EFFECT OF ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT GROWTH-PROMOTING RHIZOBACTERIA ON GLOMALIN PRODUCTION

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ABSTRACT

There is accumulating evidence that arbuscular mycorrhizal fungi (AMF) produce a glycoprotein called glomalin, which has the potential to increase soil carbon (C) and nitrogen (N) storage, thereby reducing soil emissions of carbon dioxide (CO_2) and nitrous oxide (N_2O) into the atmosphere. However, other soil microorganisms such as plant growth-promoting rhizobacteria (PGPR) that interact with AMF could indirectly influence glomalin production. The objectives of this study were to determine the effects of AMF and PGPR interactions on glomalin production and identify possible combinations of these organisms that could enhance C and N storage in the rhizosphere. The effects of AMF and PGPR interactions on pea (*Pisum sativum* L.) growth and correlations between glomalin production and plant growth also were assessed.

A series of growth chamber and laboratory experiments were conducted to examine the effect of fungal and host plant species on glomalin production by comparing the amounts of glomalin produced by *Glomus clarum*, *G. intraradices*, and *G. mosseae* in association with corn (*Zea mays* L.), in addition to examining differences in the ability of corn, pea, and wheat (*Triticum aestivum* L.) to support glomalin production by *G. intraradices*. There were no significant differences in glomalin production [measured in the rhizosphere as Bradford-reactive soil protein (BRSP)] by the three AMF species, whereas host plant significantly affected glomalin production. Specifically, higher BRSP concentrations were found in the rhizosphere of corn as compared to pea and wheat.

Additionally, the effect of long-term storage on the growth promoting traits of the PGPR strains selected; namely, *Pseudomonas cepacia* R55 and R85, *P. aeruginosa* R75, *P. putida* R105, and *P. fluorescence* R111 were investigated. These bacterial strains previously had been identified as PGPR, but had since undergone approximately twenty years of storage at -80˚C; thus, it was necessary to confirm that these strains had retained their plant growth promoting characteristics. Apparently, long-term storage had no significant adverse effect on the PGPR strains as all strains increased the total biomass of wheat significantly and demonstrated antagonism against fungal pathogens.

The possibility that spore-associated bacteria (SAB) could influence AMF associations, thereby affecting glomalin production, and subsequent crop yield potential

was assessed. This was achieved by first isolating bacteria from disinfested spores of the AMF species and determining their potential as PGPR for wheat. According to fatty acid methyl ester (FAME) profiles, four genera of bacteria were isolated from AMF spores namely; *Arthrobacter*, *Bacillus*, *Micrococcus*, and *Paenibacillus*, of which *Bacillu*s species were the most common SAB. None of these isolates, however, showed growth promoting abilities on wheat.

Based on the preliminary findings, the combined effects of the three AMF species and the five PGPR strains were examined on plant growth and glomalin production under gnotobiotic conditions using pea as the host plant. Interactions between *G. intraradices* and R75, R85, or R105 resulted in increased BRSP concentration in the mycorrhizosphere of pea. Additionally, significant interactions were observed between the AMF species and PGPR strains on BRSP concentration in pea rhizosphere under non-sterile conditions. As observed under sterile conditions, the co-inoculation of pea with *G. intraradices* and R75 or R85 increased BRSP concentrations in the rhizosphere of pea grown in non-sterile soil, although interaction effects were not significantly different from the control or when *G. intraradices* was applied alone. Significant AMF and PGPR interactions were observed to affect AMF colonization; however, the combination of these organisms did not significantly affect pea growth, nutrient uptake, and C and N storage in the plant rhizosphere. No correlations were detected between glomalin-related soil protein (GRSP), pea growth, nutrient concentrations in the plant tissue, and soil organic C and N content. This study demonstrated that although the potential exists to manipulate certain AMF and PGPR to enhance glomalin production, co-inoculation of AMF and PGPR did not enhance plant growth or C and N storage beyond that achieved by inoculation of either organism.

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LIST OF ABBREVIATIONS

ACC	1-aminocyclopropane-1-carboxylate
AMF	Arbuscular mycorrhizal fungi
ANOVA	Analysis of variance
BRP	Bradford-reactive protein
BRSP	Bradford-reactive soil protein
BSA	Bovine serum albumin
cfu	Colony forming units
DAP	Days after planting
EE-BRSP	Easily extractable Bradford-reactive soil protein
EEG	Easily extractable glomalin
EE-GRSP	Easily extractable glomalin-related soil protein
EE-IRSP	Easily extractable immunoreactive soil protein
ELISA	Enzyme-linked immunosorbent assay
FAME	Fatty acid methyl ester
GRP	Glomalin-related protein
GRSP	Glomalin-related soil protein
GWP	Global warming potential
Hsp	Heat shock protein
IAA	Indole-3-acetic acid
IRSP	Immunoreactive soil protein
LSD	Least significant difference
MHB	Mycorrhization helper bacteria
NPP	Net primary productivity
NS	No significant differences
PBS	Phosphate buffer saline
PDA	Potato dextrose agar
PGPR	Plant growth-promoting rhizobacteria
PR	Pathogenesis-related proteins
PSB	Phosphate solubilizing bacterium
SAB	Spore-associated bacteria
SOC	Soil organic carbon
SOM	Soil organic matter
STN	Soil total nitrogen
TG	Total glomalin
TSA	Trypticase soy agar
TSB	Trypticase soy broth
WAP	Weeks after planting

1. INTRODUCTION

The rhizosphere, a soil region under the direct influence of plant roots, harbours different microorganisms, and the interactions between these organisms can either benefit or hinder plant growth and development (Requena et al., 1997; Barea et al., 2005). The interactions between plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) are among the most studied due to their contributions to the productivity of agricultural systems and natural ecosystems (Requena et al., 1997; Walley and Germida, 1997; Kim et al., 1998; Adesemoye and Kloepper, 2009). Plant growth-promoting rhizobacteria enhance plant growth through nutrient uptake and control of phytopathogens (de Freitas and Germida, 1991; Vessey, 2003; Richardson et al., 2009). Similarly, AMF are known to promote plant growth and development by increasing nutrient acquisition and alleviating stress conditions of plants (Koide and Kabir, 2000; Koide and Mosse, 2004; Barea et al., 2005). Also, AMF improve soil structure and play a crucial role in soil carbon (C) storage (Zhu and Miller, 2003; Rillig and Mummey, 2006). These roles of AMF have been linked with the production of a novel fungal substance, termed glomalin (Zhu and Miller, 2003; Rillig, 2004a, b).

Glomalin, which is operationally defined and measured in soil as 'glomalin-related soil protein' (GRSP), contributes to soil aggregate formation and stabilization due to its stability and hydrophobic nature (Wright and Upadhyaya, 1998, 1999; Rillig, 2004a, b). Also, there are accumulating reports that GRSP is a major pool of soil C and N (Rillig et al., 2001; Zhu and Miller, 2003; Nichols and Wright, 2006), thus glomalin can potentially reduce soil emissions of carbon dioxide (CO_2) and nitrous oxide (N_2O) into the atmosphere. This latter role of glomalin is of major interest as the reduction of atmospheric concentration of greenhouse gases is now of paramount importance (King, 2004).

Studies involving the combined application of PGPR and AMF have shown the possibilities of using certain PGPR to stimulate the beneficial role of AMF, and vice versa (Hodge, 2000; Barea et al., 2002, 2005). These studies verify the interactions and hence it is conceivable that the combined application of PGPR and AMF could increase the production of glomalin by AMF. Although much work has been done on glomalin,

there is little knowledge on how its production can be increased using PGPR. In this context, it was hypothesized that interactions between AMF and PGPR enhance glomalin production and the storage of C and N in the rhizosphere. Therefore, the objectives of this study were to examine the effect of AMF and PGPR interactions on glomalin production; determine the possible combinations of AMF and PGPR that could enhance C and N storage in the rhizosphere; determine the effect of these interactions on pea growth; and observe the correlation between glomalin production and plant growth.

This thesis is comprised of nine chapters. Following the Introduction (Chapter 1) is the main body contained within Chapters 2 through 7. Chapter 2 is a review of the literature pertaining to rhizosphere organisms, particularly plant growth-promoting rhizobacteria and arbuscular mycorrhizal fungi. Also in this chapter is a review of glomalin, focusing on its contribution to ecosystem functions and productivity.

Chapter 3 to 5 contain the findings of preliminary experiments. Chapter 3 examines fungal and plant effects on glomalin production. In Chapter 4, the plant growth promoting characteristics of bacteria previously identified as PGPR by de Freitas and Germida (1990a, 1990b) were confirmed following several years of storage. Finally, Chapter 5 determines if AMF spore-associated bacteria (SAB) affect wheat yield, and thus may be suitable co-inoculants for enhancing glomalin production.

Chapter 6 discusses a growth chamber experiment conducted to determine potential interactions between AMF and PGPR that may affect glomalin production, plant growth, and nutrient uptake by pea under gnotobiotic conditions.

Chapter 7 discusses another growth chamber study conducted using non-sterile conditions to investigate effects of AMF and PGPR (the most effective AMF and PGPR combinations identified under sterile conditions) on glomalin production. Furthermore, Chapter 7 describes the effects of the organisms on plant growth, nutrient uptake and concentration, and C and N storage in the pea rhizosphere.

Chapter 8 is a summary of all the findings, with conclusions and recommendations for those whose interest has been heightened by this study.

Followed by Chapter 8 is the last chapter, Chapter 9, comprising of a list of literature cited.

2. LITERATURE REVIEW

2.1 The Rhizosphere

The rhizosphere may be defined as the 'heart' of the soil, as it is the zone under the direct influence of plant roots and with high populations of active microorganisms (Glick, 1995; Nelson, 2004; Barea et al., 2005; Napoli et al., 2008). In the rhizosphere, plant roots influence microbial communities by depositing photosynthates into the rhizosphere (rhizodeposition). Simultaneously, rhizosphere organisms govern plant growth and development (Nelson, 2004; Napoli et al., 2008). Although the rhizosphere was first described by Hiltner (1904), numerous studies have observed microbial and plant activities in the soil dating as far back as 400 million years (Khan, 2005; Napoli et al., 2008). Operationally, rhizosphere soils are defined as soils adhering to plant roots even with a moderate shake (Phillips and Fahey, 2008; Idris et al., 2009). Even though it may be difficult to physically separate rhizosphere and bulk soils (Hinsinger, 2005), they differ in inherent biological, chemical, and physical characteristics (Vessey, 2003; Barea et al 2005; Hinsinger, 2005). For example, higher enzyme activities (Vazquez et al., 2000), plant nutrient concentrations (Rodríguez and Fraga, 1999), soil pH (Tagliavini et al., 1995), and water repellency (Hallett et al., 2009) have been observed in the rhizosphere relative to the bulk soil. The observations are attributable to the carbon (C)-rich compounds released by plant roots into the rhizosphere and the deficiencies of these nutrient sources in the bulk soil (Fitter and Garbaye, 1994; Nelson, 2004).

2.1.1 Microbial interactions in the rhizosphere

Being a nutrient rich environment, the rhizosphere supports a diverse population of micro and macroorganisms, which form complex interactions with the plant root (Glick, 1995; Nelson, 2004; Richardson et al., 2009). Interactions among rhizosphere organisms range from competitive to mutualistic (Requena et al., 1997; Roesti et al., 2006). Concurrently, associations between rhizosphere organisms and plants could be symbiotic or parasitic depending on the type of microorganisms, and soil and environmental conditions (Walley and Germida, 1997; Vessey, 2003; Barea et al., 2005). Associations between plants and parasitic or non-parasitic deleterious bacteria and fungi are among the

detrimental ones, whereas beneficial relationships are those observed between plants and non-symbiotic or symbiotic beneficial rhizosphere bacteria and fungi, such as nitrogen-fixing bacteria, plant growth-promoting rhizobacteria (PGPR), and arbuscular mycorrhizal fungi (AMF) (Barea et al., 2005).

