Development of a Rhizosheath Selection Tool for screening perennial ryegrass (*Lolium perenne* L.) for reduced root competition against white clover (*Trifolium repens* L.) for soil phosphorus

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Abstract

Perennial ryegrass (*Lolium perenne* L.) outcompetes white clover (*Trifolium repens* L.) for soil phosphorus [P], resulting in higher P fertiliser rates to overcome the competition. Perennial ryegrass has long, dense root hairs compared to white clover, which increase the root contact with soil (soil exploration) and root surface area for P uptake. This study was conducted to test whether the selection of perennial ryegrass with reduced root hair length and density would reduce root competition for P with no detrimental effect on the perennial ryegrass growth.

A Rhizosheath Selection Tool [RST] was developed to select ryegrass populations with contrasting root hair length and density. The RST protocol used rhizosheath traits (the width and coverage of soil adhered to the extracted root system) along with the total seedling weight to select two distinct ryegrass populations. The low rhizosheath population had shorter, sparser root hairs on seminal roots and sparser root hairs on adventitious roots than the high rhizosheath population.

The two populations were then grown at five soil P levels in a glasshouse experiment and demonstrated that the rhizosheath trait selection had no detrimental effect on growth. There were no significant differences between the two population's dry weight (g) and P concentration (mg P g^{-1} dry weight). However there was evidence to indicate that the high rhizosheath population was sometimes more efficient at P uptake with a larger total P content (mg P).

The two perennial ryegrass populations were then grown with companion white clover in Olsen P 12 and 19 mg L^{-1} soil with partitions separating the shoot systems to avoid shoot competition for light. The low rhizosheath population had reduced root competition with the companion white clover. In Olsen P 12 mg L^{-1} soil, more P was partitioned to the white clover than the ryegrass, measured by total P content (mg P), and achieved a greater dry weight (g) than when grown with the HRS ryegrass. In Olsen P 19 mg L^{-1} soil there was no rhizosheath selection effect on white clover growth, possibly because of the reduced need for soil exploration by roots under the higher soil P availability.

The findings from this study confirmed that the RST for the selection of a perennial ryegrass population with a reduced capacity for root competition against white

clover for soil P is effective. Furthermore, the RST selection had no detrimental effect to the ryegrass growth. This research highlights the importance of root traits in forage plant breeding, and the potential environmental and economic benefits a low rhizosheath perennial ryegrass could have in New Zealand's pastoral farming system.

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Chapter One

Literature Review

1.1 Introduction

The New Zealand economy is highly dependent on agriculture and the climate provides good conditions for pasture based livestock feeding, traditionally using a perennial ryegrass (*Lolium perenne* L., Poaceae) and white clover (*Trifolium repens* L., Fabaceae) mixed pasture (New Zealand Government, 2016). However, perennial ryegrass outcompetes white clover for soil phosphorus [P], resulting in increased P fertiliser requirements to compensate for this competition. This root competition and the consequent elevated P fertiliser rate, has led to environmentally damaging amounts of P lost from pastoral land and entering freshwater ecosystems. It was therefore considered important to investigate the rooting structures of ryegrass and white clover, in particular the root hair characteristics of ryegrass, to determine if it was possible to produce perennial ryegrass populations with a reduced capacity for root competition against white clover for soil P.

This thesis will focus on root hair length and density in perennial ryegrass. Root hairs are critically important for soil exploration and nutrient uptake. Perennial ryegrass has long, dense root hairs that may explain its competitive advantage for soil P acquisition over white clover. Research on root hairs are difficult as there are limited methods that cause minimal disruption to the roots. The rhizosheath is defined as the soil which adhered to the root system when extracted from the soil; and the rhizosheath size correlates with root hair length and density. Therefore the rhizosheath size was used in a rapid screening method to allow for the selection ryegrass populations with contrasting root hair characteristics. The effectiveness of the selection tool and potential effect of selection on the plant required investigation. It was necessary to ensure root hair length and density were functionally suitable traits for decreasing root competition.

The information obtained from this study will be passed on to plant breeders for the purpose of adapting breeding techniques and targets for future novel ryegrass cultivars that have the potential to benefit both farmers and the environment.

Historical breeding programmes have focused on shoot morphology, as roots have been technically difficult to analyse. However there are significant potential gains to be made in agronomy through root focused breeding.

1.2 Pastoral farming in New Zealand

Early 1920s pastoral farming research promoted grass and clover mixes as high quality international seed became available to sow on soils after the removal and burning of native vegetation (Hunt & Easton, 1989). The species specific combination was later refined to perennial ryegrass and white clover (Levy, 1936). The historical agricultural use of perennial ryegrass and white clover led to an investment in breeding programmes to establish novel cultivars specific to New Zealand conditions (Charlton & Stewart, 1999; Corkill, 1949).

Perennial ryegrass and white clover pasture is the preferred mix due to their high dry matter yield, digestibility and nutritive value for livestock (Waghorn & Clark, 2004; Wilkins & Lovatt, 2011). In addition this type of pasture cover was durable to a range of management practices and was known to persist in varying environmental conditions (Allan & Keoghan, 1994). Individually, white clover is a legume with the additional benefits of fixing atmospheric nitrogen (Porqueddu *et al.*, 2016). Nitrogen fixation by white clover has been measured at 100 – 300 kg N^{-1} ha^{-1} yr^{-1} (Brock *et al.*, 1989). However low soil organic matter or high N inputs can reduce the atmospheric N fixation by clover in a mixed pasture to 30 – 130 kg N^{-1} ha^{-1} yr^{-1} (Brock *et al.*, 1989). Perennial ryegrass does not fix nitrogen but is easily established with fast seedling growth, persistent, and high quality feed for livestock particularly during cool climatic conditions (Hunt & Easton, 1989).

However, perennial ryegrass and other companion grasses outcompete white clover for soil P (Donald, 1963; Jackman & Mouat, 1972a). An increased rate of P fertiliser is required to meet the demand of perennial ryegrass before white clover's P requirement is met (Jackman & Mouat, 1972b; Ozanne *et al.*, 1976). Perennial ryegrass / white clover pasture requires three times the rate of P fertilisers than when growing the two species separately to achieve the same yield (Dunlop & Hart, 1987).

1.3 The problem

Phosphorus is a macronutrient required for plant growth as it is critical for cellular metabolic pathways and the transfer of energy (Schachtman *et al.*, 1998). However P is frequently low in availability as it is a relatively immobile nutrient in soil that readily binds to soil surfaces (Raghothama & Karthikeyan, 2005). There are also increasing concerns for the global long term availability of P and the impact a lack of supply will have on P dependent agricultural systems (Chowdhury *et al.*, 2017; Ockenden *et al.*, 2017; Williams *et al.*, 2007). Phosphorus is a non-renewable resource and cannot be synthesised (Cordell *et al.*, 2009). The current P fertiliser rates used in intensive farming systems are unsustainable, both economically for farm budgets and environmentally for the surrounding freshwater environments (Parliamentary Commissioner for the Environment, 2004).

Diffuse agricultural sources contribute 91% of the total P entering freshwater ecosystems in New Zealand, at a rate of 0.11 to 1.67 kg P ha^{-1} yr^{-1} (Gillingham & Thorrold, 2000). From agricultural land, P runoff was positively correlated with the timing and application rate of P fertiliser (Dougherty *et al.*, 2008; Hart *et al.*, 2004). The excess P was predominantly transported via overland flow from pasture as P bound to soil particles and was carried with sediment into freshwater streams and lakes (Hart *et al.*, 2004). As P is a key limiting nutrient in freshwater ecosystems the additional input causes eutrophication and exotic plant and algal growth, resulting in overall degradation to the health of freshwater ecosystems (Dougherty *et al.*, 2008; McDowell *et al.*, 2011; Ramezani *et al.*, 2016).

The freshwater resources in New Zealand hold cultural, recreational, environmental and economic value for many New Zealanders and therefore the quality and management of freshwater resources has been of significant national interest (Ministry for the Environment, 2017b). The establishment of the National Policy Statement for Freshwater Management (2014) provided Regional Councils with the authority and framework to monitor and manage freshwater resources in their region (Ministry for the Environment, 2017a). To meet the requirements of the National Policy Statement for Freshwater Management (2014) the Waikato Regional Council is currently establishing the Wai Ora - Healthy Rivers Plan

(Waikato Regional Council, 2018). The DairyNZ submission to the Wai Ora – Healthy Rivers Plan highlights the dairy industry's concern in remaining economically competitive while meeting their environmental obligation; asking for flexibility and a realistic timeframe to allow for research and innovation to provide on-farm solutions (DairyNZ, 2017).

1.4 Competition drivers

Donald (1963) reported that companion grasses (such as perennial ryegrass) were stronger competitors for soil nutrients than white clover. Jackman and Mouat (1972b) researched competition between browntop (*Agrostis tenuis* Sibth.) and white clover and identified that white clover had reduced yield and nitrogen concentration when grown with companion grasses at sub-optimal soil P concentrations. In a further study by Jackman and Mouat (1972a) the competition between the browntop and white clover was explained by root competition with browntop locating and assimilating more P from the soil. Later, the competition specifically between perennial ryegrass and white clover for soil P was confirmed to also be a result of root competition for soil P (Collins & Rhodes, 1994). It was proposed that the larger number of root tips of the companion grass had explored the soil for P, creating direct competition with white clover which had significantly fewer root tips (Jackman & Mouat, 1972a). Root volume and root hairs were also proposed as root traits contributing to the competitive advantage of companion grasses with white clover (Clarkson & Sanderson, 1970; Lewis & Quirk, 1967; Nye, 1966).

In-depth investigation into the root morphology of perennial ryegrass and white clover identified clear differences between the two species' root systems. It was well known that perennial ryegrass roots were more fibrous than white clover roots (Jacques, 1958). Evans (1977) quantified perennial ryegrass roots as longer, thinner and more frequently branching roots with longer, denser root hairs than white clover. In addition the calculated root surface area (a combination of root and root hairs) of perennial ryegrass was significantly larger than white clover's calculated root surface area (Evans, 1977). Root hairs significantly increase the surface area of the root, allowing for an increase in soil exploration for nutrients,

indicating it was root hairs that gave perennial ryegrass the competitive advantage (Barber & Silverbush, 1984; Zhu *et al.*, 2005).

Barley and Rovira (1970) conducted one of the first experiments to compare the variation in nutrient uptake within the same species of wheat (*Triticum aestivum* L.), providing an insight into the function of root hairs, and concluding that plants with the potential for long root hair length did take up more P. Their experiment involved growing the same genotype in either a compacted or an untreated soil core to control root hair growth of the plants. Bole (1973) criticised the method claiming the different compaction rates of the soil core was not considered. He then conducted an experiment with a number of wheat cultivars with known parental root hair traits and showed no relationship between root hair density and P uptake but provided no explanation for the lack of relationship. However most published reports have demonstrated the importance of root hair function in P uptake (Caradus, 1981; Evans, 1977; Simpson *et al.*, 2014). Lewis and Quirk (1967) observed a ^{32}P depleted zone surrounding the root hairs of wheat plants, indicating active absorption and uptake of the immobile nutrient by root hairs. Root hairs are single epidermal cells that elongate perpendicular to the root into the surrounding soil, increasing the root surface area into the soil (soil exploration) and the absorptive surface for P uptake (Barber & Silverbush, 1984; Datta *et al.*, 2011; Lewis & Quirk, 1967; Nye, 1966; Zhu *et al.*, 2005).

The origin of the two species provides insight into their adaptation to their initial pre-pastoralism ecosystem, resulting in their current root system differences. Perennial ryegrass originated on nutrient poor forest margin soils (Balfourier *et al.*, 2000; Scholz, 1975), and adapting their root characteristics for increased soil exploration and nutrient mining (Hill *et al.*, 2006). White clover is a natural hybrid of *Trifolium pallescens* Schreb. and *Trifolium occidentale* Coombe. which evolved on young, relatively P rich soils; consequently this species was under less selection pressure to evolve a root system adapted to infertile soils (Nichols & Crush, 2015; Papanastasis *et al.*, 2002). White clover has a higher critical P requirement needing almost double the soil P concentration than perennial ryegrass to achieve 90% of maximum yield (Kidd *et al.*, 2016; Ozanne *et al.*, 1976). The nutrient poor soils

perennial ryegrass is adapted to led to a low critical P requirement paired with increased internal vacuole storage of P (Schachtman *et al.*, 1998).

1.5 Breeding the solution

The focus of pastoral species breeding is changing towards cultivars for systems that are economically and environmentally sustainable (Abberton *et al.*, 2008; Chapman *et al.*, 2017; McDowell *et al.*, 2011; Williams *et al.*, 2007). The historical breeding focus of increasing yield to sustain intensification often resulted in an increase in root competition by perennial ryegrass against white clover, cancelling any potential gains from white clover breeding (Chapman *et al.*, 1987; Hayman, 1980; Ozanne *et al.*, 1976; Stewart, 2006).

Novel pasture cultivars including white clover with a decreased critical P requirement and a perennial ryegrass with a reduced capacity for root competition; these novel cultivars would reduce the overall P fertiliser required and therefore reduce the environmental footprint of perennial ryegrass / white clover pastures (McDowell *et al.*, 2011; Simpson *et al.*, 2014). Research continues to be conducted on white clover with a different focus on decreasing the critical P requirement, allowing the production of the same yield but at a lower soil P level. Potential traits identified in *Trifolium uniflorum* L., suggesting an interspecific hybridisation strategy with white clover (*T. repens*), may increase the responsiveness to low soil P conditions in breeding populations (Nichols & Crush, 2014, 2015; Nichols *et al.*, 2014). In combination, the development of a perennial ryegrass with reduced root competition, and a more P efficient white clover would increase the P use efficiency of the overall pastoral system.

Limited research has been conducted on below-ground traits of perennial ryegrass in comparison to above-ground traits (Crush *et al.*, 2009; Crush *et al.*, 2015; Simpson *et al.*, 2014). Rose *et al.* (2016) highlighted that a number of studies which have investigated the P use efficiency of a species have frequently failed to produce populations for breeding purposes. The lack of breeding outcomes is because of the inefficient or poor screening methods available (Rose *et al.*, 2016). Root morphology and root hair measurements also present further challenges for

researchers as methods are required that cause minimal disturbance to the root, yet accurately quantify the root traits (Downie *et al.*, 2015).

1.6 Methods for root hair investigation

Recent experimental methods to rapidly screen plants based on root hair traits for improved P use efficiency include QTL mapping (Yan *et al.*, 2004; Zhu *et al.*, 2005), *in situ* micro-photography (Vincent *et al.*, 2017), and a visual rhizosheath selection process (Delhaize *et al.*, 2012). However all methods require calibration for the investigated species and experimental focus (Jiayin *et al.*, 2017; Rose *et al.*, 2016).

The use of QTL mapping is a non-invasive screening tool, only requiring a small tissue sample (Yan *et al.*, 2004; Zhu *et al.*, 2005). The selection of QTL marker first requires identification of the genetic correlation between phenotype and genotype, using a mapping population and a method for direct assessment of root hair traits. Establishing the correlation can also be difficult as root hairs are often influenced by geneotype × environment interactions (Yan *et al.*, 2004). *In situ* micro-photography, investigated by Vincent *et al.* (2017), has shown potential with good comparisons between their data set and predictive regression models. This method does require specialised equipment and technical understanding to calibrate the model to the micro-photography images specific to the species. However, once established the *in situ* micro-photography method could provide opportunity for rapid screening and evaluation of root hair function that is not possible under conventional methods (Downie *et al.*, 2015).

The visual rhizosheath selection method has allowed for a rapid selection of seedlings for variation in root hair traits based on the rhizosheath size, with minimal equipment and disturbance to the root. The rhizosheath was defined as the soil that adheres to the roots when extracted from soil, and was first described over 100 years ago by A. G. Tansley and others (George *et al.*, 2014). The rhizosheath differs to the rhizosphere as the rhizosphere is the environment created between the root and the soil, which has been modified by the presence and activity of the root (Jiayin *et al.*, 2017; McCully, 2005). The rhizosheath size is depended on root hair traits, therefore the visual assessment of the rhizosheath traits has been used as a visual indicator for underlying root hair traits, while

imposing minimal disturbance to the root system (Delhaize *et al.*, 2012; Haling *et al.*, 2010).

Correlations have been reported between rhizosheath size and root hair length but the strength of correlation often varies depending on plant species (Haling *et al.*, 2010). A positive linear relationship between root hair length and rhizosheath size has been reported in cocksfoot (*Dactylis glomerata* L.), and phalaris (*Phalaris aquatica* L.) (Haling *et al.*, 2010), wheat (*Triticum aestivum* L.) (Delhaize *et al.*, 2012), and a weaker correlation in barley (*Hordeum vulgare* L.) (George *et al.*, 2014; Haling *et al.*, 2014). The literature shows that root hair length is an important factor in determining rhizosheath size. Fewer studies have reported on the impact of root hair density on rhizosheath size.

Delhaize *et al.* (2012) developed a novel rhizosheath selection method for wheat to rapidly select for two distinct populations with either large or small rhizosheath sizes (g soil m^{-1} root length) for the purpose of testing the two population's tolerance to soil aluminium levels. Through this rhizosheath selection method, Delhaize *et al.* (2012) were able to confirm the relationship between rhizosheath size and root hair length, suggesting that rhizosheath size could be used as an indirect selection tool for root hair length. However they did not investigate the effect of selection on root hair density. A number of experiments have taken into consideration the rhizosheath size (g soil per unit of root length) when comparing genotypes for nutrient uptake or environmental tolerance (Brown *et al.*, 2017; Gahoonia & Nielsen, 2004; George *et al.*, 2014; Haling *et al.*, 2010; Rose *et al.*, 2016). Prior to the rhizosheath selection method described in Delhaize *et al.* (2012), no studies had used the rhizosheath size to create a breeding population because of the mortality of plants used for root and dry matter measurements. The visual rhizosheath selection method has not been tested or calibrated on perennial ryegrass but the literature supports the potential for a successful adaptation of the method for the purpose of creating a breeding population of perennial ryegrass with reduced root competition for soil P.

1.7 Research aim and objectives

The aim of this thesis was to produce a perennial ryegrass population with decreased root competition against white clover for soil P with no significant negative effects on the yield and performance of the ryegrass population. This was achieved through four objectives.

