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The Potential Growth Promotion on Barley and Changes in Rhizosphere Bacterial Community Structure Introduced by HUP- Nodules of Different Pulse Crops

by

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ABSTRACT

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In this study, all inoculated pulse crops formed nodules that release H_2 to the surrounding soil (HUP- nodule). The soil around HUP- nodules showed a higher H_2 uptake rate and CO_2 fixation. The barley inoculated with soil adjacent to HUP- pulse nodules tended to have higher shoot and root growth, particularly for the barley inoculated with soil around the HUP- nodules of faba beans and dry beans. The HUP- nodules triggered alteration in the soil bacterial community. The greatest changes of bacterial community structure were also evidenced in the soil around the HUP- nodules of faba beans and dry beans. The profound changes in the bacterial community structure could be the reasons for increased growth seen on barley plants inoculated with soil around the HUP- nodules of faba beans and dry beans at a lower nitrogen level. Therefore, pulse production could potentially have positive effects on soil health and the growth of succeeding crops.

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1. GENERAL INTRODUCTION

1.1 Crop Rotation with Legumes

1.1.1 The History and Importance of Crop Rotation

Crop rotation is an ancient, but effective farming practice. It can be dated back to more than 2000 years ago in China, Greece and Rome (MacRae and Mehuys, 1985; White, 1970). In a crop rotation system, a series of crops that belong to different plant families are planted in a certain order on the same piece of land. A typical crop rotation system usually involves alternate planting of some legume species, such as soybean, alfalfa and clover, with some plants of the grass family, like corn, wheat or barley.

This farming practice was commonly used by farmers because it was well known for improving the yield of the subsequent crops compared to a monocropping system (Dabney *et al.*, 1988; Edwards *et al.*, 1988; Meese *et al.*, 1991). Studies show that maize, in a two-year rotation with soybean, had a 5 to 20% higher yield than continuous maize. Even more yield can be achieved by extending the rotation more than two years (Peterson and Varvel, 1989; Crookston *et al.*, 1991). In addition, Fyson and Oaks (1990) suggested that crop rotation not only increases the yield of succession crops, but also improves the growth of the crop plant itself.

Crop rotation practice has declined sharply since synthetic chemical fertilizers were put in use. Today, as the cost of fertilizer rises and adverse effects of fertilizers on the environment, such as water pollution caused by agricultural run-off, becomes more and

more apparent, crop rotation is again being considered and employed as an alternative by farmers (Crookston *et al.*, 1991; Bullock, 1992; Mitchell *et al.*, 1991).

1.1.2 Utilizing Legumes in Crop Rotation and Nitrogen Fixation

Fabaceae, commonly known as legume, is one of the largest flowering plant families, which contains about 18,000 species in 650 different genera, and they grow in both temperate and tropical areas (Sprent, 2001). It is well known that legume soil can increase the yield of other crops in a crop rotation system. This phenomenon was studied intensively from the 1940s to the 1970s. The studies reveal that legumes can perform biological nitrogen fixation through their root nodules. In legumes' root nodules, nitrogen-fixing bacteria converts the nitrogen gas from the air into ammonia, a substance that plants can absorb. Nitrogen is an essential nutrient for plant growth, and the amount of nitrogen in soil is usually very limited. Nitrogen fixation allows legume plants to grow better by supplying plants with extra nitrogen (Sprent, 2001; Hogh-Jenson and Schjoerring, 2001; Roper, 1983).

At the same time, the crops that grow around legumes also benefit from nitrogen fixation. In a two-year grass and legume mixed cropping system, approximately 36% of the nitrogen needed for the grass plants comes from the nitrogen fixation of the legumes (Auburn, 1998). Some of this fixed nitrogen will be released into the soil through bacterial decomposition when legumes are harvested. The residual nitrogen in soil will later help with the growth of the succeeding crops. In general, about two thirds of the nitrogen that is fixed in legumes' root nodules will become available for the growth of

later plants (Auburn, 1998). This is believed to be the main reason that legumes have been commonly used in crop rotation practice.

1.1.3 Hydrogen Gas as a By-product of Nitrogen Fixation and Its Metabolism in Soil

Nitrogen fixation counts as an important part of rotation benefits, but alone it cannot fully explain all of the positive effects of using legumes in a crop rotation system (Baldock, *et al.*, 1981; Copeland and Crookston, 1992). Some studies have quantified the left-over nitrogen from nitrogen fixation as only 25% of the crop rotation benefits of legumes (Bolton *et al.*, 1976; Fyson and Oaks, 1990). Since then, many researchers have conducted studies to search for the explanations of the remaining 75% of the legume rotation benefit.

Other possible factors that could contribute to the rotation benefits of legume plants have been proposed, such as improving soil structure, diversifying soil microbial communities, enhancing soil water-holding capacity, and breaking pest and pathogen cycles (Bullock, 1992; Lugtenberg *et al.*, 1991; Doran and Smith, 1987; Tisdall and Oades, 1982; Regnier and Janke, 1990). However, for most of those proposed factors, the underlying mechanisms are not completely understood, and the rotation benefit cannot be well-explained by any one, or any combination, of these factors.

During studies in 1970s, hydrogen gas (H_2) was found as an obligate by-product of the biological nitrogen fixation process in legumes' root nodules (Schubert and Evans 1976). Since H_2 is an energy-rich gas, this H_2 production costs about 35% of the reducing power and ATP flow that goes into nitrogen-fixing enzymes and this energy expense is

equivalent to about 5% of a plant's daily net photosynthesis gain (Hunt and Layzell, 1993; Dong and Layzell, 2002). In some legume systems, the symbiotic bacteria (rhizohia) employ an H_2 uptake system, which is able to re-oxidize almost all of the H_2 involved and recover most of the energy. This kind of legume system is usually designated as HUP+ symbioses (Uratsu *et al.*, 1982; Evans *et al.*, 1987).

Studies of the H_2 oxidation in soil have found that since the H_2 produced by nitrogen fixation is released into the soil, no detectable amount of H_2 is detectable on the soil surface (Conard and Seiler, 1979). Most H_2 was oxidized by microbes and free enzymes in the soil within about 3-4cm around legume nodules (La Favre and Focht, 1983). Some recent studies have linked the H_2 from nitrogen fixation to plant growth promotion, and it could count as part of the benefit to having legumes in crop rotation systems (McLearn and Dong, 2002; Dong *et al.* 2003).

After H_2 is released from legume nodules, the soil adjacent to the nodules develops the capacity to take up H_2 within 8 to 10 days. Several obvious changes are associated with this, such as higher H_2 oxidation kinetics (La Favre and Focht, 1983), and an increase of rhizospheric microbial biomass in the H_2 rich soil around nodules (Popelier *et al.*, 1985).

Dong and Layzell (2001) also examined the gas exchanges in H_2 treated soil. They found that oxygen uptake increased while carbon dioxide evolution decreased with increasing H_2 concentration. About 60% of the electrons from the H_2 were transferred to oxygen, and the other 40% of the reducing power went to CO_2 fixation. These results indicated that the H_2 is utilized by some agents or organisms in the soil and fix CO_2

(Dong and Layzell, 2001, 2002). The soil H_2 uptake rate was measured over 3 weeks of H_2 treatment. The soil H_2 uptake rate increased very slowly at the beginning, then increased sharply until all the H_2 was consumed and finally levelled off by the end of week three (Dong and Layzell, 2001). This H_2 uptake curve suggested that certain microbial populations could be increased in the H_2 rich soil which caused the rise of soil H_2 uptake activity.

To determine the nature of the soil H_2 uptake phenomenon, antibiotic and fungicide treatments were applied to the H_2 treated soil (McLearn and Dong, 2002). This test revealed that the organism responsible for the increasing H_2 uptake rate of H_2 treated soil, or soil adjacent to nodules, was bacteria rather than fungi (McLearn and Dong, 2002; Irvine *et al.*, 2004).

In the same study, they also found that the H_2 treated soil lost its H_2 uptake ability when the physical structure of the soil was damaged. In addition, the H_2 uptake ability of soil recovered by providing H_2 gas to the soil after the disturbance (McLearn and Dong, 2002). The phenomenon indicated that the bacteria responsible for H_2 uptake needs a special colonial structure to grow and function that could consist of long filamentous bacterial cells, such as actinomycetes (McLearn and Dong, 2002). Another study conducted by Dean (2004) found that diverse white spots in soil, that contain groups of bacterial colonies, increased in H_2 treated soil, and the soil with white spots had a much higher H_2 uptake rate compared to the control soil. These studies strongly suggested that soil H_2 oxidizing bacterial activity is the cause of the increasing H_2 uptake ability of H_2 treated soil.

1.2 Plant-Microbe Interaction in Soil

1.2.1 Diversity of Soil Microbes

As an essential part of the terrestrial ecosystem, soil is an incredibly complex and dynamic system itself. It provides habitats for a wide range of microorganisms and some low level eukaryotes, which includes bacteria, fungi, algae, protozoa, nematode, earthworms and many other small insects. One gram of soil can host thousands of different species (Pankhurst *et al.*, 1996; Bollon *et al.*, 1992). Soil microbes are often considered to play a key role in soil health and productivity because they are involved in almost every cycle and function in soil studied so far (Pankhurst *et al.*, 1996; Vessey, 2003).

The soil cycles and functions, such as: soil structure formation; decomposition of organic matter; different biochemical cycles of main elements (carbon, nitrogen, phosphorus, potassium etc.) and trace elements (iron, nickel, mercury etc.); energy and nutrient exchange; plant growth regulator metabolism; soil born diseases and so on, are crucial to plants that grow in the soil (Wall and Virginia, 1999, Arias *et al.*, 2005; Doran *et al.*, 1996; Glick *et al.*, 1998). Despite the fact that many soil cycles and functions depend on soil microbes, there is still a debate about whether the whole biodiversity of microbial species needs to be maintained in order to preserve the integrity and long-term sustainability of a soil ecosystem (Altieri, 1995; Pankhurst *et al.*, 1996; Wardle, 2002). The argument is that because the soil microbial species are extremely diverse, there is a built-in "functional redundancy" (Bianchi and Bianchi, 1995; Bardgett and Shine, 1999). In other words, the diversity of microbial species and their associated functions in soil

exceed the requirement of soil ecosystem maintenance and their functional stability (Pankhurst, 1996).

Soil microbial biodiversity studies often encounter the difficulty of recovery and identification of suitable taxonomic units to describe species (O'Donnell *et al.*, 1994; Pankhurst *et al.*, 1996). Therefore, there is little we know about the soil microbial community structures.

1.2.2 Plant-microbe Interaction and Plant Growth Promotion Bacteria in Rhizosphere

Although the majority of soil bacteria are unknown to us, the soil microbial community structure around plant roots have been studied for a long period of time. The well known factor about rhizosphere is that its microbial communities are distinctly different from bulk soil without plant roots, and rhizospheric microbial diversity is often more extensive (Giri *et al.*, 2005).

The rhizosphere is usually defined as the volume of the soil that is adjacent to and affected by the plant roots (Mantelin and Touraine, 2004). The vast surface area of plant roots provides a highly diverse habitat for a variety of microorganisms in the soil. The plant roots absorb water and soluble mineral nutrients from soil and release carbon-rich material, such as sugars, amino acids, fatty acids, sterols, vitamins and other organic chemicals into the surrounding soil (Rovira, 1979; Curl and Truelove, 1986). The organic compounds released from plant roots enrich the nutrients and soluble carbon which then facilitate the growth of microbial population in the rhizosphere (Norton and Firestone, 1991).

Besides the additional organic compound input, the activities of plant root can also influence the level of water potential, pH, oxygen content and redox potential in rhizospheric soil. Subsequently, these changes in soil properties will affect the components and activities of rhizospheric microbes (Hedley *et al.*, 1982; Bolton *et al*, 1992). Therefore, plant roots provide a unique environment to stimulate and sustain the microbial communities in rhizosphere and the structure of rhizospheric microbial communities is determined by the root exudates and physiochemical conditions in the rhizospheric soil (Marschner *et al.*, 2002; Semenov *et al.*, 1999).

On the other hand, the rhizospheric microbes can also have either detrimental or beneficial impacts on both plants and other microbes in the rhizosphere by inducing numerous plant-microbe and microbe-microbe interactions (Bowen and Rovira, 1999). Some rhizospheric microbes are considered as pathogens to plants because of the severe damage they can do to plants. In contrast, some other rhizopheric microbes are recognized for their ability to antagonize plant pathogens through competition for nutrients, stimulation of plant induced systemic resistance, and/or production of pathogen inhibitory compounds (Weller *et al.*, 2002; Van Bruggen *et al.*, 2002; Van Loon *et al.*, 1998; Van Wees *et al.*, 1999). Some beneficial rhizospheric microbes are known as biofertilizers, because they can enhance plant growth by improving the fertility status of the soil by increasing mineral and nutrient availability and employing biological nitrogen fixation (Darrah, 1993; Marschener and Römheld, 1994; Hinsinger, 1998; Reinhold-Hurek and Hurek, 1998; Yanni *et al.*, 2001).

The beneficial rhizospheric bacteria are also called plant growth promotion bacteria, which can be further divided into two groups depending on the way they interact with plant roots. One group is mostly the bacteria that belong to the genus *Rhizobium* and *Bradyrhizobium*. This group of bacteria are able to form a symbiotic relationship with legume plant roots and produce morphologically distinct structure-nodules. In the nodules, the rhizobia convert the atmospheric nitrogen gas into nitrogenous compounds which are useful to plant growth (La Favre and Focht, 1983; Welbaum *et al.*, 2004). The second group is the beneficial free-living soil bacteria which can stimulate plant growth without developing a symbiotic relationship with plant roots, and they are often referred to as plant growth promoting rhizobacteria (PGPR) (Bashan and Holguin, 1998). In the past few decades, microbiologists have been able to isolate some of the PGPR and those isolates cover a wide range of microbial genera, such as *Acetobacter, Actinoplanes, Agrobaterium, Alcaligens, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Cellulomonas, Enterobacter, Erwinia, Flavobacterium, Pasteuria, Pseudomonas, Serratia and Xanthomonas* (Tilak *et al.*, 2005). Among those genera, *Pseudomonas* and *Bacillus* are the most frequently found. Currently, some PGPRs are commercially available for farms. These can help farmers grow crops better in terms of financial and environmental cost.

1.3 Techniques of Assessing Microbial Community

1.3.1 Clone Library

Microbial populations were traditionally analyzed and characterized by physiological, morphological and metabolic traits of their isolated pure cultures or defined co-cultures

(Amann *et al*, 1995). As two essential parameters for defining microbial community structure, species richness and species evenness quantification are severely limited by conventional culture-dependent methods, simply because the majority of microorganisms cannot be cultivated in the lab (Welbaum *et al.*, 2004).

Even for the cultivatable ones, any change from their original environmental factors during the cultivation can alter the community structure. Therefore, the results obtained from a cultivated microbial product may not accurately reflect the true structure in the original environment (Osborn *et al.*, 2000).

Clone library is one of the culture-independent molecular approaches to study the diversity of microbial communities. In this method, a certain gene sequence from the total DNA of an environmental sample is amplified through polymerase chain reaction (PCR), and then inserted into a population of a well-known microbe, such as *E. coli* and yeast. The DNA fragments from the sample are propagated as the clones of microbe grow. Clone library technique is very useful and powerful to identify and characterize the dominant groups in a microbial community, but the less dominant microbial groups are often underrepresented in the library. Constructing clone library to adequately assess the complete microbial diversity in an environmental sample is also very time consuming and expensive (Hugenholtz *et al.*, 1998; Garbeva *et al.*, 2004).

1.3.2 Fluorescence in Situ Hybridization (FISH)

Originally, the situ hybridization technique was developed for cytogenetic studies and then introduced into microbiology by Giovannoni and his colleagues in 1988. They

used radioactively labeled rRNA-directed oligonucleotide probes to detect and visualize bacteria under a microscope. Fluorescent label replaced radioactive label in practice, due to its higher stability and safety level (Amann *et al.*, 1990b).

Fluorescence in situ hybridization detects nucleic acid sequence by a fluorescently labeled probe that hybridizes specifically to its complementary target sequence within the intact cell (Moter *et al.*, 1998b). Fluorescent probe can be labeled with several dyes that emit different wavelengths which enable detection of multiple target sequences in one single hybridization. FISH became a powerful tool for phylogenetic, ecological, diagnostic and environmental studies in the last couple of decades (Amann et al., 1990b). FISH has been used with water, sediment, sludge and soil samples to reveal complex microbial community structures in the environment, and the results have shown a significant improvement over the culture-dependent methods in terms of species richness and evenness. Compared to clone library, FISH also appears to be cheaper, faster, less labour intensive and a more sensitive approach (Weiss *et al.*, 1996; Llobet-Brossa *et al.*, 1998; Snaidr *et al.*, 1997 and Felske *et al.*, 1998).

Although FISH has been a very useful tool in many microbial community studies, it clearly has its own problem, limitation and pitfall. There are certain microbial groups/species that are fluorescent themselves, such as *Rhodospirillum centenum*, some *Pseudomonas spp.* and cyanobacteria (Albrecht-Buehler, 1996; Brown and Lowbury, 1996; Schönhuber *et al.*, 1999). These species can appear to have a false positive result, even when they are not hybridized with the fluorescent probes, simply because they are fluorescent and visible under the microscope for FISH analysis.

Besides the problem from autofluoresent microorganisms, the accuracy and reliability of FISH analysis for diversity in a microbial community is highly dependent on the specificity of the oligonucleotide probes (Amann et al., 1995). Therefore, a conclusive result of FISH analysis requires extensive probe design. Since the complexity and high diversity of environmental microbial community, and 95-99% of the microorganisms are unknown (Kuske *et al*, 1997; Garbeva *et al.*, 2004), designing probes for detecting the complete diversity in an environmental sample is unrealistic at the present time.

1.3.3 Fingerprinting Techniques

As increasing number of medical and environmental microbiology studies being conducted, bacterial community structure becomes a highly interesting focal point to understanding certain ecological processes and changes (Osborn et al., 2000). Fingerprinting techniques are extensively applied to bacterial community structure and dynamics analysis through distinguishing the PCR-amplified 16s rRNA genes or some other specific genes from different taxonomic groups (Osborn et al., 2000).

Most of the current fingerprinting techniques can be categorized into three different groups depending on the approaches that are used for separating the different DNA sequences. These three groups are: denaturing gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE); single strand conformation polymorphism (SSCP); and restriction fragment length polymorphism (RFLP).

DGGE and TGGE are two very similar methods because both of them differentiate PCR products based on the difference between relative helix stabilities of amplified DNA sequences in a denaturant or thermal gradient gel (Muyzer et al., 1993). These two approaches were originally developed for examining point mutations due to their relative high sensitivity. However, the different DNA fragments could have similar length which leads to similar mobility traits in gel and end up in the same band. This limits the ability of these two methods for detecting diversity. As some studies reported, only the dominant species, which is about 1-2% of the bacteria populations, can be detected by DGGE or TGGE in environmental samples (MacNaughton *et al.*, 1999).

Single strand conformation polymorphism differentiate amplifies DNA sequence by selecting different electrophoretic mobility caused by different conformations of a single strand of DNA after separating it from a double strand of DNA (Lee *et al.*, 1996). This technique is effective when used for detecting human DNA mutation, but it has a high DNA reanealing rate after the initial denaturation. This makes community analysis difficult due to the high concentration of community DNA. Another limitation of this technique is that several conformations of one product could coexist in the gel and the formation of heteroduplex DNA from PCR products with similar sequences occurs frequently. These disadvantages make SSCP unsuitable for bacterial community structure studies (Schmalenberger *et al.*, 2001; Schwieger and Tebbe, 1998).

Restriction fragment length polymorphism (RFLP) is another approach that is frequently used for analyzing bacterial population. This technique distinguishes different bacterial populations based on their 16s rRNA gene fragment length polymorphisms of

the restrictional digestion (Liu *et al.*, 1997). RFLP is one of powerful culture-independent approaches that combine PCR and rRNA-based phylogeny. It allows us to effectively identify uncultured organisms and detect bacterial population change. However, this technique cannot demonstrate the presence of specific phylogenetic groups or estimate species richness and evenness (Liu *et al.*, 1997).

Terminal restriction fragment length polymorphism (T-RFLP) is a technique that extends from RFLP. It uses the same principles of RFLP, the only difference is that T-RFLP labels one primer with fluorescent dye (Liu *et al.*, 1997). The automated DNA sequencer can determine the size of a terminal restriction fragment and quantify the amount of the fragment with fluorescent dye (Liu *et al.*, 1997). T-RFLP can be used for complex microbial communities analysis, provide a sensitive and rapid assessment for community diversity and obtain distinctive fingerprints of microbial communities. However, T-RFLP also has its own weakness. The number of microbial populations shown in the fingerprint profile depends on the abundance of each population, so the non-dominant, minor populations are not represented. Therefore, the real species diversity is underestimated. As a PCR-based technique, the biases of cell lysis, DNA extraction and PCR amplification will also be inherited by T-RFLP (Liu *et al.*, 1997; Hartmann and Widmer, 2008). Even though T-RFLP cannot detect the complete diversity of a bacterial population in an environmental sample, it is still considered the most effective and reliable approach to studying the bacterial diversity and changes in community structure of soil samples (Osborn et al., 2000; Hartmann and Widmer, 2008).

1.4 Pulse Crops and Pulse Production in Canada

1.4.1 Importance of Pulse as Food Source

Pulse crops consist of various members of the legume family, including chickpeas, peas, lentils and a variety of beans. Legumes that are grown for oil, such as soybean and peanut, are not included in the pulse group. Pulse crops are excellent sources for dietary protein, essential amino acids, complex carbohydrates and fibers, and contain many types of vitamins and minerals. Generally, pulse seeds consist of 20-30% protein, which is about double the amount in cereal, and are very low in fat and sodium content (Singh and Singh, 1992; Roy *et al.*, 2010). In total, pulse seeds provide about 10% of the world's dietary protein. In some developing countries pulse seeds make up to 80% of people's daily dietary protein because of the relative lower cost of pulse crops compared to other animal proteins, such as beef and pork (Singh and Singh, 1992).

1.4.2 Health and Medical Potential of Pulse

Besides the dietary importance, the seeds of pulse plants are also a good source for health supplement. In a cross-cultural study conducted by the International Union of Nutritional Science and World Health Organization from 1989-1996, researchers found that with every 20g pulse intake increase, the risk of death decreases 7-8% in elderly people (Darmadi-Blackerry *et al.*, 2004). Many pulse crops have medicinal properties. It has been known that a diet high in pulse foods such as peas, chickpeas and lentils can reduce the incidence of colon cancer, type-2 diabetes, LDL cholesterol and heart disease (Agriculture & Agri-Food Canada, 2006a,b, 2008). In addition, some nutritionists

recommend using pulse foods to achieve better weight loss management (Graham and Vance, 2003; Roy *et al.*, 2010).

Pulse crops are not only good health supplements when taken as food, they also contain some bioactive proteins and peptides that can potentially be used as medical treatment for certain diseases. Those bioactive proteins and peptides are antinutritional compounds when pulse seeds are consumed raw. They can cause growth suppression, diarrhea, bloating, vomiting and red blood cell agglutination (Liener *et al.*, 2002). However, those proteins and peptides have several beneficial properties on human health in their denatured forms (Roy et al., 2010). Lectin is one group of those bioactive proteins that are present in a variety of pulse crops. Studies have shown that lectins may play a key role in preventing certain cancers and activation of certain innate defense mechanisms. For example, lectins from lentils have a strong effect on reducing the onset of human hepatoma (Wang *et al.*, 2000) and lentil agglutinins can be use as therapeutic agents to treat Merkel skin carcinomas (Sames *et al.*, 2001). Other groups of bioactive compounds in pulse, such as protease inhibitors and angiotensin I-converting enzyme inhibitory peptides, have also been studied and researchers have found some promising results for potential medical treatment developments for controlling obesity, various cancers and other diseases (Ware *et al.*, 1999; Lima *et al.*, 1999; Pusztai *et al*; 1998).

1.4.3 Pulse Production in Canada

Pulse production in Canada has rapidly increased in the past 20 years, with its major locations being Saskatchewan, Manitoba and Alberta. Quebec and Ontario also have regions that focus on bean production (Pulse Canada, 2012). In western Canada, pulse

plants have been cultivated agriculturally in large quantity since the 1970s. Pulse plants have become major crops and play an important role in western Canada's economy (Saskatchewan Pulse Growers, 2008). Today, Canada is one of the leading exporters for pulse and accounted for 35% of global pulse trade in 2010. For 2011, Canada exported 4.7 million tonnes of pulse worth nearly 2.7 billion dollars in total. Canada has become the world's largest exporter for lentils and peas, and one of the top five exporters for beans (Pulse Canada, 2012). The global demands for pulse as food and livestock feed is expected to rise continuously in recent future (Agriculture & Agri-Food Canada, 2012).

1.5 Objectives of This Study

In Canada, the agricultural land that is used for pulse production increased from 1.26 million hectares in 1995 to 3.02 million hectares in 2010 (Agriculture & Agri-Food Canada, 2011). As members of the legume family, pulse plants form root nodules to fix nitrogen and play an important role in crop rotation. The large increase in pulse production can have a profound impact on soil quality in the area. Therefore, studies investigating their interaction with soil and soil microbial communities are necessary. We do not have a lot of knowledge that is specific to pulse plants, since soybean and alfalfa were used as model plants in most legume studies. The goals of this study are to investigate the impact of pulse plants on soil microbial community, especially the H_2-induced structure change in microbial community around root nodules, and their rotation benefits, which include:

1. To determine H_2 uptake status of each given pulse plant symbiosis;

2. To test the H_2 uptake rate and CO_2 exchange rate in soil adjacent to nodules;

3. To test the influence of nodules on soil microbial community structure by comparing the T-RFLP profiles from soil samples adjacent to nodules;

4. To evaluate the rotation benefit of soil adjacent to nodules from different pulse on the growth of a succeeding crop.

2. HUP STATUS OF DIFFERENT PULSE CROP NODULE AND GAS EXCHANGE IN SOIL ADJACENT TO THE NODULES

2.1 Introduction

As a by-product of nitrogen fixation, H_2 will be oxidized in 2 different ways after it is produced depending on the type of different plant-rhizobia symbioses in the root nodules of the legume plant. In some root nodules, the rhizobia possess H_2 uptake enzymes, which allow most of the H_2 to be oxidized within the nodule and recover some energy from nitrogen fixation process. This type of symbioses is named HUP+ symbioses. The other type of symbioses is called HUP- symbioses, which means the rhizobia lack a H_2 uptake enzyme and the H_2 will not be oxidized in the root nodule. For HUP- symbioses legumes, the H_2 will be released into the surrounding soil (Uratsu *et al.*, 1982; Evans *et al.*, 1987).

For a long while, HUP+ symbioses were considered superior over HUP- symbioses (which do not uptake H_2 produced from nitrogen fixation) because HUP+ can recycle the H_2, thus having a higher energy efficiency (Schubert and Evans, 1976). From an evolutionary point of view, it seems that the selection of optimal nitrogen-fixing bacteria

should prefer HUP+ symbioses. However, the majority of legume symbioses and most rhizobia that are used in agriculture lack H_2 uptake capacity and the H_2 defuses from nodules into the soil. Some studies suggested that with the energy loss from legume plants, the HUP- symbioses seems to have other beneficial influences on soil quality and succeeding crop growth (Uratsu *et al.*, 1982; Welbaum *et al.*, 2004).

The evolution of H_2 in soil after it is released from a nodule has been found to have effects on other soil properties, especially on gas exchange and H_2 oxidizing microbial population. The soil around root nodules develope H_2 uptake capacity within 8-10 days once H_2 is released from nodules and this H_2 uptake is bacterial in nature (La Favre and Focht, 1983; McLearn and Dong, 2002). The studies demonstrated that H_2 uptake of soil is often accompanied by an increased CO_2 fixation. However, both H_2 uptake and CO_2 fixation declined dramatically when glucose was added to soil. These findings suggest that certain bacteria in the soil are able to utilize H_2 as energy to convert CO_2 into carbohydrates for their own needs (Dong and Layzell, 2001; McLearn and Dong, 2002).

In this experiment, 25 different varieties from 5 different pulse plant species were planted to determine the type of symbioses (H_2 uptake status) in their root nodules and the rhizosphere soil. Soil adjacent to nodules was also collected to test their H_2 uptake rate and net CO_2 fixation.

2.2 Materials and Methods

The pulse seeds and inoculants used in this study were received from Agriculture and Agri-Food Cananda Semiarid Prairie Agricultural Research Centre in Swift Current,

Saskatchewan, Canada. The pulse seeds included 25 different varieties from peas (*Pisum sativum*), chickpeas (*Cicer arietinum*), lentils (*Lens culinaris*), faba beans (*Vicia faba*) and dry beans (*Phaseolus vulgaris*). The complete list of pulse seeds used in this experiment is shown in Table 1. The soil came from the Gerry Vermeurleu family farm in Canning, which is located in Annapolis Valley, Nova Scotia. The soil consists of 68% sand, 23.4% silt and 8.6% clay. The pH of the soil was 6.9 and the soil/water ratio is 1:2. The soil analysis is indicated in the following nutrient content of the soil: 14 mg/kg N in the form of nitrate, > 60 mg/kg of P, 106 mg/kg of K, 3 mg/kg of S, 55.4 mg/kg of Fe, 1200 mg/kg of Ca, 236 mg/kg of Mg, 24.7 mg/kg of Cu and 5.5 mg/kg of Zn.

2.2.1 Seeds Preparation and Inoculation

In actual farming practice, almost all legume crops are inoculated with commercial rhizobia to encourage nodule formation. We used peat powder based Nitrostick C culture inoculants for peas and lentils from EMD Crop Bioscience; peat powder based Nitrostick GC culture inoculants for chickpea from EMD Crop Bioscience; Becker Underwood nodulator clay phaseoli granular inoculants for dry bean; and Becker Underwood nodulator clay Viceae granular inoculants for faba bean. To ensure the seeds are only inoculated with the commercial rhizobia seeds were surface sterilized in 5% bleach for 2 minutes and then repetitively rinsed with autoclaved water before germination. After 2 days of germination in petri dishes with autoclaved water, seeds were potted in 8 inch pot from Halifax Seed. Four pots of inoculated seeds and one pot of un-inoculated seeds were planted as control for each variety. For the standards of comparison, 1 pot of soybean inoculated with JH inoculants and 1 pot of soybean inoculated with JH47 were also

Table 1: Different varieties of seeds in five pulse plant species

Species	Variety	Species	Variety
Lentil	CDC Blaze	Pea	Venture
Lentil	CDC Rouleau	Pea	Cooper
Lentil	CDC Robin	Pea	CDC Striker
Lentil	CDC Impact	Pea	CDC Handel
Lentil	CDC Imperial	Pea	CDC Meadow
Lentil	CDC Richlea	Pea	CDC Golden
Lentil	CDC Meteor	Chickpea	CDC Anna
Lentil	CDC Viceroy	Chickpea	CDC Nika
Lentil	Laird	Chickpea	CDC Vanguard
Lentil	CDC Glamis	Chickpea	CDC Luna
Lentil	CDC Sedley	Chickpea	CDC Frontier
Faba Bean	CDC Blitz	Chickpea	Amit
Dry Bean	Pintium		

planted at the same time. Soybean inoculant JH is known to form HUP+ symbioses and JH47 form HUP- symbioses with soybean plants.

2.2.2 Greenhouse Condition and Sample Collection

The pulse plants were grown in the Green Roof Facility greenhouse of Saint Mary's University. The temperature control was set between 15°C to 25°C and the photo period was set for16 hours, from 5:00 am to 9:00 pm each day. The average light intensity on the bench at night was 234.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on the left side, 247.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the middle and 130.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on the right side. The average light intensity on the bench during the day was 562.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on the left side, 533.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the middle and 545.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on the right side. About 250 ml/pot of nutrient solution without N (QUBIT manual, 1996) was applied once every 3 days after the first week of the planting. After 8 weeks of growth, the nodules were harvested for HUP status testing. The soil approximately 1cm around the nodules from inoculated plants and the rhizosphere soil around the roots from the un-inoculated plants were also carefully collected into separate 15ml centrifuge tubes.

