THE EFFECT OF ENDOPHYTIC BACTERIA ON THE ALFALFA-*Sinorhizobium* SYMBIOSIS

A Thesis Submitted to the College of
Graduate Studies and Research in Partial
Fulfillment of the Requirements for the
Degree of Masters of
Science in the Department of Soil Science
University of Saskatchewan
Saskatoon

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ABSTRACT

Although plant growth-promoting rhizobacteria (PGPR) have shown tremendous potential to be used as inoculants for many agricultural crops, they may not survive severe environmental conditions in the field which could limit their large scale applications. Endophytic bacteria, which can be recovered from inside plant tissues such as roots, stems and leaves, might overcome this limitation due to their unique ecological niche inside plant roots where they are sheltered from external environmental disturbances. Some of these bacterial endophytes have beneficial effects on their host plants and stimulate plant growth or reduce disease symptoms, apparently through mechanisms that are similar to those proposed for PGPR. The objective of this study was to assess a collection of endophytic bacteria for PGPR traits and potential use to enhance the rhizobial-legume symbiosis. Forty isolates obtained from the roots of various plants were identified by fatty acid methyl ester (FAME) analysis, and 16S RNA gene sequencing analysis. The majority (i.e., 75%) were identified as *Pseudomonas* species. Many of these isolates were able to solubilize phosphate, produce indole-3-acetic acid (IAA), produce aminocyclopropane-1-carboxylic acid (ACC) deaminase, synthesize siderophores and show antagonistic activities against several soil-borne plant pathogenic fungi under *in vitro* conditions. Selected isolates were further evaluated for the ability to enhance plant growth and nodulation of alfalfa when co-inoculated with *Sinorhizobium meliloti* under growth chamber conditions using growth pouch and potted soil assays. Results revealed that *P. putida* strain EB EE 4-25, *P. syringae* strain EB XDE 4-48, and *P. fluorescens* strain EB EE 2-23 significantly increased shoot length, root length, enhanced nodulation and increased lateral root formation of alfalfa plants in growth pouch and potted soil assays when co-inoculated with *S. meliloti* strain P102 compared to plants inoculated with *S. meliloti* strain P102 alone. Results also suggested that expression of one or more of the mechanisms, such as solubilization of phosphate, production of IAA, production of siderophores, and ACC deaminase production might have played a role in the enhancement of the alfalfa- *Sinorhizobium* symbiosis. These results suggest that some endophytic bacterial strains may be useful as biofertilizers and/or biocontrol agents in sustainable agricultural practices.

ACKNOWLEDGMENTS

First and foremost, I would like to express my gratitude, respect and appreciation to my supervisor Prof. James J. Germida. I am very grateful for your imperturbable support and encouragement throughout my study. Sir your generosity and understanding of my eastern background are invaluable commodities. I am also very grateful and would like to give special thanks to my co-supervisor Dr. Steven D. Siciliano for his guidance, encouragement, patience and support during the course of this work. My sincere thanks are extended to the members of my committee Prof. Fran Walley and Prof. Ken Van Rees for their valuable advice and comments. I would also like to thank my external examiner Dr. Russell Hynes, of the Agriculture and Agri-Food Canada, for his critical comments and suggestions.

I would like also to thank the Soil Microbiology Laboratory group for their technical assistance and great a companion especially Arlette Seib, Lori Phillips, Bobbi Helagson, and Ashley Mascarenhas.

I would like also to thank the Saudi Arabian Cultural Bureau and King Saud University for funding and providing me with a source of income, which has enabled my dream to come true.

Finally, I am gratefully thankful to my family in Saudi Arabia for their help and support.

DEDICATION

I dedicate my thesis to my Brother

Shelwan Nasser Al Otaibi (1930- Feb 28th, 2009)

who was not only a brother to me but a father.

His love, encouragement, and support have made this dream come true.

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LISTOF ABBREVIATIONS

ACC	aminocyclopropane-1-carboxylic acid
ANOVA	Analysis of variance
AMF	Arbuscular mycorrhizal fungi
BCAs	Biological control agents
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CAS	Chrome S Azurol
CFU	Colony forming units
DNA	Deoxyribonucleic acid
FAME	Fatty acid methyl ester
IAA	Indole-3-acetic acid
LB	Luria-Bertani
MIDI	Microbial ID, Inc.
OD	Optical density
PDA	Potato dextrose agar
PDYA	Potato dextrose yeast agar
PGPR	Plant growth-promoting rhizobacteria
PSM	Phosphorus solubilizing microorganisms
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rpm	Revolutions per minute
SIM	Similarity indices
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultra-VioleT

1.0 INTRODUCTION

Alfalfa or lucerne (*Medicago sativa L*) is a herbaceous perennial forage legume species that belongs to the leguminosae family. Alfalfa is called the Queen of the forage plants because it has many desirable characteristics and outstanding economic importance (Hanson et al., 1988). Alfalfa has a very deep tap root system that allows it to reach soil water at depths not accessible by other plants and this confers drought tolerance (Frame, 2005). Alfalfa is mainly used as hay and pasture to feed farm livestock because of its excellent nutritive values. It is rich in protein, calcium, and vitamin A, and it is high in energy and low in fiber. In addition, including alfalfa in crop rotation has many benefits such as improved soil nutritional status, increased soil organic matter, and reduced requirement for nitrogen (N) fertilizers for subsequent crops due to its symbiosis with *Sinorhizobium meliloti* (Hanson et al., 1988; Frame, 2005).

Alfalfa, as a member of the legume family, has the ability to form a symbiotic association with a member of the genus *Rhizobium* (*Sinorhizobium meliloti*) which supplies the plant with fixed N compounds. This association reduces the plants dependency on inorganic N fertilizers, yielding economical and environmental benefits (Prevost and Bromfield, 2003). For example, in Saskatchewan alone, the alfalfa dehydration industry (pellet and cubes) processes more than 100 000 tonnes annually from about 20 000 hectares, where, the majority is exported to Japan and South Korea to feed the livestock industry (Saskatchewan Agriculture, 2008). Additionally, alfalfa has been proposed as a suitable plant for rhizoremediation (i.e., the use of integrated plant/microorganisms systems for *in situ* soil bioremediation) because of its perennial character, deep root system and high water consumption that might carry the pollutants to the rhizosphere (Villacieros et al., 2003).

Endophytic bacteria are defined by Hallmann et al. (1997, p. 897) as “bacteria which can be isolated from surface disinfested plant tissue or extracted from inside the plant, and which do not visibly harm the plant”. Bacterial endophytes show an incredible amount of diversity not only in plant hosts but also in bacterial phyla (Bacon and Hinton, 2006). The bacterial taxa that have been isolated from the endorhizosphere niches consist of numerous genera of both Gram-negative and Gram-positive species. For example Hallmann and Berg (2006) listed 219 endophytic species comprising 71 genera, with most being members of common soil bacteria

genera such as *Enterobacter*, *Pseudomonas*, *Burkholderia*, *Bacillus*, and *Azospirillum*. However, there is great interest in endophytic biology because endophytes are still a relatively untapped source of novel natural products. Many plant species are being assessed for the diversity of endophytic bacteria within their tissues and new endophytes are continuously being reported (Hallmann and Berg, 2006; Rosenblueth and Martinez-Romero, 2006; Ryan et al., 2008). Various reports found that bacterial endophytes contribute to the growth and health of a variety of plants (Lodewyckx et al., 2002; Sessitsch et al., 2004; Hardoim et al., 2008). The mechanisms by which endophytic bacteria may enhance the growth of their host plants are believed to be similar to those proposed for plant growth-promoting rhizobacteria (PGPR) (Vessey, 2003).

Plant growth-promoting rhizobacteria (PGPR) are soil bacteria that stimulate their host plant growth (Compant et al., 2005). Plant growth-promoting rhizobacteria may affect plant growth either directly or indirectly. Mechanisms for the direct enhancement of plant growth includes: nitrogen fixation; synthesized siderophores; production of phytohormones such as indole-3-acetic acid (IAA); and facilitation of nutrients uptake from the environment surrounding the plant roots. The indirect stimulation of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more of phytopathogenic microorganisms. Biocontrol mechanisms include: competition for an ecological niche or a substrate; production of inhibitory compounds; induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens; and reduced abiotic stresses (Gary and Smith, 2005).

Rhizobium-legume symbiosis is perhaps the best known beneficial plant-microbe interactions because of the importance of N_2 fixation. In recent years, research efforts have attempted to reveal the mechanisms through which the beneficial effects of N_2 fixation can be improved (Mishra et al., 2009).

Compared to the PGPR stimulation of the *Rhizobium*-legume symbiosis, little is known about the effect of bacterial endophytes on the alfalfa-*Sinorhizobium meliloti* symbiosis. Many studies, however, indicate that endophytic bacteria may have the potential to be used as agricultural inoculants (Compant et al., 2005; Ryan et al., 2008).

We hypothesized that selected endophytic bacteria have a positive impact on the alfalfa-*Sinorhizobium* symbiosis. The hypothesis was tested through several experiments carried out in the laboratory as well as in the growth chamber in both growth pouches and in soil.

The main objectives of this study were to:

1. Select and identify endophytic bacteria;
2. Assess endophytic bacteria for some characteristics commonly found in PGPR; and
3. Assess the effect of selected endophytes on the growth and nodulation of alfalfa plants.

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2.0 LITERATURE REVIEW

2.1 The rhizosphere, rhizoplane, and endorhizosphere

The term rhizosphere was first coined and defined by the German scientist Lorenz Hiltner in 1904 as the region of soil immediately adjacent to legume roots which supports high levels of microbial activity (Fageria and Stone, 2006). Since the term rhizosphere was introduced over a hundred years ago, several definitions have been proposed to describe this area of close proximity to plant roots. More recently, the term has been broadened to include both the volume of soil influenced by the root and root tissues colonized by microbes (Morgan et al., 2005). The rhizosphere area can be divided into three different distinct zones; the rhizosphere, the rhizoplane, and the endorhizosphere (root interior). The rhizosphere is the soil zone influenced by roots exudates. The rhizoplane is the root surface, including the strongly adhering soil particles. The endorhizosphere is the interior part of the root tissues which are colonized by certain microorganisms, the endophytes (Barea et al., 2005). Pinton and Varanini (2001) argued that the soil region nearby the roots should be called the ectorhizosphere, whereas the root interior colonized by microorganisms should be termed the endorhizosphere. The rhizoplane is the root surface that separates these two distinct zones (Fageria and Stone, 2006).

In addition to the above mentioned zones of the rhizosphere, Nelson (2004) stressed the importance of the spermosphere. The spermosphere is the zone of microbial interaction around the seed that is under the influence of seed carbon deposition. The spermosphere effect might have a lasting impact on the plant health and development because associations developing on and around germinating seeds mark the first contact between plants and soil microorganisms (Nelson, 2004).

Plant roots, as well as seeds, exude various organic substrates which alter the chemical, physical, and biological characteristics of soil. Exudates are much greater in the rhizosphere region than in bulk soil. Similarly, exudation is greater at the emerging radical (spermosphere) than any other place on the seeds. Grayston et al. (1996) reported that up to 40% of the net photosynthetic carbon is exuded via root systems as different types of organic compounds, including amino acids, organic acids, and sugars. These exudation activities make the rhizosphere, rhizoplane, and spermosphere microbial hot spots.

Due to the high content of nutrients in the rhizosphere, many rhizospheric microbial communities (which might have a beneficial, neutral, or harmful activities) occur in this habitat with bacteria being the most abundant organisms (approx. 10^{10} cells per gram of soil), followed by fungi, protozoa, and nematodes (Watt et al., 2006). Among the beneficial microorganisms found in the rhizosphere are PGPR, *Rhizobium* species that colonize the leguminous plants, and arbuscular mycorrhizal fungi (AMF), which have mutualistic associations with most land plants. However, the rhizosphere also harbors harmful microorganisms including soil-borne pathogens (fungi, bacteria, and nematodes) which cause substantive damage to many important agriculture crops (Raaijmakers et al., 2009).

Being aware of the ecological uniqueness of the rhizosphere, scientists realize the importance of manipulation of rhizosphere microorganisms to improve plant growth and yield. One potential way to achieve that goal is the use of bacterial endophytes, bacteria that reside inside the plant roots without causing any apparent damage, to enhance the *Rhizobium*-legume symbiosis.

2.2 *Rhizobium*-legume symbiosis

2.2.1 Legumes

The Leguminosae family, also called the Fabaceae family, is one of the largest family of higher plants and second only to cereals in terms of agricultural and economic importance (Graham and Vance, 2003). Legumes are further classified into three sub-families; the Caesalpinioideae, the Mimosoideae and the Papilionoideae, which in total contain 750 genera and more than 19 000 species (International Legume Database & Information Services, 2009).

Legumes constitute very important food and forage crops, which are used as food for human consumption, feed for farm animals, in many industrial purposes, and in increasing agricultural productivity worldwide. Most of the above mentioned benefits are attributed to the hallmark trait of legumes to develop root nodules and fix atmospheric dinitrogen (N_2) in symbiosis with soil dwelling bacteria, collectively referred to as rhizobia (Graham and Vance, 2003).

Benefits derived from cultivating legumes are numerous, including the reduction of inorganic N fertilizers usage by farmers. For example, *Rhizobium*-legume symbioses reduce ca. 100 million metric tonnes of atmospheric N_2 to ammonia each year which is equivalent to 10

billion US dollars for N fertilizers (Howieson et al., 2008). When included in a crop rotation, legumes play important roles in pathogen control, enhancing soil stability, and improving soil organic matter.

2.2.1.1 Alfalfa

Alfalfa or lucerne (*Medicago sativa L*) is a herbaceous perennial forage legume species that belongs to the sub-family *Papilionoideae*. *Papilionoideae* comprise 13 000 woody and herbaceous species including the forage legumes. Alfalfa has a very deep tap root system that allows it to reach soil water at depths that are not accessible by other plants, conferring drought tolerance (Frame, 2005). These values have placed alfalfa in a high economic position among other legumes and non legume crops. For instance, in the United States alone, alfalfa is considered to be the third or fourth most valuable crop with an estimated value of 7 billion US dollars per year (Graham and Vance, 2003; Howieson et al., 2008).

2.2.2 Rhizobia

2.2.2.1 Root infection process

Rhizobia are soil dwelling gram-negative bacteria that have the capacity to reduce atmospheric N_2 through a symbiotic interaction with compatible host legume plants. This mutualistic relationship is initiated after a series of complex exchanges of chemical signals between the bacteria and the legume plant. Briefly, the legume plants first secrete isoflavonoids that are recognized, bind, and are activated by the compatible bacterial NodD proteins, resulting in the initiation of the nodulation genes (nod genes) (Peck et al., 2006; Chang et al., 2009). Nod genes then encode proteins that produce specific lipochito-oligosaccharides called Nod factors (NFs) that trigger the root infection process and kick off cell division in the root cortex. Other signaling chemicals are also involved in the symbiosis process development such as surface lipopolysaccharides (LPS) (Chang et al., 2009).

The formation of root nodules leads to the symbiotic relationship between the legume plant (host) and the N_2 fixing rhizobial bacterium (microsymbiont). Once inside the nodules, the bacteria receive fixed carbon compounds (nutrients) from the host as well as a favorable environment where competition with other soil microorganisms is absent (compared to the rhizosphere zone). In return, the rhizobia fix atmospheric N_2 to NH_4 and supply it to the host

plant. Therefore, the biological nitrogen fixation (BNF) process takes place inside the nodule, a low oxygen environment that allows the expression of nitrogenase enzyme which is responsible for reducing N_2 to NH_4.

Amounts of N_2 fixed in agriculture systems by rhizobia in symbiosis with legumes is estimated to be 21 million tonnes of N per year for pulse crops and in the range of 12 to 25 million tonnes N annually by forage legumes, with alfalfa having one of the highest annual N_2 fixation rates of 200 kg N ha^{-1} (Herridge et al., 2008).

2.2.2.2 Taxonomy of root nodulation bacteria

Historically, legumes are believed to have a mutualistic symbiosis with only members of the *Rhizobiaceae* in the alpha (α)-Proteobacteria. However, recent findings demonstrate that the genera *Burkholderia* and *Ralstonia* of the class beta (β) - Proteobacteria can also form nodules on legume plants and are efficient N_2 fixers (Graham, 2008). Due to the large number of legume species, their wide geographical distribution, and the advancement in modern molecular taxonomic techniques, rhizobial taxonomy is expected to undergo a rapid expansion in the future (Willems, 2006).

As of May 29, 2009 root and stem nodule bacteria currently comprise 14 genera and some 70 species and include the following genera: *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Blastobacte*r, *Bradyrhizobium*, *Burkholderia*, *Devosia*, *Ensifer*, *Herbaspirillum*, *Mesorhizobium*, *Ralstonia*, *Rhizobium*, *Methylobacterium*, *Ochrobactrum* (LPSN, 2009). As this thesis is investigating the effect of endophytic bacteria on *Sinorhizobium*-alfalfa symbiosis, an emphasis will be given below on the current taxonomy of the genus *Sinorhizobium*.

It has recently become evident from 16S rDNA comparisons that *Ensifer adhaerens* is also phylogenetically a member of the *Sinorhizobium* linage (Willems, 2006). *Ensifer adhaerens* was first proposed by Casida (1982) as a predatory bacterium that adheres and lyses other soil bacteria (Young, 2003; Willems, 2006). The genus *Sinorhizobium* was first proposed by Chen et al. (1988) and it currently contains 11 valid species (Willems, 2006). Based on a comprehensive polyphasic approach between type strains representing both *Ensifer* and *Sinorhizobium*, it has been concluded that *Ensifer* (Casida, 1982) and *Sinorhizobium* (Chen et al., 1988) were synonymous (Young, 2003; Willems et al., 2003). Therefore, the judicial commission of the international committee on systematics of prokaryotes affirms that the genus name

Sinorhizobium (Chen et al., 1988) is a later synonym of *Ensifer* (Casida, 1982), and the genus name *Sinorhizobium* is not validly published according to the bacteriological code (Tindall, 2008). Consequently, all *Sinorhizobium* spp. are renamed as *Ensifer* spp., which currently consists of 10 species (www.dsmz.de), including *Ensifer meliloti* the microsymbiont of the forage legume alfalfa.

2.3 Endophytic bacteria

Endophytic bacteria have been known for more than 120 years (Hardoim et al., 2008). In 1926, Perotti was the first to use the term endophytes to describe non-pathogenic bacteria that had been isolated from within plants, other than rhizobia (Hallmann et al., 1997; Hardoim et al., 2008). Several definitions are used to describe bacterial endophytes; however, the most widely used definition by researchers is the one defined by Hallmann et al. (1997, p. 897) as "bacteria which can be isolated from surface disinfested plant tissue or extracted from inside the plant, and which do not visibly harm the plant".

Endophytic bacteria residing inside plant tissues are thought to gain entry primarily through root cracks formed in lateral root junctions (Hardoim et al., 2008). Although the root offers the most evident site of entry, different points of entry have been proposed such as seeds and aerial portions of plants (Lodewyckx et al., 2002).

Endophytic bacteria have been isolated from every plant studied so far (Strobel et al., 2004) including both monocotyledonous and dicotyledonous plants, and ranging from woody tree species to herbaceous crop plants (Lodewyckx et al., 2002). Also, endophytic bacteria have been isolated from different plant structures such as seeds, tubers, roots, stems, leaves, and fruits (Hallmann et al., 1997). The almost 300 000 plant species that exist on our planet, only a few of these plants have been completely tested for the presence of endophytic bacteria within their tissues. Consequently, the possibility exists to find new and beneficial endophytic bacteria. Additional potential exists for the subsequent discovery of novel metabolites with many potential biotechnological applications in agriculture, medicine, pharmaceutical, and in the field of environmental protection (Strobel et al., 2004; Ryan et al., 2008).

The bacterial taxa that have been isolated from the endorhizosphere niches include numerous genera of both Gram-negative and Gram-positive species. For example, Hallmann and Berg (2006) listed 219 endophytic species including 71 genera, with most being members of

common soil bacteria genera such as *Enterobacter*, *Pseudomonas*, *Burkholderia*, *Bacillus*, and *Azospirillum* (Lodewyckx et al., 2002). However, new endophytes are continuously being reported and may be a relatively unexploited source of novel natural products. Additionally, many plant species are being tested for the diversity of endophytic bacteria within their tissues (Hallmann and Berg, 2006; Rosenblueth and Martinez-Romero, 2006; Ryan et al., 2008).

In comparison with rhizosphere and rhizoplane bacterial communities, endophytic bacteria are likely to interact more closely with their host plant because they are provided with a readily available source of nutrients and secure residency inside apoplastic intercellular spaces of plants (Rosenblueth and Martinez-Romero, 2006; Weyens et al., 2009). In return, the endophytic bacteria could enhance their host plant growth and health via various direct and indirect mechanisms.

Considering the above mentioned features of bacterial endophytes, there is increasing interest over the last few years in utilizing these bacteria as plant growth promoters and biological control agents (Hallmann et al., 1997; Rosenblueth and Martinez-Romero, 2006; Ryan et al., 2008). Endophytic bacteria are believed to enhance their host plant growth and health through similar mechanisms proposed for PGPR (Vessey, 2003). Germida et al. (1998) suggested that bacterial endophytes residing in plant roots are a subset of the communities found in the rhizosphere. Thus, it is reasonable to expect that endophytic bacteria share similar metabolic and taxonomic features to PGPR (Misko and Germida, 2002). It should be kept in mind that many of the direct and indirect mechanisms of plant growth have focused on free-living rhizobacterial strains, and much still remains to be learned from endophytic bacteria (Compant et al., 2005).

2.4 Plant growth promotion mechanisms

Several beneficial plant-microbe interactions that could enhance plant yield and health have been studied and utilized for the benefit of agricultural productivity over the last few decades. Most of these studies focused on the *Rhizobium*-legume symbiosis, AMF, and rhizospheric bacteria such as PGPR and biological control agents (BCA). Few studies have assessed the effect of endophytic bacteria on plant growth promotion, and especially on the legume-*Rhizobium* symbiosis. As mentioned in the last section, bacterial endophytes are believed

to enhance their host plant growth and development through similar mechanisms as proposed for PGPR.

2.4.1 Plant growth-promoting rhizobacteria (PGPR)

Genera of soil bacteria that exert beneficial effects upon inoculation of plants are termed PGPR (Kloepper et al., 1999). Plant growth-promoting rhizobacteria are soil dwelling free-living bacteria that may enhance plant growth and yield either directly or indirectly via several mechanisms. Antoun and Prevost (2005) estimated that about two to five percent of the rhizosphere bacteria when reintroduced by plant inoculation could be considered PGPR. Over the years, several mechanisms of plant growth enhancement by PGPR have been documented. Direct growth mechanisms include: (1) associative N_2 fixation; (2) solubilization of immobilized mineral nutrients such as phosphorus and zinc or mineralization of organic phosphorus compounds; (3) oxidation of sulfur; (4) sequestration of iron by siderophores; (5) the production of different types of phytohormones like auxins, cytokinins, and gibberellins; (6) production of amino-cyclopropane-1-carboxylic acid (ACC) deaminase (Grayston and Germida, 1991; Vessey, 2003; Lucy et al., 2004; van Loon, 2007); and (7) production of volatile growth stimulants such as acetoin and 2,3-butanediol (Ryu et al., 2003). The indirect plant growth mechanisms include: (1) antibiosis; (2) siderophore production; (3) induced systemic resistance (ISR); (4) competition for limited resource; (5) hydrogen cyanide (HCN) production; and (6) production of a wide range of cell wall degrading enzymes. Additionally, there are several ways in which PGPR can enhance plant growth indirectly such as the stimulation of legume-*Rhizobium* symbioses and plant-fungal symbioses (Whipps, 2001; Compant et al., 2005; Banerjee et al., 2006). Because numerous studies have documented that *Pseudomonas* and *Bacillus* are the most common PGPR genera, their characteristics will be briefly reviewed in the next sub-sections.

2.4.1.1 *Pseudomonas* spp.

Pseudomonas spp. are a ubiquitous group of bacteria in agricultural soils generally referred to as pseudomonads. Taxonomically, pseudomonads are aerobic, rod-shaped Gram negative bacteria with one or more polar flagella (Haas and Defago, 2005). They comprise important plant, animal and human pathogens; however, they also contain many species with well known biocontrol and plant growth promoting traits (Mercado-Blanco and Bakker, 2007).

As of May 2009, 195 species and subspecies of the genus *Pseudomonas* were validated on the Approval Lists of Bacterial Names (www.dsmz.de/bactnom/bactname.htm).

The traits that make *Pseudomonas* spp. ideal agents for use in the field as biopesticides, PGPR, and in the bioremediation of contaminated soils are their ability to utilize a wide range of organic and inorganic compounds that are exuded by seeds and roots. Furthermore, they compete aggressively with other microbes to colonize and multiply in the rhizosphere, spermosphere and the endorhizosphere environments. Also, they produce a wide range of bioactive metabolites (Weller, 2007).

