Identifying microbial communities in disease suppressive soils as a means of improving root health of potatoes

Dr Jacqueline Edwards
Victorian Department of Primary Industries (VICDPI)

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Identifying microbial communities in disease suppressive soils as a means of improving root health of potatoes

Final report for project PT 07038

George Lazarovits *et al.*

(January 2011)

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DEPARTMENT OF PRIMARY INDUSTRIES | biosciences research

Project Details

Identifying microbial communities in disease suppressive soils as a means of improving root health of potatoes.

Final report for Horticulture Australia Ltd project PT07038
January 2011

This report details research using chaperonin gene technology from the medical field to characterise the consortia of soil microbes associated with two potato cultivars and two potato growing soils in Canada. The technology was refined, and it was demonstrated that both soil directly associated with potato roots and the endophytic community within the roots harboured many potentially useful bacteria for enhancing disease suppression and promoting plant growth.

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Contents

Media Summary

This project demonstrated that cutting-edge technology used in the medical field could be useful also in the potato industry. Chaperonin gene (*cpn60*) technology used in medical research was utilised to characterise the consortia of soil microbes associated with two potato cultivars (Shepody and Yukon Gold) growing in two different soils from Prince Edward Island and Ontario, Canada. The results confirmed that the *cpn60* technology can generate a 'fingerprint' of the soil microorganism community that may be used to characterise soils in the future, leading to identification of signature microbial groups involved in disease suppression and evaluation of the impact of agricultural practices on the soil.

Bacteria with growth promoting or disease suppressive potential were sought for future development of biocontrol agents and biofertilisers for potato. The *cpn60* technology was used to determine where best to look for microorganisms of importance to plants, and showed that microbes directly associated with roots, which also encompass the endophytic community in the roots, harbour unique concentration of potentially useful bacteria for enhancing disease suppression and promoting plant growth.

The microorganism communities associated with potato roots were screened for bacteria harbouring genes involved in nitrogen-fixation and biosynthesis of the major antifungal antibiotics produced by disease suppressive fluorescent pseudomonads. The genes were detected using PCR. 1540 bacteria were isolated, of which 81 contained antibiotic genes and 63 contained nitrogen-fixing genes. The soil from Prince Edward Island yielded greater numbers of useful isolates than the Ontario soil, and Yukon Gold provided many more antibiotic-producing isolates than Shepody.

The promising isolates were further screened for inhibitory activity against known potato pathogens or growth promotion effects on potatoes. Ten of the 81 antibiotic producers were active against all six pathogens tested, and 30 inhibited at least one of the pathogens. Molecular identification showed that 18 of the antagonists were closely related to known bacteria with antagonistic activity towards plant pathogens: *Pseudomonas fluorescens, P. brassicacearum, P. chlororaphis, P. putida, Xanthomonas retroflexus, Bacillus subtilis, Enterobacter amnigenus, Arthrobacter niigatensis* and *Paenibacillus polymyxa.* Ten promising nitrogen-fixing isolates were identified as species of the nitrogen-fixing genera *Bradyrhizobium, Azospirillum* and *Xanthobacter,* four of which promoted the growth and yield of potatoes in glasshouse pot trials. The future challenge will be to determine methods of introducing or stimulating microbial communities to achieve disease suppression and growth promotion in the field. This will ultimately provide growers with a low cost, non-chemical disease management strategy for soilborne diseases. The vast majority of organisms whose sequences were read remain unidentified species indicating that an enormous wealth of novel bacteria live on potato roots. Most astonishing was the number of *Rhizobia* species that were found associated with potato roots from both soils.

Technical Summary

This project demonstrated that cutting-edge technology used in the medical field could be useful also in the potato industry. The highly complex nature of the microbial communities in soils has been a major limitation in trying to differentiate cropping soils where plant growth is enhanced versus one where productivity is limited, particularly due to disease pressure. Attempts to describe microbial communities by either traditional microbiological or molecular methods have been limited in both scale and precision.

The availability of genomics technologies offered an unprecedented opportunity to conduct more comprehensive characterizations of such communities. A novel technique for molecular diagnostics of complex microbial ecosystems using a method based on the chaperonin-60 (cpn60) sequence, in combination with high-throughput sequencing, was utilised by this project. In this study, minitubers were grown in potato field soil from Ontario and Prince Edward Island (PEI). DNA extracts of bulk soil, potato rhizosphere soil attached to the roots, roots washed free of soil, and soil attached to tubers were subjected to pyrosequencing of the cpn60 genomic marker. Over 500,000 useable sequences were recovered. The data indicated that overall microorganism populations in the bulk soils from the two sites were fairly similar, showed some changes in soils attached to roots and tubers, but populations found on washed roots were very different from those found in the rhizosphere or bulk soil. The results confirmed that the cpn60 technology generates a 'fingerprint' of the soil microorganism community that may be used to characterise soils, allowing future evaluation of how different practices impact on the soil and identification of soils with desired characteristics such as disease suppressiveness.

The second stage of the research focused on the structure and function of the different bacterial communities. Bacteria with growth promoting or disease suppressive potential were sought for future development of biocontrol agents and biofertilisers for potato. The microorganism communities associated with potato roots were screened for bacteria harbouring genes involved in nitrogen-fixation (*nifH*), and biosynthesis of the major antifungal antibiotics produced by disease suppressive fluorescent pseudomonads namely pyrrolnitrin (*prnD)*, 2,4-diacetylphloroglucinol (*phlD)* and phenazine (*phzC/D*). The genes were detected using PCR. 1540 bacteria were isolated, of which 81 contained antibiotic genes and 63 contained nitrogen-fixing genes. The soil from Prince Edward Island yielded greater numbers of useful isolates than the Ontario soil, and Yukon Gold provided many more antibiotic-producing isolates than Shepody.

The promising isolates were further characterised on the basis of *in vitro* inhibitory activity against 6 potato pathogens, production of various enymes and phytohormones, and ability to assimilate iron. These are all characteristics of known biocontrol agents and plant growth promoters. 30 of the 81 antibiotic producers inhibited at least one of the pathogens, and 10 were active against all six pathogens tested. Molecular identification showed that 18 of the antagonists were closely related to known bacteria with antagonistic activity towards plant pathogens: *Pseudomonas fluorescens, P. brassicacearum, P. chlororaphis, P. putida, Xanthomonas retroflexus, Bacillus subtilis, Enterobacter amnigenus, Arthrobacter niigatensis* and *Paenibacillus polymyxa.* Ten promising nitrogen-fixing isolates were identified as species of the nitrogen-fixing genera *Bradyrhizobium, Azospirillum* and *Xanthobacter.* Several of them promoted the growth and yield of potatoes in glasshouse pot trials. The future will be to determine methods of introducing or stimulating microbial communities to achieve disease suppression and growth promotion. This will ultimately provide growers with a low cost, non-chemical disease management strategy for soilborne diseases.

One ambitious aim of the project was to design a prototype microarray able to detect the presence of these useful organisms directly in soil. The research has been concentrating on identifying genes and their functional activities that are important for disease suppression and growth promotion that will be used on the microarray, and the complexity of this means that the prototype is still in the conceptual phase. This research, however, is being continued within the Soil Health program of the Australian Potato Research Program Phase 2.

Background and Aims

Increased microorganism populations are postulated as a mechanism for improving disease suppressiveness of soils. The selective enrichment of microbiological ecosystems has tremendous potential if it proves to be transferable to large-scale agriculture. Human medicine has already embraced the concept of probiotic disease management whereby human diseases are prevented by enriching native microflora, thereby keeping pathogens from becoming established either directly or indirectly. In plant disease suppression, bacteria are mostly identified as the primary candidates responsible for reduced pathogen activity as they most closely interact with the host plant (Sturz et al. 2000). Lievens et al. (1989) found that of 11,614 bacteria isolated from 7 crop plants, about 33% (3910) produced antifungal metabolites. Berg et al. (2002) recently commercialised a strain of *Serratia plymuthica* HRO-C48 (Rhizostar), isolated from a strawberry rhizosphere, for use as a biocontrol agent of Verticillium wilt of strawberry (Muller et al. 2004, Scherwinski et al. 2007). Krechel et al (2002) screened 440 bacteria isolated from potatoes for antagonism against *V. dahliae* and *Rhizoctonia solani*. The highest proportion of bacteria with antagonistic activity was isolated from the rhizosphere (10%).

There are several limitations in studying microbial diversity. The information available on the bacterial rhizosphere community of potato has been obtained mostly by culture-dependent approaches (Azad et al. 1987, Berg et al 2001, Kloepper et al 1980, Loper at al 1985). The majority (99%) of soil microbes are unculturable, so only 1% is currently being studied.

In the last decade, a revolution has occurred in soil microbiology that has been accelerated by the availability of inexpensive high-throughput DNA sequencing (Hall 2007). These new technologies have now been used in numerous studies to track specific microorganisms in selected ecosystems. As yet, very few attempts have been made to obtain a holistic and quantitative picture of the entire soil or root microorganism community. Hill et al. (2002) described a technology that they used to successfully identify and enumerate members of complex microbial communities (Hill et al. 2002, 2005a, b, 2006). The technology utilises a genomic segment of DNA that codes for the chaperonin proteins, which are essential for the folding and assembly of proteins in all bacteria and in the plastids and mitochondria of eukaryotes (Hemmingsen et al. 1988, Saibil and Ranson 2002). In most bacteria, a single copy gene, *cpn*60, encodes this protein and it is an informative target for microbial species identification and for phylogenetics. Hill et al. (2002) described a robust, generic, molecular method for identifying microorganisms based on amplification of a portion of the *cpn*60 gene, the “universal target” (UT), using universal, degenerate PCR primers (Goh et al. 2000). The protein-encoding *cpn*60 gene is richer in phylogenetically informative sequence variation than is the structural RNA-encoding 16S *rRNA* gene. While 16S *rRNA* gene sequences are often identical between closely related organisms (Chatellier et al. 1998), *cpn*60 sequences usually discriminate different species within a genus, and sometimes serotypes or subspecies within a species (Brousseau et al. 2001). As a single-copy gene, *cpn*60 lacks the potential for some sequencing artefacts that are encountered with the multiple-copy 16S *rRNA* gene which makes it more amenable for us to use in quantitative PCR assays. The 549-567 bp *cpn*60 UT can be sequenced easily with a single reaction, whereas the full length (~1.5 kbp) sequence of the 16S *rRNA* gene is often required to distinguish two given species, if it is possible at all. Thus, high throughput sequencing of large libraries of cloned *cpn*60 gene regions is relatively economical and this approach has been developed for identifying and enumerating members

of complex microbial communities (Hill et al. 2002, 2005a, b, c, 2006).

Any sequence-based approach to microbial species identification, however, is dependent on the availability of a sufficient collection of reference sequence data. At the moment this is a limitation for studies of soil microorganism communities as the chaperonin sequence database, cpnDB (http://cpndb.cbr.nrc.ca), contains only about 9,000 sequences, including about 1,600 reference sequences from named organisms (prokaryotic and eukaryotic). While it has been used to create phylogenetic context for microbial communities (Hill et al. 2002) and for identifying clinical isolates (Goh et al. 2000) its usefulness for identifying highly diverse communities of soils and roots has only been superficially evaluated (Lazarovits et al. 2009).

The aims of this study were (1) to examine whether the *cpn60* technology could be used to determine and compare the microbial profile of soils collected from potato farms in Prince Edward Island (PEI) and Ontario, Canada, (2) to determine where to look in the soil for the the most useful consortia of microorganisms, and (3) to identify and characterise bacteria potentially useful for disease suppression and growth promotion in potato production. The sequence to the research was as follows:

A. Evaluate whether *cpn60* technology allows detection of the changes in the microorganism communities of soils collected prior to planting potatoes, compared to those obtained from soils attached to the roots and tubers and of the bacteria community that remained on the roots after washing them in running water. The hypothesis was that in general the microorganism communities would be similar in the two soils and that changes would be detected in the community induced by the root system.

B. Screen microorganism communities from different sources (soil type, rhizosphere vs endosphere, host cultivar) for bacterial isolates demonstrating growth promotion potential (nitrogen fixation) and disease suppression potential (antibiotic productivity).

C. Characterise and identify bacterial isolates with potential usefulness in potato production: (a) antibiotic producers: characterise the antibiotic genes responsible; test for activity *in vitro* against key pathogens (*Rhizoctonia solani, Pythium ultimum, Verticillium dahliae, Fusarium oxysporum, Streptomyces scabies*); sequence and identify the organisms; (b) nitrogen-fixers: test for nitrogenase activity; sequence and identify the organisms; test selected organisms in bioassays for growth promotion effects on potatoes.

1. UNRAVELLING THE MICROBIAL ECOSYSTEM OF POTATOES USING PYROSEQUENCING AND THE *CPN60* GENE TARGET

INTRODUCTION

Waksman and Starkey (1931) in their book "The soil and the microbe" were disturbed by the fact that only marginal efforts were being made into understanding the role soil microorganism communities have on the environment, despite the recognition of their importance in the health of humans, animals, and plants. In their review of 100 years of soil microbiology, Stockdale and Brookes (2006) reiterated this lack of headway in developing an understanding or methods to manipulate soil microbiology for the benefit of plant agriculture. The primary reason for the frustration in this field is that soils contain the most complex microorganism communities on the planet and until recently, we have not had the tools to examine this ecosystem (Hall, 2007).

There are several limitations in studying microbial diversity. Spatial heterogeneity in the form of bacterial clumping in the soil, in so called "hot spots", or as influenced by the presence of plants (Wall and Virginia, 1999), where two fold increase in bacterial numbers is observed in the rhizosphere, act as limiting factors in homogeneous sampling that is required for microbial diversity studies. The inability of culturable techniques to culture the majority of the soil microbes, and as opined by Rondon *et al.* (1999; 2000) that it is likely that the 99% of unculturable microbes are phenotypically and genetically different from the 1% culturable, and only the minority of the population is represented. Culture-independent molecular-based methods are also limited by variation in lysis efficiency between different cells in the soil (Prosser 2002), separation of bacterial cells from soil aggregates (Trevors, 1998), and the method of nucleic acid extraction (Wintzingerode *et al.,* 1997). Microbial diversity studies are constrained by the taxonomic ambiguity of microbes, particularly the genetic plasticity of bacteria, allowing DNA transfer through plasmids, bacteriophages and transposons, which complicate the concept of bacterial species (Kirk *et al*. 2004).

Despite all the limiting factors, in the last decade a revolution has occurred in the understanding of microbiology that is accelerated by the availability of inexpensive high-throughput DNA sequencing. There have been many studies carried out to track specific microorganisms in selected ecosystems but very few attempts to get a holistic and quantitative picture of the entire microorganism community. In this study, we evaluated a powerful new technology that opens an avenue for examining holistically the soil and rhizosphere communities. The technology promises to provide a routine assay for hypothesis testing of the roles microorganism communities have in plant productivity.

Cpn60-based technology

Chaperonin proteins are molecular chaperones that are essential for the folding and assembly of proteins in all bacteria and in the plastids and mitochondria of eukaryotes (Hemmingsen *et al.* 1988; Saibil and Ranson 2002). In most bacteria, a single copy gene, *cpn*60, encodes this protein and it is an informative target for microbial species identification and for phylogenetics. Hill *et al.* (2002) have described a robust, generic, molecular method for identifying microorganisms, based on amplification of a portion of the *cpn*60 gene, the "universal target" (UT), using universal, degenerate PCR primers (Goh *et al.* 2000). This method has demonstrated advantages over 16S *rRNA*-based methods. The protein-encoding

*cpn*60 gene is richer in phylogenetically informative sequence variation than is the structural RNA-encoding 16S *rRNA* gene. While 16S *rRNA* gene sequences are often identical between closely related organisms (Chatellier *et al.* 1998), *cpn*60 UT sequences usually discriminate different species within a genus, and sometimes serotypes or subspecies within a species (Brousseau *et al.* 2001). Second, as a single-copy gene, *cpn*60 lacks the potential for some sequencing artefacts that are encountered with the multiple-copy 16S *rRNA* gene. This characteristic is also advantageous for quantitative PCR assays. Last, the 549-567 bp *cpn*60 UT can be sequenced easily with a single reaction, whereas the full length (~1.5 kbp) sequence of the 16S *rRNA* gene is often required to distinguish two given species, if it is possible at all. Thus, high throughput sequencing of large libraries of cloned *cpn*60 gene UT regions is relatively economical and this approach has been developed for identifying and enumerating members of complex microbial communities (Hill *et al.* 2002, Hill *et al.* 2005 a, b, c, Hill *et al.* 2006).

Any sequence-based approach to microbial species identification, however, is dependent on the availability of a sufficient collection of reference sequence data. The chaperonin sequence database, cpnDB (http://cpndb.cbr.nrc.ca), contained ~8964 sequences at the time of this study, including ~1,600 reference sequences from named organisms (prokaryotic and eukaryotic). The cpnDB also included sequences derived from previous microbial population studies and clinical and field isolates generated by the investigators' prior studies. The cpnDB has been used to create phylogenetic context for microbial communities (Hill *et al.* 2002) and for identifying clinical isolates (Goh *et al.* 2000).

The objectives of this study were (i) to identify the active bacterial populations in the rhizosphere, rhizoplane and bulk soil of potato grown in soils from Ontario and Prince Edward Island (PEI), and (ii) to evaluate whether chaperonin gene (*cpn60*) technology allows detection of the changes in the microorganism communities of soils collected prior to planting potatoes, compared to those obtained from soils attached to the roots and tubers and of the bacteria community that remained on the roots after washing them in running water. The hypothesis was that in general the microorganism communities would be similar in the two soils and that changes would be detected in the community induced by the root system.

MATERIALS AND METHODS

Soils and plants

Mini-tubers, of the variety Shepody, derived from tissue culture plants were grown in soils collected from potato farms in Ontario and PEI. The plants were harvested and the non-adhering soil was removed by manual shaking. The roots, along with the adhering soil, were transferred to 250 mL conical flasks containing 10 g of glass beads and 100 mL of Winoradsky's Salt solution. The flasks were placed on a rotary shaker and shaken at 150 rpm to dislodge the soil particles from the roots and suspend them in the solution. After shaking, the roots were removed using a forceps, and the remaining contents were transferred, through a sieve to collect the glass beads, into a centrifuge tube. The soil suspension was centrifuged at 7000 rpm for 20 mins. The supernatant was removed and the soil pellet was scooped into a weigh boat and allowed to dry at room temperature. The soil adhering to the tuber surface was brushed off using a soft paint brush. The bulk, rhizosphere and tuber soils were stored at

4°C until DNA extraction. The roots were further washed in running distilled water and stored at -20°C until DNA extraction.

DNA preparation and amplification

The soil DNA was extracted using the Power Soil DNA kit (MoBio Laboratory, CA, USA) and the root DNA using the DNeasy Plant Mini Kit (Qiagen, MD, USA). The kit manufacturer's protocol was followed during the DNA extraction. The DNA samples were sent for sequencing of the *cpn*60 chaperonin region at the National Research Council laboratory (Saskatoon) by Dr. Sean Hemmingsen. A total of nine samples were prepared. The DNA was used as template for amplification of *cpn*60 regions using our degenerate, universal primers in PCR reactions. Forward and reverse *cpn*60 universal primer mixtures were applied to amplify the region of the *cpn*60 gene corresponding to nucleotides 274-828 of the *E. coli cpn*60 sequence (Hill *et al.* 2006); the forward primer mixture was a 1:3 molar ratio of primers H279 (5'-GAI III GCI GGI GAY GGI ACI ACI AC-3') and H1612 (5'-GAI III GCI GGY GAC GGY ACS ACS AC-3') and the reverse primer mixture was a 1:3 molar ratio of primers H280 (5'-YKI YKI TCI CCR AAI CCI GGI GCY TT-3') and H1613 (5'-CGR CGR TCR CCG AAG CCS GGI GCC TT-3'). PCR products generated from the soil and root DNA samples were analyzed using pyrosequencing.

Each unique sequence was then compared to cpnDB using sequence comparison and phylogenetic bioinformatic tools. An exact or tentative identity for the origin of each sequence is made depending on the degree of similarity between the experimental sequence and the closest relative in cpnDB. Initially, a 70% homology was used to assign a name. It is realized however that at best this may identify groups of organism rather than genera or species. The pool of sequence data generated can serve as the foundation for quantitative studies of taxa that are of interest. The frequencies of specific sequences in our libraries will need to be correlated with the relative abundances of the templates in the total DNA extract. Therefore, frequencies of the amplicons are our first estimates of the frequencies of individual species in the community.

