Investigating variation in associations with belowground micro-organisms of historic and current white clover germplasm

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White clover (*Trifolium repens*) is regarded as one of the most important forage legume in temperate regions of the world, and is very important to the New Zealand pastoral industries. The symbiotic relationship between clover and the soil bacterium *Rhizobium leguminosarum* biovar *trifolii* (rhizobia) is the basis of the nitrogen (N) fixation ability of clover. White clover breeding started intensively in the late 1920's in New Zealand. Clover breeding programmes have largely focused on above ground characteristics in selection of new cultivars. This has usually been carried out in high soil nitrogen environments, due to fertilizer use. This is the first study to investigate whether plant breeding may have impacted on white clover and it's below ground associations.

Six cultivars of white clover were used. Three newer cultivars (Tribute, Kopu II, Crusader), and three older cultivars (Huia, Irrigation, Louisiana) which were paired based on physiological characteristics and growth habits. Three major below ground associations were analyzed in this project and these were i) plant interactions with rhizobia to examine whether there is preferential selection of efficacious strains of rhizobia by the older cultivars, ii) interaction with arbuscular mycorrhizal fungi (AMF) and the responsiveness of clover cultivars to different species of AMF, iii) interaction with the wider rhizosphere bacterial community, to determine whether the cultivars preferentially selected more beneficial associations in a complex soil background.

Symbiotic potential assays *in vitro* showed that the cultivars Irrigation and Louisiana produced significantly different shoot dry weight when inoculated with six different strains of rhizobia (P=0.038, and P=0.013, respectively). Cultivars Huia and Tribute

showed strong trends (P=0.052, and P=0.059, respectively). Based on plant dry weight, rhizobia groups of “highly effective” and “less effective” strains were identified for these four cultivars and used to examine if there was preferential selection of highly effective strains. Out of the four cultivars, only Huia and Louisiana had significantly more “highly effective” and significantly less “less effective” rhizobia strains occupying their nodules than was expected by chance (P<0.001).

This was the first study to examine the bacterial rhizosphere and root endophyte communities of old and new cultivars of white clover. 16S gene sequencing using Illumina HiSeq showed that the bacterial communities in the root and rhizosphere were significantly different from each other (PERMANOVA, P=0.001) in richness and diversity. Sequencing also revealed that plant morphotype was the main factor influencing bacterial community structure in the rhizosphere and roots of white clover, as cluster analysis showed that the samples grouped together according to their leaf size (PERMANOVA, P=0.001) and not by cultivar age. Illumina sequencing also showed there were differences among the pairs of cultivars in relation to the most abundant class observed. Alphaproteobacteria (42.3-52.6%) was the most abundant class for old/new matched pairs Irrigation/Crusader, and Louisiana/Kopu II, whereas the most abundant class for matched pairs Huia/Tribute was the Gammaproteobacteria (32.8%).

DGGE showed that cultivar significantly affected AMF community structure in the rhizosphere (PERMANOVA *P*=0.006). However, there was high variation among replicates of the same cultivar (P<0.05), and principal coordinate analysis did not show any grouping by cultivar.

Overall this study revealed that strains of *R. leguminosarum* vary in their efficacy when in symbiosis with different cultivars. The results suggested that older cultivars may be better able to select for more effective strains of rhizobia than newer cultivars, and may be able to exploit a wider range of rhizobia. This study also identified that the main factor affecting the bacterial rhizosphere and root communities of white clover was cultivar morphotype.

Keywords: *Trifolium repens, Rhizobium leguminosarum,* Illumina HiSeq, DGGE, host selection, rhizosphere, root endophyte community, arbuscular mycorrhizae

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1 Literature Review

1.1 Introduction

Nitrogen (N) is an essential element for plant growth because it plays a central role in the molecular make-up of DNA, RNA, ATP and proteins. Nitrogen is also an essential component of other key compounds including chlorophyll, auxin, cytokinins, alkaloids and glucosinolates. All of which play a very important part in plant growth regulation and in the intake and conversion of light into chemical energy (Andrews *et al.*, 2013).

Since the 1960's the main aim of the agricultural industry has been to maximise output per unit of land area and N fertilizer was heavily used to try and achieve this (Andrews *et al.*, 2009). Generally crop plants are only able to take up and convert around 20-40% of exogenously supplied N and the remainder is lost to the aqueous and atmospheric environment. This has become a serious concern of recent times with problems such as eutrophication becoming more apparent (Andrews *et al.*, 2009); (Andrews and Lea, 2013). Nitrate (NO_3-) and urea are very soluble, unlike ammonium (NH_4+) which binds to negative charges in the soil. Therefore, nitrate can easily run off into the surface water or flow into the groundwater (Andrews and Lea, 2013). Water contaminated by nitrate at high concentrations is a serious health risk for humans (Powlson *et al.*, 2008).

New Zealand's pastoral farming underpins its most profitable export commodities. The New Zealand dairy industry earns 26% of the country's export revenue, followed by the sheep and beef industry at 7.4% and 7.1%, respectively (Statistics New Zealand, 2016). To maintain its international competitiveness, the pastoral industry has relied on a high quality feed source that is cheap and which can be used year round – namely white clover/ryegrass based pastures (Rattray *et al.*, 2005). It has been common practise for white clover to be sown as the main legume with ryegrass, and this has been relied upon for nitrogen fixation into the pasture (Lindström *et al.*, 2010). However, it is a very common practice to use fertilizer N to increase productivity and reduce seasonal variation in feed supply (Brock and Hay, 2001).

An important aspect of a grass-clover mixture is to retain an adequate proportion of white clover in the pasture. Sufficient white clover content helps to optimize the benefits of nitrogen fixation and to make best use of the superior feed value of white clover. Other advantages to using white clover are improved sward quality and complementation of growth patterns with many commonly used grasses (Elgersma *et al.*, 2000; Rattray *et al.*, 2005). Unlike red clover, white clover grows lower to the ground and grows more laterally. Studies conducted by Eerens and Ryan (2000) in the Southland of New Zealand showed that sheep farmers could produce 25% more dry matter; 40% more carcass weight and 25% more wool from mixed ryegrass/white clover swards without exogenous N, as compared to ryegrass alone with 270 kg N/ha annually. Other estimates of the amount of N fixed annually by white clover are approximately 1.3 M tonnes in New Zealand pastures (Walker and Woodfield, 1996). This highlights white clover as a crucial, economically important member of agricultural pastoral systems.

1.2 White Clover

White clover breeding started intensively in the late 1920's in New Zealand, happening at the same time as similar programmes in various other countries such as the UK, Denmark and Australia (Caradus *et al.*, 1996a). White clover is regarded as the most important forage legume in the temperate regions of the world, and is very important to New Zealand agricultural industries (Rattray *et al.*, 2005). It is a perennial legume that spreads by creeping stolons which root at the nodes and go on to form new daughter plants (Rattray *et al.*, 2005). White clover fixes atmospheric nitrogen owing to a symbiotic relationship with *Rhizobium* bacteria in the roots. The nitrogen which is fixed, becomes available to surrounding plants once portions of clover stolons and roots die, decay, and are mineralised to release nitrogen (Eerens and Ryan, 2000). It is winter dormant and shows peak growth and N_2 fixation in early/mid summer (Andrews *et al.*, 2007).

White clover varieties are classified based on leaf size, measured on spaced plants. Usually varieties with small leaves maintain good ground cover under intensive sheep grazing. Whereas, varieties with larger leaves are better suited to swards under cattle grazing or cutting, and fodder (Andrews *et al.*, 2007). For both dairy and sheep grazing systems, it has been noticed that increasing the clover content of the pastures leads to a

significant increase in livestock production. For example, in the South Island high country, oversowing pastures with white clover and topdressing resulted in the sheep being attracted to the more palatable pastures (Rattray *et al.*, 2005). White clover is regarded as a high quality component of grazed pastures because of its nutritive value. It has higher concentrations of crude protein, water soluble carbohydrates (sugars), lower concentration of lipids, readily fermentable carbohydrates, lignin, cellulose and fiber compared to perennial ryegrass (Caradus *et al.*, 1996b; Rattray *et al.*, 2005).

1.2.1 Breeding history

White clover is an outbreeding species with individual plants usually being self-sterile. It is an allotetraploid that forms bivalents and shows disomic inheritance (Abberton and Marshall, 2005). In the early 1970's it was acknowledged that different types of white clover were needed for various environments and management systems. This led to the development of a range of cultivars, which suited to a wider range of environments (Mather *et al.*, 1996; Rattray *et al.*, 2005). Until then there was only one general purpose white clover that was being used and this was a cultivar developed in New Zealand called Grasslands Huia (Brock, 1988). Natural selection and the assistance of plant breeders have segmented the species into cultivars with distinct leaf sizes and this has increased the utility of white clover (Mather *et al.*, 1996). Small leaf type clovers are utilized best in hard grazing situations where persistence is an important factor. Large leaved clover types are more suited for lax grazing but also possess an adaptability which has allowed these species to grow in sub-tropical regions (Mather *et al.*, 1996). However, the largest use has been of the intermediate leaf type clover which is ideally represented by the Grasslands Huia cultivar.

1.3 Microbial Symbioses

1.3.1 Rhizobia

The symbiotic relationship between clover and soil bacterium *Rhizobium leguminosarum* biovar *trifolii* is the basis of the N fixation ability of clovers. The rhizobia infect clover roots and then proceed to form nodules (Rattray *et al.*, 2005). Nitrogen fixation is when the bacteroids present in the nodule convert nitrogen from the atmosphere, into plant

available nitrogen. In this symbiosis, the plant provides the bacteria with energy and nutrients, and in return benefits from the fixed nitrogen. This symbiotic relationship underpins the nitrogen economy of New Zealand pastures, and plays a crucial role in sustainable agriculture (Rattray *et al.*, 2005; Wang *et al.*, 2012).

Rhizobia are Gram-negative, aerobic bacteria, and are currently classed as α-proteobacteria (Suzaki and Kawaguchi, 2014). The development of nodules is a consequence of several different stages of interaction, each increasing with specificity for a given host-legume pairing (HIRSCH, 1992; Spaink, 2000; Downie, 2010).

The first stage of specificity in the formation of a nitrogen-fixing symbiosis is when the plant produces flavonoid signals recognised by free living soil rhizobia which in turn activates their NodD proteins. These proteins bind to the conserved Nod-box in the promoters of bacterial nodulation genes which induces their expression (Oldroyd *et al.*, 2011; Wang *et al.*, 2012). These Nod genes code for specific enzymes for the synthesis of Nod factors. The nod factors are recognized by transmembrane Nod factor receptors on the plant surface in a strain- and ecotype- specific fashion. The specificity of Nod factors lie in modifications such as the length and saturation of the acyl group (Wang *et al.*, 2012). The activation of the Nod factor receptors triggers a change in the growth of root hairs. Once the root hairs perceive Nod factors, there is an initial short interruption of symmetrical polar growth which can sometimes accompany a swelling at the root-hair tip. After this, growth resumes at the root tip to form a branch. This new growth can cause the root hair to bend back on itself, or in the case of a branch against the main part of the root hair, and in both cases leads to the bacteria becoming trapped between oppressed cell walls and forming an infection pocket (Oldroyd *et al.*, 2011). There is also a possibility that there are other mechanisms aside from Nod factors that affect nodulation specificity. Rhizobia also use surface polysaccharides to modulate host range. The plant receptors for these are currently unknown but could potentially resemble animal receptors for surface polysaccharides targeting pathogenic bacteria (Wang *et al.*, 2012).

Furthermore, in some rhizobial strains, NodD proteins also induces the expression of *TtsI*, which codes for a transcriptional regulator that binds to highly conserved promoter elements called *tts* boxes. These boxes are found upstream of operons encoding the type III secretion machinery of effectors. The recognition of effector proteins by R genes present in some varieties of plants limits hosts range, adding further specificity (Wang *et al.*, 2012). A visual diagram of the molecular dialogue that takes place in nodulation can be seen in Figure 1.1 below.

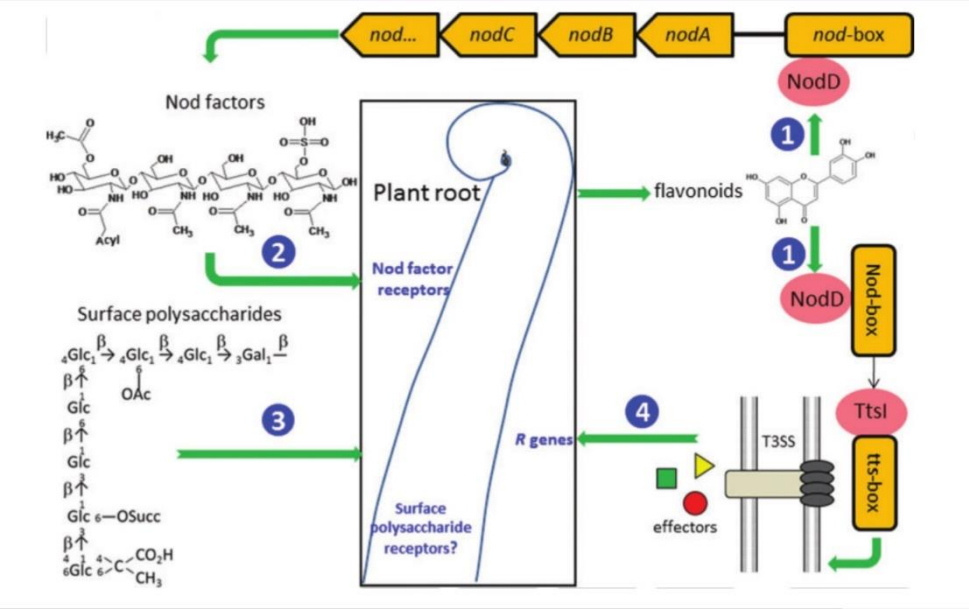

Figure 1.1 The stages of host specificity and molecular dialogue occurring between rhizobial bacteria and the plant host. Retrieved from Wang, *et al* (2012).

Rhizobia in New Zealand Soils

The rhizobia that nodulate clovers, were not present in New Zealand prior to European settlement, but were introduced most likely as dust on plants and contaminants in soil, stock hooves and agricultural equipment (Lowther, 2010). Before 1955, commercially produced rhizobia inoculants available in New Zealand were mainly for lucerne, however, by 1957 clover inoculants were also readily available (Lowther and Kerr, 2011). This occurred at approximately the same time as large scale land clearing and development began to take place, especially in acidic soils cleared out of scrub. In these sites clovers usually failed to nodulate because of the absence, or low populations, of resident

rhizobia. To overcome this issue clover seed were inoculated with rhizobia prior to sowing (Lowther, 2010) using peat slurries. Strains recommended for use in New Zealand and present in manufactured inoculants were PDDCC 2666, 2668, 2153, while strain CC 275e (PDDCC 2163) was recommended for Australian inoculants sold in New Zealand. When oversown with coated seeds, the percentage of seedlings nodulated ranged from 40-49% using these strains. This was a contrast to Australian inoculant strain TA1 which only nodulated 17% of seedlings. This low nodulation was likely due to its poor survival on seed before, or on the soil surface after oversowing. No rhizobia capable of nodulating white clover were detected on any of the sites this experiment was conducted on (Lowther and Johnstone, 1978; Lowther and Kerr, 2011).

In other studies conducted by Gaur and Lowther (1982a, 1982b) results showed that strains performed differently depending on whether the inoculated seed was sown into cultivated soil or oversown. In a cultivated situation, strain PDDCC 2163 which is a New Zealand re-isolate of the strain recommended for Australian inoculants marketed in New Zealand, showed no apparent effect on nitrogen uptake or clover growth. It resulted in a nodule occupancy of only 8 to 32% (Gaur and Lowther, 1982b). However, unlike many overseas trials conducted, Gaur and Lowther (1982b) found that the most persistent and competitive strain was PDD 4144: a New Zealand isolate of Australian strain TA1. Inoculant strains were identified by immuno-identification.

It is important to note that most of the research into strain selection of rhizobia in New Zealand was done with Grassland Huia white clover. Therefore, the efficacy of certain strains may vary based on the cultivar of white clover used. A very common measure of assessing the efficacy of rhizobia strains is by measuring the shoot dry weight (Ryle *et al.*, 1981; Sánchez *et al.*, 2014; Pereyra *et al.*, 2015). A study conducted by Ryle *et al.* (1981) showed that shoot/root ratios of nodulated plants were always higher when compared to non nodulated plants.

Clover rhizobia have now spread widely throughout New Zealand, and in a majority of situations the soil naturally contains high levels of resident rhizobia capable of nodulating white clover. Resident populations of rhizobia in New Zealand pastoral soils can range up to 1,000,000 per gram of soil (Lowther, 2010). The resident rhizobia present in the soils

are well adapted to the local conditions and compete with the introduced strains for nodule sites (Dowling and Broughton, 1986). The number of rhizobia on commercially inoculated and coated seed are usually very low, and results from trials conducted in New Zealand and overseas show that these strains form a small portion of nodules and have little or no effect on clover growth (Lowther, 2010). However, lime coating of seeds can provide a localised increase in pH to enhance nodulation in soils with lower pH where clover is oversown, especially in situations where soil liming is not economical (Lowther and Kerr, 2011). More understanding of the mechanisms involved in protecting of rhizobia from harsh conditions during the coating, storage and sowing of legume seed would result in a higher quality product for farmers when sowing.

Rhizobia partner choice and mutualism stability

In the symbiotic relationship 15-20% of the photosynthetic carbon obtained by the plant supports the growth of rhizobium containing nodules, and approximately 15% is exuded into the wider rhizosphere (Kiers and Denison, 2008). Typically, a plant associates with several rhizobial genotypes, all of whom will vary in mutualistic benefit. This can create a situation where some microbes may obtain all the benefits of being in a symbiosis while contributing less to the plant. Therefore, it would make sense for an evolutionary mechanism to be in place to reduce the fitness costs from "cheating" microbes. Kiers and Denison (2008) state that plant sanctions that discriminate among microbial partners based on their symbiotic performance are an important mechanism used to modulate the rhizobia-legume symbiosis. Several authors have argued that microbial cooperation could be maintained if plants preferentially allocated resources to nodules that contain strains of cooperative and high performing rhizobia (Simms *et al.*, 2006). A study conducted on wild *Lupinus arboreus* inoculated with a mixture of good/mediocre and mediocre/poor rhizobial strains showed that from 301 nodules, DGGE banding patterns from only two nodules suggested occupancy by more than one rhizobia. Their findings showed that of 18 plants inoculated with both good and mediocre strains, seven plants were nodulated by both strains, and the 11 remaining plants were all nodulated by the mediocre strain. However, their results showed that occupant identity accounted for a significant amount of the variation in nodule size within plants with smaller average size among nodules

occupied by the mediocre strains. These results perhaps illustrate the host's ability to limit nutrients and resources to nodules that are occupied by poor strains.

Another study conducted by Nangul *et al*. (2013) showed some specificity for rhizobia in arrow-leaf clover where 43% of nodules were occupied by a specific genotype. This work further shows the selectivity that can be displayed by clover toward a particular rhizosphere symbiont.

1.3.2 Arbuscular Mycorrhizal Fungi

In the context of ubiquity and partnerships throughout the plant kingdom, mycorrhizal relationships are the most significant plant-microbe symbiosis (Jeffries *et al.*, 2003). Of these, arbuscular mycorrhizal fungi form the most ancient and widespread mycorrhizal relationships (Vandenkoornhuyse *et al.*, 2003; Chen *et al.*, 2010).

Arbuscular mycorrhizal fungi (AMF) are symbiotic soil fungi which colonize the roots of a large majority of plants (Douds and Millner, 1999). They are globally distributed and are one of the most abundant below ground symbioses. More than 80% of plant species can form arbuscular mycorrhizal symbioses, however, only a relatively few species from a taxon known as the Glomeromycota are involved (Jeffries *et al.*, 2003). This symbiotic relationship involves a bi-directional nutrient flow, where the fungi receives carbon from the plant (this is approximately between 10-20% of net photosynthates) and the plant in return obtains mineral nutrients and water (Jeffries *et al.*, 2003; Nall, 2010). The photosynthetic C that the fungus receives, is in turn delivered to the soil via fungal hyphae. The extra-radical hyphae of AMF therefore act as a direct conduit for host C into soil and contribute directly to its C pools. This bypasses the process of decomposition and consequently stimulates the activity of other soil biota present. This phenomenon however, seems to be a selective process and particularly microbes having antagonistic activity against soil borne pathogens are stimulated (Jeffries *et al.*, 2003).

Differences in morphological and development traits of AMF have been used to classify them into seven genera and five families. However, it is not yet clear whether existing taxonomic groups, which have been based almost entirely on morphological traits are a useful indicator of their ecology (Hart and Reader, 2002). AMF have recently been reclassified into a separate fungal phylum, the Glomeromycota. Members of the

Glomeromycota typically form characteristic, tree-like structures called arbuscules in the root cortical cells of host plants, outside the plasma membrane (Facelli *et al.*, 2009). Arbuscules and intracellular coiled hyphae in root cortical cells play important roles in nutrient transfer between symbionts. One of the main nutrients transferred from the AMF to the plant partner is phosphorus (P) which is highly immobile in soil. After nitrogen, P is the second most limiting element limiting agricultural and crop production (Jeffries *et al.*, 2003; Nall, 2010). The hyphae have the ability to access pores of considerably smaller diameters than roots and this greatly increases the volume of the soil solution from which P can be absorbed as orthophosphate (Facelli *et al.*, 2009; Kaschuk *et al.*, 2009). In a study conducted by Chen*, et al.* (2010) it was found that P concentration was higher in mycorrhizal clover plants than in non-mycorrhizal plants. Also, P concentration of mycorrhizal clover plants increased significantly with increasing growth rate. AMF can also increase uptake of N from the soil, either directly or due to improvements in the plant P supply (Eason *et al.*, 2001). It has been well documented that the high-affinity Pi transporters found in the plasma membrane of extra-radical hyphae play a central role in inorganic phosphorus (Pi) uptake from the soil (Hijikata *et al.*, 2010). For host plants however, the mycorrhiza-specific plant Pi transporters localized on the periarbuscular membrane are responsible for the uptake of Pi released from the arbuscules (Hijikata *et al.*, 2010).

It is believed that sanctioning as a method to discriminate against poor rhizobia could also be a mechanism that extends to other rhizosphere mutualisms such as mycorrhizal fungi, root endophytes, and possibly other free living rhizosphere microorganisms (Kiers and Denison, 2008).

In a study conducted by Helhason, *et al.* (2002) on five woodland plants inoculated with four mycorrhizal fungi showed that, *Glomus hoi* consistently occupied a large proportion of root systems and outperformed other fungi. It improved P uptake and enhanced growth of four out of five plant species. Colonisation from the other fungal isolates was described as sparse and patchy. AMF success was measured by percentage of root colonized and plant success by phosphorus concentration in leaf, root and stem tissue. This potentially shows the specificity of cooperation displayed towards AMF by these woodland plants and could indicate possibility for a similar result in some legumes.

Another study that showed evidence of AM fungal host-plant preference was performed by Vandenkoornhuyse, *et al.* (2003). Their experiment was conducted with three co-existing grass species (*Agrostis capillaris, Festuca rubra, Pos pratensis*). The level of AM fungal diversity found between communities was analysed using T-RFLP. Their results clearly confirmed that an AM fungal host-plant preference exists, even at the level of different grass species. The AM fungal communities colonising *A. capillaris* were statistically different from the others ($P<0.05$).

Therefore, there is likely to be a host preference/ selection process present with the AMF-Plant symbiosis, however there is little research done on whether this is the case in legumes, specifically clover.

1.3.3 Plant growth promoting bacteria (PGPR)

The rhizosphere is the small area of soil directly influenced by the root. It is richer in bacteria than the surrounding bulk soil (Lugtenberg and Kamilova, 2009). The microbes in the rhizosphere benefit because the metabolites exuded by the plant roots can be utilized as nutrients. Therefore, the concentration of bacteria in the rhizosphere is 10 – 1000 times higher than that in the bulk soil. In order to exert their beneficial effects in the root environment, the bacteria need to be rhizosphere competent. For example, be able to compete well with other rhizosphere microbes for the nutrients exuded by the roots and also for space that could be potentially occupied on the roots (Lugtenberg and Kamilova, 2009). Rhizobacteria that benefit plant growth and development are called plant growth promoting bacteria (PGPR). The most studied PGPR are Gram-negative, and the most common strains are members of the fluorescent pseudomonads (Antoun *et al.*, 1998).

1.3.3.1 Pathogen inhibitors

Some bacteria can interact with plant roots and increase resistance to pathogenic bacteria, fungi and viruses. This can be done in two ways, via systemic acquired resistance (SAR), and induced systemic resistance (ISR). SAR occurs when a plant is infected by a pathogen and then develops an enhanced resistance to further pathogen attack. In the SAR state plants are primed to activate defence responses more quickly and effectively the second time they encounter pathogen attack (Conrath, 2006). An ISR

response occurs when PGPR present in the rhizosphere prime the whole plant for enhanced defence against a broad range of pathogens (Choudhary *et al.*, 2007). ISR was first discovered by the findings that resistance can be induced by the rhizobacterium *Pseudomonas* sp. strain WCS417r against *Fusarium* wilt of carnation and by selected rhizobacteria against the fungus *Colletotrichum orbiculare* in cucumber (Lugtenberg and Kamilova, 2009). ISR is dependent on jasmonic acid and ethylene signalling in the plant. There are several individual bacterial components that induce ISR, such as flagella, salicylic acid and siderophores (Kloepper *et al.*, 1980; Lugtenberg and Kamilova, 2009).

1.3.3.2 Siderophore producers

Some PGPR produce extracellular siderophores (microbial iron transport agents) which efficiently binds complex environmental iron, making it less available to native microflora. A study conducted by Kloepper, *et al.* (1980) examined the mechanisms of how a commercial inoculant of *Pseudomans fluorescens-putida* when inoculated on seed were promoting growth and increase in yields of crop plants. Their studies showed evidence that the PGPR mechanism of antagonism was by depriving native microflora of iron. The PGPR produces extracellular siderophores (microbial iron transport agents) which efficiently complex environmental iron, making it less available to native microflora.

1.3.3.3 P solubilizing bacteria

Phosphorus exists in two forms in the soil, as organic and inorganic phosphates. The ability to convert insoluble phosphate (both organic and inorganic) compounds in a form accessible to the plant is very important for PGPR to have in increasing plant yields (Hayat *et al.*, 2010). There are many bacterial strains belonging to several genera such as *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium,* and *Erwinia*, that possess the ability to solubilize insoluble inorganic phosphate compounds, with the first three genera mentioned being the most powerful P solubilizers (Hayat *et al.*, 2010). The production of organic acids, especially gluconic acid is the most common agent of mineral phosphate solubilization by bacteria like *Pseudomonas* sp., *Erwinia herbicola, Pseudomonas cepaci* and *Burkholderia cepacia*. While another common organic acid which is identified in

strains with phosphate-solublizing ability is 2-ketogluconic acid. This has been found to be present in *Rhizobium leguminosarum, Rhizobium meliloti, Bacillus firmus,* and several other unidentified soil bacteria (Hayat *et al.*, 2010).

1.3.3.4 Auxin producers

Symbiotic and non-symbiotic bacteria can promote plant growth directly via the production of plant hormones. PGPR can synthesise and export phytohormones which play a regulatory role in plant growth and development. These are known as plant growth regulators. Auxins are one of the five well-known classes of plant growth regulators and one which has received a lot of attention. The most abundant physiological auxin found in plants is indole-3-acetic acid (IAA), which is known to stimulate both rapid and long term responses in plants. It has been estimated that 80% of the bacteria isolated from the rhizosphere can produce IAA (Hayat *et al.*, 2010). The abundance of IAA in plants and the widespread ability to produce it by rhizobacteria implies this is a very important phytohormone in plant health and stability. As well as IAA, bacteria such as *Paenibacillus polymyxa* and *Azospirilla* also release other compounds in the rhizosphere such as, indole-3-butyric acid (IBA), Trp and tryptophol or indole-3-ethanol (TOL). These indirectly contribute to plant growth promotion (Hayat *et al.*, 2010).

There is little research on selection for, and exclusion of, rhizosphere bacteria. However, if selection measures occur for rhizobia and AMF, it is likely that this would be extended to PGPR as well. Kiers and Denison (2008) in their review of sanctions, cooperation and the stability of plant-rhizosphere mutualisms, conclude that cooperation between endophytic microbes and plant is plausible, and that any mechanism that selectively favours some fraction of microbes associated with a plant's roots, based on the benefits they provide the plant, would be functionally the same as sanctions from the perspective of the microbes.