Pseudomonad species characterized by production of water soluble, yellow green fluorescent pigments are of high agricultural importance. Fluorescent pseudomonads produce a wide range of secondary metabolites. Certain metabolites, including siderophores such as pyoverdine and pyochelin, play an important role in antagonism against plant root pathogenic fungi (Weller, 2007). In addition to siderophores, some pseudomonads are capable of producing a wide variety of antibiotics such as 2,4-diacetylphloroglucinol (DAPG), phenazines (PCA), pyocyanine, pyoluteorin (Plt), pyrrolnitrin (Prn), and viscosinamide (Haas and Defago, 2005). For example, *Pseudomonas* strains that produce the antifungal compound DAPG play an important role in the suppression of some soil-borne fungal diseases of wheat and barley (McSpadden Gardener et al., 2000). The HCN synthesized by pseudomonads provides biocontrol activity against particular soil-borne pathogens (Weller, 2007).

Pseudomonads may also contribute to overall plant health and development through production of different enzymes. Some of these enzymes have a role in the cycling of C, N, and P nutrients in the rhizosphere. Such examples of these enzymes are protease, urease, nitrate-and nitrite reductase, and acid-and alkaline phosphatase (Hirkala, 2006). Other cellulolytic and chitinolytic enzymes aid in the biocontrol of many plant pathogens (Garbeva et al., 2004).

2.4.1.2 *Bacillus* spp.

Bacilli, which are abundant in agricultural soils, are Gram positive, spore-forming, rod-shaped, aerobic bacteria. *Bacillus* spp. are of great economic, clinical, and industrial importance. Some bacilli species are known to cause many animals and human pathogens, and are important biological control agents of many plant pathogens (Porwal et al., 2009). Perhaps the best known example of the economic and agricultural importance of the genus *Bacillus* is *Bacillus*

thuringiensis (Bt) which accounts for over 90% of all marketed bio-insecticides, with a world-wide market of over a billion dollars annually for the control of insect pests (Emmert and Handelsman, 1999). Several studies have documented the ability of *Bacillus* spp. to enhance plant growth and to suppress a wide range of plant diseases. In fact, the genus *Bacillus* was one of the earliest candidate PGPR strains for study of their impact on plant growth in the former Soviet Union in the early and mid-twentieth century. For instance, *B. megatherium* var. *phosphoaticum* was an efficient phosphorus solubilizer (Chanway, 2002).

Several studies demonstrated the ability of *Bacillus* spp. to enhance plant growth and to suppress a wide range of plant diseases. For example, Vessey and Buss (2002) found soybean plant growth promotion upon co-inoculation of *Bacillus cereus* with *Bradyrhizobium japonicum*, through improved root development and enhanced nodulation.

2.4.2 Direct growth promotion mechanisms

The direct growth promotion of plants by endophytic bacteria occurs when the bacteria facilitate nutrient acquisition by plant roots. Several mechanisms have been proposed and include the following.

2.4.2.1 Biological nitrogen fixation

Nitrogen is an essential component of many critical plant compounds. It is a major part of all amino acids, nucleic acids, and chlorophyll. Nitrogen is considered the most limiting plant growth nutrient because atmospheric N_2, which constitutes 78% of the earth's atmosphere, can not be assimilated by higher plants directly into protein (Havlin et al., 2005). Therefore, N_2 needs to be converted to plant available N forms through the following processes: 1) BNF with different symbiotic and asymbiotic microorganisms; 2) industrial N fixation through the Haber-Bosch process that converts N_2 to ammonia under high temperature and pressure; and 3) the conversion of N_2 into N oxides by lightening in the atmosphere (Grubar and Gallowy, 2008).

As the world population continues to rise, demand for N fertilizers (which are derived by the Haber-Bosch process) is expected to increase. For instance, by 2050, about 5.5 billion inhabitants may owe their life existence to industrial N fertilizers (Crews and Peoples, 2004). However, different factors such as the increasing price of N fertilizers and the negative impact

their use has had on the environment, has lead to intensive research worldwide to reduce our dependency on chemical fertilizers (Crews and Peoples, 2004; Grubar and Gallowy, 2008). An environmentally sound and sustainable source of N as well as an economically beneficial alternative N supply strategy is the utilization of legumes which fix considerable amounts of N_2.

Biological N fixation (BNF) is the second most important biological process after the photosynthesis (Zuberer, 2005). Biological N fixation is restricted only to prokaryotic organisms. The degree of association between plant host and N_2 fixing bacteria could be either through symbiotic, endophytic, or free living associations (asymbiotic). The emphasis in this section will be on the BNF by endophytic associations; however, other N_2 fixing systems will be mentioned briefly.

The rhizobia-legume symbiosis is perhaps the most important N_2 fixing agent in agricultural systems (Herridge et al., 2008). Estimates of BNF by legumes in agricultural systems vary greatly depending on bacterial strains and host plants. Herridge et al. (2008) estimated that pulse legumes fix around 21 million tonnes (Tg $year^{-1}$) whereas in forage/pasture legumes the fixation rate is between the range 12 to 25 Tg $year^{-1}$

Unlike symbiotic associations, some free living soil bacteria in the rhizosphere of plants have the ability to fix N_2 asymbiotically. *Azotobacter*, *Beijerinckia*, *Clostridium* are examples of these bacteria (Gentili and Jumpponen, 2006). Another non-endophytic association occurs in the case of *Azospirillum* spp.. However, both systems need to compete with other microorganisms in the rhizosphere and withstand severe environmental conditions (Cocking, 2003). The growth promotion achieved by inoculating plants with these associative N_2 fixers is credited to other mechanisms other than N_2 fixation (Vessey, 2003).

Endophytic bacteria, which form intimate associations with plants, are capable of fixing N_2 in various crops without forming nodule-like structures. Bacterial endophytes residing inside the plant interior are protected from competition with other bacteria, and are supplied with nutrients directly from the host plants. In return, the plant interior, which is rich in carbon and low in O_2, provide favorable conditions for fixation of N_2 which can then be transferred by the bacteria to their hosts (Ladha and Reddy, 2003).

The best example of endophytic diazotrophs is the bacterium *Gluconacetobacter diazotrophics* which was first isolated from sugarcane in Brazil two decades ago and is now being isolated in other parts of the world. This bacterium fixes N_2 in a wide range of crops such as sugarcane, corn and wetland rice (Saravanan et al., 2008). Other genera of bacterial diazotrophs isolated from crops include *Herbaspirillum* spp. from sugarcane, maize, and rice, *Klebsiella* spp. from maize, and *Azoarcus* spp. from kallar grass (Dobbelaere et al., 2003).

The contribution of BNF by diazotrophic endophytic bacteria to N-nutrition is significant. For example, Cockin (2003) estimated that sugarcane in association with endophytic bacteria fix N_2 in the range of 26 to160 kg N ha^{-1} with an average of 100 kg N ha^{-1} in Brazilian agriculture system. Also, Kallar grass yields 20 to 40 tonnes of hay ha^{-1} $year^{-1}$ in infertile soils when inoculated with *Azoarcus* spp. (Ladha and Reddy, 2003).

2.4.2.2 Phosphate solubilization

Phosphorus (P) is one of the main macronutrients required for plant growth in relatively high amounts. Phosphorus is a major component in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) which directs the synthesis of proteins that are necessary for vigorous growth and development of reproductive parts (Havlin et al., 2005). In terms of plant nutrition, P is the second most limiting nutrient for plant growth after N especially in agricultural systems that contain N_2 fixing legumes (Richardson, 2001).

Most soils contain a high amount of P in many organic and inorganic forms, but most of it is not in a form readily available for plant uptake. Plants uptake the P from the soil solution in two anions: HPO_4^{-2} and $H_2PO_4^{-1}$ depending on the soil pH (Rodriguez and Fraga, 1999; Richardson, 2001).

To remedy P deficiencies, chemical P fertilizers are applied to soils; however, a large portion is transformed into less available forms by precipitation with aluminum (Al) and iron (Fe) oxides in acidic soils, and by calcium (Ca) in alkaline soils (Richardson, 2001). Gyaneshwar et al. (2002) projected that 75 to 90% of chemical fertilizers are rapidly precipitated after application. Considering the agricultural, economical and environmental issues with producing, managing and using P-based fertilizers, microbial communities associated with plant

rhizospheres could be utilized to the benefit of agricultural sustainability and environmental quality.

Several soil bacterial and fungal genera have the capacity to solubilize insoluble inorganic P compounds and make them available for plant uptake. Such microorganisms are referred to as phosphate solubilizing microorganism (PSM) (Rodriguez and fraga, 1999). Among bacterial genera that are considered to be members of PSM are *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aereobacter*, *Flavobacterium* and *Erwinia*. However, *Pseudomonas*, *Bacillus*, and *Rhizobium* are the superior P solubilizers (Richardson, 2001).

Phosphorus solubilizing bacteria could improve P uptake and ultimately plant development. In legume plants, P deficiency is a limiting factor for achieving high production because P is essential for nodulation and N_2 fixation (Raman and Selvaraj, 2006). Rosas et al. (2006) demonstrated that co-inoculation of soybean with *Bradyrhizobium japonicum* and *Pseudomonas putida*, a good P solubilizer, significantly enhanced root dry weight, shoot dry weight, nodule number, and dry weight of nodules compared to inoculation with *B. japonicum* alone.

Similarly, Elkoca et al. (2008) reported that co-inoculation of chickpea with *Rhizobium leguminosarum* and *Bacillus megatherium* (M-3), a P-solubilizer, in both controlled and field conditions significantly increased root and nodule dry weight, total biomass yield and total N content of shoot compared with control treatments.

2.4.2.3 Siderophores synthesis

Iron (Fe) is a cofactor for approximately 140 enzymes which catalyze many biochemical reactions. Therefore, it plays an important role for the growth and metabolism of almost all living organisms (Sharma et al., 2003; Crowley, 2006). For example, the enzyme complex nitrogenase, which mediates BNF in *Rhizobium*-legume symbioses, consists of two proteins, one of which, dinitrogenase reductase (the Fe protein), is an essential for N_2 fixation (Graham, 2005).

Iron is the fourth most abundant mineral on the earth, but the ferric ion (Fe III), the predominant form of Fe in soil, is extremely insoluble in soils at neutral pH (Glick, 1995; Mossialos and Amoutzias, 2007). Although plants produce phytosiderophores under Fe-deficient

soils, microbial siderophores show a higher affinity for Fe than phytosiderophores and might be more effective at competing for Fe in soils (Masalha et al., 2000).

Siderophores are low-molecular-weight Fe-binding compounds that are produced by many soil microorganisms under Fe deficiency conditions (Crowley, 2006). Siderophores are synthesized by microbes which chelate Fe III, and transport it back to their cells where it becomes available for microbial growth (Glick, 1995). Numerous soil bacterial and fungal genera in the plant rhizosphere are able to synthesize siderophores and up to 500 siderophore structures are known so far (Crowley, 2006). Endophytic bacteria also are reported to produce siderophores, a mechanism which may be highly important for their growth as they have to compete with their host plant cells for Fe (Sessitsch et al., 2004).

Siderophore-producing bacteria can enhance growth of their host plant either as biofertilizers (i.e., increase Fe availability in the immediate surrounding area of their host plant roots) or by their biocontrol activities (siderophores are produced that scavenge Fe from the rhizosphere of plants and disadvantage potential pathogens) (Glick, 1995).

Iron is critical for the N_2 fixation process because it is required in relatively high amounts by bacteroids, the enzyme nitrogenase, and for synthesis of leghemoglobin (Crowley, 2006). Marek-Kozaczuk et al. (1996) reported positive plant growth and symbiotic N_2 fixation upon of co-inoculation of clover with *Pseudomonas fluorescens*, which produced siderophores, and *Rhizobium leguminosarum bv. trifolii* under gnotobiotic conditions.

2.4.2.4 Phytohormone (IAA) production

Diverse bacterial and fungal species possess the ability to produce several types of plant growth regulators or phytohormones such as auxins, cytokinins, gibberellins, ethylene, and absicisic acids (ABA) (Zahir et al., 2003). These hormones are thought to change root growth patterns and morphology, resulting in greater root surface area (Vessey, 2003). Most previous research attention has focused on the role of the auxin phytohormone indole-3-acetic acid (IAA) (Glick, 1995).

Indole-3-acetic acid is a phytohormone that is said to play a central role in cell division, cell enlargement, and root initiation (Vessey, 2003). This modification of root patterns enhance root surface area and thus increase plant ability to absorb more nutrients, which in turn stimulate plant growth (Gravel et al., 2007). Production of IAA is common phenomena among many

genera of soil bacteria and fungi. Endophytic bacteria also are able to synthesize IAA (Sessitsch et al., 2004).

The phytohormone IAA appears also to be involved in the stimulation of *Rhizobium*-legume symbiosis (Spaepen et al., 2007). Indole-3-acetic acid involved in cell division and differentiation ultimately increase root length and root hair abundance providing more sites for infection and nodulation (Vessey and Buss, 2002; Spaepen et al., 2007). Molla et al. (2001) found that co-inoculation of soybean with *Azospirillum brasilense* with *Bradyrhizobium japonicum* significantly increased total root length, root number, root dry matter, root hair development, nodule number, and nodule fresh weight either by *Azospirillum* alone or its co-inoculum.

2.4.3 Indirect growth promotion mechanisms

The indirect growth enhancement of host plants by their associated beneficial microbes occurs through the lessening or suppression of phytopathogenic microorganisms in a process termed biological control, in which the BCAs produce biocontrol traits lethal to the pathogenic microorganisms or compete with them for nutrients supply and root colonization sites.

2.4.3.1 Biological control of plant pathogens

Soil-borne plant pathogens continue to be a major threat to agricultural development and productivity worldwide. Four major groups of plant pathogens are present in the soil, but only fungi and nematodes are the key players in soils, where few bacterial genera are found to be soil-borne pathogens (Raaijmakers et al., 2009). The economic damage cause by soil-borne pathogens worldwide is devastating. For example, Oerke (2005) estimated that from 2001 to 2003, an average of 7% to 15% of crop loss may be caused by soil-borne pathogenic fungi and bacteria in many agricultural crops such as wheat, rice, maize, and soybean. Also, crop losses due to nematode infection are estimated to be about 100 billion US dollars worldwide each year (Bird and Kaloshian, 2003).

Several plant disease control methods have been implemented to protect crops against a wide range of phytopathogens. These approaches include breeding for resistant cultivars; use of chemical pesticides, crop rotation, cultivation techniques, manure application, and addition of compost or other nutrients (Whipps and Gerhardson, 2007). Currently, chemical pesticides and

growing resistant cultivars are the two main methods applied in crop protection (Vassilev et al., 2006).

Due to the increasing concerns among both scientific and public communities over the environmental and health safety issues associated with using chemicals in plant protection, some chemicals have been banned (Whipps and Gerhardson, 2007). However, the use of tolerant cultivars in crop protection is still limited, especially in fruit and vegetable crops (Vassilev et al., 2006). An alternative and attractive method to control plant disease is the use of plant rhizosphere associated beneficial microorganisms, which are called Biological Control Agents (BCAs) (Whipps and Gerhardson, 2007).

Many BCAs are known to reduce the incidence and severity of plant disease. *Pseudomonas* spp. and *Bacillus* spp. are the predominant bacterial BCAs, whereas *Trichoderma* spp. is the most important fungal BCA (Gerhardson, 2002). Bacterial endophytes also exhibit antagonistic activities against a broad spectrum of fungal pathogens (Berg and Hallmann, 2006). The majority of antagonistic endophytic bacteria are Gram-negative bacteria and are dominated by the fluorescent pseudomonads, followed by members of the genus *Bacillus*, as the predominant antagonistic group among Gram-positive bacteria (Berg and Hallmann, 2006).

The modes of action of bacterial BCAs have been studied extensively for rhizospheric bacteria (e.g., PGPR) (Whipps, 2001). It is assumed that bacterial endophytes use similar mechanisms toward the control of plant pathogens (Berg and Hallmann, 2006). Some of these mechanisms are well established for antagonistic endophytic bacteria such as induced systematic resistance (Kloepper and Ryu, 2006), while much still needs to be learned about other modes of action used such as antibiosis, competition, and lysis (Berg and Hallmann, 2006).

Bacterial BCAs control plant diseases through different mechanisms, including: (1) the suppression of pathogens by the production of antimicrobial metabolites (antibiosis); (2) competition for colonization sites on roots and limited resources in the rhizosphere; competition for iron through siderophore synthesis that limit pathogen access to Fe; (3) induced systematic resistance (ISR) of their host plants; (4) hydrogen cyanide (HCN) production; (5) parasitism where beneficial bacteria inhibit plant pathogens via production of extracellular enzymes such as β-glucanase, cellulose, chitinase, pectinase, and protease that can lyse pathogen cell walls; and (6) degradation of toxins produced by plant pathogens (Whipps, 2001; Banerjee et al., 2006).

2.5. Bacterial identification and classification

A first step in biofertilizer and biopesticide development is the identification of candidate microorganisms with putative plant growth promoting traits (Hynes et al., 2008). One reason for giving priority to identification and classification of bacteria is that most bacterial species are still unknown due to our poor knowledge of bacterial ecology. Thus, the discovery of new species with novel functions is highly anticipated (Busse et al., 1996). Additionally, the rhizosphere has been found to be a natural reservoir for some opportunistic human pathogens belonging to various bacterial genera including *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Ochrobactrum*, *Pseudomonas*, *Ralstonia*, *Staphylococcus* and *Stenotrophomonas* (Berg et al, 2005). Also, early identification of a microorganism would allow comparison with prior knowledge about negative indications for commercialization which could cease further investigation and development (Whipps and Gerhardson, 2007).

Bacterial characterization and classification is based on different cultural, morphological, physiological, biochemical, chemotaxonomic, and molecular methods. Fatty acid methyl ester (FAME) analysis is a chemotaxonomic tool commonly used in the determination of bacterial species taxonomy. Fatty acid methyl ester analysis is automated, affordable, relatively easy (Slabbinck et al., 2009) and taxonomic identification is available commercially through MIDI Inc.

Over 300 fatty acids are found in bacterial cells. Bacterial fatty acids are highly conserved due to their role in cell structure and function and the presence of distinct fatty acids could be used for identification of bacteria. Fatty acid methyl ester analysis (FAME) is dependent on a library of known FAME bacterial profiles to which of profiles of unknown bacteria can be compared (Sasser, 2006). It is suggested that the by using strictly standardized conditions ensures stable and reproducible cellular fatty acid profiles (Sasser, 2006).

In recent years, as the result of the widespread use of polymerase chain reaction (PCR) and DNA sequencing, 16S ribosomal RNA (16S rRNA) gene sequencing has emerged as a more rapid, reliable, and accurate method for bacteria identification (Janda and Abbott, 2007). Although, other housekeeping genes exist, 16S rRNA is still the most common genetic marker used for bacterial phylogeny and taxonomy because: (i) it is found in almost all bacteria; (ii) its function over time has not changed; and (iii) the 16S rRNA gene is large enough for informatics purposes (Janda and Abbott, 2007). Most importantly, an enormous number of 16S rRNA gene

sequences have accumulated in public databases (Zwolinski, 2007). Similar to FAME analysis, DNA sequences must be referenced against a known set of sequences in a database. The approach of combining phenotypic and genotypic methods ensures correct identification and allows comparison of the different identification systems.

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3.0 IDENTIFICATION AND CLASSIFICATION OF ENDOPHYTIC BACTERIA ISOLATED FROM CANOLA AND WHEAT PLANTS

3.1 Preface

Plant root-associated bacteria hold great promise to enhance plant productivity and agricultural sustainability. The plant endorhizosphere niche contains a diverse population of bacterial taxa which might harbor beneficial, neutral, or harmful species. In order to develop bacterial inoculants to improve plant growth and yield under Saskatchewan soils conditions, accurate and reliable identification of bacterial isolates is an essential first step in such inoculants development program. In this study, endophytic bacterial isolates were identified using both FAME analysis and 16S rRNA gene sequence analysis. The identity of bacterial isolates obtained using these methods were compared to a public database to group them according to their biosafety level.

3.2 Introduction

The rhizosphere habitat is a hot spot for soil microorganisms due to its richness in nutrients when compared to bulk soils. Among the microbes that thrive in the rhizosphere are a subset of bacteria known as plant growth-promoting bacteria (PGPB) (Barea et al., 2005; Gray and Smith, 2005). Plant growth-promoting bacteria are either rhizospheric or endophytic and are being exploited for biotechnological applications in the agricultural industry as biofertilizer and/or biopesticide agents to improve plant growth and fitness (Lucy, 2004; Banerjee et al., 2006).

The rhizosphere, as well as the endorhizosphere, of various crop plants has been found to harbor some opportunistic human and animal pathogens that belong to various bacterial genera (Berg et al., 2005; Ryan et al., 2008). For example, potential pathogenic strains of *Stenotrophomonas maltophilia* were found to be common with the rhizosphere of alfalfa, canola, potato, and wheat (Schwieger and Tebbe, 2000; Germida and Siciliano, 2001; Berg et al., 2002). Also, strains of *Burkholderia cepacia* were detected in the majority of plant rhizospheres investigated (Berg et al., 2005).

In addition to their presence in the rhizosphere, some opportunistic human pathogens were detected inside the plant interior. For instance, Guo et al. (2002) reported that endophytic *Salmonella* strains were detected in various fruits and vegetables plants.

Thus, the identification and classification of bacteria is a vital element in any risk assessment for any potential biotechnological products (OECD, 2003) and may lead us to two important goals: 1) establish a general frame of reference; and 2) aid in predictive analysis (OECD, 2003).

As most of the bacterial species are still unknown, our knowledge about their ecology is still poor. Thus, accurate and reliable identification of bacteria is an important task in many, if not all, disciplines within microbiology (Busse et al., 1996). Traditionally, bacterial isolates were identified and classified based on phenotypic methods, including morphological, physiological, and metabolic features (Rossello-Mora and Amann, 2001). Major drawbacks of these phenotypic approaches are that they are time-consuming, tedious, and variability among strains belonging to the same species could be substantial (Busse et al., 1996; Mignard and Flandrois, 2006).

Other methods by which bacteria could be classified and identified are the chemotaxonomic methods that rely on analysis of chemical composition of bacterial cells (Busse et al., 1996) and include many features such as Gram reaction, fatty acid pattern, and whole cell protein composition (Busse et al., 1996). These methods are culture dependent which is considered a limitation of using them, as in the case of the Gram stain. Some chemotaxonomic approaches such as FAME analysis is gaining popularity and has been used increasingly by many laboratories over the last 15 years (Dawyndt et al., 2006; Slabbinck et al., 2009).

Over 300 fatty acids have been detected in bacterial cells (Kunitsky et al., 2006). Several features make fatty acid profiles a very suitable taxonomic marker, such as observed differences in chain length, position of double bounds and the binding of functional groups (Salbbinck et al., 2009). For example, fatty acids between 9 and 20 carbons in length were used to identify genera and species of bacteria (Dawyndt et al., 2006). Also, the presence of lipopolysaccharides (LPS) and hydroxy fatty acids are an indicator that the organism under investigation is Gram-negative; however, if fatty acids are branched without LPS, then the hydroxy fatty acids indicate that the bacteria are Gram-positive (Kunitsky et al., 2006). Bacterial fatty acids are genetically highly conserved, and change only over a long period of time (Kunitsky et al., 2006).

Using knowledge obtained from bacterial fatty acids profile, bacterial strains can be identified using gas chromatographic-FAME analysis that relies on an automated identification system, the Sherlock Microbial Identification System, known as the MIDI (MIDI Inc., Newark, DE) (O'Hara, 2005; Kunitsky et al., 2006). Bacterial isolates for identification are grown and processed in different steps under controlled conditions suggested by the manufacturer. Then the fatty acid composition from the organism under investigation is compared to those in a MIDI database library (Kunitsky et al., 2006).

Due to the fact that FAME analysis is affordable, relatively easy, and automated, many researchers over the world have used this technique for taxonomic identification of bacteria (Salbbinck et al., 2009). It has been found that FAME gives reproducible fatty acid profile results when controlled growth temperature and use of standardized commercially available media are followed (Sasser, 2006).

Gene sequencing of the 16S rRNA gene is a genotypic method by which bacteria can be classified and identified. 16S rRNA has been the most common genetic marker used for bacterial identification and classification for various reasons ((Janda and Abbott, 2007). First of all, its function over time has not changed. Secondly, the 16S rRNA gene is universal in bacteria. Lastly, it is large enough for informatics purposes. Using 16S rRNA has the advantage over phenotypic approaches for not requiring optimal growth conditions and cultivation of microorganisms. The 16S rRNA gene sequence has been used to identify various types of slow and fast growth bacteria, rare bacterial species, as well as a novel species of bacteria (Mignard and Flandrois, 2006).

