Exploiting heterosis in perennial ryegrass (*Lolium perenne*) through development of inbred lines, and the impact on population variability

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Abstract

Genetic improvements in the dry matter yield of perennial ryegrass via plant breeding are typically achieved through recurrent selection, delivering rates of genetic gain estimated to be in the range of 0.25 - 0.76% per year. Hybrid breeding is commonly used in self-compatible species (e.g. maize) to achieve significant yield increases through the exploitation of heterosis. However, hybrid breeding has not been used to a large extent in perennial ryegrass, due to its self-incompatibility (SI) system. However, using marker assisted selection (MAS), the alleles responsible for SI in perennial ryegrass can now be manipulated. A method has been developed which uses MAS to develop parent lines with controlled SI alleles, which are inbred for two cycles and are then crossed to create hybrids. This method provides the opportunity to exploit heterosis in perennial ryegrass breeding and for significant gains in dry matter yield.

The first experiment in this thesis aimed to investigate the expression of heterosis in F1 hybrid plants produced by this proposed novel SI hybrid breeding method. It was expected that the hybrid offspring would at least display mid-parent heterosis. Experiment one also investigated the variability in key morphological traits, in the expectation that the cycles of inbreeding would have increased genetic uniformity in the parent lines and hybrids. The hybrids did display mid-parent heterosis throughout the experiment, providing evidence that the proposed method successfully captures heterosis in the perennial ryegrass breeding cycle. Evidence of high-parent heterosis were also observed throughout the experiment, which indicates the potential to develop F1 hybrids with significant yield increases compared to current cultivars. Therefore, the method may be

commercially viable. No consistent changes in the morphological variation of the parent lines or hybrids was observed, which is a positive outcome for the ecology of perennial ryegrass in grazed pasture communities.

The second experiment investigated expression of heterosis in F1 hybrid offspring from pairs crosses with different genetic backgrounds. The amount of variation in heterosis within each F1 hybrid population was also investigated. It was expected that expression of heterosis would vary dependent on the genetic background and that there would be significant variation in expression of heterosis within each F1 population. The expression of, and variation in, heterosis was of interest because with the advent of the SI hybrid breeding method, breeders may benefit from quantifying the combining ability of their perennial ryegrass breeding pools. This would enable better selection of plants for entry into the hybrid breeding pipeline. Mid-parent and high-parent heterosis were detected, but the levels of expression were variable within, and between, the two genetic backgrounds. This supports the hypothesis that there is variation in the performance of hybrids with differing genetic backgrounds, and therefore, there would be value in quantifying the combining ability of perennial ryegrass breeding pools.

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Table of Contents

List of figures

List of tables

List of appendices

List of abbreviations

‘A x AT’	‘Alto’ x (‘Alto’ x ‘Tolosa’)
‘A x R’	‘Alto’ x ‘Rohan’
cm	Centimetres
DM	Dry matter
Emg	Emerging
g	Gram(s)
H	Hybrid
LSD	Least significant difference
ME	Metabolisable energy
mm	Millimetres
Par	Parent
Pro	Progenitor
SI	Self-incompatibility
SD	Standard Deviation
SED	Standard error of a difference between two means
YFE	Youngest fully emerged
°C	Degrees Celsius
+ ‘x’ days	Heading date is ‘x’ days later relative to the cultivar ‘Grasslands Nui’

Chapter 1 Introduction

The New Zealand dairy sector contributes $7.8 billion to New Zealand's gross domestic product (GDP) annually (Ballingall 2017). The New Zealand dairy industry is primarily pasture based; it operates in a temperate environment, using 2.3 million hectares (Stats NZ 2012b) of New Zealand's pastoral land to support 6.6 million dairy cattle (Stats NZ 2016). Pasture based systems have low costs of production and are economically sustainable, providing New Zealand dairy systems with a competitive advantage internationally (Dillon *et al.* 2005).

The most common grass species used in New Zealand pastures is perennial ryegrass (*Lolium perenne* L.), known for its high digestibility, tolerance of intensive grazing (Wilkins 1991), fast establishment and good cool-season production (Hannaway *et al.* 1997). The importance of pasture in New Zealand farm systems means that dry matter yield is a key trait of interest to plant breeders, and there is considerable focus on improving perennial ryegrass annual and seasonal production (Woodfield 1999). Conventional breeding methods, such as recurrent selection, have been used to achieve improvements in dry matter yield, however rates of genetic gain per year are currently quite low (0.76% annually post 1990 (Harmer *et al.* 2016)) relative to other forage species such as maize. It can take 10-15 years to breed a cultivar and take it to market (Lee *et al.* 2012), however, with the continuous advent of new technology, novel breeding methods are opening new avenues for plant improvement, with the potential to increase yield gains and decrease the time required to develop a new cultivar.

Hybrid breeding is a method which has long been used in plant improvement, but has not previously been possible in perennial ryegrass to the same extent as other species due to differences in the breeding system, primarily that perennial ryegrass is self-incompatible (Brummer 1999). However, the advancement of technology now offers the potential for some of these barriers to be overcome (Pembleton *et al.* 2015). Progress in the development of molecular markers for the alleles of the *S* and *Z* loci, which are responsible for controlling compatibility in perennial ryegrass, have now made it possible to predict, and therefore control, compatibility between populations using marker assisted selection (Pembleton *et al.* 2015). Theoretically, this information could allow for pairing of populations with high compatibility, and the generation of a high proportion of hybrid offspring (~83.33%) (Pembleton *et al.* 2015). This would provide the opportunity to improve rates of genetic gain in perennial ryegrass and reduce the time it takes to produce a cultivar. This is potentially highly valuable technology, however, to date, little research has been conducted on hybrids produced by this method (Inch, personal communication, 1 August 2017).

Application of the technology in commercial breeding programmes would initially involve significant additional costs (Collard & Mackill 2008). For example, initial capital costs, and also maintenance costs in genotyping large numbers of plants to quantify allele frequencies and select desirable SI genotypes (Conaghan & Casler 2011). Breeders must therefore be confident that the extra financial investment will be recovered in increased sales of cultivars that substantially outperform current cultivars in the key traits such as dry matter yield, and maximise genetic gain (Pembleton *et al.* 2015).

This confidence will come from, among other sources; clear evidence of increased trait expression, especially in dry matter yield, evidence that the novel breeding system is providing additional genetic variation to supplement existing breeding pools (i.e. is resulting in a change in allele frequencies), and also from information on the combining ability of their current germplasm, to aid selection of genepools to use in the novel breeding method.

Hence, the objective of the research presented in this thesis was to investigate the expression of heterosis in the hybrids created using this novel breeding method, investigate the genetic variability of the inbred lines and hybrids created using this novel breeding method, and investigate the expression of heterosis in the F1 progeny from pair crosses of cultivars from differing genetic origins, and therefore obtain an indication of combining ability.

Chapter 2 Literature review

2.1 Introduction

This chapter reviews perennial ryegrass breeding in a farm systems context, specifically the New Zealand pasture-based dairy system. Literature included in this review was selected to 1) provide a brief overview of the dairy industry and the role perennial ryegrass plays in it; 2) provide an in-depth review of the history of perennial ryegrass breeding, and 3) consider how novel breeding methods could play an important and evolving role in the perennial ryegrass breeding process. This is addressed in the following sections:

- An overview of dairy production
- Ryegrass species
- History of perennial ryegrass breeding in New Zealand
- Perennial ryegrass breeding objectives
- Breeding methods in perennial ryegrass
- Summary and objectives

2.2 An overview of dairy production

2.2.1 Dairy production systems

Dairy production systems vary across the developed world, from intensive feeding systems where cows are housed throughout the year and are fed a total mixed ration diet, such as those in the United States, to primarily pasture-based grazing systems, such as those found in New Zealand. Additional to the systems in the developed world, there are also millions of dairy cows used in small scale and subsistence systems in developing countries such as Brazil. The total global dairy cow population is estimated to be 274 million (Food and Agriculture Organization 2016).

When managed optimally, pasture-based systems are characterised by high milk production per hectare and low operating costs of production (Penno *et al.* 1996). Intensive housed systems have greater operating costs relative to pasture-based systems, but they are capable of greater milk production per cow (Dillon *et al.* 2005). For example, cows in the United States produce 10.3 tonnes of whole fresh milk per cow annually, whereas cows in New Zealand produce 4.2 tonnes of whole fresh milk per cow annually (Food and Agriculture Organization 2016). Pasture-based systems typically have greater profitability than systems which have a relatively greater reliance on imported feed and machinery (i.e. housed systems) of comparable size (Dillon *et al.* 1995). However, while this is desirable, both systems have advantages and disadvantages. For example; while a pasture-based system has low costs of production, farmers have relatively less control over the quality and availability of feed due to seasonal variation in climate (Clark *et al.* 1997). Intensive feed systems, while capable of higher milk yields, require a lot of

infrastructure, which has a significant expense associated with it, along with the cost of depreciation (Dillon *et al.* 2005). Pasture based systems are the most common system used in New Zealand and hence will be the focus of this review.

2.2.2 Pasture-based systems

Unlike housed systems, where feed supply is relatively consistent, pasture-based systems experience seasonal fluctuations in pasture growth, and therefore in feed supply throughout the year (Dillon *et al.* 2005). The main aim in a successful pasture-based system is to align animal demand and feed supply as closely as possible (Holmes *et al.* 1987). Pasture growth, and therefore feed supply, varies throughout the year dependent on the season, and differs from the seasonal pattern of animal demand (Figure 2.1, Holmes et al. 1987). As a result, at certain times of the year growth of pasture is insufficient to meet stock requirements and during such periods conserved pasture (i.e., hay or silage) or purchased supplements are used to maintain dry matter intake and metabolisable energy intake (Roche *et al.* 2017a). This strategy ensures that herd feed demand requirements are met, even when pasture growth is variable, however, the use of supplementary feed is more expensive than grazed pasture and so does increase costs (Holmes *et al.* 1987). Due to the cost of supplements relative to grazed pasture, any improvement in pasture dry matter yield is of economic value, particularly improvements in seasonal yield where animal demand is greater than pasture supply (e.g. early-spring). This is discussed further in section 2.2.3.

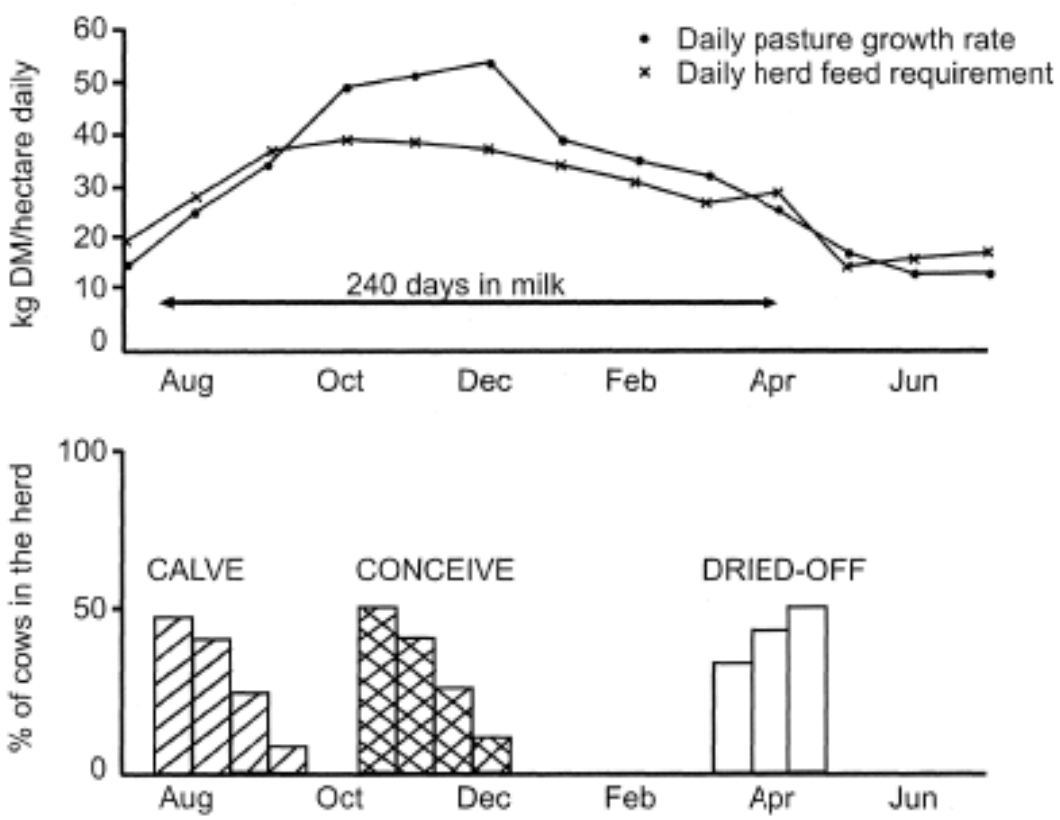


Figure 2.1 Seasonal pattern of breeding, calving and drying off and the synchrony between feed requirements and pasture growth (Holmes et al. 1987).

2.2.3 Economic importance of pasture to a dairy system

2.2.3.1 Proportion of grazed pasture

Grazed pasture is the least expensive feed available for milk production and it has been reported that systems that use a greater proportion of conserved feeds and imported feeds have greater costs of production (Dillon *et al.* 2005). The greater operating costs of production are due to high input systems requiring not only the purchase of feed, but also having a greater need for machinery (Roche *et al.* 2017b). As a result of this, the cost of milk production decreases as the proportion of grazed pasture in the cow's diet increases (Figure 2.2).

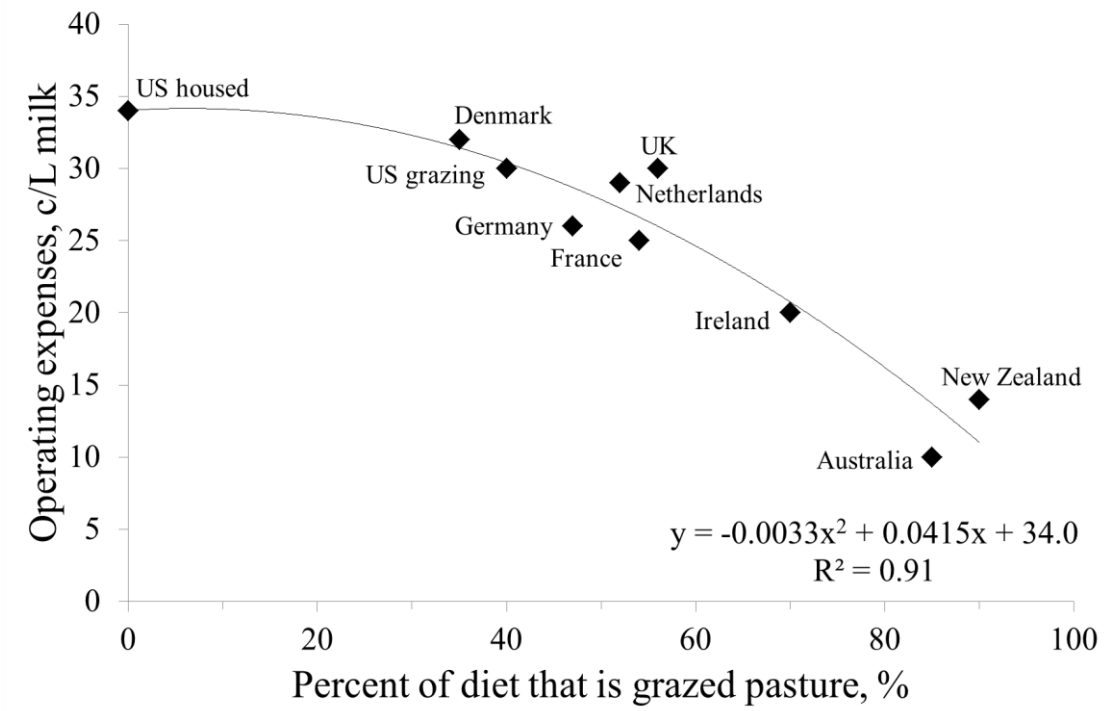


Figure 2.2 The association between the percentage of the cow's annual diet that is grazed pasture and the cost of milk production (Adapted from Dillon *et al.* (1995)).

2.2.3.2 Seasonal pasture yield

The seasonality of pasture yield is also of economic importance. The economic value of pasture varies with livestock enterprise and season, meaning that for a particular livestock enterprise, the value of additional pasture can vary significantly dependent on when the extra yield occurs relative to animal demand requirements (Doyle & Elliott 1983). Chapman et al. (2012) estimated the seasonal economic value (EV) of additional pasture for New Zealand dairy systems in four regions (Table 2.1). In general, during seasons where pasture is already in surplus relative to animal demand, the value of additional pasture is low, because this feed must be conserved (e.g. as silage) which introduces additional costs compared with grazing only. Conversely, in seasons where animal demand is greater than pasture supply, and supplements are required to meet animal

demand, the value of additional pasture is greater because it can be used to reduce the requirement for supplements. For example, in the upper North Island, additional pasture during late spring (when pasture is typically in excess supply) is of less value ($0.21/kg of additional dry matter) than additional pasture in early spring ($0.48/kg of additional dry matter), when pasture supply typically does not meet animal demand.

Table 2.1 Economic values ($/kg additional dry matter) for seasonal dry matter yields in dairy systems in four regions of New Zealand (Chapman et al. 2012).

	Region			
Season	**Upper North Island**	**Lower North Island**	**Upper South Island**	**Lower South Island**
Winter	0.30	0.37	0.45	0.40
Early spring	0.48	0.47	0.42	0.46
Late spring	0.21	0.17	0.29	0.23
Summer	0.40	0.33	0.17	0.12
Autumn	0.41	0.32	0.29	0.27

* Winter = May and June (North Island) and June and July (South Island), Early Spring = July and August (North Island) and August and September (South Island), Late Spring = September and October (North Island) and October and November (South Island), Summer = November to January (North Island) and December to January (South Island) and Autumn = February to April (North Island) and March to May (South Island).

2.2.4 Importance of plant breeding and genetics

The relationship between the proportion of pasture in a system and costs of production means that maximising the harvest of grazed pasture by the herd is important, and thus, maximising pasture yield is crucial to a farm system. Pasture yield is influenced by management, the environment, and by the genetics of the specific cultivars in the pasture. Therefore, there is significant importance placed on improving dry matter yield through

plant genetics and breeding, along with improving other traits which can contribute to greater yields, such as persistence. Additionally, the varying seasonal value of pasture means that seasonal dry matter yield is also important to breeders (Woodfield 1999). Incorporating cultivars bred for improved yield into a farm system will contribute to lower costs of production, by reducing the need for additional supplements, in turn improving farm profitability (Dillon *et al.* 1995).

Breeding for yield has been a primary trait of interest from the beginning of plant breeding. Originally, this involved using traditional methods; selecting plants that showed the best yield and using them as the parents of future generations, such as in the work of E Bruce Levy when developing the Hawkes Bay ecotype (Levy & Davies 1930). However, now, with the continual development of technology, novel breeding methods are opening new avenues for plant breeding (Pembleton *et al.* 2015), this is discussed further in section 2.6.

2.3 Ryegrass species

2.3.1 Origin and distribution of ryegrasses

Ryegrasses (*Lolium* Spp), originated from central Asia, the Mediterranean and northern Europe and there are currently considered to be 13 species in the genus, which differ in their morphological and growth characteristics (Easton 1983). In the Mediterranean, ryegrasses that are short-lived, and do not have a significant day length requirement to flower, are common, such as *L. rigidum*, an annual ryegrass (Easton 1983). However, in the more temperate environments of Europe and Asia, longer living species such as perennial ryegrass (*L. perenne*) are common (Easton 1983). This species only flowers following a period of specific temperature and day length changes (Easton 1983). While the specific floral induction requirements vary, both with cultivar and within a single population (due to genetic variation), flowering of perennial ryegrass can generally be fully induced through exposure to low temperatures (0 - 3°C) and short days (~8 hours), for a period of around 8 weeks (Cooper 1960). Additional to short lived and perennial ryegrasses, Italian ryegrass (*L. multiflorum*) grows in Mediterranean and temperate environments, and has a biennial growth cycle (Easton 1983).

Ryegrasses are now distributed in many parts of the world, in particular in North and South America, and Australia; however, there are distinct areas of these continents in which the environment is too dry, or too hot, for ryegrass to successfully persist (Easton 1983). In comparison, New Zealand is identified as having a favourable climate for ryegrasses, and *L. perenne* and *L. multiflorum* are both important species in New Zealand (Easton 1983).

2.3.2 The importance of perennial ryegrass in New Zealand

An estimated 37% of New Zealand's land mass is classified as pastoral land (~10 million hectares) (Moot *et al.* 2009), supporting 6.4 million dairy cattle, 27.6 million sheep, 3.5 million beef cattle and 835,000 deer (Stats NZ 2016). Internationally, perennial ryegrass, is one of the most commonly sown temperate perennial forage grasses (Wilkins 1991) and is the main grass species in pasture mixes in New Zealand (Lee *et al.* 2012). This is primarily due to its high digestibility, tolerance of intensive grazing, and high seed yield, meaning commercially viable volumes can be produced (Wilkins 1991). In addition to these key qualities, perennial ryegrass is also favoured due to fast establishment and good cool-season production (Hannaway *et al.* 1997).

While annual ryegrasses are typically more productive than perennial ryegrasses, due to perennial ryegrasses requiring energy to be stored in the tiller stubble to maintain future growth, there are advantages to perennial ryegrasses (Wilkins 1991). Primarily, pastures containing perennial ryegrass have lower costs of production as cultivation, weed and pest control costs are required less often. This also means less damage to the soil structure, and because of ryegrass being vigorously tillering, and having highly branched roots, soil erosion is also significantly reduced (Hannaway *et al.* 1999, Wilkins 1991).

2.3.3 Morphology of perennial ryegrass

Perennial ryegrass has dark green, shiny, smooth, hairless leaf blades, which are ridged on the upper surface (Hannaway *et al.* 1997). The leaf sheath is also hairless and is typically red at the base (Hilgendorf 1936). Beginning just below the leaves, the pseudo-

stem has an oval cross-sectional shape (Hilgendorf 1936). Perennial ryegrass has a narrow collar, with small clasping auricles and membranous (0.5-2.5mm) ligule (Hannaway *et al.* 1997) (Figure 2.3). The flowering culms of perennial ryegrass have inflorescence which have between 5 and 40 fixed, awnless spikelets (Hannaway *et al.* 1997). The roots of perennial ryegrass are highly branched, however, they are shallow (Hannaway *et al.* 1999), limiting access to water relative to deeper rooting species.

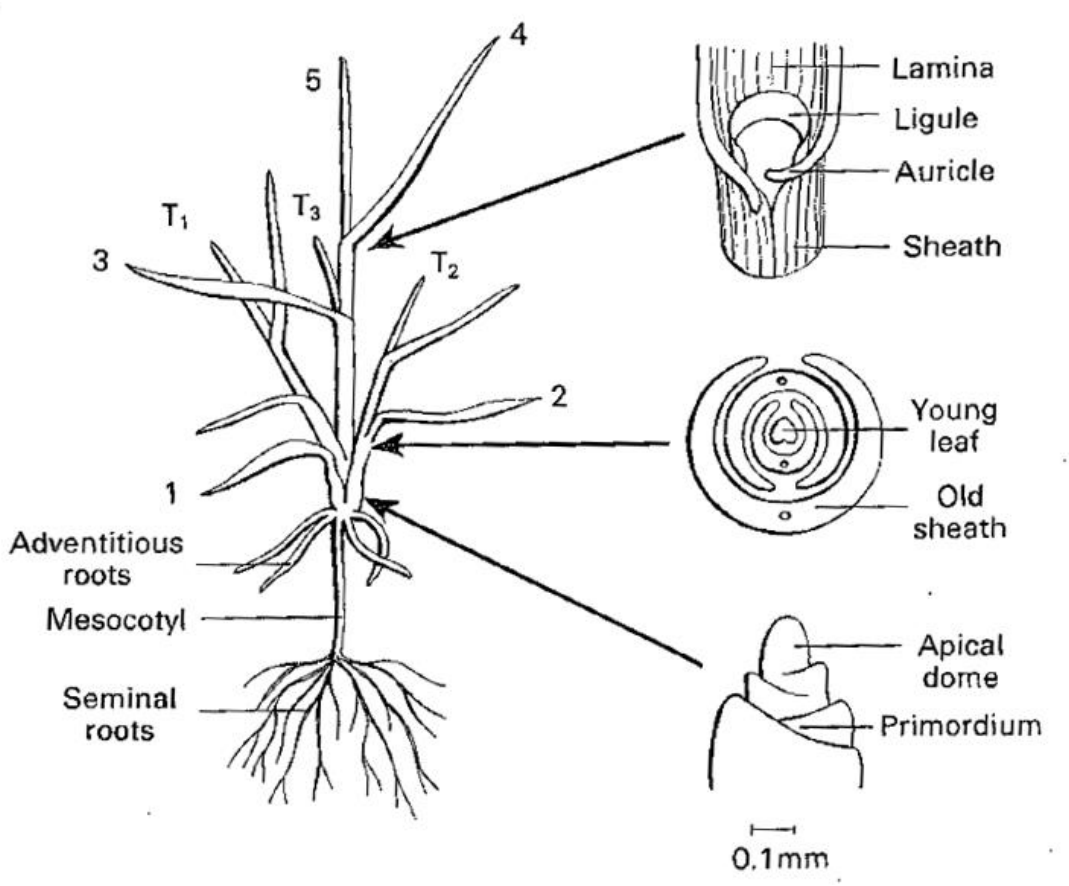


Figure 2.3 Young perennial ryegrass plant with five leaves on the main stem (four fully expanded) and three subtending daughter tillers (T1, T2 and T3). Also (from the top): the junction of lamina and sheath, a cross section of the pseudo-stem (sometimes sheaths are folded rather than rolled) and the vegetative main stem apex (Robson et al. 1988).

2.3.4 Environmental requirements

Perennial ryegrass is a temperate species, which is not well adapted to drought conditions and doesn't tolerate extreme cold well (Hannaway *et al.* 1997). It is best suited to moist, mild conditions (Hannaway *et al.* 1997) and performs best in environments which receive a minimum of 457 – 635 mm of rainfall annually (Casler 2003). However, perennial ryegrass is still vulnerable in high rainfall areas that experience summer droughts, as regular rainfall is important for perennial ryegrass to perform well (Waller & Sale 2001).

Light and temperature are the two primary factors that affect photosynthesis, and therefore, plant growth (Hannaway *et al.* 1997). At light saturation (i.e. light intensity is not a limiting factor) photosynthesis will increase with temperature from 5°C to 25°C, and the optimum temperature for growth is between 20 and 25°C (Hannaway *et al.* 1997). Photosynthesis will occur at lower temperatures, however, growth is limited (Hannaway *et al.* 1997). Perennial ryegrass does not tolerate extreme temperatures well. Dry matter production (irrespective of soil moisture) is impacted at daytime temperatures greater than 31°C, and night-time temperatures greater than 25°C (Casler 2003). Perennial ryegrass can also go dormant in hot summers (Casler 2003). As already discussed in section 2.3.1, perennial ryegrass also has day length and temperature induction requirements in order to flower (Lamp *et al.* 1990).

Perennial ryegrass performs best in high fertility soil, and nitrogen is generally the nutrient that limits growth the most (Hilgendorf 1936). Free draining soil is ideal, however, perennial ryegrass is adaptable and is capable of tolerating poorly drained soils

(Hilgendorf 1936). Adapted to a pH range of 5.0 to 8.3, perennial ryegrass tolerates acid and alkaline conditions, but performs optimally at a pH of 6.5 (Beard 1972).