RESULTS AND DISCUSSION

We set out to examine if the *cpn*60 technology can be used to determine the microbial profile of soils collected from potato farms in PEI and Ontario and whether the populations in these soils were similar or drastically different. These soils were then used to grow minitubers obtained from tissue culture plants in order to evaluate if the technology allowed for detection of the changes that occur in the microorganism communities of soils collected from soils attached to roots and tubers and the bacteria community that remained on the roots after washing in running water. The hypothesis was that overall the microorganism communities would be similar in the two soils and that we would see changes in the community as we approached the root system. The total number of useable sequences for each sample is shown in Table 1.1.

Table 1.1 The number of amplicon sequences that had >70% homology to possible genera and species in the CPN60 database.

	No. of sequences identified	No. of species identified
Bulk soil	290,000	314
Rhizosphere soil	26,000	186
Washed roots	140,000	227
Tuber soil (geocaulosphere)	55,000	246

The bacteria detected were tentatively identified, using a 70% homology cut off, for bulk soil, rhizosphere soil, root extracts and geocaulosphere soil (soil from tuber surfaces). Isolates were identified that were unique for either PEI or Ontario from each of the samples for bulk soil, rhizosphere soil, root extracts, and geocaulosphere soil. In total, 511,000 sequences were useable as they had 70% similarity to known sequences in the database, the similarity index used as the confidence limit in attaching a phylogenetic status. However, even sequences with at least 70% identity to reference sequences are very likely not giving us sufficient robustness to place the organism into genera and species. Sequences with less than 70% identity were not used but they also may have valuable information for future analysis.

At the moment the availability of reference sequence data is relatively small in the cpnDB of some ~9000 sequences, including ~1,600 reference sequences from named organisms (prokaryotic and eukaryotic), half of which were mined from public databases and half were generated by our collaborative research network. Nevertheless we now have sequences to select out specific groups and examine in greater detail the species that may be present.

Results of the top 20 most common organisms present in the PEI and Ontario soils show that in both soils *Solibacter usitatus* and *Bacterium sp. Ellin514* were the two most common organisms. Relatively little is known as to what these organisms do in soil (Galperin 2006). Interestingly, *Azospirillum brasilense* was the fourth most common organism in the Ontario soil and *Azospirillum canadense*, a bacterium originally identified in the laboratory of Lazarovits (Mehnaz et al. 2007), was also found in the top 15 isolates. Both organisms were also present in the PEI soils but in lower numbers. *Bradyrhizobium sp. BTAi1* was common to both soils. In rhizosphere soil, *Sphingopyxis alaskensis* and various *Serratia* species became more common. *Serratia* were relatively rare in bulk soil. The most dramatic changes in microorganism communities were apparent on roots, where *Pseudomonas* species, *Rhizobium* species and *Serratia* species became dominant. The increase in the prevalence of *Pseudomonas* and *Serratia* species was not unexpected; but that of populations of *Rhizobium* type organisms was. *Rhizobium* species are more commonly known as nodule-forming N-fixing symbionts associated with legumes. The role they may have in potato, a non-legume, is currently unknown and their presence was very unexpected.

The issue of spatial heterogeneity is well addressed in this study. The study compares various zones, including the bulk soil, rhizosphere, roots, and the tuber surface, and the microbial assemblage in these zones.

There are many potential beneficial agents that could be targeted as bioindicators of soil health. *Serratia plymuthica* has been developed as a biocontrol agent for Verticillium of strawberries (Rhizostar) in Europe (De Vleesschauwer and Hofte, 2007; Weissinger et al.

2009). *Telluria mixta* is a close relative of the *Herbaspirillium* spp. bacteria known to be potent nitrogen fixers in sugarcane and other plants (Schmid and Hartmann, 2003). *Rhizobium* and *Bradyrhizobium* species have been shown to control a diversity of soilborne fungal pathogens (*Fusarium solani, Macrophomina phaseolina* and *Rhizoctonia solani*) (Omar and Abd-alla 1998), although we do not see this as their primary role in soil. Hallman et al. (2001) showed that *Rhizobium etli* G12, originally isolated from the rhizosphere of potato, is an antagonist of the potato cyst nematode *Globodera pallida* and the root-knot nematode *Meloidogyne incognita.* This bacterium was the 4th most common bacterium found on roots of plants from Ontario soil. Soils associated with the tubers that formed in both soils had a rich diversity of *Rhizobium* species, not common in the bulk soils.

Generally there were relatively few organisms that were present in one soil but not in the other. Until we can get more confidence in the identification of the actual organism, the significances of the differences cannot be evaluated.

Analysis of the chaperonin gene sequences derived from microorganisms obtained from bulk soil, potato rhizosphere and washed potato roots

The initial analysis of the data attempted to link sequences with potential genera and species. A more sophisticated statistical tool was utilized to identify if we could differentiate the microbial communities in the samples analyzed. The membership and structure of the communities was analyzed using operational taxonomic units (OTU) to test community structure similarity and the probability of a statistical similarity among the soils, rhizosphere and roots. Approximately ~27,000 OTUs were represented in the samples that were sequenced.

A computer program was used which implements nonparametric estimators for the fraction and richness of OTUs shared between two communities. “Isotigs” were assembled de novo from the sequence data. A software package was designed to handle genes plus single nucleotide polymorphism (SNP) variants of genes. An Isotig can include a number of identical *cpn60* gene sequences that represent independently amplified PCR products from identical bacteria plus non-identical but very similar *cpn60* gene sequences from closely related bacteria. In this way nothing is left out of the assembly and there is no cut-off percentage that has to be met, just a length and quality requirement for a sequence read to be included.

Each Isotig consensus sequence was compared to cpnDB using the Watered-BLAST method and the best hit identified in cases where the match was 80% identity or greater.

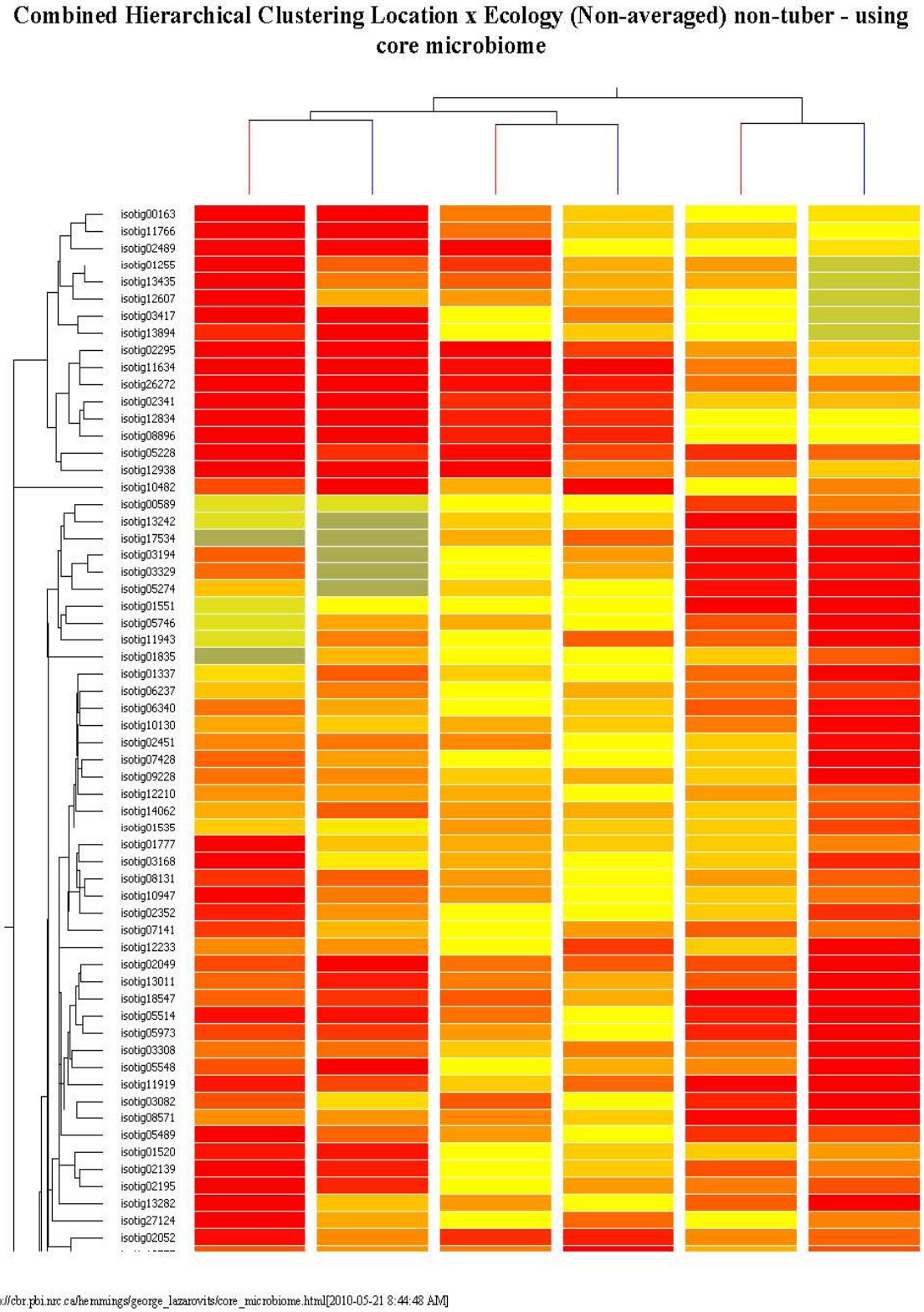
Figure 1.1 shows sequences that were present in both soil types (Ontario and PEI) and in both rhizosphere samples and both root samples. This will be referred to as the core microbiome. The sequences included in this figure represent a small fraction of the total number of different sequences seen in the experiment. The figure is a heat map (relative abundances of microbial OTUs (Isotigs)). The trees show the results of hierarchical clustering. The data reveals that the soil samples clustered together, as did the rhizosphere samples and the root samples. When samples within a site were examined, the rhizosphere and soil samples shared

similar microbial communities, but the microbial communities on the roots were significantly different from those of the soil and rhizosphere.

Based on this more thorough alignment of the 13 Isotigs shown at the bottom of Figure 1.1, Isotig 11595 and Isotig03144 were found to both represent the same bacterium, *Rhizobium leguminosarum.* The identity between the experimental sequences and the cpnDB entry (b101911) was almost 100%. The other reasonably high % identity was Isotig02433 (96% to *Arthrobacter aurescens*).

Figure 1.2 is a diagrammatic representation of microbiomes comparing total number of potential entities in various soils that were considered to be core members of microbiomes.

Figure1.1


Combined Hierarchical Clustering Location x Ecology (Non-averaged) non-tuber - using core microbiome
isotig00163
isotig11766
isotig02489
isotig01255
isotig13435
isotig12607
isotig03417
isotig13894
isotig02295
isotig11634
isotig26272
isotig02341
isotig12834
isotig08896
isotig05228
isotig12938
isotig10482
isotig00589
isotig13242
isotig17534
isotig03194
isotig03329
isotig05274
isotig01551
isotig05746
isotig11943
isotig01835
isotig01337
isotig06237
isotig06340
isotig10130
isotig02451
isotig07428
isotig09228
isotig12210
isotig14062
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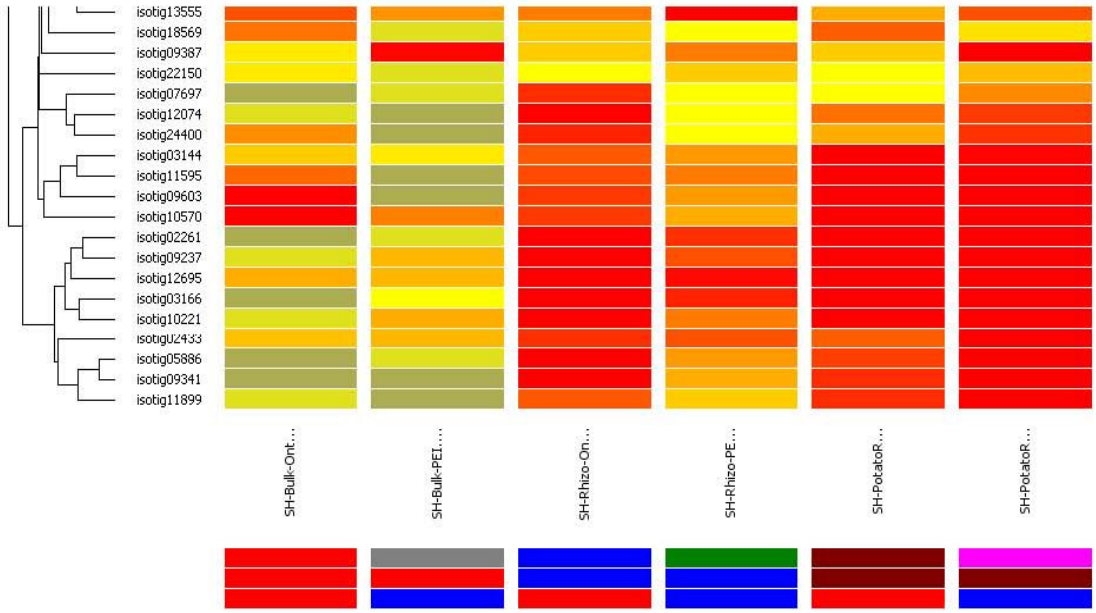

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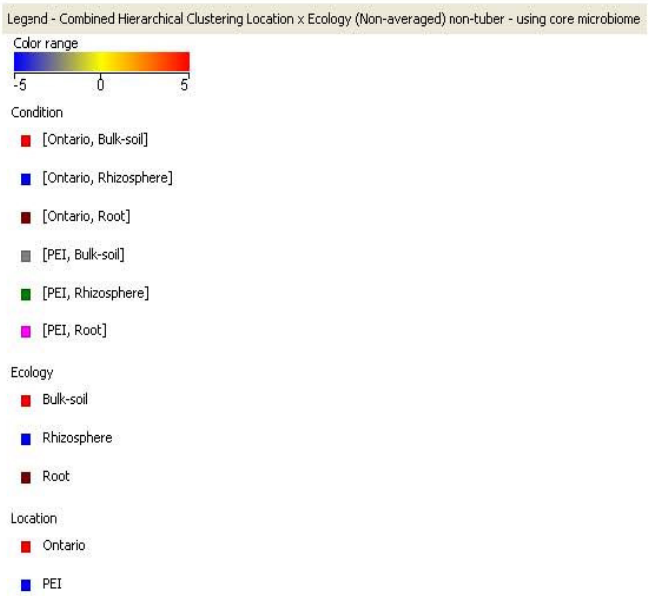

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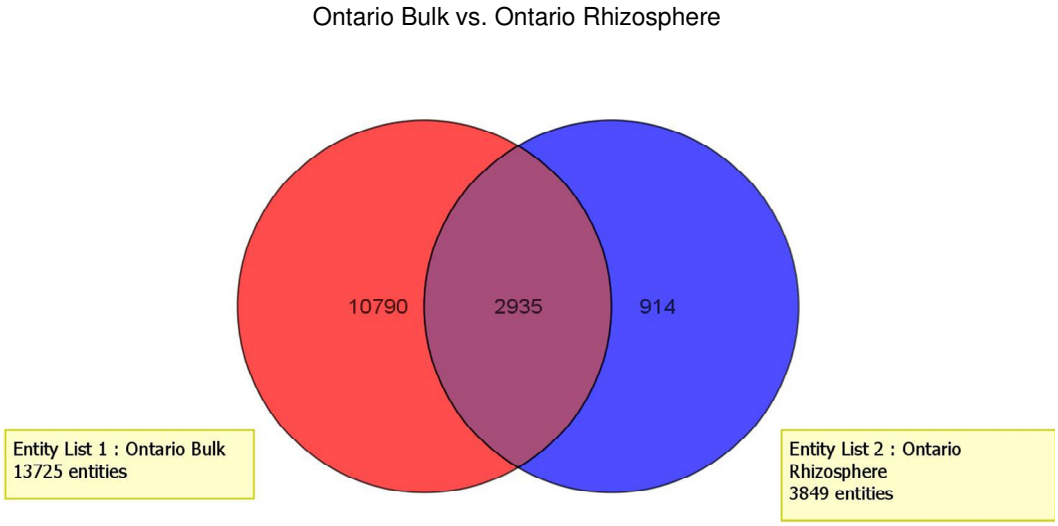
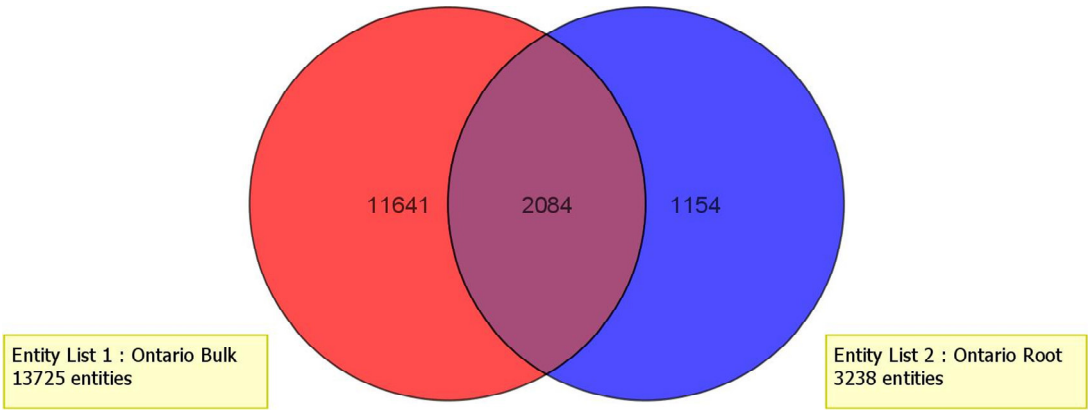
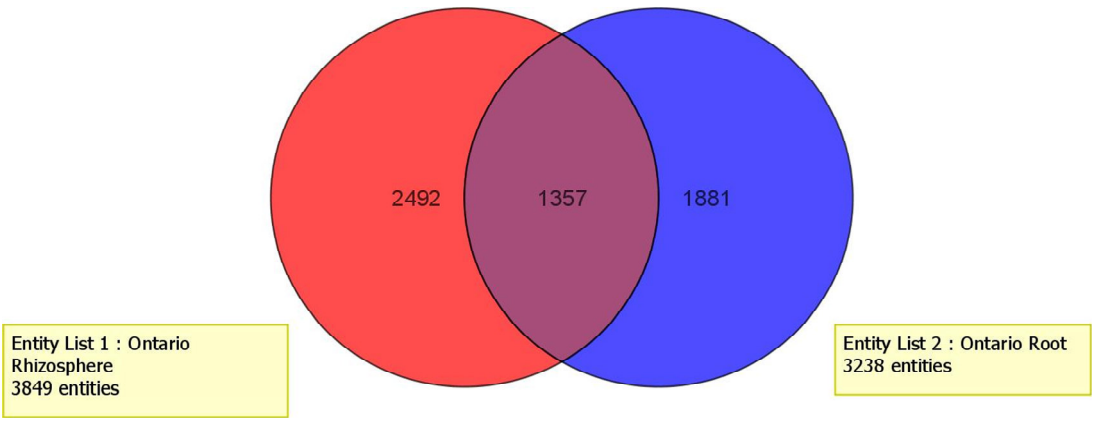
Figure 1.2 Venn diagrams of microbiomes compare total number of potential entities in various soils that were considered to be core members of microbiomes. Using such diagrams allows us to narrow the search for potential key players on potato roots. The numbers within each circle represent the number of operational taxonomic units, or entities, identified in that soil.