1. To develop a rhizosheath size selection method, specific to perennial ryegrass, for the selection of two distinct populations with either long, dense root hairs or short, sparse root hairs.
2. Investigate the underlying root hair traits, selected using the rhizosheath selection method, and the root hair traits persistence from seedling seminal roots to mature plant adventitious root systems.
3. Use a P response experiment to determine the impact of the rhizosheath selection on dry matter yield and P uptake of the two selected perennial ryegrass populations.
4. Test the functional differences of the two selected populations and their impact on root competition with white clover for soil P. This root competition trial was done to validate literature on root hair function, and investigate whether root hair traits are a plausible breeding strategy for a novel perennial ryegrass population.

1.8 Experimental material

All experiments were carried out with Grasslands Nui perennial ryegrass (*Lolium perenne*), Line: A17905 from the Margot Forde Forage Germplasm Centre, Palmerston North, New Zealand. Grasslands Nui [Nui] was accepted on the New Zealand List of Acceptable Herbage Cultivars in 1973 after field tests for yield and persistence (Armstrong, 1977; Charlton & Stewart, 1999) (Figure 1).

Figure 1: An image of Grasslands Nui perennial ryegrass from a field trial prior to commercial availability (photo from Armstrong (1977)).

1.9 Thesis outline

Chapter One: Literature review

A review of the literature relating to the competition between perennial ryegrass and white clover for soil P. This chapter also describes the function of root hairs and in particular the role of root hairs in acquiring soil P and providing perennial ryegrass with the competitive advantage over white clover. The lack of established screening methods, with minimal disturbance to the plant, was then highlighted followed by a review of current and developing screening methods. The formation of rhizosheaths and the use of the rhizosheath size selection method was then investigated for the potential to select distinct perennial ryegrass breeding populations with reduced root hair length and density, to decrease their competition against white clover for soil P.

Chapter Two: The development and assessment of a Rhizosheath Selection Tool

This chapter describes the experimental process of establishing and calibrating a rhizosheath selection protocol specific to perennial ryegrass. The experimental

process consisted of five linked experiments. Experiment One: established any endophyte infection presence in the experimental population. Experiment Two: in-depth morphological characteristics of perennial ryegrass, to determine the variation within the root architecture of the investigated perennial ryegrass population, establishing the scope for calibration of the rhizosheath selection method. Experiment Three: development of the Rhizosheath Selection Tool [RST] protocol – based on literature and the results gathered from Experiment Two, allowing for selection of seedlings with desirable rhizosheath characteristics. The fourth and fifth experiments used the plant material selected using the RST to analyse the relationship between root hair and rhizosheath size and to test whether the seminal and adventitious roots on the same plant had similar rhizosheath characteristics.

Chapter Three: Assessment of the phosphorus response of RST selected perennial ryegrass

This chapter assessed the effect the RST selection had on the selected perennial ryegrass populations created in Chapter Two, Experiment Three. Each population was grown in five soil P levels and analysed for any differences in dry weight (g), P concentration of the tissue (mg P g^{-1} dry weight) and total P assimilated (mg P).

Chapter Four: Root competition between RST selected perennial ryegrass and white clover for soil phosphorus

This chapter investigated the effect of the RST selected populations on root competition for soil P between ryegrass and white clover. Both perennial ryegrass populations, created in Chapter Two, Experiment Three, were grown with white clover for the analysis of dry weight (g), P concentration (mg P g^{-1} dry weight) and total P content (mg P) of the two species.

Chapter Five: Synthesis

A summary of the RST, its impact on the perennial ryegrass populations and overall effectiveness in reducing root competition for soil P with white clover. This chapter also highlights areas requiring further research and recommendations for the future application of the RST for perennial ryegrass.

Chapter Two

The development and use of a Rhizosheath Selection Tool

2.1 Introduction

Despite the extensive research exploring perennial ryegrass phosphorus [P] acquisition and potential for improving P use efficiency there have been limited breeding outcomes produced. Rose *et al.* (2016) attributes the limited breeding outcomes to the lack of efficient screening methods. Research involving roots can be technically difficult with many methods involving a full destructive harvest or significant disturbance to the root system (Vincent *et al.*, 2017). These methods prevent any further analysis and propagation from the same plants investigated in the trial.

Examples for cultivar yield and root morphology of agricultural crops such as alfalfa and wheat, have shown selective breeding has been successful due to the large initial within population genetic variation (Julier *et al.*, 2000; Tokatlidis *et al.*, 2004). Selective breeding within a population allows for superior parent material with desired traits which can improve the cultivar's performance (Julier *et al.*, 2000). The process of selective breeding can be sectioned into four steps, beginning with identifying the within a population variation. The second step is the development of methodology to select for the desirable traits. And thirdly testing the persistence of the trait (Tokatlidis *et al.*, 2004). A fourth step to understand the impact of selection and functional differences of the trait selected for is also necessary in selective breeding, but this will be explored in Chapters Three and Four.

To achieve the above step one (specific to this experimental aim), the variation in rooting traits of the ryegrass population must first be analysed. The variation analysis must take into account internal factors such as endophyte influence. Following this analysis, step two of developing a protocol for a Rhizosheath Selection Tool [RST] can be achieved based on any relationships between measureable root and shoot traits to rhizosheath size (Figure 2).

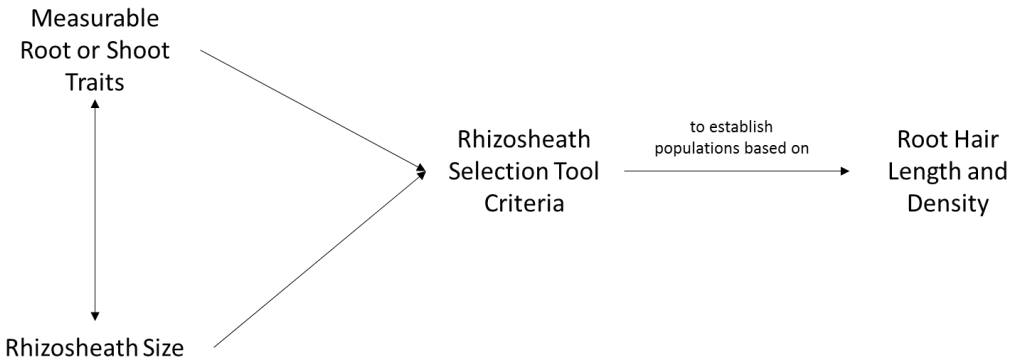

Figure 2: Design process for the development of the Rhizosheath Selection Tool

The purpose of the RST is to rapidly select for seedlings with high or low rhizosheath size with a method that does not damage the seedling. As previously defined, the rhizosheath is the soil that adheres to the circumference of the root when lifted from soil. It is hypothesised the rhizosheath size and coverage of the root is related to the length and density of root hairs. Trichoblast (root hair) cells elongate into the rhizosphere to increase the surface area of the root, coming into contact with and adhering to the soil (Datta *et al.*, 2011).

Seedling selection based on a RST is novel for perennial ryegrasses but a similar method has been performed in wheat (*T. aestivum*). Delhaize *et al.* (2012) selected germplasm based on rhizosheath size (grams of soil per metre root length) for selective breeding. Their study identified a strong, positive correlation between root hair length and rhizosheath size (Figure 3). Therefore the selection of seedlings based on the rhizosheath is hypothesised to indirectly select for variation in root hair characteristics (root hair length) with minimal disruption of the root system. However the relationship between rhizosheath size and root hair length and density for perennial ryegrass requires investigation.

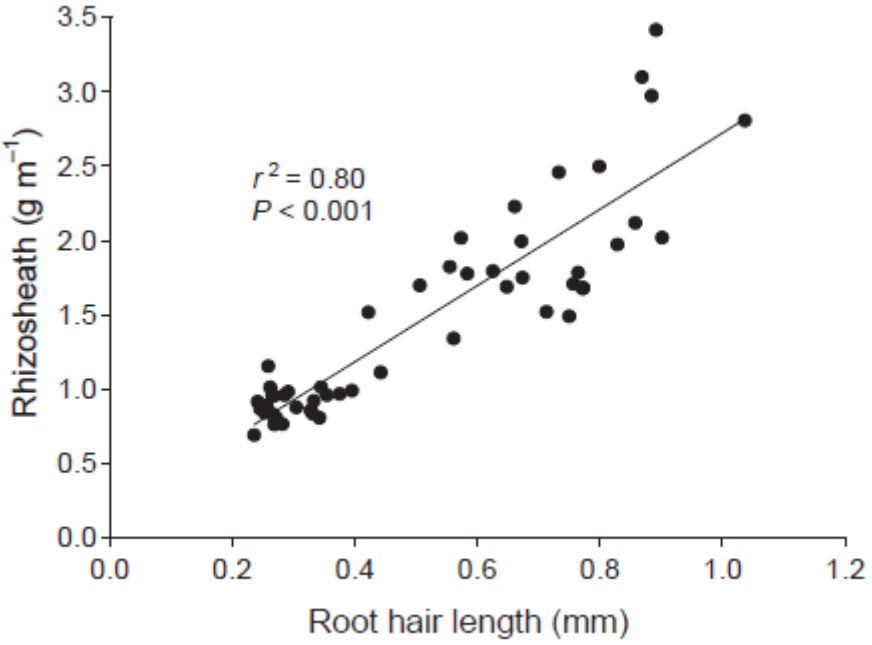

Figure 3: The strong positive correlation between root hair length (mm) and rhizosheath (g m^{-1}) in wheat (*T. aestivum*) (Delhaize *et al.*, 2012).

The third step is to assess the persistence of the selected trait in the mature plant. As the RST is designed for selection on seminal (juvenile) root systems it is therefore necessary to identify if the selected rhizosheath traits continue into the adventitious (adult) root system. The seminal root system is the primary roots that develop from the radicle of the seed and will continue to lengthen and branch (Kleeper *et al.*, 1984). Seminal roots support the juvenile seedling but are often short-lived and are replaced by adventitious roots as the plant matures (Raven *et al.*, 1992). The adventitious root system is the secondary roots that develop from nodes or the stem of the plant, not from the radicle (Kleeper *et al.*, 1984; Raven *et al.*, 1992) .

Three aims have been set for this chapter including the development of two distinct perennial ryegrass populations, one with a large rhizosheath size (with potentially long and dense root hairs) and the other with a small rhizosheath size (with potentially short and sparse root hairs). Also to investigate the correlation of root hair length and density to rhizosheath size. The final aim was to understand

the transfer of root hair traits from the seminal to adventitious root system of perennial ryegrass.

To achieve these aims there were five related experimental objectives. The order of the experimental objectives was important as the results or material produced by the previous experiment were required for the subsequent experiment.

- Investigate the percentage of endophyte presence in the population to quantify any endophyte influence. It was hypothesised that 50% of the seed was infected with endophyte as the percentage of endophyte infection of the seed was conducted prior to storage.
- Identify the root morphology variation within the ryegrass population including rhizosheath size. It was expected that the within cultivar variation would be normally distributed with a standard deviation greater than 40% of the mean.
- Develop a RST for rapid seedling selection with either large or small rhizosheaths including clear selection criteria.
- Investigate the RST effectiveness in producing two distinct populations based on rhizosheath size and use these populations to investigate the relationship between rhizosheath size and root hair length and density. As supported by literature, it was hypothesised that the rhizosheath size would have a positive correlation with the root hair length and density.
- Assess the persistence of root hair traits from seminal root systems to adventitious root systems using RST selected individuals. It was hypothesised that the root hair length and density is a persistent trait from seminal to the adventitious root system.

2.2 Method

2.2.1 Experiment One: endophyte presence

A total of 120 seeds of Grasslands Nui perennial ryegrass were germinated on damp filter paper in a petri dish for four days then planted into potting mix filled root trainers (4 cm × 4 cm × 10 cm). The experiment was planted in early January

2017, in a temperature controlled glasshouse (mean day temperature 21.6°C, night 15.8°C) under natural light. The plants were watered daily for four weeks. From four weeks after planting the seedlings had grown mature daughter tillers. A single tiller was cut low to the soil and immediately blotted by pressing the cut end of the tiller onto a nitrocellulose membrane paper leaving a circular outline of the cut end. The nitrocellulose membrane paper endophyte paper was stored in a refrigerator at 4°C until being processed for endophyte infection by an immune-detection method described in Hahn *et al.* (2003).

2.2.2 Experiment Two: in-depth morphological characteristics of the perennial ryegrass population

A large selection of > 600 Grasslands Nui perennial ryegrass seeds were germinated on damp filter paper in a petri dish for four days. A square of frost cloth was inserted into the bottom of 540 pots (4 cm X 4 cm X 8 cm) to prevent soil loss. Horotiu silt loam subsoil (Hewitt, 2010) was sieved through a 4 mm mesh and moistened to achieve 80% of soil moisture content [SMC] (~300 ml to 1 kg of air dried soil). Each pot was filled with moistened soil and a single pre-germinated seed with an emerging root was planted at a depth of 1 cm.

The pots were arranged into 10 trays, 54 pots per tray (9 X 6), and placed in two rows on a table in a temperature controlled glasshouse (early January; mean day temperature 21.6°C, night 15.8°C) under natural light (Figure 4). There was no treatment applied so tray and pot arrangement was ordered numerically by pot number. The outside edge of each tray was wrapped with silver reflective paper to mitigate any edge effect.

Figure 4: The layout of 10 trays containing a total of 540 pots; each tray wrapped with silver reflective paper.

Twenty pots were selected at random on day one, their initial weight recorded and reweighed every second day. The average change in the weight determined the volume of water that was added to all pots to maintain 80% SMC. A further five pots were randomly selected for destructive inspection of the roots during the growth period. The harvest data from these 25 pots was excluded from the final data set.

2.2.2.1 Harvest and rhizosheath measurements

From the remaining 515 seedlings planted only 430 were harvestable because of mortality or late shoot emergence. The seedlings were harvested starting at day 10 from planting, and harvesting was completed within 48 hours.

Each pot was gently tipped onto a tray and the seedling was lifted by the shoot from the soil. The seedling was gently shaken once to dislodge any non-adhered soil and the whole seedling including adhered soil was weighed and recorded. A large selection of the seedlings with soil adhered were photographed for a visual seedling assessment. The root was then washed clean of any visible adhered soil, laid on a paper towel and blotted dry once with a tissue and weighed again. The

plant was then placed into a small labelled container with 70% ethanol. Following the complete harvest the 430 seedlings were scanned for total root length, number of forks and tips, and total shoot length using an Epson Expression 1680 flatbed scanner (Epson America Inc., Long Beach, CA, United States) and software (WinRHIZO, Regent Instruments Inc., Quebec, QC, Canada) (Figure 5). Seedlings were returned and stored in their 70% ethanol containers. The weight of soil (g) that adhered to the length of root (cm) was calculated per seedling to provide a standardised measurement known as the rhizosheath size (g cm^{-1}). The WinRHIZO root and shoot traits were then compared with the rhizosheath size to identify relationships.

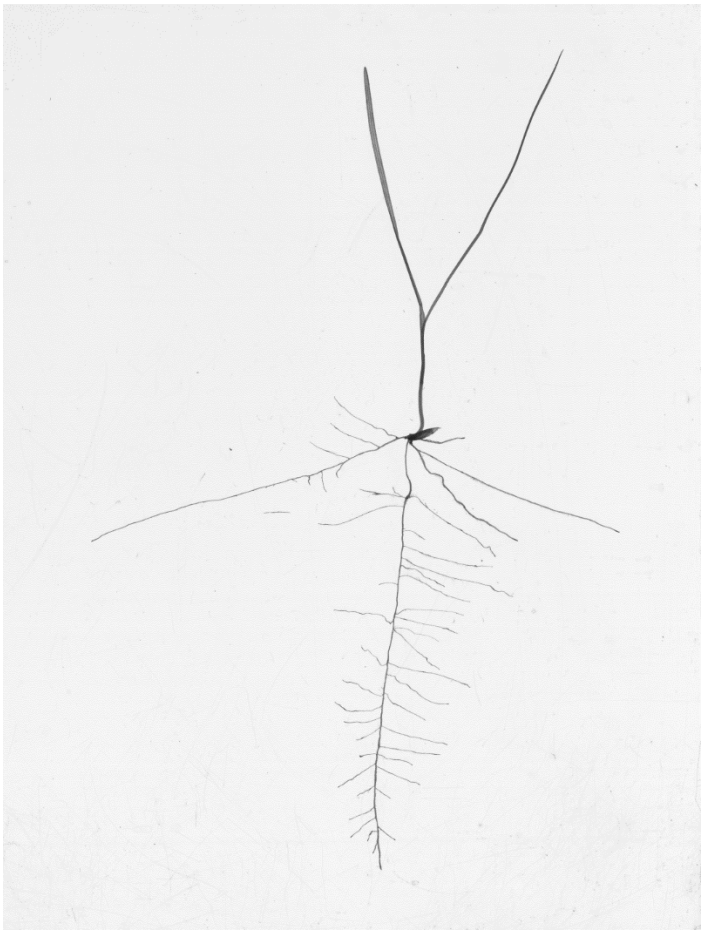

Figure 5: Example of a perennial ryegrass seedling scanned using an Epson scanner and WinRHIZO software for total root length, root branching traits (tips and forks) and total shoot length.

2.2.3 Experiment Three: development of the Rhizosheath Selection Tool

A further 250 Grasslands Nui perennial ryegrass seedlings were germinated and planted following the same procedure previously described. The pots were arranged into 4.5 trays, 60 pots per tray (10 X 6) and placed in two rows on a single table. The experiment was planted in mid-April 2017, in a temperature controlled glasshouse (mean day temperature 20°C, night 14.2°C) under natural light. There were no treatment applied so tray and pot arrangement was ordered numerically by pot number. The outside edge of the experiment pots was wrapped with silver reflective paper to mitigate edge effects. Again, 20 pots were randomly selected for weighing prior to watering to maintain 80% SMC; these 20 pots were excluded from the final harvest.