1.4 Rhizosphere and endophytic community analysis

Rhizospheric and endophytic organisms, are both known to have beneficial effects on host plants (Eevers *et al.*, 2015). Study of microbial communities can be performed using both cultivation-dependent and independent approaches (Wagner *et al.*, 1993; Tunney *et al.*, 2011). Traditionally, the analysis of soil microbial communities has relied heavily on

culture dependent techniques, using a vast range of culture media designed to maximize the recovery of different microbes present (Hill *et al.*, 2000). Rhizospheric bacteria are known to be more easily cultivable compared to endophytic species. This is because endophytes reside within plant tissue which results in exposure to a more specific and stable habitat than rhizospheric bacteria (Eevers *et al.*, 2015). However, endophytes are also more closely associated with, and dependent on the host plant.

To thoroughly investigate endophytic communities, they must be cultivable under laboratory conditions. This is not always possible and cultivation-dependent techniques can substantially underestimate the number of organisms present in and around plant tissues. The literature suggests that only 0.001% to 1% of endophytes present in plant tissues are cultivable (Eevers *et al.*, 2015). Due to the inherent limitations of culture-based methods, microbial ecologists are moving toward culture-independent methods for community analysis (Hill *et al.*, 2000).

1.4.1 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE works based on the denaturation of DNA strands based on their nucleotide sequence composition. PCR products with different compositions but of the same length will migrate different distances when they are exposed to a gradient of denaturing conditions. This results in unique DNA fingerprints when DGGE is used to separate PCR products (Nakatsu *et al.*, 2000). DGGE can target the 16S rRNA and 18S rRNA genes, for analysis of bacterial and fungal communities including AMF (Boon *et al.*, 2002; Agnelli *et al.*, 2004; Oliveira *et al.*, 2009). A study conducted by Oliveira *et al* (2002) assessed the mycorrhizal community in the rhizosphere of maize (*Zea mays* L.) genotypes with contrasting phosphorus efficiency. They found that maize genotypes had a greater influence on the rhizosphere mycorrhizal communities than the level of P in the soil. DGGE profiles from maize roots also showed bands that were present in only P efficient genotypes suggesting that P efficient maize genotypes were selectively colonised by some mycorrhizal groups.

Although there are many advantages of using DGGE, such as its ability to analyse multiple samples, its reproducibility, and speed (Kirk *et al.*, 2004), there are also limitations. These include, PCR biases, laborious sample handling, and it is estimated DGGE can only detect

1 -2% of the microbial population representing dominant species present in the environmental sample (Kirk *et al.*, 2004). It is also possible the DNA fragments of different sequences are similar in their mobility and denaturing characteristic on the polyacrylamide gel. Therefore, one band may not always be representative of one species. Although DGGE provides insight into community structure, it does not give any information regarding what is actually present, as it does not generate DNA sequence data.

1.4.2 DNA metabarcoding using next generation sequencing (NGS)

Metabarcoding is the high-throughput multi-species identification from environmental samples, and its main function is to identify taxa present in any particular environmental sample (Taberlet *et al.*, 2012). So not only does this tool help identify uncultured organisms but the multi-species nature of the technique gives an indication of the full variety of species found in a given environmental sample. Environmental samples are collected, stored at -80˚C to preserve the sample and DNA extracted. Marker genes usually on the rRNA are amplified using conserved primer pairs (Bik *et al.*, 2012). Ribosomal RNA is considered an ideal target, especially for phylogenetic relationships because it is universal and contains both highly conserved as well as variable domains (Patwardhan *et al.*, 2014). After sequencing, large databases such as SILVA, Greengenes and Ribosomal Data Project (Bacci *et al.*, 2014) make it possible to assign bacterial taxonomy. A study conducted by Edwards *et al.* (2015), using the Illumina Miseq platform to study the root microbiome of rice (*Oryza* spp.) found that in greenhouse conditions, microbiome composition varied with soil source and genotype. However, under field conditions, geographical location and cultivation practices were factors contributing to microbiome variation. This technology could also be used to study microbial communities of white clover. To date there are no published studies examining the rhizosphere and root endophytes of old and new cultivars of white clover, but NGS could provide valuable information on how plant breeding may have affected the associations of white clover.

1.5 Aims and objectives of this research

Considering the high economic importance of white clover in New Zealand, and decades of breeding focused on above ground characteristics, there has been no research to examine the impacts of this breeding on its associations. The aim of this research is to examine whether 80 years of white clover breeding has affected a new cultivar's ability to form effective below ground associations.

The finding of this research will provide information on i) whether breeding has impoverished current clover germplasm in its ability to form beneficial below ground associations or to sanction poorly performing associations and ii) will potentially identify clover lines with superior ability to form beneficial associations providing candidate lines to use in further breeding. To achieve this, three objectives were developed as outlined below:

1. To examine the relative ability of new and old* varieties of white clover to form symbioses with strains of *R. leguminosarum* that vary in ability to fix N.
2. To investigate the responsiveness of the various clover lines to different species of AMF and AMF mixtures.
3. To determine whether cultivars that formed the most interactions with effective soil micro-organisms *in vitro* were also able to do so in soil

*New and old refer to the era in which the cultivar was released.

2 Comparative interaction of six cultivars of *Trifolium repens* with microbial symbionts *in vitro*

2.1 Introduction

Genetic improvement of pasture plants through plant breeding has been pursued in New Zealand for over 75 years (Easton *et al.*, 2002). However, the focus has aimed at gaining productivity, disease resistance, and forage quality (Easton *et al.*, 2002), with little attention given to the below ground associations. Therefore, newer germplasm are in danger of, or may already be, impoverished in their ability to form these associations or to discriminate against poorly performing partners. Numbers of naturalized *Rhizobium* strains in New Zealand soils can reach 10^6 CFU/g (typically 10^3- 10^4) with populations varying from 51-102% effectiveness (relative to commercial standards). In this background of high rhizobial diversity the ability of a white clover cultivar to select effective symbionts would be advantageous. A study conducted on soybean by Kiers, Hutton *et al.* (2007) showed that older cultivars produced more seed when infected with a mixture of effective and ineffective rhizobia. They also found that when infected by symbionts varying in quality, legume defences against poor-quality partners had worsened under artificial selection (modern breeding).

Partner selection is also thought to be a key factor in stabilizing mycorrhizal symbiosis. Both plants and arbuscular mycorrhizal fungi (AMF) can preferentially allocate resources to superior partners (Bever *et al.*, 2009; Werner and Kiers, 2015). A study conducted by Bever *et al*. (2009) showed that *Allium vineale* plants in a split root system preferentially allocated photosynthetic products to the side of the root system colonized by a more mutualistic AMF. This resulted in higher spore production by the beneficial AMF compared to their non-beneficial counterparts. However, this effect was not noticed when the AMF were mixed on the same root system.

The objectives of this chapter were, (i) to rank the efficacy of six strains of *R. leguminosarum* against six different cultivars of white clover (three old and three new); (ii) to determine if there is a preferential selection by old or new cultivars of white clover to select effective strains of *R. leguminosarum* from a mixture; (iii) to investigate the

responsiveness of the six cultivars of white clover lines to different species of AMF and to AMF mixtures.

2.2 Materials and Methods

2.2.1 Cultivars of *Trifolium repens*

Six cultivars of *Trifolium repens* were chosen to compare against each other; three newer varieties and three older varieties, and from a range of large, intermediate, and small leaf sizes. These are: C20133 Grasslands Kopu II, C20132 Crusader, C21905 Grasslands Tribute, C3608 Louisiana, C2027 Grasslands Huia, and C13174 Irrigation (Table 2.1). Seeds for all the cultivars were obtained from the Margot Ford Germplasm Centre in New Plymouth, New Zealand. The new and old cultivars were matched to each other based on their leaf physiology and growth habits as shown in Table 2.1.

Cultivar details

Grasslands Kopu II (2003)

The highest producing white clover available in the large leaf class. It provides high feed quality with improved soluble carbohydrate levels and has the highest tolerance to clover root weevil amongst large leaf cultivars (Wrightson-Seeds, 2014)

Crusader (2001)

Crusader is a small leaf variety released in 2001. It has a very high total sward and clover yield for a small leaf variety. It is persistent making it an excellent grazing clover. It also grows well with pasture, and the grass in the sward will not be thinned out (Goldcrop, 2013).

Grasslands Tribute (2003)

Tribute is a medium leaved white clover released in 2003. It was bred for good drought tolerance and winter activity in Victoria, Australia from crosses with elite New Zealand germplasm. Tribute is known for its consistent performance and persistence under grazing. It shows strong stolon growth in all seasons but has especially good cool-season growth, and shows good drought tolerance in Canterbury (Woodfield, Clifford *et al*. 2003).

Irrigation (1936)

Also known as Tongala, and Ulmarra, Irrigation was first certified in 1936. It is a medium to large leaved. It has been described as similar to Grasslands Huia but larger leaved and more prostrate in growth. In New Zealand, its performance is poorer than Huia when grown in spaced plants. Irrigation also shows some resistance to rugose leaf curl virus (Caradus, 1986; Caradus and Woodfield, 1997).

Huia (1964)

Originally known as New Zealand Certified Mother seed but was renamed to Grasslands Huia in 1964. It is a medium leaved clover and has a dense shoot morphology. It shows a consistently high proportion in grass mixed swards in New Zealand. However, it is the most susceptible of New Zealand Grasslands cultivars to clover rust infection (Caradus and Woodfield, 1997).

Louisiana (1957)

Released in 1957 (Caradus and Woodfield, 1997). Louisiana has a large leaf size and its stolon growing point density is relatively low compared to Huia. It shows significantly lower clover DM yield, clover content, and stolon density under rotational grazing in New Zealand. When compared with 158 other clover varieties it was in the bottom 10% of the other lines for the proportion of clover in the sward at final harvest.

Table 2.1 Matched pairs of old and new cultivars based on leaf physiology and growth habits. Date of release in brackets

Pair	Old	New
1. (Small leaf)	Irrigation (1936)	Grasslands Crusader (2001)
2. (Medium leaf)	Grasslands Huia (1957)	Grasslands Tribute (2003)
3. (Large leaf)	Louisiana (1930s)	Grasslands Kopu II (2003)

2.2.2 Seed Preparation

Seeds were scarified by placing approximately 30 seeds at a time between two sheets of fine sand paper (Grade P150). The seeds were rubbed gently between the sheets of paper for 1 min. Once scarified the seeds were then surface sterilized by immersion in 70% ethanol for 1 min, followed by 4% sodium hypochlorite for 30 s, followed by six rinses in sterile Millipore water for 1 min each. After this, the seeds were left to soak in sterile Millipore water overnight in darkness at 4°C. The following day seeds were placed onto 10% water agar (10 g of Davis Agar per L of water). The plates were wrapped in aluminium foil and placed in an incubator at 22°C for 3-5 days to germinate.

2.2.3 Preparation of *Rhizobium leguminosarum* strains for inoculation

Six strains (1302, 316, 451, S12N10, S26N9, TA1) were selected from the Lincoln University Culture Collection based on symbiotic potential data produced as part of the MBIE programme "Improved forage legume-rhizobia performance" (Appendix 6.1).

Pure cultures contained in 1.7 mL tubes were retrieved from the -80°C freezer and thawed at room temperature for 15 min. Each 1.7 mL tube was vortexed for 10 s to ensure the contents were uniformly mixed. Using a sterile plastic loop, one loopful of the pure culture was streaked onto yeast mannitol agar (YMA; 1 g yeast extract, 4 g mannitol, 0.5 g dipotassium phosphate, 0.2 g magnesium sulphate, 0.1 g sodium chloride, 15 g agar in 1 L reverse osmosis [RO] water, autoclaved for 15 min at 121°C and 15 Psi) and incubated in darkness at 25°C for 4 d.

Once the plates had been incubated, a single colony was transferred to a 15 mL tube containing 4 mL of sterile yeast mannitol broth (YMB; 1 g yeast extract, 4 g mannitol, 0.5 g dipotassium phosphate, 0.2 g magnesium sulphate, 0.1 g sodium chloride in 1 L RO water, autoclaved for 15 min at 121°C and 15 Psi) and vortexed for 10 s to mix. A control tube was filled with 4 mL of sterile YMB. Tubes were incubated in a shaker (Labnet) for 24 h at 200 rpm at 28°C.

After incubation, the tubes were vortexed for 5 s and 1 mL was then taken from each tube for optical density reading in a spectrophotometer at 600 nm. Based on the absorbance reading, the tubes were diluted with 0.5% sterile saline to obtain a reading close to 0.167 which is approximately equivalent to a concentration of 1×10^8 cells per mL

(Weir, 2006). Based on this reading the cultures were diluted further to an absorbance reading of 0.00167 to reach approximately 1×10^6 cells per mL.

2.2.4 Experiment 1 – Symbiotic potential of *Trifolium repens* cultivars paired with different strains of *Rhizobium leguminosarum*

2.2.4.1 Preparation of tubes

Vermiculite (Exfoliators AUST PTY LTD) was pressed down into 50 mL plastic tubes and filled to the 35-mL mark. After this, each tube received 20 mL of minimal N Mc Knight solution (Calcium chloride 0.25 mL (2% w/v), Magnesium sulphate 0.25 mL (40% w/v), Potassium dihydrogen orthophosphate 1 mL (10% w/v), Potassium chloride 1 mL (15% w/v), Trace elements stock solution 0.25 mL, EDTA-$FeCl_3$ 0.75 mL (02% EDTA, <1% $FeCl_3$), Sodium hydroxide 0.25 mL (4% w/v), Millipore water 1000 mL, 100 mM ammonium nitrate 1 mL/L). The lids of the plastic tubes were loosely screwed on and the tubes were autoclaved to ensure sterility.

Once the tubes had been autoclaved, seedlings of *Trifolium repens* that had been germinated on water agar as described in Section 2.2.2 were gently placed into the tubes with long tweezers which had been sterilized by being dipped into 96% ethanol and passed through a flame. Seedlings were selected to be healthy and of as consistent size as possible. Each cultivar was replicated five times in separate tubes.

2.2.4.2 Inoculation of *Trifolium repens* cultivars

Each of the strains of *R. leguminosarum* were prepared for inoculation as described in Section 2.2.3. There was a total of eight treatments, one for each strain of *R. leguminosarum* (six treatments), along with an added nitrogen control and negative control treatment which received no extra nutrition other than the initial addition of minimal N Mc Knight solution all plants received.

Germinated seedlings were inoculated at the same time they were placed in the 50 mL tubes. To each plant in the six treatments requiring inoculation with a strain of *R. leguminosarum*, 500 µL of the respective strain was applied at the base of the plant. The nitrogen control plants received 500 µL of 0.85% NaCl solution after sowing and then 9 days later given 230 µL of sterile 1 M ammonium nitrate solution. The plants in the negative control treatment received 500 µL of 0.85% NaCl solution.

Once the seedlings had been sown, clear cellophane was wrapped around the top of tubes and a small hole pierced with a 1 mL sterile plastic pipette tip to allow for air circulation and watering. The cellophane was applied to maintain moisture in the tubes. Ten randomly selected tubes were weighed, the average weight taken, and this figure was used as the weight the tubes were watered to throughout the experiment. The plants were placed in a growth room at 16 h light and 8 h dark conditions, at 22°C and left to grow for six weeks. During the first three weeks, the plants were watered to weight with sterile Millipore water every three to four days. After three weeks, when the tallest plants were reaching the top of the tubes, the cellophane was removed. After this the plants were watered to weight every 2-3 days.

2.2.4.3 Experimental design

Five randomized blocks were set up, each block containing 56 plastic tubes containing the seedlings. Each block was made with three white plastic tube racks holing 21 tubes each. The third plastic rack in each block had only 14 tubes.

2.2.4.4 Assessment

2.2.4.4.1 Shoot dry weights and nodule count

Once the plants had grown for six weeks, the shoots were cut at the crown and placed in labelled paper bags. Each plant was carefully removed to ensure no vermiculite adhered to the shoots. The shoots were then subsequently dried in the oven at 65°C for 72 h and weighed again to obtain dry weight data. For each plant, the total number of nodules present on the root was counted.

2.2.4.4.2 Nodule strain confirmation

Roots were carefully removed from the vermiculite and washed under tap water. Three random nodules were then taken from each plant and placed in a mesh sieve and surface sterilized in a laminar flow unit by immersion in 70% ethanol for 10 s, followed by 2 min in 4% sodium hypochlorite, and 4 baths in sterile Millipore water for 30 s each.

Once sterilized each nodule was crushed using a sterile glass rod in a sterile Petri dish. Once crushed a small drop of sterile water was placed on the crushed nodule to create a wet slurry which was streaked onto yeast mannitol agar (YMA) (Appendix 6.2) using the

same glass rod. The plates were then wrapped in cellophane and placed in an incubator at 20˚C in darkness for 3-5 d. Once colonies had grown the plates were stored at 4°C until needed for direct colony PCR.

Colony PCR and Genotyping

The genotype of the strains occupying the nodules was determined by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) as described by Versalovic et al. (1991). The master mix was produced by combining 10 µL of DreamTaq™, 1 µL of ERIC1 primer (50 µM) sequence ($^{5'}$ATGTAAGCTCCTGGGGATTCAC$^{3'}$), 1 µL of ERIC2 primer (50 µM) sequence ($^{5'}$AAGTAAGTGACTGGGGTGAGCG$^{3'}$), and 7 µL of sterile water per sample. Nineteen µL of this master mix was aliquoted into a PCR tube. A sterile plastic 10 µL pipette tip was used to touch single colonies and then mixed with the aliquoted PCR reagents in each tube. The PCR was set up to run for an initial denaturation of 3 min at 95 ˚C, followed by 40 cycles of 1 min at 95˚C, 1 min at 52˚C, and 1 min at 72˚C. A final extension of 10 min at 72˚C was also included to complete the reaction.

The PCR products were separated by electrophoresis in a 1% agarose gel for 40 min at a constant 120 V. The gel was stained in ethidium bromide for 20 min and then rinsed in water for 10 min. Band patterns were then observed under UV light in a FireReader™ (UVITEC) and compared to that generated by a pure culture of the original cultured strains.

2.2.4.5 Statistical analysis

It is important to note some cultivars had mortality in some treatments which may have affected the data, this was treated as a missing value in the ANOVA analysis.

The effects of the treatments on plant dry weight, and treatment on nodule number and nodule score were subjected to analysis of variance (ANOVA) using GenStat 16th version (VSN International, Hemel Hempstead, UK). The treatment means (excluding nitrogen, and negative controls) for each cultivar were compared using Fisher's least-significant difference tests (LSD) at a P value of $P<0.1$ on GenStat 16th version (VSN International, Hemel Hempstead, UK). Bar charts of mean dry weights were prepared in R Studio (Version 1.0.143©). The groupings obtained in this experiment will be used for a test of equal proportions in experiment 2.

2.2.5 Experiment 2 – Symbiont selection by *Trifolium repens* cultivars inoculated with multiple strains of *Rhizobium leguminosarum*

2.2.5.1 Preparation of seedlings

Seedlings were grown in sterile 50 ml plastic tubes as described in Section 2.2.4.1, containing seedlings of *T. repens* that had been germinated on water agar as described in Section 2.2.2.

2.2.5.2 Inoculation of *Trifolium repens* cultivars

A total of three treatments were applied in this experiment. Treatment 1, was a mixture of the six strains of *R. leguminosarum* described in Section of 2.2.3. This mixed inoculum was prepared by adding 6 ml of each strain of *R. leguminosarum* (strains 1302, 316, 451, S12N10, S26N9, TA1) to make a final concentration of 1 x 10^6 cells per mL in 0.85% (w/v) NaCl to a 50 ml tube. To this 14 ml of 0.85% NaCl was added to reach a final volume of 50 mL. The tube was vortexed for 10 s prior to inoculating plants with 500 µL of this *R. leguminosarum* strain mixture.

Treatment 2 was a nitrogen control treatment, which received 500 µL of sterile 0.85% NaCl solution on day one, when the rhizobia treated plants received 500 µL of inoculant. Positive control plants also received 230 µL of sterile 1 M ammonium nitrate solution nine days post inoculation.

Treatment 3 was a negative control treatment. These plants also received 500 µL of sterile 0.85% NaCl solution on day one along with the positive control plants. Plants within the negative control treatment did not receive any further nutrition apart from the usual watering to weight.

Once the seedlings had been sown, they were grown for six weeks as described in Section 2.2.4.2.

2.2.5.3 Experimental Design

Five randomized blocks were set up, each block containing 18 tubes, one tube per treatment (inoculated, positive control, and negative control), per cultivar. The tubes were held in white plastic racks which hold up to 21 tubes each in three rows of six.

2.2.5.4 Assessment

2.2.5.4.1 Shoot dry and fresh weights

Once the plants had grown for six weeks, the shoots were harvested as described in Section 2.2.4.4.1.

2.2.5.4.2 Identification of strains present in nodules

Strain isolation

Strains were isolated from the first ten nodules taken from the top of the main tap root as described in Section 2.2.4.4.1. The genotype of the strains occupying the nodules was determined by ERIC-PCR as described in Section 2.2.4.4.2.

2.2.5.4.3 Statistical analysis

The effects of the treatments on plant dry weight were subjected to ANOVA using GenStat 16th version (VSN International, Hemel Hempstead, UK). Based on the ranking and groups formed in Experiment 1, differences in expected and observed nodule occupancy for each of the six cultivars were analyzed using a test of equal proportions using R Studio (Version 1.0.143©). Proportions were calculated based on an equal chance of each strain nodulating (33.33%).

2.2.6 Experiment 3 – Colonization of *Trifolium repens* cultivars by arbuscular mycorrhizal fungi (AMF)

2.2.6.1 Preparation of AMF spore inocula

Three species of AMF were obtained from the Plant Microbiology Culture Collection (Lincoln University). The species were *Acaulospora capsicula* isolated from roots of macrocarpa (*Cupressus macrocarpa*) in 2006, *Acaulospora laevis* isolated from roots of apple (*Malus domestica*) in 2006 and *Funneliformis geosporum* (syn. *Glomus geosporum*) isolated from grape (*Vitis vinifera)* in 2006. Spores were recovered from stored pot cultures (Ridgway *et al.*, 2006) using the sucrose density centrifugation method (Brundrett *et al.*, 1996). Twenty grams of each pot culture was placed into a 250 mL beaker and 200 ml tap water added. The beaker was stirred to mix the contents and left for 2 min to allow debris to settle. The beaker was then carefully poured through a stacked set of 750 µm, 500 µm, and 50 µm sieves. The contents of the 50 µm were

carefully poured into another small beaker and this liquid was then divided between four 50 mL tubes (approximately 5-10 mL each). The volume in each 50 mL tube was increased to 50 mL with tap water. The tubes were centrifuged for 5 min at 805 x g. The supernatant was discarded, the pellet re-suspended in 50% w/v sucrose and then centrifuged for 1 min at 805 × *g*. The supernatant was washed into a 50 µm sieve and rinsed thoroughly in tap water to remove sucrose. The washed contents were tipped into a plastic funnel lined with filter paper. Once filtration was complete, the filter paper was placed in a Petri dish at 4°C and stored for up to 1 week before use.

For inoculation of clover, healthy (unblemished) mature spores were collected from the Petri dishes using a super fine tip paint brush and placed onto individual pieces of filter paper (3 cm × 3 cm). Each clover cultivar received each of four treatments and they consisted of each of the three AMF species alone and a mixture consisting of equal parts of each AMF species. For the individual AMF species treatments, the inoculum was prepared by placing 15 spores on a 2×2 cm piece of filter paper. For the AMF mixture treatment, the inoculum was prepared by placing 5 spores of each of the three different AMF species (*A. capsicula, A. laevis, F. geosporum*) on a 2×2 cm piece of filter paper. Spores were examined under a stereo microscope and blemished, damaged, broken or immature spores were not selected.

2.2.6.2 Inoculation of *Trifolium repens* cultivars

This experiment was done in sterile 275 mL clear plastic cups (Budget™). To each cup five small holes for drainage were pierced in the bottom using a soldering iron. Each cup was half filled with sterile vermiculite (Exfoliators AUST PTY.LTD). Two squares of filter paper (2×2 cm) each containing 15 AMF spores of the same species were placed on the vermiculite on opposite sides of the cups. Approximately 4 cm of vermiculite was added on top of the filter paper and a mark was made on the lip of each cup to identify where the filter paper was located. Once the pots were filled with vermiculite, all pots were given 95 mL of either modified minimal N McKnight's solution or standard minimal McKnight's solution. Modified McKnight's solution in which KH_2PO_4 was substituted for hydroxyapatite (insoluble P form) to the match the molecular weight of phosphorus present in the original recipe (Appendix 6.5). McKnight solution was added to all pots receiving AMF. The soluble phosphate control received standard McKnight's and the

insoluble phosphate control received modified McKnight's solution. The soluble and insoluble P controls did not receive AMF spores but did receive empty pieces of filter paper. Each treatment was replicated 5 times.

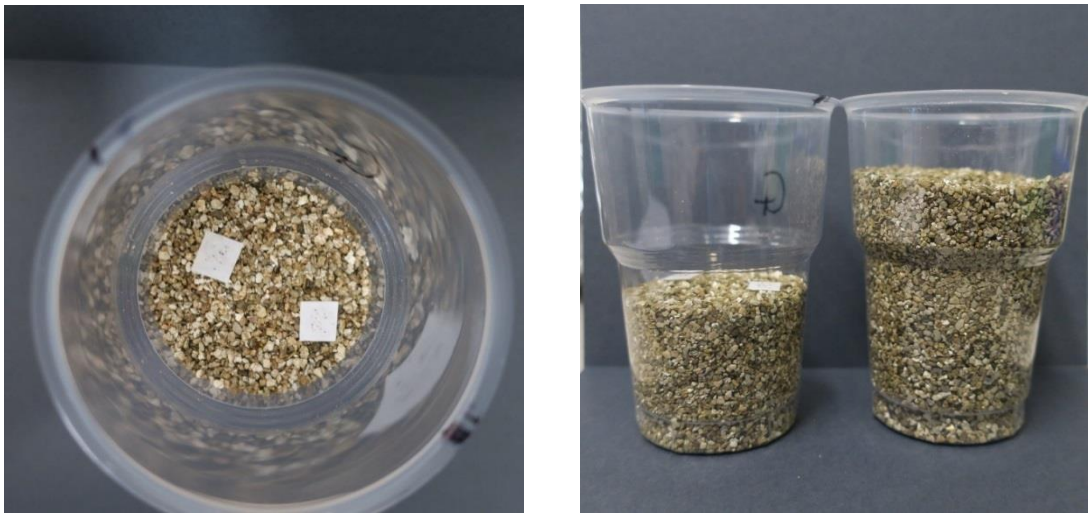

Figure 2.1 Experimental set up showing the pieces of filter paper containing spores placed inside a plastic cup with vermiculite and then covered with more vermiculite.

Five day old seedlings of similar size that had been germinated on water agar were sown into the pots directly above the filter paper. The pots were placed in a randomized complete block design in a growth room with 16 h light and 8 h dark conditions at 22 °C and left to grow for seven weeks. Pots were watered to weight every 2-3 d. in addition, as the plants had not been inoculated with rhizobia, adequate N for growth was provided by applying 920 µL of sterile 0.0625 M ammonium nitrate to each plant at 7 and 20 d after sowing.

2.2.6.3 Assessment

2.2.6.3.1 Shoot dry weights

Each plant was cut at the crown using scissors and the foliage placed in label paper bags and dried in the oven at 65˚C for 72 h. The dried shoots were then weighed to obtain dry weight data.

2.2.6.3.2 AMF colonization

Roots were washed and stored in glass universals bottles for 48 h until processed. Roots were then immersed in 10% KOH and autoclaved for 10 min. Then roots were poured out on a tea strainer and the KOH drained into a beaker. Next, the roots were rinsed in 10% HCL and then immersed in lactoglycerol blue stain (0.05% (w/v) Trypan blue in lactoglycerol (Lactic acid:glycerol:water =1:1:1 v:v:v)) overnight. The following day the stain was removed and the roots were stored in lactoglycerol. It was noted that this process did not adequately clear the roots so the procedure was repeated by re-immersing the roots in 10% KOH at 80˚C for 20 mins then staining and de-staining as previously described.

The presence or absence of mycorrhizal colonization was noted in 5 fields of view each from two random lateral roots near the bottom of the tap root under a compound microscope at 40× magnification as described by Brundrett et al. (1996) and recorded as success and fail data.

2.2.6.4 Statistical analysis

The effects of the treatments on plant dry weight were subjected to ANOVA using GenStat 16th version (VSN International, Hemel Hampstead, UK). The presence and absence of mycorrhizal colonization was analyzed using a linear regression and then with a Tukey family-wise confidence interval (95%) between the treatment levels for each cultivar in R studio (Version 1.0.143©).

2.3 Results

2.3.1 Experiment 1 Symbiotic potential of six clover cultivars with different strains of *Rhizobium leguminosarum*

Out of the six clover cultivars, only Irrigation and Louisiana produced significantly different shoot dry weight (SDW) ($P<0.05$) when inoculated with different strains of *R. leguminosarum* (Figure 2.2). Inoculation of Irrigation with *R. leguminosarum* isolate TA1 produced more SDW than inoculation with any of the other five strains ($P=0.038$). For Louisiana, *R. leguminosarum* strains 451 and 316 produced more SDW ($P=0.013$) than the other four strains. There were strong trends for Huia (P=0.052) and Tribute (P=0.059) to produce more SDW when inoculated with strain 451 and strains 451/TA1, respectively. The inoculant treatments did not affect the SDW produced by Crusader and Kopu II ($P=0.160$ and $P=0.114$, respectively).