2.3.5 Perennial ryegrass growth

2.3.5.1 Seedling development

From a seedling, perennial ryegrass develops into a plant made up of units called tillers (Griffith & Chastain 1997). Perennial ryegrass tillers vigorously forming swards (Easton 1983). Individual tillers have their own leaves and roots, however, water, carbohydrates and nutrients can be shared between all tillers in a plant as they are connected at the base of the plant (Donaghy & Fulkerson 2001).

2.3.5.2 Leaf appearance and turnover

Often referred to as a '3 leaf plant', a single perennial ryegrass leaf has a lifespan equivalent to the time it takes for 3 leaves to grow on a single tiller (Donaghy & Fulkerson 2001). In general, once a tiller has grown 3 leaves (i.e. reached the '3-leaf stage'), as the 4th leaf begins to emerge, the oldest leaf begins to die (Parsons & Chapman 2000) (Figure 2.4).

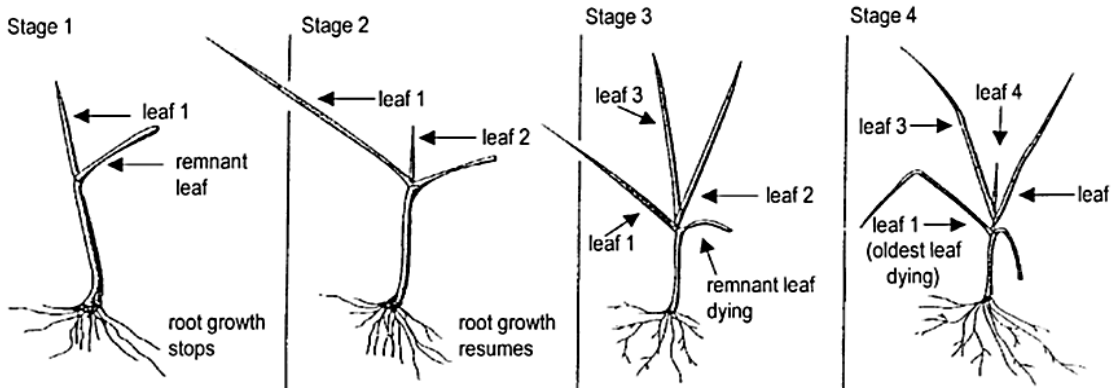


Figure 2.4 Regrowth of a ryegrass tiller following defoliation (Donaghy 1998).

2.3.5.3 Photosynthesis and plant regrowth

Photosynthesis occurs in the leaves of each tiller, producing energy in the form of simple sugars, which are used to fuel respiration and further growth of the plant (Donaghy & Fulkerson 2001). Some sugars are stored as water soluble carbohydrates (WSC) in the stubble of the tiller and are used to support plant regrowth following removal of leaves during grazing (Donaghy & Fulkerson 2001). Immediately after grazing, net energy reserves decline as WSC are used for new leaf growth (Figure 2.5). After the 1-leaf stage is reached and the rate of photosynthesis increases due to increasing leaf area, there is a net accumulation of energy reserves. This accumulation continues until around the 3-leaf stage when maximum energy reserves are reached (Figure 2.5).

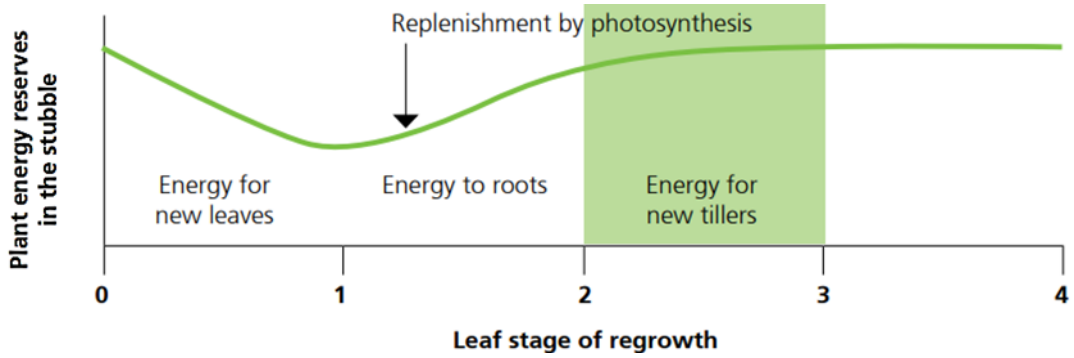


Figure 2.5 Plant energy reserve levels in the tiller stubble over a regrowth period (McCarthy *et al.* n.d.).

2.3.5.4 Management of perennial ryegrass

Dry matter yield, quality, and persistence of perennial ryegrass, are influenced by not only the natural climatic and edaphic conditions, but also by management practices (Hannaway *et al.* 1997). As already discussed, perennial plants store energy in the form of WSC in the base of the tiller (Wilkins 1991). As a result of this, grazing intensity (post-

grazing residual) and grazing interval (rotation length) play an important role in maximising pasture yield (Roche et al 2017).

Grazing intensity

The target post grazing residual for perennial ryegrass is 4 to 6 cm. If pasture is grazed to a higher residual, while it may initially result in a faster regrowth due to higher residual leaf area (Parsons & Chapman 2000), the remaining leaf is generally not as efficient in photosynthesis, and leaf senescence will occur sooner (Donaghy & Fulkerson 2001). This results in wasted feed and a decrease in nutritive value, overall negatively impacting on pasture yield and utilisation (Donaghy & Fulkerson 2001). Shading resulting from a higher residual can also have negative impacts on tillering, and therefore long term dry matter production (Donaghy & Fulkerson 2001). Conversely, if pasture is grazed below 4 cm, the plant's energy stores in the tiller stubble are depleted, impacting negatively on regrowth. Additionally, there may also be an increase in tiller death and an overall increase in plant death, negatively impacting dry matter production (Donaghy & Fulkerson 2001).

Grazing interval

If the grazing interval is too short, i.e. grazed at the 1-leaf stage, then a plants energy stores will be depleted (Donaghy & Fulkerson 2001). As discussed above, at the 1-leaf stage a plants energy reserves are being used for leaf regrowth in order to facilitate photosynthesis, and in turn be able to replenish energy reserves (Donaghy & Fulkerson 2001). However, if grazed too soon the plant does not have time to replenish reserves, and hence, does not have the energy required for regrowth (Donaghy & Fulkerson 2001).

In comparison, if grazed at the 3-leaf stage when energy stores have been replenished, the plant has sufficient energy for regrowth post-grazing (Donaghy & Fulkerson 2001). However, there can also be negative impacts if the grazing interval is too long (i.e. beyond the 3-leaf stage), as pasture quality can decrease (Donaghy & Fulkerson 2001).

2.3.6 Breeding system

Perennial ryegrass is an outcrossing species (Easton 1983), which is largely self-incompatible (Cornish *et al.* 1979). Perennial ryegrass has a self-incompatibility (SI) system that prevents self-pollination, in order to prevent inbreeding (Takayama & Isogai 2005). This ensures genetic diversity is maintained, which is important in terms of a populations ability to survive in changing conditions (Takayama & Isogai 2005).

Perennial ryegrass is also a wind pollinated species (Thorogood *et al.* 2002), which can reproduce sexually, via seed, and vegetatively, via new tillers (Wilkins 1991). It is a naturally diploid species, with 7 pairs of chromosomes (2n = 2x = 14) (Humphreys *et al.* 2010), however, plant breeders have successfully doubled chromosome numbers, to create tetraploid perennial ryegrass cultivars (Easton 1983).

2.3.6.1 Self-incompatibility

Self-incompatibility in perennial ryegrass is controlled by two unlinked, independently segregated, multi-allelic loci: the *S* and *Z* loci (Baumann *et al.* 2000). For incompatibility to occur, the alleles of both the *S* and *Z* loci in the pollen must be the same as in the recipient pistil (Baumann *et al.* 2000). An implication of multiple loci being involved in this self-incompatibility system compared with a single locus system, is that the degree

of compatibility can vary in reciprocal crosses of a pair of plants, ranging from 0, 50, 75 or 100% compatibility (Baumann *et al.* 2000). Another difference from a single locus system is that there are no dominant or recessive relationships between alleles (Baumann *et al.* 2000).

2.4 History of perennial ryegrass breeding in New Zealand

Some species from the Poaceae family, such as maize, have been under inadvertent and direct selection by humans for a centuries, however, perennial ryegrass breeding only began in the early 1900s (Wilkins 1991). The on-going selection of annual grasses has resulted in the modern plants we have today, which are significantly improved in traits such as yield, compared to the original wild types. In contrast, some of the earliest developed perennial ryegrass cultivars are still used in agriculture and breeding programmes today (Wilkins 1991).

2.4.1 New Zealand prior to settlement

Prior to settlement by humans, New Zealand was nearly completely covered in forest and scrub, aside from alpine land area above 1500 metres (Figure 2.6), an estimated 25.1 million hectares (Cumberland 1941). However, when Polynesian settlement occurred there was major destruction of both lowland and montane forest, as land was cleared by manmade fire (McGlone 1989). It is estimated that by the time of European arrival, land area covered by forest had decreased to just over half of the original area (54%), 13.7 million hectares (Cumberland 1941). When European settlement occurred, this destruction continued, in 2012 there was an estimated 6.3 million hectares of indigenous forest remaining (Stats NZ 2012a).

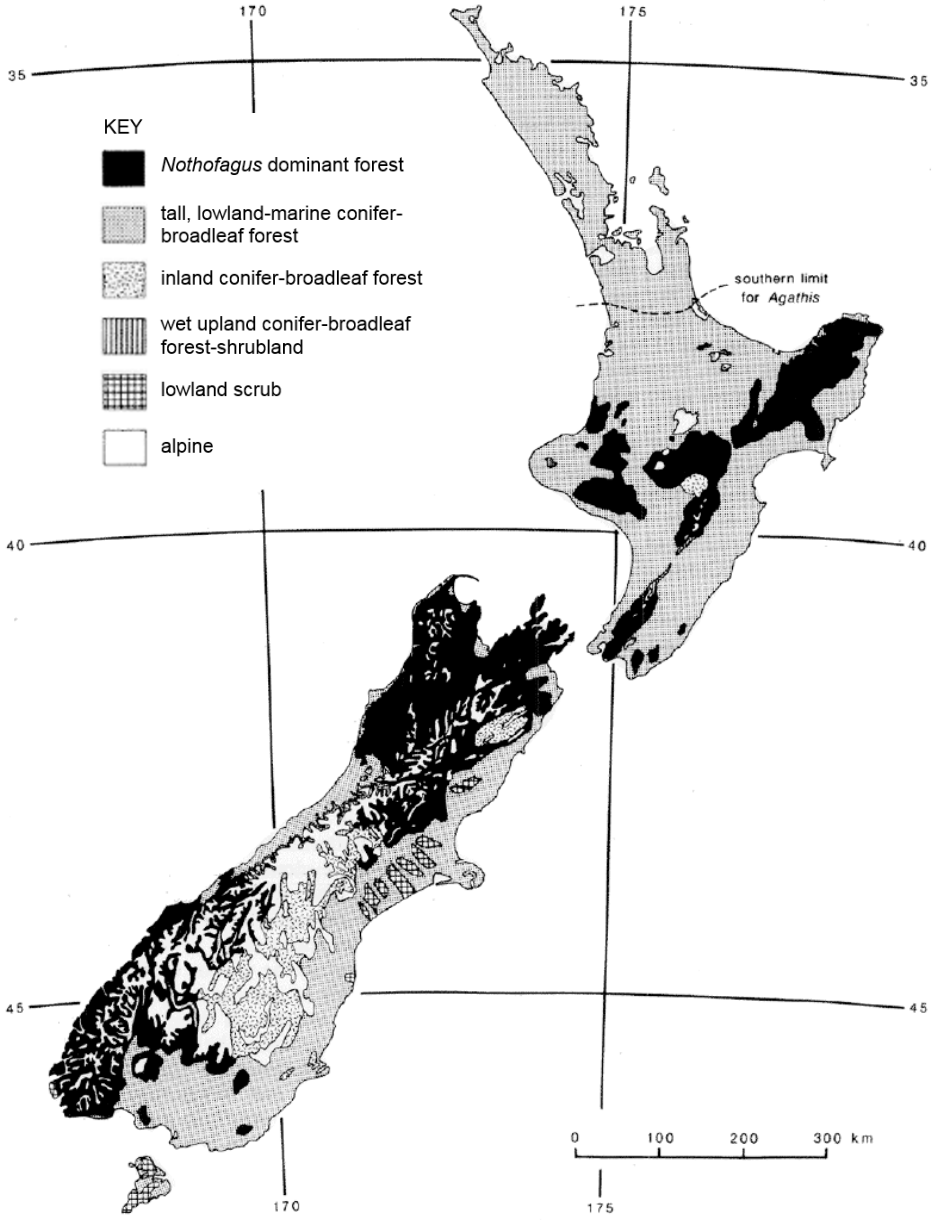


Figure 2.6 New Zealand vegetation before Polynesian settlement (McGlone 1989).

2.4.2 Introduction of perennial ryegrass to New Zealand

Perennial ryegrass germplasm was first bought to New Zealand as seed in the early 1800s by settlers from the United Kingdom (UK) (Rumball 1983). Following the cutting and burning of the forest, common European grass and clover seed was sown in the ashes, and this was the beginning of the pastures present in New Zealand today (Rumball 1983). While seed arrived from the UK, it's original source would primarily have been other countries in Europe, such as Ireland, where large scale commercial seed harvesting occurred (Stewart 2006). This seed trade continued for at least 60 years as pasture establishment in New Zealand increased, until eventually there were large enough quantities of seed being harvested locally for New Zealand to become primarily self-sufficient. By 1912, local trade was the primary source of seed in New Zealand (Stewart 2006).

2.4.3 Development of the Hawkes Bay ecotype

From the permanent pastures sown from imported UK seed, ecotypes adapted to specific local conditions throughout New Zealand gradually began to develop, as environmental pressure resulted in selection of the most well adapted genotypes from the original gene pools (Lee *et al.* 2012). Two key factors which determine whether plants within a sward survive and persist in an environment are its reseeding ability and its capability of persisting vegetatively, i.e. by tillering (Levy & Davies 1929). One ecotype in particular which was recognised to perform well under local soil and climatic conditions, and be superior to other populations, was located in the Hawkes Bay (New Zealand, North Island, east coast), and consequently named the Hawkes Bay ecotype (Easton 1983).

2.4.4 Seed certification – 1920s

With the recognition that ecotypes, such as the Hawkes Bay ecotype, were well adapted to the New Zealand climate, experiments began in the late 1890s which demonstrated that New Zealand ecotypes outperformed imported commercial lines (Stewart 2006). As a result of this finding, the government Seed Certification Scheme was developed and began operating in 1927 (NZ Ministry of Agriculture & Fisheries 1979). The scheme was administered by the New Zealand Ministry of Agriculture and Fisheries (MAF) with the aim to provide official certification of seed from New Zealand ecotypes and older pastures, to ensure 'high varietal purity' (NZ Ministry of Agriculture & Fisheries 1979). This provided farmers with confidence that the seed had strong performance and persistence (Stewart 2006). The scheme was expanded to also include certification for cultivars developed in plant breeding programs (Stewart 2006).

2.4.5 Beginning of modern perennial ryegrass breeding - 1930s

As New Zealand has a warmer winter than Europe, the initial focus of plant breeders in New Zealand was to select for perennial ryegrass which was cool-season active, which was not the case with perennial ryegrass imported from the UK. Greater persistence, leaf production and resistance to crown rust were also selected for (Easton 1983). This began in the 1930s with E. Bruce Levy, who conducted single plant experiments with the aim of understanding the makeup and variation within the Hawkes Bay ecotype, and selecting plants of a desirable 'type' in order to develop an elite strain (Levy & Davies 1930). It was from this method that a pedigree line with improved winter and spring dry matter

yield was produced, certified in 1936 and named ‘Grasslands Ruanui’ in 1964 (Easton 1983).

2.4.6 Hybrid breeding

Hybrid breeding was a method first utilised to improve growth during the cool-season (Stewart 2006). Italian ryegrass (*L. multiflorum* Lam.) was hybridised with perennial ryegrass by L. Corkill to create a short rotation hybrid, which was released in 1943 (Corkill 1953), and named ‘Grasslands Manawa’ (Stewart 2006). This ‘short rotation’ hybrid combined the fast establishment, good palatability and strong winter and early spring dry matter production of Italian ryegrass, with the permanence of perennial ryegrass (Corkill 1953). Following the success of the short rotation hybrid, Corkill then established a breeding program to develop a ryegrass which attained the yield and palatability characteristics of short rotation hybrid but had greater persistency. This programme was taken over by P.C. Barclay in 1957 (Barclay 1963). Backcrossing of ‘Grasslands Manawa’ with perennial ryegrass from the Hawkes Bay ecotype was used to achieve this objective and develop ‘Grasslands Ariki’, a long-rotation hybrid, which was released in 1965 (Stewart 2006). ‘Grasslands Ariki’ was characterised as having good persistence, palatability, and resistance to crown rust, plus greater cool-season productivity (Easton 1983). Barclay (1963) reported that Ariki out performed then available New Zealand perennial ryegrass in all seasons in Palmerston North, Kaikohe and Lincoln, and while initially only found to be of equal performance in Gore, in later experiments was shown to be superior in Southern New Zealand as well.

2.4.7 Tetraploids

Ryegrasses were first artificially doubled in chromosome number, creating tetraploids, in the 1930s by treating seedlings with colchicine (Easton 1983). Myers (1939) reported successful colchicine induced tetraploidy in perennial ryegrass, resulting in larger plants. Additional to this, Myers also reported that tetraploid plants produced larger pollen grains, providing evidence that tetraploid reproductive tissue was also formed, and that tetraploid offspring were produced from seed collected from such plants (Myers 1939).

Tetraploid plants have greater nutritive value than diploid perennial ryegrass due to having a lower ratio of cell wall to cell contents (Lambert & Litherland 2000). Tetraploid perennial ryegrasses are also more palatable relative to diploid plants, however, they also have a lower dry matter content than diploids (Wit 1959). This difference in palatability and dry matter content between tetraploid and diploid cultivars has led to the suggestion that while animals consume more of tetraploid plants due to the palatability, differences in fresh weight intake are cancelled out by the differences in dry matter content (Baert & Carlier 1988). However, contrary to this, Hageman *et al.* (1994) found that not only did tetraploid cultivars ‘Condesa’ and ‘Madera’ have a higher dry matter intake than diploid cultivar ‘Wendy’, but milk production was also greater from cows grazing the tetraploid cultivars.

Tetraploidy also presents some valuable opportunities in terms of plant genetics, masking recessive, undesirable genes and lowering the frequency in tetraploid populations, and also decreasing inbreeding depression (Simonsen 1977). Tetraploid inheritance also

reduces segregation, enabling stabilisation of hybrids between Italian ryegrass and perennial ryegrass (Breese & Thomas 1978).

Since the first experiments in 1939, tetraploidy in perennial ryegrass has been investigated thoroughly. ‘Tama’, released in 1968, was the first New Zealand tetraploid to be developed (Hunt & Easton 1989). Since then the use of tetraploidy has become common practice in perennial ryegrass breeding and there are now a number of tetraploid perennial ryegrasses on the market, such as ‘Viscount’ bred by Barenbrug Agriseeds, and ‘Base’ bred by PGG Wrightson.

2.4.8 Development of the Mangere Ecotype – 1960s

Another ecotype important to the development of perennial ryegrasses in New Zealand was identified in the Mangere district of South Auckland (Duder 1978). Trevor Ellett recognised that the ryegrass population on his 81 hectare property had good persistence in dry summers and recovered well in the autumn (Duder 1978). In the late 1950s the Mangere ecotype was compared against New Zealand bred, and imported cultivars, where it showed greater persistence after 3 years, a result supported by subsequent experiments (Duder 1978). Further yield experiments were also completed in the late 1960s and early 1970s, which also reported superior dry matter yield performance in summer and autumn (Duder 1978). In these experiments the differences in performance between the Mangere ecotype and Grasslands Ruanui and Grasslands Ariki (which both have Hawkes Bay ecotype origins) were not large, however, the ecotype clearly out performed every other cultivar in the experiment (Duder 1978). Following recognition of the potential of the ecotype, the genepool became a key source of genetics for modern breeding, and Arthur

Yates and Co Ltd first began use the genepool to select for regional strains in 1972 (Duder 1978). The perennial cultivar 'Grasslands Nui' was the first developed from the genepool and was released in 1975, followed by 'Ellett' in 1980 (Stewart 2006). Since then the Mangere ecotype has been used to breed many other cultivars, such as 'Yatsyn 1', 'Dobson', and 'Bronsyn' (Stewart 2006).

2.4.9 Endophytes

Knowledge of endophytic fungus infection in perennial ryegrass dates back to the early 1900s (Sampson 1933), and it is the endophyte *Neotyphodium lolii* which naturally infects perennial ryegrass (Easton 1999). The species has since been reclassified the genus *Epichloe* (Leuchtmann *et al.* 2014). The significance of endophyte in perennial ryegrass is the range of alkaloid metabolites that are produced. There are both positive and negative effects of the metabolites. The effects are primarily positive for pasture plants, the most significant being defence against a range of invertebrate pests and overgrazing (Easton *et al.* 2001). However, the effects on livestock production can be significantly negative, causing ryegrass staggers, reducing liveweight gain, causing heat stress and a decrease in serum prolactin levels (Easton *et al.* 2001).

Initial research found no conclusive evidence that endophyte had any effects on animal health or plant growth (Cunningham 1958, Neill 1940). Neill (1940) concluded "there is no evidence as yet that the fungus has any effect either on the rye-grass plant or on grazing animals". The true effects of endophyte were not fully understood and proven until the 1980s (Easton *et al.* 2001).

The discovery of the positive and negative effects of endophyte presence has added an extra dimension to ryegrass breeding. In the 1980's novel endophytes which do not produce the metabolites which negatively impact on livestock were developed (Fletcher 2012). Novel endophytes which have been developed include AR1, AR5, NEA2 and AR37. These are now inoculated into superior ryegrass germplasm, in order to produce cultivars which are both elite in pasture performance and have minimal negative effects on livestock (Fletcher 2012).

2.4.10 Introducing new germplasm – 1980s

As already mentioned, a key trait of interest early on in perennial ryegrass breeding was improvement of cool-season growth. Selection for late flowering cultivars (with minimal aftermath flowering) also became a key trait of interest in order to delay the decline in pasture quality that occurs with the onset of flowering, and therefore improve late spring pasture quality (Stewart & Hayes 2011). To achieve improvement in these traits new genetic material was introduced from North West Spain in the 1980s (Stewart 2006). This material is winter active and from a similar climate to New Zealand's North Island (Lee *et al.* 2012), providing a wider gene pool for selection and breeding. Additionally, the first introduced New Zealand perennial ryegrass populations lacked endophyte chemical diversity. As a result, ryegrass germplasm from overseas was also collected to expand the range of endophyte available in New Zealand (Stewart 2006).

2.4.11 Plant Variety Rights (PVR)

The Plant Variety Rights (PVR) Act was introduced in 1987 (Ministry of Business Innovation and Employment n.d.). PVR grants a breeder the exclusive rights to sell a plant variety. This protection of a breeder's efforts encourages investment in plant breeding. In order for PVR to be granted the new cultivar must be distinct, uniform and stable (Ministry of Business Innovation and Employment n.d.).

2.4.12 Current day

Currently in New Zealand there is a range of perennial ryegrass cultivars available, with differing traits and overall performance. Information on current cultivar performance is available in the DairyNZ Forage Value Index (FVI). The FVI is an independent index which ranks perennial ryegrass cultivars based their estimated economic value to dairy farmers in four regions of New Zealand (Chapman *et al.* 2017). Farmers can use the index as a tool to make informed decisions when selecting which cultivar is best suited to their region. Currently the index is solely based on seasonal dry matter yield, however, the aim is to incorporate metabolisable energy and persistence traits into the index in the future.

Many New Zealand commercial cultivars share similar genetic origins (Figure 2.7). Many cultivars, for example 'Bealey', 'Tolosa' and 'PG150', are shown to have very similar genetic origins (Figure 2.7). These similarities are likely to be primarily due to breeders using certified cultivars as the base population for breeding of new cultivars. PVR permits this so long as the new cultivar meets PVR criteria, i.e. it is distinct, uniform and stable (Ministry of Business Innovation and Employment n.d.).

Currently, novel breeding methods, such as marker assisted selection, which could be incorporated into perennial ryegrass breeding programs are emerging. It is expected that advances in breeding methods will improve the ability to select for specific traits, and reduce the time it takes to produce a cultivar. Novel breeding methods are discussed further in section 2.6.

Figure 2.7 Neighbour-joining tree of 27 cultivars from perennial ryegrass, Italian ryegrass and their hybrid (Wang et al. 2014).
Based on Nei's genetic distance calculated using the Phylip package. The scale bar indicates length of branches in Nei's genetic distance units. Cultivars of perennial ryegrass are enclosed in an oval with vertical line shading whilst cultivars of Italian ryegrass are enclosed in a rectangle with horizontal line shading.

2.5 Current breeding objectives and genetic gain

2.5.1 Breeding objectives

Currently in New Zealand there is a range of perennial ryegrass cultivars available, with differing traits, strengths, and weaknesses, dependent on the environment they are exposed to. While dry matter yield is an important trait in perennial ryegrass breeding programs, there are a number of other traits which plant breeders also focus on. Current plant breeding objectives are discussed in the following section.

2.5.1.1 Annual and seasonal dry matter yield

Since pasture is the primary source of feed in New Zealand dairy systems, increasing dry matter yield is a key trait of interest in perennial ryegrass breeding. The importance of matching seasonal feed supply to seasonal animal demand (as discussed in section 2.2.2), means that improving seasonal dry matter yield is also a trait of interest, and sometimes total annual yield is sacrificed by breeders in order to improve seasonal yield (Woodfield 1999). Improvement of yield at certain times of the year is not only beneficial for meeting animal demand (Woodfield 1999), but also has significant economic value (as discussed in section 2.2.3.2). Evidence supporting this pattern of seasonal improvement can be seen in genetic gain rates, discussed in section 2.5.2.

2.5.1.2 Quality

The metabolisable energy (ME) of a cultivar, i.e. the amount of energy a cultivar provides an animal per kg dry matter, can significantly impact animal performance (Stewart & Hayes 2011). Metabolisable energy varies due to genetic factors, such as heading date (as

already discussed in section 2.4.10) and the ratio of plant structures (in particular, pseudo-stem to leaf ratio and tiller size) (Stewart & Hayes 2011), and also due to environmental factors, such as pasture management, disease presence (e.g. crown rust (Woodfield 1999)), and seasonal growth rates (ME declines when reproductive growth occurs in spring) (Stewart & Hayes 2011). Environmental and management factors generally have a much greater impact on pasture quality than genetic variation in pasture quality (Stewart & Hayes 2011). However, in months such as summer where pasture quality is low, and supplementary feed has to be bought, even small advances in the quality of a cultivar are of value if it means the amount of supplementary feed purchased is decreased, hence quality is still a trait of focus for plant breeders (Wilkins 1991).

