H_2-OXIDIZING, PLANT GROWTH PROMOTING RHIZOBACTERIA AS SEED INOCULANTS FOR BARLEY

By Amber-Leigh Golding

A Thesis Submitted to Saint Mary's University, Halifax,
Nova Scotia, in Partial Fulfillment of the Requirements for
the Degree of Masters of Science in Applied Science.

July, 2009

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ABSTRACT

H_2-OXIDIZING, PLANT GROWTH PROMOTING RHIZOBACTERIA AS SEED INOCULANTS FOR BARLEY

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The persistence of allegedly inefficient hydrogenase uptake negative (HUP^-) legume-rhizobia associations may be accounted for by the beneficial effects of hydrogen release to soil, including the stimulation of H_2-oxidizing, plant growth promoting rhizobacteria (PGPR). Two such previously isolated strains were tested as seed inoculants for barley; there were significant differences between treatments and controls in tiller and grain head production, supported by data from greenhouse trials. TRFLP analysis of barley soil samples, supported by DNA sequencing data, successfully distinguished both species of PGPR and successful re-isolation shows that these isolates can reproduce themselves in soils and so can be used as effective inoculants with peat as the standard carrier. The development of PGPR as seed inoculants is an important step towards sustainable agriculture.

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ACC = 1-aminocyclopropane-1-carboxylate

ADP = adenosine diphosphate

AMF = arbuscular mycorrhizal fungi

ANOVA = analysis of variance

ATP = adenosine triphosphate

bp = base pairs

BSF8/20 = universal bacterial forward primer (5'-AGAGTTTGATCATGGCTCAG-3')

BSR1541/20 = bacterial universal reverse primer (5'-AGGAGGTGATCCAACCGCA--3')

BSR 534/18 = bacterial universal reverse primer (5'-CAGCAGCCGCGGTAATAC -3')

C = carbon

$CaCl_2$ = calcium chloride

CEC = cation exchange capacity

CI = confidence interval

cm = centimetre

CO_2 = carbon dioxide

$CoSO_4$ = cobalt (II) sulphate

Combo = combination of strains JM63 and JM162a

$CuSO_4$ = copper sulphate

cv. = cultivar

C1 = control 1 (sterilized peat)

C2 = control 2 (blank soil)

dH_2O = distilled water

DNA = deoxyribonucleic acid

dNTP = deoxynucleotide triphosphate

e = electron

$Fe_2(SO_4)_3$ = iron (III) sulphate

$FeCl_3$ = iron (III) chloride

g = grams (measurements) or gravity (centrifugation)

H_2 = hydrogen

H_2O = water

H_3BO_3 = boric acid

H^+ = proton

ha = hectare

HUP = hydrogenase uptake

HUP^+ = hydrogenase uptake positive

HUP^- = hydrogenase uptake negative

IAA = indole acetic acid

JM63 = *Variovorax paradoxus* strain JM63

JM120 = *Burkholderia* species strain JM120

JM162a = *Flavobacterium johnsoniae* strain JM162a

K_2HPO_4 = dipotassium phosphate

KH_2PO_4 = potassium dihydrogen phosphate

KCl = potassium chloride

KOH = potassium hydroxide

KNO_3 = potassium nitrate

K_2SO_4 = potassium sulphate

kg = kilogram

L = liter

LB = Luria broth

m = meter

m^2 = square meter

mA = milliamps

mL = millilitre

mM = millimoles

µl = microliter

µmol = micromoles

MDS = multidimensional scaling

Mg = magnesium

$MgCl_2$ = magnesium chloride

$MgSO_4$ = magnesium sulphate

$MnSO_4$ = manganese sulphate

MSA = mineral salt agar

N= nitrogen

N_2 = dinitrogen

N_2O = nitrous oxide

$NaNO_3$ = sodium nitrate

Na_2MoO_4 = sodium molybdate

NB = Nutrient broth

NH_3 = ammonia

Ni = nickel

NSAC = Nova Scotia Agricultural College

O_2 = oxygen

OD_{600} = optical density measured at wavelength of 600 nanometers

PCA = principal components analysis

PCR = polymerase chain reaction

PGPR = plant growth promoting rhizobacteria

QS = quorum sensing

RCBD = randomized complete block design

rDNA = ribosomal deoxyribonucleic acid

RE = restriction enzyme

rpm = revolutions per minute

RQ = respiratory quotient

RR = Roundup Ready

rRNA = ribosomal ribonucleic acid

SD = standard deviation

sp. = species

TRF = terminal restriction fragment

TRFLP = terminal restriction fragment length polymorphism

TTE = time to emergence

UPGMA = unweighted paired-groups using mathematical averages

v/v = volume to volume

$ZnSO_4$ = zinc sulphate

6-FAM = phosphoramidite fluorochrome 5-carboxy fluorescein

^{o}C = degrees Celsius

1.0 General Introduction

1.1 The challenge of the Agricultural Revolution

The 1950s and 1960s saw an agricultural revolution. This revolution was made possible largely by the introduction of the Haber-Bosch process (Postgate, 1998), a process that allowed for the large-scale production of inorganic nitrogen fertilizers, seemingly freeing humanity from its dependence on biological nitrogen fixation. Particularly after the Second World War, food security became a priority (Welbaum *et al.*, 2004) and traditional farming practices such as crop rotation and intercropping were downplayed in the industrialized West in favour of large-scale monocultures. It was felt that the use of chemical fertilizers (and pesticides) would maintain sufficient yields to both feed an ever-growing human population and provide fodder for increased livestock production (Welbaum *et al.*, 2004).

Over the ensuing decades, the adverse effects of inorganic fertilizers and pesticides on both human and environmental health, as well as their costs in terms of fossil fuel use and consequent climate change, have become clear. Furthermore, both decreases in yield due to soil nutrient depletion and increases in reports of soil degradation have been more recently noted (Welbaum *et al.*, 2004), suggesting that unlimited crop yields may not be feasible under conventional management practices. It is now thought that a reconsideration of more traditional farming methods, like crop rotation (Bullock, 1992), may be useful in the implementation of sustainable agriculture; however, the global population continues to rise. To ensure a sufficient food supply while simultaneously protecting the environment requires a better understanding of soil properties, soil microbiology and both plant-soil and plant-microbe interactions.

Maintaining a balance between food supply and ecological health is the driving force behind the present study.

In this section, I will explore the concepts of soil fertility and productivity, the links between the hydrogenase activity of legume-rhizobia symbioses, the role of hydrogen in soil and the mechanisms of plant growth promoting rhizobacteria (PGPR), particularly hydrogen-oxidizing bacteria. This specific area of research is especially germane for two reasons. Firstly, because the legume-rhizobia symbiosis is critically important in agriculture and has been for thousands of years (Bullock, 1992) and, secondly, because the hydrogen released from rhizobial nodules lacking a hydrogenase enzyme (HUP^- nodules) both effects important changes in soil chemistry (Dong and Layzell, 2001) and attracts H_2-oxidizing PGPR (Zhang, 2006; Maimaiti *et al.*, 2007). These PGPR possess the potential for development into commercial seed inoculants for farmers, thereby reducing the need for inorganic fertilizers, helping make agriculture more sustainable. A significant portion of the work involved in achieving these goals is dependent on the use of appropriate methodologies such as rapid screening protocols for determining nodule HUP status and molecular techniques such as the polymerase chain reaction (PCR) and DNA fragment analysis; these too are discussed below.

1.2 Soil fertility and productivity

Soil is the largest reservoir of biodiversity on the planet, harbouring massive numbers of species both eukaryotic and prokaryotic (Crawford *et al.*, 2005). It is believed that this high level of biodiversity contributes greatly to the functionality of soil, but the relationship between the two has yet to be defined (Crawford *et al.*, 2005). As

soil is the substrate utilized by most life forms, this situation should be remedied as quickly as possible; an evolutionary approach to soil ecology may be the best hope for discovering the origins of many soil processes and the means of their continued maintenance (Crawford *et al.*, 2005). Better understanding of these processes, and the physical, chemical and biological factors that sustain them, will undoubtedly lead to more efficient and sustainable soil management practices.

One area of confusion in soil science is found within the realm of soil fertility. Soil fertility is frequently defined as the ability of soil to supply essential nutrients for plant growth (Foth and Ellis, 1988) and this definition is often the basis for research into improving crop yields, for instance, the use of N_2-fixing legumes for supplying N to soils for the benefit of subsequent nonlegume crops such as rice (Oikeh *et al.*, 2008). However, soil fertility has also been seen to mean more than just nutrient supply; this term has also been used to describe other aspects of the soil such as the unimpeded decomposition of organic matter, soil biotic communities and soil physical properties conducive to plant growth (Mäder *et al.*, 2002). Soil fertility has also been directly equated with yield or the ability to provide yield, both in mechanized farming systems with high chemical inputs and low-input organic farming (Patzel *et al.*, 2000).

It has been suggested that 'soil fertility' be solely associated with definite properties of soil, whereas the term 'soil quality' be used to describe the more subjective concept of desirable soil qualities (Patzel *et al.*, 2000). Foth and Ellis (1988) use 'soil productivity' to encompass both soil fertility and all other factors beneficial to plant growth, including soil management practices. This, of course, would include such things as hydrogen treatment of soils, described by Dong and coworkers (2003) as a sort of soil

fertilization, and microbial seed inoculants, designed to improve plant growth through various mechanisms and minimize the need for chemical fertilizer inputs, sometimes referred to as 'biofertilizers' (Vessey, 2003). This separation of terms may be viewed as imperfect, as some factors placed under the umbrella of 'soil productivity' are actually involved in direct nutrient supply to plants, for example, N_2 fixation by legume rhizobia and phosphorus supplied by arbuscular mycorrhizal fungi (AMF). Other chemical and physical properties are also directly related to plant yield, such as pH, cation exchange capacity (CEC) and soil aggregation, but these would also be placed within the category of 'soil productivity' as opposed to 'soil fertility' within this regime.

The roles of plants themselves and their associated soil microorganisms in shaping the physical structure of soil must also be considered. Plant roots and soil microbes all release exudates into soils that can help soil particles bond into aggregates, polysaccharides especially (Goss and Kay, 2005). An example of this is glomalin, a hydrophobic glycoprotein secreted by AMF hyphae that adheres particularly well to mineral surfaces and has a residence time in soil of anywhere from six to forty-two years (Goss and Kay, 2005); at any given time, this protein may comprise 4% to 5% of soil carbon and may contribute to soil aggregation (Goss and Kay, 2005). Plants and microbes may also destabilize aggregates, with plant roots penetrating them or through the decomposition of organic matter by way of microbial activity (Goss and Kay, 2005).

Judging from the above, it is difficult to tell where soil fertility ends and soil productivity begins; the definition of soil fertility as merely the ability to supply nutrients to plants in the right amounts may be too narrow, whereas a concept of soil fertility based on everything conducive to plant growth may be too broad to be meaningful; the latter

would certainly be problematic as an operational definition for experimental purposes. However, the situation requires a resolution as communication between specialists in different fields increases; soil fertility to an agriculturalist will not necessarily mean the same thing as soil fertility to a soil ecologist. This could make interdisciplinary work difficult at a time when interdisciplinary approaches are being recognized as necessary to obtaining a true understanding of the most significant terrestrial habitat on earth.

1.3 The HUP status of legume-rhizobia symbioses

The most heavily investigated plant-microbe interactions are those involving relationships between plants and nitrogen-fixing microorganisms, and this is because nitrogen is the nutrient most likely to become rate limiting to plant growth under standard agricultural conditions (Postgate, 1998). These associations include those of soil-dwelling endophytic diazotrophs, which are nitrogen-fixing organisms that colonize the plant tissues of nonleguminous plants; *Gluconacetobacter diazotrophicus*, a diazotroph that colonizes sugar-rich plants such as sugarcane, is one of the more familiar examples from this group (Yousef *et al.*, 2004). However, the nitrogen-fixing association most studied, best understood and most widely applied in agriculture is the legume-rhizobia symbiosis (Welbaum *et al.*, 2004; Gray and Smith, 2005). Strains of these bacteria penetrate the root cortical cells of legumes such as soybean and pea, differentiate into bacteroids and form active, N_2-fixing root nodules (Zhang and Smith, 2002); the subsequently high amounts of nitrogen available in legume tissues explains why legumes are a better source of dietary protein than nonlegume grains such as corn and barley (Postgate, 1998).

However, even with a symbiosis as closely scrutinized as this one, certain aspects of it remain unclear. For instance, the production of hydrogen gas as an obligate, energy-rich byproduct of nitrogen fixation is a well-established fact (Schubert and Evans, 1976). Some species of rhizobia possess genes coding for a hydrogenase uptake (HUP) enzyme that allows this hydrogen to be recycled, saving the plant energy and increasing the efficiency of nitrogen fixation, resulting in higher crop yields (Dixon, 1972; Schubert and Evans, 1976; van Berkum *et al.*, 1994). These are termed HUP^+ rhizobia and they form HUP^+ symbioses with their host plants. Other rhizobia, such as those found in association with clovers and alfalfa, lack the genes for a HUP enzyme, and so they and their associations are termed HUP^- rhizobia and symbioses, respectively (Ruiz-Argüeso *et al.*, 1979). For instance, 75% of commercially grown soybean in the United States harbour *Bradyrhizobium japonicum* strains that are HUP^-, while the other 25% are HUP^+ strains (Uratsu *et al.*, 1982). Then there are the rhizobia that possess HUP genes but, for one reason or another, show little to no hydrogenase activity. An example of this phenomenon was observed in an investigation of hydrogenase activity in *Rhizobium leguminosarum*; four different cultivars of pea (*Pisum sativum*) showed varying degrees of HUP activity in their symbioses when inoculated with the same strain of *R. leguminosarum* (Bedmar *et al.*, 1983; Bedmar and Phillips, 1984). This suggests not only a plant host effect on HUP status, but a host *cultivar* effect; Bedmar and Phillips (1984) speculated that a factor transmitted from shoot to root may be responsible for this effect. Murillo and coworkers (1989) also detected a cultivar effect when they studied the relationship between lupines and their rhizobial symbionts, and there are similar reports in the literature in regards to cowpea and common bean rhizobial symbioses (Navarro *et*

al., 1993). However, there appears to have been little in the way of recent work following up on these intriguing results and a renewed research effort in this area is recommended.

1.4 The role of hydrogen in soil

A consequence of biological nitrogen fixation is the obligate production of hydrogen gas (H_2), which is released from HUP^- legume nodules to the soil as an energy-rich byproduct (Schubert and Evans, 1976; Dong and Layzell, 2001). The ATP-dependent evolution of H_2 is due to the activity of the nitrogenase enzyme that allows diazotrophs like rhizobia to fix nitrogen (Maier *et al.*, 1978). As this process seems wasteful and a loss of energy to the plant, for a time research focused on rhizobial strains possessing active uptake hydrogenase systems that recycle the H_2, increasing ATP production and reducing energy costs to the plant (Sayavedra-Soto *et al.*, 1988). It was believed for a long time that the H_2 released from HUP^- nodules simply dissipated from the soil into the atmosphere, so soil was seen to be a hydrogen source (Conrad and Seiler, 1979a). However, this same team also suggested some role for H_2-oxidizing bacteria in the consumption of soil H_2 (Conrad and Seiler, 1979b). Although they concluded the uptake of H_2 within soils was mostly due to the action of free-living hydrogenases, this at least suggested the possibility of soil as a H_2 sink. Strangely enough, this work was not immediately followed up on. Although further investigations by LaFavre and Focht (1983) showed that H_2 is consumed within soil and that hydrogen uptake occurs within one to four centimeters of HUP^- nodules, the mechanism for this uptake remained unknown. It was many years later before it was shown that the hydrogen uptake

mechanism in soils is bacterial in nature (McLearn and Dong, 2002). Further research has shown that hydrogen in soil also strongly affects soil chemistry itself. Dong and Layzell (2001) tested the effects of hydrogen in plant-free soils, to be sure the effects were not due to plants themselves, and found that approximately 60% of hydrogen's electrons are passed to oxygen, creating water (soil moisture), while the other 40% of electrons are used to fix CO_2 into carbohydrate, sequestering carbon in the soil (Dong and Layzell, 2001). The realization that H_2-oxidizing bacteria are responsible for this consumption and potentially for the increased growth and yield of nonleguminous plants in H_2-treated soils (Dong *et al.*, 2003; Maimaiti *et al.*, 2007) suggests an explanation for why seemingly wasteful HUP^- symbioses have not been selected against, either naturally or artificially (Dong *et al.*, 2003). Recent work suggests that some H_2-oxidizing bacteria may foster plant growth promotion through various mechanisms (Zhang, 2006; Maimaiti *et al.*, 2007), which will be discussed in more detail below.

1.5 Plant growth promoting rhizobacteria (PGPR)

There is a wide variety of plant growth promoting rhizobacteria (PGPR) present in soils. Many are involved in direct nutrient supply to plants; among these are the nitrogen-fixing diazotrophs. Again, the most famous nitrogen fixers are the legume rhizobia. These have been widely studied in cultivated legumes such as soybean (*Glycine max*), pea (*Pisum sativum*) and common bean (*Phaseolus vulgaris*), which serve as important food crops, as well as alfalfa (*Medicago sativa*) and clovers (*Trifolium* species) that are extensively used for livestock feed. However, the nodulation process is the same in wild legumes like *Acacia nilotica*, *Trifolium resupinatum* and *Medicago truncatula*

(Zahran, 2001), also providing crucial nitrogen to arid soils by way of biological nitrogen fixation.

Rhizobia are not the only nitrogen-fixing organisms thriving in soils. Endophytic diazotrophs that colonize nonlegume plant tissues are also capable of supplying nitrogen to their host plants, among other beneficial roles such as the production of antifungal compounds (Sturz *et al.*, 2000). *Gluconacetobacter diazotrophicus*, mentioned above, is one of the more recognizable diazotrophs. This is an endophytic species that colonizes the tissues of the graminaceous sugarcane, as well as other sugar-rich nonlegumes (Yousef *et al.*, 2004) and has been known to contribute up to 70% of the nitrogen supply in some sugarcane cultivars (Suman *et al.*, 2005). Free-living diazotrophs can also make nitrogen contributions to plants, but many, such as *Azospirillum* species, are better known for producing phytohormones like the auxin indole acetic acid (IAA), cytokinins and gibberellins (Bashan and Holguin, 1997).

Phosphorus is another nutrient that frequently limits plant growth due to poor availability, leading to deficiencies (Alikhani *et al.*, 2006; Valverde *et al.*, 2006). PGPR such as *Pseudomonas* and *Bacillus* species can promote plant growth by solubilizing phosphates, thereby supplying phosphorus to the roots for uptake (Dey *et al.*, 2004). Alikhani and coworkers (2006) have also isolated certain rhizobial strains in legumes capable of both nitrogen fixation and phosphorus solubilization. This is convenient, as phosphorus deficiencies are especially hard on legumes and their rhizobial symbionts (Alikhani *et al.*, 2006).

Outside of direct nutrient supply, as with nitrogen fixation and the mobilization of phosphorus *via* microbial solubilization (Orhan *et al.*, 2006), an important mechanism of

some PGPR is the capacity to foster plant growth by either the stimulation or suppression of specific plant hormones (Esitken *et al.*, 2006; Farag *et al.*, 2006). These phytohormones include auxins, of which IAA is the primary auxin in higher plants (Taiz and Zeiger, 1991), and cytokinins. IAA is responsible for tropisms (growth responses) and regulation of cell elongation in stems and coleoptiles, whereas cytokinins are responsible for the regulation of plant cell division, nutrient mobilization and delay of senescence (Taiz and Zeiger, 1991). In terms of hormone stimulation, Farag and coworkers (2006) revealed two different bacterial strains that released volatile compounds leading to growth promotion in *Arabidopsis thaliana*; both used cytokinin pathways, one ethylene-dependent, the other ethylene-independent (Farag *et al.*, 2006).

An example of plant hormone suppression as a mechanism of PGPR is the ability of some bacterial species to decrease plant levels of ethylene, an important phytohormone involved in many aspects of plant development including fruit ripening (Taiz and Zeiger, 1991). Inhibition of ethylene helps promote nodulation in most legumes (Hunter, 1993) and increases root elongation in nonleguminous plants; increased root elongation means better access to nutrients in soils (Shah *et al.*, 1998). Two mechanisms of ethylene inhibition (one enzymatic and the other chemical in nature) will be discussed below.

