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# **Responses of perennial ryegrass populations to grazing method and endophytic relationships**

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## Abstract

New Zealand dairy farmers rely primarily on swards based on perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) for feeding dairy cows. The direct-grazed, low-cost production system that these pastures enable provides the New Zealand dairy industry with a competitive advantage for products in the international market. As a result, the industry contributes significantly to New Zealand's export earnings and national gross domestic product. However, farmers in the upper North Island have expressed concern about the poor productivity of their perennial ryegrass pastures beyond three years post-sowing and have linked this to more-frequent occurrence of drier and warmer conditions for pasture growth during summer and autumn.

Recent research has shown that even pastures sown with new ryegrass cultivars inoculated with the most-advanced endophyte strains cannot sustain high productivity under the severe climatic conditions and increased insect pest burdens being experienced in many parts of the Waikato, Bay of Plenty and Northland. In some cases, this is due to the death of ryegrass plants and ingress of lower-producing weed species, while in other cases reasonable ryegrass plant density can be sustained, but growth rates are poor.

There is limited information on the plant population and phenological factors associated with these changes, making it difficult for farmers, agronomists and plant breeders to develop effective solutions. The study described in this thesis investigated the differences in phenotypic trait expression of perennial ryegrass plants removed from 10-year old pastures grazed by dairy cows in the Waikato ('survivor' plants) versus plants grown from the original seed lines that were used to sow the pastures and had, therefore, not been subject to any environmental selection pressure ('reference' plants). The study aimed to help inform future plant breeding efforts to utilise phenotypic changes and reselect for traits that are associated with better plant survival.

Three populations were compared: reference plants; survivors from pastures rotationally grazed by dairy cows since they were established in autumn 2011 ('control'); and survivors from pastures managed as per the control treatment, but where grazing was deferred for ~ 120 days from mid spring to mid-summer to promote new seedling recruitment two years before plants were collected from the field ('deferred'). Four perennial ryegrass cultivars were included in the design: Nui standard endophyte (SE); Commando AR37, a mid-season heading diploid; Alto AR37 a late season heading diploid; and Halo AR37 a very late heading tetraploid. Thus, 12 populations were compared.

Initially, there were 60 plants per population giving a total of 720 plants which were grown individually in pots at Lincoln University.

The first phase was a pilot study during the early acclimatisation period where progress towards equalisation of plant growth across the populations was tracked. Biomass accumulation and reproductive development were assessed from late February to late April. Substantial flowering activity was recorded in the control and deferred populations for all cultivars, but not in the reference population, illustrating the expected carry-over effects related to differences in plant source (clonal fragments from the field versus seed-derived for the reference population). Biomass accumulation was initially lower in the reference population but there were no differences between populations by day 105 when plants had tillered-out to a similar degree.

All plants were then analysed for the presence or absence of endophyte and, where endophyte was present, its genetic identity (strain). Plants with no endophyte, or with an endophyte strain that was not AR37 (or not SE, for Nui), were discarded and plant numbers rebalanced to 30 plants per population that were confirmed as 'true-to-type' for the original host genotype – endophyte strain combination sown in 2011. Four further populations of control plants (one for each cultivar; total 240 plants) were available for endophyte analysis from a site in Canterbury where the same experimental design and seed lines as used in the Waikato was established at the same time. The grazing deferral treatment was not implemented at this site.

For Waikato, the percentage of plants that were true-to-type was significantly higher in the reference population (82%) than the control and deferred populations (69-71%). Overall, Commando had significantly lower percentage true-to-type than the other cultivars (60% versus 78-82%), with a high presence of standard endophyte (22%) indicating contamination by volunteer ryegrass plants and poorer persistence of the original population. For the Canterbury control populations, mean true-to-type percentages were 85-98% across the four cultivars, i.e. generally higher than for the original seed lines.

The second phase was a detailed phenological study, where plant, leaf and tiller characteristics were measured on the true-to-type plants only, and flowering observations were conducted for 70 days starting in mid-October. Phenotypic measurements included: leaf weight, length, width, area, specific leaf area and thickness; pseudostem weight, length and width; and visual scores of rust, tillering and growth habit.

Control plants had significantly lower leaf dry weight, shorter leaves and smaller lamina area than the reference plants, with no interaction between population source and cultivar. Deferred

populations did not differ from reference populations in these traits, indicating grazing deferral 're-set' the plant phenotype back from the control state to its original state, at least for the first 18 months after deferral. There were no differences between populations in pseudostem characteristics, growth habit or tiller density scores, but reference populations showed a trend towards higher incidence of rust compared with control and deferred. A significant shift toward later flowering in the control population compared with the reference population was found for Nui, Commando and Alto, but not for Halo.

In summary, differentiation toward smaller leaves, greater disease resistance, and later flowering was observed after nearly ten years under grazing in the Waikato populations when potential contaminants were excluded and any effects due to endophyte presence/absence or variant strains were removed. It appears that the shift towards longer leaves can be reversed by deferring grazing to allow recruitment of new seedlings into the population. In contrast, shifts in rust resistance and flowering in control populations were not reversed as a result of spring/summer grazing deferral. Further investigation of the genetic factors controlling the reversibility of leaf traits could help identify breeding objectives for maintaining the persistence of yield advantages in ryegrass pastures. Future studies should also examine possible long-term benefits of this management practice to bring about phenotypic change for yield improvements and consider the duration of possible carry-over effects.

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## Chapter 1 Introduction

New Zealand dairy farming systems are world-renowned for their high production achieved with relatively low reliance on imported feed resulting in low costs of production. These attributes are facilitated by *in situ* grazing by dairy cattle of high-quality pastures which grow year-round in temperate climates that are generally favourable for pasture growth (Holmes et al. 2002). These features contribute strongly to New Zealand's competitive advantage in global dairy product markets which, in turn, explains why dairy production remains a major component of the national gross domestic product (GDP). The dairy industry is the largest value-added contributor to the New Zealand economy and remains one of New Zealand's biggest export sectors (DCANZ, 2020).

The New Zealand dairy production system is based overwhelmingly on two mutually compatible pasture species: perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) (Kemp et al. 1999). The latter is a legume which fixes nitrogen (N) from the atmosphere: some of this N is transferred to the associated grass effectively reducing reliance on artificial N fertiliser for herbage production. Perennial ryegrass grows year-round in most NZ dairy environments providing the bulk of the feed consumed by dairy cows. In 2018-19, pastures based on perennial ryegrass and grazed directly provided ~80% of the total feed consumed by the 5 million dairy cows constituting the New Zealand dairy herd (DairyNZ Economics Group, 2019), equating to ~ 20 million tonnes of feed (dry matter basis) per year. The principles and practices for managing pastures and animals in New Zealand grazing systems are well-established (e.g. Sheath et al. 1987, Matthews et al. 1999; Chapman, 2016) and widely implemented by farmers. Pasture productivity is also underpinned by a long history of forage plant breeding (Rumball, 1983). A key advancement from this sector has been the identification and breeding of different ryegrass endophyte strains which remove negative effects on animal health of the 'standard' endophyte strain while providing protection against several common insect pests (Caradus et. al., 2021).

However, New Zealand dairy pastures are continually exposed to abiotic and biotic variability, including changes in the climate, diversity in soil properties, and pressure from pests and diseases. The persistence of perennial ryegrass in pastures has recently become a serious concern for New Zealand dairy farmers in the upper North Island (e.g. Jagger 2021, McCahon et al. 2021), likely related to increased environmental stress on ryegrass especially from climate change. Two national symposiums have been held to address this emerging issue: one in 2011 (Mercer, 2011) followed by another in 2021 (Douglas, 2021). These symposia have highlighted a general lack of information on the changes that occur in the structure and function of ryegrass plants and populations as pastures age post-sowing, and how those changes are related to pasture persistence, or failure to persist

(Parsons et al., 2011). This information could be of considerable practical importance, for example by identifying management interventions that could enhance persistence, and plant traits that could be emphasised in plant selection and breeding to improve the physical survival of plants and populations. Advances in both areas would help ensure the on-going economic sustainability and competitiveness of the New Zealand dairy industry. They would also help address negative environmental sustainability effects related to pasture failure such as decreased efficiency of N use and increased N loss to the environment (Betteridge et al., 2011), reductions in soil carbon due to frequent soil disturbance associated with pasture renewal (Wall et al., 2021), and increased greenhouse gas emissions (de Klein et al. 2021).

The overall purpose of the study described in this thesis was to quantify changes in ryegrass plants and populations over 10 years post-sowing under intensive dairy cattle grazing in two contrasting environments with a view to help fill the knowledge gaps noted above. It is now clear that different perennial ryegrass/endophyte combinations produced from the past 40 years of breeding have not provided lasting solutions to the problem of persistence failure in the upper North Island (Lee et al., 2017). It appears that the cumulative pressure of successive hot and dry summer/autumn seasons, which are consistent with projections of climate change for the region, plus more-frequent and damaging insect pest outbreaks, is too great for current perennial ryegrass cultivar-endophyte combinations to withstand. In some situations, perennial ryegrass contribution to total herbage production falls to less than 20% in summer and autumn within three years post-sowing, allowing less productive weed species to invade the pasture and degrade herbage production and quality (Lee et al. 2017). Nonetheless, some ryegrass plants do survive. The characteristics of these plants and of the population that evolves over time could provide valuable insights for future research into new ryegrass breeding and management options.

The study compared surviving perennial ryegrass plants collected from a pasture persistence trial reported by (Lee et al., 2017, 2018). The same trial design was implemented at two sites: Newstead in central Waikato, and Lincoln in Canterbury. While the Canterbury site was irrigated, the Waikato site was not; this reflects common farm practice in each of the two largest dairying regions of New Zealand. The study also used different functional types of perennial ryegrass: diploid versus tetraploid cultivars, mid-season versus late-season heading dates, and new cultivars versus old cultivars.

Perennial ryegrass is an outcrossing species, thus there is genetic diversity present within any perennial ryegrass seed line (albeit limited by breeders to ensure sufficient uniformity to meet the standard required for plant variety rights registration of cultivars). Where genetic diversity is

present, natural selection can operate, resulting in distinctive genotypic and phenotypic differentiation within surviving populations (Snaydon, 1987). Therefore, it was hypothesised that plants that had survived into the tenth year of the field experiment were those from among the original gene pool that were best suited to the current environment and that their morphology would point to what is needed in plants to improve persistence. To test this hypothesis, and to establish what changes if any had occurred over time in plant morphology, the survivor plants were compared with plants grown from the original seeds used in 2011, samples of which had been stored at low temperature and humidity in the Margot Forde Germplasm Centre, AgResearch, Palmerston North.

Two research questions were addressed in the study:

- A) Were the surviving plants derived from the original sown population or were they volunteer plants not related to the original sowing?
- B) If they were derived from the original sown population, had the populations changed phenotypically? If so, what are the implications for plant breeders? Could there be phenotypic traits plant breeders should be selecting to increase survival in these increasingly stressful environments particularly in the upper North Island?

In order to answer these questions, this thesis is divided into the following chapters.

Chapter 2 reviews key literature to understand the background to the study further and identify gaps in knowledge that relate to the research questions.

Chapter 3 reports the results from a pilot study conducted using the widest possible set of ryegrass populations available from the long-term field experiment to provide an initial assessment of phenotypic variation and the sources of the variation (across environments, survivor populations, cultivars and managements).

Chapter 4 reports the results of a more-detailed phenotypic analysis restricted to only 'true-to-type' plants identified via endophyte DNA analysis as being derived from the original sowing. This analysis focussed on changes within and between populations.

Chapter 5 concludes the study with a discussion of the overall outcomes including answers to the research questions. It also includes a discussion around further suggested studies resulting from the findings of this thesis.

## Chapter 2 Literature review

### Grazing systems in New Zealand

New Zealand livestock production systems are based on direct, year-round in situ grazing of pastures with minimal reliance on imported/purchased feed. They are based on long-established principles of matching feed supply with animal demand as documented by, for example, Milligan et al. (1987), Sheath et. al. (1987) and Matthews et al. (1999). There are all types of stock fed using these pasture-based systems, but the main ones are sheep, beef, and the focus of this work, dairy cows.

The outdoor grazing systems used in New Zealand's dairy industry provide many benefits. From an economic perspective, there are lower direct and indirect feed costs which underpins the ability of the New Zealand livestock industries to compete strongly on price for products in overseas markets. From an animal welfare perspective, there are benefits associated with animal health as animals are able to roam around in a more natural environment. Many markets value outdoor, grass-fed animal products over indoor, mass-produced products from other competing countries (Neave et al., 2022). This advantage is created through the public perception that housed, grain-fed animals are less 'natural': moreover, consumers are willing to pay premiums for products made from animals 'naturally raised' (Neave et al., 2022).

### The New Zealand environment

New Zealand's climate ranges from warm subtropical in the far north to cool temperate in the deep south (Mackintosh, 2001). New Zealand's generally reliable rainfall and mild temperatures allow year-round pasture growth which strongly favours the direct grazing systems that have evolved for red meat and milk production. However, over the last decade, there has been an increase in the occurrence of severe weather events, including droughts, periods of high rainfall, extreme temperatures and increases in the presence of pests and diseases, highlighting the vulnerability of the agricultural sector to climatic variability and climate change (Cradock-Henry, 2008). These events have resulted in difficult growing conditions for pasture species such as perennial ryegrass which are best suited to cool temperate climates with limited exposure to moisture deficits (Levy, 1955).

Simultaneously, farmers in the upper North Island have noted increasing issues with ryegrass persistence over the last decade, reporting a decline in perennial ryegrass population density and production, especially where environmental conditions are marginal or unfavourable for the species (e.g. Reynolds, 2013; Jagger, 2022; McCahon et al., 2022). Drought presents an obvious direct climatic risk to perennial ryegrass and white clover (*Trifolium repens* L.) population survival because both species have limited drought avoidance/tolerance capacity (Chapman et al., 2011). Perennial

ryegrass is particularly sensitive to drought (Garwood & Sinclair, 1979). While mild moisture deficiency leads to a reduction in herbage production, under severe drought, plants may become dormant or die. The inability of perennial ryegrass to survive very dry summers obviously limits its use. However, there has been natural variation found in accessions collected from consistently dry habitats, compared with the commercial cultivars available in the market (Faville et al., 2020a).

Since 2011, the central Waikato has experienced prolonged droughts in several years, consistent with climate change predictions (Keller et al, 2021). Farmers struggling with pasture persistence have attributed much of the problem to climatic conditions (Clark, 2011). Lee et al. (2017) found that no currently available perennial ryegrass cultivar-endophyte combinations are able to withstand the sequence of hot, dry summer/autumns being experienced in parts of the upper North Island, such that ryegrass makes up a minor proportion of the pasture in summer and autumn within three years after sowing. Even old, supposedly highly persistent ryegrass cultivars with standard endophyte are unable to resist the environmental stresses. With the rate of change forecast to accelerate, plant breeders must consider future climatic and environmental conditions. From a study of climate futures for agriculture in New Zealand between 2050 and 2100, Keller et al. (2014) predicted substantial changes, especially in annual precipitation rates. For example, a 5-10% reduction in annual precipitation was predicted along the eastern side of New Zealand. Increases in national average temperatures were also predicted. These changes will have a direct impact on seasonal farm production. Increases in winter pasture growth and decreases in summer growth follow the trend of changes in temperature: dry summers markedly reduce production, even if the remaining seasons have normal precipitation (Radcliffe & Baars, 1987).

## **Perennial ryegrass attributes**

Perennial ryegrass is an obligate outcrossing, wind pollinated grass species. Distinct perennial ryegrass populations, called ecotypes, have developed in different parts of New Zealand, reflecting different farming practices and local environmental factors (Thom et al., 1998). The New Zealand pastoral dairy system is primarily based on pastures comprised of perennial ryegrass and white clover. Perennial ryegrass comprises 60% of the total volume of seed of proprietary cultivars of pasture species sold in New Zealand (information provided by Thomas Chin, New Zealand Plant Breeding and Research Association). Ryegrass-based pastures provide relatively low-cost forage that is generally of high quality as a ruminant animal feed (Clark et al. 1997). It grows well in a wide range of environmental conditions, is easy to establish and manage, has high growth rates, supports high animal performance, usually has good persistence, and forms a compatible mix with white clover (Kemp et al., 1999). Ryegrass-based pastures grow strongly in spring and autumn, and also in the

milder winters of northern New Zealand (Holmes et al., 2002). This seasonal pattern of feed supply fits well with the pattern of feed demand in spring calving of dairy herds, the standard calving period in New Zealand farm systems (Holmes et al., 2002). Thus, perennial ryegrass is a favoured feed supply in the New Zealand dairy industry.

Perennial ryegrass is described as a leafy and highly tillered plant that can produce high herbage yields of good feeding value for ruminant animals over several years (Sampoux et al., 2011). It is well-adapted to direct grazing and withstands animal treading and frequent, hard defoliation better than other temperate grasses. Perennial ryegrass growth rates peak at the time of flowering (Parsons & Chapman, 2000). The timing of initiation of heading is under strong genetic control (Stewart & Hayes, 2011), and can vary by > 40 days from earliest to latest (Stewart, 2006). Plant breeders have exploited this variability to develop cultivars with a wide range of heading dates (Sampoux et al., 2011; Ullmann et al., 2015).

The recommended seeding rates for perennial ryegrass swards in New Zealand are currently 20–25 kgs of seed per ha for diploid cultivars and 25–30 kgs/ha for tetraploids (Stewart et al., 2022). Perennial ryegrass can freely cross with *Lolium multiflorum* (Italian ryegrass), producing fertile hybrids (*L. Hybridum*) with intermediate characteristics. The two species differ in lifespan, with the Italian ryegrass being a short-lived (approximately two years) species.

## **Environmental variability and adaptation**

There are many different climatic environments in New Zealand. Pasture based grazing systems are spread across these environments and perform differently in each, making them all slightly unique. Keller et al. (2021) state that there is a high degree of regional variation in New Zealand. This fact means that considerable uncertainty remains as to how environmental interactions will occur under projected climate change trends. Under stress, such as severe defoliation, nutrient deficiency or soil water deficits, plants respond phenotypically and express their traits differently compared with non-stressful environmental conditions (Hazard et al. 2001), a process known as phenotypic plasticity (Chapman & Lemaire 1993). The different plant morphological traits that emerge under stress show us how plants are coping/dealing with stress and what changes have occurred to help the plant survive. Understanding these changes is important for future plant breeding efforts. This knowledge will help breeders identify traits that enable better pasture persistence in the face of increased stress due to climate change.

Farmers in the upper North Island have expressed concerns about the poor productivity of their perennial ryegrass pastures beyond three years. They have noted that that summers and autumns in recent years have become generally drier and warmer than normal (e.g. McCahon et al. 2021; Jagger



2021) and Glassey et al. (2021) have shown that this explains much of the poor performance observed by farmers. New Zealand's intensively managed pastoral agricultural systems, characterised by high pasture utilisation, frequent grazing, the application of fertilisers, irrigation, other management interventions, and high stocking rates, are vulnerable to climate changes due to their reliance on pasture as the primary feed source (Lee et al., 2013). Keller et al. (2021) have shown that pasture growth in New Zealand is expected to become more variable and unpredictable, driven largely by more frequent and prolonged periods where soil water supply is well below the water demand for active pasture growth. Some regions that are already dry will experience increased temperatures and drier summers that will increase pressure on water resources. Regional differences in projected climate will require greater adaptation in some places compared with others. With both the occurrence of droughts and flooding projected to increase (Keller et al., 2021), the contrast between extremes will present new challenges for pastoral agriculture. Adaptation in pasture species selection and management will be required to help farmers cope with the increasing risk of summer moisture deficits (Glassey et al. 2021).

As pasture has a lower production cost when compared to alternative feeds, maximising pasture utilisation to satisfy feed demand is a key objective for farmers looking to ensure profitability (Mills & Neal, 2021). Between 2004 and 2019, pasture eaten on New Zealand dairy farms plateaued nationally and at the regional level, and even declined in some regions (Mills & Neal, 2021). These trends raise serious concerns for New Zealand's dairy industry because the important economic advantages associated with pastoral systems identified above may be eroded in future if the trends continue.

## **Ryegrass functional types, cultivars, and traits**

### **Key traits**

Vegetative persistence is an important objective in perennial ryegrass cultivar development. Persistence encompasses the survivorship of plants and the stability of dry matter yields from sown populations (Parsons et al., 2011; O'Connor et al., 2020a). Leaf phenology is often measured to gain an understanding of how a plant is reacting to external stimuli (Barre et al., 2015). Often these traits are closely associated; for example, a plant that is densely tillered typically has smaller leaves compared to a less densely tillered plant due to the  $-3/2$  self-thinning effect (Kays & Harper, 1974). Griffiths et al. (2017) found that while yield was positively associated with a smaller tiller size, higher tiller density, and decreased leafiness, the highest yielding ryegrass types had longer, wider, and larger lamina and longer pseudo stems. As the distribution of stem, pseudo stem, and leaf in the

vertical profile of the sward influences feed intake and milk production, understanding how plants react to different management practices is of considerable importance (Hurley et al., 2009). While plant survival is important, the plant also needs to provide feed for stock in the form of herbage yield, generally measured in kg DM/ha. Plants exhibiting prostrate habits and narrower leaves have been associated with persistence in New Zealand hill pastures (Wedderburn et al., 1989). A prostrate plant growth habit means plants can avoid intense defoliation pressure by retaining more leaf below the cutting or grazing height that plants with more erect growth habits (Cashman et al., 2016), which means they retain some ability to intercept light and photosynthesise to support initial regrowth (Chapman & Lemaire, 1993). Briske (1986) used the term 'grazing resistance' to describe the ability of plants to survive and produce under grazing, and identified two components of resistance: avoidance, related to mechanisms that reduce the chances of defoliation; and tolerance, related to mechanisms that promote fast regrowth after defoliation. Compared with other temperate grass species, perennial ryegrass is better able to avoid defoliation than to tolerate defoliation (Cullen et al., 2006).