2.5.1.3 Persistence

The trait persistency can be defined as "maintenance of a desired species through time without major intervention" (Clark 2011). It is influenced by the rate at which the tiller density of a pasture declines (Wilkins 1991). After sowing of a pasture, tiller density may gradually decline, making it vulnerable to invasion of weeds and a decline in performance. Cultivars in which this occurs more slowly than others are considered to be more persistent (Wilkins 1991). Thus, persistency is dependent on both the rate of tiller death and the rate of tiller replacement, and any factor which influence these rates (Easton *et al.* 2011). In turn, dry matter yield and how often pastures need to be resown are also impacted (Wilkins 1991).

Persistence is a more important issue in environments which are at the margins of the adaptive range of perennial ryegrass, and hence cause some level of stress to the plants.

It is for this reason that it is important to select perennial ryegrass cultivars with traits adapted to the specific environment. Examples of the extremes of the adaptive range of perennial ryegrass include, environments with warmer temperatures (heat tolerance), dry summers (drought tolerance) (Easton *et al.* 2011), pest and disease problems (tolerance/resistance), extremely cold winters (freezing tolerance), and also management imposed stresses such as high grazing intensity and treading damage (Stewart & Hayes 2011). In some cases, these stresses are more complex, for example, in New Zealand during a summer drought growth rates and tillering are reduced, making pasture more vulnerable to pests, and it is the presence of endophyte that is a key factor in pasture survival and persistence. If endophyte strains that provide effective resistance to the dominant insect species are not present, then persistence can decline (Stewart & Hayes 2011). So, while increasing dry matter yield is a primary objective, selection for other traits such as those discussed above are all important in improving overall persistency and in turn long term dry matter yield.

2.5.1.4 Seed production

A crucial part of developing a new cultivar is ensuring that seed yields are sufficient to enable economic commercial production of the cultivar (Wilkins 1991). Even if breeders develop a cultivar which displays many good production traits, if it has an insufficient seed yield it will not be commercially viable (Stewart & Hayes 2011). An example of this in New Zealand is the cultivar ‘Tolosa’, bred by Barenbrug Agriseeds, which had good dry matter yield and palatability but low seed production (Stewart & Hayes 2011).

All the traits discussed above are of importance to plant breeders when breeding new cultivars. Improvement in dry matter yield not only comes from selecting for plant genetics which have a greater yield capability, but also from selection for other traits which collectively improve overall dry matter yield by creating a more productive and persistent population. Because of the importance of dry matter yield in New Zealand farm systems, several studies have been conducted to quantify the gains that have been achieved in this trait through breeding, as discussed in the following section.

2.5.2 Genetic gain in dry matter yield

Genetic gain in perennial ryegrass dry matter yield in New Zealand has been quite limited. Woodfield (1999) estimated it to be between 0.25 - 0.73% per year of plant breeding effort. However, more recently Harmer *et al.* (2016), through the analysis of 46 perennial ryegrass experiments in New Zealand and Australia, have identified that pre 1990 and post 1990 are two distinctly different periods in the breeding of perennial ryegrass, with significantly different rates of genetic gain. Prior to 1990, no significant changes in total, or seasonal, dry matter yield were detected on an annual basis (Harmer *et al.* 2016). However, post 1990, there were significant and steady increases in total dry matter yield of 105 ± 11kg DM/ha/year, a genetic gain of 0.76% annually (Harmer *et al.* 2016).

Harmer *et al.* (2016) suggested that this change in the rate of genetic gain after 1990 was due to both technical factors (e.g. endophyte technology and tetraploidy) and economic factors. A key economic factor was the development of the Plant Variety Rights act in 1987. After which private sector investment in perennial ryegrass breeding greatly

increased, leading to a more competitive commercial environment and more private breeding programmes (Harmer *et al.* 2016).

Genetic gain in seasonal dry matter was also limited prior to 1990 (Harmer *et al.* 2016). Post 1990, all seasons had significant rates of up to 1.28%. Easton *et al.* (2002) reported that while total dry matter yield was estimated to have improved by 0.4% per annum in the previous 25-30 years, genetic gain in spring yield had only increased by 0.1%, compared to 0.7% for summer-autumn, indicating that yield improvement had not been even across all seasons. This finding demonstrates the focus of perennial ryegrass breeders on selecting for increased yield in seasons which typically have lower yields, rather than seasons where forage supply is more available. While post 1990 the rate of genetic gain in perennial ryegrass dry matter yield has increased significantly, improvement in dry matter yield of perennial ryegrass remains substantially less than in maize, another commonly used forage in New Zealand farm systems (Figure 2.8).

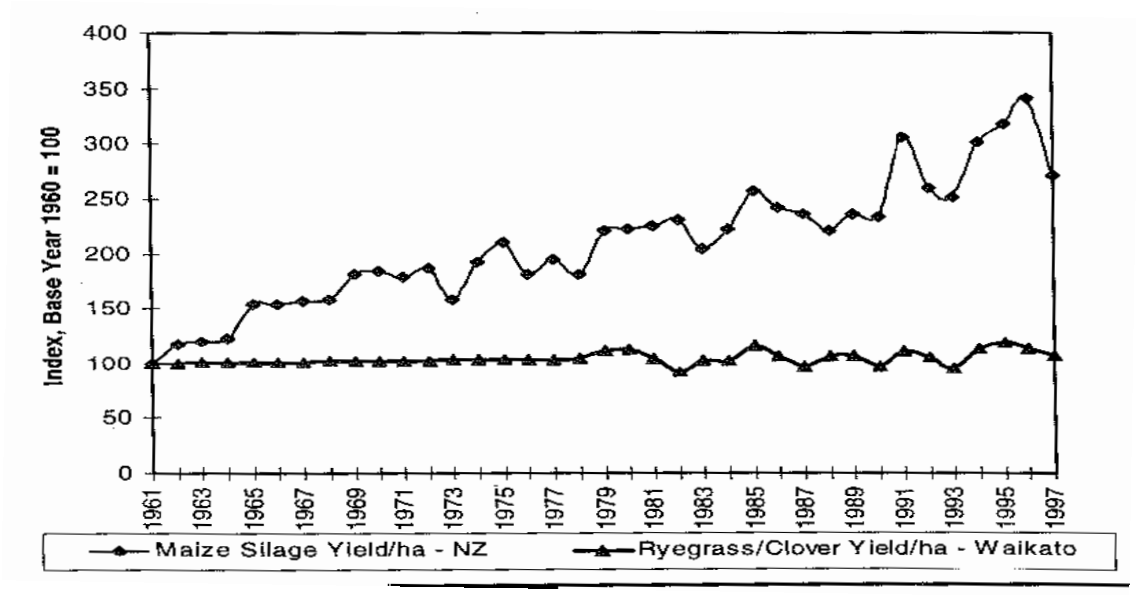


Figure 2.8 A comparison of gains in Waikato pasture yield (triangle symbols) and New Zealand maize silage yield (diamond symbols) (Deane 1999).

2.6 Breeding methods in perennial ryegrass

The most common method of achieving trait gains in outcrossing species, such as perennial ryegrass, is by intra-population improvement. This is typically done using sexual recombination of genes and selection (Humphreys *et al.* 2006). The most common method of perennial ryegrass improvement is the use of recurrent selection (Acquaah 2009). Along with the traditional method of recurrent selection, hybrid breeding has also been considered by breeders. Additionally, investigation of the use of novel breeding methods is increasing in perennial ryegrass improvement programmes, such as the use of marker assisted selection and other biotechnology techniques. These areas of perennial ryegrass breeding are outlined below.

2.6.1 Recurrent selection

Recurrent selection is a cyclic technique, in which population performance in a particular trait of interest is improved through selection of individuals which demonstrate desirable characteristics, followed by inter-crossing to produce a new generation for a further selection cycle (Acquaah 2009). This method of selection leads to a change in the population genetic structure, increasing the frequency of desirable genotypes (Brummer & Casler 2009) and through the introduction of new genotypes due to recombination (Acquaah 2009). The cyclic nature of this process means that each successive cycle should result in a shift in the population mean (Figure 2.9), while maintaining genetic diversity so that there is the potential to select for further generations (Acquaah 2009). The end result of this process is an improved population, which is released as a synthetic variety (Vogel & Pedersen 1993).

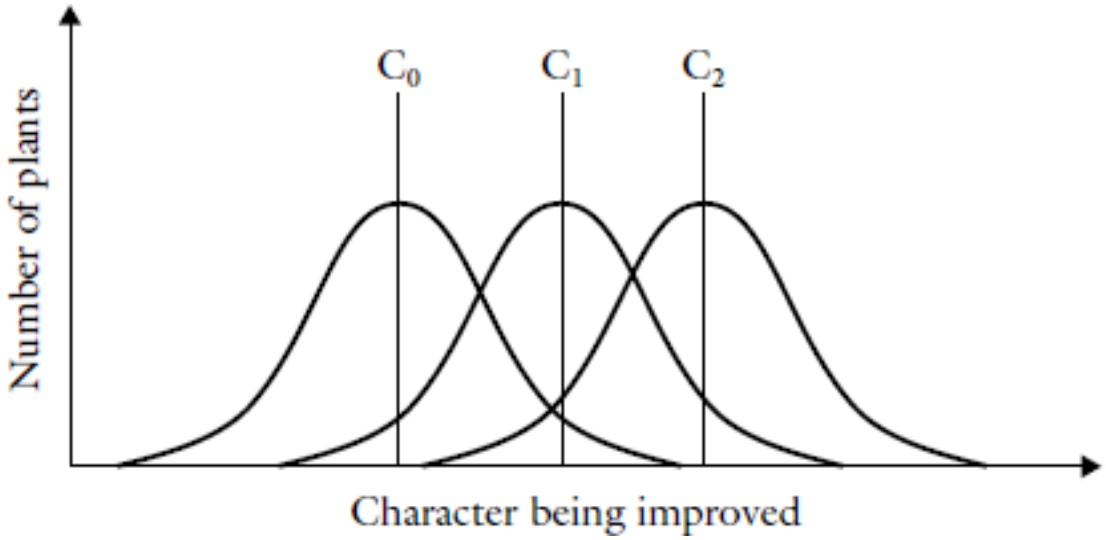


Figure 2.9 The concept of recurrent selection. C = cycle (Acquaah 2009).

There are many different variations of recurrent selection, but the two main types are phenotypic and genotypic selection. Genotypic selection can be further split into recurrent selection for general combining ability (half-sib family selection) and recurrent selection for specific combining ability (full-sib family selection) (Conaghan & Casler 2011). These recurrent selection methods are discussed below.

While there are a number of different forms of recurrent selection, all methods follow a basic outline in a single cycle, which is repeated several times dependent on the specific system:

1. Establishment of a genetically diverse base population.
2. Evaluation of the population and selection of desirable plants or families.
3. Inter-breeding of the selected plants or families to form the base population for the next cycle of selection.

In general, the average timeframe to produce a perennial ryegrass cultivar is 10-15 years (Lee *et al.* 2012).

2.6.1.1 Phenotypic recurrent selection (simple recurrent selection)

Phenotypic recurrent selection (Figure 2.10), also referred to as simple recurrent selection, is the method in which the selection criteria is purely based on plant phenotypes, and does not use any form of test crossing (Acquaah 2009). The seed of selected plants is harvested and used as the base population for the next generation. There are two main types of simple recurrent selection, uniparental, where selected plants are open pollinated, and therefore only one parent is controlled, and bi-parental, where both parents are controlled (Conaghan & Casler 2011). The method of simple recurrent selection is most effective with traits of high heritability that can easily be scored visually, and is not considered highly effective when breeding for yield gains (Acquaah 2009).

Figure 2.10 Schematic flow diagram of 1 cycle of phenotypic recurrent selection (Conaghan & Casler 2011).

2.6.1.2 Genotypic recurrent selection

Recurrent selection for general combining ability

Genotypic recurrent selection for general combining ability (Figure 2.11) is a method which assesses genetic merit, and bases selection, on the performance of the half-sib progeny of individuals in the population (Conaghan & Casler 2011). A polycross is used to randomly pollinate selected female plants and produce the half-sib families (Acquaah 2009). Evaluation and crossing of these families is then completed. Half-sib families which perform well are deemed to have good general combining ability.

Genotypic recurrent selection for general combining ability is a commonly used breeding method for perennial forage species (Acquaah 2009). The advantages of this method include the simplicity of randomly crossing genotypes in a polycross, which is easier than pair crossing, and prevents inbreeding depression (Brummer & Casler 2009). The use of clones in a polycross means it is easy to generate sufficient quantities of seed to enable evaluation of rows or swards, rather than single spaced plants, making assessments more representative of an on farm scenario (Brummer & Casler 2009).

Figure 2.11 Schematic flow diagram of one cycle of genotypic recurrent selection using half-sib families (Conaghan & Casler 2011).

Recurrent selection for specific combining ability

Genotypic recurrent selection for specific combining ability (Figure 2.12) is a method which assesses genetic merit, and bases selection, on the performance of the full-sib progeny of individuals in the population (Conaghan & Casler 2011). Unlike the half-sib method where a polycross is used, the full-sib approach uses bi-parental crosses, meaning both parents are known (Acquaah 2009). Evaluation of these families is then completed. Full-sib families which perform well are deemed to have good specific combining ability and are selected to recombine for use in future generations (Acquaah 2009).This method

is less commonly used as the aim of a synthetic variety is to combine a group of parent genotypes with good combining ability among themselves, i.e. good general combining ability, rather than specific combining ability (Acquaah 2012).

Figure 2.12 Schematic flow diagram of one cycle of genotypic recurrent selection using full-sib families (Conaghan & Casler 2011).

Evaluation and inter-crossing

Genotypic recurrent selection has a point of difference to phenotypic recurrent selection, as genotypic selection usually involves replicated assessment in multiple locations (Brummer & Casler 2009). Assessment of genotype by environment interactions results in greater heritability (and therefore genetic gain) in comparison to what can be achieved through phenotypic recurrent selection (Brummer 1999).

Following the evaluation of the half-sib or full-sib families and identification of the best families, they are inter-crossed to form the next set of families for the following cycle. The method of crossing can have a significant impact on the genetic gain per cycle (Conaghan & Casler 2011). The three main types of crossing that are used in genotypic recurrent selection are:

- Progeny test selection: once the best performing families have been selected the maternal plants from half-sib families or both the parents from full-sib families are polycrossed (Conaghan & Casler 2011). The seed from these plants is then either randomly selected, or specifically selected, from the polycross, and in the case of half-sib families is used for another polycross, or in the case of full-sib families are pair crossed (Conaghan & Casler 2011).
- Family selection: uses randomly selected plants from the remnant seed from the original cross of each of the selected families. For half-sib families, the seed is used in a polycross to produce a new set of half-sib families for evaluation (Conaghan & Casler 2011). For full-sib families, pair-crosses are made in a partial

diallel cross to produce new full sib families for evaluation (Conaghan & Casler 2011).

- Among-and-within family selection: selects the best plants from the best families. For half-sib families, this is followed by further polycrossing and for full-sib families, pair-crossing in a partial diallel (Conaghan & Casler 2011).

2.6.2 Marker assisted selection

Marker assisted selection is a tool which uses DNA markers as predictors of trait performance, enabling the identification of individuals with desirable gene profiles (Barrett *et al.* 2006), and in turn indirect selection of individuals with desirable traits (Xu & Crouch 2008). Marker assisted selection has many possible applications in plant breeding, including evaluation of breeding material, backcrossing, pyramiding, early selection of lines, and it can also be used in combination with phenotypic selection (Collard & Mackill 2008).

Marker assisted selection has some significant advantages over conventional phenotypic breeding methods, and has a positive effect on the effectiveness and efficiency of selection (Conaghan & Casler 2011). Advantages include:

- *Simplicity:* MAS is a lot simpler than phenotypic selection, and can reduce the time and resources required to screen plants (Collard & Mackill 2008). For example, it removes difficulties associated with time of year or location, or traits which are difficult to assess phenotypically (Conaghan & Casler 2011).
- *Enables earlier assessment:* MAS can assess traits at the seedling stage, speeding up the time taken to assess traits which have to be assessed at later

development stages when using phenotypic selection (Collard & Mackill 2008).

- *Increased reliability in single plant selection:* using MAS to select single plants with desirable traits is more reliable, as the genotype by environment influence experienced in phenotypic selection is removed (Collard & Mackill 2008).

However, while the development of this technique was a huge step forward in plant breeding, it has not delivered the commercial gains anticipated. Reasons for this include:

- *Limited explanation of phenotypic variation:* in general, markers only account for a small amount of the total phenotypic variation in a trait (Brummer & Casler 2009), and therefore are of limited use in achieving significant trait gains.
- *Reliability and accuracy of markers:* MAS is only as accurate as the phenotypic data which was used to identify the markers (Conaghan & Casler 2011) and the importance of accuracy increases for complex traits which are influenced by a number of markers, such as yield (Collard & Mackill 2008). Replication and population size of experiments used to collected phenotypic data play an important role in accurate mapping of markers (Beavis 1998).
- *Erosion of marker-trait associations:* recombination can result in separation of markers and genes (Collard & Mackill 2008). This means that over time the efficiency of selection using markers will decrease, and hence the rate of

genetic gain will decrease, resulting in recalibration of markers being required (Conaghan & Casler 2011).

- *Genetic background:* markers identified for a specific trait in a specific population do not necessarily correlate to, and predict, the same trait in a different genetic background (Collard & Mackill 2008).
- *Cost:* MAS has significant costs associated with it, which may outweigh the benefits when compared with phenotypic selection. The exact cost will depend on factors effecting the cost of phenotypic selection, such as the trait of interest, how it is assessed and any associated costs such as labour and resources (Collard & Mackill 2008). Additionally, there are initial capital costs, and maintenance costs, of MAS and overall, these costs need to be weighed up against the rate of genetic gain which can be achieved (Conaghan & Casler 2011).

2.6.3 Hybrid breeding and heterosis

In hybrid breeding, two genetically divergent parents are crossed to produce offspring, 'hybrids', with superior trait performance, relative to the parents though exploitation of heterosis (hybrid vigour) (Brummer 1999, Pembleton *et al.* 2015). Mid-parent heterosis refers to the performance of the offspring relative to the mean performance of the two parents, while high parent heterosis refers to the performance of the offspring relative to the highest performing parent (Barret *et al.* 2010).

Not all populations or parents cross to give the same level of heterosis (Barrett et al 2010). Populations which combine well to produce offspring with superior performance are

considered to be in separate ‘heterotic groups’ (Brummer 1999). The distinction of heterotic groups enables breeders to selectively target populations when selecting plant material for a breeding programme (Brummer 1999). Breeding programmes of maize and rye have shown that crossing parent lines from different gene pools maximise heterosis (Posselt 1993).

Hybrid breeding is a method commonly used in self-compatible species and has resulted in significant yield increases in species such as maize (as was shown in Figure 2.8). In Maize, self-pollination over several generations has been used to produce homozygous inbred lines. These lines are crossed in order to produce hybrids which exhibit strong heterosis (Duvick 2001).

However, it has not been possible to capture heterosis in perennial ryegrass to the same extent as in maize (Barret *et al.* 2010). As perennial ryegrass is an outbreeding, self-incompatible species, options are limited for producing hybrids using the conventional methods used in self-compatible species (Brummer 1999). However, methods have been proposed to capture some level of heterosis in perennial ryegrass. These include the crossing of heterogenic populations to create ‘semi-hybrids’, cytoplasmic male sterility hybrids (CMS hybrids) and the use of partially inbred parents to produce self-incompatibility hybrids.

2.6.3.1 Semi-hybrids

While recurrent selection is a form of intra-population improvement, crossing two separate populations (hybrid breeding) is a form of inter-population improvement, as the final product is a hybrid population which exploits interpopulation heterosis (Acquaah

2009). Assuming that when two populations are crossed, half of the resulting progeny are produced through inter-population crossing (i.e. crossing between the two populations) and the other half are produced from intra-population crosses (i.e. crossing within each population), the resulting population will contain 50% hybrids, referred to by Brummer (1999) as 'semi-hybrids'. The breeding method proposed by Brummer (1999) was to select within two populations from separate heterotic groups, and then polycross selected individuals from each population to produce hybrids (Figure 2.13) (Brummer 1999).

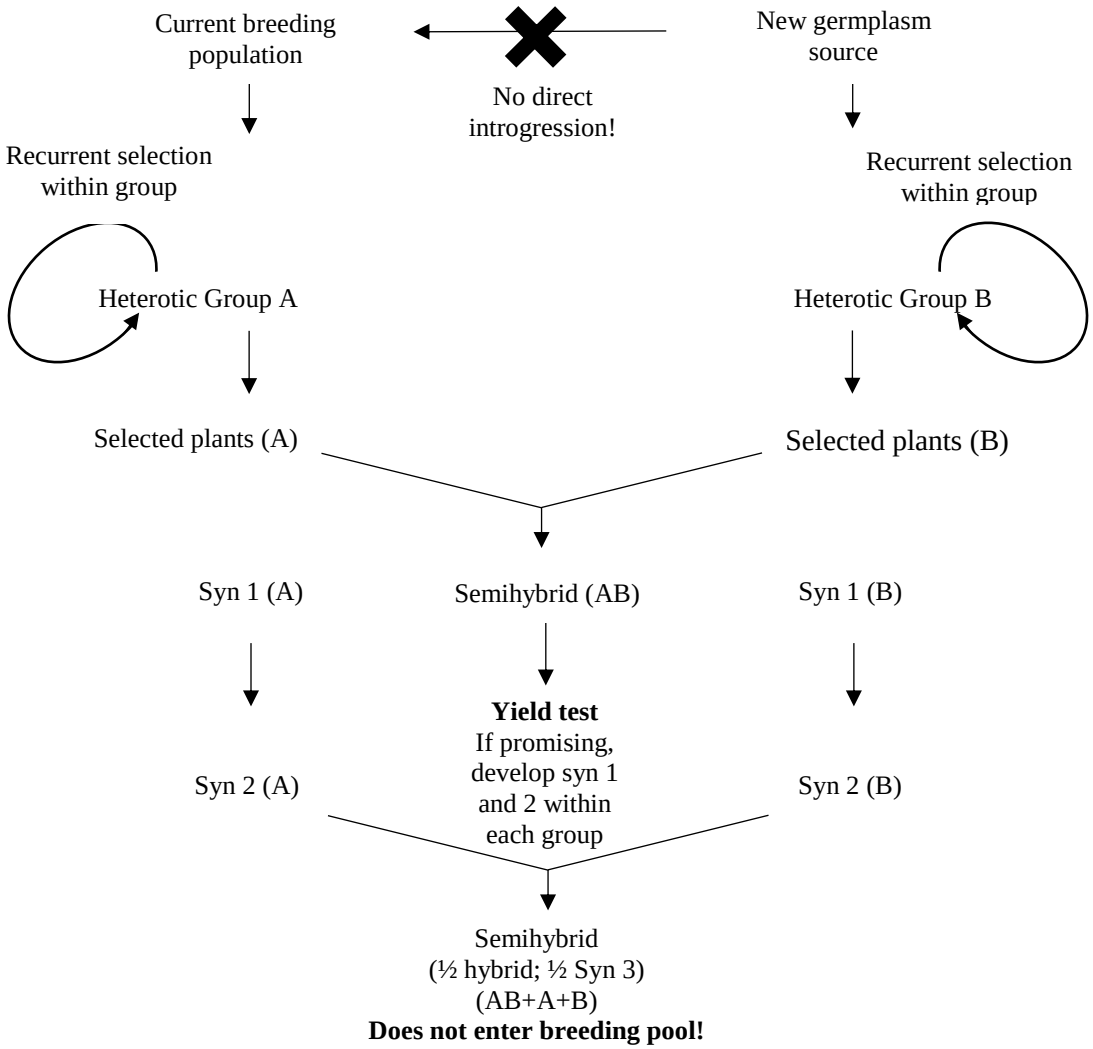


Figure 2.13 Proposed breeding scheme to make semi-hybrids expressing partial heterosis (Brummer 1999).

The potential for the use of semi-hybrid breeding systems in perennial ryegrass has been assessed in both mown swards and under grazing. Foster (1973) found that in a mown sward experiment, the best performing hybrids exhibited up to 5.5% greater yield than the mid-parent mean, and 3.6% greater than the highest yielding parent, and at particular harvests mid-parent heterosis of up to 25% was observed. Barret *et al.* (2010) found the best semi-hybrids in a grazing experiment to exhibit 7% high-parent heterosis for total dry matter yield and that there were also seasonal influences, with the expression of high-parent heterosis reaching up to 19% in spring in particular hybrids.

While there is evidence of some level of hybrid vigour exhibited in the F1 generation (first generation post initial crossing) when two populations are crossed, if the F1 generation is multiplied (i.e. allowed to randomly inter-mate) to increase seed volume, heterosis is reduced by 50% and further heterosis is lost in successive generations (Posselt 2003). Dilution of heterosis is a significant practical challenge to the production of commercially viable cultivars by this method.

2.6.3.2 Cytoplasmic male sterility hybrids

Cytoplasmic male sterility (CMS) is a maternally inherited trait which is responsible for preventing production of viable pollen, while maintaining female fertility in a plant (Levings 1993). It can occur naturally in some plant populations, or can be induced in species such as perennial ryegrass through interspecific crosses (Kiang & Kavanagh 1996). In order to successfully produce hybrid plants, pollination needs to be controlled (Horn & Friedt 1999). CMS has proved an efficient tool to do this in maize and has played an important role in its improvement, removing the need to detassle plants for hybrid

production (Levings 1993). The concept of CMS could be applied to perennial ryegrass hybrid breeding in a similar way as maize to control pollination, however, the use of CSM in forage grasses has been limited (Kiang & Kavanagh 1996). This is primarily due to the fact that commercially viable seed production is not possible (Posselt 1993). In addition, most forage grasses are outbreeding, self-incompatible species, as is the case with perennial ryegrass (Cornish *et al.* 1979). This means that the self-incompatibility system which is designed to ensure cross-pollination, and maintain heterozygosity, creates difficulty in maintaining the CMS phenotype in a population (Islam *et al.* 2014).

2.6.3.3 Self-incompatibility (SI) hybrids

England (1974) was the first to propose a hybrid breeding method based around the two-locus incompatibility system in perennial ryegrass, which theoretically results in 83% hybrid production in the F1 offspring. The proposed method was based around developing inbred perennial ryegrass lines, which could be crossed to create a hybrid population (England 1974). The theory was to ensure the compatibility within each line was relatively less than the compatibility between the two lines, so that when crossed the offspring included a high proportion of true hybrids (i.e. offspring produced from pollination between the two lines, rather than within each line) (England 1974). This method was proposed based on the assumption that the perennial ryegrass incompatibility system is not 100% effective and it is possible, with specific SI genotypes (Posselt 1993), to get some degree of inbreeding within a population (England 1974). In theory, the resulting offspring from the cross should be 83% hybrids (occurring from inter-line

crosses) and 17% inbreds (from crossing within each line) (Posselt 1993). This approach is illustrated in Figure 2.14.

Figure 2.14 Overview of the F1 hybrid breeding scheme based on restriction of SI allele diversity within two defined parental pools. Seed multiplication refers to inbreeding (Pembleton et al. 2015).

When Posselt (1993) compared 75 different SI hybrids, created from crossing 30 partially inbred lines, to the cultivar ‘Lihersa’, it was found that while ‘Lihersa’ performed better than the average of all hybrids, the top hybrids yielded 5-10% more dry matter than

‘Lihersa’. This indicated, that while there was variability in performance of hybrids, there was potential to increase dry matter yield using such a method (Posselt 1993).