Some PGPR elicit plant growth by stimulating plant defensive responses, such as the production of polyphenolic compounds, defensive enzymes and salicylic acid, an important defense compound found in higher plants (Chakraborty *et al.*, 2006; Domenech *et al.*, 2007; Saravanakumar *et al.*, 2007). PGPR may also be capable of phytopathogen suppression by directly competing with pathogens for space and resources (Matos *et al.*, 2005). Ecologists suggest that this kind of interspecific competition is most likely to

occur in microbial populations exhibiting higher diversity (Matos *et al.*, 2005). Matos and coworkers (2005) investigated microbial species diversity and its effects on the survival of the economically significant wheat pathogen *Pseudomonas aeruginosa.* The results indicated the communities least susceptible to invasion by *P. aeruginosa* were indeed those showing the highest levels of diversity; although the mechanism for this process was not revealed (Matos *et al.*, 2005), competition seems a plausible candidate.

Until recently, much of the research concerning plant pathogens has focused on the plants themselves, so more work is required to determine precisely how significant a role microbial species diversity plays in pathogen control and the various mechanisms PGPR use to exert this control (Matos *et al.*, 2005). To this end, bacterial quorum sensing (QS) in the rhizosphere has been looked into (Bloemberg and Lugtenberg, 2001; Sharma *et al.*, 2003). QS is based on the bacterial production of autoinducers (small signaling molecules), the concentration of which, when cell density reaches a critical threshold, is sufficiently high to activate bacterial genes (Sharma *et al.*, 2003); these genes allow bacterial colonies to behave in an organized, multicellular fashion (Sharma *et al.*, 2003). QS is involved in the initiation of the legume-rhizobia symbiosis (Bloemberg and Lugtenberg, 2001) and also plays a role in biocontrol, for instance, the biosynthesis of the antibiotic zwittermicin A in *Bacillus cereus* (Bloemberg and Lugtenberg, 2001). Other PGPR produce antifungal compounds in response to QS (Ping and Boland, 2004); as more signaling pathways are defined within the PGPR, they will be more exploitable within the framework of sustainable agricultural systems, both for biocontrol and as potential 'biofertilizers' (Vessey, 2003).

The PGPR of interest in the present study are the H_2-oxidizing bacteria, of which a few species have been successfully isolated, characterized and identified (Maimaiti *et al.*, 2007). With this characterization comes a new understanding of some of the ways in which PGPR increase plant growth and yield. For instance, some H_2-oxidizing bacteria are in possession of ethylene biosynthesis inhibitors such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase; this enzyme cleaves ACC, a precursor of ethylene (Taiz and Zeiger, 1991) into α-ketobutyrate, disrupting the ethylene biosynthetic pathway (Hontzeas *et al.*, 2006; Zhang, 2006). ACC deaminase activity has been observed in strains of *Variovorax paradoxus* and *Flavobacterium johnsoniae* (Zhang, 2006; Maimaiti *et al.*, 2007). Another, chemical, inhibitor of ethylene is rhizobitoxine, which blocks the action of ACC synthase, disrupting biosynthesis one step earlier than ACC deaminase (Sugawara *et al.*, 2006; Zhang, 2006). This chemical's activity had been recorded in *Bradyrhizobium elkanii*, *Bradyrhizobium japonicum* (Hunter, 1993) and *Burkholderia* species of H_2-oxidizing bacteria (Zhang, 2006; Maimaiti *et al.*, 2007). In root elongation experiments with spring wheat, some isolates produced increases of up to 254% in root length (Maimaiti *et al.*, 2007). Clearly, these PGPR hold promise in the challenge to minimize chemical fertilizer (and pesticide) use, but we must learn more about them before they can be utilized to their greatest potential.

1.6 Methodology

1.6.1 The polymerase chain reaction (PCR):

The polymerase chain reaction (PCR) technique for amplifying DNA is a simple one involving the combination of a few chemical reagents and heat; within a short time,

one molecule of DNA can be replicated into billions of identical copies (Mullis, 1990). The required reagents include deoxynucleotide triphosphates (dNTPs) correspondent with the bases adenine, guanine, cytosine and thymine that comprise DNA (Lodish *et al.*, 2004), primers, small oligonucleotides up to approximately 20 base pairs (bp) required for DNA replication, a DNA polymerase to extend primers and add bases from the dNTPs and a buffer to stabilize the reaction's pH; all these and a heat source are all that is necessary for successful DNA amplification (Mullis, 1990; Lodish *et al.*, 2004). Repeating cycles of denaturation, annealing and primer extension exponentially increase the amounts of target DNA within a couple of hours (Mullis, 1990). First used in conjunction with restriction enzyme cutting to create a more rapid screening protocol for sickle cell anemia (Saiki *et al.*, 1985), PCR is now a standard protocol in molecular biology labs (Lodish *et al.*, 2004). Some limitations of PCR include contamination, primer mismatches and primers annealing to parts of the template DNA other than the targeted area for amplification (Ishii and Fukui, 2001), but these issues can be readily overcome through careful handling, lowering annealing temperatures and ensuring that primers lack the capacity to bind to each other (Ishii and Fukui, 2001). Many of these issues arise in later cycles, so decreasing the number of cycles, and increasing the number of PCR replicates, can also be helpful in resolving PCR biases (Ishii and Fukui, 2001; Kanagawa, 2003).

1.6.2 TRFLP analysis:

Soil is a complex environment and thus difficult to study under controlled conditions so it is not surprising that most soil bacterial species have not been identified (Singh *et al.*, 2006). A single gram of soil may contain over 4000 bacterial species, most

not amenable to traditional culturing methods (Kirk *et al.*, 2004). This difficulty has led to the development of culture-free methods to investigate soil bacterial assemblages, including a group of techniques collectively termed molecular fingerprinting. These methods take genomic DNA extracted from soil samples and amplify it *via* PCR. The latest innovation in molecular fingerprinting is Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis (Liu *et al.*, 1997). The 16S rDNA is used for this technique as it is highly conserved in bacteria yet sufficiently variable to be used for taxonomic purposes; when the amplified DNA is digested with restriction enzymes (REs), the variability in cutting sites is what allows the fragments to be distinguished (Osborne *et al.*, 2006). TRFLP analysis has been used to study bacterial diversity in a wide range of environments, including soils (Tiquia *et al.*, 2002; LaMontagne *et al.*, 2003); aquatic habitats (Danovaro *et al.*, 2006) and vertebrate and invertebrate intestines (Lan *et al.*, 2004; Shinzato *et al.*, 2005). For TRFLP, the forward primer is labeled with a fluorescent dye (Liu *et al.*, 1997; Blackwood *et al.*, 2003); the amplified DNA is then digested with REs and the fragments separated by gel electrophoresis (Blackwood *et al.*, 2003) and run through a DNA analyzer, where the fluorescence intensity is read as a peak height and the peak size is given in bp (Blackwood *et al.*, 2003).

As a PCR-based method, TRFLP is subject to the same limitations as PCR itself; it is also susceptible to unique issues such as the formation of pseudo terminal restriction fragments (pseudo-TRFs) from single-stranded amplicons that cannot be digested with REs but can be removed with mung bean nuclease prior to analysis (Egert and Friedrich, 2003) and a problem termed 'TRF drift" (Kaplan and Kitts, 2003), which is the difference between observed and true fragment lengths. When TRF drift occurs due to temperature

fluctuations, it can be easily managed by maintaining a constant laboratory temperature (Kaplan and Kitts, 2003), but variations in purine content may remain and can result in observed and true fragment lengths differing by as much as seven bp (Kaplan and Kitts, 2003).

Other difficulties with TRFLP analysis lie in the area of data analysis. A variable percentage threshold method has been recently introduced (Osborne *et al.*, 2006) to ensure less abundant contributors to bacterial assemblages that nonetheless may be ecologically significant are not removed as 'noise' from datasets (Zhang, 2006). The use of multiple REs has also been suggested to improve the resolution of the technique as closely related species may produce fragments of the same length and so be impossible to differentiate from each other (Osborne *et al.*, 2006). Further, it has been posited that the use of multivariate analyses such as principal components analysis (PCA) and multi-dimensional scaling (MDS), commonly employed by other ecologists, are not only appropriate but readily applicable to TRFLP data analyzed by microbial ecologists and will increase the statistical rigour of TRFLP analysis (Rees *et al.*, 2004).

1.7 Conclusions

Further research into HUP distribution in legumes and the mechanisms determining HUP status of legume-rhizobia associations, as well as the effects of hydrogen release into soil, is required to gain a better understanding of legume-rhizobia associations and their impacts on plant growth. The more that is understood about both the legume-rhizobia symbiosis itself and the potential benefits of the H_2-oxidizing PGPR that some associations attract, the better equipped we will be to utilize both in the development and implementation of sustainable agricultural systems.

1.8 Objectives of the present study

1. To determine the efficacy of two known strains of H_2-oxidizing PGPR as seed inoculants for non-legume crop species (in this case, barley).
2. To verify that the above strains could be re-isolated and identified through TRFLP analysis, supported by DNA sequencing data.
3. To compare a crop rotation experiment using soybean with seed inoculation experiments to investigate if seed inoculation confers the same growth benefits on nonleguminous plants as crop rotation using legume species.

2.0 Hydrogen-oxidizing bacteria as plant growth promoting rhizobacteria (PGPR)

2.1 Introduction

The terms 'plant growth promoting rhizobacteria' and 'biofertilizer' have been in use in the scientific literature since the late 1970s (Vessey, 2003). Plant growth promoting rhizobacteria (PGPR) are bacteria found in the rhizosphere soil that live in, on or around plant roots. Rhizobacteria differ from bacterial populations in bulk soil in being able to colonize soils where plant root exudates can alter any number of parameters such as soil pH, water potential or pO_2 (Vessey, 2003). PGPR help stimulate plant growth through either direct nutrient supply, for example, the N_2-fixing rhizobia that colonize legumes, or indirectly through beneficial effects on plant root morphology and growth (Vessey, 2003). Biofertilizers have been defined as any number of things, from green and animal manures (Rao and Gill, 1995; Abdel-Magid *et al.*, 1995) to seaweed extracts or vitamin supplements in chemical fertilizers (Vessey, 2003). Vessey (2003) defines biofertilizers as substances containing living microorganisms that, when applied to the soil, seeds or plant parts, promote plant growth by either direct nutrient supply or by making essential nutrients such as nitrogen and phosphorus more available to plants. These biofertilizers differ from organic fertilizers in that they using living organisms, whereas organic fertilizers use organic compounds that either directly supply nutrients to soils or provide them through their decomposition (Vessey, 2003).

Not all PGPR qualify as biofertilizers; others promote plant growth by deterring pests (biopesticides) or plant pathogens (biocontrols) (Vessey, 2003). However, some PGPR can perform both roles. For instance, strains of *Burkholderia cepacia* act as biocontrols with the phytopathogenic fungal species of *Fusarium*, but they can also act as

biofertilizers for plants living in iron-poor environments through the production of siderophores (Vessey, 2003); these are smaller molecules that bind iron and form soluble complexes with it (Nielands, 1995), thereby making it available to plants.

Endophytic PGPR colonize the apoplastic spaces within plant roots; two of the better known examples are legume rhizobia and the actinorhizal *Alnus-Frankia* associations (Vessey, 2003; Berg, 1999), both of which form root nodules on the host plant. With many other endophytes, the infection mechanism is not as well characterized; it is believed that one species in particular, *Gluconacetobacter diazotrophicus*, may use *Saccharicoccus sacchari* (the pink sugarcane mealybug) and/or arbuscular mycorrhizae (AM fungi) to infect host plants such as sugarcane (Vessey, 2003).

Rhizospheric PGPR, on the other hand, tend to either live in close proximity to or simply attach themselves directly to the surfaces of plant roots, though how this is done has not been elucidated with many species (Vessey, 2003). It is the rhizospheric class of PGPR under investigation in the present study. As stated above, both *Variovorax paradoxus* and *Flavobacterium johnsoniae* have exhibited plant growth promoting properties in that they increase root elongation by decreasing ethylene levels in the host plant (Maimaiti *et al.*, 2007), providing an indirect benefit to plants by allowing them to better access soil nutrients.

One objective of the present study was to check the efficacy of these isolates as field inoculants for nonleguminous crop species. To test this, field studies with barley (*Hordeum vulgare* L., cv. Chapais) were run in the 2006, 2007 and 2008 growing seasons at the Plumdale Facility of the Nova Scotia Agricultural College (NSAC) in Truro, Nova

Scotia. Field trials were supported by barley greenhouse trials over the winter and spring of 2009. Another objective of the present study was to treat soil by planting soybean inoculated with both HUP^+ and HUP^- rhizobial strains, along with controls, and then plant a nonlegume species the following year to see if yield differences would appear between plants grown in HUP^+ plots and those grown in HUP^- plots; these data would then be compared to results from seed inoculant experiments with barley using H_2-oxidizing strains, the purpose of which was to see if seed inoculation with H_2-oxidizing PGPR gives the same benefits to plant growth as crop rotation. To this end, soybean field trials were run in the 2007 growing season but had to be repeated in 2008 due to poor germination and growth; the soybean was planted in the same location as the barley trials. Soybean seeds (*Glycine max* Merr., cv. RR Drako) were inoculated with a HUP^- (JH47) or a HUP^+ (JH) strain of *Bradyrhizobium japonicum*; these strains are isogenic, with the only difference being the insertion of a Tn5 transposon into JH47, disrupting the hydrogenase gene and rendering the strain HUP^- (Dean *et al.*, 2006). A HUP^- positive control (532C, a commercial soybean inoculant) was also employed in the experiment. Unfortunately, a nonlegume species could not be planted on these plots (see below). Molecular work was performed on soil samples from the barley trials, including TRFLP analysis and DNA sequencing, to identify strains re-isolated from the field trials; grain yield, along with tiller number or grain head number, was compared between treatments in 2007 and 2008 only, as insect damage prevented such data from being recorded in 2006. Soybean grain yields from NSAC were compared between the treatments in 2007 and 2008.

2.2 Methods and Materials

2.2.1 Preparation of barley inoculants

2.2.1.1 2006 growing season:

Liquid inoculant was used in the 2006 growing season to inoculate barley (*Hordeum vulgare* L., cv. Chapais), with seeds kindly provided by Mr. Doug MacDonald, Scientific Officer, NSAC. Bacterial cells of strains JM63 (*Variovorax paradoxus*), JM120 (*Burkholderia* sp.) and JM162f (*Flavobacterium johnsoniae*) were taken from the -80°C storage, were cultured on nutrient agar plates (11.5 g Difco nutrient agar (BDMS, Sparks, MD) in 500 mL dH_2O and then autoclaved for 30 minutes in a 533LS Vacuum Steam Sterilizer (Getinge Canada Ltd., Mississauga, ON)); these were incubated in a New Brunswick Scientific incubator/shaker (Edison, NJ) at 30°C for three days. Single colonies were picked from each of five plates per strain and used to inoculate another five nutrient agar plates; these were incubated at 28°C for 24 hours. Single colonies from each plate were then picked to inoculate 50 mL of nutrient broth for each strain (NB; Difco nutrient broth powder) and incubated at 28°C and 200 rpm overnight.

The next morning, 1 L of NB was prepared for each strain and given a 1% inoculation each of JM63, JM162f and JM120. These flasks were then incubated overnight at 28°C and 200 rpm.

Bacterial pellet was harvested via centrifugation with an IEC Multi RF centrifuge (Thermo Scientific, Waltham, MA) at 7000 g and 4°C for 20 minutes for each run until all the suspension was centrifuged. The bacterial pellets were then rinsed thoroughly with 0.85% KCl (8.5 g KCl in 1L dH_2O, autoclaved for 30 minutes) and resuspended in the KCl sterile saline solution to 5.0×10^7 cells/mL; the cell density was measured by

spectrophotometry (Genesys 20 spectrophotometer, Thermo Scientific, Waltham, MA) using the OD_{600} reading. An extra 2 L of sterile saline solution was prepared and autoclaved to use as a control. Treatments for the 2006 barley field trials were as follows: Four replicates of JM63, four replicates of JM162f, four replicates of JM120, four replicates of sterile saline solution (control 1 or C1) and four replicates of blank soil (control 2 or C2) for a total of twenty plots in a randomized complete block design (RCBD) (see Figure 3). Inoculants and sterile saline solution were stored in glass flasks in a 4°C fridge until ready for planting. On the day of planting, inoculants and control solution were transferred to 2-L plastic bottles for ease of transport and to avoid breakage.

2.2.1.2 2007 and 2008 growing seasons:

Peat inoculant was prepared for both the 2007 and 2008 growing seasons as it was seen as easier to use for seeding, as well as for ease of transport. Sterilized peat was kindly donated by Novozymes Inc., formerly Philom Bios Inc., of Saskatchewan, Canada. The peat had a moisture content of 13% and the inoculant was prepared in the following manner: Bacterial cells of strains JM63 (*Variovorax paradoxus*) and JM162a (*Flavobacterium johnsoniae*) were taken from the -80°C storage and were cultured on mineral salt agar (MSA) plates (MSA: 10% $NaNO_3$ (2.0 g/L); 12% K_2HPO_4 (1.2 g/L); 10% $MgSO_4$ (0.5 g/L); 10% KCl (0.5 g/L); 14% KH_2PO_4 (0.14 g/L); 1% $Fe_2(SO_4)_3*H_2O$ (0.01 g/L); granulated agar (15 g/L); added to 800 mL distilled water (dH_2O) and filled to 1L; autoclaved for 30 minutes) at 20mL MSA per plate. Plates were incubated in 100% H_2 for two weeks at room temperature. Bacterial cells from the plates were then used to

inoculate two 250-mL flasks with 200 mL of Luria Broth (Bacto LB, Difco) each and the flasks were incubated on a Max^{Q} 4000 incubator/shaker (Thermo Scientific, Waltham, MA) at 37°C and 120 rpm for three days. The strain JM120 was not used in the 2007 and 2008 trials because some members of the genus *Burkholderia* are pathogenic to humans, so it was thought best to not repeat use of this strain. This being the case, no sequencing of this strain, re-isolated in 2006, was done and the results of TRFLP analysis from soil-extracted DNA of this strain are not reported here.

On the morning of the fourth day, three 2-L jars and one 1.5-L flask were prepared with 7 L of LB and autoclaved for 30 minutes. These were then given a 1% inoculation with strain JM63 (20 mL for the jars and 15 mL for the flask). The 250-mL flasks with both strains continued to shake in the incubator and the jars and flask were added after autoclaving; these were incubated at 37°C and 110 rpm overnight on the same incubator/shaker. A spectrophotometry reading (OD_{600}) was taken the next morning and the following equations were used to calculate grams of peat and mL of bacterial suspension comprising that day's harvest of bacterial pellet:

1. OD_{600} * 7000 mL/22.342 = grams of peat
2. Grams of peat * 0.359552 = mL of bacterial suspension
3. Grams of peat + mL suspension = inoculant harvest/day

Bacterial suspension was poured into 16 50-mL centrifuge tubes and centrifuged at 4°C and 8000 g for 10 minutes; this was repeated until all 7 L of bacterial suspension had been centrifuged. The pellet was stored in 50-mL centrifuge tubes at 4°C. This process was repeated for two more days, after which the bacterial pellet harvested was thoroughly washed of LB by repeated centrifuge runs in ice cold 5% saline (10 minutes at 4°C and

8500 g); this was to ensure the bacteria would have to go to the plants' roots for food. The pellet was then mixed with the appropriate amount of peat (from the above calculations) to give a peat-based inoculant with a 39-40% moisture content and containing $2.0 * 10^9$ cells per gram of peat. This process was repeated with strain JM162a. The work was done in early spring of both 2007 and 2008. Approximately 400 g peat inoculant (both strains) and 400 g sterilized peat were sent to experimental farms in both Saskatchewan and Ontario, with another 200 g of each strain (and an equal amount of sterilized peat) sent to Dr. Yanping Cen at Queen's University in Kingston, ON for greenhouse trials in both years. The rest was used for the field trials at NSAC. The treatments for the 2007 and 2008 barley field trials were as follows: Four replicates of JM63, four replicates of JM162a, four replicates of JM63/JM162a (Combo), four replicates of sterile peat (control 1 or C1) and four replicates of blank soil (control 2 or C2) for a total of twenty plots in a randomized complete block design (RCBD).

2.2.2 Barley and soybean planting

2.2.2.1 2006 barley planting:

Barley plants (cv. Chapais) were planted on June 7th, 2006 in the Demonstration Garden of NSAC. 300 seeds/m^2 were planted in eight rows for each plot with an H & N small plot double-disc seeder. Each plot was 4.5 m by 1.5 m for a total of 6.75 m^2 per plot. Each plot inoculated with JM63 received 375 mL of inoculant; each plot inoculated with JM162f received 250 mL of inoculant and each plot inoculated with JM120 received 500 mL of inoculant. The saline control plots received 500 mL of sterile saline solution per plot. Inoculants and control solution were administered with plastic squeeze bottles

after being measured out in 250-mL graduated flasks. The rows were then hoed over with soil. Surface soil samples were taken from three treatment plots and a sterile saline plot. On the 19th of July, soil samples were taken from all five treatments (three strains and the two controls) from three random locations in each plot, ensuring to position the soil probe close to plant roots. The three samples, from a depth of 0 to 7.5 cm, were then mixed together for standardization. No tiller, grain head or yield data were collected from this planting; the barley was planted late and so suffered severe insect damage, resulting in extremely poor growth. However, the soil samples taken in July were used for molecular analysis.

