Sown diversity effects on yield and resistance to weed invasion: Clues to improve mixture design under climatic change in the Mediterranean

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ABSTRACT

With the aim to improve mixture design, particularly in regions vulnerable to climate change, we tested several forage communities following the biodiversity–ecosystem function (BEF) framework. We sowed monocultures and 4-species mixtures from a pool of 7 forage species in a sub-Mediterranean region (Eastern Pyrenees) and assessed the diversity effects on yield and resistance to weed invasion. The tested species included two grasses and five legumes with contrasting temporal patterns and different climatic amplitudes. The communities differed in their specific composition (mixture types) and the relative abundance of the components, following a simplex design, which allowed us to estimate separately the two components of the diversity effect: the individual species effects and that due to species interactions. Whereas monocultures performed in a highly variable way within and across harvests, both in relation to yield and weed suppression, mixture variability was narrower. Both functions increased in mixtures (with significant interaction effects between 24% and 57% for yield and 13% and 96% for weed suppression), especially in those mixtures including Mediterranean species, which showed the highest diversity effects that persisted over the three experimental years. Extreme climatic events during the experimental period might have affected not only the species' individual performances but also the strength of species interactions. Both components of diversity, identities and interactions, were key in maintaining high performances. We conclude that, under the current climate change scenario, it is important to include species in mixtures that increase resistance or resilience not only at the species level but also at the community level, through enhanced interaction effects.

1. Introduction

In the current global change scenario, agricultural systems have the challenge to increase their potential for mitigation and adaptation (IPCC, 2022) and to decrease their dependency on external inputs (Kirschenmann, 2007; Niggli et al., 2009). Increasing the diversity of agroecosystems may be a tool to respond to these challenges (Brooker et al., 2015; Haughey et al., 2018). Diverse grassland systems have resulted in enhanced yield (Finn et al., 2013; Hooper et al., 2005; Tilman et al., 2002, 2014), lower fertilizer (Nyfeler et al., 2009; Suter et al., 2021; Weisser et al., 2017) and agrochemical needs linked to reduced incidence of weeds, pests and diseases (Connolly et al., 2018; Van Ruijven et al., 2003), and reduced environmental impact, including

reduced nitrogen (N) losses through leaching (Hauggaard-Nielsen et al., 2003; Szumigalski and Van Acker, 2006) and emissions (Cummins et al., 2021; Pappa et al., 2011; Ribas et al., 2015). Diversity may also be an important adaptation strategy against climatic risks in an uncertain future, through enhanced resistance and resilience (Altieri et al., 2015; Isbell et al., 2015; Lüscher et al., 2022; Volaire et al., 2014), after extreme climatic events (Grant et al., 2014; Haughey et al., 2018; Sunil et al., 2020) or under sub-optimal environmental growing conditions (Porqueddu et al., 2016; Psyllos et al., 2022).

The changes in environmental patterns derived from the current climate change may represent an important challenge in Mediterranean areas. These are expected to be exposed to increased frequency of extreme temperatures, expanded drought periods and more irregular

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precipitation patterns (IPCC, 2022), thus being especially vulnerable to climate change. Agricultural systems will have to overcome a multitude of new biotic and abiotic stressors. The multi-faceted and uncertain challenge that climate change poses on agriculture requires other strategies besides breeding. In this context, mixtures may allow the simultaneous incorporation of several resistance traits at the community level (Dooley et al., 2015; Ehrmann and Ritz, 2014).

In forage systems, the difficulty of establishment and lack of persistence may be particularly important, affecting not only the stability of forage supply but also weed invasibility and thus forage quality. As both establishment and persistence are expected to be highly affected by environmental conditions, under increased unpredictablility, the sustainability of these systems in the Mediterranean will be exceptionally threatened (Porqueddu et al., 2016).

The use of a broader range of cultivated species or varieties to guarantee a minimum output under uncertain conditions has been known since ancient times (Altieri, 1999; Altieri et al., 2015; Vandermeer, 1989). This positive relationship between diversity and productivity is underpinned by two major components: the identity effect and the interaction effect. The former corresponds to individual species contributions and is essentially equivalent to the portfolio effect (i.e., increase the probability that at least one of the included species will respond favourably to the given conditions) (Campbell et al., 2012; Díaz and Cabido, 2001). The latter is related to species interactions owing to complementarity mechanisms (niche differentiation and facilitation) and is defined as the increase in a given function observed on top of the expected value according to the proportional contribution of the components (McIntire and Fajardo, 2014; Kirwan et al., 2007; Nyfeler et al., 2011).

The optimum composition and management of mixtures for a given agro-climatic system, or under unpredictable conditions, remain important questions of mixture design. This gap of knowledge has resulted in diverse forage systems still occupying a limited area (around 10% in Catalonia; Statistics of the Spanish Ministry of Agriculture, 2012) while the use of forage monocultures has been generalized (Peeters et al., 2006). Nevertheless, recent advances in methodological approaches, such as the one developed in the Agrodiversity experiment (Connolly et al., 2013; Kirwan et al., 2009), are setting the basis to optimize the design of mixture composition, as they test different levels of evenness, plant functional traits and sown density. Additionally, this approach allows to segregate between identity and interaction effects.

With the aim to develop forage mixtures best adapted to the current climatic conditions in Mediterranean mountains, we manipulated sown diversity using a range of species proportions in four-species mixtures, and repeated this design with five mixture types, differing in the specific forage species composition. We wanted to know: 1) if sown diversity provides advantages in terms of forage yield and resistance to weed invasion for a range of mixture types within a site; 2) if sown diversity provides stability of forage supply and resistance to weeds in the context of rainfed systems under experimental conditions; 3) if there is a mixture type best adapted to the current changing conditions in these Mediterranean mountains in which the experiment was developed; and 4) the relative importance of identity and species interactions effects in the studied responses for the different tested mixtures in order to maintain or increase crop performance.

2. Materials and methods

2.1. Selected forage species

In this study we used six forage species: two grasses (*Lolium perenne* L. var. Lacerta and *Dactylis glomerata* L. var. Accord) and four legumes (*Trifolium repens* L. var. Milo, *T. pratense* L., *Onobrychis viciifolia* Scop. var. Fakir and *Medicago sativa* L. var. Aragó), one of which (*Trifolium pratense* L.) was represented by two varieties, including an erect (*T. pratense* L. var. Merivot) and a prostrate form (wild variety native to

Swiss pastoral landscapes). For simplicity, hereafter we refer to all seven forages as species, even though the two *T. pratense* varieties correspond to the same species. All the species are commonly found in the Iberian Peninsula as cultivated species or in natural meadows except for the prostrate *T. pratense*, however, in the Pyrenees similar varieties can be found naturally in grasslands. The selected species are adapted to different climatic conditions and have different ecological/climatic amplitudes and range from typical Mid-European forage species to the most typical Mediterranean forages (species climatic preferences are shown in Table 1 along with their establishment/persistence patterns and biogeographic distribution).