This method does present some potential challenges as outlined by Pembleton *et al.* (2015). Firstly, the production of large volumes of self-fertilised seed required for this method is difficult, and the generations of inbreeding involved may also result in inbreeding depression, further enhancing the difficulty in producing large volumes of seed. Also, inbreeding to restrict SI diversity may result in inadvertent selection for genotypes with weak SI systems, making them more predisposed to self-pollination and therefore increasing within pool breeding, decreasing between pool breeding and therefore decreasing hybrid production (Pembleton *et al.* 2015).

However, progress in the development of molecular markers for the alleles of the *S* and *Z* loci which are responsible for controlling compatibility, have now made it possible to predict, and therefore control, compatibility between genotypes using marker assisted selection (Pembleton *et al.* 2015). This means that marker assisted section can now be used to select for desirable combinations of the alleles responsible for self-incompatibility (Thorogood *et al.* 2002). This provides an efficient solution to restrict SI genotypes and develop inbred lines for the production of F1 hybrids using the concept of the self-incompatibility method (Pembleton *et al.* 2015). This breeding method is currently being investigated by an international breeding company.

2.6.4 Biotechnology

Biotechnology is defined by "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use" (The Food and Agriculture Organization 2000). There is large scope for the use of biotechnology in agricultural industries, presenting opportunities to develop sustainable methods of food production which are essential with rising global demand (The Food and Agriculture Organization 2000). A key use of biotechnology in agriculture is genetic modification/engineering. Genetic engineering is the manipulation of a host species genome through random insertion of a foreign donor gene (Sticklen 2015) and presents a useful tool for accelerating the rate of trait improvement in species (Bajaj *et al.* 2010). The aim is to improve specific traits of a species, e.g. yield or pest resistance. Any species which has been genetically engineered is classed as a genetically modified organism (GMO), it can then be further classified dependent on the type of genetic manipulation.

Transgenesis is genetic manipulation in which genetic material from one species is inserted into another species which it could not naturally hybridise with (Holme *et al.* 2013). This technology was rapidly taken up two decades ago when first introduced as a method of increasing crop yields, such that 160 million hectares of transgenic crops were grown in 29 countries in 2013 (Holme *et al.* 2013).

Other biotechnology methods which do not involve mixing genetic material from species which do not naturally breed are cisgenic and intragenic technology. Cisgenesis refers to a genetically engineered organism in which the donor gene, and all regulatory sequences,

are from either the same species as the host or another species which it can naturally crossbred with, and the entire ‘cisgene’ is identical to its original state with no changes are made (Sticklen 2015, Wang & Brummer 2012). Intragenesis is similar to cisgenesis in that the donor gene and gene regulatory sequences come from the same sexually compatible gene pool. However, intragenesis also allows for new gene combinations to be created in vitro, i.e. new promoter regions, coding regions and terminal regions can be utilised from other genes (Sticklen 2015, Wang & Brummer 2012).

There are concerns with transgenic material, that if used in outcrossing species, such as perennial ryegrass, the readiness of plants to cross with other populations may result in rapid spread of transgenes (Holme *et al.* 2013). As a result, there are stringent regulations for the development of transgenic cultivars (Wang & Brummer 2012). However, as intragenesis and cisgenesis use the same gene pool as a traditional breeding system this is considered to be of less concern, and hence these methods may potentially make more progress (Holme *et al.* 2012).

2.7 Summary and objectives

2.7.1 Summary of literature review

As pasture is a low cost feed, maximising pasture yield is an important goal for farmers and plant breeders in New Zealand. To date, conventional breeding is estimated to have achieved genetic gains in dry matter yield of 0.76% annually (Harmer *et al.* 2016). Currently the average time from the beginning of a breeding programme, through to the release of a commercial cultivar, is between 10 and 15 years (Lee *et al.* 2012). Promising novel breeding methods have the potential to be incorporated into current breeding methods and improve rates of genetic gain. However, currently novel breeding methods have had minimal impact in commercial plant improvement programmes.

2.7.2 Objectives of thesis

The novel breeding tool, marker assisted selection, and the development molecular markers for the *S* and *Z* alleles, has enabled further advances in the self-incompatibility hybrid breeding method in perennial ryegrass (section 2.6.3.3). Recently four inbred lines of perennial ryegrass, derived from five progenitor cultivars and one progenitor ecotype, were produced from the breeding strategy described by Pembleton *et al.* (2015), based around the SI hybrid breeding method proposed by England (1974). All six possible crosses of the four inbred lines were performed, generating six populations with theoretically up to 83% hybrid progeny. This is the first time that hybrid lines of New Zealand perennial ryegrass cultivars have been produced, and therefore the first opportunity to assess the effects of the breeding method on population phenotypic diversity and trait expression.

The first experiment described in this thesis uses the new hybrid plant material, in which there is interest in quantifying some of the morphological traits. This experiment compared dry matter yield, and the variability in morphological traits within the progenitor cultivars, parent lines and F1 hybrid populations. The hypothesis for experiment one was that population uniformity would increase from the progenitor cultivars through to the F1 hybrids, due to the cycles of inbreeding used to generate the parent lines. The experiment sought to validate this theory and provide an indication of the extent to which uniformity increases. Due to the small number of crosses available (n = 6), it was considered unlikely that the experiment would detect clear and strong yield gains. However, it was expected that an improvement in hybrid yield relative to the mean yield of the two restricted parent lines should be observed, i.e. mid-parent heterosis.

The second experiment described in this thesis focused on using dry matter yield to assess the expression of, and variation in, hybrid vigour in the F1 progeny of full-sib plant crosses, i.e. individual pair crosses. There were two types of crosses used in the experiment with varied genetic origins, ‘Alto’ x (‘Alto’ x ‘Tolosa’) and ‘Alto’ x ‘Rohan’. With the further development of the perennial ryegrass SI hybrid breeding method (the basis of experiment one), information gathered from the second experiment could provide a useful indication of which plant crosses have the best general combining ability, and therefore give the best chances of capturing strong hybrid vigour. Additionally, it was also expected that the experiment would give an indication of the extent of variability in hybrid vigour within the F1 populations. Currently breeders have relatively little information on the general combining ability of sub-populations in their breeding pools, and therefore a limited ability to identify the best plants to enter into the SI hybrid

breeding pipeline. The hypothesis for experiment two was that it is expected that an improvement in the dry matter yield of the F1 offspring would be observed relative to the mean yield of the two individual parent plants, and additionally, that the expression of heterosis in the F1 progeny would vary dependent on the genetic origins of the parent cultivars. Furthermore, it was expected that there would be significant variation in hybrid vigour in the F1 populations.

If the theoretical expectations of the proposed SI hybrid breeding method are fulfilled in practice, there is the prospect that this method may become a key tool in future breeding programmes, with the potential to significantly improve rates of perennial ryegrass genetic gain. The information from these two experiments has the potential to improve understanding of the extent to which hybrids from the proposed breeding method match with breeding theory, and help streamline the process of selecting populations to inbreed and cross to capture heterosis.

The specific objectives of this thesis therefore are:

Experiment one:

i. Investigate if early proof of increased yield performance can be detected in F1 hybrids produced using the SI breeding method.
ii. Quantify the vegetative morphological traits, and compare the variation in the vegetative morphological traits, between progenitor cultivar, parent line and F1 hybrid populations produced using the SI breeding method.

Experiment two:

i. Quantify the expression of, and variation in, hybrid vigour in dry matter yield observed in the F1 progeny from pair crosses of cultivars from differing genetic origins.

Chapter 3 Experiment 1: Self-incompatibility hybrid breeding; the growth and morphology of hybrids compared with parent lines and progenitor cultivars

3.1 Introduction

Hybrid breeding is a method which is commonly used in self-compatible species, such as maize, to achieve significant yield increases through exploitation of heterosis (Brummer 1999). This breeding method has not previously been used to the same extent in perennial ryegrass because ryegrass is an outcrossing, self-incompatible species (Thorogood *et al.* 2002). Current perennial ryegrass breeding methods have delivered low rates of genetic gain in dry matter yield (0.76% per year post 1990 (Harmer *et al.* 2016)).

Through the development of *S* and *Z* molecular markers, self-incompatibility in perennial ryegrass can now be controlled, enabling the development of inbred lines. These lines can be crossed to create F1 hybrids which theoretically exhibit significantly increased dry matter yield relative to their parents (Pembleton *et al.* 2015). As described in Pembleton *et al.* (2015), the proposed hybrid breeding method uses marker assisted selection to control the combinations of the alleles responsible for compatibility in perennial ryegrass. Two separate lines are created from selected progenitor gene pools. In each line the diversity of the alleles responsible for compatibility are restricted to different, specific, combinations. This enables the control of compatibility within and between each line. The aim is to ensure each line can inbreed, but also that compatibility *between* the two lines is greater than compatibility *within* each line. The progenitor gene pools of the parental lines are selected to be genetically diverse. Following the use of MAS to select

desirable SI alleles, the two lines are inbred for two cycles, which increases genetic uniformity relative to current cultivars (Acquaah 2012). As the two lines have been selected to have a high degree of between line compatibility, theoretically, when crossed approximately 83% of progeny produced will be F1 hybrids resulting from between line pollination. While the remaining 17% will be inbred, occurring due to pollination within either of the parental lines (Posselt 1993). The hybrid offspring which are produced would be expected to be heterozygous and genetically uniform, due to the cycles of inbreeding in the proposed method (Acquaah 2012).

The proposed breeding method is at the early stages of development (Inch, personal communication, 1 August 2017). There has been little evaluation of the hybrid plants created by this method, thus it is not known exactly what level of hybrid vigour is to be expected. Only a small number of crosses have been completed so far (Inch, personal communication, 1 August 2017). Potentially, many crosses may need to be performed and screened to find a sub-set of hybrids that express strong hybrid vigour above the highest yielding parent in the cross (high-parent heterosis), and that outperform current commercial cultivars. However, it is expected that an improvement in the hybrid yield relative to the mean yield of the two parent lines (mid-parent heterosis) should be observed, even from a small sub-set of crosses.

Perennial ryegrass cultivars must be phenotypically uniform, in order to gain Plant Variety Rights (Ministry of Business Innovation and Employment n.d.), however current cultivars bred by traditional breeding methods are not genetically uniform (Snaydon 1978). The proposed breeding method involves cycles of inbreeding to create the parent

lines, therefore the parent line and F1 hybrid populations should increase in uniformity relative to the progenitor cultivars (Janick 1998). Hence, changes in the pattern of genetic variability within the inbred parent lines and F1 hybrid populations, relative to current cultivars, could provide a good indication of the extent to which the breeding method is succeeding in changing the structure of the populations.

Increased population uniformity could also have significant impacts on the ecology of pasture plant populations. Environmental conditions, such as climate and pasture management, are variable and the genetic variability of a population can influence its ability to adapt to environmental variation (Snaydon 1978). Therefore, this experiment focuses on quantifying several morphological variables, and the changes in the variability/uniformity of these variables, throughout the key stages of the hybrid breeding process. This information is important in helping to understand the potential impacts of using this breeding method to create commercial hybrids on the ecology of pasture plant populations.

3.2 Objectives

- Investigate if early proof of increased yield performance can be detected in F1 hybrids produced using the self-incompatibility breeding method.

- Quantify the vegetative morphological traits, and compare the variation in the vegetative morphological traits, between progenitor cultivar, parent line and F1 hybrid populations produced using the self-incompatibility breeding method.

3.3 Materials and methods

3.3.1 Treatments

Three ‘treatments’ were used to represent the different stages in the hybrid perennial ryegrass breeding process; Progenitor cultivars (n = 5 commercial cultivars, one progenitor ecotype was also used (Table 3.1), however no seed of that ecotype was available for this experiment), Inbred parent lines (n = 4) and F1 Hybrids (n = 6). The progenitor gene pools used to develop the inbred parent lines were selected from widely dispersed geographic centres of origin, in order to maximise genetic diversity, and therefore the probability of the expression of hybrid vigour in the F1 hybrids (Table 3.1). The four parent lines were crossed in all possible combinations to create the six F1 hybrids (Table 3.1).

Table 3.1 Progenitor cultivars and ecotype genetic origins and characteristics, and relationships between progenitor cultivars, ecotypes, inbred parent lines and hybrids. Heading date relative to the cultivar 'Grasslands Nui'.

Progenitor	Identified genetic origins	Characteristics
Pro1	United Kingdom and European (Stewart 2006).	Heading date: +15 days. Selected for increased levels of water soluble carbohydrates (Specialty Seeds Ltd. n.d.).
Pro2	Mangere ecotype and north west Spain (Stewart 2006).	Heading date: +14 days. Selected for summer and winter growth (New Zealand Agriseeds Limited 2007).
Pro3	Mangere ecotype and north west Spain (Stewart 2006).	Heading date: +5 days. Selected for dry matter yield in early spring and autumn, survival in drought conditions and under insect pressure (New Zealand Agriseeds Limited n.d.).
Pro4	Mangere and possibly Hawkes Bay ecotype, north west Spain (Stewart 2006) and a *Lolium perenne* x *Festuca pratensis* cross (Cropmark Seeds Australia 2001).	Heading date: +23 days. Selected high tiller density, and winter growth (Cropmark Seeds Australia 2001).
Pro5	Elite Spanish and New Zealand breeding lines (PGG Wrightson Seeds Ltd 2007)	Heading date: +20 days. Selected for dry matter yield and disease resistance (PGG Wrightson Seeds Ltd 2007).
Pro6	Belgium.	An ecotype. Unknown characteristics.

Inbred parent line	Progenitor genepools
Par1	Pro4 x Pro1
Par2	Pro3 x Pro3
Par3	Pro1 x Pro6
Par4	Pro5 x Pro2

Hybrid	Inbred parent line
H1	Par1 x Par2
H2	Par1 x Par3
H3	Par1 x Par4
H4	Par2 x Par3
H5	Par2 x Par4
H6	Par3 x Par4

3.3.2 Experimental design

Fifty seedlings of each progenitor cultivar, parent line and hybrid were potted up and placed in a glasshouse on tables. A randomised block design with 5 replicates was used in order to minimise the effects of spatial variation within the glasshouse environment. The sample size of 50 plants was decided based on the results of the power analysis (Appendix 1). This sample size assumed a minimum reduction in the standard deviation (used as an estimate of variation) from one population to another of ~37% in order to get over 80% power of detecting a change in variability between two populations (e.g. Parent population SD = 1, Hybrid population SD = 0.63, refer to Appendix 1 for further details).

Each replicate block consisted of 10 pots of each progenitor cultivar, parent line and hybrid. Within a replicate block the 10 pots of each progenitor cultivar, parent line and hybrid were blocked together so that environment variation among them was minimised and genetic variation within each line could be estimated. The experiment ran over summer and early autumn, from January through to April 2018.

3.3.3 Plant establishment and maintenance

Treatments were sown on the 16th of October 2017 at QuikStart Seedlings, Christchurch, New Zealand. The seed was sown in 64 cell Lannen trays, one seed per tray. Three trays of each of the progenitor cultivars and hybrids were sown, and four trays of the parent lines due to an expectation that germination rates of the parent line seed would be lower (n = 49 trays). This expectation was confirmed in germination results (Appendix 2). Following a period of four days in a germination chamber, which was maintained at a

temperature of 23°C, trays were then transferred into a tunnel house for 38 days. On the 27th of November 2017, one tray of each progenitor and hybrid, and two trays of each parent, were randomly selected and transported via refrigerated freight truck to the Massey University Plant Growth Unit (PGU), Palmerston North, New Zealand.

The seedlings were transplanted into 1.7 litre planter bags on the 30th of November 2017. The planter bags were filled with a soil mix of 57% Manawatu silt loam (B horizon), 29% sand and 14% seed raising mix. For every 35 litres of soil mix, 40 grams of long term fertiliser (composition presented in Appendix 3), 75 grams of short term fertiliser (composition presented in Appendix 4) and 50g of Dolomite were included. Liquid fertiliser (1g fertiliser per 1000ml; composition presented in Appendix 5) was applied at a rate of 100 ml per plant on 1st March 2018 when signs of nutrient exhaustion were observed. Capillary irrigation was used to maintain soil moisture. All pots were supplied the same amount of water, and additional overhead watering was used when required over the summer period.

Crown rust, caused by the fungus *Puccinia coronata*, was detected on the plants on February 27th 2018. Proline fungicide (active ingredient: prothioconazole) was applied at a rate of 0.3ml per litre of water to control the crown rust, applied via Knapsack sprayer, and a repeat application was applied a month later as per label recommendations. Orthene (active ingredient: Acephate) was applied at a rate of 5g per litre of water on the 12th March to control Aphids. Supra-optimal glasshouse temperatures in January placed the plants under heat stress (discussed in section 3.4.4 and section 3.5.1.2). In order to reduce the heat stress the plants were under, the plants were removed from the glasshouse and

placed in a shade house (Figure 3.1) following Harvest 2. Harvest 3 occurred at the end of this period and the plants were moved back into the glasshouse for the final growth period. To reduce the glasshouse temperature, a shade cloth was placed on top of the glasshouse for the regrowth period leading up to Harvest 4 (Figure 3.1).

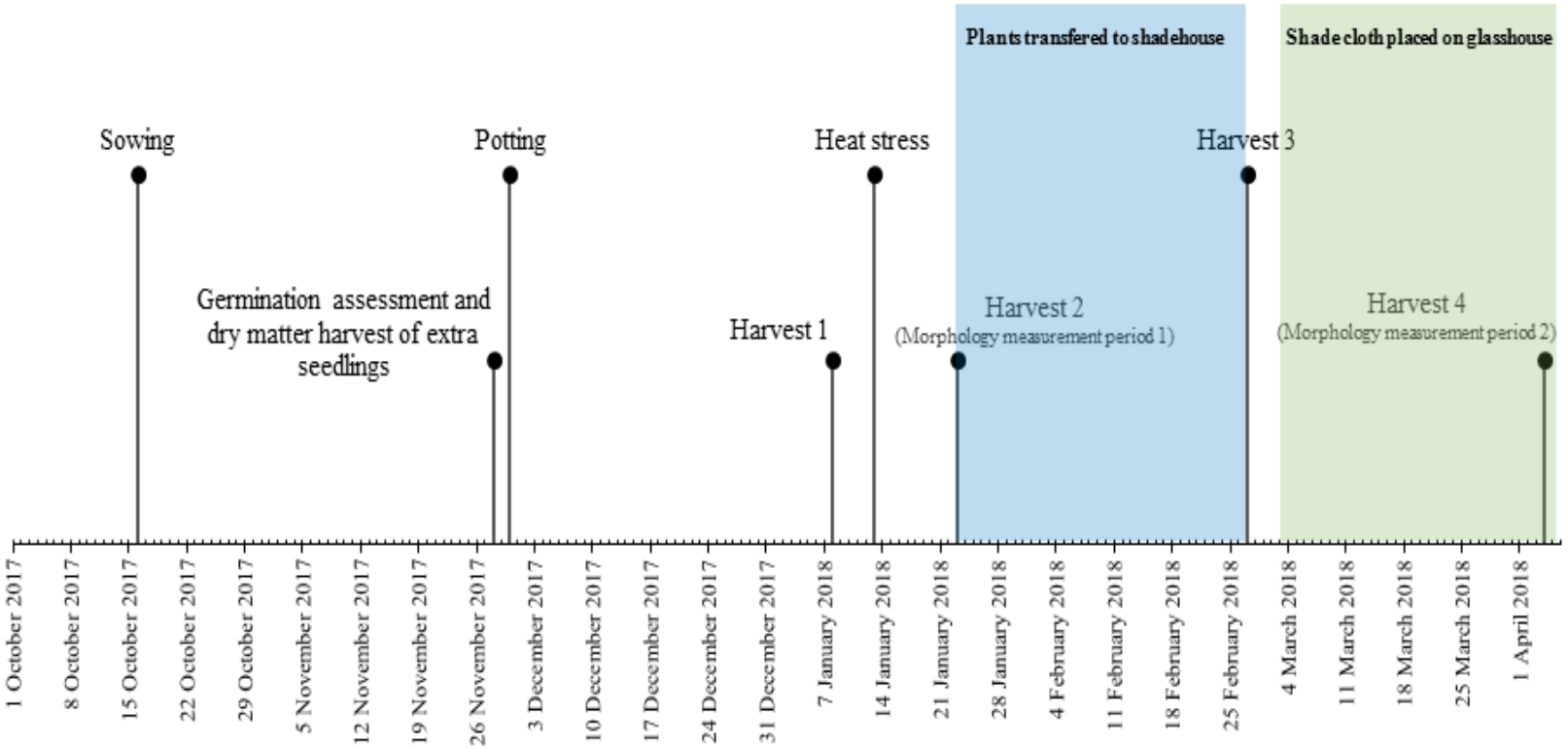


Figure 3.1 Experiment 1 timeline, October 2017 – April 2018.

3.3.4 Measurements

3.3.4.1 Establishment

In the initial germination phase, the number of seeds that germinated successfully per tray was recorded (Appendix 2). A dry matter harvest was also completed on the extra trays of seedlings which were not transported to the Massey University Plant Growth Unit (Appendix 6). This harvest was conducted on the 28th of November 2017 (plants were harvested to soil level).

3.3.4.2 Dry matter yield

All plants were cut to a standard height of 6 cm using electric shears on the 8th of January 2018, 38 days after seedlings were transplanted into pots (Harvest 1, Figure 3.1). Herbage was collected, oven-dried for a minimum of 48 hours and weighed. Dry matter yield was measured three more times over the course of the experiment (Figure 3.1), and plant morphology data were collected at the end of the regrowth cycles coinciding with Harvest 2 and 4. For the purposes of this experiment a regrowth cycle was defined as the period between cutting to the standard height and regrowth to the 2½ - 3 leaf stage. It was expected that there would be very little variation in leaf appearance interval between plants, therefore data collection and harvests occurred at the same time for all lines.

3.3.4.3 Morphology

Leaf morphology measurements were completed on 2 tillers per plant for every plant (n = 750 x 2). The following variables were measured:

Lamina width of the youngest fully emerged leaf was measured using digital calipers, at a point halfway between the ligule and lamina tip. If there was no fully emerged leaf, it was noted and the emerging leaf was measured.

Lamina length of the emerging, and youngest fully emerged, leaves were measured using a ruler, from the ligule to the lamina tip.

Pseudo-stem diameter was measured halfway up the sheath of the tiller using digital calipers.

Leaves per tiller were counted.

Total tillers per pot were counted post dry matter harvest, using the 6cm of tiller stubble.

Health status of the plants was visually scored, using a 1-5 scoring system (Appendix 7), as a result of the plants being exposed to a period of heat stress following Harvest 1 (Figure 3.1, and described in the section 3.4.4).

Lamina area was derived from the leaf width and length measures using the equation: area = 0.7 x (length x width) (Robin *et al.* 2010).

3.3.4.4 Glasshouse temperature

Temperature data for the glasshouse were collected prior to the beginning of the experiment. Temperature micro loggers were placed in a grid layout in the glasshouse to collect these data. Radiation screens were used with the micro loggers to protect them from the sun and provide a passive air flow to minimise error in data collected. This information was used to identify temperature zones and make decisions on how to position replicate blocks. Temperature data were also collected over the course of the experiment using a micro logger in a shade box with a fan for air circulation, continuously sampling glasshouse ambient air at approximately 1.5 m above ground level.

3.3.5 Statistical analysis

Using Microsoft Excel, the mean and standard deviation (as an estimate of variation) of each variable were calculated from the raw data. This was completed for each hybrid, parent line and progenitor cultivar, in each replicate block.

GenStat (2014) was used for all analyses. An analysis of variance (ANOVA) of each variable as a randomised block design, with 'Rep' as the blocking factor, was carried out. Three ANOVAs with different treatment factors were run; 1) 'Treatment' (i.e. Hybrids vs Parents vs Progenitors); 2) 'Line within Treatment' (i.e. for the Hybrids: Hybrid 1 vs Hybrid 2… etc.) and, 3) 'Line' (comparing all 15 lines; Hybrids, Parents, and Progenitors). A Fishers Protected least significant difference (LSD) multiple comparison was used to assess whether differences between the treatments and between the individual hybrids, parent lines and progenitor cultivars were significant.

In order to detect signs of hybrid vigour, the difference between the hybrid and the mid-parent mean and high parent mean were calculated. In order to test the significance of the difference between the hybrid and mid-parent mean, the difference was then divided by the SED of the hybrid-parent relationship. This SED of the hybrid-parent relationship was calculated by multiplying the SED from the Line treatment ANOVA by 0.866 (Assumptions for the value 0.866 shown in Appendix 8). Using Microsoft Excel, a t-test was then completed to calculate a P value indicating the significance of the mid-parent or high-parent heterosis. In order to test the significance of the high-parent mean, the difference was divided by the line SED and a t-test was then completed.

Not all plants were at the target leaf stage during the morphology measurement periods, and where they did not have a youngest fully expanded leaf, the width of the emerging leaf was measured instead. Hence, the analyses which have been completed for the emerging leaf width and youngest fully emerged leaf width, were not completed using full sets of data, rather the 1500 data collected for leaf width (n = 750 pots x 2 readings per pot) were split, dependent on the leaf stage of the individual plant.

3.4 Results

3.4.1 Dry matter yield

3.4.1.1 Progenitor, Parent, and Hybrid treatment means

The mean dry matter yield of the Hybrids was 53%, 45%, 19% and 15% greater than the Parents for Harvests 1 to 4, respectively (Table 3.2; the difference was significant ($P < 0.05$) for all harvests, except Harvest 4). The total dry matter yield of the Hybrids over the four harvests was 37% greater than the Parents (Table 3.2; $P < 0.001$).

The dry matter yield of the Hybrids was 17%, 5%, -1% and 7% greater than the Progenitors for Harvests 1 to 4, respectively (Table 3.2; the difference was significant at Harvest 1, $P < 0.001$) The total dry matter yield of the Hybrids was 11% greater than the Progenitors ($P < 0.001$).

The mean dry matter yield of the Progenitors was 31%, 38%, 20% and 8% greater than the Parents for Harvests 1 to 4, respectively (Table 3.2; the difference was significant ($P < 0.05$) for all harvests, except Harvest 4). The total dry matter yield of the Progenitors was 47% greater than the Parents ($P < 0.001$).

Overall, initially at Harvest 1, there were significant differences between all treatments (Table 3.2; $P < 0.001$, Hybrids > Progenitors > Parents), however differences declined over time. At Harvest 2 and 3, there was no difference between the Hybrids and the Progenitors, but both still yielded significantly more than the Parents ($P < 0.001$ and $P = 0.016$ for Harvest 2 and 3, respectively). However, at Harvest 4 there were no differences between any of the treatments. The significant yield differences at Harvest 1, compared

with Harvest 2, 3 and 4, flowed through into the total dry matter yield from the four harvests, where significant differences among all three treatments were also observed ($P < 0.001$).

3.4.1.2 Differences in lines within treatments

Significant differences in dry matter yield were also observed among the lines within each of the treatments (Table 3.2).

Differences among Hybrids

At Harvests 1, 2 and 4, significant differences in dry matter yield were observed among the hybrid lines (Table 3.2; $P < 0.05$). The difference in total dry matter yield among the hybrid lines tended towards significance ($P = 0.061$). The ranking order of the individual hybrids was variable over the four harvests, for example, Hybrid 3 was the highest yielding hybrid at Harvest 1, but was the lowest yielding at Harvest 4 (Table 3.2).