2.2.2.2 2007 and 2008 barley plantings:

Barley plants (cv. Chapais) were planted May 15th, 2007 at the Plumdale Facility at NSAC (Plumdale #5). 300 seeds/m^2 were planted in eight rows per plot with an H & N seeder as per the previous year (see Figures 3 and 5). The twenty plots were each 4.5 m by 1.5 m for a total of 6.75 m^2 per plot. 108 g of barley seed were premixed with 10.125 g of peat inoculant prior to planting. Two applications of 34-0-0 (140 kg ha^{-1} and 60 kg ha^{-1}) fertilizer were administered over the course of the growing season, and an application of Target™ herbicide (1.5 L ha^{-1}) was administered once. Soil samples (0-7.5 cm depth) were taken from the fields on June 15th following the same procedure as in 2006. Tillers were counted from 10 plants from each treatment on August 23rd and the barley was harvested on August 29th, 2007. This process was repeated the following growing season, with the barley being planted on May 27th, 2008, with soil samples taken on August 22nd. The same day soil samples were collected, barley grain heads were

counted. A row was randomly chosen in a treatment plot and heads were counted the length of one meter down each row; this was repeated five times per treatment in different plots to produce five replicates. Barley grain heads had to be counted instead of tillers for this growing season as the crop had matured by this date and it was too late to count tiller number. The barley received the same maintenance as the year before and was harvested on September 9th, 2008. Yield data, along with tiller and grain head data, for the 2007 and 2008 barley trials were provided by Mr. Doug MacDonald of NSAC and were analyzed using Minitab v. 12 (Minitab Inc., State College, PA), with graphs done in Excel (Microsoft Corporation, Redmond, WA).

2.2.2.3 2007 and 2008 soybean plantings:

Soybean seeds (*Glycine max* L. Merr., cv. RR Drako) were surface sterilized in a 10% bleach solution for 5 minutes and then air-dried before being mixed with inoculant. 126 g seed per plot were inoculated with 40 g each of strains JH and JH47 (inoculant supplied by Dr. Yanping Cen of Queen's University in Kingston, ON), 60 grams of 532C, a commercial inoculant already available in the lab (more of this was used than the JH and JH47 inoculant because it was older); all strains were *Bradyrhizobium japonicum* species strains. The remaining seeds were inoculated with 20 g sterilized peat or not inoculated at all. The sterile peat and the non-inoculated seeds were used as negative controls (control 1 or C1 being sterile peat and control 2 or C2 being blank soil) and the 532C was used as a HUP^- positive control. In 2007, the soybean was planted on June 15th at the Plumdale Facility at NSAC (Plumdale #5, next to the barley trials) in eight rows per plot in twenty plots with four replicates of each of the five treatments in a

randomized complete block design (RCBD) as with the barley (see Figures 4 and 6). Each plot was 4.5 m by 1.5 m for a total of 6.75 m^2. One application of 34-0-0 fertilizer (60 kg ha^{-1}) was administered over the course of the growing season, as was one application of Roundup Weathermax™ herbicide (1.67 L ha^{-1}); the soybean was harvested on September 14th. The sterilization protocol proved too hard on the soybean seeds, as poor germination and growth were observed. This meant no nonlegume species could be planted the subsequent year, so soybean planting was repeated in 2008; for this trial, the soybean seeds were surface sterilized with 70% ethanol and air-dried in a clean hood in the lab before mixing them with inoculant and taking them to the site for planting. The second soybean trial was planted on June 9th, 2008, received the same maintenance as the year before and was harvested on October 31st, 2008. The ethanol sterilization proved much more successful. Yield data for both the 2007 and 2008 soybean trials were provided by Mr. Doug MacDonald of NSAC and were analyzed using Minitab v. 12 (Minitab Inc., State College, PA) with graphs done in Excel (Microsoft Corporation, Redmond, WA). It should be noted that soil samples were taken from the soybean trials in the same manner as from the barley trials; strains were re-isolated from these samples and DNA was extracted from them for molecular work. However, no sequencing PCR was done from re-isolated strains and the results of TRFLP analysis are not reported here because using our primers, which target the 16S ribosomal DNA (rDNA) of bacteria, we would not have been able to tell the strains apart as these DNA sequences are too highly conserved and so too similar for comparing different strains of the same species; this would have required the use of primers targeting the

hydrogenase genes instead, which would have distinguished the strains from each other despite being the same species.

2.2.3 Re-isolation of H_2-oxidizing bacteria:

A soil dilution series was carried out for re-isolation of strains used as inoculant and to isolate H_2-oxidizing strains in samples from the control plots. Test tubes (six tubes for each treatment) containing 10 mL of dH_2O were autoclaved for 30 minutes; 0.1 g of soil from each treatment was placed in a tube and mixed with a pipette. 100 µL from this tube were transferred to the next tube and mixed and this was repeated until all six tubes were completed for all five treatments, giving each treatment a dilution series running from 10^{-1} to 10^{-11}. Three plates per dilution were prepared with MSA (see above) and 100 µL from each tube was spread onto each plate and sprinkled with a small amount of autoclaved soil to start growth; this gave 18 plates/treatment and 90 plates in total. Plates were incubated at room temperature in a 100% H_2 atmosphere for approximately two weeks, whereupon they were re-plated on fresh MSA without autoclaved soil. After another two weeks, they were quadrant-streaked on fresh MSA plates to allow for the growth of single colonies that could then be picked for sequencing PCR. This procedure was carried out in all three years.

2.2.4 Sequencing of re-isolated strains:

The best growth of isolates occurred on the 10^{-1} to 10^{-3} dilution plates in all years, so single colonies were picked from these quadrant streak plates and transferred to 0.2-mL PCR tubes containing the following 49-µL PCR mix: 40.6 µL sterile dH_2O; 5.0 µL 10X ThermoPol reaction buffer (New England Biolabs Ltd., Pickering, ON); 1.0 µL 2mM dNTP (New England Biolabs Ltd., Pickering, ON); 1.0 µL 200mM BSF8/20 forward primer (5'-AGAGTTTGATCATGGCTCAG-3') (Applied Biosystems, Foster City, CA); 1.0 µL 200mM BSR 1541/20 reverse primer (5'-AGGAGGTGGATCCAACCGCA-3') (Applied Biosystems, Foster City, CA) and 0.4 µL 5U/µL *Taq* polymerase (New England Biolabs Ltd., Pickering, ON). The protocol for all PCR reactions was as follows: Three minutes at 94°C for initial denaturing; 35 cycles of 94°C for one minute, 55°C for 45 seconds for annealing and 72°C for 45 seconds for primer extension, with a final primer extension at 72°C for ten minutes and a 4°C hold. All PCR reactions were run using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). PCR results were verified by gel electrophoresis using 1% agarose gels run at 40 V and 250 mA for three hours. The 1500 bp PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN Inc., Mississauga, ON) with the manufacturer's instructions for increased DNA yields and verified by gel electrophoresis. Purified samples were then sent to Macrogen Inc. (Seoul, Korea) for sequencing. JM63 and JM162a isolates, as well as Combo and control plot isolates, from all three years were sequenced. Sequences were aligned using Chromas™ v.1.4.5 and BLAST searches on the NCBI website were utilized for isolate identification (http://blast.ncbi.nlm.nih.gov/Blast).

2.2.5 TRFLP analysis of barley soil samples:

Soil DNA from 2006, 2007 and 2008 soil samples from barley trials was extracted using the PowerSoil Soil DNA Isolation Kit (MoBio Labs Inc., Carlsbad, CA) according to the manufacturer's instructions for increased DNA yields. Extracted DNA was then used for PCR amplification by transfer to 0.2-mL PCR tubes containing the following 49-µL PCR mix: 40.6 µL sterile dH_2O; 5.0 µL 10X ThermoPol reaction buffer (New England Biolabs Ltd., Pickering, ON); 1.0 µL 2mM dNTP (New England Biolabs Ltd., Pickering, ON); 1.0 µL 200mM fluorescently labeled forward primer 6-FAM (phosphoramidite fluorochrome 5-carboxy fluorescein)- BSF8/20 (5'-AGAGTTTGATCATGGCTCAG-3') (Applied Biosystems, Foster City, CA); 1.0 µL reverse primer BSR534/18 (5'- CAGCAGCCGCGGTAATAC-3') (Applied Biosystems, Foster City, CA) and 0.4 µL 5U/µL Taq polymerase (New England Biolabs Ltd., Pickering, ON); 1.0 µL of template DNA was added to the mixture for a total of 50 µL per reaction. The protocol for all PCR reactions was as follows: Three minutes at 94°C for initial denaturing; 35 cycles of 94°C for one minute, 55°C for 45 seconds for annealing and 72°C for 45 seconds for primer extension, with a final primer extension at 72°C for ten minutes and a 4°C hold. All PCR reactions were run on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA); these PCR reactions targeted the 16s rDNA genes, resulting in 500 bp products. PCR results were verified by gel electrophoresis on 1% agarose gels run at 40 V and 250 mA for three hours. PCR products were then purified using the QIAquick PCR Purification Kit (QIAGEN Inc., Mississauga, ON) and verified by gel electrophoresis.

Four restriction enzymes (REs) were used to digest the purified DNA: BstUI, HinfI, HaeIII and MspI. The DNA digestion reactions were run in 0.2-mL PCR tubes with the following enzyme digestion mixture: 31.0 µL of sterile dH_2O; 5.0 µL 10X Buffer #2 (New England Biolabs Ltd., Pickering, ON); 10.0 µL template DNA and 3.0 µL of RE (all REs from New England Biolabs Ltd., Pickering, ON) for a total of 49 µL per reaction.. Samples digested with HinfI, HaeIII and MspI were incubated in a 37°C water bath for four hours; samples digested with BstUI were incubated in a 60°C water bath for four hours. The reactions were stopped with the QIAquick Nucleotide Removal Kit (QIAGEN Inc., Mississauga, ON) according to the manufacturer's protocol. Results were verified by gel electrophoresis and samples were then sent to the Core DNA Services Lab, Faculty of Medicine, University of Calgary, Calgary, AB for fragment analysis. GeneMarker™ v. 1.4 (Softgenetics LLC, USA) was used for TRFLP analysis of fragment results.

2.2.6 Greenhouse barley trials

2.2.6.1 First trial:

Soil collected from NSAC in the fall of 2008 was used to prepare 20 1-gallon pots; the soil was sieved through a 2-mm sieve and mixed with sand in a 2:1 (v/v) ratio and then put into the pots in the Saint Mary's University (Halifax, Nova Scotia) greenhouse. Four pots each were labeled with one of the five treatments (JM63, JM162a, Combo, C1 (sterile peat) or C2 (no inoculant)), watered and left to sit for two days to allow soil to assume the shape of the pot. On December 28th, 2008, five groups of 12 barley seeds (cv. Chapais) were given the appropriate treatment and three seeds were

planted in each pot for a total of 60 replicates, with 12 replicates per treatment. The plants were grown with supplemental lighting of 350 μmol quanta^{-1} s^{-1} (P.L. Light Systems Inc., Beamsville, ON) in a temperature range of 24°C to 32°C and an 18-hour photoperiod; they were watered regularly with the following nutrient solution: 1000X KH_2PO_4 (34.98 g/L); 1000X K_2HPO_4 (9.93 g/L); 1000X K_2SO_4 (87.48 g/L); 1000X $MgSO_4.7H_2O$ (59.89 g/L); 1000X $MgCl_2.6H_2O$ (50.01 g/L); 1000X $CaCl_2.2H_2O$ (109.97 g/L); 1000X $FeCl_3.6H_2O$ (10.27 g/L); 1000X aqueous $MnSO_4$ (1.69 g/L); 1000X $CuSO_4.5H_2O$ (0.250 g/L); 1000X $ZnSO_4.7H_2O$ (0.287 g/L); 1000X H_3BO_3 (1.92 g/L); 1000X $Na_2MoO_4.2H_2O$ (0.121 g/L); 1000X $CoSO_4.7H_2O$ (0.056 g/L) and 1000X KNO_3 (50.55 g/L). Time to emergence (TTE) in days was recorded for each plant. At harvesting, tillers were counted, plant height was measured from root to tip and pictures were taken of five representative plants, one from each treatment (see Figure 7). Data were analyzed using Minitab v. 12 (Minitab Inc., State College, PA), with graphs done in Excel (Microsoft Corporation, Redmond, WA).

2.2.6.2 Second trial:

The soil in the pots from the first trial was kept and used for the second trial, which ran from February 25th to April 26th, 2009; all greenhouse conditions remained the same and seeds were inoculated, planted and maintained as before. Again, time to emergence was recorded and at harvest, tillers were counted, plant height was measured and another picture of five representative plants was taken (see Figure 8). Data were analyzed using Minitab v. 12 (Minitab Inc., State College, PA), with graphs done in Excel (Microsoft Corporation, Redmond, WA).

Figure 1: Map of 2007 and 2008 Barley Field Trials

	C1		63		162a		C2		Comb	
	C2		162a		63		C1		Comb	
	63		C2		C1		Comb		162a	
	63		C1		162a		C2		Comb	

South corner **Northeast corner**

Figure 1: Barley Field Trials Map, Plumdale # 5, NSAC

Legend:

63: Inoculated with strain JM63 (*Variovorax paradoxus*)
162a: Inoculated with strain JM162a (*Flavobacterium johnsoniae*)
Combo: Combination of JM63 and JM162a
C1: Sterile peat
C2: Blank control

Figure 2: Map of 2007 and 2008 Soybean Field Trials

	C1		JH		JH47		C2		532C	
	C2		JH47		JH		C1		532C	
	JH		C2		C1		532C		JH47	
	532C		C1		JH47		C2		JH	

South corner **Northeast corner**

Figure 2: Soybean Field Trial Map, Plumdale # 5, NSAC

Legend:

JH: Inoculated with strain JH
JH47: Inoculated with strain JH47
532C: Inoculated with strain 532C
C1: Sterile peat
C2: Blank control

Figure 3: Barley Plants, Greenhouse Trial #1 (a) and Barley Plants, Greenhouse Trial #2 (b)

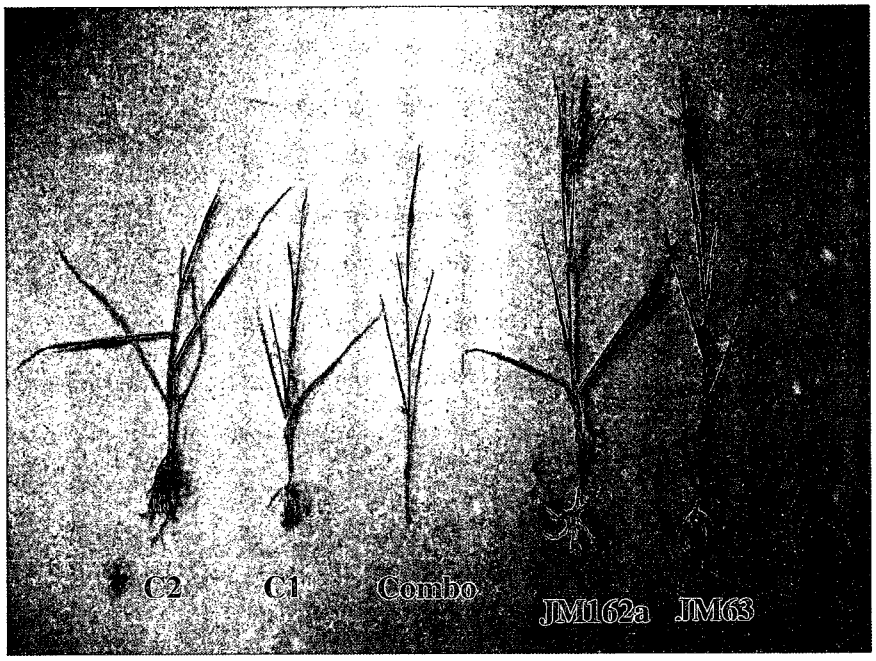

Figure 3(a): Five representative barley plants, one from each treatment, from the first greenhouse trial.

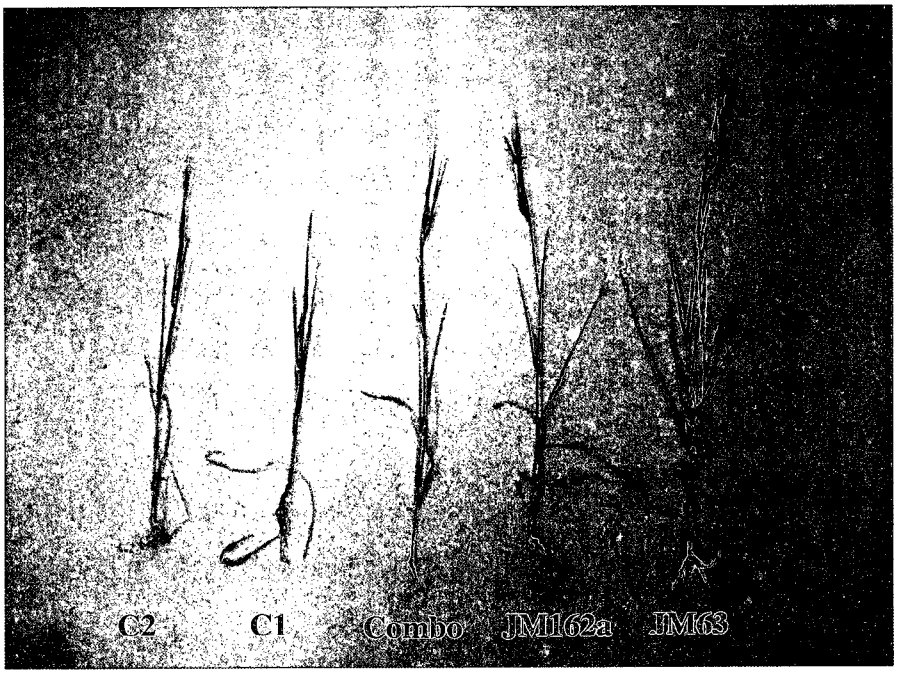

Figure 3(b): Five representative barley plants, one from each treatment, from the second greenhouse trial.

2.3 Results

2.3.1 Barley field trial data

2.3.1.1 2007 growing season:

The Anderson-Darling test for normality revealed a non-normal distribution of the 2007 mean barley yield data ($A^2 = 0.203$, $p = 0.857$), so a Kruskal-Wallis test was performed to check for differences amongst the treatments, but none were revealed ($H = 1.89$, $p = 0.755$, adjusted for ties; see Table 1 and Figure 4a). The 2007 mean tiller number data did show a normal distribution and variances were approximately equal, so a one-way ANOVA was used to find differences amongst the means. These data did reveal a significant difference ($F = 20.4$, $p = 0.000$), so Tukey's pairwise comparison tests using a family error rate of 0.05 were conducted to find out where the differences lay. There were significant differences between treatments and controls, but no significant differences within treatments or controls (see Table 2 and Figure 4b). Significance for all tests was determined at the 5% level.

Table 1: Mean Barley Yield for 2007

Sample	Mean Yield (t/ha)	Standard Deviation
JM63	5.475	0.287
JM162a	5.850	0.742
Combo	5.425	0.450
C1	5.650	0.451
C2	5.625	0.411

Table 2: Mean Barley Tiller Number for 2007

Sample	Mean Tiller Number	Standard Deviation
JM63	5.900[a]	0.876
JM162a	6.300[a]	0.675
Combo	6.200[a]	0.632
C1	4.000[b]	0.943
C2	4.300[b]	1.059

*Means with differing superscripts are significantly different

2.3.1.2 2008 growing season:

The Anderson-Darling test showed a non-normal data distribution for mean barley yield in 2008 ($A^2 = 0.559$, $p = 0.129$); the Kruskal-Wallis test showed no significant differences in yield between treatments ($H = 1.93$, $p = 0.858$, adjusted for ties; see Table 3 and Figure 5a). The mean grain head data were also not normally distributed ($A^2 = 0.457$, $p = 0.244$), so differences were revealed with Kruskal-Wallis ($H = 18.73$, $p = 0.001$), and two-tailed two-sample t-tests were run (without assuming equal variances) to see where the differences lay. Significant differences were observed both between treatments and controls and within treatments and controls (see Table 4 and Figure 5b), with JM63 outperforming all other treatments in regards to this parameter. Significance for all tests was determined at the 5% level.