2.2.3 Methylene Blue Reduction

Methylene blue reduction assays have been proven to be an effective method to test the HUP status of nodules (Haugland *et al.*, 1983; Lambert *et al.*, 1985). Methylene blue reduction solution was freshly made with 200mM iodoacetic acid, 200 mM malonic acid, 50 mM potassium phosphate, 2.5 mM magnesium chloride and 10 mM methylene blue and the pH was adjusted to 5.6 (Haugland *et al.*, 1983). The iodoacetic acid and malonic

acid act as inhibitors to prevent the respiratory electron transport processes which can interfere with the results of HUP status testing (Lambert *et al.*, 1985). For the reliability of result, methylene blue reduction assays were performed within 24 hours after the nodules were harvested. The nodules were squashed to release the enzymes, and placed about 1 cm apart on a piece of sterilized filter paper which was saturated with the methylene blue reduction solution in glass petri dishes according to variety. The petri dishes were then left to incubate in air for 10 minutes, covered with glass lids, and put into an air tight metal chamber. The chamber was vacuumed with a vacuum pump and then filled with pure H_2 (Praxair Inc.) from a high purity hydrogen cylinder. In order for the methylene blue reduction assays to work, the chamber was vacuumed and refilled with H_2 3-4 times to ensure there was no oxygen present in the chamber. After 48 hours of incubation in the pure H_2 at room temperature, the petri dishes were removed from the chamber and the results were recorded immediately by digital camera.

For HUP+ nodules, H_2 was reduced by the H_2 uptake enzyme – hydrogenase, and the excess electron had no transport path because of the inhibitors in the methylene blue solution and the lack of oxygen in the chamber. Thus, the methylene blue was reduced to colourless form. Therefore, a white circle formed around the crushed HUP+ nodules as seen in Figure 1a. For HUP- nodules, the blue colour around the nodules remained unchanged, since the hydrogenase was absent and H_2 was not reduced (Figure 1b).

2.2.4 Constructing Hydrogen Concentration Standard Curves

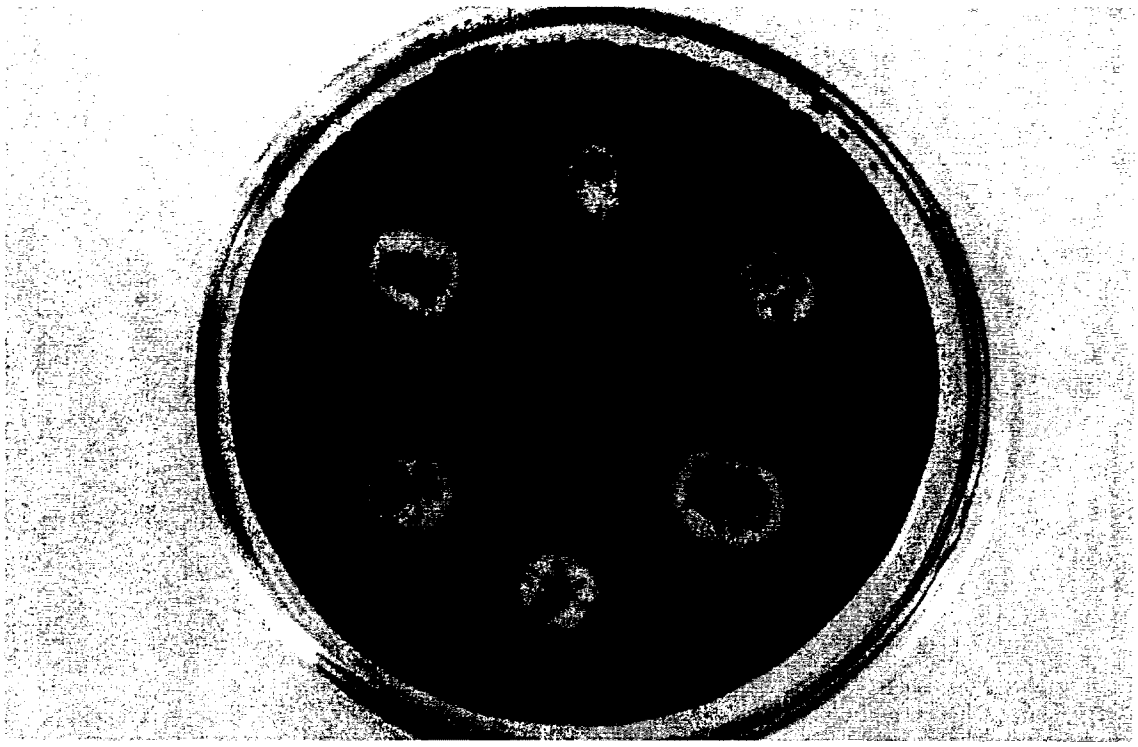
In order to calculate the H_2 concentration for H_2 uptake rate, the H_2 concentration standard curves of the measuring system need to be constructed. The construction of the

standard curve requires measurements of the electric signal voltage sent by an H_2 sensor (Model S121, Qubit System Inc., Kingston, Canada) at different known concentrations of H_2. The different concentration of H_2 gas flow was generated by electrolysis of mixed solution of H_2O and 0.1 M H_3PO_4 in a glass flask that was connected to an electric pump. One milliliter of H_2 gas at a certain concentration was injected into a chromatography (GC) column that was attached to an H_2 sensor. The sensor then transmitted an electric signal to a computer that contained a program to measure the voltage of that electric signal. The difference between the base line and the peak of the electric signal after the H_2 injection is the voltage that is generated by the 1ml injection of H_2 at a certain concentration.

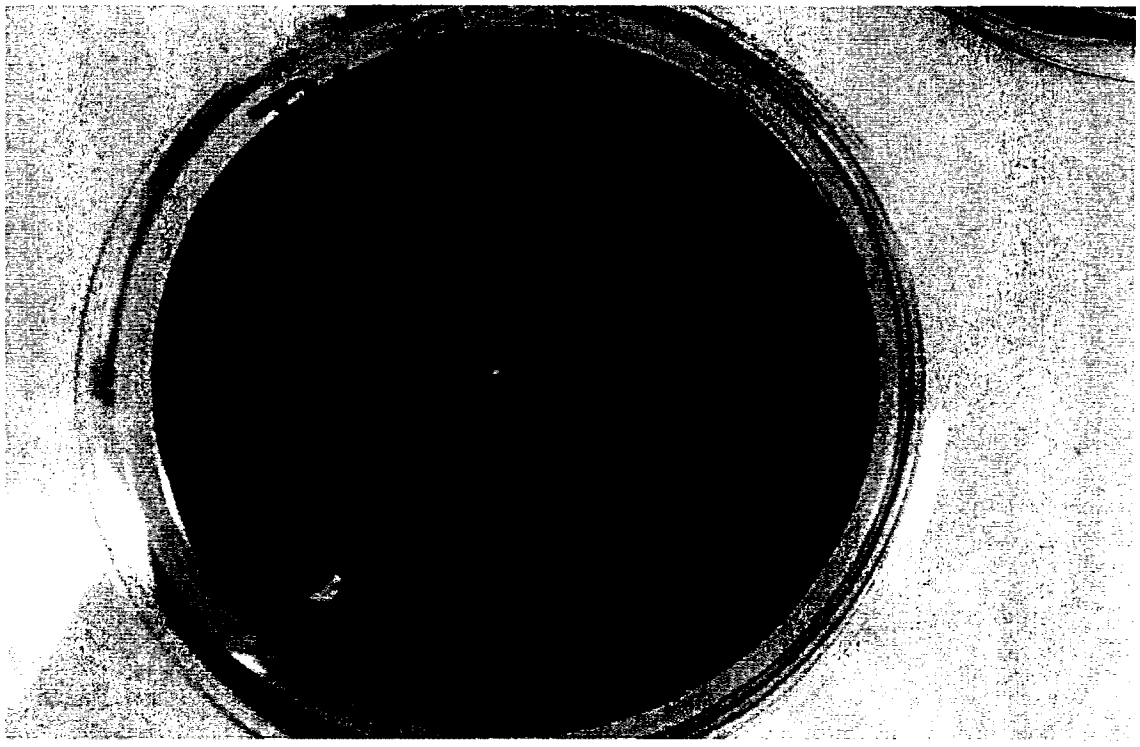
The concentration of H_2 can be changed by adjusting the electric current. An hour of settling period was allowed between each adjustment of the current in order for the H_2 concentration to reach a stable level. The flow rate, temperature and current at each voltage measurement were recorded for calculating the exact H_2 concentration according to the ideal gas laws. The voltages and their associated H_2 concentration were plotted to generate the standard curve in GraphPad Prism 5 (GraphPad Software Inc.). The R^2 of the standard curve needs to be higher than 0.99, in order to calculate the unknown H_2 concentration more precisely. The standard curve for the measuring system changes day to day because the factors that affect the standard curve, such as room temperature, air humidity and gas flow rate, vary daily. Two standard curves were constructed in this experiment.

Figure 1: The results of methylene blue reduction assays from HUP+ and HUP- nodules. (a) Methylene blue reduction assays result for HUP+ nodules from soybean plants that were inoculated with JH inoculants. The white circles around the nodules indicate that there are uptake hydrogenases in the nodules. (b) Methylene blue reduction assays result for HUP- nodules from soybean plants that were inoculated with JH47 inoculants. The absence of white circles around the nodules indicates that there are no uptake hydrogenases in the nodules.

a.



b.



2.2.5 Measuring the Hydrogen Uptake

In previous studies, the soil H_2 uptake dropped steeply when the soil was disturbed because the physical structure of the H_2 oxidizing bacteria in soil was destroyed, but the H_2 uptake ability could be recovered by treating the soil with H_2 for about 2 days after disturbance (McLearn and Dong, 2002). After the collection of soil samples, about 1g of soil from each sample was put into a 19 ml glass test tube for H_2 uptake measurement, and the rest of the sample was put into a 5ml plastic syringe for CO_2 fixation measurement. The H_2 treatment was provided to all soil samples for 2 days in order to recover the H_2 oxidizing bacteria population before any measurement. The intensity of the H_2 treatment ranged from 100 ppm to 200 ppm mixed in with air, which is the approximate amount of H_2 released by the nodules.

The H_2 uptake was measured by the GC column that was connected to the H_2 sensor. The voltages of the electric signals were plotted into the formulas for H_2 standard curve and converted to H_2 concentration in the unit of ppm.

After 2 days of H_2 treatment, the glass test tubes were sealed with air tight rubber septum stoppers. To measure the H_2 uptake rate, 1 ml of gas was taken from a constant gas flow that contains 1000 ppm of H_2, and injected into the glass tubes. After 1 minute of equalization, the initial H_2 concentration in the test tube was measured by taking 0.5 ml of gas from the test tube and then injected into the GC column. The difference between the base line and the peak of the electric signal after the H_2 injection showed the voltage of the H_2 concentration in the test tube. The measurement was taken every 30 minutes after the initial reading for 2 hours.

2.2.6 CO_2 Fixation Measurement

The CO_2 concentration was measured by a CO_2 analyzer (Model S151, Qubit Systems Inc., Kingston, Canada). The 5ml syringe with soil sample was connected to a gas flow system and the CO_2 analyzer after 2 days of H_2 treatment for recovery. The flow rate of the gas system was controlled to around 60 ml/min, and the exact gas flow rate was determined before the CO_2 level measurement of each set of soil samples. The CO_2 level was measured 4 times for each soil sample, twice with air flow and twice with 1000 ppm H_2 flow. In each type of gas flow, CO_2 measurement was taken before and after the gas flow passed through the soil sample in the syringe. The difference between these two measurements is the CO_2 fixation in that certain gas flow. The difference between the CO_2 evolution in air and the CO_2 evolution in H_2 is the net CO_2 fixation that is caused by H_2 exposure.

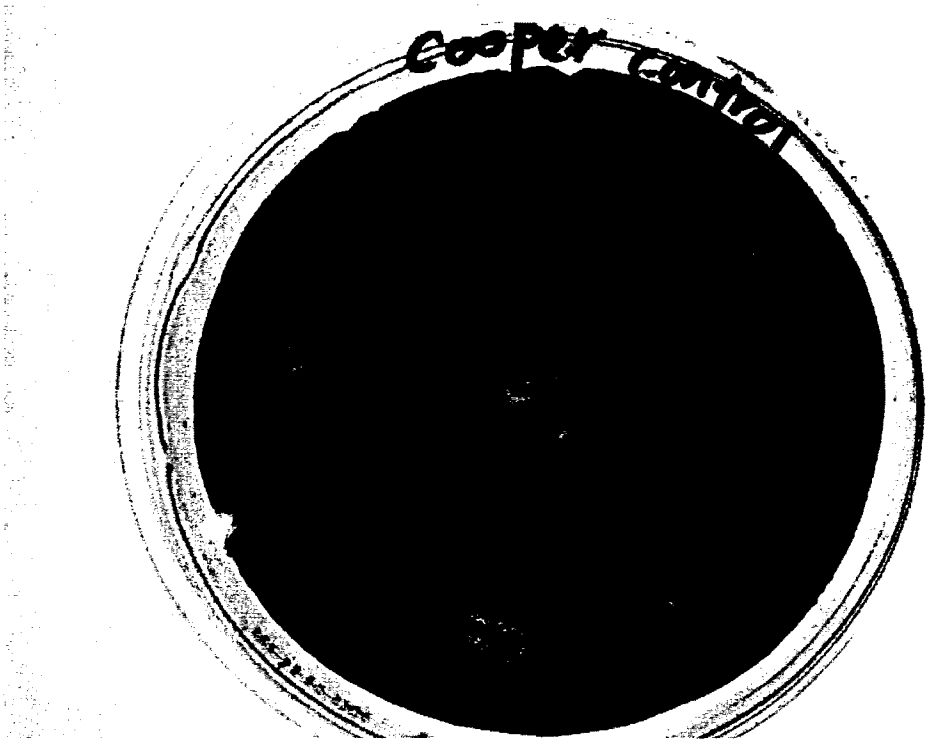
2.3 Results

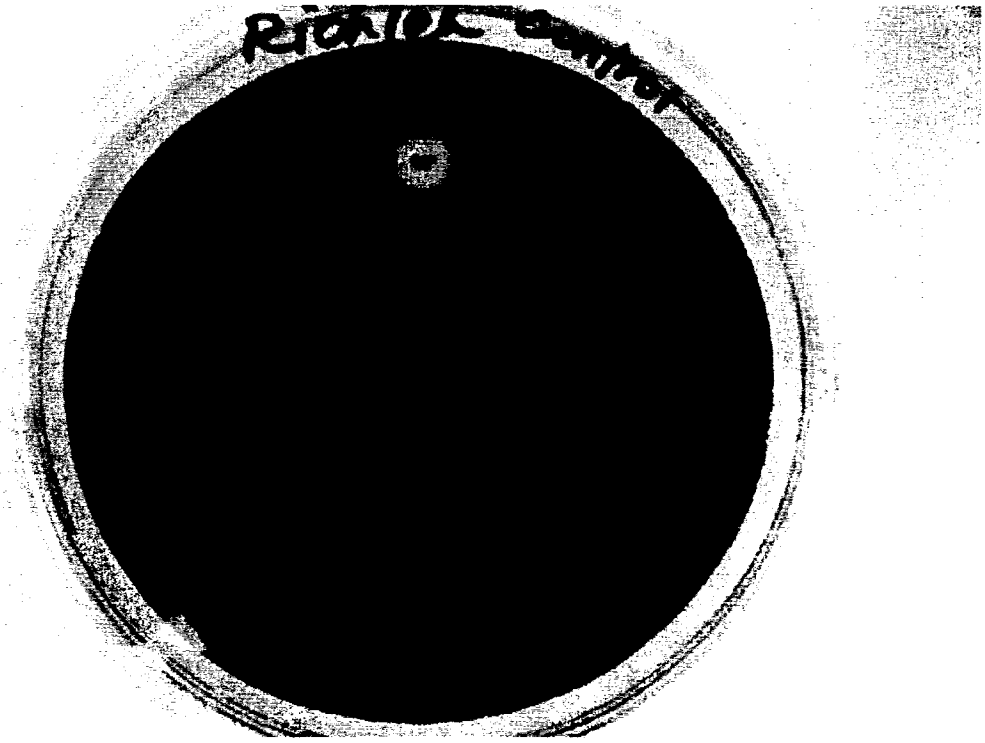
2.3.1 HUP Status of Pulse Crops

For the 25 varieties of pulse crops, all the nodules from the inoculated plants appeared to possess HUP- symbioses. Almost all the volunteer nodules from the un-inoculated control plants also formed HUP- symbioses except for a few nodules from the control plants of variety Cooper (pea) (Figure 2a) and variety CDC Richlea (lentil) are HUP+ (Figure 2b). The control plants of variety CDC Frontier (chickpea), CDC Anna (chickpea) and CDC Striker (pea) did not have visible root nodules formed. The inoculated plants tended to form significantly more nodules than their control plants by

Figure 2: The methlyene blue reduction assays for the volunteer nodules from control plants of variety Cooper and CDC Richlea. (a) Methylene blue reduction assay result for the volunteer nodules from variety Cooper control plant. Only a few nodules showed the positive result (white circle around the nodule) for uptake hydrogenase. (b) Methylene blue reduction assay result for the volunteer nodules from variety CDC Richlea control plant. Only one nodule showed the positive result (white circle around the nodule) for uptake hydrogenase.

a.





weight (Table 2).

For lentil plants, the nodules are very small in size (ranging from 1-2 mm), round in shape and densely distributed all along the roots. The root nodules of peas are relatively bigger than the nodules of lentils. The nodules of peas are rod shaped, 3-5 mm in length and 1-2 mm in width. The nodules are evenly distributed all along the roots of the pea plant. For dry bean, the nodules are round in shape, about 5-10mm in diameter and disturbed all along the roots. For faba bean and chickpea plants, the nodules range from 5-10 mm, are hand shaped, and usually cluster around the base of the stem on the main roots or are otherwise randomly distributed all along the roots.

2.3.2 Hydrogen Concentration Standard Curves

There are 2 standard curves generated for measuring the H_2 uptake activity. For each standard curve, the voltages of the electric signal for 10 different H_2 concentrations were measured, which ranged from 0-300 ppm. The independent variable x is H_2 concentration in the unit of ppm, and the dependent variable Y is the associated voltage. The first standard curve is $Y= 527.5x / (2.353-x) + 3.018$, and the R^2 equals to 0.9982 (Figure 3). The second standard curve is $Y= 406x / (2.845-x) + 6.944$, and the R^2 equals to 0.9995.

2.3.3 Hydrogen Uptake Rate

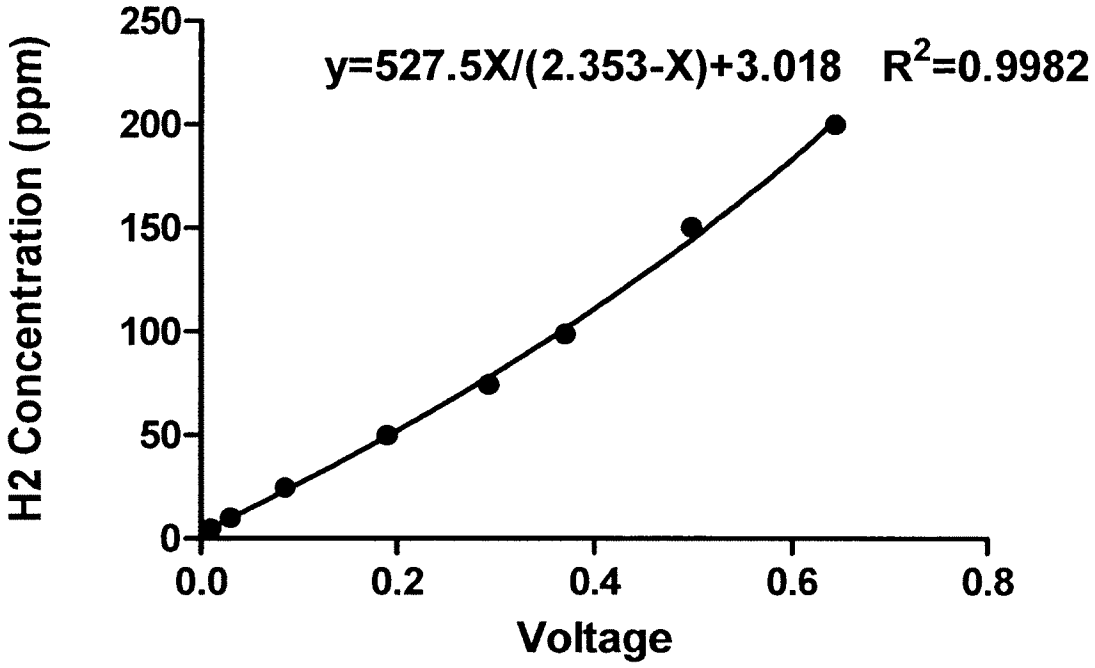
The H_2 uptake measurement showed that the soil adjacent to HUP- nodules have a higher capacity of H_2 uptake than the rhizophere soil away from nodules. The H_2 uptake was most active during the first 30 minutes after the injection of H_2 and then decreased gradually with time in most soil sample. The H_2 uptake rate was calculated according to

Table 2: The weight of nodules for each pulse variety. 1 control plant and 4 test plants were grown and measured, the weight of nodules from the test plants is showed as mean weight ± SD.

Variety	Plant	Weight (g)	% Change	P-value
CDC Viceroy	Control Test	0.4753 0.78025 ± 0.1012	64.16%	0.0092
CDC Venture	Control Test	0.2735 0.6033 ± 0.1276	120.58%	0.0141
Amit	Control Test	2.8816 4.3534 ± 0.3350	51.07%	0.0031
CDC Meadow	Control Test	0.4044 0.5126 ± 0.0114	26.74%	0.0003
CDC Vanguard	Control Test	0.9813 3.4846 ± 0.8413	255.10%	0.0095
CDC Imperial	Control Test	0.3347 0.7064 ± 0.337	111.05%	0.0002
CDC Blaze	Control Test	0.213 0.3945 ± 0.0871	85.22%	0.0251
CDC Luna	Control Test	- 0.7102 ± 0.1833	-	-
CDC Glamis	Control Test	0.3925 0.4348 ± 0.0173	10.77%	0.0164
CDC Meteor	Control Test	0.3181 0.4251 ± 0.0207	33.64%	0.0019
Cooper	Control Test	0.2994 0.4838 ± 0.0739	61.59%	0.0155
CDC Nika	Control Test	0.5607 0.9743 ±0.0798	73.76%	0.0019

CDC Rouleau	Control Test	0.0972 0.1786 ± 0.0553	83.77%	0.0602
CDC Frontier	Control Test	- 0.6593 ± 0.0921	-	-
CDC Handel	Control Test	0.0318 0.1813 ± 0.0442	469.97%	0.0066
CDC Richlea	Control Test	0.0054 0.0624 ± 0.0221	1055.09%	0.0142
CDC Blitz	Control Test	0.1882 0.5917 ± 0.1374	214.39%	0.0098
CDC Anna	Control Test	- 0.0839 ± 0.0073	-	-
Pintium	Control Test	0.596 0.8122 ± 0.1325	36.28%	0.047
CDC Robin	Control Test	0.0476 0.0878 ± 0.0121	84.35%	0.007
CDC Golden	Control Test	0.0364 0.0782 ± 0.0195	114.70%	0.0233
Laird	Control Test	0.0037 0.031 ± 0.0115	736.49%	0.0178
CDC Impact	Control Test	0.4473 0.5949 ± 0.0595	32.99%	0.0157
CDC Sedley	Control Test	0.369 0.7299 ± 0.1281	97.80%	0.0111
CDC Striker	Control Test	- 0.6839	-	-

Figure 3: The first standard curve generated for calculating H2 concentration. Each dot on the curve represents the known concentration of a H2 gas and its associated voltage of the electrical signal from H2 sensor. The higher concentration of the H2 gas has, the higher the higher voltage it generates.


y=527.5X/(2.353-X)+3.018 R2=0.9982
H2 Concentration (ppm)
250
200
150
100
50
0
0.0
0.2
0.4
0.6
0.8
Voltage

the reduction in H_2 concentration during the first 30 min of the measurement. For the soil was adjacent to HUP- nodules, the samples from variety CDC Rouleau in lentils had the lowest H_2 uptake rate (8.13 nmol/hr•g) and the highest H_2 uptake rate (58.59 nmol/hr•g) was observed in samples from variety CDC Vanguard in chickpeas.

For the rhizosphere soil from control plants, the lowest H_2 uptake rate (3.12 nmol/hr•g) was seen in samples from variety CDC Impact in lentils, and the sample from variety CDC Nika in chickpea had the highest H_2 uptake rate (18.74 nmol/hr•g). The complete list of H_2 uptake rate for each soil sample is shown Table 3.

For the group of peas, the soil adjacent to the HUP- nodules from inoculated CDC Striker plants displayed the highest H_2 uptake rate, which was 32.35 nmol/hr•g. This H_2 uptake rate is 471.98% higher than the rhizosphere soil from CDC Striker control plant. The soil adjacent to the HUP- nodules from variety CDC Vanguard had an H_2 uptake rate of 58.59 nmol/hr•g, which was the highest among the all the varieties of chickpea. This H_2 uptake rate is 459.68% higher than the rhizosphere soil from CDC Vanguard control plant. The soil adjacent to the HUP- nodules from variety CDC Glamis showed an H_2 uptake rate of 20.54 nmol/hr•g, which is the highest among all the varieties of lentil. This H_2 uptake rate is 224.51% higher than the rhizosphere soil from CDC Glamis control plant.

To compare the H_2 uptake rate of soil adjacent to HUP- nodules and the rhizosphere soil within the same variety of crop, one sample T-test was performed in Graphpad QuickCalcs Online Calculator for Scientists. The one sample T-test detected statistical significance in almost all varieties, except the soil samples from variety CDC Blaze in

lentils. With a P-value of 0.0528, the difference between H_2 uptake rates of soil adjacent to the nodules of CDC Blaze inoculated plants and the rhizosphere soil of CDC Blaze control plants was not statistically significant. The P-values of all the one sample T-tests are also listed in Table 3.

2.3.4 CO_2 Fixation

The soil CO_2 evolutions in air and in H_2 were quantified respectively and then the net CO_2 fixation for each soil sample was calculated. The net CO_2 fixation of rhizosphere soil from control plants ranged from 0-54.8 nmol/hr•g. There were 12 rhizosphere soil samples that did not display any net CO_2 fixation and the highest net CO_2 fixation among rhizosphere soil samples was observed in the CDC Viceroy (lentil) rhizospher soil. The net CO_2 fixation of soil that was adjacent to HUP- nodules ranged from 31.3-139.7 nmol/hr•g. The soil adjacent to variety CDC Blaze (lentil) HUP- nodules had the lowest net CO_2 fixation. The soil adjacent to variety CDC Blitz (Faba bean) HUP- nodules had the highest net CO_2 fixation. The net CO_2 fixation of each soil sample is listed in Table 4.

For the group of peas, the soil adjacent to the HUP- nodules from inoculated Cooper plants displayed the highest net CO_2 fixation, which was 128.7 nmol/hr•g. The soil adjacent to the HUP- nodules from variety CDC Nika had a net CO_2 fixation of 124.7 nmol/hr•g, which was the highest among the all the varieties of chickpea. The soil adjacent to the HUP- nodules from variety CDC Imperial showed a net CO_2 fixation of 122.9 nmol/hr•g, which was the highest among all the varieties of lentil.

Table 3: H_2 uptake rate of each soil sample

Variety	Soil Sample	H_2 Uptake Rate (nmol/hr•g)	% Change	P-value
CDC Viceroy	rhizosphere adjacent	11.61 22.04 ± 5.07	89.82%	0.0261
CDC Venture	rhizosphere adjacent	4.53 18.10 ± 0.8094	299.59%	0.0001
Amit	rhizosphere adjacent	9.96 55.16 ± 4.51	453.82%	0.0003
CDC Meadow	rhizosphere adjacent	9.95 19.53 ± 2.78	96.26%	0.0062
CDC Vanguard	rhizosphere adjacent	10.47 58.59 ± 4.53	459.69%	0.0002
CDC Imperial	rhizosphere adjacent	25.34 45.95 ± 11.66	81.35%	0.0385
CDC Blaze	rhizosphere adjacent	14.92 23.30 ± 5.38	56.13%	0.0528
CDC Luna	rhizosphere adjacent	7.1 26.48 ± 2.65	273.07%	0.0007
CDC Glamis	rhizosphere adjacent	6.33 20.54 ± 7.36	224.51%	0.0307
CDC Meteor	rhizosphere adjacent	13.7 22.60 ± 2.89	65.02%	0.0086
Cooper	rhizosphere adjacent	14.93 30.17 ± 4.37	102.02%	0.0061
CDC Nika	rhizosphere adjacent	18.74 30.73 ± 3.89	63.99%	0.0086

CDC Rouleau	rhizosphere adjacent	5.87 8.13 ± 0.78	38.44%	0.0104
CDC Frontier	rhizosphere adjacent	6.56 17.24 ± 0.88	162.96%	0.0002
CDC Handel	rhizosphere adjacent	12.37 21.14 ± 0.54	70.93%	0.0001
CDC Richlea	rhizosphere adjacent	8.52 16.48 ± 4.51	93.34%	0.0387
CDC Blitz	rhizosphere adjacent	4.76 20.46 ± 2.82	329.64%	0.0016
CDC Anna	rhizosphere adjacent	9.39 22.50 ± 6.45	139.62%	0.0269
Pintium	rhizosphere adjacent	7.07 19.68 ± 2.42	178.25%	0.0019
CDC Robin	rhizosphere adjacent	11.05 16.94 ± 1.52	53.24%	0.0044
CDC Golden	rhizosphere adjacent	5.44 11.39 ± 0.74	109.33%	0.0005
Laird	rhizosphere adjacent	7.41 11.96 ± 1.86	61.35%	0.0164
CDC Impact	rhizosphere adjacent	3.12 12.96 ± 2.66	315.29%	0.0051
CDC Sedley	rhizosphere adjacent	9.6 17.25 ± 1.86	79.71%	0.0037
CDC Striker	rhizosphere adjacent	5.66 32.35 ± 3.61	471.98%	0.0007

Table 4: Net CO2 fixation for each soil sample

Variety	Soil Sample	Net CO_2 Fixation (nmol/hr•g)	% Change	P-value
CDC Viceroy	rhizosphere adjacent	54.76 109.66 ± 13.01	100.25%	0.003
CDC Venture	rhizosphere adjacent	0 93.91 ± 6.19	-	0.0001
Amit	rhizosphere adjacent	0 70.61 ± 8.60	-	0.0005
CDC Meadow	rhizosphere adjacent	47.13 79.04 ± 7.39	67.70%	0.003
CDC Vanguard	rhizosphere adjacent	0 101.48 ± 3.95	-	0.0001
CDC Imperial	rhizosphere adjacent	0 122.92 ± 6.68	-	0.0001
CDC Blaze	rhizosphere adjacent	0 31.32 ± 1.51	-	0.0001
CDC Luna	rhizosphere adjacent	0 73.06 ± 8.47	-	0.0004
CDC Glamis	rhizosphere adjacent	33.64 47.21 ± 8.55	40.34%	0.0504
CDC Meteor	rhizosphere adjacent	32.82 81.31 ± 10.49	147.72%	0.0027
Cooper	rhizosphere adjacent	0 128.72 ± 11.82	-	0.0002
CDC Nika	rhizosphere adjacent	50.86 124.72 ± 7.28	145.22%	0.0003

CDC Rouleau	rhizosphere adjacent	27.5 83.06 ± 2.94	202.03%	0.0001
CDC Frontier	rhizosphere adjacent	30.02 73.87 ± 8.32	146.03%	0.0018
CDC Handel	rhizosphere adjacent	0 29.46 ± 3.23	-	0.0004
CDC Richlea	rhizosphere adjacent	0 48.46 ± 8.11	-	0.0013
CDC Blitz	rhizosphere adjacent	0 139.72 ± 11.56	-	0.0002
CDC Anna	rhizosphere adjacent	48.35 93.45 ± 10.33	93.28%	0.0032
Pintium	rhizosphere adjacent	48.63 105.92 ± 6.67	117.83%	0.0004
CDC Robin	rhizosphere adjacent	23.5 69.47 ± 7.05	195.56%	0.001
CDC Golden	rhizosphere adjacent	27.26 104.16 ± 1.86	282.16%	0.0001
Laird	rhizosphere adjacent	25.94 52.85 ± 4.51	103.72%	0.0013
CDC Impact	rhizosphere adjacent	24.1 51.71 ± 7.21	114.58%	0.0046
CDC Sedley	rhizosphere adjacent	0 46.04 ± 2.35	-	0.0001
CDC Striker	rhizosphere adjacent	0 118.02 ± 4.07	-	0.0001

To compare the net CO_2 fixation of soil adjacent to HUP- nodules and the rhizosphere soil within the same variety of crop, one sample T-test was performed in Graphpad QuickCalcs Online Calculator for Scientists. The one sample T-test detected statistical significance in almost all varieties, except the soil samples from variety CDC Glamis in lentils. With a P-value of 0.0504, the difference between H_2 uptake rates of soil adjacent to the nodules of CDC Glamis inoculated plants and the rhizosphere soil of CDC Glamis control plants was almost statistically significant. The P-values of all the one sample T-tests are also listed in Table 4.