2.2.3.1 Harvest and Rhizosheath Selection Tool criteria

The 230 remaining pots were harvested 10 days after planting. The pot was gently tipped onto a tray from which the seedling was lifted and gently shaken once to dislodge any non-adhered soil. Whole seedlings including adhered soil were weighed and recorded. Three separate rhizosheath diameters were measured using calipers and the whole root system photographed. A total of 40 seedlings (20 low rhizosheath and 20 high rhizosheath sized seedlings) were replanted into a sand tray if they met the following RST criteria (Table 1).

Table 1: The Rhizosheath Selection Tool criteria for the selection of high or low rhizosheath of perennial ryegrass seedlings

High Rhizosheath Size	Low Rhizosheath Size
Seedling weight greater that 1 g	Seedlings less than 1 g
Complete rhizosheath with adhered soil	Incomplete rhizosheath with adhered soil
Thick rhizosheath diameter: ≥ 0.85 mm	Thin rhizosheath diameter: ≤ 0.85 mm
Visible root hairs	No visible root hairs

The RST was based on the methodology of Delhaize *et al.* (2012), involving the use of a seedling's rhizosheath as a visual indicator for root hair length and density. The RST criteria of Table 1 and Figure 6 were adapted to Nui perennial ryegrass from the results and relationships investigated in Experiment Three. The seedling had to meet the threshold weight and rhizosheath coverage criteria to successfully

be selected for a population. The rhizosheath thickness and visibility of root hairs supported the selection process but was not ultimately required for selection.

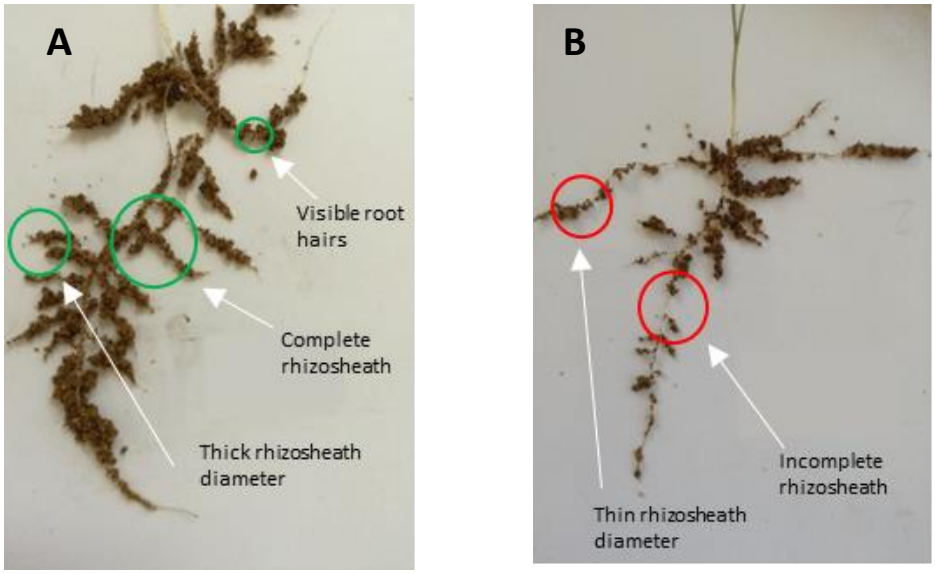

Figure 6: Two selected seedlings based on high rhizosheath size (A) and low rhizosheath size (B), both showing annotations of the Rhizosheath Selection Tool criteria.

2.2.4 Experiment Four: seminal roots

This experiment involved transplanting the HRS and LRS seedlings (20 per population) into hydroponics from which root micro-photography was captured for root hair measurements. The 40 RST selected seedlings from the previous experiment remained in the sand trays for 10 days then were transplanted into hydroponic tanks with low ionic nutrient solution (Table 2). The low ionic solution was designed based on the median nutrient concentrations of New Zealand pasture top soils (Care, 1999; Edmeades *et al.*, 1985). The pH of the low ionic nutrient solution was maintained at 5.8 – 6.3 by the addition of 10% HCl or 20% NH_4 to each tank. The low ionic nutrient solution was replaced with new solution every seven days. The transplanting into hydroponics occurred in mid-May 2017 and were plants grown in a temperature controlled glasshouse (mean day temperature 15.3°C, night 10.4°C) under natural light.

Table 2: Low ionic nutrient solution formula (Care, 1999).

Formula	g L^{-1} for stock solution	mL of stock solution per 45 L tank
Macronutrients		
NH_4NO_3	54.0	10
KNO_3	136.5	10
$MgSO_4.7H_2O$	110.8	10
$NH_4H_2PO_4$	3.1	5
NaCl	52.2	5
$CaSO_4.2H_2O$		3.5 g
Micronutrients		
$MnSO_4.4H_2O$	1.0	
$ZnSO_4.7H_2O$	1.3	
H_3BO_3	1.7	5
$CuSO_4.5H_2O$	0.2	
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.02	
EDTA FeNa	0.37	0.6

The seedlings were arranged within four tanks in a split plot design with two rows of five per tank (Figure 7). Each column contained one seedling from each population, completing one repeat [rep]. The order of placement within one population was randomised.

On top of the tank was a large cover which had pre-cut 7 cm diameter holes for bottomless pots. Seven cm diameter foam discs were cut, with a slit along the radius. The tiller was held by enclosing the foam disc around the base of the seedling, ensuring the tiller was through far enough for the tips of the root to be submerged in the solution (Figure 8). The foam discs were placed inside the pot which held the foam in place. Each tank had a pipe running along the base of the tank to release compressed air allowing each tank to remain aerated.

	Col 1	Col 2	Col 3	Col 4	Col 5	Col 6	Col 7	Col 8	Col 9	Col 10
	Tub 1					Tub 2				
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10
Row 1	1	2	2	1	1	2	1	1	2	2
Row 2	2	1	1	2	2	1	2	2	1	1
	Tub 3					Tub 4				
	Rep 11	Rep 12	Rep 13	Rep 14	Rep 15	Rep 16	Rep 17	Rep 18	Rep 19	Rep 20
Row 3	1	2	2	1	2	1	1	2	2	1
Row 4	2	1	1	2	1	2	2	1	1	2

Figure 7: The seminal root hydroponic experimental design; the grey '1' representing a low rhizosheath individual and the white '2' representing a high rhizosheath individual.

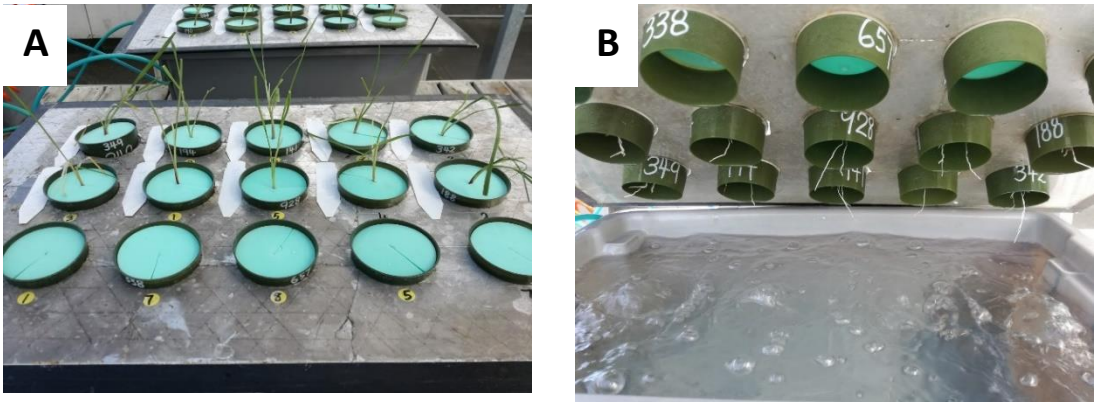

Figure 8: The hydroponic set up for the seminal root trial with each individual seedling secured within a foam disk (A), and a view of the lifted lid showing the bottomless pots with the root extending just past the pot lip to touch the low ionic nutrient solution (B).

2.2.4.1 Root hair measurements

From day 14 after transplanting into hydroponics, ¼ of the seminal root systems were removed, transported in low ionic nutrient solution and micro-photographed using a stereomicroscope equipped with a camera (Leica MZ12, Leica Microsystems, Wetzlar, Germany and AxioCam HRc, Zeiss, Gottingen, Germany). Three lateral roots from each seminal root section was photographed. The root hair images were captured using compatible software to the camera (AxioVision, Zeiss, Gottingen, Germany) and analysed with image analysis software (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA). The root hair density (number of hairs per mm root length) was measured and the length of each root

hair recorded. The root hair count began at the start of the maturation zone where the majority of root hairs had reached full length (Figure 9) and ended at either the intersection of a new emerging lateral bud or where the root hairs began to decay.

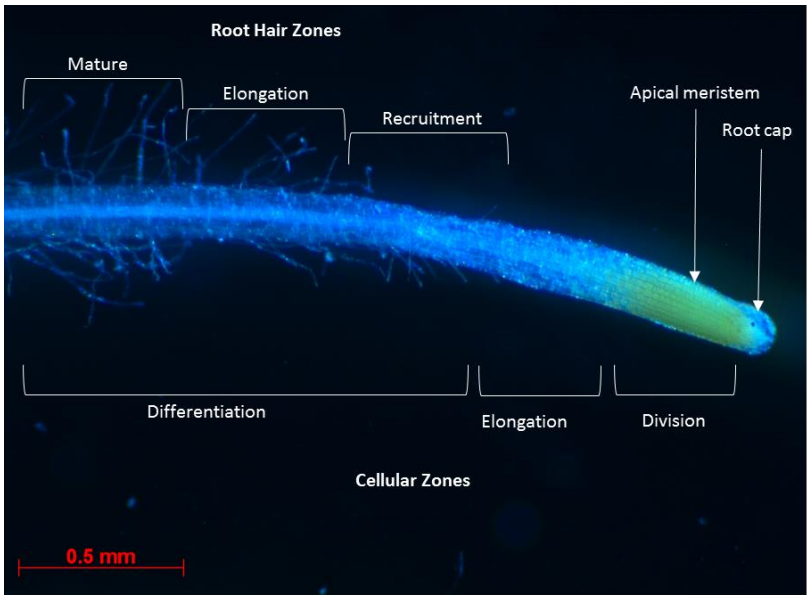

Figure 9: Root hair development zones of perennial ryegrass, separated into (from root tip) recruitment, elongation and mature root hair zones. The cellular zones refer to the root cellular function of a root tip and eventual differentiation of cells leading to root hair development (adapted from Care (1999) and Datta *et al.* (2011)).

Once the seminal root hair data were captured, the 40 seedlings were transplanted from hydroponics into 15 cm diameter pots with potting mix. These 40 seedlings provided material for the adventitious root system root hairs experiment to follow, and for Chapter Three and Four.

2.2.4.2 Calculations

The average root hair length (mm) per plant was calculated by the mean of all root hair lengths across the three lateral roots sampled for one plant. The density of root hairs (hairs per mm root length) was calculated by dividing the number of root hairs by the length of seminal root section the hairs were counted on. This was done for each of the three lateral roots then averaged per plant.

2.2.5 Experiment Five: adventitious roots

The 40 seedlings selected via the RST in the previous experiment were used to propagate material to allow for the direct comparison between the seminal and

adventitious root systems on the same genotypes. Two tillers were taken (one for hydroponic planting and the other for tiller dry weight) from each of the 40 RST selected plants. To remove the tillers, the plants were removed from their pot onto a tray. The soil was parted exposing the outer tillers of the plant and a small section of three to five tillers from the perimeter was held and pulled away from the plant. Once a gap between the small section of tillers and the plant was created, the small section of tillers was cut away leaving a maximum of 1 cm length of root attached. Unlike Experiment Four, the tillers used in the adventitious root experiment may not all be the same age. Therefore trimming the roots to 1 cm encourages new adventitious roots to develop at the same age. The remaining plant was returned to its pot and refilled with any lost potting mix. The small section was separated carefully, by hand, into individual tillers and any daughter tillers were removed. Two tillers were selected and trimmed to 10 cm in shoot length and 1 cm in root length. The roots were washed free of soil and blotted dry. The two tillers were weighed for fresh weight and planted into a sand tray in order of weighing. The tillers were watered daily for eight days during which adventitious roots were developing.

After eight days, tiller one was washed, patted dry then bagged for oven drying at 65°C for 48 hours, then weighed for the dry weight. The dried tiller's fresh and dry weight were compared between the two populations. This comparison provided an assessment to ensure the two populations at time zero begun with similar size.

The second tiller was removed from the sand tray, washed and transplanted into a 45 L hydroponic tank repeating the same hydroponic set up as the Experiment Four. The tillers were distributed in a split plot design across four hydroponic tanks with 10 plants per tank in two rows of five (Figure 10). The transplanting into hydroponics occurred in early August 2017 and were grown in a temperature controlled glasshouse (mean day temperature 16°C, night 10.2°C) under natural light. The two populations were paired and both present in each column as one rep. Again the order of placement within one population was randomised.

	Col 1	Col 2	Col 3	Col 4	Col 5	Col 6	Col 7	Col 8	Col 9	Col 10
	Tub 1					Tub 2				
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10
Row 1	1	1	1	2	2	2	1	2	1	2
Row 2	2	2	2	1	1	1	2	1	2	1
	Tub 3					Tub 4				
	Rep 11	Rep 12	Rep 13	Rep 14	Rep 15	Rep 16	Rep 17	Rep 18	Rep 19	Rep 20
Row 3	2	1	1	1	2	2	1	2	2	1
Row 4	1	2	2	2	1	1	2	1	1	2

Figure 10: The experimental design of the adventitious root experiment in hydroponics; grey '1' representing the low rhizosheath population and white '2' representing the high rhizosheath population.

Long Ashton complete nutrient solution (Hewitt, 1966) was used in the hydroponic tanks to support longer term growth for adventitious roots and has been extensively used in previous pastoral species experiments (Crush *et al.*, 2015; Nichols & Crush, 2014, 2015). The solution was mixed per 45 L tank and a pH of 5.8 – 6.3 was maintained by adding 10% HCl or 20% NH_4 into each tank sa necessary (Table 3). After 20 days the complete nutrient solution was replaced with new solution to ¾ of the tank volume to 30 L. A high humidity environment was created in the distance above the nutrient solution to the plant base. This high humidity environment induced root hair growth on developing adventitious roots that had not reached the nutrient solution. Three of these adventitious roots from each plant were cut and laid flat on a petri dish for micro-photography. The micro-photography of the adventitious root system hairs were captured on a stereomicroscope equipped with a camera (Leica M8 and Leica MC 170, Leica Microsystems, Wetzlar, Germany). The images were captured using compatible software and analysed with image analysis software (Leica Microsystems (LAS V4.8), Wetzlar, Germany; ImageJ).

Table 3: Long Ashton complete nutrient solution formula (Hewitt, 1966).

Formula	Stock Solution ($g\ L^{-1}$)	ml of stock solution per 45 L tank
Macronutrients		
KNO_3	40.4	90
$Ca(NO_3)_2$	65.6	90
$MgSO_4.7H_2O$	36.8	90
$NaH_2PO_4.2H_2O$	20.8	45
$NaNO_3$	68.0	90
Micronutrients		
$MnSO_4.4H_2O$	0.22	
$CuSO_4.5H_2O$	0.02	
$ZnSO_4.7H_2O$	0.03	
H_3BO_3	0.31	4.5
NaCl	0.59	
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.009	
$CoSO_4.6H_2O$	0.005	
EDTA FeNa	1.8	22.5

2.2.5.1 Calculations

Using ImageJ analysis, each root had 10 measurements taken for the calculation of the rhizosheath cylinder volume (Equation 1), the recruitment zone truncate cone volume (Equation 2) and the root cylinder volumes within the previous two volumes listed (Equation 3 and 4; Figure 11). The rhizosheath cylinder volume measurements started at the mature root hair zone where the root hairs became standardised in length, and continued until either the root hairs decayed, the development of a lateral root bud, or the cut from the plant ending the sample. The recruitment zone truncate cone measurements began from the first appearance of root hairs near the root tip to the start of the mature root hairs (where the rhizosheath cylinder volume measurements begun) (Figure 11Figure 9). The root cylinder volumes measurements were taken within the rhizosheath cylinder volume or the recruitment zone truncate cone volume sections. The

following calculations were performed to produce the final root hair cross sectional area.

Rhizosheath cylinder volume

(1) $$V_R = \pi(d/2)^2 h$$

V_R = Rhizosheath cylinder volume (A in Figure 11)

d = The average of the four rhizosheath width measurements[1]

h = Length of the rhizosheath section measured

[1]Four vertical white lines in Figure 11

Rhizosheath root cylinder volume

(2) $$V_{Rr} = \pi(d/2)^2 h$$

V_{Rr} = Rhizosheath root cylinder volume (B in Figure 11)

d = The average of the two root width measurements[2]

h = Length of the rhizosheath section measured

[2] Two vertical red lines in Figure 11

Recruitment zone root cylinder volume

(3) $$V_{Zr} = \pi(d/2)^2 h$$

V_{Zr} = Recruitment zone root cylinder volume (C in Figure 11)

d = The average of the two root width measurements[2]

h = Length of the recruitment zone section measured

Recruitment zone truncated cone volume

(4) $$V_Z = \frac{1}{3}\pi\,(R^2 + R \cdot r + r^2)h$$

V_Z = Recruitment zone truncated cone volume (D in Figure 11)

R = Large width measurement[3] / 2

r = Small width measurement[4] / 2

h = Length of the recruitment zone section measured

[3] Yellow line furthest from the root tip in Figure 11

[4] Yellow line closest to the root tip in Figure 11

These measurements and calculations were completed for each of the three adventitious roots per plant (60 roots per population). Following this the below

calculations were performed to remove the root cylinder volume from the rhizosheath and recruitment zone volumes to produce the exclusive volumes that the root hairs extend from the root into the soil (Equation 5 and 6). The exclusive rhizosheath cylinder volume was then divided by the length of the rhizosheath measured (h), to standardise each root by removing the variable length, producing a rhizosheath cylinder cross sectional area (Equation 7; Figure 12).