Medicago sativa and *Onobrychis viciifolia* can be classified as typical Mediterranean legumes regarding their climatic preferences and adaptability, mainly due to their drought-tolerant character (see Table 1) and will be hereafter referred to as "Mediterranean legumes". Indeed, *Medicago sativa* is one of the most widely cultivated forage legume species in the area (40% of all forage monocultures in Catalonia; Statistics of the Spanish Ministry of Agriculture, 2012). It is grown in irrigated and rainfed systems as a monoculture but also in combination with grasses (Delgado, 1984). On the other hand, *Onobrychis viciifolia* is usually grown above 600 m of altitude, substituting *Medicago sativa* in mountain rainfed areas (Delgado, 1984; Delgado et al., 2001, 2014), as a monoculture or in association with a grass, e.g., *Dactylis glomerata* (Sebastià et al., 2011). Nevertheless, its use is decreasing in the Iberian Peninsula (Delgado et al., 2001; Pujol, 1984).

2.2. Site description

The experiment was carried out in the municipality of Gósol, in the Eastern Pyrenees, Catalonia (42°13′N, 1°39′E, 1410 m.a.s.l.). Gósol is located in a valley surrounded by mountains on a relatively flat watershed, and the study site is placed in the montane altitudinal belt and has a sub-Mediterranean climate. The soil is clay textured (22.4% sand; 37.4% silt; 40.2% clay), it presents a high organic matter content (7.93%) and an alkaline pH of 8 (Kirwan et al., 2014); whereas extractable nutrients accounted for 26 mg kg^{-1} Olsen phosphorus, 303 mg kg^{-1} potassium (flame photometer determination) and 174 mg kg^{-1} magnesium (measured by atomic absorption spectrophotometry). Natural vegetation includes deciduous forests with oaks and mixed riparian forests by the river bank, but in many places the original forests have been substituted by Scots pine trees. The valley bottom is covered by forages and other crops while semi-natural grasslands occupy slopes and marginal sites.

2.3. Climatic conditions

According to models based on long-term data (1940–2005) for the study area, mean annual temperature (MAT) and mean annual precipitation (MAP) are respectively 7.1°C and 1126 mm, estimated from the database of the Confederación Hidrográfica del Ebro (CHE). Nevertheless, CHE data for the experimental period (2000–2005) estimates 8.0°C and 877.4 mm for MAT and MAP, respectively (9.2 °C and 812.1 mm according to the closest meteorological station-Gisclareny), thus exemplifying the trends of temperature increase and precipitation decrease for that region (which is indeed warming and drying at a faster rate than other areas in Catalonia according to Calbó et al., 2012) (see the Supplementary Material section 1.1: climatic conditions, for more detail).

It is worth noting that during the experimental years, both temperature and precipitation suffered anomalies. According to the Gisclareny meteorological station, the monthly average of maximum (daily) temperatures reached in 2003 values 3.9°C higher than those reached on average across the 3 previous years. Similarly, the monthly average of minimum temperatures was 4 and 6°C lower in February 2003 and 2005, compared to that in the 2000–2002 period which, in addition to low precipitation during this period (with a total between November and March less than 50% of the average in the 2000–2002 period), left the

Table 1

Biogeographic distribution (not including areas where the species have been introduced for cropping) and origin of the studied species. Species are also compared within each functional group (grasses and legumes) in terms of climatic amplitude (temperature growth conditions and water requirements) and establishment and persistence traits.*T. pratense cv prostrate characteristics are not shown as considered similar to T.pratense (erect variety).

Functional group	Species	Biogeographic distribution/ Origin	Temperature growth conditions	Water requirements	Establishment/ persistence
Grasses	*Lolium perenne* L.	Moist-temperate/ Europe, Asia and northern Africa	Limited growth when > 25ºC stopping at 35ºC. Tolerant to moderate cold temp.	Minimum annual rainfall = 457–800 mm. Not tolerant to drought.	Fast-establishing/ moderate persistence
	Dactylis glomerata L.	Mediterranean and temperate/ Eurasia	Optimum temperature = 4.3–23.8 °C. Wide climatic range, tolerant to high temp.	Minimum annual rainfall = 380 mm. Tolerant to drought.	Slow-establishing/ persistent
Legumes	*Trifolium repens* L.	Moist-temperate and Mediterranean/ Mediterranean region	Optimum temperature = 24 ºC / limited growth when > 35 ºC. Moderate tolerance to cold temp.	Minimum annual rainfall = 750 mm. Not tolerant to drought, needs regularly distributed water supply.	Slow-establishing/ persistent
	Trifolium pratense L.	Moist-temperate and Mediterranean/ SE Europe and Asia Minor	Optimum temperature = 18–25 ºC. Tolerant to cold temp.	Minimum annual rainfall = 550 – 700 mm for persistent growth, 350 mm for survival. Moderate tolerance to drought.	Fast-establishing/ limited persistence
	Medicago sativa L.	Mediterranean/ Mediterranean Basin and SW Asia	Optimum temperature = 25 ºC. Wide climatic range, Tolerant to high and low temp.	Minimum annual rainfall = 350 mm. Tolerant to drought.	Slow-establishing/ persistent
	Onobrychis viciifolia Scop.	Mediterranean and temperate (typical of montane Mediterranean areas)/SE Europe and west and south-central Asia	Optimum = 20/0 °C (day/night temperature) Tolerant to intense cold temp. but not high temp.	Minimum annual rainfall = 330 mm. Tolerant to short drought periods.	Variable establishment/ moderate persistence

References

Alemayehu et al. (2020); Cooper and Tainton (1968); Delgado (1984); Delgado et al., (2001, 2014); Frame (2005); Hannaway et al. (1999); Heuzé and Tran (2015); Heuzé et al. (2019); Hybner and Winslow (2012); Jahufer et al. (2001); Lamp et al. (1990); Lattimore and McCormick (2012); Lloveras (1999); McKenna et al. (2018); Montserrat and Capdevila (1964); Montserrat and Fillat (1984); Mora-Ortiz and Smith (2018); POWO Plants of the World Online (2019); UPNA (2019).

plants exposed to extreme temperatures without a protective snow cover. Indeed, total precipitation recorded in the climatic station was around 30% lower during the experimental period than the 6-years period, overall, it was 50% lower than the long-term average from CHE.

2.4. Experimental design

This study was established in the context of a multisite diversity experiment, the *Agrodiversity* experiment (Kirwan et al., 2014), following the biodiversity-ecosystem function modelling framework (Connolly et al., 2013), where species composition and evenness of forage mixtures were manipulated to assess diversity effects on several ecosystem functions. The experimental design of the *Agrodiversity* experiment (Finn et al., 2013; Kirwan et al., 2007, 2009) was based on the variation of the relative abundances of four species, which corresponded to four functional types, according to their nitrogen fixation ability (legumes versus grasses) crossed with different temporal developments (fast-establishing versus persistent patterns).