Strains that produced significantly more SDW ($P\leq0.05$), or those that showed a strong trend ($P\leq0.06$) for producing more SDW, were classified as 'highly effective' associations (Table 2.2). For cultivars capable of selecting symbiotic partners it was assumed that these strains would be present in a greater abundance than that solely produced by chance.

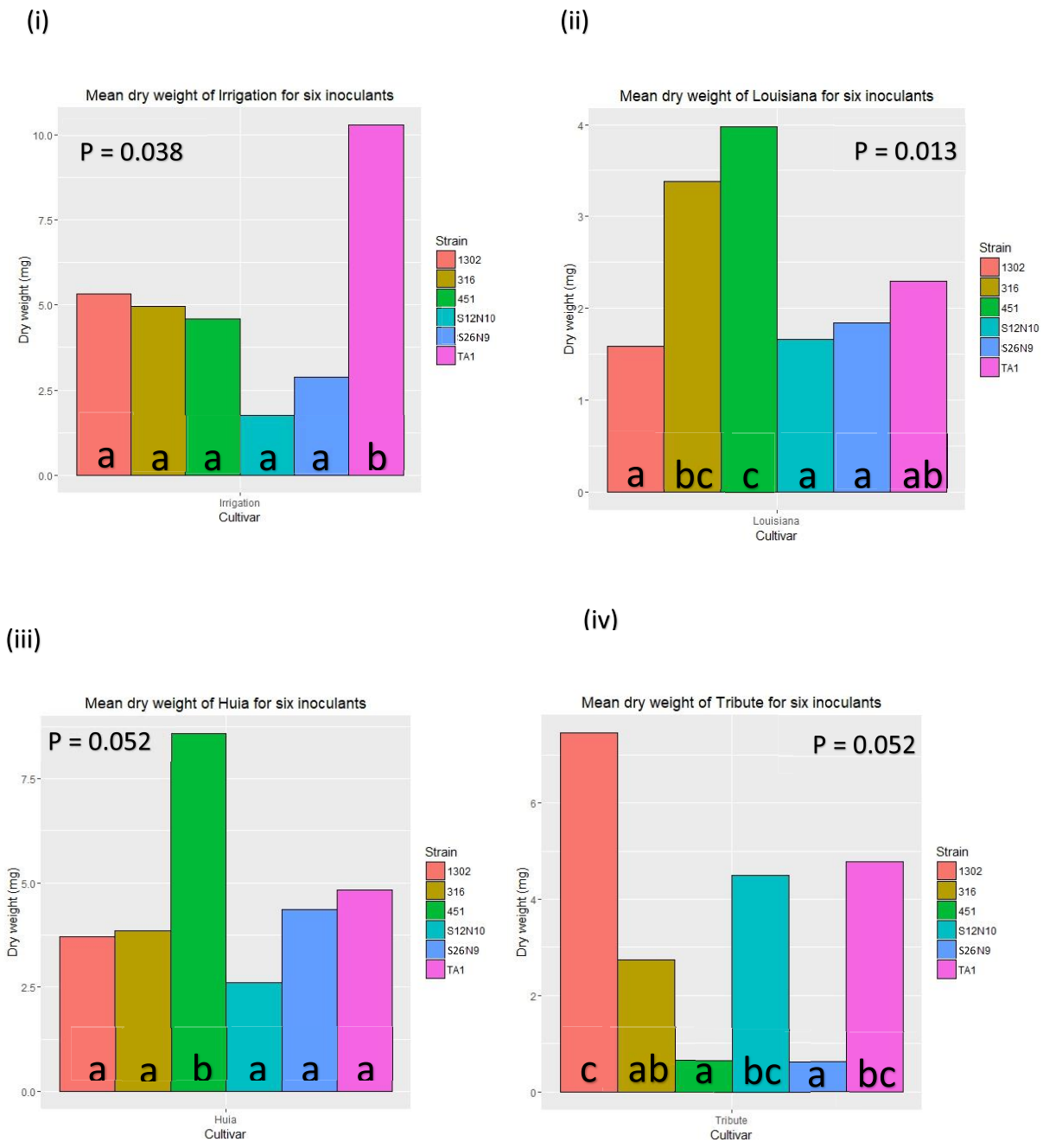

Figure 2.2 Mean dry weights for all inoculants across cultivars that showed a significant difference, or strong trend between treatments. Letters correspond to Fisher's least significant difference at the appropriate P values. Treatments with the same letters are not significantly different.

Table 2.2 Mean shoot dry weights (mg) of all six white clover cultivar inoculated with each of six strains of *Rhizobium leguminosarum*. Shaded cells are strains that are 'highly effective' (P≤0.6) when in symbiosis with a specific cultivar. Significance was determined by using Fisher's least significant LSD.

Cultivar	**Treatment**					
	1302	**316**	**451**	**S12N10**	**S26N9**	**TA1**
Huia	3.71 a	3.86 a	8.59 b	2.60 a	4.36 a	4.82 a
Tribute	7.46 c	2.74 ab	0.66 a	4.49 bc	0.62 a	4.78 bc
Irrigation	5.32 a	4.95 a	4.60 a	1.76 a	2.87 a	10.30 b
Crusader	4.20	3.46	2.13	1.24	1.20	3.12
Louisiana	1.59 a	3.38 bc	3.98 c	1.66 a	1.84 a	2.29 ab
Kopu II	6.18	4.68	5.73	1.60	2.35	2.48

2.3.1.1 Nodule numbers of *R. leguminosarum*

The total number of nodules was noted for each inoculant treatment, across all live plants. There was a significant cultivar × treatment effect on total nodule numbers (P<0.001) as the strain of *R. leguminosarum* forming the most nodules varied between cultivars. For example, strain 451 formed the most nodules on Huia, but it formed the second least on Tribute. There were no nodules found on any positive or negative control plants. Fisher's least significant difference was used to identify nodule numbers that were significantly different for each cultivar (Table 2.3).

2.3.1.2 Strain confirmation

All nodules plated for confirmation were confirmed using direct colony ERIC PCR and no bright bands other than the expected were noticed (Figure 2.3).

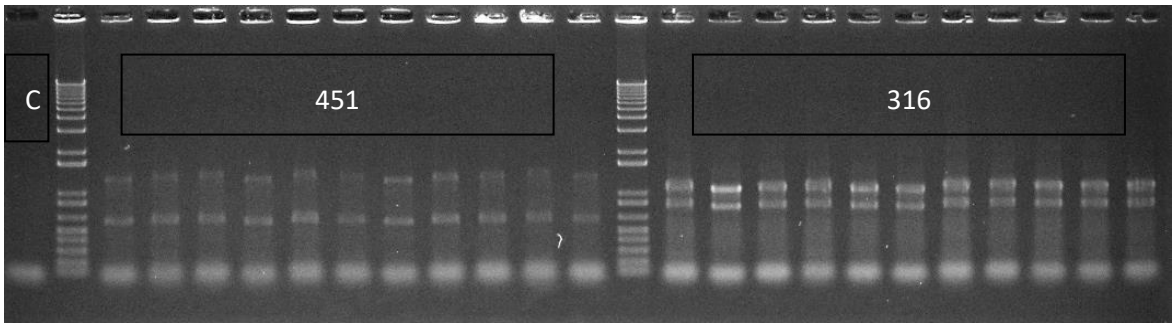

Figure 2.3 Agarose gel (1%) of ERIC-PCR products for bacteria recovered from nodules of *Trifolium repens* inoculated with strain 451 (left), and strain 316(right). 1kb – 1kb plus DNA ladder (Invitrogen) and C – negative control.

Table 2.3 Total number of nodules for all six white clover cultivar across each inoculant treatment. Across each row, values with the same letters are not significantly different at the *P* value prescribed at the end of each row

Cultivar	**Inoculant Treatment**						***LSD value***
	1302	**316**	**451**	**S12N10**	**S26N9**	**TA1**	
Huia	4.74 *a*	5.80 *ab*	17.53 *b*	8.39 *abc*	13.20 *cd*	13.0 *bcd*	*P* = 0.05
Tribute	13.3 *ab*	4.99 *a*	2.13 *a*	10.87 *ab*	1.12*a*	19.16 *b*	*P* = 0.05
Irrigation	14.36 *b*	15.60 *b*	11.86 *ab*	10.60 *ab*	5.49 *a*	16.75 *b*	*P* = 0.05
Crusader	5.21 *a*	10.60 *ab*	8.0 *ab*	12.8 *b*	5.37 *a*	10.0 *ab*	*P* = 0.1
Louisiana	9.0 *a*	15.20 *b*	9.20 *a*	10.42 *ab*	6.0 *a*	10.16 *ab*	*P* = 0.05
Kopu II	10.75 *a*	9.20 *a*	10.99 *a*	7.70 *a*	11.25 *a*	29.74 *b*	*P* = 0.05
Mean across all cultivars	10.10 *a*	10.54 *a*	10.62 *a*	10.33 *a*	7.80 *a*	16.40 *b*	*P* = 0.05

2.3.2 Experiment 2: Symbiotic selection by *Trifolium repens* cultivars inoculated with multiple strains of *Rhizobium leguminosarum*

2.3.2.1 Dry weights

There was a significant difference in dry weight between each of the three treatments (positive control, negative control, and rhizobia mixture $P < 0.001$). The plants that received the mixture of rhizobia had dry weights significantly greater than the uninoculated controls, but significantly lower than the positive control (Table 2.4). There was also a significant difference in total dry weight between cultivars ($P < 0.001$) (Table 2.5).

The total mean dry weights of Huia and Kopu II were significantly higher than those of Irrigation and Tribute. For each of the cultivar pairs only Huia/Tribute produced different dry weights. There was no significant difference in total dry weight between each of the other two cultivar pairs of Louisiana/Kopu II, and Irrigation/Crusader (Table 2.5).

Table 2.4– Total mean dry weights for each treatment. Values with different letters are significantly different at $P < 0.05$.

Treatment	Mean DW(mg)
Positive control	40.92 *c*
Rhizobia mixture	19.18 *b*
Negative control	3.11 *a*

Table 2.5 Mean dry weights of all six white clover cultivars across all three treatments. Within column, values with the same letters are not significantly different at $P < 0.05$

Cultivar	Mean DW (mg)
Huia	25.17 *c*
Kopu II	23.91 *c*
Louisiana	22.53 *bc*
Tribute	20.97 *bc*
Crusader	18.47 *ab*
Irrigation	15.36 *a*

2.3.2.2 Nodule occupancy

Rhizobium leguminosarum strain 451 was the most common nodule occupant across all the six cultivars of white clover, occupying approximately 55% of the total nodules examined. Strain TA1 was the next most abundant occupying approximately 20% of all the nodules recovered (Table 2.6).

Based on the analysis presented in Table 2.3, strains were grouped, with shaded cells identifying strain/cultivar combinations that were classed as “highly effective”. Strains that were not ‘highly effective’ were termed ‘less effective’ strains. A test of equal proportions was conducted to assess whether the number of nodules occupied by strains were significantly different than that expected by chance (equal proportions) (Table 2.7). The test of equal proportions was conducted based on each strain occupying 16.66% ($1/6^{th}$) of the nodules on each plant.

Between matched pairs Huia and Tribute, Huia nodules contained more ‘highly effective’ strains than would occur by chance ($P < 0.001$). Out of the 47 total nodules recovered from Huia, strain 451 should have occupied only 8 nodules, however 21 more nodules contained strain 451 than expected by chance ($P < 0.001$).

Table 2.6 The number of nodules occupied by each strain of *Rhizobium leguminosarum* for each of the six cultivars of white clover. The strains from some nodules were unable to be recovered, therefore in some instances the total number of nodules is less than 50. Shaded cells identify the nodules containing the 'highly effective' strains for each cultivar as observed in Experiment 1.

Cultivar	**Number of *R leguminosarum* strain present**						**Total nodules**
	1302	**316**	**451**	**S12N10**	**S26N9**	**TA1**	
Huia	4	2	29	4	1	7	47
Tribute	5	2	23	6	1	13	50
Irrigation	4	1	18	5	1	7	36
Crusader	5	3	22	2	0	14	46
Louisiana	7	2	23	3	0	8	43
Kopu II	2	2	34	4	1	5	48
Total number of nodules across cultivars	27	12	149	24	4	54	270

Table 2.7 Difference in expected versus observed nodule occupancy for 'highly effective' or 'less effective' strains across cultivars. P values in this table are generated from a test of equal proportions. Cultivars Crusader and Kopu II were omitted from the table as no significant difference in their interaction with strains had been observed in experiment 1.

Cultivar	Highly Effective Strain(s)	P value	'less effective' Strains	P value
Huia	+21	P < 0.001	-21	P <0.001
Tribute	-3	P = 0.3348	+3	P 0.2587
Irrigation	+1	P = 0.7366	-1	P = 0.8661
Louisiana	+16	P <0.001	-13	P <0.001

2.3.3 Experiment 3 Colonization of white clover cultivars with different arbuscular mycorrhizae

2.3.3.1 Shoot dry weights

The different AMF treatments did not affect plant dry weight (*P*=0.698), however, there was a slight trend of differences in dry weight between cultivars (*P*=0.101). There was no significant interaction between treatments and cultivars (*P*=0.470).

2.3.3.2 AMF colonization

The presence or absence of mycorrhizal colonization was noted in 5 fields of view each from two random lateral roots near the bottom of the tap root under a compound microscope at 40× magnification. It is important to note that there was considerable fungal (possibly AMF or oomycete) contamination in the positive and negative control plants (Figure 2.4).

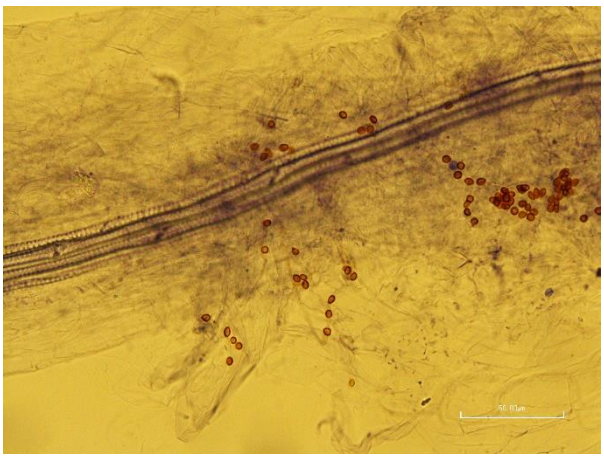

Figure 2.4 Example of the contamination seen in roots of a Crusader positive control plant. This type of contamination was seen throughout the experiment

A Tukey family-wise confidence interval (95%) showed no significant difference in AMF colonization between treatments for Huia, Tribute, or Irrigation (Table 2.8). Crusader showed significant differences in colonization between the five treatments (Figure 2.5), however in four of those differences, the control treatments have significantly more colonization than the inoculated treatment. Louisiana and Kopu II were the only matched pairs that showed significant differences in colonization (Figure 2.5).

Table 2.8 Family-wise Tukey Test *P* values for colonization differences between treatments for each of the six cultivars of white clover. Significant interactions in bold.

Treatment Pair	Huia	Tribute	Irrigation	Crusader	Louisiana	Kopu II
A. laevis - A. capsicula	0.35	0.99	0.88	**0.01**	0.42	**0.007**
F. geosporum – A. capsicula	0.99	0.99	0.97	0.97	0.89	0.97
Mixture – *A. capsicula*	1.00	1.00	0.99	0.99	**0.007**	0.99
Neg C – *A. capsicula*	0.99	0.87	0.97	**0.02**	0.10	0.98
Pos C – *A. capsicula*	1.00	1.00	0.52	**0.01**	0.19	0.36
F. geosporum – A .laevis	0.10	1.00	0.50	0.09	0.95	0.13
Mixture – *A. laevis*	0.47	0.96	0.70	**0.01**	0.43	**0.001**
Neg C – *A. laevis*	0.57	0.41	1.00	1.00	0.88	**0.001**
Pos C – *A. laevis*	0.28	0.99	0.99	1.00	0.98	0.69
Mixture – *F. geosporum*	0.94	0.99	1.00	0.99	0.08	0.89
Neg C – *F. geosporum*	0.93	0.47	0.74	0.10	0.46	0.73
Pos C – *F. geosporum*	1.00	1.00	0.18	0.08	0.70	0.89
Neg C - Mixture	1.00	0.85	0.85	**0.02**	0.99	0.99
Pos C – Mixture	0.99	0.99	0.39	**0.01**	0.91	0.18
Pos C – Neg C	0.99	0.59	0.99	0.99	0.99	0.13

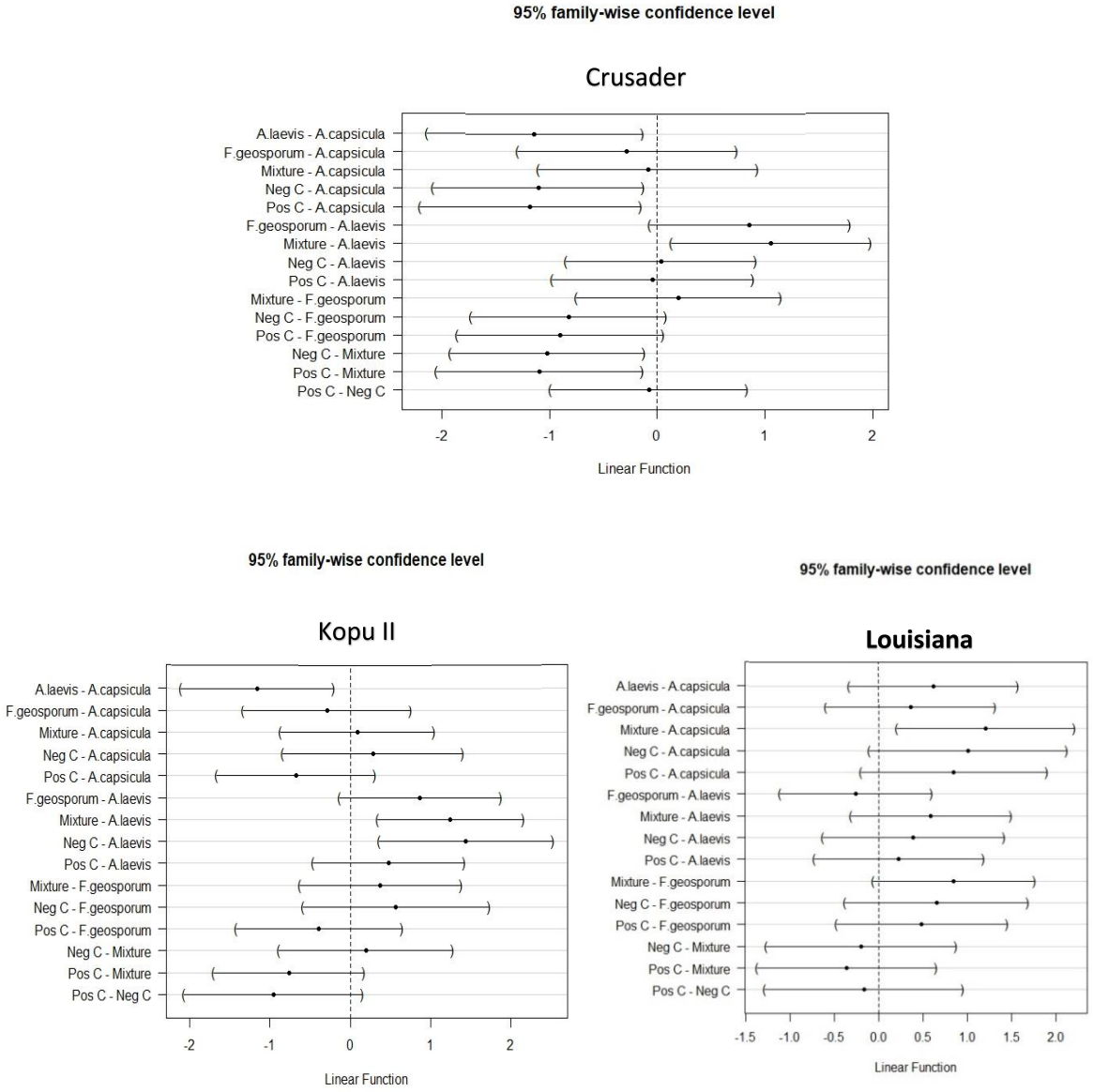

Figure 2.5 Difference between $\log_{10}$ of odds [p/(1-p)] where p=probability of success (colonization) for Crusader. Treatment differences which intersect with 0 are not significantly different. The relative proportion of colonization by the treatments in each pair is indicated by the length of the bar on the left or right side of 0. For example, in the first comparison for Crusader, *A. laevis* has significantly more colonization than *A. capsicula*.

2.4 Discussion

White clover is predominantly an outcrossing species and shows disomic inheritance. Therefore, populations of white clover are a heterogeneous mixture of heterozygous individuals. This results in high levels of genetic variation both within and between populations. This contributes to broad environmental adaption and phenotypic plasticity of white clover (Woodfield, 1996; Jahufer *et al.*, 2013). A study conducted by Jahufer, *et al*. (2013) found significant genotypic variation for a range of aboveground traits such as internode length, node number, stolon branching, and stolon thickness. To account for this variation, it is important to include high amounts of replication in experiments. The five replicates used in the experiments may not have been enough to obtain good statistical resolution, and could have been the reason for not seeing significant differences in the first benchmarking experiment. Alternatively, the opposite is also a possibility, where, the combination of the low replication and plant mortality, false positives were observed, where, with higher replication, some differences may not have been significant.

An issue that contributed to the lack of replicates was the low germination of seeds. Due to the age of the seeds, the germination percentage of the old cultivars was around 30%. These seeds were also expensive and difficult to obtain. This low germination rate made it difficult to obtain enough plants to increase the statistical resolution of the experiments. A suggestion for the future might be to plan a pilot experiment focusing on the germination rates of seeds of various white clover cultivars and adding seed germination as an added criteria for cultivar selection, or alternatively using newer seed.

The number of nodules formed by each strain when used as the sole inoculant on each of the six clover species varied. The best strain did not always produce the most nodules. For example, strain S12N10 formed the most nodules on Crusader and was the least effective strain in terms of dry weight. A similar result was reported by Sachs *et al.* (2010) who showed that when effective and ineffective bradyrhizobia were applied to *Lotus strigosis,* that the ineffective strain induced the most nodules per plant. The high number of nodules created by S12N10 and the minimal benefit to the plant host is indicative of a 'cheating' strain. A reason for the proliferation of S12N10 nodules on Crusader could be

that, because the host was growing in a low nitrogen environment survival relied on forming a symbiosis driving the initiation of nodules. Once the nodule had been formed the low N fixation by this strain may have induced the plant to initiate more nodules rather than allocate carbon to the ineffective symbiont. This is a hallmark of the sanction theory. In contrast, *R. leguminosarum* strain 451 formed the most nodules on Huia and was also the most effective strain for Huia. This finding supports the sanctions hypothesis (Kiers and Denison, 2008) which proposes that one of the ways mutualistic interactions are stabilized is when individuals preferentially reward more mutualistic (beneficial) behaviour (West *et al.*, 2002).

Some cultivars of white clover were effective with a greater number of strains, compared to others. For example, Huia was only effective with strain 451, however, Kopu II did not show any significant differences between its rhizobia treatments. Being effective with a wider range of strains is of benefit to the plant as the cultivar has a greater likelihood of forming an effective symbiosis when choice of partners is limited (Mpepereki *et al.*, 2000). However, in the case of Kopu II, there was no significant difference between the number of nodules from its least effective (S12N10) and most effective strain (1302) produced as sole inoculants. In contrast, the most effective strain for Huia nodulated significantly more than the least effective strain when applied as a sole inoculant. This would suggest that although Huia was less able to form effective symbioses with diverse strains, it can restrict nodulation by poor contributors. A reason this contrast is interesting is because a study conducted by Jahufer, *et al.* (2003) analyzing the genetic diversity and variation among 32 white clover cultivars using microsatellite (SSR) markers showed that Kopu II had a strong Huia contribution to its pedigree. A dendrogram based on cluster analysis of cultivars by SSR marker matrix, had Huia and Kopu II in adjacent groups (Jahufer *et al.*, 2003). The study also showed that Huia was one of the cultivars with the most unique microsatellite alleles (4). SSR variations in protein-coding regions can lead to a gain or loss of gene function via several means such as frameshift mutations (Li *et al.*, 2004). These unique alleles could have a role to play in the contrast seen between the two cultivars in the variety of effective partners and ability to sanction ineffective partners. There may also be other SSRs in other regions that could lead to this difference in ability to express partner choice. A possible draw back to having multiple

effective partners could be a more relaxed defence against ineffective symbioses as could have been the case with Kopu II.

Experiment one also showed different strain/cultivar combinations were more effective than others. A study conducted by Burdon, *et al.* (1999) on the variation in effectiveness of native rhizobia and species of temperate Australian Acacia showed similar results. All rhizobial isolates showed a capacity to induce nodulation on the host plants, however, the symbiotic effectiveness of the resulting relationship was variable between half-sib families. Bacterium-legume specificity was also demonstrated between *Rhizobium meliloti* and *Medicago sativa* L. genotypes and this was a significant component of the variability in plant yield (Chanway *et al.*, 1991). This could indicate that the symbiosis between rhizobia and their hosts is one in which genetic differences that can impact the mutualism may occur due to the host and the bacterium (Parker, 1995; Burdon *et al.*, 1999). The genotypic variability among the six cultivars and genetic variation among the strains chosen could be a major driver in the variation of effectiveness seen in different clover/rhizobia combinations.

When the six strains of rhizobia were inoculated as a mixture to the six cultivars, strain 451 outcompeted all the six strains being the most dominant nodule occupant across all the six white clover cultivars. Out of the 270 nodules genotyped, strain 451 occupied 149 nodules, and commercial standard TA1 was the next more prominent, occupying 54 nodules. A reason for strain 451's competitive edge could be its ability to initiate infection and nodulate quickly, and efficiently, providing it an advantage over strains that are slower. Initial nodules are known to apically suppress further nodulation in legumes (Kosslak *et al.*, 1983; Dowling and Broughton, 1986). A study conducted by Kosslak, *et al.* (1983) on the effects of pre-exposure of soybean roots to *Rhizobium japonicum* strains found that the delayed inoculation of a second strain influenced competition. When less competitive strains were inoculated before competitive strains, the proportion of nodules occupied by the less competitive was increased. Some rhizobia are also known to produce peptide antibiotics such as trifolitoxin which has resulted in significantly increased nodule occupancy values in non-sterile soil (Robleto *et al.*, 1998). There is a benefit for the strain which can nodulate first, and perhaps the success of strain 451 to nodulate can be attributed to its ability to beat its competitors for initial nodulation.

Further analysis would need to be conducted on strain 451 to see if it exploited the production of biochemical compounds as a tool to overcome competitor strains.

The competitive ability of strain 451 was highlighted by the fact that it could dominate nodule occupancy across all cultivars even when it was labelled as an "less effective" strain for a particular cultivar from Experiment 1. "Cheater" strains, as defined by Kiers and Denison (2008) are strains that are highly competitive for nodulation and abundant inside nodules but provide little or no benefit to plants. However, experiment 1 also revealed that 451 was an effective strain for cultivars e.g. Huia. Therefore, it is possible for a strain to act both as an effective mutualist and a "cheater" depending on the host plant it is inhabiting. This shows that some strains of rhizobia could have a very host specific interaction at the plant cultivar level. A study conducted by Lewis-Henderson and Djordjevic (1991) found that a *nod* gene, *nodM*, is involved in restricting the host range in a cultivar specific manner for *R. leguminosarum* strain TA1. There was also a negatively acting cultivar specificity locus *csn-1* which was identified in the study. The action of these two gene products among others could be responsible in modifying the chemical and molecular signals exuded by strain TA1 to generate an incompatible response with subterranean clover cultivar Woogenellup (Lewis-Henderson and Djordjevic, 1991). Although the expression of these genes stops TA1 from nodulating cultivar Woogenellup, there could be genetic triggers responsible for the extreme cultivar specific efficacy of strain 451, which may help dictate its role as an excellent mutualist or as a "cheater" strain.