Table 3: Mean Barley Yield for 2008

Sample	Mean Yield (t/ha)	Standard Deviation
JM63	1.200	0.408
JM162a	1.200	0.294
Combo	1.000	0.245
C1	1.175	0.236
C2	1.250	0.342

Table 4: Mean Barley Grain Heads for 2008

Sample	Mean Grain Heads	Standard Deviation
JM63	51[a]	5.48
JM162a	37[b]	8.37
Combo	33[b]	5.70
C1	28[b]	7.58
C2	18[c]	2.74

*Means with differing superscripts are significantly different

2.3.2 Soybean field trial data

2.3.2.1 2007 growing season:

The Anderson-Darling test showed a non-normal data distribution for mean soybean yield in 2007 ($A^2 = 0.307$, $p = 0.532$); a subsequent Kruskal-Wallis test revealed

no significant differences in grain yields between treatments ($H = 7.64$, $p = 0.106$; see Table 5 and Figure 6a). All tests were run at the 5% significance level.

Table 5: Mean Soybean Yield for 2007

Sample	Mean Yield (kg/ha)	Standard Deviation
JH	1503	350
JH47	1544	142
532C	970	472
C1	1100	221
C2	1382	377

2.3.2.2 2008 growing season:

The data for 2008 mean soybean yield followed a normal distribution ($A^2 = 0.902$, $p = 0.017$) and variances were approximately equal; no significant differences in mean yield between treatments were discovered with one-way ANOVA ($F = 0.45$, $p = 0.772$; see Table 6 and Figure 6b). All tests were run at the 5% significance level.

Table 6: Mean Soybean Yield for 2008

Sample	Mean Yield (kg/ha)	Standard Deviation
JH	2960	348
JH47	2712	202
532C	2632	298
C1	2940	270
C2	2664	876

Figure 4: 2007 Mean Barley Yield (a) and Mean Tiller Number (b)

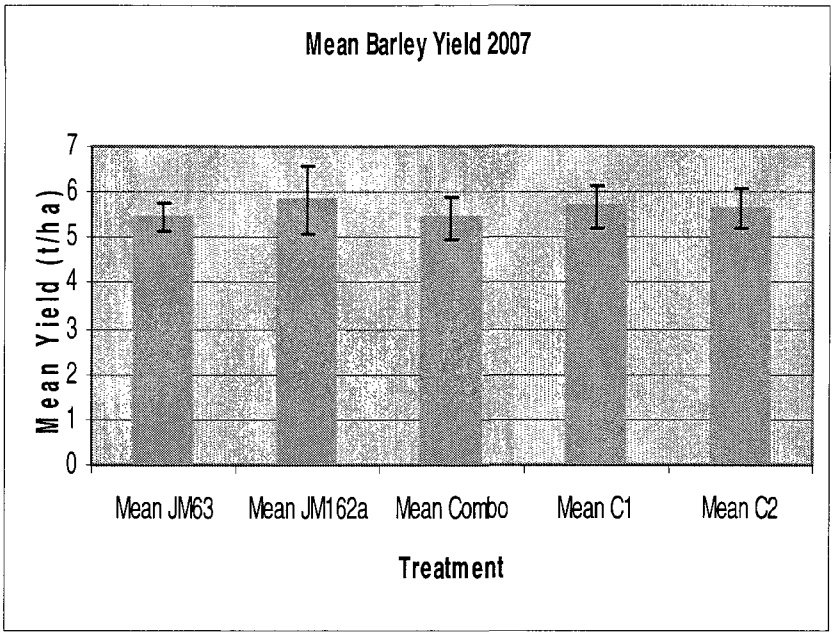

Figure 4(a): Mean barley yield for 2007 in tons ha^{-1}. Error bars are ± one standard deviation.

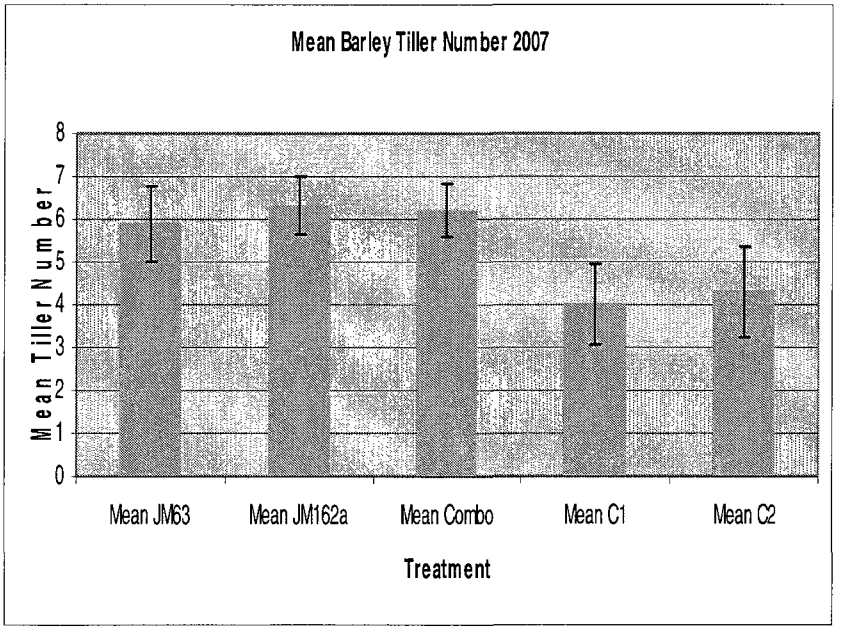

Figure 4(b): Mean tiller number for 2007. Error bars are ± one standard deviation.

Figure 5: 2008 Mean Barley Yield (a) and Mean Grain Heads (b)

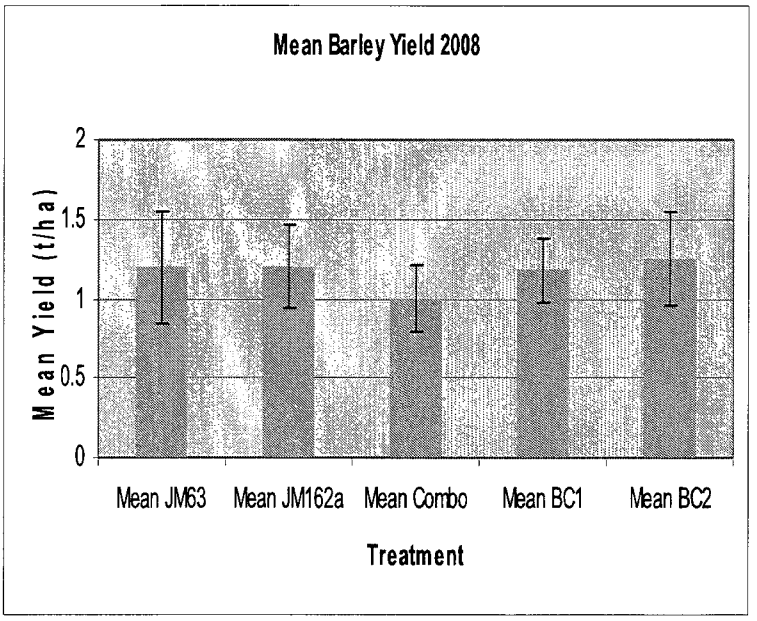

Figure 5(a): 2008 mean barley yield in tons ha^{-1}. Error bars are ± one standard deviation.

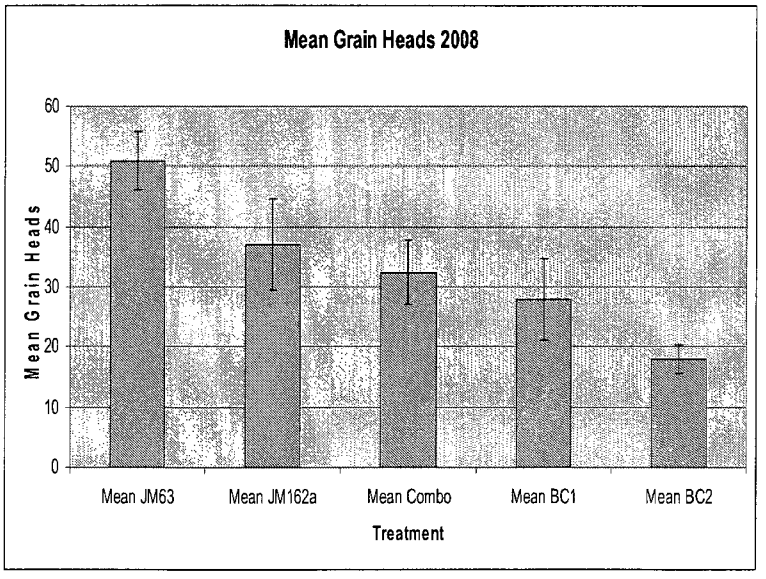

Figure 5(b): 2008 mean barley grain heads. Error bars are ± one standard deviation.

Figure 6: 2007 Mean Soybean Yield (a) and 2008 Mean Soybean Yield (b)

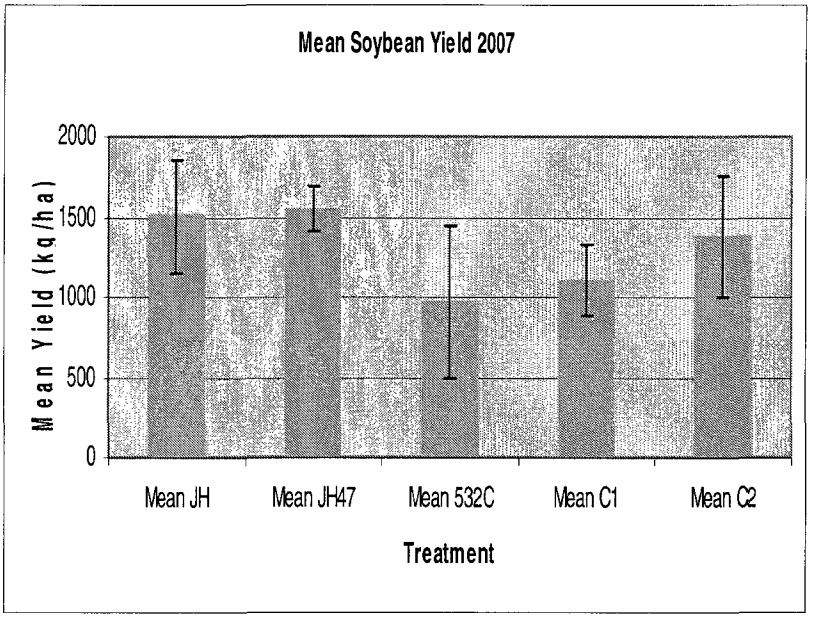

Figure 6(a): 2007 mean soybean yield in kg ha^{-1}. Error bars are ± one standard deviation.

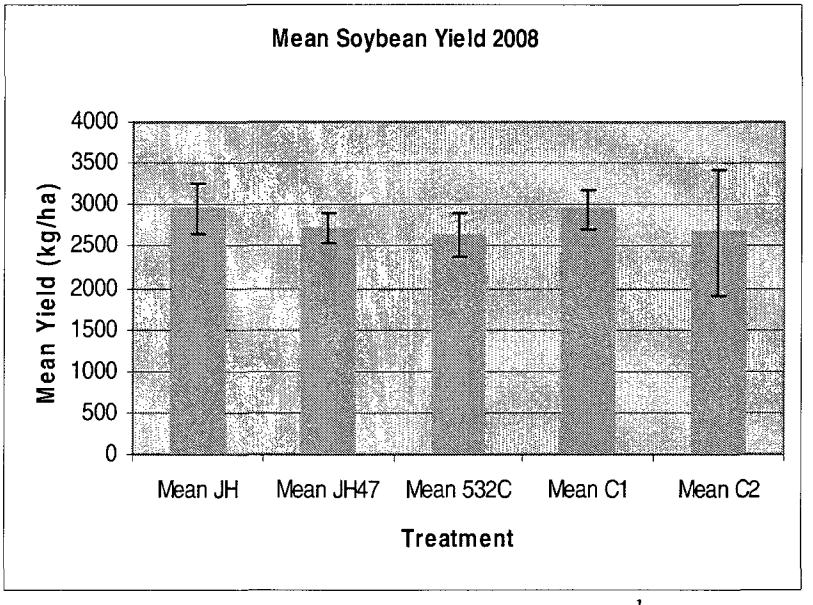

Figure 6(b): 2008 mean soybean yield in kg ha^{-1}. Error bars are ± one standard deviation.

2.3.3 DNA sequencing results:

Sample 8JM162 gave a 98% match with the isolated strain of *Flavobacterium johnsoniae* JM162a (Accession No. DQ256490 in GenBank) in the BLAST search, while samples 7JM162 and 6JM162 gave matches with this strain of 95% and 89%, respectively (see Table 7). Sample 8JM63 shared 95% similarity with the *Variovorax paradoxus* strain JM63 (Accession No. DQ256487 in GenBank), whereas samples 7JM63 and 6JM63 only shared 92% and 91% similarity, respectively, with this strain, although this is still a strong match. The 8Combo sample showed 89% similarity with JM162a but the 7Combo sample revealed an 81% similarity with JM63 (see Table 7); this is not altogether unsurprising as these samples are made up of both strains. 8BC1 shared 90% similarity with an uncultured bacterium (wkA01, Accession No. AF257804 in GenBank) and 8BC2 shared 95% similarity with an uncultured rape rhizosphere bacterium (wr0008, Accession No. AJ295469 in EMBL); interestingly, sample 6BC2 shared a 96% similarity with this same organism, while 6BC1 showed 88% similarity with an uncultured bacterial clone (G22-31, Accession No. EU153026 in GenBank). 7BC1 shared 81% similarity with a *Mycobacterium* species (T126, Accession No. FJ719354 in GenBank), while an 88% similarity between 7BC2 and an uncultured *Nevskia* species clone (woFOC R50, Accession No. EF600646 in GenBank) was revealed (see Table 7).

Table 7: Sequence Matches with Bacterial Strains in BLAST.

Sample	Similarity	Identification
8JM162	98%	JM162a (DQ256490 in GenBank); 16s rRNA partial sequence from *Flavobacterium johnsoniae*.
7JM162	95%	Same as 8JM162.
6JM162	89%	Same as 8JM162.
8JM63	95%	JM63 (DQ256487 in GenBank); 16s rRNA partial sequence from *Variovorax paradoxus*.
7JM63	92%	Same as 8JM63.
6JM63	91%	Same as 8JM63.
8Combo	89%	JM162a (DQ256490 in GenBank); 16s rRNA partial sequence from *Flavobacterium johnsoniae*.
7Combo	81%	JM63 (DQ256487 in GenBank); 16s rRNA partial sequence from *Variovorax paradoxus*.
8BC1	90%	Uncultured bacterium (wkA01, Accession No. AF257804 in GenBank); 16s rRNA partial sequence.
8BC2	95%	Uncultured rape rhizosphere bacterium (wr0008, Accession No. AJ295469 in EMBL); 16s rRNA partial sequence.
7BC1	81%	*Mycobacterium* sp. (woFOC R50, Accession No. EF600646 in GenBank); 16s rRNA partial sequence.
7BC2	88%	Uncultured *Nevskia* sp. clone (G22-31, Accession No. EU153026 in GenBank); 16s rRNA partial sequence.
6BC1	88%	Uncultured *Nevskia* sp. clone (G22-31, Accession No. EU153026 in GenBank); 16s rRNA partial sequence.
6BC2	96%	Same as 8BC2.

2.3.4 TRFLP analysis:

Areas of TRFLP profiles showed the overwhelming majority to be higher in the treatments than in the controls, meaning most of the observed peaks were observed in treatment samples as opposed to control samples. This was true for samples from all three years (see Tables 8, 9 and 10). There were only three exceptions seen in the data: The Combo sample from 2007 (7Combo) cut with MspI only took up 40.14% of the total area profile whereas the 2007 sterile peat sample (7BC1), also cut with MspI, took up 59.86% of the area (see Table 9). The 2008 Combo sample (8Combo) cut with BstUI comprised 46.55% of its total profile area but the 2008 sterile peat sample (8BC1), also cut with BstUI, took up the other 53.45% of the profile area (Table 10). Finally, the 2008 Combo sample cut with MspI (8Combo) comprised only 48.53% of its profile area, whereas the 2008 blank soil sample (8BC2), also cut with MspI, took up the other 51.47% of the profile area (Table 10). It should be noted that in these exceptional profiles, the increase in percentage area for the control treatments was always less than one times the treatment percentage area (0.67, 0.87 and 0.94, respectively). Increases in percentage area where treatment percentage areas are higher than those in controls were always over one times the percentage areas of the controls (Tables 8, 9 and 10); some treatment percentage areas were many times that of control percentage areas, for instance, the 2006 JM162 sample (6JM162) cut with HinfI produced a percentage area for the profile over 284 times larger than that of the 2006 blank soil sample (6BC2) cut with the same enzyme, comprising 99.65% of the total profile area, whereas the 6BC2 sample contributed only 0.35% to the total profile area (Table 8).

In Zhang (2006), expected cutting sites for each of the restriction enzymes used in this study were determined for strains JM63 and JM162 using sequence data; the observed cutting sites were then recorded after performing TRFLP analysis with soil samples spiked with both strains (Zhang, 2006); it is believed that multiple cutting sites for the same restriction enzyme are due to incomplete digestion, which may be due to the complex nature of the substrate the DNA was extracted from (Zhang, 2006). These results were observed even after overnight digestions in the previous study, so digestions in the present study were run for four hours as increasing the incubation time did not alleviate the situation. These observed cutting sites from the previous work were used in this study to identify these strains when used as inoculants in the current barley field trials. Similar cutting sites were observed in the present study with peaks within approximately ±2 bp of Zhang's observed sites accepted as peaks associated with the isolates used. The most significant finding of the TRFLP analysis carried out in the present study is that even the few profiles where control samples contributed more to the total percentage area of the profile than treatment samples (in bold in Tables 9 and 10), the peaks for control samples at the observed cutting sites for each strain were either much smaller than those of treatment samples or they were entirely absent in control samples (see Tables 11, 12 and 13); it is entirely possible that where peaks matching these strains do appear in control samples, it is from an indigenous bacterial population already present in the soil, as the intensity (height) and the percentage areas of these peaks were always smaller than those observed with treatment samples, indicating a much lower abundance of the strain in untreated soil samples.

Table 8: Profile area percentages for treatments *versus* controls, 2006.

RE	***6JM63 Area (%)***	***6BC1 Area (%)***	***X Increase***
BstUI	91.96	8.04	11.44
HinfI	90.61	9.39	9.65
HaeIII	98.29	1.71	57.44
MspI	92.86	7.14	13.01
RE	***6JM162 Area (%)***	***6BC1 Area (%)***	***X Increase***
BstUI	90.36	9.64	9.37
HinfI	90.69	9.31	9.75
HaeIII	97.43	2.57	37.96
MspI	93.68	6.32	14.83
RE	***6JM63 Area (%)***	***6BC2 Area (%)***	***X Increase***
BstUI	94.22	5.78	16.31
HinfI	99.65	0.35	283
HaeIII	57.40	42.60	1.35
MspI	50.41	49.59	1.02
RE	***6JM162 Area (%)***	***6BC2 Area (%)***	***X Increase***
BstUI	95.01	5.03	18.90
HinfI	99.65	0.35	284.28
HaeIII	52.87	47.13	1.12
MspI	56.29	43.71	1.29

Table 9: Profile area percentages for treatments *versus* controls, 2007.