The common experimental design of the *Agrodiversity* followed a simplex design (Cornell, 1990; Ramseier et al., 2005). In particular, the mixtures included: four communities dominated in turn by each of the four component species (with 70% of the dominant species and 10% of each of the other three); six communities co-dominated in turn by species pairs (40% of the two dominant species and 10% of each of the other two); and the centroid mixture with an even proportion (25%) of each of the four species. These communities were established at two sowing densities, the lower being a 60% of the total applied density (Table 2), which was determined following the agricultural recommendations of each location (Kirwan et al., 2014). Replication in experiments using the simplex design is across the set of continuous species proportions. In this design, estimation is based on regression methods whereas inference is based on the residual variation around the regression model fitted (Draper and Smith, 1998); thus, replication of each community is not needed.

In Gósol, the experiment was extended and a multi-experiment was established, comprising five mixture types each including four species out of the pool of seven. All mixture types included two grasses: *Lolium perenne* (fast-establishing) and *Dactylis glomerata* (persistent). In four out of the five mixture types, the third species was the legume *Trifolium repens*. In those four mixture types the fourth component was: a)

Table 2

Identity and specific composition of the sown mixtures and sowing density for each species at full rate (scaled for each species and mixture according to the included proportion, e.g., in centroid mixtures sowing densities are multiplied by 0.25).

Tp-mix	Tpp-mix	Ov-mix	Ms-mix	OvMs-mix	Sowing density (kg seed ha^{-1})
Lolium perenne	*Lolium perenne*	*Lolium perenne*	*Lolium perenne*	*Lolium perenne*	30
Dactylis glomerata	*Dactylis glomerata*	*Dactylis glomerata*	*Dactylis glomerata*	*Dactylis glomerata*	25
Trifolium repens	*Trifolium repens*	*Trifolium repens*	*Trifolium repens*		20
Trifolium pratense (erect)					20
	Trifolium pratense (prostrate)				40
		Onobrychis viciifolia		*Onobrychis viciifolia*	100
			Medicago sativa	*Medicago sativa*	30

Trifolium pratense (Tp-mix) erect variety; b) *Trifolium pratense* (Tpp-mix) prostrate variety; c) *Onobrychis viciifolia* (Ov-mix); d) *Medicago sativa* (Ms-mix). The fifth mixture type included *Onobrychis viciifolia* and *Medicago sativa*, plus the two grasses (OvMs-mix; Table 2).

Overall, the complete experimental design included the monocultures of the seven experimental species sown at two densities (totalling 14 communities), and the 11 mixtures of each of the five mixture types sown at two densities (totalling 11 $\times 5 \times 2 = 110$ communities). Thus, we established a total of 124 plots with a completely randomized distribution.

2.5. Sward management and sampling

The experiment was established in April 2003 and lasted until the autumn harvest in October 2005. Prior to sowing the field was harrowed. The communities were hand-sown in plots measuring 8.75 m^2 (3.5 $\times 2.5$), whereas distances between plots had a minimum of 20 cm. Sowing rates corresponded to those recommended for the study area (Table 2). Weeds were not removed over the duration of the experiment to allow for the natural succession in the sward. The system was rainfed. Plots were not fertilized except in 2004, when an isotopic label was incorporated as NH$_4$NO$_3$ at a dose of 40 kg N ha^{-1}.

Yield and species composition were measured in all harvests. Total yield was estimated at the same dates in all plots by harvesting 4.32 m^2 in the centre of the plots to a height of approximately 5 cm following the protocol of Kirwan et al. (2014). Fixed 0.5 × 0.5 m^2 quadrats were cut and total biomass was separated into the four sown components and weeds, to estimate the sown and unsown (i.e., weeds) species fractions. Samples were then oven-dried to constant mass at 60ºC. In the establishment year, the vegetation was harvested twice after sowing, in August and November 2003. The first harvest, often considered as a cleaning cut, eliminates annual weeds, giving competitive advantage to the forage components. In the following years, the vegetation was harvested three times in 2004 and twice in 2005. Harvest frequency mirrored the local farmer's common practice in rainfed forage systems. Namely, depending on the climatic conditions of a particular year and the concomitant vegetation development, farmers perform one or two harvests per year before the aestival drought period. The autumn regrowth, generally small, is typically grazed by livestock (an effect that here we simulated also by harvesting) (specific harvest dates can be checked in Supplementary Fig. S1).

Because of the climatic anomalies and combined extreme events during the experimental period, most plots in three out of the five mixture types were lost in the second harvest of the final experimental year. For this reason, in the autumn of 2005 we only harvested plots from the Ms-mix and the OvMs-mix mixture types.

2.6. Data analysis

We modelled total forage dry matter yield (sown and unsown species), and the segregated dry matter yield of the sown and unsown (weeds) fractions, including the five mixture types and the 3 main harvests: 2 (spring and summer) harvests in 2004, and one (summer) harvest in 2005, leaving the autumn harvest which simulates the grazed biomass and, moreover, presented high plant mortality for many plots, especially in 2005. For the Ms-mix and the OvMs-mix mixture types, we modelled total yield in the seven harvests, including the cleaning cut: two in 2003, three in 2004 and two in 2005. The most relevant models discussed in the paper are detailed in Supplementary Tables S1, S2, and S3.

Using the Generalized Diversity-Interaction (GDI) modelling approach (Kirwan et al., 2009; Connolly et al., 2013), the response variables were modelled as a function of sowing density (M), the sown proportions of the species (P_i), and the species pairwise ($P_i P_j$) and, eventually, three-way ($P_i P_j P_k$) interactions. We also tested the propensity of species to interact by raising the species product $P_i P_j$ to the power of θ (Connolly et al., 2013). Sowing density, essentially a nominal variable with two levels, was scaled to have a zero mean so that all other terms could be read directly as the effects at average density. The temporal variability across harvests and its interaction with diversity terms were assessed by considering it as a repeated variable associated to each plot as a subject (through a mixed model).

In the tested models, we can distinguish two groups of response variables: the individual species' effects and their interactions. The former, corresponds to species identity effects, while the latter expresses the effects of mixing or interaction effects, which can be studied separately and thus can be compared, or aggregated as a single term (as a global interaction or evenness effect, which in our analysis corresponded to the sum of all pairwise interactions) if differences between interaction terms are statistically negligible.

We restricted the number of three-way interactions included in the model by using the Bonferroni correction. The models finally selected included the separated pairwise interactions and $\theta = 1$. For the model including all harvests (Ms-mix and OvMs-mix), two out of the 12 possible three-way interactions emerged as important after the Bonferroni corrections.