Differences among inbred parent lines

Parent 4 had a greater dry matter yield than Parents 2 and 3 at Harvest 1 (Table 3.2; $P < 0.05$), a greater dry matter yield than Parents 1 – 3 at Harvest 2 ($P < 0.01$), and a greater total dry matter yield than Parents 1 – 3 ($P < 0.05$). At Harvest 4, Parent 1 had a lower dry matter yield than Parents 2 – 4 ($P < 0.05$).

Table 3.2 Mean dry matter yields (g DM per plant), P values and SED for treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Harvest									
	1		2		3		4		Total	
Treatment										
Hybrid	7.88	a	1.77	a	2.40	a	1.47		13.57	a
Parent	5.14	c	1.22	b	2.01	b	1.28		9.92	c
Progenitor	6.72	b	1.68	a	2.42	a	1.38		12.27	b
P value	<.001		<.001		0.016		0.131		<.001	
Line within treatment										
H1	7.40	b	1.49	b	2.12		1.51	a	12.66	
H2	7.07	b	1.87	ab	2.51		1.35	ab	12.80	
H3	9.69	a	2.23	a	2.33		1.17	b	15.41	
H4	7.49	ab	1.50	b	2.52		1.53	a	13.04	
H5	8.15	ab	1.88	ab	2.41		1.62	a	14.20	
H6	7.46	b	1.63	b	2.53		1.62	a	13.31	
P value	0.017		0.009		0.592		0.034		0.061	
SED	0.709		0.196		0.260		0.143		1.944	
Par1	5.38	ab	1.01	b	1.85		0.83	b	9.07	b
Par2	4.43	b	0.88	b	1.72		1.37	a	8.85	b
Par3	4.58	b	1.03	b	1.97		1.51	a	9.33	b
Par4	6.18	a	1.95	a	2.49		1.42	a	12.42	a
P value	0.020		0.001		0.362		0.034		0.011	
SED	0.521		0.217		0.440		0.471		0.989	
Pro1	5.73	c	1.48		2.38		1.34		10.94	b
Pro2	6.05	bc	1.59		2.30		1.30		11.24	b
Pro3	6.98	abc	1.89		2.90		1.75		13.67	a
Pro4	7.62	a	1.72		2.36		1.11		12.97	a
Pro5	7.21	ab	1.74		2.16		1.42		12.53	ab
P value	0.035		0.142		0.190		0.134		0.011	
SED	0.614		0.154		0.301		0.229		0.755	
Line										
P value	<.001		<.001		0.089		0.004		<.001	
SED	0.625		0.195		0.329		0.197		0.920	

3.4.1.3 Individual hybrid dry matter yields relative to the Progenitors

The level of individual hybrid performance relative to the Progenitors ranged from an increase in total dry matter yield of 3% (Hybrid 1) up to 26% (Hybrid 3) (Table 3.3). The top 3 hybrids for total dry matter yield, relative to the Progenitors, were Hybrid 3, 5 and 6. All six hybrids yielded greater than the mean of the Progenitors at Harvest 1 (Table 3.3). At Harvest 2 and 3, only three of the hybrids yielded greater than the Progenitors, and at Harvest 4, four of the hybrids out yielded the Progenitors (Table 3.2).

The consistency of yield production over the four harvests varied from hybrid to hybrid. While Hybrid 3 appeared to perform well relative to the Progenitors at Harvest 1, and overall when comparing the total dry matter yields, over time yield significantly reduced to the point where at Harvest 3 and 4, Hybrid 3 was yielding less than the Progenitors. While there were some hybrids which maintained dry matter production better relative to Hybrid 3, each of the six hybrids yielded less than the Progenitors in at least one harvest.

Table 3.3 Hybrid dry matter yield relative to the mean of the Progenitors.

	Harvest									
	1		2		3		4		Total	
	g DM	%	g DM	%	g DM	%	g DM	%	g DM	%
Hybrid										
H1	7.40	110	1.49	89	2.12	87	1.51	109	12.66	103
H2	7.07	105	1.87	111	2.51	103	1.35	98	12.80	104
H3	9.69	144	2.23	133	2.33	96	1.17	84	15.41	126
H4	7.49	111	1.50	89	2.52	104	1.53	111	13.04	106
H5	8.15	121	1.88	111	2.41	99	1.62	117	14.20	116
H6	7.46	111	1.63	97	2.53	104	1.62	117	13.31	108
Progenitor	6.72		1.68		2.42		1.38		12.27	

3.4.1.4 Heterosis

At Harvest 1 and 2, all hybrids, with the exception of Hybrid 6 at Harvest 2, had a greater dry matter yield than the mean of their two parents, i.e. displayed mid-parent heterosis (Table 3.4; $P<0.001$). However, at Harvest 3 only Hybrid 2 and 4 displayed mid-parent heterosis, and at Harvest 4 only Hybrid 1 displayed mid-parent heterosis. All six of the hybrids displayed mid-parent heterosis for total dry matter yield ($P < 0.001$ for Hybrids 1 - 5 and $P = 0.003$ for Hybrid 6).

All hybrids had greater dry matter yield than their highest yielding parent, i.e. high-parent heterosis at Harvest 1 (Table 3.4; $P < 0.05$). Hybrids 1, 2 and 4 displayed high-parent heterosis at Harvest 2 ($P < 0.05$). However, there were no hybrids which displayed high-parent heterosis at Harvest 3 or 4. For total dry matter yield, Hybrids 1-4 displayed high-parent heterosis ($P < 0.05$).

Table 3.4 Summary of mid-parent heterosis (g DM), high-parent heterosis (g DM) and P values. A significant P value indicates the hybrid is significantly greater than its mid-parent mean or high-parent mean.

	Harvest									
	1		2		3		4		Total	
	g DM	P value	g DM	P value	g DM	P value	g DM	P value	g DM	P value
Mid-parent heterosis										
H1	2.50	<.001	0.55	0.002	0.33	0.246	0.41	0.020	3.70	<.001
H2	2.09	<.001	0.85	<.001	0.60	0.041	0.19	0.281	3.60	<.001
H3	3.91	<.001	0.75	<.001	0.16	0.581	0.04	0.809	4.67	<.001
H4	2.99	<.001	0.54	0.002	0.68	0.020	0.10	0.580	3.95	<.001
H5	2.85	<.001	0.46	0.009	0.31	0.287	0.22	0.198	3.57	<.001
H6	2.08	<.001	0.14	0.404	0.30	0.296	0.16	0.367	2.44	0.003
High-parent heterosis										
H1	2.02	0.002	0.48	0.017	0.27	0.419	0.14	0.484	3.59	<.001
H2	1.69	0.009	0.84	<.001	0.54	0.108	-0.15	0.444	3.47	<.001
H3	3.51	<.001	0.28	0.158	-0.16	0.628	-0.25	0.203	2.99	0.002
H4	2.91	<.001	0.47	0.020	0.55	0.098	0.03	0.888	3.71	<.001
H5	1.97	0.003	-0.08	0.702	-0.08	0.812	0.20	0.320	1.78	0.058
H6	1.28	0.045	-0.32	0.108	0.04	0.900	0.11	0.569	0.89	0.338

3.4.2 Morphological variables

There was a significant effect of treatment on all morphological variables except the youngest fully emerged leaf length and youngest fully emerged leaf area in Measurement period 1 and 2, and emerging leaf length, stem diameter and emerging leaf area in Measurement period 2 (Table 3.5).

3.4.2.1 Emerging leaf length

In Measurement period 1 the length of the emerging leaf of the Parents was shorter than the Progenitors and Hybrids (Appendix 9; $P < 0.001$). There was no effect of treatment on the length of the emerging leaf at Measurement period 2. There were also significant differences in emerging leaf length among the Hybrid and Parent lines in the first measurement period.

3.4.2.2 Youngest fully emerged leaf length

There was no effect of treatment on the length of the youngest fully emerged leaf at either measurement period (Appendix 10). There were significant differences among lines within the three treatments at Measurement period 1 and among the parent lines in Measurement period 2.

3.4.2.3 Emerging leaf width

In Measurement period 1 the Hybrids had a wider emerging leaf than the Progenitors and the Parents (Table 3.5; $P = 0.004$). In Measurement period 2 the Hybrids had a wider

emerging leaf than the Progenitors (Table 3.5; P = 0.041). There were also significant differences among the Parent lines at the first measurement period.

3.4.2.4 Youngest fully emerged leaf width

In Measurement period 1, the Hybrids had a wider youngest fully emerged leaf than the Progenitors (Table 3.6; P = 0.035). In Measurement period 2, the Hybrids and the Parents had a wider youngest fully emerged leaf than the Progenitors (Table 3.6, P = 0.004). There were also significant differences among lines within the three treatments, except for the Progenitors in the first measurement period.

3.4.2.5 Stem diameter

In Measurement period 1, the Hybrids and Parents had a greater stem diameter than the Progenitors (Appendix 11; P = 0.006). There was no effect of treatment on stem dimeter in Measurement period 2.

3.4.2.6 Number of tillers per plant

At both measurement periods, the Hybrids and the Progenitors had a greater number of tillers per plant than the Parents (Table 3.7; P < 0.001). There were also significant differences among lines within the three treatments, except for the Hybrids in the first measurement period.

3.4.2.7 Leaves per tiller

In Measurement period 1, the Progenitors had more leaves per tiller than the Parents (Appendix 12; P= 0.036). In Measurement period 2, the Parents had more leaves per tiller

than the Progenitors and Hybrids, and the Progenitors had more leaves per tiller than the Hybrids (Appendix 12; $P < 0.001$). There were also significant differences among the Hybrid lines in the first measurement period, and among the Parent lines in the second measurement period.

3.4.2.8 Emerging leaf area

In Measurement period 1, the Hybrids and Progenitors had a greater (Table 3.8; $P < 0.001$) estimated emerging leaf area than the Parents. There were no differences between the emerging leaf area of the Hybrids compared to the Progenitors, however, the Hybrids did still have a larger leaf area in both measurement periods, with an estimated leaf area of 3.78 cm^2 relative to 3.63 cm^2 in Measurement period 1, and 2.71 cm^2 relative to 1.98 cm^2 in Measurement period 2. There was no effect of treatment in Measurement period 2. There were also significant differences the Hybrid and Parent lines in the first measurement period.

3.4.2.9 Youngest fully emerged leaf area

Treatment did not have an effect on the estimated youngest full emerged leaf area in Measurement period 1 or 2 (Table 3.9). While there were no significant differences, the Hybrids youngest fully emerged leaf area was larger compared to the Progenitors in both measurement periods, with an area of 3.99 cm^2 relative to 3.72 cm^2 in Measurement period 1, and 3.67 cm^2 relative to 3.33 cm^2 in Measurement period 2. There were differences among lines within the three treatments, except for the Progenitors in the first measurement period.

3.4.2.10 Health status

In Measurement period 1 the Hybrids and Progenitors had a greater mean health status (Appendix 13; both with a mean health status of 3.7) than the Parents (3.1; $P < 0.001$). In Measurement period 2 the Hybrids had a greater mean health status (3.5) than the Parents (3.3; $P = 0.037$). There were also significant differences among the Hybrid and Parent lines in the first measurement period.

Table 3.5 Summary of mean plant morphology variables P values for treatment, lines within treatment and line.

	Morphology measurement period									
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
	Emg leaf length		**YFE leaf length**		**Emg leaf width**		**YFE leaf width**		**Stem diameter**	
Treatment	<.001	0.799	0.065	0.344	0.004	0.041	0.035	0.004	0.006	0.071
Line within treatment										
Hybrid	<.001	0.240	0.005	0.213	0.061	0.777	<.001	<.001	0.675	0.261
Parent	<.001	0.064	<.001	0.018	<.001	-	0.003	0.048	0.887	0.144
Progenitor	0.507	0.242	0.028	0.068	0.289	0.256	0.569	0.019	0.316	0.211
Line	<.001	0.208	<.001	0.013	<.001	0.051	<.001	<.001	0.146	0.045
	Tillers per plant		**Leaves per tiller**		**Health status**		**Emg leaf area**		**YFE leaf area**	
Treatment	<.001	<.001	0.036	<.001	<.001	0.037	<.001	0.068	0.243	0.149
Line within treatment										
Hybrid	0.338	0.013	0.008	0.060	0.006	0.460	0.003	0.744	0.003	0.018
Parent	0.023	0.004	0.336	0.020	0.002	0.507	<.001	-	<.001	0.038
Progenitor	0.004	0.016	0.088	0.125	0.084	0.916	0.746	0.750	0.064	0.039
Line	<.001	<.001	0.006	<.001	<.001	0.366	<.001	0.128	<.001	0.003

Table 3.6 Mean emerging leaf width (mm), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	3.35	a	2.44	a
Parent	3.02	b	2.35	ab
Progenitor	3.14	b	2.16	b
P value	0.004		0.041	
Line within treatment				
H1	3.09		2.38	
H2	3.12		2.42	
H3	3.58		2.45	
H4	3.08		2.58	
H5	3.86		2.36	
H6	3.36		2.47	
P value	0.061		0.777	
SED	0.281		0.247	
Par1	2.64	c	2.16	
Par2	2.89	b	2.71	
Par3	2.72	bc	1.84	
Par4	3.84	a	2.69	
P value	<.001		-	
SED	0.103			
Pro1	3.33		1.87	
Pro2	3.09		2.21	
Pro3	3.32		2.26	
Pro4	3.05		2.21	
Pro5	2.94		2.26	
P value	0.289		0.256	
SED	0.204		0.218	
Line				
P value	<.001		0.051	
SED	0.209		0.243	

Table 3.7 Mean youngest fully emerged leaf width (mm), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	3.41	a	2.58	a
Parent	3.32	ab	2.61	a
Progenitor	3.21	b	2.44	b
P value	0.035		0.004	
Line within treatment				
H1	3.07	d	2.48	b
H2	3.19	cd	2.45	b
H3	3.45	bc	2.66	ab
H4	3.27	cd	2.45	b
H5	3.88	a	2.82	a
H6	3.61	ab	2.63	ab
P value	<.001		<.001	
SED	0.157		0.078	
Par1	2.94	b	2.41	b
Par2	3.31	b	2.78	a
Par3	3.12	b	2.61	ab
Par4	3.92	a	2.66	ab
P value	0.003		0.048	
SED	0.210		0.116	
Pro1	3.23		2.64	a
Pro2	3.26		2.44	a
Pro3	3.32		2.43	a
Pro4	3.02		2.14	b
Pro5	3.24		2.53	a
P value	0.569		0.019	
SED	0.185		0.131	
Line				
P value	<.001		<.001	
SED	0.173		0.119	

Table 3.8 Mean number of tillers per plant, P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	83	a	70	a
Parent	63	b	49	b
Progenitor	82	a	72	a
P value	<.001		<.001	
Line within treatment				
H1	81		69	b
H2	90		86	a
H3	87		69	b
H4	82		70	b
H5	82		59	b
H6	74		67	b
P value	0.338		0.013	
SED	7.060		6.380	
Par1	65	a	54	ab
Par2	51	b	34	c
Par3	66	a	63	a
Par4	71	a	47	bc
P value	0.023		0.004	
SED	5.780		6.130	
Pro1	69	c	67	ab
Pro2	72	c	63	b
Pro3	92	ab	88	a
Pro4	100	a	81	ab
Pro5	80	bc	60	b
P value	0.004		0.016	
SED	7.620		8.260	
Line				
P value	<.001		<.001	
SED	6.700		6.910	

Table 3.9 Mean emerging leaf area (cm^2), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	3.78	a	2.71	
Parent	2.92	b	2.32	
Progenitor	3.63	a	1.98	
P value	<.001		0.068	
Line within treatment				
H1	3.03	cd	3.37	
H2	3.46	bcd	2.21	
H3	5.16	a	2.51	
H4	2.58	d	2.52	
H5	4.57	ab	2.87	
H6	3.87	bc	2.76	
P value	0.003		0.744	
SED	0.579		0.714	
Par1	2.39	b	1.62	
Par2	2.19	b	3.70	
Par3	2.16	b	1.53	
Par4	4.95	a	2.43	
P value	<.001		-	
SED	0.311		-	
Pro1	3.75		1.87	
Pro2	3.67		1.55	
Pro3	3.95		2.17	
Pro4	3.38		1.88	
Pro5	3.42		2.42	
P value	0.746		0.750	
SED	0.487		0.805	
Line				
P value	<.001		0.128	
SED	0.477		0.691	

Table 3.10 Mean youngest fully emerged leaf area (cm^2), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	3.99		3.67	
Parent	3.69		3.57	
Progenitor	3.72		3.33	
P value	0.243		0.149	
Line within treatment				
H1	3.19	b	3.76	abc
H2	3.96	ab	3.21	bc
H3	4.62	a	3.79	ab
H4	3.27	b	3.17	c
H5	4.56	a	4.13	a
H6	4.32	a	3.94	a
P value	0.003		0.018	
SED	0.385		0.294	
Par1	3.22	b	3.01	b
Par2	2.93	b	4.36	a
Par3	2.75	b	3.23	b
Par4	5.85	a	3.69	ab
P value	<.001		0.038	
SED	0.585		0.431	
Pro1	3.56		3.54	a
Pro2	3.95		3.23	ab
Pro3	3.85		3.46	a
Pro4	3.18		2.51	b
Pro5	4.08		3.90	a
P value	0.064		0.039	
SED	0.305		0.404	
Line				
P value	<.001		0.003	
SED	0.441		0.405	

3.4.3 Variability morphological traits

At the treatment level, the amount of variability (as explained using the standard deviation of the sample of 50 plants per line, see section 3.3.5) in leaf width, leaf length, leaf area and stem diameter did not differ at either measurement period (Table 3.11). These data are presented in the appendices (Appendix 14 - 20). The Progenitors had a greater amount of variation in leaves per tiller than the Hybrids in Measurement period 2 (Table 3.12; P = 0.047). The Progenitors and the Parents had a greater amount of variation in the number of tillers per plant compared with the Hybrids in Measurement period 2 (Table 3,13; P = 0,004). The Hybrids had less variation in health status than the Parents, and Progenitors, in Measurement period 1 (Table 3.14; P < 0.001), and both the Hybrids and Progenitors had less variation in health status than the Parents in Measurement period 2 (Table 3.14; P < 0.001).

Table 3.11 Summary of variability (estimated using sample standard deviation) in plant morphology measurements P values for treatment, lines within treatment and line.

	Morphology measurement period									
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
	Emg leaf length		**YFE leaf length**		**Emg leaf width**		**YFE leaf width**		**Stem diameter**	
Treatment	0.274	0.420	0.695	0.751	0.743	0.180	0.780	0.898	0.057	0.357
Line within treatment										
Hybrid	0.089	0.107	0.761	0.004	0.003	0.110	0.020	0.204	0.181	0.086
Parent	0.009	0.219	0.018	0.683	0.228	-	0.111	0.064	0.031	0.388
Progenitor	0.253	0.002	0.297	0.964	0.034	0.044	0.045	0.006	0.315	0.363
Line	0.003	0.021	0.041	0.356	0.007	0.004	0.002	0.018	0.004	0.193

	Tillers per plant		**Leaves per tiller**		**Health status**		**Emg leaf area**		**YFE leaf area**	
Treatment	0.153	0.004	0.173	0.047	<.001	<.001	0.660	0.052	0.881	0.291
Line within treatment										
Hybrid	0.449	0.657	0.637	0.362	0.335	0.184	0.001	0.066	0.140	0.056
Parent	0.017	0.052	0.343	0.801	0.515	0.132	0.013	-	0.120	0.319
Progenitor	0.137	0.001	0.504	0.238	0.081	0.220	0.940	0.605	0.045	0.489
Line	0.018	<.001	0.438	0.141	0.007	0.001	0.003	0.328	0.044	0.096

Table 3.12 Estimate of variation in number of leaves per tiller, P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period		
	1	2	
Treatment			
Hybrid	0.36	0.45	b
Parent	0.41	0.53	ab
Progenitor	0.35	0.57	a
P value	0.173	0.047	
Line within treatment			
H1	0.38	0.37	
H2	0.34	0.38	
H3	0.31	0.43	
H4	0.31	0.62	
H5	0.42	0.40	
H6	0.41	0.50	
P value	0.637	0.362	
SED	0.080	0.123	
Par1	0.45	0.55	
Par2	0.37	0.56	
Par3	0.40	0.50	
Par4	0.42	0.50	
P value	0.343	0.801	
SED	0.044	0.072	
Pro1	0.33	0.47	
Pro2	0.40	0.54	
Pro3	0.38	0.47	
Pro4	0.26	0.67	
Pro5	0.39	0.69	
P value	0.504	0.238	
SED	0.090	0.119	
Line			
P value	0.438	0.141	
SED	0.073	0.111	

Table 3.13 Estimate of variation in number of tillers per plant, P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	28		23	b
Parent	27		29	a
Progenitor	31		29	a
P value	0.153		0.004	
Line within treatment				
H1	33		25	
H2	25		22	
H3	27		23	
H4	29		25	
H5	26		19	
H6	27		25	
P value	0.449		0.657	
SED	3.820		4.420	
Par1	35	a	32	
Par2	20	b	27	
Par3	27	ab	36	
Par4	25	b	21	
P value	0.017		0.052	
SED	3.800		4.990	
Pro1	23		21	c
Pro2	28		25	bc
Pro3	40		43	a
Pro4	33		31	c
Pro5	30		26	bc
P value	0.137		0.001	
SED	6.090		4.210	
Line				
P value	0.018		<.001	
SED	4.663		4.453	

Table 3.14 Estimate of variation in health status, P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	0.52	b	0.54	b
Parent	0.76	a	0.85	a
Progenitor	0.68	a	0.57	b
P value	<.001		<.001	
Line within treatment				
H1	0.61		0.59	
H2	0.49		0.37	
H3	0.39		0.45	
H4	0.48		0.55	
H5	0.56		0.64	
H6	0.58		0.62	
P value	0.335		0.184	
SED	0.075		0.115	
Par1	0.80		0.67	
Par2	0.66		1.17	
Par3	0.88		0.79	
Par4	0.69		0.75	
P value	0.515		0.132	
SED	0.164		0.207	
Pro1	0.47		0.42	
Pro2	0.60		0.52	
Pro3	0.98		0.72	
Pro4	0.68		0.62	
Pro5	0.78		0.57	
P value	0.081		0.220	
SED	0.139		0.125	
Line				
P value	0.007		0.001	
SED	0.133		0.152	

3.4.4 Glasshouse temperature

In January, the air inside the glasshouse reached a peak temperature of 39°C due the glasshouse cooling fans not being able to compensate for high outdoor temperatures (Figure 3.2). A shade cloth was placed on top of the glasshouse for the regrowth period leading up to Harvest 4, this resulted in a reduction in glasshouse temperatures (Figure 3.2).

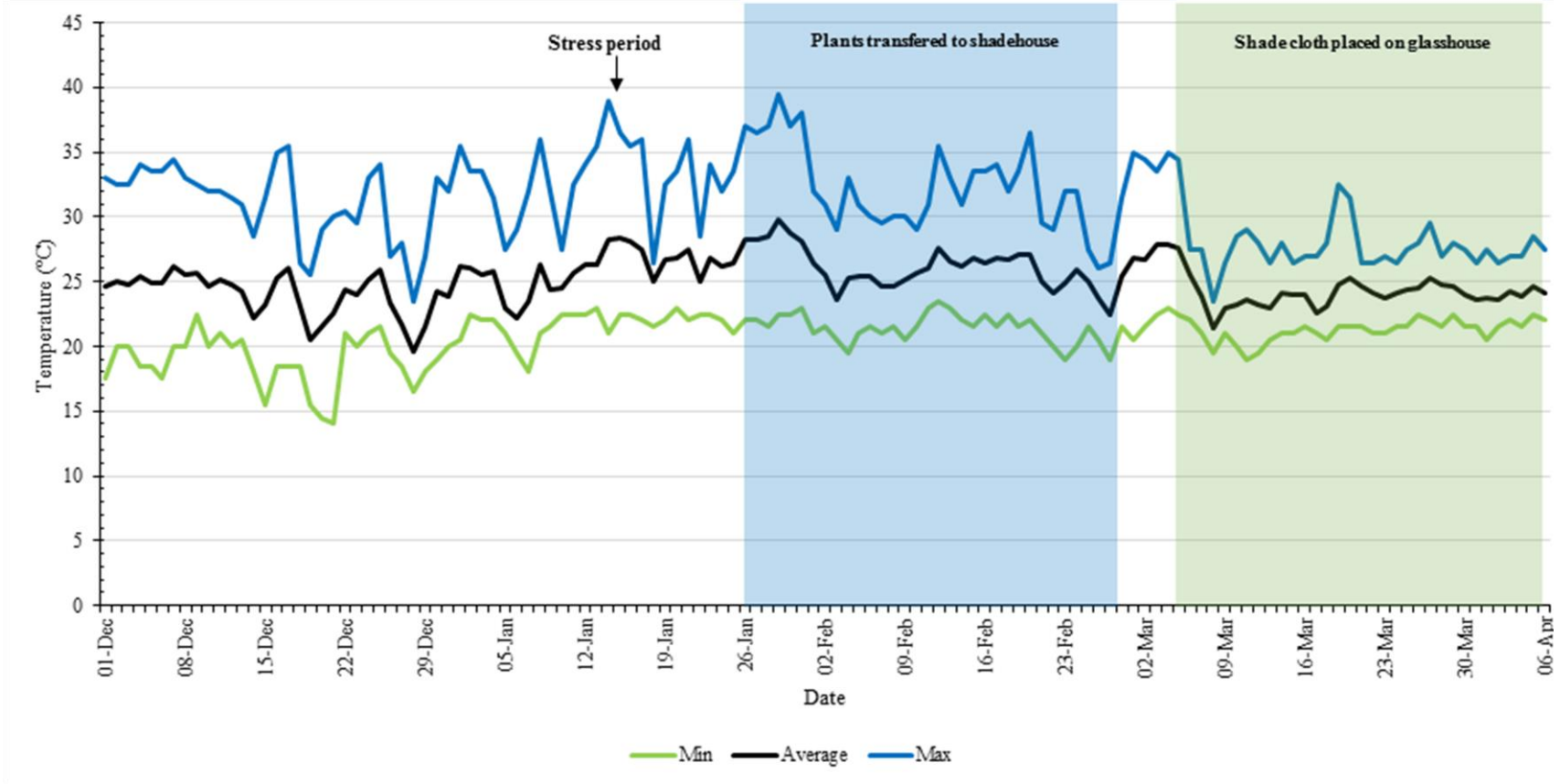


Figure 3.2 Glasshouse maximum, minimum and average temperature (°C) from December 2017 to April 2018.

3.5 Discussion

Four key questions underpinning this research are:

1. Does the SI hybrid method result in heterosis, i.e. do the F1 hybrids yield more than the mean yield of the two parent lines (mid-parent heterosis)?
2. Does the SI hybrid breeding method result in *significant* heterosis that is useful for plant improvement, i.e. do the F1 hybrids yield greater than the highest performing parent (high-parent heterosis) and current commercial cultivars?
3. What are the morphological determinants of any difference in dry matter yield observed?
4. Are changes in the genetic uniformity of a hybrid population (relative to current commercial cultivars), due to the cycles of inbreeding, overserved? This is of importance for helping to understand the practicality, and likely success, of the proposed method in producing commercial cultivars that persist in the variable environment of a grazed pasture.