Exclusive recruitment zone truncate cone volume

(5) $$V_{EZ} = V_Z - V_{Zr}$$

V_{EZ} = Exclusive recruitment zone truncate cone volume

V_Z = Recruitment zone truncated cone volume (D in Figure 11)

V_{Zr} = Recruitment zone root cylinder volume (C in Figure 11)

Exclusive rhizosheath cylinder volume

(6) $$V_{ER} = V_R - V_{Rr}$$

V_{ER} = Exclusive rhizosheath cylinder volume

V_R = Rhizosheath cylinder volume (A in Figure 11)

V_{Rr} = Rhizosheath root cylinder volume (B in Figure 11)

Rhizosheath cylinder cross sectional area

(7) $$CS = V_{ER} / h$$

CS = Rhizosheath cylinder cross sectional area (Figure 12)

V^{ER} = Exclusive rhizosheath cylinder volume

h = Length of the rhizosheath section measured

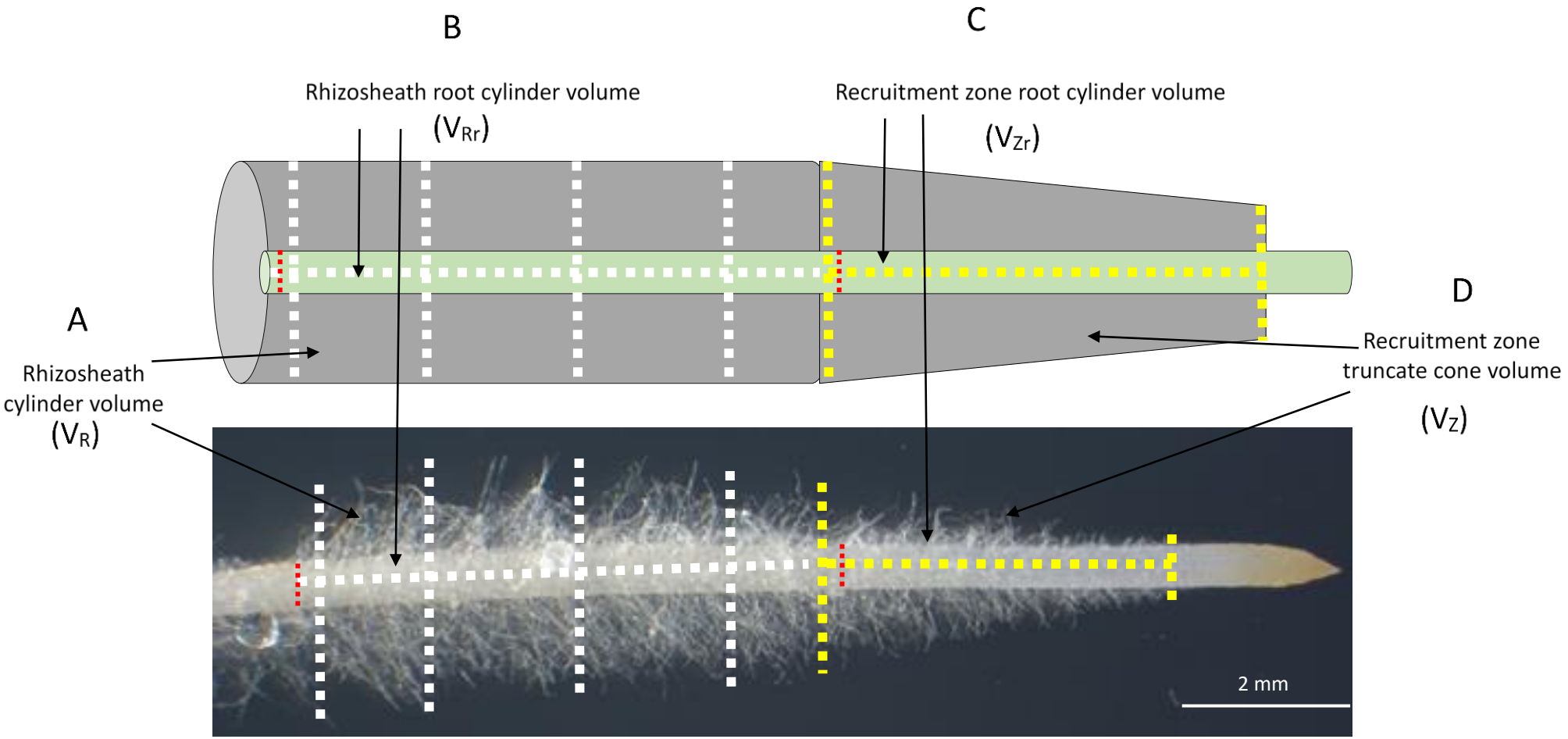

Figure 11: Adventitious root measurements (dotted lines) for the calculation of the rhizosheath cylinder volume (A) the two root cylinder volumes (B and C), and the recruitment zone truncate cone volume (D).

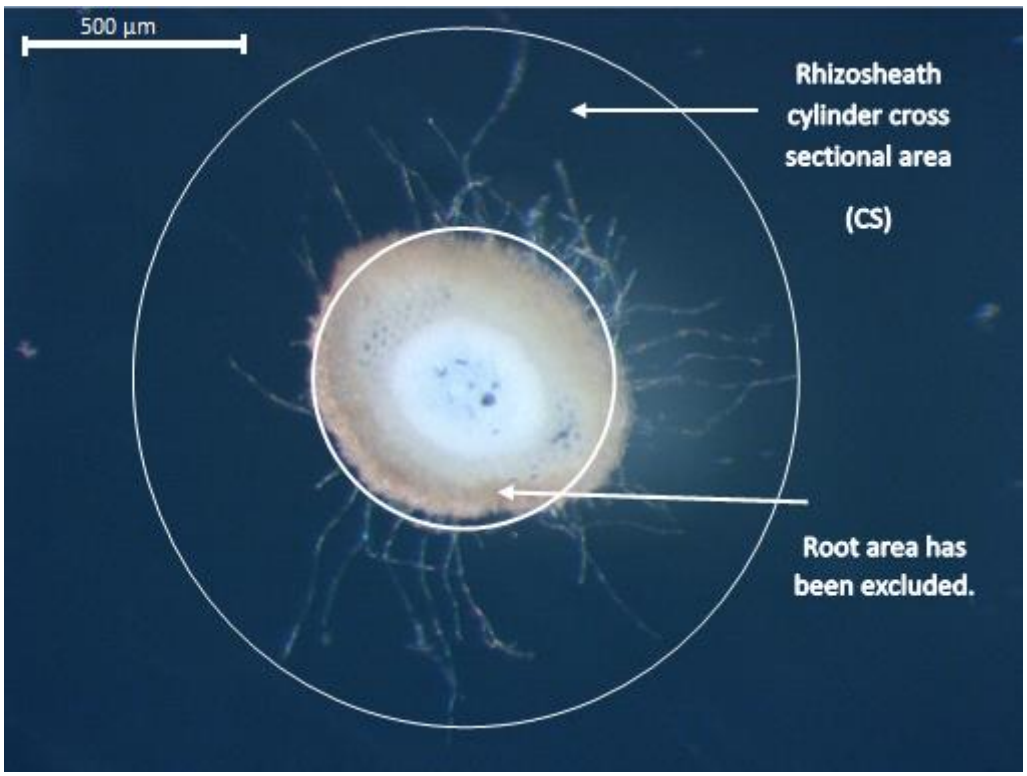

Figure 12: A cross section of a root with root hairs extending into the surrounding environment, illustrating the root hair cross sectional area from which the root area (centre) has been excluded.

2.2.5.2 Root hair density score

Root hair density of each root were given a score of 1 to 4, 1 being the sparsest to 4 being the densest. The scoring was based on the visual density of root hairs and referenced to one of the following four images (Figure 13). This was completed for each of the three roots per plant (60 roots per population) then averaged per plant.

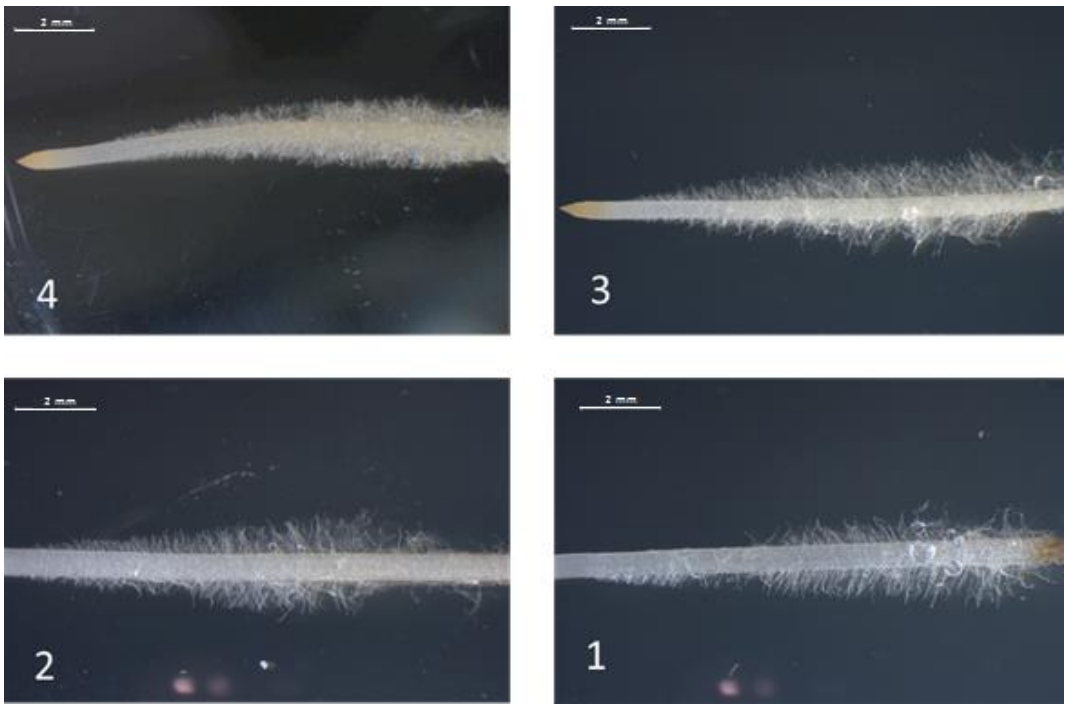

Figure 13: Reference images for the adventitious root system hair density visual score; increasing in root hair density from 1 to 4.

2.2.6 Statistical analysis

The data were analysed using Microsoft Excel 2013 (Microsoft Corporation, Washington, United States) and GenStat 18th edition (VSN International Ltd, Oxford, Great Britain). The data were processed by Analysis of Variance (ANOVA). The ANOVA data was structured by population and blocked by plant ID (as the three roots per plant ID). The residuals were inspected for any divergence from the assumptions of normality and constant variance. Transformations were not required. The relationship between root hair length and density was analysed by a linear regression. Statistically significant results were reported when the probability value [*P*] was < 0.05.

2.3 Results

2.3.1 Experiment One: endophyte presence

All endophyte blots returned a pale pink negative result. There was no wild type or other endophyte present in the Nui perennial ryegrass seed used in any of these experiments.

2.3.2 Experiment Two: in-depth morphological characteristics of the perennial ryegrass population

As hypothesised the seedling root and shoot traits were normally distributed with a standard deviation of the mean ≥ half that of the mean itself (Table 4). With the exception of total shoot length, each of the analysed traits were also positively skewed to the right due to outliers, some which had a value that was three times greater than the interquartile range.

Table 4: A statistical summary of five root and shoot traits expressing the variation within the Nui cultivar.

Trait	Mean	Standard Deviations	Minimum Value	Maximum Value
Total Root Length (cm)	54.2	25.3	2.3	132.6
Forks (count)	92.7	56.2	0	330
Tips (count)	68.7	33.8	1	197
Total Shoot Length (cm)	12.0	2.5	3.3	21.2
Rhizosheath Size (g cm^{-1})	0.05	0.03	0.001	0.21

2.3.3 Experiment Three: development of the Rhizosheath Selection Tool

The rhizosheath size had no relationship to the number of tips or forks (Figure 14 A and B). Likewise the total shoot length also had no correlation to the rhizosheath size (Figure 14 C), providing no above ground indication to the rhizosheath size before lifting the seedling from the soil. Therefore, branching traits and total shoot length will not be incorporated into the RST criteria.

There is evidence of a positive trend with increasing rhizosheath size resulting in an increase in weight (Figure 14 D). However, from a rhizosheath size of 0.1 g cm^{-1} (with an approximate total weight of 5 g) the data begins to expand from the line of best fit. Due to this trend the seedling weight can be included into the RST criteria; seedlings <1 g are likely to have a low rhizosheath size (<0.01 g cm^{-1}) and vice versa for selecting high rhizosheath seedlings.

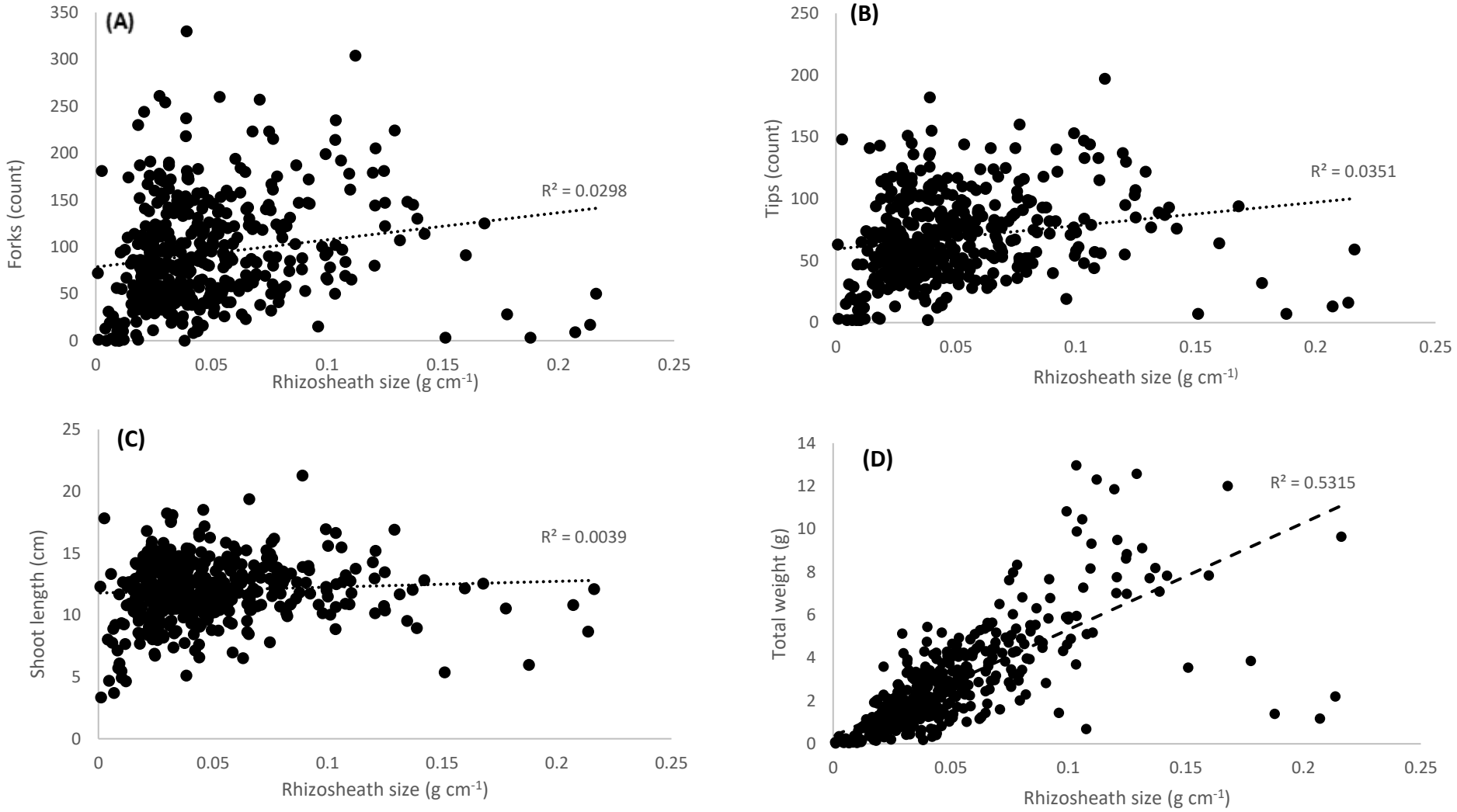

Figure 14: The root (A and B), shoot (C), and total weight of seedling including adhered soil (D) measurements and their correlation to the rhizosheath size within the experimental population.

The rhizosheath visual assessment resulted in clear trait differences between the two populations (Figure 15). The LRS frequently had thin rhizosheath width, incomplete soil coverage of the root and simple, infrequently branching root structures. In contrast, the HRS seedlings had thick rhizosheath widths, complete soil coverage of the entire root and often a higher frequency of branching. When removed from the pot the HRS individuals often had visible root hairs on roots that were grown against the side of the pot. As few quantitative relationships had been identified, the RST criteria is based on seedling weight and visual identifiers of rhizosheath size.

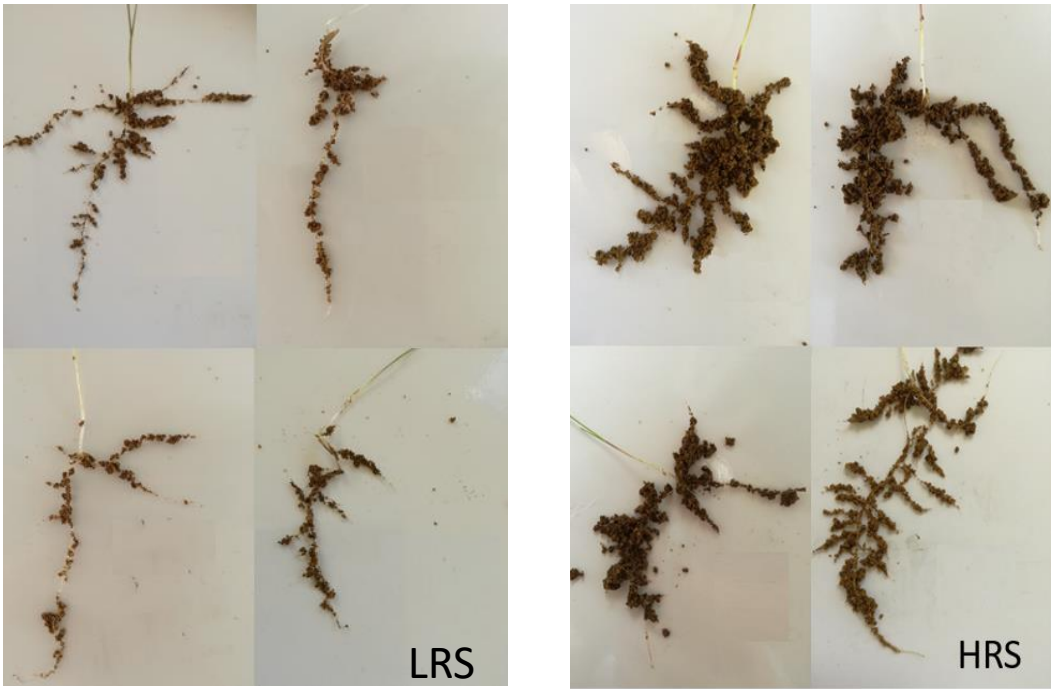

Figure 15: Visual assessment of the low and high rhizosheath sized seedlings.