To illustrate the differences in species performances (percentual relative performances) and the importance of the diversity effects, we estimated the responses from the model for the different mixture types. We estimated the responses for each of the seven monocultures, and for the centroid community of each mixture type, characterized by an even proportion of all included species. We also estimated the expected centroid responses assuming no diversity effect, which correspond to the average monoculture performances. All analyses were conducted with SAS (9.1; SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Species performance and persistence patterns

There was a higher stability in the response of mixtures compared to monocultures for sown species yield and resistance to weed invasion, with monocultures showing a higher variability than mixtures for both responses in the three main harvests (spring and summer 2004 and summer 2005 harvests, Figs. 1, 2, 3 and Supplementary Tables 4 and 5). Sown-species yield of the seven forage species ranged from 381 to 3290 DM (dry matter) kg ha^{-1} in the most productive harvest (spring 2004) (Fig. 2). Monoculture responses in terms of sown species yield (Fig. 2) and resistance to weed invasion (Fig. 3) changed throughout the different harvests (see also Supplementary Figures 2 and 3 for sown species yield and weed biomass evolution per treatment and year). For instance, the most productive species in the most productive harvest (spring 2004) was the erect variety of *Trifolium pratense*, but this species was one of the least productive components in 2005 (Fig. 2). Conversely, *Medicago sativa* showed the lowest productivity in spring 2004, yielding only 10.2% of the erect *T. pratense*; nonetheless, its productivity gradually increased, rating among the best-performing species in 2005 (Fig. 2). These temporal patterns reflect differences in establishment ability and persistence among the seven species. Overall, the following species acted as fast-establishing: *Lolium perenne*, *Trifolium pratense* (both varieties) and *Onobrychis viciifolia*, while *Dactylis glomerata* and *Medicago sativa* acted as persistent species. *Trifolium repens* was initially sown as a persistent legume, nonetheless, all *Trifolium* species performed very poorly in the last year of the experiment (Fig. 2), after a very dry and cold winter (detail in Supplementary Fig. S1). Seasonal patterns within a year could also be recognized in sown species distributions: within 2004, *Onobrychis viciifolia* attained maximum yield in the spring harvest, while *Medicago sativa* performed better in the summer harvest (Fig. 2).
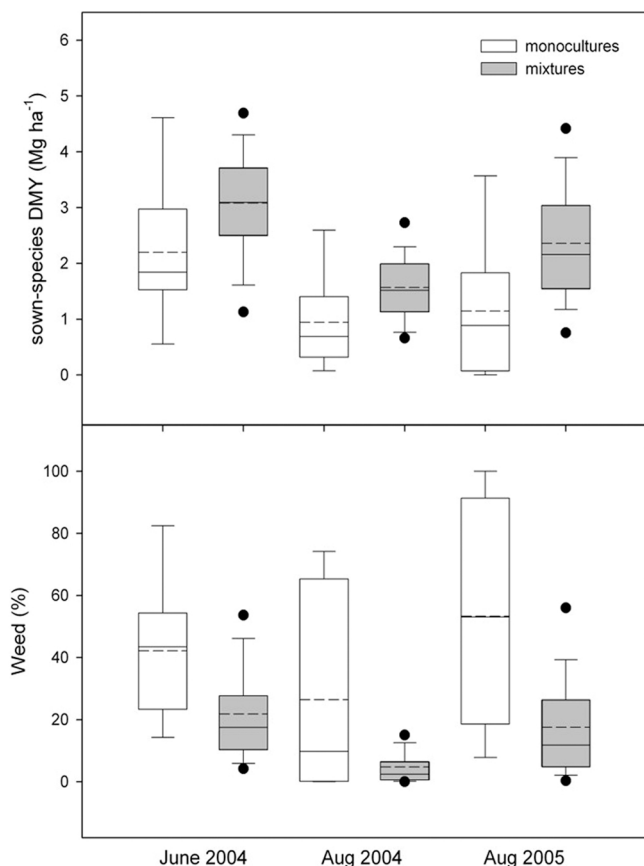
Fig. 1. Dry matter yield of the sown fraction (Mg ha^{-1}) and weed proportion (%), boxplots for the three main harvests and for all monocultures (n = 14, corresponding to the 7 species at two densities) and mixtures (n = 110; including all mixtures of the 5 studied mixture types). The solid and dashed lines show the mean and median, respectively. Dots show the 5th and 95th percentiles.

3.2. Species diversity benefits on yield and stability for the studied mixture types

Along the three main harvests mixtures consistently provided higher total and sown-species yields than expected from the relative contribution of the component species, as a result of a positive and significant global interaction effect on yield (P < 0.0001 for the total and sown-species yield models, with all pairwise interactions grouped as a single term). For each mixture type, the interaction effect on sown-species yield persisted along the three main harvests (P ≤ 0.0004, Table 3) with the exception of the Tp-mix, for which it was significant in 2004 (P < 0.0001 and P = 0.031 in spring and summer respectively) but not significant in 2005 (P = 0.95; Fig. 2, Table 3). As a result of the interaction effect, within mixture types, the sown-species yield of the centroid communities (mixtures with maximum evenness and thus a maximum expression of this effect) was higher than that expected from averaging the corresponding monoculture yields, for all mixture types and harvests except the one mentioned above (Tp-mix in 2005; Fig. 2). In addition, for the three harvests all centroid communities performed better than the average of the seven tested monocultures overall the multi-experiment (P ≤ 0.0071 for all comparisons; Fig. 2, Supplementary Table S6) except the Tp-mix in 2005. This mixture type shifted from being the highest yielding in spring 2004 to the least productive one in summer 2005. Even so, it still gave yields around the seven monocultures average (non-significantly different; Fig. 2, Supplementary Table S6).

Transgressive overyielding, i.e., centroid mixture yielding above the best-performing component in the mixture (Trenbath, 1974), emerged only for the OvMs-mix in summer 2004 (P = 0.03; Fig. 2, Supplementary Table S7). Although transgressive overyielding was not generalized, in spring 2004 all other centroid mixtures showed yields around that of the best-performing monoculture, which in 2004 was *T. pratense* (NS for all comparisons; Fig. 2, Supplementary Table S8). In summer 2004, the same trend was found, except for the Tpp-mix centroid, which gave lower yields than the best monoculture, namely the erect *T. pratense* (P = 0.010; Fig. 2, Supplementary Table S8). In 2005 only the centroids including *O. viciifolia* or *M. sativa*, or both species gave yields in the range of the best monoculture (thus non-significantly different), but this was then *Dactylis glomerata* (Fig. 2, Supplementary Table S8).

In the spring harvest of 2004, the difference in yield between the most and the least productive monoculture was as high as 89% (relative to the maximum); however, the difference between the most and the least productive centroid of the studied mixture types was of only 24%. This suggests a higher stability of mixtures compared to monocultures. In summer 2004 and in 2005, the difference between monocultures reached 94% and 99.9%, but the difference between centroids only increased with time up to 34% in summer 2004 and 56% in 2005. Moreover, for the Ov-mix, Ms-mix and OvMs-mix mixture types the difference between centroids was consistently below 20%.