### **Heading date**

The term "heading date" indicates the onset of anthesis, which results in a reduction in forage quality due to a higher stem to leaf ratio (Byrne et al., 2017). Perennial ryegrass cultivars are marketed according to their heading date, indicated the number of days after 'day 0' when ~50% of tillers have initiated stem elongation. 'Day 0' corresponds to point where 50% of tillers have initiated elongation in the cultivar Nui, which is used as the mid-season standard for relative heading date. Day 0 is generally taken to occur on 22<sup>nd</sup> October in Canterbury (Lee et al., 2012). Most New Zealand perennial ryegrass cultivars fall in the range 0 (mid-season) to +25 (very late season) days. Some early season cultivars (-3 to -6 days) have been bred in the past but are no longer traded commercially (Lee et al. 2012). Mid-season cultivars have relatively strong early spring growth and are generally recommended for environments where there is a risk of late spring/early summer growth being restricted by moisture stress (Stewart et al., 2022). Conversely, late or very late heading varieties are better suited to areas with reliable spring growth through into early or mid-summer, and/or where irrigation is available (Stewart et al., 2022).

Reproductive tillers reduce pasture quality because they contain high quantities of structural carbohydrates and vascular tissue making the plants less digestible for animals, thereby leading to reduced pasture intake (O'Donovan & Delaby, 2005). Variation within a cultivar for aftermath heading would be problematic for farmers as it increases the timespan of reproductive tiller appearance and their associated negative effects on pasture quality (Lee et al., 2012; Wims et al.,

2017). Due to its significant effects on other traits, breeding has always aimed to exploit natural variation in the heading date (Lee et al., 2012). Sward growth and herbage quality may also differ between early and late-heading cultivars when defoliated frequently; the lower digestibility of early heading types persists well into summer (Laidlaw, 2005). This is why heading dates and aftermath heading are traits of crucial importance in variety development (Byrne et al., 2017). The development of later flowering ryegrasses, such as Grasslands Impact and Quartet, which allow pastures to remain leafy and of higher quality later into the spring, has been of value to the New Zealand dairy industry (Stewart, 2006). Ryegrasses with reduced aftermath heading provide high quality leafy pasture in summer (Woodfield & Easton, 2004).

### **Rust resistance**

Another key trait targeted in perennial ryegrass breeding is disease resistance. Scientists have found a significant positive association between perennial ryegrass persistence and infection with novel strains of endophytic fungi which increase disease resistance to crown rust (*Puccinia coronata f. sp. lolii*) and improve tiller survival (Easton et al., 2011; O'Connor et al., 2020a). Crown rust can have a detrimental effect on ryegrass plants. Mattner and Parbery (2007) found that mature plants from rust-infected parents in Italian ryegrass had fewer tillers, as well as fewer and smaller leaves. Pathogens, such as rust, reduce the longevity of the host and the reproductive ability of the host and its offspring. There is also a lack of information on key plant traits associated with vegetative persistence due to a limited numbers of long-term field trials (Tozer et al., 2014).

Crown rust is caused by the *Puccinia coronata Corda f. sp. lolii* fungus and has a destructive effect on perennial ryegrass (Schubiger et al., 2010). Given the economic impact of crown rust infections, improvement of genetic resistance to this disease is one of the major goals in ryegrass breeding programmes (Wilkins & Humphreys, 2003). Lancashire and Latch (1966) concluded that crown rust may be an important factor limiting pasture production. Crown rust drastically reduces the value of susceptible cultivars by decreasing dry matter up to 37% and green tissue by 94% (Clarke & Eagling, 1994). It ultimately reduces the plant's yield and nutritional quality.

### **Ploidy**

Perennial ryegrass is naturally a diploid species (2n) with seven pairs of chromosomes (Easton, 1983). Artificially doubling the chromosome number to create tetraploid varieties (4n) is a breeding technique that has been used by ryegrass breeders in New Zealand since the 1950s (Stewart, 2006). Compared with diploids, tetraploids have larger cells with higher water content which leads to plants with larger leaves and tillers, but a tendency to lower tiller density (Lee et al., 2012). Thus the ploidy

of a cultivar can be important to understand the morphological and physiological features of the cultivar. Diploid and tetraploid perennial ryegrass cultivars may have contrasting responses to stresses due to differences in their morphological, physiological, and agronomic traits (Burns et al., 2012). For example, (Tozer et al., 2017) observed that feeding by grass grub larvae reduced root biomass and total plant biomass by 34% and 25% respectively in a tetraploid perennial ryegrass compared with a control treatment (no insect feeding), but there was no effect of the insect in a diploid cultivar. They concluded that the lower tiller density and total biomass of the tetraploid compared with the diploid, combined with greater susceptibility to insect pest damage, may compromise persistence of tetraploids in grazed pastures. Similarly, Popay et al. (2003) found that both ploidy and species affected the amount of Argentine stem weevil (a common pasture pest in New Zealand) damage to plant tillers. Annual and hybrid cultivars were considerably more damaged than two perennial cultivars.

Another difference between tetraploids and diploids is their relative 'palatability' to grazing animals. Hearn et al. (2021) documented that tetraploid varieties are more suitable for grazing than diploids as they tend to have advantageous nutritive and morphological characteristics, including higher values for dry matter digestibility, and increased levels of leaf in the sward, all of which lead to greater herbage utilisation (Byrne et al., 2018). Tozer et al. (2014) suggested that tetraploids with lower tiller density and larger tillers than diploids may have limited ability to recover from periods of moisture stress. Laidlaw (2005) also found that diploids have consistently higher tiller densities than tetraploids. The possibility of limited recovery from drought makes it important to include tetraploid varieties into studies that consider drought to determine just how these cultivars are affected by moisture stress.

### **Perennial ryegrass cultivars**

Breeding to improve the performance of pasture species began in New Zealand in the 1920's when selection programmes using ryegrass, clover, cocksfoot and timothy plants collected from wild populations across the country and from commercial supplies (Rumball, 1983). The term 'cultivar' means cultivated variety. The most commonly used breeding approach in New Zealand has been recurrent phenotypic selection where many genotypes of plants from the same species are screened for specific traits and the best individuals are crossed in open pollination. The progeny from these poly-crosses is then further screened and (generally) crossed with adapted material before an elite group of ~6-10 plants is selected for the production of nucleus seed which is multiplied to produce a minimum of 40 tonnes of seed for sale through retail outlets. From the mid-1970s, New Zealand dairy pastures were based on the cultivars Nui, Ellett and Yatsyn which were all derived from the

‘Mangere’ ecotype collected from a dairy farm in South Auckland in the 1960s (Stewart, 2006). Nui is still used as a benchmark for traits such as heading date, but is no longer maintained as a proprietary cultivar with full seed certification and quality assurance credentials. However, the ‘Mangere’ ecotype is still used in New Zealand breeding programmes because it is well-adapted to New Zealand grazing environments (Armstrong, 1975) and thus useful for back-crossing into new selections to ensure adaptability.

Due to the popularity of perennial ryegrass, there has been significant commercial investment into breeding programmes in New Zealand since c. 1980. As a result, in New Zealand, 30 to 40 different cultivars of perennial ryegrass are traded commercially (Chapman et al., 2017). The cultivars are all bred with specific traits highlighted to allow for the ‘best fit’ for different regions of the country. According to the forage value index (DairyNZ, 2021) for the upper South Island, ‘Halo AR37’ cultivar is one of the best options for the environment. In contrast, in the upper North Island, ‘Maxsyn NEA4’ is considered one of the best options.

## Endophyte

In New Zealand, perennial ryegrass seed is almost always infected with an endophyte. The endophyte present in ryegrass (*Epichloë festucae* var. *lolii*, formerly *Neotyphodium lolii*, Leuchtman et al., 2014)) grows intercellularly as sparsely separate hyphae which are present in the leaf sheaths and flowering stems of the plant. They are rarely found in the leaf blades and do not occur in the roots (di Menna et al., 2012). *Epichloë* fungal endophytes have been found in over 50 species that cover a vast range of ecosystems around the world (Semmartin et al., 2015). These endophytes form symbiotic relationships with many cool-season temperate grasses such as perennial ryegrass. The use of an appropriate *Epichloë* endophyte can improve ryegrass persistence through the production of secondary metabolites that reduce herbivory by insects (Popay & Hume, 2011). It has been suggested that under different environmental conditions, due to global climate change, microbial symbionts will be essential to ensure plants can deal with novel or stressful conditions (Dastogeer, 2018; Redman et al., 2011).

Naturally occurring endophytes, commonly known as wild-type or standard endophytes, release alkaloids such as ergovaline and peramine that deter pests and protect the plants from predators. These alkaloids are not always beneficial in farming systems; some that are produced by the endophyte, such as lolitrem B and ergovaline, cause serious animal health issues such as ryegrass staggers and heat stress via vasoconstriction (Thom et al., 2013). Alkaloid levels in endophyte infected perennial ryegrass are affected by season (Ball et al., 1995), environment (Lane et al., 1997), and plant genotype (Spiering et al., 2005). While newly developed novel endophytes such as AR37 do not

contain the alkaloids lolitrem B and ergovaline that cause animal health issues, they do contain alkaloids related to lolitrem B called epoxy-janthitrems which also act as insect pest deterrents (Thom et al., 2013). The AR37 endophyte provides perennial ryegrass with protection from Argentine stem weevil, root aphid (*Aploneura lentisci*), African black beetle (*Heteronycus arator*), pasture mealybug, and porina (*Wiseana cervinata*). Infected plants thus have an agronomic advantage (Thom et al., 2014; Thom et al., 2013).

Although the effects of the plant–endophyte interaction are a prime focus of study for ecologists and agronomists (Canals et al., 2008; Rodriguez et al., 2004), research on the mechanisms of endophyte transmission and the factors that affect its viability within the host are limited or have focused solely on the survival of the endophyte within the seed (Canals et al., 2008; Welty et al., 1987).

Perennial ryegrass cultivars can be inoculated with different endophyte strains that form better symbiotic relationships than other types (Popay et al., 2003). Once established in a newly-sown pasture, the frequency of endophyte infection remains stable over time. For example, Faville et al. (2020b) reported limited and largely non-significant variation in infection rates in survivor ryegrass populations compared with reference populations grown from the original seed lines five years after sowing in three different environments. However, the dynamics of host plant–endophyte interactions with respect to alkaloid profiles and effects on insects, plant survival and animals are complex (Malinowski & Belesky, 2006; Caradus et al., 2021). Both the plant host strain and endophyte strain, and interactions between the two plus with the environment can modify the quantity and type of alkaloids produced to deter pests (Ball et al., 1995; Popay et al., 2003).

Therefore, perennial ryegrass research should explicitly include endophytes as a factor, as they interact so strongly with the host plant that they affect almost all aspects of plant performance, including persistence. Without these relationships, perennial ryegrass swards are less likely to persist and more likely to perform poorly in New Zealand pastures. There is also evidence of direct beneficial endophyte effects on plant physiology (Malinowski & Belesky, 2006) which could improve plant tolerances to stresses such as drought (e.g. He et al., 2017b; Kane, 2011). However the evidence is not clear cut and most research into grass endophyte relationships emphasises the benefits for insect pest protection.

## Persistence in perennial ryegrass swards

Regrassing represents a significant cost to pastoral farmers. When pastures fail to persist, farmers either have to resow paddocks or leave them as low producing, low quality pasture (Daly et al., 1999). Either way, a cost is incurred. When pastures are not persisting to their full potential, the need for renewal becomes more frequent than best practice, with Tozer et al. (2010) explaining that pasture renovation costs are high. As re-sowing pastures is expensive, alternative management strategies are needed that will slow pasture decline (Brazendale et al., 2011) or improve the percentage of perennial ryegrass in a pasture (Graham et al., 2000). Poor persistence of perennial ryegrass in dairy pastures is a significant issue in parts of New Zealand (Tozer et al., 2011). Genetic improvement of pasture persistence is beneficial for the farmers: a cultivar that maintains yields and survives longer, decreases the frequency and expenses associated with pasture renewal (Malcolm et al., 2014).

A 2009/10 survey of 776 Waikato and Bay of Plenty dairy farmers found that 46% of respondents were dissatisfied with the performance of their pastures after 3 years post-sowing, with 20% responding that they were 'very dissatisfied' (Kelly et al., 2011). It is estimated that New Zealand sheep and beef farmers renew about 2% of pasture land area on their farms each year while dairy farmers renew 5–6% of pasture land area annually (Sanderson & Webster, 2009). At these rates, farmers are seemingly expecting pastures to remain productive for more than 20 years before reseeding (Daly et al., 1999). However, there is clear evidence that newly-sown ryegrass pastures are lasting in a productive state for only 2-4 years in many parts of the upper North Island, with climatic stresses being a major driver of this problem (Lee et al., 2017; McCahon et al., 2021). From data reported by Kelly et al. (2011), it can be estimated that problems with pasture persistence were driving regrassing rates of about 20% per year in Waikato/Bay of Plenty in the late 2010s. The Pasture Persistence Symposium held in 2011 (Mercer, 2011) was a response to increased farmer dissatisfaction with persistence. From a practical perspective, the persistence of the yield advantage gained by sowing a new pasture is what matters to farmers (Parsons et al., 2011). The expected yield advantage can only persist if the sown species continues to contribute a high percentage of total herbage mass, which obviously requires the physical survival of the sown plant. In a study of pasture persistence in four regions (Waikato, Taranaki, Northland, and Canterbury), Tozer et al. (2010) found that the pastures in the Waikato had reached 35% unsown species invading the paddocks within four years of sowing. Most swards gradually become invaded by weed species, with consequent decline in their nutritional value and dry matter yield. This invasion proceeds much more slowly where persistent grasses are sown (Wilkins & Humphreys, 2003). While the 2011 pasture persistence symposium stimulated more research into the occurrence and causes of persistence

failure, there remains a gap in the knowledge as to how changing environmental conditions will affect persistence of our current cultivars in the future and what new knowledge or technology is needed to help manage the problem.

Farmers frequently report ryegrass population density decline (Tozer et al., 2014), especially where environmental conditions are marginal or become unfavourable for the sown species (Chapman et al., 2011). When the ryegrass population is depleted, its contribution to total pasture dry matter yield falls, to be replaced by volunteer weed species, as illustrated in a 5-year study reported by Lee et al. (2017, 2018). This markedly changes the seasonal pattern of pasture growth and composition, especially in northern regions whereby the pasture is completely dominated by unsown summer-active C<sub>4</sub> species in the warmer months, with some contribution still from ryegrass in winter and spring (Lee et al., 2017). Effective weed management measures have always been crucial for successful agricultural production (Ghanizadeh & Harrington, 2019). Weeds are a threat to pasture production in New Zealand as they reduce the quality and quantity of the fodder produced for the animals. A plant is regarded as a weed when it has not been intentionally sown from seed (Ghanizadeh & Harrington, 2019). The problem is that in pastures, weeds compete with pasture grasses and legumes for resources such as water, light, and mineral nutrients (Grice & Campbell, 2000). These weed species in pastures can replace the pasture plants through competition, resulting in decreased pasture persistence (Bourdôt & Saville, 2002).

### **Grazing management for perennial ryegrass**

Grazing management has been shown to affect perennial ryegrass persistence (Graham et al., 2000). Beattie (1994) stated that well-targeted grazing management may give ryegrass a competitive edge by reducing weed invasion, and that autumn deferment could be used to help control volunteer annual grasses.

Dairy grazing systems are designed to grow large yields of digestible forage, generally grass-legume mixtures, and harvest a high proportion of the herbage grown directly by the cow. Intensive grazing systems use rotational grazing practices, wherein the cows rotate around a sequence of paddocks. This grazing method allows pasture feed availability to be monitored and allocated to stock to meet their feed demand, and/or to deal with pasture surpluses (e.g. in spring) by closing some paddocks from grazing and conserving the surplus as silage or hay (Holmes, 1987). The overall pasture cover (pasture mass, kg DM/ha) of the farm can be controlled by adjusting rotation length (the interval between successive grazing events for a paddock), pre-grazing pasture mass and post-grazing mass, which also helps maintain the quality of pasture for animal feeding (Holmes, 1987; Matthews et al., 1999).



By contrast, in continuous grazing systems such as set stocking, it is not possible to establish and maintain a consistently optimum average post grazing residual (i.e., high utilisation), because some areas of the pasture become overgrazed or grazed excessively whereas other areas are not adequately utilised. Patch grazing creates high spatial variability in pasture mass and quality, and limits the ability to control pasture intake (Chapman et al., 2007). The regrowth curve of perennial ryegrass is well-suited to the rotational grazing system, and simple rules of thumb for the timing of grazing to optimise plant regrowth and animal intake have been developed to help farmers manage their systems, e.g. the leaf stage rule (Fulkerson & Donaghy, 2001).

### **Use of silage to conserve spring surpluses**

In spring and summer, surplus pasture can be conserved as silage or hay and offered to animals during periods of low pasture growth, particularly to nonlactating cows during winter (Milligan et al., 1987). This practice is a key part of feed planning for the pasture-based systems used on New Zealand dairy farms. It enables farmers to transfer feed from season to season, and helps to control imported feed costs. Deferring grazing in spring is another way of transferring energy (feed) from season to season, similar to silage making, but without the associated machinery and labour costs.

### **Deferred grazing**

Pasture deferral is an old concept which has recently received renewed interest among farmers in dryland conditions experiencing a climate shift toward lower rainfall over the summer periods. Although deferred grazing has been used to rejuvenate pastures and manage the spring surplus, its impact on profitability is still unknown. The timing is critical, as allowing the desirable species to produce seedheads both enables the pasture species to reseed and promotes new tillering from existing plants in the autumn after the deferred period (Tozer et al., 2021). Some dairy farmers defer selected paddocks from late spring-summer to shift spring surplus feed into late summer and early autumn without having to make pasture silage or hay (Harris et al., 1999). There is also the added benefit of pasture regeneration via the recruitment of new seedlings from perennial ryegrass (L'Huillier & Aislabie, 1988). This process is achieved by recognising the importance of tiller demography and reproductive physiology in our primary perennial pasture species (Tozer et al., 2021). Managing surplus pasture, particularly during spring when there is an increase in stem and reproductive material, is crucial, so that pasture quality, at a farm scale, can be maintained.

Deferral has been investigated as a grazing method to increase pasture persistence by allowing reserves to build up which stimulates an increase in tiller density. The resulting increased pasture quality is caused by a decreased stocking rate during the spring and summer period on deferred paddocks and an increase in stocking rates on other parts of the farm. This practice effectively

enables better control of pasture quality. Deferral results in the higher utilisation of spring feed, therefore maintaining pasture quality. The deferred paddocks provide a feed wedge at the end of summer which can be particularly useful in drought years (Harris et al., 1999).

Studies have shown that clover in pasture benefits greatly from this grazing method after the first autumn graze, when pastures open up (Tozer et al., 2020). Thus far, deferral grazing reports seem to mainly refer to dry stock farms (Tozer et al., 2021). It is thus of interest to examine whether this grazing method would work for dairy pastures in environments prone to drought such as the Central Waikato (Griffiths et al., 2021).

### **Consequences of over-grazing and under-grazing**

There is a fine balance between harvesting the pasture grown but not undermining future growth potential by grazing too severely (i.e., over-grazing) and not underfeeding cows to the detriment of milk production. Brougham (1957) established the typical sigmoidal pattern of regrowth of ryegrass after defoliation, whereby herbage accumulates relatively slowly in the first days post-grazing while plants rebuild leaf area, then accelerates before levelling off when the rate of senescence of old leaf material has increased to equal the rate at which new leaf is being produced – a point known as ‘ceiling yield’ (Parsons & Chapman, 2000). The regrowth pattern will differ depending on how much pasture (kg DM/ha or leaf area) is left following the previous grazing (Parsons et al., 1988), commonly referred to the ‘post-grazing residual’. Over-grazing, or reducing the pasture to very low mass by grazing or cutting, will extend the period of low growth rates at the start of the regrowth period (Parsons et al., 1988; Lee et al., 2008) and reduce total annual DM production if repeated over successive events (Chapman, 2016). This finding means that post grazing residuals are very important for future growth.

Under-grazing, the converse of over-grazing, also has consequences for pasture growth, quality and animal production. Leaving high grazing residuals means ceiling yield is reached early in the regrowth period (Parsons et al., 1988) and implies fast grazing rotations (Chapman, 2016) which bring a risk of underfeeding stock. Increased dead material occurs as a result of under-grazing with tiller shading occurring and a build-up of rank/low quality plant material leading to poor quality feed. Pasture quality is negatively associated with grazing severity in the previous defoliation: thus, quality declines with higher residuals (Lee et al., 2008) and animal intake (DM, energy and protein) can be reduced as a result. The pasture intake of dairy cows is one of the biggest factors in milk production and profitability, and the efficiency of pasture use in relation to the factors described above is very important for overall systems performance (Holmes et al., 2002).

## Differentiation in perennial ryegrass

Differentiation in plant populations is caused by a change in genetic structure which is reflected in plant phenotypic changes. Differentiation is a form of natural selection in response to the environment (e.g., stress) and causes changes in the genetic structure of plant populations. These changes can occur in multiple traits such as dry matter production, flowering date, plant height, leaf size, tillering, and disease susceptibility. The traits can be modified by grazing management, fertiliser, and the abundance of other species (Snaydon, 1978). Differentiation is an important process underpinning plant breeding, as it has traditionally been exploited by using ecotypes such as the Mangere ryegrass ecotype which has been widely used in the development of New Zealand perennial ryegrass cultivars (Stewart, 2006; Lee et al., 2012). Shifts in the genetic structure of survivor populations have been proposed as one of the reasons for poor pasture persistence because these shifts tend to favour lower yielding plants and thus overall pasture production declines (Parsons et al., 2011). This can be viewed as a persistence failure, since the initial yield advantage of a new pasture is eroded over time (Parsons et al., 2011). In this case, persistence failure may arise from yield decline of the newly sown sward over time with no, or minimal, change in population density (Snaydon, 1978).