Beneficial rhizosphere organisms promote plant growth by increasing nutrient uptake and alleviating biotic and abiotic stress conditions of plants (Vessey, 2003; Barea et al., 2005; Richardson et al., 2009). For example, *Rhizobium* species contribute significantly to the nitrogen (N) nutrition of leguminous plant through atmospheric N_2 fixation (Vessey, 2003). Rhizosphere organisms such as AMF and PGPR increase the bioavailability of essential nutrients, especially phosphorus (P), through the solubilization and mineralization of nutrients from organic and inorganic sources (Koide and Kabir, 2000; Hodge et al., 2001; Tawaraya et al., 2006; Idris et al., 2009; Richardson et al., 2009). Also, these organisms improve plant health by controlling the growth of plant pathogens and inducing systemic resistance in plants (George et al., 1995; Ramamoorthy et al., 2001; Weller et al., 2002). As a consequence, numerous studies have proposed the manipulation of rhizosphere organisms to enhance plant productivity, re-establish degraded habitats, and phytoremediate polluted soils (Requena et al., 1997; Biro et al., 2000; Khan, 2005; Adesemoye et al., 2008).

2.2 Plant Growth-Promoting Rhizobacteria

Plant growth-promoting rhizobacteria are beneficial inhabitants of the rhizosphere, found in association with plant roots (Kloepper and Schroth, 1978; Kloepper et al., 1989; Vessey, 2003; Lucy et al., 2004). Although beneficial rhizosphere bacteria have been identified prior to the naming of these organisms, identification by Kloepper and Schroth, (1978) heightened the interest of other researchers (Vessey, 2003; Lucy et al., 2004). The beneficial effects of PGPR on various crops including cabbage (*Brassica oleracea* L.), canola (*Brassica rapa* L.), corn (*Zea mays* L.), potato (*Solanum tuberosum* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.) have been reported (Burr et al., 1978; de Freitas and Germida, 1990a; Germida and de Freitas, 1994; de Freitas et al., 1997). Tree crops, such as highbush blueberry (*Vaccinium corymbosum*), mulberry (*Morus alba*), and sweet cherry (*Prunus avium* L.) also benefit from PGPR (De Silva et al., 2000;

Sudhakar et al., 2000; Esitken et al., 2006). Consequently, a number of growth chamber and field studies have been conducted to study the modes of action and mechanisms used by PGPR to stimulate plants growth and development (Vessey, 2003; Lucy et al., 2004).

Plant growth-promoting rhizobacteria enhance plant productivity via many different modes of action including increasing seedling emergence, shoot and root growth, nutrient content, seed yield and protein concentration, and simulating ripening and senescence of plants after maturity (Kloepper et al., 1988; Dashti et al., 1997; Dobbelaere et al., 2002). Even though the mechanisms involved in growth promotion by PGPR are not completely understood (Cattelan et al., 1999; Nelson, 2004), atmospheric N_2 fixation, phytohormone production, antagonism against pathogens, enhancement of plant nutrient uptake such as P solubilization, and stimulation of beneficial activities of other rhizosphere organisms are frequently reported (Glick, 1995; de Freitas et al., 1997; Lucy et al., 2004).

As reviewed by Davison (1988), beneficial effects of PGPR on plant growth can be classified as direct or indirect. For example, by removing hazardous chemicals and inhibiting the growth of deleterious microorganisms (biocontrol) in the rhizosphere, PGPR indirectly stimulate plant growth (Davison, 1988). Plant growth-promoting rhizobacteria reduce growth of pathogens through the production of antibiotics and siderophores (Thomashow et al., 1990; Glick, 1995; Whipps, 2001). In contrast, the direct effects reflect the ability of PGPR to promote plant growth in the absence of pathogens or other rhizosphere microorganisms. Often, plant growth promotion is achieved through a combination of mechanisms (Glick, 1995; Nelson, 2004; Richardson, et al., 2009). Also, by using these mechanisms, PGPR influence the symbiotic association between plant and other microorganisms including AMF and nodule forming *Rhizobium* species (Vessey, 2003). The most studied PGPR are the fluorescent pseudomonads, though other beneficial rhizosphere bacteria including non-fluorescent pseudomonads, *Azotobacter, Bacillus, Enterobacter,* and *Serratia* species have been identified (Kloepper et al., 1989; Vessey, 2003; Lucy et al., 2004; Richardson et al., 2009).

2.2.1 Direct effects of plant growth-promoting rhizobacteria on plant growth

Nitrogen is one of the most important elements for plant production; however, most organisms cannot use atmospheric N_2 directly. As a result, N frequently is a limiting

nutrient (McCormick, 1988). The role of bacteria in biological N_2 fixation has been reported as early as 1800s (Burris, 1998), and there are reports that certain PGPR promote growth, mainly because of their role in N_2 fixation (Vessey, 2003). For example, *Bacillus polymyxa*, found in wheat rhizosphere, was regarded as a PGPR due to its ability to fix N_2 (Omar et al., 1996). In fact, a number of studies report N_2 fixing rhizobia such as *Bradyrhizobium* and *Rhizobium* species as PGPR due to their atmospheric N_2 fixation even if they do not possess other PGPR qualities, such as hormone production and thus, should not be regarded as PGPR (Adesemoye and Kloepper, 2009). Although some researchers have selected PGPR by virtue of observed nitrogenase activity, this activity may not relate to growth promotion by PGPR (Vessey, 2003). For example, in a study conducted by Cattelan et al. (1999), the five isolates that showed nitrogenase activities did not promote soybean (*Glycine max* (L.) Merr.) growth. Thus N_2 fixation may not be an important trait of PGPR (George et al., 1995; Mantelin and Touraine, 2004), and PGPR do not necessarily contribute substantially to plant N nutrition (Richardson et al., 2009).

Apart from the reported atmospheric N_2 fixation by PGPR, some PGPR increase the availability of other essential plant nutrients, such as P (Glick, 1995; Vessey, 2003; Adesemoye and Kloepper, 2009). Phosphorus is a limiting soil nutrient because a considerable fraction of total soil P is organic P or insoluble inorganic P and cannot be absorbed by plants (Koide and Kabir, 2000). Phosphorus is mainly absorbed by plants in the orthophosphate forms; namely, monobasic ($H_2PO_4^-$) and diabasic (HPO_4^{2-}) P (Rodríguez and Fraga, 1999; Vance et al., 2003; Vessey, 2003). Additionally, soil iron (Fe) and aluminum (Al) oxides form Fe and Al-hydroxylated surfaces that retain P, and at high pH, calcium carbonate reduces bioavailability of soluble inorganic P in soil solution (Vance et al., 2003). Also, P applied as fertilizer is easily immobilized, further increasing P deficiencies (Dey, 1986, cited by Rodríguez and Fraga, 1999). Therefore, mineralization of organic P and solubilization of inorganic P by PGPR is an important aspect of their association with plants (Rodríguez and Fraga, 1999).

Some PGPR solubilize insoluble inorganic and organic P compounds by secreting organic acids and enzyme phosphatases, respectively (Kim et al., 1998; Rodríguez and Fraga, 1999; Adesemoye and Kloepper, 2009). De Freitas et al. (1997) and Idris et al.

(2009) are among several researchers that have demonstrated solubilization of tricalcium and rock phosphate by PGPR. De Freitas et al. (1997) related rock phosphate solubilization by *Bacillus* and *Xanthomonas* species to the synthesis of organic acids by the PGPR. Organic acid acidifies microbial substrates which induce proton exchange for calcium ions (Ca^{2+}), thereby releasing soluble P forms from mineral phosphate (Rodríguez and Fraga, 1999). The production of organic acids such as acetic, citric, lactic, oxalic, gluconic, and succinic acids have been reported (de Freitas et al., 1997; Kim et al., 1998; Rodríguez and Fraga, 1999; Adesemoye and Kloepper, 2009), with gluconic acid being the most common (Rodríguez and Fraga, 1999). De Freitas and co-workers (1997) also related P solubilization by PGPR to phosphatase activity. Plant growth-promoting bacteria hydrolyze phosphoesters and phosphoanhydrides through the production of acid and alkaline phosphatases (de Freitas et al., 1997; Rodriquez and Fraga, 1999; Adesemoye and Kloepper, 2009), a process known as mineralization of organic P (Rodriquez and Fraga, 1999). Nevertheless, not all P solubilizing bacteria increase P uptake and P concentration in plant tissues. The study by de Freitas et al. (1997) showed that P solubilization by *Bacillus* and *Xanthomonas* species did not enhance P content of canola; rather the PGPR used other mechanisms such as hormone production to stimulate plant growth.

Calcium (Ca), Fe, magnesium (Mg), and potassium (K) are among other essential nutrients supplied by PGPR to their host (Glick, 1995; Lucy et al., 2004; Khan, 2005; Orhan et al., 2006). Some PGPR enhance nutrient availability via production of siderophore. For example, iron though abundant in soil, cannot be assimilated directly by plants (Glick, 1995), certain organisms, such as the fluorescent pseudomonads, produce an iron chelator (siderophore) which increases Fe availability for plant uptake. Siderophores are yellow-green fluorescent pigments with high affinity for Iron (III) (Fe^{3+}) and are capable of reducing this Fe form to Iron (II) Fe^{2+} which can be absorbed by plant cells. This mechanism is especially important under Fe limiting conditions (Kloepper et al., 1980; Glick, 1995).

It is well established that PGPR produce phytohormones that stimulate plant growth (Glick, 1995; Dobbelaere et al., 2002; Vessey, 2003). In fact, the plant growth promoting abilities of PGPR are often related to the production of these growth regulators (Glick,

1995). Auxin [e.g., Indole-3-acetic acid (IAA)] is one of the important hormones produced by PGPR (Glick, 1995; Dobbelaere et al., 2002; Richardson et al., 2009). By increasing lateral roots and roots hair formation, IAA expands the root surface area, and allows greater exploration of soil regions for nutrients (de Freitas et al., 1997; Dobbelaere et al. 2002; Erturk et al., 2010). Dobbelaere et al. (2002) related yield increases of spring wheat to root development by IAA produced by *A. brasilense*. Idris et al. (2009) linked the colonizing and growth promoting abilities of some strains of *Bacillus cereus* to IAA production. Furthermore, beneficial effects of IAA-producing PGPR on root growth increases colonization sites for other beneficial microorganisms, such as AMF and symbiotic N_2 fixers (Vessey, 2003).

Cytokinins, gibberellins, and ethylene are other hormones produced by PGPR. Although cytokinins reduce the growth of lateral roots, they increase cell division to facilitate root hair formation and plant growth (Silverman et al., 1998; Dobbelaere et al. 2002). Gibberellins are involved in the formation of lateral roots and stimulate root elongation (Richardson et al., 2009). Ethylene is another important hormone that governs root growth, and increases senescence and fruit ripening in plants (Glick et al., 2007a, b). Early ripening and drying induced by ethylene is vital, especially in temperate regions (Dobbelaere et al., 2002). Ethylene production also has been attributed to plant resistance to pathogens (Glick et al., 2007b), thus contributing to plant growth. Nevertheless, cytokinins, gibberellins, and ethylene are not as characterized as auxin and further studies are required to understand the mechanisms by which these hormones promote plant growth (Richardson et al., 2009).

Studies have shown that factors, such as bacteria type, levels of hormone produced by the bacteria, and plant response to these levels, determine the actual effects of growth regulators on plant productivity (Glick, 1995; Cattelan et al., 1999; Dobbelaere et al., 2002). For example, overproduction of IAA reduce plant growth (Glick, 1995; Dobbelaere et al., 2002; Richardson et al., 2009). Interestingly, some PGPR synthesize enzymes to reduce the levels of phytohormones, thereby maintaining desirable concentrations of these phytostimulators for plant growth (Glick, 1995). The enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase is produced by some PGPR and increases plant growth through the inhibition of ethylene production (Glick et al., 1998;

Cattelan et al., 1999; Lugtenberg and Kamilova, 2009). At high concentrations, ethylene hinders seedling emergence and root elongation which reduces plant growth (Glick, 1995). Hormone production by PGPR is an important aspect of their plant growth promoting abilities; however, more studies are required to understand the functional roles of the growth regulators (Richardson et al., 2009).