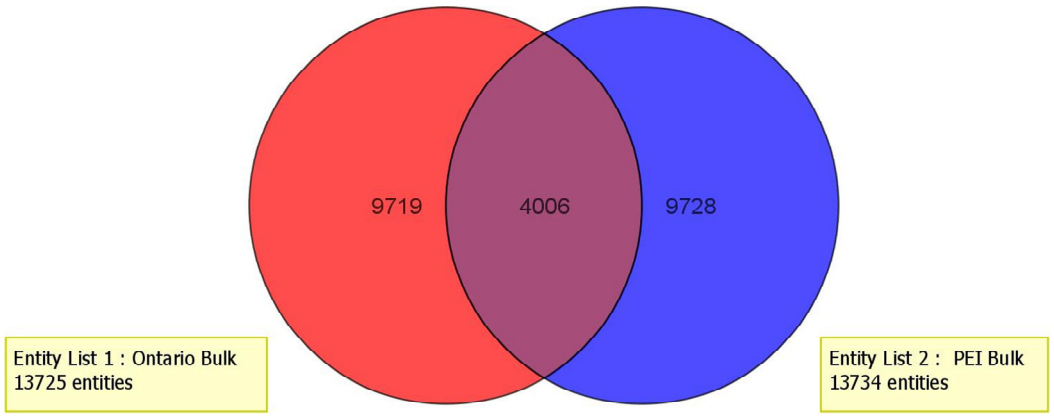
Ontario Bulk vs Root



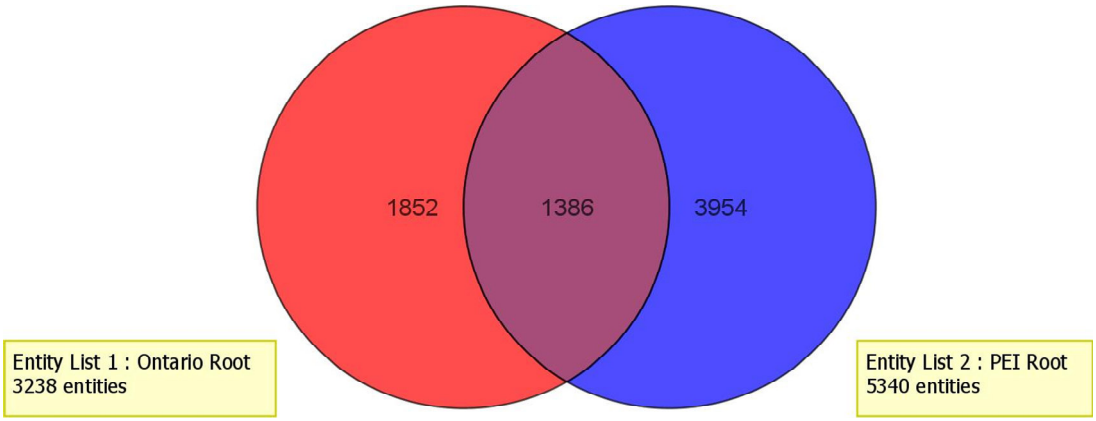
Ontario Rhizosphere vs. Root



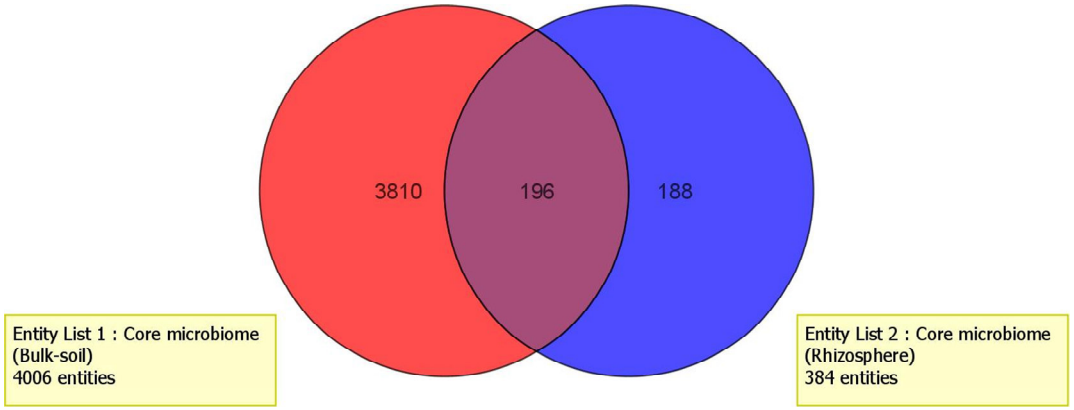
Ontario Bulk Soil vs PEI Bulk Soil



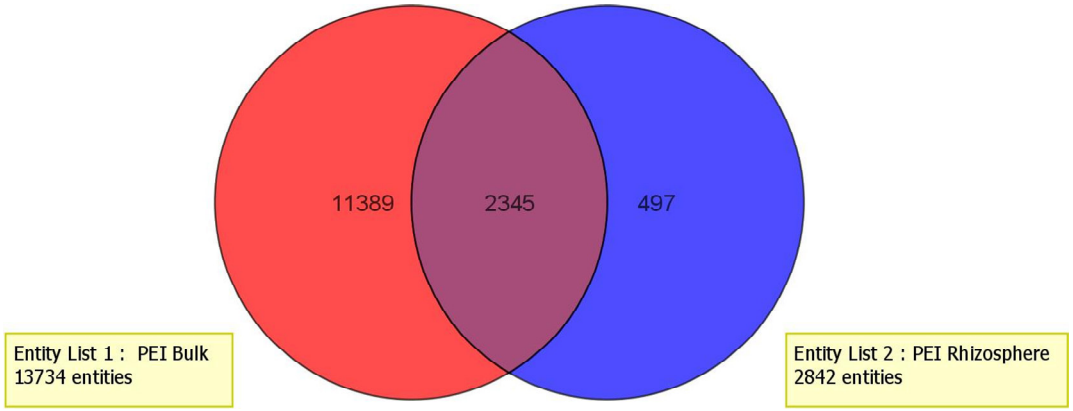
Ontario Root vs. PEI Root



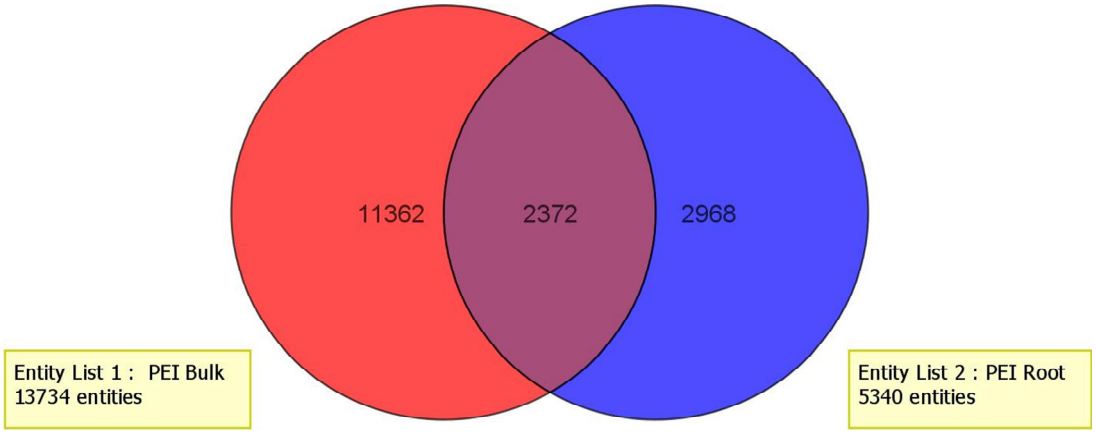
Core Microbiomes (Ontario and PEI) Bulk vs. Rhizosphere



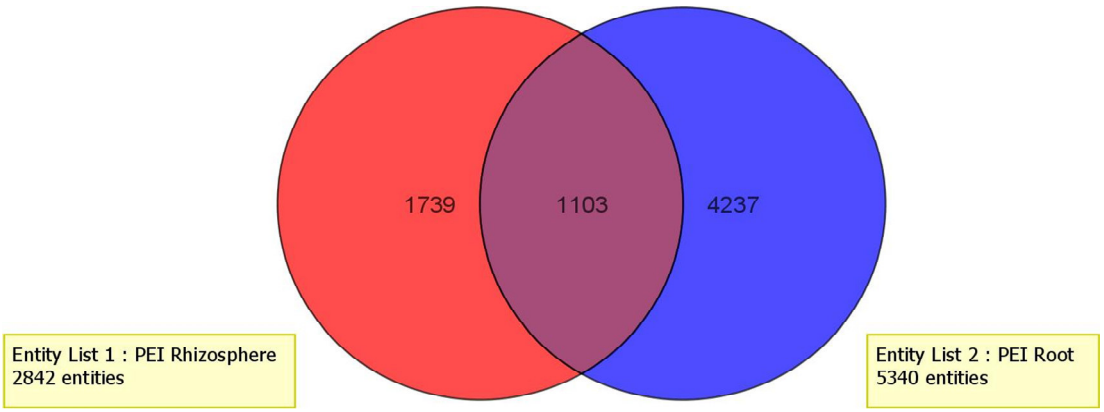
PEI Bulk vs. PEI Rhizosphere



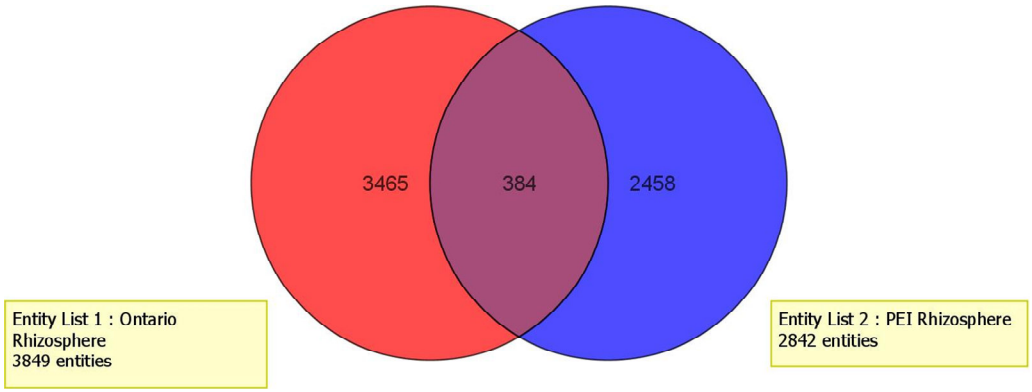
PEI Bulk vs. Root

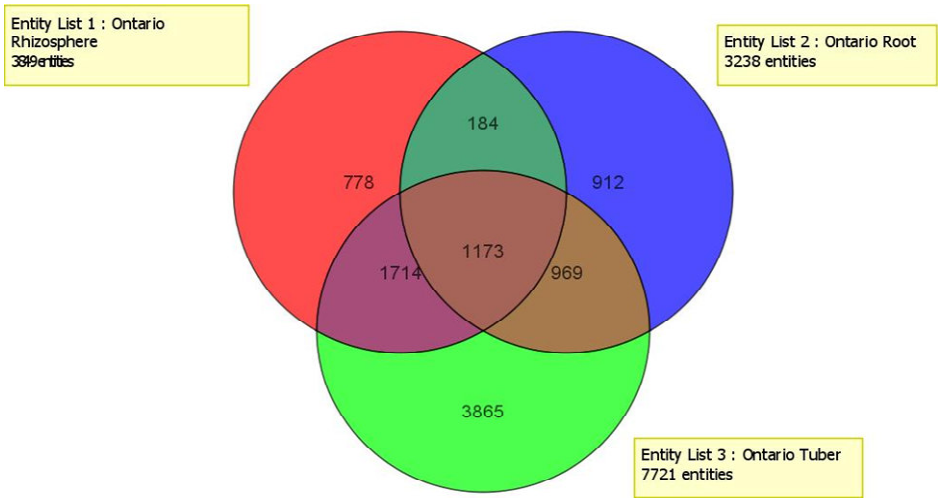


PEI Rhizosphere vs. Root

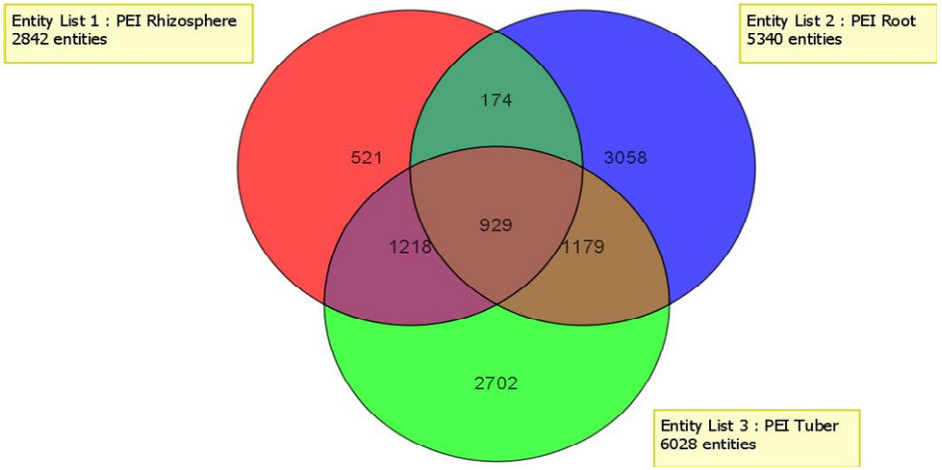


Ontario Rhizosphere vs. PEI Rhizosphere





Ontario Rhizosphere, Root, Tuber



PEI Rhizosphere, Root, Tuber

Stockdale and Brookes (2006) indicated that studies of single soil organisms, while useful in specialized cases such as Rhizobia and mycorrhizae, do not yield information on the functioning of the soil ecosystem as most soil processes depend upon interactions between entire suites of organisms. Studies such as this, particularly when more microorganisms are placed into the database, can generate a fingerprint of a soil microbial community and provide the measurement of the "forest rather than an individual tree" as indicated by Stockdale and Brookes. It will also provide a means to evaluate how the soil microbial community is influenced by agricultural practices and climate change.

Sustainable agriculture will require producers to better manage their soil microbial communities in order to minimize losses caused by soilborne pathogens. This will likely involve processes that return specific nutrients to soil that enrich the desired organism by way of crop rotations, green manures, composts etc. The process will require years to achieve rather than months or weeks, but once attained is expected to be fairly easy and inexpensive to maintain. Through molecular genetic approaches we can now fingerprint soils and identify the desirable microorganism residents. Generation of such fingerprints will make it possible to move forward with knowledge based decisions systems as to how to achieve conditions that benefit plant growth.

2. UNDERSTANDING THE RHIZOSPHERE AND ENDOPHYTIC BACTERIAL COMMUNITIES OF POTATO AS INFLUENCED BY THE HOST VARIETY AND SOIL TYPE

INTRODUCTION

Plant roots regulate the soil microbial community in their immediate vicinity (rhizosphere) through a wide variety of chemicals (root exudates) secreted into the soil (Nardi et al. 2000). These root exudates are used by soil microbes for energy and biomass production, while for plants this interaction generates the basis for growth through availability of mineral nutrients, production of plant hormones, degradation of phytotoxins and suppression of soil-borne pathogens (Bais et al. 2006). Root exudate composition varies with plant species, plant age and soil type (Hertenberger et al. 2002). Depending on their chemical composition, root exudates activate different microbial genes and growth of different microbial populations. Bacteria respond differently to the compounds released by the plant root, and thus different compositions of root exudates are expected to select different rhizosphere communities (Garbeva et al., 2004). Conversely, rhizosphere bacteria will also influence plants, as many promote plant growth via chemical signals such as auxins, gibberellins, glycolipids and cytokinins. Genera such as *Pseudomonas, Agrobacterium, Bacillus, Variovorax, Phyllobacterium* and *Azospirillum* are among the most efficient plant growth-promoting bacteria (Bertrand et al., 2001). For example, *Azospirillum brasilense* can exert a positive effect on the growth of common bean and soybean, and *Agrobacterium tumefaciens* can have a strong effect on plant root development (Burdman et al., 1997; Molla et al., 2001). The composition of root exudates is strongly affected by the plant developmental stage, which in turn can affect rhizosphere communities over time (Yang and Crowley, 2000).

In Chapter 1, differences were observed between the rhizosphere, rhizoplane and bulk soil bacterial communities of soil from PEI and Ontario. This current study focused on the assembly and function of these different bacterial communities. With regards to function, we focused on the free-living diazotrophs (nitrogen-fixers) and disease suppressive antibiotic producing pseudomonads.

Atmospheric forms of nitrogen unavailable to plants enter soil ecosystems and are converted to available forms through biological nitrogen fixation. This is mostly carried out by so called ‘diazotrophs’ (nitrogen-fixing bacteria). Various species across bacterial taxonomic groups possess the ability to reduce the atmospheric nitrogen to ammonia through the activity of the enzyme nitrogenase. A well known and documented biological nitrogen fixation system is the association between leguminous plants and some rhizobia species. In addition to this symbiotic interaction, there have been numerous evidences that indigenous nitrogen fixing bacteria are present in the rhizosphere which also colonize the tissues of non-leguminous plants. Besides the improvement in nitrogen nutrition of plants and in some cases significant reduction in nitrogen fertilizer application, diazotrophic bacteria are important for plant growth, plant health and stress resistance. In Brazil, continuous sugarcane cultivation over very long periods without nitrogen fertiliser has been attributed to nitrogen fixing bacteria that live in the stem and root tissue of sugarcane (Reis et al. 2007).

Direct antagonism of pathogenic organisms through antibiosis is one of the mechanisms by which disease suppressive bacteria achieve disease control. Antibiosis is mediated through

the production of a chemically heterogeneous group of organic, low molecular mass compounds (Raaijmakers et al., 2002) such as phenazine, pyrrolnitrin and 2,4-diacetylphloroglucinol. Many studies on the role of antibiotics in biocontrol of plant pathogens have led to the isolation and characterization of numerous antibiotics from various bacterial genera. Some prominent examples are: control of multiple pathogens such as *Pythium aphanidermatum*, *Botryodiplodia theobromae* and *Alternaria solani* by phenazine produced by *P. chlororaphis* PA23 (Kavitha et al., 2005); control of *Rhizoctonia solani* in poinsettia by pyrrolnitrin produced by *Burkholderia cepacia* (Hwang et al., 2002), and control of *Gaeumannomyces graminis* var *tritici* in wheat by 2,4-diacetylphloroglucinol produced by *P. fluorescens* Q8r1-96 (Raaijmakers and Weller, 2001).

The aims of this study were to determine whether (1) these bacteria originate from the host plant or the soil, (2) the soil type and the host plant influence the structure and activity of the bacterial communities, and (3) to identify and characterize potential plant growth promoters/biocontrol antagonists.

MATERIALS AND METHODS

Soil preparation and planting

Two soils, one (BC) from Prince Edward Island and the other (V4) from Alliston, Ontario, were used for a greenhouse trial. Some of each field soil (V4 and BC) was autoclaved three times at 121^{o}C and 22 psi. Potato varieties, Yukon Gold and Shepody, were planted in the autoclaved (V4A and BCA) and non-autoclaved (V4 and BC) field soils in 6" pots. Shepody mini-tubers and Yukon Gold cut tuber pieces containing the "sprouting eye" were used. The plants were allowed to grow for 6 weeks, then harvested for collection of rhizosphere soil and roots for bacterial isolation. The field soil collected prior to planting was considered as bulk soil.

Isolation of rhizosphere bacteria

For each variety x soil type (autoclaved or field) combination, there were 3 replicates and 4 plants per replicate. The rhizosphere soils from the 4 plants of each replicate were combined during harvest, of which 10 g was suspended in 90 mL of WSSA (Winogradsky's Salt solution with 0.1% agar) in stomacher bags. The suspensions were shaken at 200 rpm for 30 minutes and later stomached at medium speed for 30 s. Ten-fold serial dilutions were made from the stomached suspensions and 100 µL was plated onto selective media (R2A, PAF, NFM and LGI). The R2A medium was selected for its capacity to support the growth of a large bacterial population, especially slower growing bacteria, while the PAF medium is specific for Pseudomonads, and the NFM and LGI media favour the free-living nitrogen fixing diazotrophs. In addition, the suspensions were used to inoculate 5 ml of NFM and LGI semi-solid media. All media were amended with cycloheximide (100 µg/ml) to prevent fungal growth. The plates and vials were incubated at 28^{o}C and colonies counted.

Isolation of root endophytes

The roots were washed in running distilled water and excess water was removed with paper towels. Five grams of root tissue for each replicate, 1.25 g from each plant, was used for the isolation procedure. The roots were surface sterilized for 5 min in 1.5% NaOCL (bleach)

followed by 5 min in 70% ethanol and five 1 min washes in sterile water. The 5th wash solution was plated onto LB agar to check the efficiency of the sterilization process. The roots were cut into 1 cm pieces using sterile scissors and pulverized. Five grams of the pulverized homogenate was suspended in 45 mL of saline solution and subjected to ten-fold serial dilution, followed by plating onto solid and semi-solid media as described above.

Determination of nitrogen fixing ability of microorganisms

Cell cultures from the semi-solid NFM and LGI vials were streaked onto their respective solid medium plates and incubated for five days at 28^{o}C. Based on different morphology types, a total of 360 colonies were selected from all treatments and semi-solid media. These colonies were lysed and screened by multiplex PCR for *nifH* and *recA* genes (Table 2.1). PCR amplicons were visualized on agarose gel; isolates which showed amplification of the expected fragment size were further screened for the activity of nitrogenase enzyme (see Chapter 3).

Acetylene reduction assay (ARA) was also performed to determine the presence and growth of free-living nitrogen fixing bacteria in the rhizosphere and root tissues of the two selected potato cultivars. Nitrogenase enzyme, responsible for nitrogen fixation, is also known to reduce acetylene (C_2H_2) to ethylene ($C_2H_{4)}$ which provides a useful assay for the detection and quantification of the N_2-fixation process. For this assay, 3.5 ml (10% of head space) air was removed from the three-days incubated vials containing NFM and LGI semi-solid media inoculated with rhizosphere and root tissue suspensions. The air was replaced with 3.5 ml of acetylene followed by another 3-5 days incubation at 28^{o}C. After this, the gas mixtures in the vials were taken and analysed by gas chromatograph for the reduction of acetylene to ethylene.