The outcomes of differentiation in newly-sown pastures may provide insights into the traits that are important for persistence which breeders could then target. O'Connor et al. (2020a) observed that plants exhibiting prostrate growth habits and narrower leaves are positively associated with persistence. The prostrate growth habit means plants can avoid the consequences of intense grazing pressure whereas the erect types are preferentially grazed by stock (Cashman et al., 2016) and must regrow from a lower residual leaf area compared with prostrate plant types. O'Connor et al. (2020a) found that Commando was less subject to directional selection when compared with other ryegrass cultivars. The differences reported to occur between cultivars corresponds with the results of Cashman et al. (2016) who also found that directional selection of persistent plants varied between cultivars. This suggests that some plant populations are more robust and less subject to directional selection than others in specific environments.

In another comprehensive study of the changes in ryegrass plant phenotype and genotype over time, Faville et al. (2020b) found that neither the phenotype or genotype of ryegrass plants surviving in pastures 5 years after sowing did not differ from that of plants grown from remnant seed of the same seed lines. This was the case for two cultivars (Nui and Alto) sown in three different environments (Northland, Waikato and Canterbury). Faville et al. (2020b) concluded that factors other than population genetic structure are responsible for poor pasture persistence, such as

grazing management, climatic conditions and insect pest damage. They also noted that the results do not support the idea that 'new' ryegrass cultivars (e.g. Alto) are more susceptible to persistence failure through plant mortality than 'old' cultivars (e.g. Nui) and therefore that ryegrass breeding in New Zealand has not exacerbated the persistence problem.

The Faville et al. (2020b) study used plants from the same experimental sites from which plants were collected for this study. Key differences between the studies include age of the swards from which survivor plants were collected (nearly ten years versus five years), the cultivars used (Commando AR37 and Halo AR37 were included in the present study in addition to Nui and Alto, with Halo being a late flowering tetraploid), sward management (a deferred grazing management intervention was included in the present study), and the plant traits that were measured (for example, reproductive development was measured in the current study, but not by Faville et al., 2020b).

The use of agricultural inputs to alter low yielding environments to suit cultivars with increased yield potential such as perennial ryegrass cultivars is not sustainable in pastoral agriculture (Hazard et al., 2001). The cultivars need to be bred to cope and produce in these low yielding environments. This goal can be achieved by identifying key traits that may increase the plant's survival. The cost of inputs has increased, relative to the value of outputs, and there is increasing concern about the effect of such inputs on the environment. Adaptation of forage cultivars to their environment has thus become a major focus in plant breeding (Hazard et al., 2001). Morphogenetic adaptation may be the secret to unlocking survival trait expression in plant populations: Hazard et al. (2001) found that morphogenetic differences between populations remained constant across environments with differential mortality under the most severe environments/conditions. The key is identifying those traits that have differentiated from the population would enable the survival of certain plants within the population under a low yielding/harsher environment. This would allow plant breeders to target these traits to ensure cultivars can maintain these differentiated trait expressions and increase the plant's survival. Maintaining these key traits in commercial cultivars presents a challenge for plant breeders going forward.

### **Carry over effects**

The term "environmental 'carry over' effects" refers to the effects seen as a result of the environment previously encountered by a plant, on the growth of a plant itself, or its offspring (Bullock et al., 1993). There has been very little research on the possible conditioning of plants. Those studies which have been conducted have indicated two types of 'carry-over' effects. The one of most interest suggests that exposure to a specific environment could induce heritable, directional changes in phenotypic expression of the sexual progeny (Durrant, 1958; Durrant, 1962, 1971). Carry-

over effects may only cause short-term phenotypic change. Where it exists, carry-over has important consequences for plant ecology since it increases the heterogeneity of the environment encountered by a plant in a way not usually considered, or that is difficult to measure. This process can also be identified as morphological adaptation where there are structural changes which give the plant a greater chance at survival in particular environmental conditions (Hazard et al., 2001). Morphogenetic differences between ryegrass populations can determine their competitive ability and persistence in a given environment (Hazard et al., 2001).

## **Gaps in knowledge and study objectives**

There is conflicting information in the literature around the extent to which genotypic and phenotypic changes occur in perennial ryegrass populations over time. In particular, this applies to information on key perennial ryegrass traits associated with vegetative persistence for which there is a very limited number of long-term trials available from which to establish trends (O'Connor et al., 2020a). Long-term trials are best when evaluating the diversity of persistence as changes within the population can be recorded over time periods that are relevant to farming (Parsons et al., 2011). Most plant breeding trials only last for 18 months to 3 years, after which time, selections are made. This time period limits the amount and variation of environmental stresses to which the plants are exposed (Kerr et al., 2012).

This study reported in this thesis investigated the differences in phenotypic trait expression between a survivor population planted 10 years ago, and a reference population of plants newly re-established from seed of the original seed lines that had not been subject to any environmental selection pressure. With 'carry-over' effects highly likely to occur in the early stages of the study, any obvious differences can be recorded. In this study, phenotypic observations are applied to the same control population sown in 2011 vs a deferred grazing population to study possible management effects on the changes in phenotypic structure and whether these occur using deferral grazing. In particular, this study investigated the environmental effect on the elite perennial ryegrass germplasm and examined whether trait differences between them can be detected. The study also established the genetic identity of individual plants used for detailed phenotypic analysis using endophyte genomic analysis and examined the possibility of an endophyte effect on the expression of phenotypic traits in the surviving population over time. The overall intention was to help inform future plant breeding efforts by providing more information on phenotypic factors and traits that appear to be associated with long term survival in ryegrass pastures.

## Chapter 3 Pilot study

### Introduction

Plant phenology can serve as a bio-indicator of climate change whereby changes in plant phenology can indicate changes in the environment surrounding a plant or population (Menzel et al., 2020). Changes to plant phenology are important for identifying how plants are coping with environmental change and what plant breeders need to work towards to increase persistence in cultivars of New Zealand pasture species. Biomass is very important when selecting what pasture species should be used on the farm. The biomass is essentially what makes the money, as this is the feed for stock on the farm. Pasture is the cheapest form of feed available and represents New Zealand's competitive advantage. However, if a plant is unable to survive in the environment it has been sown in, there will be no production. The survival of a plant population within a certain environment (in this case, the harsh Waikato region), is critical to the success of the farm business using it for production. Each input needed to help the pasture survive, means a reduction in profit on the other side. As Hazard et al. (2001) have explained, "The use of agricultural inputs to alter low yielding environments to suit cultivars with increased yield potential such as perennial ryegrass cultivars, is not sustainable in pastoral agriculture". This statement is even more relevant with the implementation of new environmental regulations and greater emphasis and increased awareness of environmental impacts. Identifying traits that are linked to plant survival are key to the success of New Zealand farming practices and maintaining our competitive advantage against the other dairy producers around the world.

There has been a gap identified in long-term trials that address survivorship of plant populations in the field. There are very studies that examine exposure to a particular environment over long periods of time; thus, changes to population trait expression have not been well documented in the scientific literature. Questions around survivorship of plant populations and what and how much physiological change a plant population endures over time (in this case, in the Waikato region) need to be addressed. Plant populations can adapt. When using clonal material to explore these questions, the 'permanent' changes in a population must be isolated from the short-term 'carry over' effects of the ambient environment from which survivor plants are removed. The growth of clonal cuttings, or ramets' can be influenced by the environment that the plant they were removed from was experiencing (Bullock et al. 1993). Carry-over effects were considered highly likely to occur in the early stages of this study. Therefore, background measurements of biomass and seed head

development were taken during an initial phase of the overall study to identify major differences between populations and how those differences changed over time. The traits chosen for this first phase are important to farmers and industry experts as they influence the amount of feed and the quality of feed available at certain times of the year. Because of the timing of collection and transplanting of clonal material from the field, the measures of reproductive development were confined to aftermath heading. Aftermath heading is a key trait that plant breeders have been targeting in commercial cultivars of perennial ryegrass in New Zealand and other temperate regions (Stewart & Hayes, 2011).

The objectives of phase one of the study were to record the time sensitive trait expressions and compare them with a seed population that had the original gene expression of these treatment populations from ten years ago. The aim was to record changes in the survivor populations from the original seed population, as a result of time and a harsher environment. This Chapter provides a foundation for the next phase by assessing what was happening in the plants immediately after removal from the Waikato and to lay the groundwork for the traits selected in the next phase where more-detailed phenotypic data were collected: the harvest phase.

## **Methods**

### **Plant populations**

The plant populations used for the overall study were drawn from a field experiment described by Lee et al. (2017, 2018). This experiment compared the long-term persistence of four perennial ryegrass cultivars, initially established from five different sowing rates. The experiment was set up at three locations using exactly the same design and ryegrass seed lines. It was sown at each location in April 2011.

The perennial ryegrass cultivars included in the experiment were the Nui with standard endophyte (Nui SE, Commando with AR37 endophyte (Commando AR37), Alto AR37 and Halo AR37.

Commando, Alto, and Halo were released commercially in the 2000's. Commando is a diploid with a similar flowering date to Nui and was bred from New Zealand ecotypes. Alto is a diploid that flowers 14 days after Nui and is based on late flowering material from Nui and material from North-West Spain. Halo is a tetraploid that is very late flowering: it flowers 25 days after Nui. It is also partly based on material from North-West Spain (Chapman et al., 2022). Ryegrass was sown with white clover in the experiment, using the clover cultivars Weka and Kotare. All four ryegrass cultivars were included in this study.

Only two of the three experimental locations were used in the study: DairyNZ Scott farm at Newstead, Waikato; and Lincoln University Research Dairy Farm, Lincoln, Canterbury. Plant phenology and phenotypic measurements in this Chapter and Chapter 4 were only carried out on the ex-Waikato populations. Ex-Canterbury populations were used only for the endophyte presence and identity analysis described in Chapter 4. The pasture treatments were established in plots of 540 m<sup>2</sup>, replicated 4 times. Pastures were grazed by dairy cattle between ~7 and 12 times per year at intervals typical for rotation grazing management in the regions and depending on growing conditions.

The original seed rate treatments were discontinued after 3-4 years so are not considered in this study. However, in spring-summer 2018-19, a deferred grazing treatment was implemented across all four cultivars at the Waikato site, as described by Griffiths et al. (2021). Starting in October 2018, the main plots were split to leave equal areas within each replicate to either continue under past grazing management, or to withhold (defer) grazing for ~ 120 days. Grazing deferral was timed to maximise heading according to heading date: thus, it started and finished ~ 3 weeks later earlier for Alto and Halo than for Nui and Commando. Survivor plants were collected from both management treatments, referred to as 'control' for the continuation of normal grazing and 'deferred' for the grazing deferral treatment. There was no grazing deferral at the Canterbury site, therefore plant populations were only available for the 'control' treatment.

In addition, plants were grown from seed of the four cultivar seed lines used to establish the experiment, which had been stored under low temperature and humidity in the Margot Forde Germplasm centre at AgResearch Grasslands, Palmerston North since soon after the experiments were sown. These plants then formed four further populations of 'reference' plants against which changes in phenotype or phenology in the survivor populations could be compared.

In total, therefore, 16 populations were available for the overall study, as shown in Table 1. Sixty plants were initially collected or established from seed for each population, giving a total of 960 plants. The ex-Canterbury plants were not used in the initial phase, therefore the number of plants relevant to this Chapter was 720.

#### *Key trends in the field experiments*

At the Waikato site, in the summer of 2014/15 and autumn 2015 (3.5 to 4 years after sowing) the perennial ryegrass content of total pasture dry matter fell to ~20% for all cultivars (compared with 60-70% in previous years) (Lee et al. 2017). It increased in the following winter and spring, but fell to low levels again in successive summer-autumn periods, replaced by weed species and, to a lesser



extent, white clover (Lee et al. 2017). By the autumn of 2016, perennial ryegrass tiller density had declined to  $\sim 1500/m^2$ , compared with  $4000-6000/m^2$  in the first few years (Lee et al., 2018). At the Canterbury site, ryegrass contribution to total pasture DM remained at 80-90% and tiller density at  $\sim 7000/m^2$  (Lee et al., 2018).

Table 1 Overview of the 16 treatment populations used in this study. Populations 1-4 were established from seed of the original seed line used to establish the experiments in 2011. The seed for the reference plants was sourced from the Margot Forde Germplasm Centre. Populations 5 – 16 inclusive were survivor plants collected from a field experiment at two locations: Waikato and Canterbury. For the Canterbury site, survivors were only available from the ‘control’ treatment.

Cultivar x endophyte combination	Treatment			
	Reference	Control ex Waikato	Deferred	Control
			ex Waikato	ex Canterbury
Alto AR37	1	5	9	13
Commando AR37	2	6	10	14
Halo AR37	3	7	11	15
Nui SE	4	8	12	16

It was concluded that none of the ryegrass-endophyte combinations were able to withstand the stressful environmental conditions experienced at the Waikato site (Lee et al., 2018), where mean monthly temperatures generally exceeded the long-term average (Figure 1). In the summer of 2018/19, there was a 14% increase in the average temperature compared with the 30-year average (Figure 1).

As well as high temperatures, rainfall was also below average particularly in the summers of 2012/13 and 2013/14 (Figure 2). In 2015/16, the total average rainfall for the period was 58% below the 30-year average and rainfall was also lower than the 30-year average in the following year. In 2017/18, there was 68% higher rainfall compared to the 30-year average. From 2019 onwards, rainfall amounts showed a downward trend, with 55% lower rainfall in October 2020 compared to the 30-year average.

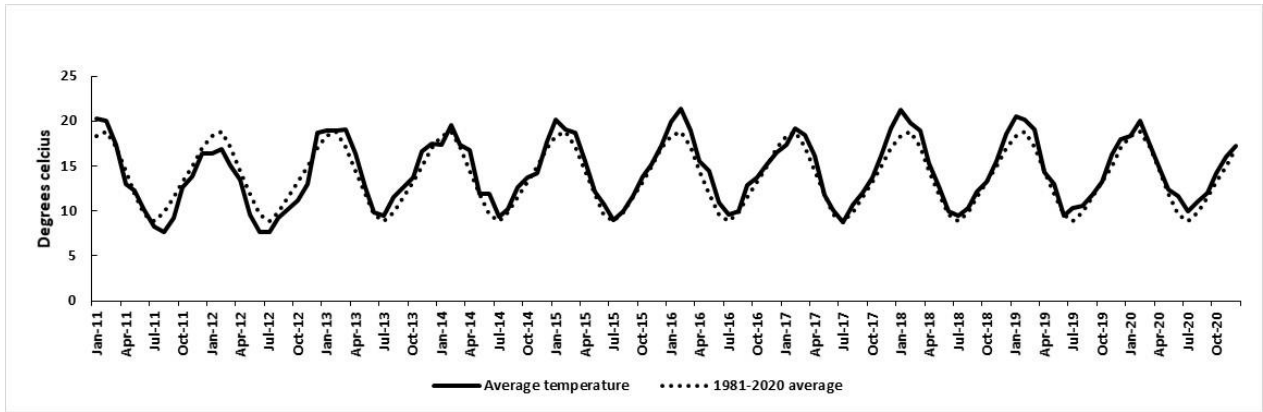


Figure 1. Waikato mean monthly average temperatures (°C) compared with 1981-2010 averages. Data obtained from CliFlo, NIWA.

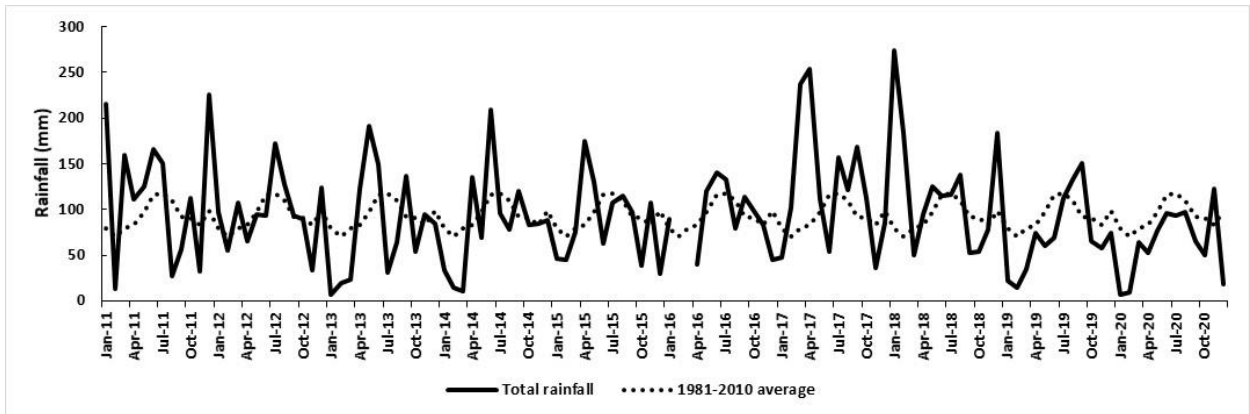
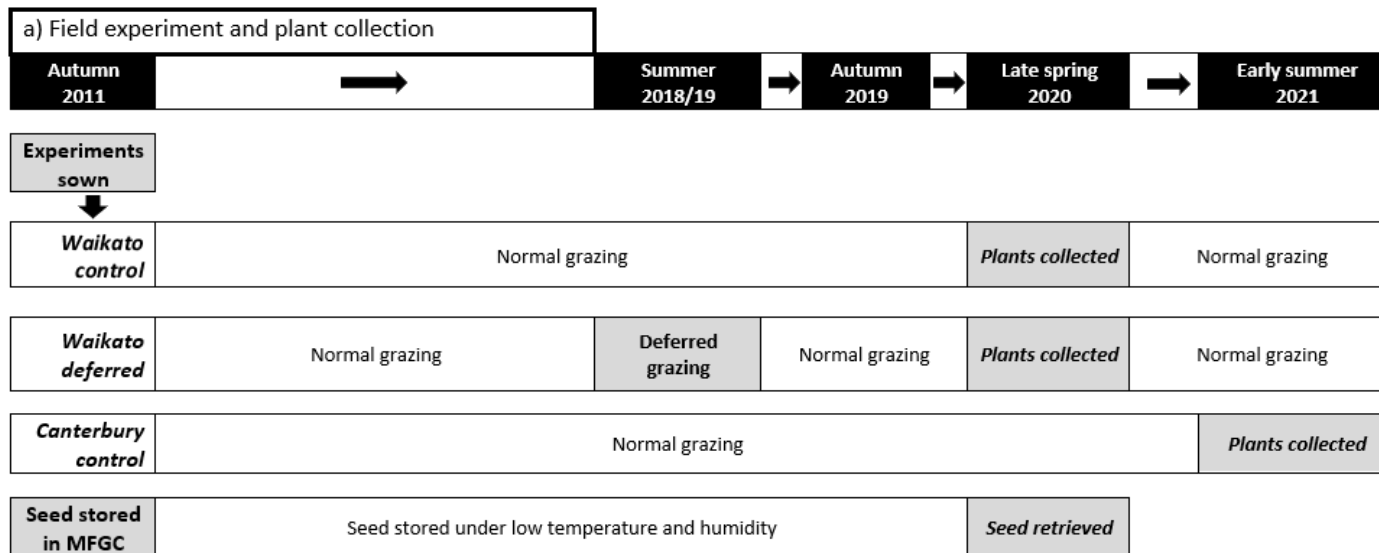


Figure 2. Waikato monthly total rainfall (mm) compared with 1981-2010 averages. Data obtained from CliFlo, NIWA.

In the deferred grazing treatment at Waikato, the aim of replenishing ryegrass populations through natural seed set was achieved, with tiller density in autumn 2019 (following the end of the deferral period) increasing to nearly 6,400/m<sup>2</sup> compared with 2,830/m<sup>2</sup> in the control treatment (Griffiths et al. 2021). Most of this increase came from the emergence of new seedlings from seed drop in the preceding summer. While tiller density in the deferred pastures more than halved in the following 12 months, it was still nearly double that in the control treatment and perennial ryegrass DM yield from winter 2019 to autumn 2020 was 62% greater in deferred than control (Griffiths et al., 2021).

### Timelines

Figure 3 shows the key timelines covering the sowing of the original trial in 2011 through to the point when plants were collected from the field and seed was retrieved from storage (Figure 3a), and the sequence of events in the polit study (Figure 3b).



b) Pilot study plant management and measurements

Nov 2020	Dec 2020	Jan 2021	Feb 2021	Mar 2021	Apr 2021
Plants transplanted into pots	Seedhead counts initiated	Plants moved outdoors. First biomass harvest	Second seedhead count and biomass harvest	Third seedhead count and biomass harvest	Fourth and final seedhead count and biomass harvest

Figure 3. Timing of key events for a) the field experiments and plant collections, and b) plant culture and measurement in the pilot study. MFGC = Margot Forde Germplasm Centre.

## Plant collection and trial design

On 4<sup>th</sup> of November 2020, 15 divots were cut with a knife from random locations in each of the 4 replicate plots of each cultivar by management (control, deferred) combination at the Waikato site, giving a total of 60 for each population. The divots were transferred to Lincoln, where they were individually broken up and two ryegrass tillers, with roots attached, were removed. The two clonal fragments from each divot were transplanted together into 2.5l pots on 18<sup>th</sup> November 2020. The soil medium was an 8–9-month mixture containing 400L bark, 100L pumice, 2500g Osmocote Exact, 500g horticultural lime, and 500g of hydraflo wetting agent. The potted transplants were stored in the glasshouse and any older leaves were trimmed to 10 cm to avoid dehydration. After an adjustment period of 2-3 days, the plants were shifted into the shade house where they were hand-watered daily to establish and grow out.

On the 5<sup>th</sup> of December 2020, 150 seeds of each cultivar from the batches stored in the MFGC were planted into cell trays, watered and placed into the glasshouse for germination. On the 16<sup>th</sup> of December, two seedlings were transplanted into each of 60 2.5l pots per cultivar. The same growing medium as described above was used.

In mid-December, the ex-field clonal fragments and reference plant (ex-seed) seedlings were combined and arranged into four replicate blocks of a 15x15 Latin square formation inside the shade house. Each population was present once in each row and column of each replicate. Because there were only 12 reference, control and deferred populations, three further seed lines were used as filler plants. These plants were from either Nui AR37, Nui SE or a Norway ecotype, all drawn from accessions at MFGC and germinated at the same time as the Nui, Commando, Alto and Halo seeds. In late December 2000, pots were randomly thinned to one plant, except when in instances where one plant of the pair was struggling to survive, in which case, the stronger plant was retained and the other discarded.