2.4 Discussion

The control plants of variety CDC Frontier (chickpea), CDC Anna (chickpea) and CDC Striker (pea) did not have visible root nodules formed. This means that commercial inoculants are a necessity to ensure the formation of nitrogen-fixing nodules. The methylene blue reduction assays suggested that all the pulse plants would form HUP- nodules with commercial inoculants and all pulse plants would also tend to form HUP- nodules under natural conditions without artificial inoculation. The results of this experiment match the findings of previous studies, which confirms that the natural selection of optimal nitrogen-fixing bacteria prefer to form HUP- symbioses, although the HUP+ symbioses have higher energy efficiency for legume plants (Uratsu et al., 1982; Welbaum et al., 2004). These results offer the theoretical foundation for the next experiments of this study, which investigate the beneficial effects of the H_2 released from HUP- nodules on crop rotation and soil properties.

The construction of the standard curve is a time consuming process due to a long stabilizing period after changing the current from one level to another for the generation of a different H_2 concentration. Therefore, only two H_2 concentration standard curves were constructed and used to measure the soil H_2 uptake in this experiment, although the standard curve can vary day to day. For each set of soil samples, one standard curve was chosen to measure the H_2 uptake. After the soil samples were treated with H_2 for 2 days to recover the H_2 uptake activity, the voltage and the current of H_2 treatment were measured again before measuring the soil H_2 uptake. The concentrations that were calculated from the two standard curves at the same voltage were compared to the actual H_2 concentration for the treatment, and then the standard curve that generated the concentration closer to the actual measurement at the time was chosen.

Based on previous studies, the H_2 released from nodules will be absorbed by bacteria in the surrounding soil with increased soil CO_2 fixation (Dong and Layzell, 2001; McLearn and Dong, 2002; Stein *et al.*, 2005). Therefore, the H_2 uptake and the CO_2 of soil can be an indication of the level of H_2 oxidizing bacteria activity. The H_2 uptake of each soil sample was measured in order to determine which pulse plants have higher stimulation of the H_2 oxidizing bacteria population. The results of soil H_2 uptake indicate that the soil around the HUP- nodules has a higher H_2 uptake than the associated rhizosphere soil away from nodules and bulk soil. This matches the findings of other studies. The H_2 uptake rate of each soil sample varied in a wide range due to the daily change of standard curve of the system. Therefore, the percentage of change is more appropriate in comparison to different groups of soil sample. Since the soil sample from

25 varieties of pulse plants were not collected and tested on the same day, we cannot compare the H_2 uptake rate among all soil samples from different varieties. However, the test and control soil samples from the same variety of pulse plant were measured on the same day, and the direct comparison of H_2 uptake rate from soil samples that belong to the same variety of pulse plant is validated.

The result of soil net CO_2 fixation also showed a similar pattern as the H_2 uptake rate measurement. The soil adjacent the HUP- nodules had significantly higher net CO_2 fixation than the associated rhizosphere soil. The results of this experiment support the findings of previous studies that the soil has a higher H_2 uptake capacity coupled with an increased CO_2 fixation after the soil is exposed to H_2 for a prolonged period (Dong and Layzell, 2001; McLearn and Dong, 2002; Stein *et al.*, 2005). However, the results of soil net CO_2 fixation reveal that the soil that with the highest H_2 uptake rate does not necessarily have the highest net CO_2 fixation. Soil samples used in previous studies were directly treated with H_2 gas without the presence of different types of pulse plants, while the soil samples tested in this experiment were plant rhizosphere soil and soil adjacent to root nodules. The existence of plant-soil interaction was well known, though the details about all mechanisms involved are not clearly understood yet. With the presence of plants, a wide range of different chemical compounds is secreted into the soil through roots, and then a variety of different microbes and small insect populations are stimulated. The difference between the ranking of soil H_2 uptake rate and net CO_2 fixation could be caused by the different microbial communities and small organism population, as well as their different functions in the soil.

In this experiment, a number of the control pulse plants planted were not equal to the number of their associated inoculated test plants due to the limited space in the greenhouse. Only one pot of control plant and four pots of inoculated test plants were planted for each variety of pulse crops. Therefore, the most appropriate and legitimate statistical analysis is one sample T-test. Although the sample size is very small and there only one control sample was used for each variety of pulse plant, the trends seen in nodule HUP status, soil H_2 uptake rate and soil net CO_2 fixation are very consistent across all the varieties. The one-sample tests also demonstrated that the difference seen between the control sample and test sample is significant in almost every variety. Based on the reasons stated above, the current analysis of this experiment is overall reliable. That is to say, the improvements for future experiment of this topic could be achieved by increasing the sample size for each target variety the study focuses on in order to conduct a more complex analysis and then draw a more reliable conclusion.

3. GROWTH PROMOTION OF SOIL ADJACENT TO PULSE NODULES ON BARLEY

3.1 Introduction

In a series of studies, H_2 treated soil and legume soil were tested, under greenhouse and field conditions, for their impacts on plant growth. The results showed that the biomass (dry weight) of 7-week old soybean spring wheat, barley and canola were 15% to 48% higher in the H_2 treated soil than those planted in the regular air treated soil. In addition, the tiller number of 7-week old barley and spring wheat in the field trial

increased 36% and 48% respectively, compared to the control plants, when H_2 treated soil was applied to seedlings (Dong, *et al.*, 2003).

Exposure to H_2 gas causes H_2 oxidizing bacteria to grow rapidly in soil and many studies have shown that soil with high H_2 oxidation activities can promote plant growth (Fyson and Oaks, 1990; Dong *et al.*, 2003; Peoples *et al.*, 2008). Unfortunately, it is not known what kind of H_2 oxidizing bacteria are responsible for the plant growth promoting effects. Therefore, it is necessary to isolate and characterize soil H_2 oxidizing bacteria in order to fully understand their metabolic and physiological interaction with plants.

Maimaiti and her colleagues (2007) attempted to isolate aerobic H_2 oxidizing bacteria from soils around HUP- soybean nodules and H_2 treated soils. There are 19 strains of aerobic H_2 oxidizing bacteria that were successfully isolated, and most of them belong to genera *Variovorax*, *Burkholderia* and *Flavobacterium* according to conventional identification tests and 16S rDNA sequence analysis (Maimaiti *et al*, 2007). They also tested the isolates for plant growth promotion. The results showed that all isolates have the ability to stimulate root elongation of spring wheat seedlings up to 250% after 2 days of growth and increased plant biomass of *Arabidopsis thaliana* by 11-27% over the same time period (Maimaiti *et al*, 2007). By combining several other studies, it becomes obvious that soil H_2 oxidizing bacteria can stimulate plant growth through utilizing a considerable amount of energy released from H_2 oxidation in the soil (Dong *et al.*, 2003; Maimaiti *et al.*, 2007; Peoples *et al.*, 2008).

The underlying mechanisms involved in the plant growth promotion effect of H_2 oxidizing bacteria are not clear, but it seems that some plant growth regulators, such as

phytohormones, may play a role in plant growth promotion. Ethylene is known as a plant growth hormone that can inhibit cell division, DNA synthesis and the growth of the meristems of roots, shoots and axillary buds (Burg, 1973). There are studies that have demonstrated some H_2 oxidizing bacterial strains from *Variovorax* and *Burkholderia* are able to lower the plant-produced ethylene level (Glick *et al.*, 1995, 1998; Belimov *et al.*, 2001). Within the strains of H_2 oxidizing bacteria that were isolated by Maimaiti (2007), activity of an ethylene inhibitor – ACC deaminase was found in *Variovorax paradoxus* and *Flavobacterium johnsoniae* (Zhang, 2006; Maimaiti et al., 2007). Rhizobitoxine is another ethylene inhibitor that has also been reported in strains of the genus *Burkholderia* (Zhang 2006; Maimaiti et al. 2007). Therefore, it is reasonable to hypothesize that H_2 oxidizing bacteria can promote plant growth by lowering the ethylene level in plants. Since H_2 oxidizing bacteria is a taxonomically diverse group, to understand the exact mechanisms of plant growth promotion in each kind of bacteria more in-depth studies need to be done.

Since most pulse crops have not been studied for their impact on crop rotation and soil quality, this experiment was designed to test the contribution of pulse plants on crop rotation benefit. More specifically, this experiment will examine the growth promotion of soil adjacent to pulse nodules on succeeding crops. Barley was planted as the succeeding crop after the harvest of pulse plants.

3.2 Materials and Methods

3.2.1 Seed preparation and Inoculation

After the measurement of soil H_2 uptake and CO_2 fixation had been done, the soil samples were kept for the inoculation of barley seeds. The variety of barley used in this experiment is AC Metcalfe, and it was received from Agriculture and Agri-Food Cananda, Semiarid Prairie Agricultural Research Centre in Swift Current, Saskatchewan, Canada. The barley seeds were washed thoroughly with autoclaved water several times and then germinated with autoclaved water in petri dishes for 2 days before planting. The germinated barley seeds were planted in 8 inch pots with bulk soil from the Gerry Vermeurleu family farm in Annapolis Valley, Nova Scotia. Each barley seed was inoculated with 1 ml of soil sample collected from either the rhizosphere of control pulse plants or soil adjacent to the HUP- nodules of inoculated pulse plants.

The barley plants were grown in the Green Roof Facility greenhouse of Saint Mary's University. The temperature control was set between 15°C and 25°C and the photo period was set for 16 hours, from 5:00 am to 9:00 pm each day. The average light intensity on the bench at night was 234.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on the left side, 247.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the middle and 130.2 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on the right side. The average light intensity on the bench during the day was 562.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on the left side, 533.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the middle and 545.6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on the right side. About 250ml/pot of nutrient solution was applied once every 3 days starting one week after planting. To ensure that 3 barley plants would survive the early seeding stage, 5 germinated barley seeds were planted in each pot. Two weeks after planting the extra barley plants were pulled out. After 8 weeks of growth, the above ground biomass and the root mass of barley plants were harvested, and then dried in a drying oven at 80 °C for 2 days. The tiller number and the shoot dry

weight of each barley plant were recorded. The dry weight of the root mass for each pot was also measured.

3.2.2 Trial Design

Two trials of crop rotation for testing plant growth promotion were conducted in this experiment. The first trial was a screening trial where all 25 pulse varieties and the soil samples collected from those pulse plants were tested. Since each pulse variety had 1 pot of control plants and 4 pots of inoculated test plants, 1 pot of barley inoculated by the control pulse plant rhizosphere soil and 4 pots of barley inoculated by the soil adjacent to HUP- nodules of the test pulse plants for each pulse variety were planted. One pot of barley was planted in bulk soil without any inoculation as a negative control for each pulse variety's soil sample. Half strength Hoagland solution was used as fertilizer and three levels of N in fertilizer (0.1 mM, 0.5 mM and 5mM) was applied to the barley plants in the first trial due to the accidental miscalculation when we switched from pure KNO_3 chemical to an agricultural graded N fertilizer source (27-0-0).

In the second trial, 4 different varieties from 4 different pulse species were chosen according to the H_2 uptake rate of their soil sample. The variety that has the highest soil H_2 uptake rate in each species was selected, except for the species lentil. These 4 varieties of pulse were planted for 8 weeks in order to collect fresh soil samples for the inoculation of barley seeds. Due to the accidental change in N level during the first trial, two levels of N fertilizer (0.1 mM and 0.5 mM) were applied on the barley plants in the second trial to see if the inoculated barley plants responsed differently to different N levels. At each N level 4 pots of barley inoculated with the rhizosphere soil of the control pulse plants and

4 pots of barley inoculated with soil adjacent to the HUP- nodules of test pulse plants were grown for each pulse variety. Four pots of barley without any inoculation and 4 pots of barley inoculated with H_2 treated soil were set up as negative and positive controls of each N level.

3.2.3 Root Mass Collection

The root mass collection process was a time consuming and labor intensive procedure. The fibrous roots of the barley plants in each pot were very dense and intertwined, so the soil was held tight by the roots in the pot. The only way to get a complete root system was to remove the soil and roots as a whole from the pot, and then wash away the soil with a constant slightly pressured water flow in a sieve that had a fine mesh metal screen. After most of the soil was washed away, the root mass was then transferred into a fine mesh metal strainer for further cleaning. The root mass was gently shaken within the strainer immersed in water to get rid of the finer soil and sand particles. Most of the root fragments that broke off during the wash were collected by the sieve and the strainer and then put in with the rest of the major root mass.

3.3 Results

3.3.1 The First Trial

There was no clear overall pattern shown in the results of the first trial for the rotation benefit of the soil adjacent to pulse HUP- nodules. However, the first 5 sets of barley that were given 0.1 mM N fertilizer showed that the barley inoculated with soil adjacent to pulse HUP- nodules had a higher dry weight of shoots and roots than the

barley inoculated with the rhizosphere soil of the control pulse plants and the barley grown in bulk soil (Figure 4 and Figure 5). Four of the first 5 sets showed that the barley inoculated with the soil adjacent to pulse HUP- nodules had more tillers than the barley inoculated with the rhizosphere soil of control pulse plants (Figure 6). Three of the first 5 sets showed that the barley inoculated with the soil adjacent to pulse HUP- nodules had more tillers than the barley grown in bulk soil (Figure 6). The increase in tiller number, dry weight of shoots and dry weight of roots are higher between the barley inoculated with soil adjacent to pulse HUP- nodules and the barley grown in bulk soil.

There was a miscalculation that the 5 mM N level in the fertilizer solution was too high. The 0.5 mM N level was applied as an alternate N test level to latter sets of barley. Under 0.5 mM and 5 mM of N levels, the differences among barley inoculated with soil adjacent to pulse HUP- nodules, barley inoculated with the rhizosphere soil of control pulse plants and barley grown in bulk soil are not consistent across all sets. In the set for CDC Meteor, the barley inoculated with soil adjacent to HUP- nodules grew better than the barley inoculated with the rhizosphere soil of the CDC Meteor control plants and the barley grown in bulk soil in all 3 measurements (Figure 4, Figure 5 and Figure 6). In the sets for CDC Nika and CDC Sedley, the barley inoculated with soil adjacent to HUP- nodules had higher dry weight in both shoots and roots than the barley inoculated with the rhizosphere soil of their control pulse plants and the barley grown in bulk soil (Figure 4 and Figure 5). In other sets, the barley inoculated with soil adjacent to HUP- nodules did not necessarily grow better than the barley inoculated with the rhizosphere soil of control pulse plants and the barley grown in bulk soil. They even show a decrease in

Figure 4: The percentage change of dry weight of shoots from barley inoculated with the soil adjacent to HUP- pulse nodules compared to barley inoculated with the rhizosphere soil of control pulse plants and barley grown in bulk soil for all 25 pulse varieties.

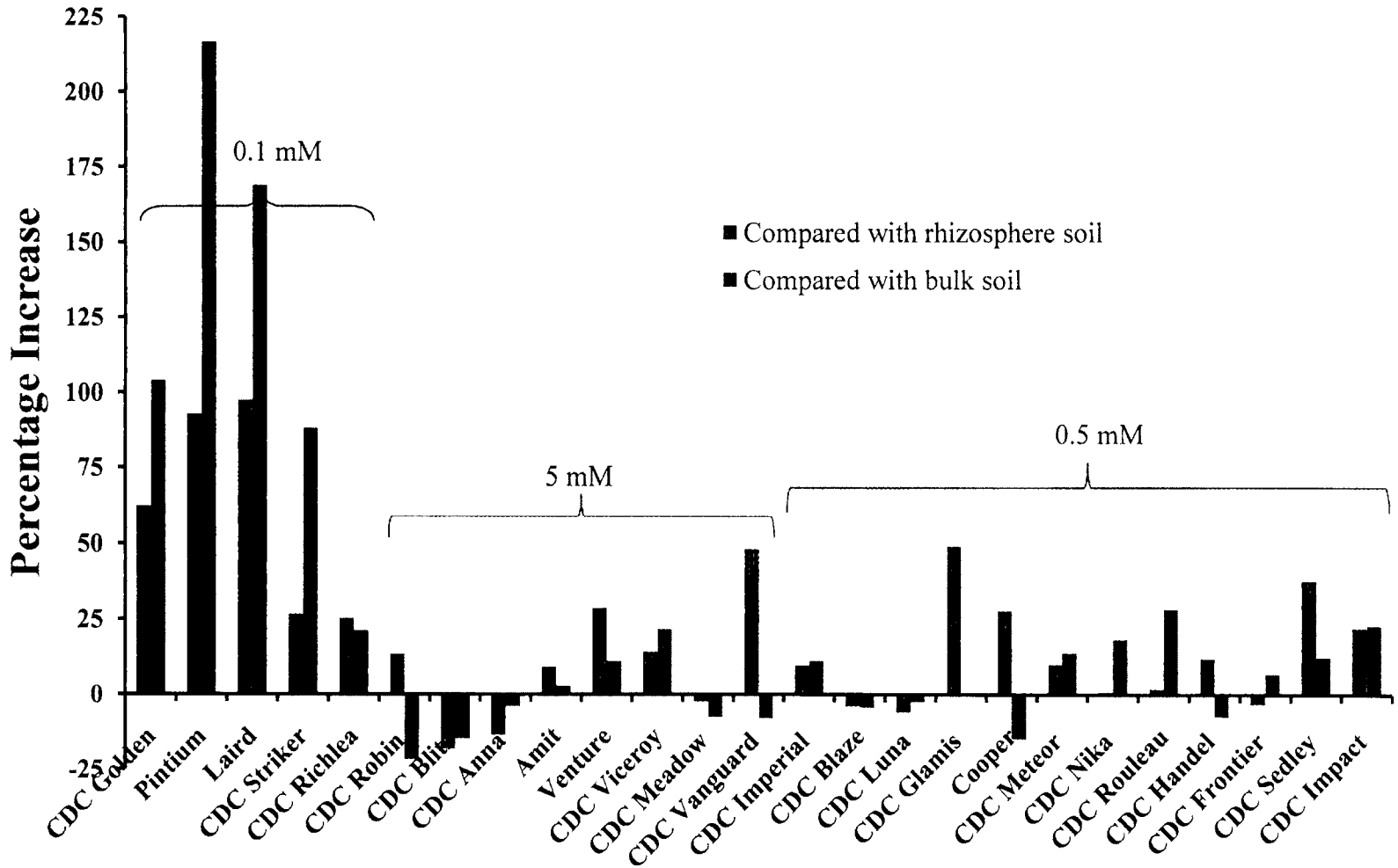

Percentage Increase
225
200
175
150
125
100
75
50
25
0
-25
0.1 mM
5 mM
0.5 mM
Compared with rhizosphere soil
Compared with bulk soil
CDC Golden
Pintium
Laird
CDC Striker
CDC Richlea
CDC Robin
CDC Bli
CDC Anna
Amit
Venture
CDC Viceroy
CDC Meadow
CDC Vanguard
CDC Imperial
CDC Blaze
CDC Luna
CDC Glamis
Cooper
CDC Meteor
CDC Nika
CDC Rouleau
CDC Handel
CDC Frontier
CDC Sedley
CDC Impact

Figure 5: The percentage change of dry weight of roots from barley inoculated with soil adjacent to HUP- pulse nodules compared to barley inoculated with the rhizosphere soil of control pulse plants and barley grown in bulk soil for all 25 pulse varieties.

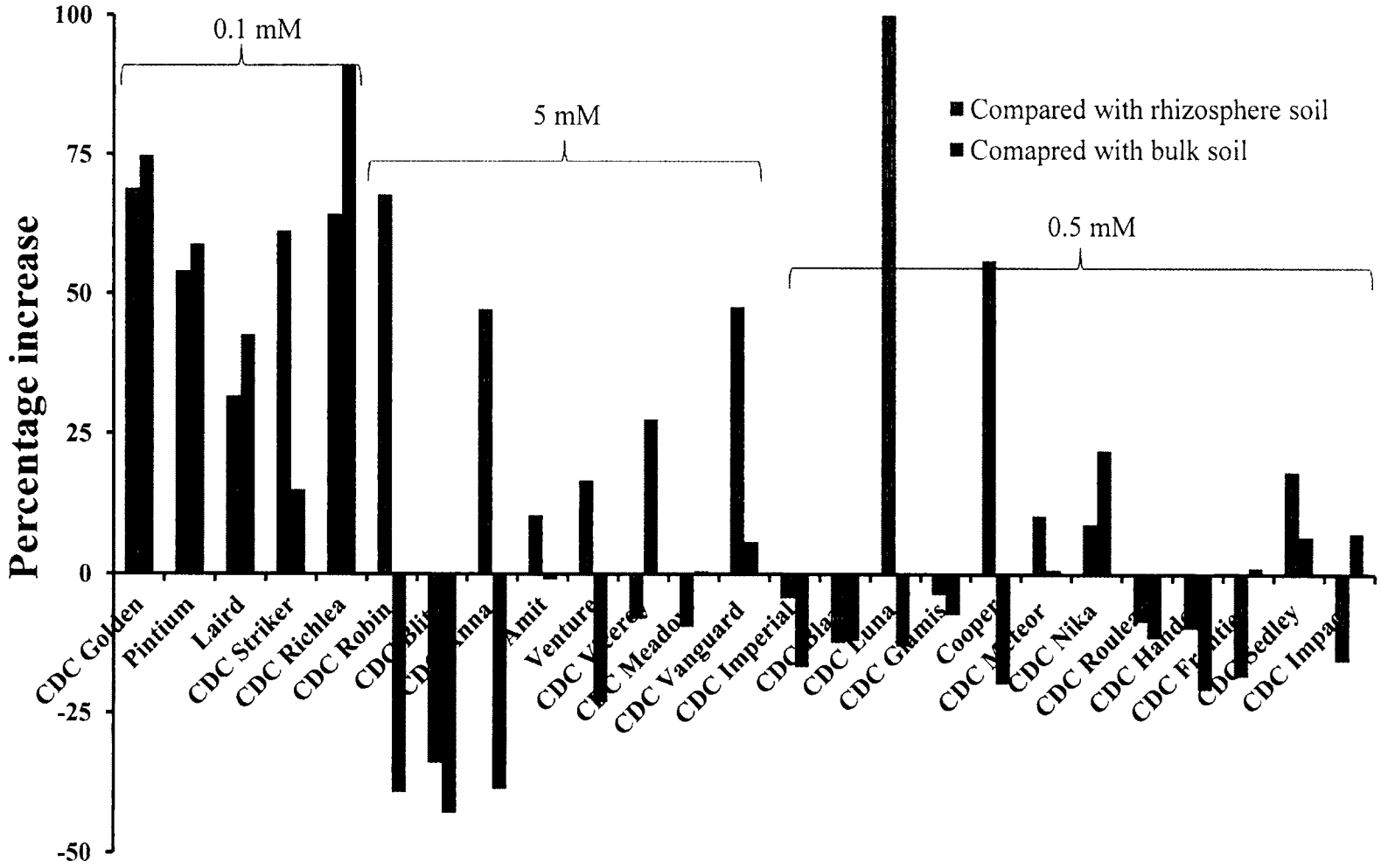
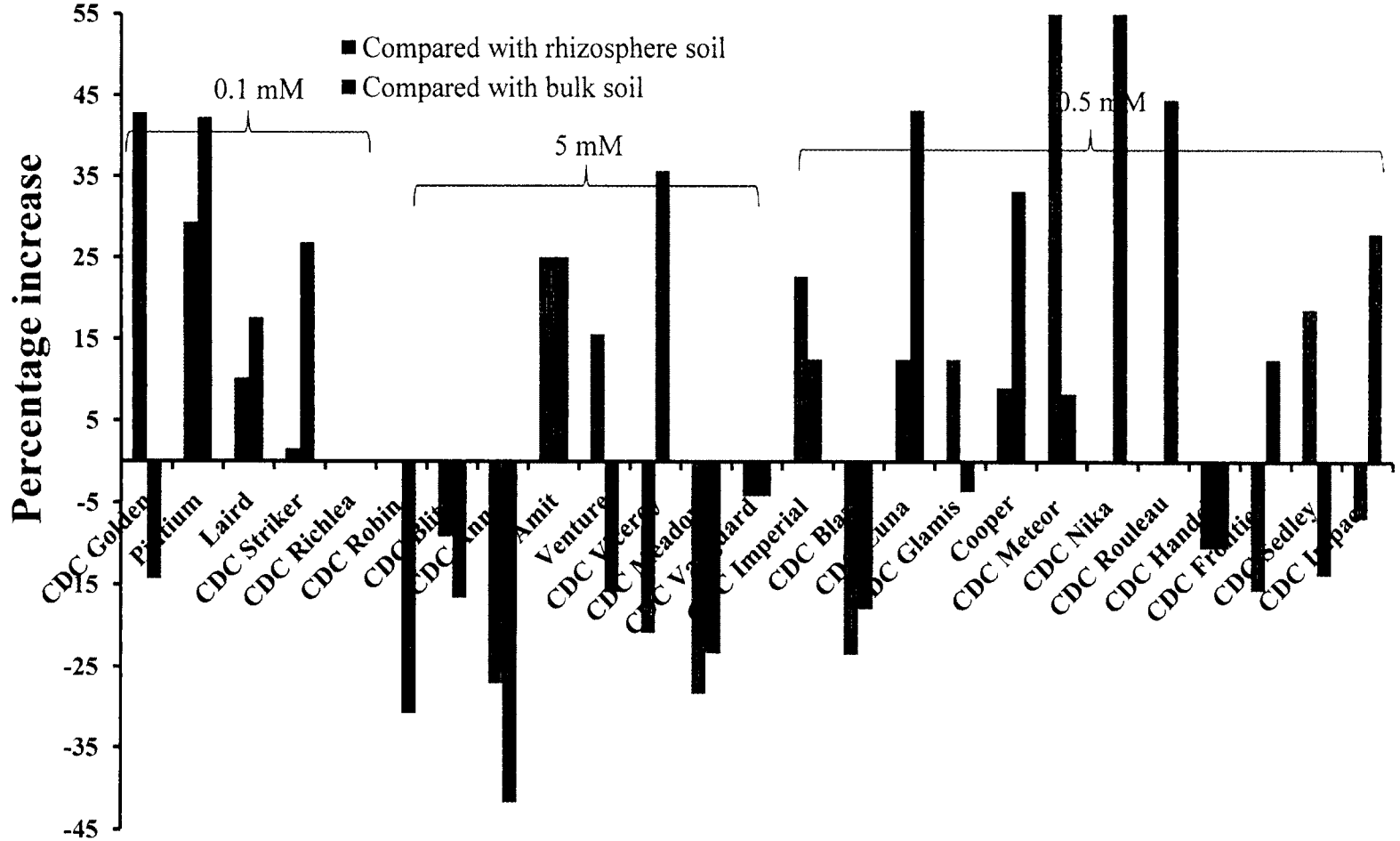

100
75
50
25
0
-25
-50
Percentage increase
0.1 mM
5 mM
0.5 mM
Compared with rhizosphere soil
Comapred with bulk soil
CDC Golden
Pintium
Laird
CDC Striker
CDC Richlea
CDC Robin
Amit
Venture
CDC Vanguard
CDC Imperial
CDC Luna
Cooper
CDC Nika
CDC Sedley

Figure 6: The percentage change of tiller number from barley inoculated with soil adjacent to HUP- pulse nodules compared to barley inoculated with the rhizosphere soil of control pulse plants and barley grown in bulk soil for all 25 pulse varieties.


Percentage increase
55
45
35
25
15
5
-5
-15
-25
-35
-45
Compared with rhizosphere soil
Compared with bulk soil
0.1 mM
5 mM
0.5 mM
CDC Golden
Laird
CDC Striker
CDC Richlea
CDC Robin
Amit
Venture
Imperial
Cooper
CDC Meteor
CDC Nika
CDC Rouleau
CDC Sedley

certain measurements.

3.3.2 The Second Trial

The soil samples of variety CDC Vanguard from chickpea, variety CDC Striker from pea, variety Pintium from dry bean and variety CDC Blitz from faba bean were selected for the second trial for the rotation benefit of pulse crops on barley because the soil samples from the test plants of these varieties had the highest H_2 uptake rate within their own species group.

At the level of 0.1 mM N, a few general trends can be observed from a basic statistical comparison. The average tiller number of barley inoculated with soil adjacent to HUP- nodules of test pulse plants is higher than the average tiller number of barley inoculated with the rhizosphere soil of control pulse plants and the barley grown in bulk soil (Figure 7). The average tiller number of barley inoculated with H_2 treated soil is higher than the average tiller number of barley grown in bulk soil (Figure 7). The average dry weight of shoots from barley inoculated with soil adjacent to HUP- nodules of test pulse plants is higher than the dry weight of shoots from barley inoculated with the rhizosphere soil of control pulse plants and the barley grown in bulk soil (Figure 8). The average dry weight of shoots from barley inoculated with H_2 treated soil is higher than the average dry weight of shoots from barley grown in bulk soil (Figure 8). The average dry weight of roots from barley inoculated with soil adjacent to HUP- nodules of test pulse plants and barley inoculated with H_2 treated soil are higher than the average dry weight of roots from barley inoculated with the rhizosphere soil of control pulse plants and the barley grown in bulk soil (Figure 9). A further and more accurate statistical

Figure 7: The number of tillers from barley inoculated with soil adjacent to HUP-pulse nodules, barley inoculated with the rhizosphere soil of pulse control plants, barley grown in bulk soil and barley inoculated with H2 treated soil for the 4 selected pulse varieties at the 0.1 mM N level in second trial. Error bar: standard error; Test: barley inoculated with soil adjacent to pulse nodules; Control: barley inoculated with rhizosphere from control plants; Bulk: barley grew in bulk soil; H_2 treated: barley inoculated with H_2 treated soil.