DNA extraction and PCR-based detection of fragments of the biosynthetic genes involved in nitrogen fixation and antifungal antibiotic production

Five hundred grams of soil was used for DNA extraction, which was performed using the manufacturer's protocol (FastDNA Soil Extraction Kit, MP Bio). The DNA was further purified using the GeneClean system (MP Bio). Both the pre and post-GeneClean samples were quantified using a NanoDrop spectrophotometer. Initially, the soil community DNA (original stock and 1:10 dilution of the stock) was used for the PCR screening for the detection of fragments of genes involved in the biosynthesis of nitrogenase and the antibiotics pyrrolnitrin (PRN), 2,4-diacetylphloroglucinol (DAPG) and phenazine (PHZ) (Table 2.1). Based on positive results from the community DNA screening, bacterial isolates picked from the plates (R2A, PAF, NFM and LGI) for each potato variety x soil combinations were screened for the detection of these biosynthetic genes. Bacterial suspensions were made by suspending the colony in 50 mg of 0.1 mm zirconia beads and 150 µL of filter sterile MilliQ water. The suspensions were subjected to intense agitation using a FastPrep instrument (setting 6 for 20 s), followed by centrifugation at 12,000 rpm for 1 min. The supernatant was transferred to a fresh tube and subjected to 5-fold dilution. The diluted suspensions were used as templates for PCR. Following detection of the genes, the isolates were pure-cultured and screened again for the presence of the gene fragments they had earlier tested positive for.

RESULTS

Isolation of rhizosphere bacteria
For cultivar Shepody, no significant differences in numbers of bacterial colonies were observed between the autoclaved and non-autoclaved V4 soil, but significant differences were observed between the autoclaved and non-autoclaved BC soil using PAF and LGI media. For cultivar Yukon Gold, significant differences in numbers of bacterial colonies were observed for the autoclaved and non-autoclaved V4 soils using the PAF and NFM media, but no significant difference was observed for the BC soil (Figure 2.1).

Overall, Shepody had higher numbers of bacterial colonies than Yukon Gold, especially using PAF and NFM media.

Isolation of root endophytes
Lower endophytic colony counts were observed for both Shepody and Yukon Gold, as compared to the rhizosphere counts, ~ logCFU 8 vs ~logCFU 4.5, respectively (Figure 2.2).

For Shepody, no significant differences in counts were observed for any of the media, although a trend was evident for slightly higher counts from the autoclaved soil as compared to the non-autoclaved for both V4 and BC. For Yukon Gold, no significant differences were observed among the soil or the media type.

Acetylene reduction assay (ARA) and PCR-based detection of *nifH* gene fragments in isolates from semi-solid media, and identification of ten nitrogen-fixing bacterial isolates
Rhizosphere suspensions inoculated into semi-solid media yielded more nitrogen-fixing bacteria than the root tissue suspensions, as indicated by ARA (7 vs 3) (Table 2.2). The acetylene reduction by these isolates correlated with PCR detection of the *nifH* gene (Figure 2.3). Eight out of ten isolates retrieved from the semi-solid media came from the NFM medium (Table 2.2). The ten nitrogen-fixing bacteria that tested positive with both PCR screening and ARA were selected for identification by 16S RNA gene sequencing. Sequences were aligned with those present in the relevant databases using the nucleotide-nucleotide blastn search tool from NCBI (http://www.ncbi.nih.gov/) and the sequence match tool of the Ribosomal Database Project II (RDP -http://rdp.cme.msu.edu/). The original sources of the ten ARA/*nifH* positive bacterial isolates and their identity based on closest affiliations are shown in Table 2.3. They showed between 97-100% similarities in the 16S *rRNA* gene sequences of already known nitrogen-fixing bacteria species belonging to three bacterial genera: *Bradyrhizobium, Azospirillum* and *Xanthobacter*.

Table 2.1 List of PCR conditions used for detection of fragments of the biosynthetic genes involved in nitrogen fixation and antifungal antibiotic production.

		PCR conditions						
Primers	**Target**	**Initial Denat.**	**Denat.**	**Ann.**	**Ext.**	**Final Ext.**	**Cycles**	**Reference**
PolF/PolR	*nifH*	95°C 5 min	94°C 30 sec	57°C 30 sec	72°C 1 min	72°C 7 min	35	Poly et al. 2001
PRND1/PRND2	PRN	95°C 2 min	95°C 1 min	67°C 1 min	72°C 2 min	72°C 7 min	35	de Souza et al. 2003
PCa2a/PCa3b	PHZ	94°C 2 min	94°C 1 min	67°C 45 sec	72°C 2 min	72°C 7 min	35	Raaijmakers et al. 1997
B2BF/BPR4	DAPG	95°C 3 min	94°C 1 min	58°C 45 sec	72°C 2 min	72°C 7 min	35	McSpadden Gardener et al. 2001
PHZ1/PHZ2	PHZ	94°C 2 min	94°C 1 min	58°C 45 sec	72°C 2 min	72°C 7 min	35	Delaney et al. 2001

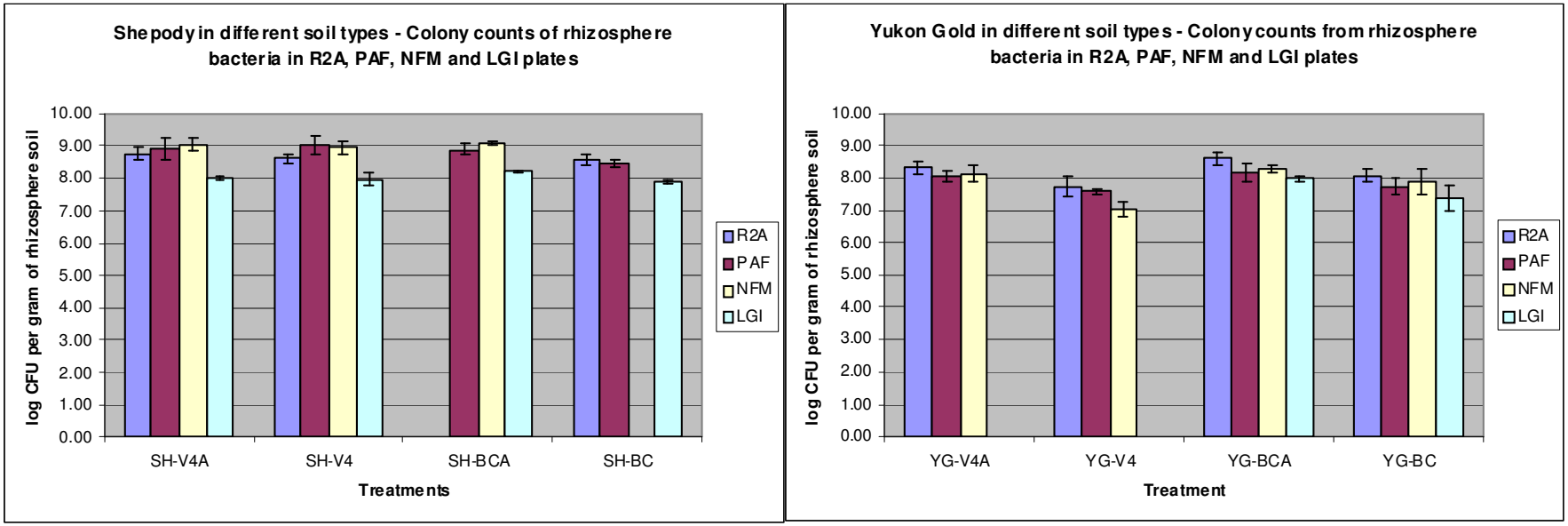


Figure 2.1 Rhizosphere bacterial colony counts of Shepody and Yukon Gold grown in different types of soils

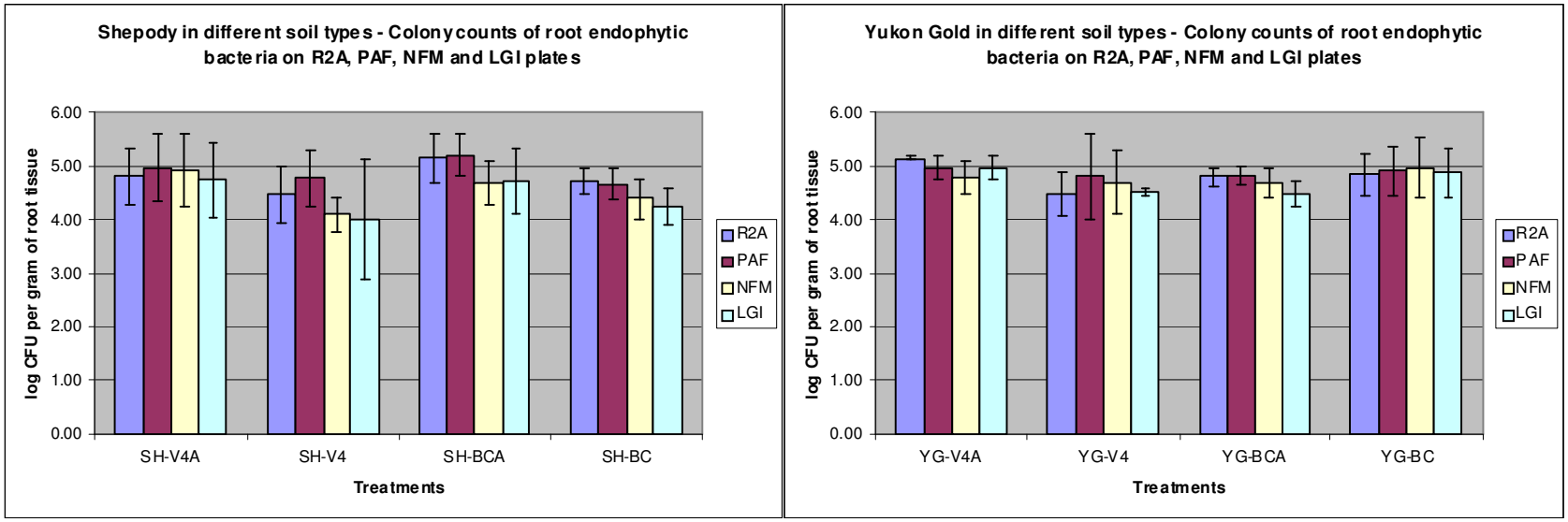


Figure 2.2 Endophytic bacterial colony counts for Shepody and Yukon Gold grown in different types of soils

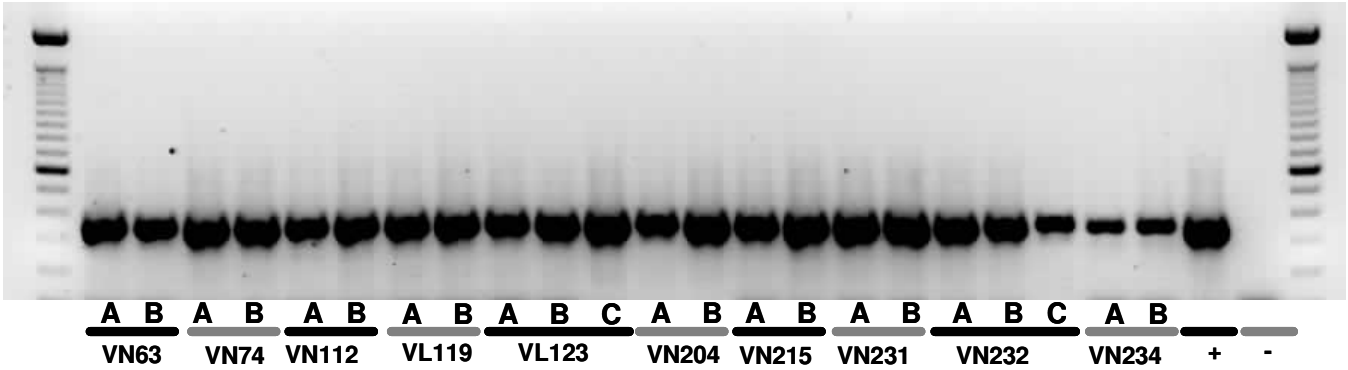


Figure 2.3 PCR detection of *nifH* gene in ARA positive bacterial isolates obtained from semi-solid media inoculated with root tissue and rhizosphere suspensions of Shepody and Yukon Gold from the different soil types.

Table 2.2 Detection of ethylene by gas chromatography in LGI and NFM semisolid media inoculated with rhizosphere and root tissue suspensions from Shepody and Yukon Gold cultivated in the different soil types. Three replicates per treatment were analyzed.

		Shepody	Yukon Gold	
	Soil type	**NFM**	**LGI**	**NFM**
Rhizosphere	V4A	+ + - (3)	- - -	+ + -
	V4	+ - - (2)	- - -	+ - -
	BCA	+ + -	- - -	+ + -(2)
	BC	- - -	- - -	+
Root endosphere	V4A	ND	- - - (2)	+ (1)
	V4	ND	- - -	- - -
	BCA	ND	+ + -	+ - -
	BC	ND	- - -	+ - -

ND, indicates not determined; +, detection of ethylene; -, no detection of ethylene. In parenthesis are numbers of *nifH*-carrying bacterial isolates retrieved from the semi-solid media.

Table 2.3 Origin of the ten ARA/*nifH* positive bacterial isolates and their identification by 16S *rRNA* gene sequencing

Isolate ID	**Potato cultivar**	**Soil type**	**Source of isolation**	**Closest bacterial affiliates**	**% similarity**
VL119	Yukon Gold	V4A	Root endosphere	*Bradyrhizobium japonicum*	99%
VL123B	Yukon Gold	V4A	Root endosphere	*Bradyrhizobium japonicum*	99%
VN112B	Yukon Gold	V4A	Root endosphere	*Azospirillum brasilense*	100%
VN204B	Shepody	V4	Rhizosphere	*Xanthobacter flavus*	100%
VN 215A	Shepody	V4	Rhizosphere	*Azospirillum brasilense*	100%
VN231B	Shepody	V4A	Rhizosphere	*Xanthobacter flavus*	100%
VN232A	Shepody	V4A	Rhizosphere	*Xanthobacter flavus*	100%
VN234C	Shepody	V4A	Rhizosphere	*Azospirillum lipoferum*	97%
VN63	Yukon Gold	BCA	Rhizosphere	*Azospirillum brasilense*	99%
VN74	Yukon Gold	BCA	Rhizosphere	*Azospirillum lipoferum*	100%

PCR-based detection of DNA fragments of genes involved in nitrogen fixation and antifungal antibiotic biosynthesis in bacteria isolated from potato rhizosphere on different types of solid media

The *nifH* gene was detected in all soil types and cultivars but at varying band intensities (Figure 2.4). Shepody tended to enrich the population of nitrogen fixing bacteria compared to Yukon Gold. Detection of the *nifH* gene was higher in autoclaved soils than in field soils, irrespective of cultivar.

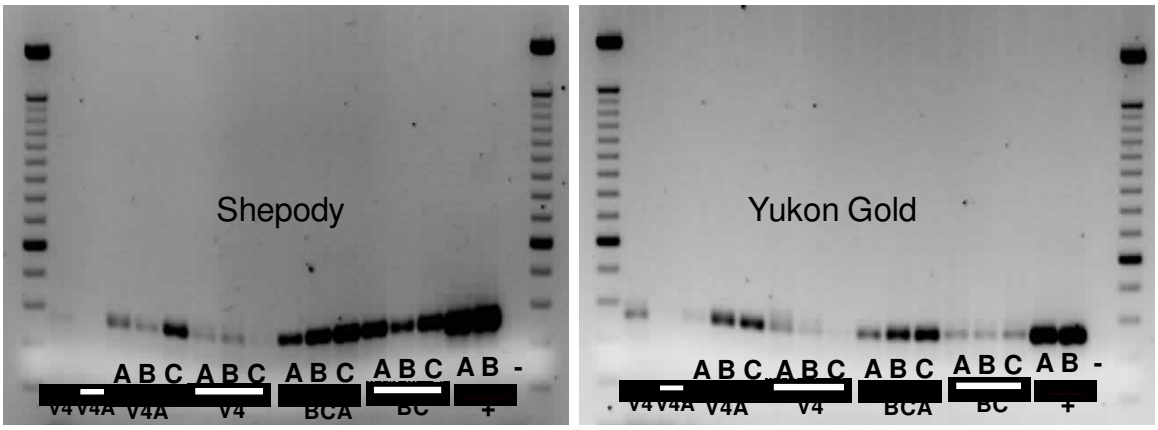


Figure 2.4. PCR detection of *nifH* gene in community DNA extracts obtained from Shepody and Yukon Gold rhizosphere samples

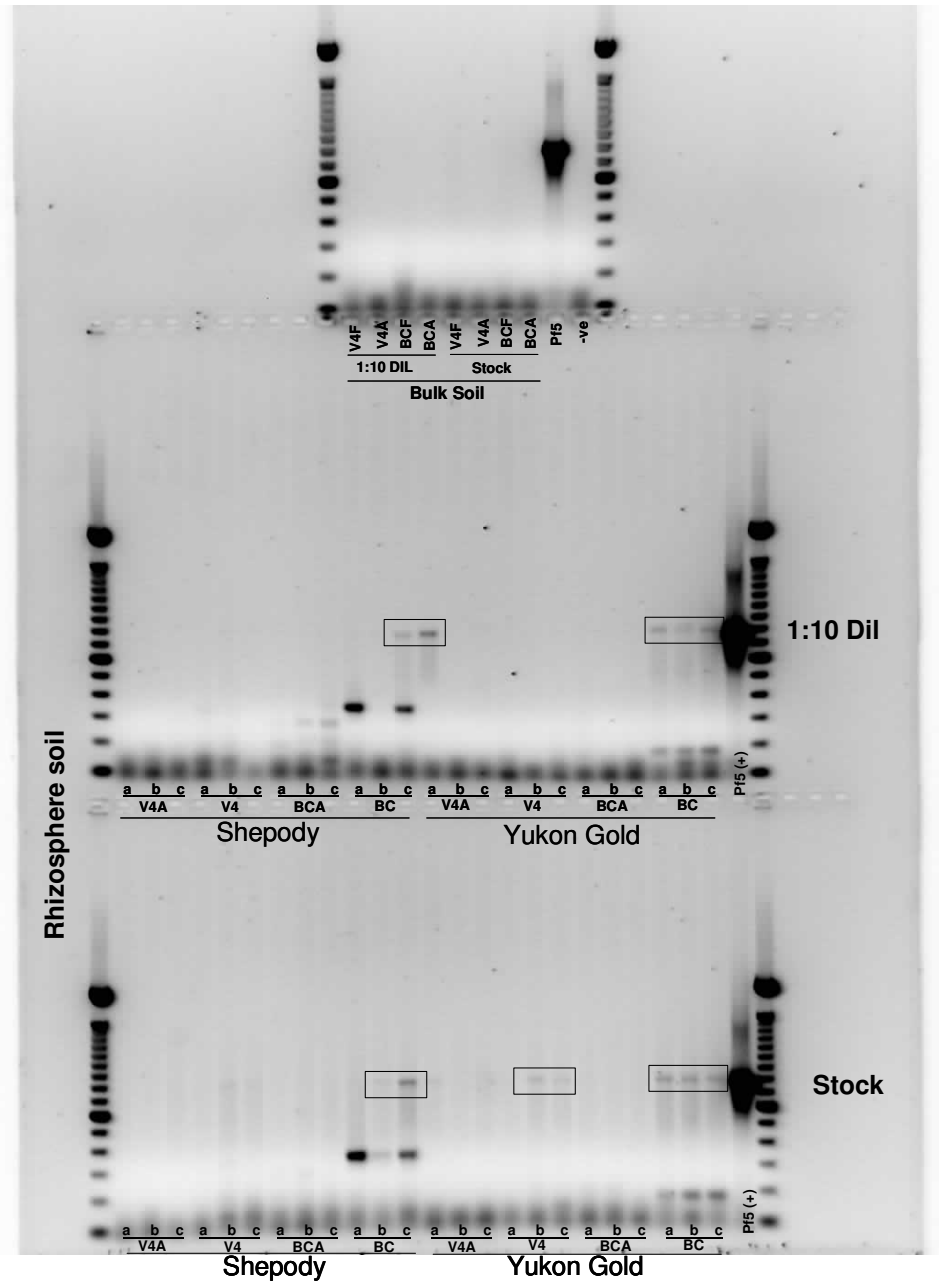


Figure 2.5 PCR detection of *prnD* biosynthetic gene fragments in soil community DNA extracts obtained from Shepody and Yukon Gold rhizosphere samples

No *prnD* biosynthetic gene fragments (786 bp) were detected in the bulk soil, but they were detected in the rhizosphere field soils (Figure 2.5). Yukon Gold favoured the presence of *prnD*-harbouring bacteria; both V4 and BC rhizosphere soils tested positive, compared with BC rhizosphere alone for Shepody