RE	***7JM63 Area (%)***	***7BC1 Area (%)***	***X Increase***
BstUI	77.10	22.90	3.37
HinfI	51.72	48.28	1.07
HaeIII	60.17	39.83	1.51
MspI	54.86	45.14	1.22
RE	***7JM162 Area (%)***	***7BC1 Area (%)***	***X Increase***
BstUI	69.27	30.73	2.25
HinfI	52.31	47.69	1.10
HaeIII	54.46	45.54	1.20
MspI	56.05	43.95	1.28
RE	***7Combo Area (%)***	***7BC1 Area (%)***	***X Increase***
BstUI	88.00	12.00	7.33
HinfI	61.51	38.49	1.60
HaeIII	58.68	41.32	1.42
MspI	**40.14**	**59.86**	**0.67**
RE	***7JM63 Area (%)***	***7BC2 Area (%)***	***X Increase***
BstUI	98.33	1.67	58.84
HinfI	66.87	33.13	2.02
HaeIII	73.16	26.84	2.73
MspI	99.63	0.36	272.59
RE	***7JM162 Area (%)***	***7BC2 Area (%)***	***X Increase***
BstUI	97.27	2.73	35.57
HinfI	67.15	32.85	2.04
HaeIII	70.23	29.77	2.36
MspI	98.40	1.60	61.50
RE	***7Combo Area (%)***	***7BC2 Area (%)***	***X Increase***
BstUI	99.17	0.83	119
HinfI	67.94	32.06	2.12
HaeIII	73.27	25.65	2.86
MspI	95.67	4.33	22.11

Table 10: Profile area percentages for treatments *versus* controls, 2008

RE	***8JM63 Area (%)***	***8BC1 Area (%)***	***X Increase***
BstUI	99.53	0.47	209.48
HinfI	77.97	22.03	3.54
HaeIII	53.16	46.84	1.13
MspI	66.95	33.05	2.03
RE	***8JM162 Area (%)***	***8BC1 Area (%)***	***X Increase***
BstUI	56.01	43.99	1.27
HinfI	77.93	22.07	3.53
HaeIII	57.37	42.63	1.35
MspI	92.15	7.85	11.75
RE	***8Combo Area (%)***	***8BC1 Area (%)***	***X Increase***
BstUI	**46.55**	**53.45**	**0.87**
HinfI	79.94	20.06	3.99
HaeIII	64.06	35.94	1.78
MspI	56.03	43.97	1.27
RE	***8JM63 Area (%)***	***8BC2 Area (%)***	***X Increase***
BstUI	59.93	40.07	1.49
HinfI	78.37	21.63	3.62
HaeIII	56.73	43.27	1.31
MspI	63.07	36.93	1.71
RE	***8JM162 Area (%)***	***8BC2 Area (%)***	***X Increase***
BstUI	75.32	24.68	3.05
HinfI	77.04	22.96	3.36
HaeIII	58.80	41.20	1.43
MspI	92.88	7.12	13.04
RE	***8Combo Area (%)***	***8BC2 Area (%)***	***X Increase***
BstUI	55.03	44.97	1.22
HinfI	79.39	20.61	3.85
HaeIII	62.85	37.15	1.69
MspI	**48.53**	**51.47**	**0.94**

Table 11: Comparison of treatment and control samples at cutting sites specific to strains JM63 and JM162a, 2006

6JM63 vs. 6BC1	*Delta*	*X Increase*	*6JM63 vs. 6BC2*	*Delta*	*X Increase*
BstUI			**BstUI**		
62.5	2.035	3.657	62.5	2.371	5.763
102	1.460	3.329	102	1.707	4.959
222.4	2.356	∞	222.4	2.414	∞
384.4	3.994	∞	384.4	3.460	6.471
HinfI			**HinfI**		
320.1	9.129	3.901	320.1	15.171	∞
321.6	13.794	∞	321.6	9.100	∞
HaeIII			**HaeIII**		
63.2	1.335	∞	63.2	0.041	1.053
72.6	0.739	∞	72.6	0.035	1.082
217.5	9.775	∞	217.5	1.890	1.506
MspI			**MspI**		
81.2	1.650	∞	81.2	0.344	1.388
275.5	N/A	N/A	275.5	N/A	N/A
483.6	N/A	N/A	483.6	N/A	N/A
6JM162 vs 6BC1	*Delta*	*X Increase*	*6JM162 vs. 6BC2*	*Delta*	*X Increase*
BstUI			**BstUI**		
62.5	2.539	3.550	62.5	3.052	5.593
102	0.962	∞	102	0.775	4.277
222.4	2.895	∞	222.4	3.044	∞
384.4	3.771	∞	384.4	3.121	4.698
HinfI			**HinfI**		
320.1	6.333	3.134	320.1	10.160	176.804
321.6	5.340	∞	321.6	5.867	∞
HaeIII			**HaeIII**		
63.2	2.959	4.697	63.2	1.928	∞
72.6	0.558	∞	72.6	0.329	2.149
217.5	4.722	∞	217.5	2.572	2.062
MspI			**MspI**		
81.2	2.805	∞	81.2	0.900	1.560
275.5	N/A	N/A	275.5	N/A	N/A
483.6	N/A	N/A	483.6	N/A	N/A

Table 12: Comparison of treatment and control samples at cutting sites specific to strains JM63 and JM162a, 2007.

7JM63 vs. 7BC1	*Delta*	*X Increase*	*7JM63 vs. 7BC2*	*Delta*	*X Increase*
BstUI			**BstUI**		
62.5	1.261	2.073	62.5	2.565	5.722
102	0.078	1.048	102	2.148	∞
222.4	1.312	∞	222.4	4.224	∞
384.4	3.460	∞	384.4	4.413	∞
HinfI			**HinfI**		
320.1	0.340	1.151	320.1	0.872	1.532
321.6	0.870	1.126	321.6	33.244	7.766
HaeIII			**HaeIII**		
63.2	1.349	∞	63.2	0.145	1.127
72.6	0.040	1.104	72.6	0.358	∞
217.5	1.269	1.424	217.5	22.172	7.154
MspI			**MspI**		
81.2	3.103	∞	81.2	1.267	∞
275.5	N/A	N/A	275.5	N/A	N/A
483.6	N/A	N/A	483.6	N/A	N/A
7JM162 vs. 7BC1	*Delta*	*X Increase*	*7JM162 vs. 7BC2*	*Delta*	*X Increase*
BstUI			**BstUI**		
62.5	3.247	2.859	62.5	6.116	7.891
102	2.388	∞	102	2.242	∞
222.4	3.158	∞	222.4	4.429	∞
384.4	3.619	∞	384.4	5.075	∞
HinfI			**HinfI**		
320.1	4.014	∞	320.1	2.310	∞
321.6	9.169	∞	321.6	35.703	7.766
HaeIII			**HaeIII**		
63.2	0.441	∞	63.2	0.341	∞
72.6	0.560	∞	72.6	0.433	∞
217.5	0.009	1.002	217.5	28.092	10.162
MspI			**MspI**		
81.2	0.324	∞	81.2	0.568	∞
275.5	N/A	N/A	275.5	N/A	N/A
483.6	0.767	∞	483.6	1.347	∞

Table 12 (continued).

7Combo vs. 7BC1	*Delta*	*X Increase*	*7Combo vs. 7BC2*	*Delta*	*X Increase*
BstUI			**BstUI**		
62.5	2.082	4.380	62.5	2.789	12.089
102	0.208	1.246	102	1.183	∞
222.4	3.287	∞	222.4	3.705	∞
384.4	3.469	∞	384.4	3.910	∞
HinfI			**HinfI**		
320.1	6.519	∞	320.1	3.613	∞
321.6	9.027	∞	321.6	33.849	7.76
HaeIII			**HaeIII**		
63.2	1.007	∞	63.2	0.686	1.870
72.6	0.151	1.403	72.6	0.411	∞
217.5	3.706	∞	217.5	26.651	10.186
MspI			**MspI**		
81.2	1.281	∞	81.2	3.052	∞
275.5	N/A	N/A	275.5	N/A	N/A
483.6	N/A	N/A	483.6	N/A	N/A

Table 13: Comparison of treatment and control samples at cutting sites specific to strains JM63 and JM162a, 2008.

8JM63 vs. 8BC1	*Delta*	*X Increase*	*8JM63 vs. 8BC2*	*Delta*	*X Increase*
BstUI			**BstUI**		
62.5	2.392	22.315	62.5	9.021	8.143
102	1.283	48.818	102	0.139	1.210
222.4	1.626	∞	222.4	0.222	1.271
384.4	4.673	∞	384.4	0.349	1.148
HinfI			**HinfI**		
320.1	6.356	4.363	320.1	6.550	4.767
321.6	6.714	5.634	321.6	8.205	∞
HaeIII			**HaeIII**		
63.2	0.358	1.713	63.2	0.584	1.910
72.6	1.033	4.048	72.6	0.708	3.234
217.5	0.397	1.263	217.5	15.752	12.062
MspI			**MspI**		
81.2	0.419	5.676	81.2	0.480	∞
275.5	N/A	N/A	275.5	N/A	N/A
483.6	N/A	N/A	483.6	N/A	N/A
8JM162 vs. 8BC1	*Delta*	*X Increase*	*8JM162 vs. 8BC2*	*Delta*	*X Increase*
BstUI			**BstUI**		
62.5	0.446	∞	62.5	39.708	253.295
102	0.189	∞	102	3.947	60.107
222.4	0.179	∞	222.4	3.976	63.925
384.4	N/A	N/A	384.4	N/A	N/A
HinfI			**HinfI**		
320.1	8.705	5.183	320.1	8.780	8.780
321.6	5.990	3.555	321.6	6.220	6.220
HaeIII			**HaeIII**		
63.2	0.870	3.007	63.2	0.091	1.088
72.6	0.961	∞	72.6	0.750	∞
217.5	1.976	2.818	217.5	13.392	6.599
MspI			**MspI**		
81.2	1.976	30.324	81.2	2.059	∞
275.5	N/A	N/A	275.5	N/A	N/A
483.6	N/A	N/A	483.6	N/A	N/A

Table 13 (continued).

8Combo vs. 8BC1	*Delta*	*X Increase*	*8Combo vs. 8BC2*	*Delta*	*X Increase*
BstUI			**BstUI**		
62.5	0.524	3.915	62.5	37.067	181.711
102	0.344	3.045	102	2.748	19.395
222.4	0.099	∞	222.4	1.649	57.885
384.4	N/A	N/A	384.4	N/A	N/A
HinfI			**HinfI**		
320.1	4.896	3.504	320.1	5.027	3.828
321.6	7.637	6.094	321.6	7.167	4.760
HaeIII			**HaeIII**		
63.2	0.185	1.720	63.2	0.076	1.277
72.6	0.351	8.919	72.6	0.351	∞
217.5	2.181	2.741	217.5	12.336	5.556
MspI			**MspI**		
81.2	4.872	∞	81.2	5.644	∞
275.5	N/A	N/A	275.5	N/A	N/A
483.6	N/A	N/A	483.6	N/A	N/A

2.3.5 Greenhouse trial data

2.3.5.1 First trial:

The Anderson-Darling test revealed a non-normal distribution of the mean tiller number data ($A^2 = 0.619$, $p = 0.081$) and a significant difference in tiller number was revealed with a Kruskal-Wallis test ($H = 47.80$, $p = 0.000$; see Table 14 and Figure 7a), so two-tailed two-sample t-tests were run (without assuming equal variances) to discover where the differences lay. There were significant differences between treatments and controls and within treatments but no significant differences within controls, with JM63 outperforming all other treatments in regards to this parameter (see Table 14 and Figure 7a). Data for mean time to emergence followed a normal distribution ($A^2 = 0.844$, $p = 0.028$) with approximately equal variances and one-way ANOVA revealed no significant differences in time to emergence between any of the five treatments ($F = 0.12$, $p = 0.974$; see Table 15 and Figure 7b).

The data for mean height also followed a normal distribution ($A^2 = 2.080$, $p = 0.000$), but the variances were unequal so a Kruskal-Wallis test was run and a significant difference was found ($H = 38.65$, $p = 0.000$). Two-tailed two-sample t-tests revealed where the differences were located; there were significant differences between treatments and controls and within treatments but no significant differences within the controls (see Table 16 and Figure 7c). All tests were run at the 5% significance level. The germination rate for plants in the first greenhouse trial was 57 out of 60 seeds or 95%.

Table 14: Mean Tiller Number, First Barley Greenhouse Trial

Sample	Mean Tiller Number	Standard Deviation
JM63	7.833[a]	0.937
JM162a	5.833[b]	1.267
Combo	5.167[b]	0.718
C1	3.000[c]	1.537
C2	2.917[c]	0.996

*Means with differing superscripts are significantly different.

Table 15: Mean Time to Emergence, First Barley Greenhouse Trial

Sample	Mean TTE (days)	Standard Deviation
JM63	5.917	2.610
JM162a	5.333	2.229
Combo	5.500	2.316
C1	5.917	3.288
C2	5.833	2.623

Table 16: Mean Plant Height, First Barley Greenhouse Trial

Sample	Mean Height (cm)	Standard Deviation
JM63	59.22[a]	5.55
JM162a	55.70[a]	5.58
Combo	49.73[b]	4.16
C1	29.77[c]	16.19
C2	38.51[c]	14.68

*Means with differing superscripts are significantly different.

Figure 7: Mean Tiller Number (a), Time to Emergence (b) and Height (c), Greenhouse Barley Trial #1

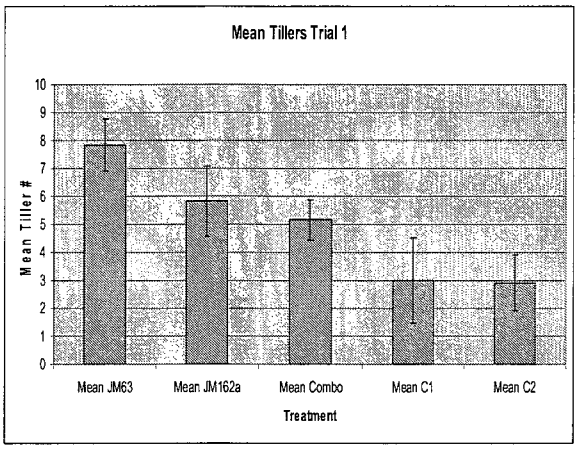

Figure 7(a): Mean tiller numbers for the first barley greenhouse trial. Error bars are ± one standard deviation.

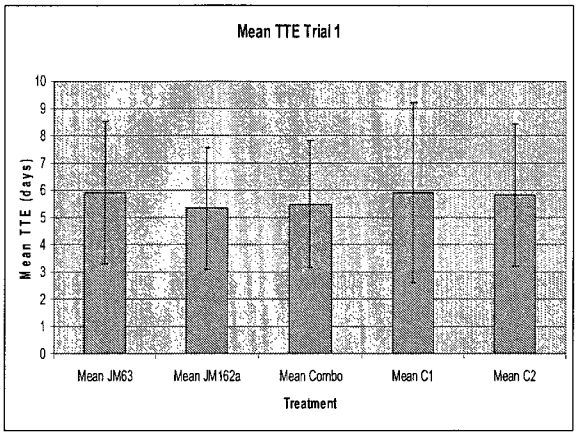

Figure 7(b): Mean time to emergence (days) for the first barley greenhouse trial. Error bars are ± one standard deviation.

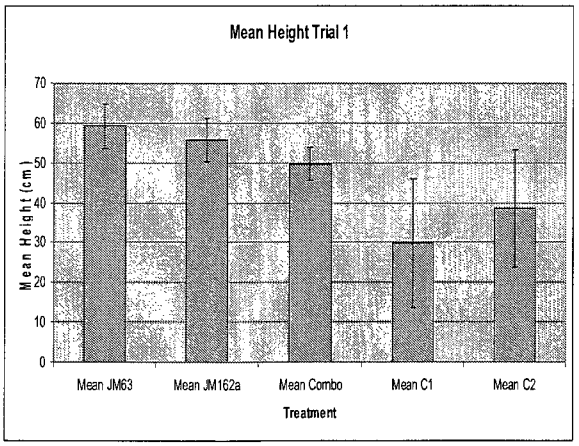

Figure 7(c): Mean height (cm) at end of the first barley greenhouse trial. Error bars are ± one standard deviation.

2.3.5.2 Second trial:

The data for mean tiller number followed a normal distribution and variances were approximately equal ($A^2 = 1.534$, $p = 0.001$); a one-way ANOVA revealed a significant difference in tiller number between treatments ($F = 93.20$, $p = 0.000$), so Tukey's pairwise comparisons with a family error rate of 0.05 were used to see where the differences were located. There were significant differences in tiller number both between treatments and controls and within treatments but no significant difference within the controls (see Table 17 and Figure 8a).

Mean time to emergence data also followed a normal distribution ($A^2 = 1.066$, $p = 0.008$) and variances were approximately equal; one-way ANOVA revealed a significant difference ($F = 3.48$, $p = 0.013$) but Tukey's pairwise comparisons with a family error rate of 0.05 showed only one significant difference, that between JM63 and C2 (see Table 18 and Figure 8b). The mean height data again followed a normal distribution ($A^2 = 1.024$, $p = 0.01$) but the variances were unequal so a Kruskal-Wallis test was performed, revealing a significant difference in mean height ($H = 31.78$, $p = 0.000$, adjusted for ties; see Table 19 and Figure 8c). Two-tailed two-sample t-tests (not assuming equal variances) revealed that there were significant differences in mean height between treatments and controls, except for between Combo and C2; there were no significant differences within treatments except between JM63 and Combo, and there were no significant differences within controls. All tests were run at the 5% significance level; the germination rate for the second barley greenhouse trial was 60 out of 60 seeds germinated or 100%.

Table 17: Mean Tiller Number, Second Barley Greenhouse Trial

Sample	Mean Tiller Number	Standard Deviation
JM63	8.250[a]	0.754
JM162a	7.250[b]	1.055
Combo	5.333[c]	0.778
C1	3.833[d]	0.718
C2	3.000[d]	0.603

*Means with differing superscripts are significantly different.

Table 18: Mean Time to Emergence, Second Barley Greenhouse Trial

Sample	Mean TTE (days)	Standard Deviation
JM63	3.583[a]	1.311
JM162a	4.250	1.960
Combo	4.750	1.960
C1	5.250	1.960
C2	5.750[b]	1.913

*Means with differing superscripts are significantly different.

Table 19: Mean Plant Height, Second Barley Greenhouse Trial

Sample	Mean Height (cm)	Standard Deviation
JM63	60.77[a]	5.74
JM162a	57.13[a]	6.20
Combo	50.43[b]	10.36
C1	39.23[b]	9.43
C2	44.15[c]	10.19

*Means with differing superscripts are significantly different.

Figure 8: Mean Tiller Number (a), Time to Emergence (b) and Height (c), Greenhouse Barley Trial #2

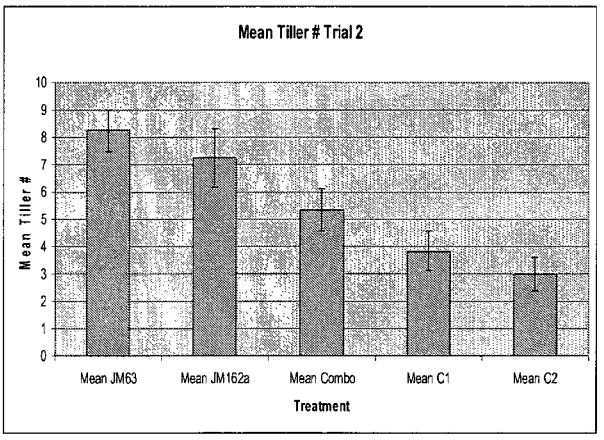

Figure 8(a): Mean tiller numbers for the second greenhouse barley trial. Error bars are ± one standard deviation.

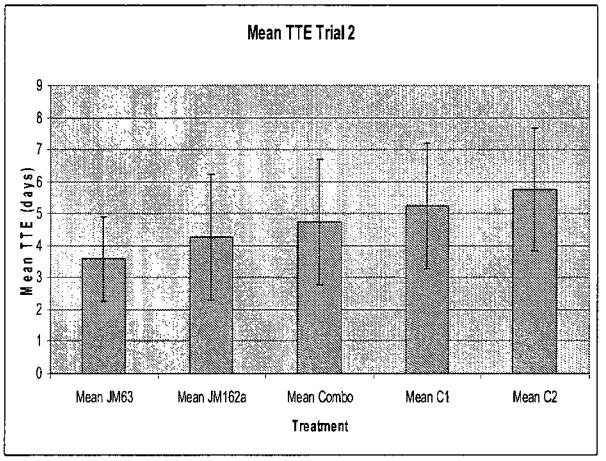

Figure 8(b): Mean time to emergence (days) for the second greenhouse barley trial. Error bars are ± one standard deviation.

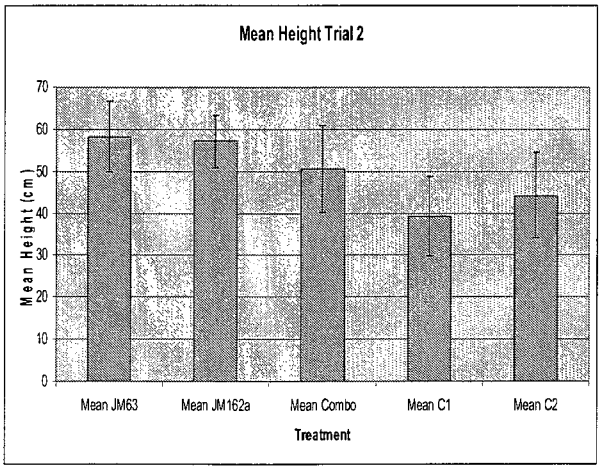

Figure 8(c): Mean height (cm) for the second greenhouse barley trial. Error bars are ± one standard deviation.