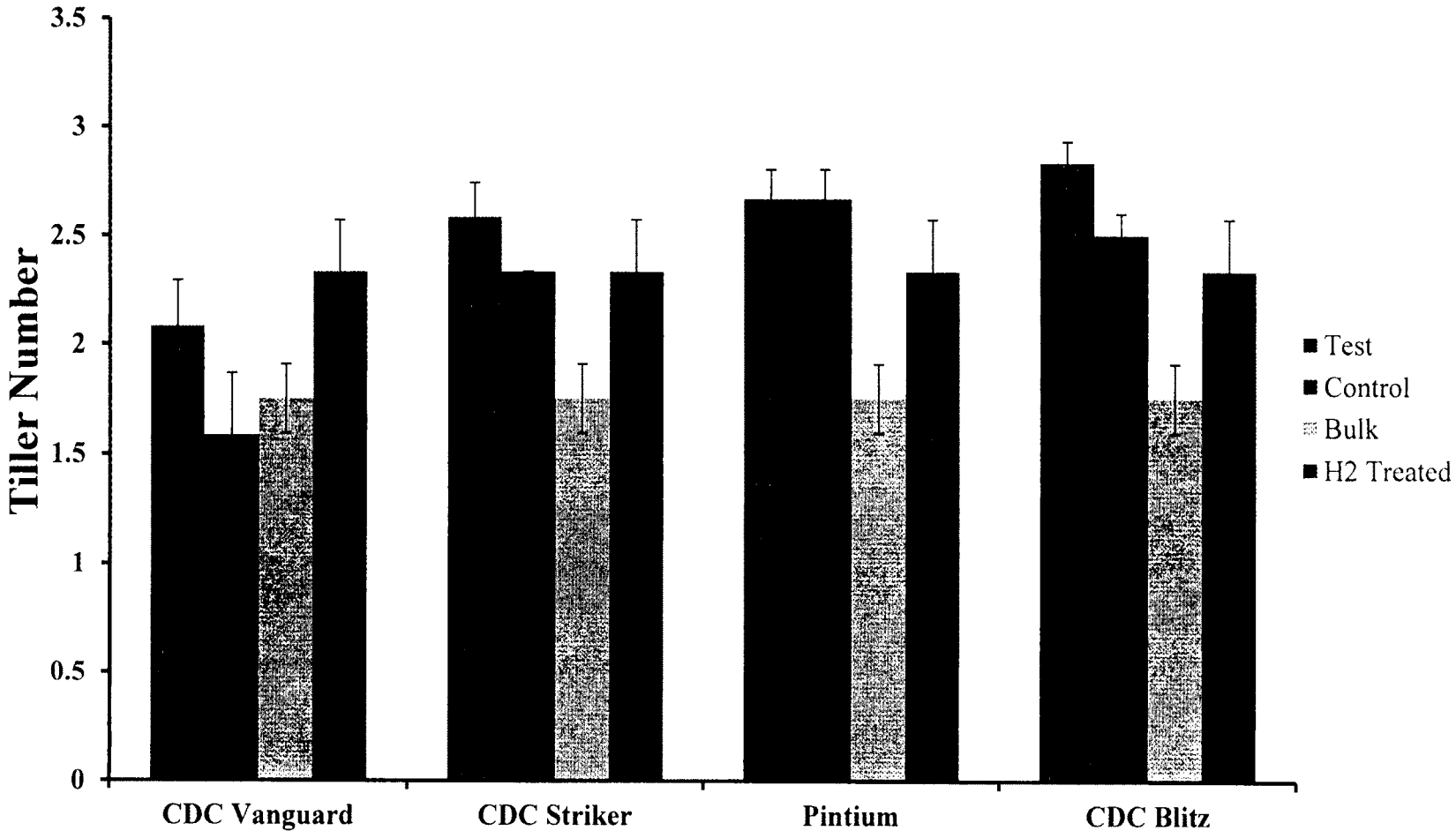

Tiller Number
3.5
3
2.5
2
1.5
1
0.5
0
CDC Vanguard
CDC Striker
Pintium
CDC Blitz
Test
Control
Bulk
H2 Treated

Figure 8: The dry weight of shoots from barley inoculated with soil adjacent to HUP- pulse nodules, barley inoculated with the rhizosphere soil of pulse control plants, barley grown in bulk soil and barley inoculated with H2 treated soil for the 4 selected pulse varieties at the 0.1 mM N level in the second trial. Error bar: standard error; Test: barley inoculated with soil adjacent to pulse nodules; Control: barley inoculated with rhizosphere from control plants; Bulk: barley grew in bulk soil; H_2 treated: barley inoculated with H_2 treated soil.

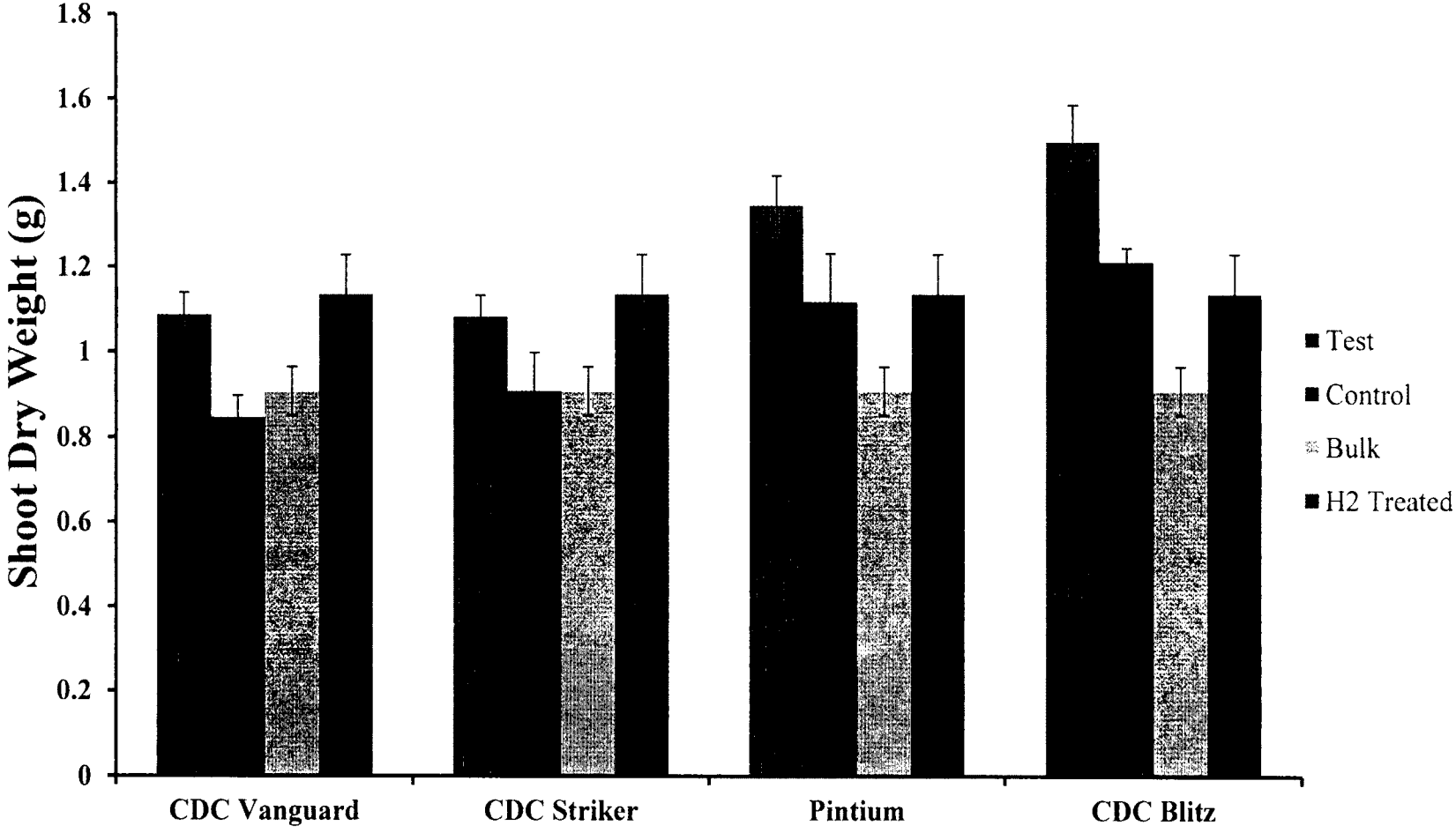
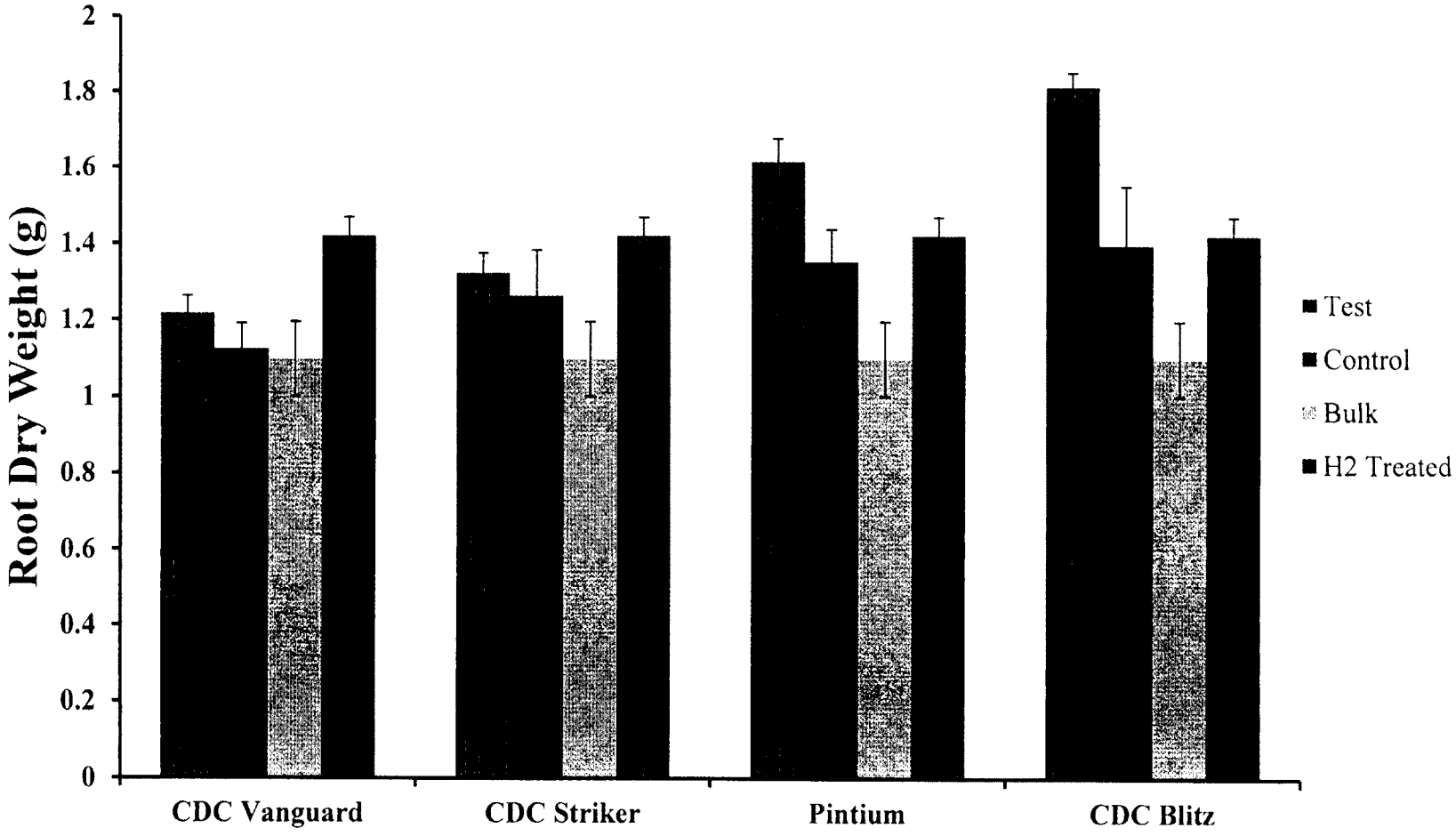

Shoot Dry Weight (g)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CDC Vanguard
CDC Striker
Pintium
CDC Blitz
Test
Control
Bulk
H2 Treated

Figure 9: The dry weight of roots from barley inoculated with soil adjacent to HUP-pulse nodules, barley inoculated with the rhizosphere soil of pulse control plants, barley grown in bulk soil and barley inoculated with H2 treated soil for the 4 selected pulse varieties at the 0.1 mM N level in second trial. Error bar: standard error; Test: barley inoculated with soil adjacent to pulse nodules; Control: barley inoculated with rhizosphere from control plants; Bulk: barley grew in bulk soil; H_2 treated: barley inoculated with H_2 treated soil.


Root Dry Weight (g)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CDC Vanguard
CDC Striker
Pintium
CDC Blitz
Test
Control
Bulk
H2 Treated

analysis (Fisher's least significant difference test) was performed following ANOVA.

For the barley inoculated with soil samples from CDC Vanguard at 0.1 mM N level, the P-value of ANOVA for the number of tillers on each barley plant among different soil inoculation treatments is 0.142, which is not significant. This means that the number of tillers on each barley plant among different soil inoculation treatments is not different overall. The detail statistical analysis is shown in Table 5. The difference is only seen in the comparison between the barley inoculated with H_2 treated soil and barley inoculated with the rhizosphere soil of CDC Vanguard control plants (Table 5, $P = 0.038$, $n = 4$). The P-value of ANOVA for shoot dry weight is 0.024. The barley inoculated with H_2 treated soil has a significantly higher dry weight of shoots compared to the barley grown in bulk soil (Table 6, $P = 0.03$, $n = 4$) and the barley inoculated with the rhizosphere soil of CDC Vanguard control plants 9 (Table 6, $P = 0.009$, $n = 4$). The barley inoculated with soil adjacent to HUP- nodules of CDC Vanguard test plants has a significantly higher dry weight of shoots than the barley inoculated with the rhizosphere soil of CDC Vanguard control plants (Table 6, $P = 0.023$, $n = 4$). The P-value of ANOVA for root dry weight is 0.021. The dry weight of roots from barley inoculated with H_2 treated soil is higher than the dry weight of roots from barley inoculated with the rhizosphere soil of CDC Vanguard control plants (Table 7, $P = 0.009$, $n=4$) and the barley grown in bulk soil (Table 7, $P = 0.005$, $n=4$).

Table 5: Multiple comparison of tiller number from barley inoculated with soil samples of CDC Vanguard plants at the 0.1 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Number of Tiller	P-value
CDC Vanguard CDC Vanguard Control	2.0833 ± 0.4194 1.5833 ± 0.5693	0.145
CDC Vanguard Bulk	2.0833 ± 0.4194 1.75 ± 0.3191	0.319
CDC Vanguard H_2 Treated	2.0833 ± 0.4194 2.3333 ± 0.4714	0.451
CDC Vanguard Control Bulk	1.5833 ± 0.5693 1.75 ± 0.3191	0.613
CDC Vanguard Control H_2 Treated	1.5833 ± 0.5693 2.3333 ± 0.4714	0.038*
H_2 Treated Bulk	2.3333 ± 0.4714 1.75 ± 0.3191	0.094

Table 6: Multiple comparison of the dry weight of shoots from barley inoculated with soil samples of CDC Vanguard plants at the 0.1 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Shoot Dry Weight (g)	P-value
CDC Vanguard CDC Vanguard Control	1.0873 ± 0.1035 0.8468 ± 0.1028	0.023*
CDC Vanguard Bulk	1.0873 ± 0.1035 0.9069 ± 0.1138	0.074
CDC Vanguard H_2 Treated	1.0873 ± 0.1035 1.135 ± 0.1845	0.616
CDC Vanguard Control Bulk	0.8468 ± 0.1028 0.9069 ± 0.1138	0.528
CDC Vanguard Control H_2 Treated	0.8468 ± 0.1028 1.135 ± 0.1845	0.009*
H_2 Treated Bulk	1.135 ± 0.1845 0.9069 ± 0.1138	0.03*

Table 7: Multiple comparison of the dry weight of roots from barley inoculated with soil samples of CDC Vanguard plants at the 0.1 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Root Dry Weight (g)	P-value
CDC Vanguard	1.2164 ± 0.0943	0.351
CDC Vanguard Control	1.1235 ± 0.1327	
CDC Vanguard	1.2164 ± 0.0943	0.237
Bulk	1.0974 ± 0.1952	
CDC Vanguard	1.2164 ± 0.0943	0.054
H_2 Treated	1.4209 ± 0.0923	
CDC Vanguard Control	1.1235 ± 0.1327	0.789
Bulk	1.0974 ± 0.1952	
CDC Vanguard Control	1.1235 ± 0.1327	0.009*
H_2 Treated	1.4209 ± 0.0923	
H_2 Treated	1.4209 ± 0.0923	0.005*
Bulk	1.0974 ± 0.1952	

For the barley inoculated with soil samples from CDC Striker at 0.1 mM N level, the P-value of ANOVA for the number of tillers on each barley plant among different soil inoculation treatments is 0.022. The average number of tiller from barley grown in bulk soil is significantly lower than the average number of tillers from barley inoculated with soil adjacent to HUP- nodules of CDC Striker test plants (Table 8, P = 0.004, n=4), barley inoculated with the rhizosphere soil of CDC Striker control plants (Table 8, P =0.027, n = 4) and the barley inoculated with H_2 treated soil (Table 8, P = 0.027, n = 4). The P-value of ANOVA for shoot dry weight is 0.109. There is no significant difference found in the average dry weight of shoots among all treatment groups (Table 9). The P-value of ANOVA for root dry weight is 0.102. The only significant difference observed in the average dry weight of roots appeared between the barley inoculated with H_2 treated soil and the barley grown in bulk soil (Table 10, P = 0.019, n = 4).

For the barley inoculated with soil samples from Pintium at 0.1 mM N level, the P-value of ANOVA for the number of tillers on each barley plant among different soil inoculation treatments is 0.008. The average number of tillers from barley grown in bulk soil is greatly lower than the average number of tillers from the barley inoculated with soil adjacent to HUP- nodules of Pintium test plants (Table 11, P = 0.003, n=4), barley inoculated with the rhizosphere soil of Pintium control plants (Table 11, P =0.003, n = 4) and the barley inoculated with H_2 treated soil (Table 11, P = 0.033, n = 4). The P-value of ANOVA for shoot dry weight is 0.027. The average dry weight of shoots from barley inoculated with soil adjacent to HUP- nodules of Pintium test plants is higher than the average dry weight of shoots from barley grown in bulk soil (Table 12, P = 0.004, n = 4).

Table 8: Multiple comparison of tiller number from barley inoculated with soil samples of CDC Striker plants at the 0.1 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Number of Tiller	P-value
CDC Stirker	2.5833 ± 0.3191	0.3
CDC Striker Control	2.3333 ± 0	
CDC Stirker	2.5833 ± 0.3191	0.004*
Bulk	1.75 ± 0.3191	
CDC Stirker	2.5833 ± 0.3191	0.3
H_2 Treated	2.3333 ± 0.4714	
CDC Striker Control	2.3333 ± 0	0.027*
Bulk	1.75 ± 0.3191	
CDC Striker Control	2.3333 ± 0	1
H_2 Treated	2.3333 ± 0.4714	
H_2 Treated	2.3333 ± 0.4714	0.027*
Bulk	1.75 ± 0.3191	

Table 9: Multiple comparison of the dry weight of shoots from barley inoculated with soil samples of CDC Striker plants at the 0.1 mM N level

Inoculation Treatment	Shoot Dry Weight (g)	P-value
CDC Stirker CDC Striker Control	1.0812 ± 0.1014 0.9078 ± 0.1784	0.126
CDC Stirker Bulk	1.0812 ± 0.1014 0.9069 ± 0.1138	0.124
CDC Stirker H_2 Treated	1.0812 ± 0.1014 1.135 ± 0.1845	0.62
CDC Striker Control Bulk	0.9078 ± 0.1784 0.9069 ± 0.1138	0.993
CDC Striker Control H_2 Treated	0.9078 ± 0.1784 1.135 ± 0.1845	0.052
H_2 Treated Bulk	1.135 ± 0.1845 0.9069 ± 0.1138	0.052

Table 10: Multiple comparison of the dry weight of roots from barley inoculated with soil samples of CDC Striker plants at the 0.1 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Root Dry Weight (g)	P-value
CDC Stirker	1.3215 ± 0.1058	0.634
CDC Striker Control	1.2633 ± 0.2367	
CDC Stirker	1.3215 ± 0.1058	0.085
Bulk	1.0974 ± 0.1952	
CDC Stirker	1.3215 ± 0.1058	0.421
H_2 Treated	1.4209 ± 0.0923	
CDC Striker Control	1.2633 ± 0.2367	0.19
Bulk	1.0974 ± 0.1952	
CDC Striker Control	1.2633 ± 0.2367	0.211
H_2 Treated	1.4209 ± 0.0923	
H_2 Treated	1.4209 ± 0.0923	0.019*
Bulk	1.0974 ± 0.1952	

Table 11: Multiple comparison of tiller number from barley inoculated with soil samples of Pintium plants at the 0.1 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Number of Tiller	P-value
Pintium	2.6667 ± 0.2722	1
Pintium Control	2.6667 ± 0.2722	
Pintium	2.6667 ± 0.2722	0.003*
Bulk	1.75 ± 0.3191	
Pintium	2.6667 ± 0.2722	0.195
H_2 Treated	2.3333 ± 0.4714	
Pintium Control	2.6667 ± 0.2722	0.003*
Bulk	1.75 ± 0.3191	
Pintium Control	2.6667 ± 0.2722	0.195
H_2 Treated	2.3333 ± 0.4714	
H_2 Treated	2.3333 ± 0.4714	0.033*
Bulk	1.75 ± 0.3191	

Table 12: Multiple comparison of the dry weight of shoots from barley inoculated with soil samples of Pintium plants at the 0.1 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Shoot Dry Weight (g)	P-value
Pintium Pintium Control	1.3443 ± 0.1415 1.1168 ± 0.2251	0.085
Pintium Bulk	1.3443 ± 0.1415 0.9069 ± 0.1138	0.004*
Pintium H_2 Treated	1.3443 ± 0.1415 1.135 ± 0.1845	0.11
Pintium Control Bulk	1.1168 ± 0.2251 0.9069 ± 0.1138	0.109
Pintium Control H_2 Treated	1.1168 ± 0.2251 1.135 ± 0.1845	0.883
H_2 Treated Bulk	1.135 ± 0.1845 0.9069 ± 0.1138	0.085

The P-value of ANOVA for root dry weight is 0.003. The barley grown in bulk soil has a lower average dry weight of roots than the barley inoculated with soil adjacent to HUP- nodules of Pintium test plants (Table 13, P = 0.000, n=4), barley inoculated with the rhizosphere soil of Pintium control plants (Table 13, P =0.034, n = 4) and the barley inoculated with H_2 treated soil (Table 13, P = 0.01, n = 4). The average dry weight of roots from barley inoculated with soil adjacent to HUP- nodules of Pintium test plants is significantly higher than that of barley inoculated with the rhizosphere soil of Pintium control plants (Table 13, P = 0.029, n = 4).

For the barley inoculated with soil samples from CDC Blitz at 0.1 mM N level, the P-value of ANOVA for the number of tillers on each barley plant among different soil inoculation treatments is 0.003. The barley grown in bulk soil has fewer tillers on average than the barley inoculated with soil adjacent to HUP- nodules of CDC Blitz test plants (Table 14, P = 0.000, n = 4), barley inoculated with the rhizosphere soil of CDC Blitz control plants (Table 14, P =0.006, n = 4) and the barley inoculated with H_2 treated soil (Table 14, P = 0.023, n = 4). The average number of tillers from the barley inoculated with soil adjacent to HUP- nodules of CDC Blitz test plants are significantly higher than the average number of tillers from the barley inoculated with H_2 treated soil (Table 14, P = 0.045, n = 4). The P-value of ANOVA for shoot dry weight is 0.001. The barley grown in bulk soil has a lower average dry weight of shoots than the barley inoculated with soil adjacent to HUP- nodules of CDC Blitz test plants (Table 15, P = 0.000, n = 4), barley inoculated with the rhizosphere soil of CDC Blitz control plants (Table 15, P =0.011, n = 4) and the barley inoculated with H_2 treated soil (Table 15, P = 0.043, n = 4). The average

dry weight of shoots from the barley inoculated with soil adjacent to HUP- nodules of the CDC Blitz test plants is higher than the average dry weight of shoots from the barley inoculated with the rhizosphere soil of the CDC Blitz control plants (Table 15, P =0.015, n = 4) and the barley inoculated with H_2 treated soil (Table 15, P = 0.004, n = 4). The P-value of ANOVA for root dry weight is 0.002. The barley grown in bulk soil has a lower average dry weight of roots than the barley inoculated with soil adjacent to HUP- nodules of the CDC Blitz test plants (Table 16, P = 0.000, n = 4), barley inoculated with the rhizosphere soil of the CDC Blitz control plants (Table 16, P =0.049, n = 4) and the barley inoculated with H_2 treated soil (Table 16, P = 0.035, n = 4). The average dry weight of shoots from the barley inoculated with soil adjacent to HUP- nodules of the CDC Blitz test plants is higher than the average dry weight of shoots from the barley inoculated with the rhizosphere soil of the CDC Blitz control plants (Table 16, P =0.01, n = 4) and the barley inoculated with H_2 treated soil (Table 16, P = 0.014, n = 4).

Table 13: Multiple comparison of the dry weight of roots from barley inoculated with soil samples of Pintium plants at the 0.1 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Root Dry Weight (g)	P-value
Pintium	1.6145 ± 0.1229	0.029*
Pintium Control	1.3518 ± 0.1692	
Pintium	1.6145 ± 0.1229	0.000*
Bulk	1.0974 ± 0.1952	
Pintium	1.6145 ± 0.1229	0.093
H_2 Treated	1.4209 ± 0.0923	
Pintium Control	1.3518 ± 0.1692	0.034*
Bulk	1.0974 ± 0.1952	
Pintium Control	1.3518 ± 0.1692	0.528
H_2 Treated	1.4209 ± 0.0923	
H_2 Treated	1.4209 ± 0.0923	0.01*
Bulk	1.0974 ± 0.1952	

Table 14: Multiple comparison of tiller number from barley inoculated with soil samples of CDC Blitz plants at the 0.1 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Number of Tiller	P-value
CDC Blitz CDC Blitz Control	2.8333 ± 0.1925 2.5 ± 0.1925	0.161
CDC Blitz Bulk	2.8333 ± 0.1925 1.75 ± 0.3191	0.000*
CDC Blitz H_2 Treated	2.8333 ± 0.1925 2.3333 ± 0.4714	0.045*
CDC Blitz Control Bulk	2.5 ± 0.1925 1.75 ± 0.3191	0.006*
CDC Blitz Control H_2 Treated	2.5 ± 0.1925 2.3333 ± 0.4714	0.469
H_2 Treated Bulk	2.3333 ± 0.4714 1.75 ± 0.3191	0.023*

Table 15: Multiple comparison of the dry weight of shoots from barley inoculated with soil samples of CDC Blitz plants at the 0.1 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Shoot Dry Weight (g)	P-value
CDC Blitz CDC Blitz Control	1.4963 ± 0.17333 1.2096 ± 0.0655	0.015*
CDC Blitz Bulk	1.4963 ± 0.17333 0.9069 ± 0.1138	0.000*
CDC Blitz H_2 Treated	1.4963 ± 0.17333 1.135 ± 0.1845	0.004*
CDC Blitz Control Bulk	1.2096 ± 0.0655 0.9069 ± 0.1138	0.011*
CDC Blitz Control H_2 Treated	1.2096 ± 0.0655 1.135 ± 0.1845	0.473
H_2 Treated Bulk	1.135 ± 0.1845 0.9069 ± 0.1138	0.043*

Table 16: Multiple comparison of the dry weight of roots from barley inoculated with soil samples of CDC Blitz plants at the 0.1 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Root Dry Weight (g)	P-value
CDC Blitz CDC Blitz Control	1.8133 ± 0.0732 1.395 ± 0.3103	0.01*
CDC Blitz Bulk	1.8133 ± 0.0732 1.0974 ± 0.1952	0.000*
CDC Blitz H_2 Treated	1.8133 ± 0.0732 1.4209 ± 0.0923	0.014*
CDC Blitz Control Bulk	1.395 ± 0.3103 1.0974 ± 0.1952	0.049*
CDC Blitz Control H_2 Treated	1.395 ± 0.3103 1.4209 ± 0.0923	0.852
H_2 Treated Bulk	1.4209 ± 0.0923 1.0974 ± 0.1952	0.035*

At the level of 0.5 mM N, the barley inoculated with H_2 treated soil had more tillers than the barley grown in bulk soil (Figure 10). The barley inoculated with soil adjacent to HUP- nodules of test pulse plants had a higher dry weight of shoots than the barley inoculated with the rhizosphere soil of control pulse plants in most of the cases, except the barleys inoculated with soil samples from CDC Vanguard plants (Figure 11). The barley inoculated with H_2 treated soil also had a higher dry weight of shoots than the barley inoculated with the rhizosphere soil of control pulse plants in all 4 sets of barley (Figure 11). The barley inoculated with H_2 treated soil and barley inoculated with soil adjacent to HUP- nodules of the test pulse plants had a higher dry weight of shoots and more roots mass than the barley grown in bulk soil across all 4 sets of barley (Figure 11 and Figure 12). Fisher's least significant difference test was performed for further and more detail results after ANOVA test.

For the barley inoculated with soil samples from CDC Vanguard at the 0.5 mM N level, the P-value of ANOVA for the number of tillers on each barley plant among different soil inoculation treatments is 0.829. There is no significant difference found in tiller numbers among the different treatments (Table 17). The P-value of ANOVA for shoot dry weight is 0.037. The barley inoculated with H_2 treated soil had a significantly higher average dry weight of shoots than barley inoculated with soil adjacent to HUP- nodules of the CDC Vanguard plants (Table 18, $P = 0.022$, $n = 4$), barley inoculated with the rhizosphere soil of the CDC Vanguard control plants (Table 18, $P = 0.039$, $n = 4$) and barley grown in bulk soil (Table 18, $P = 0.008$, $n = 4$). The P-value of ANOVA for root dry weight is 0.093. The barley inoculated with H_2 treated soil had more roots than the

Figure 10: The number of tillers from barley inoculated with soil adjacent to HUP-pulse nodules, barley inoculated with the rhizosphere soil of pulse control plants, barley grown in bulk soil and barley inoculated with H2 treated soil for the 4 selected pulse varieties at the 0.5 mM N level in the second trial. Error bar: standard error; Test: barley inoculated with soil adjacent to pulse nodules; Control: barley inoculated with rhizosphere from control plants; Bulk: barley grew in bulk soil; H_2 treated: barley inoculated with H_2 treated soil.

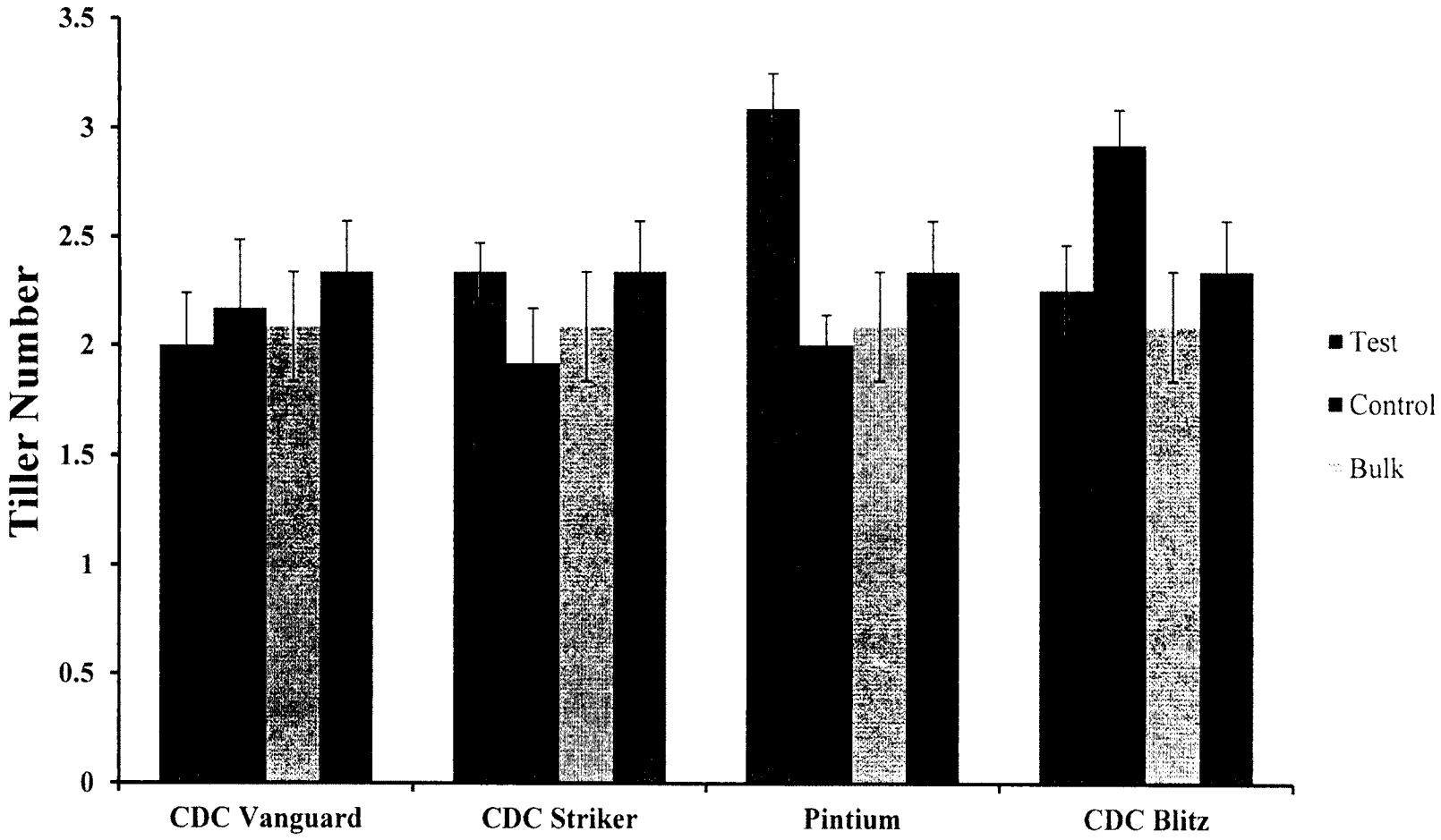

Tiller Number
3.5
3
2.5
2
1.5
1
0.5
0
CDC Vanguard
CDC Striker
Pintium
CDC Blitz
Test
Control
Bulk

Figure 11: The dry weight of shoots from barley inoculated with soil adjacent to HUP- pulse nodules, barley inoculated with the rhizosphere soil of pulse control plants, barley grown in bulk soil and barley inoculated with H2 treated soil for the 4 selected pulse varieties at the 0.5 mM N level in the second trial. Error bar: standard error; Test: barley inoculated with soil adjacent to pulse nodules; Control: barley inoculated with rhizosphere from control plants; Bulk: barley grew in bulk soil; H_2 treated: barley inoculated with H_2 treated soil.