2.2.2 Indirect effects plant growth-promoting rhizobacteria on plant growth

The realization that PGPR improve plant health through the control of phytopathogens increased the number of studies focusing on disease and pest management practices involving the use of PGPR (Ramamoorthy et al., 2001). In fact, Kropp et al. (1996) observed that the main mechanism by which *P. chlororaphis* increased emergence of spring wheat was through its biocontrol activities. Some PGPR control phytopathogens through the production of siderophores, antibiotics, enzymes, hydrogen cyanide, and organic acids (Thomashow et al., 1990; Glick, 1995; Richardson et al., 2009). Siderophores, such as pseudobactin, reduce the numbers and activities of pathogenic bacteria and fungi by depriving them of Fe^{3+} (Kloepper et al., 1980; Glick, 1995; Richardson et al., 2009). Although some deleterious organisms also produce siderophores, they produce lower quantities, or produce siderophores with reduced affinity for Fe^{3+} relative to the PGPR (Kloepper et al., 1980).

Another important mechanism used by PGPR in the control of pathogens is the production of antibiotics, such as phenazine-1-carboxylic acid, pyocyanine, and pyrrolnitrin (Weller, 1988; Thomashow et al., 1990; Ramamoorthy et al., 2001), and enzymes including β-1,3-glucanase and chitinase (Glick, 1995). These compounds lyse the cells of pathogenic fungi (Chet and Inbar, 1994; Glick, 1995) and degrade toxic compounds synthesized by the detrimental organisms (Ramamoorthy et al., 2001). Plant growth-promoting rhizobacteria also reduce pathogenic organisms by producing volatile antifungal compounds or by competing with them for nutrients and colonization sites (Cattelan et al., 1999; Ramamoorthy et al., 2001). It follows that PGPR that have antifungal properties may also affect beneficial associations, such as AMF. In their review, Ramamoorthy et al. (2001) discussed how some PGPR reduce the population of insects (e.g. stripped cucumber beatle, *Acalyma vittatum*) and nematodes (e.g.

Meloidogyne incognita, a root knot nematode) by hindering the growth and development of these organisms. For more effective control of disease causing organisms, the authors advised using mixtures of biocontrol PGPR.

2.2.3 Factors affecting plant growth-promoting rhizobacteria

Despite the frequently observed beneficial attributes of PGPR, their performances are unpredictable (Germida and Walley, 1996; Dobbelaere et al., 2002; Lucy et al., 2004). Unfortunately, the specific causes of these inconsistencies in PGPR effects are yet to be identified. Nevertheless, several studies have reported the influence of soil physical, chemical, and biological properties on PGPR activities (Burr et al., 1978; de Freitas and Germida, 1992; Requena et al., 1997). Although associations between PGPR and other soil beneficial organisms, such as certain AMF and *Rhizobium* species can result in synergistic growth promotion (Requena et al., 1997; Vazquez et al., 2000), a number of organisms hinder PGPR activities (Kropp et al., 1996). Kropp et al. (1996) reported *P. chlororaphis* strain O6 that demonstrated antagonism to *Fusarium culmorum* on a growth medium under laboratory conditions, but failed to suppress the activities of fungal pathogens under field conditions. However, another isolate of the same strain, *P. chlororaphis* 2E3, inhibited growth of pathogens under both laboratory and field conditions, thereby promoting the emergence of spring wheat. The study of Kropp and co-workers (1996) implied that some deleterious microorganisms may inhibit biocontrol and emergence promotion by certain PGPR strains.

In a pot study conducted to examine effects of two *Pseudomonas* species on the growth and N_2 fixation (measured using acetylene reduction technique) of field bean (*Phaseolus vulgaris* L.) and pea, de Freitas et al. (1993) noted varying effects of the PGPR species on the plants. For example, *P. syringe* R25 adversely affected growth of bean plants, but enhanced the growth of pea plants. These observations may be crucial when selecting PGPR for plant growth promotion because of the possibilities that PGPR of a certain crop may negatively affect the productivity of another. Other studies have noted the influence of plant species, growth stage, exudate production, and plant interactions with other soil organisms on PGPR activities (de Freitas and Germida, 1992; Germida and Walley, 1996; Roesti et al., 2006; Strigul and Kravchenko, 2006).

Soil type, nutrient, organic matter, and moisture content also affect PGPR functions (Burr et al., 1978; de Freitas and Germida, 1992). Burr et al. (1978) observed reductions in PGPR populations under dry soil conditions, and noted that high moisture content inhibited PGPR. The findings of de Freitas and Germida (1990a, 1992) indicate that some PGPR exhibit stimulatory effects in low fertility soil compared with a fertile soil. Similarly, Strigul and Kravchenko (2006) found that PGPR have higher growth promoting abilities in relatively low N soils compared with soils with higher N levels. They explained that at low N concentrations, indigenous microorganisms grow at a slower rate which reduces the competition between the organisms and PGPR for other nutrient sources. These authors also noted that the combined application of organic fertilizer and PGPR reduced the survival of PGPR because the fertilizer increased the growth rate of the indigenous microorganisms in the rhizosphere.

Since PGPR can be specific in their growth promotion effects, Nelson (2004) suggested screening PGPR isolates for traits that may enhance their competence in the rhizosphere of the host of interest under certain soil and environmental conditions. In addition, genetically modified PGPR may have to be developed to increase their rhizosphere competence through the introduction of certain beneficial traits, thereby facilitating the utilization of PGPR to enhance productivity (Nelson, 2004). Ramamoorthy et al. (2001) also advised the use of PGPR strains with different biocontrol activities that can synergistically reduce the activities of various pests and pathogens. They proposed that such an approach would increase PGPR effectiveness in the control of soil pathogens.

2.3 Arbuscular Mycorrhizal Fungi

The association between plants and AMF is one of the most important symbioses on earth, linking the root and the soil system (Koide and Mosse, 2004). Arbuscular mycorrhizal symbiosis is possibly the oldest and the most abundant plant-microbe association on earth (Simon et al., 1993; Smith and Read, 1997). Arbuscular mycorrhizal fungi belong to the phylum Glomeromycota, and order Glomales (Simon et al., 1993; Schüßler et al., 2001). They are unique endomycorrhizae (i.e., fungi found within plant roots) distinguished from other mycorrhizas, such as ectomycorrhiza and ericoid by

structures such as arbuscules and vesicles (Smith and Read, 1997). Primarily, nutrient and C exchanges between AMF and plant occur in the arbuscules, while the vesicles, where present, are a storage organ. Also, AMF possess intraradical hyphae located within the host and extraradical hyphae found outside the root, in the soil environment. Collectively, the, arbuscules, vesicles, and intraradical hyphae are regarded as the intraradical mycelium, and the collection of extraradical hyphae is known as extraradical mycelium (Smith and Read, 1997).

In AMF-plant symbioses, AMF translocate nutrients from soil to plant through the extraradical mycelium, and in return, the plant supplies AMF with C in the form of photosynthates; about 5 to 85% of C depending on the plant species and its dependence on the association (Treseder and Allen, 2000). Apart from nutrient uptake, the extraradical mycelium also is involved in spore formation and initiation of root colonization (Brundrett, 1991; Smith and Read, 1997). Spores, hyphae, and colonized root and organic matter are propagules of AMF (Brundrett, 1991).

Mosse (1953) was the first to culture and identify an AMF on strawberry (*Fragaria spp*. L.), named *Endogone mosseae*, now called *Glomus mosseae* (Koide and Mosse, 2004). The identification of the AMF by Mosse (1953) spurred the interest of many researchers in investigating the association between plants and AMF (Koide and Mosse, 2004). Over the years, several studies have shown the contributions of AMF to ecosystem functioning and productivity (Wright and Upadhyaya, 1998; van der Heijden et al., 1998b; Koide and Kabir, 2000; Hodge, 2001; Rillig, 2004a; Richardson et al., 2009). Arbuscular mycorrhizal fungi support plant establishment through the supply of nutrients, especially the less available ones including P (Koide and Kabir, 2000). They alleviate biotic and abiotic stress from their host by inhibiting the growth of pathogenic organisms and increasing plant resistance to drought and other unfavourable conditions (Barea et al., 2005). By increasing plant nutrient content and influencing rhizodeposition by the plant, AMF modify plant growth and alter rhizosphere processes (Richardson et al., 2009). As a result, AMF play crucial roles in the soil system. In addition, AMF and their products such as glomalin, directly contribute to soil structural formation (Rillig and Mummey, 2006). By virtue of their roles in soil aggregate formation and stabilization, they increase C and N storage (Zhu and Miller, 2003; Rillig, 2004a), implicating them in the reduction

of greenhouse gas emissions. With these beneficial roles of AMF, it is conceivable that among the numerous soil organisms, AMF play a key role in ecosystem processes.

2.3.1 Importance of arbuscular mycorrhizal fungi to plant ecology

One of most important contributions of AMF to ecosystem function and productivity is the uptake and transfer of plant nutrients. Phosphorus acquisition is a major contribution of AMF to plant growth and development. Phosphorus is an essential nutrient, but may be limiting; a considerable fraction of soil P is in organic forms, and a percentage of the inorganic P is insoluble. Both the organic P and the insoluble inorganic P are unavailable for plant uptake (Koide and Kabir, 2000). Only a few plants on their own are capable of producing organic acids and phosphatases to hydrolyze and release P (Tawaraya et al., 2006). However, Tawaraya and co-workers (2006) demonstrated that AMF in associations with the plants increase P availability by solubilizing the insoluble fraction of inorganic P, which significantly increased P uptake by onion (*Allium cepa* L.) and consequent P concentration in the plant tissue.

Using a split-dish *in vitro* carrot mycorrhizal system developed by St Arnaud et al. (1996), Koide and Kabir (2000) found that AMF are capable of hydrolyzing organic P sources, and are able to translocate the P obtained to plant roots. Their findings indicate that mycorrhizal plants have access to organic P sources and can successfully compete with soil microorganisms for P. However, the authors pointed out that AMF utilize organic P in a slower rate compared with inorganic P. Nonetheless, because a large percentage of total P is organic P, AMF contributions to plant P uptake through the solubilization of organic P remains substantial (Koide and Kabir, 2000). Recently, van der Heijden (2010) showed that AMF reduced P lost by leaching in microcosms established with a sandy textured soil that is susceptible to nutrient loss. Thus, AMF have the capacity to increase available soil P and reduce losses of the P.

Although AMF influence on plant N nutrition is not as high as in the case of P (George et al., 1995; van der Heijden, 2010), they give their host access to different forms of N, thereby increasing plant N uptake (Hodge et al., 2001; Govindarajulu et al., 2005). Hodge et al. (2001) demonstrated the ability of an AMF to decompose organic matter and acquire N from the organic source. They also showed that AMF increased the

diffusion rate of N to its host. Hence, mycorrhizal plants have additional access to N sources compared to non-mycorrhizal plants. The most exciting part of their findings is that AMF could be saprophytic, especially when decomposition is required for nutrient uptake. While exploring N transfer by AMF, Govindarajulu et al. (2005) showed that AMF transfer a substantial amount of N from the soil to the root system. Using a proposed model, the authors explained that extraradical hyphae of AMF take up inorganic N, and transfer it to intraradical hyphae as amino acids (mainly arginine). The intraradical hyphae decompose the amino acids to access the C, and then transfer the remaining N as ammonium to the host plant. These authors are among several that have demonstrated the remarkable contributions of AMF to N uptake by plants.

Nitrogen and P are not the only nutrients transferred by AMF; the ability of AMF to supply micronutrients to the host plant has been documented (Smith and Read, 1997; Ryan and Graham, 2001). Studies have shown that AMF increase zinc (Zn) uptake by plants by increasing its bioavailability in the soil (Ryan and Graham, 2001). Because only a small fraction of Zn is supplied by most crops, Zn deficiencies in the human diet have been a concern; therefore, AMF indirectly improve the nutritional value of human food through Zn acquisition (Welch and Graham, 1999; Ryan and Graham, 2001; Rillig, 2004b). Other micronutrients acquired by AMF for plant use include copper (Cu) and Fe (Smith and Read, 1997; Liu et al., 2000). At low P concentrations, Liu and co-authors (2000) observed higher Cu, Fe, and Zn content in corn inoculated with *G. intraradices* than a non-mycorrhizal plant, even without the additions of micronutrients. Roesti et al. (2006) reported a two-hundred percent increase in Fe content of wheat grain when inoculated with AMF compared with the uninoculated control. Ryan and Graham (2001) expressed the need be cognizant of AMF functions in the uptake of Zn and other micronutrients.

