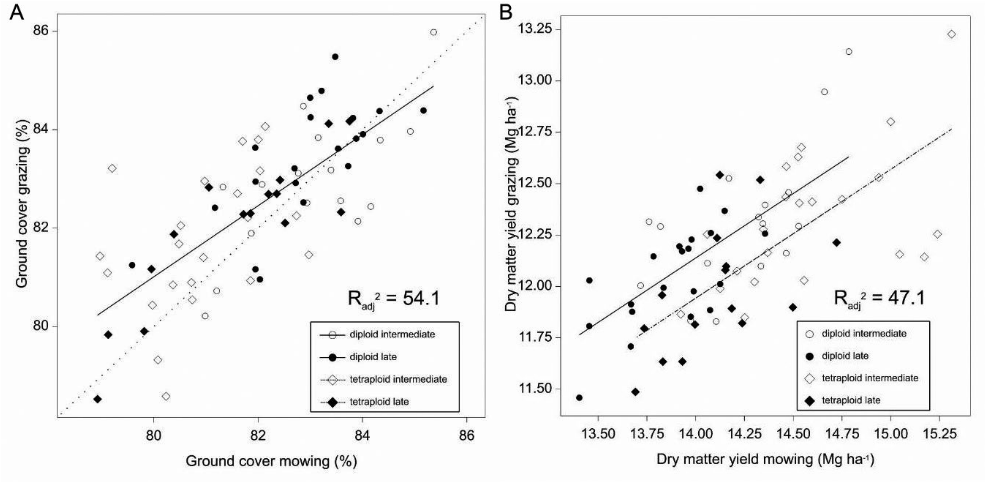

Figure 1 (A). Relation between Ground Cover (%) under mowing and under grazing for individual varieties. The dotted line represents y=x. (B) Relation between Annual Dry Matter Yield (Mg ha^{-1}) under mowing and under grazing for individual varieties.

Table 1. Average grass growth of grazing cuts in kg ha^{-1} d^{-1} ($P<0.001$) and stemmy regrowth (%; average, minimum, maximum; $P<0.001$) in early summer for varieties of the Recommended List 2018, results of testing 2010-2017.

	Growth (kg DM ha^{-1} d^{-1})			**Stemmy regrowth (%)**	
	Intermediate	**Late**		**Intermediate**	**Late**
Diploid	48.5 b	50.8 a	Diploid	15 (11-21) b	9 (4-15) a
Tetraploid	46.7 c	47.8 bc	Tetraploid	15 (10-19) b	15 (9-20) b
Lsd5%	1.3		Lsd5%	2.6	

Therefore, late-heading varieties might still be preferred by farmers even though the annual DMY is slightly lower (Figure 2A). The heading dates of listed varieties of the intermediate group were spread equally in time, unlike of the late group (Figure 2B).

The results show that the varieties in the late group, especially diploid varieties, had advantages for grazing, like good summer growth and less stemmy regrowth, but they did not realise an early growth in spring. With the specific parameters of the varieties, farmers are able to create mixtures of varieties for grazing. It is recommended that further examination be made to determine whether very late varieties have further advantages for grazing.

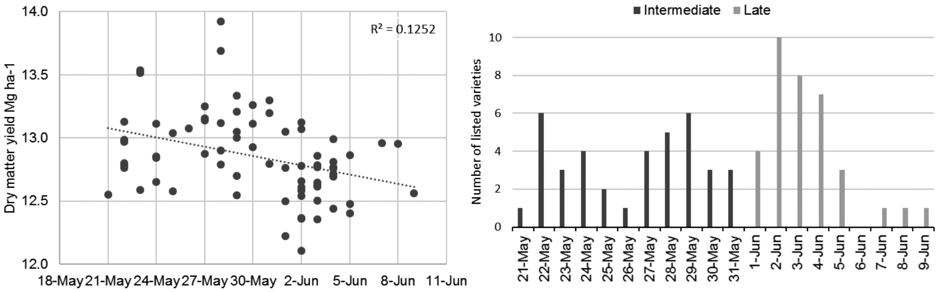

Figure 2. (A) Relation between heading date and annual DMY of *Lp* varieties of the Recommended List 2019. (B) Number of listed varieties per heading date.