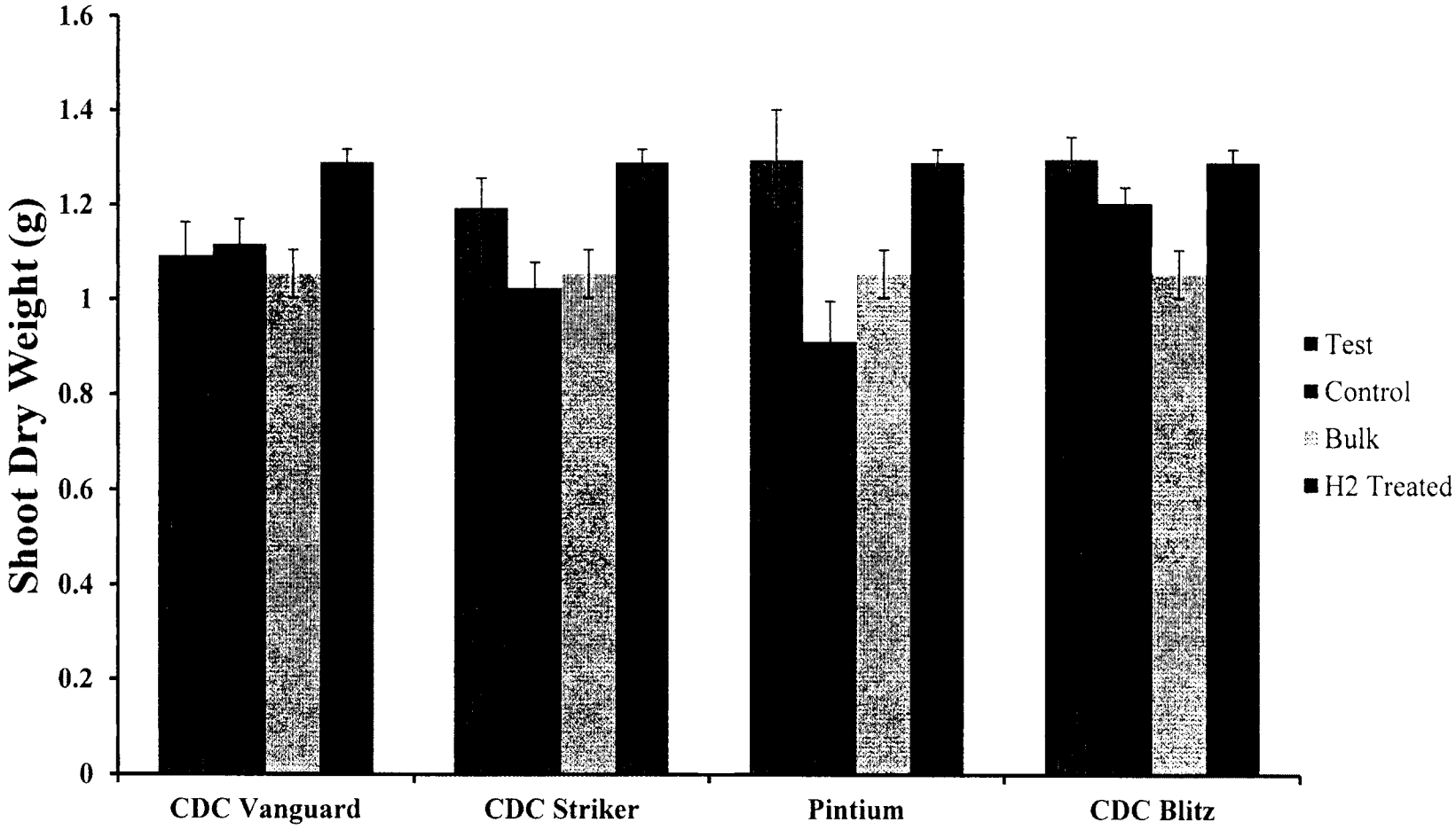

Shoot Dry Weight (g)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CDC Vanguard
CDC Striker
Pintium
CDC Blitz
Test
Control
Bulk
H2 Treated

Figure 12: The dry weight of roots from barley inoculated with soil adjacent to HUP- pulse nodules, barley inoculated with the rhizosphere soil of pulse control plants, barley grown in bulk soil and barley inoculated with H2 treated soil for the 4 selected pulse varieties at the 0.5 mM N level in the second trial. Error bar: standard error; Test: barley inoculated with soil adjacent to pulse nodules; Control: barley inoculated with rhizosphere from control plants; Bulk: barley grew in bulk soil; H_2 treated: barley inoculated with H_2 treated soil.

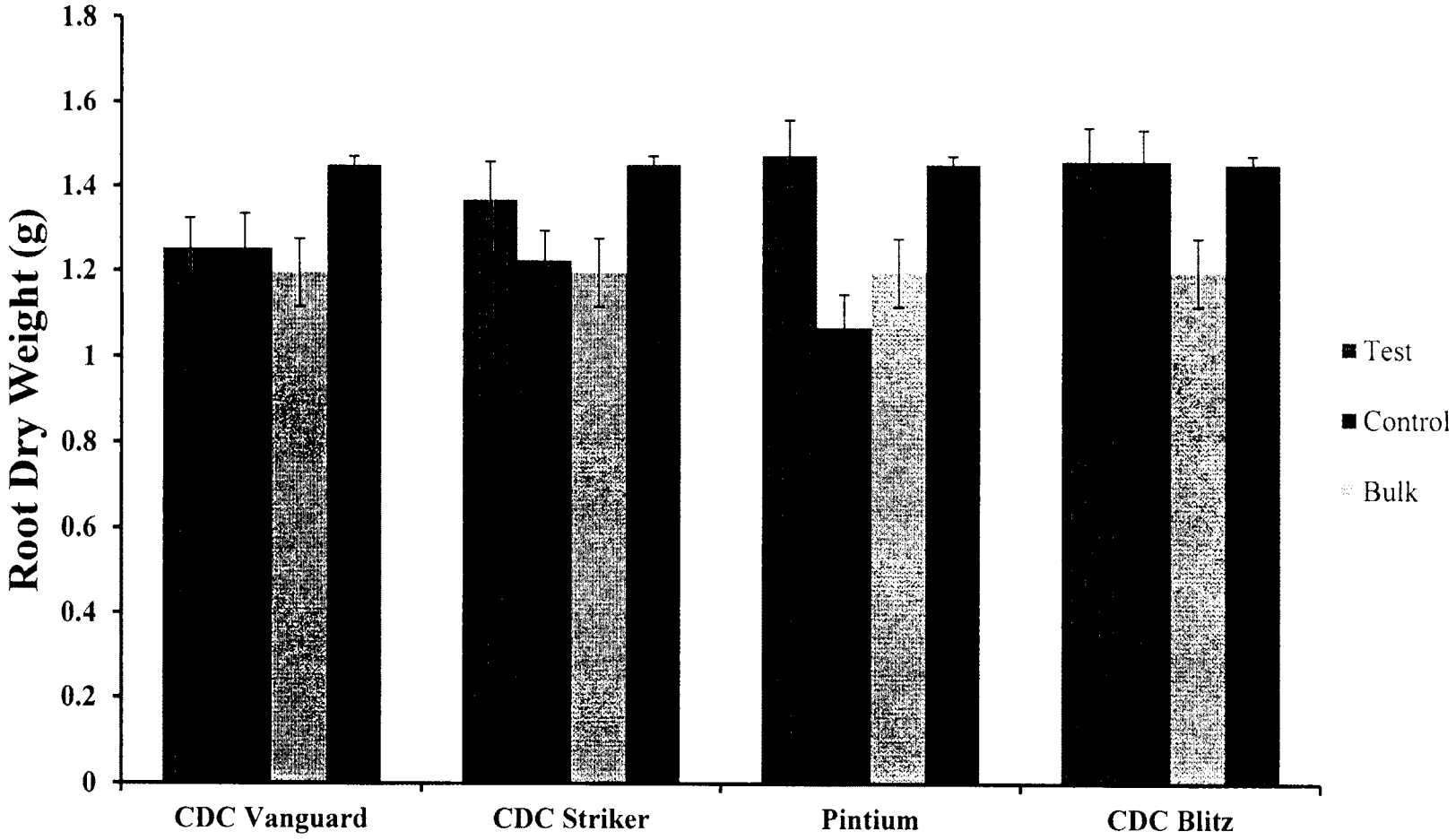

Root Dry Weight (g)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CDC Vanguard
CDC Striker
Pintium
CDC Blitz
Test
Control
Bulk

Table 17: Multiple comparison of tiller number from barley inoculated with soil samples of CDC Vanguard plants at the 0.5 mM N level

Inoculation Treatment	Number of Tiller	P-value
CDC Vanguard CDC Vanguard Control	2.000 ± 0.4714 2.1667 ± 0.6383	0.661
CDC Vanguard Bulk	2.000 ± 0.4714 2.0833± 0.500	0.826
CDC Vanguard H_2 Treated	2.000 ± 0.4714 2.3333 ± 0.4714	0.387
CDC Vanguard Control Bulk	2.1667 ± 0.6383 2.0833± 0.500	0.826
CDC Vanguard Control H_2 Treated	2.1667 ± 0.6383 2.3333 ± 0.4714	0.661
H_2 Treated Bulk	2.3333 ± 0.4714 2.0833± 0.500	0.513

Table 18: Multiple comparison of the dry weight of shoots from barley inoculated with soil samples of CDC Vanguard plants at the 0.5 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Shoot Dry Weight (g)	P-value
CDC Vanguard CDC Vanguard Control	1.0923 ± 0.1412 1.1166 ± 0.1048	0.75
CDC Vanguard Bulk	1.0923 ± 0.1412 1.0530 ± 0.1017	0.607
CDC Vanguard H_2 Treated	1.0923 ± 0.1412 1.2890 ± 0.0557	0.022*
CDC Vanguard Control Bulk	1.1166 ± 0.1048 1.0530 ± 0.1017	0.409
CDC Vanguard Control H_2 Treated	1.1166 ± 0.1048 1.2890 ± 0.0557	0.039*
H_2 Treated Bulk	1.2890 ± 0.0557 1.0530 ± 0.1017	0.008*

Table 19: Multiple comparison of the dry weight of roots from barley inoculated with soil samples of CDC Vanguard plants at the 0.5 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Root Dry Weight (g)	P-value
CDC Vanguard CDC Vanguard Control	1.2536 ± 0.1421 1.2527 ± 0.1641	0.992
CDC Vanguard Bulk	1.2536 ± 0.1421 1.1956 ± 0.1600	0.559
CDC Vanguard H_2 Treated	1.2536 ± 0.1421 1.451 ± 0.0389	0.063
CDC Vanguard Control Bulk	1.2527 ± 0.1641 1.1956 ± 0.1600	0.565
CDC Vanguard Control H_2 Treated	1.2527 ± 0.1641 1.451 ± 0.0389	0.062
H_2 Treated Bulk	1.451 ± 0.0389 1.1956 ± 0.1600	0.021*

barley grown in bulk soil (Table 19, P = 0.021, n = 4).

For the barley inoculated with soil samples from CDC Striker at the 0.5 mM N level, the P-value of ANOVA for the number of tillers on each barley plant among different soil inoculation treatments is 0.499. There is no significant difference observed in tiller numbers among the different treatments (Table 20). The P-value of ANOVA for shoot dry weight is 0.01. The barley inoculated with H_2 treated soil had a higher average dry weight of shoots than the barley inoculated with the rhizosphere soil of the CDC Striker control plants (Table 21, P = 0.003, n = 4) and barley grown in bulk soil (Table 21, P = 0.007, n = 4). The barley inoculated with soil adjacent to HUP- nodules of the CDC Striker plants also had a higher average dry weight of shoots than the barley inoculated with the rhizosphere soil of the CDC Striker control plants (Table 21, P = 0.036, n = 4). The P-value of ANOVA for shoot dry weight is 0.079. The barley inoculated with H_2 treated soil had a higher average dry weight of roots than the barley inoculated with the rhizosphere soil of the CDC Striker control plants (Table 22, P = 0.042, n = 4) and barley grown in bulk soil (Table 22, P = 0.026, n = 4).

For the barley inoculated with soil samples from Pintium at the 0.5 mM N level, the P-value of ANOVA for the number of tillers on each barley plant among different soil inoculation treatments is 0.01. The barley inoculated with soil adjacent to HUP- nodules of Pintium plants had significantly more tillers than the barley inoculated with the rhizosphere soil of Pintium control plants (Table 23, P = 0.003, n = 4), barley grown in bulk soil (Table 23, P = 0.004, n = 4) and the barley inoculated with H_2 treated soil

Table 20: Multiple comparison of tiller number from barley inoculated with soil samples of CDC Striker plants at the 0.5 mM N level

Inoculation Treatment	Number of Tiller	P-value
CDC Stirker CDC Striker Control	2.3333 ± 0.2722 1.9167 ± 0.500	0.211
CDC Stirker Bulk	2.3333 ± 0.2722 2.0833± 0.500	0.444
CDC Stirker H_2 Treated	2.3333 ± 0.2722 2.3333 ± 0.4714	1
CDC Striker Control Bulk	1.9167 ± 0.500 2.0833± 0.500	0.607
CDC Striker Control H_2 Treated	1.9167 ± 0.500 2.3333 ± 0.4714	0.211
H_2 Treated Bulk	2.3333 ± 0.4714 2.0833± 0.500	0.444

Table 21: Multiple comparison of the dry weight of shoots from barley inoculated with soil samples of CDC Striker plants at the 0.5 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Shoot Dry Weight (g)	P-value
CDC Stirker	1.1921 ± 0.1263	0.036*
CDC Striker Control	1.0226 ± 0.1092	
CDC Stirker	1.1921 ± 0.1263	0.077
Bulk	1.0530 ± 0.1017	
CDC Stirker	1.1921 ± 0.1263	0.202
H_2 Treated	1.2890 ± 0.0557	
CDC Striker Control	1.0226 ± 0.1092	0.68
Bulk	1.0530 ± 0.1017	
CDC Striker Control	1.0226 ± 0.1092	0.003*
H_2 Treated	1.2890 ± 0.0557	
H_2 Treated	1.2890 ± 0.0557	0.007*
Bulk	1.0530 ± 0.1017	

Table 22: Multiple comparison of the dry weight of roots from barley inoculated with soil samples of CDC Striker plants at the 0.5 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Root Dry Weight (g)	P-value
CDC Stirker CDC Striker Control	1.3671 ± 0.1819 1.2233 ± 0.1423	0.177
CDC Stirker Bulk	1.3671 ± 0.1819 1.1956 ± 0.1600	0.113
CDC Stirker H_2 Treated	1.3671 ± 0.1819 1.451 ± 0.0389	0.419
CDC Striker Control Bulk	1.2233 ± 0.1423 1.1956 ± 0.1600	0.787
CDC Striker Control H_2 Treated	1.2233 ± 0.1423 1.451 ± 0.0389	0.042*
H_2 Treated Bulk	1.451 ± 0.0389 1.1956 ± 0.1600	0.026*

Table 23: Multiple comparison of tiller number from barley inoculated with soil samples of Pintium plants at the 0.5 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Number of Tiller	P-value
Pintium	3.0833 ± 0.3191	0.003*
Pintium Control	2.000 ± 0.2722	
Pintium	3.0833 ± 0.3191	0.004*
Bulk	2.0833± 0.500	
Pintium	3.0833 ± 0.3191	0.022*
H_2 Treated	2.3333 ± 0.4714	
Pintium Control	2.000 ± 0.2722	0.775
Bulk	2.0833± 0.500	
Pintium Control	2.000 ± 0.2722	0.264
H_2 Treated	2.3333 ± 0.4714	
H_2 Treated	2.3333 ± 0.4714	0.397
Bulk	2.0833± 0.500	

Table 24: Multiple comparison of the dry weight of shoots from barley inoculated with soil samples of Pintium plants at the 0.5 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Shoot Dry Weight (g)	P-value
Pintium Pintium Control	1.2939 ± 0.2127 0.9108 ± 0.1691	0.003*
Pintium Bulk	1.2939 ± 0.2127 1.0530 ± 0.1017	0.04*
Pintium H_2 Treated	1.2939 ± 0.2127 1.2890 ± 0.0557	0.964
Pintium Control Bulk	0.9108 ± 0.1691 1.0530 ± 0.1017	0.199
Pintium Control H_2 Treated	0.9108 ± 0.1691 1.2890 ± 0.0557	0.004*
H_2 Treated Bulk	1.2890 ± 0.0557 1.0530 ± 0.1017	0.043*

Table 25: Multiple comparison of the dry weight of roots from barley inoculated with soil samples of Pintium plants at the 0.5 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Root Dry Weight (g)	P-value
Pintium	1.4726 ± 0.1664	0.001*
Pintium Control	1.0692 ± 0.1508	
Pintium	1.4726 ± 0.1664	0.016*
Bulk	1.1956 ± 0.1600	
Pintium	1.4726 ± 0.1664	0.83
H_2 Treated	1.451 ± 0.0389	
Pintium Control	1.0692 ± 0.1508	0.223
Bulk	1.1956 ± 0.1600	
Pintium Control	1.0692 ± 0.1508	0.002*
H_2 Treated	1.451 ± 0.0389	
H_2 Treated	1.451 ± 0.0389	0.023*
Bulk	1.1956 ± 0.1600	

(Table 23, P = 0.022, n = 4). The P-value of ANOVA for shoot dry weight is 0.007. The barley inoculated with soil adjacent to HUP- nodules of Pintium plants had a much higher average dry weight of shoots than the barley inoculated with the rhizosphere soil of Pintium control plants (Table 24, P = 0.003, n = 4) and the barley grown in bulk soil (Table 24, P = 0.04, n = 4). The barley inoculated with H_2 treated soil also had a higher average dry weight of shoots than the barley inoculated with the rhizosphere soil of Pintium control plants (Table 24, P = 0.004, n = 4) and barley grown in bulk soil (Table 24, P = 0.043, n = 4). The P-value of ANOVA for root dry weight is 0.003. The barley inoculated with soil adjacent to HUP- nodules of Pintium plants had a much higher average dry weight of roots than the barley inoculated with the rhizosphere soil of Pintium control plants (Table 25, P = 0.001, n = 4) and barley grown in bulk soil (Table 25, P = 0.016, n = 4). The barley inoculated with H_2 treated soil also had a higher average dry weight of roots than the barley inoculated with the rhizosphere soil of Pintium control plants (Table 25, P = 0.002, n = 4) and barley grown in bulk soil (Table 25, P = 0.023, n = 4).

For the barley inoculated with soil samples from the CDC Blitz at the 0.5 mM N level, the P-value of ANOVA for the number of tillers on each barley plant among different soil inoculation treatments is 0.085. The barley inoculated with the rhizosphere soil of the CDC Blitz control plants had significantly more tillers than the barley grown in bulk soil (Table 26, P = 0.019, n = 4) and the barley inoculated with the rhizosphere soil of the CDC Blitz control plants also had near significantly more tillers than the barley inoculated with the soil adjacent to HUP- nodules of the CDC Blitz plants (Table

26, P = 0.05, n = 4). The P-value of ANOVA for shoot dry weight is 0.004. The barley grown in bulk soil had a significantly lower average dry weight of shoots than the barley inoculated with soil adjacent to the HUP- nodules of the CDC Blitz plants (Table 27, P = 0.001, n = 4), the barley inoculated with the rhizosphere soil of the CDC Blitz control plants (Table 27, P = 0.022, n = 4) and the barley inoculated with H_2 treated soil (Table 27, P = 0.001, n = 4). The P-value of ANOVA for root dry weight is 0.042. The barley grown in bulk soil had a significantly lower average dry weight of roots than the barley inoculated with soil adjacent to the HUP- nodules of the CDC Blitz plants (Table 28, P = 0.017, n = 4), the barley inoculated with the rhizosphere soil of the CDC Blitz control plants (Table 28, P = 0.017, n = 4) and the barley inoculated with H_2 treated soil (Table 28, P = 0.02, n = 4).

Table 26: Multiple comparison of tiller number from barley inoculated with soil samples of CDC Blitz plants at the 0.5 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Number of Tiller	P-value
CDC Blitz CDC Blitz Control	2.25 ± 0.4194 2.9167 ± 0.3191	0.05
CDC Blitz Bulk	2.25 ± 0.4194 2.0833± 0.500	0.596
CDC Blitz H_2 Treated	2.25 ± 0.4194 2.3333 ± 0.4714	0.79
CDC Blitz Control Bulk	2.9167 ± 0.3191 2.0833± 0.500	0.019*
CDC Blitz Control H_2 Treated	2.9167 ± 0.3191 2.3333 ± 0.4714	0.081
H_2 Treated Bulk	2.3333 ± 0.4714 2.0833± 0.500	0.43

Table 27: Multiple comparison of the dry weight of shoots from barley inoculated with soil samples of CDC Blitz plants at the 0.5 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Shoot Dry Weight (g)	P-value
CDC Blitz CDC Blitz Control	1.2956 ± 0.0946 1.2040 ± 0.0649	0.138
CDC Blitz Bulk	1.2956 ± 0.0946 1.0530 ± 0.1017	0.001*
CDC Blitz H_2 Treated	1.2956 ± 0.0946 1.2890 ± 0.0557	0.911
CDC Blitz Control Bulk	1.2040 ± 0.0649 1.0530 ± 0.1017	0.022*
CDC Blitz Control H_2 Treated	1.2040 ± 0.0649 1.2890 ± 0.0557	0.166
H_2 Treated Bulk	1.2890 ± 0.0557 1.0530 ± 0.1017	0.001*

Table 28: Multiple comparison of the dry weight of roots from barley inoculated with soil samples of CDC Blitz plants at the 0.5 mM N level (* significant in comparison, $\alpha = 0.05$)

Inoculation Treatment	Root Dry Weight (g)	P-value
CDC Blitz CDC Blitz Control	1.4593 ± 0.1567 1.4591 ± 0.1454	0.998
CDC Blitz Bulk	1.4593 ± 0.1567 1.1956 ± 0.1600	0.017*
CDC Blitz H_2 Treated	1.4593 ± 0.1567 1.451 ± 0.0389	0.932
CDC Blitz Control Bulk	1.4591 ± 0.1454 1.1956 ± 0.1600	0.017*
CDC Blitz Control H_2 Treated	1.4591 ± 0.1454 1.451 ± 0.0389	0.934
H_2 Treated Bulk	1.451 ± 0.0389 1.1956 ± 0.1600	0.02*

3.4 Discussion

3.4.1 The First Trial

As a screening trial, there was no complex or detail statistical analysis conducted for interpretation of the results for the first trial of the rotation benefit experiment. Only the percentage change between 2 treatments was calculated. The results of all 3 measurements in the first trial showed a considerable amount of fluctuation and no general overall patterns were observed (Figure 4, Figure 5 and Figure 6). This fluctuation could be the actual result of different treatments on the seeds, but it could be caused by the different levels of N in the fertilizer solution since the level of N in the fertilizer was accidentally changed when we switched the sources of N. Therefore, the results of this trial cannot be used as a base to select the pulse varieties for the second trial of rotation benefit.

The H_2 uptake rate of the soil samples that were used to inoculate the barley seeds became the reasonable standards for selecting the pulse varieties to plant for the second trial since the purpose of this experiment was to test the effects of soil bacterial community change, mainly H_2 oxidizing bacteria, around HUP- pulse nodules on crop rotation. Although the results of the first trial did not show any general pattern of affect on barley growth, the barley grown at the 0.1 mM N level seems to contain a consistent trend, which suggests that the barley inoculated with the soil adjacent to HUP- pulse nodules grows better than the barley inoculated with rhizosphere soil of the pulse control plants (Figure 3, Figure 4 and Figure 5). Therefore, 2 levels of N in fertilizer solution

were used in the first trial, 0.1 mM and 0.5 mM, were applied in the second trial to confirm the trend seen in first trial under the section of the 0.1 mM N level.

3.4.2 The Second Trial

In the second trial, the varieties of lentil were left out of the experiment. The reason for not including the lentil species is that the nodules of all the lentil varieties are very small and distribute densely all along the roots, which makes the collection of soil samples around the nodules difficult and less accurate. Therefore, only 4 pulse species were used in the second trial and the variety that offers the highest soil H_2 uptake rate around HUP- nodules was selected for each pulse species.

The barley that inoculated with the sample soil did not perform differently at 2 different N levels. However, the results of the second trial indicate that different pulse species respond to 2 different N levels differently. Overall, the barley inoculated with the soil adjacent to HUP- pulse nodules and the barley inoculated with H_2 treated soil grow better than the barley inoculated with the rhizospere soil of pulse control plants and the barley grown in bulk soil at both the 0.1 mM N level and the 0.5 mM N level, although the difference is not necessarily significant.

For the barley inoculated with soil samples from the CDC Vanguard at the 0.1 mM N level, the barley inoculated with the soil adjacent to the CDC Vanguard HUP- nodules grow better than the barley inoculated with the rhizosphere soil of the CDC Vanguard control plants and the barley grown in bulk soil in all 3 measurements (tiller, shoot and root). However, the growth of barley inoculated with soil adjacent to the CDC Vanguard

HUP- nodules remained relatively unchanged, while the other 3 treatment groups showed increased growth at the 0.5 mM N level. The barley inoculated with the rhizosphere soil of the CDC Vanguard control plants had a slightly higher tiller number and dry weight of shoots than the barley inoculated with the soil adjacent to the CDC Vanguard HUP- nodules. The growth of barley grown in bulk soil catches up to the barley inoculated with the soil adjacent to the CDC Vanguard HUP- nodules in every measurement. The changes in growth under 2 different N levels in different treatment groups could possibly be explained by the different bacterial communities in the soil samples that were used to inoculate the barley seeds. The soil adjacent to the CDC Vanguard HUP- nodules may contain some bacteria that can fix N or better utilize the N in the soil, which helps that barley grow better than the group inoculated with the rhizosphere soil of the CDC Vanguard control plants and the barley grown in bulk soil at a lower N level (Evans *et al.*, 1987; Lechner and Conrad, 1997). Once the N level is increased, the N-fixing bacteria or N-utilizing bacteria could be inhibited. Therefore, the extra N from the fertilizer solution replaced the bacteria's function in N utilization and the growth of the barley inoculated with the soil adjacent to the CDC Vanguard HUP- nodules remained the same at both N levels. For other 3 treatment groups, the growth of barley was limited by the low N level and lack of help from N-fixing soil bacteria or N-utilizing soil bacteria. When the level of N increases, the growth of the barley in these 3 treatment groups also increases.

For the barley inoculated with soil samples from CDC Striker, the growth of barley increased in shoots and roots, and the general growth patterns among different treatment groups were similar at 0.1 mM N and 0.5 mM N. The barley inoculated with the soil

adjacent to CDC Striker HUP- nodules and the barley inoculated with H_2 treated soil grew better than the barley inoculated with the rhizosphere soil of the CDC Striker control plants and the barley grown in bulk soil at both N levels. These observations suggest that the difference in growth seen among different treatment groups is probably driven by the H_2 oxidizing bacteria in the soil adjacent to the CDC Striker HUP- nodules and H_2 treated soil, since the major difference among soil samples is the exposure to H_2 gas. The difference in bacteria communities introduced by the presence of the CDC Striker plant probably had no or little effect on the growth of barley in this case.

For the barley inoculated with soil samples from Pintium, the barley inoculated with the soil adjacent to Pintium HUP- nodules and the barley inoculated with the rhizosphere soil of Pintium control plants grew better at the 0.1 mM N level than the barley with the same inoculation treatments at the 0.5 mM N level, in terms of having a slightly higher dry weight of shoots and roots. The barley inoculated with H_2 treated soil and the barley grown in bulk soil showed the opposite trend. The barley inoculated with H_2 treated soil and the barley grown in bulk soil had a little higher dry weight of shoots and roots at the 0.5 mM N level than their comparison groups at the 0.1 mM N level. These 2 opposite trends indicate that the soil with the presence of Pintium plants contains certain bacteria that help succeeding barley grow better and the bacteria work at their optimal level when the level of N is very low. The barley inoculated with the soil adjacent to Pintium HUP- nodules and the barley inoculated with H_2 treated soil showed higher growth on shoots and roots than the other 2 treatment groups at both N levels. However, the barley inoculated with the soil adjacent to the Pintium HUP- nodules had higher shoot and root

growth than the barley inoculated with the H_2 treated soil at the 0.1 mM N level while the shoots and roots for these two groups at the 0.5 mM N level are almost equal. This suggests that H_2 oxidizing bacteria and some other growth promoting bacteria positively influence succeeding barley together at a low N level, but when the level of N rises some growth promoting bacteria were inhibited. Therefore, H_2 oxidizing bacteria become the main force of growth promotion.

For the barley inoculated with soil samples from CDC Blitz, the change among different treatments groups at 2 different levels of N display a similar pattern as the barley inoculated with the soil samples from Pintium, except the barley inoculated with the rhizosphere soil of the CDC Blitz control plants. The dry weight of roots slightly increased in the barley inoculated with the rhizosphere soil of the CDC Blitz control plants when the N level increased from 0.1 mM to 0.5 mM. Due to lack of H_2 oxidizing bacteria in the rhizosphere soil of the CDC Blitz control plants, the higher root dry weight for the barley inoculated with the rhizosphere soil of the CDC Blitz control plants was possibly driven only by a higher N level. This pattern implies that the higher dry weight of shoots and roots seen in the barley inoculated with the soil adjacent to the CDC Blitz HUP- nodules at the 0.1 mM N level was possibly caused by H_2 oxidizing bacteria, and part of these H_2 oxidizing bacteria can also fix N (Evans *et al.*, 1987; Lechner and Conrad, 1997). Therefore, when the N level raises from 0.1 mM to 0.5 mM, the N fixing H_2 oxidizing bacteria could be inhibited and contribute less to plant growth promotion. It results in a slight drop in the shoot and root dry weight for the barley inoculated with the soil adjacent to the CDC Blitz HUP- nodules.