Even though DNA:DNA hybridization, a genomic method used to measure the genetic similarity between two species, has been proposed for defining species and genus, difficulties with performing this technique has made the 16S rRNA gene the new gold standard for definition of species (Clarridge III, 2004). It has been generally accepted by taxonomists that a percent identity score of $\geq$ 97% and $\geq$ 99% is required to classify bacteria to genus and species, respectively (Petti, 2007). However, a major limitation of the 16S rRNA gene sequence is its inability to sufficiently discriminate among all bacterial phyla (Janda and Abbott, 2007). For example, *Bacillus psychrophilus* and *B. globisporus* share >99.5% sequence similarity, yet they are distinguishable by DNA homology (Janda and Abbott, 2007). Also, the 16S rRNA

sequencing can not differentiate species within the *P. fluorescens*, *P. putida*, or *P. syringae* complexes (Ait Tayeb et al., 2005).

The successful identification and classification of endophytic bacteria would represent a significant resource for further plant-bacterial studies under Saskatchewan soil conditions. Therefore the objectives of this study were: (i) to confirm the taxonomic identification of bacterial endophytes using FAME analysis and 16S rRNA gene sequencing; (ii) to compare the identification obtained with the 16S rRNA gene sequencing to those obtained with FAME analysis; and (iii) to group bacterial isolates based on their risk assessment.

3.3 Materials and Methods

3.3.1 Origin of bacterial isolates

The bacterial isolates used in this study were pure cultures originally obtained from the culture collection of the Soil Microbiology Laboratory at the department of Soil Science at the University of Saskatchewan. These bacteria were previously isolated from canola and wheat plants from different field sites in Saskatchewan as mentioned earlier (Germida et al., 1998; Misko and Germida, 2002). The bacteria were previously identified using FAME analysis (Germida et al., 1998; Misko and Germida, 2002). Forty endophytic bacterial isolates were initially selected on the basis of their FAME identification for this study and are summarized in Table. 3.1. Information about bacterial source is provided in Appendix 1.

Table 3.1. List of endophytic bacterial isolates used in this study.

Serial #	Isolate code	FAME Identification	Similarity Index (SIM) †
1	EB EE 4-36	*Pseudomonas corrugata*	0.861
2	EB EE 2-28	*Pseudomonas fluorescens*	0.816
3	EB XDE 4-48	*Pseudomonas viridiflava*	0.833
4	EB XDE 4-33	*Pseudomonas putida*	0.795
5	EB IE 1-32	*Pseudomonas chlororaphis*	0.711
6	EB EE 3-78	*Stenotrophomonas maltophilia*	0.429
7	WQE 2-28	*Pseudomonas chlororaphis*	0.604
8	EB QE 3-37	*Pseudomonas savastanoi pv. fraxinus*	0.810
9	EB EE 4-25	*Pseudomonas putida*	0.708
10	EB QE 4-19	*Pseudomonas corrugata*	0.791
11	WQE 2-25	*Pseudomonas putida*	0.603
12	WQE 3-1	*Pseudomonas marginalis*	0.537
13	WQE 2-8	*Pseudomonas syringae*	0.924
14	WEE 2-30	*Bacillus pumilus*	0.795
15	EB IE 1-43	*Pseudomonas syringae*	0.836
16	KCE 3-7	*Bacillus megaterium*	0.523
17	EB FE 1-59	*Pseudomonas chlororaphis*	0.773
18	EB FE 2-45	*Pseudomonas putida*	0.673
19	EB FE 2-92	*Pseudomonas corrugata*	0.827
20	EB EE 2-16	*Pseudomonas viridiflava*	0.897

Table 3.1. Continued.

Serial #	Isolate code	FAME Identification	Similarity Index (SIM) †
21	EB EE 2-18	*Pseudomonas cichorii*	0.841
22	EB EE 2-23	*Pseudomonas syringae*	0.613
23	MF QE 1-69	*Pseudomonas putida*	0.939
24	MF EE 1-44	*Pseudomonas syringae*	0.782
25	MF IE 3-83	*Bacillus megaterium*	0.781
26	MF FE 2-21	*Pseudomonas syringae*	0.693
27	MF EE 1-34	*Pseudomonas putida*	0.711
28	MF FE 3-69	*Flavobacterium johnsoniae*	0.403
29	MF IE 2-50	*Arthrobacter ilicis*	0.907
30	MF IE 2-30	*Bacillus cereus*	0.169
31	MF FE 1-18	*Pseudomonas syringae*	0.674
32	MF FE 3-64	*Pseudomonas putida*	0.517
33	MF EE 4-4	*Pseudomonas chlororaphis*	0.517
34	MF XDE 3-57	*Pseudomonas syringae*	0.690
35	MF EE 2-122	*Pseudomonas corrugata*	0.805
36	MF EE 4-19	*Pseudomonas putida*	0.627
37	MF XDE 1-18	*Pseudomonas fluorescens*	0.511
38	MF XDE 1-6	*Pseudomonas chlororaphis*	0.741
39	MF XDE 3-65	*Pseudomonas syringae*	0.672
40	KCP 1-52	*Pseudomonas chlororaphis*	0.916

†: A numerical value that tells how closely the fatty acid composition of an unknown isolate to the mean of the fatty acid composition of the control strains used to generate the FAME library entry as its match (Kunitsky et al., 2006)

Stock cultures were maintained in 20% glycerol at -80 ˚C. All strains were cultured in 50 mL of 1/10 strength TSB (3 g L^{-1} H_2O:Difco Laboratories Inc. Detroit, Michigan, USA) at room temperature for 48 h with continuous agitation at 150 rpm on a G-24 environmental gyratory shaker (New Brunswick Scientific, N.J. USA).

3.3.2 Bacterial identification and classification

Bacterial isolates were initially identified using FAME analysis by comparing to known library database using MIDI Microbial Identification Software (Sherlock TSBA Library version 3.80; Microbial ID, Inc. Newark, NJ, USA). Subsequent identification of bacterial isolates was performed via the 16S rRNA gene sequence analysis.

3.3.2.1 Fatty acids methyl ester (FAME) analysis

Isolates were identified based on FAME analysis and analyzed by gas chromatography (GC), using the MIDI system (Microbial Identification Systems, Inc., Newark, NJ, USA) as described by Germida et al. (1998). Briefly, isolates were grown on solidified TSB plates at 28 ˚C for 24 h and bacterial cells (ca. 40 mg) were collected. Methanolic NaOH solution (1 mL) (15% [w/v] NaOH in 50% [v/v] methanol) was added and cells were saponified at 100 ˚C for 30 min. Esterification of fatty acids were performed with 2 mL of 3.25 N HCl in 46% (v/v) methanol at 80 ˚C for 10 min. The FAMEs were extracted into 1.25 mL of 1:1 (v/v) methyl-*tert*-butyl ether-hexane, and the organic extract washed with 3 mL of 1.2% (w/v) NaOH before analysis using a gas chromatograph (GC). The GC (Hewlett-Packard 5890A) was equipped with a flame ionization detector and a capillary column Ultra 2-Hewlett Packard No. 19091B-102 (cross-linked 5% phenyl-methyl silicone; 25 m, 0.22 mm ID; film thickness, 0.33 μm; phase ratio, 150) with N_2 as the carrier gas. The FAME peaks were automatically integrated by a Hewlett-Packard 7673 integrator and bacterial isolates named using the MIDI Microbial Identification Software (Sherlock TSBA Library version 3.80; Microbial ID, Inc. Newark, NJ, USA). The FAME profile of *Xanthomonas maltophilia* ATCC 13637 was used as a reference for the MIDI determinations. Strains with a similarity index (SIM) of less than 0.3 were not considered tentatively identified (Sasser, 2006).

3.3.2.2 16S rRNA gene sequence analysis

The procedure of Philips (2008) to carry the 16S rRNA gene sequence analysis was followed. Isolates from stock cultures were streaked on 1/10 strength TSA plates to obtain pure colonies. Purified isolates were then inoculated into 150 µL of Luria-Bertani (LB) broth medium in a 96-well microtitre plate by just touching the loop to the colony and carefully mixing into the media in the well to prevent the production of dense cultures, which were grown overnight at 28 ˚C. Ninety microliters of each culture grown in the LB plate was then pipetted into each well of a sterile 96-well PCR plate containing 10 µL sterile anhydrous glycerol into each well. Cultures were mixed with glycerol by repeatedly pipetting up and down. Plates were then sealed with adhesive cover and were frozen at -20 ˚C until ready for sequencing. Near full-length 16S rRNA gene fragments were sequenced directly from isolates using the bacterial primers PB36 (5'-AGRGTTTGATCMTGGCTCAG-3'; Saul et al. 2005) and PB38 (5'-GKTACCTTGTTACGACTT-3'; Saul et al. 2005). Sequencing of both strands was performed at the Plant Biotechnology Institute, Saskatoon, SK, Canada using the AB 3730xl capillary electrophoresis DNA analyzer (Applied Biosystems, Foster City, CA).

Each 16S rRNA gene sequence was submitted for comparison to the GeneBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1997).

3.3.3 Risk grouping of bacterial isolates

After the identity of bacterial isolates were determined by FAME analysis and/or 16S rRNA gene sequencing. The public database, the German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de), was used for grouping of bacteria into risk groups (Berg et al., 2005).

3.4 Results

3.4.1 Identification of bacteria by FAME analysis

Fatty acid methyl ester analysis was determined for 40 endophytic isolates that have been previously identified by FAME analysis (Misko and Germida, 2002). When compared with the previous FAME identification, 35 of 40 isolates were in agreement on the genus level based on the current FAME analysis (Table 3.2). Five isolates were previously identified as *Pseudomonas corrugata* EB EE 4-36, *Pseudomonas fluorescens* EB EE 2-28, *Pseudomonas putida* EB EE 4-25, *Pseudomonas marginalis* WQE 3-1, and *Pseudomonas viridiflava* EB EE 2-16. The confirmation of their analysis based on FAME identification has resulted on the following identity *Bacillus cereus*, *Bacillus cereus, Bacillus cereus, Stenotrophomonas maltophilia*, and *Chromobacterium violaceum*, respectively. At the species level, only 20% (N= 8) of the examined isolates identity were in agreement at the species level based on their reconfirmation by FAME analysis (Table 3.2).

It should be noted that several isolates had similarity index values less than 0.3, which is considered tentatively unidentified as described by the manufacturer of MIDI system (Kurtinesy et al., 2006).

3.4.2 Identification of endophytic bacteria using 16S rRNA gene sequencing

All of 40 endophytic isolates that were characterized by FAME analysis as mentioned above were further characterized by 16S rRNA gene sequencing. However, seven isolates (17.5%) sequences were not obtained, despite repeated attempts (Table. 3.2).

Among the 33 isolates that were successfully sequenced, 30 isolates belong to the genus *Pseudomonas*, while three others belong to the genus *Stenotrophomonas* (Table. 3.2). Generally, both methods have highly correlated identification agreement at the genus level, but in some incidences they agreed at the species level as in the case of the strains EB EE 3-78, and WQE 3-1 which were both were identified as *Stenotrophomonas maltophilia* by both FAME analysis and 16S rRNA gene sequencing (Table. 3.2). Similarly, the strains MF FE 2-21, and MF FE 1-18 were identified as *Pseudomonas putida* by both methods.

Table 3.2. Identification of endophytic bacterial isolates by FAME analysis and 16S rRNA gene sequencing.

Isolate code †	FAME Identification ‡	SIM §	FAME Identification	SIM	16S rRNA Identification ¶	(%) Sequence Similarity	Phylogenetic group
EB EE 4-36	*Pseudomonas corrugata*	0.861	*Bacillus cereus*	0.183	*Pseudomonas fluorescens*	99	γ- proteobacteria
EB EE 2-28	*Pseudomonas fluorescens*	0.816	*Bacillus cereus*	0.305	*Pseudomonas fluorescens*	99	γ- proteobacteria
EB XDE 4-48	*Pseudomonas viridiflava*	0.833	*Pseudomonas chlororaphis*	0.460	*Pseudomonas syringae*	99	γ- proteobacteria
EB XDE 4-33	*Pseudomonas putida*	0.795	*Pseudomonas fluorescens*	0.028	*Pseudomonas putida*	99	γ- proteobacteria
EB IE 1-32	*Pseudomonas chlororaphis*	0.711	*Pseudomonas putida*	0.259	*Pseudomonas fluorescens*	99	γ- proteobacteria
EB EE 3-78	*Stenotrophomonas maltophilia*	0.429	*Stenotrophomonas maltophilia*	0.286	*Stenotrophomonas maltophilia*	95	γ- proteobacteria
WQ 2-28	*Pseudomonas chlororaphis*	0.604	*Pseudomonas putida*	0.259	*Pseudomonas fluorescens*	98	γ- proteobacteria
EB QE 3-37	*Pseudomonas savastanoi pv. fraxinus*	0.810	*Pseudomonas chlororaphis*	0.383	*Pseudomonas mandelii*	98	γ- proteobacteria
EB EE 4-25	*Pseudomonas putida*	0.708	*Bacillus cereus*	0.237	No match		
EB QE 4-19	*Pseudomonas corrugata*	0.791	*Pseudomonas chlororaphis*	0.492	*Pseudomonas fluorescens*	99	γ- proteobacteria
WQE 2-25	*Pseudomonas putida*	0.603	*Pseudomonas chlororaphis*	0.177	*Pseudomonas fluorescens*	98	γ- proteobacteria
WQE 3-1	*Pseudomonas marginalis*	0.537	*Stenotrophomonas maltophilia*	0.410	*Stenotrophomonas maltophilia*	97	γ- proteobacteria

Table 3.2. Continued.

Isolate code †	FAME Identification ‡	SIM §	FAME Identification	SIM	16S rRNA Identification ¶	(%) Sequence Similarity	Phylogenetic group
WQE 2-8	*Pseudomonas syringae*	0.924	*Pseudomonas syringae*	0.422	*Pseudomonas fluorescens*	95	γ- proteobacteria
WEE 2-30	*Bacillus pumilus*	0.795	*Bacillus pumilus*	0.660	No match		Firmicutes
EB IE 1-43	*Pseudomonas syringae*	0.836	*Pseudomonas chlororaphis*	0.573	*Pseudomonas fluorescens*	95	γ- proteobacteria
KCE 3-7	*Bacillus megaterium*	0.523	*Bacillus psychrosacch*	0.680	No match		Firmicutes
EB FE 1-59	*Pseudomonas chlororaphis*	0.773	*Pseudomonas acidovorans*	0.646	*Pseudomonas* sp.	95	γ- proteobacteria
EB FE 2-45	*Pseudomonas putida*	0.673	*Pseudomonas chlororaphis*	0.582	*Pseudomonas putida*	84	γ- proteobacteria
EB FE 2-92	*Pseudomonas corrugata*	0.827	*Pseudomonas syringae*	0.615	*Pseudomonas putida*	98	γ- proteobacteria
EB EE 2-16	*Pseudomonas viridiflava*	0.897	*Chromobacterium violaceum*	0.869	*Pseudomonas fluorescens*	98	γ- proteobacteria
EB EE 2-18	*Pseudomonas cichorii*	0.841	*Pseudomonas putida*	0.596	*Pseudomonas fluorescens*	98	γ- proteobacteria
EB EE 2-23	*Pseudomonas syringae*	0.613	*Pseudomonas putida*	0.605	*Pseudomonas fluorescens*	99	γ- proteobacteria
MF QE 1-69	*Pseudomonas putida*	0.939	*Pseudomonas chlororaphis*	0.168	*Pseudomonas fluorescens*	99	γ- proteobacteria
MF EE 1-44	*Pseudomonas syringae*	0.782	*Pseudomonas chlororaphis*	0.304	*Pseudomonas fluorescens*	98	γ- proteobacteria

Table 3.2. Continued.

Isolate code †	FAME Identification ‡	SIM §	FAME Identification	SIM	16S rRNA Identification ¶	(%) Sequence Similarity	Phylogenetic group
MF IE 3-83	*Bacillus megaterium*	0.781	*Bacillus megaterium*	0.831	No match		Firmicutes
MF FE 2-21	*Pseudomonas syringae*	0.693	*Pseudomonas putida*	0.683	*Pseudomonas putida*	99	γ- proteobacteria
MF EE 1-34	*Pseudomonas putida*	0.711	*Pseudomonas acidovorans*	0.786	*Pseudomonas fluorescens*	99	γ- proteobacteria
MF FE 3-69	*Flavobacterium johnsoniae*	0.403	*Flavobacterium johnsoniae*	0.490	No match		Bacteroidetes
MF IE 2-50	*Arthrobacter ilicis*	0.907	*Arthrobacter aurescens*	0.764	No match		Actinobacteria
MF IE 2-30	*Bacillus cereus*	0.169	*Bacillus cereus*	0.493	No match		Firmicutes
MF FE 1-18	*Pseudomonas syringae*	0.674	*Pseudomonas putida*	0.438	*Pseudomonas putida*	98	γ- proteobacteria
MF FE 3-64	*Pseudomonas putida*	0.517	*Pseudomonas chlororaphis*	0.629	*Pseudomonas fluorescens*	98	γ- proteobacteria
MF EE 4-4	*Pseudomonas chlororaphis*	0.517	*Pseudomonas putida*	0.827	*Pseudomonas fluorescens*	100	γ- proteobacteria
MF XDE 3-57	*Pseudomonas syringae*	0.690	*Pseudomonas corrugata*	0.333	*Pseudomonas fluorescens*	99	γ- proteobacteria
MF EE 2-122	*Pseudomonas corrugata*	0.805	*Pseudomonas putida*	0.647	*Pseudomonas fluorescens*	99	γ- proteobacteria
MF EE 4-19	*Pseudomonas putida*	0.627	*Pseudomonas putida*	0.520	*Pseudomonas flavescencens*	97	γ- proteobacteria

Table 3.2. Continued.

Isolate code †	FAME Identification ‡	SIM §	FAME Identification	SIM	16S rRNA Identification ¶	(%) Sequence similarity	Phylogenetic group
MF XDE 1-18	*Pseudomonas fluorescens*	0.511	*Pseudomonas chlororaphis*	0.706	*Pseudomonas fluorescens*	98	γ- proteobacteria
MF XDE 1-6	*Pseudomonas chlororaphis*	0.741	*Pseudomonas chlororaphis*	0.620	*Stenotrophomonas maltophilia*	99	γ- proteobacteria
MF XDE 3-65	*Pseudomonas syringae*	0.672	*Pseudomonas putida*	0.816	*Pseudomonas putida*	98	γ- proteobacteria
KCP 1-52	*Pseudomonas chlororaphis*	0.916	*Pseudomonas syringae*	0.812	*Pseudomonas fluorescens*	99	γ- proteobacteria

†: For additional information about bacterial isolate code please consult Appendix 1

‡: Endophytic bacterial isolates were identified using fatty acids methyl ester (FAME) analysis.

§: Similarity index, A numerical value that tells how closely the fatty acid composition of an unknown isolate to the mean of the fatty acid composition of the control strains used to generate the FAME library entry as its match (Kunitsky et al., 2006)

¶: Endophytic bacterial isolates were identified using 16S rRNA gene sequencing.

3.4.3 Risk grouping of bacterial isolates

All strains were grouped into risk group 1 (e.g., microorgainsms which are not known to cause any harmful effect against human or/and animals) except five strains. These strains were EB EE 3-78, MF XDE 1-6 and WQE 3-1 which were identified as *S. maltophilia*, strain MF FE 3-69 which was identified as *Flavobacterium johnsoniae* and strain MF IE 2-30 which was identified as *Bacillus cereus* (Table 3.2).

3.5 Discussion

The plant endorhizosphere contains a remarkable diversity of bacteria that could be utilized for agriculture as biofertilizer and/or biopesticide to enhance plant growth and development (Weyens et al., 2009). Among the bacterial genera found within plant tissues are *Arthrobacter*, *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, *Flavobacterium*, *Enterobacteriaceae* and many other groups (Berg and Hallmann, 2006).

The first step toward choosing and developing microbial inoculants is accurate and reliable identification and characterization of potential candidate microorganisms (Hynes et al., 2008; Berg, 2009). Some of the above mentioned bacterial genera contained many human and animal pathogenic species. For example, *S. maltophilia* has been associated with different plant rhizospheres, and has shown a tremendous potential role in many biotechnological applications such as BCA of plant pathogens in agriculture, and in bioremediation of contaminated soils (Berg et al., 1999). However, they have the potential to act as an opportunistic human pathogen (Berg, 2009).

Stenotrophomonas maltophilia has caused significant fatality cases in certain patient populations, especially in those who are severely debilitated, immunocompromised or suffering from cystic fibrosis (CF) or HIV infections (Berg et al., 1999; Ribbeck-Busch et al., 2005). Therefore, it is essential to exclude potential pathogenic bacteria in the early steps of any screening program.

In the current study, the identity of selected bacterial endophytes isolated from inside the roots of field-grown canola and wheat plants were examined using FAME analysis and 16S rRNA gene sequencing. These bacterial isolates were identified by FAME analysis previously as shown in (Table 3.2).

My results have shown that, when isolates whose identity was determined by FAME analysis previously (Table 3.2) were reconfirmed again by FAME analysis in this study, 88% of isolates agreed at the genus level. Only 20% of the examined isolates identity agreed at the species level. These findings may result from issues associated with the FAME database, as in the case of many commercial biochemical systems, such as the need to update the FAME profiles within the database where different important genera are not represented sufficiently with many species (O'Hara, 2005; Hirkala, 2006).

Of the 32 pseudomonad isolates identified using FAME analysis, only two isolates, *P. putida* MF EE 4-19, and *P. syringae* WQE 2-8, agreed at the species level. This might be because of the high genetic diversity of this genus (Ait Tayeb et al., 2008; Tourkya et al., 2009). In agreement with my results, Slabbinck et al. (2009) argued that the uncertainties in the taxonomic position of many *Pseudomonas* spp. is one of the major reasons of the lower resolution power of FAME analysis.

As in the case with the genus *Pseudomonas*, the genus *Bacillus* is a diverse and heterogeneous group of organisms (Slabbinck et al., 2008). My results indicated that reproducible FAME profiles were achieved as four out of five *Bacillus* isolates were re-identified as the same species. These results correlated with previous reports indicating that reproducible FAME profiles could be obtained when standardized environmental conditions are followed (Bertone et al., 1996; Sasser, 2006). Several reports documented that FAME analysis has the potential to discriminate between species within the genus *Bacillus* (Kämpfer, 1994; Slabbinck et al., 2008; Slabbinck et al., 2009). However, FAME analysis can not differentiate some closely-related species within the *Bacillus cereus* and *Bacillus subtilis* groups (Slabbinck et al., 2008).

Comparison between FAME analysis and 16S rRNA gene sequence methods of bacterial isolates in this study, demonstrate that both methods agreed completely in terms of genus identification, when isolates for which sequences were not obtained were excluded. However, at the species level the 16S rRNA identification agreed with the FAME identification only 10% of the time. Similar results were obtained by Hirkala (2006). Such discrepancies between the two methods in identification of isolates at the species level may be attributed to the reasons that have been mentioned earlier regarding the FAME analysis.

All sequences obtained in my study using the 16S rRNA gene belong to two genera; *Pseudomonas* (*n*= 30) and *Stenotrophomonas* (*n*= 3). Two strains WQE 3-1 and MF XDE 1-6 were identified by FAME as *Pseudomonas marginalis* and *Pseudomonas chlororaphis*, respectively. Their identities were confirmed by 16S rRNA gene sequencing as *S. maltophilia*. This is not surprisingly because the taxonomy of the genus *Pseudomonas* has changed considerably over the last few years mainly due to the use of the 16S rRNA gene sequence which transferred many *Pseudomonas* spp. to other genera within the Proteobacteria phylum, and among them the genus of *Stenotrophomonas* (Ait Tayeb et al., 2008). In addition, 16S rRNA

gene sequencing sometimes fails to distinguish closely-related species because the conservative character of this gene prevents detection of small changes in base sequences (Mulet et al., 2010).

The identification of pseudomonads to the species level has proven to be difficult. The resolution of species within the *P. fluorescens*, *P. putida*, *P. syringae*, and *P. stutzeri* complexes can not be discriminated even when the finest phenotypic systems such as Biotype-100 strips are used (Meyer et al., 2008).

Several isolates (*n*= 7) did not generate sequences despite repeated attempts. Different factors could affect the success of the sequencing. For example, Izmailou et al. (2002) reported that such failure might arise during the preparation of sequencing template, when conducting and separating sequence reaction, and when analyzing the detectors data stream to produce the base sequence. Nelson et al. (2002) reported that growing bacterial cultures to high cell density could negatively inhibit the amplification reaction. They also observed that growing bacterial cells in rich media may inhibit the amplification process.