An automatic irrigation system, consisting of two oscillating sprinklers, watered the plants for 30 minutes each morning. On the 20<sup>th</sup> of January, all plants were shifted outside into full sunlight and ambient, remaining in their original Latin square configurations. Sprinklers were used to water the plants daily.

Figure 4 shows the arrangement of individual plants in the Latin square design, which is also shown in Plate 1. The average temperature, wind speed, and light levels were measured using a weather station situated on site (seen in the middle left of Plate 1, and in Plate 2).

Rep 1															Rep 2														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240
16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255
31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270
46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285
61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300
76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315
91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330
106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345
121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360
136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375
151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390
166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405
181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420
196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435
211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450
Sprinkler															Sprinkler														
Rep 3															Rep 4														
451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690
466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705
481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720
496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735
511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750
526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765
541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780
556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795
571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810
586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825
601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840
616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855
631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870
646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885
661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	886	887	888	889	890	891	892	893	894	895	896	897	898	899	900

Figure 4. Layout of Latin square stationed outside at the Lincoln University plant nursery with four replicates.



Plate 1. Layout of the Latin square formation of plants outdoors at Lincoln University Horticulture Nursery.



Plate 2. The weather station measured average temperature, wind speed, and light levels.

During the time the plants were outside, the temperature ranged from 5 degrees to 25 degrees (Figure 5).

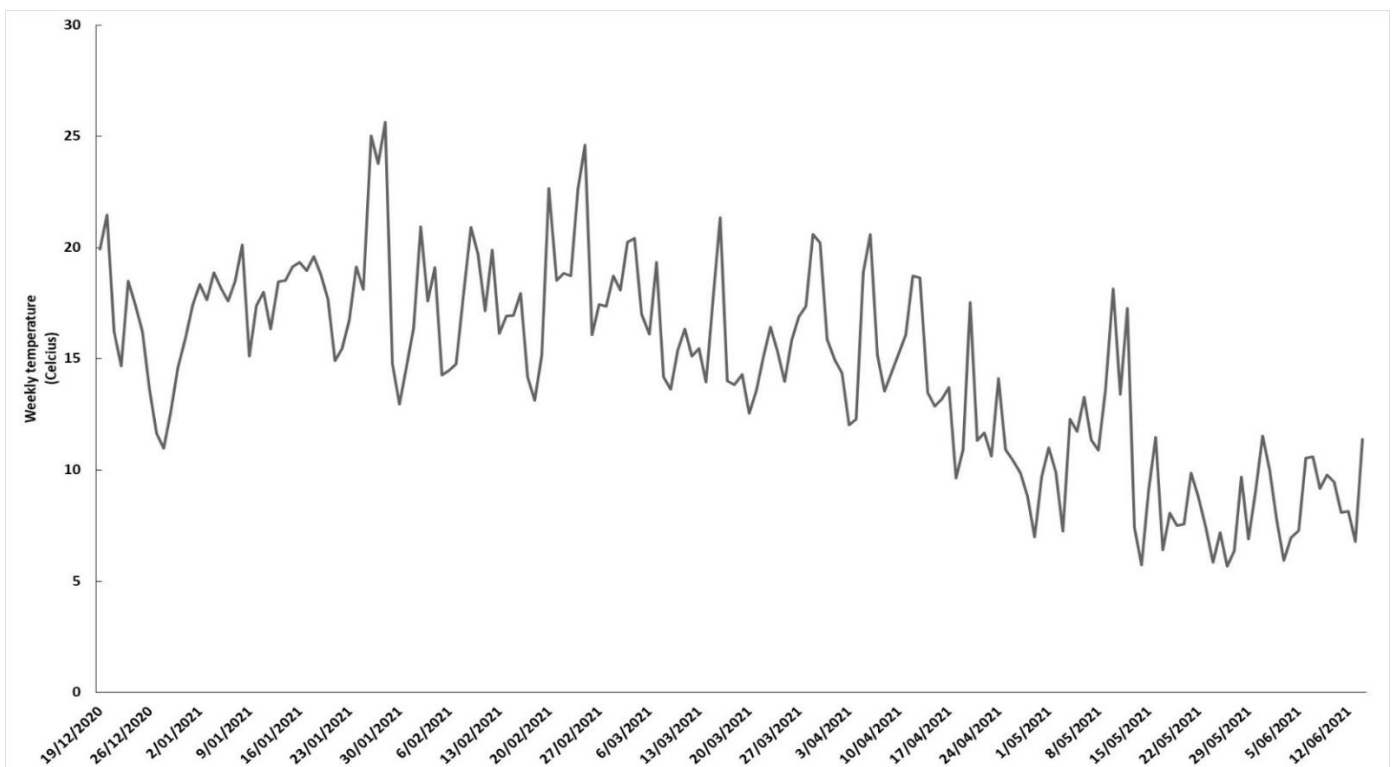


Figure 5. Mean daily temperature recorded by the weather station (Plate 2), recorded whilst the plants were in the Latin square arrangements at the Lincoln University nursery from mid-December 2020 through until final harvest in mid-June 2021.



Clonal fragments from the Canterbury experimental site were collected in February 2021, and transplanted in exactly the same way as for the reference and Waikato treatment plants. Because they were collected ~ 3 months later than the other populations, the 60 pots per cultivar were retained in the shade house to accelerate their growth to the point where they were sufficiently close in size and tiller density to the rest of the study plants for inclusion in the endophyte analysis described in Chapter 4. No phenotypic or phenological measurements were collected from these plants during the overall study.

## Measurements

Counts of the number of reproductive stems per plant began on 22<sup>nd</sup> December 2020 (Figure 3b). This process consisted of counting the number of reproductive stems present on each of the ex-Waikato plants in the Latin square: the reference plants had not reached the stage where reproductive growth was evident, though some stem elongation occurred later in the measurement period. The presence of the first visible node on an elongating stem was the baseline for including a stem as 'reproductive' in the count. The number of stems that had reached this baseline or beyond (through to full seed head emergence) was counted on each plant on each occasion.



Plate 3. Example of perennial ryegrass plant state before and after cutting to 7 cm height and collection of herbage for drying and weighing. Elongating stems that would be included in the reproductive counts are visible on the pre-cut plant on the left.

On 18<sup>th</sup> January 2021, the ex-Waikato plants were cut for the first time, to approximately 7 cm above the surface of the growth medium (Plate 3). The cut herbage was collected, dried at 65 degrees for two days, and then weighed. Plants grown from seed were not cut as they were not yet big enough to warrant trimming. A second count of reproductive stems was also taken on 18<sup>th</sup> January.

From 23<sup>rd</sup> February 2021, all plants were trimmed monthly to 7 cm height, to mimic grazing and maintain and regulate plant growth. On 23<sup>rd</sup> February, another reproductive tiller count was conducted on all plants. Monthly cuts and reproductive stem counts continued until the size of the reference plants caught up with the control and deferred plants and all the plants had acclimatised to the growing conditions. For the last cut, the leaf biomass was separated from the seed head biomass, and these were weighed separately. This phase ceased on the 6<sup>th</sup> of April 2021 with the last cut and count occurring on this day.

## Statistical analysis

Statistical analyses were performed using SAS 9.4 (2016, SAS Institute Inc, Cary, NC, USA). Data for total harvested (above 7 cm) biomass were subjected to repeated analysis of variance (ANOVA, Proc Mixed) measures. The model included cultivar, treatment, time, and all their interactions as fixed effects. The unit of analysis was the replicate mean in all cases and replicate was considered the random effect. The analysis used a heterogeneous variance first-order autocorrelation structure (type=ARH(1)). The analysis was then run again for reproductive stem counts, either including or excluding the reference plants. For the final harvest where leaf and stem were separated, leaf biomass and reproductive stem biomass were analysed separately to test for the effects of cultivar, treatment, and their interaction when the reference plants were included or excluded from the analysis. For all variables, ANOVA was followed by Tukey t-tests for significant differences in pairwise comparisons of cultivar and treatment effects across and within time points. If required, data were transformed by  $\log_{10}$  or square root to achieve homogeneity of variances. Significance was declared if  $P < 0.05$ .



## Results

The significance of differences between treatments and cultivars and their interactions are summarised in Table 2.

Table 2 Statistical significance (Fprob) of main effects and interactions in biomass harvest (g DM/pot) and flowering attributes (reproductive stems per plant, and g DM/pot of reproductive stem 105 days after moving plants outdoors).

Factor	Total plant biomass yield	Reproductive attributes			
		Reproductive stem number		Reproductive stem biomass (day 105 only)	
		Including reference plants	Excluding reference plants	Including reference plants	Excluding reference plants
Cultivar	0.2507	<.0001	<.0001	0.0053	0.0018
Treatment	<.0001	<.0001	0.8084	<.0001	0.3273
Time	<.0001	<.0001	<.0001		
Cultivar x treatment	0.8768	0.0003	0.0005	0.0115	0.0118
Cultivar x time	0.2626	0.0166	0.0014		
Treatment x time	<.0001	<.0001	0.7621		
Cv x trt x time	0.0638	0.2804	0.7336		

### Biomass yield

There were no significant differences between cultivars in biomass yield per pot for any of the harvest dates, and none of the interactions involving cultivar were significant (Table 2).

The significant treatment x time interaction (Table 2) is shown in Figure 6. Overall, the biomass per plant increased at each harvest interval. Initially (day 28), the reference plants grew much less than the survivors ( $P < 0.001$ ); however, 11% more DM was removed from the reference plants than from the control and deferred plants at day 56 ( $P < 0.05$ ). Thereafter, biomass yield did not differ between treatments (Figure 6).

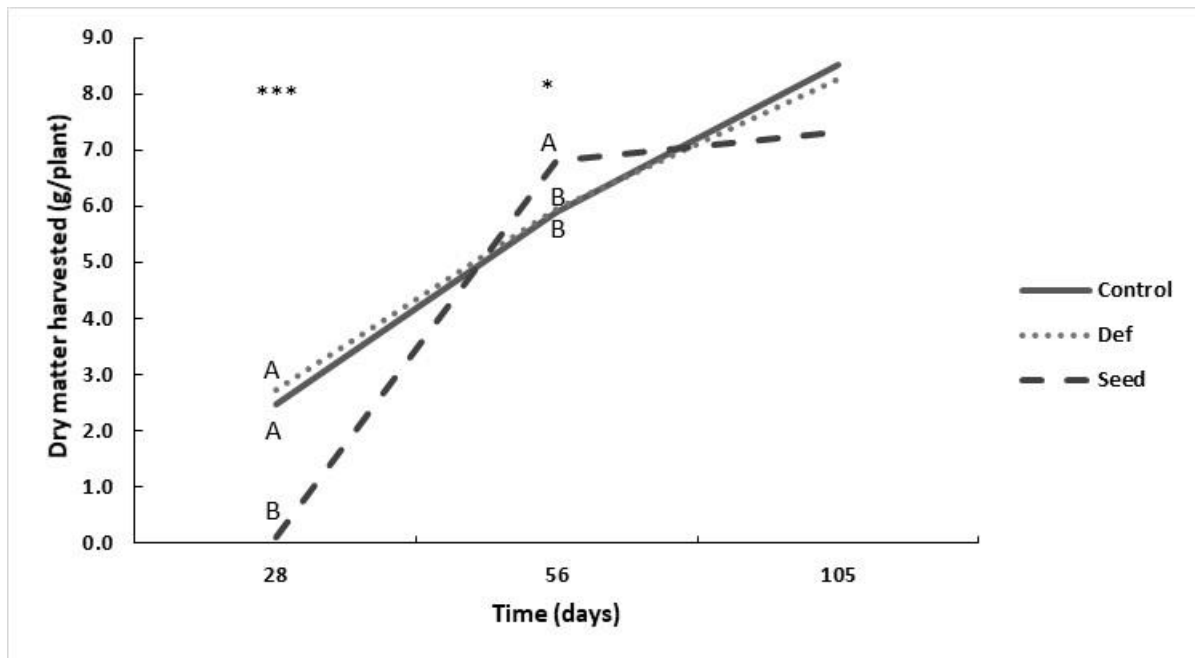


Figure 6. Treatment effects on the dry matter harvested per plant in days after 20<sup>th</sup> January when the plants were transferred to the outdoor environment. The legend shows control and deferred survivor populations and the reference plant populations ('Seed'). Means with a common letter at a given time do not differ according to Tukey multiple means comparisons ( $P > 0.05$ ). The asterisks indicate significance of the treatment effect (\*\*\*) =  $P < 0.001$ , \* =  $P < 0.05$ ).

### Number of reproductive stems

The treatment x time interaction ( $P < 0.001$  when reference plants were included, Table 2) is shown in Figure 7. No reproductive stems were present on reference plants. They produced no reproductive stems at day 28 and day 56; a few emerged on these plants by day 105, but much less than in the control and deferred treatment plants which displayed substantial continuous stem development. The control and deferred treatments did not differ from each other at any stage ( $P > 0.05$ , Fig 7).

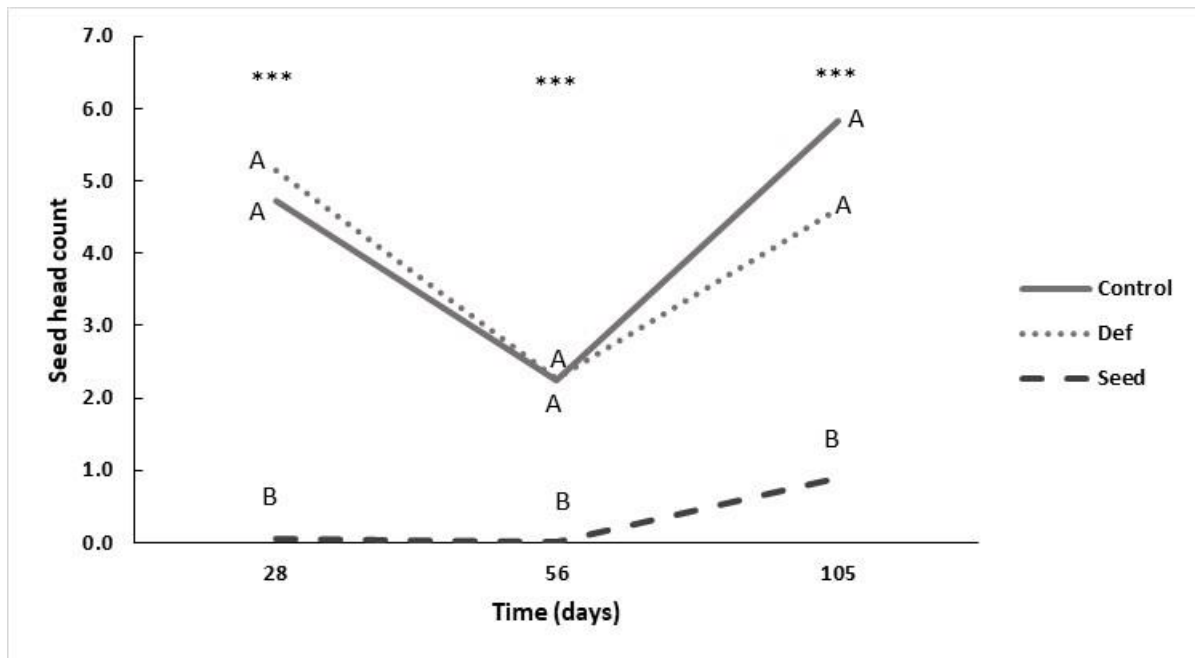


Figure 7. Treatment effects on the number of reproductive stems per plant (seed head counts) in days after 20<sup>th</sup> January when the plants were transferred to the outdoor environment. The legend shows control and deferred survivor populations and the reference plant populations ('Seed'). Means with a common letter at a given time do not differ according to Tukey multiple means comparisons ( $P > 0.05$ ). The asterisks indicate significance of the treatment effect ( $*** = P < 0.001$ ).  $***$ ).

The cultivar x time interaction (statistically significant, with or without inclusion of reference plants, Table 2) is shown in Figure 8. Initially (day 28), Halo had 45% fewer reproductive stems per plant than Alto ( $< 0.01$ ); with Commando, and Nui did not differ from each other, or from Halo and Alto. There was no difference among cultivars at day 56, but on day 105 Halo had, on average, 80% fewer reproductive stem per plant than the other three cultivars (Figure 8).

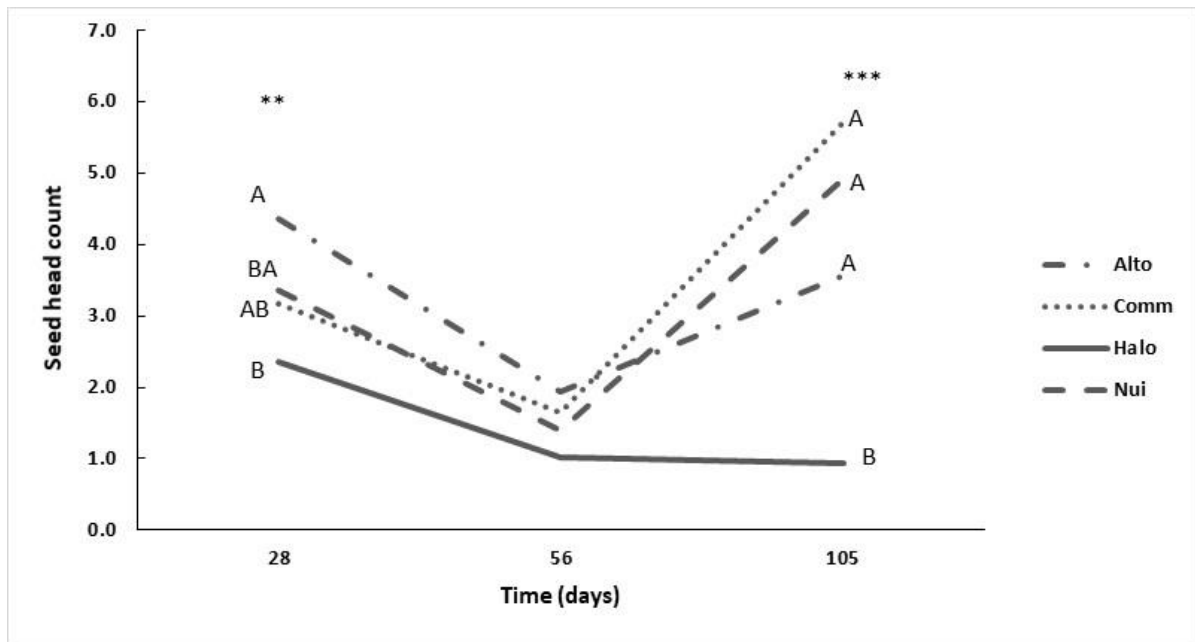


Figure 8. Cultivar effects on the number of reproductive stems per plant (seed head counts) in days after 20<sup>th</sup> January when the plants were transferred to the outdoor environment. Means with a common letter at a given time do not differ according to Tukey multiple means comparisons ( $P > 0.05$ ). The asterisks indicate significance of the treatment effect (\*\*\*) =  $P < 0.001$ ; \*\* =  $P < 0.01$ ).

The cultivar x treatment interaction (inclusion of reference plants,  $P < 0.001$ , Table 2) is shown in Figure 9. Plants in the Alto deferred population had 48% more reproductive stems per plant compared with the Alto control population, whereas Commando showed the opposite response to these treatments with 63% more reproductive stems in the control population compared with the deferred population (Figure 9). The number of reproductive stems did not differ between treatments for Halo and Nui.

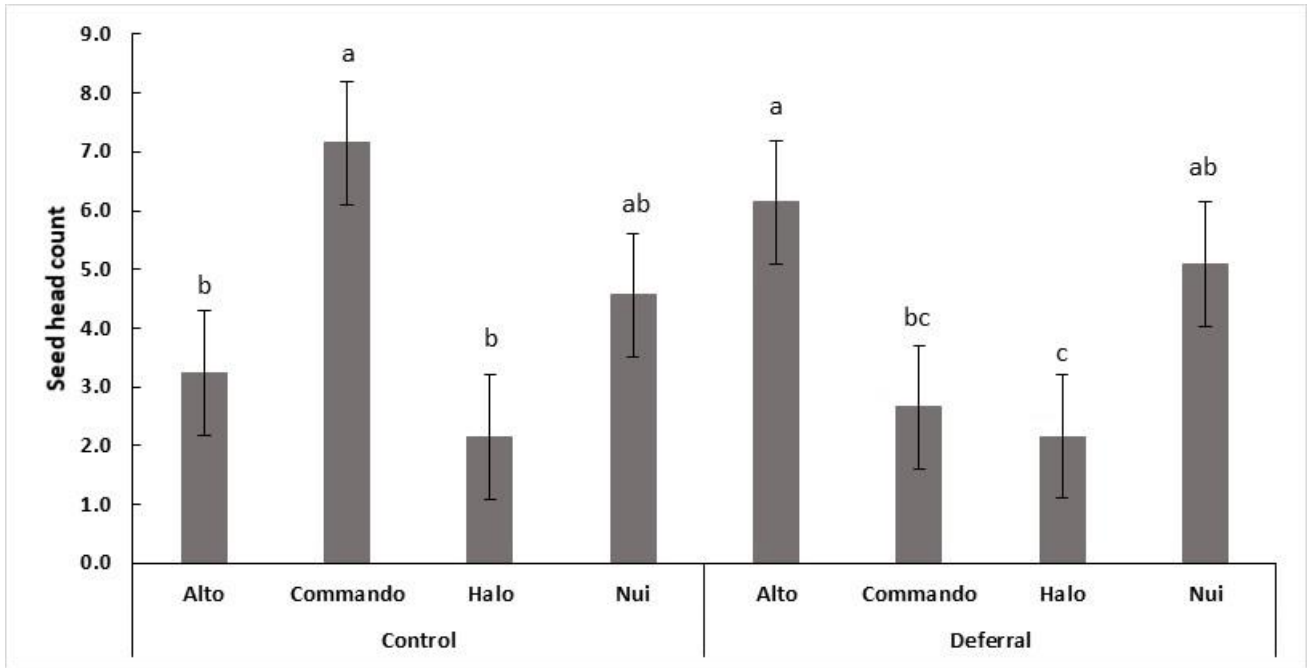


Figure 9. Cultivar and treatment effects on the mean (of three measurement points) number of reproductive stems per plant (seed head counts). Means with a common letter do not differ according to Tukey multiple means comparisons ( $P > 0.05$ ). Bars equal one standard error.