Conclusion

The information on performance of individual varieties under grazing and cutting trials is relevant to support farm management. The Dutch variety testing system is suitable for selecting varieties for both cutting and grazing management. It is recommended that additional information on varieties should be presented in the Dutch Recommended List of Varieties to support farmers' decisions on selecting varieties for grazing.

Acknowledgements

The authors acknowledge the grass breeders and the Dutch breeders association Plantum for the use of the data of the variety testing for the Dutch Recommend List of Varieties.

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‘From research – for practice’: translating research findings for practical application

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Introduction: The prolonged drought in 2018 has emphasised again how changes in temperature and precipitation and the occurrence of extreme events directly affect agricultural systems such as grasslands. Thus, translating research findings for practical application is crucial, in particular to support resilient forage production systems that ensure productivity under future climate. Although drought effects on grasslands have been studied in numerous scientific experiments, findings have typically been published only in scientific literature unavailable for practice or policy-making. Thus, there is a lack of adequate translation and communication of research findings into the practical context.

Materials and methods: Using data from several multi-year summer drought experiments in Switzerland (e.g. Burri *et al.*, 2014; Gilgen and Buchmann, 2009; Hofer *et al.*, 2016), we (1) summarised observed responses of grassland vegetation and soils to experimental drought and (2) translated them for practice to support grassland management decisions in the future.

Results: Grassland responses to drought varied considerably among individual studies. Thus, simple generalisations based on single studies are impossible and recommendations for practice need to be based on a comprehensive evaluation of multiple studies taking into account environmental and management aspects (Table 1). We rely on established channels and networks to disseminate these results to different stakeholder groups. Examples are low threshold activities like hands-on activities at fairs for the general public, information leaflets and field days for practitioners, and scientific discourse with governmental actors.

Table 1. Responses from drought experiments in Swiss grasslands and their applicability.

Variable	Response	Universal applicability
Short-term yield	Mostly negative but positive responses also occur	Restricted to regions as response is site-dependent; species or functional group composition is relevant
Annual yield	Moderate to no response	High resilience of grasslands to drought can be generalised
Photosynthesis/stomatal conductance	Mostly species-dependent decrease	Not directly relevant for practice
Weed pressure	Increase possible	Generalisation difficult as weeds are often not considered explicitly; sward diversity is relevant

Conclusion: Only when appropriately translated for practical application and distributed through tailored channels such as the Swiss Grassland Society (AGFF), regional extension services and other established farmers’ networks, research findings on drought effects can contribute directly to achieving resilient forage production systems in Switzerland.

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The Sown Grassland Cup: a competition as training tool for mixture choice and management

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Introduction: Many young farmers enjoy joining breeders, ploughing or crop competitions. The Sown Grassland Cup (SGC) provides the opportunity for young farmers to compete in the field of grassland management. We organise the SGC with the aim of sensitizing farmers to the importance of professional grassland management for efficient forage production.

The game: The SGC is open to agricultural school classes and young farmer teams. Each team chooses a seed mixture among the Swiss Standard Mixtures (Suter *et al.*, 2019). In August, the organisers (Strickhof with the help of Agroscope) sow the chosen mixtures on three plots (18 m^2) per team, following a randomized complete block design with three replicates. For the next one and a half years, the teams monitor their plots via the Internet and decide on the timing of cutting and fertilizer applications, as well as on the form and amount of fertilizer. The teams send their decisions by e-mail to the organisers who carry out the fieldwork.

Materials and methods: During the first year after sowing, forage yield is measured at each cut, as well as forage digestibility, crude protein and crude fibre content. The content of net energy for lactation (NEL) in the forage is then calculated from the forage quality parameters and multiplied by the yield in order to obtain the net energy yield per hectare (GJ NEL ha^{-1}).

Results and discussion: Seven teams participated in the last SGC. The three best teams obtained 19% more NEL ha^{-1} than the three weakest teams (Table 1, $P<0.05$). Among the four teams that chose the mixture SM240 (Suter *et al.*, 2019), two were among the three winners, but one was among the losers (Table 1). Thus, mixture choice is not the sole decision influencing NEL yield. The results were echoed by the agricultural press, and the teams and organisers hold a lively debate about management factors that could explain the results.