The best-performing monoculture averaged across harvests was *Trifolium pratense* with 2198 DM kg ha^{-1} (non-significantly different from *Dactylis glomerata, Lolium perenne* and *Onobrychis viciifolia)*. When averaged across harvests, the OvMs-mix centroid showed transgressive overyielding, that is, the yield of this community (2890 DM kg ha^{-1}) was higher than that of the best monoculture also considered across harvests, *Trifolium pratense* (P = 0.038). The Ov-mix and Ms-mix centroids gave average yields across harvests in the range of the best monoculture (*T. pratense*, P > 0.20).

3.3. Species diversity benefits on resistance to weeds

The advantages of sown diversity for yield described above emerged also in terms of resistance to invasion (Figs. 2 and 3). On one hand, as observed for yield, monocultures showed high variability in terms of weed invasion, with mixtures showing a decrease in the variability of weeds proportion in comparison (Fig. 1). *T. pratense* (erect) showed the lowest weed biomass (Fig. 3) and proportion (14%, 0% and 54% in the two 2004 and in the 2005 harvests respectively). Generally, all *Trifolium* species performed better in terms of resistance to weeds at the beginning of the experiment than at the end in 2005 (Fig. 3). The most invaded monocultures were those of *M. sativa* and *O. viciifolia*, particularly in 2004 (Fig. 3) reaching a weed proportion of 56% and 66%, respectively.

There was a general negative interaction effect (with all interaction terms grouped as a single term) on weed biomass (P < 0.0001), which resulted in a decrease of weed invasion in mixtures relative to the expected one according to monoculture weed biomass (Fig. 3). Moreover, this effect was significant individually for most mixture types, either when comparing with the average of the mixture's corresponding monocultures (except for Tp- and Tpp-mix; Fig. 3) or with the average of all seven monocultures (Supplementary Table S9). As observed for sown-species yields (Fig. 2), in spring 2004 all centroid mixtures showed similar weed biomasses (maximum 32% difference), in spite of the considerable range in invasibility existing for monocultures (75%; Fig. 3). In summer 2004, there were considerable differences in weed biomass within monocultures (99%) and within centroids (83%). Nevertheless, in centroids, weed biomass was below 63 DM kg ha^{-1} in all cases, while in monocultures weed biomass reached 671 DM kg ha^{-1}. In 2005, differences were 48% and 36% for monocultures and centroids respectively. We did not observe transgressive over-suppression in centroid mixtures, i.e., weed biomasses in mixtures lower than the least invaded of all tested monocultures, but within each mixture type, weed biomass of the centroid didn't exceed that of the least invasible mixture component (Supplementary Tables S10 and S11).
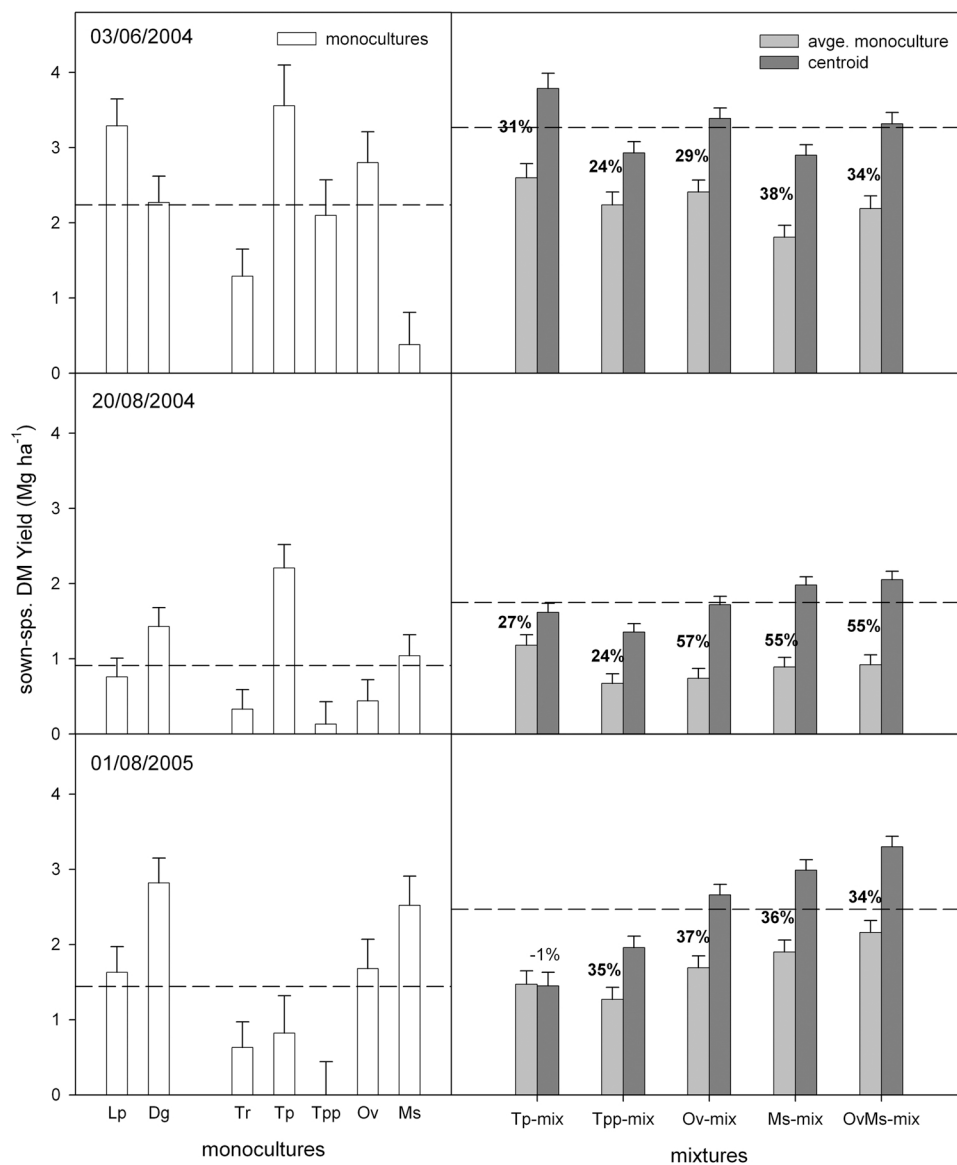
Fig. 2. Dry matter yield of the sown species fraction (Mg ha^{-1}) in the three main harvests. Estimates (+SE) at average density for each monoculture (open bars) and for each centroid of the 5 mixture types, without including the interaction effect (average of corresponding monocultures; light grey) and including it (dark grey). The interaction effect is expressed relative to the total centroid value (%). Significant effects are expressed in bold. Dashed lines indicate the global averages across monocultures and across centroid mixtures (including the interaction effect). Lp: *Lolium perenne*; Dg: *Dactylis glomerata;* Tr: *Trifolium repens*; Tp: erect *Trifolium pratense*; Tpp: prostrate *Trifolium pratense*; Ov: *Onobrychis viciifolia*; Ms: *Medicago sativa*. See Table 2 for mixture type composition.