2.3.4 Experiment Four: seminal roots

The seminal root trial was designed with material from the two divergent RST selected populations. The HRS had 37% longer root hairs than the LRS population (P <0.001; Figure 16 A). There was also a statistically significant difference in the root hair density, with the HRS root hairs being 30% denser than the LRS population (P <0.001; Figure 16 B).

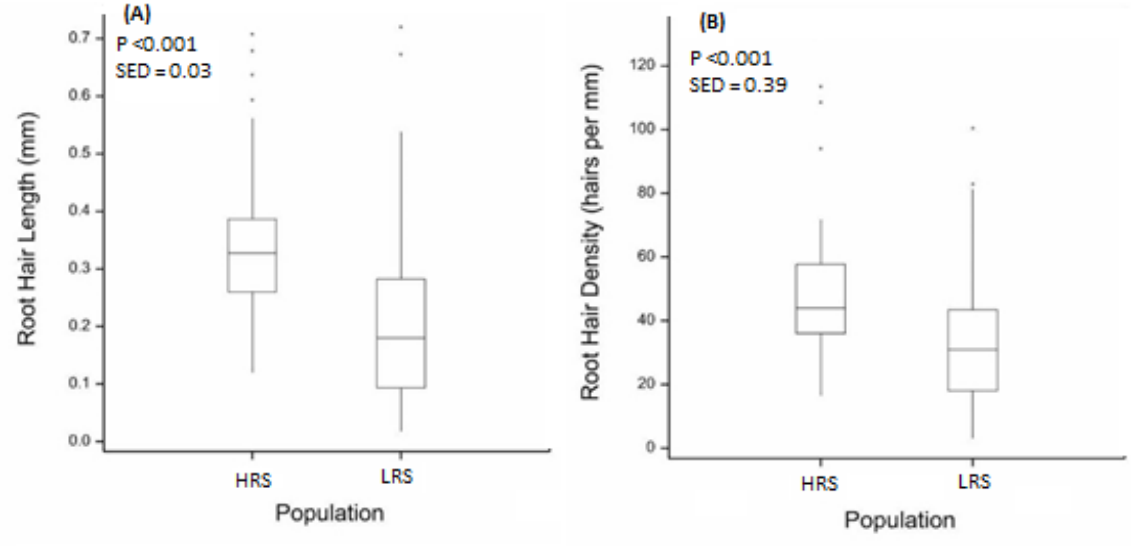

Figure 16: The high rhizosheath (HRS) and low rhizosheath (LRS) population's root hair length (A) and density (B) on the seminal root system. SED = standard error of difference of the means; the small represent individual outliers with values up to three times greater than the interquartile range.

A simple linear regression model was fitted to predict the root hair density based on root hair length (R^2 = 0.2224; Figure 17). The overall regression was statistically significant (*P* = 0.002) and accounted for 20.2% of the variation in root hair density. There was evidence that the intercept of the linear regression differed between populations (test of different intercepts given the same slope: P = 0.027) but no evidence that the slope does differ (test of different slopes allowing different intercepts: *P* = 0.689).

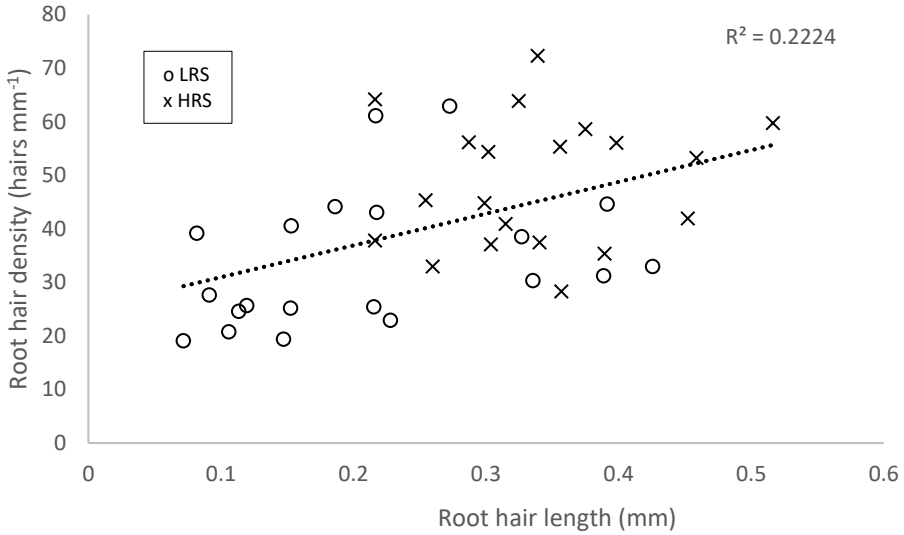

Figure 17: The simple linear relationship between seminal root system root hair length and density of the high rhizosheath sized (×) and the low rhizosheath sized (o) population.

2.3.5 Experiment Five: adventitious roots

The material for the adventitious root hair experiment was derived via tiller propagation from the two RST selected populations. Of the spare tillers taken at the beginning of the experiment, there was no statistically significant difference in the fresh or dry weight between the HRS and LRS populations (P = 0.647 and 0.905 respectively). This supports the assumption that both populations begun with similar tiller weights.

There was no statistically significant difference in the rhizosheath cylinder cross sectional area between the two populations (P = 0.969); indicating no difference in root hair length (Figure 18 A). However there was a significant difference between the two population's mean density scores with the HRS being 15% denser than the LRS (P = 0.038; Figure 18 B).

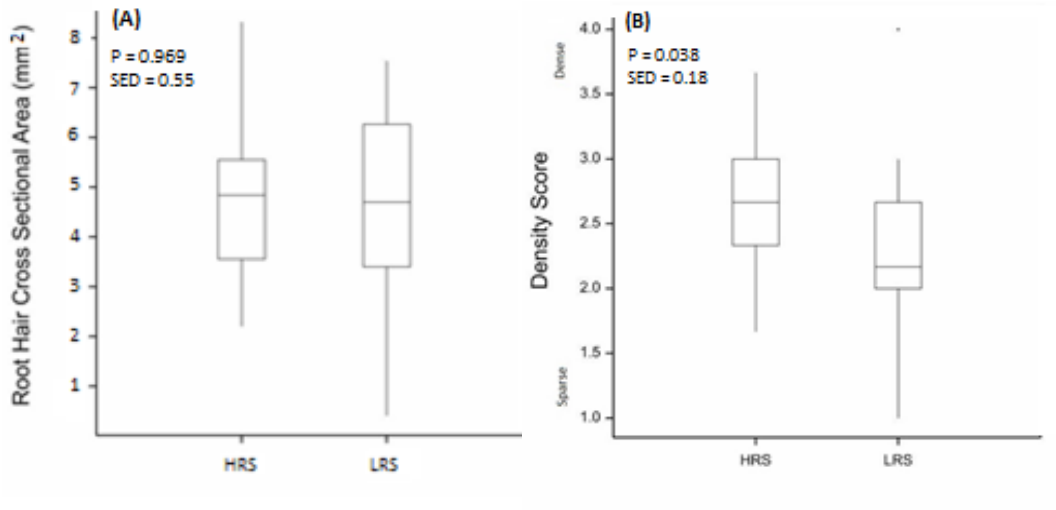

Figure 18: Adventitious root system root hair cross sectional area (A) and root hair density score (B) difference between the two populations. SED = standard error of difference of the means; the small circle above a box plot represents an individual outlier.

The exclusive root hair recruitment zone truncate cone volume (with the root cylinder volume excluded) showed no statistically significant difference between the HRS and LRS population's means (*P* = 0.555). The mean length of the recruitment zone was also not statistically significantly different between the HRS and LRS population (*P* = 0.254). Indicating no difference in the root hair recruitment characteristics between the two populations.

A simple linear regression model was fitted to predict the root density based on root hair length (R^2 = 0.0145; Figure 19). The overall regression was not statistically significant (*P* = 0.46) and was unable to account for any of the variation in root hair density scores.

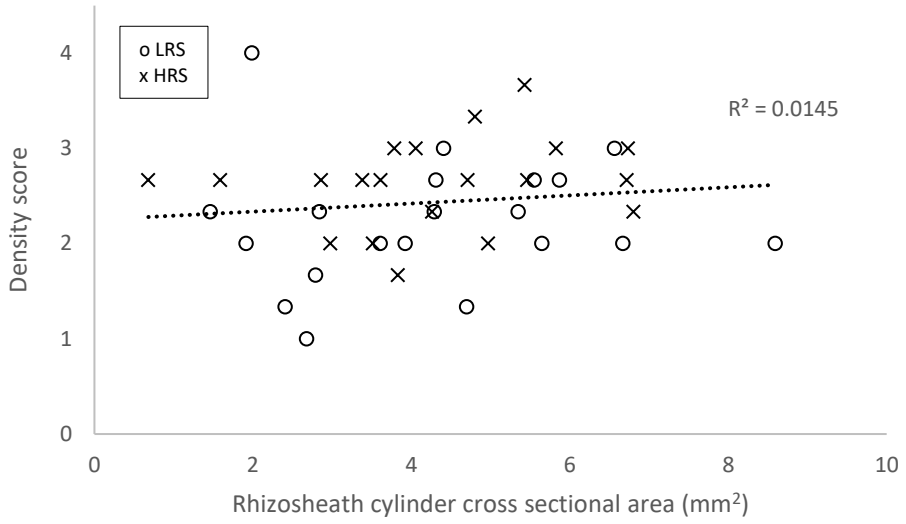

Figure 19: The indicated Root hair length (rhizosheath cylinder cross sectional area) and density of adventitious roots showing no linear relationship, with 'x' representing the high rhizosheath population and 'o' representing the low rhizosheath population.

2.4 Discussion

The RST criteria produced two distinct populations based on their rhizosheath size, which meets the first aim set for this chapter. These two populations provided the material for both the seminal and adventitious root system trials. This material subsequently confirmed a relationship between root hair length and density, and rhizosheath size; the second aim set for this chapter. Furthermore, the selected material gave insight into the transferability of root hair traits from seminal to adventitious root systems, the final aim of this chapter.

2.4.1 Endophyte

The presence of an endophyte does not influence the partitioning of resources; a number of trials have reported no significant difference in the root/ shoot ratio in perennial ryegrass with or without endophyte presence (Crush *et al.*, 2004; Latch *et al.*, 1985). However Crush *et al.* (2004) found a potential endophyte influence on the root distribution pattern of Grasslands Samson perennial ryegrass. This was supported by Malinowski and Belesky (2000) which suggested that indole alkaloids produced by the endophyte has the potential to regulate plant growth. Indole alkaloids interfere in the production of secondary metabolites, impacting the host plant's physiology and rooting distribution patterns (Malinowski & Belesky, 2000).

Due to the potential influence endophyte could have on perennial ryegrass rooting architecture, the quantity of seed with positive endophyte infection was a factor that required investigation. As all 120 seed tested for endophyte presence returned negative it has been assumed that the whole population was endophyte negative, despite the 40% endophyte infected hypothesis. Long term storage conditions of the seed can result in a 100% endophyte negative population (Rolston *et al.*, 1986). Because of the 100% endophyte negative result, it is assumed the variation present within the rooting architecture for these trials has not been affected by the presence of endophytes nor any association to indole alkaloids effect.

2.4.2 Within cultivar variation

The morphological variation within the perennial ryegrass population supports the development of the RST criteria. As all the seedlings were grown under the same conditions and time period, it was assumed that the variation in root architecture is attributed to genetic variation, which is the basis for selective breeding (Julier *et al.*, 2000). From the examination of root architecture traits of 430 individuals it was clear there was large within population variation following a normal distribution, as hypothesised. The large variation was expressed by the standard deviation of each trait being greater than half the mean (Table 4). Perennial ryegrass is an outcrossing species with mechanisms to prevent self-pollination (Thorogood *et al.*, 2002). Outcrossing encourages genetic diversity within a population. Therefore it was expected for the studied perennial ryegrass population to also have a large variation.

The morphological investigation also highlighted a number of traits including total shoot length and branching traits (tips and forks) were independent from rhizosheath size, providing no correlation (Figure 14). This limited the development of the RST criteria as few measurable root or shoot traits could be incorporated. Young shoots often do not express significant variation as the shoot requires time to respond to root effects (Rahnama *et al.*, 2011). Likewise the genes expressing root branching traits may be independent from the genes expressing root hair length and density, but this hypothesis requires further investigation.

2.4.3 Rhizosheath Selection Tool criteria development

The RST criteria was developed based on the methodology of Delhaize *et al.* (2012), using the rhizosheath size as a visual indication of root hair length and density. The data set of the 430 seedlings indicated low rhizosheath size seedlings in the lower quartile weighed <1 g. Likewise, the seedlings in the upper quartile with large rhizosheaths frequently weighed >1 g (Figure 14 DFigure 3). A visual assessment of rhizosheath coverage was also tested (Figure 15). The low rhizosheath selected seedlings had patchy soil adhesion whereas the high rhizosheath seedling roots were completely surrounded with adhered soil. Some high rhizosheath seedlings also presented obvious root hairs exposed where the root was pressed against the pot. For a seedling to be selected it must have met both the weight and coverage criteria; the visual presence or absence of root hairs was an additional criteria that supported the selection decision but was not required for each selection. Three caliper measurements of the rhizosheath diameter were also recorded for each of the 40 selected plants in an effort to quantify the rhizosheath thickness, however this process was time consuming and reduced the potential for rapid screening. Future adaptations of the RST methodology should include a visual rhizosheath thickness assessment to replace of caliper measurements.

2.4.4 Rhizosheath Selection Tool outcomes

The two populations, selected by the RST criteria, had statistically significant differences in root hair length and density (Figure 16); supporting the hypothesis that the RST was effective in selecting two distinct populations based on rhizosheath size. As hypothesised the HRS population had longer (0.338 mm) and denser (48.8 hairs mm^{-1}) root hairs than the LRS population (0.212 mm and 33.9 hairs mm^{-1}) on average. This also indicates that rhizosheath size is dependent on root hair length and density; a finding supported by Delhaize *et al.* (2012) for wheat and Haling *et al.* (2010) for wheatgrass (*Thinopyrum ponticum* Z.) and phalaris (*Phalaris aquatica* L.). As described earlier, the rhizosheath is the soil that has adhered to the root once lifted from the soil (Haling *et al.*, 2010). Therefore the longer and denser the root hairs the greater the root surface area, increasing

the amount of soil that could adhere to the root, resulting in a larger rhizosheath. Likewise, shorter and sparse root hairs resulted in a smaller rhizosheath.

The RST has established that rhizosheath size can be used as an indirect visual indication of root hair length and density of perennial ryegrass. With minimal seedling disturbance, this method provides an essential selection tool for breeding programmes. Selective breeding methods for root traits are difficult to develop due to the destructive nature or disturbance caused by plant measurements. A direct measure of root hair length and density involves micro-photography and analysis which is time consuming and requires further root disturbance. Although subjective, the RST allows for rapid selection of high or low root hair length and density characteristics from a large population. There will be disturbance to the plant during root up-lifting, but the plant remains intact for replanting.

It is predicted that the overall pastoral demand on soil P can be reduced through the development of an RST and selective breeding for LRS populations. Root hairs increase the surface area of roots, allowing for further exploration of the soil for immobile nutrients such as P. As mentioned in Chapter One, perennial ryegrass is efficient at acquiring soil P, depleting soil resources faster than white clover (Dunlop & Hart, 1987). The difference in root architecture including perennial ryegrass' long and profuse root hairs, and therefore larger rhizosheath size, allows perennial ryegrass to be more efficient and more competitive against white clover for soil P (Dunlop & Hart, 1987; Simpson *et al.*, 2014). Due to this competition, P fertiliser is typically applied to pastures in excess as perennial ryegrass must be fully supplied before white clover has the ability to acquire the remainder. Therefore the ability to select for a small rhizosheath size (and consequently shorter and less dense root hairs) should make it possible to select for reduced competition for soil P by ryegrass against white clover. By boosting white clover production relative to ryegrass there are potential gains to decrease both the amount of P and N fertiliser required in the pastoral system because of the increased nitrogen fixation by the white clover (Stewart, 2006).

2.4.5 Continuation of root hair traits from seminal to adventitious root systems

The third aim of this chapter was to identify the transferability of root hair traits from seminal to adventitious root systems. It was hypothesised that both root hair length and density are transferable between root systems, remaining proportionate in length and density on each of the root systems. As described, seminal roots are the primary and short lived root systems developed from the radicle. Unlike the adventitious root system which is long lived and considered the mature root system. The RST was based on seminal roots of young seedlings but for practical application of the trait being selected for it is the adventitious roots that are predominant in the plant's life. Therefore it is important to know if the root hair traits that are being selected for were shared between the two root types.

There was evidence of a linear relationship between root hair density explained by root hair length in seminal root systems but not in adventitious root systems (Figure 17 and Figure 19). Furthermore, the root hair density traits of each population selected in the seminal root system type were also expressed in the adventitious root system type; with the HRS remaining statistically significantly denser than the LRS. However the root hair length traits selected for was not expressed in the adventitious root system type, with no significance between the two populations.