In summary, the comparison between the barley inoculated with the soil adjacent to the HUP- pulse nodules and the barley inoculated with the rhizosphere soil of pulse control plants is similar to the comparison between the barley inoculated with H_2 treated soil and the barley grown in bulk soil in most cases in the experiment. According to previous studies, certain groups of H_2 oxidizing bacteria in H_2 treated soil can promote plant growth (Dong *et al.*, 2003; Maimaiti *et al.*, 2007; Peoples *et al.*, 2008). Therefore, it is reasonable to speculate that the higher growth in the barley inoculated with the soil adjacent to the HUP- pulse nodules compared to the barley inoculated with the rhizosphere soil of pulse control plants is partially caused to by the H_2 oxidizing bacteria in the soil around the HUP- nodule since the nodules release H_2 into the soil. The different patterns of response to the 2 levels of N between the barley inoculated with the soil adjacent to the HUP- pulse nodules and the barley inoculated with H_2 treated soil also suggest that the bacterial communities in the soil around nodules are different from the bacterial communities in H_2 treated soil due to the plant's exertion. Different plant species interact with soil differently according their specific needs for nutrients, water and air. Then, different soil bacterial communities are facilitated and triggered to respond to these needs (Marschner *et al.*, 2002; Semenov *et al.*, 1999). The different trends observed between barley groups that were inoculated with the soil adjacent to HUP- nodule of different pulse species at different levels of N could possibly be the results of different bacterial communities existing in the soil around different pulse nodules. Moreover, most bacterial communities facilitated by pulse HUP- nodules are more beneficial to succeeding crops when the N level is low, which means that the benefit of including pulse plant in rotation cycles could be maximized by lowering the

use of chemical N fertilizers for the succeeding crops. Based on the results, faba bean seems to have the most positive effects on succeeding barley than other pulse species. The statistical analysis on this rotation benefit experiment is preliminary and relatively liberated due to the small sample size allowed in our facility. In the results, the observations from this experiment need to be further tested in larger scale experiments. Future study on this direction should increase the sample size to obtain more reliable results. There 3 groups of control barleys were used in this experiment: barley inoculated with rhizosphere soil of control pulse plant, barley inoculated with H_2 treated soil and barley planted in bulk soil without any inoculation. Ideally, the absolute equivalent control to the barley inoculated with soil adjacent to HUP- nodules of pulse plant should be the barley inoculated with soil adjacent to HUP+ nodules. However, no or very few HUP+ nodules were found in the experiment. Therefore, using soil adjacent with HUP+ nodules was not an option. Since the soil adjacent to pulse nodules could possibly had little higher amount of N than other soil sample, using two different levels of N in fertilizer solution could also test for the role of N in the H_2 induced rotation benefit.

4. MICROBIAL COMMUNITY CHANGE INDUCED BY NODULES

4.1 Introduction

The H_2 released from HUP- legume nodules is absorbed by the soil within 3-4 cm around the nodules (La Favre and Focht, 1983; Dong and Leyzell, 2001). It has also been reported that the soil bacterial populations increased in H_2 treated soil and the soil adjacent to HUP- nodules of soybean and alfalfa (Popelier *et al.*, 1985; Cunningham *et*

al., 1986). The soil bacteria were proven to be the main cause of the H_2 uptake in soil (McLean and Dong, 2002). The soil bacteria that can utilize the H_2 for their own benefit are called the H_2 oxidizing bacteria.

The H_2 oxidizing bacteria is not the name of a particular taxonomic group, and comprises species from a diverse taxa of Knallgas bacteria, nitrogen fixing bacteria and photosynthetic microorganisms (Evans *et al.*, 1987; Lechner and Conrad, 1997). As the name suggests, the group of aerobic H_2 oxidizing bacteria is characterized by their ability to oxidize H_2 gas or to use H_2 as an energy source to grow chemolithoautrophically, and they are most likely to occur in places where both H_2 and O_2 are available (Aragno and Schlegel, 1992).

From the current understanding of this bacterial group, it includes the representatives of alpha (α), beta (β), gamma (γ), subclasses of Proteobacteria as well as bacteria from the Cytophaga–Flavobacterium–Bacteroides (CFB) group (Friedrich and Schwartz, 1993; Lechner and Conrad, 1997; Stein *et al.*, 2005). According to the results of some studies, β-, γ-Proteobacteria and the CFB group benefit from soil H_2 treatment, but not α-Proteobacteria (Stein *et al.*, *2005*). Most rhizobia belong to α-Proteobacteria, and some of rhizobia, such as *Rhizobium* and *Bradyrhizobium*, are known to grow autotrophically with H_2 (Watson and Tabita, 1997). On the other hand, Stein and his colleagues (2005) found that α-Proteobacteria did not respond to H_2 treatment in their experiment and this could potentially be explained by a selective effect to β- and γ-Proteobacteria. The results of these studies suggest that the H_2 metabolism in soil can drastically alter the soil bacterial community structure.

Despite this, the rhizosphere is an extremely diverse and dynamic living environment for a wide range of microorganisms and the interactions between soil bacteria and plants are very complex. These plant-soil bacterial interactions play important roles in maintaining soil quality and fertility (Lin *et al*, 2004). The composition and functions of soil bacteria can be easily influenced by various abiotic and biotic factors, such as different agricultural practices, plant growth and water and air content in soil (Bever *et al*, 1997). Since the H_2 released from HUP- legume nodules can alter the soil bacterial community structure and potentially have profound impacts on soil quality and plant growth, how much the soil bacterial community changes in the complex environment of the rhizosphere, because of the H_2 released from nodules, and whether the soil bacterial community structure changes to the same extent when different legume species (pulse crops in this case) are grown when the H_2 is released from different HUP- nodules needs to be investigated.

Terminal restriction fragment length polymorphism (T-RFLP) analysis is a cultural independent approach to assessing complex microbial communities. This technique can distinguish different bacterial populations based on their PCR amplified 16s rRNA gene fragment length polymorphisms of the restriction digestion (Liu *et al.*, 1997). One of the PCR primers is labeled with fluorescent dye for the automated DNA sequencer to determine the size of terminal restriction fragment and quantify the amount of fragment with fluorescent dye (Liu *et al.*, 1997). T-RFLP analysis has been proven to be an effective and reliable method to study bacterial community structure change in marine samples, animal fecal samples and soil samples (Moeseneder *et al.*, 1999; Kaplan *et al.*,

2001; Dunbar *et al.*, 2000). Therefore, the 16S rRNA gene T-RFLP analysis was employed in this experiment to better understand the change of soil bacterial community structure caused by different pulse nodules.

4.2 Materials and Methods

4.2.1 Soil DNA Extraction

The soil samples collected from Variety CDC Vanguard, CDC Striker, Pintium and CDC Blitz were used for T-RFL analysis. For each sample, the total soil DNA was extracted from about 0.25 g of soil by using an UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, California, USA). The alternative protocol provided by the company was followed to reach maximum yield of DNA. The soil was put into the 2 ml bead solution tubes and gently vortexed for a few seconds to mix the soil and solution. Then 60 µl of solution S1, which contains SDS to aid cell lysis, was added and mixed by inverting the tubes several times. About 200 µl of inhibitor removal solution (IRS) was added to precipitate humic acids and other PCR inhibitors. The bead tube was then secured horizontally and vortexed at maximum speed for 10 minutes. After the vortex, the bead tube was centrifuged for 30 seconds at 10,000× g, and the cell debris, soil particles, beads and humic acids formed a pellet at the bottom of the tube. The supernatant was transferred to a clean 2 ml microcentrifuge tube. To remove the protein content, 250 µl of solution S2 was added, then vortexed for 5 seconds and incubated at 4°C for 5 minutes. The tube was centrifuged at 10,000× g for 1 minute after the incubation. All supernatant was transferred to a clean 2 ml microcentrifuge tube and then mixed with 1.3 ml solution S3 for binding DNA to the filter membrane later. About 700

μl of the mixture of supernatant and solution S3 was loaded on a spin filter and centrifuged for 1 minute at 10,000× g. Each soil sample usually took 3 loads for all DNA to bind on the filter membrane. The flow through was discarded and the spin filter was centrifuged again for 1 minute at 10,000× g to further remove the excess solution mixture. For further cleaning of the extracted DNA, 300 μl of ethanol based solution S4 was loaded in the spin filter and centrifuged at 10,000× g for 30 seconds. The flow through was discarded. To reach the maximum purity of DNA, this washing step was repeated 3 times. Then the spin filter was centrifuged once more at 10,000× g for 1 minute. The DNA was eluted into a clean 1.5 ml microcentrifuge tube by adding 50 μl of solution S5 (sterile elution buffer) to the centre of filter membrane and centrifuged at 10,000× g for 30 seconds. The spin filter was discarded. A gel electrophoresis was performed to check the integrity of the extracted DNA. The products of three replicate DNA extractions were combined to limit the random bias on soil bacteria selection.

4.2.2 Polymerase Chain Reaction (PCR) for Amplifying 16S rRNA Gene

The 16S rRNA genes from each soil sample were amplified through PCR with a pair of bacterial universal primers: forward primer BSF8/20 with fluorescent dye 6-FAM (phosphoramidite fluorochrome 5-carboxyfluorescein) labeled at the 5' terminus (6-FAM-5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer BSR534/18 (5'-ATTACCGCGGCTGCTGGC-3'). The expected length of PCR product is 527 base pair.

Although most PCR inhibitors were removed during the DNA extraction process, the level of residual PCR inhibitors in the extracted DNA was still high enough to affect the activity of DNA polymerase, and then negatively influence the result of the PCR.

Dilution of the DNA template was required to improve the efficiency of the PCR. The optimal DNA template dilution ration was determined as 1:10 (DNA volume: PCR reaction volume) after running several PCR trials and comparing their PCR product gel electrophoresis profiles.

The total volume of each PCR reaction was 25 µl, which contained 17.45 µl of PCR water (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada), 2.5 µl of 10× ThermoPol Reaction Buffer (New England Biolabs Ltd, Pickering, ON, Canada), 2.5 µl of 2 mM dNTP (New England Biolabs Ltd, Pickering, ON, Canada), 1 µl of 20 µM 6-FAM-5'-BSF8/20 (Eurofins MWG Operon, Huntsville, AL, USA), 1 µl of 20 µM BSR534/18 (Eurofins MWG Operon, Huntsville, AL, USA), 0.2 µl of 5 U/µl Taq DNA polymerase (New England Biolabs Ltd, Pickering, ON, Canada), 0.25 µl of extracted DNA, 0.1 µl of 3% BSA (Fermentas Canada Inc. Burlington, ON, Canada). After mixing all the reactants, the PCR was carried out in a 2720 Thermal Cycler (Applied Biosystems Life Technologies Corporation, Carlsbad, CA, USA). The PCR cycling condition was set at 3 minutes of denaturating stage at 95°C, an amplifying stage of 35 cycles that consisted of 60 seconds at 95°C, 35 seconds at 58°C for annealing and 35 seconds at 72°C for extension and 10 minutes at 72°C for the final extension. Gel electrophoresis was conducted after the PCR to ensure the target DNA fragments were amplified. To minimize PCR-induced random biases, 16 PCR reactions were pooled together for each soil sample. The PCR products were purified by the QIAquick PCR Purification Kit (QIAGEN Inc, Mississauga, ON, Canada) before performing the restriction enzyme digestion.

4.2.3 Restriction Enzyme Digestion

Four restriction enzymes BstUI, HinfI, HaeIII and MspI were used to digest the purified PCR products in order to obtain 4 separate terminal restriction fragment (TRF) profiles for each soil sample. The restriction enzyme digestion reactions were run in 1.5 mL microcentrifuge tubes with the following enzyme digestion mixture: 5.0 μL 10X Buffer #4 (New England Biolabs Ltd., Pickering, ON, Canada); 500 ng of purified PCR products; 20 U of 1 of the 4 restriction enzymes (all restriction enzymes from New England Biolabs Ltd., Pickering, ON, Canada); and PCR water (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada), for a total of 50 μL for each reaction. Samples digested with HinfI, HaeIII and MspI were incubated in a 37°C water bath and samples digested with BstUI were incubated in a 60°C water bath over night (approximately 16 hours) to ensure complete digestion. For each restriction enzyme 3 replicate reactions were set up and pooled together to minimize the artificial biases. The reactions were stopped with the QIAquick Nucleotide Removal Kit (QIAGEN Inc., Mississauga, ON, Canada) according to the manufacturer's protocol. The products of enzyme digestion were then sent to the Core DNA Services Lab, Faculty of Medicine, University of Calgary, AB Canada for fragment analysis.

4.2.4 Generation of TRF Profiles and Data Sets

The TRF profiles were loaded and read by the GeneMarker V-1.4 software (SoftGenetics LLC, PA, USA). Each TRF was described in 3 aspects: fragment length in nucleotides, which is the apex position of each peak on a base pair (bp) scale relative to a

DNA size ladder (GeneScan 500 LIZ Size Standard in this case); the peak height at apex; and the area under the peak in fluorescence units. The area of a peak was calculated by integrating the fluorescence under that peak, and the total area of a profile was the amount of the areas of all peaks between 50bp and 500bp.

The TRF profiles were grouped into 4 different data sets according to the restriction enzyme used. To compare the individual TRF profiles within each data set, a peak plate was generated as a standard that consists of all the peaks from each individual TRF profile in the data set. After compared to the peak plate, the reading of the individual TRF profiles for each data set was exported into Microsoft Office Excel to format the data for standardization of the TRF profile.

4.2.5 Standardization of TRF Profiles

For more reliable further analysis, the variable percentage threshold method reported by Osborne and his colleagues (2006) was applied to standardize all TRF profiles within a data set. By using this method, a unique percentage threshold value of each profile was calculated by using a divisor to dive the total area of each TRF profile in the data set. For each TRF profile, the peaks that contribute less than their unique percentage threshold value in the profile (area of the peak/total area of the profile compared to the unique percentage threshold) were considered as background noise and removed from the profile. A series of gradient divisors were generated by running the program TRFLPdemo in software Matlab 7.1 (MathWorks Inc. Natick, MA, USA). The program TRFLPdemo was written by Luo, F. (Master student graduate from Computer Science department of Saint Mary's University) and Zhang, Y. (Master student graduate from Biology department of

Saint Mary's University). The divisors started with 100 times the mean total area of all profiles within the same data set and increased with an interval of 1.00×10^6. At each divisor the number of remaining peaks from each TRF profile after the standardization was plotted against the total area of the original TRF profile, a best fitted power function curve was generated and R^2 of the power was calculated by Matlab 7.1. The divisor that resulted in the most random relationship between the remaining peaks and the total area of the original profile was picked as the optimal divisor, which was indicated by an R^2 that was closest to zero.

4.2.6 Comparison of TRF Profiles

The standardized TRF profiles within a data set were aligned and the TRFs that have similar fragment sizes were identified and grouped together into a bin based on the bin table report that was generated by the GeneMarker V-1.4 software. All TRFs within the same bin were assigned with their average fragment size and represented 1 peak, and the range of a binned peak was the average fragment size ± 0.4 bp. A composite list of binned peaks was created for all the TRF profiles within the same data set. For each TRF profile a binned peak that was present in both the composite list and the TRF profile was denoted as 1, a binned peak that was present in the composite list but absent in the TRF profile was denoted as 0. The data set was transformed into a binary matrix in which the rows represented binned peaks and the columns represented the individual TRF profiles. The Jaccard coefficient was used to estimate the similarity between each two individual TRF profiles based on the pdist function in Matlab 7.1. The agglomerative hierarchical clustering was performed under the rule of unweighted average distance by applying the

Jaccard coefficient to the linkage function in Matlab 7.1. At the end, the hierarchical binary cluster tree was generated by plotting the result of linkage function under the dendrogram function in Matlab 7.1. The cophenetic correlation coefficient, which indicates the accuracy of the dendrogram compared to the data set, was also calculated through the function of cophenet during the process of creating the dendrogram. The closer the cophenetic correlation coefficient is to 1, the more accurately the dendrogram reflects the data set.

The weighted dendrogram was also generated in Matlab 7.1 through a series of steps similar to the unweighted dendrogram creation, except for the step of assigning a binned peak that was present in both the composite list and the TRF profile as 1. In this step, a binned peak that was present in both the composite list and the TRF profile was assigned the fraction the area of that peak represented in total area of that particular standardized TRF profile (binned peak area/total area of the standardized TRF profile).

4.3 Results

4.3.1 Standardization of TRF Profiles

An optimal divisor was calculated for each data set and listed in Table 29. The curves of the power function between the number of remaining peaks and the total area of original profiles were much flatter and linear (the red linear lines) when the optimal divisors applied were compared to the curves that resulted from other divisors (Figure 13, Figure 14, Figure 15 and Figure 16). The R^2 of the power curves generated from the optimal divisor are really close to 0 (Table 29), which means that the variable percentage

threshold (Table 30) derived from these optimal divisor can standardize the TRF profiles properly.

4.3.2 Comparison of TRF Profiles

Eighteen TRF profiles (9 soil samples and 2 replicates for each sample) were generated and formed a data set for each restriction enzyme. After the 4 individual data sets were standardized, a complex data set was constructed by combining all 4 standardized data sets together for comprehensive TRF profile analysis. Therefore, 5 data sets in total were created. For each data set, unweighted and weighted dendrograms were generated for TRF profile comparison. The unweighted dendrogram only counts the presence and absence of TRFs, while the weighted dendrogram takes into account the proportion of each TRF in the whole profile.

Table 29: Optimal divisors for T-RFLP data sets and R squares of power curves derived from optimal divisors

	BstUI	HaeIII	HinfI	MspI
Optimal Divisor	6.92×10^{7}	6.26×10^{7}	5.36×10^{7}	3.76×10^{7}
R Square	9.80×10^{-5}	-5.79×10^{-5}	3.76×10^{-5}	-7.73×10^{-4}

Table 30: Variable percentage threshold for T-RFLP profiles in each data set

Soil Sample	BstUI		HaeIII		HinfI		MspI	
	Total Area	Threshold (%)	Total Area	Threshold (%)	Total Area	Threshold (%)	Total Area	Threshold (%)
Striker-1	2.24×10^5	0.32	2.28×10^5	0.36	2.27×10^5	0.42	2.54×10^5	0.68
Striker-2	1.75×10^5	0.25	3.32×10^5	0.53	1.91×10^5	0.36	2.15×10^5	0.57
Str Con-1	2.03×10^5	0.29	3.15×10^5	0.5	2.18×10^5	0.41	1.24×10^5	0.33
Str Con-2	2.02×10^5	0.29	2.24×10^5	0.36	1.91×10^5	0.36	1.77×10^5	0.47
Vanguard-1	9.24×10^5	0.13	0.54×10^5	0.09	0.56×10^5	0.1	0.45×10^5	0.12
Vanguard-2	9.20×10^5	0.13	0.91×10^5	0.15	0.82×10^5	0.15	0.33×10^5	0.09
Van Con-1	5.72×10^5	0.08	0.70×10^5	0.11	0.61×10^5	0.11	0.57×10^5	0.15
Van Con-2	5.54×10^5	0.08	1.17×10^5	0.19	0.58×10^5	0.11	0.22×10^5	0.06
Blitz-1	3.35×10^5	0.48	3.42×10^5	0.55	2.39×10^5	0.45	2.18×10^5	0.58
Blitz-2	2.33×10^5	0.34	3.55×10^5	0.57	2.10×10^5	0.39	2.17×10^5	0.58
Blitz Con-1	2.19×10^5	0.32	3.47×10^5	0.55	2.73×10^5	0.51	2.01×10^5	0.53
Blitz Con-2	3.07×10^5	0.44	3.01×10^5	0.48	2.84×10^5	0.53	1.47×10^5	0.39
Pintium-1	2.53×10^5	0.36	1.28×10^5	0.2	0.97×10^5	0.18	1.48×10^5	0.39
Pintium-2	2.41×10^5	0.35	1.28×10^5	0.2	1.43×10^5	0.27	1.01×10^5	0.27
Pin Con-1	1.46×10^5	0.21	1.16×10^5	0.19	0.87×10^5	0.16	1.09×10^5	0.29
Pin Con-2	1.19×10^5	0.17	2.00×10^5	0.32	0.96×10^5	0.18	1.04×10^5	0.28
Bulk-1	1.83×10^5	0.26	1.36×10^5	0.22	1.50×10^5	0.28	1.42×10^5	0.38
Bulk-2	1.35×10^5	0.2	2.19×10^5	0.35	1.41×10^5	0.26	1.31×10^5	0.35

Figure 13: Estimation of the optimal divisor for the calculation of variable percentage threshold for BstUI data set. Nine curves generated by the calculation of different divisors were shown as the following: □, $Z\times10^2+7.3x10^7$; ×, $Z\times10^2+6.3x10^7$; ○, $Z\times10^2+5.3x10^7$; +, $Z\times10^2+4.3x10^7$; ✱, $Z\times10^2+3.3x10^7$; ▷, $Z\times10^2+2.3x10^7$; ◁, $Z\times10^2+1.3x10^7$; ◆, $Z\times10^2+0.3x10^6$ (Z: the mean total area for the data set. The optimum divisor was shown as '◆', which resulted in the minimum R square (almost zero) of the power function which means the weakest relationship between the total area on the original T-RFLP patterns and the numbers of remaining peaks after normalized by the threshold based on that divisor.

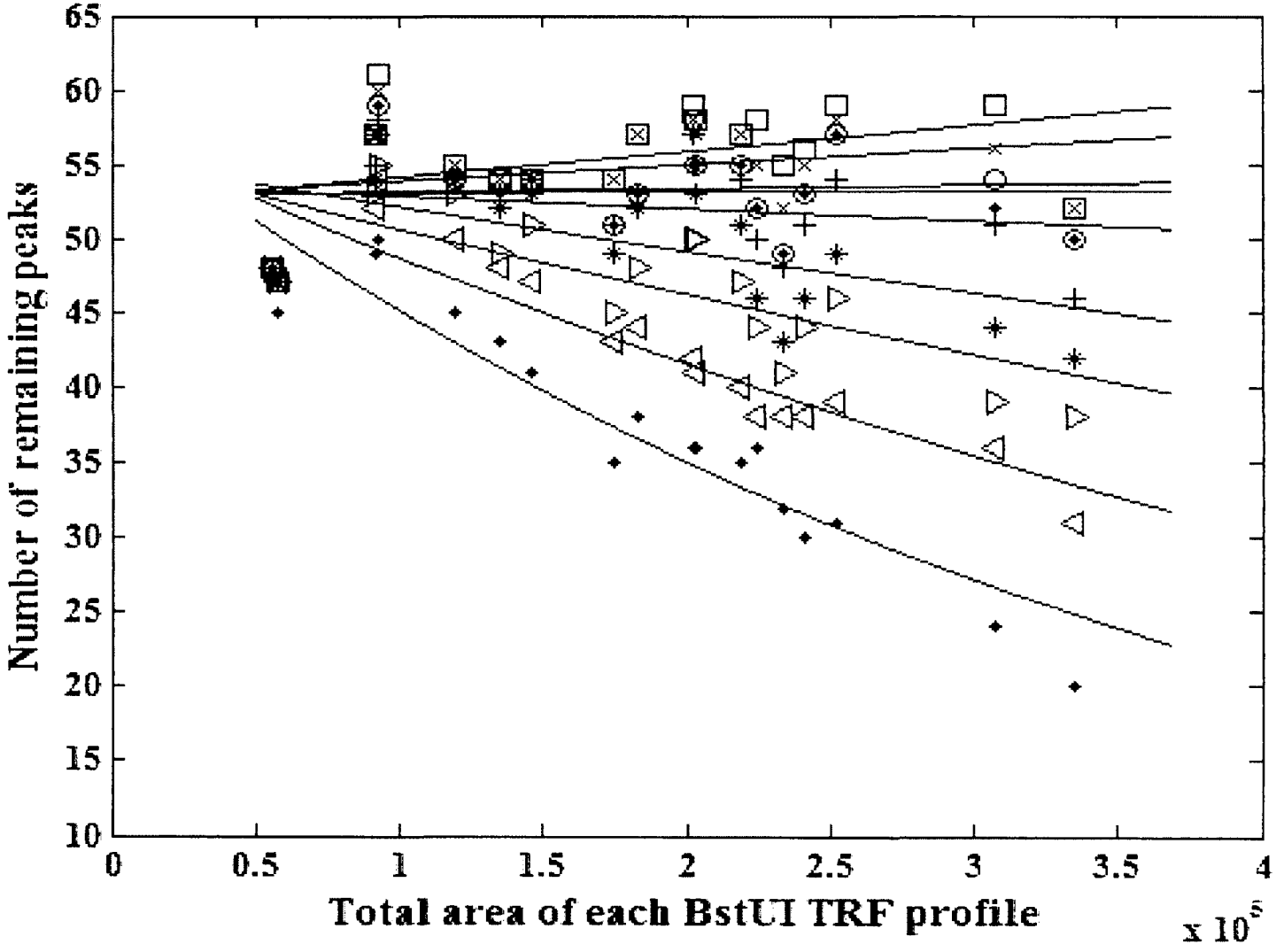

Number of remaining peaks
65
60
55
50
45
40
35
30
25
20
15
10
0
0.5
1
1.5
2
2.5
3
3.5
4
Total area of each BstUI TRF profile
x 10^5

Figure 14: Estimation of the optimal divisor for the calculation of variable percentage threshold for HaeIII data set. Nine curves generated by the calculation of different divisors were shown as the following: □, $Z\times10^2+7.3x10^7$; ×, $Z\times10^2+6.3x10^7$; ○, $Z\times10^2+5.3x10^7$; +, $Z\times10^2+4.3x10^7$; ✱, $Z\times10^2+3.3x10^7$; ▷, $Z\times10^2+2.3x10^7$; ◁, $Z\times10^2+1.3x10^7$; ◆, $Z\times10^2+0.3x10^6$ (Z: the mean total area for the data set. The optimum divisor was shown as '◆', which resulted in the minimum R square (almost zero) of the power function which means the weakest relationship between the total area on the original T-RFLP patterns and the numbers of remaining peaks after normalized by the threshold based on that divisor.

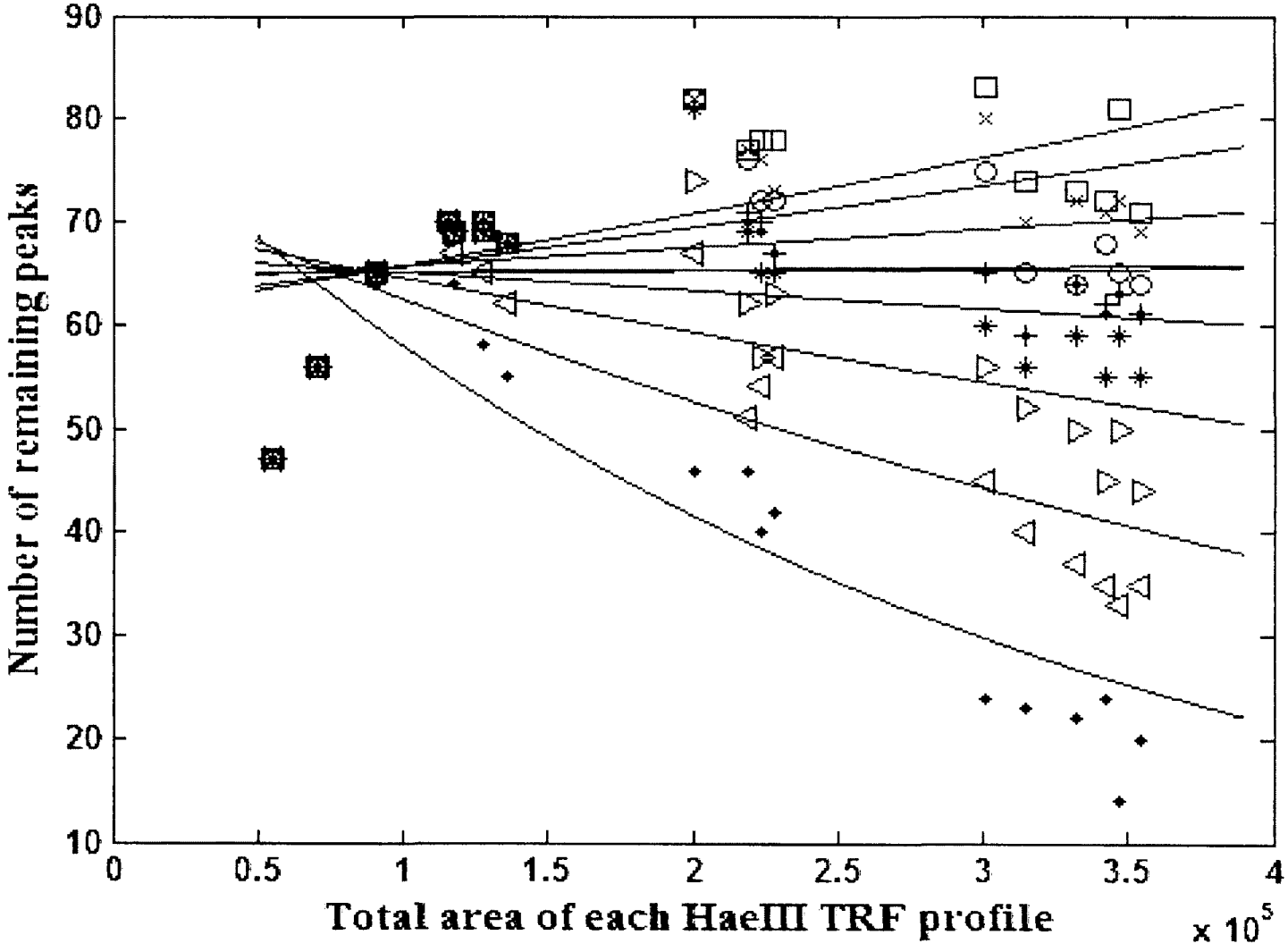

Number of remaining peaks
90
80
70
60
50
40
30
20
10
0
0.5
1
1.5
2
2.5
3
3.5
4
Total area of each HaeIII TRF profile
x 10^5

Figure 15: Estimation of the optimal divisor for the calculation of variable percentage threshold for HinfI data set. Nine curves generated by the calculation of different divisors were shown as the following: □, $Z\times10^2+7.3x10^7$; ×, $Z\times10^2+6.3x10^7$; ○, $Z\times10^2+5.3x10^7$; +, $Z\times10^2+4.3x10^7$; ✱, $Z\times10^2+3.3x10^7$; ▷, $Z\times10^2+2.3x10^7$; ◁, $Z\times10^2+1.3x10^7$; ◆, $Z\times10^2+0.3x10^6$ (Z: the mean total area for the data set. The optimum divisor was shown as '◆', which resulted in the minimum R square (almost zero) of the power function which means the weakest relationship between the total area on the original T-RFLP patterns and the numbers of remaining peaks after normalized by the threshold based on that divisor.