3.4. Differences in diversity benefits among mixture types

Despite the general beneficial diversity effect on the studied functions, there were differences in the strength of the tested species interactions that resulted in differences in the total interaction effect for the studied mixture types (Table 3), as witnessed by a significance of the χ^2 comparison test of segregating the global interaction term in individual species interactions, with P = 0.01 and P = 0.003 for the sown-species yield and weed biomass models. Mixtures including at least one Mediterranean legume (*O. viciifolia* and *M. sativa*) showed the highest interaction effects across harvests resulting in the best performances (Figs. 2, 3 and Table 3). For the sown-species yield, the mixture including the erect *T. pratense* showed the highest interaction strength in the year after establishment (first harvest of 2004), when this species also attained the best performances, but it dropped after that moment, being nil in 2005 (NS; Fig. 2, Table 3). As a result, considered globally across harvests, this mixture showed the minimum interaction effect (Table 3). For the rest of the mixture types the changes in the interaction effect across harvests were less important (P > 0.001 for all P_iP_j ×harvest interactions, excluded by the Bonferroni correction). Regarding weed invasion resistance, the mixtures with Mediterranean legumes, and specifically those including *O. viciifolia* (Ov- and OvMs-

mix), showed the strongest effects on weed invasion reduction (through a negative and significant interaction effect) when compared with the other mixture types (against Tp-, Tpp-, and Ms-mix, P < 0.013 for all comparisons; Table 3, Supplementary Table S12), while this species (*O. viciifolia*) showed the highest weed invasion in monoculture (Fig. 3).

3.5. Persistence of diversity benefits in two Mediterranean mixture types

A detailed analysis including all seven harvests across the three years, for the Ms-mix and OvMs-mix mixture types, reveals that the interaction effects on total yield (sown species plus weeds) emerged after the first harvest (cleaning cut) and persisted until the last harvest (Fig. 4), all being positive and significant (P < 0.041; Fig. 4, Supplementary Table S13). In this model, two out of the twelve three-way interaction terms were significant, namely, *L.perenne*×*D.glomerata*×*O. viciifolia* and *L.perenne*×*O.viciifolia*×*M.sativa*. The interaction effects for the two mixture types were singularly high in August 2005 (Supplementary Table S13), with a significant evenness×harvest interaction (P = 0.0016), although in relative terms they contributed to total yield particularly in autumn (Fig. 4) with a contribution above 48% for all autumn harvests and for both mixture types.
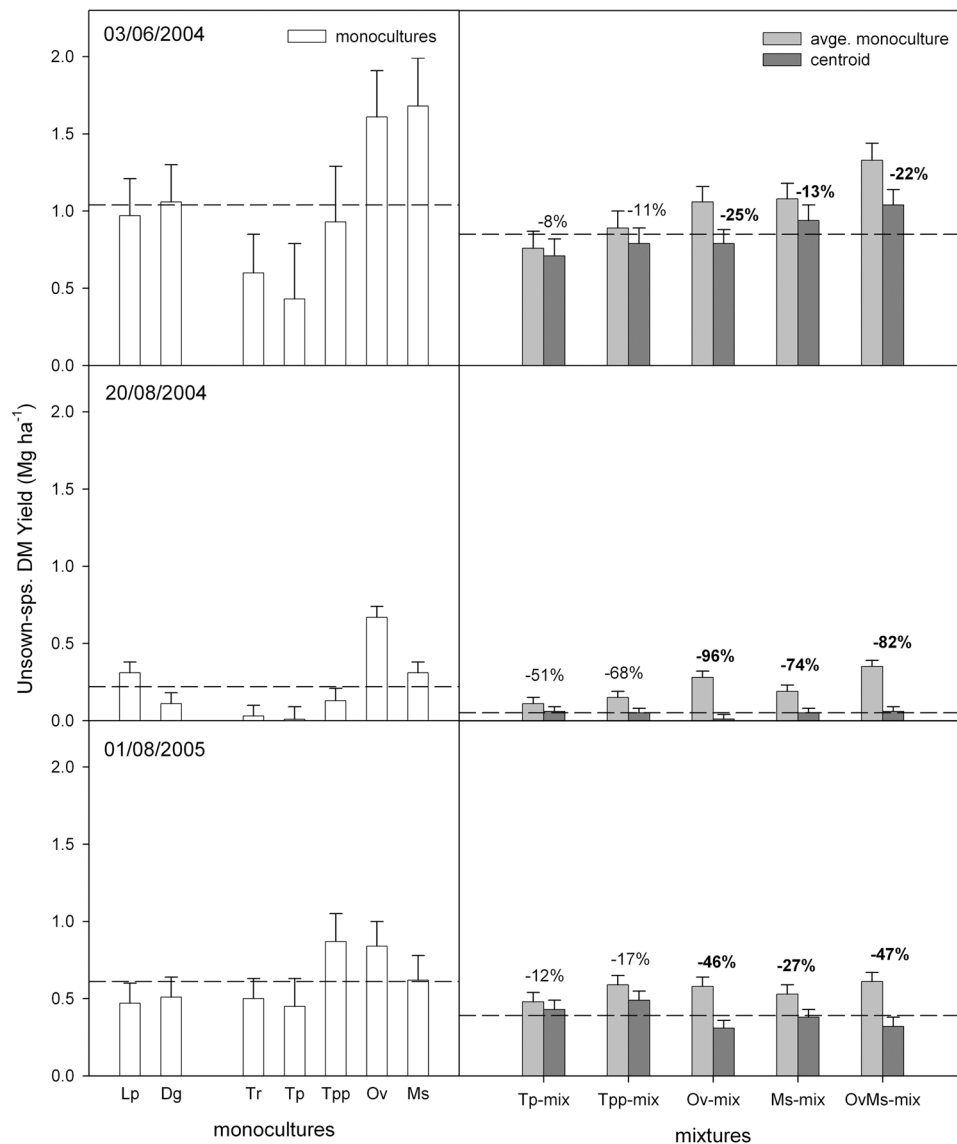
Fig. 3. Unsown species (weeds) biomass (Mg ha^{-1}) in the three main harvests. Estimates (+SE) at average density for each monoculture (open bars) and for each centroid of the 5 mixture types, without including the interaction effect (average of included monocultures, light grey) and including it (dark grey). The interaction effect is expressed as the decrease relative to the expected centroid value without the interaction effect (%). Significant effects are expressed in bold. Dashed lines indicate the global averages across monocultures (in the left-hand side panels) and across centroid mixtures (including the interaction effect) in the right-hand side panels. Lp: *Lolium perenne*; Dg: *Dactylis glomerata;* Tr: *Trifolium repens*; Tp: erect *Trifolium pratense*; Tpp: prostrate *Trifolium pratense*; Ov: *Onobrychis viciifolia*; Ms: *Medicago sativa*. See Table 2 for mixture type composition.