Arbuscular mycorrhizal fungi establishment influences plant and water relations (Augé et al., 2001; Hallett et al., 2009). Hallett and co-workers (2009) observed that the rhizosphere of a wild-type tomato plant was drier than the mycorrhizal-defective mutant due to stomatal changes caused by the AMF association with the plant. In addition, these authors found that transpiration rate was higher in the wild-type tomato plant compared with the mycorrhizal-defective mutant. The observations of Hallett et al. (2009) can be

related to water and nutrient uptake by plants since transpiration drives water and nutrient transfer from soil to plant roots (Marschner and Dell, 1994). Also, the study implied that AMF enhanced plant water and nutrient use efficiency by modifying plant stomata and transpiration rate.

There is ample evidence that AMF protect plants against phytopathogens by: reducing the numbers and activities of pathogens (Linderman, 1994; Slezack et al., 1999); competing with pathogens for colonization sites, nutrients, and photosynthates (Slezack et al., 1999; Linderman, 1994); and by improving plant resistance to disease (St Arnaud et al., 1995, cited by Slezack et al., 1999). The major ways AMF perform the latter role is by inducing the release of pathogenesis-related (PR) proteins by the host plant (Collinge et al., 1994), and increasing nutrient acquisition of the affected plant to compensate for the damage caused by the pathogen (Smith and Read, 1997; Slezack et al., 1999). However, biocontrol of AMF is dependent on the degree of mycorrhization (Slezack et al., 1999). At a high percent colonization, Slezack et al. (1999) found that *G. mosseae* significantly decreased the disease index of *Aphanomyces euteiches*, regardless of the density of the pathogenic inoculum, whereas when AMF colonization was low, the AMF exhibited no protection against the pathogen.

Despite the numerous reports of AMF on plants, studies have shown that the known beneficial effects of mycorrhizal symbioses on plant productivity are a mere estimate and other functional roles are yet to be determined (Rillig, 2004a; Govindarajulu et al., 2005).

2.3.2 Importance of arbuscular mycorrhizal fungi to soil ecology

Although plants are important in soil aggregate formation (Rillig and Mummey, 2006; Hallett et al., 2009), the role of AMF is as vital (Rillig et al., 2002; Rillig, 2004a, b; Rillig and Mummey, 2006). Because AMF symbiosis influences plant physiology such as root-to-shoot ratio, nutrient content, and rhizodeposition, plant effects on soil aggregate formation, to a large extent, are governed by AMF activities. By influencing the root system, AMF enhance the enmeshment and entanglement of soil particles by the plant roots and root hairs (Rillig and Mummey, 2006). Roots are known to exert some pressure on soil particles, thereby aligning and binding the particles together to facilitate soil aggregate formation (Miller and Jastrow, 1990; Rillig and Mummey, 2006; Hallett et al.,

2009). Arbuscular mycorrhizal fungi influence the amount of pressure applied by the plant root through their effects on root density and branching pattern (Rillig and Mummey, 2006).

Rillig and Mummey (2006) gave an overview of the direct contributions of AMF to soil aggregation. Arbuscular mycorrhizal fungi are involved in certain biophysical, biochemical, and biological processes that interrelate to promote stable aggregate formation. Like plant roots, AMF hyphae enmesh and entangle soil particles and microaggregates into macroaggregates. Additionally, glomalin-related soil protein (GRSP), mucilages, polysaccharides, and other compounds produced by AMF partake in soil aggregate formation by facilitating micro and macroaggregate formation. Glomalin-related soil protein, in particular, coats these aggregates and increases their stability (Wright et al., 2007), hence preventing disintegration of the aggregates. It is interesting that AMF involvement in aggregate formation may be to the advantage of the AMF (Rillig and Steinberg, 2002). Using glass beads, Rillig and Steinberg (2002) demonstrated that AMF hyphae grew better in a well aggregated soil simulated by large beads compared with a less aggregated soil (i.e., simulated by small beads). In addition, these authors observed that AMF produced higher amounts of glomalin (which is thought to be a stressed-induced glycoprotein) in the less aggregated soil than in the well aggregated soil. Their study implies that under less favourable conditions, AMF may be investing more of their plant derived C to glomalin production than to hyphal growth. Thus, it is possible that AMF facilitate the formation of a suitable environment through aggregate stabilization rather than the environment 'costing' AMF their C.

Furthermore, Rillig and Mummey (2006) explained that AMF stimulate the activities of other soil organisms that play vital roles in soil aggregation by increasing the quantity and quality of root exudates or by serving as a nutrient source and substrate for these organisms. For example, bacteria that inhabit spore and hyphae of AMF (Walley and Germida, 1996; Budi et al., 1999; Xavier and Germida, 2003a) may contribute to microaggregates formation. Also, the Rillig and Mummey (2006) reported that AMF facilitate the burrowing and soil binding activities of some macroorganisms (e.g., earthworm) by serving as a prey or by providing suitable environments for the organisms.

Carbon storage necessitated by the rising concentration of carbon dioxide (CO_2) has become a major focus in recent times (Janzen et al., 1998; Lal, 2004). By virtue of their role in soil aggregate formation, AMF contribute to soil C storage. A large fraction of soil C is labile and can be easily decomposed when exposed to microbes, especially under high temperature and moisture (Janzen et al. 1992; Janzen et al., 1998; Smith and Almaraz, 2004). However, when these labile C fractions are stored in soil aggregates, they are better protected and decompose less than when in bulk soil (Six et al., 2002; Rillig, 2004a). Generally, all attributes of AMF facilitate C storage; while the intraradical mycelium enhances CO_2 fixation and rhizodeposition by plants, the extraradical mycelium promotes the storage of the acquired C in aggregates (Zhu and Miller, 2003; Rillig, 2004a). Additionally, because erosion is a main channel of soil organic carbon (SOC) losses (Lal, 2003; Smith and Almaraz, 2004), AMF can reduce C lost via erosion through the formation of water stable aggregates. A well structured soil is less susceptible to wind and water erosion compared with a 'poorly' structured soil (Brady and Weil, 1999).

Apart from facilitating C storage, AMF also contribute directly to C storage as they represent a considerable fraction of microbial biomass C (Zhu and Miller, 2003; Rillig, 2004a; Rillig and Mummey, 2006). Arbuscular mycorrhizal fungi contribute up to 900 kg ha^{-1} C and 1.45 Mg C ha^{-1} to the recalcitrant soil C pool, through mycelium and glomalin production, respectively (Zhu and Miller, 2003; Rillig, 2004a). The authors explained that some plant C can be stored in chitinous walls of AMF for up to 68 years (Zhu and Miller, 2003), and the residence time of GRSP in soils varies from 12 to 60 years (Rillig et al., 2001).

2.3.3 Factors affecting arbuscular mycorrhizal fungi

Factors affecting AMF and their symbiotic association with plants can be classified as biotic and abiotic. The biotic factors include plant and microbial effects on AMF, while the abiotic factors are soil and climatic effects (Abbott and Robson, 1991). Effects of perturbations resulting from human activities such as tillage, grazing, mining, and other land use change also are important factors influencing the beneficial functions of AMF (Abbott and Robson, 1991; Brundrett et al., 1996a; Bohrer et al., 2001). Since AMF are

obligate endosymbionts, plants are a major factor governing AMF functions. Several studies have reported increases in AMF colonization due to higher population of total number of mycorrhizal plants (Miller, 1987), or population of a particular host (Newman et al., 1981). The latter case implies that some plants associate more with certain AMF species than others. An example is the findings of Schenck and Kinlock (1980) who found more spores of *Glomus spp* in the rhizosphere of monocotyledonous crops [bahia grass (*Paspalum notatum* Flagge), corn, sorghum (*Sorghum vulgare* Pers)] than dicotyledonous crops [cotton (*Gossypium hirsutum* L.), peanut (*Arachis hypogea* L.), soybean].

Also, there are reports that plant growth stage affects AMF abundance and establishment (reviewed by Abbott and Robson, 1991). For example, Giovannetti (1985) reported a higher percentage of root length colonized by *Gigaspora spp* during the flowering stage than other growth stages. The observation may be attributable to the ability of the plant to regulate the levels of AMF they associate with at a certain point by altering C allocation to AMF (Abbott and Robson, 1991). Thus, AMF colonization levels determined at harvest may not reflect the actual levels of mycorrhization over a growing period since AMF colonization may be greater during a plant's active growth stage than at maturity (Abbott and Robson, 1991). Conversely, Nogueira and Cardoso (2007) reported a reduction in the metabolic activity of AMF mycelium during the reproductive stage of soybeans due to the C drain from roots to shoots.

Establishment of AMF symbioses is dependent on soil biological, physical and chemical characteristics (Abbott and Robson, 1991; Brundrett et al., 1996b; Bohrer et al., 2001; Treseder and Turner, 2007). Nehl et al. (1998) related reductions in AMF colonization of field-grown cotton to the adverse affect of some deleterious rhizobacteria on cotton. However, Garbaye (1995) and Xavier and Germida (2003a) are among several authors that reported certain bacterial species that enhance mycorrhization and growth of mycorrhizal plant. Also, it has been observed that high soil nutrient concentrations, particularly P, reduce AMF functions (Liu et al., 2000; Bohrer et al. 2001; van der Heijden, 2010). The study by Liu et al. (2000) showed that Zn, Cu, and Fe uptake by *G. intraradices*-inoculated corn decreased at high concentrations of P and micronutrients. High sodium content has been shown to decrease AMF colonization (Kim and Weber,

1985). However, the effects of soil characteristics such as soil pH, nutrient concentration and salinity cannot be generalized (Abbott and Robson, 1991; Treseder and Allen, 2000). For example, in a study to determine the cause of slow AMF colonization of cotton, Nehl et al. (1998) found that soil N and P content had no direct influence on AMF colonization. However, the authors pointed out that N and P content of soil may have an indirect effect on AMF colonization through their influence on the host plant.

Supplying plant nutrients through fertilization and manure application reduces plant dependency on AMF for nutrient uptake (Johnson et al., 1997; Treseder and Allen, 2000; van der Heijden, 2010), thereby reducing C allotted to AMF by the plant. Nevertheless, fertilizer applications do not always negatively affect AMF colonization (Abbott and Robson, 1991). Furthermore, lime application affects AMF abundance by influencing the soil pH (Abbott and Robson, 1991; Brundrett et al., 1996b). Interestingly, the pH change typically has no effect on percent AMF colonization; rather it changes the AMF-host relationship, i.e., AMF species associating with a certain plant (Abbott and Robson, 1991). Hamel et al. (1994), however, found no significant effect of a soil pH change on AMF abundance even though liming increased the pH of the studied soil. The observation was related to the adaptability of certain AMF to soil pH changes.

The influence of cropping systems on AMF is well-documented (Abbot and Robson, 1991; Hamel, 1996). It has been reported that conventional tillage, inclusion of a fallow period into a cropping system, crop rotation, and grazing affect AMF establishments (Hamel, 1996; Johnson et al., 1997; Bohrer et al., 2001). Tillage reduces the initiation of AMF colonization by disrupting hyphal growth or altering plant composition. Furthermore, the disruption of the hyphal network by tillage may affect nutrient uptake and supply from AMF to the host (Johnson et al., 1997). Also, to repair damage caused by tillage, AMF may require more C from the host, thereby increasing the cost of the symbioses to the plant (Fitter and Garbaye, 1994; Johnson et al., 1997; Purin and Rillig, 2007). In addition, crop rotation and fallow period decrease AMF density and inoculum potential (ability of an AMF propagule to initiate and maintain a symbiotic association with the plant) (Abbott and Robson, 1991; Brundrett, 1996b; Hamel, 1996). Finally, nutrient patchiness caused by animal grazing through fecal deposition affects AMF abundance and diversity (Bohrer et al., 2001).

Even though a number of the aforementioned factors may negatively influence AMF symbioses (Abbott and Robson, 1991; Brundrett, 1996b; Hamel, 1996; Bohrer et al., 2001), a study by Brundrett et al. (1996a) showed that AMF propagules are readily found in disturbed sites. Development of AMF is in order of days (Slezack et al., 1999); in as much as there are dispersal agents of the fungi, and the sites conditions favour the establishment of plant species with minimal dependence on AMF symbioses (Brundrett, 1996b). Additionally, AMF propagules, such as spores and colonized root litter are known to survive disturbances (Brundrett, 1991); therefore, these resistant propagules can easily form symbiotic association with succeeding plants in a disturbed habitat (Brundrett et al., (1996a)

Other factors that govern AMF symbioses are climatic factors including amounts of rainfall and sunlight (Bohrer et al., 2001), and elevated CO_2 concentration (Rillig et al., 1999; Treseder and Allen, 2000). Effects of burning also have been observed on AMF-plant associations due to temperature increases attributed to fire (Abbott and Robson, 1991; Knorr et al., 2003). Nonetheless, Brundrett et al. (1996a) reported a non-significant effect of burning on the number and distribution of AMF propagules.