The lack of transferability of root hair length between root system types is likely due to differences in the environmental conditions each root system was grown, known as a genotype × environment interaction (Zhu *et al.*, 2005). Results by Haling *et al.* (2010) indicated root hair length of phalaris varied with increasing soil acidity levels, whereas root hair density remained relatively constant. This suggested root hair density is more tolerant of genotype × environment interactions than root hair length, and was able to maintain the same density level with changing environmental conditions (Haling *et al.*, 2010).

Despite both the perennial ryegrass population's seminal and adventitious root hair measurements obtained via hydroponic growth environments, the seminal

root systems were grown in low ionic nutrient solution whereas the adventitious roots were grown in Long Ashton complete nutrient solution. The change to Long Ashton complete nutrient solution provided a higher nutrient concentration to support longer term growth to allow for adventitious roots to develop. The limitation of P in an environment has been reported to increase root hair length by a factor of three (Bates & Lynch, 1996; Datta *et al.*, 2011). Therefore changing the environment induced a genotype × environment interaction which influenced root hair length but had little impact on root hair density

The continuation of root hair traits from one root system to the other may not be as important as first believed. There is a considerable amount of time during the establishment period of a plant where both root systems are present. This establishment period is significant to the plant as it will be resource demanding (Collins & Rhodes, 1994). If the seedling can acquire more soil nutrients, it will have a greater chance at establishing in space for light resources (White, 1973).

Overall, further investigation of transferability of root hair traits between root system types, with the same environmental conditions, is required. In addition the heritability of selected root hair traits onto offspring also requires investigation.

2.5 Conclusion

The three chapter aims of creating two distinct populations, investigating the rhizosheath to root hair length and density relationship, and beginning to understand the root hair trait's transferability between root systems have been achieved. The absence of endophyte infection and the large variations within the Nui perennial ryegrass cultivar supported the development of the RST criteria. The RST criteria is based on seedling weight and visual indications of the rhizosheath coverage, with aid from rhizosheath width and root hair visibility. The RST has successfully selected two populations with significant differences in root hair length and density. These two populations, HRS and LRS, provided an insight into the positive relationship between rhizosheath size and root hair length and density. This has allowed the rhizosheath size to be an indirect selection tool, with minimal disturbance to the seedling, for the preferred root hair length and density. The transferability of these root hair traits, between root system types, occurred

for root hair density but not root hair length. The lack of transferability of root hair length was attributed to the increase in nutrient (including P) concentration, causing a genotype × environment interaction. However the significance of the transferability of root hair traits requires further investigation as the establishment of an individual may be of higher importance.

Chapter Three

Assessment of the phosphorus response of RST selected perennial ryegrass

3.1 Introduction

Pasture plant breeding has a long history in New Zealand with the establishment of the Grasslands site in Palmerston North, New Zealand in 1927 (Williams *et al.*, 2007). The initial breeding focus was yield driven and although new challenges for pastures suitable for low-input systems are gaining attention, yield remains a high priority (Chapman *et al.*, 2017; Williams *et al.*, 2007). Farm production is driven by producing feed for livestock, and increasing pasture yield and nutritive value is the most economic method to meet increasing livestock feed demands (Chapman *et al.*, 2017; Macdonald *et al.*, 2017). Therefore, for customer adoption of a novel perennial ryegrass cultivar the cultivar must maintain or improve on the yield of existing cultivars in addition to exhibiting any other novel traits (Chapman *et al.*, 2017; Williams *et al.*, 2007).

The Rhizosheath Selection Tool [RST] was used to select two distinct populations based on rhizosheath traits (Chapter Two, Experiment Three). The low rhizosheath sized [LRS] population has shorter and sparse seminal root system root hairs, and sparse adventitious root system root hairs compared to the high rhizosheath sized [HRS] population. A full root morphology assessment was carried out in Chapter Two. It was hypothesised that the rhizosheath traits of the LRS population reduced soil exploration and P uptake. The rhizosheath traits of the LRS population would reduce the overall P fertiliser requirements for a combined species pasture due to a reduction in root competition against white clover for soil P (explored in Chapter Four).

However it was first necessary to validate the role of rhizosheath traits in soil P uptake and assess effects of the RST selection on dry matter yield under reduced soil P conditions. The impact of selecting perennial ryegrasses based on rhizosheath was examined in this chapter through a phosphorus response experiment. Each population was grown at five P levels and data on the population

differences in dry weight and P assimilated into roots and shoots, and the partitioning of resources was collected.

3.2 Method

3.2.1 Plant material

Two populations of Grasslands Nui perennial ryegrass were pre-selected using the RST developed in Chapter Two, Experiment Three. These were a HRS population and a LRS population, each with 20 individuals per population. Each plant was grown in five soil P levels consisting of Olsen P 7, 10, 12, 14 and 20 mg L^{-1}, abbreviated to P1 to P5 respectively.

Six tillers were taken from each of the HRS and LRS population plants. The tiller propagation method used the same procedure as in Chapter Two, Experiment Five.

After eight days of growth in sand trays when new adventitious roots had emerged, one tiller from each plant was washed free of sand, blotted dry and oven dried 65°C for 48 hours then reweighed for fresh and dry weight comparisons. Of the five remaining tillers were planted into one of five pots, each with a different P level resulting in 200 pots in total.

3.2.2 Soil and experimental design

The baseline soil was 4 mm sieved Horotiu silt loam subsoil (Hewitt, 2010) with an additional 0.17 g of $CaHPO_4$ per kg of air dried soil. The soil was then amended by mixing with either sand or $CaHPO_4$ (for P5) to achieve one of the five P levels (Table 5); mixed in batches of 6 kg of soil, enough to fill five pots. To achieve the P5 Olsen P level, the $CaHPO_4$ was increased to 0.25 g per kg of air dried soil.

To mix $CaHPO_4$ through the soil, the 6 kg of soil was weighed and spread out onto a plastic sheet. The $CaHPO_4$ was evenly sprinkled on top from one bag; soil was also put into a bag and shaken and returned to the pile to include any residue left in the bag. The spread out soil was then thoroughly mixed, then approximately 1.25 kg of soil was scooped into each pot, filling each group of pots progressively and evenly from the mixture (Figure 20). Four additional pots of each P level, without plants, were maintained alongside the main experiment for later P

analysis. Post-harvest the additional soil pots were air dried, then 500 g from each of the four pots at the same P level were combined and sent to a commercial testing laboratory for Olsen P analysis.

Table 5: The ratio of baseline soil (4 mm sieved Horotiu silt loam subsoil with addition of $CaHPO_4$) to sand to achieve the five Olsen P levels.

P Level	$CaHPO_4$ per kg of air dried soil	Soil : Sand	Olsen P level (mg L^{-1})
P1	0.17 g	1 : 4	7
P2	0.17 g	1 : 2	10
P3	0.17 g	1 : 1	12
P4	0.17 g	2 : 1	14
P5	0.25 g	1 : 0	20

Figure 20: The mixing of Horotiu silt loam subsoil with $CaHPO_4$ on a plastic sheet; inverting the soil from left to right piles to ensure a complete mix.

The pots were arranged in a split plot design across four tables with 50 pots per table (Figure 21). The experiment was planted in mid-August 2017, in a

temperature controlled glasshouse (mean day temperature 17.2°C, night 12°C) under natural light. Each column was one replicate and had one pot of each soil P level with one tiller planted into each pot; all the tillers in a column were clonal replicates of a single genotype. The P level order was randomised within each column and the two populations were randomised across the columns. All pots were watered daily. Three times a week each pot received 100 mL of Long Ashton complete nutrient solution minus phosphate so that no nutrients other than P varied (Table 3).

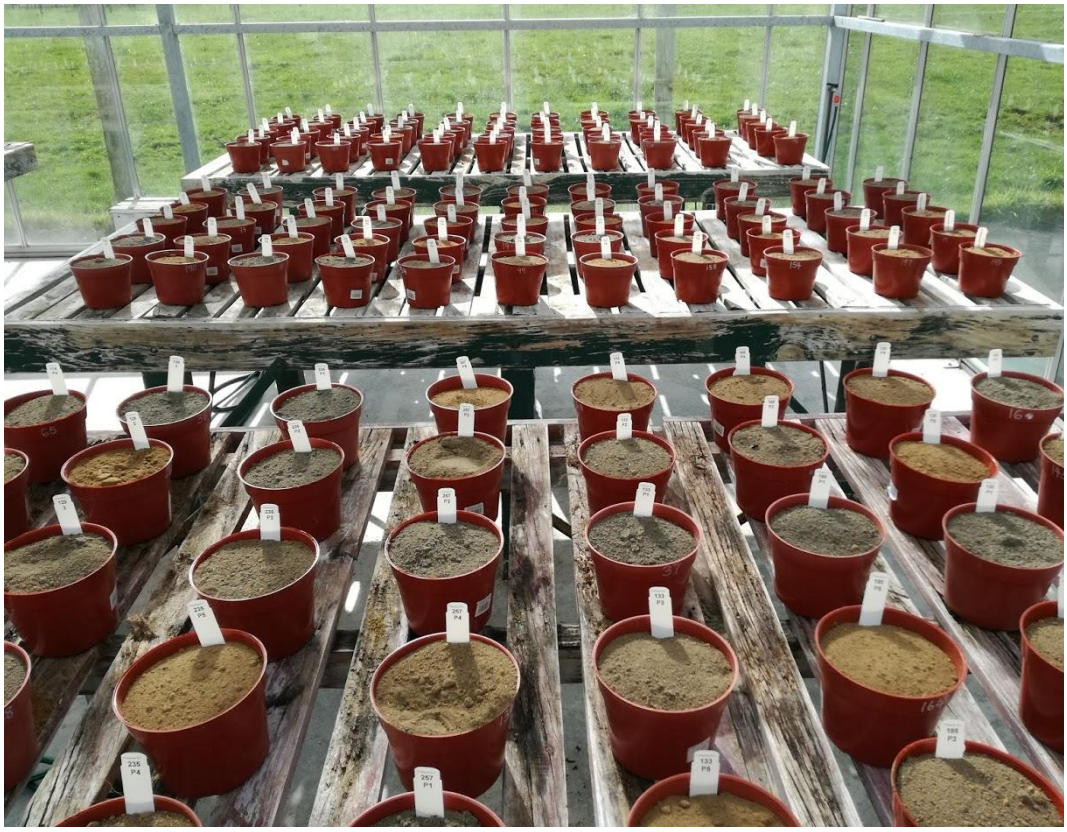

Figure 21: The experimental layout of the phosphorus response experiment. Each column of five pots was one replicate (five soil P levels × one genotype). The order of the high rhizosheath size and low rhizosheath size populations were randomised per column.

3.2.3 Harvest

After 45 days of growth the shoots were trimmed to 5 cm above the soil surface. Each pot's trimmings were placed into labelled bags and oven dried for 48 hours at 65°C and weighed.

After 70 days of growth the full destructive harvest began and was completed in 48 hours. The roots were washed by removing the pot, submerging the roots in

water and shaking. The roots were then gently teased out to dislodge any adhered soil then were again washed in clean water to remove any remaining soil. The roots and shoots were then separated, blotted dry with a paper towel and placed into a labelled bag. All roots and shoots were dried at 65°C for 48 hours and weighed.

The trimmed shoot material taken on day 45 was added to the respective shoot samples. Each sample was ground in an Udy mill to pass through a 1 mm screen and captured in a labelled bag. The grinding procedure began with the lowest P level (P1) and worked up to the highest (P5). The mill was cleaned using a brush and vacuum cleaner between each sample within a P level and between P levels the mill was dismantled with all components thoroughly cleaned. The ground samples were then sent to Eurofins NZ Laboratory Services Limited for P concentration analysis.

3.2.4 Statistical analysis

The fresh and dry weight of the sixth tiller taken from each of the 40 plants at the beginning of the experiment was calculated and compared via an Analysis of the Variance [ANOVA] between the two populations.

The harvest results produced dry weight (g) and P concentration (mg P g^{-1} of dry weight) of the roots and shoots grown at five P levels. The dry weight and P concentration were multiplied together to give the total P content (mg P) per sample. The root/ shoot ratio were also calculated for dry weight, P concentration and total P content, per plant by dividing the root by the shoot. The root/ shoot ratios were compared via ANOVA for population effect within each P level. These comparisons provided an insight into the partitioning of resources within populations at different P levels.

The ANOVA comparisons were processed using GenStat 18th edition (VSN International Ltd, Oxford, Great Britain) and Microsoft Excel 2013 (Microsoft Corporation, Washington, United States). The ANOVA data were structured by population and blocked by parent material (clonal tillers or stolons) per column. The residuals of AVOVA comparisons were inspected for any divergence from the assumptions of normality and constant variance. Transformations were not

required. Statistically significant results were reported when the probability value [*P*] was ≤ 0.05.

3.3 Results

3.3.1 Initial tiller fresh weight and dry matter

There was no statistically significant difference between the two population's initial tiller fresh weight and dry matter (*P* = 0.74 and 0.5 respectively). The lack of statistically significant difference supports the assumption that the tiller size was the same for both populations when the experiment started.

3.3.2 Comparisons

The main effect of P level presented a statistically significant effect on dry weight (g), P concentration (mg P g^{-1} dry weight) and total P content (mg P) for both HRS and LRS populations (*P* = <0.001). Both populations followed the same P response pattern for dry weight and P concentration with no statistically significant difference between the HRS and LRS population (Figure 22 A - D). When pairwise comparisons were made, the HRS had a consistently larger root and shoot total P content (mg P) than the LRS population, and this difference was significant for three out of ten comparisons (Figure 22 E - F).

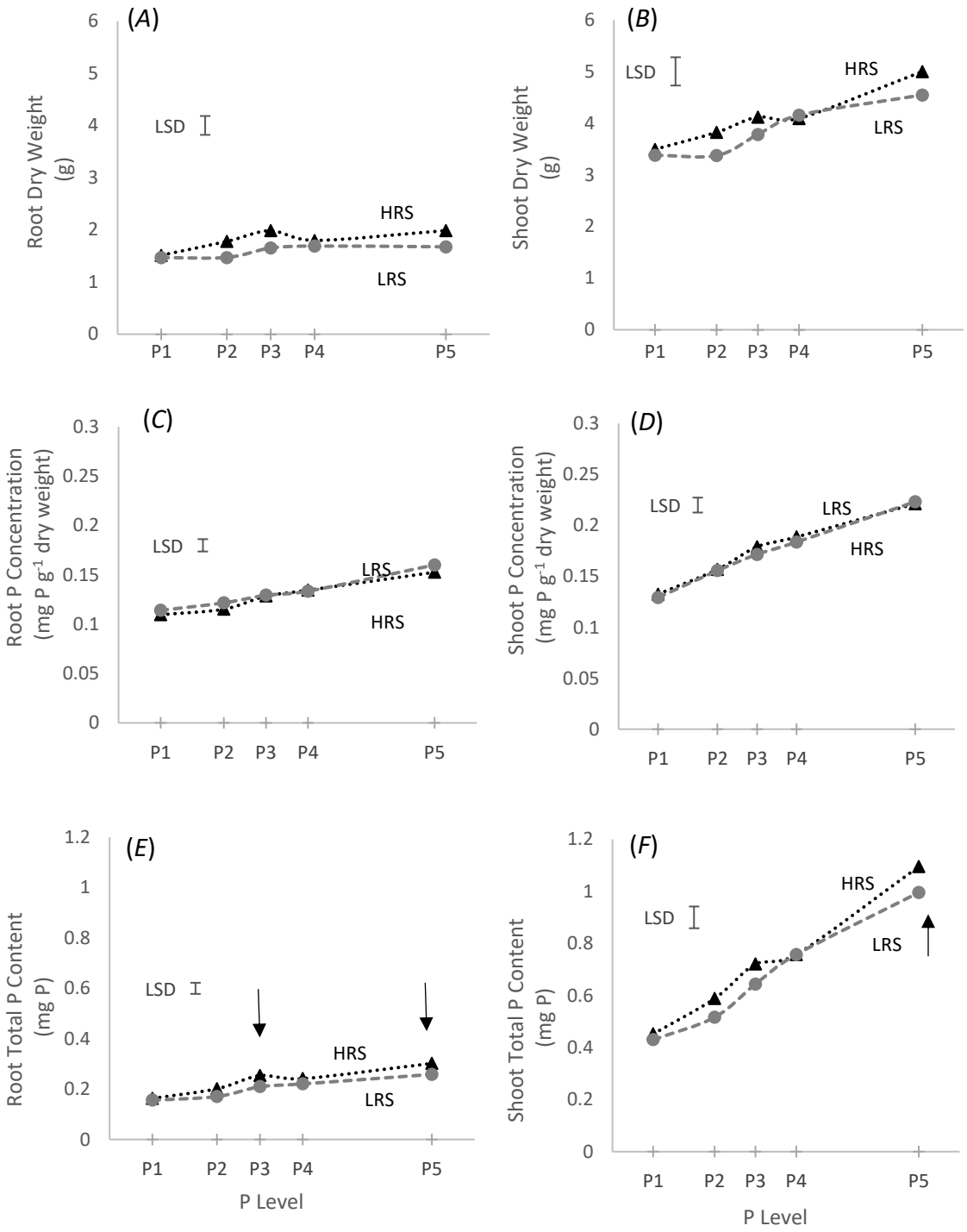

Figure 22: Effect of soil phosphorus level on both root and shoot for dry weight (A and B), P concentration (C and D), and total P content (E and F) of the high rhizosheath size (HRS) and low rhizosheath size (LRS) population. Arrow indicates a statistically significant difference between the two populations at the corresponding soil phosphorus level (P = 0.05). LSD = Least significant difference of means.

3.3.3 Partitioning

There were no significant differences between the two populations at the same P level in the partitioning of dry weight, P concentration or total P content (Table 6). The root/ shoot ratio of all measured traits (dry weight, P concentration and total P content) overall decreased with increasing soil P level, P1 to P5, for both populations.

Table 6: The mean root/shoot ratio of dry weight, P concentration and total P content for the high rhizosheath and low rhizosheath populations grown from clonal tiller cuttings to 70 days in five soil P levels. *P* = Probability value; LSD = Least significant difference of means.