4. Discussion

4.1. Insurance effects and species interactions

4.1.1. Species establishment and persistence patterns

In our experiment, the used species generally followed the predicted temporal patterns selected in the design, including fast-establishing species, which suffered from a limited persistence, and persistent species, which tended to establish slowly (Fig. 2). But apart from these specific trends, the extremely harsh climatic conditions given during the experiment, seem to have affected both the species establishment and persistence patterns, with a stronger effect on the *Trifolium* species, even though they are naturally found in the study area. These effects must have been particularly important for *T. repens*, commonly showing a slow-establishment and persistent development (Finn et al., 2013; Hycka, 1973). In Mediterranean irrigated systems this species has been found to dominate the mixtures one or two years after establishment (Hycka, 1973), but it is known to be highly affected by water deficit (Neal et al., 2009), which occurred during the experimental years (mean annual precipitation across the 3 years being 50% lower than the long-term average, as explained above).

Thus, the different temporal patterns of the studied species, but also the differential species responses to the harsh climatic conditions that took place during the experiment, seem to be behind the huge differences in species performances (white bars in Figs. 2 and 3). This was the case of the difference in the performance of *T. pratense* and *O. viciifolia,* both acting as fast-establishing, but *O. viciifolia* showed a higher persistence in 2005 compared to *T. pratense*, which thus showed less adaptation to the given harsh conditions.

Despite these differences in monoculture performances, mixtures showed a stabilizing effect (light grey bars in Figs. 2 and 3). The basic effect behind this stability is the portfolio effect, i.e., that of averaging species with contrasting responses, traditionally used as an insurance strategy under unpredictable conditions (Altieri et al., 2015). This is essentially a probabilistic effect, but a positive effect can also be targeted through the inclusion of specifically aimed traits. In this sense, the inclusion of species with different temporal patterns (i.e., different seasonal or inter-annual optimums) has been used to balance the changes in forage supply due to temporal variability (Fukai and Trenbath, 1993; Lithourgidis et al., 2011; Yu et al., 2015). Indeed, in experiments assessing grass-legume binary mixtures, where the temporal complementarity axis was missing (both being fast or slow-establishing), mixture performance suffered a temporal variability associated with that of the included species (Hycka, 1975). Other interesting contrasting

Table 3
Interaction effects expressed as the sown-species yield increase or weed biomass decrease (both in Mg ha^{-1}) for each mixture type. We show estimates ± standard errors for the maximum interaction effects, given for the centroid communities (maximum evenness).

	sown DMY increase (Mg ha^{-1})			weed biomass decrease (Mg ha^{-1})		
	estimate ± SE	P		estimate ± SE	P	
Tp-mix	0.53 ± 0.20*	< 0.0001	a	-0.058 ± 0.052	0.26	a
Tpp-mix	0.69 ± 0.19	0.0004	a,b	-0.10 ± 0.052	0.054	a
Ov-mix	0.98 ± 0.19	< 0.0001	b,c	-0.27 ± 0.051	< 0.0001	b
Ms-mix	1.09 ± 0.19	< 0.0001	c	-0.14 ± 0.051	0.0066	a
OvMs-mix	1.13 ± 0.19	< 0.0001	c	-0.29 ± 0.051	< 0.0001	b

[*] The interaction effect for the different mixture types did not change significantly with harvest after the Bonferroni correction, except for the Tp-mix. For this reason, for Tp-mix we give an average value of the interaction effect across the three studied harvests. For Tp-mix, the estimates±SE and P-values for the three studied harvests are respectively: 1.19 ± 0.28 and P < 0.0001 in June 2004; 0.43 ± 0.20 and P = 0.031 in Aug 2004; and − 0.02 ± 0.26 and P = 0.95 in Aug 2005.

patterns, such as resistance versus survival traits (Lüscher et al., 2022), could be included simultaneously to widen the community response plasticity.

4.1.2. Interaction effects

The advantages of mixing go beyond the portfolio or identity effect, exceeding the monocultures average due to species interactions, which in our study resulted in an extra effect above (below for weed biomass) the expected monoculture average (Figs. 2, 3 and 4). The biological mechanism behind this positive interaction effect could be simply the substitution effect of the less persisting species by the surviving species,

Fig. 4. Total (sown species + weeds) dry matter yield (Mg ha^{-1}) for the centroid mixtures of the Ms-mix and the OvMs-mix mixture types, for the seven harvests (h1-h7) along the three years. The grey fraction, which is only shown when significative (p < 0.05), corresponds to the interaction effect added to the expected centroid value according to monoculture performances (white fraction). Note that h2, h5 and h7 correspond to autumn harvests. See Table 2 for mixture composition.

because of shifts in competitive advantage under different environmental conditions. Contrastingly, in addition to this substitution effect, the interaction effect can reflect an advantage associated with other complementarity and facilitation mechanisms, which are the basis of the ecological theory regarding species coexistence (Homulle et al., 2022; Li et al., 2014).

The final effect of mixing will depend on the sum of identity and interaction effects, and the possible trade-off between the two groups of terms. Interestingly, in our study, the highest interaction potentials occurred between species with modest performances, thus with a trade-off between the two diversity components. Probably, under the climatic anomalies suffered by the swards, the inclusion of species with a wide climatic amplitude, and especially those adapted to cope with drought (Mediterranean legumes), was key in the persistence of the mixture components and thus its interaction potential, even though they didn't exhibit the highest responses individually. Contrastingly, *M. sativa* has been reported to yield and suppress weeds with almost a total reliance on identity effects (Picasso et al., 2008).

Regarding diversity advantages under drought conditions, some authors report functional group complementarity as the main driver of resistance and resilience in ley grasslands (Finn et al., 2018; Grange et al., 2021) while other studies point to both identity and functional diversity as key factors (Komainda et al., 2020). On another direction, Ergon et al. (2018) concluded that biodiversity at all levels is crucial as an adaptation strategy to drought.

4.1.3. Differences in strength and persistence of species interactions across mixture types

As reported above, despite the generalized beneficial interaction effect across mixture types (on both studied functions), mixtures including at least one Mediterranean legume exhibited higher advantages due to mixing. As we have explained, these differences are derived from the range in strengths of the tested species interactions (Figs. 2, 3 and 4; Table 3).

Regarding interaction effects persistence across harvests, except for Tp-mix, these persisted even for the mixture including *T. repens* and the prostrate *T. pratense*, even though both species presented a major decline by the end of the experiment. Although ecologically speaking most mixtures were consistently advantageous in terms of a significant interaction effect above the expected monoculture average, from an agronomical point of view only the mixtures equalling or overyielding the best monoculture or mixture would be considered of interest. For the Tpp-mix, although the interaction effect persisted across harvests, it was not enough to compensate the low identity effect (basically of the prostrate *T. pratense* variety), and thus offered less agronomical interest.