Summarily, any factor, either biotic or abiotic, that stimulates photosynthesis (e.g., adequate rainfall) or increases plant dependency on AMF (e.g., limited soil nutrient) will increase AMF abundance and colonization, and vice versa. More studies, however, are required to address the direct effects of perturbation on AMF rather than linking with the effect on the host plant. In addition, care must be taken when recommending cropping practices in favour of AMF symbioses (e.g., reduction of nutrient application) as some may not be profitable for farmers (Ryan and Graham, 2001). Recently, Adesemoye et al. (2008) reported the likelihood of utilizing AMF with other organisms and fertilizer to benefit soil microbes, humans, and the environment.

2.3.4 Glomalin

Glomalin is a component of the spore and hyphal wall of AMF (Wright and Upadhyaya, 1999; Driver et al., 2005) discovered in 1996 by Wright and co-workers while identifying monoclonal antibodies for AMF (Nichols and Wright, 2004). Glomalin was thought to be exuded by the living fungus (Wright and Upadhyaya, 1996) until

Driver and co-workers (2005) found that glomalin is only released by an AMF into the soil environment during hyphal turnover and after the death of the fungus. Glomalin, though still not biochemically defined, is an N-linked glycoprotein composed of 3 to 5% N, 36 to 59% C (Lovelock et al., 2004a; Schindler et al., 2007), 4 to 6% hydrogen, 33 to 49% oxygen, and 0.03 to 0.1% P (Schindler et al., 2007). Glomalin also contains 0.8 to 8.8% Fe (Wright and Upadhyaya, 1998; Rillig et al., 2001), which may be responsible for the reddish colour of glomalin extracts (Wright and Upadhyaya, 1998). Glomalin is a stable compound, insoluble in water and resistant to heat degradation (Wright et al., 1996). Because it is glue-like in nature and attaches to horticultural film and soil surfaces, glomalin is likely hydrophobic in its native state (Wright and Upadhyaya, 1998, 1999). Apart from the Glomeromycota, no other fungal group produces this glycoprotein in significant amounts (Wright et al., 1996; Wright and Upadhyaya, 1996).

Glomalin has been found in agricultural, grassland, forest, desert, and non-cultivated soils (Wright and Upadhyaya, 1996; Rillig et al., 2003; Nichols and Wright, 2004; Antibus et al., 2006; Bai et al., 2009). Glomalin concentrations of over 100 mg g^{-1} of soil were recorded in tropical forest soils of Hawaii (Rillig et al., 2001) and values lower than 1 mg g^{-1} soil were obtained in soils of a desert ecosystem in Mu US sandland, China (Bai et al., 2009). Values up to 21 mg g^{-1} soil were obtained from Scottish woodland soils (Wright and Upadhyaya, 1998). Although most of these findings are based on glomalin in the A horizon, both B and C horizon contain glomalin (Rillig et al., 2003) and it can be found to a depth of 140 cm in the soil profile (Harner et al., 2004). Harner and co-workers (2004) detected the glycoprotein in floodplain soils, river water and river foam. In fact, river foam contained 9.66 mg g^{-1} of glomalin in freeze-dried foam.

2.3.4.1 Glomalin extractions and quantifications

Currently, glomalin is operationally defined based on its extraction procedure, due to the challenge of identifying its biochemical structure (Wright et al., 1996; Rillig, 2004b). Glomalin is extracted from hyphae and soil in sodium citrate solution by autoclaving for thirty to sixty minutes or more (Wright et al., 1996; Wright and Upadhyaya, 1996). The extraction procedure varies depending on what fraction of glomalin is of interest; either easily extractable or total glomalin. Easily extractable glomalin (EEG) is obtained by

placing soil samples in 20 m*M* sodium citrate solution (pH 7) and autoclaving at 121˚C for thirty minutes, while total glomalin (TG) is removed from soil using 50 m*M* sodium citrate solution (pH 8) for sixty minutes. Extraction of TG usually occurs within an hour; however, more time may be required (Wright and Upadhyaya, 1998).

Apart from the differences in extractant concentrations and extraction duration, TG is extracted until supernatant is colourless or straw-coloured, which can be achieved after autoclaving for three to five cycles, though up to seven (Wright and Upadhyaya, 1998) and nine (Rillig et al., 2003) extraction cycles have been reported. Using a centrifuge, soil from which glomalin is extracted is pelleted immediately after autoclaving to ensure the glomalin extract is free of soil particles when decanting the supernatant. Because of it proteinous nature, extracts are stored at 4˚C (Wright et al., 1996). Also, these authors advised that any analysis should be done within two to four weeks as glomalin does degrade.

Additionally, glomalin is defined by the method employed to quantify it (Wright et al., 1996; Rillig, 2004b). Bradford protein analysis is a common method for protein quantification (Bradford, 1976). The Bradford assay is based on the principle that a dye (Coomassie Brilliant Blue G-250) binds with proteins and changes the dye colour from red to blue (Bradford, 1976; Wright et al., 1996). The degree of colour change, read by a spectrophotometer at a wavelength of 590 nm (A_{590}) as optical density, can be related to protein concentration in a glomalin extract using a standard of known concentration of protein. The standard is prepared in a range of 1.25 to 5 µg bovine serum albumin (BSA) in phosphate buffer saline (PBS). The equation of the regression line generated by plotting optical density against BSA values is then used to calculate protein concentration in glomalin extracts as Bradford-reactive soil protein (BRSP) for TG and easily extractable Bradford-reactive soil protein (EE-BRSP) for the EEG fraction (Rillig, 2004b).

An indirect enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody MAb32B11 developed against crushed spores of *G. intraradices* (Wright et al., 1996) is also employed to quantify glomalin. In ELISA, an anti-glomalin antibody (MAb32B11) is added to the glomalin extract and subsequently binds to an antigenic site (i.e., a site in which antibodies are induced) of glomalin. Another antibody, biotinylated anti-mouse

IgM antibody, is added to bind to the antigenic site of the MAb32B11. Then, a solution containing a protein (e.g., ExtAvidin) and an enzyme (e.g., peroxidase) is added, followed by the addition of a colour developer. The protein molecules bind to the biotin in the anti-mouse IgM antibody, and the enzyme reacts with a substrate molecule in the colour developer to produce a blue-green colour. The degree of colour change is determined using a spectrophotometer at 405 or 410 nm and compared with a standard to calculate glomalin concentrations. The standard curve in a range of 0.005 and 0.04 µg is prepared using glomalin obtained from pot cultures or soil samples with 100% immunoreactivity (Wright et al., 1996; Nichols and Wright, 2004; Rillig, 2004b).

Total glomalin quantified using ELISA is regarded as immunoreactive soil protein (IRSP) and the easily extractable fraction is named easily extractable immunoreactive soil protein (EE-IRSP) (Wright and Upadhyaya, 1998; Rillig, 2004b). Rillig (2004b) explained these terminologies (EE-BRSP, BRSP, EE-IRSP, and IRSP) in his review and cautioned against the use of the word glomalin to describe the soil fraction of glomalin. Because the extraction procedure does not eliminate other soil proteins, Rillig (2004b) suggested 'glomalin-related soil protein' (GRSP) to be used instead, which is widely accepted. Table 1, modified from Rillig (2004b), describes each term to ensure clarity. In addition, some fractions of glomalin extracted from other sources (e.g., the root) would have similar terms. For instance glomalin extracted from plant roots using the Bradford assay is Bradford-root protein (Rosier et al., 2008).

Usually, glomalin values obtained from the ELISA technique are compared with the Bradford values to determine percentage of immunoreactive protein in glomalin extract (i.e., immunoreactivity). Immunoreactivity is calculated by dividing ELISA values by the Bradford values and multiplying by 100. The higher the percentage, the more immunoreactive the glomalin fraction (Wright et al., 1996; Nichols and Wright, 2004; Rillig, 2004b).

Several studies have reported that EE-GRSP is more immunoreactive compared with GRSP. Wright and co-workers speculated that EE-GRSP was the freshly produced fraction and less bound to soil particles relative to GRSP (Wright et al., 1996; Wright and Upadhyaya, 1996, 1998). Their assumptions were supported by higher correlation between aggregate stability and EE-GRSP (Wright and Upadhyaya, 1996, 1998).

Nevertheless, these speculations have been proven incorrect (Steinberg and Rillig, 2003). Steinberg and Rillig (2003) found that without a living host and mycorrhizal production, IRSP decomposed by about 46%, whereas EE-IRSP increased up to five times within 150 days. If indeed EE-GRSP is the fresh glomalin fraction, it should not increase without mycorrhizal production, and is expected to decompose faster if it was less bound to soil particles compared with the 'older GRSP fraction'. Generally, EE-GRSP and GRSP differ in their responses to treatments and environmental factors (Rillig et al., 2001, 2003; Lutgen et al., 2003). For example, Lutgen et al. (2003) reported 54% seasonal changes in EE-IRSP, while IRSP concentrations were constant over the six month study period. To date, the differences between GRSP and EE-GRSP in their productions and functions are yet to be identified (Rillig et al., 2001, 2003; Lutgen et al., 2003).

After six years of suppressing mycorrhizal symbioses through fungicide application, Wilson et al. (2009) found that EE-BRSP and BRSP levels were reduced by 18% and EE-IRSP and IRSP reduced by 53 and 76%, respectively. Thus, the effect of AMF suppression was more evident on the immunoreactive fraction that is more specific for glomalin. Their study demonstrated that IRSP may be a better indicator of glomalin and more related to AMF. In addition, Rosier et al. (2008) reported that some treatment effects observed using the ELISA technique may not be detected by the Bradford assay. For example, when comparing the effect of AMF inoculation on Bradford-reactive protein in the root of *Bromus inermis*, Rosier and co-workers (2008) found that *Entrophospora colombiana* and *G. intraradices* had effects similar to the uninoculated control. However, the immunoreactive fraction of the root protein was increased significantly by the AMF species compared to the control. These studies confirmed that the ELISA technique is more sensitive and specific for GRSP quantification (Wright and Upadhyaya, 1999).

Although the Bradford assay is not specific for glomalin, positive and significant correlations are usually found between Bradford and ELISA values (Wright and Upadhyaya, 1996, 1998, 1999; Harner et al., 2004). Additionally, the Bradford assay may be more precise than the ELISA technique because the Bradford assay requires less pipetting (Rosier et al., 2008). Also, the Bradford assay is cheaper and faster, and less technical and laborious compared with the ELISA technique. Consequently, the Bradford

assay is the more common method for glomalin quantification. Rillig et al. (2003), Bedini et al. (2009), and Kohler et al. (2009a, b) are among several authors who have quantified GRSP using only the Bradford assay.

Table 2.1. Current terminologies for glomalin and their definitions (modified from Rillig, 2004b).