Table 1. Net energy yield realized by the farmer teams during the first year after sowing.[1]

Team	Energy yield (GJ NEL ha^{-1})		Mixture
A	111.7	a	SM240
B	108.4	a	SM320
C	108.3	a	SM240
D	105.0	ab	SM240
E	92.3	b	SM300
F	91.6	b	SM240
G	91.1	b	SM323

[1] Means followed by a common letter are not different at the 5% level (Duncan MRT). Mixture composition is given in Suter *et al.* (2019).

Conclusion: Results clearly show that managerial decisions about fertilisation and timing of cutting, and not only mixture choice, influence the amount and quality of forage from sown grasslands. This occurred even if each team tried to optimise its management to win the cup. Using the farmers' own decisions to discuss these effects greatly helps reach the intended audience.

Suter D., Kessler W., Thomet E., Frick R. and Lüscher A. (2019) Grass-clover mixtures in practice: key-factors of success. *Grassland Science in Europe* 24, this volume.

REDCap, an inter-profession cooperation to improve and promote sown grasslands for sustainable dairy goat systems in western France

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Introduction: Increasing feed self-sufficiency and grassland use leads to reduced feeding costs and reduced negative environmental impacts. In the west of France, a research and development scheme for high performance and sustainable goat farming has been created: REDCap and Patuchev (Caillat and Jost, 2015). This encompasses (1) experiments in experimental units (Patuchev) and on farms (REDCap), (2) exchanges between scientists, advisers and breeders to support the emergence of innovations, and (3) the dissemination of new knowledge on these topics. Greater use of sown grassland with legumes (e.g. multi-species sward with a significant share of alfalfa and clover) is one solution to improve sustainability and protein self-sufficiency on goat farms. However, there are few references available on their use in western France for this kind of goat farming systems.

The scheme: To design and assess productive and perennial goat-adapted multi-species swards, three trials were set up on farms (REDCap) and at Inra (Patuchev) since 2012. These multi-species swards were developed by goat breeders, advisers, industry and researchers. In 2012, 2015 and 2017, 40 fields were sown by 25 breeders in western France and later followed up. Results on botanical diversity and yield evolution of these multi-species swards are available (Jost *et al.*, 2017). A major question now is: how to involve stakeholders in a participative approach to improve the adoption of mixture cultivation by goat breeders?

Different tools have been used for five years:

- Both farm fields and experimental units have been evaluated (evolution of botanical diversity, yields, field management and goat behaviour) under a large range of soils and utilisation in order to facilitate their take-up by goat breeders.
- Both breeders and advisers are involved in the evaluation of each field. This improves their skills on grassland management.
- Dissemination of results at meetings on farms and experimental units, and on social networks. Since 2017, the use of Instagram ('redcap_pme') allows breeders to follow the evolution of each field (pictures, technical information, botanical diversity) from a distance.
- Mixtures have also been developed with industry (Jouffray-Drillaud). Thanks to exchanges with seed companies, a supply for goat breeders could be developed more easily.

Conclusion: The transfer of results to stakeholders is essential. It is a strong challenge. Distance and lack of time often prevent links from being created. The social network 'redcap_pme' on Instagram is an interesting way to fulfil this challenge. Co-building mixtures with breeders also encourages the dissemination of our work.

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Performance evaluation of the collaboration between R&D organization and SME: a case study approach

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Introduction: The performance management of research and development (R&D) has been extensively debated in the literature. Implementation of managerial tools such as Balanced Scorecard (BSC) applied to collaborative projects between R&D organizations and small and medium-sized enterprises (SME) is being neglected. There is also little knowledge about the long-term effect of such projects on the competiveness the organizations (Khoshnevis and Teirlinck, 2018; Nepelski and Piroli, 2018). The aim of this paper is to contribute to empirical knowledge about the implementation of strategic management tools in the evaluation of the collaboration between R&D organizations and SMEs, with a primary focus on the BSC.

Materials and methods: In line with Chiesa *et al.* (2009), Cuganesan *et al.* (2012) and Bremser and Barsky (2004) a reference framework was developed that identifies three levels of R&D performance management: the project level, the company level and the market level. A set of interrelated financial and also non-financial indicators were developed. When examining one particular management tool a case study methodology is recommended in the literature. The field study was conducted over a period of 24 months for an R&D project concerning the breeding of a unique variety of a protein legume and newly developed population chips for detection of allelle gene variants. The project was implemented by Agriculture Research Troubsko and an SME. Desk research, interviews and observations were implied.