4.2. Selecting mixtures components under climatic uncertainty

4.2.1. How do environmental conditions affect diversity effects?

The harsh climatic conditions that took place during the experiment might have affected not only the individual species' performances but
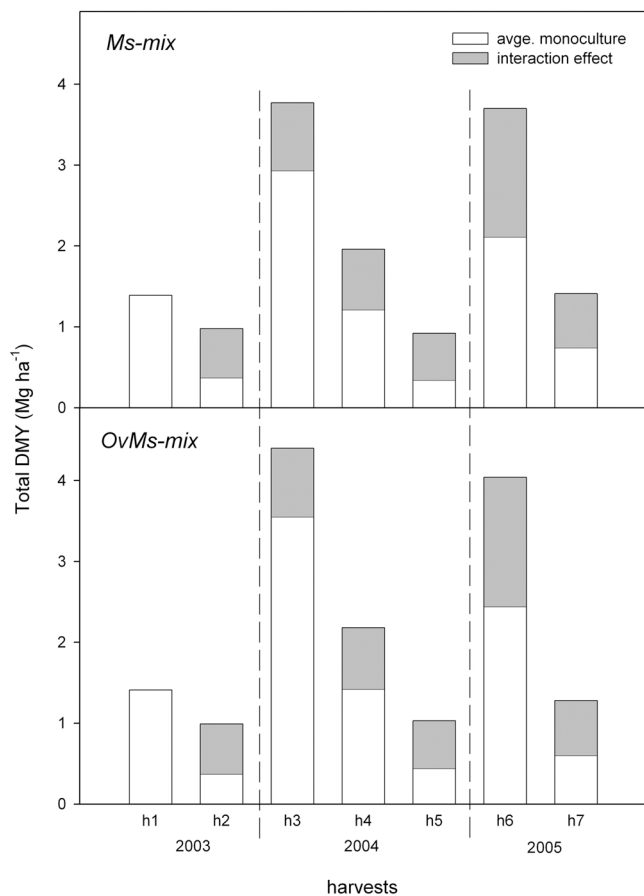
also the species' interactions, probably resulting in the differences in interaction strengths observed across mixture types (Section 4.1.3). This effect could also explain the differences in diversity effects of the same mixtures across sites and studies. Indeed, the Tp-mix showed important and sustained diversity effects resulting in transgressive overyielding in most sites of the *Agrodiversity* experiment (Finn et al., 2013; Kirwan et al., 2007), while in our site it was important in the first 2004 harvest, but it disappeared in the following (Table 3). In experiments carried out in irrigated systems in the Ebro Valley, this mixture type was found to be the best mixture (Hycka, 1973).

In Gósol, with a historical precipitation above 1000 mm, we expected a good performance and persistence of both Tp-mix and Ms-mix, but under the drought and frost experienced during the experimental years, mixtures including species with broader climatic amplitudes, such as *M. sativa*, performed better than mixtures with *Trifolium* species, particularly *T. repens*, which may become relegated to irrigated systems even in mountain areas. Nonetheless, it must be noted that Finn et al. (2018) found out that the tested mixture including *T. repens* and *T. pratense* outperformed monocultures when an experimental drought was imposed in two temperate regions (however, *T. repens* proportion in mixture declined in one of the two locations). In Mediterranean areas, where water scarcity is forecast to increase, drought resistance is a target trait to pursue. In this regard, and accordingly to our results, *M. sativa* and *O. viciifolia* have been found to outperform several species (including the ones in the present study) in terms of drought resistance (Mueller-Harvey et al., 2019). Indeed, in early experiments in the seventies, grass-legume mixtures with *M. sativa* and *O. viciifolia*, were considered appropriate for the extremely harsh conditions of a continental Mediterranean climate in the Ebro Valley, overyielding the best-performing species (*M. sativa*), but *O. viciifolia* showed a lack of adaptation below 600 m (Hycka, 1974), as found in several studies (Delgado, 1984; Delgado et al., 2014; Montserrat and Fillat, 1984). In the lowlands, *O. viciifolia* could be substituted by *T. pratense*, particularly under irrigated systems. In the *Agrodiversity experiment,* the grass-legume mixture including these two legumes (*T. pratense* and *M. sativa*) gave significant diversity effects in two out of three sites in which it was sown (Kirwan et al., 2007) but it failed in France, probably due to the extreme climatic events experienced in most European countries in 2003 (Olesen and Bindi, 2004). Binary grass-legume mixtures including *M. sativa* outperformed those including *T. pratense* in Greek mountain systems (Hadjigeorgiou, Thanapoulos, 2004), while the reverse was found in Mid-European conditions (Kleen et al., 2011), according to a better adaptation of *M. sativa* in Mediterranean conditions. Contrastingly, Ergon et al. (2018) advocate for both *T. pratense* and *M. sativa* as key components in Mediterranean mixtures to avoid drought-related yield reductions.

The differences in diversity effects listed above may be explained by the environmental variability underlying the studies. Ecological theory predicts that species interactions may change under stressful conditions (Kikvidze and Armas, 2010). Notably, Lüscher et al. (2022) have also identified the severity of stressful events such as droughts as a key factor to understand the importance of species interactions, concluding that complementary effects are maximized at moderate drought levels whereas identity effects play a major role at severe droughts.

4.2.2. From selected adapted cultivars to selected adapted mixtures

The development and use of adapted cultivars are key in Mediterranean regions (Hycka, 1974), especially considering that many forage species have been developed in temperate regions (Volaire et al., 2009). Nevertheless, the trade-off between identity and interaction effects observed in our study stresses the importance of selecting species that maximize the sum of the two effects (Connolly et al., 2013; Kirwan et al., 2007, 2009; Lüscher et al., 2022). If for monocultures we would select species with high identity effects, for mixtures we should include species with high individual, but also with high interaction potential. As a result, the genetic material to be included in mixtures may differ from

the varieties to be used in monoculture (Volaire et al., 2014), and its selection should be done in mixture (Atwood and Garber, 1942).

5. Conclusions

In this study we provide sound evidence on the forage mixtures' advantages over monocultures in terms of increased yield, resistance to weed invasion and the stability of both functions. This effect may be key for rainfed production systems, which will be particularly threatened by extreme temperatures and irregular precipitation patterns, such as those experienced during the development of our experiment. Nevertheless, our results suggest that, although interaction effects tend to occur for most mixture compositions, not all of them overyield the best-adapted species. Moreover, there can be a trade-off between identity and interaction effects, thus, we should select species that maximize the sum of both diversity components, as happened in our experiment with mixtures including at least one Mediterranean legume. In the context of climate change, it is crucial to explore species interactions and related mechanisms that have a special role under harsh conditions. The selection of the best mixture composition and management for a particular agro-climatic environment will require the study of identity and interaction effects, through GDI models, under different environmental conditions, and for multiple functions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2023.108601.

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