Cultivar age may have an impact on the ability of a white clover cultivar to select for, and discriminate against, effective and ineffective strains of rhizobia. Among the three matched pairs of white clover cultivars, a difference in strain selection was seen between Huia and Tribute (Table 2.8). When presented with a strain mixture inoculant, Huia (an older cultivar) had 21 more "best performing" strains occupying nodules than expected by chance (P <0.001), whereas, its match pair Tribute did not have significantly more "best performing" strains occupying its nodules (P=0.335). This result indicates that clover plants may be able to display a degree of partner choice. A study on *Medicargo Trancatual* and two strains of *Sinorhizobium meliloti* displaying different nitrogen fixation phenotypes, showed in a split root experiment that pre-infection partner choice was

displayed by the host plant. The side of the root associating with the N fixing strain, formed a greater number of nodules than the non-fixing strain (Gubry-Rangin *et al.*, 2010). A study conducted on soybean by Kiers, *et al.* (2007) showed that older cultivars were more competent when infected with a mixture of effective and ineffective rhizobia. Older cultivar produced more seeds in such environments, suggesting their benefit could be a result of partner choice and sanctions against "cheater" strains. This suggested that when infected by symbionts varying in quality, legume defences against poor-quality partners had worsened under artificial selection. Among these three old and new pairs of clover cultivars, the comparison of Huia and Tribute support this hypothesis.

Difference in nodule occupancy by strains in experiments one and two were most likely due to the interaction between host and plant. However, differences in cell numbers added to the pottle system could also have created some variation. Cell numbers were calculated using the method of Weir (2006) which measures optical density (OD) at 600 nm, with OD_{600} of 0.167 equivalent to 1×10^8 cells per mL. Previous work in the Lincoln University "rhizobia group" has shown that these OD_{600} readings are relatively accurate (Ridgway pers.comm) and plating of strains during the experiments confirmed this. During the course of the first two experiments fungal growth/contamination was seen on positive control plants. This was only found in the positive control plants and did not seem to affect their growth. This could have been due to the added nitrogen. Contamination must have been air borne as the plants were always watered with sterile water. The air conditioning unit in the growth room in which the plants were grown may be in need for a clean. Growing the tubes in a concealed growth chamber next time may help in reducing chances for contamination. A growth chamber would also decrease the amount of foot traffic of other people moving around the growth room where soil based experiments were also being run simultaneously.

In the third experiment, whenever the mixture treatment was significantly different from another treatment in regards to AMF colonization, the mixture treatment always had a lower amount of colonization. A study conducted by Engelmoer, Behm *et al.* (2014) using *in vitro* root organ cultures and two closely related AMF species *Rhizophagus irregularis* and *Glomus aggregatum*, showed that competitive interactions between AMF species reduced the overall fungal abundance. Competition was most intense for resources

within the host, where both species negatively affected each other's abundance. This could be a reason why the only significant difference seen with the mixture treatment was where there was significantly less colonization. In some cases the colonization in the control plants was more than the treatment. This could possibly indicate there was only one major fungal contaminant present in the uninoculated controls and therefore did not need to compete with other microorganisms.

There was a large amount of contaminant non-septate hyphae observed in the roots of plants in experiment three, with no significant difference in colonization between the positive and negative control plants compared to all the inoculated treatments. The non septate hyphae is likely to be oomycete. Oomycetes are similar to fungi in growth and morphology, however, they always have nonseptate hyphae and are common pathogens of seedlings (Fawke *et al.*, 2015). Oomycete sporangia release swimming zoospores as a means of dispersal through water (Birch *et al.*, 2012). This water borne dispersal could have been an avenue for infection for this experiment. The large amount of McKnights solution required for the experiment was made in buckets which were wiped with ethanol, and produced using sterile chemicals. However, non autoclaved Millipore water was used, and also non sterlised Millipore water was used for watering. It was assumed that the filters in the Millipore system would exclude oomycetes, however, once the solutions were made up, they were also left in the buckets overnight, providing an opportunity for contamination to occur.

Another assumption that was made is that the cups which were bought came sterile from the manufacturer. No extra steps such as exposing to UV or soaking in bleach was performed to ensure complete sterility, which could have helped minimize contamination. The plants were also grown in a growth room which had soil experiments running simultaneously and did not have a source of ventilation and high traffic areas around the pots when people would be watering or checking other experiments.

Due to the heavy extent of the contamination seen in the positive and negative controls, the results of this experiment cannot be analysed with much integrity. A reason for such obvious contamination in the trial could be that AMF are known to have little, or no, host specificity (Zhu *et al.*, 2000). Less specificity means it is a lot easier for a microorganism to infect and affect the growth of a plant. Due to the clear presence of fungal

contamination, the results seen could be heavily skewed by the invading organisms. A way to safeguard for this and improve the resolution of the results to better understand what is affecting the plants would be to incorporate molecular identification once the experiment is over to make sure exactly what was infecting the plant roots.

2.5 Conclusions

In summary, Experiment 1 identified highly effective rhizobial strain-host combinations. When applied in mixtures some rhizobia strains were found in greater numbers than would occur by chance demonstrating that for some cultivar/ strain combinations a selection process for nodule entry occurred. However, this was not observed in all cultivars suggesting that cultivar type may have been a driver for rhizobia selection *in-vitro*. For example, the paired cultivars of Huia and Tribute were different in their ability to select highly effective partners, with Huia better able to do this than Tribute. Experiment three was inconclusive due to the large amount of oomycete contamination.

3 The rhizosphere and root microbiome of *Trifolium repens*

3.1 Introduction

Agricultural soils are rich in microbial communities with bacterial populations varying from 10^2 to 10^{11} per gram of soil. The rhizosphere is the thin (3-5 mm) zone of soil near the roots, which is home to an extremely high number of microorganisms, and is considered to be one of the most dynamic interfaces on earth (Philippot *et al.*, 2013). However, only a small subset of this microbial community is successful in colonizing plant roots (Hartmann *et al.*, 2009; Gaiero *et al.*, 2013). Endophytic and rhizospheric microbes can benefit plants in many ways, including improving nutrient supply, protecting against pathogens, helping to withstand high soil temperature, and producing phytohormones that could benefit the host (Kiers and Denison, 2008). Regulation of the community composition of bacteria colonizing roots can be strictly controlled by the plant. The ability of plants to discriminate among partners based on symbiotic performance has been observed for the legume-rhizobia interaction and thus may exist in more rhizosphere mutualisms, and root endophytism (Kiers and Denison, 2008).

As many organisms in the rhizosphere and roots of plants are not culturable outside their host (Hill *et al.*, 2000), the study of these communities is most effectively conducted using molecular tools. For several years, the most common approach has been denaturing gradient gel electrophoresis (DGGE). In this process, nested PCR with taxa specific primers containing a GC clamp are arrayed on a denaturing gel. This technique has been used to characterize the microbial community structure of several legumes (Sharma *et al.*, 2005). A study conducted by Sharma *et al.* (2005) using DGGE found distinct profiles between the rhizosphere bacterial communities of faba beans, peas, and white lupin. More recently DGGE has been superseded by next generation sequencing (NGS) platforms, which provides a deeper resolution by detecting more species with greater accuracy than DGGE (Yu *et al.*, 2015; Qin *et al.*, 2016). However due to low cost, expected low diversity and the lack of suitable AMF specific primers, DGGE is still used as a molecular tool to provide an overview of AMF community structure. A study conducted by Wigley *et al.* (2017) used Illumina sequencing to investigate the microbiome inside lucerne nodules.

Their study found that the genus *Sinorhizobium* was the dominant genus in nodules, comprising 90-99% of all sequences, but also showed that other taxa were present.

Results from the previous chapter show there are differences between some old and new cultivar pairs in the efficacy of rhizobial partners chosen. Therefore, there may also be some differences in the wider microbial community recruited by the cultivars to the rhizosphere and roots. The objectives of this chapter were to, (i) investigate differences in the microbial communities recruited into the rhizosphere and roots of old and new cultivars of white clover when grown in the same soil; (ii) examine the AMF community structure of the different cultivars of white clover; (iii) investigate the symbiotic potential of the strains of rhizobia recruited by cultivar pair Huia and Tribute grown in the same soil.

3.2 Materials and methods

3.2.1 Cultivars of *Trifolium repens*

Six cultivars of *Trifolium repens* were used as described in Section 2.2.1.

3.2.2 Seed preparation

Seeds were prepared for sowing as described in Section 2.2.2.

3.2.3 Experiment 4

3.2.3.1 Soil

White clover cultivars were grown in soil obtained from Lincoln University paddock H18 (Coordinates -43.648762, 172.461245). Soil test results are in Appendix 7.1. The soil was collected from the west side of the paddock (Figure 3.1) using a spade. The top layer of grass was removed and only ~30 cm of the top soil was collected into plastic bags. After collecting the soil was passed through a 20 mm sieve to remove stones and plant debris and stored in a large plastic container for one week before use.

Figure 3.1 Aerial view of paddock H18 (highlighted in red) where soil was collected from. Image obtained from google maps satellite on 15/08/2017**.**

3.2.3.2 Experimental Design

White clover seedlings that had been germinated on water agar as described in Section 2.2.2 were sown into 500 mL black square plastic pots (Interworld Plastics). Pots were filled to the top with the sieved soil, and one seedling was planted in the center of the pot. Seedlings were healthy and of as consistent size as possible. Each cultivar was replicated 10 times. Ten randomized blocks were set up, each consisting of six pots.

The plants were left to grow for 12 weeks (November 16th 2016 till February 1st 2017) in the Lincoln University shade house and were watered with tap water when necessary.

3.2.3.3 Harvest

3.2.3.3.1 Shoot dry weights

Once the plants had grown for twelve weeks. Each plant was carefully removed to ensure no soil adhered to the shoots. The shoots were cut at the crown, placed in labelled paper bags, dried in the oven at 65°C for 72 h and weighed to obtain shoot dry weights.

3.2.3.3.2 Rhizosphere soil collection

Roots were carefully removed from the pots and gently shaken to remove loosely adhering soil, any soil that remained attached to the root was termed rhizosphere soil.

Each root was placed in a sterile 50 mL plastic tube which was filled to 35 mL with sterile Millipore water. Tubes were vigorously shaken for 2 min to wash the rhizosphere soil from the roots. The roots were then removed, rinsed under tap water and set aside for further use. The tubes were centrifuged for 5 min at 805 x g and the supernatant discarded. The tubes containing the rhizosphere soil were stored at -80°C until needed for DNA extraction.

3.2.3.3.3 Root harvest and fresh weight

After rhizosphere soil collection, the roots were washed under tap water for approximately 5 s, blotted dry with a paper towel and weighed (fresh weight). Three plants were randomly selected from each cultivar and two nodules were cut at random from each root. Random plants and nodules per plant were selected by assigning each a number and then using a random number generator (www.randomizer.org) to identify plants/nodules for sampling. Nodules were surface sterilized and plated onto YMA as described in Section 2.2.4.4.2.

A piece of lateral root (approximately 5-10 cm) was placed in a sterile 1.7 mL plastic tube filled with 20% glycerol and stored at -80°C until use.

3.2.3.4 PMA cross linking

Root pieces were surface sterilized as described in Section 2.2.4.4.2. Root tissue was then treated with propidium monoazide (PMA) before DNA extraction. PMA is a membrane-impermeable dye that selectively penetrates cells with damaged membranes. Once PMA penetrates the cell it intercalates DNA upon exposure to intense visible light. This results in the DNA from dead cells not being amplified by PCR (Nocker *et al.*, 2007). PMA treatment after surface sterilization but prior to DNA extraction ensured that amplification was mainly of DNA from viable fungal and bacterial endophytes.

For PMA treatment the sterile root pieces were soaked in 500 µL sterile water using transparent 1.7 mL tubes (Axygen, USA) and 1.25 µL 20 mM PMA (Biotium Inc., USA) added. The samples were incubated in darkness for 5 min and then exposed to a 650-W halogen light for 5 min. Samples were placed on ice to avoid overheating from the lamp. The tubes were turned every 4 min to ensure even light exposure to all parts of the root.

3.2.3.5 DNA extraction

Root DNA was extracted using the PowerPlant™ DNA isolation kit (MOBIO laboratories, USA) following the manufacturer's instructions. One gram of root tissue was crushed and ground to a fine powder with liquid nitrogen, of which up to 50 mg of was used for DNA extraction. The DNA was stored at -20˚C.

Approximately 0.25 g of rhizosphere soil was used for DNA extraction. DNA was extracted using the Qiagen DNeasy ® Powersoil ® DNA extraction kit as per the manufacturer's instructions. Extracted DNA was stored at -20˚C.

3.2.3.6 Investigation of AMF community structure using DGGE

Extracted rhizosphere DNA from Section 3.2.4.5 was stored at -20°C for PCR-DGGE analysis. To investigate the genetic diversity of AMF, 18S rRNA fragments of AMF were amplified using a nested PCR strategy. In the first PCR reaction used primers AML1/AML3, the 1 µL of PCR products from this reaction were used as a template for the second reaction using primers NS31-GC/GLO1 (Higo *et al.*, 2015).

DGGE was performed with a Cipher DGGE Electrophoresis system (CBS Scientific). The gel was prepared in the following way: Gel bond (SERVA, Germany) was placed on the DGGE glass plates before pouring the gradient gel. A linear gradient gel from 30% to 45% of denaturants was poured using 8% (w/v) polyacrylamide gel (acrylamide/bis solution, 37.5:1). Each gradient solution contained 0.0012% (v/v) tetramethylethylenediamine (TEMED) and 0.07% (w/v) ammonium persulfate (APS) (Biorad, New Zealand) as catalysts for polymerization of acrylamide and bis-acrylamide. After 30-40 min of gradient gel polymerization, approximately 2-3 mL of stacking gel solution of 0% denaturant polyacrylamide gel (acrylamide/bis solution, 37.5:1), 0.002% TEMED and 0.1% APS were poured onto gradient gel and the DGGE gel comb inserted. The prepared gel was placed in the Cipher DGGE Electrophoresis system tank containing 0.5 x TAE.

Ten µL of PCR product was mixed with 10 µL of loading dye (0.5% bromophenol blue, 0.5% xylene cyanol and 70% glycerol in ddH_20) and loaded into a single well on the gradient gel. The gels were run in 0.5 x TAE buffer for 16 h at 90 V and 60˚C. After the running process had finished, the glass plates were removed and the gels were soaked in 200-250 mL of fixative solution (40% ethanol, 2% acetic acid in water) for 3 min. The gels

were stained using 200-250 mL of silver stain solution (0.1% (w/v) silver nitrate) for 10 min. The silver stain was decanted and the gels were rinsed using Millipore water for 2 min. Gels were developed with 200-250 mL of developer solution (3% (w/v) sodium hydroxide and 0.01% (v/v) formaldehyde solution in Millipore water) for 30-40 min. After developing the gels were soaked in 200-250 mL of Cairns' preservation solution (25% ethanol, 10% glycerol in water) and subsequently placed in a drying oven at 65˚C for 24 h. The dried gel was scanned and the converted file (.jpeg) used for further analysis of the microbial communities.

3.2.3.7 Investigation of root endomicrobiome and rhizospheric microbial community using Illumina Hiseq metabarcoding

The taxonomic composition of the root microbiome (both internal and rhizosphere) was produced by amplicon sequencing of total bacteria libraries using the Illumina HiSeq platform by Novogene, Beijing, China.

The hypervariable V3-V4 region (expected size approximately 300 bp) of the 16S rRNA gene from the rhizosphere and root bacterial endophytes was amplified according to the protocol described by Klindworth *et al.* (2012) using primers F341 (5'- CCT AYG GGR BGC ASC AG-3') and R806 (5'- GGA CTA CNN GGG TAT CTA AT-3'). These primers had been extended to include a unique barcode and Illumina cell flow adaptors (Appendix 7.2.3). Details of unique barcode combinations are in Appendix 7.2.4. The PCR mixture contained 12.5 µL 2 x KAPA HiFi HotStart ReadyMix (Kapa Biosystem, South Africa), 5 µL each of forward and reverse primer (1 mM), and 2.5 µL of genomic DNA (5 ng/ µL). The following PCR conditions were used: initial denaturation at 95˚C for 3 min, followed by 35 cycles consisting of denaturation (98˚C for 20 s), annealing (55˚C for 15 s), and extension (72˚C for 40 s) and a final extension step at 72°C for 1 min.

PCR products (5 µL) were separated by electrophoresis on a 1% w/v agarose gel in 1 x TAE buffer (40 mM Tris acetate, 2 mM Na_2EDTA, pH 8.5) at 10 V/cm for 45 min alongside a 1kb+ DNA ladder (Invitrogen). Gels were stained with ethidium bromide (0.5 µg per mL 1 x TAE) for 15 min, rinsed in RO water and photographed under UV light in a FireReader™ (UVITEC) to verify the PCR had worked. The root samples produced a single band, and were purified using a PCR clean up kit from Qiagen (MOBIO laboratories, USA) according to the manufacturer's instructions. The soil rhizosphere samples displayed multiple bands

on the gel. The band of the desired size range (440-480 Bp) was excised using a sterile scalpel on a UV gel box, placed in a sterile 1.7 ml plastic tube and the PCR product recovered using a Zymoclean™ Gel DNA Recovery Kit (Zymo-Research, USA).

A fungal 18s library was also prepared for sequencing (Appendix 7.2.6), however, not enough data was generated from the sequencing and thus was excluded from further analysis.

The DNA concentration of the purified PCR products was measured using the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Based on these concentrations the products were pooled into two tubes (tube one was all 16S samples, and tube two was the ITS2 samples) based on their relative concentrations to make a final 1.5 μg of DNA in each tube as specified by the sequencing company (Novogene, Beijing, China).

3.2.3.8 Statistical Analysis

The cultivar differences in plant dry weight, were subjected to analysis of variance (ANOVA) using GenStat 16th version (VSN International, Hemel Hempstead, UK).

The raw 16S sequencing data was analysed with assistance from Aurelie Laugraud (Bioinformatician, AgResearch). Paired-end reads were assembled using Flash v1.2.11 and unpaired sequences were discarded (Magoč and Salzberg, 2011). The sequences were trimmed and separated according to their barcodes using Flexbar v2.4 (Dodt *et al.*, 2012). Reads were clustered to operational taxonomy units (OTUs) on the open source software package QIIME 1.8.0 (http://qiime.org) followed by taxonomic assignment of representative sequences with a 80% threshold based on the reference database Greengenes release gg_13_8_99 (DeSantis *et al.*, 2006). All reads assigned to chloroplasts and mitochondria were removed prior to further analysis. The OTU table generated was rarified to the sample with the lowest number of read counts by randomly selecting subsets of sequences using a script provided as part of the QIIME 1.8.0 software (Caporaso *et al.*, 2010). Reads assigned as chloroplasts, mitochondria, and "unassigned" were removed in QIIME, and low abundance reads (<10) and reads present <0.01% were removed using USEARCH V1.8. To calculate the alpha diversity, bacterial richness was estimated using the number of observed OTUs and the bacterial diversity was

determined using the Simpsons Index in USEARCH V8.1 in QIIME 1.8.0 open source software package. The taxonomy bar charts were generated and visualized using R studio version 1.0.143©. Beta diversity was assessed by performing principal coordinate analysis based on calculating the Bray Curtis distance matrix using Primer 7 software (Primer-E Ltd, Plymouth Marine Laboratory, UK).

Analysis of the AMF communities was performed using Phoretix 1D Pro Gel Analysis (Totallab, UK). A matrix based on the presence/absence of bands generated from Phoretix binary data was analyzed using Primer version 7 (Primer-E Ltd, Plymouth Marine Laboratory, UK) multivariate software package. Resemblance matrices for community profiles were built by calculating similarities between each pair of samples using Jaccard ordination were generated to interpret multivariate distance between samples and factors. PERMANOVA tests were used to test the statistical difference between AMF communities between samples. The number of bands per lane was used as a diversity indicator of AMF taxa richness. AMF richness was analysed with a general linear model (GLM) to determine the significance of factors and followed by Fisher's ad-hoc analysis.

3.2.4 Experiment 5 – Symbiotic potential of rhizobia strains isolated from the nodules of Huia and Tribute, from Experiment 4.

3.2.4.1 Preparation of seedlings

Seedlings of cultivars Huia and Tribute were germinated on water agar as described in Section 2.2.2. Seedlings were placed in sterile 50 mL plastic tubes as described in Section 2.2.4.1. Only Huia and Tribute seeds were used in this experiment in order to achieve high replication (15) with the amount of remaining seed available.

3.2.4.2 Inoculation of *Trifolium repens* cultivars

Six nodules were plated from each of the two cultivars (2 nodules from each of 3 replicate plants) and the symbiotic potential of each of the 12 strains were tested individually, along with a positive and negative control treatment. These comprised the 14 treatments.

The nodules from plants grown in field soil were surface sterilized and plated as described in Section 2.2.4.4.2. Resultant colonies were sub cultured three times to ensure purity and single colonies were selected for re-inoculation and assessing symbiotic potential.

Inoculum from each of the 12 strains was prepared as described in Section 2.2.3.

The tubes were prepared as described in Section 2.2.4.1 and the tube inoculated as described in Section 2.2.4.2.

Once the seedlings had been sown, they were grown for six weeks in conditions described in Section 2.2.4.2.

3.2.4.3 Experimental design

Fifteen randomized blocks were set up, each block containing 28 tubes, one tube per treatment (12 inoculated, positive control, and negative control), per cultivar. The tubes were randomly placed in two white plastic racks. Plants were watered to weight with sterile Millipore water every two days.

3.2.4.4 Assessment

3.2.4.4.1 Shoot dry weights

Once the plants had grown for six weeks, the shoots were harvested as described in Section 2.2.4.4.1.

3.2.4.4.2 Statistical analysis

The effects of the strains on plant dry weight for each treatment were subjected to ANOVA using GenStat 16th version (VSN International, Hemel Hempstead, UK). Treatment differences were compared using Fisher's Protected LSD at 0.05.

3.3 Results

3.3.1 Experiment 4 shoot DW and root FW

There was no significant difference in shoot dry weight (*P* =0.386), or root fresh weight (P =0.987) between the six clover cultivars (Table 3.1).

Table 3.1 Mean shoot dry weight (mg), and root fresh weight (mg) for each of six white clover cultivars grown in the same soil for 12 weeks.

Cultivar	Mean Shoot DW (mg)	Mean Root FW (mg)
Huia	263	623
Tribute	201	546
Irrigation	315	623
Crusader	237	630
Louisiana	323	659
Kopu II	206	617

3.3.1.1 Root and rhizosphere bacterial community structure using Illumina HiSeq metabarcoding

A total of 2,002,903 reads were generated after initial quality filtering and excluding unmatched paired end reads. Chloroplast, mitochondria and “unassigned” reads were excluded from the OTU table before further analysis and these accounted for 16% of the total reads. A total of 1,689,775 reads remained and these were divided among a total of 88,211 OTUs. The total number of reads per cultivar, roots and rhizosphere are presented in Table 3.2. An average of 172,736, and 108,878 reads were obtained from the root (min=8051, max=145893), and rhizosphere (min=15051, max=60503), respectively.

Bacterial community richness was much higher in the rhizosphere than inside plant roots (Table 3.3). The bacterial community structure was significantly different in the roots and the rhizosphere (PERMANOVA, *P*=0.001), forming two distinct clusters (Figure 3.2). Therefore, the data was split into these two groups for further analysis. Low abundance OTUs were classified as those with less than 10 reads (Yeoh *et al.*, 2017) and were discarded from analysis.

Table 3.2 Number of sequences per cultivar and replicate plant from root (a) and rhizosphere (b) samples generated by Illumina Hiseq.

(a)

	Cultivar					
Plant Root	**Huia**	**Tribute**	**Irrigation**	**Crusader**	**Louisiana**	**Kopu II**
1	116522	85253	47865	54422	66807	69630
2	31459	145893	22590	50826	8051	32227
3	89559	48067	22980	37353	37174	69828
Total	237540	279123	93435	142601	112032	171685

(b)

	Cultivar					
Plant Rhizosphere	**Huia**	**Tribute**	**Irrigation**	**Crusader**	**Louisiana**	**Kopu II**
1	48182	60503	31521	31875	31296	48811
2	23802	48764	15868	29264	15051	43904
3	45015	53424	27188	26265	28205	44331
Total	116999	162691	74577	87404	74552	137046

Table 3.3 The average richness and Simpson index for non-rarefied root (a) and rhizosphere (b) OTUs of the six white clover cultivars. Richness is the number of OTUs observed. Simpson index shows probability that two randomly selected reads will belong to the same OTU. Values close to 1 indicate that a single large OTU dominates the sample, small values indicate that the reads are distributed over many OTUs and hence, higher diversity.

(a) Plant Root	Cultivar					
	Huia	**Tribute**	**Irrigation**	**Crusader**	**Louisiana**	**Kopu II**
Richness	289	301	157	249	201	386
Simpson's index	0.461	0.487	0.485	0.465	0.478	0.457

(a) Plant Rhizosphere	Cultivar					
	Huia	**Tribute**	**Irrigation**	**Crusader**	**Louisiana**	**Kopu II**
Richness	4242	4721	3564	3850	3498	4414
Simpson's index	0.005	0.005	0.005	0.008	0.006	0.006

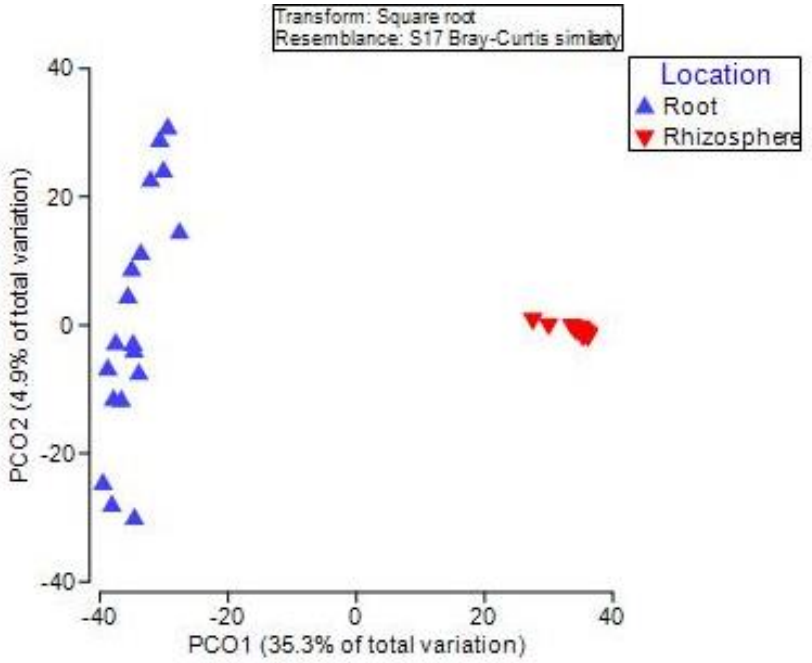

Figure 3.2 Principal coordinate plot showing the difference between root and rhizosphere bacterial communities for six cultivars of white clover after removal of chloroplast, mitochondria, and "unassigned" reads.

3.3.1.2 Root endomicrobiome analysis

Greenegene id Denovo64224 (*Rhizobium leguminosarum*), and Denovo33832 (genus *Rhizobium*) were dominant OTUs in the roots and when combined they accounted for 97.9% of the total root reads (502877 and 483648 reads, respectively). These OTUs were likely to have originated mainly from nodules included in the DNA extraction process and were common to all cultivars. Thus, they were excluded from further analysis to allow a focus on the composition of other bacterial endophytes within the roots of the different cultivars. Low abundance reads, those which occurred at ≤0.1% were also removed (Yeoh *et al.*, 2017), leaving a total of 18842 reads, and 137 OTUs. Differences in phylum composition between each of the cultivars and the replicate plants is shown in Figure 3.3. Proteobacteria were the dominant phyla (51-96.3%) in 17 out of the 18 plants. Plant 1 of cultivar Crusader differed from the other plant samples with the phyla Bacteroidetes being most abundant (75%) (all of which belonged to the genus *Chitinophaga*).