### Reproductive stem biomass

The cultivar effect on reproductive stem dry matter harvested per plant at day 105 (statistically significant with or without including the reference plants, Table 2) is shown in Figure 10. On average, Halo produced 65% less reproductive stem biomass than Commando and Nui, while Alto did not differ from any of the other cultivars.

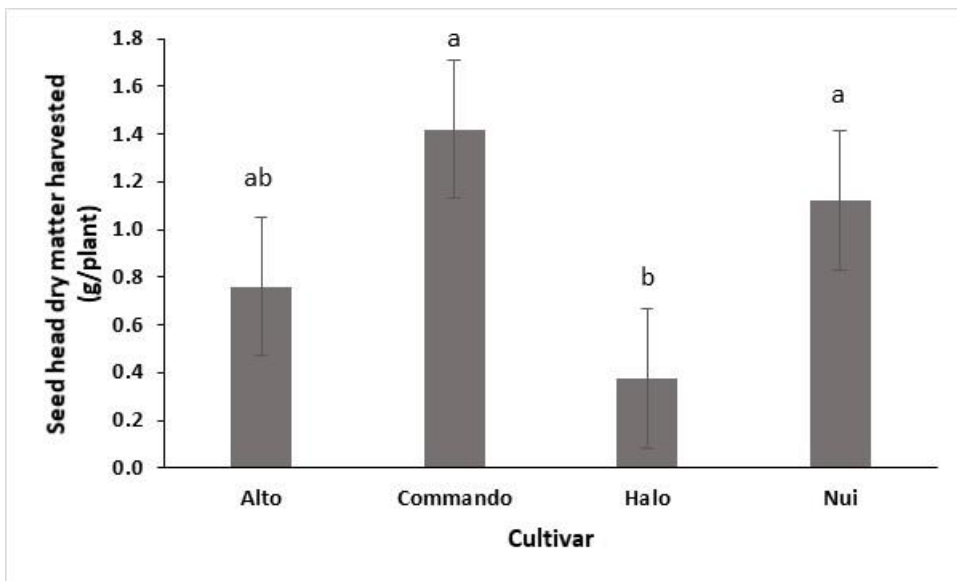


Figure 10. Effect of cultivar on reproductive stem biomass per plant at 105 days after plants were moved outdoors. Means with a common letter do not differ according to Tukey multiple means comparisons ( $P>0.05$ ). Bars equal one standard error.

The cultivar x treatment interaction for reproductive stem biomass (statistically significant with or without including the reference plants, Table 2) is shown in Figure 11. Plants from the Commando control treatment produced three times more reproductive stem mass than plants from the Commando deferred treatment. No significant differences between control and deferred were observed for the other cultivars.

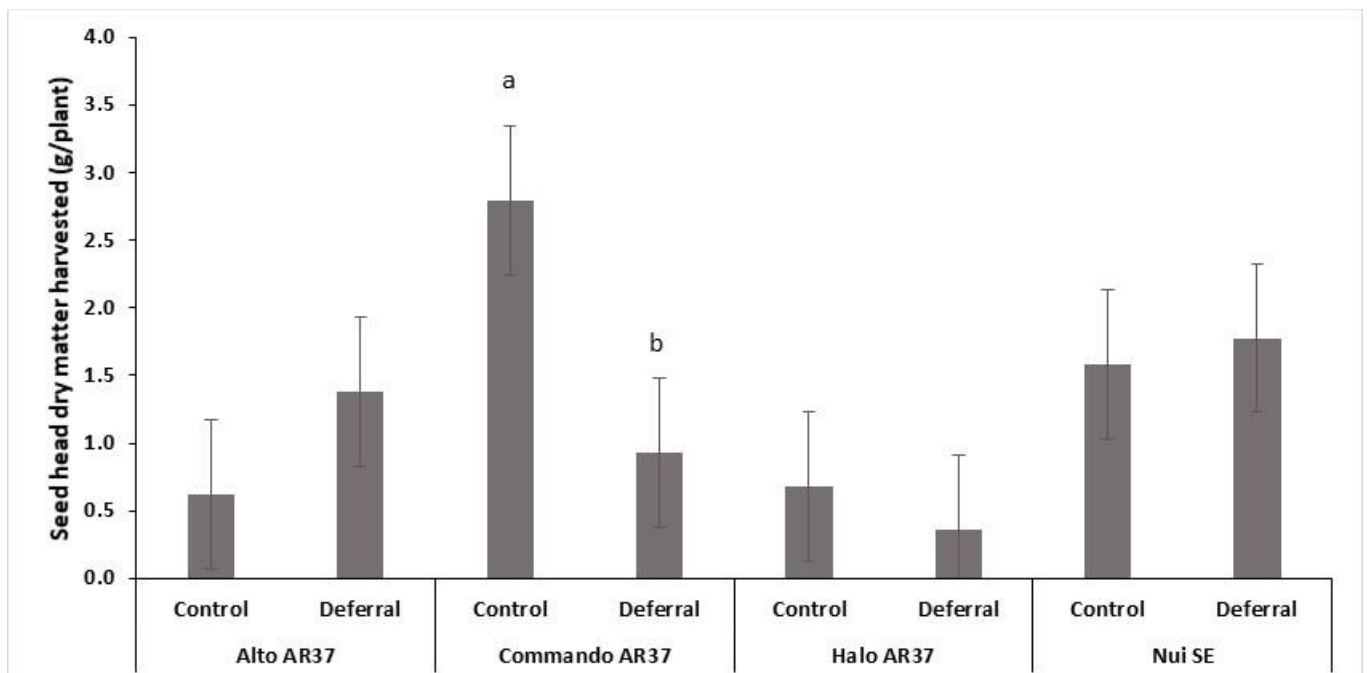


Figure 11. Interaction between cultivar and treatment in reproductive stem biomass per plant 105 days after plants were moved outdoors. Letters indicate where differences between treatments within cultivars occurred according to Tukey multiple means comparisons ( $P<0.05$ ). Bars equal one standard error.

## Discussion

This phase of the study encompassed the early establishment of the plants during which they were acclimatising to the outdoor environment. The plants came from different sources: seed and seedlings (reference treatment), versus vegetative transplants from the field (control and deferred populations). Thus, they were not starting from the same point with regard to the amount of roots and shoots available to support further growth. The transplanted plants would have been subject to carry-over effects from their field environment, which take time to dissipate (Bullock et al., 1993) Hence, there were two main points of interest in the results:

- a) the trajectory toward the expected equilibration of all plants toward a similar mass and physiological state; and
- b) whether there were any early indications of differences between the control and deferred plants which might be useful for deciding which variables to focus on in the next study phase.

### Biomass yield

As expected, the reference plant population had low biomass yield after establishment: the seedlings were still catching up to the clonal transplants at day 28. However, by day 56, the biomass of the reference treatment plants had increased and was greater than the other two treatments at this point. It is important to note that the clonal transplants were placed outside on the 22<sup>nd</sup> of Nov and the reference plants stayed in the glasshouse until the 15<sup>th</sup> of Dec. This was to allow the growth of the seedlings to catch up to that of the transplants. This rapid growth phase in the reference plants was likely because these plants were started in the glasshouse and remained there until they were transferred to the outdoor environment. Leishman and Westoby (1994) stated that conditions in the glasshouse were more favourable in terms of soil moisture, air temperatures, and humidity for plant establishment. The glasshouse is designed to provide the best growing conditions for seedlings. This environment allowed them to grow with fewer environmental stresses than the control and deferred plants which were exposed to variable temperature outdoors (Figure 5). The glasshouse enabled less interrupted growth. When transferred outside, the reference plants were growing vigorously, such that, by day 105, there were no differences in biomass between the treatments: this result suggests that the plants were in the same stages of growth and that the reference plants had reached a similar state of growth equilibrium as the control and deferred plants. Figure 7, however, would contradict that statement, as the reference plants were producing fewer reproductive stems than the other two treatments at day 105. This result suggests that while

these plants may have been at the same growth state, they were not at the same developmental stage. The control and deferred plants were clearly carrying some signal from their field environment that resulted in continued flowering when removed to the common environment at the nursery. The results from day 105 confirmed the expectation that the reference plants would lag behind the others at the start, but that possible carry over effects would dissipate over time. The plants from the deferred treatment were just as productive as the control plants over the first three months. This lack of difference could be due to the plants being in an adjustment period: they were moved to a new environment during their establishment phase.

## **Reproductive stem development**

It is likely that the reference plants did not have enough time to gain an environmental signal to start flowering before these measurements were taken. The Waikato survivors experienced these environmental cues such as day length change in the field before they were removed. They were then subject to further day length change when they were translocated to longer days in Canterbury (NIWA, 2010). Day 105 showed the start of an increase in seed head counts (Figure 7): however, with no more collections after this time point, it is difficult to say whether the differences were due to the reference population starting from seed or whether flowering was stimulated for some reason in the control and deferred treatments. The substantial amount of reproductive stem produced in the control and deferred plants into March and April was not representative of 'normal conditions' where aftermath heading usually does not extend beyond summer. The relatively low reproductive stem number and weight in Halo isn't consistent with what is observed in the field in the Waikato where this cultivar is similar to others including Alto in spring and summer seed head development (Chapman et al., 2022). Whether or not shifts in flowering have occurred can only be determined by taking 'true-to-type' plants through a full main flowering season following vernalisation in winter and spring.

According to plant breeders' information, Commando and Nui have the earliest heading dates of the four cultivars (Stewart, 2006; Lee et al. 2012). However, at day 28, Alto had produced similar numbers of reproductive stems per plant to the earlier heading date cultivars (Figure 8). Compared to the other cultivars, Alto is considered a late season cultivar with a heading date of +14 days (Lee et al., 2012). Therefore, it was not expected to see Alto reproductive development matching the early season cultivars. This result could be due to stress; it is possible that the plants received different environmental signals with the translocation from the Waikato to Canterbury and that this was reflected in the data collected soon after. On day 56 there was no difference in the reproductive stem numbers per plant of the four cultivars (Figure 8). By day 105 (early April), reproductive stem



development had increased in all cultivars, with the exception of Halo. The spike at day 105 was the result of aftermath heading (Lee et al., 2012; Wims et al., 2014). Commando is known for high aftermath heading followed by Nui (Stewart, 2006). Alto was in the middle, with lower aftermath heading while Halo had the lowest, consistent with the expectation of aftermath heading being lower in the later heading date cultivars (Lee et al., 2012).

Climate and plant phenology are intimately linked (Menzel et al., 2020). This means that evaluating changes in plant phenology is a powerful way to assess the impact of climate change on plant populations. The cultivar by treatment interaction results for reproductive stem number per plant indicated that Commando and Alto responded to the control and deferral treatments in completely opposite directions (Figure 9). On average, the Alto control plants produced significantly fewer reproductive stems per plant compared with the deferred population whereas the reverse was true for Commando. Subsequent analysis of the endophyte status and identity in the control and deferred plants showed there was contamination by 'off types' (i.e. plants with an endophyte strain other than AR37, especially standard endophyte) in several survivor populations. These results are presented in more detail in Chapter 4, but of relevance to the cultivar x treatment interaction observed here, it is notable that standard endophyte was found in 28% of plants in Alto deferred versus 12% in Alto control. If the standard endophyte-containing volunteers were early flowering old ryegrass types similar to Nui, then the higher proportion of them in the Alto deferred treatment may explain the higher number of reproductive stems that developed in this population compared with the control treatment.

Significantly, the Commando population showed the opposite response to Alto, with significantly higher seed head counts in the control population on average over the experimental period (Figure 9). Contaminants were present in the Commando populations too (as discussed in Chapter 4); however they were present in high proportions of the total sample population in both the control and deferred treatments: 42% and 30% respectively. The difference between 42% and 30% may explain some of the difference in reproductive stem number between control and deferred, but not all, especially since Commando is also an early flowering type, with Mangere ecotype in its breeding background (Lee et al., 2012). . There have been many reports of changes in flowering phenology as a result of directional changes to the climate (CaraDonna et al., 2014; Fitter & Fitter, 2002; Forrest et al., 2010). To examine the hypothesis that flowering patterns has shifted, it would be necessary to repeat the study after the reference plants had had at least a year to grow while, at the same time, any carry-over effects in the control and deferred plants had time to dissipate. Such measurements would allow for the full effect of the three treatment to be examined.

In light of the significant differences observed in reproductive stem number and biomass in this preliminary phase, flowering behaviour was analysed as part of the more-detailed phenotypic and phenological study reported in the next Chapter. This at least allowed time for all plants to go through a full winter and spring and receive the winter cold temperature conditioning, followed by increasing daylength in spring, that initiates flowering in this species (Cooper, 1960).

## **Concluding points**

- There were no effects on plant biomass yield as a result of the control and deferred treatments in this preliminary phase.
- Growth rate of the reference plants relative to the control and deferred plants was similar after 105 days of growth outdoors, indicating reasonable equilibration of plant state across treatments during the preliminary phase.
- However, large differences in reproductive development between the reference and survivor populations show that carry-over effects were still present, and these must be considered in interpretation of results of more-detailed phenotypic comparisons.
- The presence of off types (non-true-to-type) could have affected the results from the preliminary study; thus, endophyte analysis is necessary to identify true-to-type plants for more detailed phenotypic measurements.
- There were early indications of differences between the control and deferred plants which were useful for deciding which variables to focus on in the next phase of the study.
- In this preliminary stage, the interaction between cultivar and treatment (control, deferred) in number of reproductive stems per plant highlights the need to monitor flowering patterns closely in the next phase of the work.

# Chapter 4 Phenotypic differentiation

## Introduction

The identification of traits that allow for the survival and persistence of New Zealand perennial ryegrass cultivars, is becoming more and more crucial. This need is caused by regulations for reduced environmental impact and the high costs of re-grassing pastures. As Grafton and Manning (2017) have shown, the median re-grassing cost for a Canterbury dairy farm in 2017 was \$13,370. With further increases in costs for services and consumer goods, it is likely that these costs have risen in the years since this survey was undertaken.

Menzel et al. (2020) stated that climate and plant phenology are intimately linked; thus, the need to understand the trait adaptations occurring in a population as a result of climate change becomes increasingly important. The Waikato field experiment from which survivor plants were collected for this study ran for nearly 10 years after sowing before the plants were collected. During that time, several very dry and warm summer/autumns were experienced (Figures 1 and 2), consistent with climate change projections for the upper North Island of New Zealand (Keller, et al., 2021; Glassey et al., 2021). Few studies have spanned this time frame, with the notable exception of O'Connor et al. (2020a, b), who identified positive associations between persistence and tiller number, reproductive tiller number, lamina sheath length, and dry weight. O'Connor et al. (2020a) noted that genetic shifts over time were cultivar specific. Thus, it is reasonable to expect some phenotypic differentiation could be found in the populations studied here which may provide useful insights for plants breeders regarding persistence-related traits that could help improve pasture resilience to climate change.

Chapter Three identified some differences between the control and deferred treatments in reproductive stem number per plant. These were broadly consistent with O'Connor et al. (2020b) but also inconsistent in that differences were in opposite directions for two of the cultivars, Commando and Alto. However, it is not clear if those differences are true reflections of phenotypic differentiation because there were clearly carry-over effects on flowering which meant that the survivor populations could not be compared directly with the reference plant population. Also, it was possible that the presence of volunteer ryegrass plants among the survivors, not related to the original seed lines sown, could have affected the results.

The aim of the experiment reported in this Chapter was to identify traits associated with survival and to measure how they differed from the reference plant population after being exposed to the Waikato environment for 10 years. There was also cause to explore differences between the control

population and the deferral population to examine if there had been any changes due to grazing methods and the natural re-seeding event that was facilitated by deferring grazing. Importantly, the first step was to confirm the identity of the plants in the control and deferred treatments, and select only ‘true-to-type’ plants to ensure that the comparisons were not biased by the presence of different numbers of different plant off-types. This was achieved through endophyte DNA strain analysis, in which the survivor plants from the Canterbury site of the field experiment were also included, enabling a comparison of the persistence of the original populations in two very different climatic environments.

## Materials and methods

A timeline of key stages in the phenotypic/phenological study is shown in Figure 12. There were three main steps: endophyte analysis to assess the endophyte status and genetic integrity of the survivor populations; leaf and pseudostem measurements plus scores of plant habit and rust incidence; and monitoring of flowering in spring and early summer. Throughout the second and third phases, the plants selected from the endophyte analysis as ‘true-to-type’ remained outdoors in their original replicate formation but not in a Latin Square since more than half of the original plants were removed, including the ‘filler plants’.

Mar 2021	Apr 2021	May 2021	Jun 2021	Jul 2021	Aug 2021	Sep 2021	Oct 2021	Nov 2021	Dec 2021
Plant material sampled for endophyte DNA analysis	Plants maintained outdoors	30 true-to-type plants per population selected for detailed measurement	Leaf lamina and pseudostem measurements conducted, plus growth habit, tillering and rust scoring	Plants maintained outdoors with regular trimming and fertiliser application			Weekly flowering scores conducted		

Figure 12. Timeline of the detailed phenotypic/phenological study, including initial screening of all plants used in Chapter 3 for endophyte presence and genetic identity.

### Endophyte DNA analysis to determine population genetic integrity

Methods described by Faville et al. (2020b) were used for endophyte genotyping. The analysis was conducted in the AgResearch plant genomics laboratory located at Palmerston North. In this method, endophyte DNA was extracted from approximately 100 mg of fresh ryegrass pseudostem material removed from the lower-most 2-3 cm of pseudostem of a single tiller from each plant in all populations shown in Table 1. The Canterbury control survivor plants were still in the glasshouse at this stage, while the Waikato survivors and reference plants were outdoors. However, the

endophyte DNA methods are highly sensitive and simply require some pseudostem material; the age or growing conditions of the plant do not affect the analysis (M. Faville, AgResearch Grasslands, pers. comm.).

DNA extracts were typed for the endophyte strain using the simple sequence repeat (SSR) marker B11. This marker discriminates between the AR37 endophyte strain, other commercial endophyte strains, and the 'wild-type' or standard endophyte (SE). Thus, using the genomic analysis, it was possible to distinguish between AR37 and non-AR37 infections in the Alto, Commando, and Halo populations. It was also possible to identify the strain of the non-AR37 infection. Any 'volunteer' contaminants that might have invaded any of the cultivars from old seed buried in the soil are likely to contain the SE strain. While these can potentially be identified for the three AR37 cultivars, it is not possible to distinguish between SE sown from the original seed lines and SE in volunteer plants for the Nui cultivar used in the field experiment.

The results from the endophyte analysis were used to select plants for the next phase of the study to ensure that the phenotypic measurements were collected from only 'true-to-type' plants for Alto, Commando and Halo. The plants that were infected with the endophyte that was inoculated in the original seed lines (AR37 for Commando, Alto and Halo; SE for Nui) were identified as 'true-to-type.' Other endophyte strains detected at low levels included AR1, NEA variants, and an unknown strain.

In one of the 16 populations (Table 1), only 50% of plants (i.e., n=30) were confirmed as true-to-type. To keep the study balanced for plant numbers per population, all other populations of Commando, Alto and Halo were reduced to 30 plants each by firstly discarding plants that contained no endophyte, then plants containing an endophyte strain other than AR37, then taking a random selection of the remaining true-to-type plants. The same procedure was used for Nui, except that plants with AR37 were discarded (n = 9 out of 60 in the control treatment, 5 in the deferred treatment, and 2 in the reference population).

The total plants available for the next phases was therefore 360 (12 populations x 30 plants per population). Some plants died between the detailed phenotypic/phenological measurement and the flowering period in spring and early summer, but this was usually no more than one or two in any given population.

## **Phenotypic measurements**

Four weeks after the last maintenance cut that occurred on the 4<sup>th</sup> of June 2021, plant tiller density, growth habit and rust incidence were scored, and phenotypic measurements were conducted (Table 3). Plants were scored on a scale of 1-5 for tiller density and 1-3 for growth habit and rust. Pictures

were taken (see Appendix B, C and D) and used to standardise the scoring, with one person scoring all the plants to reduce variability in the scores. Plants were then cut in half using a hacksaw to access the middle tillers of each plant (see Plates 4b and 4c). The centre was the site from where tillers for measurements were harvested, to provide better representation of the whole plant compared with tillers growing at the periphery of the plant where intra-plant competition for light and space is much less. Two tillers with at least two fully expanded, or nearly fully expanded, leaves were chosen. The tiller was cut from the plant at the point where it was physically connected to other tillers, which was essentially at the crown of the plant from which multiple tillers were growing. The lamina of youngest fully expanded leaf of both tillers was removed using a sharp blade at the ligule incision point and both laminae were used for the leaf measurements listed in Table 3 (Plate 4e). The pseudostem of both tillers, comprising the material from the ligule incision point to the cut base of the tiller, was retained for measurement of the pseudostem variables listed in Table 3.

Once lamina and pseudostem measurements were completed, the two laminae per plant and the two pseudostems per plant were placed in paper envelopes then dried for 48 hours in a 60°C oven before dry weights were recorded. Specific leaf area ( $\text{cm}^2/\text{g}$  dry weight) was derived by dividing by leaf area by leaf dry weight, and leaf thickness was calculated using dry mass and leaf area data (Table 3).

Table 3 Measurements taken during the phenotypic sampling in June 2021.