Several criteria were used to exclude potential opportunistic pathogens at early stages of the inoculant development program such as grouping in risk groups by consulting a public database [e.g., the German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de)] growth of bacteria at 37 ˚C, or using pathogenicity assays (Berg, 2009). In this study, the grouping of bacterial isolates into risk groups was used because it is easier, affordable, and knowledge already exists in such databases. Five strains were grouped at level 2. These strains belong to the genera *Stenotrophomonas* (*n*= 3), *Flavobacterium* (*n*= 1) and *Bacillus* (n=1). These findings would limit their further development as agricultural inoculants.

In summary, further taxonomic tools should be employed to differentiate between closely-related species as in the case with the genera *Pseudomonas* and *Bacillus*. Such tools might include the use of different highly conserved genes such as *rpoB* and *gyrB* or siderotyping as in the case of *Pseudomonas* spp.

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4.0 CHARACTARIZATION OF PLANT GROWTH-PROMOTION TRAITS OF ENDOPHYTIC BACTERIA

4.1 Preface

In the previous chapter the identity of endophytic bacterial isolates was determined and then they were grouped according to their health risk level. Bacterial identification and classification is an important step at the beginning of an inoculant development program and it should be combined with other tests to fully reveal the whole potential capacity of these endophytic bacteria to be used as inoculants to improve plant growth. To date, little is known about the mechanisms by which endophytic bacteria might enhance plant growth and yield. Thus, to address this knowledge gap, this study screened endophytic bacteria for different direct and indirect plant growth-promoting mechanisms commonly found in soil rhizobacteria.

4.2 Introduction

Plants have developed numerous associations with a wide range of soil microorganisms, including associated plant microbes. Bacterial endophytes are still a relatively unexploited reserve for the discovery of isolates with novel plant growth promotion traits (Mendes et al., 2007; Ryan et al., 2008).

Endophytic bacteria are defined as bacteria that reside within interior healthy plant tissues without causing obvious damage to their host plant health (Bacon and Hinton, 2006; Ryan et al., 2008). Endophytic bacteria exhibited a wide diversity not only in plants species occupied but also in the different taxa involved (Taghavi et al., 2009). So far over 219 endophytic bacterial species, representing 71 genera, have been isolated. Of them are common soil bacteria such as *Enterobacter*, *Pseudomonas*, *Burkholderia*, *Bacillus*, and *Azospirillum* (Hallmann and Berg, 2006). However, there is so much interest in the biology of bacterial endophytes that new endophytes species are continuously being reported (Rosenblueth and Martinez-Romero, 2006).

Over the last few years, there is a growing interest in utilizing endophytic bacteria as plant growth promoting biofertilizers or biological control agents (Hallmann et al., 1997; Martinez-Romero, 2006; Ryan et al., 2008). This is because bacterial endophytes are likely to interact more closely with their host plant where they are provided with secure shelter and a

continuous supply of nutrients inside the intercellular space of plants (Rosenblueth and Martinez-Romero, 2006; Weyens et al., 2009).

Endophytic bacteria are believed to stimulate their host plant growth and development through mechanisms similar to those proposed for PGPR, either directly via increasing the plants ability to acquire nutrients or indirectly by preventing the proliferation of pathogenic microorganisms that suppress plant growth (Long et al., 2008; Ryan et al., 2008).

The direct growth promotion mechanisms by PGPR include: N_2-fixation providing increased N to plants; iron (Fe) sequestration through siderophores; solubilization of organic and inorganic phosphorus (P) compounds that otherwise are not readily available for plant uptake; sulfur oxidization; and the production of several types of phytohormones such as IAA which play an important role in increasing root surface area at different stages in plant developments (Vessey, 2003; Rosenblueth and Martinez-Romero, 2006).

Indirectly, PGPR increase host plant yield via the following mechanisms: competition with pathogens for colonization sites on roots; production of antimicrobial metabolites that suppress plant pathogens growth (antibiosis); preventing plant pathogen access to iron nutrition by exerting siderophores; induced systematic resistance (ISR) of their host plants; production of hydrogen cyanide (HCN), a broad-spectrum antimicrobial compound; and through of hyperparasitic activity by producing lytic enzymes (e.g.,chitinase, protease, and β-1,3-glucanase) that degrade cell walls components of pathogens (Whipps, 2001; Compant et al., 2005).

The objective of this study was to characterize endophytic bacteria for several direct and indirect plant growth-promoting traits. Isolates that possess some of these traits might be suitable candidates as inoculants to improve the alfalfa-*Sinorhizobium* symbiosis.

4.3 Materials and Methods

4.3.1 Source of bacteria

The bacterial isolates (*n*= 40) used were described in Section 3.2.1.

4.3.2 Phosphate solubilization

The ability of bacterial strains to solubilize phosphate was assessed using a potato-dextrose yeast agar (PDYA, pH 7.0) medium containing freshly precipitated calcium phosphate [i.e., 50 mL sterile 10% (w/v) K_2HPO_4 and 100 mL sterile 10% (w/v) $CaCl_2$ was added per litre

sterile PDYA media to produce a precipitate of $CaHPO_4$ as described by de Freitas et al. (1997)]. Bacterial cultures were grown as described in Section 3.2.1, streaked in the centre of PDYA-CaP plate, and incubated at 28 ˚C. Phosphate solubilization activity was assessed up to 14 d by measuring the zone of clearing surrounding the developed bacterial colony. An autoclaved bacterial culture was used as a negative control.

4.3.3 Siderophores production

Siderophore synthesis by bacterial strains was detected using Chromo Azurol-S (CAS) medium as described by Deol (1992). CAS reacts with iron giving a blue color to the medium. Siderophores form more stable complexes with iron than does CAS. Therefore, when siderophores are produced by an isolate on a medium containing CAS, the color of the medium changes from blue to orange. Chrome Azurol-S agar plates (pH 6.8) were prepared. Isolates were grown as described in Section 3.2.1, streaked on the CAS plates, and incubated at 28 ˚C for 3 d. The appearance of orange pigmentation around colonies indicated putative siderophore production. An autoclaved bacterial culture was used as a negative control, and isolate *Pseudomonas syringae* R55 from the Soil Microbiology Laboratory culture collection was used as a positive control.

4.3.4 Indole-3-acetic acid (IAA) production

Bacterial isolates were screened for production of indole-3-acetic acid (IAA) as outlined by Bric et al. (1991). Briefly, liquid cultures were prepared in 200 mL Erlenmeyer flasks containing 50 mL Luria-Bertani (LB) medium containing the following: Bacto-Tryptone (Difco Laboratories Inc. Detroit, Michigan, USA), 10 g L^{-1}; yeast extract, 5 g L^{-1}; NaCl, 5 g L^{-1}; and amended with 5mM L-tryptophan. The pH was adjusted to 7.5 with 1 N NaOH before autoclaving. Cultures were grown on a gyratory shaker (150 rpm) at 28 ˚ C for 72 h. Cultures were then streaked in grid format on Luria-Bertani agar medium containing 15 g L^{-1} Bacto-agar (Difco Laboratories Inc. Detroit, Michigan, USA). After 48 h of inoculating the bacteria on the surface of the LB agar plates, Whatman no. 2 filter paper (Whatman International Ltd., England) was overlaid immediately and incubated at 28˚C. After 72 h, the filter paper was removed from the plate and placed in a Petri dish soaked with Salkowski's reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid). Pink-color development was allowed to develop at room temperature for about

30 min. Isolate *Pseudomonas cepacia* R85 from the Soil Microbiology Laboratory culture collection was used as a positive control, and an autoclaved bacterial culture was used as a negative control.

4.3.5 ACC deaminase enzyme activity of endophytic bacteria strains

The ability of endophytic bacterial isolates to utilize ACC as a sole N source was tested as described by Glick et al. (1995) with some modifications. Bacterial isolates were inoculated into a 50 of mL 1/10 strength TSB. The isolates were incubated at room temperature for 48 h with continuous agitation at 150 rpm on a gyratory shaker. Then a loopful of the culture was streaked onto agar plates of DF salt minimum medium (Dworkin and Foster, 1958) supplemented with 3 mM ACC as the N source. Plates were then incubated up to 7 d, and checked for bacterial growth which indicated the production of ACC deaminase enzyme. An autoclaved bacterial culture was included in the experiment as a negative control.

4.3.6 Seed germination assay

This assay was carried out in Petri plate lined with sterile filter paper (Whatman International Ltd., England) and filled with 5 mL distilled water. The procedure of Carrillo et al. (2002) was followed, with several modifications. Alfalfa seeds that were free from obvious damage were selected and surface sterilized by soaking in ethanol (95% v:v) for 30 s and then a sodium hypochlorite solution (1.2% w:v) for 5 min, followed by 10 rinses in sterile tap water. Bacterial strains were inoculated into flasks containing 50 mL sterile 10% TSB, as mentioned earlier in Section 3.2.1. After 48 h of growth, the optical densities were determined using a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co. Inc., NY USA). The bacterial culture was centrifuged at 8400 g for 10 min at 20 °C to pellet the bacteria. Cells were resuspended in a 50 mL phosphate buffer saline (PBS) twice to wash and remove excess nutrients. The bacterial pellet were resuspended in 10 mL sterile distilled water, shaken for 10 min, and a 10 mL aliquot was transferred to a flask containing the surface-sterilized alfalfa seeds. Flasks were then shaken for 30 min to allow bacteria to adhere to the seed coat. Seeds and inoculant were poured through sterile Buckner funnels to remove excess inoculant. Twenty seeds were aseptically transferred to corresponding Petri plates, stacked and placed in a dark place at room temperature. Seeds were checked for germination after 4 to 7 d of incubation. This

experiment had five replicates per isolate. One non-inoculated, surface-disinfected seed treatment was used as a control.

4.3.7 *In vitro* screening for antagonistic activity

Bacterial isolates were screened *in vitro* for their antifungal activity towards different soil-borne plant pathogenic fungi on agar plates as described by Sessitsch et al. (2004). Fungal strains tested were *Rhizoctonia solani*, *Fusarium oxysporum, Fusarium sporotrichioides,* and *Fusarium sambucinum* were isolated from diseased pulse crops grown in Saskatchewan and were kindly provided by Dr. Sabine Banniza, of the Department of Plant Science, University of Saskatchewan. Fungal isolates were maintained on potato dextrose agar (PDA) (Difco Laboratories Inc, Detroit, MI, USA) and transferred to Petri dishes containing fresh PDA to produce fungal mycelium plugs. Bacterial isolates were grown as described in Section 3.2.1 and 10 µL aliquot were spotted at opposite ends of PDA agar plates near the edge, and incubated at 27 ºC for 48 h. An agar plug (5 mm diameter) containing a fresh fungal mycelium was placed in the center of these pre-inoculated plates, which were incubated at room temperature. Fungal inhibition was evaluated by measuring radial growth of fungi towards the bacterial colony after 7 to 10 d. Plates with only fungi without bacterial cultures were used as controls.

Inhibition activity was assessed based on agar diffusion of excreted bacterial metabolites and assigned to one of the following categories: 0) no inhibition; 1) contact inhibition; 2) = 1-5 mm wide zone of inhibition; 3) = 5-10 mm wide zone of inhibition; and 4) ≥10 mm wide zone of inhibition.

4.3.8 Production of extra-cellular enzyme

To test for enzyme production, bacterial isolates were inoculated into 3 mL of 1/10 strength tryptic soy broth (TSB) in 12-mm × 75-mm test tubes. The inoculated tubes were incubated at 28 ˚C and shaken at 150 rpm for 24 to 48 h until the broth became visually turbid (Misko and Germida, 2002).

The assays for amylase, cellulose, mannanase and xylanase hydrolysis were performed using azurine-crosslinked (AZCL) substrates (Megazyme, Ireland). To 1/10 strength TSB, 0.5 g L^{-1} of powdered AZCL appropriate for the test was added before autoclaving (Misko, 2002).

The substrates for α-amylase, cellulase, β-mannanase, and endo-xylanase activity were AZCL-amylose, AZCL-He-cellulose, AZCL-galactomannan (carob) and AZCL-xylan (oat spelts), respectively. Clearing zones around colonies after incubation on plates for up to 7 d at 28 ˚C were assumed to indicate enzymes positive activities (Misko, 2002).

Chitinase activity was screened by plating onto chitin-agar-plates (CA) containing: nutrient broth (Difco) 1.62 g, NaCl 0.5 g, M9 salts 6 g, Chitin 2 g, $CaCl_2$ 14.7 g, $MgSO_4$ 246.6 g, Thiamin-HCl 1 g, Agar Difco) 15 g, and distilled water 1 L. After incubation for 5 d at 28 ˚C, a clear halo indicated chitinase activity, as described by Berg et al. (2000).

Lipase production was determined by plating onto lipase-agar-plates containing: peptone 10 g L^{-1}, NaCl 5 g L^{-1} , $CaCl_2$ 0.1 g L^{-1}, Tween 80 1 mL, agar (Difco) 20 g L^{-1}. Plates were incubated for 5 d at 28 ˚C. Clear halos around the colonies indicate lipase production (Kumar et al., 2005).

Protease activity was determined by casein degradation using 50.0 g L^{-1} of skim milk base (Difco, Detroit, MI, USA) in 1/10 strength TSA. Plates were then incubated for 5 d at 28 ˚C. Clearing halo around colonies indicated casein hydrolysis (Misko, 2002). Urease production was assessed using Christiansen urea substrate. Production of a red-violet color around the colony indicates urease hydrolysis (Misko, 2002).

The activity of acid and alkaline phosphatase were assessed on Petri plates containing 0.01% phenolphthalein (VWR, Mississauga, ON, Canada) added to 1/10 strength TSA. After 48 h incubation at 28 ˚C, colonies were exposed to a vapor of 25% ammonia. A positive response for production of enzyme was a red colony and a red halo surrounding the colony (Smibert and Krieg, 1994; Misko and Germida, 2002).

4.3.9 Pigment production

Isolates were also screened for fluorescent pigment production. Isolates were grown on King's B medium and PDA medium and incubated for 3 d, and then were checked for pigment production under UV light source.

4.4 Results

4.4.1 Phosphate solubilization, siderophores production, and IAA production

About 75% of the endophytic isolates (*n*= 30 of 40) solubilized phosphate as indicated by the presence of clearing zone around the bacterial colony on the PDYA-CaP medium (Table 4.1). Isolates *Pseudomonas syringae* EB XDE 4-48, *P. fluorescens* EB IE 1-32, *P. fluorescens* EB IE 1-43, *P. fluorescens* EB EE 2-18, *P. fluorescens* MF EE 2-122 and *P. fluorescens* K CPE 1-52 were the best P-solubilizers (Figure 4.1). Surprisingly, none of the bacilli isolates tested (*n*= 4), as well as *Flavobacterium johnsoniae* MF FE 3-69 and *Arthrobacter ilicis* MF IE 2-50, solubilized P. Isolate *S. maltophilia* MF XDE 1-6 solubilized P with a moderate degree of efficiency.

Table 4.1. Plant growth-promoting traits detected for 40 endophytic bacterial isolates.

Isolate code †	Bacterial I.D. ‡	P-solubilization §	Siderophore production ¶	IAA synthesis #	ACC activity ††
EB EE 4-36	*Pseudomonas fluorescens*	++	-	W	+
EB EE 2-28	*Pseudomonas fluorescens*	+++	+	-	-
EB XDE 4-48	*Pseudomonas syringae*	++++	+	M	-
EB XDE 4-33	*Pseudomonas putida*	++	+	-	+
EB IE 1-32	*Pseudomonas fluorescens*	++++	+	M	-
EB EE 3-78	*Stenotrophomonas maltophilia*	-	+	W	-
WQ 2-28	*Pseudomonas fluorescens*	+++	-	W	-
EB QE 3-37	*Pseudomonas mandelii*	+	+	-	+
EB EE 4-25	*Pseudomonas putida* *	+++	-	S	+
EB QE 4-19	*Pseudomonas fluorescens*	++	+	W	+
WQE 2-25	*Pseudomonas fluorescens*	+++	+	M	-
WQE 3-1	*Stenotrophomonas maltophilia*	-	+	-	-

Table 4.1. Continued

Isolate code †	Bacterial I.D. ‡	P-solubilization §	Siderophore production ¶	IAA synthesis #	ACC activity ††
WQE 2-8	*Pseudomonas fluorescens*	++	+	W	-
WEE 2-30	*Bacillus pumilus* *	-	-	W	-
EB IE 1-43	*Pseudomonas fluorescens*	++++	+	W	+
KCE 3-7	*Bacillus psychrosacch* *	-	-	W	-
EB FE 1-59	*Pseudomonas* sp.	+	+++	-	-
EB FE 2-45	*Pseudomonas putida*	++	++	W	+
EB FE 2-92	*Pseudomonas putida*	+	+	M	-
EB EE 2-16	*Pseudomonas fluorescens*	-	+	W	-
EB EE 2-18	*Pseudomonas fluorescens*	++++	++	W	-
EB EE 2-23	*Pseudomonas fluorescens*	-	++	M	-
MF QE 1-69	*Pseudomonas fluorescens*	++	+	W	+
MF EE 1-44	*Pseudomonas fluorescens*	+	++	W	-

Table 4.1. Continued.

Isolate code †	Bacterial I.D. ‡	P-solubilization §	Siderophore production ¶	IAA synthesis #	ACC activity ††
MF IE 3-83	*Bacillus megaterium* *	-	+	-	-
MF FE 2-21	*Pseudomonas putida*	+	++	W	+
MF EE 1-34	*Pseudomonas fluorescens*	+++	+	M	-
MF FE 3-69	*Flavobacterium johnsoniae* *	-	+	W	-
MF IE 2-50	*Arthrobacter ilicis* *	-	-	-	++
MF IE 2-30	*Bacillus cereus* *	-	-	W	-
MF FE 1-18	*Pseudomonas putida*	+++	+	W	+
MF FE 3-64	*Pseudomonas fluorescens*	++	+	W	-
MF EE 4-4	*Pseudomonas fluorescens*	+++	+	-	-
MF XDE 3-57	*Pseudomonas fluorescens*	++	+	W	-
MF EE 2-122	*Pseudomonas fluorescens*	++++	+	M	+
MF EE 4-19	*Pseudomonas fluorescens*	+++	+	W	-

Table 4.1. Continued.

Isolate code †	Bacterial I.D. ‡	P-solubilization §	Siderophore production ¶	IAA synthesis #	ACC activity ††
MF XDE 1-18	*Pseudomonas fluorescens*	+	-	-	-
MF XDE 1-6	*Stenotrophomonas maltophilia*	++	+	W	-
MF XDE 3-65	*Pseudomonas putida*	+++	+	W	+
KCPE 1-52	*Pseudomonas fluorescens*	++++	-	W	-

†: For additional information about bacterial isolate code please consult Appendix 1

‡: Bacteria were identified using 16S rRNA gene sequencing., except for those isolates followed by an asterisk (*), which were identified by FAME analysis.

§: -, no phosphate solubilization; +, 0-10 mm zone clearing; ++, 10-15 mm zone clearing; +++, 15-20 mm zone clearing; and ++++, 20-25 mm clearing zone around the colony.

¶: +, indicates change in color of medium from blue to orange, positive for siderophore production; -, indicates no change in color of medium, negative for siderophore production.

#: Production of indoles as detected by colorimetric method (-, negative; S=Strong; M= Moderate; W=Weak reaction developed in filter paper after soaked in Salkowski's reagent).

††: +, indicates the ability to use ACC as sole nitrogen source; -, indicates no growth with ACC as sole nitrogen source.

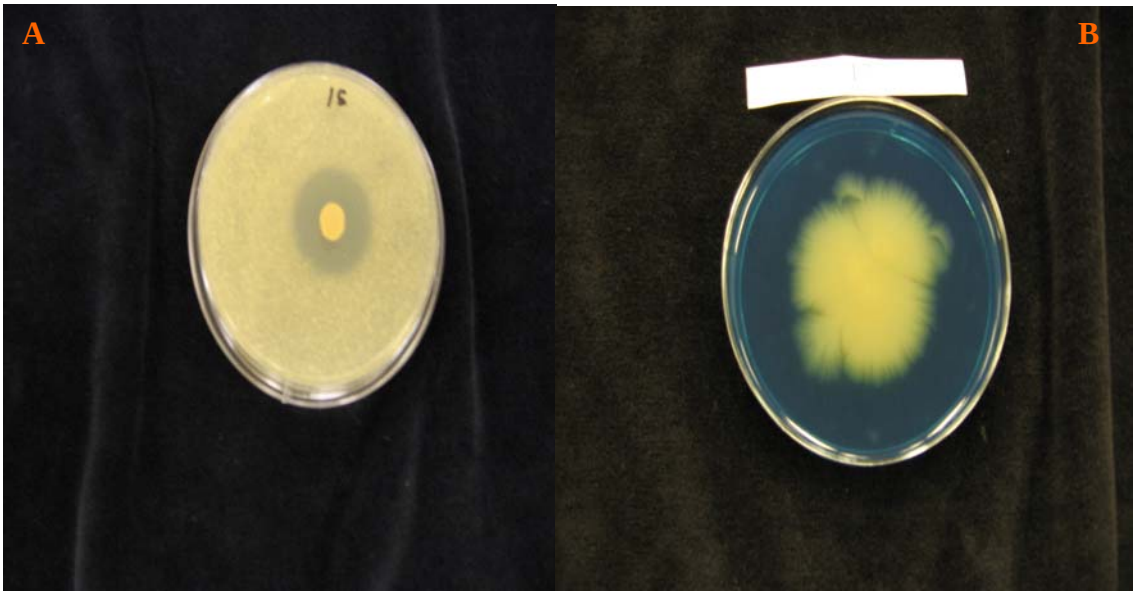

Figure 4.1 Colored or cleared zones on test plates indicating (A) phosphate solubilization by *Pseudomonas fluorescens* strain EB IE 1-43 on the PDYA-CaP medium, and (B) siderophore production by *Pseudomonas* sp. strain EB FE 1-59 on CAS media plate.

Siderophore production by endophytic isolates was a common trait. Seventy-eight percent of the isolates (*n*= 31 of 40) synthesized siderophores under iron-deficient condition when tested on CAS media plates (Table 4.1). Only one isolate, *Pseudomonas* sp. strain EB FE 1-59, was a very good siderophore producer according to this qualitative assay (Figure 4.1). Other isolates, representing different genera, exhibited a moderate or low ability to synthesis siderophores. As in the case with P-solubilization assay, *A. ilicis* strain MF IE 2-50 was unable to produce siderophores.

About 80% of the isolates tested produced IAA as indicated by the colony plate lift method (Table 4.1). However many of these isolates (*n*= 23 of 40) gave a weak reaction on the filter paper test, which indicated low IAA production. Several isolates appear to be moderate IAA producers, and only *P. putida* strain EB EE 4-25 exhibited strong reaction. Again, *A. ilicis* strain MF IE 2-50 was unable to produce IAA.

4.4.2 Ability of bacterial isolates to utilize ACC deaminase.

The capacity of endophytic isolates to utilize ACC deaminase as the sole source of N was evaluated using DF salt minimum medium plate. Out of 40 isolates tested, ca. 35% (*n*= 14 of 40) grew in DF salt minimum medium supplemented with ACC as the only source of N, indicating the presence of ACC deaminase (Table 4.1). However, all but one of the isolates showed poor growth in this medium after 7 d of incubation. The only isolate exhibiting moderate growth was *Arthrobacter ilicis* MF IE 2-50.

4.4.3 Effect bacterial endophytes on alfalfa seed germination

The effect of bacterial inoculation on alfalfa seed germination was examined using a Petri plate assay. After the 7 d incubation period, the number of germinated seeds was determined in each treatment. Most of the bacterial isolates tested had no effect on seed germination as between 95 to 99% of seeds germinated. Only isolate *P. putida* EB FE 2-45 seemed to inhibit seed germination (84%).

4.4.4 *In vitro* screening for antagonistic activity

The antagonistic activity of all 40 endophytic isolates toward four soil-borne fungal plant pathogens(i.e., *Rhizoctonia solani*, *Fusarium sambucinum*, *Fusarium oxysporum*, and *Fusarium sporotrichioides*) was tested in a plate assay using PDA media.

About 23% of endophytes tested (*n*= 9/40) exhibited antifungal activity toward the soil-borne pathogen *R. solani* with different degrees of efficiency (Table 4.2). For example, the isolate *P. putida* EB FE 2-45 was found to be the most effective in inhibiting the mycelial growth of *R. solani*, followed by *P. fluorescens* strain MF EE 4-4, and *P. fluorescens* strain MF XDE 3-57. The rest of the isolates exhibited weak antagonizing abilities.

Ten isolates (25%) exhibited antifungal activities against the soil-borne pathogen *F. sambucinum* (Table 4.2). Two isolates, *P. fluorescens* EB EE 2-18 and *P. fluorescens* EB EE 2-23, were very effective inhibitors of fungal growth. Also, *Bacillus cereus* strain MF IE 2-30 showed a good ability to suppress fungal mycelial growth.