Terminology	Description
Glomalin	A yet to be identified putative gene product of arbuscular mycorrhizal fungi
Glomalin-related soil protein (GRSP)	Total soil glomalin fraction, possibly contains other soil protein; fraction of soil glomalin extracted repeatedly using 50 m*M* sodium citrate solution (pH 8) and autoclaving at 121˚C for 60 min until glomalin extract is straw-coloured
Easily extractable glomalin-related soil protein (EE-GRSP)	Fraction of soil glomalin extracted once using 20 m*M* sodium citrate solution (pH 7) and autoclaving at 121˚C for 30 min
Bradford-reactive soil protein (BRSP)	Glomalin-related soil protein quantified using the Bradford assay, measures all protein in glomalin extract
Easily extractable Bradford-reactive soil protein (EE-BRSP)	Easily extractable glomalin-related soil protein quantified using the Bradford assay, measures all protein in glomalin extract
Immunoreactive soil protein (IRSP)	Glomalin-related soil protein quantified using an indirect enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody MAb32B11, specific for glomalin, though may cross-react with other soil protein
Easily extractable immunoreactive soil protein (EE-IRSP)	Easily extractable glomalin-related soil protein quantified using an indirect enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody MAb32B11

2.3.4.2 Roles of glomalin

The long-term effect of AMF on aggregate stabilization (Miller and Jastrow, 2000) may partly be credited to glomalin production by the fungi (Rillig et al., 2001; Rillig, 2004a, b; Rillig and Mummey, 2006). A number of authors have demonstrated the roles of glomalin in soil aggregate stabilization (Wright and Upadhyaya, 1998; Wright and Anderson, 2000; Rillig et al., 2002; Wright et al., 2007). For example, Wright and Anderson (2000) found a positive correlation between GRSP concentrations and soil aggregate water stability across a variety of soils under different cropping systems and management practices. Using the path analysis model, Rillig et al. (2002) showed that the direct effect of GRSP on aggregate stability was higher than the total (direct and indirect) effect of hyphae on soil aggregate stability, but similar to the total root effect. Since soil aggregation governs water, nutrient content, and gaseous exchanges in soil (Rillig and Mummey, 2006), glomalin could play a crucial role in soil aeration and drainage, plant nutrient uptake, and productivity (Nichols and Wright, 2004).

Because of its role in aggregate stability, glomalin facilitates soil C storage (Zhu and Miller, 2003, Rillig et al., 2004). Rillig et al. (2001) found that glomalin accounted for 4 to 5% of total C and N in Hawaiian soils. Also, they reported the contributions of the glycoprotein to total C were greater than microbial biomass C. Their observation may be due to the slow turnover rate of glomalin and its ability to accumulate in soil (Wright and Upadhyaya, 1996; Rillig et al., 2001; Steinberg and Rillig, 2003). Recently, Wilson et al. (2009) observed reductions in soil C and N content due to AMF suppression, and related it to significant decreases in AMF hyphae and GRSP concentrations. They speculated that decreases in AMF hyphae and GRSP concentrations led to the losses of C and N protected in macroaggregates by reducing aggregate stabilization. Additionally, while studying the roles of glomalin in the sequestration of heavy metals, Cornejo et al. (2008) found that GRSP levels correlated strongly and positively to SOC as reported by other researchers (Franzluebbers et al., 2000; Zhu and Miller, 2003). Apparently, the study by Cornejo et al. (2008) was the first to show that GRSP could account for up to 89% of the SOC. Nevertheless, not much is known about the direct influence of glomalin on organic C storage, since most of its relation to C storage is by virtue of stabilizing aggregates (Feeney et al., 2004).

A number of studies have reported the contributions of glomalin to phytoremediation (González-Chávez et al., 2004; Cornejo et al., 2008). While examining the roles of glomalin in heavy metals sequestration of two polluted soils, González-Chávez et al. (2004) stated the potential of glomalin in reducing availability and toxicity of 'potentially toxic elements' such as Cu, cadmium (Cd), and lead (Pb). Furthermore, Cornejo et al. (2008) reported GRSP to bind to about 28% Cu and 6% of Zn in a soil heavily polluted with these heavy metals. From their study, it appears that the higher the concentration of the pollutant, the higher the ability of GRSP to bind to them and make the pollutants unavailable. These studies are proof of significant contributions of AMF to phytoremediation through glomalin production. Cornejo et al. (2008) found correlations between GRSP and heavy metal concentrations in the soil. They explained that toxicity induced stress by metals may be enhancing glomalin production by AMF (Purin and Rillig, 2007).

Glomalin has been linked with heat shock protein 60 (hsp60), which are proteins produced by eukaryotic and prokaryotic cells when under environmental related stress conditions, such as increased temperatures, pH change, and starvation (Gadkar and Rillig, 2006; Purin and Rillig, 2007). Using tandem liquid chromatography-mass spectrometry, Gadkar and Rillig (2006) demonstrated that the amino acid sequences of glomalin are related to hsp60, thereby confirming the speculations by other studies (Rillig and Steinberg, 2002; Driver et al., 2005) that glomalin may be serving a protective function for AMF as a stress-induced protein. Relating glomalin with heat shock protein clarifies how stress imposed by heavy metals may rapidly increase glomalin production by AMF and GRSP concentrations in polluted soils (Cornejo et al., 2008). In exploring glomalin production as one of the mechanisms employed by AMF to alter their environment, Rillig and Steinberg (2002) demonstrated that glomalin production decreased as AM fungal growing space increased. Their study was the first to show that unfavourable growing conditions may enhance glomalin production by AMF. Thus, they argued that glomalin is produced by AMF for AMF use, and functional roles of glomalin in soil are secondary (Purin and Rillig, 2007) or coincidental. It is conceivable that glomalin performs a protective function in a living fungus since AMF allocates many of it resources (mainly C and N) to glomalin production (Rillig and Steinberg, 2002).

2.3.4.2.1 The potential for manipulating glomalin to reduce greenhouse gas emissions

Because this study is a part of a larger project focusing on the reduction of greenhouse gas emissions, particularly CO_2 and N_2O, it is pertinent to mention the potential for manipulating glomalin to reduce the emissions of these gases into the atmosphere. The increasing atmospheric concentrations of greenhouse gases have become a major threat to mankind, mainly because of the annual mean temperature increases attributed to the emissions of these gases (King, 2004). Although the atmospheric concentration of CO_2 is higher, N_2O has 298 times more global warming potential (GWP) (Forster et al., 2007). Over the past one hundred and forty years global temperature has risen by 0.6˚C, and the IPCC (2001) report showed that it could rise up to 5.8˚C by 2100. Consequently, strategies are taken to decrease the annual emissions of these gases by reducing sources and increasing sinks (Pennock, 2005).

Through land use change, the agricultural sector has become a major contributor to global warming (Janzen, 2004; Smith and Almaraz, 2004). Currently, the atmospheric concentration of CO_2 has risen above the 370 ppm predicted by Keeling and Whorf (2002), and higher levels have been speculated (IPCC, 2001). Although fossil fuel combustion accounts for a large percentage of increases in CO_2, expansion of agriculture through practices such as deforestation and conventional tillage is depleting the soil organic matter (SOM) and releasing CO_2 into the atmosphere (Gregorich et al., 1998; Lal, 2004). The physical disturbances caused by agricultural practices expose SOC that previously was protected from microbial decomposition. Consequently, microbial decomposition of SOC results in the release of considerable amounts of CO_2 into the atmosphere (Janzen, 2004; Smith and Almaraz, 2004).

Because CO_2 emissions from soil are mainly due to poor management practices that destroy the soil structure (Janzen et al., 1998; Lal and Kimble 2000; Lal, 2004), any practice that promotes aggregate formation and stabilization will reduce soil C losses. It is interesting to note that the desirable effects of minimum tillage on soil structure have been linked to higher glomalin concentrations in these aggregated soils (Wright and Upadhyaya, 1998, Wright and Anderson, 2000). Janzen et al. (1992, 1998) expressed the need to be cognizant that most changes observed in SOC occur at the labile fraction. As a

result, when conditions are favourable, such as when soil is moist and warm, the accumulated C fraction can be depleted easily and lost to the atmosphere as CO_2 (Smith and Almaraz, 2004). A large portion of glomalin is recalcitrant and resistant to enzymatic degradation (Wright et al., 1996; Purin and Rillig, 2007); consequently, it follows that glomalin will contribute more to the stable C pools as opposed to the labile fraction.

According to Agriculture and Agric-Food Canada (1998), agricultural practices are responsible for 70% of total N_2O emissions. Nitrous oxide is a by-product of nitrification and an intermediate product of denitrification (Hutchinson and Davidson, 1993). While nitrification is the oxidation of ammonium ion (NH_4^+) or ammonia (NH_3) to nitrate (NO_3^-) through nitrite (NO_2^-), denitrification is an anaerobic process involving the reduction of nitrate to nitrite, nitrite to nitrous oxide, and nitrous oxide to dinitrogen gas (N_2). Because of its stability and slow decomposition in the atmosphere, N_2O has an atmospheric lifetime of about 120 years (IPCC, 1996). Furthermore, N_2O emissions have been related to the destruction of the ozone layer (Crutzen, 1970; World Meteorological Organization (WMO), 1999). Of all the agricultural sources of this potent gas, soil is a major source (IPCC, 1996). Agricultural soils contributed to about 68% of the mean N_2O emissions (58.1 Gg N_2O-N yr^{-1}) in Canada between 1990 and 2005 (Rochette et al., 2008).

Glomalin can contribute immensely in reducing the release of N_2O into the atmosphere. Apart from being a N pool (Nichols and Wright, 2006), glomalin may diminish N losses by the indirect influence on nitrification and denitrification. For example, at saturation, a poorly aggregated soil becomes anaerobic, which stimulates denitrification. Apparently, management practices developed to reduce N_2O emissions will increase glomalin production. For example, minimizing N fertilizer and manure application (Desjardins et al., 2001; Grant et al., 2004) will benefit mycorrhizal symbioses (Treseder and Turner, 2007) and thus increase glomalin production and its beneficial roles in soil. It is crucial that we develop a better understanding of the direct impact of glomalin in C and N storage, so that these speculations can be ascertained.

2.3.4.3 Factors governing glomalin-related soil protein concentration

Since the discovery that AMF are in symbiotic association with most plant species, it has been observed that the relationship is only completely mutual when AMF supply

nutrients required by plant in exchange for plant C (Koide and Mosse, 2004). The more dependent a plant is on mycorrhizal symbioses, the more C is allotted to AMF by the plant. In fact, plant C allocation to AMF can be up to 85% for some plant species (Treseder and Allen, 2000). Because a considerable amount of C allocated to AMF is used in glomalin production, plant productivity and photosynthate allocation will govern glomalin production by AMF (Treseder and Turner, 2007). Plants are, therefore, a major determinant of glomalin production.

Violi et al. (2007) reported significant positive effects of plant growth rate and nutrient status on glomalin production. Additionally, Rosier et al. (2008) related differences in glomalin-related protein (GRP) concentrations in the root of smooth brome (*Bromus inermis*), Sudan grass (*Sorghum bicolor*), and narrowleaf plantain (*Plantago lanceolata*) to length of the growth period and differences in host plant species. Under field conditions, the plant effect may be more pronounced than those observed in controlled environments with single AMF or plant species (Treseder and Turner, 2007). For example, Wilson et al. (2009) reported the possible influence of plant biomass, biomass allocation, and litter quality and quantity on GRSP concentration. This may be attributable to the dependence of AMF on host plant abundance and net primary productivity (NPP) (Treseder and Turner, 2007).

In their review, Treseder and Turner (2007) highlighted that environments limited in water and nutrients, where plants are more likely to depend on AMF symbioses, should be rich in glomalin. In contrast, when examining the distribution and accumulation of BRSP in floodplain soil, Harner et al. (2004) found that BRSP levels in the downstream region were high compared with those in the upstream region. The differences in BRSP level were related to higher soil nutrient concentration at the downstream favouring plant growth and AMF colonization. In addition, Wilson and co-workers (2009) demonstrated that N fertilizer application significantly increased EE-IRSP and IRSP fractions, though it had no effect on EE-BRSP and BRSP. Immunoreactive glomalin fractions tend to be higher in fertile soil than in low fertility soil due to rapid AMF turnover in fertile soils (Lovelock et al., 2004a). Thus, although low nutrient status has been shown to enhance AMF associations (Liu et al., 2000; Bohrer et al. 2001), high soil nutrient content can enhance glomalin production by increasing the fungal turnover (Lovelock et al., 2004a).

In an effort to determine the roles of PGPR and AMF in improving aggregate stability under water-stress conditions, Kohler et al. (2009a) found no significant effect of drought on EE-BRSP concentrations in the rhizosphere of lettuce (*Latuca sativa* L.) inoculated with *G. intraradices*. However, drought increased EE-BRSP concentrations in soils inoculated with *P. mendocina* and the uninoculated control. Atmospheric CO_2 concentrations are another important factor that may influence glomalin levels (Rillig et al., 1999) because elevated CO_2 influences the C cycle. Elevated CO_2 concentration increases NPP by increasing photosynthetic rates, water, light and nutrient use efficiency, and by reducing stomata conductance and transpiration (Drake et al., 1997). Nonetheless, a study by Kohler et al. (2009a) demonstrated that elevated CO_2 had no significant effect on EE-BRSP levels.