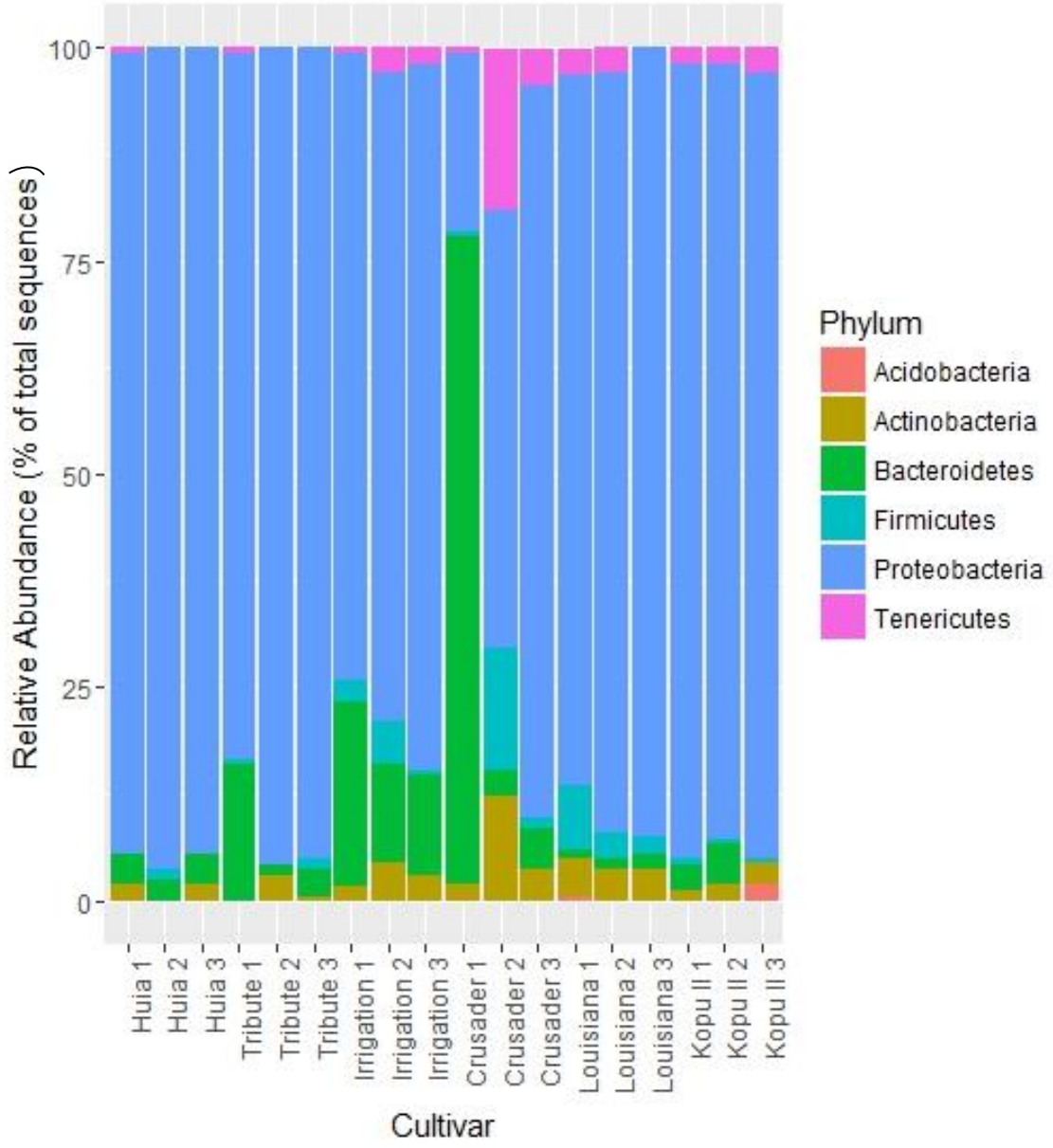

Figure 3.3 Community structure of root endophytic bacteria in all samples of white clover show by metabarcoding with Illumina HiSeq 16S rRNA amplicon sequencing at phylum level.

Plant cultivar was the major driver of the root endomicrobiome (PERMANOVA *P*=0.001 (Figure 3.4). When grouped according to their morphology (matched pairs), the root endophytic bacterial communities between the three groups was significantly different (PERMANOVA *P*=0.001). However, there were no significant differences within matched pairs, Huia/Tribute, Irrigation/Crusader, and Louisiana/Kopu II (PERMANOVA *P*=0.214, *P*=0.292, and *P*=0.098, respectively). Although there was a trend (*P*=0.098) for a difference between Louisiana/Kopu II.

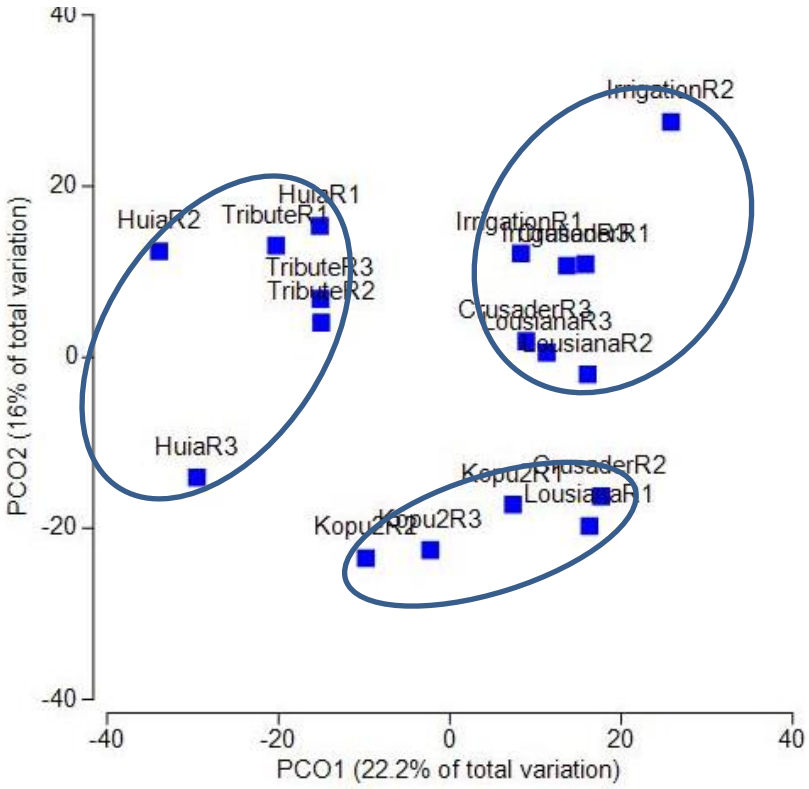

Figure 3.4 Principal coordinate plot showing rough groupings of the white clover cultivars. Bacterial read numbers were rarefied to 162 (as the smallest number of reads). Three circles outline the groupings.

Bacterial read numbers were rarefied to the smallest number of reads for each of the three pairs of matched clover cultivars and differences the root bacterial communities at the phylum (Figure 3.5) and class (Figure 3.6) levels determined. The bacterial phylum Proteobacteria was the most abundant in all three groups (67.4 - 91.8%). Less abundant

phyla were the Actinobacteria (2.3 - 3.1%), Firmicutes (0.4 - 4.2%), and Acidobacteria (0-0.3%). At the class level, Alphaproteobacteria were the most abundant in the Irrigation/Crusader and Louisiana/Kopu II matched pairs, ranging from (42.3-52.6%). The most abundant class for the Huia/Tribute pair was Gammaproteobacteria (32.8%). The second most abundant class was different for each of the three matched pairs, being Alphaproteobacteria for Huia/Tribute (32.4%), Saprospirae for Irrigation/Crusader (21.2%) and Betaproteobacteria for Louisiana/Kopu II (38.5%).

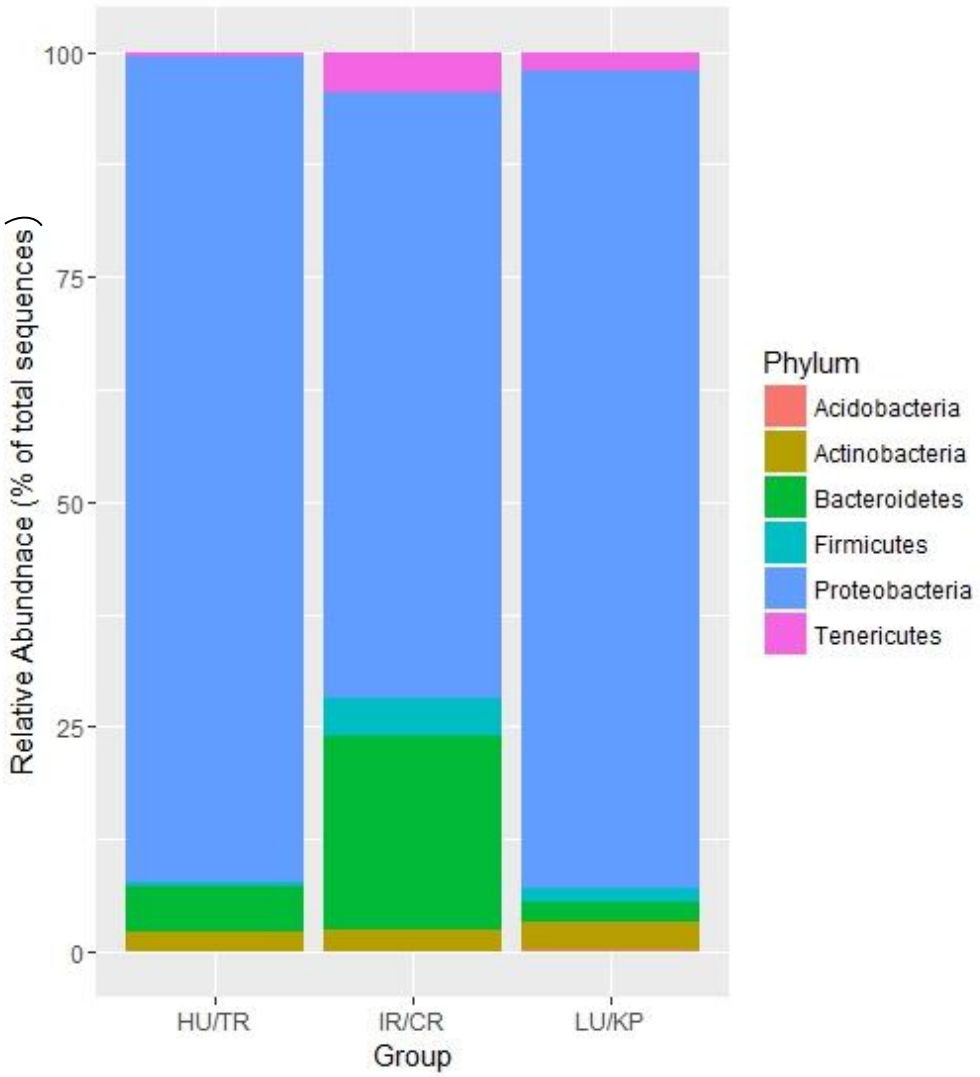

Figure 3.5 Community structure of endophytic bacteria in the roots of the three different paired groups of white clover as shown by metabarcoding with Illumina HiSeq of the 16S rRNA amplicon at the phylum level. Bacterial reads were rarified to 696, 162, and 311 as the smallest number of reads for each of the three groups (Huia/Tribute [HU/TR], Irrigation/Crusader[(IR/CR], Louisiana/Kopu II [(LU/KP]), respectively.

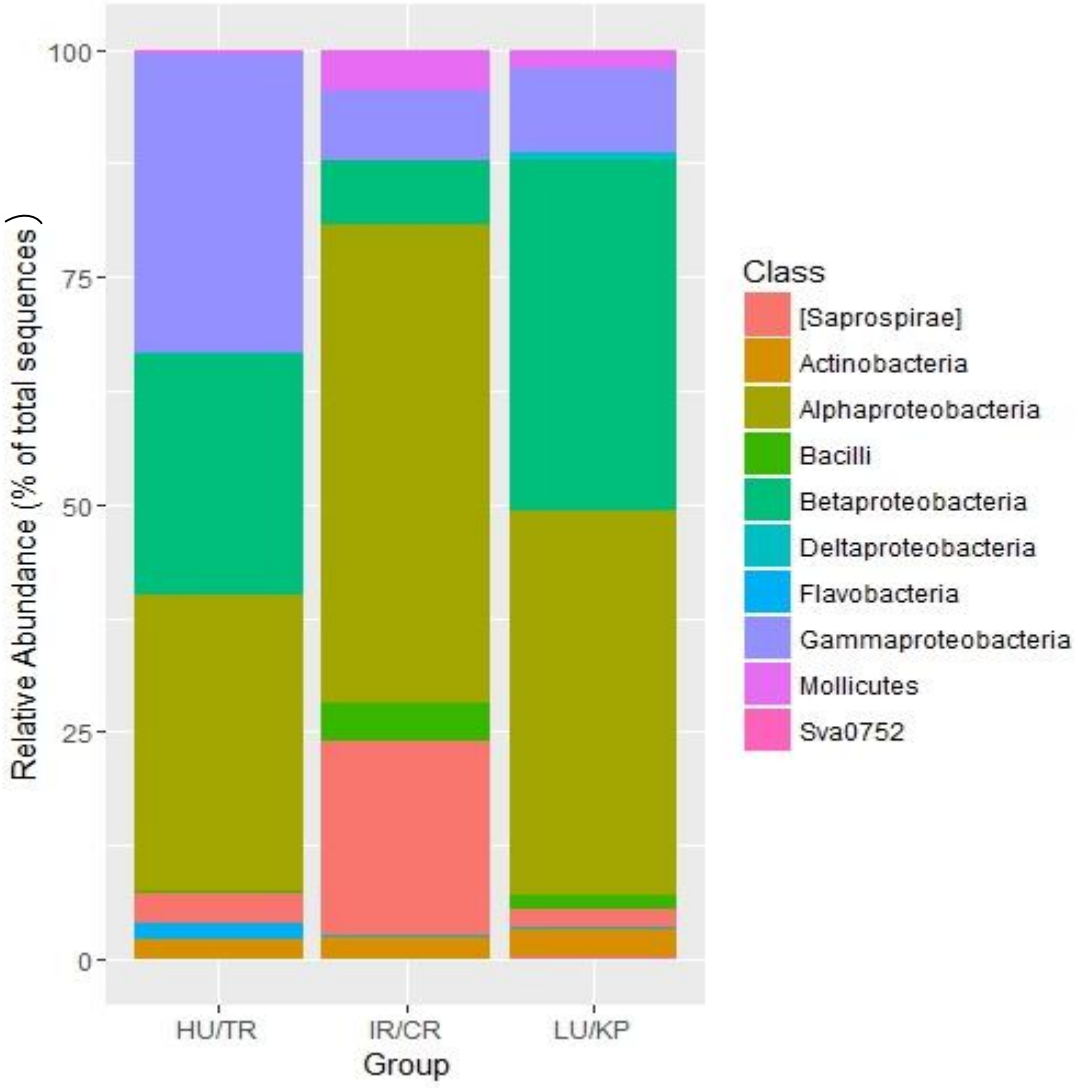

Figure 3.6 Community structure of endophytic bacteria in the roots of the three different paired groups of white clover as shown by metabarcoding with Illumina HiSeq of the 16S rRNA at the class level. Bacterial reads were rarified to 696, 162, and 311 as the smallest number of reads for each of the three groups (Huia/Tribute [HU/TR], Irrigation/Crusader [IR/CR], Louisiana/Kopu II [LU/KP]), respectively.

3.3.1.3 Rhizosphere bacterial community analysis

In, total 263761 reads representing 345 bacterial OTUs and were detected in the rhizosphere soil of the six cultivars after the removal of low abundance OTUs present at ≤0.1% relative abundance (Yeoh *et al.*, 2017). Read numbers were rarefied to 5898 as the lowest number of reads per sample to determine the bacterial community of the rhizosphere of white clover at phylum level (Figure 3.7). The phylum Proteobacteria was the most abundant in all the samples (53.8 - 69.2%). Phylum Actinobacteria, Acidobacteria, and Bacteroidetes were the next two most abundant taxa in all the samples (6.8 - 19.4%, 4.6 - 14.9%, and 2 - 13.1%, respectively). These four taxa collectively represented 91.5% of taxa present in rhizosphere soil of all six cultivars of white clover. Analysis of the rhizosphere bacterial communities showed that plant cultivar significantly affected bacterial community composition in the rhizosphere (PERMANOVA P=0.001). The Principal coordinate plot appeared to show some clustering of cultivars according to physical morphology (Figure 3.8). When grouped into these physiological pairs, there was significant difference between the rhizosphere community structure between the three groups (PERMANOVA P=0.001). Bacterial read numbers were rarefied to the smallest number of reads for each of the three pairs of matched clover cultivars and differences the rhizosphere bacterial communities at the phylum (Figure 3.9) determined. The bacterial phylum Proteobacteria was the most abundant in all three groups (59.5 - 63.5%). The next most abundant phyla were the Actinobacteria (11.9 – 15.2%). The third most abundant phyla were Acidobacteria (9.8 – 10.9%) for pairs Huia/Tribute and Irrigation/Crusader. The phyla Bacteroidetes were the third most abundant for cultivars Louisiana/Kopu II (Figure 3.9).

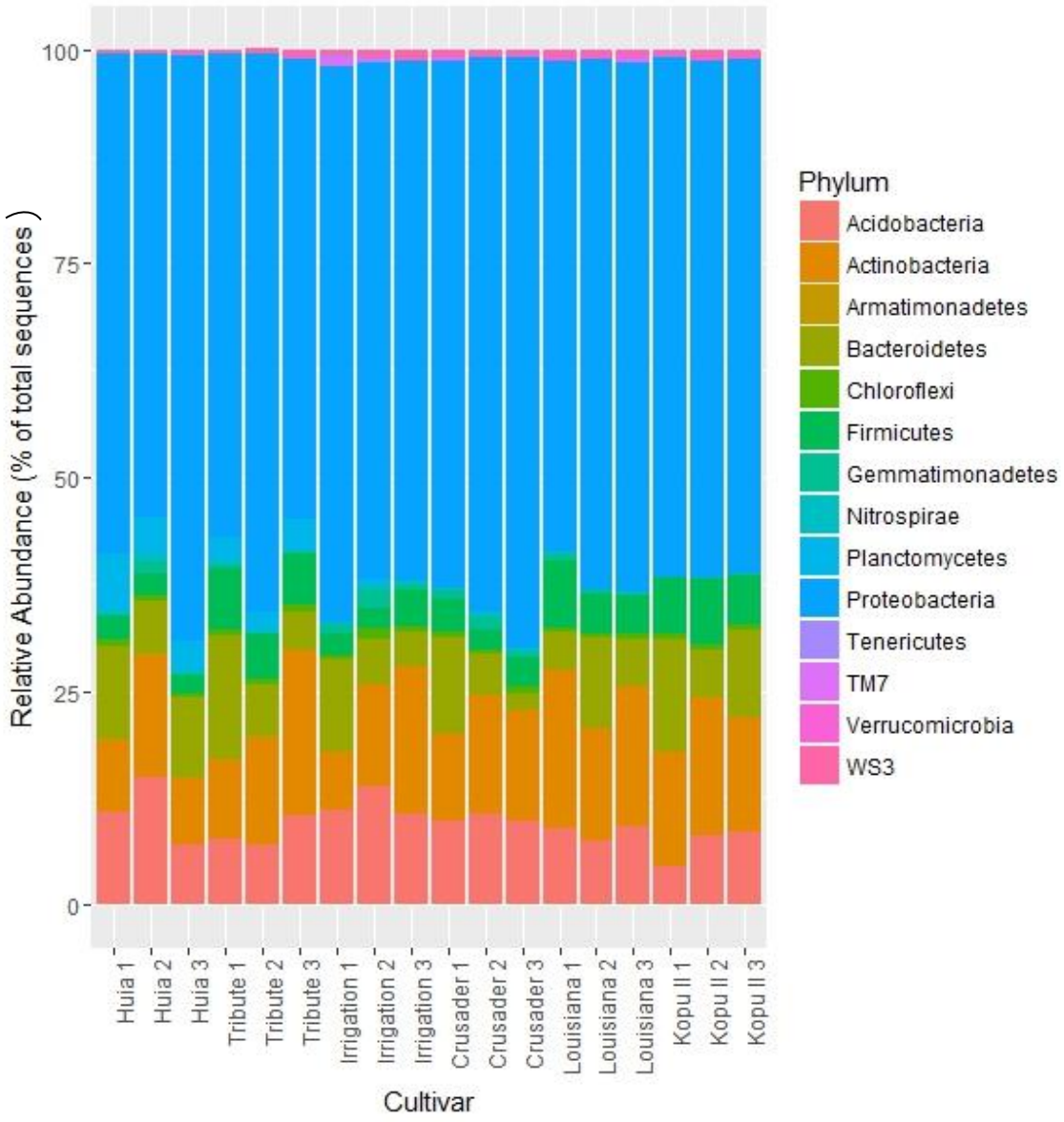

Figure 3.7 Community structure of rhizosphere bacteria in all samples of white clover shown by metabarcoding with Illumina HiSeq 16S rRNA amplicon sequencing at phylum level.

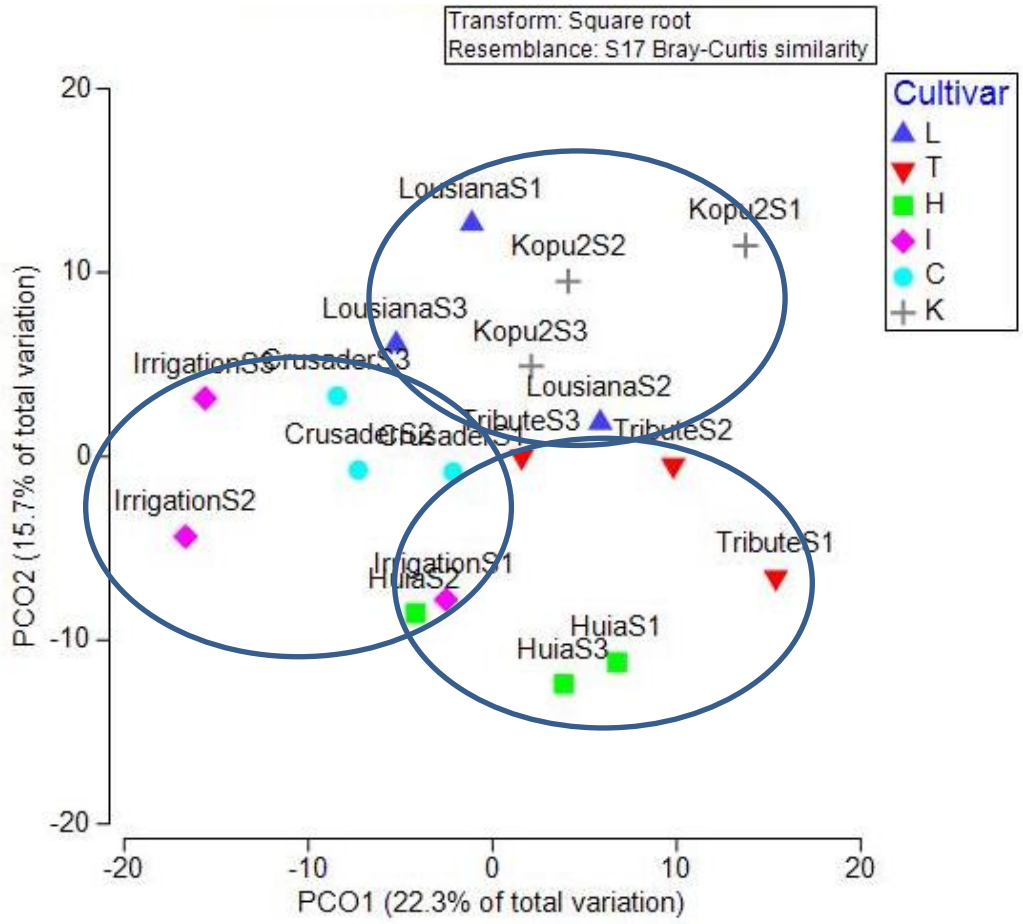

Figure 3.8 Principal coordinate plot showing square root transformed Bray-Curtis similarity between white clover cultivars based on the rhizosphere bacterial community. Reads were rarefied to 5898 as the smallest number of reads. Circles show the clustering of cultivars according to physiology.

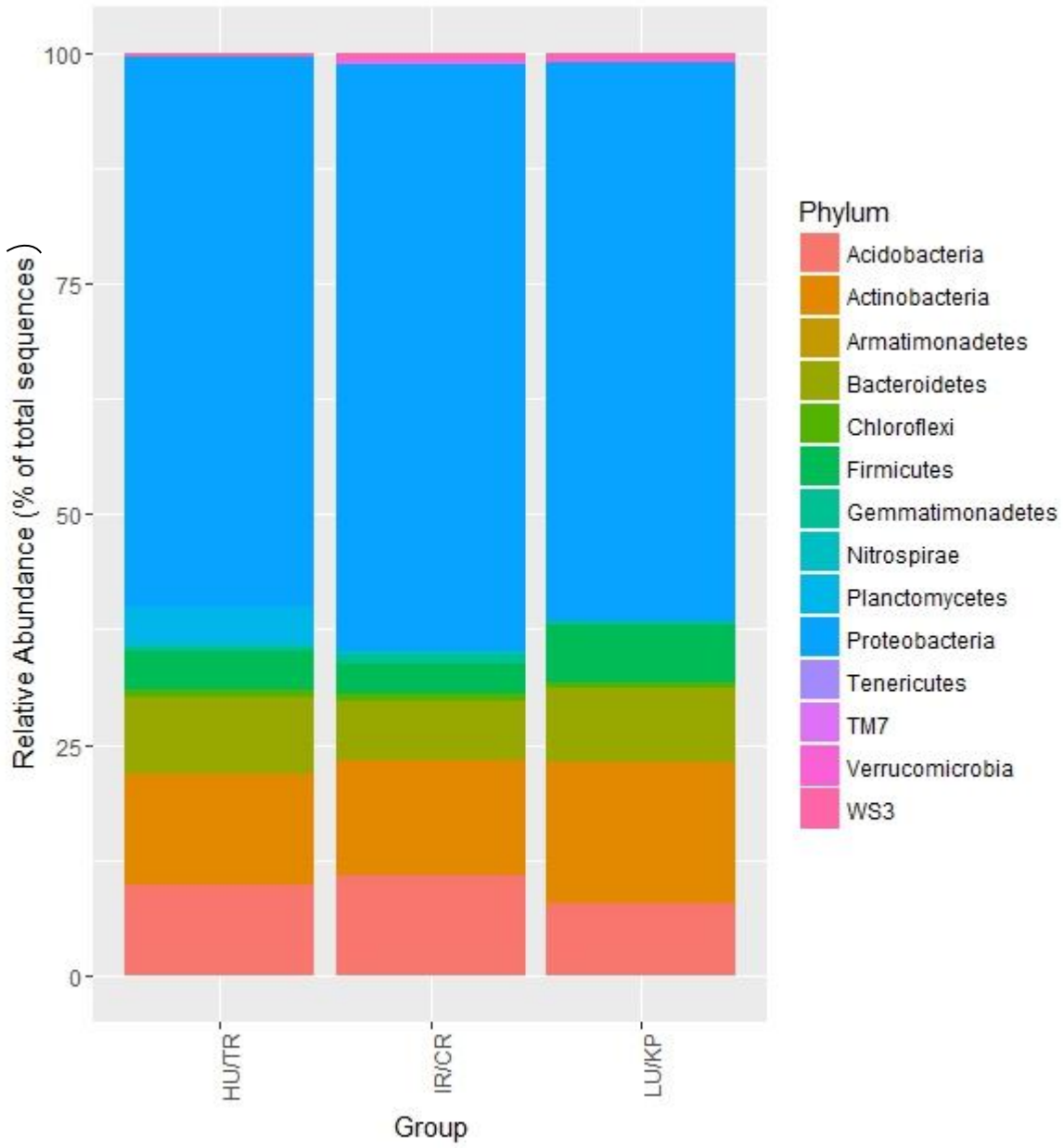

Figure 3.9 Community structure of bacteria in the rhizosphere of the three different paired groups of white clover as shown by metabarcoding with Illumina HiSeq of the 16S rRNA amplicon at the phylum level. Bacterial reads were rarified to 8988, 5898, and 6425 as the smallest number of reads for each of the three groups (Huia/Tribute [HU/TR], Irrigation/Crusader[(IR/CR], Louisiana/Kopu II [(LU/KP]), respectively.