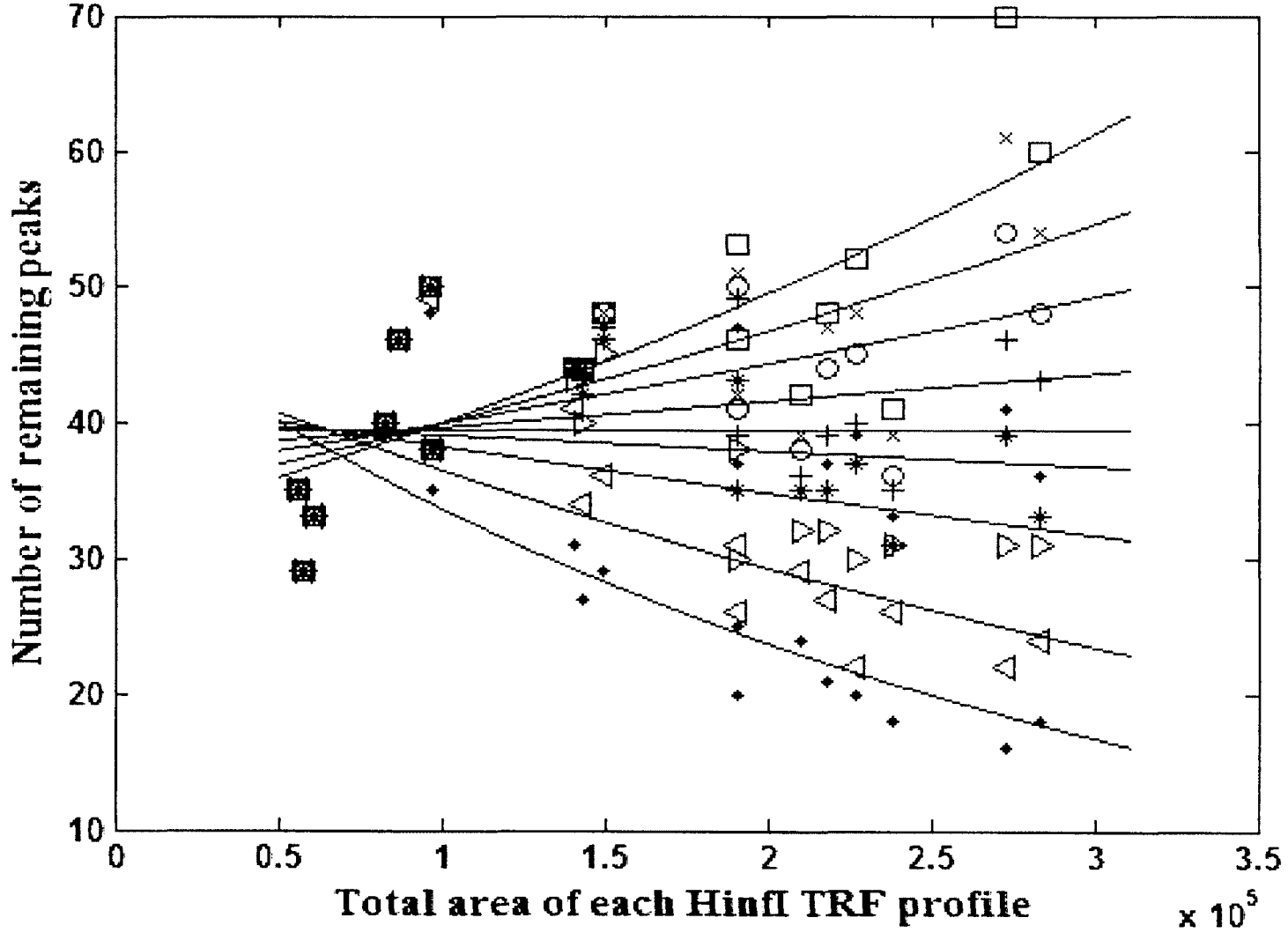
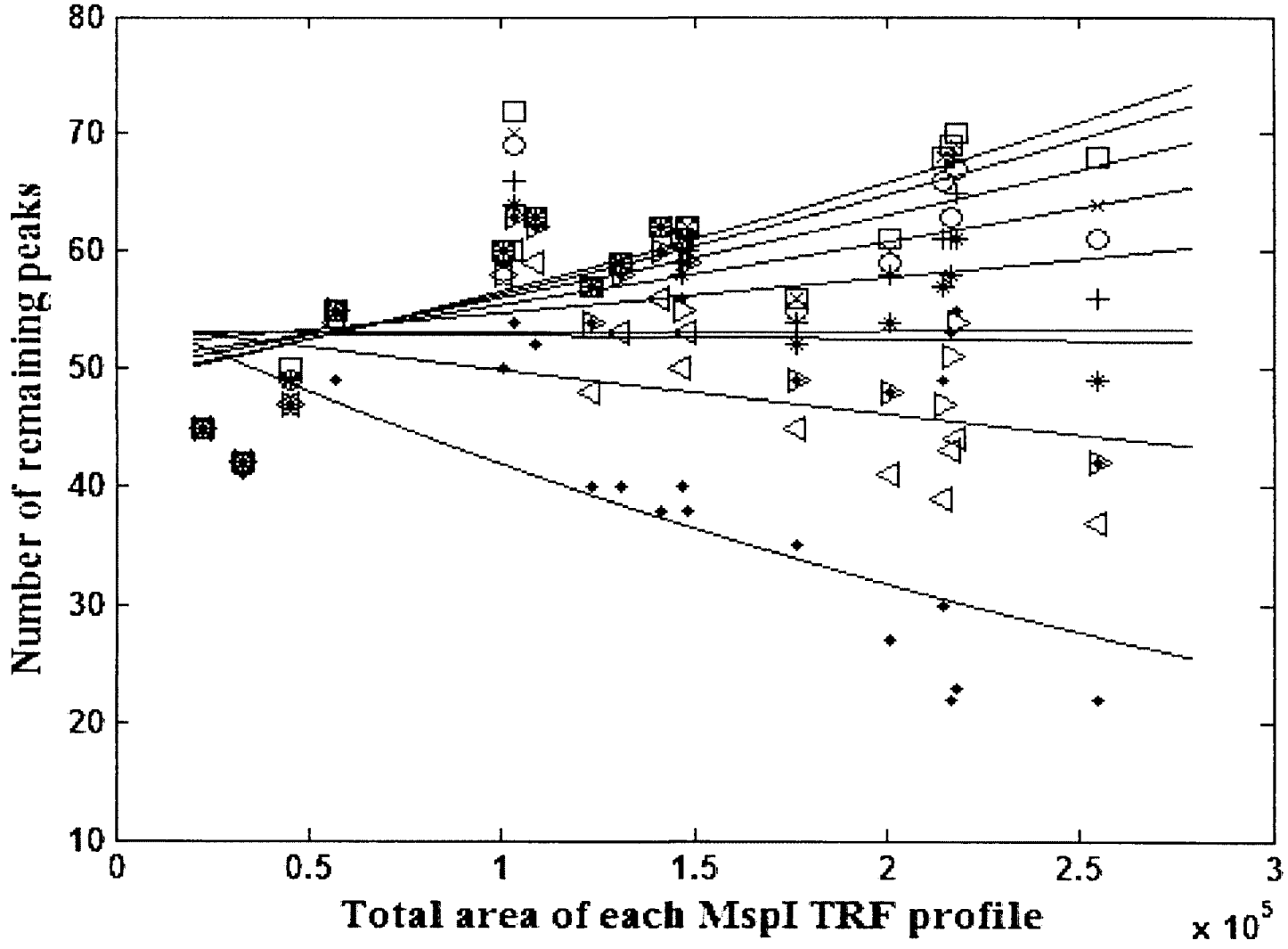

Number of remaining peaks
70
60
50
40
30
20
10
0
0.5
1
1.5
2
2.5
3
3.5
Total area of each HinfI TRF profile
x 10^5

Figure 16: Estimation of the optimal divisor for the calculation of variable percentage threshold for MspI data set. Nine curves generated by the calculation of different divisors were shown as the following: □, $Z\times10^2+7.3x10^7$; ×, $Z\times10^2+6.3x10^7$; ○, $Z\times10^2+5.3x10^7$; +, $Z\times10^2+4.3x10^7$; ✱, $Z\times10^2+3.3x10^7$; ▷, $Z\times10^2+2.3x10^7$; ◁, $Z\times10^2+1.3x10^7$; ◆, $Z\times10^2+0.3x10^6$ (Z: the mean total area for the data set. The optimum divisor was shown as '◆', which resulted in the minimum R square (almost zero) of the power function which means the weakest relationship between the total area on the original T-RFLP patterns and the numbers of remaining peaks after normalized by the threshold based on that divisor.


Number of remaining peaks
80
70
60
50
40
30
20
10
0
0.5
1
1.5
2
2.5
3
Total area of each MspI TRF profile
x 10^5

For the data set from the digestion reaction with BstUI enzyme, the unweighted dendrogram showed that the soil samples from the rhizosphere of uninoculated pulse plants have different bacterial communities compare to the soil adjacent to HUP- nodules of inoculated pulse plants for most of the varieties and the 2 replicates from the same soil sample are very similar, except the soil samples from variety CDC Striker plants (Figure 17). The bulk soil also has different bacterial community structure than all other soil samples, but it is relatively more similar to the soil samples collected from CDC Striker plants (Figure 17). The weighted dendrogram showed similar patterns as the unweighted dendrogram, expect that the large difference between 2 replicates of the same soil sample were seen in the soil adjacent to HUP- nodules of inoculated CDC Blitz plants and the rhizosphere soil of uninoculated CDC Vanguard plants (Figure 18). The cophenetic correlation coefficients for the unweighted dendrogram and weighted dendrogram are 0.8971 and 0.8714 respectively.

For the data set from the digestion reaction with HaeIII enzyme, the unweighted dendrogram indicated that the soil samples from the rhizosphere of uninoculated pulse plants have different bacterial community structure compare to the soil adjacent to HUP- nodules of inoculated pulse plants for most of the varieties, and the 2 replicates from the same soil sample are very similar, except the soil samples from variety CDC Vanguard plants (Figure 19). The bacterial communities in soil samples that collected from the plants of same pulse species are more similar to each other than the bacterial communities in the soil samples collected from the plants of other pulse species (Figure 19). The bacterial community in bulk soil is different than the bacterial communities in

Figure 17: The unweighted dendrogram structures of TRF profile comparisons for BstUI data set.

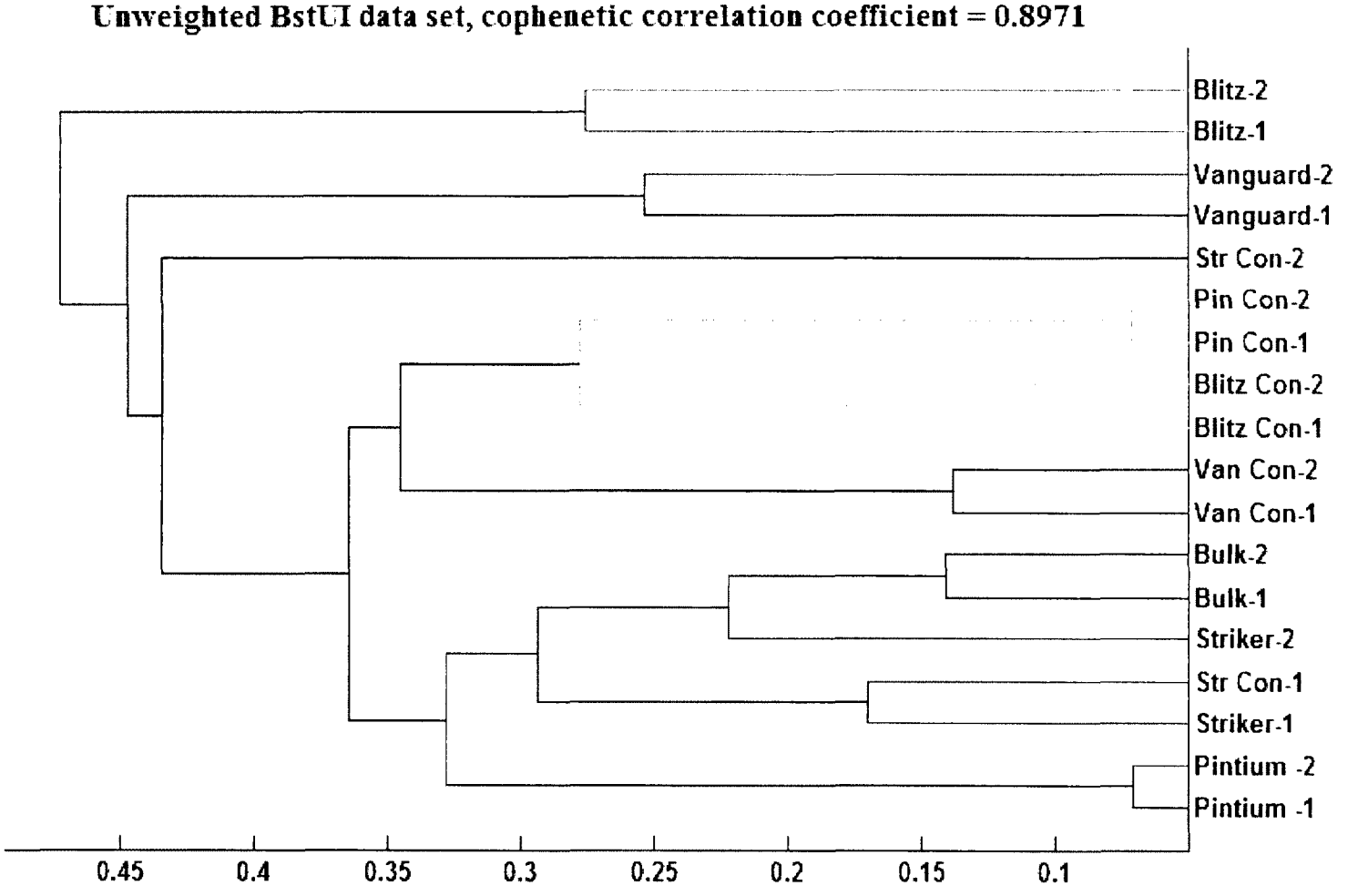

Unweighted BstUI data set, cophenetic correlation coefficient = 0.8971
Blitz-2
Blitz-1
Vanguard-2
Vanguard-1
Str Con-2
Pin Con-2
Pin Con-1
Blitz Con-2
Blitz Con-1
Van Con-2
Van Con-1
Bulk-2
Bulk-1
Striker-2
Str Con-1
Striker-1
Pintium -2
Pintium -1
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1

Figure 18: The weighted dendrogram structures of TRF profile comparisons for BstUI data set.

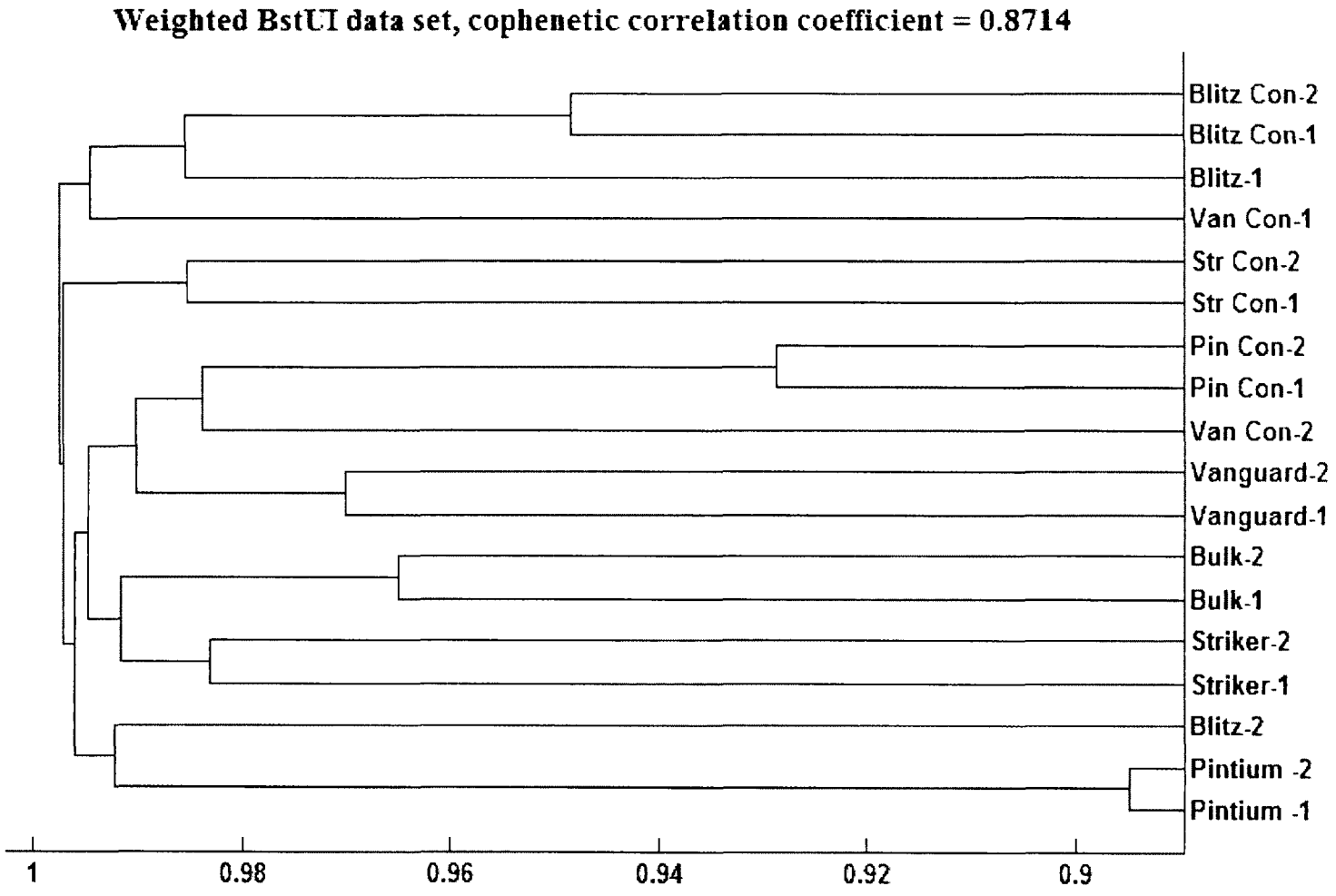

Weighted BstUI data set, cophenetic correlation coefficient = 0.8714
Blitz Con-2
Blitz Con-1
Blitz-1
Van Con-1
Str Con-2
Str Con-1
Pin Con-2
Pin Con-1
Van Con-2
Vanguard-2
Vanguard-1
Bulk-2
Bulk-1
Striker-2
Striker-1
Blitz-2
Pintium -2
Pintium -1
1
0.98
0.96
0.94
0.92
0.9

Figure 19: The unweighted dendrogram structures of TRF profile comparisons for HaeIII data set.

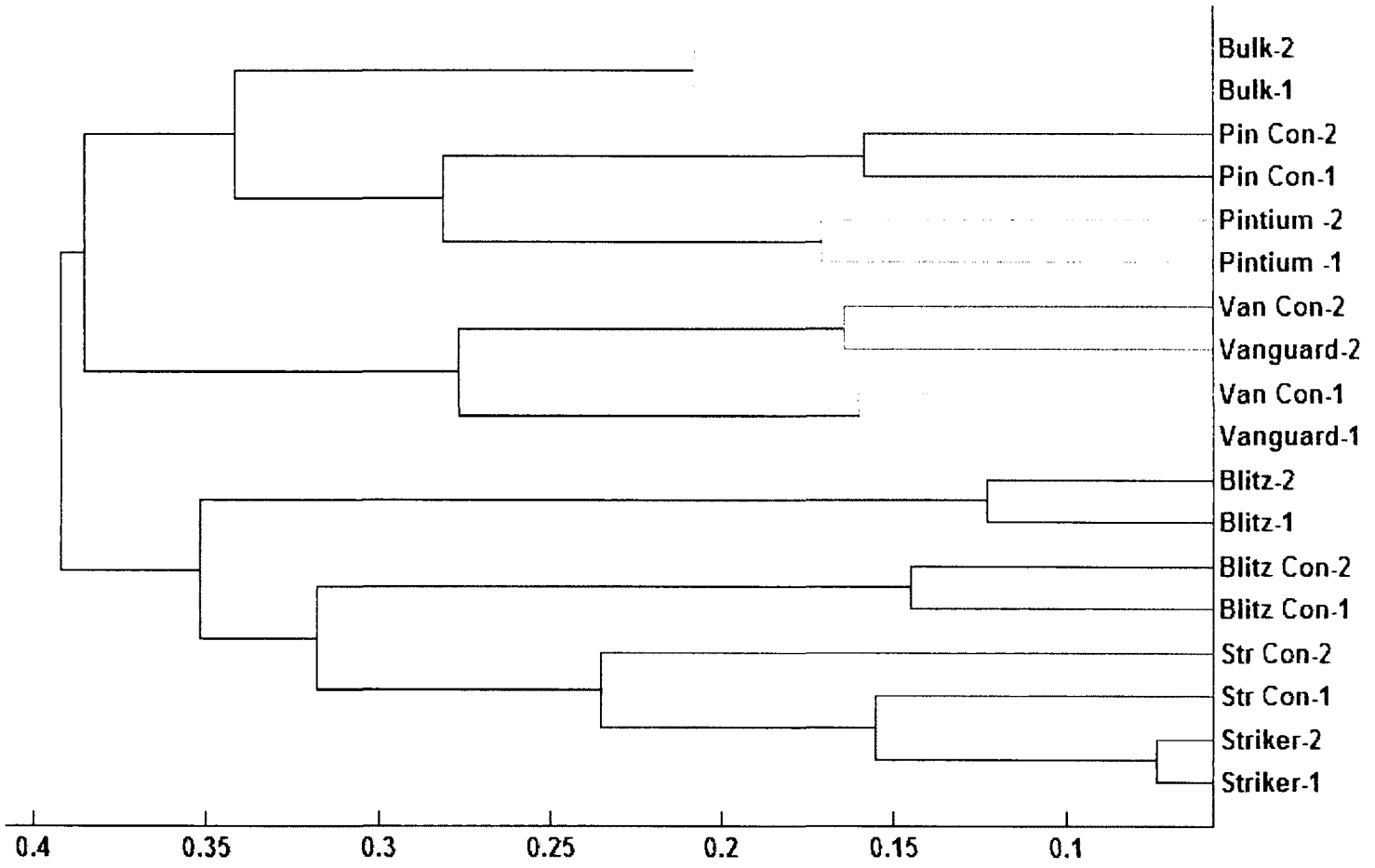
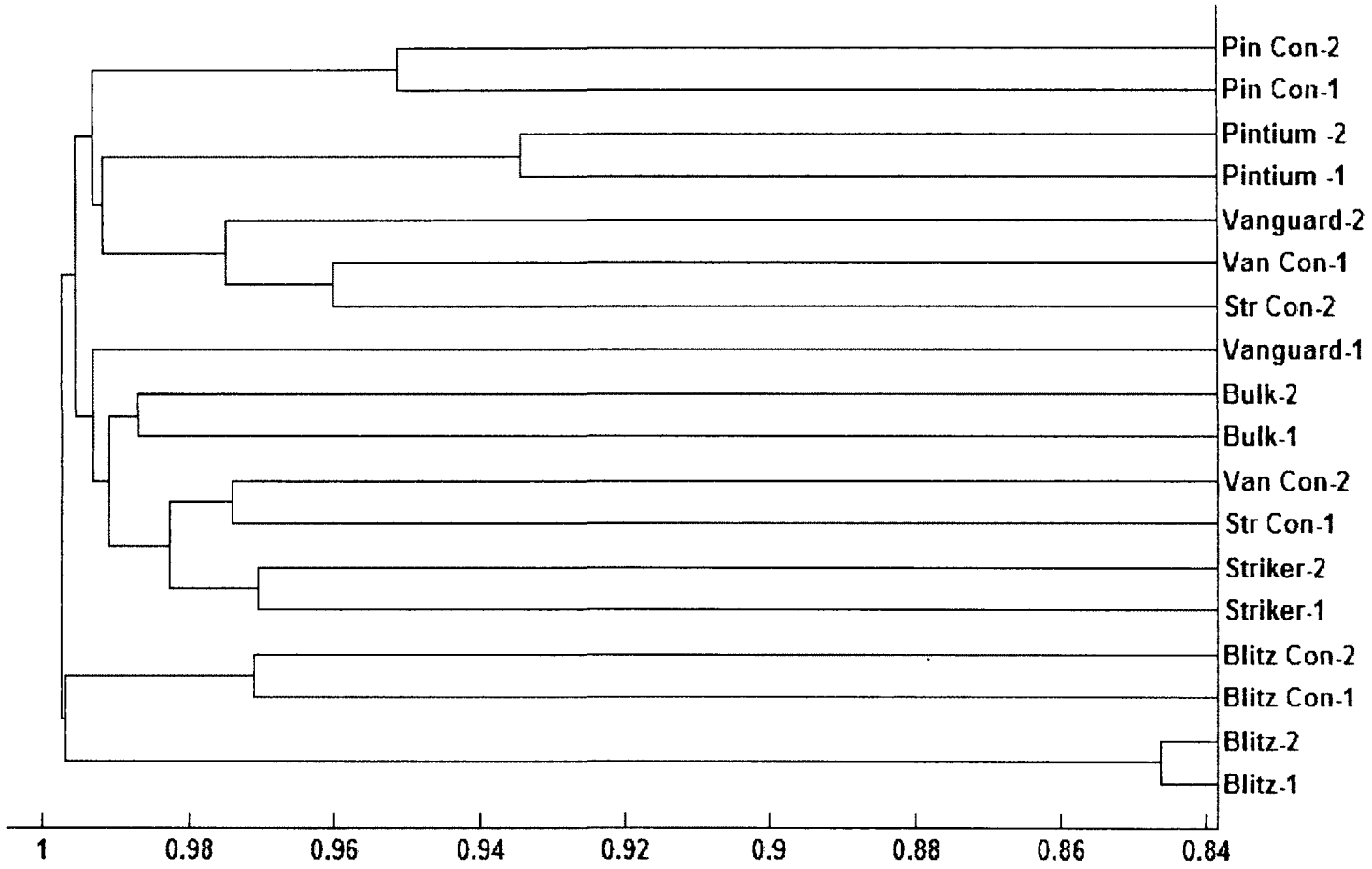

Unweighted HaeIII data set, cophenetic correlation coefficient = 0.7453
Bulk-2
Bulk-1
Pin Con-2
Pin Con-1
Pintium -2
Pintium -1
Van Con-2
Vanguard-2
Van Con-1
Vanguard-1
Blitz-2
Blitz-1
Blitz Con-2
Blitz Con-1
Str Con-2
Str Con-1
Striker-2
Striker-1
0.4
0.35
0.3
0.25
0.2
0.15
0.1

Figure 20: The weighted dendrogram structures of TRF profile comparisons for HaeIII data set.

Weighted HaeIII data set, cophenetic correlation coefficient = 0.9056



all other soil samples, but it is relatively more similar to the bacterial communities in the soil samples from the plants of Variety Pintium (Figure 19). The weighted dendrogram showed that bulk soil has different bacterial community from all other soil samples (Figure 20). The soil samples from the rhizosphere of uninoculated pulse plants have different a bacterial community structure compared to the soil adjacent to HUP- nodules of inoculated pulse plants, and the 2 replicates from the same soil sample are very similar for Variety Pintium and CDC Blitz (Figure 20). Moreover, the bacterial communities in the soil samples for Variety CDC Blitz are more similar to each other than to the bacterial communities in the soil samples collected from other pulse varieties (Figure 20). The bacterial communities in the soil samples for Variety CDC Vanguard and the rhizospher soil are difficult to distinguish from each other and differences were also seen within the 2 replicates of these 3 kinds of soil samples (Figure 20). The cophenetic correlation coefficients for the unweighted dendrogram and weighted dendrogram are 0.7453 and 0.9056 respectively.

For the data set from the digestion reaction with the HinfI enzyme, the unweighted dendrogram indicated that the soil samples from the rhizosphere of uninoculated pulse plants have different bacterial community structures compared to the soil adjacent to HUP- nodules of inoculated pulse plants for all the pulse varieties, and the 2 replicates from the same soil sample are very similar, except the soil adjacent to the HUP- nodules of inoculated Pintium plants (Figure 21). The bacterial communities in the soil samples from the same pulse species are more similar to each other than to the bacterial communities in the soil samples collected from other pulse species for CDC Vanguard

Figure 21: The unweighted dendrogram structures of TRF profile comparisons for HinfI data set.

Unweighted HinfI data set, cophenetic correlation coefficient = 0.8399

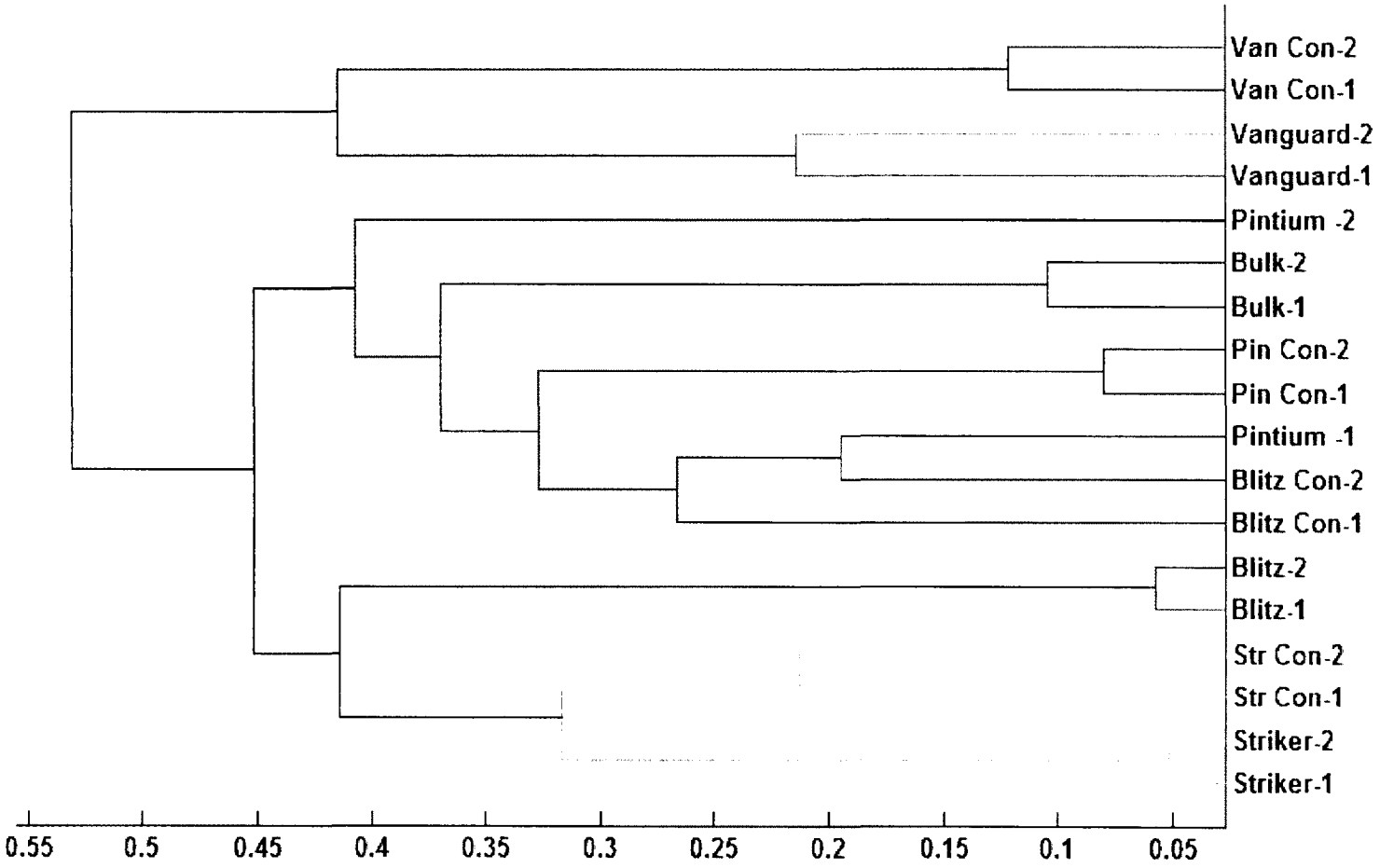
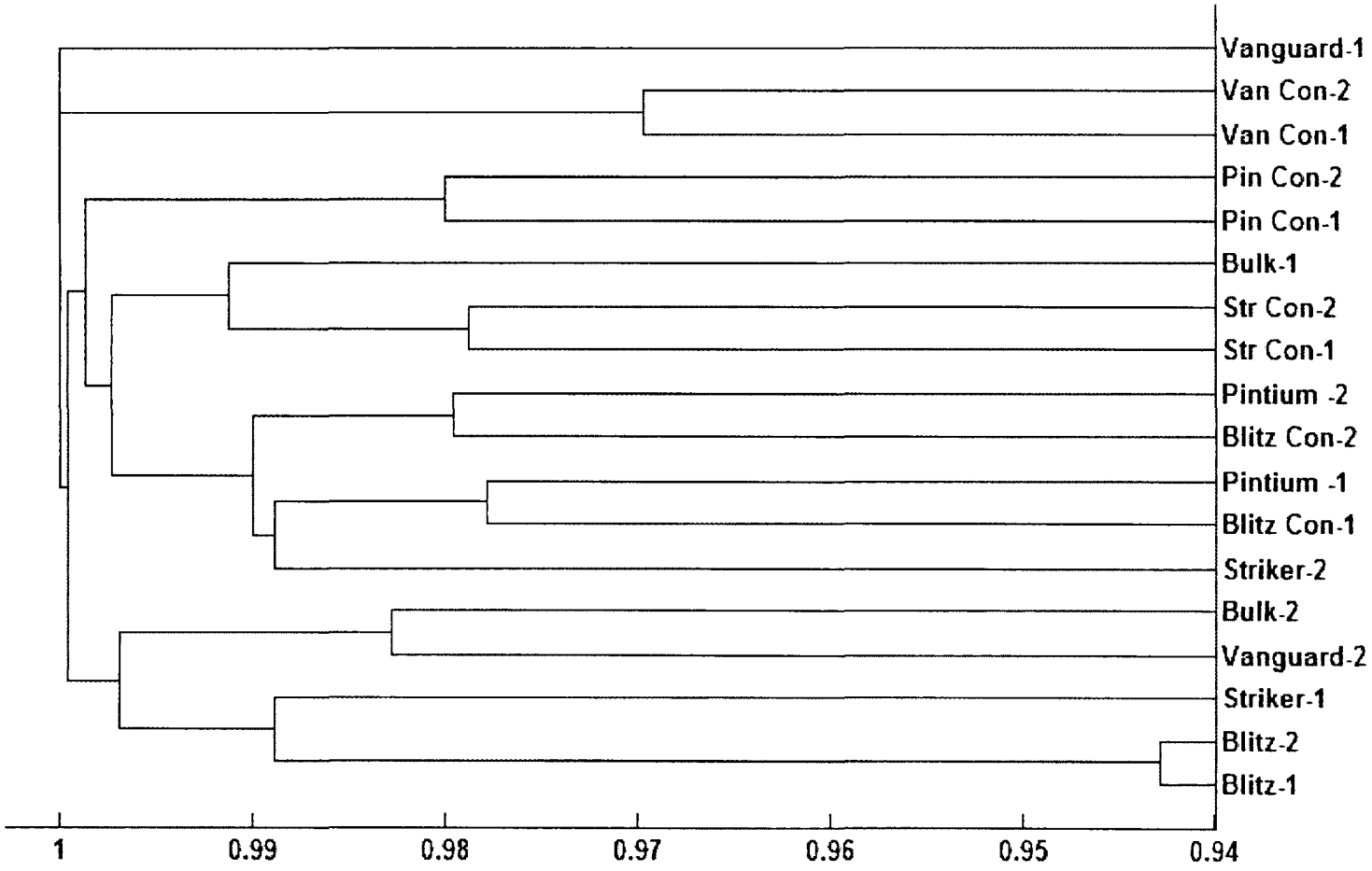


Figure 22: The weighted dendrogram structures of TRF profile comparisons for HinfI data set.