Soil organisms that enhance mycorrhization may increase glomalin production. As a result, the so called 'mycorrhization-helper bacteria' (MHB) (Garbaye, 1994) have the potential to increase glomalin production by stimulating spore germination, hyphal growth, and AMF establishment. Interestingly, some of these bacteria are *Bacillus* and *Pseudomonas* species that possess growth promoting traits (Bharadwaj et al., 2008a). Plant growth-promoting rhizobacteria may enhance glomalin production directly by promoting mycorrhizal symbioses (Toro et al. 1997; Barea et al., 2005), or indirectly through their beneficial effects on the AMF host plant (Vessey, 2003; Lucy et al., 2004). Nonetheless, some associations between AMF and supposed 'beneficial bacteria' can be detrimental on AMF and its host plant by inhibiting mycorrhizal symbioses (Walley and Germida, 1997). Alternatively, microorganisms may facilitate the release of glomalin into the soil environment by inducing stress on AMF (Purin and Rillig, 2007) or through the decomposition of AM fungal hyphae (Rillig and Mummey, 2006).

Macroorganisms such as nematodes and collembola also may influence glomalin production positively or negatively. For example, by grazing on AM fungal mycelium, collembola may reduce the proliferation and spread of hyphae. Additionally, grazing may disrupt nutrient uptake and transfer by AMF to the host plant (Fitter and Garbaye, 1994). As a result, AMF become a C drain to its host and this may eventually affect the symbiotic association between AMF and the host plant, thereby diminishing glomalin production. On the other hand, grazing may stimulate rapid and extensive re-growth of

AMF hyphae (Fitter and Garbaye, 1994), thereby increasing glomalin production. Furthermore, Fitter and Garbaye (1994) explained how some macroorganisms may promote AMF establishment by facilitating the dispersal of AMF propagules, and the spread of AMF propagules could increase glomalin production. The numerous aforementioned biotic influences on AMF functions and productivity lead to the exciting possibilities that both detrimental and beneficial associations between AMF and other organisms may benefit glomalin production. As previously discussed, unfavourable conditions such as stress imposed on AMF by its environment, perhaps including predators, may increase C allocation to glomalin production (Rillig and Steinberg, 2002; Purin and Rillig, 2007). Although not commonly reported, soil inhabitants will influence glomalin production by AMF. Investigators need to explore this area of research.

Cropping systems and land management practices affect GRSP levels (Wright et al., 1999; Rillig et al., 2003; Wright et al., 2007). After a three-year period of converting from ploughed tillage to no-till, Wright et al. (1999) detected substantial increases in GRSP concentrations. Even with the conversion from conventional tillage to no-till, GRSP levels in the studied soil were lower than levels in undisturbed grassland (Wright et al., 1999). A study by Rillig et al. (2003) also indicated lower GRSP concentrations in agricultural soils relative to native forest and afforested soils. Tillage reduces glomalin production and enhances its decomposition by decreasing vegetation and AMF abundance (Treseder and Turner, 2007).

Other factors that influence glomalin production are fire and landscape position. Since fire decreases vegetation and AMF abundance, soil nutrient content, and microbial activities, it is expected that fire could reduce GRSP concentrations (Treseder and Turner, 2007). However, fire may become desirable if it stimulates plant re-growth, and productivity (Treseder and Turner, 2007). Interestingly, Knorr and co-workers (2003) reported no significant effect of prescribed fire on the amount of GRSP in soils collected from two burned forested watersheds located in southern Ohio. They explained that the 5 to 10% increases in soil temperature at the studied site may not affect AMF activity. However, they emphasized that only the standing pools were determined, so it was difficult to explore any increases or decreases in glomalin levels that fire might have caused between the sampling periods. Similarly, Wilson et al. (2009) observed that fire

alone had no significant effect on GRSP concentrations except that it significantly reduced IRSP concentration.

Influences of landscape position on soil glomalin concentrations have been documented (Knorr et al., 2003; Harner et al., 2004). Although, Knorr et al. (2003) found no significant effect of landscape position on EE-IRSP, they reported highest IRSP levels in lower slope, and lowest IRSP concentration in upper slope position. They related their observations to higher host plant diversity at the lower slope relative to the upper slope position. A study conducted by Harner et al. (2004) supported the findings of Knorr and co-workers (2003); they observed significantly lower BRSP concentrations in the losing reach (upstream region) of the studied floodplain compared with that measured from the gaining reach (downstream region) that is finer textured and with more nutrients.

Interestingly, AMF abundance does not always correlate with glomalin production (Treseder and Turner, 2007). For example, Lutgen et al. (2003) found no correlations between EE-BRSP or BRSP concentrations and AMF parameters, including percent vesicle and arbuscule colonization, AMF colonization, colonized root length, and hyphal length. Bai et al. (2009) also observed non-significant correlations between EE-BRSP or BRSP concentrations and percent arbuscular, hyphal, vesicular, and total AMF colonization. These researchers related their findings to the gap between hyphae and glomalin decomposition because the residence time of these AMF variables differ in soil significantly (Rillig et al., 2003; Steinberg and Rillig, 2003). Besides, under unfavourable conditions, AMF may transfer a considerable amount of C for hyphal growth to glomalin production (Rillig and Steinberg, 2002). Thus, hyphal length may relate inversely to glomalin production. Nonetheless, the lack of correlation between glomalin and AMF variables cannot be generalized as there are few exceptions. For instance, Violi et al. (2007) found a positive and linear correlation between *Scutellospora heterogama* propagule density and glomalin concentration, although they reported no significant correlation between *G. intraradices* propagules density and the concentration of the glycoprotein.

Arbuscular mycorrhizal activity also may not correspond to the levels of glomalin. For example, the 12 non-cultivated soils studied by Wright and Upadhyaya (1996) had GRSP concentrations in a range of 4.4 to 14.8 mg g^{-1} soil, and they observed that AMF activities

in these soil were low at the time of sampling. Despite the observation, several studies have demonstrated that AMF differ in their influence on GRSP levels (Lovelock et al., 2004b; Nichols and Wright, 2004). In fact, differences between isolates also have been reported (Bedini et al., 2009). Bedini et al. (2009) observed that inoculation of *Medicago sativa* plants with *G. mosseae* IMA1 had a greater effect on BRSP relative to the other *G. mosseae* isolate, *G. mosseae* AZ225C, but found no significant differences between *G. mosseae* and *G. intraradices* on this glomalin fraction. Wright and Anderson (2000) linked differences in AMF hyphae to variations in amount of GRSP obtained from sites under no-till and plow tillage. Additionally, Lovelock and co-workers (2004b) showed that glomalin concentrations may be higher in coarse hyphae compared to fine hyphae. Therefore, AMF may produce glomalin in different concentrations (Wright et al., 1996).

Summarily, GRSP concentration measured at a certain point (in space or time) is dependent on the rate of glomalin production by AMF, and the rate at which GRSP is decomposed (Rillig, 2004b; Treseder and Turner, 2007). As a result, any factor that affects glomalin production and GRSP decomposition including the history of the site will influence GRSP levels. These observations are indications that glomalin production by AMF can be maximized and its decomposition minimized.

2.4 Interactions between Plant Growth-Promoting Rhizobacteria and Arbuscular Mycorrhizal Fungi

There is documentary evidence that the symbiotic associations of plants with AMF are influenced by a number of rhizosphere organisms including PGPR (Fitter and Garbaye, 1994; Walley and Germida, 1997; Barea et al., 2002, 2005; Vessey, 2003). In a similar way, the activities of PGPR are dependent on their associations with other rhizosphere organisms, especially the commonly found AMF (Germida and Walley, 1996; Hodge, 2000; Roesti et al., 2006). A number of these studies found that the beneficial associations between AMF and PGPR enhance plant growth and nutrient uptake (Kim et al., 1998; Rodríguez-Romero et al., 2005; Roesti et al., 2006; Adesemoye and Kloepper, 2009). For example, the dual inoculation of *G. etunicatum* and *Enterobacter agglomerans* resulted in the highest plant weight and N and P uptake of tomato in the study of Kim et al. (1998). Vazquez et al. (2000) observed that the effect of *A. brasilense* on shoot and

root weight of corn was only beneficial when co-inoculated with *G. deserticola*. Rodríguez-Romero et al. (2005) and Wu et al. (2005) also reported similar observations. Rodríguez-Romero and co-workers (2005) found that the co-inoculation of *G. manihotis* and *Bacillus* species increased banana growth and nutrient uptake, although the interaction effect was not significantly greater than the bacterial treatment. Interaction effects of these organisms also have been observed on the uptake of micronutrients. Meyer and Linderman (1986) showed increases in the concentrations of Al, Cu, Fe, Ni, and Zn in a subterranean clover (*Trifolium subterraneum* L.) shoot when *P. putida* was co-inoculated along with native AMF species. This effect was higher than the individual effect of the PGPR or AMF.

The beneficial interactions observed between AMF and PGPR on plant growth are based on few known mechanisms (Toro et al., 1997; Barea et al., 2002, 2005; Richardson et al., 2009). Certain PGPR produce some phytohormones that enhance AMF colonization by increasing root surface area and susceptibility of plant to AMF hyphae penetration (Toro et al., 1997; Barea et al., 2002). Toro et al. (1997) reported a phosphate solubilizing bacterium (PSB), *B. subtilis*, that stimulated root colonization by *G. intraradices*, even though there was a significant reduction in the population of the introduced bacterium. Furthermore, some PGPR inhibit the growth of pathogens that may interfere with AMF symbiotic association with plants (Vessey, 2003). Garbaye (1994) described these beneficial bacteria as MHB. Interestingly, some of these MHB inhabit the cytoplasm, spore, and hyphae of AMF (Bianciotto et al., 1996; Walley and Germida, 1996; Xavier and Germida, 2003a).

Beneficial effects of AMF on PGPR are usually related to the ability of AMF to serve as a bridge between P solubilized by PGPR and the host plant (Toro et al., 1997; Barea et al., 2002, 2005). Barea et al. (2005) reported the involvement of some AMF species in the establishment of beneficial rhizobacteria such as the PSB. Additionally, by virtue of their roles in aggregate stabilization, AMF provide habitable pore space for bacteria (Rillig and Mummey, 2006), including PGPR, thereby enhancing PGPR growth promoting characteristics. In combination, AMF and PGPR modify soil nutrient concentration ratios and nutrient mobility to facilitate nutrient retention in the plant tissue

(Toro et al., 1997). Finally, they both influence microbial community composition to benefit their host plant (Roesti et al., 2006).

Although beneficial associations between AMF and PGPR are frequently observed, neutral and detrimental ones also have been found (Germida and Walley, 1996; Walley and Germida, 1997; Kim et al., 1998; Vazquez et al., 2000; Wu et al., 2005; Adesemoye and Kloepper, 2009). Kim and co-authors (1998) reported a PSB, *E. agglomerans,* that had no influence on the percentage of tomato root colonized by *G. etunicatum*. These authors also found that the AMF had no significant effect on colony forming units (cfu) of the bacteria except at 75 days after planting (DAP). Additionally, at 75 DAP, P concentrations in the mycorrhizal and bacterial treated soil were similar to the uninoculated control, and none of these treatments enhanced P concentration in the dry tissue of tomato. Similarly, Vazquez et al. (2000) reported an *A. brasilense* and a *P. fluorescens* strain that had no effect on percentage of root colonization by *G. deserticola* and native AMF species. In a study conducted by Wu et al. (2005), co-inoculation of rhizobacteria together with *G. intraradices* or *G. mosseae* had no effect on the height of corn. Results of these studies suggest that PGPR may not necessarily influence AMF establishments, and vice versa. Also, these studies showed that some interactions between AMF and PGPR could be ineffective on plant growth.

Several studies have demonstrated detrimental effects between AMF and PGPR (Fitter and Garbaye, 1994; Walley and Germida, 1997; Wu et al., 2005). For example, a study by Wu and co-authors (2005) indicated that some AMF species interfered with PGPR colonization. They found that the population of the P and K solubilizers decreased as root colonization by *G. mosseae* increased. Recently, Dwivedi et al. (2009) also observed a reduction in bacterial populations following an AMF treatment. Likewise, negative effects of certain strains of PGPR on AMF have been reported (Walley and Germida, 1997). In their study, a strain of *P. cepacia* reduced spore germination, percent colonization, and root length of spring wheat colonized by *G. clarum*. These observations may be linked with the reported antagonistic effects of certain PGPR on AMF (Walley and Germida, 1997) or competition between mycorrhizal fungi and rhizobacteria (Fitter and Garbaye, 1994).