Trait	Background	Method	Plant part
Tiller density score	The density of tillers can provide a key agronomic evaluation of a plant's productivity. Tillers are the units from which the plant produces leaves. More tillers often mean more leaves.	Score plants from 1 (not many tillers) - 5 (many tillers).  Modified from Griffiths et al. (2017) method of tiller density.  Visual	Whole plant
Plant growth habit score	The growth habit of a perennial ryegrass plant can be influenced by external stimuli. The way a plant grows is important for feed utilisation of the grazing stock. It is also important for the plant's survival, as more erect plants can have higher growing points. Stock grazing to lower levels (such as sheep) could thus affect persistence.	Scoring the growth habit of the whole plant. 1= prostrate growth, 2= normal tufty growth, 3= erect growth.  Visual (O'Connor et al., 2020a)	Whole plant
Leaf rust score	Leaf rust is a disease commonly found on stressed plants. The plants in our experiment have travelled to a new environment, so the plants are expected to be stressed.	Rust score from 1 (very little rust) - 3 (high levels of rust).  Visual	Whole plant
Biomass	Biomass cuts indicate the growth rate and how much the yield the plant produces in a season.	Weighing dried cuts of each plant over different intervals.	Dried leaves cut down to 7cm.
Reproductive tiller count	The number of reproductive tillers a plant produces indicates stress.	Counting the reproductive tillers on each plant before each biomass cut.	Seed heads
Leaf SPAD	The SPAD reading is an indication of the chlorophyll content or 'greenness' of the leaf. The amount of chlorophyll in a leaf is closely related to the N levels of the plant.	Using a SPAD-502 Plus meter from Konica Minolta Sensing Ltd, Osaka, Japan (D'Oliveira et al., 2020).	Leaf blade
Leaf length	Leaf length contributes to leaf characteristics. In general, a longer leaf means more biomass for the animal to consume. There is also more surface area for the plant to capture light for photosynthesis.	Measured on the lamina of the youngest fully expanded leaf from a tiller. The length of this lamina was measured from the ligule to the tip (Griffiths et al., 2017).	Lamina of the youngest, fully expanded leaf.
Leaf fresh mass	The mass of the fresh lamina used for leaf thickness assumption.	Weighing fresh laminae on a scale.	Lamina of the youngest, fully expanded leaf.
Leaf dry matter content	The amount of leaf dry matter the animal is digesting and using for growth.	Weighing dry laminae on a scale and subtracting this from the fresh weight to get the DMC (Dry matter content) of the leaf.	Lamina of youngest, fully expanded leaf.

Leaf width	The rationale behind the leaf width measurement is similar to that for leaf length.	Measured from the same lamina as the one used for the length measurement but using callipers. Leaf is bent at the mid-way point where it is measured from (Griffiths et al., 2017).	Lamina of youngest, fully expanded leaf.
Specific leaf area	Higher SLA indicates thinner, less dense leaves relative to biomass. Occurs when plants are recovering from defoliation. A good way for the plant to rebuild, optimising carbon to catch the light for photosynthesis.	Lamina Area= 0.7 (l x w). SLA= Lamina area/lamina dry mass. Unit: SLA cm <sup>2</sup> leaf area/g DM. (Griffiths et al., 2017; Robin et al., 2010)	Lamina of youngest, fully expanded leaf.
Leaf thickness	A thicker leaf indicates better persistence if drought occurs.	Leaf thickness = (SLA x LDMC) <sup>-1</sup> LDMC (leaf dry matter content) = leaf DM/leaf FM (Vile et al., 2005).	Lamina of youngest, fully expanded leaf.
Pseudostem diameter	Key measure of perennial ryegrass morphology. The wider the diameter, the thicker the pseudostem. Plants that grow fast put relatively high investment in leaf, not in the stem. Persistent plants may form a more substantial stem that includes storage carbohydrates and protein.	Measured with callipers at the midpoint of the leaf sheath (Griffiths et al., 2017).	The pseudostem of the tiller. The tiller was randomly chosen from the middle of the plant that was cut in half. .
Pseudostem length	Another measure of pseudostem performance. Persistent plants may form a shorter, thicker pseudostem that includes storage carbohydrates and protein.	Measuring the distance between the base and the ligule of the youngest, fully expanded lamina with a ruler (Griffiths et al., 2017).	The pseudostem of the tiller used. The tiller was randomly chosen from the middle of the plant that was cut in half.
Pseudostem dry mass	Persistent plants may form a more substantial stem that includes storage carbohydrates and protein.	Weighing dried pseudostem on scales (Griffiths et al., 2017).	The pseudostem of the tiller used. The tiller was randomly chosen from the middle of the plant that was cut in half. .



a) Plants used for the measurements



b) Bisecting plants to access central tillers



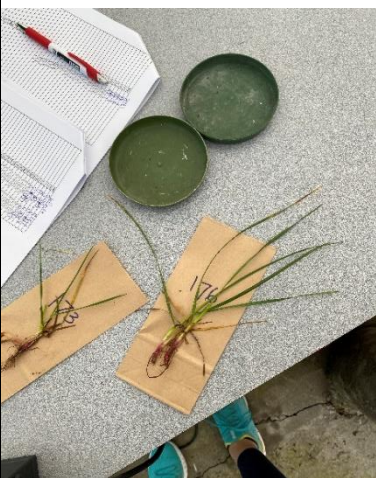
c) Example of a bisected plant



d) choosing tillers for measurements



e) Example of tillers used for measurements



f) Measurements in progress



Plate 4. Illustration of the methods used for detailed leaf and pseudostem measurements.

## Flowering

Flowering dynamics was monitored in spring/early summer using a scoring scale method adapted from Table 1 in Moore et al. (1991). Each plant was scored according to the stage of flowering on a scale from 2 (no evidence of stem elongation on any tillers) to 10 (anthesis) (Table 4). The most-advanced reproductive stems found on a plant at each observation date became the score for that plant; more than one stem at that most-advanced stage of development needed to be present to assign that score. Thus, if only one stem with a visible flag leaf was present, the score assigned would be 5. If two or more flag leaves were present, the score would be 6 (Table 4). Only the stage of reproductive development was assessed; the number of reproductive stems was not monitored.

Table 4 Scoring system for monitoring stage of flowering in the ryegrass populations. Based on Moore et al. (1991).

Visual identification	Score
No nodes visible	2
First node visible, hard to find	3
First node visible, easy to find	4
Multiple nodes easily visible	5
Flag leaf visible	6
Spikelet just emerging	7
Spikelet fully emerged	8
Peduncle visible	9
Anthesis	10

## Statistical analysis

Two analyses were performed for the percentage of 'true-to-type' endophyte in the populations, both using the GENMOB procedure in SAS: cultivar x treatment interaction using reference, Waikato control and Waikato deferred populations; and cultivar x site interaction, using Waikato control and Canterbury control populations. For phenotypic data, analysis of variance (GenStat v21.1) was used to examine differences among the 12 populations (reference, Waikato control, Waikato deferred by 4 cultivars) for the traits described in Table 3. Data were tested for the assumption of homogeneity of variances and transformed using square root or natural log (ln) where necessary. before analysis Various multiple means comparison methods were used including Bonferroni, Tukey, and LSD with significance levels declared at  $P < 0.05$ . Cultivar, cultivar x endophyte effect, and the cultivar x treatment effects were reported. Flowering scores were subjected to repeated analysis of variance (ANOVA, Proc Mixed) measures using SAS software. The model included cultivar, treatment, time, and all their interactions as fixed effects. Replicate was included as the random effect. A heterogeneous variance first-order autocorrelation structure (type=ARH(1)) was used.

## Results

### Endophyte analysis

A summary of the results from the endophyte analysis is presented in Table 5 for each cultivar - treatment combination. In the reference plant populations, 93, 78, 82 and 92% of plants contained an endophyte (all strains combined) in Alto, Commando, Halo and Nui respectively, of which the sown endophyte was the dominant strain. When the original seed lines were tested for endophyte presence in 2011, the respective infections rates were slightly lower at 84, 74, 78 and 87%, but followed essentially the same rank order among the cultivars (Lee et al. 2018).

Table 5 Percentage of plants of each cultivar in each population (n = 58-60 per combination) containing no endophyte ('Nil endo'), the sown endophyte strain ('Sown endo': AR37 or, for Nui, SE), standard endophyte (or, for Nui, AR37), and other endophyte strains.

Cultivar		Treatment/population			
		Reference	Waikato		Canterbury
			Control	Deferred	Control
Alto AR37	Nil endo	6.7	10.2	13.3	1.8
	Sown endo	90.0	78.0	58.3	98.2
	SE	1.7	10.2	26.7	0
	Other	1.7	1.7	1.7	0
Commando AR37	Nil endo	21.7	6.8	16.1	0
	Sown endo	73.3	50.8	53.6	96.7
	SE	1.7	42.4	25.0	3.3
	Other	3.3	0	5.4	0
Halo AR37	Nil endo	18.3	6.9	11.1	3.3
	Sown endo	76.7	81.0	81.5	90.0
	SE	5.0	8.6	3.7	5.0
	Other	0	3.4	3.7	1.7
Nui SE	Nil endo	8.3	3.4	5.2	8.3
	Sown endo	86.7	74.1	84.5	85.0
	AR37	3.3	15.5	8.6	1.7
	Other	1.7	6.9	1.7	5.0

The percentage of 'true-to-type' plants, based on agreement between the endophyte strain detected and the sown strain ranged from 50.8% to 98.2%. The main 'off-type' present was SE, ranging from 1.7% to 42.4% in the Waikato populations and 0 to 5% in the Canterbury populations. AR37 was detected in 8.6% and 15.5% of the Nui deferred and Nui control populations in the

Waikato, indicating some cross-contamination across plots in the field. This was not the case in Canterbury (Table 5).

*Treatment x cultivar interaction: Waikato*

The interaction between treatment (reference, Waikato control and Waikato deferred populations) and cultivar was not statistically significant. The main effects of treatment and cultivar were significant ( $P < 0.05$  in both cases). When meaned across cultivars, the percentage of plants that were 'true-to-type' for the inoculated endophyte strain was significantly higher in the reference population than in the control and deferred populations (82% versus 69-71%, Figure 13).

Contamination by SE infected plants was particularly pronounced in the Commando control and deferred populations, and the Alto deferred population (Table 5).

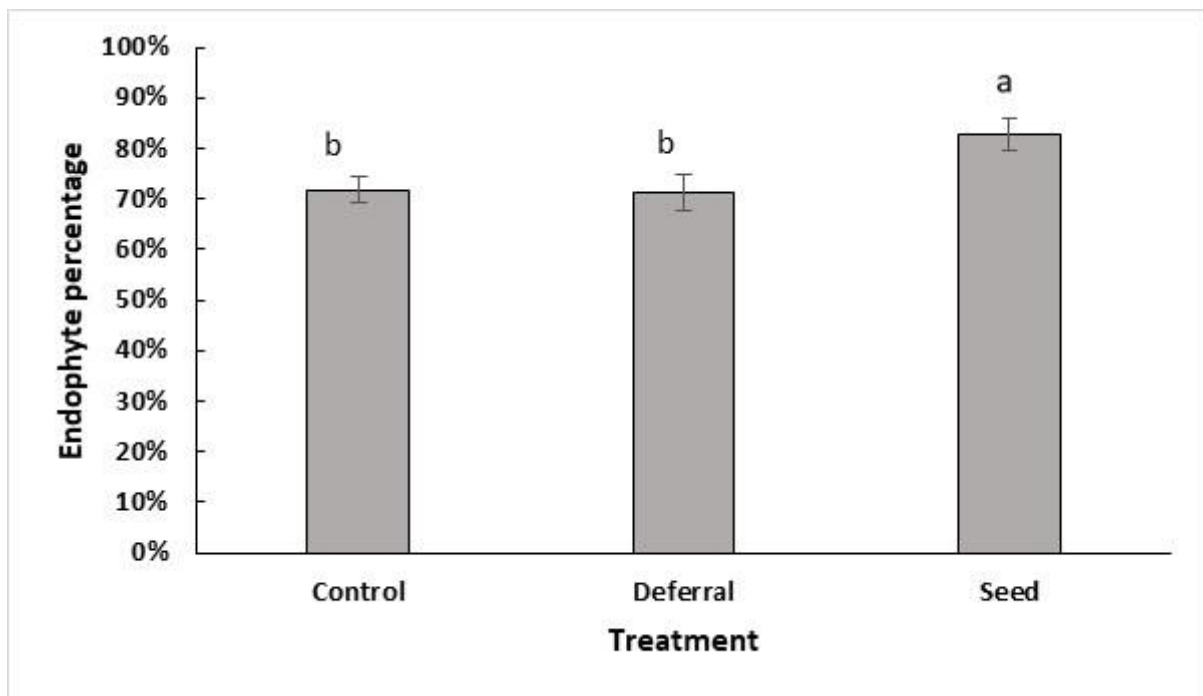


Figure 13. The effect of treatment on the 'true-to-type' endophyte percentages in the Waikato control and deferred survivor populations, and in the reference plant populations ('Seed'). Means with the same letter are not significantly different according to Tukey multiple means comparison ( $P > 0.05$ ). Bars equal  $\pm$  one SE.

When meaned across treatments at the Waikato site, true-to-type endophyte frequency was significantly lower for Commando AR37 (59%) compared with the other three cultivars (75-82%, Figure 14).

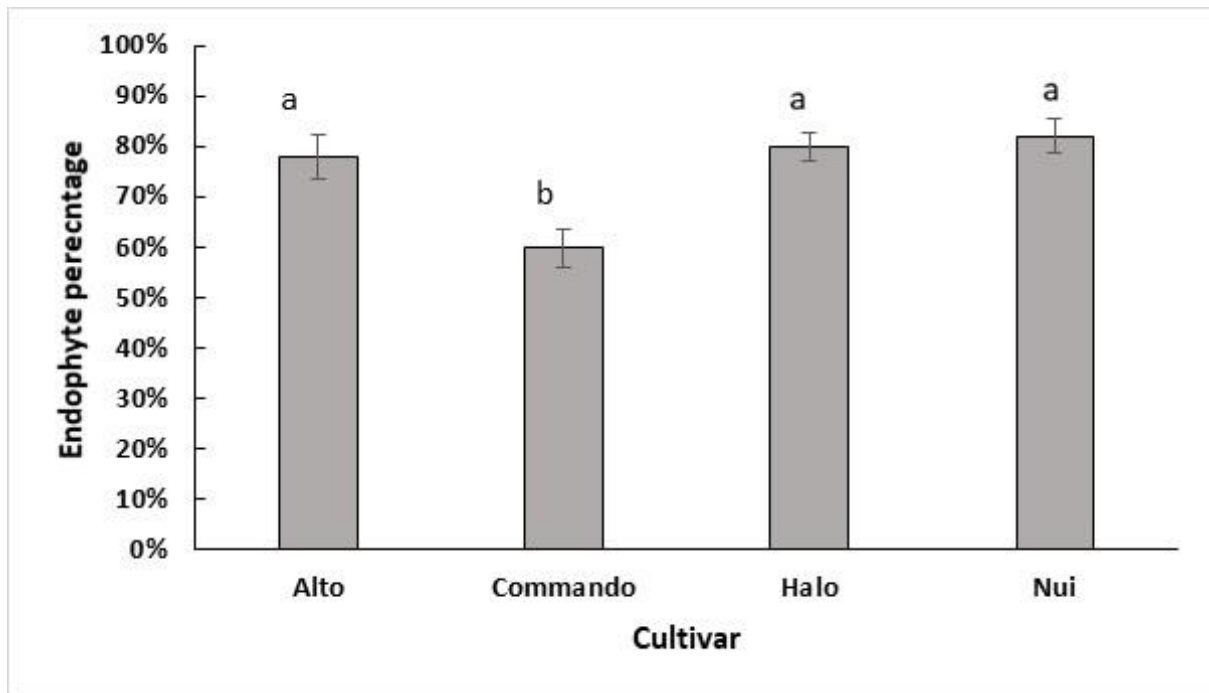


Figure 14. Effect of cultivar on the ‘true-to-type’ endophyte percentages of the four cultivars (means of the reference plant, Waikato control and Waikato deferred populations). Means with the same letter are not significantly different according to Tukey multiple means comparison ( $P > 0.05$ ). Bars equal  $\pm$  one SE.

*Cultivar x site interaction*

The interaction between cultivar and site was statistically significant ( $P < 0.01$ ). The overall percentage of plants testing ‘true-to-type’ was higher in the Canterbury control population than the Waikato control population (92.5 versus 70.5%) (Table 5, Figure 15). The percentage of plants testing true-to-type for Alto AR37, Commando AR37 and Halo AR37 was significantly higher in the Canterbury populations than the Waikato populations (98 v 78%; 96 v 51%; and 90 v 81% for the respective cultivars; Figure 15). There was a trend toward a similar effect for Nui.

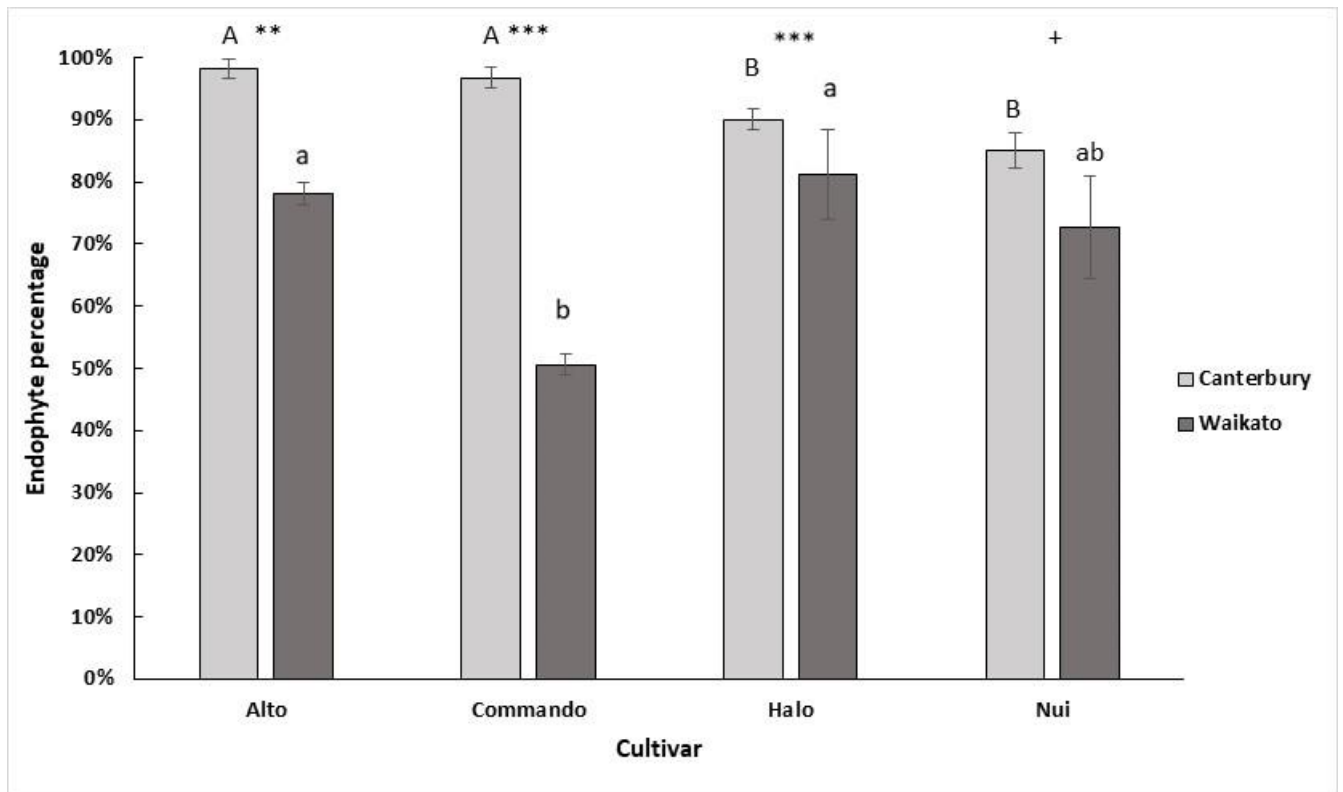


Figure 15. Interaction between cultivar and environment in the percentage of ‘true-to-type’ plants in the control treatment populations from Waikato and Canterbury. Means with the same letter in upper case (Canterbury) or lower case (Waikato) are not significantly different according to Tukey’s multiple means comparison. Differences between sites within cultivars are indicated: \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , +  $P < 0.1$ . Bars are  $\pm$  one standard error.

## Plant phenotypic traits

There were no significant interactions between treatment and cultivar for any of the leaf and pseudostem traits, or the growth habit, tiller density and rust scores. There was a trend toward an interaction for rust scores ( $P=0.056$ ). This indicated a scaling interaction, whereby the size of the difference in rust score between the Nui reference plants (2.2) and Nui control or deferred plants (1.6) was much greater than in other cultivars.

Main effects and means for all traits and scores are presented in Tables 6, 7 and 8. In the cultivar comparison, there was a highly significant difference between cultivars for all leaf traits, except for leaf dry matter content, specific leaf area, and leaf thickness (Table 6). Fresh leaves of Halo were 38.8% heavier than Commando and 18-26% heavier than Alto and Nui, respectively. In the treatment comparison, leaf fresh weight of plants in the control treatment was 15% less than in the deferred treatment, with the reference population intermediate (Table 6). Cultivar and treatment differences

were similar for leaf dry weight though, with the exception that leaf weight of plants in the control was significantly less than in the reference population. Leaf dry matter percentage was lower in the deferred plants than the reference and control plants, which reduced the difference between control and deferred in the leaf weight traits: the difference between the means for these two treatments was significant for fresh weight, but not for dry weight.

Leaves were ~8% shorter in the control population than in the reference and deferred populations, a relative difference that carried through to mean lamina area. Leaves of Halo and Nui were ~ 11% longer on average than Commando, with Alto intermediate (Table 6). The rank order of cultivars for lamina area followed the same pattern. There was a significant effect of cultivar (but not treatment) on leaf width which contributed to differences between cultivars in lamina area.

Table 6 Means for leaf fresh weight, leaf dry weight, leaf length, leaf width, lamina area, leaf dry matter content, specific leaf area, and leaf thickness  
Means with a common letter are not statistically different according to the Bonferroni multiple means comparison test ( $P < 0.05$ ) after ANOVA analysis  
except for lamina area which was derived using  $LSD_{0.05}$ . s.e. = standard error.