3.3.1.4 AMF community analysis using DGGE

A total of 90 different bands (taxa) were identified by DGGE (Appendix 7.2.7). The number of bands produced per cultivar ranged from 28 (Crusader plant 2) to 49 (Crusader plant 1 and Irrigation plant 2). Clover cultivar affected AMF community composition (PERMANOVA P=0.006) (Figure 3.10). Replicate samples from some cultivars were not significantly different from each other, such as Louisiana, whereas others were significantly different from each other, such as Huia. Figure 3.11 shows that all three Tribute replicates are similar and are significantly different from the other cultivars (P<0.05). Crusader sample 2 had approximately 20 less bands than the other Crusader replicates (Table 3.4) and therefore the samples did not group (Figure 3.11). There was no significant difference in AMF community composition between the three old and three new cultivars (PERMANOVA P=0.338)

Table 3.4 Number of bands seen from each sample on the DGGE gel to identify AMF community composition associated with the different plant replicates for each of the six clover cultivars

	Cultivar					
Plant replicate	**Huia**	**Tribute**	**Irrigation**	**Crusader**	**Louisiana**	**Kopu II**
1	42	37	35	49	47	45
2	46	36	49	28	41	47
3	34	38	45	44	39	36

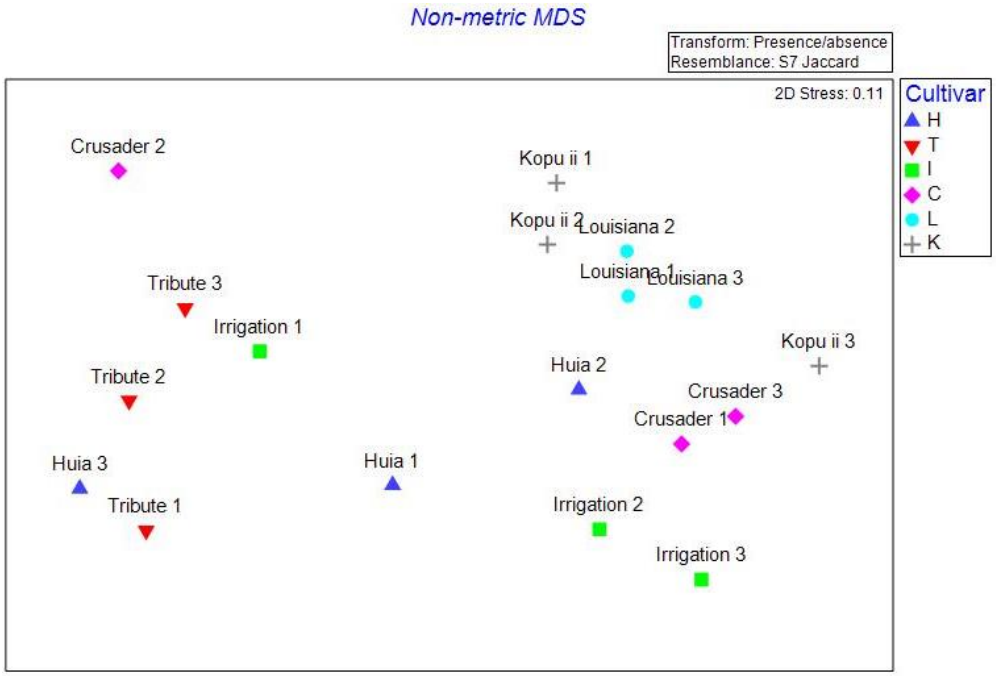

Figure 3.10 Nonmetric multidimensional scaling (MDS) plot showing AMF communities from the rhizosphere of different white clover cultivars.

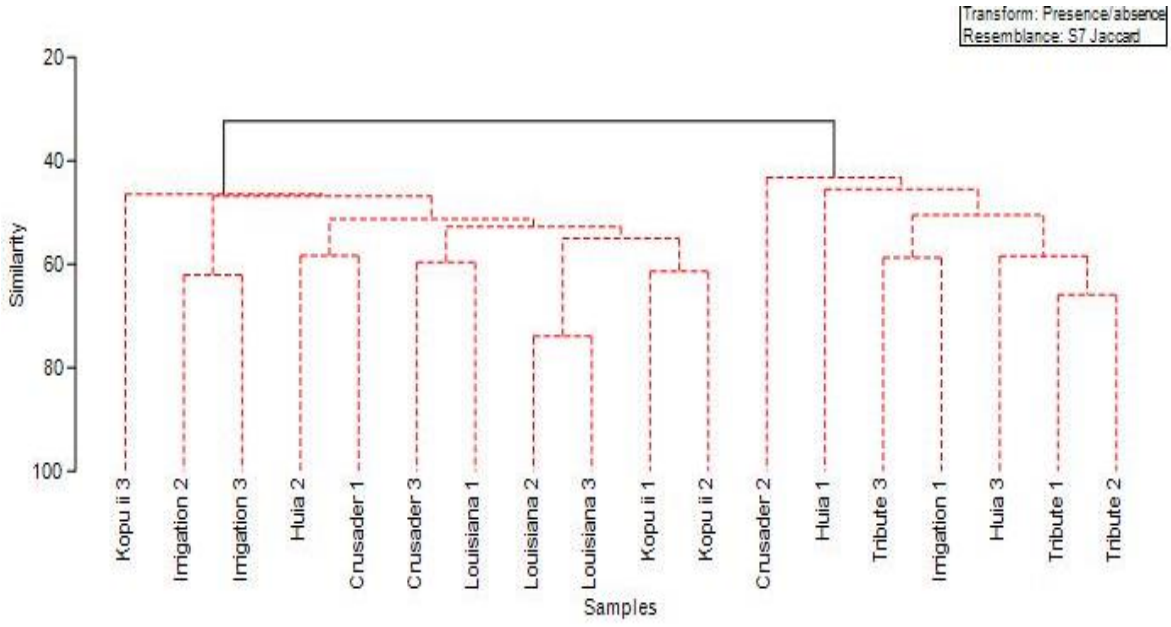

Figure 3.11 Similarity profile analysis at 95% threshold of AMF from the rhizosphere of six white clover cultivars. Samples on different solid lined branches are significantly different, *P* <0.05. Those separated by red dashed lines are not significantly different from each other.

3.3.2 Experiment 5 symbiotic potential of rhizobia strains isolated from roots of clover cultivars Huia and Tribute.

The positive and negative controls were removed before analysis. There was a significant difference in mean plant dry weight between the two white clover cultivars (*P*<0.001) (Huia 5.78 mg and Tribute 4.18 mg). There was also a significant difference in plant dry weight caused by the different inoculant treatments (*P*<0.001), but no significant treatment x cultivar interaction (*P*=0.179). The best performing strains for Huia were a strain isolated from its own root and one from the roots of Tribute (Figure 3.13). The best performing strain with Tribute was isolated for its own roots (Figure 3.14). In both cultivars the worst performing strain was T5.

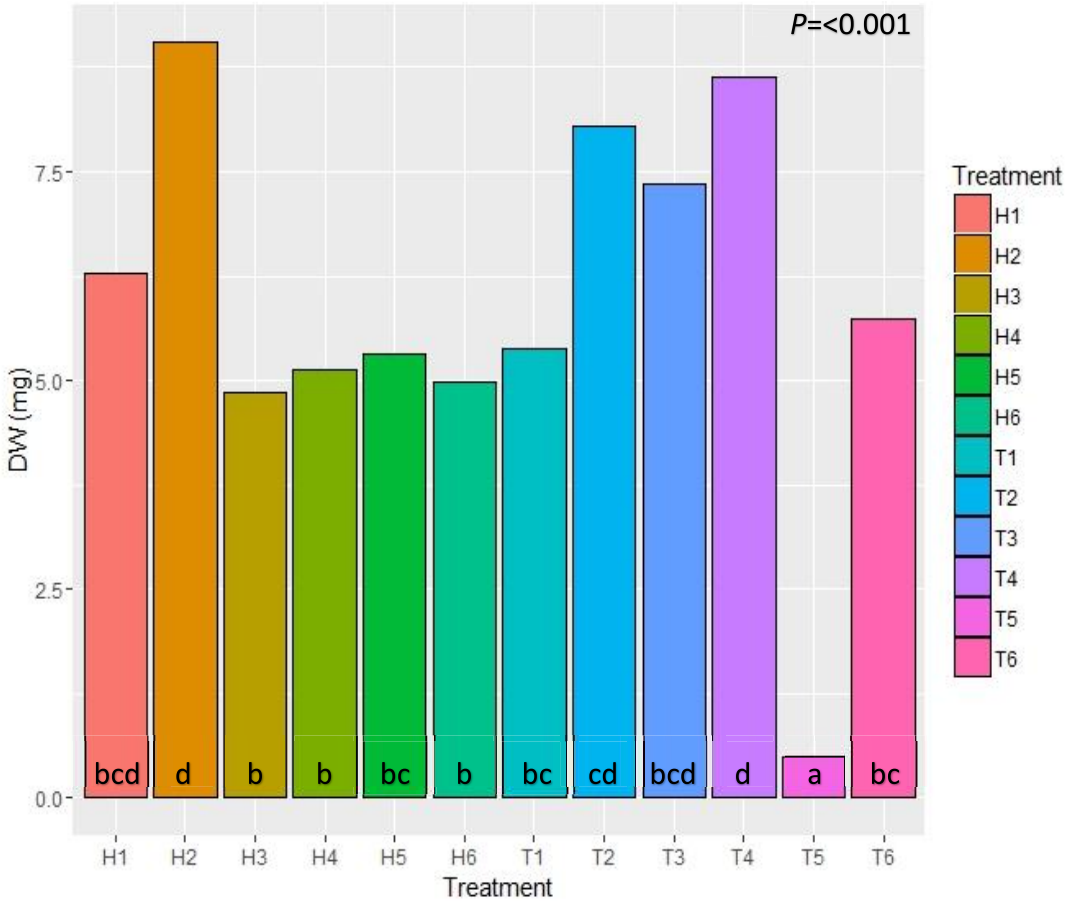

Figure 3.12 Mean dry weights (mg) for all inoculants on white clover cultivar Huia. Letters correspond to Fisher's least significant difference at the appropriate P value. Treatments with the same letters are not significantly different. H1-H6 represent rhizobia isolated from 6 different Huia nodules, and T1-T6 represent rhizobia isolated from 6 different Tribute nodules.

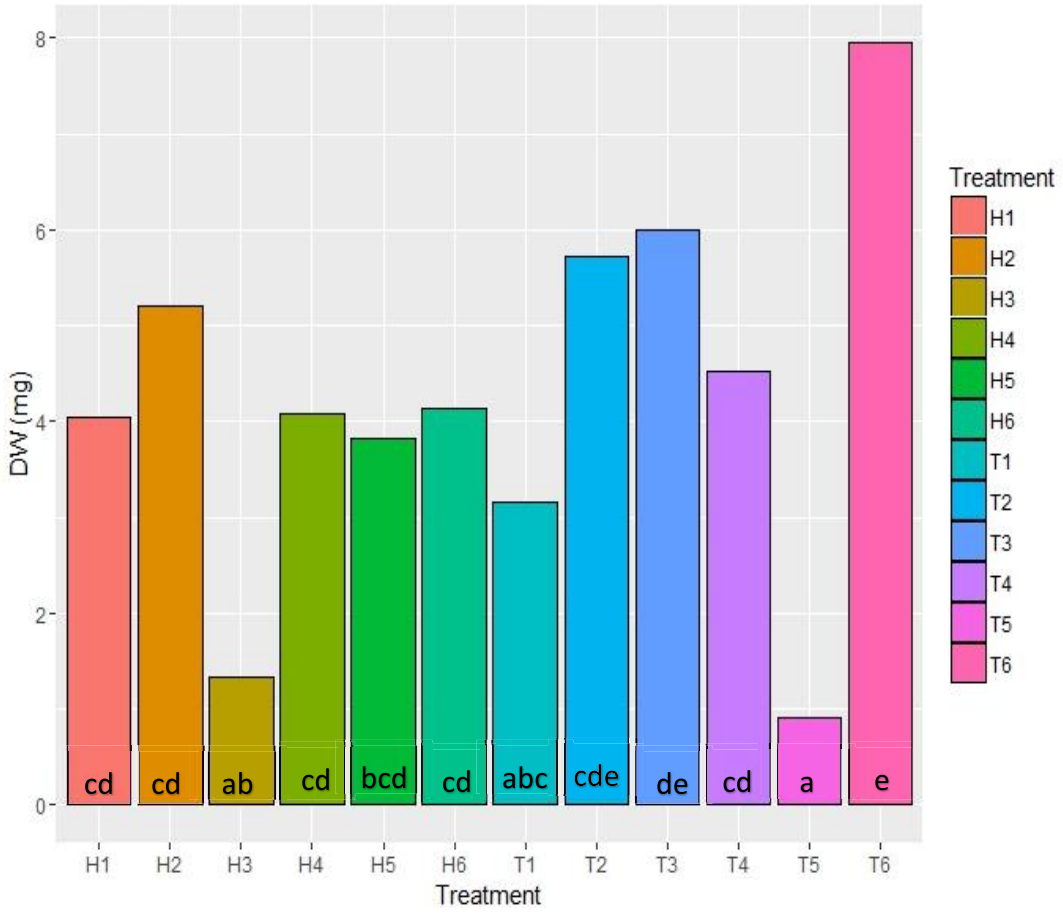

Figure 3.13 Mean dry weights (mg) for all inoculants on white clover cultivar Tribute. Letters correspond to Fisher's least significant difference at the appropriate P value. Treatments with the same letters are not significantly different. H1-H6 represent rhizobia isolated from 6 different Huia nodules, and T1-T6 represent rhizobia isolated from 6 different Tribute nodules.

3.4 Discussion

Global research has shown that plants are metaorganisms, and that the microorganisms that inhabit them play important roles in their physiology and ecology. This is the first study to characterize the diversity and structure of the bacterial root endophytic and, bacterial and AMF rhizosphere communities of different "old" and "new" cultivars of white clover. Understanding whether cultivar affects the structure of microbial communities in the root will help to understand the effects of breeding and plant physiology on microbial recruitment below ground. At the outset of this work it was hypothesized that there would be a difference in the microbial communities present in the roots of "old" versus "new" white clover cultivars. To undertake this work, culture independent molecular tools NGS and DGGE were used.

The three most common taxa found in the rhizosphere of the white clover plants were *Proteobacteria, Actinobacteria,* and *Acidobacteria*. This was consistent with other studies as it is these taxa that usually dominate in the rhizosphere (Weinert *et al.*, 2011; Yeoh *et al.*, 2017). The three most abundant phyla associated as endophytes of roots were *Proteobacteria, Actinobacteria*, and *Bacteroidetes*. This general dominance of phyla in the rhizosphere and roots of white clover is similar to that found in other legumes such as soya bean and alfalfa (Xiao *et al.*, 2017).

The high throughput sequencing showed a distinct difference in bacterial community structure (Figure 3.3) and diversity (Table 3.3) between the rhizosphere and root microbiome of white clover. Community richness was greater in the rhizosphere than in the roots. It has been relatively well established in the literature that only a small subset of the soil microbial community is successful in colonizing plant roots (Hartmann *et al.*, 2009; Gaiero *et al.*, 2013). A study conducted by Xiao *et al.* (2017) characterizing the microbiomes of different belowground compartments (nodule and root endophytes, rhizosphere, and root zone) of soya bean and alfalfa also showed that different bacterial communities were associated with these distinct habitats. Their study also showed the diversity of bacterial communities in the rhizosphere and root zone were much higher than those of the nodule and root endophytes.

In this study the bacterial root and rhizosphere communities of old and new cultivars were not significantly different. It is believed that, generally, as plant domestication has decreased plant genetic diversity, that this has affected the ability of plants to establish beneficial associations with rhizosphere microbes (Pérez-Jaramillo *et al.*, 2016). Decreased ability to associate with symbionts has been demonstrated for legumes. Mutch and Young (2004) showed that pea and broad bean were much more limited in the strains of rhizobia they could interact with as compared to their wild relatives which, in contrast, were able to exploit the diverse rhizobial community present in soil. The lack of a significant difference between old and new cultivars of white clover in the current study may be due to the high amount of genetic variability within white clover cultivars. White clover unlike soy bean and other commonly studied legumes, is an outcrossing plant, which introduces genetic variability into the experiment. Therefore, the age difference between cultivars may not be long enough to notice major differences in recruitment of a bacterial community to the roots and rhizosphere. Perhaps, specifically examining the rhizobial diversity would have been more likely to demonstrate differences between old and new cultivars, as was indicated in the previous chapter. Amplifying and sequencing the nodulation genes (such as *nodC*) which are known to be highly variable between taxa (Laguerre *et al.*, 2001; Bontemps *et al.*, 2005) may have identified differences in the rhizobial associations of the six cultivars.

The results from this study showed that the bacterial communities between the paired white clover cultivar groups were clustered more loosely in the principal coordinate analysis of the rhizosphere, than those recovered from the interior of the root (Figure 3.5, Figure 3.9). It is known that plants play an active role in recruiting microorganisms from the bulk soil into the rhizosphere through root exudates (Bais *et al.*, 2006). This showed that although there was differential recruitment of rhizosphere bacterial communities there was greater specificity in root endophytic bacterial communities. This could be because plants are able to actively regulate their endophytic associations. One way plants regulate endophyte communities is by producing compounds whose function is to suppress unrestrained growth and pathogenicity by microbes (Bacon and White, 2016). The ability of plants to adjust root exudate composition can vary among plant species and

genotype (Lareen *et al.*, 2016), and therefore this could explain the distinct differences in microbial communities between cultivar groups.

The results from this study revealed that the morphology of the cultivar was a significant factor affecting the bacterial root endophytes and rhizosphere community of white clover. White clover cultivars grouped together within their matched leaf sizes in the principal coordinate plots for the roots and rhizosphere (Figure 3.5, Figure 3.9). Studies have shown plant developmental stage is a strong driver shaping the rhizobacterial community structure (Chaparro *et al.*, 2014; Pérez-Jaramillo *et al.*, 2016). A study conducted by Chaparro *et al.* (2014) showed that Arabidopsis plants can select a subset of microbes at different stages of development. Given that the paired cultivars were chosen based on their similarity in morphology it may be that their parallel developmental stages made their microbiomes more similar to each other. It is possible that a particular leaf size physiology allows a plant to release a specific set of exudates which alter the rhizobacterial and consequently root endophytic community. Further work would need to be done to identify the similarities between specific root exudates produced by these cultivars of white clover to ascertain what compounds could be responsible in driving rhizobacterial community differences.

Illumina HiSeq showed that plant cultivar significantly affected the microbial community structure of the roots and rhizosphere of white clover. A study conducted by Weinert *et* al. (2011) showed that for three different potato cultivars, a subset of detected OTUs was cultivar-specific. Similar cultivar-dependent effects in the rhizobacterial communities in the rhizosphere of young potato plants has also been shown (İnceoğlu *et al.*, 2011). The study conducted by İnceoğlu *et al.* (2011) also found that the cultivar dependent bacterial community structures disappeared in later development stages of the plants, such as during flowering and senescence. In the current study, plants were harvested well before they were fully mature or new flowering. Perhaps analysing the bacterial community of the roots and rhizosphere of flowering white clover plants could possibly reveal other changes in community structre.

Principal coordinate analysis showed that there was some variability in the microbial communities of the root and rhizosphere within cultivar replicates (Figure 3.5, Figure 3.9). Some of this variation could be attributed to the genotypic variability present within

white clover (Gustine and Huff, 1999; Jahufer *et al.*, 2013). Plant genotype is known to regulate the rhizosphere microbiome between plant species and also between different genotypes of the same species (Weinert *et al.*, 2011; Pérez-Jaramillo *et al.*, 2016). A study conducted by Aira *et al.* (2010) on maize showed that plant genotype strongly modified the structure of microbial communities in the rhizosphere of maize plants. Their study showed plant rhizosphere communities also strongly reacted to other variables such as fertilizer treatments. There is no genotypic relationship between morphological matched cultivar pairs, and the results of PCO analysis show much closer clustering based on morphology indicating genotype is not the most defining variable of white clover root and rhizosphere microbial communities.

Another reason for variation in the replicates could be the amount of root tissue used for DNA extraction as the first lateral root, regardless of size and length, was taken for each plant. Using a set weight or having a set root order from which to cut could have further enhanced the agreement between replicates. To get a broader and accurate understanding of the root and soil rhizosphere community, more plant replicates and/or triplicate PCRs for each sample could have been conducted and pooled together, therefore being more representative of the root.

Crusader plant one had a much higher abundance of the phyla *Bacteroidetes* (genus *Chitinophaga)* in its roots than was observed for the other two replicates and among every other plant cultivar. Bacteroidetes are one of the most abundant phyla in the roots and leaves of *Arabidopsis thaliana* (Bodenhausen *et al.*, 2013). Recently a study conducted by Shaffer *et al.* (2017) showed that *Chitinophaga* can be endohyphal, and tested its effects on substrate use by its host, a seed-associated strain of *Fusarium keratoplasticum*. For 62% of the substrates the fungal strain harbouring the bacterium significantly outperformed the cured strain. This was measured by hyphal density and respiration (Shaffer *et al.*, 2017). Therefore, it could be possible it entered the plant root in the hyphae of a fungi. The present study also found that the high levels of *Chitinophaga* found in the roots of Crusader plant one did not raise abundance of the genus in the rhizosphere. Results showed that the plant dry weight, and shoot fresh weights were not significantly different for Crusader from other cultivars. This suggested that even though Crusader plant 1 had a higher abundance of *Bacteroidetes* in the roots,

this did not hinder plant shoot and root growth. Thus, the significance of this taxa in Crusader plant 1 is unclear.

Illumina Hiseq revealed that cultivar pair Huia/Tribute had a higher relative abundance of gammaproteobacteria in the roots compared to the other cultivars. Gammaproteobacteria are known to be opportunistic colonizers of nodules induced by rhizobia (Ibáñez *et al.*, 2009). A study conducted by Ibanez *et al.* (2009) isolated three strains of Gammaproteobacteria from nodules and found they were unable to induce nodule formation on *Arachis hypogaea* L. plants, however, when co-inoculated with an ineffective *Bradyrhizobium* strain, they were observed colonizing pre-formed nodules. Gammaproteobacteria and can also provide benefits to hosts via the production of organic compounds such as L-ascorbic acid and, indole acetic acid which plays a central role in plant growth and development (Ghosh *et al.*, 2015; Kumar Ghosh *et al.*, 2015). Out of the three match pairs, Huia/Tribute had the lowest abundance of Tenericutes, of which some species can be pathogenic (Lee *et al.*, 2000).

This study also used DGGE as a molecular tool to analyze the structure of AMF communities across the rhizosphere of the six white clover cultivars. Although considered an older technique, there are advantages and disadvantages associated with DGGE. DGGE is relatively cost effective, and can be good to provide a broad overview of microbial community structure (Cleary *et al.*, 2012). Some disadvantages of DGGE are that for instance, it is likely DGGE will only detect a part of the total diversity from samples as most of the DGGE bands will be composed of the more abundant species (Mühling *et al.*, 2008). Another problem could be that one DGGE band can represent multiple species and it is also possible for one species to be represented by many bands (Dowd *et al.*, 2008). This could lead to misinterpretation of the community profiles. Due to low cost, expected low diversity and the lack of suitable AMF specific primers for NGS, DGGE was chosen as the molecular tool to provide an overview of AMF community structure in the rhizosphere of white clover.

DGGE analysis showed that cultivar was a significant factor influencing the composition of AMF in the rhizosphere of white clover plants. However, unlike the clustering seen in the bacterial root endophyte and rhizosphere communities, the clover cultivars did not cluster. The MDS plot (Figure 3.10) and dendrogram (Figure 3.11) showed that cv. Tribute

grouped separately from the other cultivars. This indicated that rhizospheric bacterial community was not strongly linked to AMF community structure. A study conducted by Becklin *et al.* (2011) showed that host identity impacted rhizosphere fungal communities associated with alpine plant species. Their study showed that AMF community composition was influenced by host identity as richness varied among host species. Alternatively, there could be a non-mycorrhizal fungal symbiont, such as a helper bacteria, associated with Tribute roots that could have had large impact on the AMF community structure. A study conducted on young pea plants has shown a stimulatory bacterial isolate, enhanced AMF-colonization for *Glomus clarum* as well as isolates which inhibit the germination of spores and infectivity of AMF (Xavier and Germida, 2003).

As discussed previously, the genetic diversity within white clover is large. A reason for not seeing better separation and grouping, between and within cultivar replicates could be the low amount of replication used for studying the AMF community structure. Increasing the replication could be critical in improving the statistical resolution of the study and could uncover other factors influencing AMF community structure among white clover. Another reason for the lack of any groupings could be that AMF are spatially distinct on the root and some occupy larger sections more vigorously than others (Dodd *et al.*, 2000; Hart and Reader, 2002). Species of *Glomus* tend to be the first colonizers of any root system in a mixed soil inocula and the spread of extraradical hyphae are also unique to different species (Dodd *et al.*, 2000). Therefore, it is possible that due to the competitive nature of *Glomus* species these may have been the first to colonize and spread extraradical hyphae and because the plants were harvested fairly young, other species of AMF did not get a chance to establish a significant hyphal presence. A study conducted by Dodd *et al.* (2000) also showed difference in extraradical hyphae of six species of AMF grown under the same conditions. Sampling from the rhizosphere soil could be biased to the AMF that invest strongly in the growth and distribution of extraradical hyphae and thus may have reduced the resolution of the experiment.

In the last experiment of this chapter the ability of two clover cultivars to select high performing symbionts was explored by recovering isolates from the nodules and then measuring their symbiotic potential on either Huia or Tribute. The results showed that Huia produced more shoot dry weight than Tribute irrespective of which cultivar the

rhizobia strain had originated from. Huia also performed better with more strains of rhizobia than Tribute. A study conducted by Mutch and Young (2004) showed that wild progenitors of broad bean and pea cultivars were better able to exploit a wider range of rhizobial symbionts. The results from the current study show that Huia is a more capable of utilizing a diverse range of rhizobial symbionts. This confirmed the results seen in Section 2.2, where Huia had significantly more associations with effective strains of rhizobia than Tribute. However, it was assumed that all strains used for re-inoculation were different and the current experiment could have been improved by genotyping each strain.

3.5 Conclusions

In summary this study was the first to describe the bacterial community structure of the root and rhizosphere of old and new white clover cultivars. This chapter shows that there is strong difference between the bacterial communities in the rhizosphere and root of white clover cultivars. It indicated that plant developmental stages are strong drivers of microbial community structure, especially for the endomicrobiome. DGGE showed no difference between old and new cultivars or clustering of cultivars in relation to AMF community structure in the rhizosphere. Although there was no apparent difference between old and newer cultivars it was observed that in the matched Huia/Tribute pairing that the older cultivar, Huia, was able to effectively utilize a broader range of rhizobial genotypes.

4 Concluding discussion

The overall aim of this thesis was to examine whether ~80 years of white clover breeding has affected the ability of newer cultivars to form effective below ground associations. This was tested by:

1. Examining if there was a preferential selection by either “old” or “new” cultivars of white clover to select effective strains of *R. leguminosarum* from a mixture.
2. Investigating the responsiveness of six cultivars of white clover to different species of AMF and AMF mixtures
3. Examining the root endophytic and rhizosphere bacterial communities of old and new cultivars.
4. Examining the rhizosphere AMF community structure

In New Zealand, clover breeding programmes have focused on above ground characteristics in selection of new cultivars. This has been carried out with a background of high nitrogen soil environments, due to fertilizer use. There is evidence in the literature which suggests that breeding has decreased the ability of some legumes, such as soybeans, to defend against poor-quality mutualists (Kiers *et al.*, 2007). This is the first study to investigate whether plant breeding may have impacted white clover and it’s below ground associations.

The ability of old and new cultivars to select effective strains of rhizobia from a mixture was investigated *in vitro* in Chapter 2. Genotyping of *R. leguminosarum* strains recovered from ten nodules from each white clover plant was used to determine which strains had occupied nodules from the mixture of highly effective and less effective strains. The results showed that two out of the three older cultivars (Huia and Louisiana) of white clover selected significantly more highly effective strains and significantly fewer less effective strains than would be expected by chance. No new cultivars demonstrated this capability. This suggested that plant breeding may have affected a cultivars ability to discriminate among rhizobial partners of varying efficacies. These findings were consistent with the findings of Kiers *et al.* (2007) who found soybean defences against poor-quality rhizobia have worsened under artificial selection. Though genotyping of 10 randomly selected nodules showed differences between cultivars, an improvement to the

experiment could be to genotype all the nodules on each plant. This would give a more complete understanding on the extent of partner choice by each cultivar. Another improvement would be to record the size and coloring of all the nodules. This would provide an added layer of information to complement the nodule occupancy data. Nodule size and colour can be good estimates of how much resources (carbon) are allocated to the nodule, and whether a particular nodule is fixing nitrogen (Maier and Brill, 1976).

Chapter 2 also showed that strain 451 was a very competitive nodulator, and was the most dominant strain found in the nodules of all the cultivars. The presence of such an aggressive competitor strain may lower the resolution of the experiment to identify strain selection by the hosts. Using a bigger mixture of strains with similar competitive abilities would provide a more even basis for selection. Therefore, it would be interesting to run the experiment again without the addition of strain 451. Further investigation could look at genetic basis of the competitiveness of strain 451, even though it was not most beneficial strain on all cultivars. Such work may identify traits that could be selected for in commercial inoculants, improving their success when deployed in the field.

In Chapter 2, the responsiveness of the six white clover cultivars to AMF and AMF mixtures was also examined. No conclusive results were obtained from this experiment due to the high amount of contamination (possibly oomycete) that was observed. Improvements to the experiment could be, sterilizing the pots in which the plants were grown as well as the media. Using sterile Millipore water when watering and preparing the minimal nutrient solutions to reduce the risk of contamination from a water borne source. Growing the plants in a dedicated soil-less controlled growth chamber instead of a growth room where other soil based experiments were run simultaneously would also reduce the risk of contamination from the air.

This was the first study to examine the bacterial rhizosphere and root endophyte communities of old and new cultivars of white clover. Illumina HiSeq showed that, for white clover, there was a distinct difference between the bacterial communities of the root and rhizosphere. Other studies of the microbiome of plants have not shown such a distinct separation between the root and rhizosphere microbial communities (Jin *et al.*, 2014; Xiao *et al.*, 2017). The distinct separation observed in the current study could have

been because no other parts of the plant were sampled and included in the analysis. Studies have also shown plant genotype and age shape the leaf and root microbiomes of wild perennial mustard (Wagner *et al.*, 2016). As that study indicated that the microbial communities of the roots and leaves were linked, it would be interesting to examine the microbial communities in the leaves of white clover alongside the root and rhizosphere to see if such a relationship was also true for white clover. Analysis was not conducted on the microbial community structure of the bulk soil, however, such analysis might have shown which microbial communities were selected for in the rhizosphere against a background control. This may have further highlighted differences seen between the root and rhizosphere microbial communities of the six cultivars.