P level	Dry Weight (g)		P Concentration (mg P g^{-1} dry weight)		Total P Content (mg P)	
	HRS	LRS	HRS	LRS	HRS	LRS
P1	0.440	0.421	0.837	0.879	0.364	0.357
P2	0.458	0.427	0.742	0.788	0.347	0.328
P3	0.464	0.428	0.727	0.758	0.367	0.322
P4	0.441	0.405	0.725	0.728	0.319	0.292
P5	0.397	0.361	0.696	0.72	0.274	0.258
P	0.969		0.706		0.831	
LSD	0.07		0.07		0.06	

3.4 Discussion

The material of the two populations were selected based on their rhizosheath size and characteristics via the RST established in Chapter Two. The HRS population maintained a higher total P content (mg P) than the LRS population, when compared within P level, for both root and shoot tissues although this was only statistically significantly different on three out of ten of these comparisons (Figure 22 E and F). As previously mentioned the root structure of the HRS population had denser adventitious root system root hairs, which should have increased the root surface area into the soil (soil exploration) and the absorptive surface for P uptake (Barber & Silverbush, 1984; Zhu *et al.*, 2005). In addition perennial ryegrass has a relatively low critical P requirement for growth and large capacity to accumulate P (McNaught, 1970; Schachtman *et al.*, 1998; Simpson *et al.*, 2014; Smith *et al.*, 1985). Perennial ryegrass was originally adapted to nutrient poor soils on forest margins (Scholz, 1975). This origin was believed to drive the evolution of root traits for increased soil exploration and the low critical P requirement of perennial

ryegrass (Hill *et al.*, 2006). Furthermore, the ability to increase P vacuole storage and later mobilise stored P under deficient conditions (Schachtman *et al.*, 1998). Perennial ryegrass can accumulate up to 0.9 mg P g^{-1} dry weight without change in dry weight yield (Smith *et al.*, 1985). The mean P concentration of perennial ryegrass at maximum yield is 0.32 mg P g^{-1} dry weight (McNaught, 1970). Therefore perennial ryegrass can accumulate and tolerate up to three times the P concentration required for maximum yield. For these reasons perennial ryegrass is a successful competitor in nutrient rich soils such as agricultural land. The HRS has a greater soil exploration ability than the LRS and these differences have been expressed in the higher total P content (mg P) present in the HRS tissues at varying soil P levels.

The RST selection had no measurable impact on the dry weight or P concentration as there was no significant difference between the two populations across all five P levels (Figure 22 A - D). Both populations followed an expected pattern of increasing dry weight and P concentration with increasing soil P levels for both root and shoot. The same pattern of P response has been reported for other temperate pastoral species including white clover, yorkshire fog (*Holcus lanatus* L.) and annual grasses (*Vulpia* spp.) (Caradus, 1981; Hill *et al.*, 2006; Nichols & Crush, 2015). This pattern is due to plant growth and metabolic activity being heavily reliant on P (Raghothama & Karthikeyan, 2005). Both perennial ryegrass populations responded similarly suggesting the two populations have similar internal P requirements for growth, regardless of their rhizosheath traits.

The population treatment, of the HRS and LRS populations, did not present any statistically significant differences for the traits measured. This indicates the RST method had no overall impact as both populations performed with minimal difference. The total P content does indicate that the HRS population is more efficient at P uptake, which is supported by the underlying literature, however would require further experimental investigation of the interaction.

The soil P treatment had a significant impact on the plant growth, regardless of population. The mixed ratios of soil to sand to achieve the five P levels will have caused variation in soil texture, but has had minimal effect on the plant growth (McLaren & Cameron, 1993). Root growth and morphology influenced by nutrient

availability, soil water content and temperature (McLaren & Cameron, 1993). As the plants were watered daily, given Long Ashton complete nutrient solution minus phosphate and grown in temperature controlled glasshouse conditions, the variation in sand to soil ratio textures would have had a minimal effect on plant growth, restricting the difference in growth to the effect of P availability.

3.4.1 Partitioning

All resources and nutrients other than P were supplied to the experimental plants, isolating soil P levels as the only limiting resource. Both populations followed the same pattern of partitioning more resource to the shoots than roots as the soil P availability increased (Table 6). Partitioning of biomass (dry weight) and resources within a plant is a function of balancing the trade-off to increase plant growth efficiency as resources allocated to a certain part cannot be used elsewhere (Schippers & Olff, 2000). As a limited soil resource becomes more available the biomass aboveground will increase, therefore increasing photosynthate production (Tilman, 1985). Likewise, when a soil resource becomes limited it can trigger a nutrient-stress response to allocate more resources towards the roots (Hill *et al.*, 2006; Schippers & Olff, 2000). The transportation of resources into the roots can increase the plants ability to locate soil nutrients and reduce nutrient loss from shoot turnover or grazing (Berendse & Elberse, 1990; Schippers & Olff, 2000). Therefore the root/ shoot ratio pattern expressed by the two populations followed the expected pattern. The RST selection has had no detrimental influence on either population as there were no statistically significant differences between the two populations in resource partitioning.

3.5 Conclusion

The total P content of the two populations did differ indicating the HRS population is more efficient at P uptake. This interaction is supported by the selected rhizosheath traits and warrants further experimental investigation. The RST selection overall had no negative impact on the growth or function of the two populations. Both populations expressed typical responses in the comparison of the means and partitioning of resources at the different soil P levels. The results show that it is possible to select perennial ryegrass for reduced rhizosheath size

without compromising its ability to acquire enough soil P to grow at low soil P levels. The effects of reducing the rhizosheath size of perennial ryegrass on root competition for soil P between perennial ryegrass and white clover will be tested in Chapter Four.

Chapter Four

Root competition between RST selected perennial ryegrass and white clover for soil phosphorus

4.1 Introduction

Perennial ryegrass outcompetes white clover for soil phosphorus [P] resources (explored in Chapter One). The long, dense root hairs of ryegrass allows for efficient soil exploration and uptake of soil P resulting in root competition. The Rhizosheath Selection Tool [RST], developed in Chapter Two, allowed for the selection of two distinct ryegrass populations, a high rhizosheath (HRS) and a low rhizosheath size (LRS) population. The rhizosheath size correlated positively with root hair length and density. Therefore the LRS population is hypothesised to decrease root competition with companion white clover.

The aim of this experiment was to investigate the role rhizosheath size had on root competition between the two species for soil P. The two ryegrass populations were grown with white clover at two P levels. The shoot growth response of the two species was compared and analysed for RST selection effects. It was hypothesised that the white clover grown with the LRS ryegrass would acquire more P and grow larger (dry weight) than the white clover grown with the HRS ryegrass. It was assumed the LRS ryegrass populations would have a reduced soil P exploration capacity compared with the HRS plants and therefore reduce competition with its companion white clover for the same soil P resource. It was expected that there would be no measurable difference between the two ryegrass populations as a result of the RST selection, as reported in Chapter Three.

4.2 Method

4.2.1 Experimental material

White clover was grown with two RST selected ryegrass populations, at two soil P levels, to assess the impact the RST had on promoting soil P acquisition and the growth of white clover. The two perennial ryegrass populations were developed using the RST designed in Chapter Two, Experiment Three. The LRS had shorter

and sparser seminal root system root hairs with sparse adventitious root system root hairs compared to the HRS. The white clover cultivar was Grasslands Kopu II (*T. repens*); Accession number: C25624; Margot Forde Forage Germplasm Centre, Palmerston North, New Zealand.

Fifteen of the 20 RST selected plants available per population were used in the experiment. The five LRS individuals with the longest root hair length on seminal root systems and five HRS individuals with the shortest root hair length on seminal root systems were discarded to increase the difference between the populations.

Five tillers were collected from each of the HRS and LRS population plants and propagated as in Chapter Two, Experiment Five. After five days of growth in sand trays when new adventitious roots were developing, one tiller from each plant was washed free of sand, blotted dry and oven dried 65°C for 48 hours then reweighed. The four remaining tillers were washed and planted into soil in pairs with a pair of white clover stolon tip cuttings, resulting in four plants per pot and 60 pots in total.

White clover seeds were scarified and left to germinate for five days in a petri dish on a moistened filter paper. Thirty five randomly selected germinated seeds (with a protruding radicle) were planted into potting mix in mid-June and grown in a temperature controlled glasshouse (mean day temperature 18°C, night 12°C) with natural light and additional day extension lighting (6 – 8 am and 4 – 6pm) for eight weeks.

From 30 of the white clover stock plants, four – five stolons were cut on an angle behind the second node of the developing bud on each plant. When possible an additional fifth stolon was taken for the purpose of being dried and weighed to calculate initial dry weight. All open leaves on all stolon tip cuttings were cut away from the stolon to reduce transpiration. The stolons were washed free of soil, blotted dry and weighed for initial fresh weight. All stolons were then planted into a sand tray and covered with an elevated plastic sheet to increase humidity. The stolons were watered daily for five days during which adventitious roots were developing. After five days, the fifth stolon was washed free of sand, blotted dry then oven dried at 65°C for 48 hours.

The four clonal white clover stolons and four clonal ryegrass tillers were paired, planting two of each species in one pot of soil with an Olsen P of 12 mg L^{-1} [P12], and the remaining planted into soil with an Olsen P of 19 mg L^{-1} [P19]. These two pots formed one replicate [rep], with 15 reps per ryegrass population. The shoots of each plant were separated by a perspex divider to reduce shoot competition between them (Figure 23).

Figure 23: A single pot containing two ryegrass and two white clover plants, separated by a perspex divider to reduce shoot competition.

4.2.2 Soil and experimental design

The root competition trial also used 4 mm sieved Horotiu silt loam subsoil (Hewitt, 2010) at Olsen P levels 12 and 19 mg L^{-1}. The soil had an initial Olsen P of 12 mg L^{-1}. To achieve the Olsen 19 mg L^{-1} soil, the soil had 0.5 g $CaHPO_4$ added to each pot (2.5 g of $CaHPO_4$ to 6 kg of soil). The mixing of soil and $CaHPO_4$ followed the procedure described in Chapter Three. Additional pots of each P level, without plants, were maintained alongside the main experiment for soil P analysis. Post-harvest the additional soil pots were air dried, then 500 g from each pot at the same soil P level were combined and sent to a commercial testing laboratory for Olsen P analysis.

The experiment was planted in late August 2017, in a temperature controlled glasshouse (mean day temperature 18°C, night 12°C) under natural light with the 60 experimental pots arranged on one table (Table 7). All pots were watered daily. Three times a week each pot received 100 ml of Long Ashton complete nutrient solution minus phosphorus so that no nutrients other than P varied (Table 3).

Table 7: The experimental design for the 60 root competition pots. The 1 and 2 represent phosphorus level one (Olsen P 12 mg L^{-1}) and phosphorus level two (Olsen P 19 mg L^{-1}). The grey filled boxes represents the low rhizosheath population and no fill represents the high rhizosheath population. A clonal pair from one population (eg: Column 1, Row 1 & 2) share the same parent material and have been duplicated to grow in both phosphorus levels.

	Table Column									
Table Row	1	2	3	4	5	6	7	8	9	10
1	2	1	1	1	2	1	1	2	2	2
2	1	2	2	2	1	2	2	1	1	1
3	1	1	1	2	2	1	2	1	2	2
4	2	2	2	1	1	2	1	2	1	1
5	1	2	2	2	2	1	1	2	1	1
6	2	1	1	1	1	2	2	1	2	2

4.2.3 Harvest

After 56 days growth the shoots of the ryegrass were trimmed to 5 cm above the soil surface. The shoot trimmings from the two ryegrass plants per pot were bulked together into labelled bags and oven dried for 48 hours at 65°C and weighed.

After 68 days of growth, all plants were trimmed to 2 cm above soil height. The shoot material was grouped into labelled bags by species per pot, oven dried for 48 hours at 65°C, then weighed. The trimmed ryegrass material from day 56 was added to the respective harvested material for ryegrass total shoot dry matter.

The samples were then grouped by P level, plant species and then population (LRS or HRS). Each sample was ground in an Udy mill to pass through a 1 mm screen and collected in a labelled bag. The grinding procedure began with the lowest P level (P12) and worked up to the highest (P19). The mill was cleaned using a brush and vacuum cleaner between each sample within a P level × species combination. Between P level × species combinations the mill was dismantled with all

components thoroughly cleaned. The ground samples were then sent to Eurofins NZ Laboratory Services Limited for P concentration analysis.

4.2.4 Statistical analysis

The weights taken of the spare tillers and stolons at the beginning of the experiment were used to compare initial fresh and dry weight. The fresh weight, and separately the dry weight, of this sub group were compared between ryegrass and white clover using an analysis of variance [ANOVA].

From the harvest of the shoots, the dry matter (g) and the P concentration (mg P g^{-1} dry matter) of each species per soil P level was obtained. The total P content (mg P) was calculated by multiplying dry matter and P concentration. This calculation was carried out for each sample (species × P level × ryegrass population).

Initial data analysis was performed using Microsoft Excel 2013 (Microsoft Corporation, Washington, United States), prompting ANOVA which was performed on each set of samples (species × P level × ryegrass population) using GenStat 18th edition (VSN International Ltd, Oxford, Great Britain). The data were structured by the treatments: (species × P level × ryegrass population), blocked by reps (clonal material × P level) and subplot by species. The residuals were inspected for any divergence from the assumptions of normality and constant variance. Transformations were not required. Statistically significant results were reported when the probability value [*P*] was ≤ 0.05.

4.3 Results

4.3.1 Initial material weight and dry matter

The subset of ryegrass tillers were statistically significantly larger in fresh weight and dry weight than the subset of white clover stolons taken at the beginning of the experiment ($P = 0.01$). These significant differences support the assumption that the ryegrass was initially larger than the white clover at the start of the experiment.

4.3.2 Dry weight

At the low soil P level (P12) the white clover produced a greater average dry weight (g) when grown with the LRS ryegrass population than the HRS ryegrass population (P = 0.05). There was no significant difference between the two ryegrass populations at P12 (Figure 24).

At the high soil P level (P19) the white clover dry weights were statistically significantly larger than the dry weight produced by the ryegrass they were paired with, which was visually noticeable (Figure 25). The ryegrass dry weight decreased with an increase in soil P level, with the HRS ryegrass decreasing in dry weight by 29% (P = 0.05).

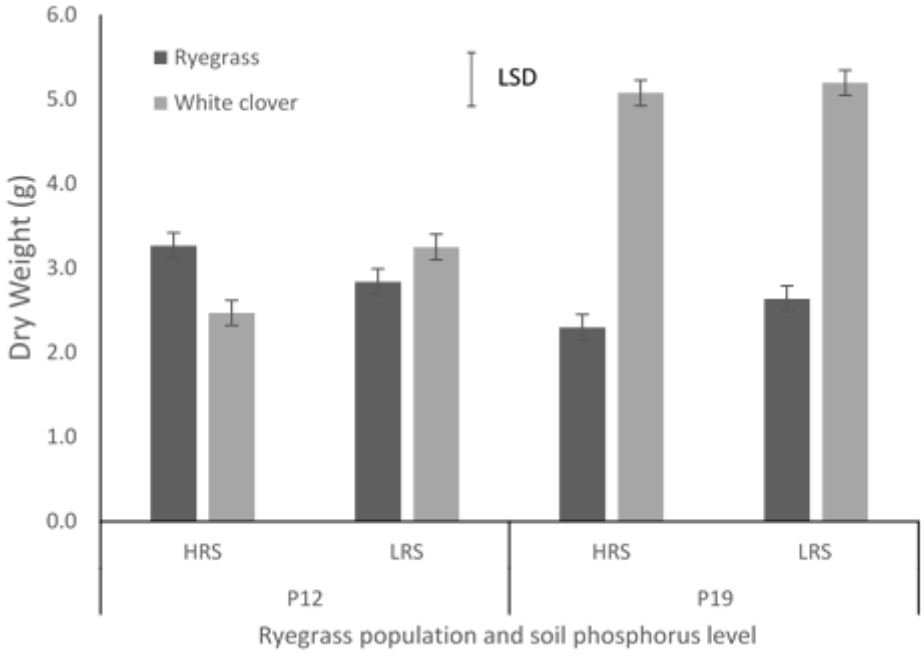

Figure 24: The combined treatment effect of species (perennial ryegrass and white clover), population (high rhizosheath size perennial ryegrass [HRS] and low rhizosheath size perennial ryegrass [LRS]) and phosphorus level (Olsen P 12 mg L^{-1} [P12] and Olsen P 19 mg L^{-1} [P19]) on the shoot dry matter. The error bars indicate the standard error of differences of means. LSD, least significant difference of means at $P < 0.05$.