2.3.6 Between Trials Data Analysis

2.3.6.1 2007 Barley Field Trial *versus* 2008 Barley Field Trial:

There were insufficient replicates in both trials to successfully execute a one-way ANOVA between both years, so two-sample t-tests (without assuming equal variances) were run instead to compare the two years. There were significant differences between all five treatments between the first and second field trials (see Tables 1, 3 and 20 and Figures 4a, 5a and 9a).

Table 20: Comparison of 2007 and 2008 Mean Barley Yields with Two-Sample T-tests

Sample	T Statistic	P value
JM63	17.13	0.0000
JM162a	11.66	0.0007
Combo	17.27	0.0000
C1	17.58	0.0000
C2	16.37	0.0000

The mean barley yields in 2007 were significantly higher than those observed in 2008; presumably this is due to the poor growth occurring in the second field trial; all tests were run at the 1% significance level to lend increased rigour to the results.

2.3.6.2 2007 Soybean Field Trial *versus* 2008 Soybean Field Trial:

For this between years analysis there were also insufficient replicates to perform ANOVA analysis, so again, two-sample t-tests without assuming equal variances were

used to compare differences in mean soybean yield between 2007 and 2008. There were significant differences in mean soybean yield between the two years, with the mean 2008 soybean yield being much higher than that observed in 2007; the only lack of a significant difference between years was with the 2007 and 2008 C2 samples, though this result would have been considered significant at the 5% significance level. This reflects the patchy germination and growth of soybean in the first field trial (see Tables 5, 6 and 21 and Figures 6a, 6b and 9b). All tests were run at the 1% significance level to lend additional rigour to the results.

Table 21: Comparison of 2007 and 2008 Mean Soybean Yields with Two-Sample T-tests

Sample	T Statistic	P value
JH	-5.90	0.0010
JH47	-9.47	0.0001
532C	-5.95	0.0010
C1	-10.54	0.0001
C2	-2.69	0.027

Figure 9: Mean 2007 and 2008 Barley Yields (a) and Mean 2007 and 2008 Soybean Yields (b)

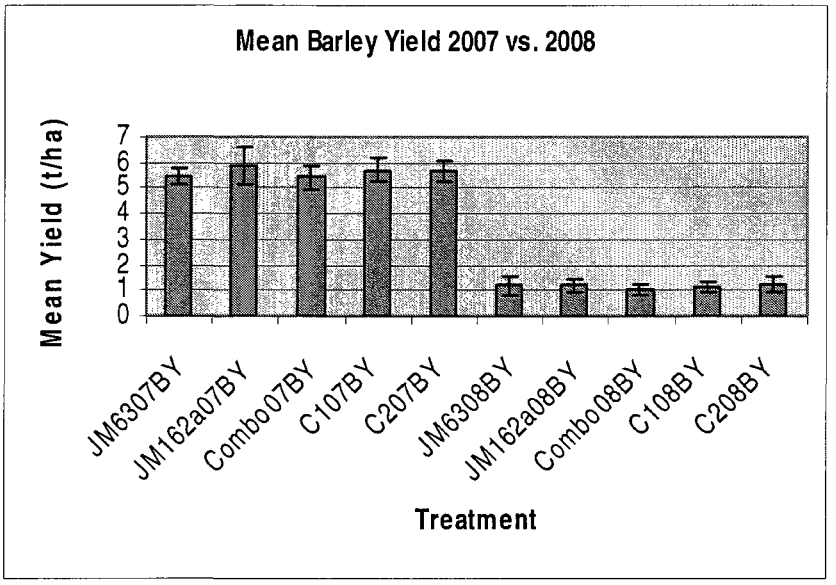

Figure 9(a): Comparison of 2007 and 2008 mean barley yields. Error bars are ± one standard deviation.

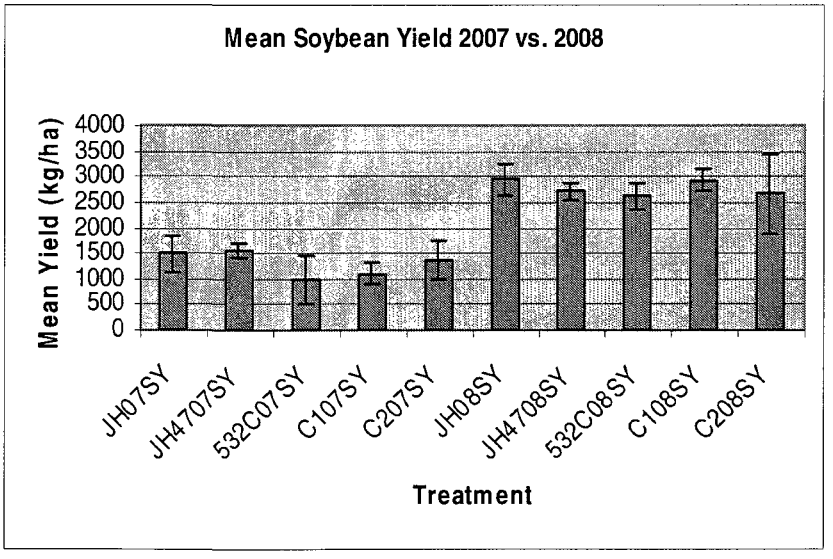

Figure 9(b): Comparison of 2007 and 2008 mean soybean yields. Error bars are ± one standard deviation.

2.3.6.3 Barley Greenhouse Trial 1 *versus* Barley Greenhouse Trial 2:

One-way ANOVA analysis showed little difference between the first and second greenhouse trials for any of the three parameters tested (see Table 22 and Figure 10). The only significant differences observed were mean tiller number for JM162a, where the mean was significantly higher in the second trial than in the first ($F = 8.86$, $p = 0.007$), and mean time to emergence for JM63, where the mean was significantly higher in the first trial than in the second trial ($F = 7.66$, $p = 0.011$). All tests were run at the 5% significance level.

Table 22: Comparison of First and Second Barley Greenhouse Trials with ANOVA

Sample	F Statistic	P value
JM63 Tiller Number	1.144	0.243
JM162a Tiller Number	8.86	0.007
Combo Tiller Number	0.30	0.591
C1 Tiller Number	2.89	0.103
C2 Tiller Number	0.06	0.807
JM63 TTE	7.66	0.011
JM162a TTE	1.60	0.219
Combo TTE	0.73	0.401
C1 TTE	0.36	0.552
C2 TTE	0.01	0.930
JM63 Height	1.19	0.287
JM162a Height	1.42	0.246
Combo Height	0.05	0.828
C1 Height	3.06	0.094
C2 Height	1.20	0.286

Figure 10: Mean Tiller Number, Greenhouse Trials 1 and 2 (a), Mean Time to Emergence, Greenhouse Trials 1 and 2 (b) and Mean Plant Height, Greenhouse Trials 1 and 2 (c)

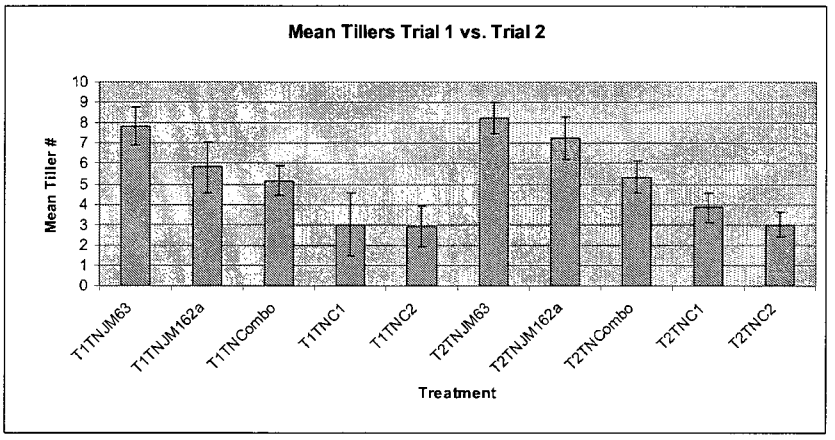

Figure 10(a): Comparison of mean tiller number between greenhouse trials 1 and 2. Error bars are ± one standard deviation.

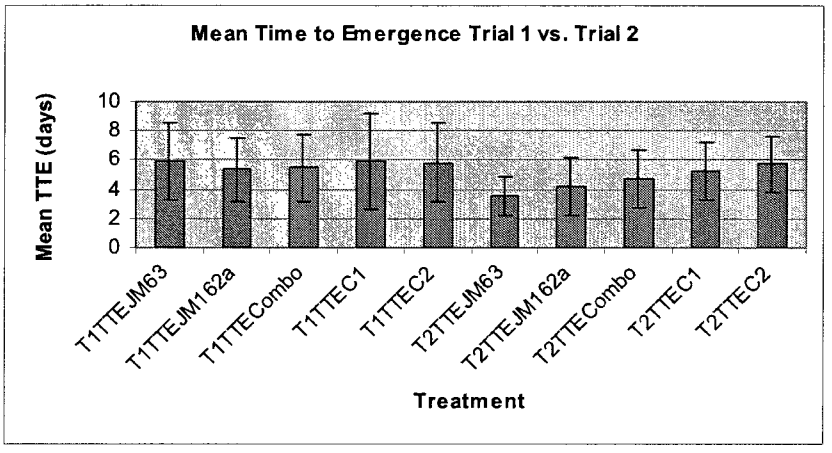

Figure 10(b): Comparison of mean time to emergence between greenhouse trials 1 and 2. Error bars are ± one standard deviation.

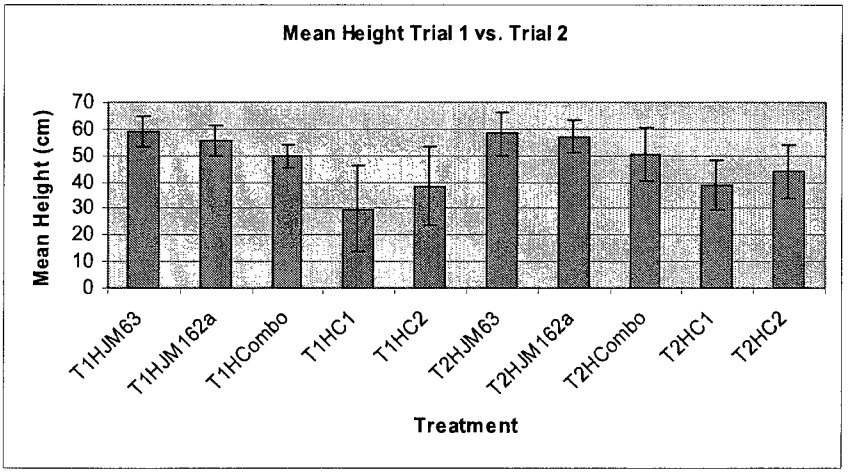

Figure 10(c): Comparison of mean height between greenhouse trials 1 and 2. Error bars are ± one standard deviation.

2.4 Discussion

2.4.1 Barley field trials:

No significant differences in mean barley yield were observed between the five treatments in either 2007 or 2008. In 2007, the plants lodged (tipped over) at least a week before harvesting. This can be a problem with older, taller cultivars such as Chapais (Caldwell, personal communication) and is one reason why China began experimenting with semi-dwarf varieties of rice in the 1950s (Cheng *et al.*, 2007). There were significant differences between treatments and controls in regards to tiller number in the 2007 trial (see Figure 4b and Table 2), but what precisely the relationship is between tillering and grain yield has yet to be clarified; increased tillering is supposed to indicate increased plant growth (Dong, personal communication) but until a strong correlation at least is established between tiller number and grain yield, it would make more sense to focus on number and size of grain heads in subsequent experiments. Regardless, the grain yield results from 2007 (see Figure 4a and Table 1) are suspect due to the lodging that occurred that season.

The mean barley yield results for 2008 are no more reliable than those for 2007 (see Figure 5a and Table 3). The plants suffered poor growth in 2008 due to an unspecified cause. A rotational difficulty or poor seed vigour were suggested as possible causes (MacDonald, personal communication); the former may be a possibility but the latter seems unlikely as the seeds used in the 2008 field trial were the same as those used in both greenhouse trials, where no growth disturbances were observed. The aerial parts of the plant showed no signs of insect or pathogen damage, but the root systems were never looked at; representative sampling from all five treatments should have been

performed to check for signs of root system dysfunction but as this was not done, it is not possible to say whether or not belowground conditions were responsible for the poor growth. A treatment effect was noted in 2008 with respect to grain head production (see Figure 5b and Table 4), with JM63 outperforming all other treatments, but again the results are unreliable. Due to these difficulties, the objective of investigating these strains' efficacy as seed inoculants was not met.

2.4.2 Soybean field trials:

The soybean trials were supposed to repeat the work of Dean and coworkers (2006) but germination and growth in the 2007 soybean field trial were poor due to the procedure used for seed surface sterilization. Using a 10% bleach solution for this purpose is a perfectly valid protocol but it needs to be carried out quickly and with care under controlled laboratory conditions; these seeds were hastily poured into a large, non-graduated bucket filled with water and bleach and agitated. As the bleach was poured by eye, the concentration of the solution could easily have been over or under 10%. The seeds were then left to air dry in non-sterile conditions after being pat down with non-sterile paper towel. Many seeds lost their seed coats and many others simply became waterlogged. At this point, the experiment should have been stopped and planting rescheduled for the following week, which for soybean would not have been a problem; this would have left time to properly prepare the seeds in the lab before planting. Unfortunately, the experiment went forward and the results are not surprising. Thus, this field season was wasted and the soybean planting had to be repeated in 2008 instead of planting barley in the plots as originally planned. No significant differences in mean

yield between the five treatments were observed in 2007 (see Table 5 and Figure 6a) but the data are irrelevant in the face of the plants' failure to thrive.

In 2008, the soybean seeds were surface sterilized in the lab by spraying them with 70% ethanol followed by air drying them in a sterile clean hood. This procedure was followed by inoculation (also carried out in the lab) and the seeds were then stored at 4°C until planting. This soybean trial was far more successful in terms of germination and growth and this is reflected in the increased yields. However, there were again no significant differences in mean yield between the five treatments (see Table 6 and Figure 6b); it was expected that there would be significant differences in yield between treatments and controls and a lack of one suggested the possibility of volunteer rhizobia in the control plots that may have migrated during the course of the previous year's planting. Due to this possibility, no barley could be planted here the following year and the experiment was abandoned; as the experiment could not go forward, the presence of volunteer rhizobia was not actually verified. Thus, the objective of comparing crop rotation and non-legume seed inoculation with H_2-oxidizing bacterial strains was also not met.

2.4.3 Sequencing of re-isolated strains:

The lowest percentage match observed in the DNA sequencing data is that for the 2007 Combo soil re-isolation, which showed an 81% similarity to strain JM63 (Accession DQ256487 in GenBank; see Table 7). The highest similarity seen was with the 2008 JM162a soil re-isolation, showing a 98% similarity with strain JM162a (Accession DQ256490 in GenBank; see Table 7). None of the samples gave a 100% similarity result, although sequences from both the forward and reverse primer samples

were exactly matched; this may be due to biases or artifacts from the PCR and similarities may well have reached 100% (or at least higher levels than seen here) if the isolates had been sequenced following cloning procedures instead of being sequenced directly from PCR samples.

The control samples matched three previously uncultured strains: a rape rhizosphere bacterium, an unidentified bacterium and a *Nevskia* species clone, as well as a *Mycobacterium* species (see Table 7). These previously uncultured species have now been successfully cultured on MSA plates in a 100% H_2 environment. What remains is to identify and characterize these isolates and to test them for plant growth promoting properties, probably through root elongation experiments as performed with previously isolated strains, including the two used in the present study (Maimaiti *et al.*, 2007) and, if they do show beneficial effects on plant growth, to determine the mechanism whereby they exert these effects, as has also been done previously (Zhang, 2006; Maimaiti *et al.*, 2007).

2.4.4 TRFLP analyses:

As stated previously, molecular work was carried out with strain JM120, a *Burkholderia* species strain used in the 2006 barley field trial, but results were not reported here as this strain was not used in subsequent field trials as some strains in this genus are human pathogens. The overall profile areas of treatment samples were larger than in control samples (see Tables 8, 9 and 10), probably due to the artificially created overabundance of strains introduced to the soil from the seed inoculation; indeed, the inoculants were designed to achieve just this effect through outcompeting other, indigenous, strains. Most important were the differences in peak intensity (height) and

area at the expected cutting sites for strains JM63 and JM162a; peaks for treatment samples at these sites were always larger than in control samples and in many cases, no peak was observed in control samples at these specific sites (see Tables 11, 12 and 13). These results support the findings of Zhang (2006), whose work showed these peaks corresponding to these two strains after spiking soil samples in the lab with both species, a process analogous to inoculating seeds and then planting them in soil. For all three years' worth of TRFLP data, only three profiles showed control samples with larger overall area percentages than treatment samples: 7Combo cut with MspI *versus* 7BC1 cut with MspI; 8Combo cut with BstUI *versus* 8BC1 cut with BstUI and 8Combo cut with MspI *versus* 8BC2 cut with MspI (see Tables 8, 9 and 10). These results involve two different control samples (BC1 and BC2) cut with two different restriction enzymes (BstUI and MspI), but what all three have in common is that they all occur in samples where the two strains were combined, suggesting the strains may have competed with each other, reducing each other's abundance and allowing other species to show more growth in the soil. However, even in these profiles, results at expected cutting sites for each enzyme, specific to each strain, showed either smaller peaks in control samples compared to treatment samples or else no peak at that cutting site was observed in the control sample (see Tables 11, 12 and 13). These results strengthen the argument for using TRFLP to both compare whole bacterial assemblages in different environmental samples, as was done in Zhang (2006), and to pinpoint already known isolates within a single bacterial community, as has been done here.

2.4.5 Greenhouse barley trials:

The greenhouse trials with barley were run as a support for the field trials and again tiller number was used as an indication of improved plant growth, though plant height is probably a more useful tool for gauging plant growth. What should have been used, along with plant height and time to emergence, was root length, which is strongly correlated plant growth; it was root elongation that was initially used to test the plant growth promoting effects of both JM63 and JM162a and should have replaced tiller number as a test parameter. However, this was not the case and significant differences were observed between treatments and controls in regards to both tiller number and height, indicating a treatment effect. In the first trial, there were also significant differences observed between JM63 and JM162a with respect to both tiller number and height, though not for time to emergence (see Tables 14, 15 and 16 and Figure 7a, b and c). However, in the second trial, these two strains only differed in regards to tiller number (see Tables 17, 18 and 19 and Figure 8a, b and c). There were no significant differences between the two controls for any of the three parameters tested. Although there were no problems with germination or growth in the greenhouse trials, using tiller number as a test parameter makes the results problematic. Admittedly, in greenhouse trials, plants cannot always be grown to full maturity so testing grain head production is not necessarily as feasible as it is out in the field, but using height and root length, as opposed to tiller number, is advised for further repetition of these experiments.

2.4.6 Between trials data analysis:

Significant differences were noted between the first and second barley field trials, with the mean yields in the first trial significantly higher than those seen in the second trial; this reflects the poor barley growth in 2008 (see Table 20 and Figure 9a). The opposite was seen in the analysis of the 2007 and 2008 soybean trials, with mean 2008 soybean yields being much higher than those in 2007; like the barley, this is due to the poor germination and growth of soybean in the first field trial (see Table 21 and Figure 9b).

The only significant differences noted between the first and second greenhouse barley trials were in tiller number for JM162a, where mean tiller number was less in the first trial than in the second (see Tables 14 and 17 and Figure 10a), and in mean time to emergence for JM63, where seeds emerged more quickly in the second trial than in the first for this treatment (see Tables 15 and 18 and Figure 10b). Both of these differences may be due to lower temperatures in the greenhouse during the first trial, which ran from late December, 2008 to late February, 2009; temperatures in the greenhouse increased over the course of the second trial, which ran from late February to late April, 2009. However, as these were the only two significant differences observed between both trials for all three parameters tested, natural variation still seems a more plausible explanation. No significant differences were observed between trials in regards to plant height at harvesting with either the treatments or the controls (see Tables 16 and 19 and Figure 10c).

3.0 Conclusions

The soybean experiment was unsuccessful in that there was no opportunity to grow a nonlegume species in the original soybean plots due to poor germination and growth in the first soybean trial; the lack of any differences in yield between any of the five treatments in the second trial suggested the presence of volunteer rhizobia left in the plots after the first trial. It is believed to be unlikely that the 532C, a commercial inoculant, worked poorly in nodulating soybean; thus, if there were no volunteer rhizobia left in the plots than differences in yield between treatments and controls should have been observed. With this in mind, any thought of planting a nonlegume species in these plots was abandoned as results from such an experiment would not have been reliable under these circumstances. As this objective was not met in the present study, this experiment should be attempted again to see if planting nonlegume species in soil previously planted with soybean harbouring HUP^- rhizobia gives similar results to inoculating nonlegume seeds with H_2-oxidizing bacteria.