Chapter 3 showed that cultivar age did not influence the root and rhizosphere bacterial communities of white clover. Instead the results showed that plant morphotype was a significant factor and suggested there was a link between the development stage of a particular morphotype and bacterial communities recruited into the roots and rhizosphere. This link could be further investigated by running the experiment for a longer period of time, and sampling key developmental time points, such as flowering, seed set and stolon growth. Having more information across different growth stages would improve resolution of the degree to which plant development affects bacterial communities in the roots and rhizosphere. It would also be interesting to analyze the root exudates produced by the different cultivars to verify whether they contribute to host mediated selection of microbial communities (Aulakh *et al.*, 2001; Kidd *et al.*, 2001).

DGGE analysis of the AMF community in the rhizosphere of white clover plants showed there was a significant cultivar effect but no grouping by cultivar pairs. It was likely that *Glomus* species were the first to colonize and generate extra-radical hyphae, therefore limiting opportunity for other AMF to establish their presence on root surfaces. A time course or harvesting and analyzing more of the rhizosphere soil may have improved this experiment. In addition, extracting DNA from the whole root system and identifying the AMF community may also have been a better approach.

In experiment 5 of Chapter 3, only matched pair cultivars Huia and Tribute were chosen to assess the symbiotic potential of the rhizobial symbionts retrieved from nodules. These two cultivars were chosen because these are the current and former “benchmark”

cultivars of white clover used in New Zealand agriculture. The results showed that Huia was able to effectively utilise a wider range of rhizobial strains than its matched pair, Tribute. Similar results have been noted in other legumes, where wild cultivars were better able to exploit the rhizobial symbionts in the soil than domesticated cultivars (Mutch and Young, 2004). Seed germination, and availability was also an important factor in the selection choice of these cultivars, due to the replication required. Sufficient seed was available for these two cultivars but not others.

Genetic variation played a big role in the results of this thesis. Significant differences between the six individual rhizobia strains was not seen for all cultivars in Experiment 1. This is likely due to the low replication used for this experiment. This was important as this was the main benchmarking experiment from which the strain rankings were made for experiment 2. Improving the replicate number to 15 or 20 could have decreased the variation seen and provided a more robust ranking of rhizobia strains for each cultivar to carry forward to experiment 2. Another way to reduce the effect of white clover genetic variation in this experiment would be to propagate replicates using stolon cuttings. Variation among replicates was also visible in the Chapter 3 in the PCOs for the root and rhizosphere bacterial communities, and the AMF rhizosphere communities. An improvement to the experimental design for the NGS and DGGE experiments would also be to increase the number of replicates. However, increasing replication would also increase the financial cost of the experiment.

In summary, this study confirmed reports that strains of *R. leguminosarum* vary in their efficacy when in symbiosis with different cultivars. The results also supported the hypothesis that plant breeding has caused a relaxation in rhizobia partner selection by newer cultivars of white clover. The findings of this study also show that the bacterial communities of the root and rhizosphere are strongly affected by cultivar morphotype. Several promising areas of future research were identified, such as the mechanistic basis for the observed differences in rhizobia partner selection, and the mechanism of why morphotype is a driver of bacterial communities in the root and rhizosphere.

5 References

Abberton, M. T. and Marshall, A. H. 2005. Progress in breeding perennial clovers for temperate agriculture. *The Journal of Agricultural Science*, **143**, 117-135.

Agnelli, A., Ascher, J., Corti, G., Ceccherini, M. T., Nannipieri, P. and Pietramellara, G. 2004. Distribution of microbial communities in a forest soil profile investigated by microbial biomass, soil respiration and DGGE of total and extracellular DNA. *Soil Biology and Biochemistry*, **36**, 859-868.

Andrews, M. and Lea, P. 2013. Our nitrogen 'footprint': the need for increased crop nitrogen use efficiency. *Annals of Applied Biology*, **163**, 165-169.

Andrews, M., Lea, P., Raven, J. and Azevedo, R. 2009. Nitrogen use efficiency. 3. Nitrogen fixation: genes and costs. *Annals of Applied Biology*, **155**, 1-13.

Andrews, M., Raven, J. and Lea, P. 2013. Do plants need nitrate? The mechanisms by which nitrogen form affects plants. *Annals of Applied Biology*, **163**, 174-199.

Andrews, M., Scholefield, D., Abberton, M. T., McKenzie, B. A., Hodge, S. and Raven, J. A. 2007. Use of white clover as an alternative to nitrogen fertiliser for dairy pastures in nitrate vulnerable zones in the UK: productivity, environmental impact and economic considerations. *Annals of Applied Biology*, **151**, 11-23.

Antoun, H., Beauchamp, C. J., Goussard, N., Chabot, R. and Lalande, R. 1998. Potential of Rhizobium and Bradyrhizobium species as plant growth promoting rhizobacteria on non-legumes: effect on radishes (Raphanus sativus L.). *Plant and soil*, **204**, 57-67.

Aulakh, M., Wassmann, R., Bueno, C., Kreuzwieser, J. and Rennenberg, H. 2001. Characterization of root exudates at different growth stages of ten rice (Oryza sativa L.) cultivars. *Plant Biology*, **3**, 139-148.

Bacci, G., Bazzicalupo, M., Benedetti, A. and Mengoni, A. 2014. StreamingTrim 1.0: a Java software for dynamic trimming of 16S rRNA sequence data from metagenetic studies. *Molecular Ecology Resources*, **14**, 426-434.

Bacon, C. W. and White, J. F. 2016. Functions, mechanisms and regulation of endophytic and epiphytic microbial communities of plants. *Symbiosis*, **68**, 87-98.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S. and Vivanco, J. M. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.*, **57**, 233-266.

Bever, J. D., Richardson, S. C., Lawrence, B. M., Holmes, J. and Watson, M. 2009. Preferential allocation to beneficial symbiont with spatial structure maintains mycorrhizal mutualism. *Ecology letters*, **12**, 13-21.

Bik, H. M., Porazinska, D. L., Creer, S., Caporaso, J. G., Knight, R. and Thomas, W. K. 2012. Sequencing our way towards understanding global eukaryotic biodiversity. *Trends in Ecology & Evolution*, **27**, 233-243.

Birch, P. R., Coates, M. E. and Beynon, J. L. 2012. Evolution of Virulence in Oomycete Plant Pathogens. *Evolution of Virulence in Eukaryotic Microbes*, 385-403.

Bodenhausen, N., Horton, M. W. and Bergelson, J. 2013. Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. *PloS one*, **8**, e56329.

Bontemps, C., Golfier, G., Gris-Liebe, C., Carrere, S., Talini, L. and Boivin-Masson, C. 2005. Microarray-based detection and typing of the Rhizobium nodulation gene nodC: potential of DNA arrays to diagnose biological functions of interest. *Applied and Environmental Microbiology*, **71**, 8042-8048.

Boon, N., De Windt, W., Verstraete, W. and Top, E. M. 2002. Evaluation of nested PCR–DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiology Ecology*, **39**, 101-112.

Brock, J. 1988. Evaluation of New Zealand bred white clover cultivars under rotational grazing and set stocking with sheep. *In*: Proceedings of the New Zealand Grassland Association. New Zealand Grassland Association. **Vol. 49**. p 203-206.

Brock, J. and Hay, M. 2001. White clover performance in sown pastures: A biological/ecological perspective. *In*:Proceedings of The Conference-New Zealand Grassland Association. p 73-84.

Brundrett, M., Bougher, N., Dell, B. and Grove, T. 1996. Working With Mycorrhizas in Forestry and Agriculture.

Burdon, J., Gibson, A., Searle, S. D., Woods, M. and Brockwell, J. 1999. Variation in the effectiveness of symbiotic associations between native rhizobia and temperate Australian Acacia: within-species interactions. *Journal of Applied Ecology*, **36**, 398-408.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K. and Gordon, J. I. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, **7**, 335-336.

Caradus, J., Hay, R. and Woodfield, D. 1996a. The positioning of white clover cultivars in New Zealand. *Special Publication - Agronomy society of New Zealand*, 45-50.

Caradus, J., Woodfield, D. and Stewart, A. 1996b. Overview and vision for white clover. *Special Publication - Agronomy society of New Zealand*, 1-6.

Chanway, C. P., Turkington, R. and Holl, F. B. 1991. Ecological Implications of Specificity between Plants and Rhizosphere Micro-organisms. *In:* A. H. F. M. Begon and A.

Macfadyen, (eds). Advances in Ecological Research: Academic Press, **Volume 21**, 121-169

Chaparro, J. M., Badri, D. V. and Vivanco, J. M. 2014. Rhizosphere microbiome assemblage is affected by plant development. *The ISME journal*, **8**, 790.

Chen, M., Yin, H., O'Connor, P., Wang, Y. and Zhu, Y. 2010. C: N: P stoichiometry and specific growth rate of clover colonized by arbuscular mycorrhizal fungi. *Plant and Soil*, **326**, 21-29.

Choudhary, D. K., Prakash, A. and Johri, B. N. 2007. Induced systemic resistance (ISR) in plants: mechanism of action. *Indian Journal of Microbiology*, **47**, 289-297.

Cleary, D. F., Smalla, K., Mendonça-Hagler, L. C. and Gomes, N. C. 2012. Assessment of variation in bacterial composition among microhabitats in a mangrove environment using DGGE fingerprints and barcoded pyrosequencing. *PloS One*, **7**, e29380.

Conrath, U. 2006. Systemic Acquired Resistance. *Plant Signaling & Behavior*, **1**, 179-184.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P. and Andersen, G. L. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and environmental microbiology*, **72**, 5069-5072.

Dodd, J. C., Boddington, C. L., Rodriguez, A., Gonzalez-Chavez, C. and Mansur, I. 2000. Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. *Plant and Soil*, **226**, 131-151.

Dodt, M., Roehr, J. T., Ahmed, R. and Dieterich, C. 2012. FLEXBAR—flexible barcode and adapter processing for next-generation sequencing platforms. *Biology*, **1**, 895-905.

Douds, D. D. and Millner, P. D. 1999. Biodiversity of arbuscular mycorrhizal fungi in agroecosystems. *Agriculture, Ecosystems & Environment*, **74**, 77-93.

Dowd, S. E., Sun, Y., Secor, P. R., Rhoads, D. D., Wolcott, B. M., James, G. A. and Wolcott, R. D. 2008. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. *BMC Microbiology*, **8**, 43.

Dowling, D. and Broughton, W. 1986. Competition for nodulation of legumes. *Annual Reviews in Microbiology*, **40**, 131-157.

Downie, J. A. 2010. The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. *FEMS Microbiology Reviews*, **34**, 150-170.

Eason, W. R., Webb, K., Michaelson-Yeates, T., Abberton, M., Griffith, G., Culshaw, C., Hooker, J. and Dhanoa, M. 2001. Effect of genotype of *Trifolium repens* on

mycorrhizal symbiosis with *Glomus mosseae*. *The Journal of Agricultural Science*, **137**, 27-36.

Easton, S., Amyes, J., Cameron, N., Green, R., Kerr, G., Norriss, M. and Stewart, A. 2002. Pasture plant breeding in New Zealand: where to from here? *In*: Proceeding of The Conference - New Zealand Grassland Association. p 173-180.

Eerens, J. and Ryan, D. 2000. Managing white clover for productive and profitable sheep farming in Southland. *In*: Proceedings of The Conference - New Zealand Grassland Association. p 137-140.

Eevers, N., Gielen, M., Sánchez-López, A., Jaspers, S., White, J., Vangronsveld, J. and Weyens, N. 2015. Optimization of isolation and cultivation of bacterial endophytes through addition of plant extract to nutrient media. *Microbial Biotechnology*, **8**, 707-715.

Elgersma, A., Schlepers, H. and Nassiri, M. 2000. Interactions between perennial ryegrass (*Lolium perenne L.*) and white clover (*Trifolium repens L.*) under contrasting nitrogen availability: productivity, seasonal patterns of species composition, N2 fixation, N transfer and N recovery. *Plant and Soil*, **221**, 281-299.

Facelli, E., Smith, S. E. and Smith, F. A. 2009. Mycorrhizal symbiosis—overview and new insights into roles of arbuscular mycorrhizas in agro-and natural ecosystems. *Australasian Plant Pathology*, **38**, 338-344.

Fawke, S., Doumane, M. and Schornack, S. 2015. Oomycete interactions with plants: infection strategies and resistance principles. *Microbiology and Molecular Biology Reviews*, **79**, 263-280.

Gaiero, J. R., McCall, C. A., Thompson, K. A., Day, N. J., Best, A. S. and Dunfield, K. E. 2013. Inside the root microbiome: bacterial root endophytes and plant growth promotion. *American Journal of Botany*, **100**, 1738-1750.

Gaur, Y. and Lowther, W. 1982b. Competition and persistence of strains of *Rhizobium trifolii* in relation to inoculation level and lime pelleting on white clover sown into cultivated soil. *New Zealand journal of Agricultural Research*, **25**, 277-280.

Ghosh, P. K., De, T. K. and Maiti, T. K. 2015. Ascorbic acid production in root, nodule and Enterobacter spp.(Gammaproteobacteria) isolated from root nodule of the legume Abrus precatorius L. *Biocatalysis and Agricultural Biotechnology*, **4**, 127-134.

Gubry-Rangin, C., Garcia, M. and Béna, G. 2010. Partner choice in *Medicago truncatula–Sinorhizobium* symbiosis. *Proceedings of the Royal Society of London B: Biological Sciences*, **277**, 1947-1951.

Gustine, D. L. and Huff, D. R. 1999. Genetic variation within among white clover populations from managed permanent patures of the Northeastern USA. *Crop Science*, **39**, 524-530.

Gweon, H. S., Oliver, A., Taylor, J., Booth, T., Gibbs, M., Read, D. S., Griffiths, R. I. and Schonrogge, K. 2015. PIPITS: an automated pipeline for analyses of fungal internal transcribed spacer sequences from the Illumina sequencing platform. *Methods in Ecology and Evolution*, **6**, 973-980.

Hart, M. M. and Reader, R. J. 2002. Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. *New Phytologist*, **153**, 335-344.

Hartmann, A., Schmid, M., Van Tuinen, D. and Berg, G. 2009. Plant-driven selection of microbes. *Plant and Soil*, **321**, 235-257.

Hayat, R., Ali, S., Amara, U., Khalid, R. and Ahmed, I. 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. *Annals of Microbiology*, **60**, 579-598.

Higo, M., Isobe, K., Matsuda, Y., Ichida, M. and Torigoe, Y. 2015. Influence of sowing season and host crop identity on the community structure of arbuscular mycorrhizal fungi colonizing roots of two different gramineous and leguminous crop species. *Advances in Microbiology*, **5**, 107.

Hijikata, N., Murase, M., Tani, C., Ohtomo, R., Osaki, M. and Ezawa, T. 2010. Polyphosphate has a central role in the rapid and massive accumulation of phosphorus in extraradical mycelium of an arbuscular mycorrhizal fungus. *New Phytologist*, **186**, 285-289.

Hill, G., Mitkowski, N., Aldrich-Wolfe, L., Emele, L., Jurkonie, D., Ficke, A., Maldonado-Ramirez, S., Lynch, S. and Nelson, E. 2000. Methods for assessing the composition and diversity of soil microbial communities. *Applied Soil Ecology*, **15**, 25-36.

HIRSCH, A. M. 1992. Developmental biology of legume nodulation. *New Phytologist*, **122**, 211-237.

Ibáñez, F., Angelini, J., Taurian, T., Tonelli, M. L. and Fabra, A. 2009. Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria. *Systematic and Applied Microbiology*, **32**, 49-55.

İnceoğlu, Ö., Al-Soud, W. A., Salles, J. F., Semenov, A. V. and van Elsas, J. D. 2011. Comparative analysis of bacterial communities in a potato field as determined by pyrosequencing. *PLoS One*, **6**, e23321.

Jahufer, M., Barret, B., Griffiths, A. and Woodfield, D. 2003. DNA fingerprinting and genetic relationships among white clover cultivars. *In*: Proceedings of the New Zealand Grassland Association. New Zealand Grassland Association. **Vol. 65**. p 163-169.

Jahufer, M., Dunn, A., Baird, I., Ford, J., Griffiths, A., Jones, C., Woodfield, D. and Barrett, B. 2013. Genotypic variation for morphological traits in a white clover mapping population evaluated across two environments and three years. *Crop Science*, **53**, 460-472.

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K. and Barea, J.-M. 2003. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biology and Fertility of Soils*, **37**, 1-16.

Jin, H., Yang, X.-Y., Yan, Z.-Q., Liu, Q., Li, X.-Z., Chen, J.-X., Zhang, D.-H., Zeng, L.-M. and Qin, B. 2014. Characterization of rhizosphere and endophytic bacterial communities from leaves, stems and roots of medicinal *Stellera chamaejasme L. Systematic and Applied Microbiology*, **37**, 376-385.

Kaschuk, G., Kuyper, T. W., Leffelaar, P. A., Hungria, M. and Giller, K. E. 2009. Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? *Soil Biology and Biochemistry*, **41**, 1233-1244.

Kidd, P., Llugany, M., Poschenrieder, C., Gunse, B. and Barcelo, J. 2001. The role of root exudates in aluminium resistance and silicon-induced amelioration of aluminium toxicity in three varieties of maize (*Zea mays L.*). *Journal of Experimental Botany*, **52**, 1339-1352.

Kiers, E. T. and Denison, R. F. 2008. Sanctions, cooperation, and the stability of plant-rhizosphere mutualisms. *Annual review of Ecology, Evolution, and Systematics*, 215-236.

Kiers, E. T., Hutton, M. G. and Denison, R. F. 2007. Human selection and the relaxation of legume defences against ineffective rhizobia. *Proceedings of the Royal Society of London B: Biological Sciences*, **274**, 3119-3126.

Kirk, J. L., Beaudette, L. A., Hart, M., Moutoglis, P., Klironomos, J. N., Lee, H. and Trevors, J. T. 2004. Methods of studying soil microbial diversity. *Journal of Microbiological Methods*, **58**, 169-188.

Kloepper, J. W., Leong, J., Teintze, M. and Schroth, M. N. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature*, **286**, 885-886.

Kosslak, R. M., Bohlool, B. B., Dowdle, S. and Sadowsky, M. J. 1983. Competition of *Rhizobium japonicum* strains in early stages of soybean nodulation. *Applied and Environmental Microbiology*, **46**, 870-873.

Kumar Ghosh, P., Kumar Sen, S. and Kanti Maiti, T. 2015. Production and metabolism of IAA by *Enterobacter* spp. (Gammaproteobacteria) isolated from root nodules of a legume *Abrus precatorius* L. *Biocatalysis and Agricultural Biotechnology*, **4**, 296-303.

Laguerre, G., Nour, S. M., Macheret, V., Sanjuan, J., Drouin, P. and Amarger, N. 2001. Classification of rhizobia based on nodC and nifH gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. *Microbiology*, **147**, 981-993.

Lareen, A., Burton, F. and Schäfer, P. 2016. Plant root-microbe communication in shaping root microbiomes. *Plant Molecular Biology*, **90**, 575-587.

Lee, I.-M., Davis, R. E. and Gundersen-Rindal, D. E. 2000. Phytoplasma: phytopathogenic mollicutes. *Annual Reviews in Microbiology*, **54**, 221-255.

Lewis-Henderson, W. R. and Djordjevic, M. A. 1991. A cultivar-specific interaction between *Rhizobium leguminosarum* bv. trifolii and subterranean clover is controlled by nodM, other bacterial cultivar specificity genes, and a single recessive host gene. *Journal of Bacteriology*, **173**, 2791-2799.

Li, Y.-C., Korol, A. B., Fahima, T. and Nevo, E. 2004. Microsatellites within genes: structure, function, and evolution. *Molecular Biology and Evolution*, **21**, 991-1007.

Lindström, K., Murwira, M., Willems, A. and Altier, N. 2010. The biodiversity of beneficial microbe-host mutualism: the case of rhizobia. *Research in Microbiology*, **161**, 453-463.

Lowther, B. 2010. Clover Inoculation in New Zealand. Retrieved from http://www.4nutrition.co.nz/downloads/Rhizobia_Bill_Lowther%20report_FINAL_May_2010.pdf

Lowther, W. and Johnstone, P. 1978. Effect of strains of *Rhizobium trifolii* on the establishment of oversown white clover (Trifolium repens). *Soil Biology and Biochemistry*, **10**, 293-295.

Lowther, W. and Kerr, G. 2011. White clover seed inoculation and coating in New Zealand. Retrieved from https://www.grassland.org.nz/publications/nzgrassland_publication_1594.pdf

Lugtenberg, B. and Kamilova, F. 2009. Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology*, **63**, 541-556.

Magoč, T. and Salzberg, S. L. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, **27**, 2957-2963.

Maier, R. and Brill, W. J. 1976. Ineffective and non-nodulating mutant strains of Rhizobium japonicum. *Journal of Bacteriology*, **127**, 763-769.

Mather, R., Melhuish, D. and Herlihy, M. 1996. Trends in the global marketing of white clover cultivars. *Special Publication-Agronomy Society of New Zealand*, 7-14.

Mpepereki, S., Javaheri, F., Davis, P. and Giller, K. 2000. Soyabeans and sustainable agriculture: promiscuous soyabeans in southern Africa. *Field Crops Research*, **65**, 137-149.

Mühling, M., Woolven-Allen, J., Murrell, J. C. and Joint, I. 2008. Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. *The ISME Journal*, **2**, 379-392.

Mutch, L. A. and Young, J. P. W. 2004. Diversity and specificity of *Rhizobium leguminosarum* biovar *viciae* on wild and cultivated legumes. *Molecular Ecology*, **13**, 2435-2444.

Nangul, A., Moot, D. J., Brown, D., Ridgway, H. J. 2013. Nodule occupancy by *Rhizobium leguminosarum* strain WSM1325 following inoculation of four annual *Trifolium* species in Canterbury, New Zealand. *New Zealand Journam of Agricultural Research, **56**, 215-223*

Nakatsu, C. H., Torsvik, V. and Øvreås, L. 2000. Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. *Soil Science Society of America Journal*, **64**, 1382-1388.

Nall, V. 2010. Role of mycorrhizae in rhizosphere processes and phosphorus dynamics, (Doctoral dissertation thesis, Lincoln University, Christchurch, New Zealand). Retrieved from https://hdl.handle.net/10182/1803

Nocker, A., Sossa-Fernandez, P., Burr, M. D. and Camper, A. K. 2007. Use of propidium monoazide for live/dead distinction in microbial ecology. *Applied and Environmental Microbiology*, **73**, 5111-5117.

Oldroyd, G. E., Murray, J. D., Poole, P. S. and Downie, J. A. 2011. The rules of engagement in the legume-rhizobial symbiosis. *Annual Review of Genetics*, **45**, 119-144.

Oliveira, C. A., Sá, N. M., Gomes, E. A., Marriel, I. E., Scotti, M. R., Guimarães, C. T., Schaffert, R. E. and Alves, V. M. 2009. Assessment of the mycorrhizal community in the rhizosphere of maize (*Zea mays* L.) genotypes contrasting for phosphorus efficiency in the acid savannas of Brazil using denaturing gradient gel electrophoresis (DGGE). *Applied Soil Ecology*, **41**, 249-258.

Parker, M. P. 1995. Plant fitness variation caused by different mutualist genotypes. *Ecology*, **76**, 1525-1535.

Patwardhan, A., Ray, S. and Roy, A. 2014. Molecular Markers in Phylogenetic Studies-A Review. *Journal of Phylogenetics & Evolutionary Biology*.

Pereyra, G., Hartmann, H., Michalzik, B., Ziegler, W. and Trumbore, S. 2015. Influence of rhizobia inoculation on biomass gain and tissue nitrogen content of *Leucaena leucocephala* seedlings under drought. *Forests*, **6**, 3686-3703.

Pérez-Jaramillo, J. E., Mendes, R. and Raaijmakers, J. M. 2016. Impact of plant domestication on rhizosphere microbiome assembly and functions. *Plant Molecular Biology*, **90**, 635-644.

Philippot, L., Raaijmakers, J. M., Lemanceau, P. and Van Der Putten, W. H. 2013. Going back to the roots: the microbial ecology of the rhizosphere. *Nature Reviews Microbiology*, **11**, 789.

Powlson, D. S., Addiscott, T. M., Benjamin, N., Cassman, K. G., de Kok, T. M., van Grinsven, H., L'hirondel, J.-L., Avery, A. A. and Van Kessel, C. 2008. When does nitrate become a risk for humans? *Journal of Environmental Quality*, **37**, 291-295.

Qin, Y., Fu, Y., Dong, C., Jia, N. and Liu, H. 2016. Shifts of microbial communities of wheat (*Triticum aestivum* L.) cultivation in a closed artificial ecosystem. *Applied Microbiology and Biotechnology*, **100**, 4085-4095.

Rattray, P. V., By, C. and SFF, S. F. F. 2005. Clover management, research, development and extension in the New Zealand pastoral industries. *A review of R&D literature and R&D opportunities, extension initiatives and extension opportunities. Sustainable Farming Fund Report [ISBN 0-478-29827-7]*.

Ridgway, H., Kandula, J. and Stewart, A. 2006. Optimising the medium for producing arbuscular mycorrhizal spores and the effect of inoculation on grapevine growth. *New Zealand Plant Protection*, **59**, 338.

Robleto, E. A., Kmiecik, K., Oplinger, E. S., Nienhuis, J. and Triplett, E. W. 1998. Trifolitoxin production increases nodulation competitiveness of *Rhizobium etli* CE3 under agricultural conditions. *Applied and Environmental Microbiology*, **64**, 2630-2633.

Ryle, G., Arnott, R. and Powell, C. 1981. Distribution of dry weight between root and shoot in white clover dependent on N2 fixation or utilizing abundant nitrate nitrogen. *Plant and Soil*, **60**, 29-39.

Sánchez, A. C., Gutiérrez, R. T., Santana, R. C., Urrutia, A. R., Fauvart, M., Michiels, J. and Vanderleyden, J. 2014. Effects of co-inoculation of native *Rhizobium* and *Pseudomonas* strains on growth parameters and yield of two contrasting *Phaseolus vulgaris* L. genotypes under Cuban soil conditions. *European Journal of Soil Biology*, **62**, 105-112.

Shaffer, J. P., U'Ren, J. M., Gallery, R. E., Baltrus, D. A. and Arnold, A. E. 2017. An Endohyphal Bacterium (Chitinophaga, Bacteroidetes) Alters Carbon Source Use by *Fusarium keratoplasticum* (F. solani Species Complex, Nectriaceae). *Frontiers in Microbiology*, **8**.

Sharma, S., Aneja, M., Mayer, J., Munch, J. and Schloter, M. 2005. Characterization of bacterial community structure in rhizosphere soil of grain legumes. *Microbial Ecology*, **49**, 407-415.

Simms, E. L., Taylor, D. L., Povich, J., Shefferson, R. P., Sachs, J., Urbina, M. and Tausczik, Y. 2006. An empirical test of partner choice mechanisms in a wild legume–rhizobium interaction. *Proceedings of the Royal Society of London B: Biological Sciences*, **273**, 77-81.

Spaink, H. P. 2000. Root nodulation and infection factors produced by rhizobial bacteria. *Annual Review of Microbiology*, **54**, 257.

Suzaki, T. and Kawaguchi, M. 2014. Root nodulation: a developmental program involving cell fate conversion triggered by symbiotic bacterial infection. *Current Opinion in Plant Biology*, **21**, 16-22.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C. and Willerslev, E. 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, **21**, 2045-2050.

Tunney, M., Klem, E., Fodor, A., Gilpin, D., Moriarty, T., McGrath, S., Muhlebach, M., Boucher, R., Cardwell, C. and Doering, G. 2011. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. *Thorax*, **66**, 579-584.

Unkovich, M., Herridge, D., Peoples, M., Cadisch, G., Boddey, B., Giller, K., Alves, B. and Chalk, P. 2008. Measuring plant-associated nitrogen fixation in agricultural systems Australian Centre for International Agricultural Research (ACIAR)

Vandenkoornhuyse, P., Ridgway, K., Watson, I., Fitter, A. and Young, J. 2003. Co-existing grass species have distinctive arbuscular mycorrhizal communities. *Molecular Ecology*, **12**, 3085-3095.

Wagner, M., Amann, R., Lemmer, H. and Schleifer, K.-H. 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Applied and Environmental Microbiology*, **59**, 1520-1525.

Wagner, M. R., Lundberg, D. S., Tijana, G., Tringe, S. G., Dangl, J. L. and Mitchell-Olds, T. 2016. Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. *Nature Communications*, **7**, 12151.