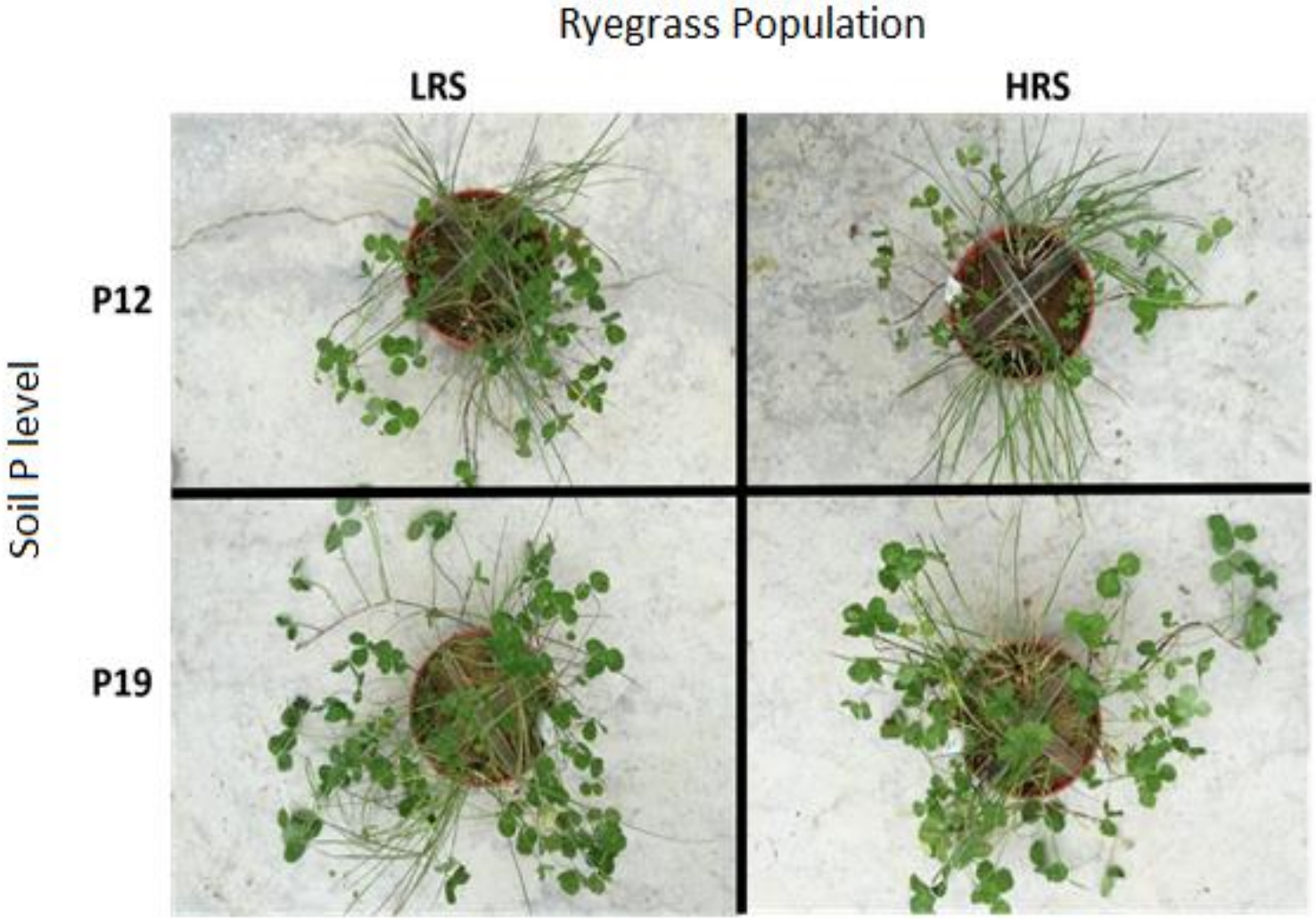

Figure 25: A visual comparison of the shoot size at day 68 before the final harvest. Each pot consisting of two clonal white clover and two clonal ryegrass with either a low rhizosheath [LRS] or a high rhizosheath [HRS] selected root system, grown in a soil phosphorus level Olsen 12 mg L^{-1} [P12] or Olsen 19 mg L^{-1} [P19].

4.3.3 Phosphorus concentration

At the high soil P (P19) the ryegrass assimilated a greater concentration of P (mg P g^{-1} dry weight) than the white clover it was paired with (P = 0.05). At P12 there was no difference in P concentration between species or population (Figure 26).

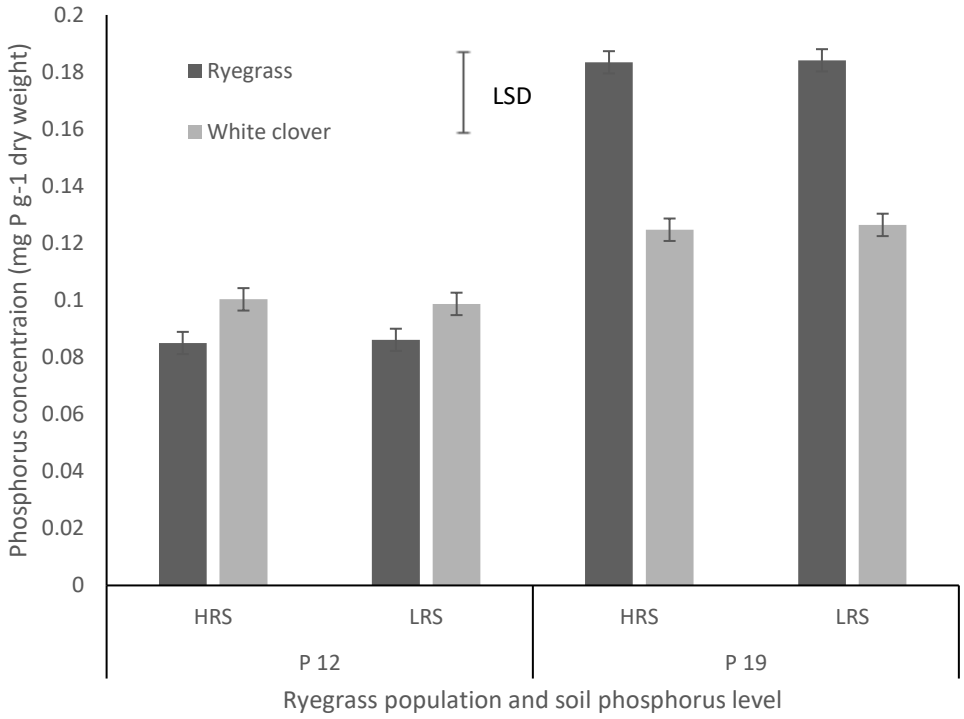

Figure 26: The combined treatment effect of species (perennial ryegrass and white clover), population (high rhizosheath size perennial ryegrass [HRS] and low rhizosheath size perennial ryegrass [LRS]) and phosphorus level (Olsen P 12 mg L^{-1} [P12] and Olsen P 19 mg L^{-1} [P19]) on the shoot phosphorus concentration. The error bars indicate the standard error of differences of means. LSD, least significant difference of means at $P < 0.05$.

4.3.4 Total P content

The mean white clover total P content (mg P) was statistically significant larger, at P19, than the paired ryegrass. No differences in means between species or population were reported at P12.

At P12, the ratio of total P content of white clover / ryegrass was significantly different between the two populations (P = 0.039). The LRS treatment partitioned more total P content to white clover (white clover : ryegrass = 1.78) than the HRS treatment (white clover : ryegrass = 0.97), evident in Figure 27, P12.

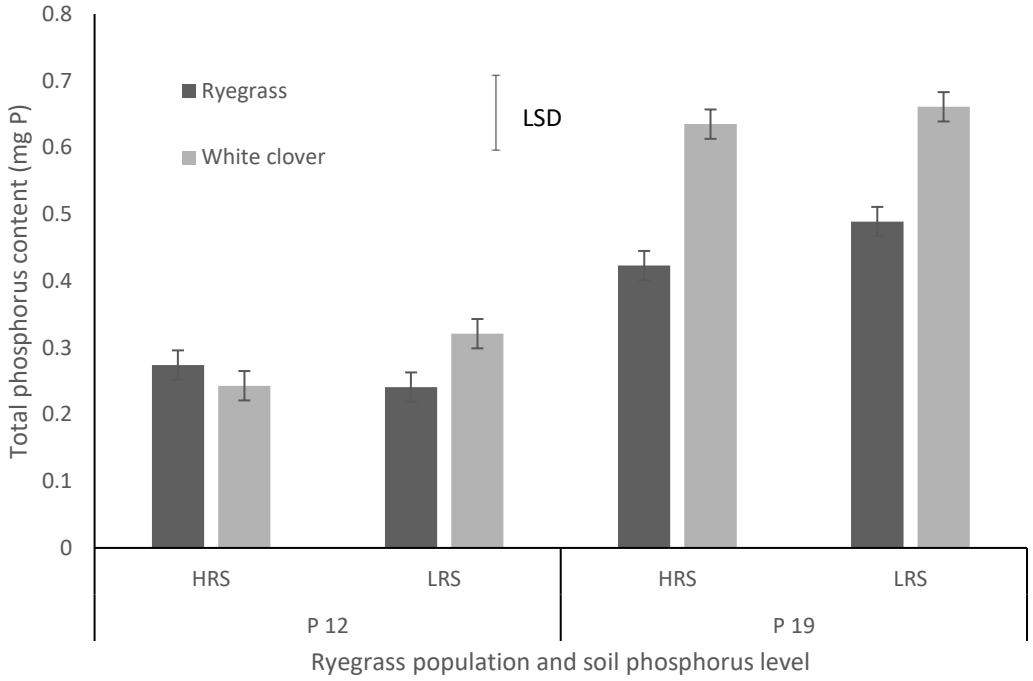

Figure 27: The combined treatment effect of species (perennial ryegrass and white clover), population (high rhizosheath size perennial ryegrass [HRS] and low rhizosheath size perennial ryegrass [LRS]) and phosphorus level (Olsen P 12 mg L^{-1} [P12] and Olsen P 19 mg L^{-1} [P19]) on the total shoot phosphorus content. The error bars indicate the standard error of differences of means. LSD, least significant difference of means at $P < 0.05$.

4.4 Discussion

When grown in low P soil conditions (P12), the white clover responded with a greater mean dry weight (g) and larger white clover / ryegrass ratio of total P content (mg P) when grown with LRS ryegrass. This positive response by white clover when grown with the LRS ryegrass population, indicates the RST selection

for LRS ryegrass had a reduced competitive capacity against white clover than the HRS selected ryegrass in low soil P conditions, supporting the initial hypothesis.

As described in Chapter Two, the HRS population had longer and denser seminal root system root hairs and denser adventitious root system root hairs than the LRS population. The shorter and sparse root hair characteristics of the LRS population had reduced the soil explorative ability for P uptake, therefore reducing the root competition with the paired white clover for the same soil P resource. Phosphorus is a crucial macronutrient for plant growth; therefore by reducing the competition by ryegrass for soil P, the white clover was able to increase P uptake (Raghothama & Karthikeyan, 2005; Schachtman *et al.*, 1998). This was evident by the larger white clover / ryegrass ratio of total P content matched by the greater dry weight of the white clover when grown with LRS ryegrass, at P12.

Furthermore there were no reported differences between the two ryegrass populations' performances at a low soil P. This result had been indicated in Chapter Three, suggesting the RST selection has had no measurable impact on the ryegrass dry weight yield when grown in low soil P conditions.

When the same genotypes as above were grown in a higher soil P level (P19) the P concentration and total P content increased. This pattern was expected and had been observed in other pasture species (Caradus, 1981; Crush *et al.*, 2015; Hill *et al.*, 2006). Although the ryegrass populations began initially with a larger fresh weight and dry weight of cuttings (P <0.01), the white clover overcame this difference and was significantly larger in dry weight than ryegrass at P19. However, the ryegrass (regardless of rhizosheath trait) had a significantly higher P concentration also at P19. This was where the total P content provided key insights to the total P uptake and internal partitioning of P by each species, as it was a function of dry weight × P concentration.

At P19, the white clover had a significantly greater total P content than ryegrass and therefore a greater overall P uptake rate. The white clover's total P content was a result of its significantly larger dry weight (yield) but with low P concentration per gram of dry weight. In comparison, the ryegrass had significantly less dry weight but with a significantly higher P concentration per gram of dry weight. Therefore despite the significantly high P concentration

achieved by ryegrass, the overall P uptake was low due to low dry weight produced, when grown at P19.

The higher P concentration per g of dry weight of ryegrass highlights the species' ability to accumulate and store P, as the high concentration had not resulted in an increase in dry weight produced. Ryegrass is known to internally store P in vacuoles under high soil P availability for later mobilisation (Schachtman *et al.*, 1998). This was suggested to be in response to environmental adaptation to forest margins soil types with fluctuating soil P availability (Balfourier *et al.*, 2000; Scholz, 1975). However both ryegrass populations presented a similar response to high P soils. There was no measurable effect of rhizosheath trait as no within species differences were reported. At adequate soil P availability (P19), soil exploration was not as necessary for the uptake of P (Hill *et al.*, 2006; Nye, 1966). Therefore any benefit of the rhizosheath selection in reducing root competition, had been removed in high P soil conditions.

Interestingly, the dry weight produced by the ryegrass populations decreased with an increase in soil P availability. This result does not follow the expected trend of increase in yield (dry weight) with increasing soil P as reported for other temperate pasture species (Caradus, 1981; Hill *et al.*, 2006; Nichols & Crush, 2015). The HRS population had a significant decline of 29% dry weight from P12 to P19. This suggests a potential rhizosheath selection effect on the ryegrass function. A higher metabolic cost had been linked with maintaining a larger rhizosheath size, resulting in a decrease in dry matter production (Brown *et al.*, 2017; Zhang *et al.*, 2018). However this relationship has not been well supported in the literature, requiring further investigation. Specifically for this experiment, the influence of white clover growth on decreasing ryegrass growth also requires further investigation. This should include an analysis of root growth in response to the interspecies competition and variation in soil P availability as this data was not captured in this experiment.

4.5 Conclusion

When grown with LRS ryegrass, white clover achieved a greater dry weight and larger white clover / ryegrass ratio of total P content at the low soil P level. This

was due to the decrease in root competition by LRS ryegrass for the same soil P resource. The increase in white clover growth at P12 came at no cost to the LRS ryegrass as it experienced no reduction in dry matter itself when compared to HRS. The increase in soil P to P19 removed any beneficial rhizosheath selection traits as soil exploration for P uptake was not heavily relied on. Both species mostly presented expected responses in dry weight, P concentration and total P content to an increase in soil P availability, with ryegrass having a greater P concentration but white clover achieving the greatest overall P uptake. However at P19 the HRS ryegrass experienced a 29% decline in dry matter from P12. Further research is required to investigate the rhizosheath selection influence on the growth response of ryegrass and white clover when grown together. However it was promising that the LRS selection was performing with the desirable outcome to reduce the root competition against white clover at a reduced soil P level without detrimental effect on its own growth.

Chapter Five

Synthesis

5.1 Summary

The aim of this study was to test whether it was possible to select a perennial ryegrass population with reduced root competition against white clover for soil phosphorus [P] with no significant detrimental effects on the yield and performance of the ryegrass population.

This aim was achieved through the development of the Rhizosheath Selection Tool [RST] which allowed for the selection of two distinct perennial ryegrass populations. The high rhizosheath size [HRS] and low rhizosheath size [LRS] population were selected based on contrasting rhizosheath traits which correlated with root hair length and density, as described in Chapter Two. Chapter Three, a P growth response experiment demonstrated that the rhizosheath selection had no significant detrimental effect on either population's growth. The two populations did not differ in dry weight (g) or P concentration (mg P g^{-1} dry weight). However there was evidence that the HRS was more effective at P uptake than the LRS population with occasionally larger total P content (mg P). The two populations were then grown with white clover (Chapter Four), supporting the hypothesis that the LRS population would decrease root competition with companion white clover for soil P. This was evident as white clover grown with LRS ryegrass achieved a significantly higher dry weight (g) and larger partitioning of P to the white clover than the ryegrass, measured by total P content (mg P), than when grown with the HRS ryegrass, at low soil P availability.

5.2 Outcomes and further investigation

Chapter Two: The analysis of 430 perennial ryegrass seedlings provided insight into the variation of root traits within the population. It was the understanding of this variation and the relationship of root traits to rhizosheath size (g soil per mm root length) that allowed for the development of the RST criteria. The RST criteria predominantly used rhizosheath width, soil coverage and total seedling weight (including adhered soil) to select the LRS and HRS populations. Through

hydroponic growth, the correlation of the seminal root system root hair length and density to the selected rhizosheath traits was confirmed. The selected populations were then returned, with adventitious root systems, to a hydroponic setup for the investigation of the continuation of root hair traits between root systems. Only root hair density remained significantly different between the two populations for both root systems. However a change in the experimental protocol meant the two root systems were grown in different nutrient solution. The lack of trait transferability was attributed to the sensitivity of root hair length to environmental change. Further research on the relationship of root hair traits between seminal and adventitious root system is required. The heritability of root hair traits was not tested, but requires determining before advocating the rhizosheath trait as a breeding tool. The RST has potential to be calibrated and tested on alternative cultivars. A broader selection of germplasm has the potential to contain a larger range of traits, further emphasising the rhizosheath selection effect in reducing root competition capacity of grasses.

Chapter Three: The rhizosheath selection had no significant effect on the two ryegrass populations. The two populations were tested for growth response in soil with Olsen P levels of 7, 10, 12, 14 and 20 mg L^{-1}. There was no significant difference in dry weight (g) and P concentration (mg P g^{-1} dry weight) between the two populations. Both populations followed expected growth and partitioning responses to increasing soil P availability which have been reported for other temperate pasture species. The HRS population did however have a significantly larger total P content (mg P) on three occasions which was attributed to its rhizosheath traits and increased ability for soil exploration. This indicates the HRS was potentially more efficient at P uptake that the LRS population, however further investigation is warranted to be conclusive. The two populations are theoretically expected to present greater difference at low soil P levels, when root exploration is critical for P uptake; this is another hypothesis that requires further investigation.

Chapter Four: The two ryegrass populations were grown with companion white clover in Olsen P 12 and 19 mg L^{-1} soil, with the shoot systems separated to avoid shoot competition for light. At the low soil P availability, the LRS was reported to

have decreased root competition with white clover for soil P as a result of its smaller rhizosheath traits. The white clover grown with the LRS ryegrass had a significantly larger dry weight (g) and more P was partitioned to the white clover than the ryegrass, measured by total P content (mg P), than the white clover grown with the HRS ryegrass. In the high soil P availability, there was no rhizosheath trait effect on the growth of white clover. This was attributed to the reduced requirement for soil exploration and potential for competition by roots as soil P was more readily available. Interestingly the HRS ryegrass population significantly decreased by 29% in dry weight with the increase in soil P availability, which was not an expected result. This suggests the maintenance of the HRS requires a higher metabolic cost than the LRS, however this requires further investigation. The LRS population's dry weight remained stable with the increase in soil P availability. Overall there was evidence to support the hypothesis that the LRS population exhibited decreased root competition against white clover for soil P, but only at lower soil P availability.

5.3 Practical applications

The development and testing of the RST were designed as preliminary experimental investigations for a potential plant breeding protocol. This study highlighted the potential for the LRS population to decrease root competition against white clover for soil P, suggesting a strategy for developing grasses suitable for a low P fertiliser input system. The reduction of P fertiliser use on farm would be economically beneficial for farm budgets and environmentally sustainable by reducing P runoff into freshwater ecosystems. The findings of this study also highlight the unutilised potential of root traits for breeding purposes, and recommends investigations of the heritability of rhizosheath traits.

Further work should include the genetic mapping of the two distinct populations to identify potential QTL markers for the rhizosheath traits. This would improve the accuracy of the RST and allows rapid screening for beneficial rhizosheath traits this would progress a breeding programme for perennial ryegrass suitable for low P systems.

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