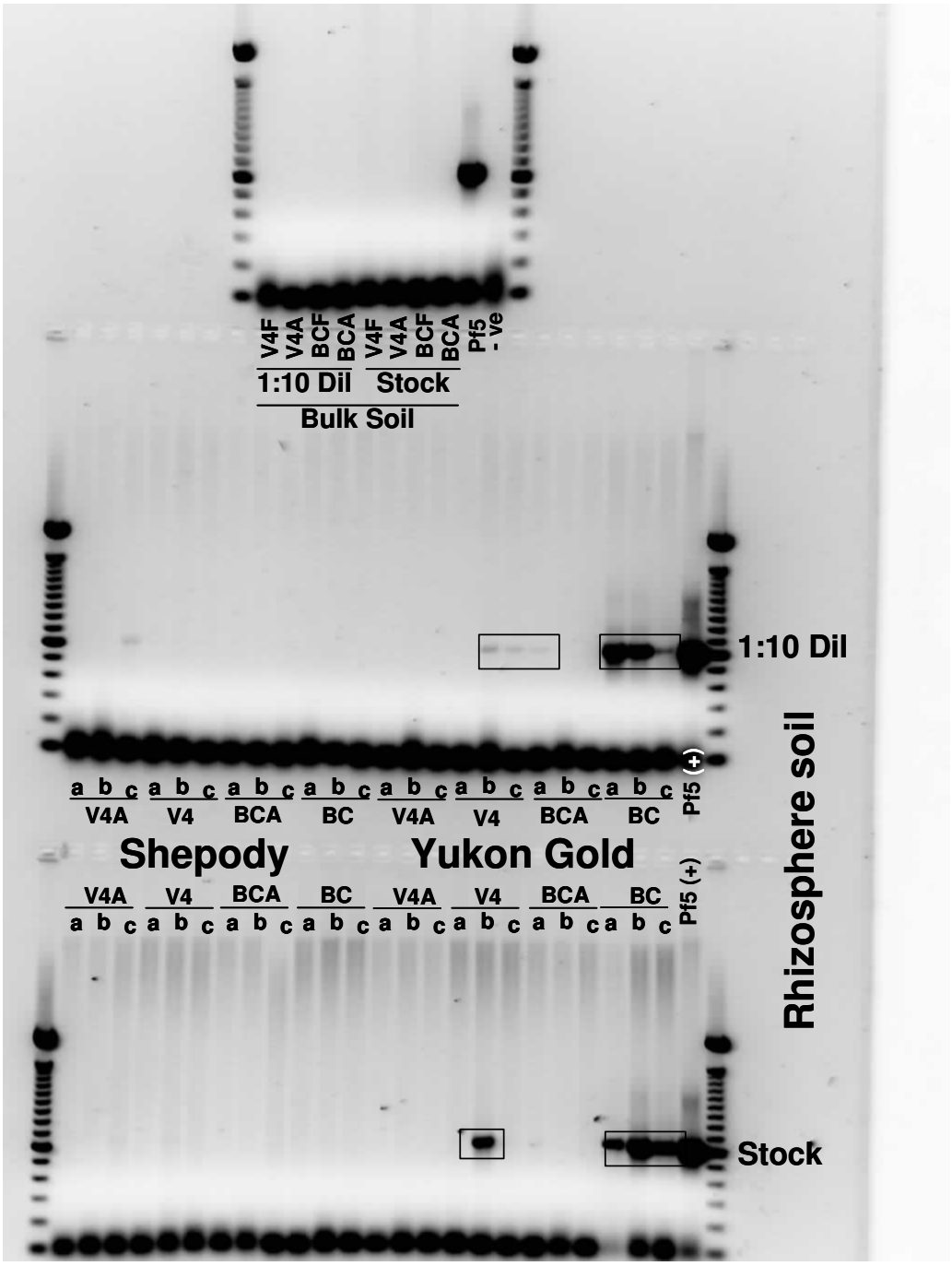


Figure 2.6 PCR detection of *phlD* biosynthetic gene fragments in soil community DNA extracts obtained from Shepody and Yukon Gold rhizosphere samples

No *phlD* gene products were detected in the bulk field soil (Figure 2.6). Detection was better with the 1:10 dilution than with the original stock DNA extracts. The detection of the *phlD* gene fragment (635 bp) was more consistent in the rhizosphere field soils than the autoclaved soils. Yukon Gold had higher and more consistent detection than Shepody.

A total of 1540 isolates from the different variety/soil type/medium combinations were selected for PCR screening. Out of 900 isolates screened for the presence *nifH* and *recA* gene fragments, 73 bacteria containing these gene fragments were detected (Table 2.4). Out of 315 isolates screened for the presence of *phlD*, *prnD*, *pltC* and *phzC/D* gene fragments, 2, 3 and 14 bacteria potentially containing *phlD*, *phzC/D* and *prnD*, respectively, were detected (Table 2.4).

Table 2.4 Origin (soil type and media) of isolates that tested positive to carrying *nifH*, *recA*, *phlD*, *prnD*, *pltC* and *phzC/D* genes using PCR-based detection

Potato cultivars	***Soil type***	***Medium***	***nifH/recA***	***phlD***	***prnD***	***pltC***	***phzC/D***
Shepody	V4A	R2A	3				
		PAF	4				
		NFM	1				
		LGI	2				
	V4	R2A	8				
		PAF	3				
		NFM	1				
		LGI	1				
	BCA	R2A	8				
		PAF	3				
		NFM	7				
		LGI	7				
	BC	R2A	1	ND	1		
		PAF	4	ND	5		
		NFM					
		LGI	-	ND	1		
Yukon Gold	V4A	R2A					
		PAF					
		NFM					
		LGI					
	V4	R2A	6				
		PAF	1				
		NFM	9				
		LGI	4				
	BCA	R2A					
		PAF					
		NFM					
		LGI					
	BC	R2A		ND		ND	ND
		PAF		ND	2	ND	3
		NFM		1	2	ND	*
		LGI		1	4	ND	*

ND – No detection; * - non-specific amplifications under optimization

Cultivar differences

Both cultivars yielded similar numbers of nitrogen-fixing isolates, but Yukon Gold yielded the majority of the isolates that contained antibiotic production genes (Table 2.5). These results demonstrate clear cultivar differences, and suggest that Yukon Gold may harbour many beneficial bacteria, in particular 2,4-DAPG producers.

Table 2.5 Bacterial isolates containing genes involved in nitrogen fixation (*nifH*) and antibiotic production (*phlD*, *prnD*, and *phz)* and their source (ie. from the rhizosphere or root tissue (endosphere) of Shepody and Yukon Gold potatoes grown in different soils). BC and BCA: Prince Edward Island field soil and autoclaved field soil; V4 and V4A: Ontario field soil and autoclaved field soil.

		nifH		***phlD***		***prnD***		***phz***	
Cultivar	**Soil**	**Rhizo**	**Endo**	**Rhizo**	**Endo**	**Rhizo**	**Endo**	**Rhizo**	**Endo**
Shepody	V4A	4	0	-	-	-	-	-	-
	V4	4	18	-	2	-	-	-	-
	BCA	2	1	3	-	-	-	-	-
	BC	0	1	-	-	9	-	-	-
Yukon Gold	V4A	2	5	-	26	-	-	-	-
	V4	3	3	-	-	-	-	-	-
	BCA	8	6	-	-	-	-	3	-
	BC	1	5	1	10	16	1	10	-

Rhizo = rhizosphere, endo = endosphere.

DISCUSSION

The rhizosphere is a hot spot for microbial activity and this was observed from our colony count results, where the rhizosphere had higher bacterial counts than the bulk soil or the endosphere. This was to be expected, as the root exudates associated with the rhizosphere are rich in nutrients making it a more conducive environment for bacteria to thrive than the bulk soil or the endosphere. The endosphere is a niche environment favouring specially-adapted bacteria. These bacteria must find a mode of entry into the plant tissue and have the ability to overcome the plant's natural defence to establish and survive inside the host tissue. The selective PCR screening, targeting specific nitrogen-fixing and antibiotic production genes, demonstrated that the abundance of these specialised bacteria within the endosphere was at least equal to that found in the rhizosphere.

The observation that bacterial numbers were higher on roots grown in autoclaved soil compared with non-autoclaved field soil was unexpected as the autoclaving process would have eliminated heat-sensitive miroorganisms. It is likely the autoclaving process killed the resident microbial population in the soil, as well as releasing some soluble nutrients from the organic matter, thereby creating an energy source for bacteria introduced with the tubers. These bacteria could then rapidly multiply and establish as they had no competition. The R2A medium was selected for its capacity to support the growth of a large bacterial population, especially slower growing bacteria, while the PAF medium is specific for Pseudomonads, and the NFM and LGI media favour the free-living nitrogen fixing diazotrophs. We expected to see higher counts in the R2A across all the treatments, but surprisingly observed equally high numbers on the PAF and NFM plates. Probably these media were not as selective as we thought or there were high numbers of Pseudomonads and diazotrophs among the overall bacterial community, both in the rhizosphere and endosphere, which is unlikely. This needs to be examined further.

As expected, in the acetylene reduction assays the rhizosphere yielded higher numbers of nitrogen-fixing bacteria than the endosphere suspensions. The correlation of ARA to the presence of the *nifH* gene fragment reiterated the earlier established fact that the presence of the gene mostly correlates with its activity (McSpadden Gardener et al. 2001), in this case the biosynthesis of the nitrogenase enzyme and the reduction of acetylene to ethylene. The ten isolates showed between 97-100% similarities to 16S *rRNA* gene sequences of already known nitrogen-fixing bacteria species belonging to three bacterial genera: *Bradyrhizobium, Azospirillum* and *Xanthobacter*. The isolation of higher numbers of diazotrophs from NFM than LGI can be explained by its higher bacterial counts and also as a better choice for selecting diazotrophs. A higher detection of the nitrogenase gene in autoclaved soil compared to non-autoclaved soils suggests the pre-existence or colonization of the tubers/tuber surface (geocaulosphere) by nitrogen fixing bacteria prior to sowing, as the tubers were not surface sterilized before sowing.

The presence of bacteria harboring *phlD* gene fragments of the 2,4-diacetylphloroglucinol biosynthetic cluster and those harboring the *prnD* gene fragment of the pyrrolnitrin biosynthetic cluster were detected only in non-autoclaved field soil. This is a very interesting observation, suggesting that these antibiotic-producing bacteria are not tuber borne but native soil residents. Of the two varieties, Yukon Gold favored the growth of both DAPG and PRN

producers, and Shepody favoured the PRN producers alone. This was not influenced by soil, as Yukon Gold grown in both V4 and BC non-autoclaved soils favoured the detection of both DAPG and PRN producers. We suggest that the more robust plants and root systems of Yukon Gold, observed during the growth period and harvest, enabled the availability of more nutrients in the form of root exudates favouring the growth of these antibiotic-producing bacteria. Also, we propose that there could be a variety specificity correlation for the growth and detection of these antibiotic producers. Of the two soils, BC soil was more favourable to growth of the nitrogen-fixing and antibiotic-producing bacteria, as observed by the high intensity of amplification of the PCR products. This may be due to the chemical composition of the soil, but this was not analyzed.

3. RHIZOSPHERE AND ENDOPHYTIC BACTERIA ASSOCIATED WITH TWO POTATO CULTIVARS: CHARACTERIZATION OF ISOLATES CARRYING THE NIFH AND/OR ANTIBIOTIC SYNTHETIC GENES

INTRODUCTION

In the earlier chapters, we discussed how a total of 1540 bacteria were isolated from two potato cultivars (Shepody and Yukon Gold) and two soil types (BC and V4) on four selective media (R2A, PAF, LGI and NFM). When the 1540 isolates were subjected to PCR screening, the *nifH* gene involved in nitrogen fixation was detected in 63 isolates, but due to problems encountered in culturing some of the isolates, only 58 were successfully retained as stock cultures. Genes involved in antibiotic production were detected in 81 of the isolates: *phlD* (2,4-diacetylphloroglucinol) was present in 42 isolates, *prnD* (pyrrolnitrin) in 26, and *phz* (phenazine) was found in three of the isolates.

This chapter presents further characterization of these bacterial isolates based on nitrogenase enzyme activity, antagonistic activity towards plant pathogens, production of siderophores, hydrolytic enzymes, phosphate solubilizing enzyme and indole acetic acid; BOX-PCR fingerprint grouping and 16S *rRNA* sequencing.

Isolates which showed amplification of the expected fragment size for the *nifH* gene were further screened by acetylene reduction assay (ARA) for the activity of nitrogenase enzyme, indicating that the *nifH* gene is functional. Nitrogenase enzyme, responsible for nitrogen fixation, is also known to reduce acetylene (C_2H_2) to ethylene ($C_2H_{4)}$ which provides a useful assay for the detection and quantification of the N_2-fixation process.

Isolates which showed amplification of the expected fragment sizes for the antibiotic genes were tested for antagonist activity using *in vitro* dual culture competition assays with selected plant pathogen isolates.

Iron is an essential growth element for all living organisms and the scarcity of its bio-available form in soil habitats results in competition (Loper and Henkels**,** 1997). Siderophores, low molecular weight compounds with high iron affinity, are produced by some microorganisms (also by most biocontrol agents and some plant growth promoting rhizobacteria, PGPR) to solubilise and competitively acquire ferric ion under iron-limiting conditions, thereby making iron unavailable to other soil microorganisms which cannot grow for lack of it (Haas and Defago, 2005).

Protein, chitin and s-1,3-glucan are the major structural components of most plant pathogenic fungi. Antagonists invade pathogens by excretion of extracellular enzymes that can lyse pathogen cell walls. Such extracellular enzymes include chitinases, proteases and s-1,3-glucanases.

Production of plant growth hormones, such as auxin, by some plant growth promoting rhizobacteria (PGPR) have been implicated in enhancing plant shoot and root growth. Indole acetic acid is one of the most physiologically active auxins and it is a common product of L-tryptophan metabolism by several microorganisms.

Different combinations of the above mentioned mechanisms as well as production of phosphate solubilizing enzyme have been reported to be responsible for biocontrol activities or plant growth promotion in some rhizobacteria strains. Thus, our collection of nitrogen fixing bacteria and antibiotic positive bacteria were screened for these mechanisms.

MATERIALS AND METHODS

Acetylene reduction assay (ARA)

The 58 *nifH* positive isolates in the collection were subjected to acetylene reduction assay (ARA) in order to determine the activity of the nitrogenase enzyme. The isolates were grown in semi-solid CCM media in vials and were incubated for five days at 28°C. After three days of incubation, 3.5 mL air (10% of head space) was removed from the vials and replaced with 3.5 mL of acetylene, followed by further 3-5 days incubation at 28°C. The gas mixtures in the vials were then removed and analysed using gas chromatography for the reduction of acetylene to ethylene, indicating activity of the *nifH* gene.

Determination of *in vitro* antagonistic activity towards plant pathogens

In vitro antagonistic potential of 2,4-DAPG or pyrrolnitrin or phenazine positive isolates (81 in total) towards plant pathogens was determined in dual culture assays. The isolates were screened against five different plant pathogens that are known to cause disease in potato and other crop plants. The pathogens were *Fusarium oxysporum*, *Rhizoctonia solani*, *Pythium ultimum*, *Streptomyces scabies* and two strains of *Verticillium dahliae*. *F. oxysporum* and *V. dahliae* are causal agents of wilt in potato while *S. scabies* causes common scab disease of potato tubers. *R. solani* causes black scurf on tubers and cankers on underground stems and stolons of potato plants. *P. ultimum* causes seedling damping off and tuber rot of potatoes.

Activity against *S. scabies* or *V. dahliae* was determined by spreading spore suspensions of the pathogens on YME and Czapek dox agar plates respectively; after drying, bacterial cells were streaked on the plates. Inhibition against other pathogens was tested on Waksman agar (WA). Four 6-mm agar disks containing grown mycelia of F. *oxysporum or R. solani* were placed approximately 3.5 cm (distance) apart on a WA dish. Bacterial isolates were streaked between the agar disks. Zones of inhibition around the isolates were measured after 5-7 days of incubation at 20°C. All isolates were tested in two replicates on different plates and the tests were carried out three times. The *nifH* positive isolates were not screened for *in vitro* inhibitory activity against phytopathogens.

Screening of bacterial isolates for production of siderophores, cell-wall degrading enzymes and phosphate solubilizing enzyme

The ability of the antagonistic bacterial isolates to produce siderophores when grown under Fe^{3+}-limiting conditions was evaluated in a Chrome azurol S (CAS) plate assay (Schwyn and Neilands, 1987). Fresh cultures of the isolates were streaked on the blue coloured CAS plates. When Fe^{3+} is removed from the chrome azurol S complex by high-affinity siderophores, the medium changes colour from blue to orange. The occurrence of orange halos around colonies was determined after 48 - 72 hours of incubation at 28^{o}C.

The isolates were screened for the production of hydrolytic enzymes (cell-wall degrading enzymes) in plate assays. β -glucanase activity was tested using one-tenth volume of TSA containing 0.1% chromogenic azurine-dyed, cross-linked (AZCL) substrates (Megazym). Formation of blue haloes was recorded after 5 days of incubation (Berg et al., 2002). Protease activity (casein degradation) was determined by zone-clearing in skim milk agar (50 ml of sterilized skim milk mixed at 55°C with 50 ml of one-fifth volume of Trypticase Soy Broth and 4% agar) after 5 days of incubation at 28°C.

Production of phosphate solubilizing enzyme was determined on NBRIP agar (Nautiyal, 1999); the clearing zone around the bacterial cell was recorded after 7 days of incubation at 28°C.

Screening of bacterial isolates for production of indole acetic acid (IAA)
A colorimetric method was employed to detect the production of indole acetic acid among our collection of nitrogen fixing bacteria as well as antibiotic positive isolates. Briefly, each bacterial isolate was inoculated in the respective medium of growth (LB, CCM or YEM) with 2 g tryptophan per litre and incubated for 2 – 3 days depending on rate of growth. Cultures were centrifuged at 13,000 rpm for 2 mins and 3 ml of the cell free supernatant was mixed with 3 ml of Salkwoski's reagent (50 ml, 35% perchloric acid; 1 ml 0.5 $FeCl_3$). After two hours of incubation at room temperature, the optical density of mixtures with pink color development, indicating IAA production, was measured by spectrophometer at 530 nm wavelength. The concentration of IAA was determined using a standard graph generated from the optical densities of different known concentrations of indole acetic acid (5, 10, 15, 20, 25, 30, 60 ppm).

BOX-PCR fingerprinting
To determine genetic diversity among our collection of free-living nitrogen-fixing and antibiotic-producing bacterial isolates, interspersed repetitive DNA sequences present in the bacterial genome were analysed by BOX-PCR fingerprinting technique. BOX profiles for most of the isolates were generated by PCR amplification of extracted genomic DNA with BOXAIR primer. Amplification reactions were performed in volumes of 50 µL, containing 3 µM of BOXAIR primer, 250 µM of each dNTPs, 1 X PCR reaction buffer, 3 mM $MgCl_2$, bovine serum albumin, 1 unit of ampliTaq DNA polymerase (Bioscience), 1 µl of genomic DNA was added as template. Amplification was performed in a Biorad Thermal Cycler with an initial denaturation step of 7 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 53°C and 8 min at 65°C, and a final elongation step of 15 min at 65°C. 12 µl of the amplified products were loaded onto a 1.5% agarose gel in TAE buffer, post-stained with GelRed (Biotium, CA) and visualized under UV light in a Geldoc. Three lanes loaded with 1-Kb DNA ladder (Invitrogen) at the two extreme ends and the middle of gel served as standards in each electrophoresis gel.

BOX-PCR profiles were compared using the software package GelCompar II version 5.6 (Applied Maths, Kortrijk, Belgium). Cluster analysis generated from the profiles was analysed. Isolates with similar BOX-PCR fingerprints above 70% similarity according to the cluster analysis were further checked visually and considered to be the same strain, thus were assigned to the same group.

16S *rRNA* gene sequencing
16S *rRNA* PCR products amplified from genomic DNA of representative isolates selected from each BOX group were sequenced and aligned with those present in the relevant databases using the nucleotide-nucleotide blastn search tool from NCBI (http://www.ncbi.nih.gov/) and the sequence match tool of the Ribosomal Database Project II (RDP - http://rdp.cme.msu.edu/).