Growth of *F. oxysporum*, was inhibited by 28% of endophytic isolates tested (*n*= 11 of 40). Three isolates, *P. fluorescens* EB EE 4-36, *P. fluorescens*, EB EE 2-28, and *P. syringae* EB XDE 4-48, exhibited the strongest antifungal activity against the fungal pathogen followed by isolate *B. cereus* MF IE 2-30.

The soil-borne pathogen *F. sporotrichioides*, was most susceptible to inhibition by endophytic bacteria as 33% of the endophytes (*n*= 13 of 40) inhibited fungal growth. For example, *B. cereus* strain MF IE 2-30, and *P. fluorescens* strain MF EE 4-4 showed good antifungal activities with a clearing zone of inhibition between 5 to10 mm. However, the rest of the isolates exhibited weak inhibitory effects toward the pathogen (Table 4.2).

It should be noted that several bacterial endophytes exhibited antifungal activities against all or most of the soil-borne pathogenic fungi tested. For example, isolates *B. cereus* MF IE 2-30 and *P. fluorescens* EB EE 2-23 were antagonistic toward all pathogens under the bioassay test. Other isolates demonstrated biocontrol ability toward two or three pathogens (Table 4.2).

Table 4.2. ***In vitro*** antagonism against several soil-borne pathogenic fungi by 40 endophytic bacterial isolates.

Isolate code †	Bacterial I.D. ‡	Antagonism §			
		Rhizoctonia solani	*Fusarium sambucinum*	*Fusarium oxysporum*	*Fusarium sporotrichioides*
EB EE 4-36	*Pseudomonas fluorescens*	0	0	4	1
EB EE 2-28	*Pseudomonas fluorescens*	0	0	4	1
EB XDE 4-48	*Pseudomonas syringae*	0	0	4	1
EB XDE 4-33	*Pseudomonas putida*	0	0	0	0
EB IE 1-32	*Pseudomonas fluorescens*	0	0	1	0
EB EE 3-78	*Stenotrophomonas maltophilia*	0	0	0	1
WQ 2-28	*Pseudomonas fluorescens*	0	0	0	0
EB QE 3-37	*Pseudomonas mandelii*	0	0	0	0
EB EE 4-25	*Pseudomonas putida* *	0	2	1	0
EB QE 4-19	*Pseudomonas fluorescens*	0	0	0	0
WQE 2-25	*Pseudomonas fluorescens*	0	0	0	0
WQE 3-1	*Stenotrophomonas maltophilia*	0	0	0	1

Table 4.2. Continued.

Isolate code †	Bacterial I.D. ‡	Antagonism §			
		Rhizoctonia solani	*Fusarium sambucinum*	*Fusarium oxysporum*	*Fusarium sporotrichioides*
WQE 2-8	*Pseudomonas fluorescens*	0	0	0	0
WEE 2-30	*Bacillus pumilus* *	0	0	0	0
EB IE 1-43	*Pseudomonas fluorescens*	0	1	0	0
KCE 3-7	*Bacillus psychrosacch* *	0	0	0	0
EB FE 1-59	*Pseudomonas* sp.	0	0	0	0
EB FE 2-45	*Pseudomonas putida*	4	1	0	0
EB FE 2-92	*Pseudomonas putida*	1	1	1	0
EB EE 2-16	*Pseudomonas fluorescens*	0	0	0	1
EB EE 2-18	*Pseudomonas fluorescens*	0	4	0	1
EB EE 2-23	*Pseudomonas fluorescens*	1	4	1	2
MF QE 1-69	*Pseudomonas fluorescens*	0	2	1	0
MF EE 1-44	*Pseudomonas fluorescens*	0	0	1	0

Table 4.2. Continued.

Isolate code †	Bacterial I.D. ‡	Antagonism §			
		Rhizoctonia solani	*Fusarium sambucinum*	*Fusarium oxysporum*	*Fusarium sporotrichioides*
MF IE 3-83	*Bacillus megaterium* *	0	0	0	0
MF FE 2-21	*Pseudomonas putida*	1	0	0	1
MF EE 1-34	*Pseudomonas fluorescens*	0	0	0	0
MF FE 3-69	*Flavobacterium johnsoniae* *	0	0	0	0
MF IE 2-50	*Arthrobacter ilicis* *	0	0	0	0
MF IE 2-30	*Bacillus cereus* *	1	3	3	3
MF FE 1-18	*Pseudomonas putida*	0	0	0	0
MF FE 3-64	*Pseudomonas fluorescens*	1	0	0	0
MF EE 4-4	*Pseudomonas fluorescens*	3	0	1	3
MF XDE 3-57	*Pseudomonas fluorescens*	2	1	0	1
MF EE 2-122	*Pseudomonas fluorescens*	0	0	0	0
MF EE 4-19	*Pseudomonas fluorescens*	1	1	0	0

Table 4.2. Continued.

Isolate code †	Bacterial I.D. ‡	Antagonism §			
		Rhizoctonia solani	*Fusarium sambucinum*	*Fusarium oxysporum*	*Fusarium sporotrichioides*
MF XDE 1-18	*Pseudomonas fluorescens*	0	0	0	0
MF XDE 1-6	*Stenotrophomonas maltophilia*	0	0	0	1
MF XDE 3-65	*Pseudomonas putida*	0	0	0	0
KCPE 1-52	*Pseudomonas fluorescens*	0	0	0	0

†: For additional information about bacterial isolate code please consult Appendix 1

‡: Bacteria were identified using 16S rRNA gene sequencing, except for those isolates followed by an asterisk (*), which were identified by FAME analysis.

§: Inhibition of fungal growth on PDA medium; 0, no inhabitation; 1, contact inhabitation; 2, 1-5 mm wide zone of inhabitation; 3, 5-10 mm wide zone of inhabitation; and 4, more than 10 mm wide zone of inhabitation.

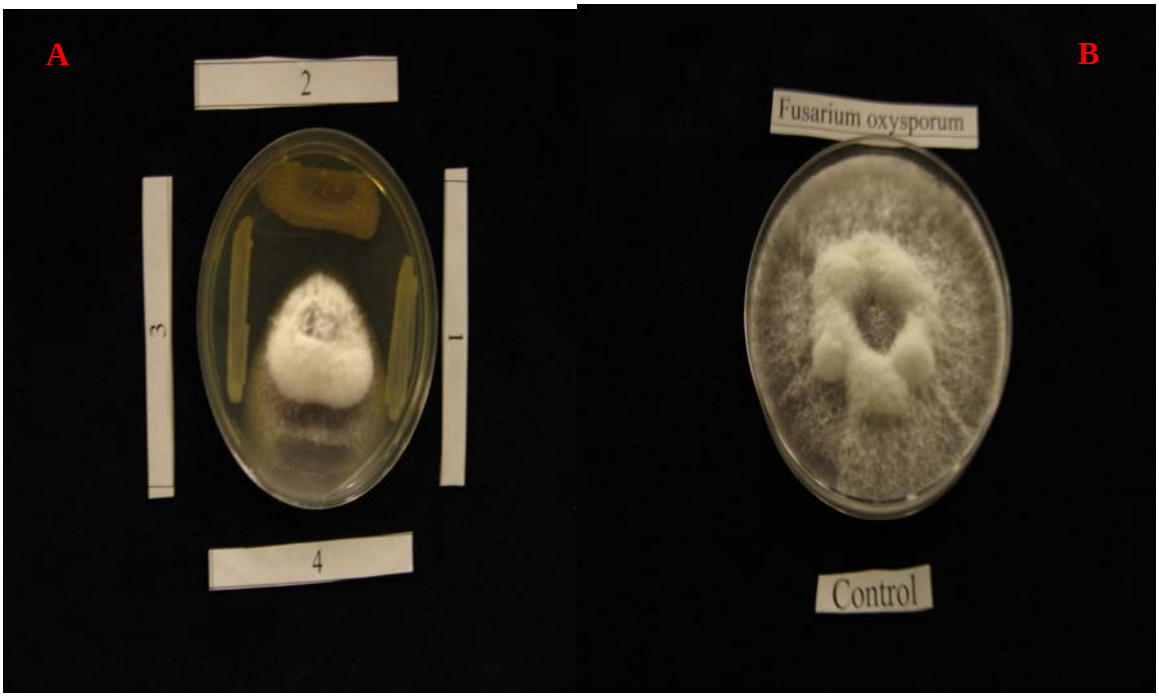

Figure 4.2 ***In vitro*** inhibition of *Fusarium oxysporum* by selected endophytic isolates (A). Isolates were as follows: (1) *Pseudomonas fluorescens* strain EB EE 4-36; (2) *Pseudomonas fluorescens* strain EB EE 2-28; (3) *Pseudomonas syringae* strain EB XDE 4-48; and (4) *Pseudomonas putida* EB XDE 4-33. Note that control PDA plate inoculated with fungus alone was entirely overgrown by the fungal pathogen with no inhibition zones (B).

4.4.5. Production of extra-cellular enzyme

Selected bacterial entophytes were characterized for their production of extra-cellular enzymes important for biocontrol and nutrient cycling (Table 4.3). In general, these isolates produced a wide spectrum of enzymes. Urease production was found in all endophytes tested. Also, protease were found in 78% of the isolates (*n*= 31 of 40). Another enzymes expressed by many endophytes (13 of 40) was acid phosphates. Nine isolates were positive for mannanase (23%), but only four, two, and two isolates were positive for xylanase, cellulase, and amylase enzymes, respectively (Table. 4.3). None of the endophytes produced chitinase and lipase (Table 4.3).

Among all endophytes tested for enzymes production isolates, *P. putida* EB FE 2-45, and *F. johnsoniae* MF FE 3-69, produced most of the enzymes under investigation (Table 4.3). Also, the isolate *P. flavescencens* MF EE 4-19 produced four different enzymes (Table 4.3).

4.4.6 Pigment production

Isolates were checked for pigment production using King's B, and PDA media. When grown on King's B medium, a medium specific for fluorescent Pseudomonads, 19 isolates were found to fluorescence under UV light indicating yellowish and greenish pigments (Table 4.3). When grown on PDA medium, 13 isolates were found to produce diffusible pigments, some of which have a blue green color (Figure 4.3).

Table 4.3. Extra-cellular enzyme production and pigment diffusions by 40 endophytic bacterial isolates.

Isolate code †	Bacterial I.D. ‡	Enzymes §									Pigments ¶	
		Chit-	Ure-	Lip-	Mann-	Cell-	Xyla-	Amyl-	Phos-	Prot-	King B	PDA
EB EE 4-36	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	+	+	+
EB EE 2-28	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	+	+	+	+
EB XDE 4-48	*Pseudomonas syringae*	-	+	-	-	-	-	-	-	-	-	-
EB XDE 4-33	*Pseudomonas putida*	-	+	-	-	-	-	-	-	-	+	-
EB IE 1-32	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	+	+	-
EB EE 3-78	*Stenotrophomonas maltophilia*	-	+	-	-	-	-	-	+	+	-	-
WQ 2-28	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	+	+	-	-
EB QE 3-37	*Pseudomonas mandelii*	-	+	-	+	-	-	-	-	+	+	+
EB EE 4-25	*Pseudomonas putida* *	-	+	-	-	-	-	-	+	+	-	-
EB QE 4-19	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	+	-	-
WQE 2-25	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	+	+	-

Table 4.3. Continued.

Isolate code †	Bacterial I.D. ‡	Enzymes §									Pigments ¶	
		Chit-	Ure-	Lip-	Mann-	Cell-	Xyla-	Amyl-	Phos-	Prot-	King B	PDA
WQE 3-1	*Stenotrophomonas maltophilia*	-	+	-	-	-	-	-	+	+	-	-
WQE 2-8	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	-	-	-
WEE 2-30	*Bacillus pumilus* *	-	+	-	-	-	+	-	-	+	-	+
EB IE 1-43	*Pseudomonas fluorescens*	-	+	-	+	-	-	-	-	+	+	-
KCE 3-7	*Bacillus psychrosacch* *	-	+	-	+	-	-	-	+	+	-	-
EB FE 1-59	*Pseudomonas* sp.	-	+	-	-	-	-	-	-	+	+	-
EB FE 2-45	*Pseudomonas putida*	-	+	-	+	+	+	+	+	+	+	-
EB FE 2-92	*Pseudomonas putida*	-	+	-	-	-	-	-	-	+	+	+
EB EE 2-16	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	+	+	-	+
EB EE 2-18	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	+	+	+	+
EB EE 2-23	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	+	+	+	+

Table 4.3. Continued.

Isolate code †	Bacterial I.D. ‡	Enzymes §									Pigments ¶	
		Chit-	Ure-	Lip-	Mann-	Cell-	Xyla-	Amyl-	Phos-	Prot-	King B	PDA
MF QE 1-69	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	+	+	+
MF EE 1-44	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	-	+	-
MF IE 3-83	*Bacillus megaterium* *	-	+	-	-	-	-	-	-	-	-	+
MF FE 2-21	*Pseudomonas putida*	-	+	-	-	-	-	-	-	+	-	-
MF EE 1-34	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	+	+	-
MF FE 3-69	*Flavobacterium johnsoniae* *	-	+	-	+	+	+	+	-	+	-	+
MF IE 2-50	*Arthrobacter aurescens*	-	+	-	+	-	-	-	-	+	-	-
MF IE 2-30	*Bacillus cereus*	-	+	-	+	-	-	-	-	+	-	-
MF FE 1-18	*Pseudomonas putida*	-	+	-	-	-	-	-	-	+	-	-
MF FE 3-64	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	+	-	-
MF EE 4-4	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	-	-	-

Table 4.3. Continued.

Isolate code †	Bacterial I.D. ‡	Enzymes §									Pigments ¶	
		Chit-	Ure-	Lip-	Mann-	Cell-	Xyla-	Amyl-	Phos-	Prot-	King B	PDA
MF XDE 3-57	*Pseudomonas fluorescens*	-	+	-	+	-	-	-	+	+	-	-
MF EE 2-122	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	-	+	-
MF EE 4-19	*Pseudomonas flavescencens*	-	+	-	+	-	-	-	+	+	+	+
MF XDE 1-18	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	-	-	-
MF XDE 1-6	*Stenotrophomonas maltophilia*	-	+	-	-	-	-	-	+	+	-	-
MF XDE 3-65	*Pseudomonas putida*	-	+	-	-	-	-	-	-	+	+	+
KCP 1-52	*Pseudomonas fluorescens*	-	+	-	-	-	-	-	-	-	+	-

†: For additional information about bacterial isolate code please consult Appendix 1

‡: Bacteria were identified using 16S rRNA gene sequencing, except for those isolates followed by an asterisk (*), which were identified by FAME analysis.

§: Enzymes plate assays, with positive (+) or negative (-) enzyme production. Abbreviations represents the following enzymes: Chit: chitinase; Ure: urease; Lip: lipase; Mann: mannase; Cell: cellulase; Xyla: xylanase; Amyl: amylase; Phos: phosphates; Prot: protease.

¶: Pigments production on King's B, and PDA media under UV light source, with positive (+) or negative (-) pigmentation

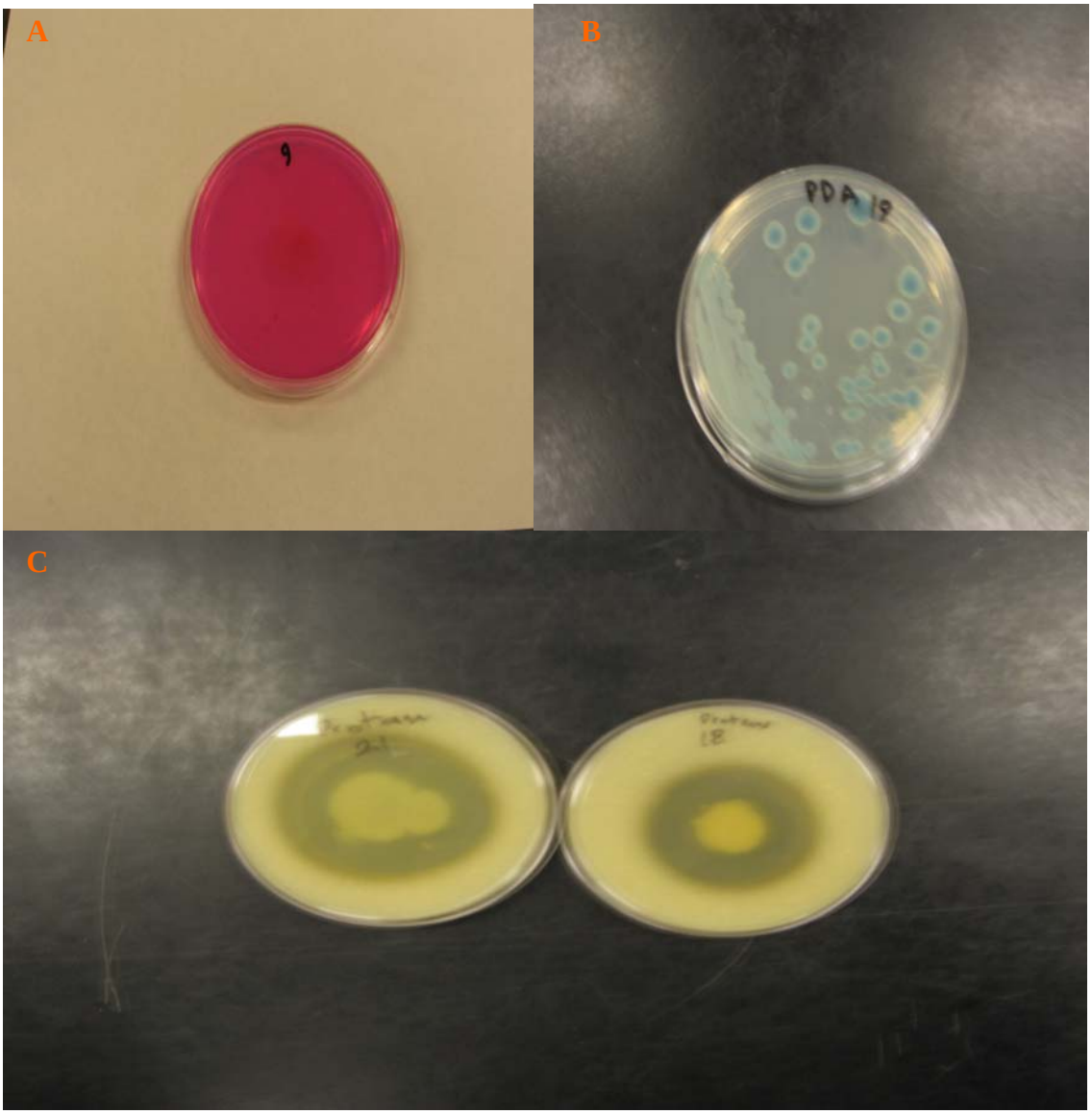

Figure 4.3 Ability of some endophytic bacterial isolates to produce extra-cellular enzyme and pigment on test plates indicating (A) phosphates enzyme production by *Pseudomonas putida* strain EB EE 4-25, (B) blue green pigments on PDA agar media by *Pseudomonas putida* strain EB FE 2-92, and (C) protease enzyme production by *Pseudomonas fluorescens* strain EB EE 2-18 (on the left side) and *Pseudomonas putida* strain EB FE 2-45 (on the right side).

4.5 Discussion

The plant rhizosphere is a dynamic and complex habitat where various microorganisms interact with plants in beneficial, neutral, or deleterious ways (Morgan et al., 2005). Among the beneficial plant associated bacteria, bacterial endophytes are isolated from within inside the plant root, a niche that has been defined as the endorhizosphere. It has been suggested that endophytic bacteria hold great promise to enhance agricultural productivity and sustainability. Several reports found that bacterial endophytes improve the growth and health of many agricultural crops (Hardoim et al., 2008; Ryan et al., 2008). Endophytic bacteria are believed to promote their host growth and fitness through direct and indirect mechanisms similar to those proposed for PGPR (Vessey, 2003).

Phosphate solubilization is considered as one of the direct growth enhancement mechanisms of PGPR. Soils usually contain high amounts of P, but it's availability to plants is limited due to its precipitation with other elements depending on the soil pH (Rodriguez and Fraga, 1999). Several groups of microorganisms have the ability to solubilize P and make it readily available for plant uptake. For example, soil rhizobacteria and soil fungi are well known for their capacity to solubilize P (de Freitas et al., 1997; Rodriguez and Fraga, 1999). However, little is known about P-solubilization by bacterial endophytes. My results show that 75% of isolates tested were found to be able to solubilize P. In accordance with my findings, Kuklinsky-Sobral et al. (2004) found that 49% out of 373 endophytic isolates were capable of P-solubilization. In contrast, Long et al. (2008) screened 77 endophytes for their ability to solubilize P, but only six isolates were found to be able to solubilize inorganic phosphate. These findings indicate that phosphate solubilization is a possible direct mechanism of plant growth promotion by endophytic bacteria.

Another important direct growth enhancement mechanism is IAA production by plant associated-bacteria. IAA, a type of auxin, is involved in many physiological processes in plant development such as cell elongation and division, tissue differentiation, and root initiation (Gravel et al., 2007). IAA is usually produced by various soil microbes including endophytes (Kuklinsky-Sobral et al., 2004; Sessitsch et al., 2004). In agreement with previous reports, my study found that 80% of isolates tested were able to synthesis IAA in plate assays. IAA production is widespread among endophytes compared to rhizobacteria in sugarcane and soybean plants (Kuklinsky-Sobral et al., 2004; Mendes et al., 2007).Thus, endophytes that

possess the ability to produce IAA could be a suitable candidate to be used as inoculants for agricultural crops because the vital role this hormone plays in changing root morphology and plant development.

Over 77% of the isolates tested in my study were able to synthesis siderophores. Siderophore production is considered as another direct mechanism of plant growth. Siderophores are produced by various plant root-associated microorganisms under Fe III-deficient conditions. Other researchers also reported siderophore production by endophytes from different plants. For example, Barzanti et al. (2007) reported that 83% of endophytes isolated from *Alyssum bertolonii* Desv. (Brassicaceae), a nickel hyperaccumulator plant, produced siderophores. Similarly, Sessitsch et al. (2004) found that 77% out 35 endophytes isolated from field-grown potato plants possessed siderophores. These results demonstrated that siderophores production is a common phenomenon among endophytic bacteria from a wide range of crops and non-agricultural plants. More importantly, it has been speculated that siderophore synthesis is an important trait for bacterial endophytic growth as they have to compete with plant cells for Fe supply (Sessitsch et al., 2004).

Finally, endophytic bacteria are thought to elicit plant enhancement by another direct mechanism, ACC deaminase production, which can lower plant ethylene levels in plant tissues. Long et al. (2008) found that 23 out of 77 endophytes which were isolated from different parts of black nightshade (*Solanum nigrum*) were found to have ACC deaminase activity. My results also agree with their finding as 14 isolates possessed ACC deaminase. Hardoim et al. (2008) hypothesized that ACC deaminase-producing endophytes might be excellent plant-growth promoters because their ACC deaminase activities inside plant cells may alleviate plant stress by effectively blocking ethylene production before its oxidation by the plant ACC oxidase.

Indirectly, bacterial endophytes may improve host plant growth by preventing or reducing the negative impact of phytopathogens through diverse mechanisms, a process known as biological control. Recently, more interest is in utilizing bacterial endophytes as BCA is because of the unique ecological niche they occupy, as they colonize internal cells of plants, which offers them an advantage to tolerate many detrimental environmental factors. This could be considered a second defense line inside plant internal tissues against soil-borne phytopathogens (Liu et al., 2009). My results show that several strains of *Pseudomonas* and *Bacillus* were able to suppress mycelial growth of several soil-borne pathogens. Numerous

studies have demonstrated the ability of endophytes to control many different phytopathogens in a wide range of plant crops (Whipps, 2001). For example, Liu et al. (2009) reported that an endophytic isolate *B. subtilis*, which was isolated from wheat roots, was able to inhibit the mycelium growth of numerous plant pathogenic fungi under *in vitro* conditions.

The mechanisms by which these isolates inhibited pathogenic fungal mycelial growth are not fully well-understood. The modes of action involved with bacterial biocontrol of pathogenic fungi include: siderophore production, antibiosis, induced resistance, competition, parasitism and extra-cellular enzymes production (Whipps, 2001). Gerhardson (2002) demonstrated the difficulty of fully clarifying the modes of action in detail for most BCAs because of the diversity of mechanisms involved in biocontrol action.