Walker, T. and Woodfield, D. 1996. The value of N-fixation to pastoral agriculture in New Zealand. *Special Publication - Agronomy Society of New Zealand*, 115-118.

Wang, D., Yang, S., Tang, F. and Zhu, H. 2012. Symbiosis specificity in the legume–rhizobial mutualism. *Cellular Microbiology*, **14**, 334-342.

Weinert, N., Piceno, Y., Ding, G.-C., Meincke, R., Heuer, H., Berg, G., Schloter, M., Andersen, G. and Smalla, K. 2011. PhyloChip hybridization uncovered an enormous bacterial diversity in the rhizosphere of different potato cultivars: many

common and few cultivar-dependent taxa. *FEMS Microbiology Ecology*, **75**, 497-506.

Weir, B. 2006. Systematics, specificity, and ecology of New Zealand Rhizobia, (Doctoral dissertation thesis, University of Auckland, Auckland, New Zealand) retrieved from ResearchSpace@ Auckland

Werner, G. D. and Kiers, E. T. 2015. Partner selection in the mycorrhizal mutualism. *New Phytologist*, **205**, 1437-1442.

West, S., Kiers, E. T., Pen, I. and Denison, R. 2002. Sanctions and mutualism stability: when should less beneficial mutualists be tolerated? *Journal of Evolutionary Biology*, **15**, 830-837.

Wigley, K., Moot, D., Wakelin, S. A., Laugraud, A., Blond, C., Seth, k., Ridgway, H. 2017. Diverse bacterial taxa inhabit root nodules of lucerne (*Medicago stiva* L.) in New Zealand pastoral soils. *Plant and Soil.* **420**, 253-262

Woodfield, D. 1996. Breeding strategies for developing transgenic white clover cultivars. *Special Publication-Agronomy Society of New Zealand*, 125-130.

Xavier, L. J. and Germida, J. J. 2003. Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. *Soil Biology and Biochemistry*, **35**, 471-478.

Xiao, X., Chen, W., Zong, L., Yang, J., Jiao, S., Lin, Y., Wang, E. and Wei, G. 2017. Two cultivated legume plants reveal the enrichment process of the microbiome in the rhizocompartments. *Molecular Ecology*, **26**, 1641-1651.

Yeoh, Y. K., Dennis, P. G., Paungfoo-Lonhienne, C., Weber, L., Brackin, R., Ragan, M. A., Schmidt, S. and Hugenholtz, P. 2017. Evolutionary conservation of a core root microbiome across plant phyla along a tropical soil chronosequence. *Nature Communications*, **8**, 215.

Yu, L., Zhang, W., Liu, L. and Yang, J. 2015. Determining microeukaryotic plankton community around Xiamen Island, southeast China, using Illumina MiSeq and PCR-DGGE techniques. *PLoS One*, **10**, e0127721.

Zhu, Y.-G., Laidlaw, A., Christie, P. and Hammond, M. 2000. The specificity of arbuscular mycorrhizal fungi in perennial ryegrass–white clover pasture. *Agriculture, Ecosystems & Environment*, **77**, 211-218.

6 Appendices for Chapter 2

6.1 Data from "Improved forage legume-rhizobia performance" MBIE trial

Table 6.1 Mean shoot DWs *Rhizobium leguminosarum* strains on white clover cultivar Tribute from the MBIE trial. Strains with a * were tested on white clover cultivar S10.

Strain	DW (mg)
TA1	12.82
451	16.02
1302	19.15
316	15.53
S12N10 *	0.4
S26N9 *	1.1

6.2 Yeast Mannitol Agar

1 g yeast extract

4 g mannitol

0.5 g dipotassium phosphate

0.2 g magnesium sulphate

0.1 g sodium chloride

15 g agar

1 l water

Autoclave for 15 minutes at 121°C and 15 Psi

6.3 Water Agar

10 g Davis Agar

1 l water

Autoclave for 15 minutes at 121°C and 15 Psi

6.4 Yeast Mannitol Broth

1 g yeast extract

4 g mannitol

0.5 g dipotassium phosphate

0.2 g magnesium sulphate

0.1 g sodium chloride

1 l water

Autoclave for 15 minutes at 121°C and 15 Psi

6.5 McKnight's nutrient solution

Stock solutions (Unkovich *et al.*, 2008)	
Chemical	
Calcium Chloride	20g/l
Magnesium sulfate	400g/l
Potassium dihydrogen orthophosphate	100g/l
Potassium chloride	150g/l
Trace elements stock solution	
-Boric acid	2.86g
-Manganese sulfate	2.03g
-Zinc sulfate	0.222g
-Copper sulfate	0.079g
-Molybdic acid	0.09g
Added together and diluted to 1l	
EDTA stock solution (stored in dark)	
-Ethylene diamine tetra-acetic, sodium salt (EDTA	2g
-60% wt/vol ferric chloride solution	16.8 ml
Added together and diluted to 1l	
Sodium hydroxide	40g/l
Ammonium nitrate	8g/l

Each stock solution was prepared by being dissolved in distilled water and autoclaved for 15 minutes at 121°C and 15 Psi

McKnight's Min N (for 1l working solution)	
Calcium chloride	0.25ml
Magnesium sulfate	0.25ml
Potassium dihydrogen orthophosphate	1ml
Potassium chloride	1ml
Trace elements stock solution	0.5ml
EDTA stock solution	0.75ml
Sodium hydroxide	0.25ml
Ammonium nitrate	1ml

Diluted in 1 l distilled water autoclaved for 15 minutes at 121°C and 15 Psi

McKnight's insoluble P altered recipe (for 1 l working solution)

Same as McKnight's Min N, but Potassium dihydrogen orthophosphate replaced with 123.1 mg hydroxyapatite.

6.6 Experiment 1 ANOVA

6.6.1 ANOVA result of the effect of six *Rhizobium leguminosarum* treatment on the dry weight of white clover cultivar Huia

Source	DF	SS	MS	F	P
Treatment	5	1.068×10^{-04}	2.137×10^{-05}	2.99	0.052
Residual	13	9.279×10^{-05}	7.138×10^{-06}		
Total	22	1.935×10^{-04}			

6.6.2 ANOVA result of the effect of six *Rhizobium leguminosarum* treatments on the dry weight of white clover cultivar Tribute

Source	DF	SS	MS	F	P
Treatment	5	$1.762x10^{-04}$	$3.523x10^{-05}$	4.05	0.059
Residual	6	$5.217x10^{-05}$	$8.695x10^{-06}$		
Total	15	$2.212x10^{-04}$			

6.6.3 ANOVA result of the effect of six *Rhizobium leguminosarum* treatment on the dry weight of white clover cultivar Irrigation

Source	DF	SS	MS	F	P
Treatment	5	$2.168x10^{-04}$	$4.335x10^{-05}$	3.68	0.038
Residual	10	$1.178x10^{-04}$	$1.178x10^{-05}$		
Total	19	$2.429x10^{-04}$			

6.6.4 ANOVA result of the effect of six *Rhizobium leguminosarum* treatment on the dry weight of white clover cultivar Crusader

Source	DF	SS	MS	F	P
Treatment	5	$4.071x10^{-05}$	$8.142x10^{-06}$	1.83	0.160
Residual	17	$7.558x10^{-05}$	$4.446x10^{-06}$		
Total	26	$1.168x10^{-04}$			

6.6.5 ANOVA result of the effect of six *Rhizobium leguminosarum* treatment on the dry weight of white clover cultivar Louisiana

Source	DF	SS	MS	F	P
Treatment	5	2.482×10^{-05}	4.964×10^{-06}	4.11	0.013
Residual	17	2.055×10^{-05}	1.209×10^{-06}		
Total	26	4.769×10^{-05}			

6.6.6 ANOVA result of the effect of six *Rhizobium leguminosarum* treatment on the dry weight of white clover cultivar Kopu II

Source	DF	SS	MS	F	P
Treatment	5	8.927×10^{-05}	1.785×10^{-05}	2.22	0.114
Residual	13	1.044×10^{-04}	8.033×10^{-06}		
Total	22	2.215×10^{-04}			

6.6.7 ANOVA result of the effect of six *Rhizobium leguminosarum* treatment on the number of nodules formed on white clover

Source	DF	SS	MS	F	P
Cultivar	5	252	50.45	1.87	0.321
Treatment	5	776	155.14	3.650	<0.001
Cultivar x Treatment	25	1720	68.07	1.601	<0.001
Residuals	104	4421	42.51		

6.7 Experiment 2 ANOVA and Sample proportions test

6.7.1 ANOVA result showing the effect of positive control, negative control, and *Rhizobium leguminosarum* mixture on the dry weight of white clover

Source	DF	SS	MS	F	P
Cultivar	5	5.560×10^{-4}	1.110×10^{-4}	2.602	<0.001
Treatment	2	0.0172	0.0086	201.391	<0.001
Cultivar x Treatment	10	1.310×10^{-4}	7.300×10^{-5}	1.710	0.1001
Residuals	58	0.002480	4.300×10^{-4}		

6.7.2 Sample proportions test – Huia

6.7.2.1 Huia – "Highly effective" strains

```
data:  29 out of 47, null probability 0.16
X-squared = 69.6809, df = 1, p-value < 2.2e-16
```

6.7.2.2 Huia – "Less effective" strains

```
data:  18 out of 47, null probability 0.83
X-squared = 63.4317, df = 1, p-value = 1.66e-15
```

6.7.3 Sample proportions test – Tribute

6.7.3.1 Tribute – "Highly effective" strains

```
data:  5 out of 50, null probability 0.16
X-squared = 0.9301, df = 1, p-value = 0.3348
```

6.7.3.2 Tribute – "Less effective" strains

```
data:  45 out of 50, null probability 0.83
X-squared = 1.2757, df = 1, p-value = 0.2587
```

6.7.4 Sample proportions test – Irrigation

6.7.4.1 Irrigation – "Highly effective" strains

```
data:  7 out of 36, null probability 0.16
X-squared = 0.1132, df = 1, p-value = 0.7366
```

6.7.4.2 Irrigation – "Less effective" strains

```
data:  29 out of 36, null probability 0.83
X-squared = 0.0284, df = 1, p-value = 0.8661
```

6.7.5 Sample proportions test – Louisiana

6.7.5.1 Louisiana – "Highly effective" strains

```
data:  23 out of 43, null probability 0.16
X-squared = 42.2177, df = 1, p-value = 8.166e-11
```

6.7.5.2 Louisiana– "Less effective" strains

```
data:  20 out of 43, null probability 0.83
X-squared = 38.0295, df = 1, p-value = 6.968e-10
```

6.8 Experiment 3 – AMF

6.8.1 ANOVA results showing the effect of single and mixed AMF treatment on 6 cultivars of white clover

Source	DF	SS	MS	F	P
Cultivar	5	0.00126	2.519×10^{-4}	1.886	0.101
Treatment	5	4.030×10^{-4}	8.058×10^{-5}	0.603	0.698
Cultivar x Treatment	25	0.00335	1.338×10^{-4}	1.002	0.470
Residuals	135	0.0180	1.336×10^{-4}		

7 Appendices for Chapter 3

7.1 Soil test report

Hill Laboratories
BETTER TESTING BETTER RESULTS

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Hamilton 3240, New Zealand

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Fax +64 7 858 2001
Email mail@hill-labs.co.nz
Web www.hill-labs.co.nz

ANALYSIS REPORT Page 1 of 5

Client:	Lincoln University	**Lab No:**	1651796	shpv1
Address:	C/- Agriculture & Life Sciences Division	**Date Received:**	21-Sep-2016	
	PO Box 84	**Date Reported:**	27-Sep-2016	
	Lincoln 7647	**Quote No:**	80465	
	CANTERBURY	**Order No:**	LU436455	
		Client Reference:		
Phone:	03 325 3803	**Submitted By:**	Celine Blond	

Sample Name: H18 **Lab Number:** 1651796.1
Sample Type: SOIL Mixed Pasture (S1)

Analysis		Level Found	Medium Range	Low / Medium / High
pH	pH Units	6.0	5.8 - 6.2	
Olsen Phosphorus	mg/L	10	20 - 30	
Potassium	me/100g	0.43	0.40 - 0.60	
Calcium	me/100g	4.5	4.0 - 10.0	
Magnesium	me/100g	0.65	1.00 - 1.60	
Sodium	me/100g	0.12	0.20 - 0.50	
CEC	me/100g	10	12 - 25	
Total Base Saturation	%	56	50 - 85	
Volume Weight	g/mL	1.11	0.60 - 1.00	
Aluminium ($CaCl_2$ Extractable)	mg/kg	1.2	0.0 - 3.0	
Base Saturation %		K 4.3	Ca 44	Mg 6.4 Na 1.2
MAF Units		K 10	Ca 6	Mg 16 Na 6

The above nutrient graph compares the levels found with reference interpretation levels. NOTE: It is important that the correct sample type be assigned, and that the recommended sampling procedure has been followed. R J Hill Laboratories Limited does not accept any responsibility for the resulting use of this information. IANZ Accreditation does not apply to comments and interpretations, i.e. the 'Range Levels' and subsequent graphs.

7.2 Experiment 4 ANOVA

7.2.1 ANOVA result showing the effect of white clover cultivar on DW for plants grown in soil

Source	DF	SS	MS	F	P
Treatment	5	121774	24355	1.075	0.386
Residual	48	1087229	22651		

7.2.2 ANOVA result showing the effect of white clover cultivar on DW for plants grown in soil

Source	DF	SS	MS	F	P
Treatment	5	63658	12732	0.122	0.987
Residual	45	4701804	104485		

7.2.3 Barcoded HPLC purified primers used for Illumina sequencing of 16S rRNA

Type (barcode + linker + direction)	Code	Primers (5'-3') (Barcode+linker+Primer)
2 + F341	2F	CGATGTGACCTAYGGGRBGCASCAG
5 + F341	5F	ACAGTGGACCTAYGGGRBGCASCAG
17 + F341	17F	GTAGAGGACCTAYGGGRBGCASCAG
36 + F341	36F	CCAACAGACCTAYGGGRBGCASCAG
38 + F341	38F	CTAGCTGACCTAYGGGRBGCASCAG
44 + F341	44F	TATAATGACCTAYGGGRBGCASCAG
14 + R806	14R	AGTTCCGTGGACTACNNGGGTATCTAAT
26 + R806	26R	ATGAGCGTGGACTACNNGGGTATCTAAT
37 + R806	37R	CGGAATGTGGACTACNNGGGTATCTAAT
1 + R806	1R	ATCACGGTGGACTACNNGGGTATCTAAT
11 + R806	11R	GGCTACGTGGACTACNNGGGTATCTAAT
3 + R806	2R	TTAGGCGTGGACTACNNGGGTATCTAAT

7.2.4 List of white clover root and rhizosphere samples and barcode combination used

Cultivar	Location	Replicate	Forward Barcode	Reverse barcode	Qubit reading (ng/ml)
Huia	Root	1	2F	1R	189
Huia	Root	2	2F	3R	148
Huia	Root	3	2F	11R	86.1
Tribute	Root	1	2F	14R	119
Tribute	Root	2	2F	26R	0
Tribute	Root	3	2F	37R	186
Irrigation	Root	1	3F	1R	257
Irrigation	Root	2	3F	3R	186
Irrigation	Root	3	3F	11R	242
Crusader	Root	1	3F	14R	335
Crusader	Root	2	3F	26R	154
Crusader	Root	3	3F	37R	183
Louisiana	Root	1	17F	1R	67.5
Louisiana	Root	2	17F	3R	167
Louisiana	Root	3	17F	11R	115
Kopu II	Root	1	17F	14R	54
Kopu II	Root	2	17F	26R	160
Kopu II	Root	3	17F	37R	13.5
Huia	Rhizosphere	1	36F	1R	58.3
Huia	Rhizosphere	2	36F	3R	33
Huia	Rhizosphere	3	36F	11R	24.3
Tribute	Rhizosphere	1	36F	14R	52.7
Tribute	Rhizosphere	2	36F	26R	75.7

Tribute	Rhizosphere	3	36F	37R	62.8
Irrigation	Rhizosphere	1	38F	1R	143
Irrigation	Rhizosphere	2	38F	3R	93.4
Irrigation	Rhizosphere	3	38F	11R	97
Crusader	Rhizosphere	1	38F	14R	166
Crusader	Rhizosphere	2	38F	26R	154
Crusader	Rhizosphere	3	38F	37R	116
Louisiana	Rhizosphere	1	44F	1R	110
Louisiana	Rhizosphere	2	44F	3R	137
Louisiana	Rhizosphere	3	44F	11R	91.8
Kopu II	Rhizosphere	1	44F	14R	73.7
Kopu II	Rhizosphere	2	44F	26R	124
Kopu II	Rhizosphere	3	44F	37R	93.1

7.2.5 Script for NGS analysis

```
!/bin/bash
# join pair end read
/home/laugrauda/FLASH-1.2.11/./flash Tube_2_Bac_1.clean.fq.gz
Tube_2_Bac_2.clean.fq.gz 2>&1 | tee flash.log
# demultiplex
flexbar -b ../barcodeF.txt -r out.extendedFrags.fastq -bo 6 -bu -be LEFT_TAIL -t F --threads
12 -o > forward.flexbarout
for i in F_barcode*.fasta
do
#assign barcodes
flexbar -b ../barcodeR.txt -r $i -bo 6 -bu -be RIGHT_TAIL -t $i --threads 10 -o >
R.$i.flexbarout
done

cat F_barcode_2.fasta_barcode_1Reversed.fasta
F_barcode_1.fasta_barcode_2Reversed.fasta > HuiaS1.fasta
cat F_barcode_2.fasta_barcode_3Reversed.fasta
F_barcode_3.fasta_barcode_2Reversed.fasta > HuiaS2.fasta
cat F_barcode_2.fasta_barcode_11Reversed.fasta
F_barcode_11.fasta_barcode_2Reversed.fasta > HuiaS3.fasta
cat F_barcode_2.fasta_barcode_14Reversed.fasta
F_barcode_14.fasta_barcode_2Reversed.fasta > TributeS1.fasta
```

```
cat F_barcode_2.fasta_barcode_26Reversed.fasta
F_barcode_26.fasta_barcode_2Reversed.fasta > TributeS2.fasta
cat F_barcode_2.fasta_barcode_37Reversed.fasta
F_barcode_37.fasta_barcode_2Reversed.fasta > TributeS3.fasta
cat F_barcode_5.fasta_barcode_1Reversed.fasta
F_barcode_1.fasta_barcode_5Reversed.fasta > IrrigationS1.fasta
cat F_barcode_5.fasta_barcode_3Reversed.fasta
F_barcode_3.fasta_barcode_5Reversed.fasta > IrrigationS2.fasta
cat F_barcode_5.fasta_barcode_11Reversed.fasta
F_barcode_11.fasta_barcode_5Reversed.fasta > IrrigationS3.fasta
cat F_barcode_5.fasta_barcode_14Reversed.fasta
F_barcode_14.fasta_barcode_5Reversed.fasta > CrusaderS1.fasta
cat F_barcode_5.fasta_barcode_26Reversed.fasta
F_barcode_26.fasta_barcode_5Reversed.fasta > CrusaderS2.fasta
cat F_barcode_5.fasta_barcode_37Reversed.fasta
F_barcode_37.fasta_barcode_5Reversed.fasta > CrusaderS3.fasta
cat F_barcode_17.fasta_barcode_1Reversed.fasta
F_barcode_1.fasta_barcode_17Reversed.fasta > LousianaS1.fasta
cat F_barcode_17.fasta_barcode_3Reversed.fasta
F_barcode_3.fasta_barcode_17Reversed.fasta > LousianaS2.fasta
cat F_barcode_17.fasta_barcode_11Reversed.fasta
F_barcode_11.fasta_barcode_17Reversed.fasta > LousianaS3.fasta
cat F_barcode_17.fasta_barcode_14Reversed.fasta
F_barcode_14.fasta_barcode_17Reversed.fasta > Kopu2S1.fasta
cat F_barcode_17.fasta_barcode_26Reversed.fasta
F_barcode_26.fasta_barcode_17Reversed.fasta > Kopu2S2.fasta
cat F_barcode_17.fasta_barcode_37Reversed.fasta
F_barcode_37.fasta_barcode_17Reversed.fasta > Kopu2S3.fasta
cat F_barcode_36.fasta_barcode_1Reversed.fasta
F_barcode_1.fasta_barcode_36Reversed.fasta > HuiaR1.fasta
cat F_barcode_36.fasta_barcode_3Reversed.fasta
F_barcode_3.fasta_barcode_36Reversed.fasta > HuiaR2.fasta
cat F_barcode_36.fasta_barcode_11Reversed.fasta
F_barcode_11.fasta_barcode_36Reversed.fasta > HuiaR3.fasta
cat F_barcode_36.fasta_barcode_14Reversed.fasta
F_barcode_14.fasta_barcode_36Reversed.fasta > TributeR1.fasta
cat F_barcode_36.fasta_barcode_26Reversed.fasta
F_barcode_26.fasta_barcode_36Reversed.fasta > TributeR2.fasta
cat F_barcode_36.fasta_barcode_37Reversed.fasta
F_barcode_37.fasta_barcode_36Reversed.fasta > TributeR3.fasta
cat F_barcode_38.fasta_barcode_1Reversed.fasta
F_barcode_1.fasta_barcode_38Reversed.fasta > IrrigationR1.fasta
cat F_barcode_38.fasta_barcode_3Reversed.fasta
F_barcode_3.fasta_barcode_38Reversed.fasta > IrrigationR2.fasta
cat F_barcode_38.fasta_barcode_11Reversed.fasta
F_barcode_11.fasta_barcode_38Reversed.fasta > IrrigationR3.fasta
```

```
cat F_barcode_38.fasta_barcode_14Reversed.fasta
F_barcode_14.fasta_barcode_38Reversed.fasta > CrusaderR1.fasta
cat F_barcode_38.fasta_barcode_26Reversed.fasta
F_barcode_26.fasta_barcode_38Reversed.fasta > CrusaderR2.fasta
cat F_barcode_38.fasta_barcode_37Reversed.fasta
F_barcode_37.fasta_barcode_38Reversed.fasta > CrusaderR3.fasta
cat F_barcode_44.fasta_barcode_1Reversed.fasta
F_barcode_1.fasta_barcode_44Reversed.fasta > LousianaR1.fasta
cat F_barcode_44.fasta_barcode_3Reversed.fasta
F_barcode_3.fasta_barcode_44Reversed.fasta > LousianaR2.fasta
cat F_barcode_44.fasta_barcode_11Reversed.fasta
F_barcode_11.fasta_barcode_44Reversed.fasta > LousianaR3.fasta
cat F_barcode_44.fasta_barcode_14Reversed.fasta
F_barcode_14.fasta_barcode_44Reversed.fasta > Kopu2R1.fasta
cat F_barcode_44.fasta_barcode_26Reversed.fasta
F_barcode_26.fasta_barcode_44Reversed.fasta > Kopu2R2.fasta
cat F_barcode_44.fasta_barcode_37Reversed.fasta
F_barcode_37.fasta_barcode_44Reversed.fasta > Kopu2R3.fasta
```

#qiime workflow

#rename sequence and concatenate file

```
for i in *[RS][1-3].fasta
do
perl /dataset/Wakelin_burkholderia/archive/john/data_release/raw_data/parse.pl $i
qiimeInput.fasta
done
```

#Processing steps through to OTU table

```
pick_de_novo_otus.py -i qiimeInput.fasta -o otus
```

#remove OUT belonged to chloroplast, mitochondria, and unassigned

```
filter_taxa_from_otu_table.py -i out_table.biom -o filtered_otu.biom -n
c__Chloroplast,f__mitochondria,p__unassigned
```

#filter OUT's with <10 reads

```
./usearch -otutab_trim filtered_otu.txt -min_otu_size 10 -output trimmed.txt
```

#Run alpha diversity analysis

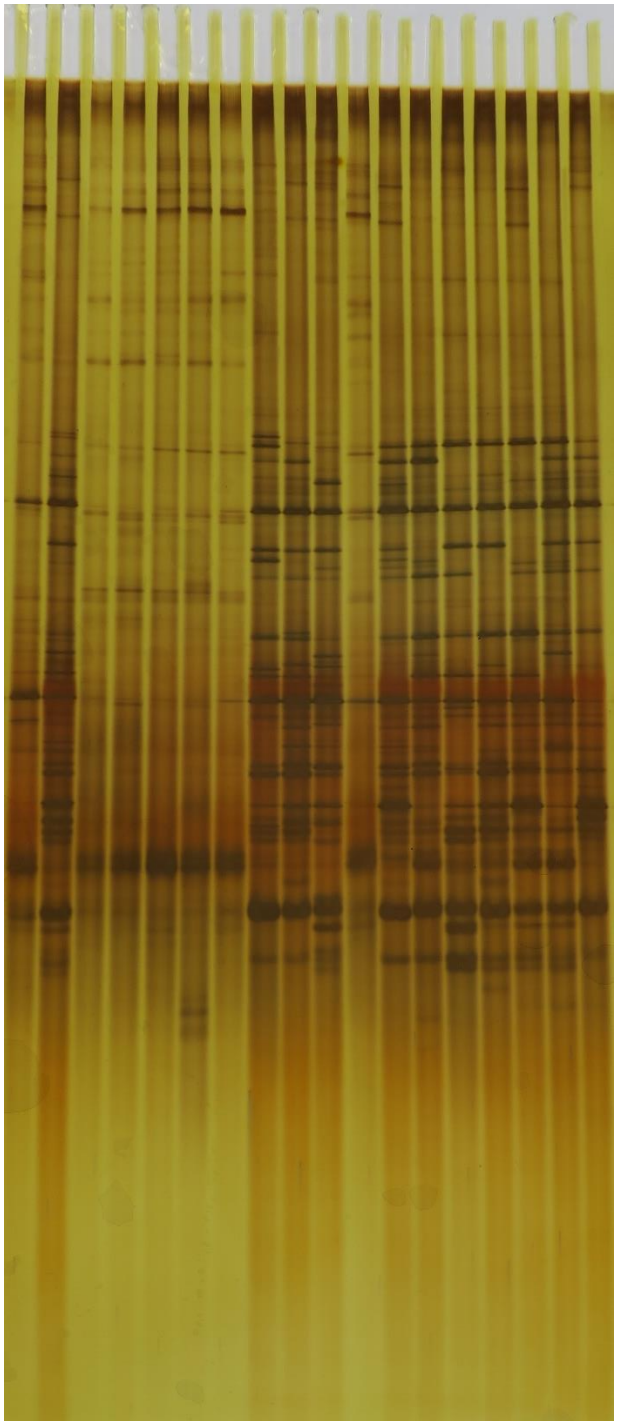
```
./usearch -alpha_div trimmed.txt -output alpha.txt
```

7.2.6 Fungal 18S Illumina sequencing

A library of the fungal internally transcribed spacer 2 (ITS2) was constructed utilizing the fITS7 as forward (5'-GTGARTCATCGAATCTTTG-3'), and ITS4 as reverse (5'-TCCTCCGCTTATTGATATGC-3') primers which annealed to the 5·8S and LSU rRNA genes, respectively (Gweon *et al.*, 2015). These primers had been extended to include a unique barcode and Illumina cell flow adaptors. The following PCR conditions were used: initial denaturation of 30 s at 95˚C, followed by 30 cycles of 30 s at 95˚C, 30 s at 52˚C and 2 min at 72˚C with a final extension of 10 min at 72˚C.

Although these samples passed the quality control at the sequencing facility, it generated a very small amount of poor data which was not used for further analysis or included in this thesis.

7.2.7 DGGE gel of AMF communities



Lanes from left to right: Huia 1, Huia 2, Huia 3, Tribute 1, Tribute 2, Tribute 3, Irrigation 1, Irrigation 2, Irrigation 3, Crusader 1, Crusader 2, Crusader 3, Louisiana 1, Louisiana 2, Louisiana 3, Kopu 1, Kopu 2, Kopu 3.

7.3 Experiment 5 ANOVA

7.3.1 ANOVA result showing the effect of rhizobia strains isolated from cultivars Huia and Tribute on DW of both cultivars

Source	DF	SS	MS	F	P
Cultivar	1	231.48	231.48	15.72	<0.001
Treatment	11	1088.89	98.99	6.72	<0.001
Cultivar x Treatment	11	225.62	20.51	1.39	0.179
Residuals	190	2797.30	14.72		

7.3.2 ANOVA result showing the effect of rhizobia strains isolated from cultivars Huia and Tribute on DW of Huia

Source	DF	SS	MS	F	P
Treatment	11	851.46	77.41	5.20	<0.001
Residual	77	1147.27	14.90		
Total	102	1772.26			

7.3.3 ANOVA result showing the effect of rhizobia strains isolated from cultivars Huia and Tribute on DW of Tribute

Source	DF	SS	MS	F	P
Treatment	11	615.82	55.98	3.99	<0.001
Residual	99	1389.18	14.03		
Total	124	2098.38			