RESULTS AND DISCUSSION

Of the 58 *nifH* positive isolates tested by ARA, 47 were able to convert acetylene to ethylene. Of the 81 antibiotic positive isolates screened, 30 isolates showed inhibitory activity towards at least one of the six tested phytopathogens; 26 isolates were active against *S. scabies* (Fig. 3.1), 18 against *R. solani*, 13 against *F. oxysporum*, 17 against *P. ultimum* while 22 and 25 isolates showed inhibitory activity towards *V. dahliae* Vd1 and Vd2 respectively (Table 3.1). Isolates with activity towards more than one or all pathogens were found. Ten of these isolates showed broad activity against all six pathogens, five of the isolates were active towards five pathogens while another five isolates inhibited four pathogens (Table 3.1). The cultivar Yukon Gold yielded the most antagonists (24 out of the 30 isolates), 17 of which originated from BC soil and 7 from V4 soil. Approximately 20 out of the 30 antagonists were isolated from endophytic tissue of potato plants, of which 17 were from Yukon Gold in both of the two soil types. The majority of these Yukon Gold inhabiting bacteria contained the gene involved in biosynthesis of 2,4-diacetylphloroglucinol *(phlD)*. The phenazine gene (*Phz*) was detected in only five of the antagonists; surprisingly again they were isolated from Yukon Gold. Only three of the antagonists harboured the *nifH* gene, and again they were from Yukon Gold. Potential pyrrolnitrin producers were also found in Yukon Gold. Only six of the antagonists were isolated from Shepody, of which five were carrying pyrrolnitrin biosythethic gene (*Prn*).

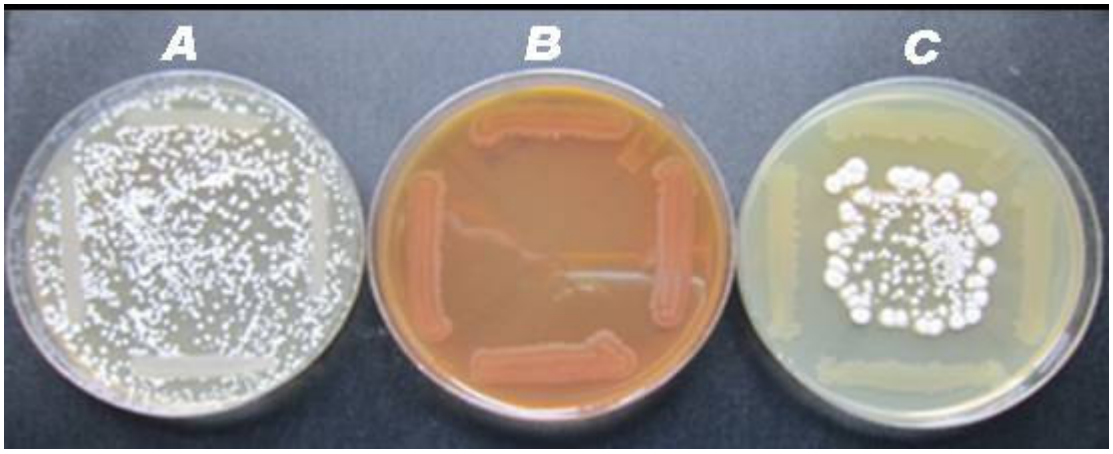
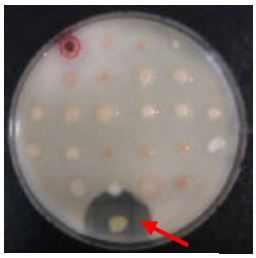
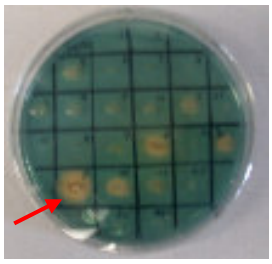


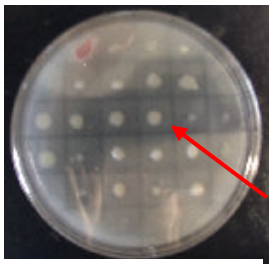
Figure 3.1 Dual culture assay to determine inhibition of *Streptomyces scabies* by bacteria isolated from rhizosphere and root endophytic tissues of potato plants: A - no inhibition, B - complete inhibition, C- moderate inhibition



Protease activity
(clear halo)



Siderophore production
(haloes with yellow colouration)



Phosphatase activity
(clear haloes)

Figure 3.2 Plate assays for enzymatic activity and siderophore production

The siderophore-, enzyme- and indole acetic acid-assays showed that siderophores, protease and phosphatase were prominently produced among the antagonistic isolates (Table 3.1, Fig.3.2), although these traits were also found in some antibiotic positive bacteria that did not demonstrate antagonistic activity to any of the tested pathogens. None of the antagonists could degrade chitinase and only two were positive for glucanase. Less than half (11 out of 30) of the antagonists produced indole acetic acid (IAA) under our test conditions, and only two of these were also 2,4-DAPG producers.

Table 3.1 Characteristics of isolates containing antibiotic biosynthetic genes

Isolate ID	BOX Group	Antagonistic activity						Enzyme activity					PCR detection genes		Partial 16S rRNA gene sequencing (closest hit and % similarity)
		SS	RS	FO	PU	VD1	VD2	Sid	Pro	Gl	PH O	IAA	Antibiotic	*nifH*	
BC11	1	-	-	-	-	-	-	+	-	-	+	+	Phz	-	
BC 3		-	-	-	-	-	-	+	-	-	-	+	PRN	-	
BC 41	2	-	-	-	-	-	-	+	-	-	+	+	PRN	-	
BC Pf5	3							+		-	+	+	DAPG, PRN	-	*Pseudomonas fluorescens Pf-5*
BC 95	4	-	-	-	-	-	-	+	+	+	-	+	PRN	-	
BC 10	5	-	-	+	-	-	-	-	-	-	+	+	Phz	-	
BC 9	6	-	-	-	-	-	-	-	-	-	+	+	Phz	-	
BC 98	7	-	-	-	-	-	-	+	+	-	-	+	PRN	-	
BC 21	8	-	-	-	-	-	+	+	+	-	-	+	PRN	-	*Xanthomonas retroflexus (100%)*
BC 107	9	-	-	-	-	-	-	+	-	-	-	+	PRN	-	
BC 109		-	-	-	-	-	-	+	-	-	-	+	PRN	-	
BC 22	10	-	n/a	n/a	n/a	n/a	n/a	+	n/a	-	-	-	PRN	-	
BC 23		-	n/a	n/a	n/a	n/a	n/a	-	n/a	-	-	-	PRN	-	
BC 31		-	n/a	n/a	n/a	n/a	n/a	n/a	n/a	-	-	+	PRN	-	
BC 88	11	-	-	-	-	-	-	+	+	-	-	+		-	
BC 111	12	-	-	-	-	-	-	-	-	-	+	+	PRN	-	
BC 102	13	++	-	-	-	-	-	+	-	-	+	+	PRN	-	*Pseudomonas putida (100%)*
BC 103		++	-	-	-	-	-	+	-	-	+	+	PRN	-	
BC 113	14	-	-	-	-	-	-	+	-	-	+	+	PRN	-	
BC 114		-	-	-	-	-	-	+	-	-	+	+	PRN	-	
BC 80		-	-	-	-	-	-	+	-	-	+	+	DAPG	+	
BC 17	15	-	-	-	-	-	-	-	-	-	-	-	Phz	-	
BC 96	16	-	-	-	-	-	-	+	-	-	+	+	PRN	-	
BC 97		-	-	-	-	-	-	+	-	-	+	+	PRN	-	
BC 93A	17	-	-	-	-	-	-	+	-	-	-	+	PRN	-	
BC 94	18	-	-	-	-	-	-	n/a	-	-	-	+	PRN	-	
BC 101	19	-	-	-	-	-	-	+	-	-	+	+	PRN	+	
BC 36	20	NPG	+	+	+	+++	+++	+	+	-	+	-	DAPG	-	*Pseudomonas brassicacearum (99%)*
BC 38		NPG	+	+	+	+++	+++	+	+	-	-	-	DAPG	-	
BC 34	21	NPG	+	+	+	+++	+++	+	+	-	-	-	DAPG	-	
BC 64	22	NPG	-	-	+	+++	+++	+	+	-	+	-	DAPG	-	
BC 66		+++	-	-	++	NPG	+++	+	+	-	+	-	DAPG	-	*P. fluorescens (100%)*
BC 60		+++	-	-	+	++	+++	+	-	-	+	+	DAPG	-	

BC 67		+++	-	-		NPG	+++	+	+	-	+	-	DAPG	-	
BC 37		NPG	+	+	+	+++	+++	+	+	-	+	-	DAPG	-	
BC 33		NPG	+	+	+	+++	+++	+	+	-	+	-	DAPG	-	
BC 35		NPG	+	+	+	+++	+++	+	+	-	+	-	DAPG	-	
BC 32		NPG	+	+	++	+++	++	+	+	-	+	-	DAPG	-	*P. fluorescens (100%)*
BC 16		+++	+	+	++	+++	+++	+	+	-	+	-	DAPG	-	*P. fluorescens (100%)*
BC 39		NPG	+	+	+	+++	+++	+	+	-	+	-	DAPG	-	
BC 40		NPG	+	+	+	+++	+++	+	+	-	+	-	DAPG	-	*Pseudomonas chlororaphis (100%)*
BC 90	23	+++	++	++	-	+	++	+	+	+	-	+	PRN	-	*Bacillus subtilis (100%)*
BC 99	24	-	-	-	-	-	-	-	-	+	-	-	PRN	-	
BC 110	25	-	-	-	-	-	-	-	-	-	-	-	PRN	-	
BC 7	26	++	-	-	-	-	+	-	-	-	+	-	Phz	+	*Enterobacter amnigenus (100%)*
BC 8		+++	-	-	-	-	+	-	-	-	+	-	Phz	+	
BC 2	27	-	-	-	-	-	-	-	-	+	-	-	PRN	-	
BC 44	28	-	-	-	-	-	-	-	-	-	-	+	PRN	-	
BC 45A			-	-	-	-	-	+	-	-	-	+	PRN	-	
BC 1		-	-	-	-	-	-	-	-	-	-	+	PRN	-	
BC 83	29	-	+	-	-	NPG	NPG	+	+	-	+	+	DAPG	-	
BC 87		++	+	-	-	NPG	NPG	+	+	-	+	-	PRN	-	*Pseudomonas fluorescens (100%)*
BC 19	30	+++	++	-	+	++	+++	+	+	-	+	-	Phz	-	*Pseudomonas fluorescens (98%)*
BC 24B	31	++	+	-	+	+	+	+	+	-	+	+	PRN	-	*Pseudomonas fluorescens (100%)*
BC 18	32	+	-	-	-	-	-	+	-	-	-	+	Phz	-	*Pseudomonas putida (100%)*
BC 13	33	-	-	-	-	-	-	-	+	+	-	+	Phz	-	
BC 14		-	-	-	-	-	-	-	+	-	-	+	Phz	-	
BC 4		-	-	-	-	-	-	-	+	+	-	+	PRN	-	
BC 6		-	-	-	-	-	-	-	+	+	-	+	PRN	-	
BC 25		+	+	-	+	++	++	+	+	-	+	+	PRN	-	*Arthrobacter niigatensis (99%)*
BC 27		-	-	-	-	-	-	n/a	-	+	-	+	PRN	-	
BC 24A		-	-	-	-	-	-	-	-	+	-	+	PRN	-	
BC 26		-	-	-	-	-	-	n/a	-	+	-	+	PRN	-	
BC 15	34	-	-	-	-	-	-	-	+	-	+	-	DAPG	-	*Pseudomonas brassicacearum (99%)*
BC 50	35	-	-	+	-	-	-	n/a	-	-	-	-		-	*Streptomyces yanglinensis (97%)*
BC 43	36	-	-	-	-	-	-	n/a	-	-	-	-	PRN	-	
BC 46		-	-	-	-	-	-	+	-	-	-	-	PRN	-	
BC 45B			-	-	-	-	-	+	-	-	-	-	PRN	-	
BC 12	37	-	-	-	n/a	-	n/a	-	-	-	-	+	Phz	-	
BC 42	38	-	-	-	-	-	-	+	-	-	-	-	PRN	-	
BC 53	39	-	-	-		-	-	-	+	-	+	-	PRN	-	
BC 81	40	-	-	-	-	-	-	+	+	-	-	+	DAPG	-	
BC 91	41	++	++	-	-	++	++	-	+	+	-	-	PRN	+	*Paenibacillus polymyxa (100%)*
BC 89B	42		-	-	-		-	+	+	+	-	-	PRN	-	
BC 89A		-	-	-	-	-	-	+	+	+	-	+	PRN	-	
BC 92	43	-	-	-	-	-	-	+	-	-	-	-	PRN	-	
BC 48A			-	-	-	-	-	+	-	+	-	+	DAPG	-	
BC 47	44	-	-	-	-	-	-	-	-	-	-	-	PRN	-	
BC 49	45	-	-	-	-	-	-	-	-	-	-	-	DAPG	-	
BC 5	46	-	-	-	-	-	-	-	-	-	-	+	PRN	-	
BC 51	47	-	-	-		n/a	-	-	-	-	-	-	PRN	-	
BC 20	48	+++	+	-	++	+++	+++	+	+	-	+	+	PRN	-	*Pseudomonas putida (99%)*
BC 48B			-	-	-	n/a	-	+	-	-	-	+	DAPG	-	

Protease- Pro, Glucanase-Glu, Phosphatase-PHO siderophores –Sid, indole acetic acid –IAA, 2,4-diacetylphloroglucinol- DAPG, pyrrolnitrin - PRN, phenazine -Phz, nitrogenase reductase – *nifH,* BOX group based on 70% similarity cut-off, + indicates positive, - negative. For antagonistic activity zone inhibition of 0.1-0.5 cm indicated by +, 0.6 -1 cm = ++, greater than 1 cm = +++, NPG = complete inhibition of pathogen, SS = *S. scabies*, RS = *R. solani*, FO = *F. oxysporum*, PU= *P. ultimum*, VD1 & VD2= *V. dahliae,* n/a = not available.

Table 3.2 Characteristics of *nifH* positive isolates

Isolate ID	Potato cultivar	Soil Type	Plant part	PCR detection of *nifH* gene	ARA	IAA	Pro	Sid	Glu	PHO	Partial 16S rRNA gene sequencing (closest hit and % similarity)
VL 119	YG	V4A	RE	+	+	-	-	-	-	-	*Bradyrhizobium japonicum (99%)*
VL 123B	YG	V4A	RE	+	+	-	-	NG	-	-	*Bradyrhizobium japonicum (99%)*
VN 112B	YG	V4A	RE	+	+	+	-	-	-	-	*Azospirillum brasilense (100%)*
VN 204B	SH	V4	RHZ	+	+	-	-	-	-	-	*Xanthobacter flavus (100%)*
VN 215A	SH	V4	RHZ	+	+	+	-	-	-	-	*Azospirillum brasilense (100%)*
VN 231B	SH	V4A	RHZ	+	+	-	-	-	-	-	
VN 232A	SH	V4A	RHZ	+	+	-	-	-	-	-	*Xanthobacter flavus (100%)*
VN 234c	SH	V4A	RHZ	+	+	+	-	+	-	-	
VN 63	YG	BCA	RHZ	+	+	+	-	-	-	-	
VN 74	YG	BCA	RHZ	+	+	+	-	-	-	-	*Azospirillum lipoferum (100%)*
Rhz L3	SH	BCA	Rhz	+	+	+	-	-	-	-	*Bradyrhizobium liaoningense (98%)*
Rhz L33	SH	BCA	Rhz	+	+	+	-	-	-	-	
Rhz P17A	SH	V4	Rhz	-	+	+	-	-	-	-	*Brevundimonas bullata (100%)*
Rhz R40	SH	V4	Rhz	+	-	+	-	N/A	N/A	N/A	*Stenotrophomonas humi (100%)*
Rhz P14A	SH	V4A	Rhz	+	+	-	-	NG	+	-	
Rhz P14C	SH	V4A	Rhz	+	+	-	-	+	-	-	*Paenibacillus borealis (97%)*
Rhz L15	YG	BC	Rhz	+	-	+	-	NG	NG	+	
Rhz L12	YG	BCA	Rhz	+	+	+	-	NG	-	-	
Rhz L30	YG	BCA	Rhz	+	+	-	-	NG	-	-	
Rhz L34	YG	BCA	Rhz	+	+	-	-	NG	-	-	*Bradyrhizobium japonicum (99%)*
Rhz L39A	YG	BCA	Rhz	+	+	+	-	+	-	-	*Stenotrophomonas humi (100%)*
Rhz L39B	YG	BCA	Rhz	-	-	+	N/A	N/A	N/A	N/A	
Rhz L44	YG	BCA	Rhz	+	+	-	-	NG	-	-	
Rhz L3A	YG	V4A	RHZ	-	+	-	-	NG	+	-	
Rhz L3B	YG	V4	Rhz	+	+	-	-	-	+	-	*Chitinophaga soli (86%)*
Rhz N15A	YG	V4	Rhz	-	+	+	-	-	-	-	
Rhz N15B	YG	V4	Rhz	+	+	+	-	-	-	-	*Variovorax boronicumulans/paradoxus (96%)*
Rhz P8	YG	V4	Rhz	+	+	+	-	+	-	+	*Obesumbacterium proteus (93%)*
Rhz	YG	V4A	Rhz	+	+	+	-	-	-	-	

L18B											
RE L19	SH	BC	RE	+	-	+	N/A	N/A	N/A	N/A	
RE R28	SH	BCA	RE	+	-	+	-	NG	+	-	
RE L31	SH	BCA	RE	+	-	-	-	NG	-	-	
RE L33	SH	BCA	RE	+	+	-	-	NG	-	-	*Bradyrhizobium japonicum (99%)*
RE L37	SH	BCA	RE	+	+	-	N/A	N/A	N/A	N/A	
RE L38	SH	BCA	RE	-	+	+	-	NG	-	-	*Bradyrhizobium japonicum (100%)*
RE L40	SH	BCA	RE	+	+	+	-	NG	-	-	*Bradyrhizobium betae (97%)*
RE L7	SH	BCA	RE	+	+	-	-	NG	-	-	
RE P12	SH	V4	RE	+	+	+	-	+	-	+	*Paenibacillus polymyxa (98%)*
RE R19	SH	V4	RE	+	+	+	-	+	-	+	*Rhizobium radiobacter (99%)*
RE R22	SH	V4	RE	+	-	+	-	+	-	+	
RE R32	SH	V4	RE	+	-	+	-	+	-	+	*Rhizobium radiobacter (100%)*
RE R33	SH	V4	RE	+	+	+	-	+	-	+	
RE L8	YG	BC	RE	+	+	+	-	+	-	+	*Rhizobium radiobacter (100%)*
RE R10	YG	BC	RE	+	+	+	-	+	-	+	
RE R15	YG	BC	RE	-	+	+	-	+	-	+	*Rhizobium radiobacter (100%)*
RE R2	YG	BC	RE	-	+	+	-	+	-	+	
RE R7	YG	BC	RE	+	+	+	-	+	-	+	*Rhizobium radiobacter (100%)*
RE L21	YG	BCA	RE	+	+	-	-	NG	-	-	*Paenibacillus borealis/Bradyrhizobium betae (95%)*
RE L29	YG	BCA	RE	+	+	-	-	NG	-	-	
RE L33	YG	BCA	RE	+	-	-	-	NG	-	-	
RE L42	YG	BCA	RE	+	+	-	-	-	-	-	*Achromobacter xylosoxidans (99%)*
RE R22	YG	BCA	RE	+	+	-	N/A	N/A	N/A	N/A	
RE R29	YG	BCA	RE	-	-	+	-	+	-	+	
RE P5	YG	V4	RE	-	+	-	-	+	-	-	*Mycobacterium confluentis (95%)*
RE R14	YG	V4	RE	-	-	-	N/A	N/A	N/A	N/A	
RE R35	YG	V4	RE	-	+	+	-	+	NG	+	*Ensifer adhaerens (100%)*
RE N9	YG	V4A	RE	+	+	-	-	NG	-	-	*Bradyrhizobium canariense (97%)*
RE R17	YG	V4A	RE	-	+	+	+	NG	+	-	

SH- Shepody, YG-Yukon Gold, BCA- autoclaved BC soil, V4A-autoclaved V4 soil, RE- root endophytic tissue, Rhz- rhizosphere, ARA- acetylene reduction assay, IAA-Indole acetic acid, Sid - siderophores, Glu-glucanase PHO – phosphatase, + positive activity/production, - negative activity/production, NG- no growth, N/A-not available.

Table 3.2 shows characteristics of the 58 potential free-living nitrogen-fixing bacteria in our collections. Approximately 63 *nifH* positive bacteria were isolated initially, but were reduced to 58 due to problems encountered with growing 6 of the isolates. Of the 58 *nifH* positive bacteria which were isolated from the two soils, 34 were associated with the cultivar Yukon Gold while 24 isolates were associated with cultivar Shepody. Activity of the nitrogenase gene, as determined by the reduction of acetylene gas to ethylene, was detected in 47 of the 58 isolates. Protease activity was rare among the *nifH* positive isolates, with only one positive isolate. Siderophores, phosphatase and glucanase production were found in 17, 14 and 5, respectively, of the tested isolates.