Results: The case study provides interrelated indicators based on a BSC and also a system explaining how to measure and evaluate the effect of collaborative projects from both R&D organizations and SME perspectives. The output indicators measure particularly the added value perceived by customers as a result of highly professional services, customer satisfaction with a new product or number of new project ideas.

Conclusion: This paper presents a tool that can evaluate the effect of the cooperation between practice and research, and can enhance further cooperation. We conclude that although collaborative research projects are complex, these case examples can be a good guide for a number of R&D organizations and SMEs, and can be utilized in various environments.

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Variety testing for grassland species – advanced recordings and analysis

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Introduction: In Austria, new varieties of grassland species are typically tested under standardized conditions at different sites to determine the most suitable ones. The main evaluation criteria are heading time, resistance to plant diseases and weed infestation, re-growth capacity, winter hardiness and yield. Forage quality, however, is only considered by means of crude protein content. To meet the increasing requirements in animal nutrition and also addressing the challenges of climate change, advanced studies and recordings of quality parameters are therefore relevant and these aspects are the subject of this study.

Materials and methods: Variety testing trials at AREC Raumberg-Gumpenstein have been extended in terms of duration and forage qualitative analysis. A strong focus was given to red clover and alfalfa of which selected cultivars were implemented twice in the respective testing trials. For these subsets four different harvesting dates were defined both for the first and second growth and numerous parameters like mean stage by count (MSC) and weight (MSW; not presented here) and some functional traits (length and weight of plants, stem diameter, leaf proportion) were recorded for each of 40 plants per cultivar and sampling date (Sanderson *et al.*, 1989). Advanced chemical analysis (e.g. protein and crude fibre fractionation) were carried out and will provide decision-making support. Analyses of variance and Pearson correlation analyses were performed using SPSS Statistics, version 25.

Results: In contrast to the commonly used BBCH-scheme, estimation of vegetation stage by MSC showed a stronger differentiation between tested red clover cultivars (Table 1). MSC-values were significantly linked with plant length, total plant weight and stem diameter ($P<0.001$). Two of the observed cultivars (Van and Fregata) clearly differed from the others concerning these plant functional traits but also showed a significant lower leaf proportion compared to Carbo and Panova. Plant length, total plant weight and stem diameter were significantly correlated with each other ($0.79<R\leq0.85$).

Table 1. Average vegetation stage and functional traits for red clover cultivars at the time of first harvest (8-6-17).[1]

Cultivar (ploidy level)	BBCH[2]	MSC	Plant length (cm)	Total plant weight (g)	Leaf proportion (weight-%)	Stem diameter (mm)
Carbo (4n)	61	3.9 a	58.4 a	6.88 a	30.5 a	3.7 a
Van (2n)	61	5.0 a	68.8 b	7.43 a	22.8 b	3.7 a
Panova (4n)	60	3.9 a	56.6 a	6.16 a	29.8 a	3.6 a
Fregata (4n)	64	4.8 a	69.7 b	7.04 a	23.4 b	4.0 a

[1] Means with same letter are not different at 5% level (Duncan's MRT).

[2] BBCH was standardly estimated for the total plot.

Conclusion: MSC and plant functional traits provide important information that may substantially support the characterization and selection of cultivars for different purposes in grassland management. These functional traits will be linked with outstanding quality parameters to find whether trait measurements could (partly) replace expensive forage quality parameters.

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Establishment of a farm network to investigate the long-term impacts of leys in rotations

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Introduction: Integration of temporary leys and livestock in crop production systems has the potential to provide benefits to both arable and livestock systems. A ley is a perennial forage crop suitable for grazing by livestock, which is grown for a limited number of years, in contrast to permanent pasture. Most leys in the UK consist of grass species, a mixture of grass and clover species, or a mixture of grass, clover and herbal species. Commonly used herbal species include chicory, sainfoin and birdsfoot trefoil and herbal leys can contain a mixture of up to 20 species, with herbs making up 5-40% of the mix. Continuous arable cropping with annual soil cultivation and little or no inputs of organic materials have led to reductions in soil organic matter (SOM) content. By taking land out of annual cultivation and increasing the return of organic matter in the form of root and litter turnover, as well as via the grazing animal, temporary leys have the potential to increase SOM levels (e.g. Johnston *et al.*, 2009).