It has been noted that PDA media, which was used in this study as dual-culture assay to screen for a potential BCA, is rich in a C-, N- and Fe (III) (Bevivino et al., 1998). Therefore, competition can not be considered as a possible mechanism of biocontrol as the antagonistic bacteria and the fungal pathogen does not have to compete for nutrients because the PDA medium provides plenty of food to the microbes growing on it (Idris et al., 2007). Similarly, siderophore production as a mode of action may not be involved in the inhibition of the pathogens growth as PDA is rich in Fe. Thus, other possible modes of action responsible for the inhibitory effect that have been observed in this study may include antibiosis (Bevivino et al., 1998) as well as HCN production and many other volatile products (Idris et al., 2007). Also, synthesis of fungal cell-wall degrading enzymes might be responsible for biocontrol activity as some of the isolates tested showed the ability to produce a wide spectrum of these enzymes (Faltin et al., 2004).

More recently, biological control activity of an antagonistic isolate is thought to occur not only from a single mode of action but rather from a combination of different mechanisms (Compant et al., 2005). *Serratia plymuthica*, which effectively controls the *Rhizoctonia* in lettuce, is considered a model example of a BCA that employs various modes of action including chitinolytic, glucanolytic, and proteolytic enzymatic activities (Faltin et al., 2004). The presence of more than one mode of action in some isolates tested in my study is considered an important trait for BCA to be effective toward a wide range of pathogens (Idris et al., 2008).

In conclusion, several endophytic isolates tested in this study exhibit one or more of both direct and indirect plant growth-promoting mechanisms. Some of these isolates have the

potential to be further exploited as plant growth promoters either as biofertilizers or as biopesticides under Saskatchewan soil conditions.

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5.0 THE EFFECT OF SELECTED ENDOPHYTIC BACTERIAL ISOLATES ON THE ALFALFA-*SINORHIZOBIUM* SYMBIOSIS

5.1 Preface

In the previous chapter the endophytic bacterial strains were screened for different putative direct and indirect plant growth-promoting mechanisms. Some of these mechanisms are believed to play an important role in improving nodulation and subsequent N_2 fixation in legume plants when co-inoculated with rhizobial strains. This has been tested extensively with rhizospheric bacteria over the last two decades. Endophytic bacteria, due to their unique ecological nature, have recently been investigated as biofertilizer agents to enhance plant growth. To date little is known about the effect of co-inoculating legume plants with endophytic bacteria and rhizobia. Thus, this study assessed the impact of inoculating alfalfa with *Sinorhizobium meliloti* and selected endophytic bacterial isolates under controlled growth chamber conditions.

5.2 Introduction:

Alfalfa (*Medicago sativa L*) is a herbaceous perennial forage legume plant that is grown extensively worldwide. Many desirable characteristics have made alfalfa a preferred forage legume to grow. First of all, it has a very deep tap root system that contributes to the improvement and conservation of the soil structure (Rosas et al., 2006). Secondly, the livestock industry used alfalfa as hay and pasture to feed farm animals because of the quality nutrients that it provides as its rich in protein, calcium, and vitamin A. Thirdly, incorporating alfalfa in crop rotation has profound impact on soil fertility such as improved soil nutritional status, increased soil organic matter, and reduce N fertilizers for subsequent crops (Hanson et al., 1988).

Alfalfa, as a legume species, has the ability to form a symbiotic relationship with *Sinorhizobium meliloti*. This association benefits the plant by fixing N_2 compounds which reduce inorganic N fertilizers usage by farmers, and minimizes the negative impact caused by these inorganic fertilizers on the environment (Prevost and Bromfield, 2003; Howieson et al., 2008). The rate of the N_2 fixed by alfalfa is estimated to be 200 kg N ha^{-1} yr^{-1} (Herridge et al., 2008).

Nodulation and subsequent N_2 fixation are crucial factors that establish the productivity of alfalfa. In the recent past, more research has been conducted to understand the mechanisms by

which the beneficial effect of legume- *Rhizobium* symbiosis can be enhanced (Mishra et al., 2009).

Different soil microorganism groups were evaluated as co-inoculants to improve the legume-*Rhizobium* symbiosis efficiency; however, most of the research focused on the use of PGPR (Bai et al., 2003; Weyens et al., 2009), followed by AMF (Xavier and Germida, 2002). Several studies have tested the effect of inoculation of PGPRs with rhizobia on the growth and nodulation of different legume plants including alfalfa (Rosas et al., 2006), bean (de Freitas et al., 1993; Figueiredo et al., 2008), lentil (Chanway et al., 1989), pea (Kumar et al., 2001), red clover (Marek-Kozaczuk and Skorupska, 2001), and soybean (Cattelan et al., 1999; Bai et al., 2003).

Modes of action employed by PGPR to enhance the legume-*Rhizobium* symbiosis are not fully understood (García et al., 2004), but are thought to include: (1) stimulating the root development in the host plant by releasing various types of phytohormones such as auxins, cytokinins, gibberellins, and ethylene, thus providing more sites for infection and nodulation (Banerjee et al., 2006); (2) enhancing the production of more flavonoid signal molecules in the legume plant that in turn are responsible for inducing nodulation genes (*nod* gene) in rhizobia and thus initiating the legume-rhizobia symbiosis (Mishra et al., 2009); (3) secretion of secondary metabolites such as tabtoxinine-β-lactam, a toxin relased by *P. syringae*, (Knight and Langston-Unkefer, 1988) and B vitamins, also produced by *Pseudomonas* sp. (Marek-Kozaczuk and Skorupska, 2001) that improved the alfalfa-rhizobia and red clover-rhizobia symbiosis, respectively (Vessy, 2003).

More recently, there is a growing interest in utilizing bacterial endophytes as plant growth promoters. This is because these endophytes occupy a unique ecological niche within the plant root interior, which might favor them because they are protected from severe environmental conditions and supplied with readily available sources of nutrients without competition with other microbes (Rosenblueth and Martinez-Romero, 2006; Weyens et al., 2009). The mechanisms by which these endophytes may enhance their host plant growth are thought to be similar to those proposed to PGPRs; however, their intimate relationship with host plant might allow other plant growth-promotion mechanisms (Hallmann et al., 1997; Bai et al., 2003).

To date, little is known about the interaction of bacterial endophytes with legume plants. Therefore, the objective of this study was to evaluate the effect of selected endophytic bacterial strains on the alfalfa-*Sinorhizobium* symbiosis under growth chamber conditions.

5.3 Materials and Methods

5.3.1 Source of bacteria

Seven bacterial strains used in this study were selected out from 40 endophytic isolates based on their ability to exhibit one or more of plant growth-promotion mechanisms as shown in the previous chapter (Chapter 4). The characteristics of these seven isolates are summarized in Table 5.1.

Sinorhizobium meliloti strain P102 was kindly provided by Dr. Marry Leggett, Novozymes (formerly Philom Bios Inc.).

5.3.2 Bacterial growth conditions

The endophytic bacterial isolates were grown in 50-mL 1/10 strength TSB medium at 28˚C for 48 h on a gyratory shaker (150 per min). The *S. meliloti* strain was grown in 50-mL Yeast Extract Mannitol Broth (YEMB) medium at 28˚C on a gyratory shaker (150 rpm) for 96 h. This yields approximately 10^8 rhizobial cells per mL.

Bacterial suspensions were concentrated by centrifugation (15 min at 5000 g), washed three times in PBS, and resuspended in 10 mL sterile tap water to yield approximately 10^8 cfu per mL. Mixed inoculants were made by combining equal amounts (10 mL) of the appropriate bacterial strains before seed inoculation.

Table 5.1. Characteristics of endophytic bacterial isolates used in this study.

Isolate Code †	Bacterial I.D ‡	P- solubilization §	Siderophore production ¶	IAA synthesis #	ACC activity ††	Antagonistic activities ‡‡
EB XDE 4-48	*Pseudomonas syringae*	++++	+	M	-	+
EB EE 4-25	*Pseudomonas putida* *	+++	-	S	+	+
EB FE 1-59	*Pseudomonas* sp.	+	+++	-	-	-
EB EE 2-18	*Pseudomonas fluorescens*	++++	++	W	-	+
EB EE 2-23	*Pseudomonas fluorescens*	-	++	M	-	+
MF IE 2-30	*Bacillus cereus* *	-	-	W	-	+++
MF XDE 1-18	*Pseudomonas fluorescens*	+	-	-	-	-

†: For additional information about bacterial isolate code please consult Appendix 1

‡: Bacteria were identified using 16S rRNA gene sequencing., except for those isolates followed by an asterisk (*), which were identified by FAME analysis.

§: -, no phosphate solubilization; +, 0-10 mm zone clearing; ++, 10-15 mm zone clearing; +++, 15-20 mm zone clearing; and ++++, 20-25 mm clearing zone around the colony.

¶: +, indicates change in color of medium from blue to orange, positive for siderophore production; -, indicates no change in color of medium, negative for siderophore production.

#: Production of indoles as detected by colorimetric method (-, negative; S=Strong; M= Moderate; W=Weak reaction developed in filter paper after soaked in Salkowski's reagent).

††: +, indicates the ability to use ACC as sole nitrogen source; -, indicates no growth with ACC as sole nitrogen source.

‡‡: Inhibition of fungal growth on PDA medium. -, indicates no inhabitation; +, indicates fungal growth Inhibition. For more regarding antagonisms activities of bacterial strains please consult Table 4.2 in Chapter 4 (p. 84-87).

5.3.3 Seed bacterization

Alfalfa seeds were surface-sterilized by soaking in ethanol (95% v:v) for 30 s and a sodium hypochlorite solution (1.2% w:v) for 10 min, followed by 10 rinses in sterile tap water. The surface-sterilized seeds were placed in bacterial suspension for 4 h to insure colonization during seed germination (de Freitas et al., 1993).

Nine treatments were used in both the growth pouch and the pot experiments and include (1) control; (2) *Sinorhizobium meliloti* strain P102 alone; (3) *Pseudomonas syringae* EB XDE 4-48 + *S. meliloti* P102; (4) *P. putida* EB EE 4-25 + *S. meliloti* P102; (5) *P.* sp. EB EE 1-59 + *S. meliloti* P102; (6) *P. fluorescens* EB EE 2-18 + *S. meliloti* P102; (7) *P. fluorescens* EB EE 2-23 + *S. meliloti* P102; (8) *Bacillus cereus* MF IE 2-30 + *S. meliloti* P102; and (9) *P. fluorescens* MF XDE 1-18 + *S. meliloti* P102.

5.3.4 Growth pouch study

Seed growth pouches (GP) (Northrup King Co, Minneapolis, MN, USA) were filled with 30 mL sterilized half-strength N-free Hoagland's nutrient solution, and were sterilized at 120ºC for 20 min. Replicates of each treatment were performed (ten seeds per pouch and five pouches per treatment). After germination the seedlings were thinned to four per pouch and maintained in a growth chamber at 18ºC a 16/18-h light/dark cycle. The plants were grown for 30 d, and at the end of the incubation period the pouches were opened and the root length were measured manually using a ruler, and shoot length, nodule were counted and number of lateral roots were determined.

5.3.5 Potted soil study

The soil used in this study was an A horizon of a Black Chernozem collected from Central Butte, Saskatchewan. The soil was air-dried, sieved (<5 mm mesh), and analyzed by ALS Laboratory, Saskatoon, Canada. The soil characteristics were: pH, 7.8; conductivity, 0.1 mS cm^{-1}, organic matter, 2.1 (%), and nutrients (kg ha^{-1}), NO_3-N, 16.8; P, 33.6; K, 571.2; SO_4-S, 12.32. The soil (0.5 kg) was placed in 500-cm^3 Styrofoam pots. Ten inoculated seeds were planted in each pot with five pots per treatment. After emergence, the seedlings were thinned to four per pot and maintained in a growth chamber at 24ºC under a 14-h light to 10-h dark cycle. Pots were watered regularly to maintain 60% field-capacity throughout the experiment. After 75

d of growth, roots length was measured manually using a ruler, and shoot length, nodules were counted, and number of lateral roots was determined. Plants were harvested and, root and shoot dry weight were measured.

5.3.6 Statistical analysis

The growth chamber studies were carried out in a completely randomized design and data were presented as means and standard errors, and the differences between treatments were analyzed using one-way ANOVA at a 5% significant level with post hoc analysis (Tukey's test) using SPSS software (version 17.0.1; SPSS, Chicago, IL).

5.4 Results

5.4.1 Growth pouch study

5.4.1.1 Effect of bacterial endophytes on alfalfa plant's shoots and roots length

Pseudomonas putida strain EB EE 4-25 significantly increased the shoot length of alfalfa plants when co-inoculated with *S. meliloti* strain P102 compared to alfalfa inoculated with rhizobia alone (Figure 5.1). Similar effects were observed with strains *P. syringae* EB XDE 4-48, and *P. fluorescens* EB EE 2-23. All bacterial treatments had significant effects on plant shoot compared to the uninoculated plant treatment (Figure 5.1).

Pseudomonas putida strain EB EE 4-25, *P. fluorescens* EB EE 2-23, and *P. syringae* EB XDE 4-48 significantly improved alfalfa plant root length when co-inoculated with rhizobia compared to the rhizobial treatment alone, respectively (Figure 5.2). However, no significant differences ($p<0.05$) were found with other dual bacterial treatments compared to the single rhizobial treatment (Figure 5.2). All bacterial treatments had significant effects on plant root length compared to the uninoculated alfalfa plants.

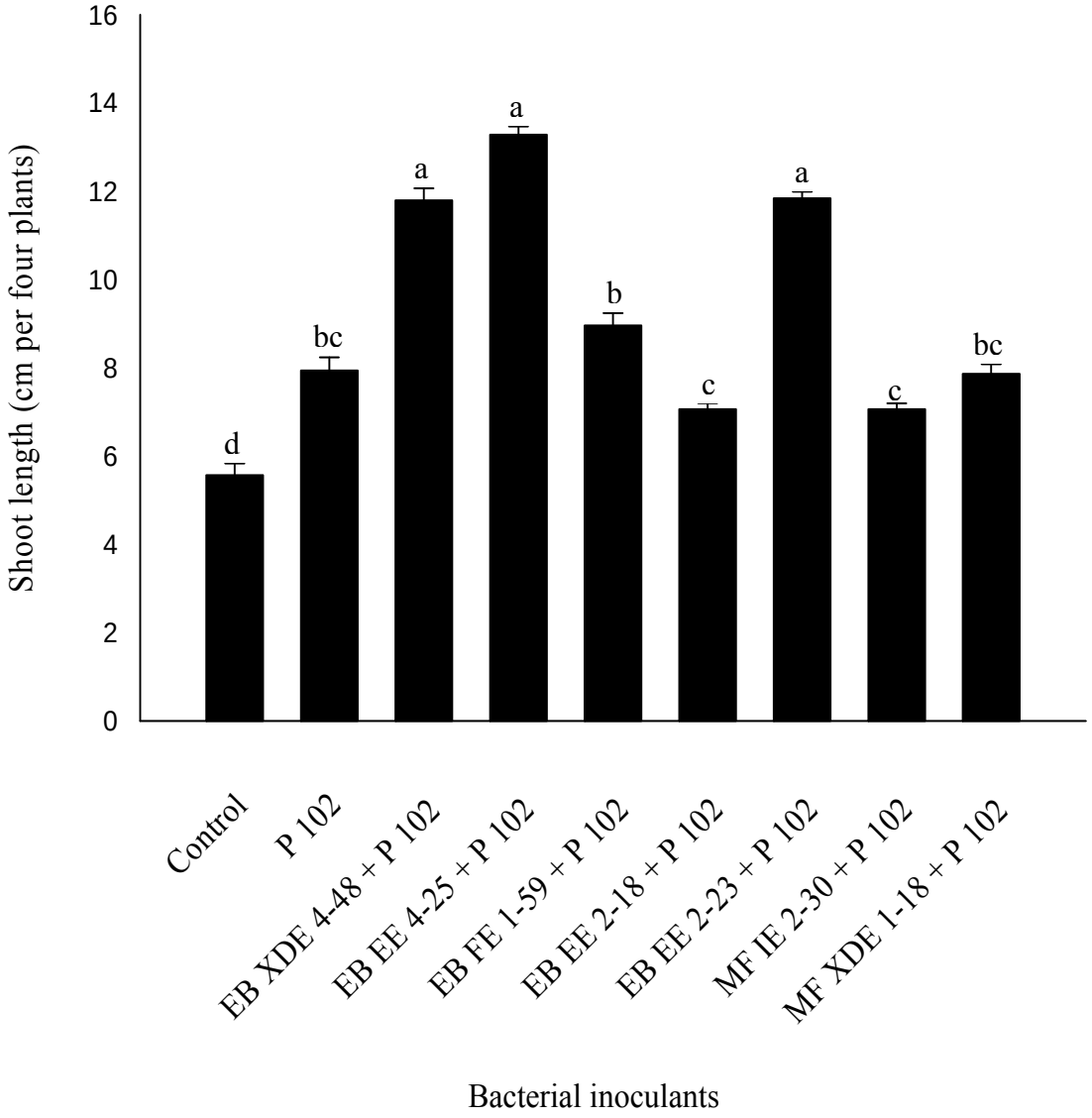

Figure 5.1 Effect of selected endophytic bacterial isolates on the shoot length of alfalfa plants when co-inoculated with *Sinorhizobium meliloti* strain P102 when grown in growth pouches. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

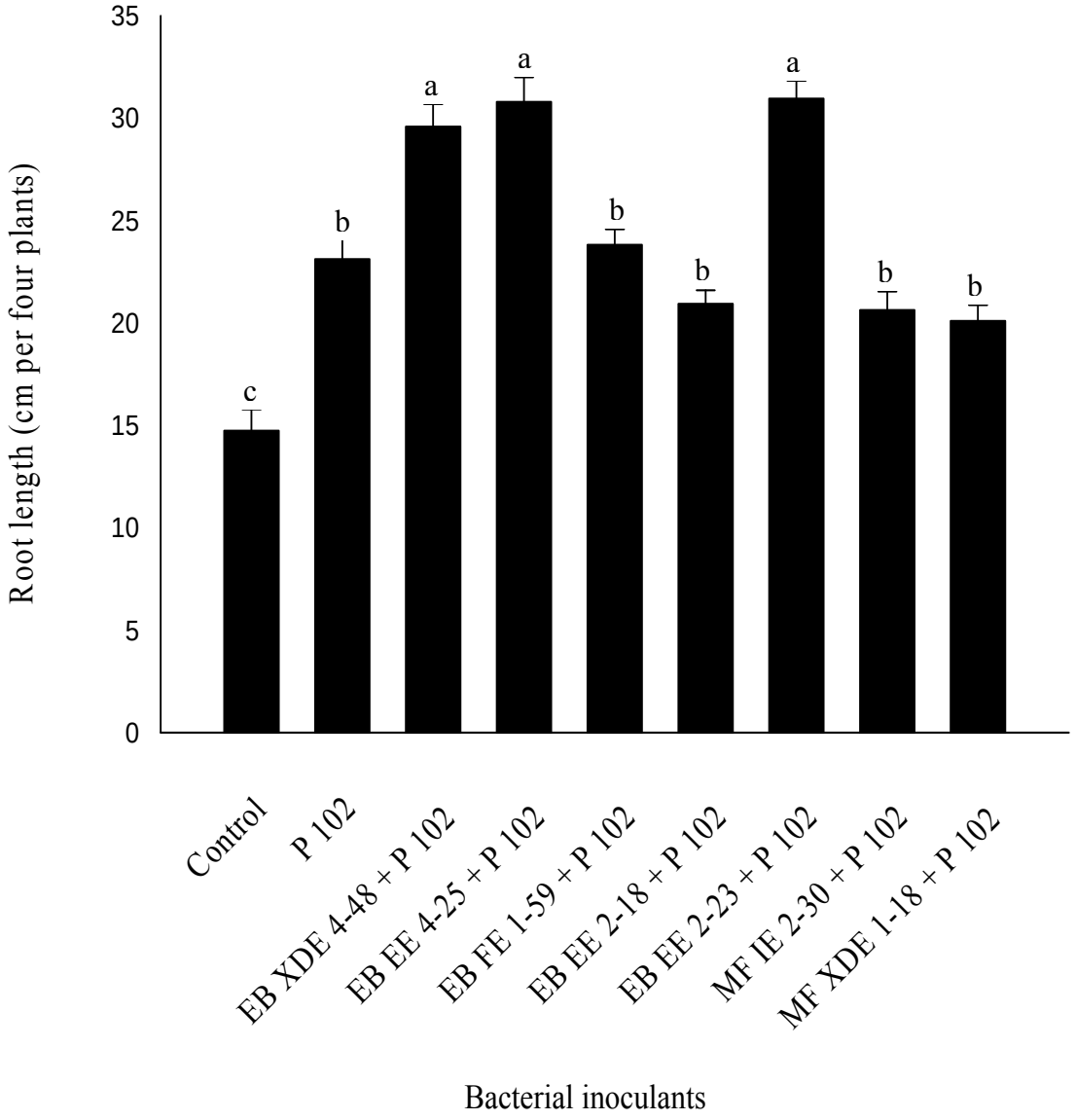

Figure 5.2 Effect of selected endophytic isolates on the root length of alfalfa plants when co-inoculated with *Sinorhizobium meliloti* strain P102 when grown in growth pouches. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

5.4.1.2 Effect of endophytic bacteria on lateral root formation and nodules number

The number of lateral roots of alfalfa plants was significantly greater with plants co-inoculated with *P. putida* strain EB EE 4-25 and *S. meliloti* strain P102 compared to seed treated with *S. meliloti* strain P102 alone (Figure 5.3). Similarly, co-inoculation of alfalfa plants with *P. syringae* strain EB XDE 4-48 and *S. meliloti* strain P102 significantly ($p<0.05$) increased the number of lateral roots compared to single rhizobial treatment (Figure 5.3). However, *Pseudomonas* sp. strain EB FE 1-59, *P. fluorescens* strain EB EE 2-18, *P. fluorescens* EB EE 2-23, and *P. fluorescens* MF XDE 1-18 did not had any significant effect on lateral roots formation when combined with *S. meliloti* strain P102 compared to inoculation of alfalfa plants with *S. meliloti* alone (Figure 5.3). *Bacillus cereus* strain MF IE 2-30 did, however, significantly decrease the number of lateral roots of alfalfa plants when co-inoculated with *S. meliloti* strain P102 compared to inoculation of alfalfa plants with *S. meliloti* alone (Figure 5.3). In fact, *P. fluorescens* strain EB EE 2-18, and *B. cereus* strain MF IE 2-30 did not significantly affect lateral root formation when combined with *S. meliloti* strain P102 compared to the uninoculated plant treatment (Figure 5.3).

Co-inoculation of alfalfa plants with *P. putida* strain EB EE 4-25 and *S. meliloti* strain P102 significantly ($p<0.05$) increased the nodule formation on the roots of alfalfa plants when compared to rhizobial treatment alone (Figure 5.4). Also, treated alfalfa seed with dual inoculants of *S. meliloti* and *P. syringae* strain EB XDE 4-48, *P. fluorescens* strain EB EE 2-23, and *P.* sp. strain EB FE 1-59 significantly enhanced the number of nodules on the plant roots compared to plants treated with rhizobia alone (Figure 5.4). No statistically significant ($p<0.05$) difference was observed with plants co-inoculated with *P. fluorescens* strain EB EE 2-18 and *S. meliloti* strain P102 compared to single rhizobia inoculation. Similar results was observed with plants co-inoculated with *B. cereus* strain MF IE 2-30 and *S. meliloti* strain P102 when compared to plants treated with *S. meliloti* alone (Figure 5.4). All bacterial treatments significantly increased the nodules number on alfalfa plants compared to the uninoculated alfalfa plants (Figure 5.4).