Cluster analysis generated from the BOX-PCR profiles showed that the collection of antibiotic isolates are quite diverse as the 81 antibiotic positive isolates could be divided into 48 groups (Table 3.1) using 70% similarity cut–off. Sixteen of the groups contained at least one bacterium with antagonistic activity. Eighteen of the antagonistic isolates were selected for 16S *rRNA* gene analysis and could be assigned to 10 bacteria species: *Pseudomonas fluorescens, P. brassicacearum, P. chlororaphis, P. putida, Xanthomonas retroflexus, Bacillus subtilis, Enterobacter amnigenus, Arthrobacter niigatensis* and *Paenibacillus polymyxa.*

BOX group 22, which was the largest group, contained 11 isolates originating from Yukon Gold grown in both V4 and BC soils. All isolates in this group are root endophytes, tested 2,4-DAPG positive and showed multiple antagonistic activities to at least four of the tested pathogens. 16S *rRNA* gene sequence analysis of four members of this group demonstrated 100% similarity to either *P. fluoresecens* or *P. chlororaphis*.

BOX group 33 was the second largest group, containing 8 isolates, all from Yukon Gold and BC soil. Six of the isolates were positive to pyrrolnitrin but only one was active against five of the tested pathogens. The one isolate sequenced from this group had 99% similarity to *Arthrobacter niigatensis*. Other BOX groups consisted of 1-3 isolates.

Isolates carrying the *nifH* gene were also quite diverse. The 27 isolates selected for 16S *rRNA* gene sequencing could be assigned to 13 different bacteria genera (Table 3.2). Several of these genera contain species known to fix nitrogen such as *Bradyrhizobium, Azospirillum, Xanthobacter, Stenotrophomonas Paenibacillus, Rhizobium, Achromobacter, Mycobacterium* and *Ensifer*. However, several isolates aligned with the bacteria genera *Brevundimonas, Chitinophaga, Variovorax* and *Obesumbacterium* which have not previously been reported to contain any members with nitrogen fixing potential.

4. ASSESSING THE GROWTH PROMOTING POTENTIAL OF FREE-LIVING NITROGEN-FIXING BACTERIAL ISOLATES ON POTATOES IN GLASSHOUSE BIOASSAYS

INTRODUCTION

This chapter reports on three greenhouse experimental trials aimed at assessing the growth promoting potential of selected nitrogen-fixing bacterial isolates on potato grown in pasteurized and non-pasteurized field soils with or without fertilizer amendments. Ten isolates were selected from our collection of free living nitrogen fixing bacterial isolates for testing in these bioassays (Table 4.1).

Table 4.1 Characteristics of the 10 free-living nitrogen fixing bacteria isolates selected for greenhouse experiments

Isolate ID	Source of isolation			Closest bacterial affiliates	IAA	Siderophores
	Potato cultivar	Soil type	Plant part	16S *rRNA* gene sequencing (% similarity)		
L38	Shepody	BCA	RE		+	NG
NV215A	Shepody	V4	Rhz	*Azospirillum brasilense* (100%)	+	-
P17A	Shepody	V4	Rhz		+	-
VL123B	Y. Gold	V4A	RE	*Bradyrhizobium japonicum* (99%)	ND	NG
VN204B	Shepody	V4	Rhz	*Xanthobacter flavus* (100%)	-	-
VN232A	Shepody	V4A	Rhz	*Xanthobacter flavus* (100%)	-	-
VN 74	Y. Gold	BCA	Rhz	*Azospirillum lipoferum* (100%)	+	-
VN112B	Y. Gold	V4A	RE	*Azospirillum brasilense* (100%)	+	-
VN119	Y. Gold	V4A	RE	*Bradyrhizobium japonicum* (99%)	-	-
VN234C	Shepody	V4A	Rhz	*Azospirillum lipoferum* (97%)	+	+

Protease, glucanase, and phosphatase activity was not detected in any of the selected isolates

EXPERIMENT 1: EFFECT OF TUBER INOCULATION WITH POTENTIAL GROWTH PROMOTING ISOLATES ON PLANT GROWTH

MATERIALS AND METHODS

Four identified isolates of free-living nitrogen-fixing bacteria (*Azospirillum lipoferum*, NV74 & VN234C; *Bradyrhizobium japonicum*, VL123B; *Xanthobacter flavus*, VN232A), two from Yukon Gold and two from Shepody, were chosen to be assessed for their ability to promote the growth of potato in a pot trial. One unidentified isolate (L38) was also included, as well as one well-characterized free-living nitrogen-fixing bacterium (*Gluconacetobacter azotocaptans* DS1) isolated from corn plants in one of our previous studies.

Three day old cultures of each bacterial isolate were used to inoculate irradiated peat obtained from Becker Underwood (Saskatoon, Canada). The peat was used as an inoculant carrier to enhance delivery and adhesion of the bacterial cells on the potato tuber surface. Inoculated peat was incubated for two days, after which it was used to coat surface-sterilized potato minitubers (Yukon Gold). The minitubers were planted in either coarse sand or pasteurized field soil obtained from an experimental site at Delhi,

Ontario. There were eight replicates per treatment and soil type. Five weeks after sowing, the leaf chlorophyll content of the two topmost fully opened leaves per plant was measured using a chlorophyll meter (SPAD-502, Soil–Plant Analysis Development (SPAD) Section, Minolta Camera Co., Osaka, Japan). The plants were then harvested as follows. The plant shoots were cut off and weighed, and the roots were washed in running water to remove any adhering soil. The dry weight of the shoots and roots was determined by oven drying at 70^{o}C for three days. The plants grown in coarse sand were stunted with very small leaves, possibly through lack of nutrients, and therefore only the results obtained from pasteurized field soil are presented here.

RESULTS

The highest chlorophyll content, as measured using SPAD, was found in plants inoculated with the corn isolate, DS1, but there were no significant differences between any of the inoculated or uninoculated control plants (Fig. 4.1).

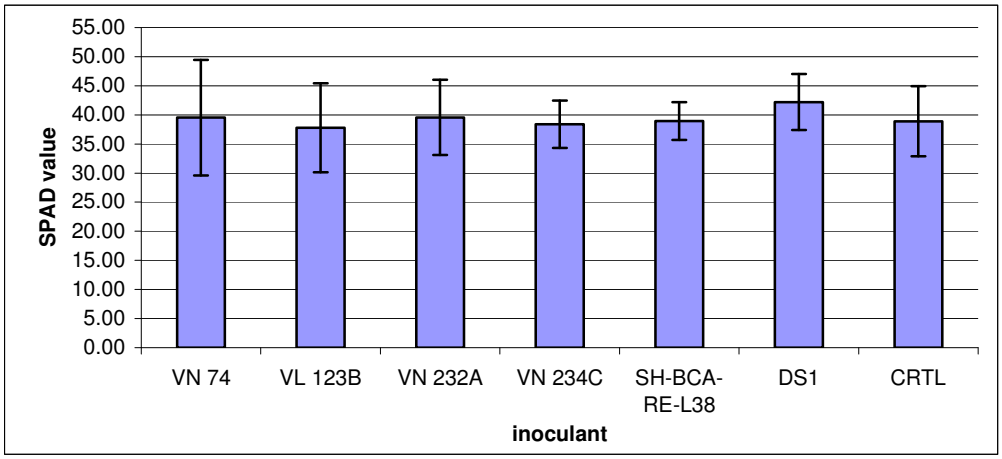


Figure 4.1 SPAD values (a measure of plant chlorophyll content) of inoculated and non-inoculated potato plants grown in pasteurized field soil. The SPAD value per treatment is the mean measurement of 8 plants, two leaves per plant.

With the exception of L38, all inoculants increased potato shoot and root fresh weight by as much as 25% when compared to the non-inoculated control plants. The highest response was due to VN232A, followed by VL123B. However, the observed differences were not statistically significant (Fig. 4.2, shoot fresh weight data only).

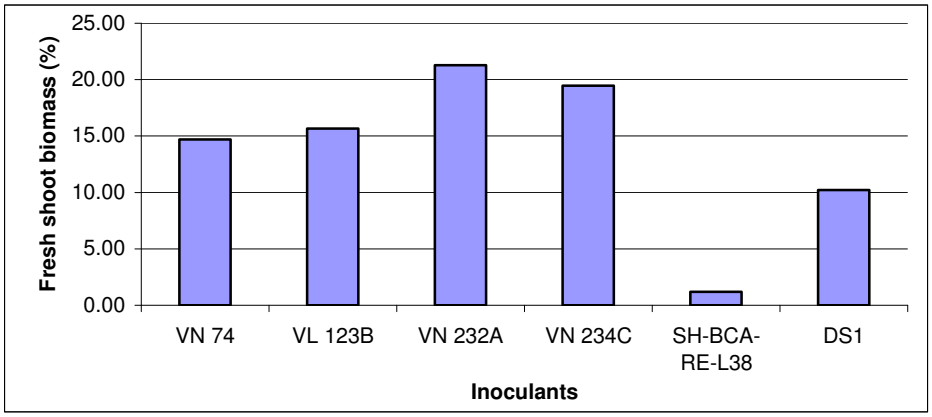


Figure 4.2 Increase in fresh shoot biomass (as % of uninoculated control plants) in response to nitrogen-fixing inoculants.

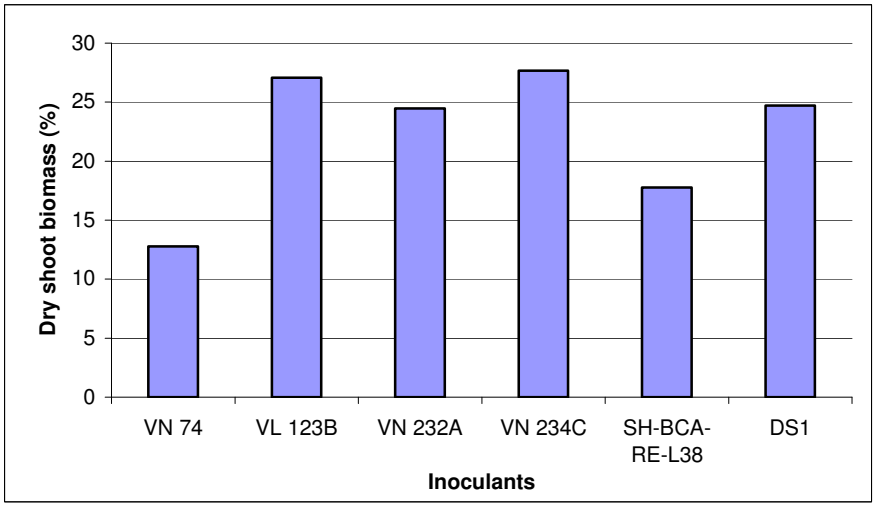


Figure 4.3 Increase in shoot dry weight, as % of uninoculated control plants, in response to nitrogen-fixing inoculants.

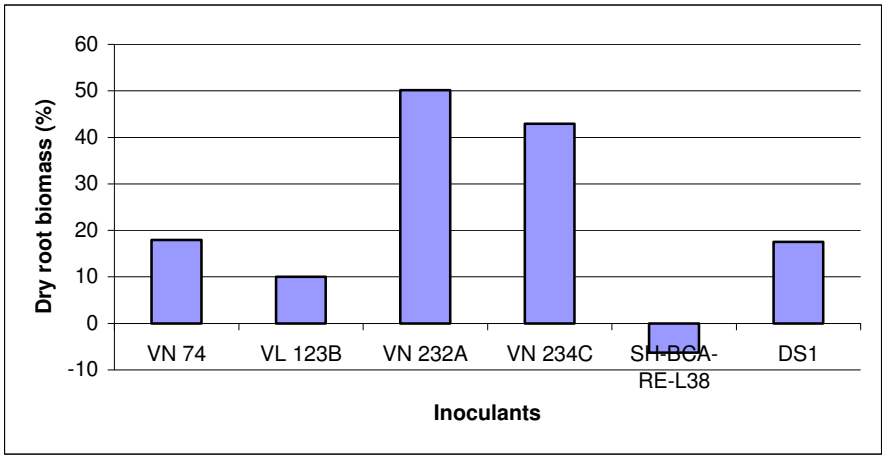


Figure 4.4 Increase in root dry weight, as % of uninoculated control plants, in response to nitrogen-fixing inoculants.

The highest shoot dry weights were recorded in plants inoculated with VN234C, followed by VL123B. Isolate L38 reduced the root dry weight by 6% (Fig. 4.3). Inoculation with VN232A resulted in the highest root dry weight, followed by VN234C (Fig. 4.4). VN232A (*Xanthobacter flavus*) and VN234C (*Azospirillum lipoferum*) were bacteria isolated from the rhizosphere of Shepody, while VL123B (*Bradyrhizobium japonicum*) was a root endophyte isolated from Yukon Gold. All three isolates originated from autoclaved V4 soil (V4A).

EXPERIMENT 2: EFFECT OF GROWTH PROMOTING BACTERIA ON PLANT GROWTH IN PASTEURIZED VERSUS NON-PASTEURIZED FIELD SOIL

MATERIALS AND METHODS

The second experimental trial was conducted in pasteurized and non-pasteurized field soil with four of the same isolates (L38, VL123B, VN74, VN232A) plus another five free-living nitrogen fixing bacteria isolates (NV215A, P17A, VL119, VN112B, VN204B) and a strain of *Burkholderia phytofirmans* E24, which has been previously reported to promote growth of potato. The aim of the experiment was to determine the ability of the inoculants to promote the growth of potato plants in pasteurized and non-pasteurized field soil. The soil was collected from AAFC sites at Delhi and divided into two parts. One part was partially sterilized through pasteurization at 80°C for 2 hours, the other part was not subjected to pasteurization. Both soils were used in the greenhouse experiment. The closest matches according to 16S *rRNA* sequencing of the isolates are shown in Table 4.1.

Approximately 2 ml of 48 hour old cultures of each isolate grown in LB or YEM broth at 28°C were used to inoculate 4 g of autoclaved peat (obtained from Becker Underwood, Saskatoon, Canada). The inoculated peat samples were incubated for 4 days, and then 1 g of each was serially diluted in sterile saline solution to determine the concentration of bacteria. The remaining 3 g of each inoculated peat sample was mixed with 3 ml of 15% solution of Gum Arabic and used to coat surface-sterilized sprouting minitubers (cv. Yukon Gold). The coated minitubers were planted at about 2 cm depth in

plastic coffee cups filled with 450 g of pasteurized or non-pasteurized field soils. There were eight replicates per treatment. Leaf chlorophyll content was measured with a SPAD chlorophyll meter 2 weeks after planting as described for the previous experiment. Plants were harvested at 6 weeks after planting and tuber mass, shoot and root dry mass per plant were determined.

RESULTS

The leaf chlorophyll content of most of the plants grown in pasteurized soils was higher than their counterpart in non-pasteurized field soil (Fig. 4.5). However, for each of the soils, none of the inoculants significantly increased leaf chlorophyll content above that of the non-inoculated control.

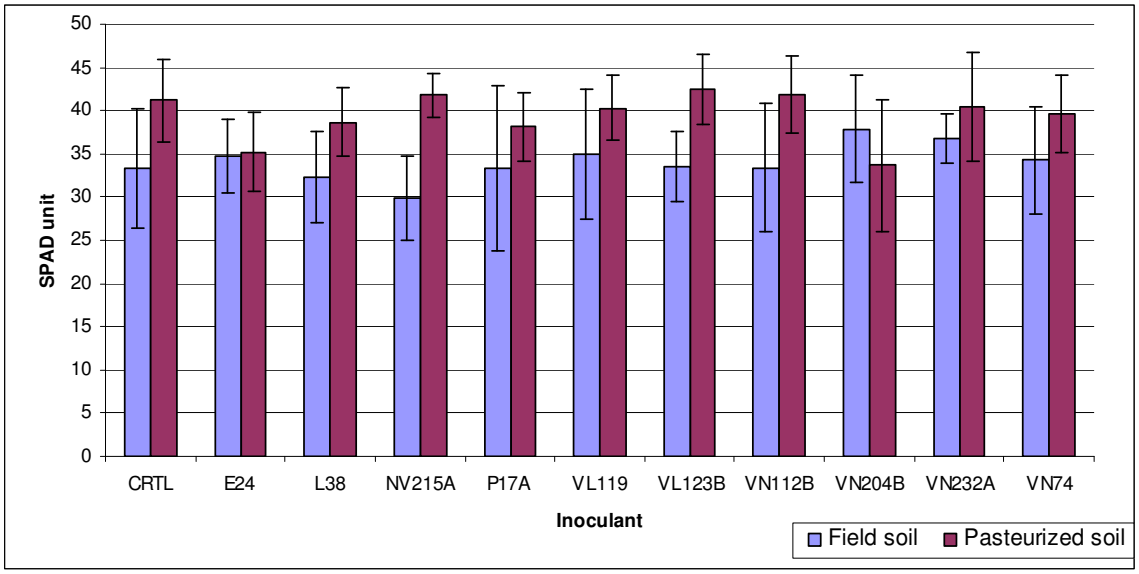


Figure 4.5 Leaf chlorophyll content (SPAD) of inoculated potato plants grown in pasteurized and non-pasteurized field soil (control was uninoculated).

Although the plants grown in pasteurized soils were healthier and stronger than their counterparts in non-pasteurized field soil, for most of the assessments, the differences between non-inoculated control and inoculated plants were more pronounced in potato plants grown in non-pasteurized field soils than in pasteurized soil (Fig. 4.6, 4.7 and 4.8). It is possible that the unculturable microorganisms in the non-pasteurized soil had a greater effect on plant growth than inoculants alone in pasteurized soil, or that there is some synergistic effect between them.

In non-pasteurized field soil, most of the bacterial inoculants were capable of increasing potato shoot, root and tuber yield when compared to the untreated control plants. The shoot dry weight per plant increased by 18 - 39%, root dry weight by 4 - 52% and average tuber weight per plant by 7 - 94%. In pasteurized soil, the percentage increases were smaller: shoot dry weight per plant (1 - 10%), root dry weight per plant (4 - 10%) and average tuber weight per plant (3 - 31%).

In the non-pasteurized field soil, inoculants VN204 and E24 significantly increased shoot dry weight by 39% and 30% respectively. Similarly, root dry weight was significantly increased by the inoculants VL38 (52%), VN215 (46%), VN74 (31%) and NV204B (28%), and tuber yield was almost doubled by VN215 and VL123. Tuber yield was decreased, however, by inoculants VN74 and L38 (Fig. 4.8).

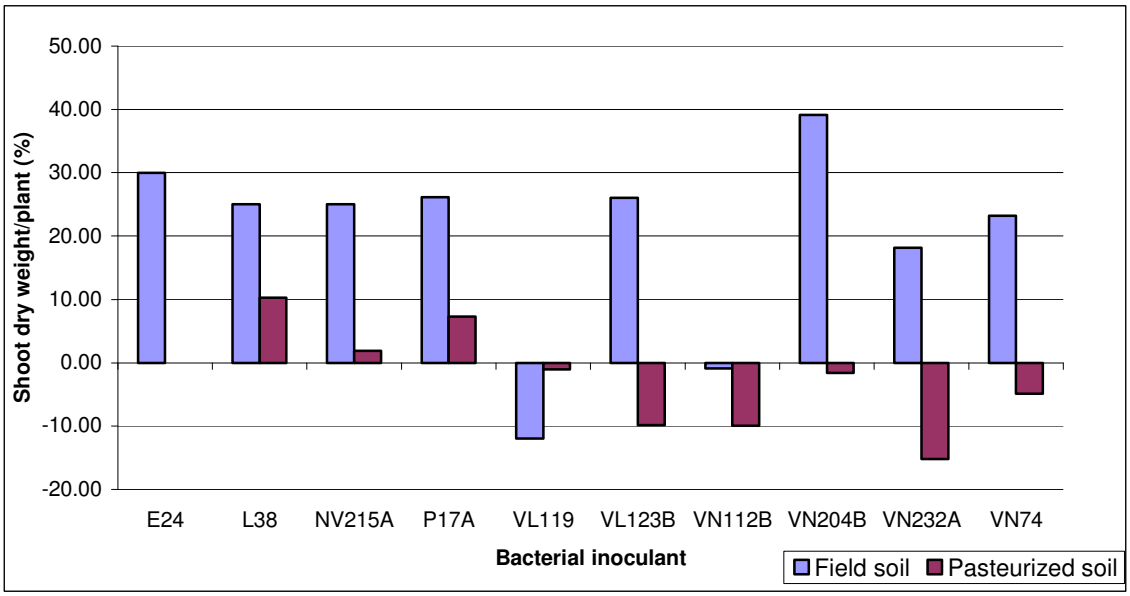


Figure 4.6 Percentage change in shoot dry weight of inoculated potato plants grown in pasteurized and non-pasteurized field soil. On the y-axis, 0 = uninoculated control.