With the aim of exploring these four questions, this discussion is structured around the following headings:

- Dry matter yield
    - Progenitor, Parent, and Hybrid treatment means
    - Pattern and persistency of heterosis
    - Individual hybrid performance relative to the Progenitors
    - Heterosis
    - Differences among inbred parent lines
- Morphological determinants of dry matter yield
- Variability in morphological traits

3.5.1 Dry matter yield

3.5.1.1 Progenitor, Parent, and Hybrid treatment means

The mean dry matter yield of the Hybrids was greater than the Parents for all harvests (except Harvest 4), and for the total yield from the four harvests. This indicates that the proposed breeding method does result in F1 hybrids with some level of heterosis relative to the parent lines that were crossed to create them.

Furthermore, the dry matter yield of the Hybrids was greater than the Progenitors at Harvest 1 and for total dry matter yield, which provides evidence that the proposed hybrid breeding method could lead to hybrids that substantially exceed the yield of current commercial cultivars. For the proposed breeding to be commercially successful, it must produce F1 Hybrids with a dry matter yield significantly greater than that of the best performing commercially available cultivars (Brummer 1999).

While the total dry matter yield was different between all three treatments, it needs to be acknowledged that Harvest 1 occurred after a longer regrowth period, relative to the other 3 harvests. As a result, Harvest 1 contributes more to the total dry matter yield, and therefore the total yield is influenced more significantly by Harvest 1 than the other three harvests.

3.5.1.2 Pattern and persistency of heterosis

The results showed a clear pattern of decreasing heterosis over the four harvests, such that the strong increases in yield of the Hybrids at Harvest 1 had completely dissipated by Harvest 4. Three possible explanations include:

Firstly, the longer growth period prior to Harvest 1 compared to the later harvests may have provided more time for expression of heterosis. In the seedling dry matter yield harvest six weeks post sowing (Appendix 6), there were no differences between the three treatments, supporting the idea that regrowth interval may play a role in heterosis expression. This raises the question, that if Harvest 2, 3 and 4 were given a longer regrowth period, would greater differences in dry matter yield among all three treatments have emerged?

Secondly, the initial yield advantages of heterosis are susceptible to loss over time. Such patterns of diminishing yields have been claimed in perennial ryegrass selected for low respiration (Robson *et al.* 1988), however there are no published data from other studies that address this phenomenon in perennial ryegrass bred to capture heterosis. While hybrid breeding has been a key method used in many crops and vegetables to increase yield, knowledge of the mechanisms behind heterosis is lacking (Fujimoto *et al.* 2018), however it is likely that if such a phenomenon existed in heterosis it would have been previously encountered in cereal breeding. The complex nature and lack of understanding of these mechanisms means that the persistency of heterosis needs further investigation. If heterosis in perennial ryegrass does deteriorate with time, then this could present a significant problem with adopting the proposed breeding methodology.

Thirdly, as a result of the significant increase in dry matter production observed in the Hybrids and the fact that experiment duration was extended for an additional regrowth period (discussed in the following section: effect of heat stress), nutrient exhaustion in the pots of small soil volume may have occurred. Signs of nutrient exhaustion were

observed in March and as a result plants were supplied with liquid fertiliser. This effect would have occurred first in the highest yielding lines.

Effect of heat stress

One factor which also needs to be considered when hypothesising about the cause of the pattern of heterosis observed, is the extreme temperatures which occurred over the course of the experiment. The plants were exposed to a period of heat stress when the glasshouse reached a high of 39°C between Harvest 1 and 2 (Figure 3.2). The optimum temperature for perennial ryegrass is between 20 and 25°C (Hannaway *et al.* 1997), and dry matter production is affected at daytime temperatures greater than 31°C and night-time temperatures greater than 25°C, regardless of soil moisture (Casler 2003). This period of heat stress, along with sustained temperatures above the optimal range for perennial ryegrass (Figure 3.2), likely had a significant effect on total dry matter yield measured at Harvest 2, and also potentially had carryover effects on dry matter yield at Harvest 3 and 4.

The period of heat stress in mid-January had three key implications for the experiment. Firstly, the target leaf stage of 2-½ - 3 leaves was not achieved at Harvest 2, due to lower growth rates relative to the expected rates of growth used to schedule the timing of the harvest. As a result, Harvest 2 (and Morphology Measurement period 1) occurred at an earlier leaf stage than planned; an average of 1.6 leaves per tiller. Secondly, plant health status was assessed in each measurement period (Appendix 7 and Appendix 13), to determine the extent of the impact of the heat stress on the populations and have the option of using this information to help explain the results. It was observed that the Parents were

more significantly impacted by the heat stress than the Progenitors and Hybrids, this was expected due to the cycles of inbreeding used resulting in inbreeding depression. Thirdly, the decision was made to include a 'recovery period' after Harvest 2 (Measurement period 1), to give the plants time to recover following the stress period.

While the recovery period was included in the experiment design to minimise the impact of the heat stress on the overall results of the experiment, the stress period may still have had an ongoing impact on Harvest 3 and 4 (and Measurement period 2). Therefore, this also presents a fourth possible explanation for the pattern of heterosis described earlier and raises the question as to whether the decrease in heterosis could have been caused, or partially caused, by the heat stress the plants experienced in mid-January. These possible explanations would need to be considered in further studies.

3.5.1.3 Individual hybrid performance relative to the Progenitors

Comparing the total yield of the individual hybrids to the average yield of the Progenitors (i.e. a representation of current commercial cultivars), it can be seen that the performance of the individual hybrids, relative to the mean of the Progenitors, ranges from an increase in dry matter yield of 3% (Hybrid 1) up to 26% (Hybrid 3). These same hybrids are also being tested in field trials conducted by a New Zealand breeding company. Preliminary results from field trials indicate similar yield differences between the hybrids and their progenitors as seen in this glasshouse experiment. Similar performance rankings were observed in the field trials, with Hybrid 5, Hybrid 6 and Hybrid 3 being the top 3 performing hybrids (Inch, personal communication, 28 September 2018). Varying levels of heterosis have also been observed in other species such as maize, and is dependent on

the parents used in the cross and the particular trait that is being measured (Stupar *et al.* 2008). The results of this study with perennial ryegrass confirm the expectation that not all six of the hybrids would have superior yield performance compared to current cultivars, and that it will take time to produce and identify high performing hybrids. Although, the performance of the hybrids was variable, the results are promising. In fact, given the level of heterosis observed in Hybrid 3 compared to the mean of the Progenitors, and the shorter generation interval of this breeding method compared to recurrent selection (which can take an average of 10 - 15 years to produce a commercial cultivar (Lee *et al.* 2012)), this method provides the potential for a step change in the dry matter yield of perennial ryegrass.

While Hybrid 3 performed well relative to the Progenitors at Harvest 1, its yield advantage declined significantly over time to the extent that at Harvests 3 and 4 it yielded less than the Progenitors. While increased dry matter yield is desirable in developing a commercial cultivar, selection for a hybrid which maintains its dry matter production advantage over time (i.e. persistency of yield) would also be important. Hence, the type of response seen in Hybrid 3 may not be the most desirable path forward. While some of the six hybrids in the experiment maintained their relative dry matter production better than Hybrid 3, yield still decreased to less that the Progenitors in the final harvests. This further supports the original hypothesis that in order to find a subset of hybrids which have superior performance, and have a set of traits which make them potentially suitable for commercial production, it is likely that a large number of crosses would need to be completed and evaluated.

3.5.1.4 Heterosis

The same pattern of decreasing heterosis was observed for both mid-parent and high-parent heterosis for the hybrids. As discussed in section 3.5.1.2, due to the period of heat stress in January, conclusions cannot be drawn regarding the cause of the trend of decreasing yield over time. Further studies would need to be completed to be able to draw conclusions on this.

While varying levels of heterosis were observed, the mid-parent heterosis results clearly indicate that the proposed breeding method successfully captured heterosis. Furthermore, the high-parent heterosis results showed potential for hybrids to out yield current cultivars, and therefore the potential for the proposed method to be commercially viable.

The level of heterosis evident in the hybrid plants was generated from the parental pools being inbred for two generations, prior to being crossed to create the hybrids. This presents the question: if this level of heterosis can be observed from only two cycles of inbreeding, then could even greater heterosis be achieved with further cycles? Additionally, if such levels of increased dry matter production are possible, what would this mean from a farm systems perspective?

3.5.1.5 Differences among inbred parent lines

Inbred parent line, Parent 4 had the greatest dry matter yield in all harvests, except for Harvest 4, and had the greatest total dry matter yield. While Parent 4 did not yield greater in all harvests, the results signal superior performance relative to the other three lines. An interesting connection between Parent 4 and the hybrids is that it is a common parent in

the top 3 performing hybrids, Hybrid 3, Hybrid 5, and Hybrid 6. This indicates that this parent line, primarily originating from north west Spain and the Mangere ecotype (New Zealand), has the genetic potential for high dry matter yield and has good general combining ability with the three other parent lines.

3.5.2 Morphological determinants of dry matter yield

The general trend of dry matter production observed in the experiment was that the Hybrids had a greater dry matter yield than the Progenitors, which had a greater dry matter yield than the Parents. This was expected due to the cycles of inbreeding involved in this method, resulting in inbreeding depression of the parent lines, but heterosis in the hybrids. However, this raises the question, *what were the key morphological determinants of the dry matter yield differences?*

For many of the variables, significant differences were detected in one measurement period but not the other. On some occasions, the measured variable was unexpectedly greater for the Parent lines than the Progenitors. However, number of tillers per plant was one variable which was consistently greater in the Progenitors compared to the Parents in both measurement periods. Therefore, number of tillers per plant was a source of the difference in dry matter yield between these two treatments in this experiment. Similarly, tiller number per plant was greater for the Hybrids compared with the Parents, and this was consistent in both measurement periods.

While this indicates that number of tillers per plant was consistently larger in the Hybrids and Progenitors, when compared to the Parents, and therefore was a source of the increased dry matter in this experiment. There was no difference in the number of tillers

per plant between the Hybrid and Progenitors. However, the leaf width of the Hybrids was greater than the leaf width of the Progenitors in both measurement periods. This indicates that larger leaf area, facilitated by greater leaf width, was a source of increased dry matter in the Hybrids relative to the Progenitors in this experiment. When leaf area was analysed the results did not show a significant difference between the Hybrids and the Progenitors. However, leaf areas tended to be greater for the Hybrids than the Progenitors, in both measurement periods. While this difference was not significant, the data trends suggest that that the greater leaf width of the Hybrids facilitates a larger leaf area (and also leaf weight). This morphological difference may explain the difference in dry matter yield between the Hybrids and the Progenitors.

3.5.3 Variability in morphological traits

Due to the cycles of inbreeding used in this breeding method, the genetic uniformity of the Progenitors, Parents and Hybrids are of interest. Genetic diversity is important for a populations ability to survive in changing conditions (Takayama & Isogai 2005). In theory, inbreeding results in an increase in genetic uniformity (Janick 1998). This could mean that commercial hybrids produced using this method, may be more vulnerable to changes in the environment, compared to current cultivar populations. However, there were few significant and consistent differences in the uniformity of the morphological traits between the three generations of plant material. This is a positive finding as significant heterosis without the potential ecological impacts hypothesised, removes one potential issue with this methodology, and makes the method more likely to be commercially viable.

The current breeding method only used two cycles of inbreeding when developing the inbred parent lines, which has been shown to achieve significant heterosis in some cases. The finding of little change in the uniformity of the hybrid populations compared to the progenitor populations poses the question: could additional cycles of inbreeding further increase heterosis without a significantly negative impact on genetic variability? Capturing even more heterosis through further cycles of inbreeding, and producing a commercial hybrid which still maintained sufficient genetic diversity, would most certainly be an achievement for plant breeders, and a possible step change in the rate of genetic gain in dry matter yield. This is a potential avenue for further investigation.

Another consideration is that while this was a large experiment, it may not have been of sufficient statistical power to detect changes in the variability of the morphological plant traits. With a sample size of 50 plants per progenitor cultivar, parent line and hybrid, it was estimated there would be sufficient statistical power (80%) to detect a change in the genetic variability between two populations of a minimum of 37% (Appendix 1). Therefore, if changes in the variability were less, then they may not have been detected in this experiment. By analysing the changes in variation at the treatment level (i.e. Hybrids vs Parents vs Progenitors) sample size was increased (e.g. Hybrid treatment = 6 hybrids x 50 plants per hybrid) which should theoretically have given more power to detect changes in variation between the three generations. However, a larger scale experiment may still be necessary to determine whether or not there are significant effects on the morphological variation. The data generated in this experiment would be useful for future experimental power analyses, since they provide an example of the variance that can be expected in these types of studies.

Chapter 4 Experiment 2: Combining ability; the influence of genetic origins on heterosis

4.1 Introduction

Forage plant breeders have always been interested in developing breeding methods which successfully capture heterosis (section 2.6.3). Heterosis can be expressed by most heterozygous forage species and can occur in the F1 progeny of both individual crosses and population crosses (Brummer 1999). This means that not only can methods which incorporate the use of inbred lines, such as the proposed SI hybrid breeding method in perennial ryegrass (experiment 1), be used to capture heterosis, but population crosses can also be used (e.g. semi-hybrids, discussed in section 2.6.3.1) (Brummer 1999). Regardless of the hybrid breeding method, not all crosses express the same level of heterosis (Brummer 1999). Brummer (1999) suggested that to capture high levels of heterosis successfully and consistently in F1 progeny, it is necessary to identify populations that will combine well (which are genetically divergent at the loci related for yield performance), i.e. identify heterotic groups within perennial ryegrass breeding pools, as is done by maize breeders. In perennial ryegrass there has been little investigation into identifying and defining heterotic groups. Hence, currently breeders have little quantitative information on the general combining ability, from which to judge which lines of perennial ryegrass, if crossed with specified other lines, might lead to the highest amount of heterosis. This information would be helpful for all methods of hybrid breeding, and with progress in the SI hybrid breeding method (experiment 1), breeders

would benefit further by being able to make informed selection of the best populations, that would maximise heterosis, to enter into the hybrid breeding pipeline.

Some investigation of the combining abilities of perennial ryegrass populations has previously been completed, such as Barret *et al.* (2010) (discussed in section 2.6.3.1). Additionally, O'Connor *et al.* (2015) investigated heterosis in full-sib progeny from individual pair crosses. The offspring of the pair crosses from this study were observed to perform better than the parents. High parent heterosis ranged from 12.1 – 17.8% in medium flowering crosses, and 7.1 – 20.7% in late flowering crosses. In this experiment the full-sib progeny were compared to random population samples of the parent cultivars, rather than to the specific individual parents, and the F1 progeny were also assessed as a population rather than as individual plants.

With the aim of building on such studies, this experiment was developed to explore expression of heterosis, and variation in expression of heterosis, in the F1 progeny from individual pair crosses relative to clonal ramets of the two parents (i.e. identical genetic material to the parents but with similar aged tillers to the seedlings). This allowed an investigation of the specific combining ability of the two plants used in each pair cross, and upon averaging this data, an indication of the general combining ability of the cultivars used. An additional aim was to investigate the variability in heterosis within the populations.

It was hypothesised that the F1 offspring would display improved dry matter yield relative to the mean yield of the two individual parent plants and that the F1 progeny from crosses

of different genetic backgrounds would express different levels of heterosis. It was also expected that there would be significant variation in heterosis in the F1 population.

4.2 Objective

- Quantify the expression of, and variation in, heterosis in dry matter yield observed in the F1 progeny from pair crosses of cultivars with differing genetic origins.

4.3 Materials and methods

4.3.1 Treatments

There were two 'treatments' in this experiment, two contrasting crosses with different genetic origins.

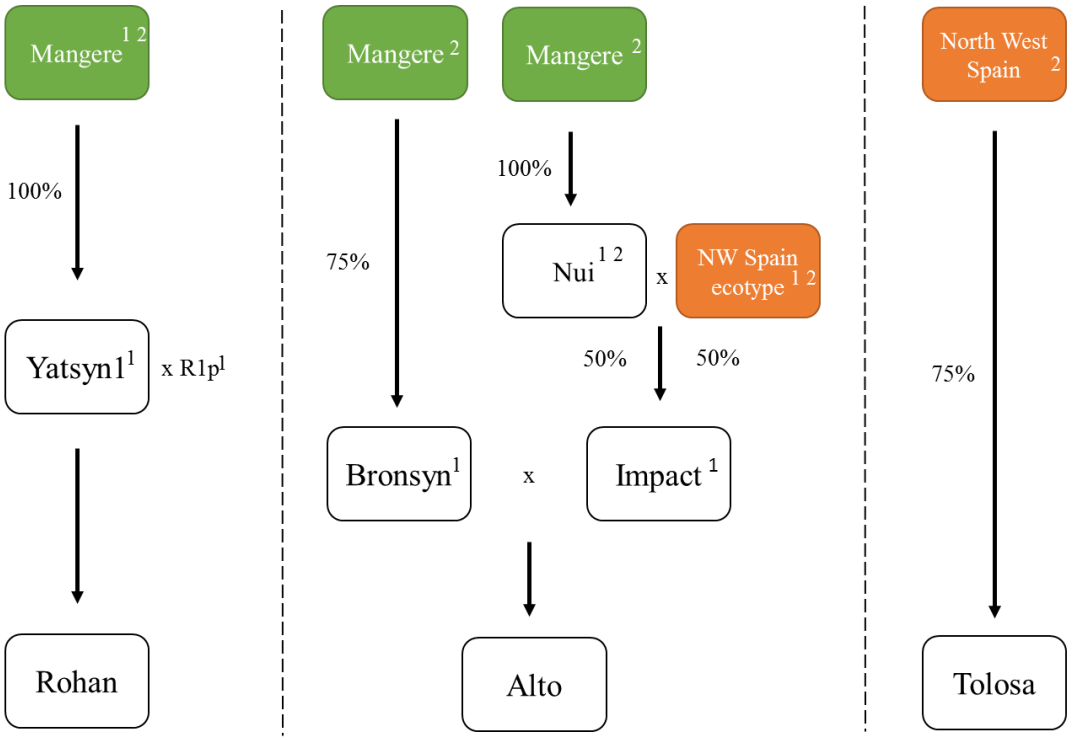
Cross 1: 'Alto' x ('Alto' x 'Tolosa') (abbreviated to 'A x AT')

Cross 2: 'Alto' x 'Rohan' (abbreviated to 'A x R')

Cross 1 has relatively more genetic variability between the parents, as Tolosa originates from north west Spain (Table 4.1; Figure 4.1), while Cross 2 has relatively less genetic variability between the parents, both 'Alto' and 'Rohan' having significant Mangere ecotype origins (Table 4.1; Figure 4.1).

Table 4.1 Summary of cultivar pedigree and key characteristics.

Progenitor	Identified genetic origins	Characteristics
'Alto'	Mangere ecotype and north west Spain (Stewart 2006).	Selected for late heading date, summer and winter growth (New Zealand Agriseeds Limited 2007).
'Rohan'	Mangere ecotype (Stewart 2006).	Selected for persistence (New Zealand Agriseeds Limited 2011).
'Tolosa'	North West Spain (Stewart 2006).	Selection for winter growth, rust resistance, and palatability (New Zealand Agriseeds Limited 2001)



1 IP Australia, Plant Breeders Rights (PBR) database

2 Stewart (2006)

Percentages estimated by Stewart (2006)

Figure 4.1 Identified genetic origins of 'Alto', 'Rohan' and 'Tolosa'.

4.3.2 Experimental design

Both crosses ('A x AT' and 'A x R') were replicated eight times using pair crosses (i.e. one individual plant from one cultivar, paired with an individual plant from another cultivar). Twelve seedlings from each pair cross were germinated (Appendix 21). Two clones of each seedling and parent plant were used in the experiment. In the glasshouse there were two replicate blocks, each containing one clone of each of the parent plants and each of the F1 seedlings. A randomised design was used in order to minimise any effects of spatial variation within the glasshouse environment.

The basic structure of this experiment was therefore:

- 2 types of crosses: ‘A x AT’ and ‘A x R”
- 8 pair crosses from each cross
- 12 seedlings from each pair cross
- 2 clones of each seedling

4.3.3 Plant establishment and maintenance

Parent plants

Seed germination of the parent plants was completed by Barenbrug Agriseeds, Christchurch, New Zealand. The parent plants were planted in single cell seedling trays and germinated in mid-December 2016. On the 24th of January 2017, the plants were placed in vernalisation cabinet for 3 months. The plants were removed on the 24th of April 2017 and placed in a glasshouse, where the temperature was maintained at 15°C during the day and night. Once the plants were large enough, they were transplanted into pots and the temperature was increased (20°C during the day and 15°C overnight). At ear emergence, the plants were paired up and pollen proof bags were placed over each pair. Seed was harvested in September 2017. Once the seed had been harvested the parent plants were cut back and allowed to regrow in the glasshouse. The plants were harvested as required in order to keep them healthy prior to the experiment. Thrive fertiliser (composition presented in Appendix 22) was applied on the 13th and 20th of November 2017. Two applications of Mavrick insecticide (active ingredient: tau-fluvalinate) were applied as a foliar spray to control aphids.

Because the experiment involved different generations of plant material, (existing mature plants used in the pair crosses and the F1 seedlings) all plant material used in the experiment was generated through clonal propagation. This meant the parent plants produced new tillers, and thus provided plant material comprising tillers of similar age and development to the seedlings. Clonal propagation was also required to generate genetically identical replicates of each parent plant and seedling, providing replication in the experiment. The parent plants were cloned in late January (Cloning 1, Figure 4.2). Cloning involved splitting off three tillers (and the root mass of those tillers) from each parent plant, clipping the leaves to 2cm to reduce evapotranspiration, and transplanting all three tillers into a pot to create a clonal replicate. Four clonal replicates of each parent plant were made, and were placed in a shade house. While in the shade house, the plants were watered by overhead sprinkling. After 6 weeks growth, the parent clones were re-cloned (cloning 2, Figure 4.2). Due to limited resources, only three clones of each parent plant were made in the second cloning, two clones for the experiment and one clone as a replacement in case either of the two experimental clones were unsuccessful. The clones were then put in the glasshouse with capillary irrigation. The soil mix used included 53% Manawatu silt loam, 23% sand and 23% seed raising mix. In every 30 litres of soil mix 25 g of long term fertiliser (composition presented in Appendix 2), 75 g of short term fertiliser (composition presented in Appendix 3) and 60 g of Dolomite were included.

F1 offspring

Seed germination of the F1 offspring was also completed by Barenbrug Agriseeds. The F1 seeds were planted into seedling trays, one seed per tray, on the 16th of November

2017. The trays were then placed in a glasshouse where the temperature was maintained at 20°C during the day and 15°C at night. Overhead watering occurred twice a day for five minutes. The seedlings were trimmed as required to encourage tillering.

On the 24th of January 2018 the seedlings were transported via refrigerated freight truck to the Massey University Plant Growth Unit (PGU), Palmerston North, New Zealand. The seedlings were transplanted into individual 1.7 litre planter bags and transferred into a shade house, where the plants were watered overhead. Six weeks after the seedlings were potted (at the same time as the parent plants were re-cloned), clonal ramets of the seedlings were created, as described above (cloning 2, Figure 4.2).

Crown rust was detected on the parent plants and seedlings on February 27th. Two applications of Proline fungicide (active ingredient: prothioconazole) were applied following the same procedure used in experiment 1.

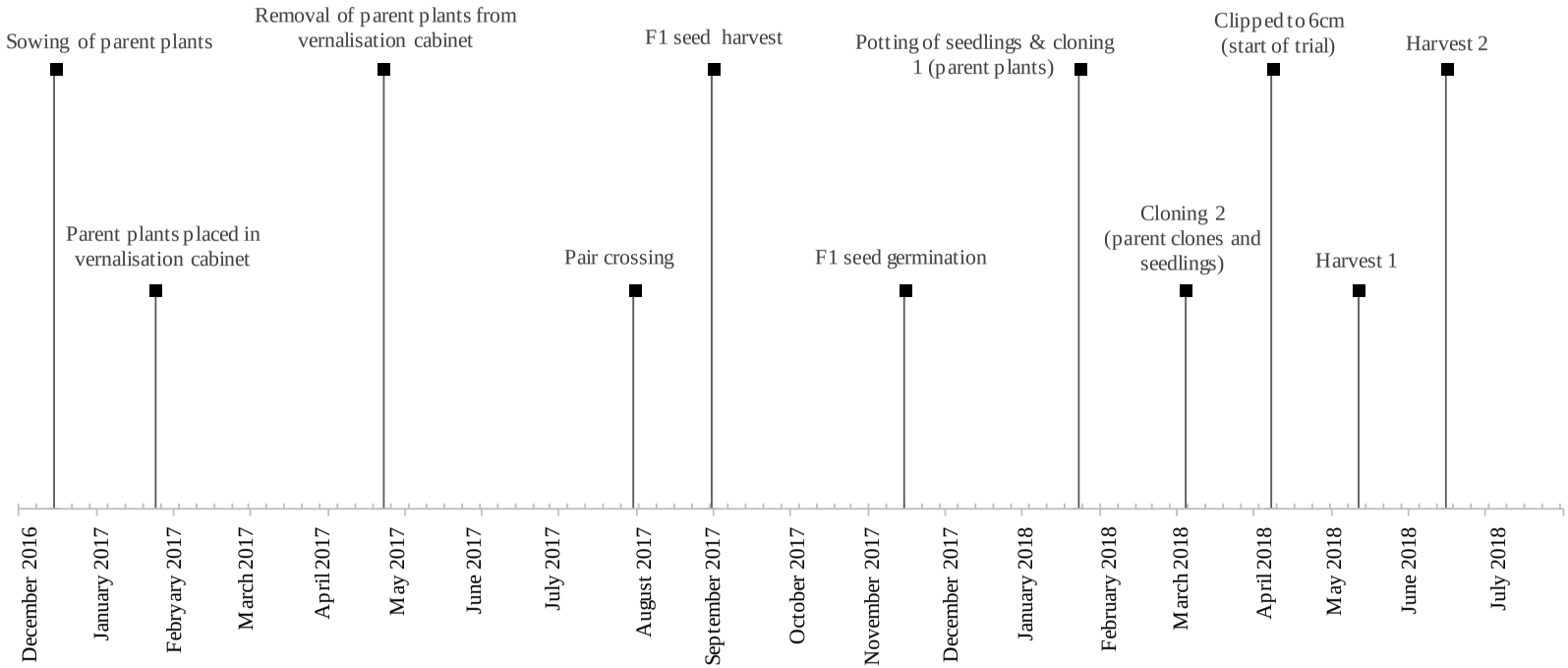


Figure 4.2 Experiment 2 timeline, May 2017 to July 2018.

4.3.4 Measurements

On the 10th of April all clones were cut to a standard height of 6cm (Figure 4.2), this was the beginning of the first regrowth period. The dry matter yield of each plant was measured at the end of two regrowth periods (Figure 4.2). Plants were harvested to 6cm, herbage was collected, oven-dried for a minimum of 48 hours and weighed. For the purposes of this experiment a regrowth cycle was defined as the period between cutting to the standard height and regrowth to the 2 ½ - 3 leaf stage.

4.3.5 Statistical analysis

All analyses were performed using SAS 9.4 and SAS/STAT 14.3 (SAS Institute Inc, 2016). Significance was declared if $P \leq 0.05$. Residuals were checked for homogeneity of variance and no transformation was needed.

Hybrid yield - Yields from the 2 clones of each parent plant were averaged to obtain the best estimate of the true yield value for each plant. The mid-parent mean of each pair cross and harvest was then calculated using the following equation:

Equation 1:

$$\left(\frac{Mean\ yield\ of\ parent\ 1 + mean\ yield\ of\ parent\ 2}{2}\right)$$

The highest yielding parent in each pair cross was also identified. The amount of mid-parent and high-parent heterosis was then calculated for each the 12 F1 seedlings from each pair cross at each harvest, i.e. the difference between the mid-parent mean or high

parent mean and the mean yield of the F1 seedling. It was this mid-parent heterosis and high-parent heterosis value for each seedling which was then analysed.