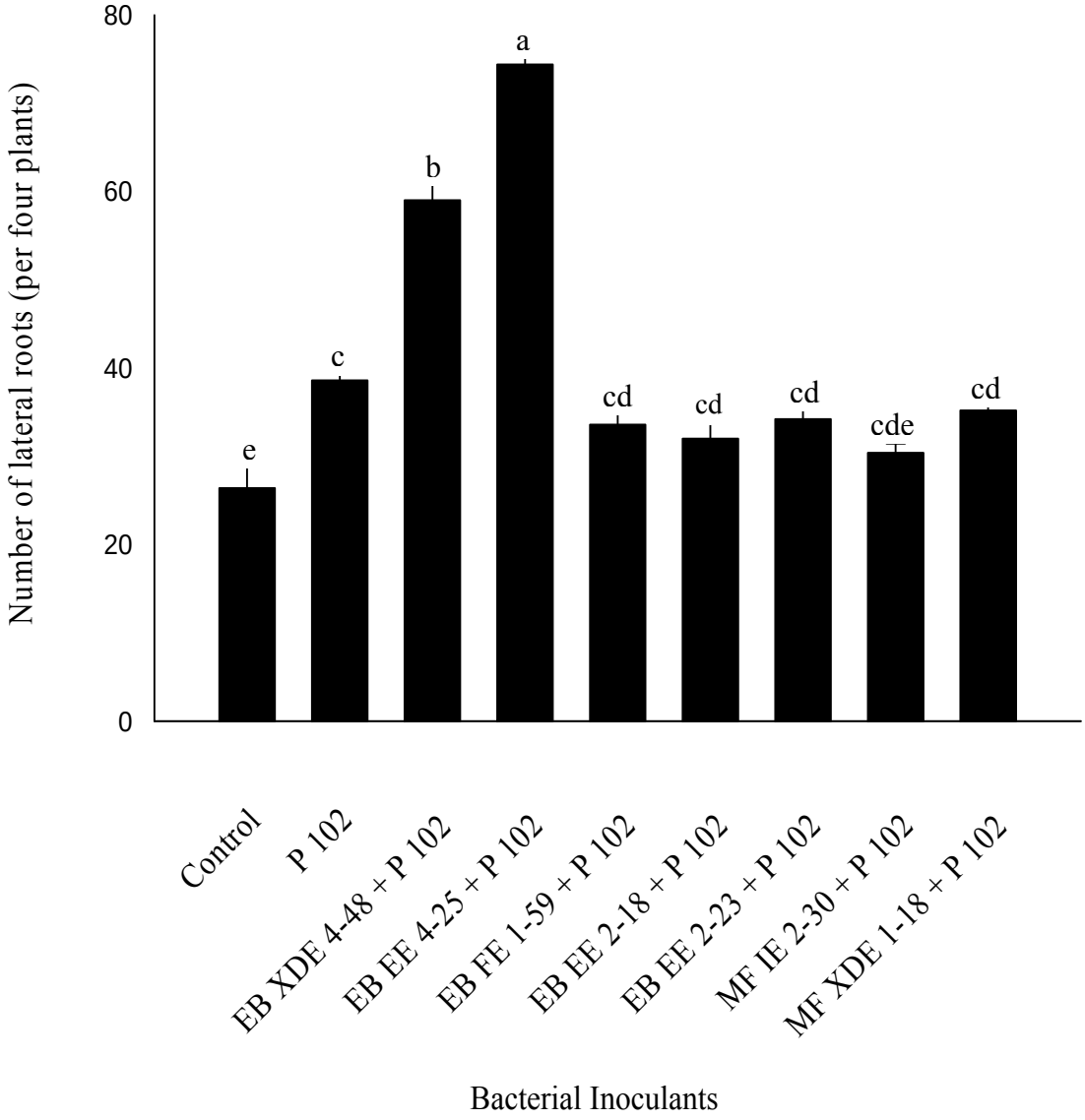

Figure 5.3 Effect of selected endophytic isolates on lateral roots formation of alfalfa plants when co-inoculated with *Sinorhizobium meliloti* strain P102 when grown in growth pouches. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

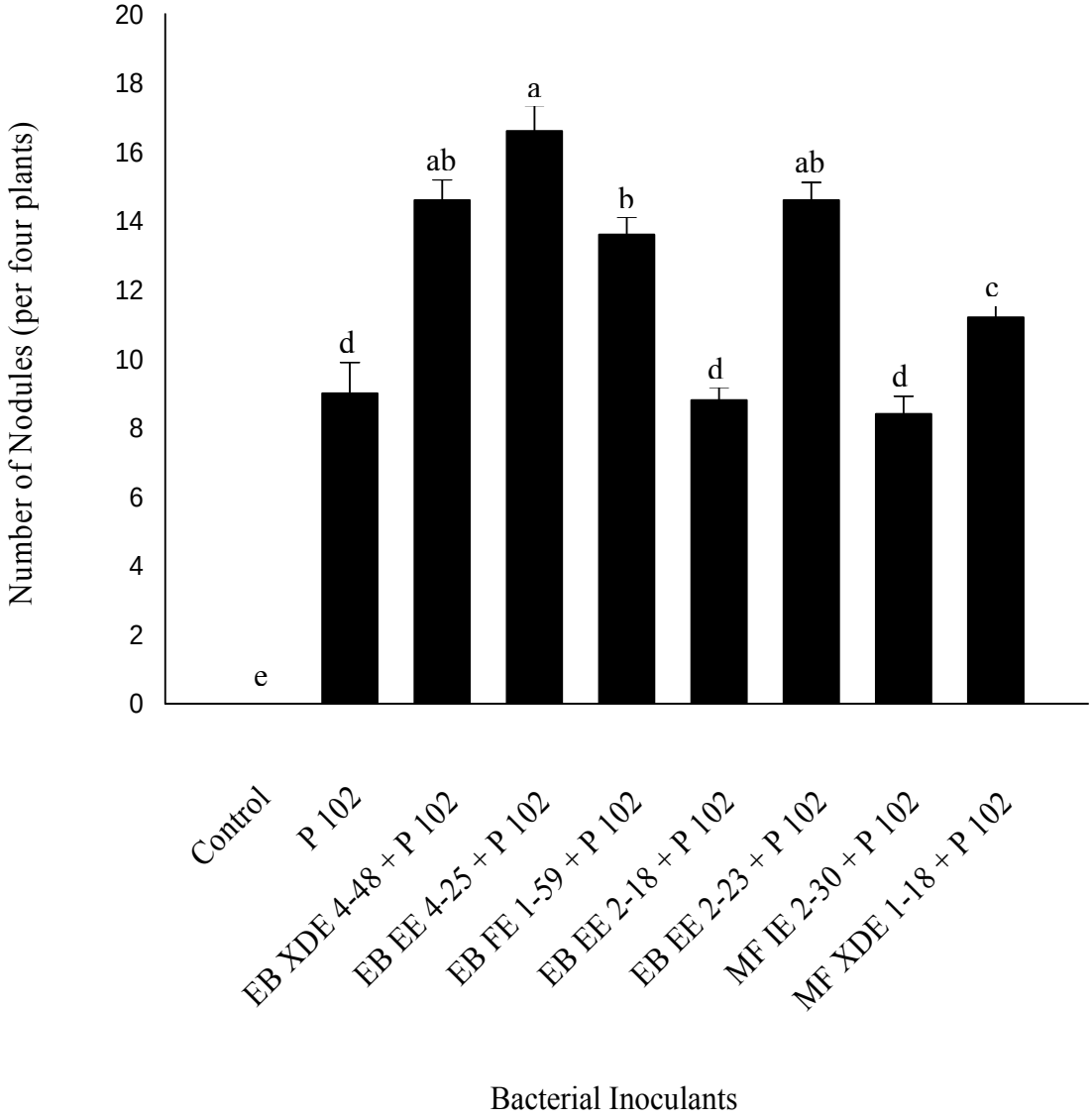

Figure 5.4 Effect of selected bacterial isolates on the nodule formation of alfalfa plants when co-inoculated with *Sinorhizobium meliloti* strain P102 when grown in growth pouches. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

5.4.2 Potted soil experiment

5.4.2.1 Effect of bacterial endophytes on alfalfa plant's shoots and roots length

Co-inoculation of alfalfa plants with endophytic bacterial isolates and *Sinorhizobium meliloti* significantly increased the shoot and root length in some bacterial treatments. For example, maximum shoot length was produced by the combination of *S. meliloti* P102 with *P. syringae* EB XDE 4-48, followed by *P. putida* EB EE 4-25, and *P.* sp. EB EE 1-59, respectively (Figure 5.5). These combinations significantly increased the shoot length of alfalfa plants than those of single inoculation with the *Rhizobium* strain alone and the control treatment (Figure 5.5). Also, co-inoculation of alfalfa with *S. meliloti* and *P. fluorescens* EB EE 2-18, and *P. fluorescens* EB EE 2-23 gave significant better results when compared with rhizobial treatment alone and the control treatment. However, two bacterial treatments, *B. cereus* MF IE 2-30, and *P. fluorescens* MF XDE 1-18, did not significantly affect the plant shoot growth when compared with the single *Rhizobium* treatment (Figure 5.5).

Pseudomonas fluorescens EB EE 2-23, when combined with *S. meliloti*, significantly increased the root growth when compared with all other bacterial treatments (Figure 5.6). Also, four other bacterial inoculants (EB XDE 4-48, EB EE 4-25, EB EE 1-59, and EB EE 2-18) showed similar effects on the root length growth when co-inoculated with *S. meliloti* and significantly enhanced root length compared to *Rhizobium* strain alone and the control treatment (Figure 5.6). Again, *B. cereus* MF IE 2-30, and *P. fluorescens* MF XDE 1-18, did not significantly increase the plant root length when compared with the single *Rhizobium* treatment (Figure 5.6).

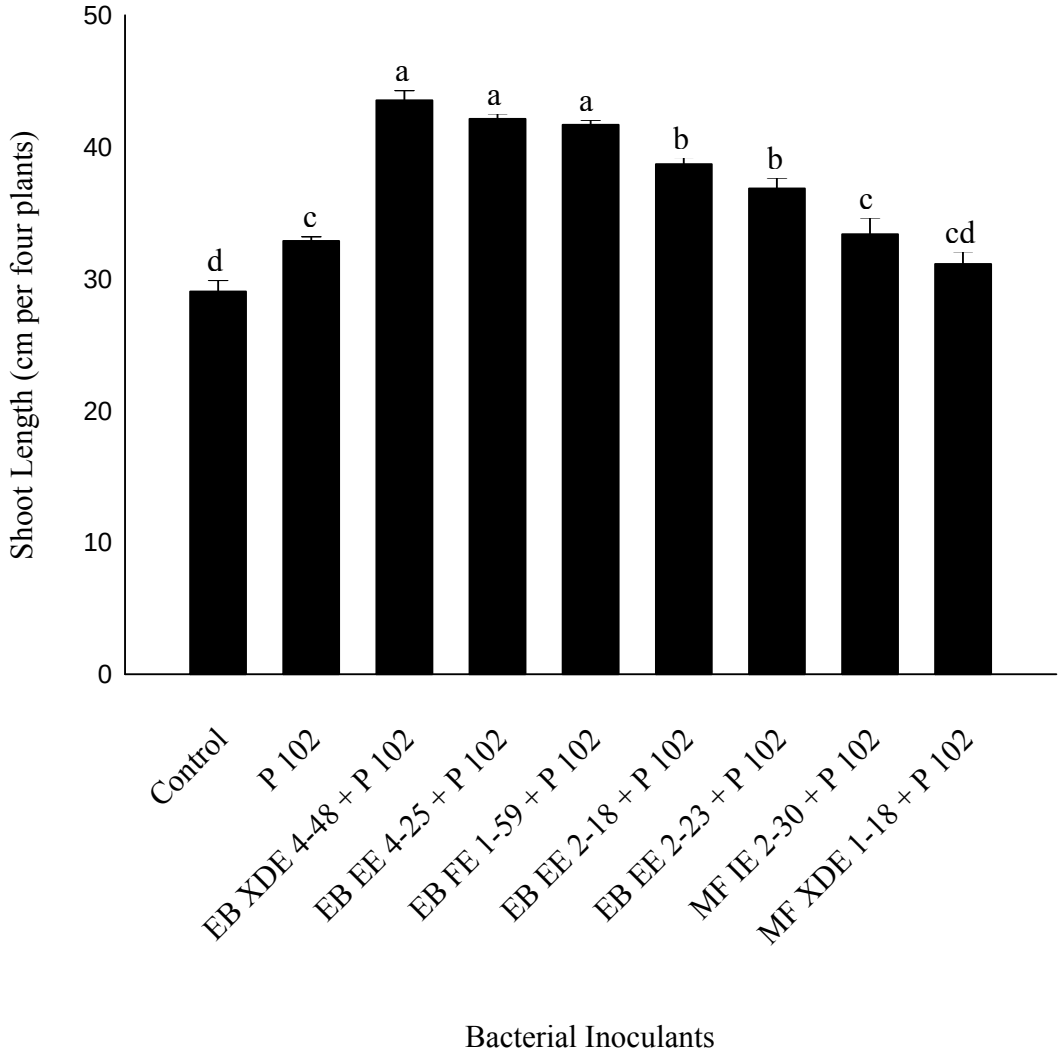

Figure 5.5 Effect of selected endophytic bacterial isolates on the shoot length of alfalfa plants when co-inoculated with *Sinorhizobium meliloti* strain P102 when grown in potted soil in the growth chamber. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

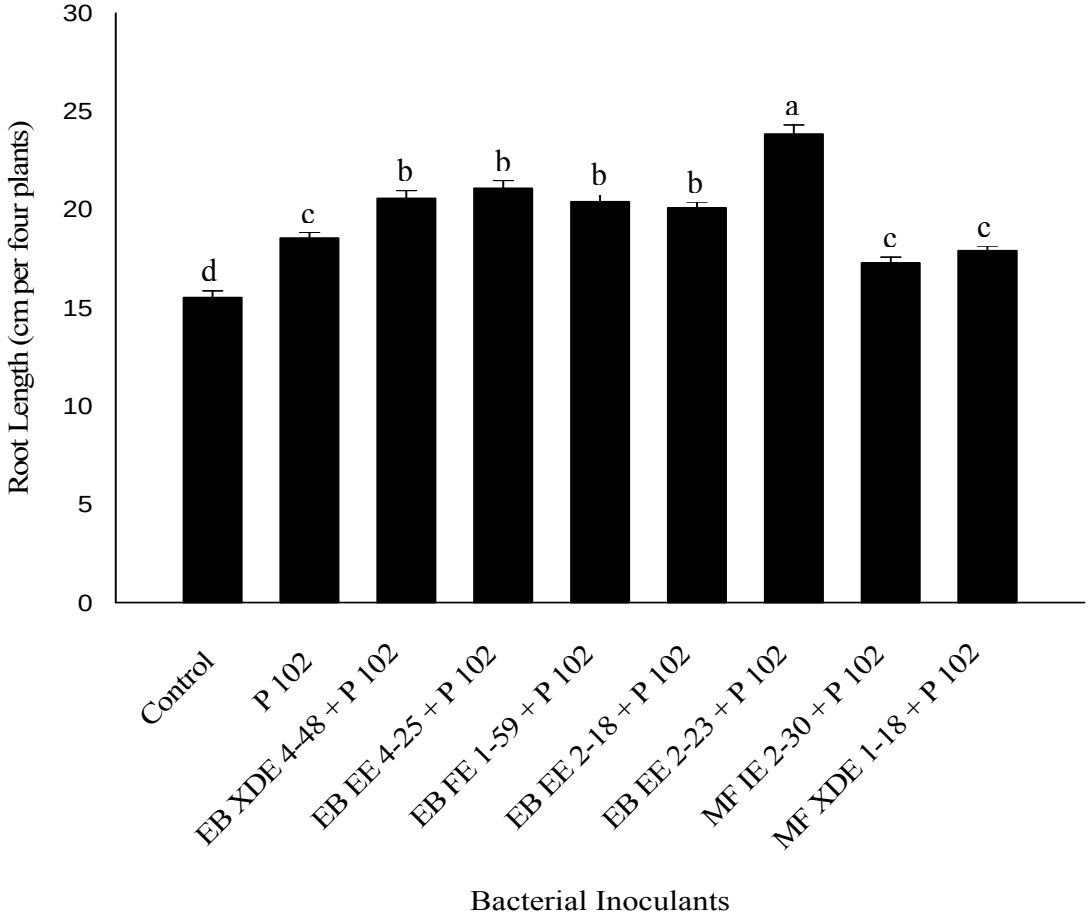

Figure 5.6 Effect of selected endophytic isolates on the root length of alfalfa plants when co-inoculated with *Sinorhizobium meliloti* strain P102 when grown in potted soil in the growth chamber. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

5.4.2.2 Effect of bacterial endophytes on alfalfa plant's shoots and roots weights

Co-inoculation of alfalfa with *P. syringae* EB XDE 4-48 and *S. meliloti* significantly ($p<0.05$) increased shoot weight compared to plants inoculated with *S. meliloti* alone (Figure 5.7). Similar effects were found with four other bacterial treatments but with less degree of efficiency (*P. putida* EB EE 4-25, *P.* sp EB FE 1-59. *P. fluorescens* EB EE 2-18, and *P. fluorescens* EB EE 2-23). All bacterial treatments were significantly different when compared to the uninoculated plant (Figure 5.7).

Only three bacterial treatments were significantly different ($p<0.05$) regarding their impact on the plants root weight when co-inoculated with *S. meliloti.* These bacterial inoculants were *P. syringae* EB XDE 4-48, *P. putida* EB EE 4-25, and *P. fluorescens* EB EE 2-23 (Figure 5.8). Rhizobial treatment alone with *S. meliloti* P102 and when co-combined with *P. fluorescens* MF XDE 1-18 did not significantly increase root weights compared to the uninoculated alfalfa plant treatment (Figure 5.8).

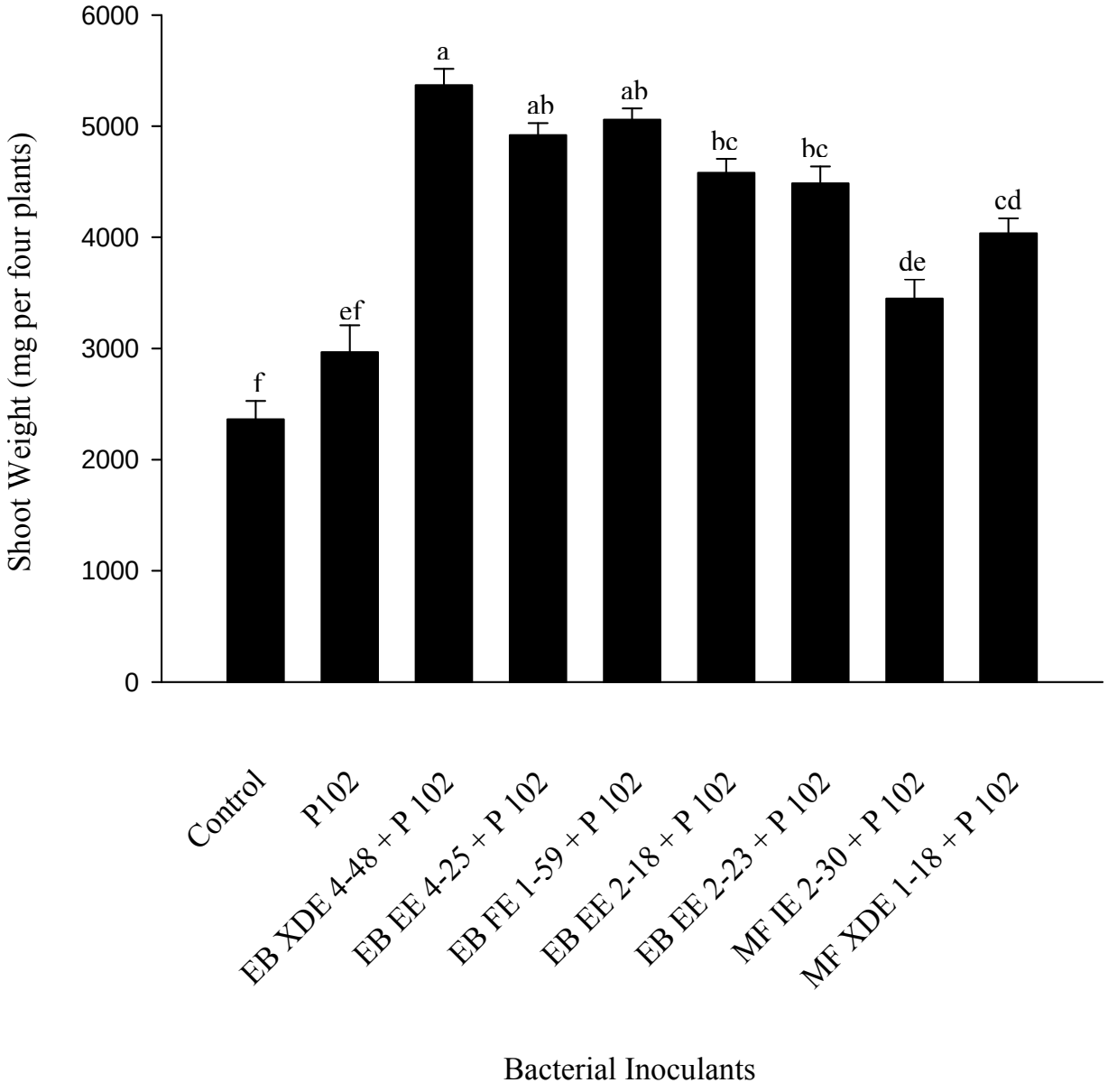

Figure 5.7 Effect of selected bacterial isolates on the shoot weight of alfalfa plants when co-inoculated with *Sinorhizobium meliloti* strain P102 when grown in potted soil in the growth chamber. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

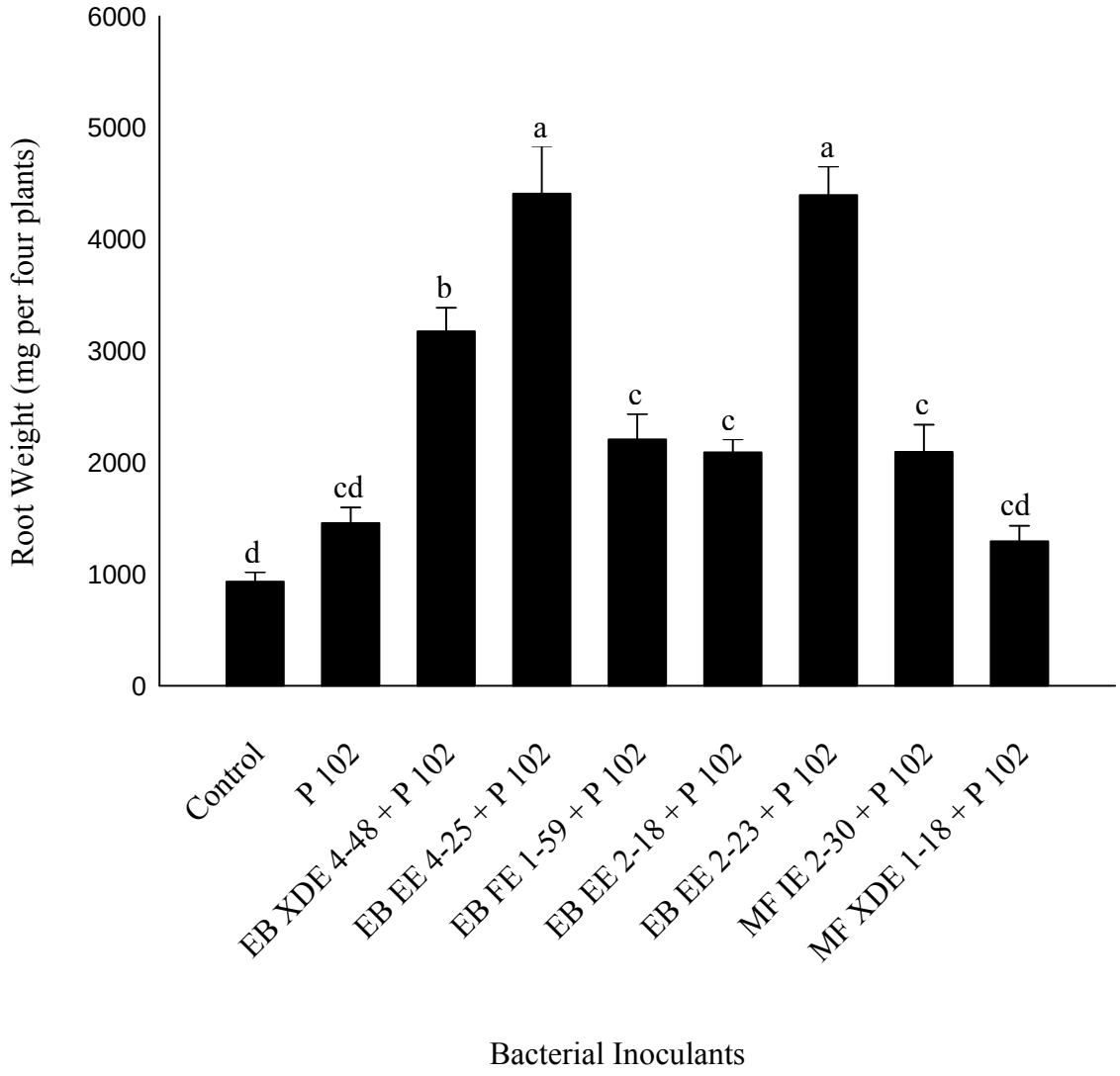

Figure 5.8 Effect of selected endophytic bacterial isolates on root weight of alfalfa plants when co-inoculated with *Sinorhizobium meliloti* strain P102 when grown in potted soil in the growth chamber. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

5.4.2.3 Effect of endophytic bacteria on lateral root formation and nodules number

Pseudomonas putida EB EE 4-25 and *P. fluorescens* EB EE 2-23 significantly increased lateral roots number of alfalfa plants when co-inoculated with *S. meliloti* compared with *S. meliloti* P102 alone (Figure 5.9). Co-inoculation of *P. syringae* EB XDE 4-48, *P. spp* EB FE 1-59, *P. fluorescens* EB EE 2-18, *B. cereus* MF IE 2-30, and *P. fluorescens* MF XDE 1-18 with *S. meliloti* had no effects on lateral roots number compared to plants treated with *S. meliloti* alone (Figure 5.9). All bacterial treatments were significantly different when compared to uninoculated alfalfa.