The main focus of the present study was to test the efficacy of two H_2-oxidizing strains as inoculants for promoting the growth of a nonlegume crop, in this case barley. The field trials presented here are both problematic in terms of lodging in the first season and poor growth in the second. They are also problematic, as are the greenhouse trials, in using tiller number as a test parameter. For subsequent field trials, the author suggests using size and number of grain heads, as well as plant height and root length at maturity, as test parameters; these would all have to be performed using representative sampling of plants from all five treatments prior to harvesting. However, the molecular work shows that the strains used in this study can survive and reproduce in peat as a standard carrier,

so if they can be shown to increase yields, they will be ideal as seed inoculants from this perspective. New strains isolated from control soil samples can also now be cultured and sent for sequencing for identification and experiments can be run to check for potential PGPR properties in these strains. This study also served to support the results of Zhang (2006), which showed that TRFLP is a powerful molecular tool that can be reliably used to compare soil bacterial communities from different samples and to pinpoint isolates used in experiments such as the barley field experiments.

The present study derives its objectives from the goals of sustainable agriculture and a better understanding of soil microbiology, plant-microbe and plant-soil interactions will all work towards the creation of healthier, more productive agroecosystems with environmental and economic benefits for everyone. The development of seed inoculants for nonleguminous crop species may lessen the need for chemical fertilizer inputs, thereby minimizing environmental damage and mitigating climate change by the consequent drop in fossil fuel use required for fertilizer production.

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APPENDIX A

Representative TRFLP Profiles of Treatments and Controls Cut with All Four Restriction Enzymes

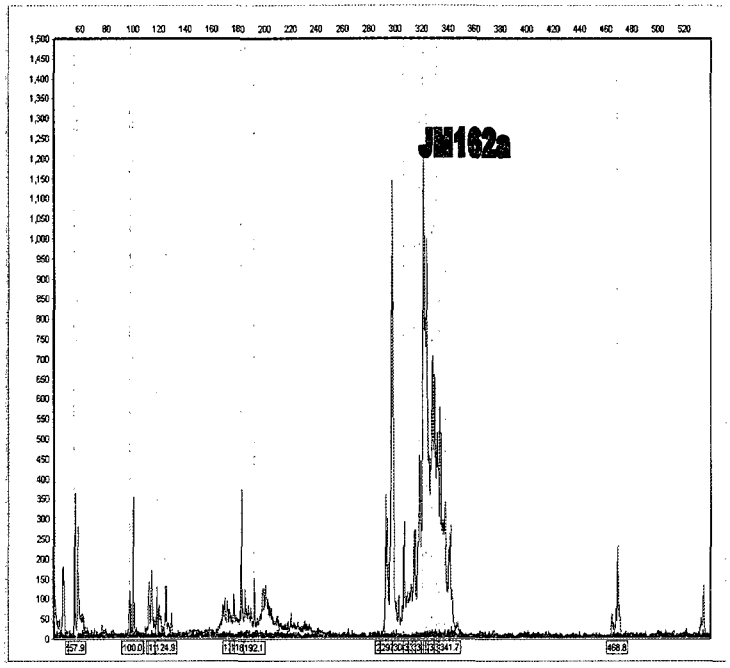


Figure 11a: TRFLP, JM162a from 2007, cut with HinfI

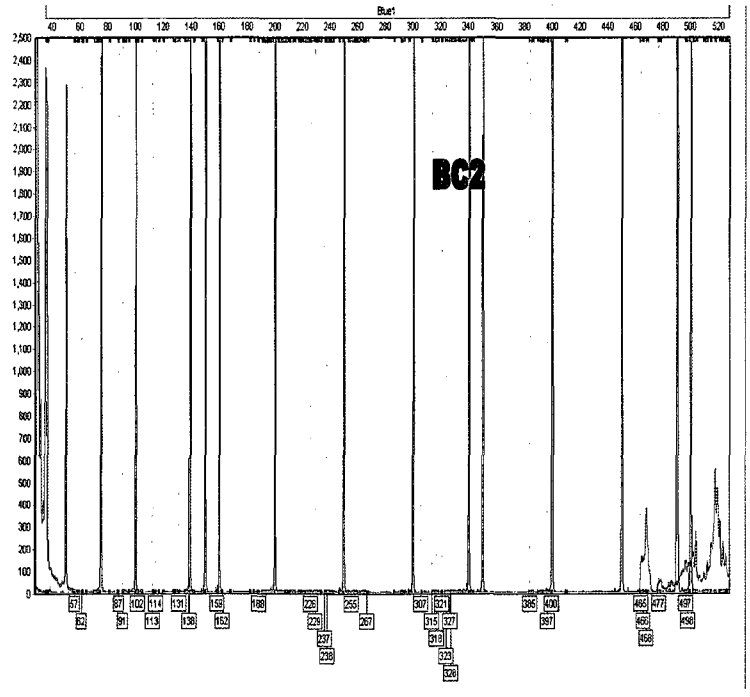


Figure 11b: TRFLP, BC2 (control 2) from 2007, cut with HinfI

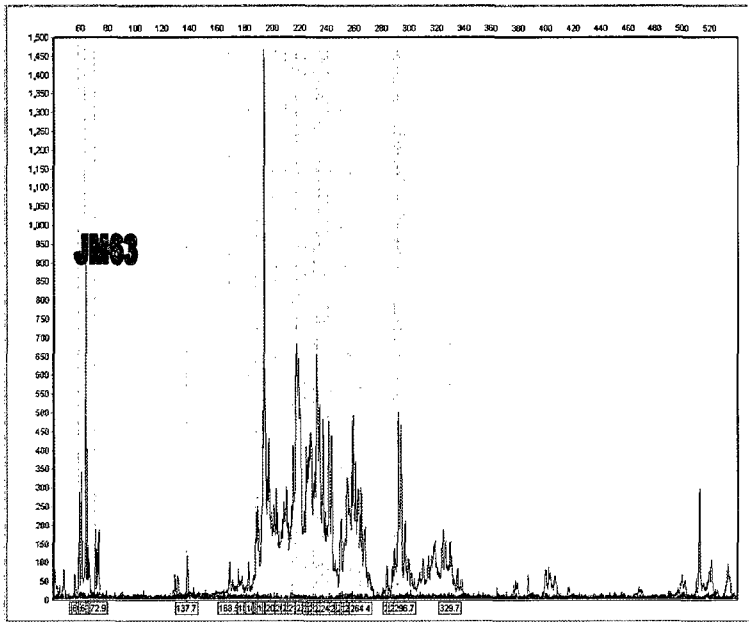


Figure 12a: TRFLP, JM63 from 2007, cut with HaeIII

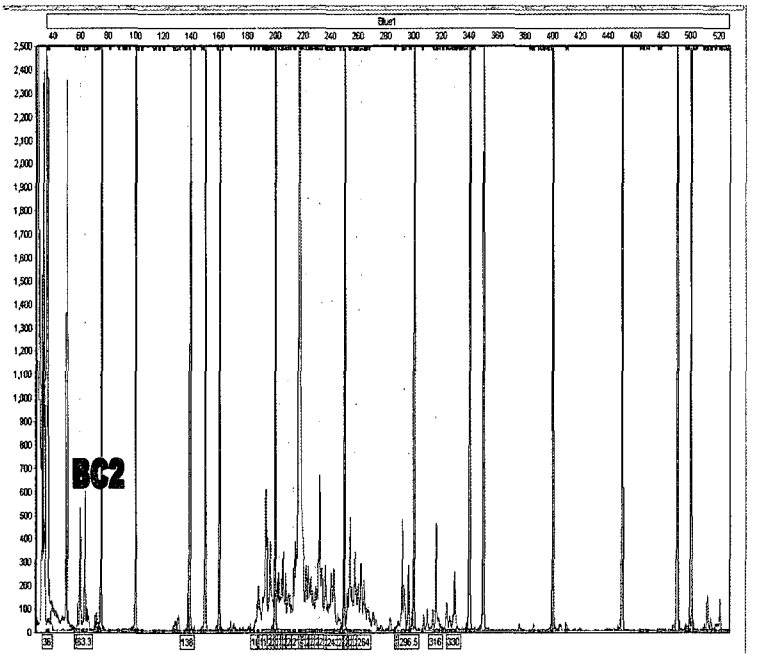


Figure 12b: TRFLP, BC2 (control 2) from 2007, cut with HaeIII

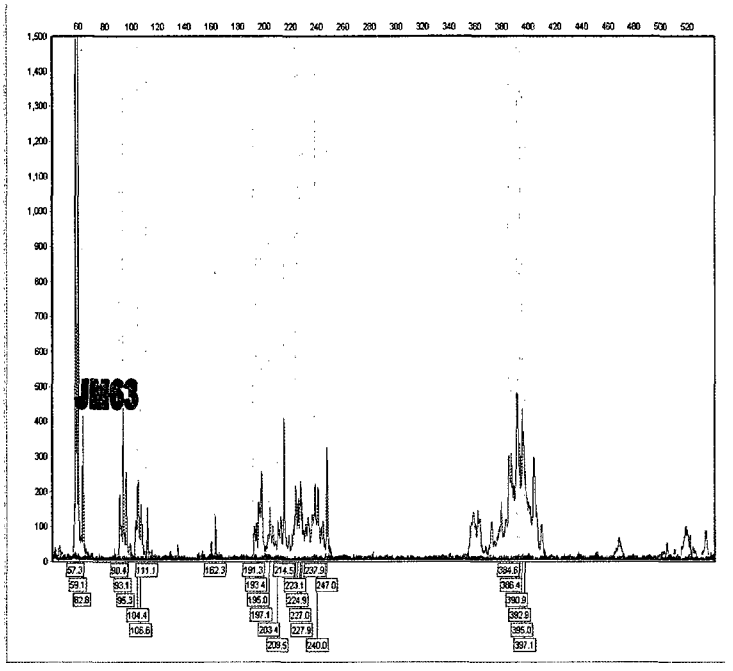


Figure 13a: TRFLP, JM63 from 2006, cut with BstUI

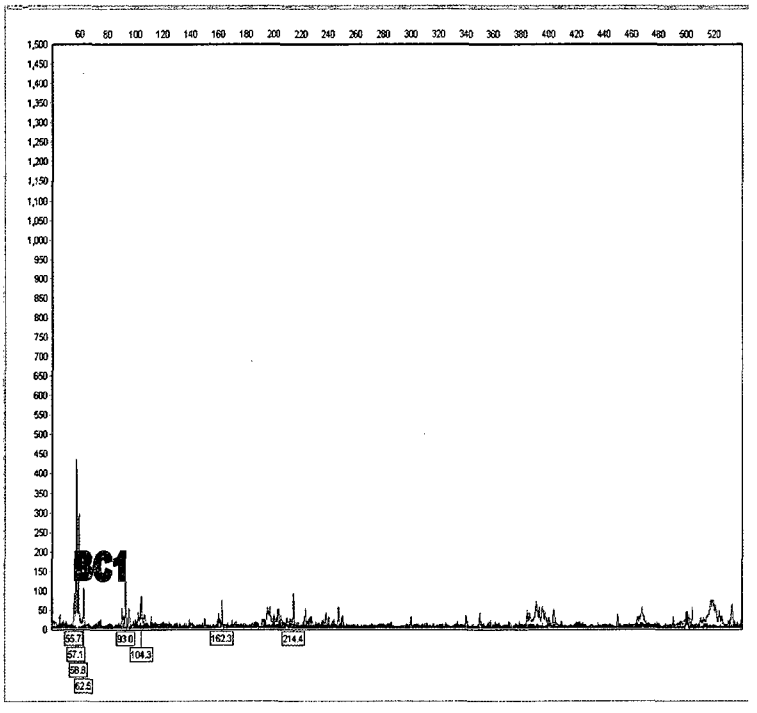


Figure 13b: TRFLP, BC1 (control 1) from 2006, cut with BstUI

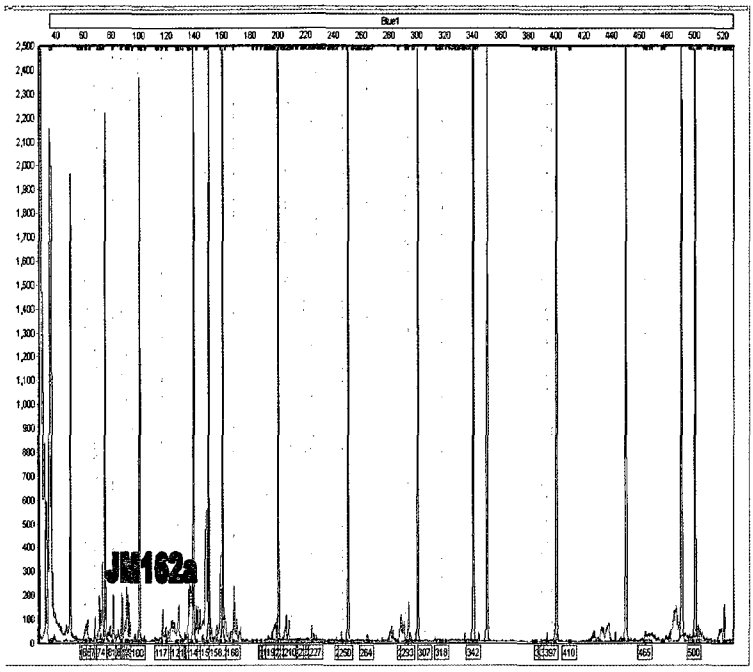


Figure 14a: TRFLP, JM162a from 2008, cut with MspI

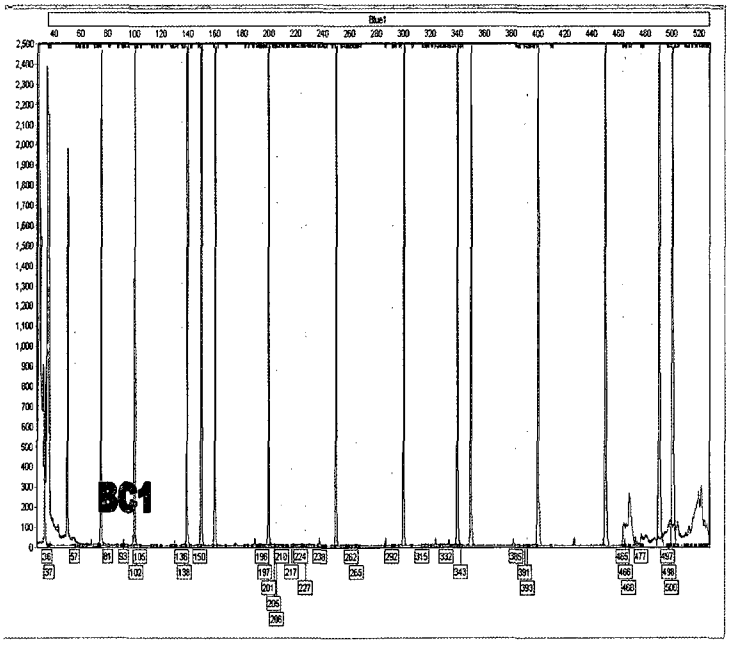


Figure 14b: TRFLP, BC1 (control 1) from 2008, cut with Msp

Appendix B

Selection of HUP status in legume-rhizobia symbioses

1.0 Introduction

Associations between plants and nitrogen-fixing microorganisms are the most intensively studied because nitrogen is the nutrient most likely to become rate-limiting to plant growth under standard agricultural conditions (Postgate, 1998) and the legume-rhizobia symbiosis is the best characterized of these associations (Welbaum *et al.*, 2004; Gray and Smith, 2005). However, even with the close attention paid to this long-utilized symbiotic system (Bullock, 1992), aspects of this crucial plant-microbe interaction remain unclear. One such facet is the uptake hydrogenase (HUP) status of individual symbioses between different legume species and their respective rhizobial partners.

It has been known for some time that hydrogen gas is an energy-rich, obligate byproduct of biological nitrogen fixation (Schubert and Evans, 1976), with the nitrogen fixation being carried out *via* the enzyme nitrogenase, found in all nitrogen-fixing prokaryotes, including rhizobia (Postgate, 1998). Some rhizobia possess genes coding for an uptake hydrogenase or 'HUP' enzyme that allows produced hydrogen to be recycled, saving the plant energy and increasing the efficiency of nitrogen fixation; this is believed to result in higher plant yields (Dixon, 1972; Schubert and Evans, 1976; van Berkum *et al.*, 1994; Baginsky *et al.*, 2005) and the associations formed between these rhizobia and their host plants are termed HUP^+ symbioses. However, many other rhizobia lack the necessary genes or gene expression (Bedmar *et al*, 1983; Murillo *et al.*, 1989) for such a functional hydrogenase enzyme, for instance those associated with clovers and alfalfa (Ruiz-Argüeso *et al.*, 1979). Consequently, hydrogen is released from these legume root nodules and diffuses into the soil; such symbioses are termed HUP^-. The energy costs of this hydrogen release to individual plants are significant. The CO_2

evolution from nodules uses up approximately 20% to 25% of a plant's net photosynthesis (Layzell *et al.*, 1979) and 70% of this is utilized by the nitrogenase enzyme (Layzell *et al.*, 1988). As the production of H_2 is responsible for about 33% of the electron flow through nitrogenase, this process alone consumes 5% to 6% of an individual legume plant's net photosynthesis (Hunt and Layzell, 1993; Dong and Layzell, 2001). It has been proposed that the loss of energy represented by the release of hydrogen from root nodules harbouring HUP^- rhizobial strains is offset by the positive effects of hydrogen on soil chemistry (Dong and Layzell, 2001) and the stimulation of hydrogen-oxidizing plant growth promoting rhizobacteria (PGPR) in the plant rhizosphere (Dong *et al.*, 2003); these benefits are discussed in more detail below, and they certainly help elucidate why such symbioses persist, both in nature and under cultivation. The question, then, is why some plants are selected for a HUP^- association while a HUP^+ status is selected in many other symbioses; one hypothesis is that root morphology and plant life cycle are somehow involved.

Much of the work to date examining the HUP status of legume-rhizobia symbioses has concentrated on cultivated legumes important in agriculture. This study instead focused on wild legumes, with the hypothesis being that legumes requiring fresh root growth on a regular basis (annuals/biennials) will engage in HUP^- symbioses due to their beneficial effects on nodulation and root elongation, whereas legumes with more permanent root systems (perennials) are more likely to engage in HUP^+ associations, where energy recycling is more of an advantage to the plant. To investigate the role of root morphology and plant life cycle in determining HUP status in symbioses, a field survey of wild legumes in Nova Scotia, Canada was undertaken whereby wild plants

were collected from various locales in the province and their nodules tested for hydrogenase activity. This field work is supplemented by a literature review of HUP status in cultivated legumes and other nitrogen-fixing microorganisms, as well as a look at the fate and effects of hydrogen in soil and H_2-oxidizing PGPR.

1.1 Materials and Methods

1.1.1 Plant Material Collection:

Overall, thirteen species of wild legumes were collected from various locales within Nova Scotia at a sampling rate of between five and ten plants per species (see Table 1). Plants were carefully removed from their substrates with a trowel digging to a depth of approximately 2 to 5 inches (5 to 12.5 cm) with the soil kept attached to the roots; this was done to ensure that root nodules remained intact. Plants were transported back to the lab in plastic bags and root nodules were immediately removed from the plants, washed and tested for their HUP status.

1.1.2 Methylene Blue Reduction Assay:

All nodules were thoroughly washed in H_2O in labeled petri dishes to remove soil particles and superfluous plant matter. Washed nodules were then placed in small labeled seed germination plates with filter paper that had been soaked with Methylene Blue reduction dye (2 mL 200mM iodoacetic acid; 2 mL 200mM malonic acid; 0.01 mL 2.5 mM $MgCl_2*6H_2O$; 1.74 mL 50 mM K_2HPO_4; 0.20 mL 10 mM Methylene Blue dye, and KOH to a pH of 5.6 (Zhang, 2006; Lambert *et al.*, 1985)). Between five and ten nodules from each plant species were spread out on each of the plates, crushed and left to incubate in air for 15 minutes. Afterwards, they were placed in 100% H_2 gas overnight at

room temperature (18-20°C). Plates were removed the next morning and photographed with a Canon PowerShot S21S digital camera. HUP status was determined by the presence or absence of colourless zones around each nodule, with presence representing HUP^+ and absence indicating HUP^- (see Figure 1).