Weighted HinfI data set, cophenetic correlation coefficient = 0.8237



and CDC Striker (Figure 21). The bacterial community in bulk soil is different to the bacterial communities in all other soil samples (Figure 21). The weighted dendrogram showed that the bacterial communities in the 2 replicates of rhizosphere soil collected from uninoculated CDC Vanguard plants, uninoculated Pintium plants, uninoculated CDC Striker plants and the soil adjacent to the HUP- nodules of inoculated CDC Blitz plants are very similar to each other (Figure 22). For the rest of the soil samples, a relatively large difference exists between 2 replicates of the sample and no clearly grouping pattern was found (Figure 22). The cophenetic correlation coefficients for the unweighted dendrogram and weighted dendrogram are 0.8399 and 0.8237 respectively.

For the data set from the digestion reaction with the MspI enzyme, the unweighted dendrogram showed that the rhizosphere soil from uninoculated pulse plants and the soil adjacent to the HUP- nodules of inoculated pulse plants have different bacterial community structures for all the pulse varieties (Figure 23). The bacterial community in bulk soil is more similar to the bacterial community in the soil adjacent to the HUP- nodules of inoculated Pintium plants (Figure 23). A large difference was seen between 2 replicates for the rhizosphere soil of Variety CDC Blitz, CDC Striker and the soil adjacent to the HUP- nodules of inoculated CDC Vanguard plants (Figure 23). The weighted dendrogram suggests that in the rhizosphere soil from uninoculated pulse plants and the soil adjacent to the HUP- nodules of inoculated pulse plants there are different bacterial community structures for all the pulse varieties (Figure 24). The difference between the rhizosphere soil from uninoculated CDC Striker plants and the soil adjacent to the HUP- nodules of inoculated CDC Striker plants is much smaller than the difference

Figure 23: The unweighted dendrogram structures of TRF profile comparisons for MspI data set.

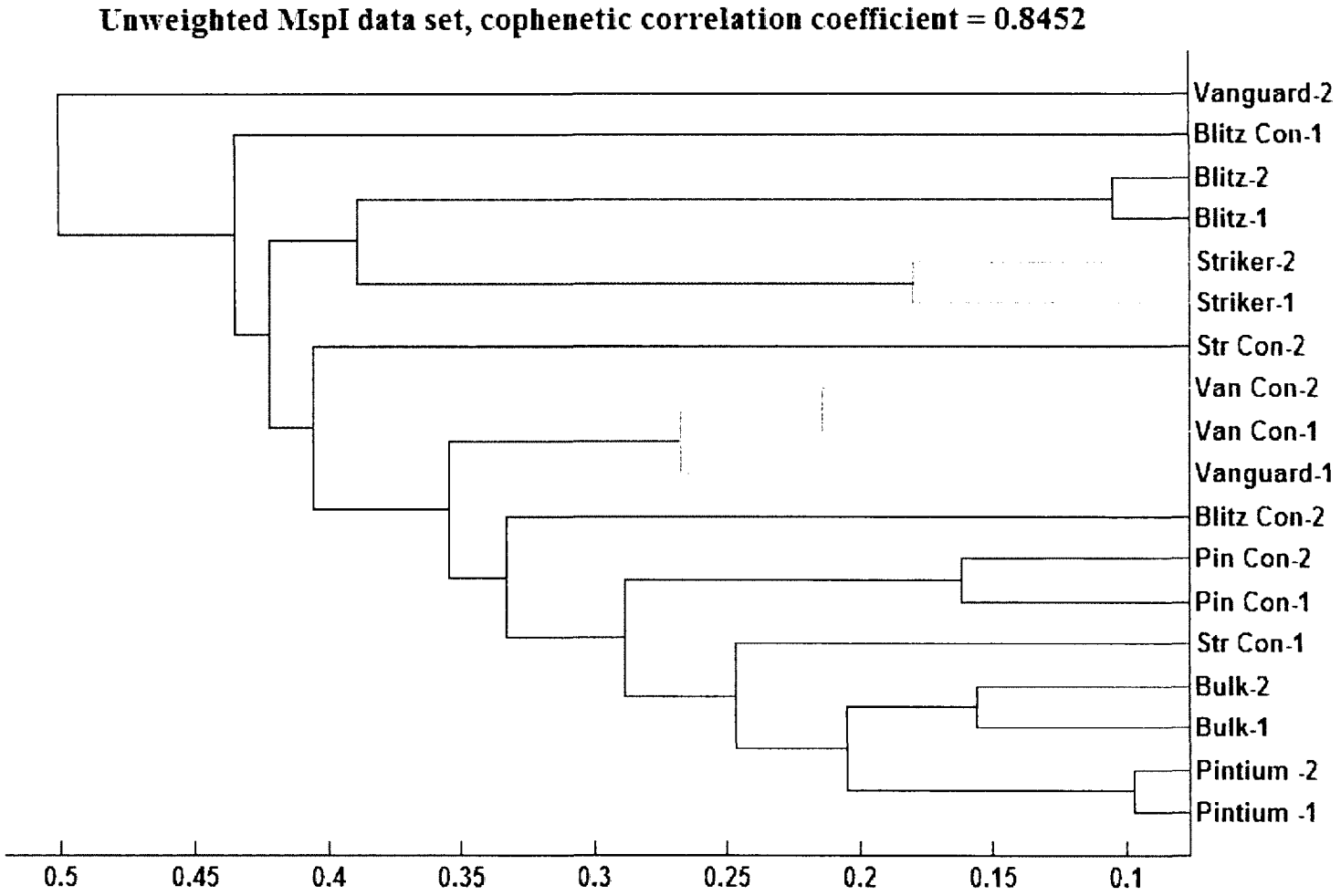
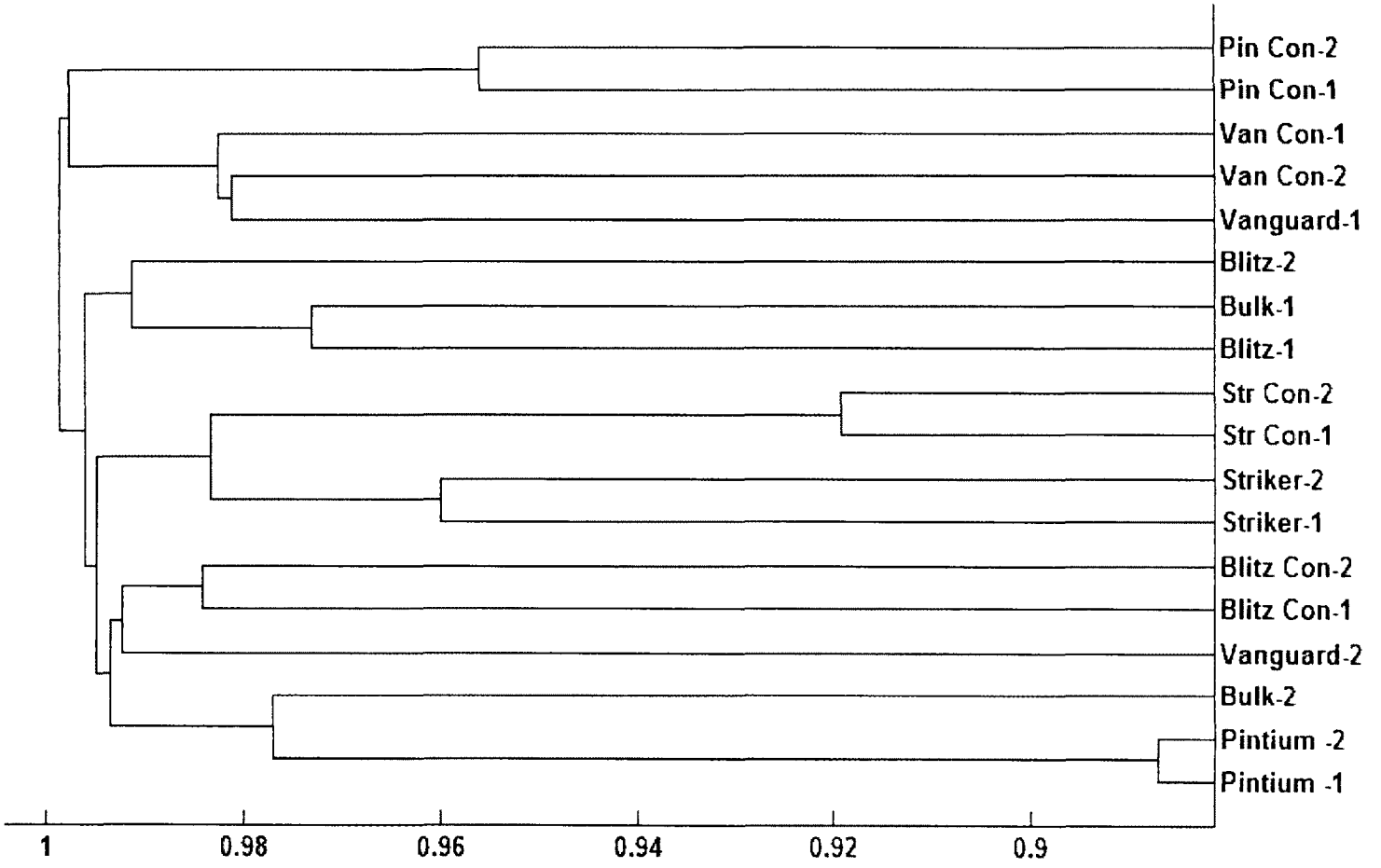

Unweighted MspI data set, cophenetic correlation coefficient = 0.8452
Vanguard-2
Blitz Con-1
Blitz-2
Blitz-1
Striker-2
Striker-1
Str Con-2
Van Con-2
Van Con-1
Vanguard-1
Blitz Con-2
Pin Con-2
Pin Con-1
Str Con-1
Bulk-2
Bulk-1
Pintium -2
Pintium -1
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1

Figure 24: The weighted dendrogram structures of TRF profile comparisons for MspI data set.

Weighted MspI data set, cophenetic correlation coefficient = 0.8555



between the rhizosphere soil from uninoculated Pintium plants and the soil adjacent to the HUP- nodules of inoculated Pintium Striker plants (Figure 24). A significant difference was found between 2 replicates of bulk soil and soil adjacent to the HUP- nodules of inoculated CDC Vanguard plants, while the difference between 2 replicates of soil adjacent to the HUP- nodules of inoculated CDC Blitz plants is relatively small (Figure 24). The cophenetic correlation coefficients for the unweighted dendrogram and weighted dendrogram are 0.8452 and 0.8555 respectively.

For the combined data set, the unweighted dendrogram indicated that the soil samples from the rhizosphere of uninoculated pulse plants have different bacterial community structures compared to the soil adjacent to the HUP- nodules of inoculated pulse plants and the 2 replicates from the same soil sample are very similar for all the pulse varieties (Figure 25). The bacterial communities in the soil samples from the same pulse species are more similar to each other than to the bacterial communities in the soil samples collected from other pulse species, except the soil samples from Variety CDC Blitz (Figure 25). The bacterial community in bulk soil can be distinguished from all other soil samples, but is relatively closer to the bacterial communities in the soil samples of Pintium plants (Figure 25). In the weighted dendrogram, the rhizosphere soil from uninoculated pulse plants and the soil adjacent to the HUP- nodules of inoculated pulse plants have different bacterial community structures for all the pulse varieties (Figure 26). The bacterial communities are more closely related among the soil samples collected from the same species for CDC Vanguard and CDC Striker (Figure 26). For Variety Pintium and CDC Blitz, the difference between the bacterial communities in the

Figure 25: The unweighted dendrogram structures of TRF profile comparisons for combined data set.

Unweighted combined data set, cophenetic correlation coefficient = 0.842

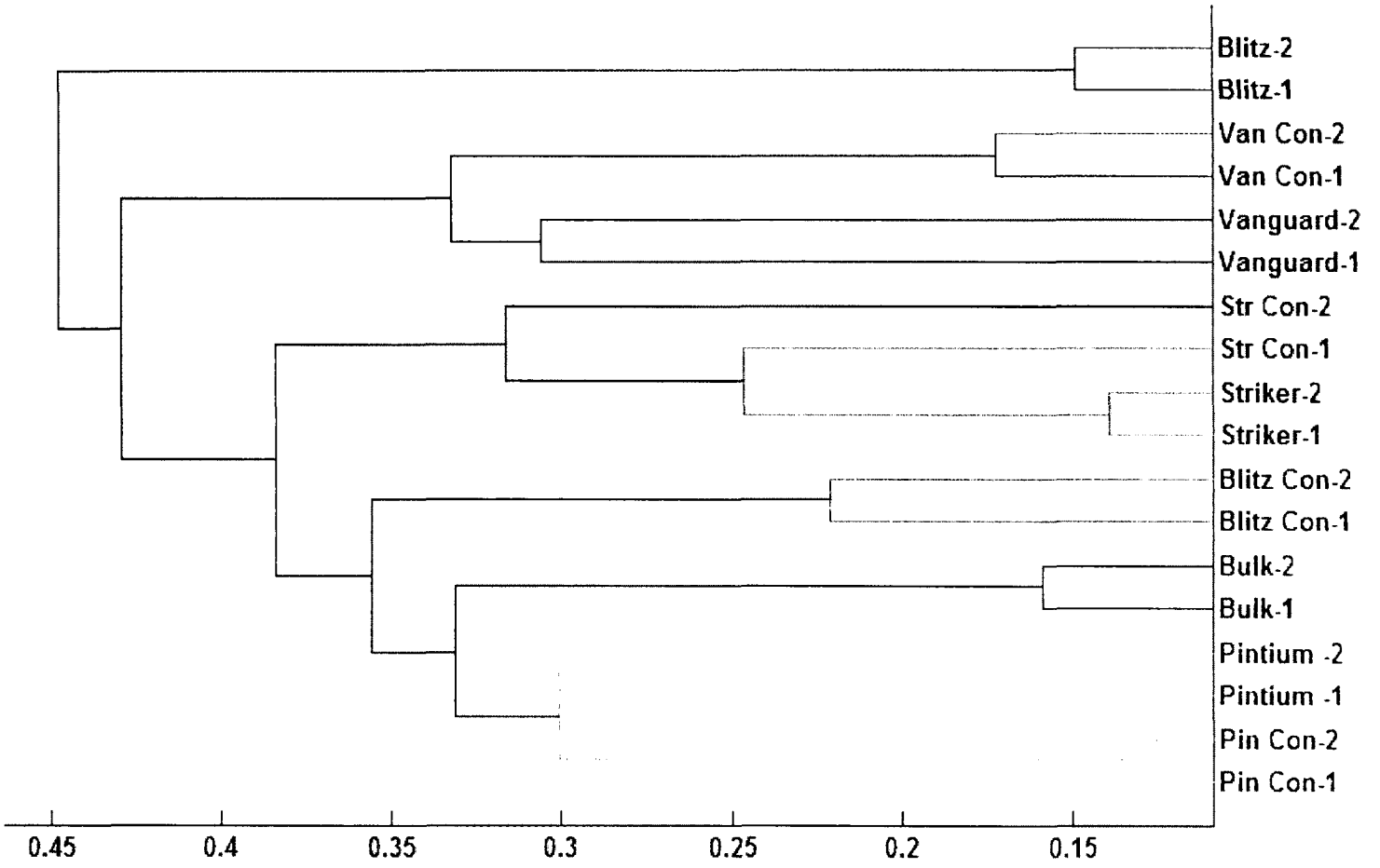
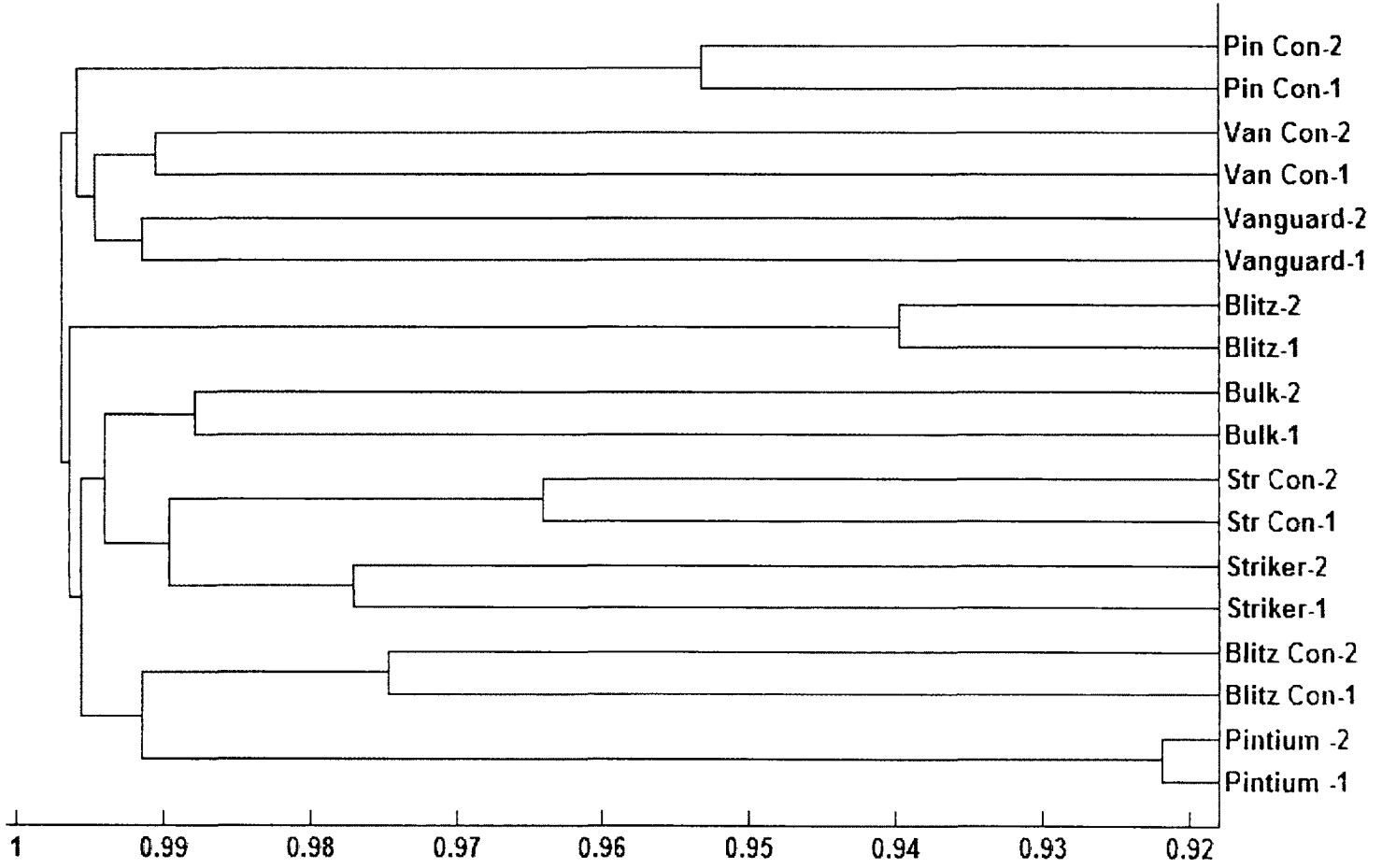


Figure 26: The weighted dendrogram structures of TRF profile comparisons for combined data set.

Weighted combined data set, cophenetic correlation coefficient = 0.9325



rhizosphere soil from uninoculated plants and the soil adjacent to the HUP- nodules of inoculated plants is significant (Figure 26). The bacterial community in bulk soil can be distinguished from all other soil samples, but is relatively closer to the bacterial communities in the soil samples of CDC Striker plants (Figure 26). The cophenetic correlation coefficients for the unweighted dendrogram and weighted dendrogram are 0.842 and 0.9325 respectively.

4.4 Discussion

4.4.1 Method for Standardizing TRF Profiles

In TRF profiles there are always some background noise peaks that resulted from either artifacts or the small difference in the amount of DNA that was loaded which cannot be accurately controlled. These background noise peaks can have an influence on the similarity analysis of bacterial community structures in different soil samples and lead to a false conclusion about changes in bacterial community structure. To minimize the negative influence of the background noise peaks on the analysis a few methods were developed for standardizing TRF profiles such as the constant percentage threshold (Sait *et al.*, 2003), the constant baseline threshold (Dunbar *et al.*, 2001) and the variable percentage threshold (Osborne *et al.*, 2006).

For the constant percentage threshold method, a series of percentages of total area of each profile were tested until a minimum percentage was reached. This minimum percentage should result in the weakest relationship between the number of remaining peaks and total area of the original profile (Sait *et al.*, 2003). The disadvantage of this method is that the background noise peaks in the profiles with higher total peak area have

an increasing possibility to be undetected while the small actual peaks in the profiles with lower total peak area are more likely to be removed. In the constant baseline method, a series of certain peak area cutoff baselines were set for all profiles in the data set, such as 50 fluorescence units (FU), 100 FU etc, instead of using the percentage of total area (Dunbar *et al.*, 2001). This method has similar weaknesses as the constant percentage threshold.

In comparison to the 2 methods above, the variable percentage threshold is a more proper approach for standardizing TRF profiles. In this method, a unique percentage threshold is generated for each profile in the data set by dividing the total area of each profile by the optimal divisor that yields the weakest relationship between the number of remaining peaks and the total area of the original profile (Osborne *et al.*, 2006). This method takes into account the difference in total area among each profile which results in a more reasonable balance between the removal of background noise peaks and retaining the small real peaks. Therefore, the variable percentage threshold method was chosen for the standardization of TRF profile in this study.

4.4.2 The Use of Multiple Restriction Enzymes

As an extremely diverse ecosystem, several thousands of bacterial species exist in each gram of soil (Curits and Sloan, 2004). However, the number of remaining peaks in each standardized TRF profile generated from about 0.25 g of soil is less than 100. Therefore, each TRF peak must present more than one species or group of bacteria which indicates that different bacterial species or groups could very likely have the same length of DNA fragments from a digestion with single restriction enzyme. Thus, using a single

restriction enzyme for T-RFLP analysis of a bacterial community structure cannot generate convincible and reliable results. The use of multiple restriction enzymes on T-RFLP analysis was proven to give a more accurate conclusion about bacterial community structure (Engebretson and Moyer, 2003). Based on this principle, 4 restriction enzymes were applied to the T-RFLP analysis in this experiment.

4.4.3 Comparison of TRF Profiles from Different Soil Samples

The number of peaks in each TRF profile generated by the different restriction enzymes was similar to each other. This indicates that the information about the bacterial diversity revealed by each restriction enzyme is not greatly different. However, the dendrograms derived from the data sets associated with 4 restriction enzymes showed that different restriction enzymes can generate different bacterial community patterns. There was only one common pattern seen across all dendrograms of single restriction enzyme digestion, which is the soil samples from the rhizosphere of uninoculated pulse plants have different bacterial community structures compared to the soil adjacent to the HUP- nodules of inoculated pulse plants.

Since the data generated from 1 restriction enzyme cannot offer conclusive patterns about the soil bacterial community structures, the combined data set generated from the digestion reactions of all 4 restriction enzymes was analyzed to give a comprehensive understanding about the difference between the bacterial community structures in different soil samples.

The T-RFLP analysis suggested that the bacterial communities in the soil samples collected from 4 different pulse species are all different from each other, and also different from the bacterial community in the bulk soil. Within the same pulse species, the soil adjacent to the HUP- nodules of the inoculated pulse plant has different bacterial community compared to the rhizosphere soil from the uninoculated control pulse plant. This finding suggests that the H_2 released from the HUP- pulse nodules did have effects on rhizosphere bacterial community. However, the difference among plant species has greater impacts on the rhizosphere bacterial community than the H_2 released from nodules. According to Zhang (2006), the presence of plants in soil has significant influence on the rhizobacterial community structure compared to H_2 treatment in the same soil in both greenhouse and field experiments, which in part agrees with the findings of this study. On the other hand, only one legume species (soy bean) was used in Zhang's study, and the effects of different legume species on soil bacterial community structure was not investigated. Based on current knowledge, the symbiotic relationship between plants and soil microbes is plant host specific, which means that the different plant species trigger different plant-soil bacteria interaction that depends on their needs for water and nutrients (Marschner *et al.*, 2002; Semenov *et al.*, 1999). Therefore, it is not surprising that different pulse species influence the soil bacterial communities differently.

In most cases, the bacterial community difference among soil samples from different pulse species is larger than the difference seen among the soil samples from the same pulse species, except for the soil samples from Variety CDC Blitz in faba bean. The difference of the soil bacterial communities between the soil adjacent to the HUP-

nodules of CDC Blitz plants and the rhizosphere soil of the uninoculated CDC Blitz control plants is the largest among all pulse species in both the unweighted and weighted dendrograms. This difference matched the result seen in the second trial of rotation benefit at the 0.1 mM N level, which indicates that faba bean probably has the highest rotation benefit among the 4 pulse species for the succeeding crops due to the H_2-induced soil bacterial community changes in both species composition and population size.

For Variety Pintium in dry bean, the unweighted dendrogram suggests that the bacterial group composition in the soil adjacent to the HUP- nodules of inoculate plants and the rhizosphere soil of the uninoculated control plants is very similar. However, the weighted dendrogram indicated that the proportional change of certain bacterial groups between Pintium soil samples was quite large. Combining the trends seen on barley inoculated with soil samples collect from Pintium plants in the second trial of rotation benefit at both 0.1 mM and 0.5 mM N levels, this proportional change of certain bacterial groups in soil bacterial community could probably be the reason for the observed succeeding crop growth promotion, since the population change of certain bacteria in soil could trigger functional changes of the entire soil bacterial community and different types of plant – soil microbe interaction (Marschner *et al.*, 2002; Semenov *et al.*, 1999).

5. GENERAL SUMMARY

The present study focused on the determination of HUP status for the nodules from different pulse varieties, the contribution of HUP- nodules from different pulse plants on rotation benefit and the changes in rhizosphere bacterial community structure induced by

different pulse nodules, especially for the rotation benefit induced by the H_2 that is released from HUP- pulse nodules.

The nodules from inoculated pulse plants were determined to be HUP- nodules across all 25 pulse varieties in the experiment. Most volunteer nodules from uninoculated control pulse plants were also HUP- nodules, except for a few nodules from the uninoculated control plants of Variety CDC Richlea and Variety Cooper. In general, all 25 varieties of pulse plants adopted HUP- symbiosis rather than HUP+ symbiosis, which supports the finding of previous studies about the HUP- status of other legume species. The soil adjacent to the HUP- nodules of inoculated pulse plants has been found to have significantly higher H_2 uptake rate and CO_2 fixation than the rhizosphere soil of uninoculated control pulse plants since the H_2 was released from the nodules during N fixation and absorbed by the soil around the nodules. However, the higher H_2 uptake rate did not correlate with the higher CO_2 fixation in the same soil sample, which indicates that there are other H_2 oxidizing pathways other than fixing CO_2.

The results of the rotation benefit experiment showed the trend that barley inoculated with soil adjacent to the HUP- nodules of inoculated pulse plants grew better than the barley inoculated with the rhizosphere soil of uninoculated control pulse plants and the barley grown in bulk soil. The different growth patterns in response to the different N levels for the same inoculation treatment suggests that the different soil samples used for the barley inoculation probably have different bacterial communities and that these bacterial communities are more beneficial for succeeding crops when the N level is low. The benefits offered by the bacterial communities that associated with certain pulse

species can not be replaced by higher level of N fertilizer application. The most obvious rotation benefit was evidenced in the barley inoculated with the soil adjacent to the HUP- nodules of inoculated Pintium and CDC Blitz plants.

The T-RFLP analysis suggested that the soil adjacent to the HUP- nodules of the inoculated pulse plants have a different bacterial community compared to the rhizosphere soil from the uninoculated control pulse plants for all 4 pulse species. This indicates that the H_2 released from HUP- pulse plant nodules did trigger changes in rhizosphere bacterial community structures. However, the scale of the changes varies according to the species of pulse plant. For chickpeas (CDC Vanguard) and peas (CDC Striker), the differences between the bacterial communities in the soil adjacent to the HUP- nodules of the inoculated pulse plants and the rhizosphere soil from the uninoculated control pulse plants are relatively small in terms of species composition and population size. The greatest alteration in rhizosphere bacterial community structure (both species composition and population size) was evidenced between the soil adjacent to the HUP- nodules of the inoculated plants and the rhizosphere soil from the uninoculated control plants of faba bean (CDC Blitz). The soil adjacent to the HUP- nodules of the inoculated plants and the rhizosphere soil from the uninoculated control plants of dry bean (Pintium) have similar bacterial species composition, but a considerable amount of variation in bacterial population size was also found between soil samples.

Overall, the 25 varieties of pulse crops tested in this study prefer to form HUP- symbiosis over HUP+ symbiosis. The H_2 released from HUP- pulse nodules was consumed by certain bacterial groups in the soil around the nodules as an energy source.

This plant-soil bacteria interaction altered the rhizosphere bacterial community structures, which could have a profound influence on the growth of succeeding crops. The strongest impacts on rhizosphere bacterial community structure and the growth of succeeding barley plants were seen in the soil samples collected from bean (CDC Blitz) and dry bean (Pintium). Combining the findings of the rotation benefit experiment and the T-RFLP analysis, H_2 oxidizing bacteria are most likely to play a role in rotation benefit of pulse crops, and the different growth patterns in response to different N levels for the same soil inoculation could potentially be the result of the H_2 induced bacterial community changes and the its derived alterations in bacterial functions.

The findings in this study are preliminary for most of the pulse crops, and more experiments are needed for a more reliable conclusion. The sample sizes used in experiments across the entire study were small due to limited greenhouse space, time frame and labor availability. Therefore, the sample sizes need to be improved in future studies. The exact details about how the species composition and bacterial population changed in the soil samples are still unknown. Future studies should investigate which bacterial groups were altered by HUP- nodules and whether their functions in soil changes after alteration. The key players in soil bacterial community for rotation benefit needed be identified and studied, and then inoculants for promoting plant growth could be developed in future. Field experiment should also be conducted to test our findings in real farming practices.

Reference

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