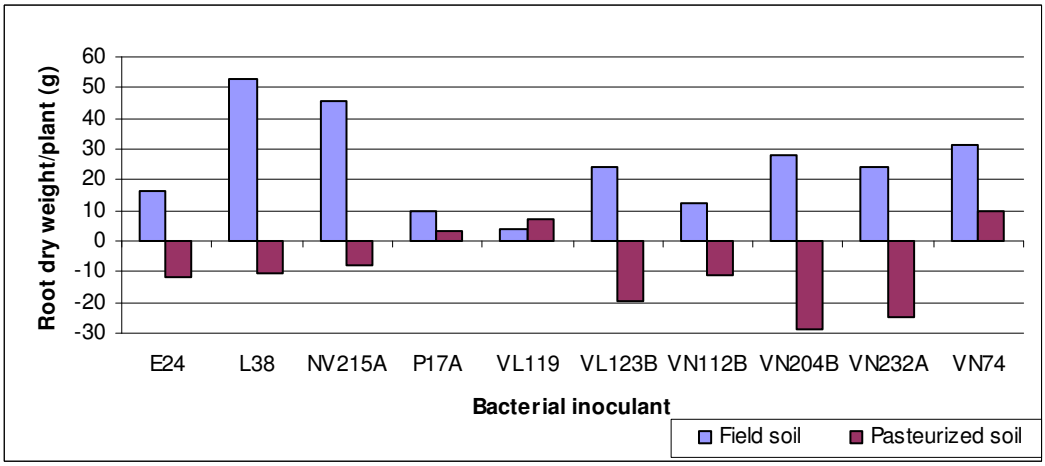


Figure 4.7 Percentage change in root dry weight of inoculated potato plants grown in pasteurized and non-pasteurized field soil. On the y-axis, 0 = uninoculated control.

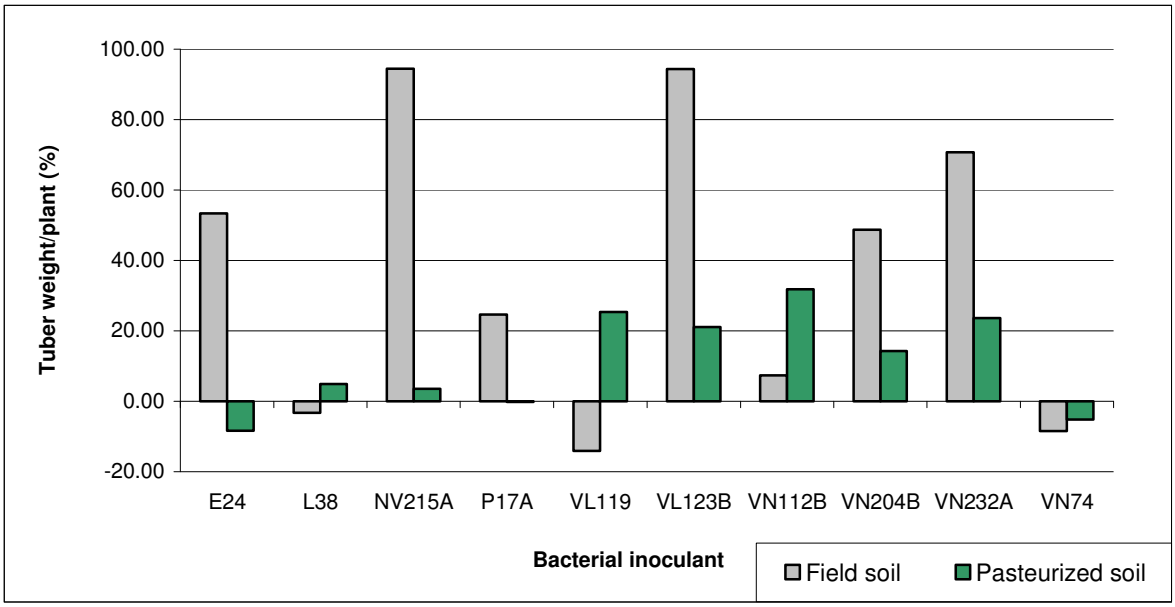


Figure 4.8 Percentage change in tuber weight of potato plants grown in pasteurized and non-pasteurized field soil. On the y-axis, 0 = uninoculated control.

EXPERIMENT 3: EFFECT OF INOCULANT APPLICATION AND FERTILIZERS ON PLANT GROWTH

MATERIALS AND METHODS

The third greenhouse experiment was conducted to determine whether the performance of the inoculants could be enhanced by a second inoculation after emergence and whether their effect is modified in the presence of fertilizers. The inoculants used were E24 (*Burkholderia phytofirmans*), NV215A, P17A, VL123B, VN204B, VN234C and VN74, and their closest matches according to 16S *rRNA* sequencing of the isolates are shown in Table 4.1.

The experimental conditions were similar to experiment 2, with the following modifications:

- Only non-pasteurized field soil was used.
- P and K fertilizers were added to the soil in the form of triple superphosphate @ 100 kg P/ha and potash @ 100 kg K/ha.
- In addition to tuber inoculation prior to sowing, plants were inoculated with 10 ml of their respective bacterial inoculant suspension two weeks after seedling emergence at the rate of 107 CFU/ml.
- P and K fertilizers were added to the soil in the form of triple superphosphate @ 100 kg P/ha and potash @ 100 kg K/ha.
- As the inoculants are nitrogen-fixing bacteria, their performance with and without the addition of nitrogen fertilizer was tested ("+N" and "-N" respectively). Nitrogen was applied to the "+N" treatment as a split dose of ammonium nitrate: 30 kg N/ha prior to sowing and 60 kg N/ha at 2 weeks after seedling emergence.

Plants were assessed as before, and harvested at 7 weeks after planting.

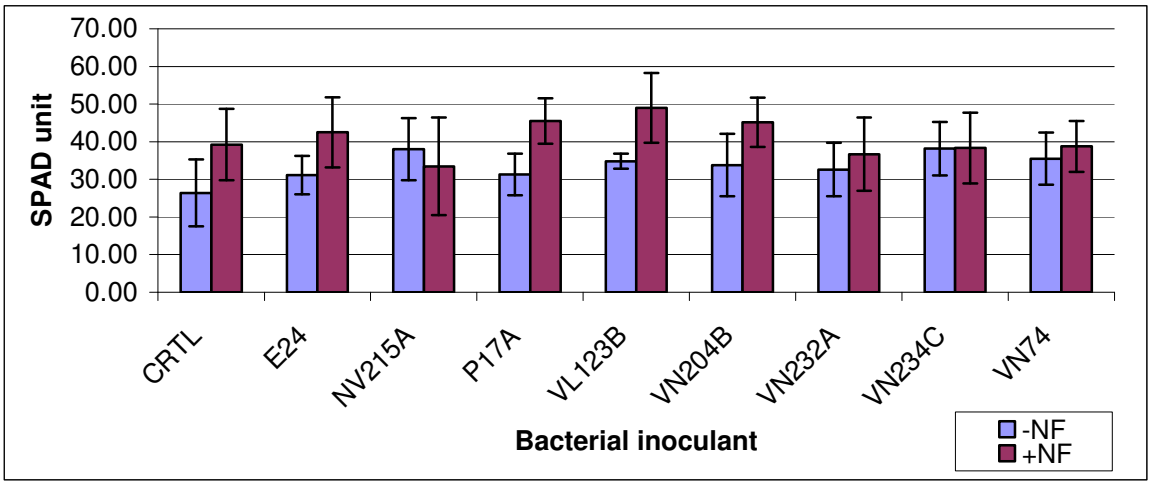


Figure 4.9 Leaf chlorophyll content from inoculated and non-inoculated potato plants supplemented with or without N fertilizer

In this experiment, all growth parameters measured were higher in plants with N amendment than their counterpart grown in soil without N application, indicating the importance of nitrogen in plant nutrition and growth. However, comparison between inoculated plants and non-inoculated control plants grown in the same soil showed a higher promotion of leaf chlorophyll content, shoot dry mass and tuber weight by inoculants when the soil was not amended with N fertilizer (Fig. 4.9, 4.10 and 4.11). This indicates that there might be a reduction in the growth promoting activity of the bacterial inoculants with increased soil available nitrogen, perhaps some form of feedback mechanism.

When nitrogen fertilizer was not applied, the highest shoot dry mass and chlorophyll content were found in VN234C treated plants. VL123B, VN215A and VN74 were among the next best inoculants that enhanced chlorophyll content and shoot dry mass. Conversely, all inoculated plants grown without added N had reduced root dry mass compared to the control plants (data not shown).

In the presence of nitrogen fertilizer, the leaf chlorophyll content was only increased in plants inoculated with VL123, VN204, P17A and E24, and shoot dry weight was highest in NV74 inoculated plants, followed by P17A, and VN234C.

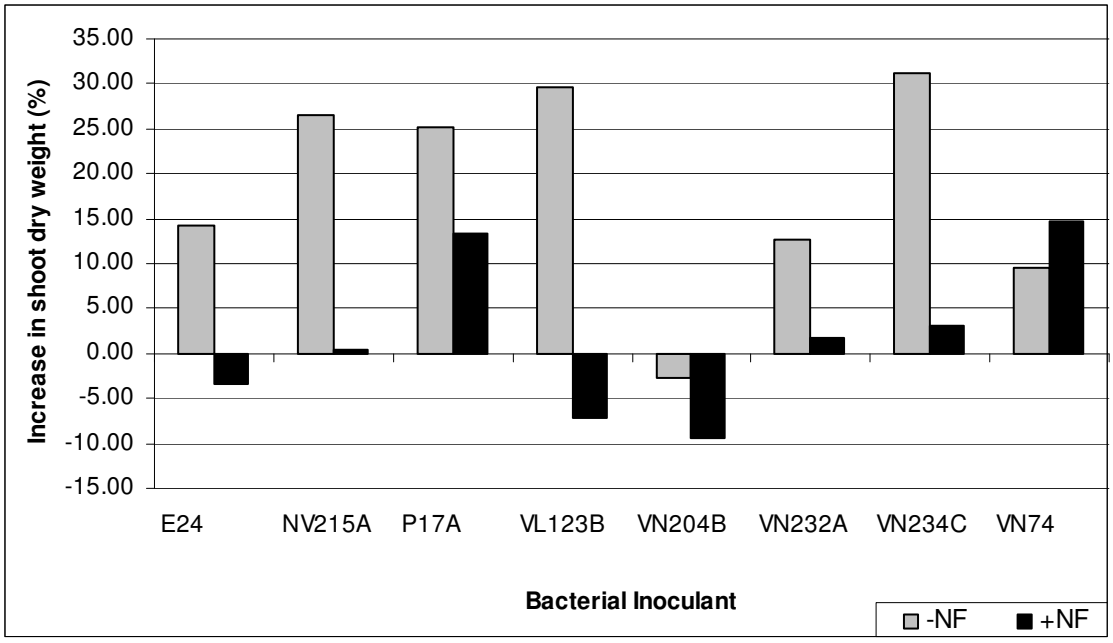


Figure 4.10 Percentage increase in shoot dry mass obtained from inoculated plants supplemented with or without nitrogen fertilizer

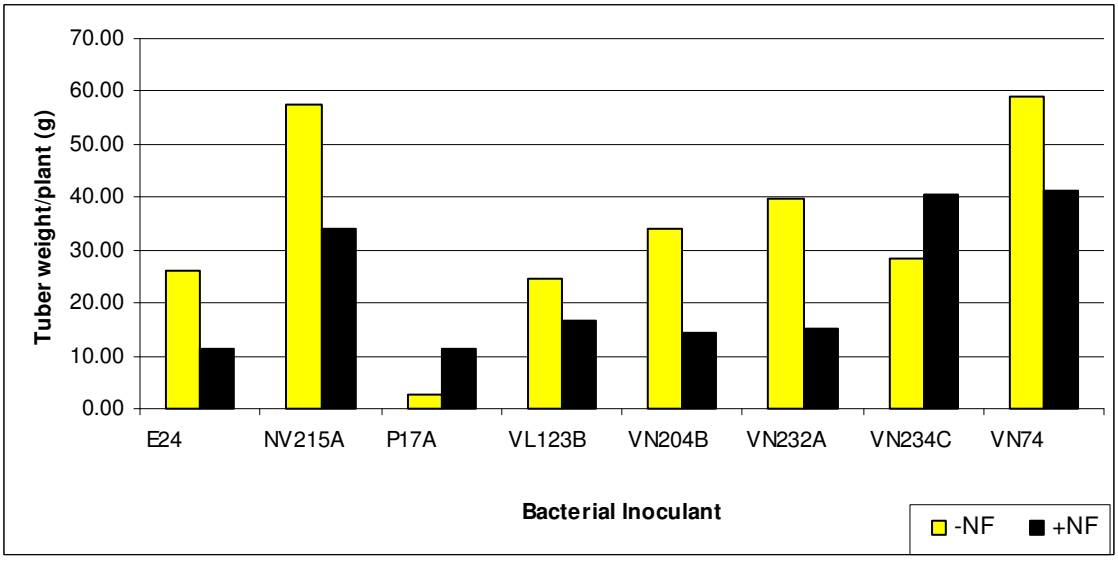


Figure 4.11 Percentage increase in tuber weight obtained from inoculated plants supplemented with or without nitrogen fertilizer.

As observed for most of the inoculants in experiment 2, potato tuber weight per plant was higher in all inoculated treatments than the non-inoculated control treatment and this was regardless of whether nitrogen fertilizer was applied or not (Fig 4.11). However, with the exception of P17A and VN234C treated plants, differences in tuber weight between inoculated plants and the control plants were much higher in soil that was not amended with nitrogen fertilizer compared to their counterparts in nitrogen amended soil, suggesting again a reduction in growth promoting effect of most of the bacteria when nitrogen fertilizer was applied. Increase in tuber weight varied between 2% and 58% in treatments

without nitrogen application, with the highest tuber yield in VN74 inoculated plants (58%), followed by NV215 (57%) and VN232A (39%) treated plants. Tuber weight was also highest in VN74 treated plants (41%) when N fertilizer was applied, followed by VN234C (40%) and VN215A (33%) treated plants.

DISCUSSION

Ten of the most promising nitrogen-fixing bacterial isolates were tested in three consecutive glasshouse experiments for growth-promoting effects on potatoes. Two isolates were unknown bacteria, but the others were identified using 16S *rRNA* sequencing as species known to promote growth in other crop plants. Two isolates each of *Azospirillum brasilense* and *A. lipoferum* were tested. These two species have been reported to improve yields of many crops, such as canola (Bertrand et al 2001) and beans (Burdman et al. 1997), and have been developed into biofertilizer products in countries such as India and China (a Google search on Azospirillum biofertilizer returned 10,700 hits). In addition to nitrogen fixing, they are also known to synthesize phytohormones such as indole acetic acid (Zimmer 1988), and all 4 isolates used here tested positive in the indole acetic acid assays described in Chapter 3. Two isolates of *Bradyrhizobium japonicum* were included in the experiments. This species is a nitrogen-fixing symbiont with soybean (*Glycine max*), so we were intrigued to find it associated with potato roots. The final two isolates included in the bioassays were *Xanthobacter flavus,* also a known nitrogen-fixer.

In the first experiment five isolates were tested and of these, *A. lipoferum* (VN234C), *B. japonicum* (VL123B) and *X. flavus* (VN232A) all promoted increased plant biomass. Pasteurized field soil proved a better growing medium for these bioassays than coarse sand. The second experiment was expanded to include nine isolates, and pasteurised versus non-pasteurised field soil was explored. Although the plants grew well in pasteurized soil, non-pasteurized soil proved better for differentiating the growth-promoting effects of the isolates. In this bioassay, *A. brasilense* isolate NV215, *B. japonicum* isolate VL123B and both *X. flavus* isolates all performed well, increasing plant biomass and tuber yields. *A. lipoferum* isolate VN74 decreased tuber yields, even though it increased shoot and root biomass. In the third experiment, non-pasteurized field soil was used for the bioassay and the addition of nitrogen on the isolates' performance was examined. In all cases, the addition of nitrogen masked the effect of the isolates, and it is possible that nitrogen may inhibit the growth-promoting properties of the isolates, although this was not tested. In the absence of nitrogen, *A. brasilense* isolate NV215, *A. lipoferum* isolates VN74 and VN234C, *B. japonicum* isolate VL123B and *X. flavus* VN232A all increased both biomass and tuber yield. *X. flavus* VN204B increased tuber yield but decreased shoot biomass.

In summary, the growth-promoting effects of the nitrogen-fixing bacterial isolates were most pronounced in the absence of additional nitrogen. Two isolates performed well in all three experiments: *B. japonicum* isolate VL123B and *X. flavus* VN232A; two isolates performed well in the two experiments in which they were tested: *A. brasilense* isolate NV215 and *A. lipoferum* isolate VN234C, and two isolates gave inconsistent results: *X. flavus* VN204B and *A. lipoferum* isolate VN74.

Technology Transfer

Industry seminars and articles:

- Dr George Lazarovits visited DPI Victoria collaborators in February 2010. He presented his research findings to grower meetings in Warragul and Ballarat.
- Dr. George Lazarovits, Managing soilborne disease of potatoes using ecologically based approaches International Potato Expo, Charlottetown Civic Centre, Canada, Friday, February 26, 2010
- Lazarovits, G. 2008. Presented a paper on "The Biology of the soil and soil health". at the Ontario Potato School, Feb.26 2008, Guelph
- 'Ear to the Ground' in Potatoes Australia, April/May 2010, pp. 22-23
- 'Potato Utopia' in Potatoes Australia, August/September 2010, pp. 24-27

Scientific presentations and papers:

- George Lazarovits invited to visit and advise the Institute of Plant and Environment Protection at the Beijing Academy of Agriculture and Forestry Science. Presented 4 lectures "Ecological approaches for management of soilborne plant diseases" at various institutes in China including Beijing Academy of Agricultural and Forestry Sciences, China Agricultural University, Nanjing Agricultural University, Shanghai Jiaotong University. November 2-18, 2008
- George Lazarovits, Raj Ramarathnam and Sean Hemmingsen. Unravelling the rhizosphere using the *cpn60* genomic marker and a 454 sequencer. 8th International Plant Growth Promoting Rhizobacter Workshop, Portland, Oregon, May 4 2009.
- George Lazarovits, Raj Ramarathnam and Sean Hemmingsen. Unraveling soil communities using the cpn60 genomic marker and pyrosequencing. VII International Symposium on Chemical and non-Chemical Soil and Substrate Disinfestation, September 13-18th 2009, Leuven, Belgium
- Lazarovits, G. (2010) Managing soilborne disease of potatoes using ecologically based approaches. American Journal of Potato Research. 87 (5): 401-411. An invited article.

Recommendations

- Chaperonin gene sequencing proved to be an excellent tool for the study of soil microbial communities associated with potato production. As a result of this research, we demonstrated that technology already available and used in the medical field has tremendous potential for application in potato research. For example, *Rhizobium* species, normally associated with legumes as nitrogen-fixing symbionts, were discovered for the first time associated with potato roots. A large number of these and other interesting bacteria have been collected and their potential should be explored further.
- By using these molecular genetic approaches, particularly when more microorganisms are placed into the database, we can generate a fingerprint of a soil microbial community and identify the desirable microorganism residents. Generation of such fingerprints will make it possible to move forward with knowledge based decision systems as to how to achieve conditions that benefit plant growth. It will also provide a means to evaluate how the soil microbial biomass is influenced by agricultural practices and climate change.
- We now know where to look for microorganisms with the potential for developing into biocontrol agents and biofertilizers. The microbial communities associated with potato roots contain far more potentially useful microorganisms than bulk soil. Several bacterial isolates were identified with disease suppressive and growth promotion potential and these should be further screened and developed.
- Some of this research is being continued as part of the Soil Health program within phase 2 of the Australian Potato Research Program. Work in progress includes:
    - Greenhouse experiments to determine the potential of eight bacteria (positive for antibiotic genes) on the suppression of common scab disease of potato caused by *S. scabies*.
    - Further greenhouse experiments to assess the growth promoting effect of selected nitrogen-fixing isolates on potato
    - HPLC analysis to determine the active inhibitory metabolites (or antibiotics) in the culture filtrates of the 30 antagonistic bacterial isolates, in order to understand their mechanism of activity.
- Future research should continue to identify the key genes in these organisms and their functional activities in order to deploy them into microarrays. For example, the antibiosis genes could be deployed into a microarray to identify disease suppressive soils; the nitrogen-fixation genes may help plant nutrition and be a key bioindicator for the performance of certain cultivars in soils.
- This project clearly demonstrates the benefits of international collaboration in providing access to cutting-edge research and technology that can be captured for the benefit of the Australian potato industry. It is highly recommended that this approach to R&D continues.

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