1.2 Results

Of all the wild legumes tested, along with a sample of cultivated alfalfa from the Nova Scotia Agricultural College (NSAC) in Truro, Nova Scotia, only *Securigera varia* tested positive for hydrogen uptake activity in whole nodule preparations (see Figure 1 and Table 1). These field data, which also reveal the HUP^- status of all clovers tested, are in agreement with the results from the literature (emphasizing cultivated legumes), which show *Vicia* species, *Medicago sativa* and *Trifolium repens* all harbouring HUP^- rhizobia (see Table 2).

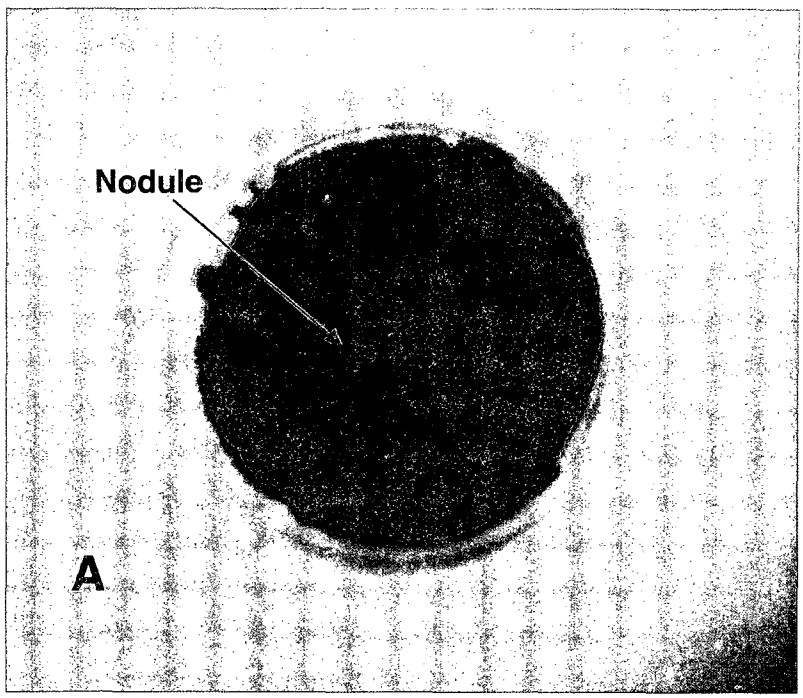
As for root morphology, all the clovers tested exhibited delicate root structures, especially the lateral roots, even the perennial *T. repens*; however, clovers are propagated through seed and stoloniferous growth, not *via* their root stocks (USDA NRCS, 2008). Both *Lupinus* species (*L. polyphyllus* and *L. nootkatensis*) showed more robust root growth, as is expected from perennials, but they die back to thick rhizomes in the fall and are largely propagated by seed (USDA NRCS, 2008). *Medicago sativa* showed a very deep taproot, but it too propagates by seed and actually shows high seed abundance, as does *Lotus corniculatus*, a prodigious seed producer and another perennial with a heavy root system (USDA NRCS, 2008). The roots of the groundnut, *Apios americana* Medic, did not appear especially robust; this plant is also a perennial, but it spreads *via* tubers, not root stocks (USDA NRCS, 2008). The vetches *Vicia sepium* and *Vicia cracca*

exhibited strong taproots and very branched lateral roots, but the lateral roots were fine in structure and did not look as though they would persist well over winter; this is consistent with the literature, which states that despite low seed abundance, they are mostly seed propagated (USDA NRCS, 2008). Both *Lathyrus maritimus* Bigelow and *Securigera varia* exhibited very robust root morphology, with very heavy, deep taproots and thick lateral roots, but again, only *S. varia* showed a positive result for a functional hydrogenase.

Table 1: HUP Status of Wild NS Legumes

Genus	Species	Common Name	HUP Status	Location
Trifolium	*T. repens*	White clover	(–)	Halifax, NS
	T. repens	White clover	(–)	Marie Joseph, NS
	T. pratense	Red clover	(–)	Halifax, NS
	T. pratense	Red clover	(–)	Marie Joseph, NS
	T. pratense	Red clover	(–)	Dartmouth, NS
	T. campestre Schreber	Low hop clover	(–)	Halifax, NS
	T. arvense	Rabbit foot clover	(–)	Halifax, NS
Lupinus	*L. polyphyllus* Lindl	Garden lupine	(–)	Halifax, NS
	L. nootkatensis	Lupine	(–)	Newport, NS
Medicago	*M. sativa*	Alfalfa/Lucerne	(–)	Truro, NS
Apios	*A. americana* Medic.	Ground-nut	(–)	Fancy Lake, NS
Lotus	*L. corniculatus* L.	Birds foot-trefoil	(–)	Halifax, NS
Lathyrus	*L. maritimus* (L.) Bigelow	Beach Pea	(–)	Little Tancook, NS
Vicia	*V. cracca*	Tufted vetch	(–)	Halifax, NS
	V. cracca	Tufted vetch	(–)	Newport, NS
	V. sepium	Hedge vetch	(–)	Dartmouth, NS
	V. sepium	Hedge vetch	(–)	Marie Joseph, NS
Securigera	*S. varia*	Crown vetch	(+)	Halifax, NS

Figure 1: HUP$^+$ nodules of *Securigera varia* (A) and HUP$^-$ nodules of *Trifolium pratense* (B)


Nodule
A

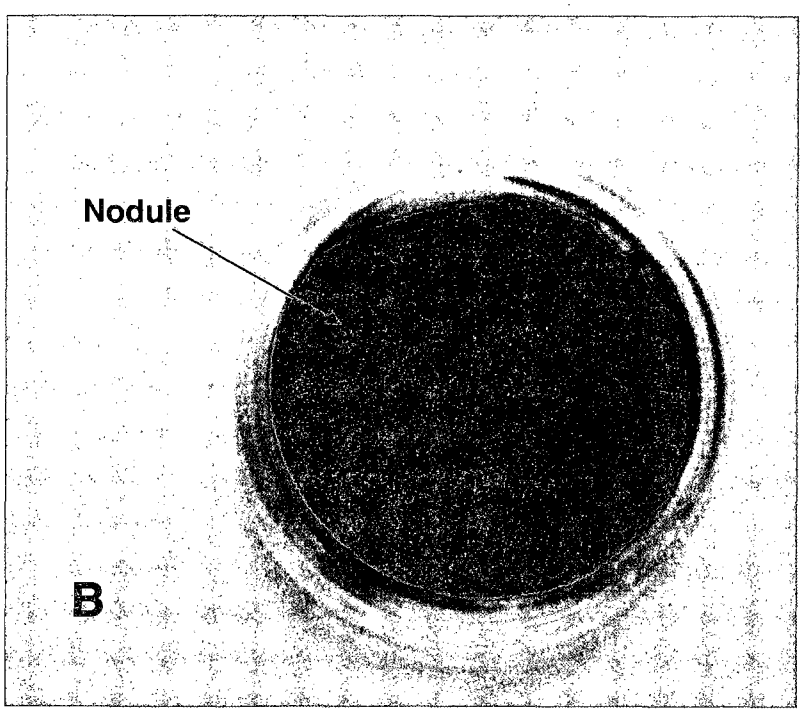

Nodule
B

Table 2: Results from the Literature

Species (Plant/Bacteria)	HUP Status	*Reference
Vicia faba (*Rhizobium* sp.)	–	Dixon, 1972
Vicia bengalensis (*Rhizobium* sp.)	–	Dixon, 1972
Pisum sativum (*Rhizobium leguminosarum*)	+/–[a]	Bedmar et al., 1983; Bedmar and Phillips, 1984 (Begum et al., 2001)
Medicago sativa (*Rhizobium meliloti*)	–	Ruiz-Argüeso et al., 1979 (Bromfield, 1984)
Trifolium repens (*Rhizobium leguminosarum* bv. *Trifolii*)	–	Ruiz-Argüeso et al., 1979 (Badenoch-Jones et al., 1985)
Glycine max (*Bradyrhizobium japonicum*)	+/–[b]	Uratsu et al., 1982
Vigna unguiculata (*Rhizobium* sp.)	+/–[c]	Schubert et al., 1977, 1978; LaFavre and Focht, 1983 (Zablotowicz and Focht, 1981)
Phaseolus vulgaris (*R. leguminosarum*, *R. etli* and *R. tropici*)	+/-[d]	Navarro et al., 1993 (van Berkum et al., 1996)
Lupinus spp. (*Rhizobium lupini*)	+/-[e]	Murillo et al., 1989 (Werner and Oberlies, 1975; Einarsson et al., 1993)
Frankia spp.	+	Mohapatra et al., 2006; Sellstedt and Lindblad, 1990
Cyanobacteria spp.	+	Bothe et al., 1977; Tamagnini et al., 2002
Azospirillum brasilense	+	Berlier and Lespinat, 1980.
Azotobacter vinelandii	+	Hyndman et al., 1953.
Free-living diazotrophs	+	Ackrell et al., 1966.

a 33% *Pisum sativum* symbioses are HUP^+
b 25% *Glycine max* symbioses are HUP^+
c 78% *Vigna unguiculata* symbioses are HUP^+
d 43% *Phaseolus vulgaris* symbioses are HUP^+
e 40% *Lupinus* spp. symbioses are HUP^+
* References in brackets state the rhizobial species infecting each host plant species; where there is no bracket the listed reference indicates the species.

1.3 Discussion

1.3.1 Functions of hydrogenases:

Many species of bacteria possess hydrogenase enzymes, including *Escherichia coli*, various *Clostridia* species (Ackrell *et al.*, 1966) and, of course, nitrogen fixers such as the actinorhizal *Frankia* species, cyanobacteria and rhizobia (see Table 2). In non-diazotrophic prokaryotes, hydrogenases may allow for chemolithotrophic growth with hydrogen as the sole energy source (Mohapatra *et al.*, 2006). In nitrogen fixers, the proposed functions of an active hydrogenase include recycling hydrogen for ATP production to support nitrogen fixation, protection of nitrogenase from damaging O_2 concentrations and/or protection of nitrogenase from H_2 levels that may inhibit nitrogen fixation (Benson *et al.*, 1980).

For the free-living HUP^+ cyanobacteria, many of which are found in aquatic or extreme environments (Tamagnini *et al.*, 2002), it is believed the primary function of the hydrogenase enzyme is to recycle the hydrogen produced during nitrogen fixation as an energy-saving mechanism (Bothe *et al.*, 1977). In *Alnus-Frankia* symbioses, a HUP^+ phenotype also appears to be the norm (Sellstedt and Lindblad, 1990), quite possibly for the same reason. In rhizobia, the situation is more complex. For instance, up to 75% of commercially grown soybean in the United States harbour *Bradyrhizobium japonicum* strains that exhibit the HUP^- phenotype, whereas only 25% are HUP^+ (Uratsu *et al.*, 1982). As stated above, many rhizobial strains are deficient in hydrogenase genes, and others may not express the genes they do possess (see Table 2); a thorough understanding of symbiotic HUP status, in both natural and agricultural ecosystems, is a worthy target of more concentrated research efforts.

1.3.2 Fate and effects of hydrogen in soil:

The loss of hydrogen to the soil from legume root nodules lacking a (functional) hydrogenase has traditionally been viewed as a disadvantage of HUP^- symbioses relative to their HUP^+ counterparts (Schubert and Evans, 1976) and the HUP^+ associations are still often presented as more ideal for agricultural purposes as they may increase the nitrogen content of the host plant (Baginsky *et al.*, 2005). This perspective was partially based on the assumption that once hydrogen is released to the soil, it, like other gases such as CO_2 and N_2O, is emitted into the atmosphere. It was subsequently discovered that the hydrogen never actually leaves the plant-soil system (Conrad and Seiler, 1979) and that it is consumed within 1 to 4 cm of the originating root nodules (LaFavre and Focht, 1983).

Further research has investigated the fate of hydrogen in soil. Dong and Layzell (2001) discovered that in plant-free, hydrogen-treated soils, approximately 60% of the hydrogen reacts with O_2. The other 40% of hydrogen electrons are used to reduce CO_2 into organic material, thereby offering a potentially significant mechanism for promoting soil carbon sequestration (Dong and Layzell, 2001). For a graphical representation of the distribution of the reducing power of hydrogen in soil, please see Figure 2.

Subsequent work has determined that the uptake mechanism of hydrogen in soil is bacterial in nature (McLearn and Dong, 2002). It has lately been shown that the bacteria responsible for this uptake are various species of H_2-oxidizing bacteria, many of which have exhibited plant growth promoting properties. These potential PGPR may represent yet a further benefit of hydrogen release from HUP^- nodules (Dong *et al.*, 2003), and a few species have been successfully isolated in the laboratory (Maimaiti *et al.*, 2007).

1.3.3 Mechanisms of H_2-oxidizing PGPR:

It has been suggested that only 25% of the observed legume rotation benefit can be accounted for by nitrogen inputs to the soil (Bullock, 1992). The plant growth promoting activities of the aforementioned H_2-oxidizers may help to account for at least part of the remaining 75% (Dong *et al.*, 2003). As mentioned previously, some H_2-oxidizers have been isolated, characterized and identified and their mechanisms of plant growth promotion investigated. Maimaiti and co-workers (2007) and Zhang and co-workers (2009) discovered the presence of 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity in *Variovorax paradoxus* and *Flavobacterium johnsoniae*, and rhizobitoxine activity in *Burkholderia* spp.; both ACC deaminase and rhizobitoxine interfere with the biosynthetic pathway of the phytohormone ethylene, an important hormone involved in many aspects of plant development (Taiz and Zeiger, 1991). Ethylene inhibition promotes nodulation in most legumes (Hunter, 1993) and increases root elongation in nonleguminous plants (Maimaiti *et al.*, 2007); increased root elongation gives plants better access to soil nutrients (Shah *et al.*, 1998), and increased nodulation leads to increased nitrogen fixation in legumes, both of which promote plant growth. ACC deaminase is an enzyme that cleaves ACC, a precursor of ethylene, into α-ketobutyrate, disrupting ethylene biosynthesis (Hontzeas *et al.*, 2006). Rhizobitoxine, a chemical inhibitor, blocks the action of ACC synthase, thus interrupting ethylene synthesis one step earlier in the pathway than ACC deaminase (Sugawara *et al.*, 2006).

1.3.4 H_2-oxidizing PGPR and plant life cycles:

Investigations into HUP^- legume-rhizobia symbioses have shown that these symbioses release hydrogen to the soil, changing the soil microbial population and stimulating the growth of H_2-oxidizing PGPR (Zhang *et al.*, 2009). As stated above, some of these PGPR have been successfully isolated in the lab and have been shown to possess ACC deaminase or rhizobitoxine activity (Maimaiti *et al.*, 2007). ACC deaminase and rhizobitoxine both inhibit ethylene synthesis in plants, resulting in increased root elongation and quicker root growth (Zhang *et al.*, 2009; Maimaiti *et al.*, 2007), as discussed in detail above. The point here is that those plants needing to establish root systems early in the season may find it more conducive to select for rhizobial symbionts lacking a hydrogenase in order to take advantage of the benefits of hydrogen release into the plant rhizosphere. This may explain why some plants select rhizobia that do not possess genes for hydrogenases in the first place, and why other plants may affect their rhizobial partners in such a way as to inhibit expression of such genes when rhizobia do possess them. This survey of wild legumes showed that many uncultivated legumes also engage in HUP^- symbioses despite many of them being perennials. In the course of the abovementioned survey, not even the very hearty perennial Beach Pea (*Lathyrus maritimus* Bigelow) showed a positive result for hydrogenase activity in its root nodules; the only legume tested that did show a HUP^+ symbiosis with its rhizobial partners was *Securigera varia* (formerly *Coronilla varia*). This plant possesses an extremely robust root system that is more than capable of overwintering, very much like the Beach Pea. However, the Beach Pea grows in very sandy substrates and is found close to bodies of

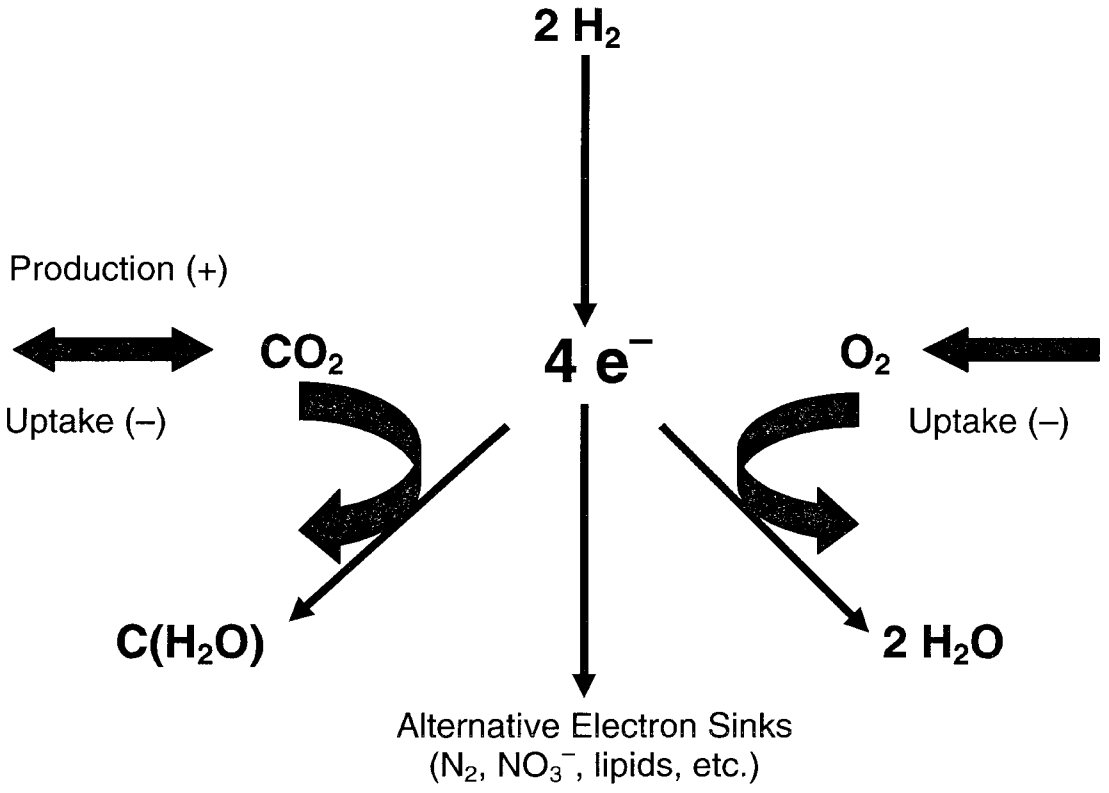
salt water, indicating some degree of salt tolerance; whether these conditions may affect the HUP status of its rhizobial symbioses remains to be investigated.

1.3.5 Conclusions:

The above delineates why HUP^- symbioses persist, and although no clear relationship between root morphology and/or mode of propagation with HUP status was revealed during the course of this survey, it may still be somewhat helpful in explaining how these associations form. However, the fact that, within the same species of legume, both HUP^+ and HUP^- associations are encountered indicates that other factors are also coming into play. For instance, it has been noted that nickel forms a part of many bacterial hydrogenases, including those of rhizobia (Kim and Maier, 1990) and seems to be required not only for hydrogenase activity, but for its very transcription (Brito *et al.*, 2000). It has further been reported that the hydrogenase activity of *Rhizobium leguminosarum* in symbiosis with pea plants may be limited by the availability of nickel in agricultural soils (Ureta *et al.*, 2005). Also, apparent host effects have been observed for both *Lupinus* (Murillo *et al.*, 1989) and *Lotus* (Monza *et al.*, 1997) species. Even more remarkable are the observed host cultivar effects in symbioses between *R. leguminosarum* and *Pisum sativum* (Bedmar *et al.*, 1983; Bedmar and Phillips, 1984; Dixon, 1987) and Bedmar and Phillips (1984) actually proposed the potentiality of a shoot factor transmitted to plant roots as being responsible for hydrogenase gene expression in rhizobia possessing hydrogenase genes. It is possible that very subtle differences in root morphology or variations in seed production between cultivars may be involved. One thing is certain: all aspects of this plant-soil-microbe system will have to

be thoroughly investigated before we come to a complete understanding of HUP status in legume-rhizobia symbioses. This is a worthy goal, one which will allow for safer, more economically and environmentally sustainable agricultural practices around the globe.

Figure 2: A summary of the reactions within H_2-oxidizing microorganisms in soil


2 H2
Production (+)
CO2
4 e−
O2
Uptake (–)
Uptake (–)
C(H2O)
2 H2O
Alternative Electron Sinks
(N2, NO3−, lipids, etc.)