	Treatment					Cultivar					
	Reference	Control	Deferral	<i>P</i>	s.e.	Alto	Commando	Halo	Nui	<i>P</i>	s.e.
Leaf fresh weight (mg)	103.5 ab	96.7 b	114.3 a	0.018	7.08	95.3 bc	87.6 c	129.3 a	107.1 ab	<.001	8.18
Leaf dry weight (mg)	22.8 a	20.5 b	22.0 ab	0.044	0.89	20.5 bc	18.6 c	25.7 a	22.6 b	<.001	1.03
Leaf length (mm)	132.7 a	121.7 b	130.2 a	0.003	3.07	122.3 b	120.9 c	136.6 a	133.1 a	<.001	3.55
Leaf width (mm)	3.6	3.7	3.7	0.425	0.06	3.6 bc	3.4 c	4.0 a	3.7 b	<.001	0.07
Lamina area (mm <sup>2</sup> )	344.0 a	317.9 b	345.8 a	0.044	11.89	312.9 bc	295.4 c	384.8 a	350.6 ab	<.001	13.73
Leaf dry matter content	0.22 a	0.21 a	0.19 b	0.004	0.007	0.21	0.21	0.20	0.21	0.243	0.008
Specific leaf area (mm <sup>2</sup> /mg)	15.20	15.56	15.79	0.183	0.316	15.57	15.92	15.04	15.55	0.136	0.365
Leaf thickness (mg/mm <sup>2</sup> )	0.29	0.30	0.33	0.07	0.015	0.30	0.29	0.33	0.30	0.179	0.017



Table 7 Means for pseudostem dry weight, pseudostem length, and pseudostem width from Waikato-derived plants. Means with a common letter are not statistically different according to the Bonferroni multiple means comparison test ( $P < 0.05$ ) after ANOVA analysis, except for leaf lamina area which was examined using  $LSD_{0.05}$ . s.e. = standard error.

	Treatment					Cultivar					
	Reference	Control	Deferral	<i>P</i>	s.e.	Alto	Commando	Halo	Nui	<i>P</i>	s.e.
Pseudostem dry weight (mg)	44.20	42.14	43.14	0.641	2.17	43.54	39.82	46.83	42.44	0.064	2.51
Pseudostem length (mm)	3.873	3.810	3.835	0.070	1.23	3.824	3.821	3.892	3.822	0.056	1.42
Pseudostem width (mm)	2.822	2.817	2.814	0.985	0.04	2.824 <b>ab</b>	2.699 <b>b</b>	2.892 <b>a</b>	2.856 <b>a</b>	0.004	0.05

Table 8 Means for growth habit score (1= prostrate growth, 2= normal tufty growth, 3= erect growth), tiller density score (refer to methods section for picture references), crown rust score (1= no rust, 2= moderate rust, 3= full rust). Means with a common letter are not statistically different according to the Bonferroni multiple means comparison test ( $P < 0.05$ ) after ANOVA analysis except for lamina area which was derived using  $LSD_{0.05}$ . s.e. = standard error.

	Treatment					Cultivar					
	Reference	Control	Deferral	<i>P</i>	s.e.	Alto	Commando	Halo	Nui	<i>P</i>	s.e.
<b>Growth habit</b>	2.20	2.10	2.10	0.19	0.06	2.19	2.14	2.02	2.21	0.066	0.07
<b>Tiller density</b>	4.00	3.90	3.90	0.76	0.09	4.10 a	3.93 ab	4.00 ab	3.82 b	0.05	0.10
<b>Leaf rust</b>	1.72 a	1.53 b	1.45 b	<.001	0.066	1.54 b	1.50 b	1.43 b	1.79 a	<.001	0.07

There was only one significant effect in pseudostem traits: for cultivar, in pseudostem width, where Halo and Nui were significantly different from Commando with Alto intermediate (Table 7). There was a trend toward a treatment effect on pseudostem length: pseudostems were shorter in the control plants than in the reference plants, with deferred plants intermediate. Trends were also apparent for the cultivar effect in pseudostem dry weight and length.

The strongest differences among the plant score data were for rust: plants from the reference population, and plants of Nui, were carrying high leaf rust loads than plants from the other treatments and cultivars (Table 8). Alto plants had a higher tiller density score than plants of Nui, and there was a trend toward a more prostrate growth habit in the Halo plants compared with the other cultivars.

### **Flowering attributes**

There were statistically significant effects of cultivar ( $P < 0.001$ ), treatment ( $P < 0.01$ ), day ( $P < 0.001$ ) and cultivar x treatment x day ( $P < 0.05$ ).

Flowering started earliest in Nui and Commando, followed by Alto and Halo, as expected (Figure 16). The rate of progression through to flowering score  $\sim 8$  (seed head fully emerged) was similar for all cultivars but, for the first 30 days after 18<sup>th</sup> October, Nui plants were  $\sim 1.5$  scores ahead of Alto and  $\sim 2.5$  scores ahead of Halo. Maximum flowering scores were reached on day 43 in Nui and Commando, and day 71 in Alto, Halo reached a mean flowering score of 9.8 by the end of the assessment period on day 71.

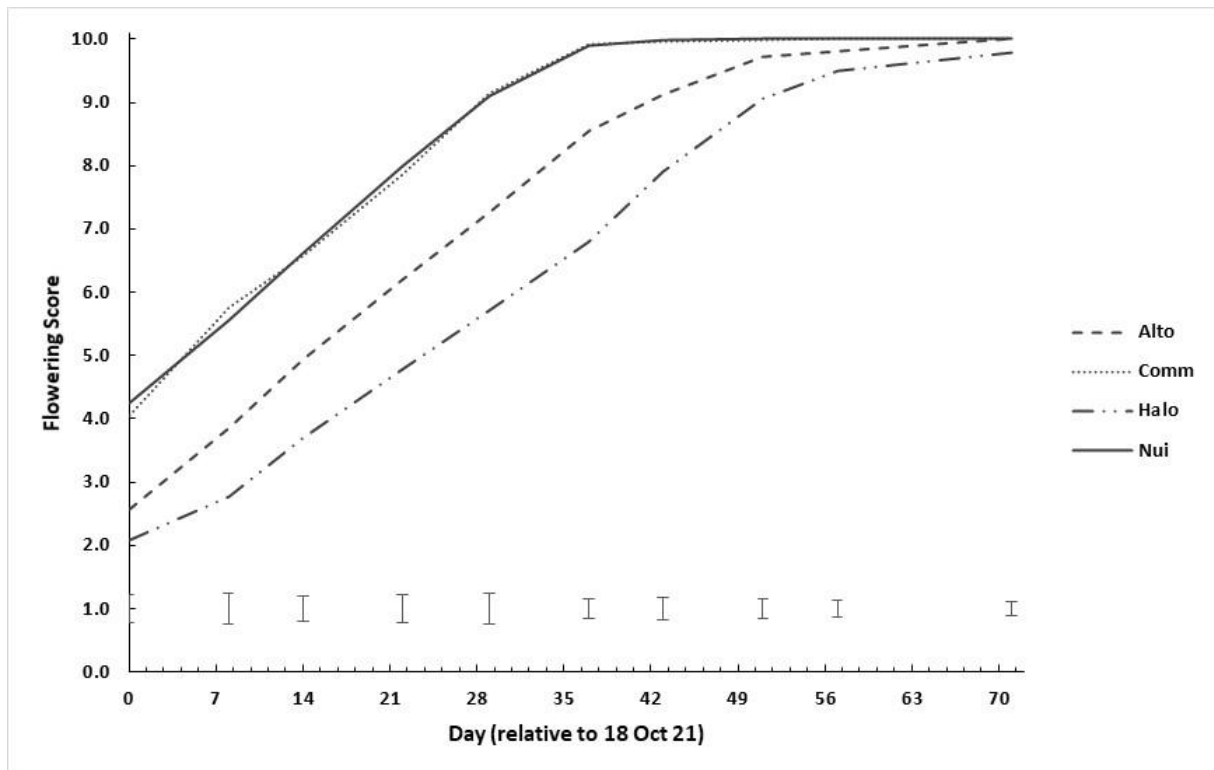


Figure 16. Progression of flowering as assessed by mean flowering score for the four cultivars from 18<sup>th</sup> October. Values are means across the reference, Waikato control and Waikato deferred populations. Bars are  $\pm$  one standard error.

Plants from the control and deferred populations flowered later than plants from the reference population (Figure 17). Although the interaction of treatment x day was non-significant, in the post-hoc Tukey adjusted means test (from day 0 to day 37), the plants from the reference population were at least 0.5 score ahead of plants from the other two treatments. From day 37 onwards, there was no difference between the three treatments in flowering score ( $P > 0.05$ , Figure 17). Interpolation of the weekly scores indicated that plants in the control and deferred treatments reached flowering score 8 six days later than plants from the reference population (~ day 32 versus ~ day 26).

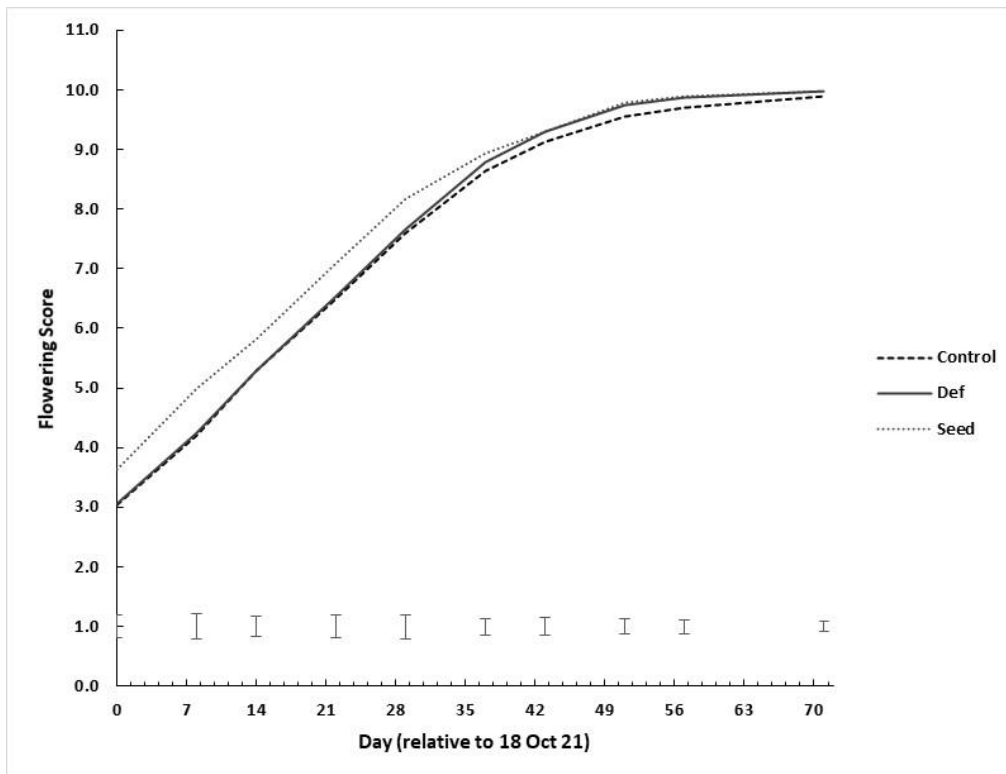


Figure 17. Progression of flowering as assessed by mean flowering score for the reference ('Seed'), control and deferred ('Def') treatments from 18<sup>th</sup> October. Values are means across the reference, Waikato control and Waikato deferred populations. Bars are  $\pm$  one standard error.

Figure 18 shows the treatment effect over time for each cultivar. Commando and Nui reached anthesis at approximately day 36 after 18<sup>th</sup> October. For Commando populations, reference plants were consistently 0.1 – 1.0 score ahead of the control and deferred plants and reached the maximum flowering score  $\sim$  6 days earlier. For Halo populations, there was very little difference in flowering score between the treatments throughout the assessment period.

Nui and Alto displayed different flowering patterns among the treatments. In Alto, the deferred plants were initially intermediate between the control and reference plants, but after day 22 there was no difference between deferred and reference, and both were 0.5 to 1.0 score ahead of the control population plants.

In Nui, the deferred plants were consistently 0.6 to 1.4 scores behind the reference plants during the first 30 days after 18<sup>th</sup> October. However, the control treatment tracked the deferred treatment for the first 10-15 days before taking an intermediate track between the reference and deferred populations.

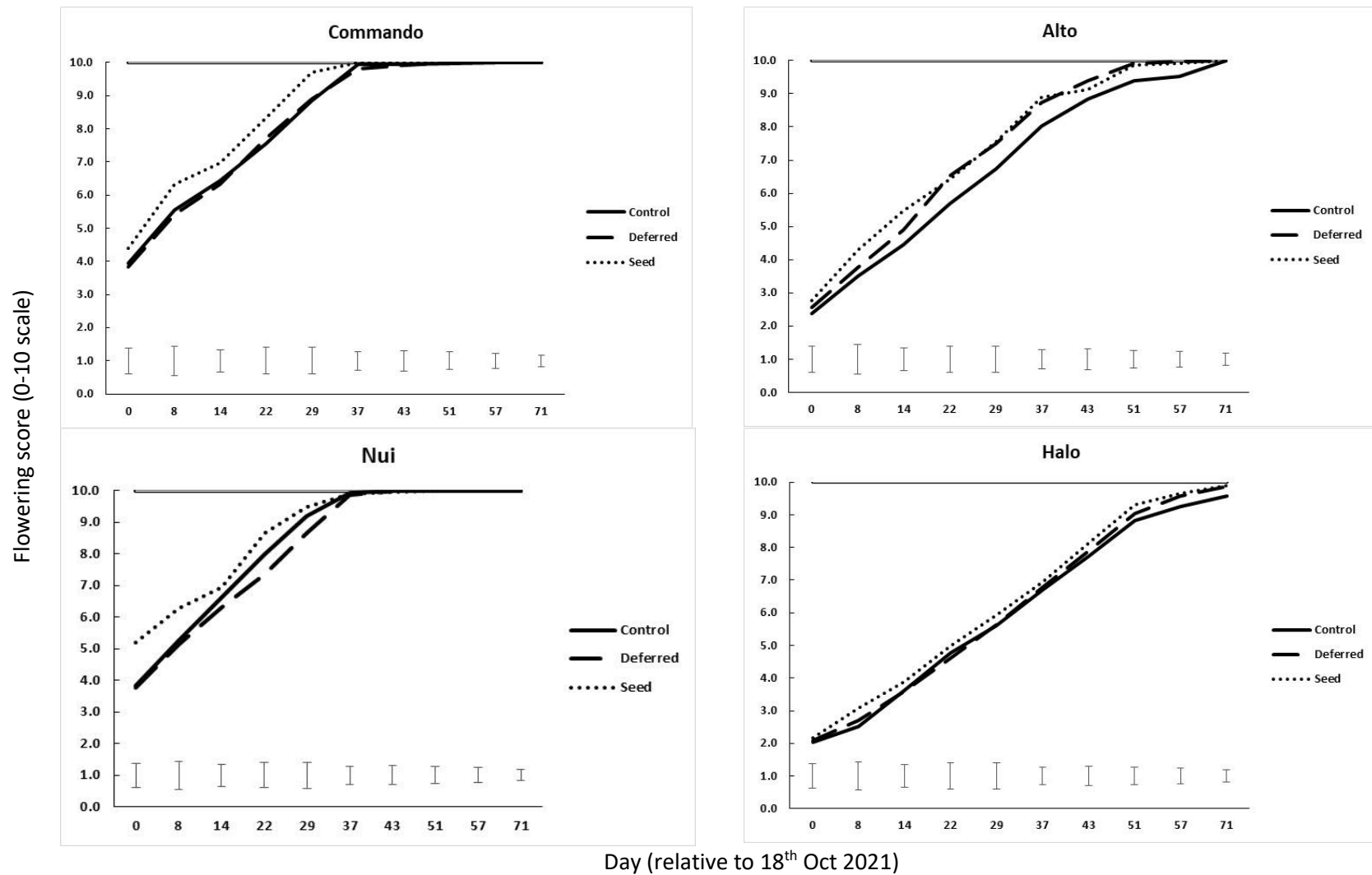


Figure 18. Cultivar x treatment x day interaction in flowering scores of the reference ('Seed'), Waikato control and Waikato deferred populations. Bars =  $\pm$  one standard error.

## Discussion

### Endophyte strain identity and population genetic integrity

The survivor plants used in this study were derived from a field experiment in its tenth year post-sowing in the Canterbury and Waikato regions. The endophyte–grass relationship is generally mutualistic and, once established, stays with the plant and its offspring (Clay & Schardl, 2002). The endophyte grows intercellularly and is vertically transmitted from mother plant to seed, with the ability to remain viable for many years if seed is stored properly (Bylin et al., 2016). However, there was a possibility that over time, perennial ryegrass populations could become infected with a different endophyte compared to the original strain through recruitment of ‘off type’. This is more likely to occur when the original ryegrass population has become depleted through plant mortality therefore opening gaps in the pasture and reduced competition for volunteer seedlings. The endophyte percentages measured here help determine if, and to what extent, how these processes have occurred during ten years after sowing. Knowledge of the cultivar and its associated endophyte in the seed sown in 2011 allowed a direct comparison of the genetic integrity of the populations by taking a snapshot of the endophyte types surviving in the plants when they were collected in 2020. If the original endophyte still occurred in the population today, it can be assumed that those were plants that were derived from the originally sown pasture. If high frequencies of true-to-type plants are found in the old pasture, it can be assumed that the original cultivar has survived (persisted) well.

Although some ‘true-to-type’ endophyte integrity was generally lost over time in the Waikato control and deferred populations (Figure 13) it is important to note that deferral itself did not lower the true-to-type endophyte percentage compared with the control treatment. Thus, it seems that the endophyte was effectively transmitted through seed into the seedling plants that established in the deferred treatment from natural re-seeding. The cause of the general decline in genetic integrity is most likely due to volunteer contamination of the pasture, introducing different types of endophyte or plants with no endophyte at all (Bluett et al., 2004). Burggraaf and Thom (2000) stated that the main sources of contamination of pastures are from surviving vegetative ryegrass plants, seed on the soil surface after a natural reseeding, seed buried in the soil, and seed transferred via hay, machinery, and dung. Over the period of eight years when the pastures were in the ground, the chances for volunteer plants to invade increased, and along with this, a decrease in the frequency occurrence of the sown cultivar-endophyte combination was seen (Figure 13).

The Nui SE (Table 5) results suggest that volunteer plants were most the likely source of contamination since the occurrence of plants with AR37 in the Nui control and Nui deferred populations shows that seed was being transferred between plots. The ~ 8-15% occurrence of AR37 plants in the Nui populations could be taken as a base rate of cross-contamination within the trial across plot boundaries. The much higher frequencies of SE-infected plants in Commando AR37 control and deferred populations (25-45%), and in Alto deferred (~25%), indicated that there was also a contamination from old residual seed, most likely of mid-season flowering material that was used in Waikato dairy pastures until relatively recent times. It is also possible that the small percentage of SE-infected plants in the reference seed lines (Table 5) had a survival advantage compared with AR37 infected plants and therefore gradually increased as a percent of the surviving pasture over time. Genetic analysis of the plants themselves would be required to distinguish between these possible pathways.

Because host-endophyte relationships are stable over time in the vegetative plant, the difference in endophyte percentages seen in this study are most likely due to differences in plant persistence, not endophyte survival. Endophyte plant associations are adapted to their native range of environmental growth conditions; this result implies that environmental change can disturb the balance of the interaction, resulting in negative endophyte effects on plant growth (Hesse et al., 2003; Kane, 2011). This finding could be a factor in reduced sown plant occurrence alongside reduced endophyte content. Canals et al. (2008) have stated that because the endophyte–grass symbiosis is dynamic and always involves a metabolic cost to the plant, the relationship may shift from mutualistic to antagonistic if plant fitness is not increased. In short, an endophyte infection can actually be destructive to an already struggling plant; this claim is exemplified by Commando in the Waikato environment in this study.

Commando appears to be prone to contamination from volunteer plants (Figure14). As a result of the endophyte analysis (Table 5) it was expected that AR37 would be associated with Commando, but the SE endophyte had a higher percentage of the associated endophyte in the sample sent for analysis (Table 5). This strongly suggests that Commando is less persistent in the Waikato compared with the other cultivars included in the study. Lee et al. (2018) also found that Commando had lower endophyte infection percentages over the first four years of the trial, although endophyte strains in plants weren't assessed at that time. It therefore seems that Commando could not maintain sufficient tiller density and vigorous growth to prevent gaps opening in the original pasture. These gaps would have allowed volunteer plants that contain different endophytes, in this case, mostly wild-type (Tozer et al., 2011), to establish. He et al. (2017a) showed that although Commando has a high summer yield potential when irrigated, it suffered a large reduction in yield under water deficit.



This result is reflected in the evidence provided in Figure 15, in the comparison of Commando between Waikato and Canterbury. Commando retained high true-to-type endophyte at the Canterbury site, but at the Waikato site only half of the survivors were true-to-type for the sown endophyte. This finding clearly suggests an interaction between environment and persistence in this cultivar which was not evident in other cultivars especially the tetraploid Halo AR37.. Contamination with other ryegrass/endophyte associations needs to be minimised to ensure that farmers achieve the expected benefits of using a novel endophyte-infected perennial ryegrass pasture, such as the absence of ryegrass staggers and control of pasture pests (Bluett et al., 2004). There is a possibility that pasture pests contribute to Commando's poor persistence, along with the environment; however, this was not tested within this study.