ANOVA - For the initial analysis, a hierarchical ANOVA model was used, including cross (i.e. 'A x AT' and 'A x R'), seedling, clone, and harvest, and all their interactions as fixed effects, and pair and its interactions with cross, seedling, and clone as random effects. Hence, the 8 pairs in each background were used as the error term for testing the overall effect of cross. Results are presented as least-squares means and 95% confidence interval. A significant interaction between harvest and cross was detected, as shown in (Appendix 23), and as a result, in further analyses, Harvest 1 and 2 were analysed separately.

Consistency between seedlings and pairs – To quantify how similar seedlings were within each pair, the raw mean and standard deviation were calculated for mid parent and high parent heterosis for each background, harvest, and pair (across the 12 F1 seedlings). Similarly, for quantifying how similar pairs were within each cross, the raw mean and standard deviation were calculated for mid-parent and high-parent heterosis for each cross and harvest (across the 8 pair means).

Effect of pairs – Mid-parent and high-parent heterosis for each cross were analysed using a mixed models approach to repeated measures analysis of variance. The model included pair, harvest and their interaction as fixed effect and seedling as random effect. The results of the main effect of pair for the 'A x AT' cross, and the effect of pair for each separate harvest for the 'A x R' cross, are presented as least-squares means and 95% confidence interval. A Tukey test was used for pairwise comparisons.

In addition to the main analyses described above, nested random effects analysis of variance was completed for mid-parent heterosis and high-parent heterosis. Pearson correlations were used to describe the associations between yields of different clones and harvests.

4.4 Results

4.4.1 Heterosis

4.4.1.1 Mean mid-parent and high-parent heterosis

The offspring from the 'A x AT' cross consistently displayed mid-parent heterosis (Table 4.2). The dry matter yield of the F1 offspring from the 'A x AT' cross was greater than the mid-parent mean at Harvest 1 (+ 0.40 g DM; P = 0.0509) and Harvest 2 (+ 0.45 g; P = 0.0269). Individual offspring exhibited inconsistent mid-parent heterosis across the two harvests, however, the average performance of the 'A x R' offspring indicated no evidence of mid parent heterosis (Table 4.2). High-parent heterosis was not detected in the offspring from either cross, in either harvest (Table 4.2).

Table 4.2 Mean mid-parent and high-parent heterosis (g DM per clone) and P values for each cross.

		Mid-parent heterosis		High-parent heterosis	
	Harvest	**g DM**	**P value**	**g DM**	**P value**
Background					
'A x R'	1	-0.30	0.1578	-1.22	<.0001
	2	0.24	0.2442	-0.42	0.1568
	Mean	-0.025	0.9033	-0.8177	0.0276
'A x AT'	1	0.40	0.0509	-0.14	0.6423
	2	0.45	0.0269	0.01	0.9725
	Mean	0.42	0.0721	-0.064	0.8344

4.4.1.2 The effect of genetic background

The difference in mid-parent heterosis between the two crosses at Harvest 1 was significant (Table 4.3; P = 0.0176), the 'A x AT' offspring had greater mid-parent heterosis than the 'A x R' offspring. While high-parent heterosis was not detected for

either cross at Harvest 1, the dry matter yield of the 'A x R' offspring was lower than the high-parent at Harvest 1, while the dry matter yield of the 'A x AT' offspring was similar to the high-parent (Table 4.2). This difference in expression of high-parent heterosis between the two crosses was significant (Table 4.3; P = 0.0111). There were no differences in the expression of mid-parent, or high-parent, heterosis between the offspring of the two crosses at Harvest 2.

Table 4.3 P values for comparison of the mean mid-parent heterosis between backgrounds.

	P value	
	Mid-parent heterosis	**High-parent heterosis**
Harvest		
1	0.0176	0.0111
2	0.4560	0.3046

4.4.2 Variation in heterosis

The 'A x R' offspring displayed a large amount of variation in the expression of mid-parent heterosis at Harvest 1 (Table 4.4; SD = 0.91 g DM), compared to Harvest 2 (SD = 0.44 g DM). The level of variation in the expression of heterosis observed at Harvest 1 and 2 for the 'A x AT' offspring was relatively consistent (SD = 0.55 and 0.48 g DM, respectively).

The expression of high-parent heterosis in 'A x R' offspring was more variable at Harvest 1 (SD = 1.37) than Harvest 2 (SD = 0.67). The 'A x AT' offspring had a consistent variability in expression of high-parent heterosis across the two harvests (SD = 0.75 g DM).

Table 4.4 Variation in mid-parent and high-parent heterosis (g DM per plant)

		Standard deviation	
Harvest	**Background**	**Mid-parent heterosis**	**High-parent heterosis**
1	'A x R'	0.91	1.37
2	'A x R'	0.44	0.67
1	'A x AT'	0.55	0.75
2	'A x AT'	0.48	0.75

Pair had a significant effect on both mid-parent and high-parent heterosis for both crosses ($P < 0.001$, Appendix 23). The offspring of five of the 'A x AT' pairs displayed mid-parent heterosis (Figure 4.3), while the offspring of three pairs displayed high-parent heterosis (Figure 4.4). Of the 'A x R' crosses, only the offspring of Pair 6 displayed mid-parent or high-parent heterosis at both harvests (Figure 4.5 and 4.6). The poor performance of the offspring of the 'A x R' Pair 3 at Harvest 1 compared with Harvest 2 was notable.

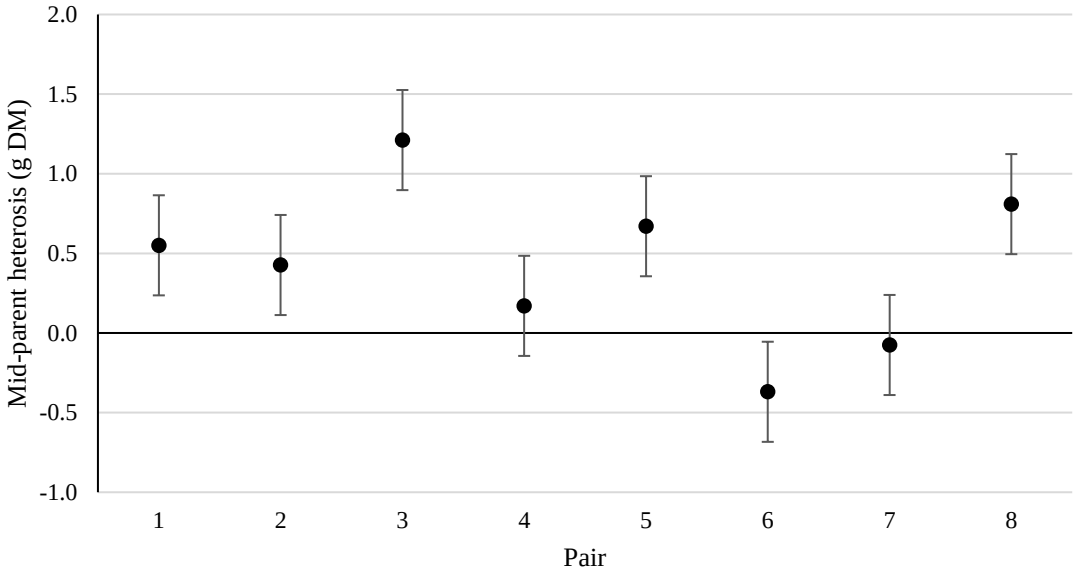


Figure 4.3 'Alto' x ('Alto' x 'Tolosa') pair crosses offspring mid-parent heterosis. Error bars represent 95% confidence intervals. The main effect (mean of Harvest 1 and 2) is plotted as there was no difference between Harvest 1 and 2 (Appendix 24).

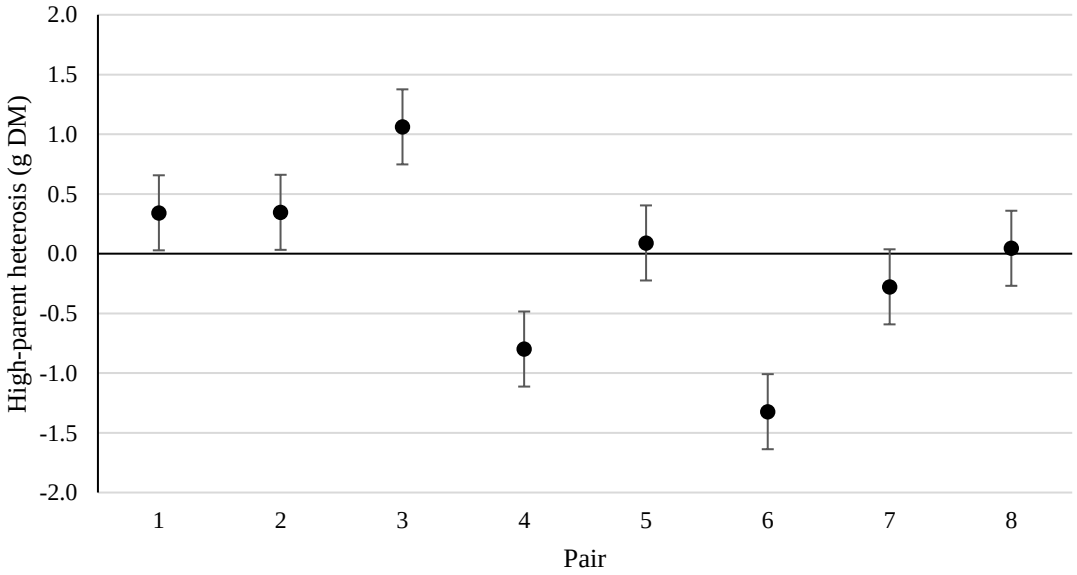


Figure 4.4 'Alto' x ('Alto' x 'Tolosa') pair crosses offspring high-parent heterosis. Error bars represent 95% confidence intervals. The main effect (i.e. mean of Harvest and 2) is plotted as there was no difference between Harvest 1 and 2 (Appendix 24).

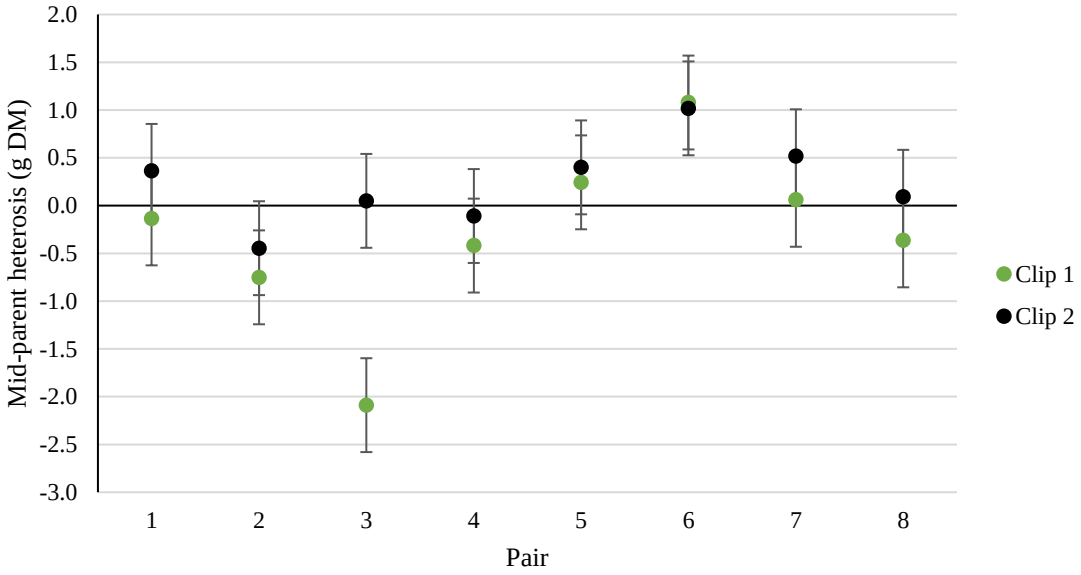


Figure 4.5 'Alto' x 'Rohan' pair crosses offspring mid-parent heterosis. Error bars represent 95% confidence intervals. Harvest 1 and Harvest 2 plotted separately due to there being a significant effect of harvest (Appendix 24).

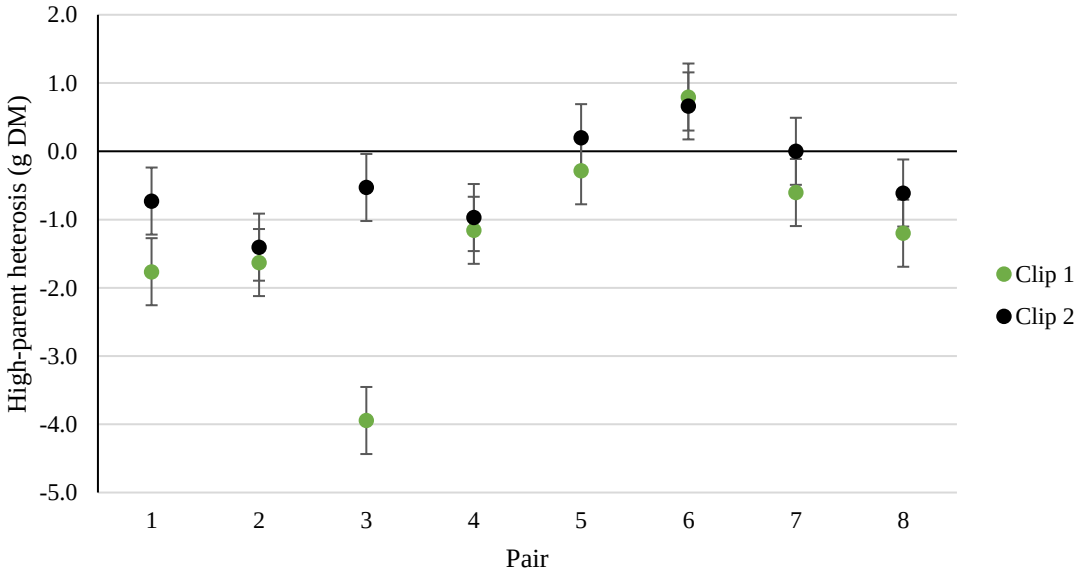


Figure 4.6 'Alto' x 'Rohan' pair crosses offspring high-parent heterosis. Error bars represent 95% confidence intervals. Harvest 1 and Harvest 2 plotted separately due to there being a significant effect of harvest (Appendix 24).

4.4.3 Sources of variation in heterosis

The major source of variation in mid-parent heterosis was between clones of the same seedling (genetically identical), which accounted for 44.8 and 68.5% of total variation at Harvest 1 and 2, respectively (Table 4.5). Pair (25.4 and 16.7%, at Harvest 1 and 2, respectively) and seedling (21.4 and 14.8%, at Harvest 1 and 2, respectively) were also important sources of variation in mid-parent heterosis. Cross was the smallest source of variability in mid-parent heterosis accounting for 8.5 and 0% of variability, at Harvest 1 and 2, respectively.

Clone accounted for 30.0 and 52.4 % of total variability in high-parent heterosis at Harvest 1 and 2, respectively. Pair accounted for 40.8 and 34.8 % of variability in high-parent heterosis at Harvest 1 and 2, respectively. While seedling accounted 14.3 and 11.3% in Harvest 1 and 2, respectively. Cross was a small source of variability accounting for 14.8 and 1.5% in Harvest 1 and 2.

The Pearson correlation (Appendix 25) between Clone 1 and 2 for mid-parent heterosis was 0.52 and 0.32 ($P < 0.0001$) at Harvest 1 and 2, respectively, while the Pearson correlation between Clone 1 and 2 for high-parent heterosis was 0.66 and 0.48 ($P < 0.0001$) at Harvest 1 and 2, respectively.

Table 4.5 Sources of variance in mid-parent heterosis and high parent heterosis (expressed as a percentage (%)).

	Mid-parent heterosis		High-parent heterosis	
	Harvest 1	**Harvest 2**	**Harvest 1**	**Harvest 2**
Variance Source				
Total	100.0	100.0	100.0	100.0
Cross	8.5	0.0	14.8	1.5
Pair	25.4	16.7	40.8	34.8
F1 seedling	21.4	14.8	14.3	11.3
Clone	44.8	68.5	30.0	52.4

4.4.4 Glasshouse temperature

Average daily temperature within the glasshouse gradually decreased over the course of Experiment 2, from an average of 24°C to 14°C (Figure 4.7). Maximum glasshouse temperatures were below 30°C for the length of the experiment, with the exception of one day in mid-May. No negative plant responses to temperature were observed.

Figure 4.7 Glasshouse maximum, minimum and average temperature (°C) from April to June 2018.

4.5 Discussion

4.5.1 Heterosis

4.5.1.1 Mid-parent and high-parent heterosis

It was expected that mid-parent heterosis would be evident in the offspring from both types of crosses. However, mid-parent heterosis was consistently observed only in the offspring of the 'A x AT' cross. While the offspring of the 'A x R' pair crosses displayed variable performance across the two harvests – only one pair cross out of eight resulted in mid-parent heterosis. Overall, mid-parent heterosis was observed, however, it was variable within and between the two crosses.

In terms of producing commercially viable hybrids, it is high-parent heterosis that is important to plant breeders, i.e. the offspring produced must out-yield the better performing parent plants, and the cultivars that are currently on the market (Brummer 1999). While overall there was no significant high-parent heterosis, there were signs of high parent heterosis in individual pairs from both crosses, for example, 'A x AT' Pairs 1, 2 and 3 and 'A x R' Pair 6. This provides evidence that, while the average performance of a population cross may show limited high-parent heterosis, there is variation in the performance of individuals within a population. This was the case for offspring from both the more genetically divergent and less divergent crosses. Thus, in order to capture maximum heterosis, additional to using populations from different heterotic groups (i.e. populations known to have good *general* combining ability), there should be value in selecting specific plants from such populations, which have good *specific* combining ability, when selecting plants to feed into the hybrid breeding pipeline (experiment 1).

4.5.1.2 The effect of genetic background

The mid-parent heterosis expressed by the offspring from the ‘A x AT’ crosses in Harvest 1 was greater than the offspring of the ‘A x R’ crosses. There was no difference in the expression of mid-parent heterosis in Harvest 2, and high-parent heterosis in both harvests. However, the offspring of ‘A x AT’ crosses tended to express greater heterosis than the offspring of the ‘A x R’ crosses. This result supports the hypothesis that crossing cultivars from different genetic backgrounds can lead to different levels of heterosis. The greater heterosis displayed by the ‘A x AT’ cross relative to the ‘A x R’ cross suggests that there is likely a difference in the general combining ability of the cultivars used in the two crosses, which causes the difference in the performance of the offspring. The ‘A x AT’ cross had more genetic variability between parent cultivars relative to the ‘A x R’ cross (Figure 4.1). This supports the suggestion of Brummer (1999) that geographically separated populations would be a likely obvious source of heterotic groups.

The scale of this experiment was limited, and hence the number of crosses, pairs, seedlings and clones enrolled in the experiment was restricted. It is recommended that future experiments of this nature include a greater number of pairs and clonal replication, in order to provide greater power to detect significant differences between the different types of crosses.

4.5.2 Variation in expression of heterosis

The performance of individual pair crosses within each type of cross varied, presumably due to variation in plant genetics within cultivars and specific combining ability. While the variability in heterosis was estimated to be less than 1 gram (except for high-parent heterosis in Harvest 1), this level of variability in expression of heterosis could still significantly impact whether any expression of heterosis is detected in a population of F1 progeny. For example, there was an estimated variability in mid-parent heterosis of 0.55 g DM in the offspring from the 'A x AT' crosses in Harvest 1, however, the mean level of mid-parent heterosis observed for that cross was 0.40 g DM. Therefore, F1 progeny from the cross could potentially display no heterosis (i.e. 0.40 g – 0.55 g = -0.15 g). This finding further supports the importance of selecting specific plants with good *specific* combining ability to capture maximum heterosis, in addition to selecting populations from different heterotic groups. This experiment only compared eight pairs per type of cross. It is recommended that future experiments should increase the number of pair crosses used, to gain a better understanding of the variability in heterosis within a population.

Heterosis expression in the offspring of the 'A x R' crosses varied between the two harvests, while it was consistent in the offspring of the 'A x AT' crosses. Upon closer inspection of the data, it became clear that a key source of the large variation seen at Harvest 1 for the 'A x R' cross, and also a likely cause of the significant difference between the two harvests, was that Pair 3 had a lower yield than all other pairs in that harvest.

4.5.3 Sources of variation in expression of heterosis

A large source of the variation in both mid-parent and high-parent heterosis was the clonal replication. To neutralise any potential effects of plant age on dry matter yield as much as possible, it was necessary to generate clones of the parent plants to get experiment material which had tillers of a similar age to the F1 progeny (and was also important to enable replication of the genetic material). This was achieved by splitting tillers off the main plant, harvesting the leaves and repotting the tillers. This process put stress on the tillers. While overall the cloning process had a high success rate, it was observed that the clones remained 'dormant' for a period before resuming growth post cloning. All clones were potted with the same number of tillers from the parent plant, however, the time it took for the clones to recover and resume growth (i.e. the time they were 'dormant'), varied. As tiller number increases exponentially in the initial growth phase of a plant (Robson 1973), only a small difference in the time individual clones took to resume growth would likely have resulted in a significant difference in the number of tillers per clone by the time the experiment began. Possible reasons for the difference in recovery time of clones could have been genetic ability to deal with the stress of the cloning, and also variability in soil moisture, temperature, or nutrient status.

In this experiment, available resources were limited, which restricted the number of clones that could be generated for each plant. There were only sufficient clones to provide for a single replacement of clones which did not establish successfully. Based on observations from this experiment, it is recommended that for future experiments of this nature, a greater number of clones are generated for each plant, to increase replication,

and therefore reduce variation due to clones, and increase statistical power. Additionally, a greater number of clones for each plant, would allow the possibility of selecting plants with a similar the number of tillers at the beginning of the experiment. It is also recommended that in future experiments, that tiller number per clone be measured prior to each harvest, so that dry matter yield on a per tiller basis can be calculated.

A significant proportion (16.7 - 40.8%) of variation due to the pair was expected. There is variation in the genetic yield capabilities of each seedling within each of the cultivars used in the cross. Additionally, there is variation in the specific combining ability of the two randomly selected plants used in each pair. These two factors contribute to the variation observed in the amount of heterosis expressed in the hybrid offspring.

The cross (i.e. 'A x AT' or 'A x R') only accounted for a small proportion of the variability in heterosis observed in the experiment. This indicates that while the genetic origins of the plants used in a cross (i.e. what heterotic group they belong to) is important, in comparison, genetic variation within a population is substantial and selection of specific plants with good *specific* combining ability from within populations is important, and potentially has a much more significant impact. In terms of hybrid breeding, a potential implication of this is that plant breeders will need to develop, not only an understanding of heterotic groups, but also of individual genotypes within a single heterotic group.

Chapter 5 General conclusions

5.1 Experiment 1

Hybrids produced by the self-incompatibility hybrid breeding method displayed mid-parent heterosis which provided clear evidence that the proposed breeding method can successfully capture heterosis. Furthermore, evidence of high-parent heterosis indicates that the breeding method could lead to the development of hybrids that substantially exceed the yield of current commercial cultivars, and therefore that the method may be commercially viable. However, a clear pattern of decreasing heterosis over the four harvests was observed in this experiment. Definitive conclusions could not be made about the cause of this pattern of decreasing heterosis from this experiment and further studies to investigate this observation are required.

Variability in the dry matter yield performance among the six hybrids was observed, confirming the expectation that development of hybrids for commercial sale would take time, and require screening of a large number of crosses to identify hybrids which display desirable levels of heterosis. However, despite the limited number of hybrids in the experiment, there was evidence that some hybrids (e.g. Hybrid 3, which had a total dry matter yield 126% of the mean of the progenitor cultivars) could substantially out yield current elite ryegrass cultivars. In combination with the short amount of time required to produce a new cultivar from this breeding method, this indicates that this SI method may have the potential to produce a step change in perennial ryegrass dry matter yield genetic gain.

The hybrids used in this experiment were created by crossing parent lines that were inbred for two cycles prior to crossing. A question for further consideration in future studies is: if the level of heterosis observed in this experiment was produced from only two cycles of inbreeding, then could even greater heterosis be achieved with further inbreeding cycles? Consideration of the impacts of achieving such levels of heterosis at a farm systems level, and how to capture economic value of additional feed, would also be of value.

The performance of Parent 4, which was a common parent in, Hybrid 3, Hybrid 5, and Hybrid 6, demonstrates the importance of parent pool selection and combining ability in producing successful hybrids. Given the promising signs for success with this proposed method, plant breeders may need to gain a better understanding of the variation in combining ability among their perennial ryegrass breeding pools.

The parent lines exhibited lower yield than the hybrids and progenitors, which was expected due to the cycles of inbreeding used to create them. Number of tillers per plant was a source of the difference in dry matter yield between the parents and other treatments in this experiment. Greater leaf width, facilitating larger total leaf area, was a source of the difference in dry matter yield between the Hybrid and the Progenitor lines in this experiment.

It was hypothesised that the use of inbreeding in the proposed method would result in a more uniform population. This could result in commercial hybrids that may be more vulnerable to changes in the environment, compared to current cultivar populations, and therefore that are less persistent. However, significant changes in the variability of the

morphological traits between the three generations of plant material were not observed. Three key points emerged from this result:

1. This is a positive finding in terms of producing commercial hybrids, since significant heterosis without potential ecological impacts makes the method much more likely to be viable to take to market.
2. Given no significant change in the genetic uniformity of the parent lines and hybrids after two cycles of inbreeding was detected, could further cycles of inbreeding further increase heterosis without a significant negative impact on morphological variability? This is a potential avenue for further investigation.
3. This experiment was as large as possible (750 plants) given the available resources, and was designed as a preliminary investigation in to morphological variability. However, to confirm the findings of this preliminary experiment and to try and quantify smaller changes in variability which could not be detected by the sample size used in this experiment, in future experiments the sample size of each hybrid, parent line and progenitor cultivar population should be increased to give greater power.

5.2 Experiment 2

With progress in the SI hybrid breeding method for perennial ryegrass (Experiment 1), it was considered that breeders may further benefit from the proposed method by quantifying the general combining ability of their breeding pools. By doing so, breeders would be able to make more informed decisions around selection of populations to enter into the hybrid breeding pipeline for *S* and *Z* allele screening, and maximise heterosis.

Mid-parent heterosis and high-parent heterosis were observed in the F1 hybrids produced from both types of pair crosses, however it was variable within, and between, the two crosses. Overall, the level of heterosis tended to be different between the two populations in this experiment. This trend suggests that there is a difference in the *general* combining ability of the two crosses, and therefore, supports the idea that there would be value in quantifying the combining ability of perennial ryegrass breeding pools in order to make selections which maximise heterosis.

Evidence of variation in the performance of individual pair crosses within each of the two types of crosses was observed. Evidence of high-parent heterosis in specific pairs within the two crosses, even when the mean of the crosses showed no high-parent heterosis, indicates there would be value in selecting specific plants, which have good *specific* combining ability, in addition to selecting populations known to have good *general* combining ability.