Farm network: In February 2018, ADAS, the UK Agriculture and Horticulture Development Board (AHDB) and the Department for Environment Food and Rural Affairs (Defra) launched the 'Grass and herbal leys farm network' in the UK. The network is a partnership between farmers, researchers and industry, and provides a platform to investigate:

- Changes in SOM and soil health from introducing temporary leys across a range of soil types and rainfall areas.
- Subsequent changes to SOM and soil health following destruction of the temporary ley and return to arable production.
- The effectiveness of leys in controlling blackgrass in 'problem' fields.

There are currently (in November 2018) 187 individuals registered with the network; 44% are farmers, 18% on-farm advisors or agronomists, 12% researchers and 26% 'other'.

Survey: An on-line ley management survey was used to collect information on ley duration, ley management, and establishment and destruction methods. Around 75% of the farmers who completed the survey had leys as part of their rotation, either as part of a mixed farming system or for beef/lamb production, with the leys both cut for silage and grazed. About 80% of farmers included legumes/herb species in the ley seed mix (8% had pure grass swards), with leys typically in place for 3-5 years (73%). Just under half of farmers with leys used plough-based cultivation to establish arable crops after the ley, 28% used minimum tillage and 26% direct drill. More than 90% of farmers with leys said improvements in soil quality were an important factor in their use of leys and 53% said the leys helped them control blackgrass.

Conclusion: The network provides a valuable platform for current and future projects investigating the long-term rotational benefits of leys. 43 farmers with leys in the rotation have joined the network and said they would be willing to take part in monitoring on their farms. Further information on the network is available at http://www.adas.uk/services/Grass-and-herbal-leys-farm-network

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Reflections on stakeholder engagement in basic research on soil microbial communities in production grasslands

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Introduction: BIOINVENT, an ongoing research project studying the diversity and functioning of soil microorganisms in grassland ecosystems across a Pan-European transect ranging from Sweden, Germany, and Switzerland to Portugal with Azores, comprises an outreach stream with work packages for Stakeholder Engagement. Farmers and research teams both contribute to the project. This interaction is here reflected upon to give hints on how the interaction can be optimized in future research projects.

Materials and methods: A wise choice of level of participation has been recommended to balance benefits and pitfalls (Baker and Chapin III, 2018). The role of farmers in this project is mainly to give access to their fields and to answer questions about field management; thus 155 farmers giving access were included. The research teams are responsible for conducting the research but also for sharing their results. During the conduction phase, spontaneous discussions and questions appeared in the interaction between farmers and researchers. Thus, no quantitative data were collected and consequently no statistics was done.

Results: Basic research does not always solve the farmers' prevailing problems, and it was an important aspect that farmers proposed open questions and concerns not considered by BIOINVENT (e.g. role of EU liquid manure regulation), which provides options for prospective research projects (Figure 1). Furthermore, researchers' and farmers' time frames are not aligned; while researchers publish their results in scientific journals at the end of the project, farmers expect preliminary results during the project implementation phase. To manage this, a stringent communication strategy was used. One strategy was to send out farm-based data sheets to the participating farmers (Figure 1).

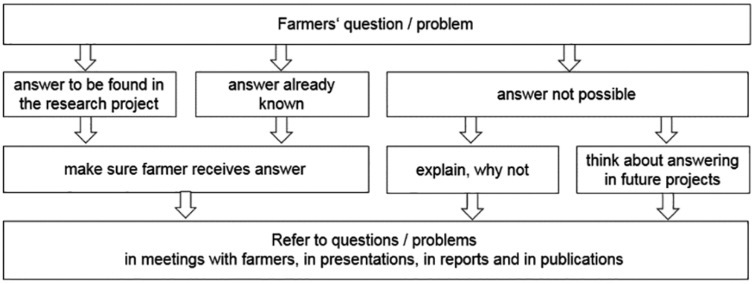

Figure 1. Different ways on how research teams can respond to farmers' questions or problems.

Conclusion: Communication between research teams and farmers needs to be formulated in a stringent communication strategy. The fulfilment of the actions in the strategy should be assessed. The strategy should also include measures to meet unexpected topics arising in the interaction between actors in a research project. Professional moderation of communication strategy and assessment is advised, to improve and secure the mutual obligations.

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