The results in Figure 15 indicate genetic integrity of the survivor populations was significantly higher at the Canterbury site when compared with the Waikato site. Considering that the differences are most likely caused by volunteer species contamination through poor persistence of the originally sown population, the likely reason for the differences between the original sites would be irrigation. Woodward et al. (2020) found irrigation to be the most effective strategy for preventing perennial ryegrass failure in the Waikato region. Waikato's low summer rainfall (Figure 2) helps explain the apparent poorer persistence seen in this study when compared to Canterbury. Based on trends in dry matter yield and seasonality in pasture growth rates in the original experiment, Lee et al. (2017) concluded that the environment dominated all other factors at the Waikato site and exceeded tolerance thresholds for the persistence of the perennial ryegrass cultivar sown in 2011. This has significant implications for the expectations of farmers in the persistence of their perennial ryegrass plants in locations similar to the Waikato environment, where Lee et al. (2017) found that ryegrass failed to persist into the fourth year after sowing. In contrast, the Canterbury site had more stable ryegrass production, and this reflected in the endophyte results presented here. The Waikato site showed strong evidence of population decline, especially in the Commando cultivar (Figures 14 and 15).

The Canterbury site was irrigated (Lee et al., 2018), meaning that the pastures growing at this site experienced minimal water stress. The graphs of rainfall and temperature for the Waikato site show the stresses the Waikato survivor plants were exposed to and when (Figures 1 and 2). There were years of very low rainfall over the period that these plants grew in the Waikato environment. Some of the years also had particularly high rainfall. The years of lower rainfall occurred just before the years of higher rainfall, creating the optimum opportunity for volunteer plants to invade the pastures. The gaps opened in the drier periods provided space for the volunteer seed to germinate

when the higher rainfall accompanied by the warmer temperatures followed, allowing the volunteer plants to establish successfully in the pastures.

In addition to physical loss of plants from the original sowing, the phenotypic attributes of survivor plants could also have changed over time as a result of the environment (including the grazing management to which they were exposed). To test for this, it was essential to use only plants that had come from the original seed lines to allow for accurate representation of the surviving populations and exclude volunteer or nil endophyte plants. For this reason, the endophyte analysis that was conducted at the beginning of this phase of the study was important in identifying plants to use for the phenotypic measurements, in addition to the information it provided regarding what has happened to these populations over time.

### **Plant phenotypic comparison**

The objective of this experiment was to identify phenotypic traits associated with survival, focussing on the differences between treatments. The cultivar effects were investigated to examine whether the cultivars were responding as would be expected based on their breeding background. This study does not reflect on-farm results due to the constraints that accompany a pot study. There is also the possibility that carry-over effects could still be occurring due to the plants being moved from a different environment. It was expected that the plants would have acclimatised before the measurements were undertaken, but the possibility of some carry-over effects from the Waikato environment influencing the results cannot be ruled out entirely.

In the plant leaf characteristics (Table 6), the cultivar results all reflected the expected cultivar differences. As Halo is a tetraploid, it was expected that all leaf traits in Halo would be expressed at a higher level than in the diploid cultivars. This assumption is based on tetraploids having a greater ratio of cell content to cell wall (Stewart & Hayes, 2011), thus resulting in greater digestibility, crude protein (CP), and water soluble carbohydrate concentration. The expression of Halo followed this trend with all key leaf trait values being greater than in the other cultivars (Table 6).

A key result from this study was the difference in leaf length due to treatment (Table 6). Plants grown from the original seed lines had longer leaves than the survivor control population. Significantly, the deferred treatment reset this trend back to the longer leaves seen in the reference population. This result implies the suppression of leaf growth occurring in the control population as a result of the Waikato environment. It is possible that during the period following the deferral period until the collection of the clonal fragments from the field, the original genetic suppression of this trait had been released, suggesting that this suppression is a result of time and is reversible. This

gives reason to believe that the leaf length trait is genetically conserved as an important trait for the survival of perennial ryegrass. Sampoux et al. (2011) found that longer leaves could be a means of increasing spring dry matter yields. Longer leaves improve cumulative intercepted radiation during re-growth and consequently increase photosynthate accumulation under frequent defoliation. The results of this study support Hazard et al. (2006) who found that short leaves were selected under frequent defoliation. This finding helps explain the longer leaves in the reference plant population: leaves were shorter under frequent grazing in the control treatment. Hazard et al. (2006) states that as a result of frequent defoliation, short-leaved plants tend to be slower-growing and lower-yielding. In perennial ryegrass, leaf length has great genetic variability (Rhodes, 2009) and high heritability (Hazard & Ghesquiere, 1995). A combination of erect growth and long leaves provide the highest yields due to the light reaching tillers and leaves through the canopy (Rhodes, 2009). In plants with prostrate growth and smaller leaves, the uppermost leaves receive light at above saturation, resulting in a wastage of quantum energy; in contrast, lower leaves receive little light due to shading (Rhodes, 2009). As the growth habit in our study did not differ among the treatments, these finding could not be tested. The absence of differences in the dry matter cuts discussed in Chapter Three among the treatments could support these findings; however, information on the growth habit of all of the plants used in that study would have been necessary to fully understand the effect.

The longer leaves grown on plants from the reference and deferred populations compared with the control population explains the greater lamina area in the former since lamina area is a function of length and width (Griffiths et al. 2017). The deferral treatment seemed to result in more productive leaves compared to the control treatment for key traits such as leaf fresh weight, leaf dry weight, leaf length, lamina area, and dry matter content (Table 6). Whilst the leaves in the deferral treatment had higher fresh weight, the leaves in the control and deferral treatments did not differ from each other in their dry weights. This finding suggests that the deferral treatment results in bigger, lusher leaf growth that is caused by a higher water percentage in the leaves.

Although small, the difference in dry matter content among treatments is significant. This finding supports the notion of improved water status in the deferred treatment. Generally, a plant that produces less dry matter content would not be favoured as a feed in the New Zealand dairy industry. Lower dry matter feeds require stock to eat more to achieve dry matter intake to support milk production (Holmes et al., 2002).

This raises the question of whether the deferred plants are more persistent/resilient as a result of increased water in their leaves. If so, could a more persistent/resilient plant outweigh the economic cost of needing more feed due to less DM (as a result of deferral) to keep up with DM intakes to

support the farm? Taken together, these results suggest the need for further research on the effects of deferral on plant water status.

The pseudostem of the plants did not seem to be affected by the treatments (Table 7). The trend toward shorter pseudostems in the control treatment is consistent with the shorter leaves produced on these plants, since leaf length and pseudostem length are closely and positively related in perennial ryegrass (Davies, 1993). A cultivar effect on pseudostem width was expressed and this was expected as per breeding backgrounds. The tetraploid (Halo) showed larger values for pseudostem characteristics alongside Nui and Alto which have proven to be middle of the range for the results of this study. Commando had the smallest pseudostem characteristics. This result was not surprising as Commando has smaller tillers compared with the other cultivars.

Nui is not a highly selected cultivar. It was selected from the Mangere ecotype and went through a few selection cycles before being brought to the market. Therefore, it has not been through as much selection as the other three cultivars (Armstrong, 1977). This is a very likely reason for the higher crown rust scores seen in the Nui cultivar compared with the other three cultivars, even though rust was a selection factor for the cultivar. Crown rust, *Puccinia coronata* f. sp. *lolii*, commonly infects perennial ryegrass regardless of where it is grown. It drastically reduces the value of susceptible cultivars by decreasing dry matter by up to 37% and green tissue by 94% (Clarke & Eagling, 1994).

The treatment effects observed here may indicate that crown rust is a mortality factor for perennial ryegrass. Whilst the reference plant population contained higher levels of rust, a shift in the control population towards less rust was seen over time due to the environment. The deferral treatment maintained this shift, indicating a gene elimination effect causing a permanent shift towards more crown rust resistance in field-grown plants over time.

In this study, rust scoring was completed purely on sight and self-identification of the virus. There is a chance that some of the symptoms that were ascribed to rust identification could have been caused by other diseases. The barley yellow dwarf virus and ryegrass mosaic virus (Wilkins & Catherall, 1977) have both been prominent in ryegrass in past years. Whilst there is a chance that these have been mis-identified, on occasion, as rust, it still seems clear from the results that there was a shift in survivor populations to better disease resistance.

## **Flowering patterns**

Differences among the cultivars in the timing of the various stages of flowering were exactly what would be expected based on the breeder's information summarised by Stewart (2006) and Lee et al. (2012) Each cultivar had a heading date representative of its breeding selection (Humphreys &

O'Kiely, 2007). Of the four cultivars, Nui and Commando have the earliest heading dates (Lee et al. 2012). This fact explains their higher flowering scores throughout the spring/summer period. These two cultivars reached anthesis around day 35 (22<sup>nd</sup> November) while Alto reached anthesis around day 70 (27<sup>th</sup> November) with Halo not far behind (Figure 16). These results suggest that the true effects of treatments are captured in the data with little effect of other outside influences. There is always the risk that a carryover effect from the environment these plants were taken from has not been completely removed. It would therefore be important to repeat the flowering assessment on the same plants in the consecutive year, to ensure that they have actually stabilised, and that the results truly reflect the effect of the treatments.

Another key result of the study is the amount that each cultivar shifted in flowering score as a result of the environment (Figure 18). For example, the Commando control population shifted towards later flowering. The environment in which the control population developed seemingly favoured later initiation of reproductive and the deferred population held this change. This result suggests that the change is favourable and fixed, presumably because it increases the survival of the population. Changes in reproductive phenology have been reported in other species by CaraDonna et al. (2014), Fitter and Fitter (2002) and Forrest et al. (2010) who have all shown that these changes are a result of changing climatic conditions. By contrast, the phenotypic shift towards shorter leaves in the control population was not permanent, because the deferred populations aligned with the reference populations, not the control plants.

In Nui, the control population moved to slightly later flowering initiation compared with the reference population, and the deferred population moved even further away. This was somewhat consistent with what was observed in Commando. However, the results for Nui are still potentially confounded by volunteer contamination since the original Nui seed line was inoculated with SE, and there was evidence of SE volunteers in other sown populations, as discussed above. Thus, it is not possible to know if the Nui survivor plants were truly derived from the original sowing in 2011. Nonetheless, there is evidence that the two early flowering cultivars both shifted towards later flowering over the 10-year period in the Waikato environment suggesting that that better survival may occur with a later flowering date in this environment.

Many plant species have been shown to differ with respect to the onset of reproductive initiation as a result of climatic influences (Love & Mazer, 2021; Park & Mazer, 2018). Love and Mazer (2021) stated that variation in phenological sensitivity within species may be high and that shifts in the timing of reproduction across a species can result in populations advancing or delaying their

flowering date more quickly than others. In the Alto population, the control population shifted to later flowering, whereas the deferred population did not maintain this and moved back towards the original flowering pattern shown in the seed population. This finding suggests that the original timing of flowering initiation in Alto was a good 'fit' for the Waikato environment. Similarly, the latest flowering cultivar in this study, Halo, showed no evidence of a shift in flowering date. The extent to which shifts can occur within cultivars will, presumably, be controlled to some extent by the amount of genetic diversity within the final parent plant selection and original nucleus seed crop. Halo may be an example of a cultivar with relatively narrow genetic diversity leaving limited scope for natural selection as a result of environmental factors. Again, it would be interesting to study this issue in the consecutive year to see if these results truly reflect the effect on the plants or if the results reflect a carryover effect associated their original environment. As Love and Mazer (2021) have shown, the sensitivity of plant phenology to climate changes could improve our ability to predict short- and long-term changes to the phenology of plant populations. Such predictions would provide a better understanding of regions identified to experience greater or lesser shifts with perennial ryegrass, to enable farmers to manage accordingly and breeders to refocus their plant selection criteria to increase the expression of persistence-related traits.

## **Concluding points**

- The true-to-type endophyte frequency of the control populations was sustained in the survivor plants from the deferral treatment. This result shows that the endophyte was successfully transmitted into the new plants in the deferred treatment.
- Commando AR37 had lower frequency occurrence of true-to-type endophyte in the control and deferred treatments compared with the reference population, indicating relatively poor persistence of this cultivar in the Waikato environment.
- There was an environmental effect on the frequency occurrence of true-to-type endophyte with survivor populations from Canterbury having a much more stable genetic integrity than survivor populations from Waikato.
- The control population showed significantly lower values for the key trait of leaf length compared with the reference and deferred populations. While there was a shift to shorter leaves in the control population, the deferred population showed a shift back to the original trait values in the reference population.
- Higher fresh weight, dry weight, leaf length, lamina area, and dry matter content all point to plants in the deferred population having improved water status compared with plants in the control and reference populations.

- There was a shift toward lower incidence of leaf rust in the control and deferred populations compared with the reference population, indicating that disease resistance could have been a factor causing loss of plants from the original sowing.
- There was a significant shift toward later flowering in the control and deferred populations compared with the reference population, in the order of 6-8 days.
- However, the shift in flowering was cultivar specific: it was pronounced and consistent in Commando, but not in Halo. There was a suggestion that in Alto, grazing deferral moved the mean flowering time forward again from the control population, towards that of the reference population.

## Chapter 5 Overall conclusions and outlook

A key objective of this study was to inform future plant breeding efforts with information on phenotypic changes in populations and traits that appear to be associated with survival. In doing this, the following questions were raised;

- A) Are the surviving plants derived from the original sown population or based upon volunteer plants?

In the harsher of the two environments included in the study, the dryland Waikato region, the percentage of survivor plants that were true-to-type for the sown endophyte decreased compared with the reference plants, irrespective of treatment or cultivar. At the irrigated Canterbury site, the true-to-type percentage actually increased slightly from the reference population showing that the presence of endophyte is positive for long-term survival.

There was one stand-out effect of cultivar in the data set, which was Commando showing significantly lower than expected endophyte percentages compared with the other cultivars in the Waikato. This suggests that this cultivar persisted poorly at this site and many ryegrass plants present in these swards nearly ten years after sowing were volunteers, probably via residual seed from past pastures grown at the site. Halo and Alto populations mostly consisted of plants derived from the original population: however, overall ryegrass proportions in all treatments fell to very low levels within four years post-sowing (Lee et al., 2017) showing that perennial ryegrass in general is unable to maintain long-term dominance of swards in this environment.

- B) If they are derived from the original sown population, have these populations changed phenotypically and if so, what are the implications for plant breeders? Could there be phenotypic traits plant breeders should be selecting to increase survival in harsh environments?

Compared to the control, deferral yielded a small increase in leaf size and a small decrease in dry matter content. In fact, deferral resulted in a reset of leaf length to the level of the seed-derived plants (Table 6, Figure 19). The fact that the control population had smaller leaves is most likely a result of the continued intensive grazing pressure in that treatment favouring defoliation avoidance traits in surviving plants (Briske, 1986). A lower dry matter content in the deferral plants indicates a higher plant water status, which places this treatment at an advantage for persistence in harsher environments such as the Waikato.



In contrast to the leaf length trait, phenotypic shifts toward greater rust resistance and later flowering were sustained from the control treatment into the deferral treatment (Figure 19). These results indicate that there could have been genetic differentiation within the survivor populations whereby plants with low relatively low rust resistance and/or earlier flowering from among the original genetic diversity were eliminated via some form of natural selection. Since Faville et al. (2020b) found no evidence of genetic shifts in the same populations five years after sowing, this shift may have occurred later, after the point where ryegrass production in general declined steeply (~ 4 years after sowing; Lee et al., 2018).

Later flowering dates in the Waikato survivor plants suggests that selection of later flowering material for harsher environment such as the Waikato may be beneficial for ryegrass persistence whilst also producing leafy green feed into the spring period (Woodfield & Easton, 2004). Plants in both the Commando and Alto control populations moved through the reproductive stages 6-8 days later than plants in the reference populations. Alto has a standard flowering date of +14 days, so the fact that it also showed a shift toward later flowering indicates that a flowering date of ~ +20 days may be optimum for this environment. However, Halo, with a standard flowering date of +25 days, didn't show any evidence of a phenological shift. More information on the extent of genetic diversity for flowering present in the original seed lines would be needed to interpret these results fully. It is possible that Halo had little original diversity for natural selection processes to operate upon, while both Commando and Alto had enough baseline diversity to allow the 6-8 days shift that was observed, but no more.

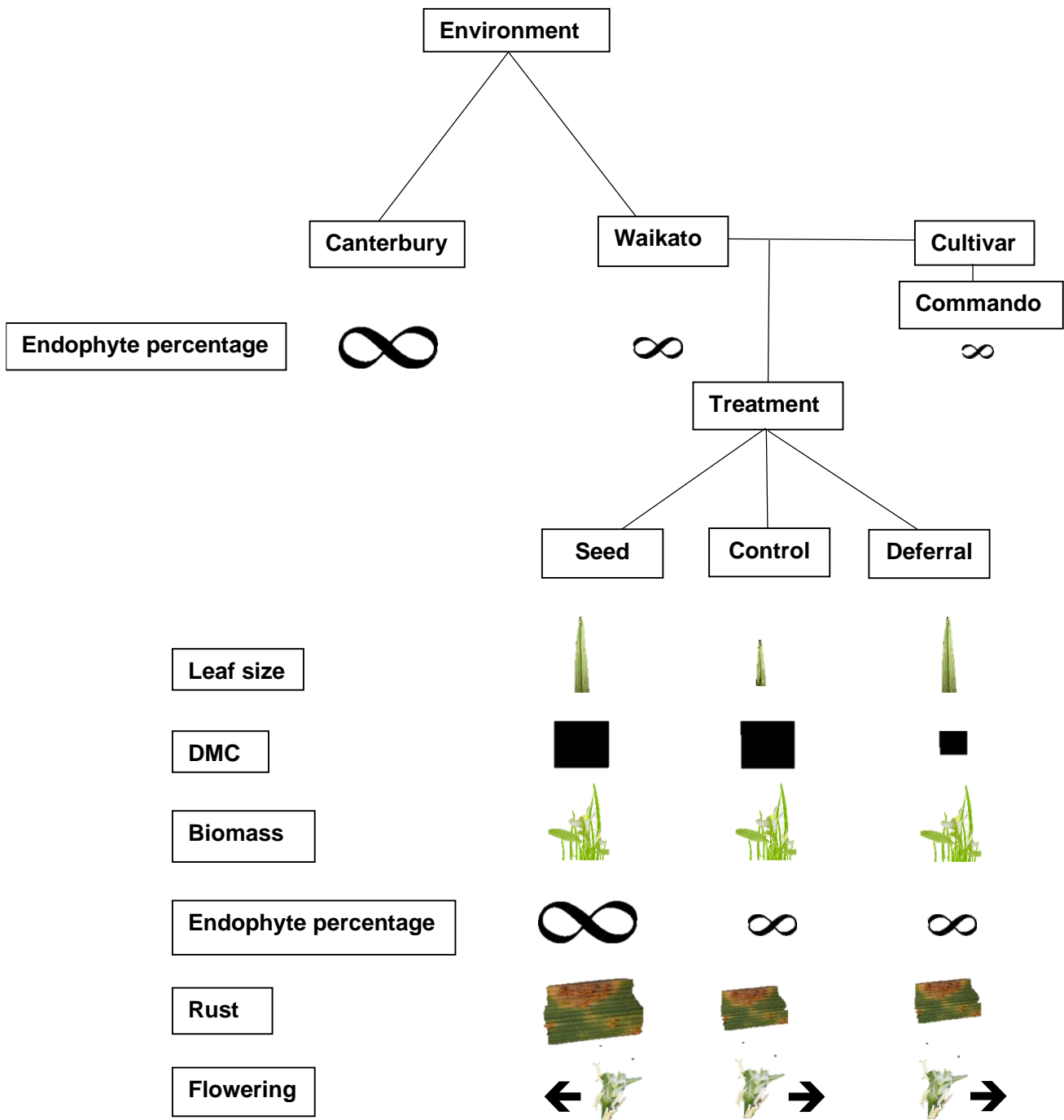


Figure 19. Visual depiction of key findings from Chapters 3 and 4 with respect to genetic integrity of ryegrass populations as assessed by endophyte true-to-type percentage, leaf traits, biomass, rust incidence and time of flowering. DMC = leaf dry matter content, size of the symbol reflects the results, flowering arrows symbolise the direction of the shift in flowering. The three treatments are control and deferred survivor populations and the reference plant populations ('Seed').

## Limitations and recommendations for future research

Pot studies are generally more reproducible and repeatable, and results are simpler to interpret, than field studies (Passioura, 2006). However, extrapolating results to field situations can be problematic, due to differences in abiotic and biotic conditions experienced by plants growing in the field (Passioura, 2006). Therefore, the value of potential mitigation strategies identified in studies such as those conducted here requires confirmation under field conditions before being promoted for consideration in plant breeding programmes. Apart from the pilot phase, there were no further dry matter yield harvests conducted, yet ultimately yield is what matters for farmers/feeding cows (Stewart & Hates, 2011). Thus, the findings from this work do not directly correlate with on farm conditions and further trials in the field would be required.

'Carry over' effects could have contributed to the results of the detailed phenological, and phenotypic analysis described in Chapter 4. Due to time constraints, it was not possible to take plants through a longer adaptation phase before phenotype and phenology were analysed to ensure that the results were a true representation of the effects of the environment on plant traits. The fact that a clear difference in leaf traits between control and deferred populations was detected in Chapter 4 suggests that, while some background carry-over effects may still have been present, the study design was sufficiently robust to pick up real effects of the environment on plant characteristics.

It would have been interesting to pursue the biomass cuts that occurred in the preliminary part of the current study for a longer period. The trend seen at day 105 (Figure 6) shows that a difference between the reference populations and the survivor populations could have been emerging but it was not possible to continue the cuts into the next phase of the study. Long-term studies could be used to fully identify the continuing effects on biomass/yield, including seed head mass, under the three different treatments. Future field studies using mini-swards could also examine possible population differences in yield. These mini swards should be grown from clonal transplants because yields in pots or as spaced plants in the field differ from yields in a sward on farm.

The shift in flowering to later flowering initiation could indicate an adaptation mechanism in the Waikato environment. The decrease in seed heads could be correlated with this change in timing. To examine this hypothesis, it would be beneficial if the study could be repeated after the seed plants had at least a year to grow. With the seed population at the same developmental stage as the reference plants, the measurements would then allow for the full effect of the seed treatment to be examined.

## Chapter 6 References

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# Appendix

## A. Tiller density score references



1













4





5



## B. Leaf rust score references

The most rust





Some rust





No rust





## C. Growth habit scores references

### 1. Prostrate growth





2. 'Normal' tufty growth





### 3. Erect growth