Future experiments of this nature would require a much greater scale than was possible in this experiment. To quantify the combining ability of populations so that breeders could

have confidence in making decisions based on the results, the number of pairs, seedlings, and clones in the experiment would need to be increased, along with assessing a much larger range of types of crosses than just the two assessed in this preliminary study. Additionally, the clonal ramets used in this experiment contributed to a high proportion of the variability in heterosis. In order to reduce the variation associated with the clonal ramets in future experiments, it is proposed, that in addition to increasing the number of clones of each plant, ensuring each clonal ramet has the same number of tillers at the beginning of the experiment. Counting the number of tillers per plant prior to each harvest would also be of value so that dry matter yield on a per tiller basis could be calculated.

5.3 Concluding remarks

In summary, the results of this work show potential yield advantages in hybrids produced by the SI hybrid breeding method, which could result in a step change in genetic gain in dry matter yield of perennial ryegrass (experiment 1). With evidence which supports the proposed breeding method, further work is now required to identify specific parental lines which, when crossed, maximise hybrid vigour to produce hybrids which perform significantly, and consistently, greater than current commercial cultivars. Evidence of differences in expression of heterosis in hybrids with differing genetic origins (experiment 2) indicates that identification, and selection, of such parental lines will be aided by developing a better understanding of the combining ability of perennial ryegrass populations. Once the combining ability of populations has been quantified, this information can be used to inform selection of breeding material to feed into the SI hybrid breeding method. Overall, the results of this work are very promising and indicate the proposed method could have a significant impact on the genetic gain of perennial ryegrass, and therefore have a significant positive impact on the New Zealand dairy industry.

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Appendices

Appendix 1. Experiment 1 power analysis

As one of the objectives of this experiment was to compare the variation within and between the treatments in the key plant morphological traits, a rigorous power analysis was unable to be completed, as an estimate of the expected variance is required for a power analysis and no previous data of this nature has been collected. There was also the added complication that a smaller difference in variance would be expected between the parent lines and F1 hybrids than the progenitor cultivars and parent lines. Additionally, the difference in variance may also depend on which trait is measured.

Based on this, a power analysis was completed assuming a range of standard deviation ratios (i.e. changes in variability, as standard deviation was used as an estimate of variability), in order to gain an understanding of what sample sizes would be required to have at least 80% power of detecting a difference in variance between populations, at probability of $P < 0.05$ (Table A1). Based on the results of this power analysis, and also considering the practical limitations to what experiment size was manageable given available resources, a sample size of 50 plants was been selected (total of 750 plants). This assumed a minimum reduction in the standard deviation (estimated variability) of ~37% from one population to another (e.g. SD of progenitor population = 1 and SD of parent population = 0.6325).

Table A1. Partial power analysis

Number of plants per 'population'	Standard deviation ratio	Power using Levenes test	Example of standard deviations	
			SD of population 1	SD of population 2
30	0.50	92.5	1	0.50
30	0.63	59.9	1	0.63
30	0.75	29.6	1	0.75
30	0.80	19.82	1	0.80
40	0.50	97.3	1	0.50
40	0.63	74.4	1	0.63
40	0.75	36.8	1	0.75
40	0.80	23.2	1	0.80
50	0.50	99.1	1	0.50
50	0.63	83.4	1	0.63
50	0.75	48.3	1	0.75
50	0.80	30.6	1	0.80
100	0.50	100	1	0.50
100	0.63	97.8	1	0.63
100	0.75	74.1	1	0.75
100	0.80	53.2	1	0.80
150	0.50	100	1	0.50
150	0.63	99.9	1	0.63
150	0.75	89.1	1	0.75
150	0.80	72.9	1	0.80
200	0.50	100	1	0.50
200	0.63	100	1	0.63
200	0.75	96.4	1	0.75
200	0.80	84.8	1	0.80

Values in yellow provide a minimum of 80% power of detecting a difference between populations at a given SD ratio (i.e. at a given reduction in variability)

Appendix 2. Mean germination rate (%), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Germination rate (%)	
Treatment		
Hybrid	82	a
Parent	58	b
Progenitor	84	a
P value	<.001	
Line within treatment		
H1	86	ab
H2	92	a
H3	85	b
H4	87	ab
H5	73	c
H6	68	c
P value	0.003	
SED	3.438	
Par1	68	a
Par2	55	b
Par3	71	a
Par4	39	c
P value	0.002	
SED	5.918	
Pro1	86	ab
Pro2	81	b
Pro3	88	a
Pro4	74	c
Pro5	90	a
P value	0.002	
SED	2.018	
Line		
P value	<.001	
SED	4.482	

Appendix 3. Long term fertiliser: Woodace 18-2.2-8.3

Total Nitrogen (N)		18.0%
1.0%	Ammoniacal Nitrogen	
1.9%	Nitrate Nitrogen	
13.0%	Water insoluble Nitrogen*	
2.1%	Urea Nitrogen	

Phosphorus	(P)	2.2%
Potassium	(K)	8.3%
Magnesium	(Mg)	0.3%
Sulpher	(S)	1.0%
Iron	(Fe) Actual	0.3%
Manganese	(Mn)	0.3%
Zinc	(Zn)	0.2%

Nutrient Sources:	Ammonium Phosphate
	Isobutylidene Diurea
	Potassium Nitrate
	Sulphate of Potash
	Magnesium Sucrate
	Iron Sucrate
	Manganese Sucrate
	Zinic Sucrate

* 13.0% Slowly Available Nitrogen from Isobutylidene Diurea

Appendix 4. Short term fertiliser: Woodace 14-6-11.6

Total Nitrogen (N)			14.0%
	2.8%	Ammoniacal Nitrogen	
	5.8%	Water Insoluble Nitrogen	
	2.7%	Urea Nitrogen	
	2.7%	Other Water Soluble Nitrogen*	
Phosphorus		(P)	6.0%
Potassium		(K)	11.6%
Magnesium		(Mg)	1.0%
Sulpher		(S)	4.0%
Iron		(Fe) Actual	1.0%
Manganese		(Mn) Actual	0.5%
Nutrient Sources:		Ammonium Phosphate	
		Ammonium Sulphate	
		Isobutylidene Diurea	
		Methylene Urea	
		Urea	
		Sulphate of Potash	
		Magnesia	
		Dolomite	
		Magnesium Oxide	
		Ferrous Sulphate	
		Ferrous Oxide	
		Manganous Oxide	
		Mananese Sulphate	
Chlorine		(C) not more than	2.0%

* 17.1% Slowly Available Nitrogen from Isobutylidene Diurea

Appendix 5. Liquid fertiliser: Peters professional 20-9-17 + Trace

Nitrogen (N)			20%
	4.5%	Nitrate nitrogen	
	2.4%	Ammonium nitrogen	
	13.1%	Urea nitrogen	
Phosphorus (P) as ammonium phosphate (Soluble in neutral ammonium citrate and water)			8.7%
	16.5%	Potassium (K) as potassium nitrate (Water soluble)	

Trace elements are completely water soluble		
Iron chelated by DTPA	Fe	0.12%
Manganese chelated by EDTA	Mn	0.06%
Boron	B	0.02%
Copper chelated by EDTA	Cu	0.015%
Zinc chelated by EDTA	Zn	0.015%
Molybdenum	Mo	0.01%
Lead (Pb)		1.17mg/kg
Cadmium (Cd)		below detectable levels
Mercury (hg)		below detectable levels

EC Value:	0.8mS/cm at 1.0g/L
	200ppm Nitrogen

Appendix 6. Mean seedling dry matter yield (g DM per seedling), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Dry matter yield (g DM per seedling)
Treatment	
Hybrid	0.19
Parent	0.18
Progenitor	0.19
P value	0.920
Line within treatment	
H1	0.17
H2	0.18
H3	0.23
H4	0.16
H5	0.23
H6	0.18
P value	0.841
SED	0.076
Par1	0.17
Par2	0.20
Par3	0.13
Par4	0.22
P value	0.157
SED	0.027
Pro1	0.18
Pro2	0.16
Pro3	0.17
Pro4	0.19
Pro5	0.24
P value	0.777
SED	0.062
Line	
P value	0.897
SED	0.061

Appendix 7. Health status criteria

Score	Description
1	Dead – no green leaf sprouting on any tiller – 100% brown.
2	Some green but majority of plant dead.
3	‘Spindly’ bleached/yellow lamina tips and a small amount of dead material.
4	Fairly healthy – slight bleaching/yellowing on lamina tips, no dead material.
5	Healthy plant - no major visible damage or only very minimal damage on lamina tips.

Appendix 8. Mid-parent heterosis standard error of the difference (SED) calculation.

Standard error of the difference (sed) =sqrt ($se1^2$ +$se2^2$)

Where se1 and se2 are the standard error of the mean (se) for each of two lines being compared.

As the replication is the same for each line, the standard error of the mean is the same for each line based on the pooled residual standard deviation.

i.e. sed = sqrt(se^2+se^2)

= sqrt (2) *se

Comparing a line to the mean of its two parents (i.e. the mid-parent mean) doubles the replication, therefore:

Standard error of the parent mean = se/sqrt (2)

Sed for comparing a line with the mid-parent mean (sed_l_vs_p) = sqrt (se^2 +se^2/2)

= sqrt (1 + ½) *se

Ratio of the two standard errors of differences:

sed_l_vs_p/sed= (sqrt (1 + ½) *se) / (sqrt (2) *se)

=sqrt (1.5)/sqrt (2)

=sqrt (0.75)

=0.866

i.e. sed_l_vs_p= 0.866*sed

Appendix 9. Mean emerging leaf length (mm), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period		
	1		2
Treatment			
Hybrid	107.60	a	101.50
Parent	92.60	b	101.80
Progenitor	104.90	a	98.50
P value	<.001		0.799
Line within treatment			
H1	97.90	bc	113.10
H2	98.50	bc	92.00
H3	144.40	a	108.90
H4	81.70	c	93.10
H5	113.90	b	101.50
H6	109.40	b	100.60
P value	<.001		0.240
SED	8.630		9.730
Par1	96.60	b	89.30
Par2	78.50	c	120.90
Par3	73.70	c	97.00
Par4	121.50	a	99.90
P value	<.001		0.064
SED	8.030		10.710
Pro1	104.00		99.00
Pro2	111.70		99.60
Pro3	103.60		105.40
Pro4	98.00		81.30
Pro5	107.20		110.10
P value	0.507		0.242
SED	7.650		12.560
Line			
P value	<.001		0.208
SED	8.360		12.210

Appendix 10. Mean youngest fully emerged leaf length (mm), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	164.80		200.10	
Parent	151.50		190.20	
Progenitor	162.80		192.00	
P value	0.065		0.344	
Line within treatment				
H1	146.70	bc	212.30	
H2	172.90	a	186.40	
H3	188.00	a	200.90	
H4	142.10	c	183.50	
H5	167.80	ab	206.40	
H6	171.20	a	211.10	
P value	0.005		0.213	
SED	11.170		14.020	
Par1	155.50	b	173.40	b
Par2	125.10	b	221.50	a
Par3	125.50	b	174.70	b
Par4	199.80	a	191.50	ab
P value	<.001		0.018	
SED	13.970		14.210	
Pro1	156.50	bc	191.90	
Pro2	170.90	ab	187.50	
Pro3	162.10	abc	197.90	
Pro4	147.30	c	161.90	
Pro5	177.40	a	220.70	
P value	0.028		0.068	
SED	8.780		18.190	
Line				
P value	<.001		0.013	
SED	12.660		16.360	

Appendix 11. Mean stem diameter (mm), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period		
	1		2
Treatment			
Hybrid	1.70	a	1.50
Parent	1.69	a	1.53
Progenitor	1.52	b	1.40
P value	0.006		0.071
Line within treatment			
H1	1.68		1.59
H2	1.82		1.54
H3	1.72		1.41
H4	1.66		1.49
H5	1.68		1.63
H6	1.62		1.35
P value	0.675		0.261
SED	0.124		0.126
Par1	1.66		1.31
Par2	1.68		1.68
Par3	1.70		1.58
Par4	1.74		1.56
P value	0.887		0.144
SED	0.070		0.149
Pro1	1.69		1.57
Pro2	1.59		1.42
Pro3	1.50		1.37
Pro4	1.43		1.26
Pro5	1.40		1.37
P value	0.316		0.211
SED	0.150		0.124
Line			
P value	0.146		0.045
SED	0.134		0.130

Appendix 12. Mean number of leaves per tiller, P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	1.64	ab	2.20	c
Parent	1.58	b	2.68	a
Progenitor	1.69	a	2.42	b
P value	0.036		<.001	
Line within treatment				
H1	1.65	ab	2.12	
H2	1.77	a	2.18	
H3	1.51	b	1.94	
H4	1.77	a	2.33	
H5	1.62	ab	2.35	
H6	1.54	b	2.28	
P value	0.008		0.060	
SED	0.076		0.137	
Par1	1.54		2.48	b
Par2	1.57		2.93	a
Par3	1.69		2.78	ab
Par4	1.52		2.51	b
P value	0.336		0.020	
SED	0.100		0.140	
Pro1	1.60		2.38	
Pro2	1.71		2.63	
Pro3	1.72		2.20	
Pro4	1.83		2.42	
Pro5	1.60		2.45	
P value	0.088		0.125	
SED	0.086		0.149	
Line				
P value	0.006		<.001	
SED	0.088		0.155	

Appendix 13. Mean health status, P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	3.66	a	3.54	a
Parent	3.11	b	3.30	b
Progenitor	3.68	a	3.42	ab
P value	<.001		0.037	
Line within treatment				
H1	3.32	d	3.52	
H2	3.86	ab	3.64	
H3	3.90	a	3.60	
H4	3.50	cd	3.60	
H5	3.56	bcd	3.34	
H6	3.80	abc	3.52	
P value	0.006		0.460	
SED	0.153		0.155	
Par1	3.18	ab	3.28	
Par2	2.60	c	3.18	
Par3	3.10	b	3.56	
Par4	3.54	a	3.18	
P value	0.002		0.507	
SED	0.177		0.280	
Pro1	3.60		3.36	
Pro2	3.72		3.44	
Pro3	3.52		3.46	
Pro4	3.58		3.38	
Pro5	4.00		3.48	
P value	0.084		0.916	
SED	0.171		0.152	
Line				
P value	<.001		0.366	
SED	0.174		0.196	

Appendix 14. Estimate of variation in emerging leaf width (mm), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	0.62		0.28	
Parent	0.58		0.22	
Progenitor	0.58		0.22	
P value	0.743		0.180	
Line within treatment				
H1	0.63	ab	0.10	
H2	0.43	bc	0.46	
H3	0.67	a	0.37	
H4	0.42	c	0.25	
H5	0.73	a	0.20	
H6	0.82	a	0.28	
P value	0.003		0.110	
SED	0.101		0.098	
Par1	0.51		0.18	
Par2	0.44		0.22	
Par3	0.57		0.22	
Par4	0.79		0.26	
P value	0.228		-	
SED	0.166			
Pro1	0.67	a	0.37	a
Pro2	0.67	a	0.12	b
Pro3	0.62	a	0.04	b
Pro4	0.39	b	0.34	a
Pro5	0.57	ab	0.22	ab
P value	0.034		0.044	
SED	0.080		0.052	
Line				
P value	0.007		0.004	
SED	0.121		0.075	

Appendix 15. Estimate of variation in youngest fully emerged leaf width (mm), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	0.67		0.42	
Parent	0.64		0.41	
Progenitor	0.66		0.41	
P value	0.780		0.898	
Line within treatment				
H1	0.67	abc	0.42	
H2	0.54	bc	0.38	
H3	0.91	a	0.43	
H4	0.49	c	0.33	
H5	0.76	ab	0.53	
H6	0.68	abc	0.43	
P value	0.020		0.204	
SED	0.116		0.075	
Par1	0.70		0.38	
Par2	0.53		0.38	
Par3	0.52		0.35	
Par4	0.82		0.55	
P value	0.111		0.064	
SED	0.128		0.072	
Pro1	0.57	b	0.33	c
Pro2	0.65	b	0.38	bc
Pro3	0.66	ab	0.44	ab
Pro4	0.58	b	0.38	bc
Pro5	0.82	a	0.50	a
P value	0.045		0.006	
SED	0.079		0.040	
Line				
P value	0.002		0.018	
SED	0.104		0.065	

Appendix 16. Estimate of variation in emerging leaf length (mm), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	45.70		42.50	
Parent	41.80		40.80	
Progenitor	46.70		38.90	
P value	0.274		0.420	
Line within treatment				
H1	42.70		52.40	
H2	39.50		32.60	
H3	54.70		45.80	
H4	35.20		42.20	
H5	51.70		38.20	
H6	50.20		43.40	
P value	0.089		0.107	
SED	7.260		6.540	
Par1	44.70	ab	36.30	
Par2	33.70	b	48.40	
Par3	32.70	b	34.40	
Par4	56.00	a	44.20	
P value	0.009		0.219	
SED	6.270		7.140	
Pro1	40.70		40.20	b
Pro2	51.70		35.10	cb
Pro3	48.60		49.70	a
Pro4	39.40		29.90	c
Pro5	53.10		39.60	b
P value	0.253		0.002	
SED	7.300		4.010	
Line				
P value	0.003		0.021	
SED	6.660		6.310	

Appendix 17. Estimate of variation in youngest fully emerged leaf length (mm), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period			
	1		2	
Treatment				
Hybrid	38.20		42.00	
Parent	38.00		42.90	
Progenitor	40.60		40.20	
P value	0.695		0.751	
Line within treatment				
H1	42.50		46.70	ab
H2	35.60		28.50	c
H3	36.90		51.80	a
H4	36.00		37.50	bc
H5	35.00		45.30	ab
H6	43.40		41.90	ab
P value	0.761		0.004	
SED	7.350		5.130	
Par1	31.30	b	43.60	
Par2	34.60	b	37.30	
Par3	28.80	b	39.70	
Par4	57.20	a	50.90	
P value	0.018		0.683	
SED	8.290		11.760	
Pro1	35.80		38.00	
Pro2	39.90		39.70	
Pro3	41.20		39.30	
Pro4	35.10		42.50	
Pro5	51.10		41.40	
P value	0.297		0.964	
SED	7.840		6.600	
Line				
P value	0.041		0.356	
SED	7.510		7.700	

Appendix 18. Estimate of variation in stem diameter (mm), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period		
	1		2
Treatment			
Hybrid	0.25		0.25
Parent	0.30		0.28
Progenitor	0.27		0.25
P value	0.057		0.357
Line within treatment			
H1	0.19		0.25
H2	0.29		0.23
H3	0.26		0.24
H4	0.22		0.31
H5	0.28		0.29
H6	0.28		0.20
P value	0.181		0.086
SED	0.042		0.039
Par1	0.42	a	0.26
Par2	0.26	b	0.28
Par3	0.29	b	0.24
Par4	0.24	b	0.33
P value	0.031		0.388
SED	0.056		0.054
Pro1	0.32		0.26
Pro2	0.27		0.20
Pro3	0.29		0.23
Pro4	0.22		0.24
Pro5	0.27		0.31
P value	0.315		0.363
SED	0.044		0.056
Line			
P value	0.004		0.193
SED	0.044		0.049

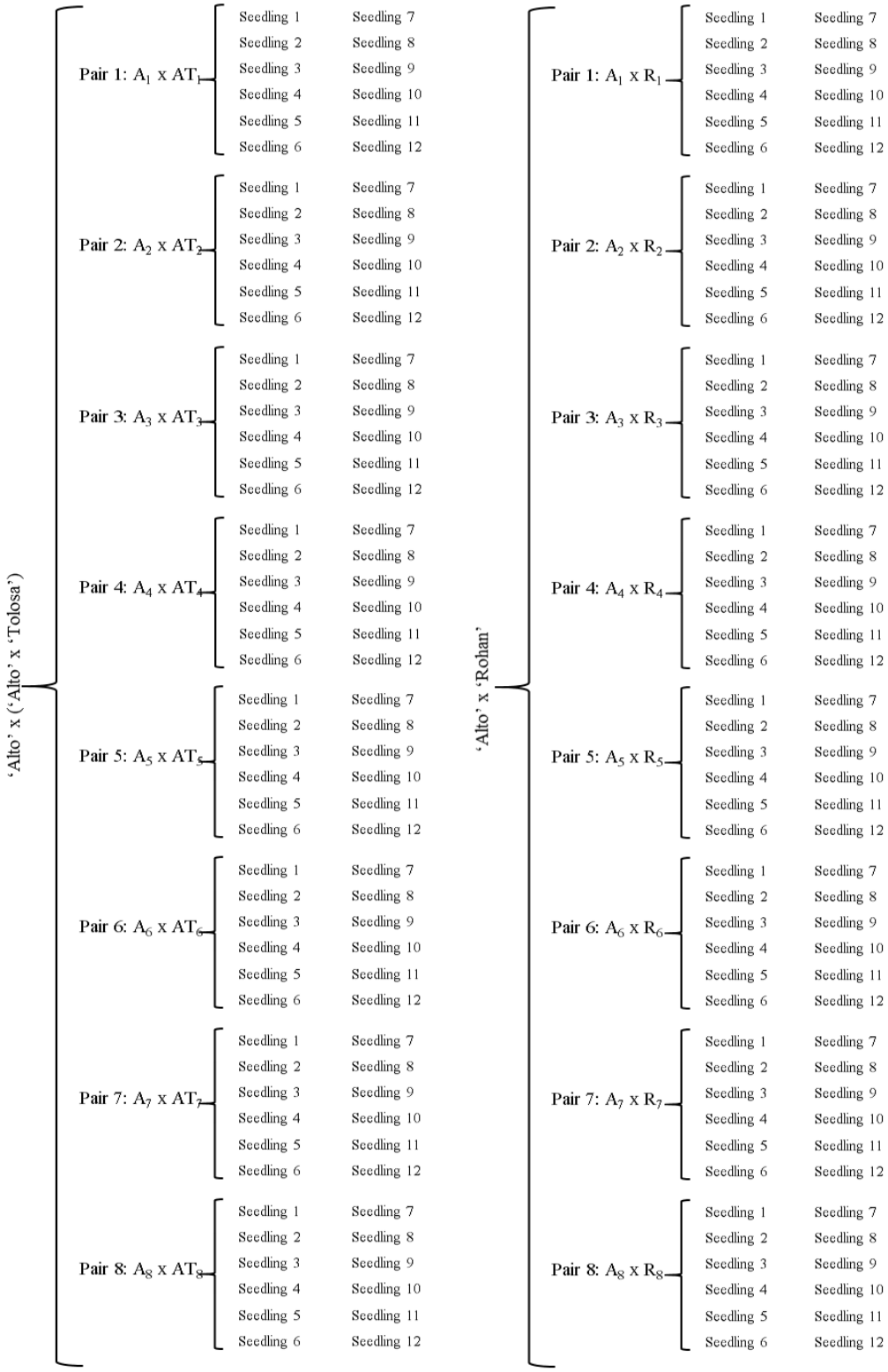
Appendix 19. Estimate of variation in emerging leaf area (cm^2), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period		
	1		2
Treatment			
Hybrid	1.24		0.88
Parent	1.14		0.73
Progenitor	1.31		0.52
P value	0.660		0.052
Line within treatment			
H1	1.24	bc	1.12
H2	0.80	cd	0.87
H3	1.84	a	0.73
H4	0.75	d	0.69
H5	1.15	bcd	0.71
H6	1.66	ab	0.82
P value	0.001		0.066
SED	0.250		0.093
Par1	0.98	b	0.50
Par2	0.48	b	0.68
Par3	0.93	b	0.68
Par4	2.19	a	0.86
P value	0.013		-
SED	0.443		-
Pro1	1.31		0.87
Pro2	1.43		0.51
Pro3	1.40		0.25
Pro4	0.99		0.75
Pro5	1.41		0.59
P value	0.940		0.605
SED	0.414		0.456
Line			
P value	0.003		0.328
SED	0.374		0.299

Appendix 20. Estimate of variation in youngest fully emerged leaf area (cm2), P values and SED for treatments, lines within treatments and lines. Means for treatments, or for lines within treatments, with a common letter are not significantly different (LSD 0.05).

	Morphology measurement period	
	1	**2**
Treatment		
Hybrid	1.29	1.17
Parent	1.22	1.23
Progenitor	1.29	1.07
P value	0.881	0.291
Line within treatment		
H1	1.23	1.24
H2	1.03	0.89
H3	1.57	1.17
H4	0.93	0.96
H5	1.55	1.45
H6	1.39	1.32
P value	0.140	0.056
SED	0.273	0.189
Par1	1.07	1.08
Par2	1.05	1.25
Par3	0.84	1.02
Par4	1.92	1.56
P value	0.120	0.319
SED	0.437	0.301
Pro1	0.94	0.98
Pro2	1.24	0.96
Pro3	1.43	1.23
Pro4	1.13	0.94
Pro5	1.69	1.22
P value	0.045	0.489
SED	0.232	0.216
Line		
P value	0.044	0.096
SED	0.322	0.220

Appendix 21. Experiment 2 experimental design. Two clones of each plant were used in the experiment.



Appendix 22. Thrive fertiliser

Total Nitrogen (N)			14.0%
	1.2%	Ammonium	
	12.8%	Urea Nitrogen	
Phosphorus		(P)	2.6%
Potassium		(K)	21.0%
Sulpher		(S)	9.7%
Magnesium		(Mg)	0.5%
Iron		(Fe)	0.18%
Manganese		(Mn)	0.01%
Zinc		(Zn)	0.004%
Boron		(B)	0.004%
Molybdenum		(Mo)	0.001%
Copper		(Cu)	0.0003%

Appendix 23. The effects of background, harvest, and background by harvest interactions on heterosis.

	P value	
	Mid-parent heterosis	**High-parent heterosis**
Effect		
Background	0.1570	0.1137
Harvest	<.0001	<.0001
Background*Harvest	<.0001	<.0001

Appendix 24. Effects of pair, harvest, and pair by harvest interactions on mid-parent heterosis.

There were no significant effects on mid-parent or high parent heterosis of harvest, or harvest by pair interaction, for the 'A x AT' background. In the 'A x R' background harvest had a significant effect on mid-parent and high-parent heterosis ($P < 0.001$). There was also significant effect on mid-parent and high-parent heterosis of harvest by pair interaction ($P = 0.0014$ and $P < 0.001$ respectively). The specific pair had a significant effect on both mid-parent and high-parent heterosis in both backgrounds ($P < 0.001$, Table A24).

Table A24. Effects of pair, harvest, and pair by harvest interactions on mid-parent heterosis.

	Mid-parent P value		High-parent P value	
	'A x AT'	**'A x R'**	**'A x AT'**	**'A x R'**
Effect				
Pair	<.0001	<.0001	<.0001	<.0001
Harvest	0.7050	<.0001	0.2091	<.0001
Harvest*Pair	0.8817	0.0014	0.4309	<.0001

Appendix 25. Pearson Correlation

Mid-parent heterosis

	Harvest 1 Clone 1	Harvest 1 Clone 2	Harvest 2 Clone 1
Harvest 1 Clone 2	0.52 <.0001		
Harvest 2 Clone 1	0.64 <.0001	0.37 <.0001	
Harvest 2 Clone 2	0.38 <.0001	0.82 <.0001	0.32 <.0001

High-parent heterosis

	Harvest 1 Clone 1	Harvest 1 Clone 2	Harvest 2 Clone 1
Harvest 1 Clone 2	0.66 <.0001		
Harvest 2 Clone 1	0.63 <.0001	0.44 <.0001	
Harvest 2 Clone 2	0.46 <.0001	0.79 <.0001	0.48 <.0001