Pseudomonas sp. strain EB FE 1-59, *P. syringae* EB XDE 4-48, and *P. putida* EB EE 4-25 had the highest effects on nodules number formation on alfalfa plants when co-inoculated *S. meliloti*, respectively (Figure 5.10). Co-inoculation of alfalfa plants with *P. fluorescens* EB EE 2-18, *P. fluorescens* EB EE 2-23, *P. fluorescens* MF XDE 1-18 and *S. meliloti* did not significantly increase the nodules number on alfalfa plants compared to plants inoculated with *S. meliloti* alone (Figure 5.10). Interestingly, *B. cereus* MF IE 2-30 when co-inoculated with *S. meliloti* decreased the nodule number on alfalfa plants compared to plants treated with *S. meliloti* alone (Figure 5.10). All bacterial treatments significantly increased the nodules number on alfalfa plants compared to the uninoculated alfalfa plants (Figure 5.10).

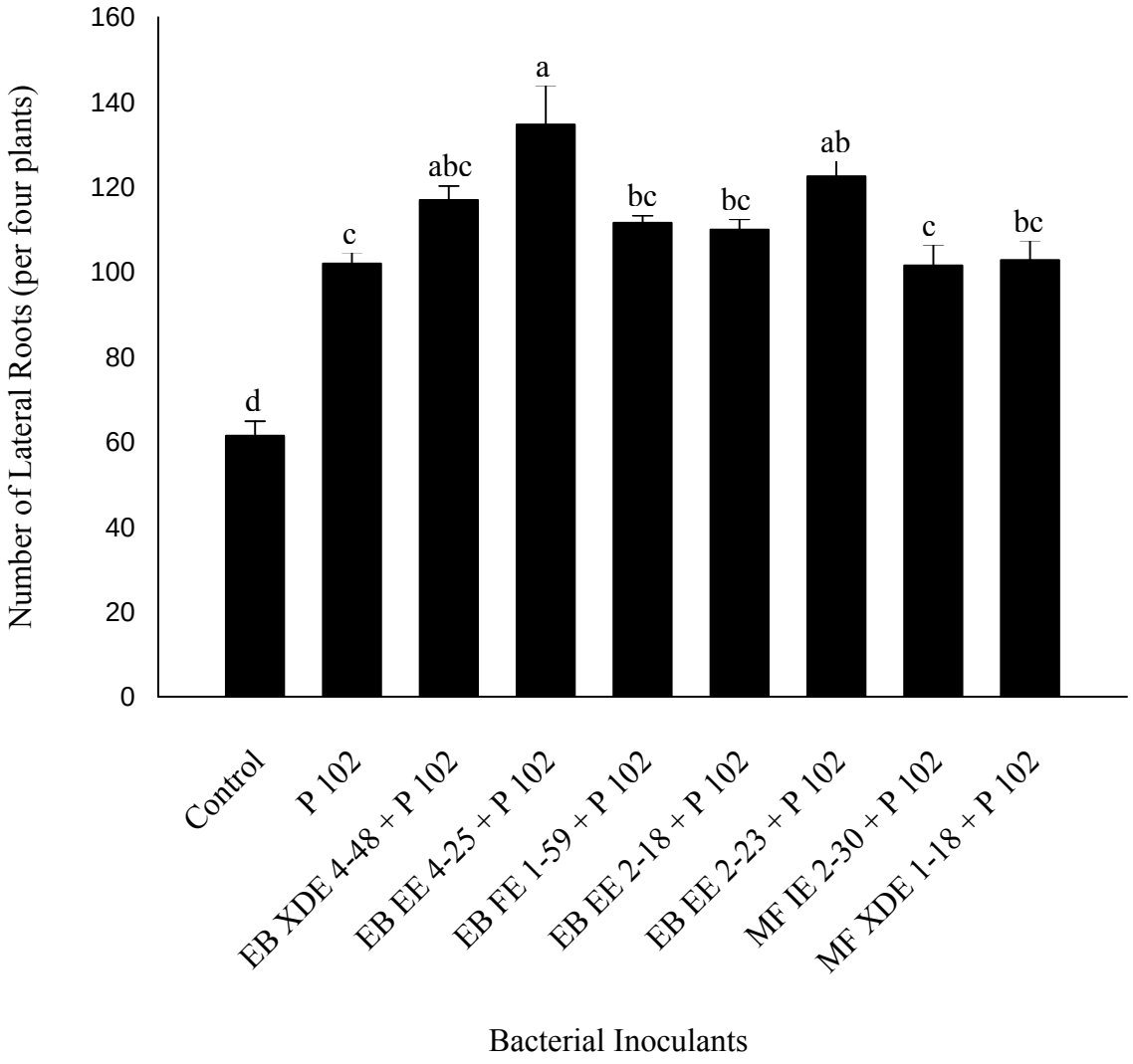

Figure 5.9 Effect of selected endophytic isolates on lateral roots formation of alfalfa plants when co-inoculated with *Sinorhizobium meliloti* strain P102 when grown in potted soil in the growth chamber. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

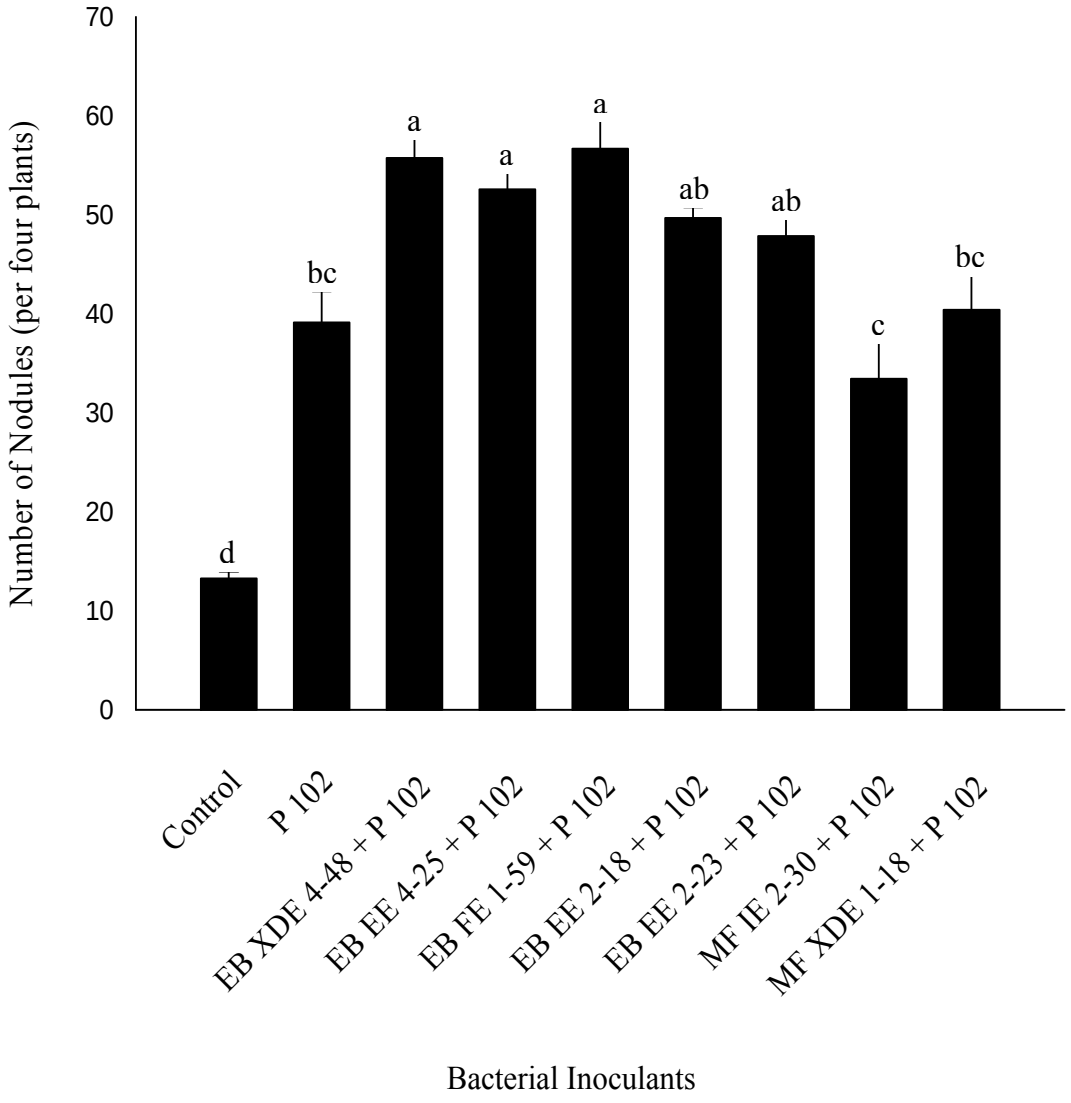

Figure 5.10 Effect of selected bacterial isolates on the nodule formation of alfalfa plants when co-inoculated with *Sinorhizobium meliloti* strain P102 when grown in potted soil in the growth chamber. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

5.5 Discussion

The legume-*Rhizobium* symbiosis is perhaps the best known beneficial symbiotic relationship. Legume cultivation is considered to be a critical component in sustainable agricultural systems (Howieson et al., 2008) because legumes fix N_2 symbiotically with rhizobia thus reducing plant dependence on chemical N fertilizers (Herridge et al., 2008).

The enhancement of legume-*Rhizobium* symbiosis is of great agricultural importance as it improves N availability in sustainable crop production (Bai et al., 2003). Over the last two decades, several PGPR strains, have been used in combination with rhizobia to inoculate legume seeds and were found to improve the N_2 fixation, nodulation, growth and development of roots of various legume plants when compared with plants inoculated with rhizobial strains alone (Chanway et al., 1989; de Freitas et al., 1993; Dashti et al, 1998; Bai et al., 2003; Figueiredo et al., 2008). However, the major drawbacks that limit the large scale application of PGPR are that these bacteria might not always survive the detrimental effects of various environmental conditions in the field (Glick et al., 2007). One possible way to overcome this limitation may be the use of endophytic plant growth-promoting bacteria (Sturz and Nowak, 2000; Glick et al., 2007).

In the recent past, endophytic bacteria have shown great potential to be used as agricultural inoculants to improve plant growth and productivity of many crops (Compant et al., 2005; Ryan et al., 2008; Weyens et al., 2009). However, few studies have assessed their impact on legume-*Rhizobium* symbiosis.

In this study, the effect of selected endophytic bacterial strains on the alfalfa-*Sinorhizobium* symbiosis was investigated. My results showed that several endophytic strains had an overall positive impact on the alfalfa-*Sinorhizobium* symbiosis in both the growth pouch assay and potted soil experiment.

Pseudomonas putida strain EB EE 4-25 improved the shoot length, root length, enhanced nodulation and increased lateral root formation of alfalfa plants in growth pouch and potted soil assays when co-inoculated with *S. meliloti* strain P102 compared to plants inoculated with *S. meliloti* strain P102 alone. The possible mechanisms of action that resulted in such plant growth could be attributed to any one or a combination of the following plant growth-promoting traits. In the present study strain EB EE 4-25 was able to use ACC as a sole N source, produce the

phytohormone IAA, solubilize inorganic P compounds, and show antagonistic activities against several soil pathogenic fungi (Table 5.1).

In the pot experiment, the soil used was deficient in P and soil pH (7.8) was conducive for phosphate solubilization, therefore; phosphate solubilization by strain EB EE 4-25 (*P. putida*) may play a role in enhancing alfalfa plant growth. Dey and co-workers (2004) speculated similar mechanisms behind the better growth of peanut plants inoculated with PGPR strains that were able to solubilize P. In the growth pouch, the ability of strain EB EE 4-25 to solubilize P could enhance plant P uptake. In legume plants, P is essential for nodulation, and subsequent N_2 fixation (Raman and Selvaraj, 2006). Rosas et al. (2006) reported that *P. putida* strain SP22 had increased root dry weight, shoot dry weight, and number of nodules of soybean plant when co-inoculated with *Bradyrhizobium japonicum* compared with plants inoculated with *B. japonicum* alone. Similar findings have been reported by Elkoca et al. (2008).

Another mode of action employed by endophytic bacteria to enhance alfalfa growth is the production of phytohormones. Most of the attention has been focused on the phytohormone (IAA) that is known to play a central role in cell division, cell enlargement, and root initiation (Vessey, 2003). Among all strains tested in this study, *P. putida* strain EB EE 4-25 was the best producer of IAA as assessed using the plate-left colorimetric method. Enhanced nodulation of alfalfa plants in both growth pouch and potted soil studies when co-inoculated by *P. putida* strain EB EE 4-25 and *S. meliloti* strain P102 might be related to the ability of strain EB EE 4-25 produce IAA. It has been speculated that production of IAA by bacteria could enhance the legume-*Rhizobium* symbiosis by increasing root length and root hair modulation which provide more sites for infection, and nodulation and subsequently enhance plant development (Vessey, 2003; Spaepen et al., 2007).

Another mechanism by which *P. putida* strain EB EE 4-25 could enhance the alfalfa-*Sinorhizobium* symbiosis is the production of ACC deaminase. The plant hormone ethylene has been reported to be an inhibitor of rhizobial nodulation of legumes when locally produced in plants in response to plant roots infection by rhizobia (Glick et al., 2007; Saleem et al., 2007). Bacterial strains containing ACC deaminase, that hydrolyzes ACC (the immediate precursor of ethylene), to ammonia and α-ketobutyrate, lowered the ethylene level and enhanced nodulation of legumes. For example, Shaharoona et al. (2006) reported that PGPR strains containing the ACC deaminase increased nodulation and subsequent N_2 fixation of mung bean plants upon co-

inoculation with *B. japonicum* compared to plants inoculated with *B. japonicum* alone. Also, increased numbers of nodules and dry weight in peanut plants inoculated with *P. fluorescens* isolate possessing ACC deaminase activity under field conditions has been reported by Dey and co-workers (2004). Additionally, endophytic *Burkholderia* sp. strain MSSP exhibiting ACC deaminase activity has been found to increased nodulation of the legume plant *Mimosa pudica* (Pandey et al., 2005).

Pseudomonas syringae strain EB XDE 4-48, and *P. fluorescens* strain EB EE 2-23 enhanced alfalfa plants growth in growth pouches and potted soil assays when co-inoculated with *S. meliloti* strain P102 compared to plants inoculated with *S. meliloti* strain P102 alone. Modes of action employed by these two strains which resulted in such plant growth could be attributed to similar mechanisms possessed by *P. putida* strain EB EE 4-25, reported previously. For example, *P. syringae* strain EB XDE 4-48 was able to produce IAA, solubilize inorganic P compounds, and show antagonistic activities against several soil pathogenic fungi. Also, *P. fluorescens* strain EB EE 2-23 was found to produce IAA, and exhibit antagonistic activities against several soil pathogenic fungi. Unlike strain EB EE 4-25, *P. syringae* strain EB XDE 4-48, and *P. fluorescens* strain EB EE 2-23 were able to produce siderophores (Table 5.1). Siderophores are low-molecular-weight organic molecules that show high affinity for Fe (III) ions (Barzanti et al., 2007). Siderophores are found to be produced by many soil microorganisms including endophytic bacteria (Sessitsch et al., 2004; Barzanti et al., 2007). Siderophore production may play a role in promoting nodule formation and subsequently N_2 fixation and plant growth because it is required in relatively high amounts by the enzyme nitrogenase (Vessey, 2003; Crowley, 2006). For example, Dey et al. (2004) attributed peanut plant enhancement upon inoculation with PGPRs to a possible role of siderophore production by *P. fluorescens* strains that enhanced nodule dry weight and subsequently N_2 fixation under both pot and field trials. Similar observations were reported for soybean plants by Cattelan et al. (1999).

Pseudomonas fluorescens strain EB EE 2-18 improved several parameters of alfalfa plants such as shoot length, shoot weight and root length when co-inoculated with *S. meliloti* strain P102 under potted soil conditions only. Even though this strain showed a strong ability to solubilize P, produce siderophores, and produce IAA, it did not promote plant growth similar to the enhancement observed when alfalfa plants were inoculated with *P. fluorescens* strain EB EE 2-23, which did not solubilize P. This suggests that P availability was not a limiting factor within

this study. Alternatively, *P. fluorescens* strain EB EE 2-23 might produce other plant hormones such as cytokinins and gibberellins that could promote root development which provides more sites for root infection by rhizobia and also increases the ability of plant to uptake mobile nutrients, thus increasing plant growth (Cattelan et al., 1999; Bai et al., 2003; Vessey, 2003). However, endophytic bacterial strains used in this study were not screened for these traits. These reasons may also explain the alfalfa plants response to co-inoculation with *P.* sp. strain EB FE 1-59 and *S. meliloti* strain P102.

Bacillus cereus strain MF IE 2-30 did not improve alfalfa plants growth under both growth pouch and potted soil conditions when co-inoculated with the rhizobial strain. This strain was negative for all PGPR mechanisms tested for except antagonistic activities toward several plant pathogenic fungi. However, when alfalfa plants were grown in growth chambers, no disease symptoms were observed, thus, biological control could be excluded as a plant growth mechanism. A similar conclusion was drawn by Bai and co-workers (2003). Similarly, *P. fluorescens* strain MF XDE 1-18 did not enhance alfalfa plant growth when co-inoculated with *S. meliloti* strain P102; this was not surprising because it was negative for all PGPR traits and it showed low ability to solubilize P using an *in vitro* plate method.

In conclusion, several endophytic bacterial strains tested in this study showed plant growth enhancement effects. Possible mechanisms of plant growth employed by these bacteria may include IAA production, solubilization of P, siderophores production, ability to produce ACC deaminase. However; it was difficult to determine what the exact mechanism was behind such a growth of alfalfa plants as most of the endophytes tested exhibited more than one PGPR trait. A co-inoculant containing both endophytic bacteria and *S. meliloti* could be developed as a biofertilizer agent under Saskatchewan soil conditions.

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6.0 SUMMARY AND CONCLUSION

Over the last two decades extensive research has been conducted to evaluate PGPR strains as potential inoculants for use in agriculture. However, a major limitation of these PGPRs is that they might not always survive aggressive competition with indigenous soil microorganisms under the field condition which limits any further applications (Glick et al., 2007). Endophytic bacteria are thought to overcome this problem as they reside inside plant roots and are thus avoid competition with other microbes for source of nutrients (Glick et al., 2007; Ryan et al., 2008).

Endophytic bacteria might enhance their host plants via direct and indirect plant growth mechanisms. Direct modes of action include phosphate solubilization, IAA production, N_2 fixation, ACC deaminase production, sulfur oxidation, and production of siderophores. Indirect plant growth mechanisms include the ability of bacteria to inhibit the growth of phytopathogens.

Determining the identity of bacterial endophytes strains used in this study was accomplished using FAME analysis and 16S rRNA gene sequence analysis. Most of the isolates belonged to the genus *Pseudomonas* (75%). Comparison between results obtained using these methods revealed that highly correlated identification agreement was achieved at the genus level; however, at the species level, both methods agreed only a few times (Chapter 3). The proper identification of these endophytic bacteria would serve as a significant resource for further application (as biofertilizers and/or biopesticides) in agriculture. Such a step would exclude any harmful bacterial agents at early stage in any inoculant development program.

The results obtained indicated that endophytic isolates exhibited various plant growth mechanisms. In this study, 40 endophytic bacterial isolates were screened for various direct and indirect plant growth mechanisms. Many of these bacteria possessed more than one direct plant growth promoting traits. For example, *P. fluorescens* strain EB QE 4-19 was found to produce siderophore, solubilize P, produce IAA, and produce ACC deaminase. Similarly, isolates EB IE 1-43 (*P. fluorescens*), EB FE 2-45 (*P. putida*), MF QE 1-69 (*P. fluorescens*), MF FE 2-21 (*P. putida*), MF FE 1-18 (*P. putida*), MF EE 2-122 (*P. fluorescens*), and MF XDE 3-65 (*P. putida*) were also found to possess these plant growth promoting traits (Chapter 4). On the other hand, some isolates were only able to exhibit one mode of action; for instance, *B. megaterium* strain

MF IE 3-83 was only able to produce siderophore. Similarly, *P. fluorescens* strain MF XDE 1-18 was only positive for P solubilization (Chapter 4).

Some endophytes were found to antagonize several soil-borne pathogenic fungi under *in vitro* conditions, which is considered an indirect plant growth mechanisms. For example, *B. cereus* strain MF IE 2-30 was able to inhibit the growth of four different pathogenic fungi. *P. fluorescens* strain EB EE 2-23 inhibited the growth of all pathogenic fungi under investigation (Chapter 4).

The growth chamber study (Chapter 5) was conducted to evaluate the impact of endophytic bacteria on alfalfa growth and development. We hypothesized that selected endophytic bacterial strains would enhance alfalfa- *Sinorhizobium* symbiosis.

Several endophytic isolates (from Chapter 3 and Chapter 4) were selected to evaluate their impact on the alfalfa-*Sinorhizobium* symbiosis under growth chamber conditions based on their identification, their ability to possess one or more direct plant growth promoting mechanisms, and their ability to control plant pathogens (Chapter 5).

The findings from the growth chamber study clearly demonstrated the ability of several selected endophytic bacterial strains to stimulate the alfalfa-*Sinorhizobium* symbiosis. For example, *P. putida* strain EB EE 4-25 improved various parameters of alfalfa plants such as shoot length, root length, nodulation and lateral root formation in growth pouch and potted soil assays when co-inoculated with *S. meliloti* strain P102. Similar results were observed for *P. syringae* strain EB XDE 4-48, and *P. fluorescens* strain EB EE 2-23.

The above findings support the hypothesis that selected endophytic bacterial strains would enhance alfalfa- *Sinorhizobium* symbiosis under growth chamber conditions. The possible mechanisms behind such enhancement could be attributed to one or more of the following traits: ACC deaminase production, phosphate solubilization, IAA production, and production of siderophores.

Further characterizations of these strains are required to elucidate their exact modes of action. For example, developing mutants of these strains with decreased ACC activity, IAA production, phosphate solubilization, and siderophores production could be one possible way. Tagging these strains with a reporter gene [e.g., green fluorescent protein (GFP)] would help in understanding their root colonization pattern in the rhizosphere and could be another way to

select strains that are able to compete with other microorganisms and persist in the root environment.

Finally, the effect of these strains on the alfalfa- *Sinorhizobium* symbiosis should be evaluated under the field conditions where environmental conditions are much more complex than under the growth chamber.

7.0 APPENDICES

Appendix A: Bacterial isolates code:

Isolate	Isolate code refers to
EB EE 4-36 EB EE 2-28 EB XDE 4-48 EB XDE 4-33 EB IE 1-32 EB EE 3-78 EB QE 3-37 EB EE 4-25 EB QE 4-19 EB IE 1-43 EB FE 1-59 EB FE 2-45 EB FE 2-92 EB EE 2-16 EB EE 2-18 EB EE 2-23	EB indicates the Eyebrow site, the second letter or letters indicates the plant cultivar (E= Excel, F= Fairview, I= Innovator, Q=Quest, XD= Exceed). The third letter indicates the root location (E=Endophytic). The first number indicates which of four replicates the isolate came from. The second number is a randomly assigned number.
WQE 2-28 WQE 2-25 WQE 3-1 WEE 2-30 WQE 2-8	W, indicates the Watrous site, the second letter or letters indicates the plant cultivar (E= Excel, F= Fairview, I= Innovator, Q=Quest, XD= Exceed). The third letter indicates the root location (E=Endophytic). The first number indicates which of four replicates the isolate came from. The second number is a randomly assigned number.
KCE 1-52 KCE 3-7	K indicates Kernen site, the second letter indicates the plant cultivar (C= CDC Teal). The third letter indicates the root location (E=Endophytic). The first number indicates which of four replicates the isolate came from. The second number is a randomly number

APPENDIX A. Continued.

Isolate code	Isolate code refers to
MF QE 1-69 MF EE 1-44 MF IE 3-83 MF FE 2-21 MF EE 1-34 MF FE 3-69 MF IE 2-50 MF IE 2-30 MF FE 1-18 MF FE 3-64 MF EE 4-4 MF XDE 3-57 MF EE 2-122 MF EE 4-19 MF XDE 1-18 MF XDE 1-6 MF XDE 3-65	MF indicates the Melfort site, the second letter or letters indicates the plant cultivar (E= Excel, F= Fairview, I= Innovator, Q=Quest, XD= Exceed). The third letter indicates the root location (E=Endophytic). The first number indicates which of four replicates the isolate came from. The second number is a randomly assigned number