Detrimental associations that exist between these beneficial organisms can also affect their host plant. For example, Germida and Walley (1996) reported reductions in root length and weight of spring wheat due to the inoculation of certain PGPR strains. The observation was attributed to the negative effects of some PGPR species on beneficial relationships between plants and indigenous microorganisms, especially the ubiquitous AMF. Requena and co-authors (1997) reported that the dual inoculation of *G. coronatum* with a native or an exotic rhizobacteria reduced N content of *A. cytisoides* compared to when the PGPR were applied alone. Although Wu et al. (2005) found that the co-inoculation of *G. intraradices* with a consortium of three beneficial bacteria increased N content in corn tissue; the beneficial interactions were not found when *G. mosseae* was paired with the bacteria.

Interestingly, not all detrimental associations between AMF and PGPR have undesirable effects on the host plant. For instance, under non-sterile conditions, Walley and Germida (1997) observed that the co-inoculation of *G. clarum* with *P. putida* R104 reduced the detrimental effect of the PGPR on spring weight. As a result, the PGPR had an effect comparable to the uninoculated control. These authors demonstrated that some PGPR inhibited spore germination and reduced AMF colonization, but those inhibitory effect may be desirable if the AM fungus is actually detrimental (i.e., parasitic). As a result, an interaction that seems detrimental may in fact benefit the plant or at least protect the plant from detrimental associations. In contrast, a beneficial association between detrimental organisms may inhibit plant growth. In general, all these authors demonstrated that AMF and PGPR interaction effects are unpredictable, and plant response to these effects is inconsistent. Microbial, soil, and other environmental factors are commonly used to explain these variabilities (Vazquez et al., 2000; Roesti et al., 2005).

The interaction effects between AMF and PGPR are not limited to plants; a number of researchers have implicated these organisms in the removal of potentially toxic elements (González-Chávez et al., 2004) in the soil (Barea et al., 2002; Khan, 2005). Plant growth-promoting rhizobacteria enhance mycorrhizoremediation of contaminated soils and water (Khan, 2005). Mycorrhizoremediation may be defined as the involvement of AMF in the reduction or removal of contaminants from affected soils and water by stimulating plant

growth and nutrient uptake (Khan, 2005). Plant growth-promoting rhizobacteria facilitate mycorrhizoremediation by increasing plant root surface area for AMF colonization, modifying microbial community associations with the mycorrhizal root, and inducing the transfer of these contaminants to the rhizosphere where they can be easily accessed and taken up by plants (Barea et al., 2002, 2005).

Furthermore, AMF and PGPR influence soil physical and biochemical characteristics such as aggregate formation and stabilization. Although bacterial effects on soil aggregate formation are found primarily on microaggregates while mycorrhizal effects are more evident on macroaggregates (Rillig and Mummey, 2006), together these organisms increase the formation of stable aggregates in the soil (Rillig and Mummey, 2006). Nevertheless, Kohler et al. (2009b) found no significant interaction between *G. mosseae* and *P. mendocina* in their effects on aggregate stability of the lettuce rhizosphere. However, the authors demonstrated that the dual inoculation of *G. mosseae* and *P. mendocina* significantly increased the quantity of EE-BRSP in the plant rhizosphere compared to when *G. mosseae* was applied alone. Apparently, when applied singly, the effect of the bacterium was similar to that of *G. mosseae*. In another study, Kohler et al. (2009a) related increases in GRSP levels to the inoculation of *P. mendocina* under a water-limiting condition. To my knowledge, their study is the first to examine AMF and PGPR effects on GRSP concentrations. Adding to the appeal of Purin and Rillig (2008), more studies should explore bacterial influences on AMF fitness and functions such as glomalin production by the fungi.

3. DETERMINATION OF ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT EFFECTS ON GLOMALIN PRODUCTION

3.1 Introduction

Studies have indicated that the species of arbuscular mycorrhizal fungi (AMF) present in the soil is an important factor governing glomalin-related soil protein (GRSP) concentrations (Rillig et al., 2001; Nichols and Wright, 2004; Bedini et al., 2009). In a study to characterize glomalin production by AMF hyphae during active root colonization, Wright et al. (1996) observed that the amount of protein extracted per unit weight of hyphae varied among genera and between species of *Gigaspora gigantea, Glomus etunicatum,* and *G. intraradices.* Also, Wright and Upadhyaya (1999) reported significant differences in total glomalin produced by *Gi. rosea, G. caledonium*, and *G. intraradices*. In this study, the quantity of Bradford-reactive soil protein (BRSP) produced by *Gi. rosea* and *G. caledonium* was significantly greater than that produced by *G. intraradices*, although the differences were not significant when glomalin was quantified using an enzyme-linked immunosorbent assay (ELISA) technique. Bedini et al. (2009) also reported significant differences in glomalin production between isolates of *G. mosseae*. In addition, Lovelock et al. (2004b) observed differences in the yield of immuonreactive glomalin among *Acaulospora morrowiae*, *Gi. rosea, G. etunicatum*, and *G. intraradices* with *A. morrowiae* producing the highest concentration of glomalin and *G. intraradices* producing the lowest.

Host plant and productivity are among other determinants of glomalin production by AMF (Rillig et al., 2002; Treseder and Turner, 2007). Rillig et al. (2002) observed plant effects on the immunoreactive easily extractable glomalin fraction while determining the effects of five plant species on glomalin production and soil aggregation by AMF. Also, Nichols and Wright (2004) reported significant differences in the levels of GRSP in the rhizosphere of corn and crimson clover (*Trifolium incarnatum.* L.) inoculated with *G. etunicatum.* The results of these studies indicate that glomalin production can be influenced by fungal species and host plant in which the fungus is propagated. Therefore, it is possible to select the most efficient AMF and host plant for glomalin production. The objectives of this study were to compare the amount of glomalin produced by *G. clarum*,

G. intraradices, and *G. mosseae*, and determine differences in the ability of corn (*Zea mays* L.), pea (*Pisum sativum* L.), and wheat (*Triticum aestivum* L.) to support glomalin production by *G. intraradices*.

3.2 Materials and Methods

3.2.1 Arbuscular mycorrhizal fungi spore propagation

Pure starter cultures of *G. mosseae* (Nicolson and Gerd) Gerd and Trappe, *G. intraradices* (Schenck and Smith), and *G. clarum* (Nicolson and Schenck) supplied by Agriculture and Agri-Food Canada were propagated as described by Talukdar (1993) using corn as the host. *Glomus* species were selected because of their abundance in Saskatchewan soils (Talukdar and Germida, 1993) and worldwide (Blaszkowski, 1989). For example, *G. intraradices* is known as a generalist fungus; it associates with a number of plant species and is found in many ecosystems (Öpik et al., 2003; Scheublin et al., 2004).

Soil-sand mix was prepared by mixing a low phosphorus (P) Elbow soil (Calcareous Dark Brown Chernozem), collected from the top 15 cm of a soil from the Bradwell Association, with coarse silica sand in equal proportion (1:1 w/w); the mix was amended with modified Hoagland solution (Hoagland and Arnon, 1938; Millner and Kitt, 1992) (minus P; 50 mL kg^{-1}), and sterilized (autoclaved twice for 1h at 121˚C at an interval of 24 h) to eliminate indigenous AMF. The physical and chemical properties of the autoclaved growth media were determined by ALS laboratory (Saskatoon, SK) and were as follows: 23.5 μg NO_3-N g^{-1}; 26.3 μg P g^{-1}; 588.0 μg K g^{-1}; 33.0 μg SO_4-S g^{-1}; 1.1 μg Cu g^{-1}; 149.5 μg Mn g^{-1}; 3.8 μg Zn g^{-1}; 1.3 μg B g^{-1}; 19.0 μg Fe g^{-1}; 25.8 μg Cl g^{-1}; pH 7.3; conductivity 0.5 mS cm^{-1}.

Two kilograms of sterilized soil-sand mix was placed into a 15-cm diameter pot. Soil moisture was maintained at 70% moisture holding capacity and the plants were watered daily with sterile distilled water to constant weight. Corn seeds were surface-sterilized with 75% (v/v) ethanol for 1 min, followed by 10% (w/v) sodium hypochlorite solution for 5 min and then washed eight times with sterile distilled water. The seeds were aseptically germinated on 1.5% water agar; four uniform corn seedlings were transferred into transplant holes and inoculated with AMF spores using the funnel technique

described by Merge and Timmer (1982). Corn was chosen as the nurse crop because it is mycotrophic and highly responsive to mycorrhizal propagules regardless of low propagule densities (Liasu and Shosanya, 2007). The funnel technique ensures that growing roots are colonized by AMF.

Plants were grown using a 16 h day / 8 h night cycle. The day and night temperatures were 24˚C and 21˚C, respectively. The light intensity in the growth chamber during the growth period ranged from 290 to 350 µE m^{-2} s^{-1}. Pots were supplied with modified Hoagland solution (Millner and Kitt, 1992) (50 mL kg^{-1} soil-sand mix) every week for eight weeks. At maturity (90 days after planting) corn shoots were cut off and the soil containing AMF propagules was left in the growth chamber for one week to allow the soil to dry and spores to mature, and then stored at 4˚C.

Spores were extracted from 20 g samples of root-soil mixtures using the wet-sieving method described by Dandan and Zhiwei (2007). The soil samples were suspended in 150 mL of tap water and stirred for 2 min with a magnetic stirrer. The soil suspension was allowed to settle for 10 s, and soil fraction between 53 µM and 425 µM was collected using sieves of appropriate mesh size. The soil samples were resuspended in 150 mL of tap water to repeat the procedure. The filtrate was transferred to 50 mL centrifuge tubes with a fine stream of water, centrifuged first in tap water for 5 min at 1270 × g to remove floating organic debris from the soil, and then in 50% sucrose for 1 min after shaking vigorously to separate the spores from denser soil particles. The sucrose supernatant was poured through a 53 µM sieve, and the trapped spores were washed with tap water to remove the sucrose, with the aid of a vacuum filter. The spores were rinsed into a small Petri dish, and were used as infective propagules; 100 spores of the appropriate AMF were used to inoculate each seedling.

3.2.2 Growth medium preparation and planting

Growth medium preparation and planting were done as described by Wright and Upadhyaya (1999) with some modifications. To ensure no contamination, GRSP was pre-extracted from 2 kg of a 1:1 mixture of coarse sand and fine sand by saturating the mix with 50 m*M* sodium citrate solution (pH 8.0) and autoclaving at 121˚C for 1 h. The extract was decanted, and the sand-mix thoroughly washed with tap water followed by

deionized water. After extracting GRSP, the sand-mix was air dried and autoclaved at 121˚C for 1h on two consecutive days.

As reported by Wright and Upadhyaya (1999), approximately 300 mL (450 ± 5 g) of autoclaved soil-sand mix (1:1) was packed into a 40 μm nylon mesh (Sefar America, Inc., Chicoutimi, QC) pouch positioned at the centre of a 15 cm diameter pot (Figure 3.1). The enclosed soil-sand mix was intended to represent the rhizosphere (soil region around the root). The nylon mesh restricts the plant's roots but allows hyphae to pass through, thereby restraining the growth of plant roots into the autoclaved media. Two kilograms of sterilized sand mix was transferred to the surroundings of the nylon mesh pouch, filling the remaining pot volume. This sand mix represented the mycorrhizosphere (soil region around the hyphae).

Seeds were surface sterilized by immersing in 70% (v/v) ethanol for 2 min, transferring to 1.2% (v/v) sodium hypochlorite for 10 min, and rinsing 10 times in sterile tap water. Sterile seeds were aseptically transferred onto 1.5% (w/v) water agar in sterile Petri dishes and allowed to germinate in the dark at 27˚C. Four uniform seedlings were aseptically placed at the centre of the enclosed autoclaved media with 100 spores of the appropriate AMF inoculum. One hundred milliliters of a half-strength Hoagland nutrient solution (Millner and Kitt, 1992) was supplied to each pot every week, starting at four weeks after planting (WAP), to replenish the soil nutrients.

The physical and chemical properties of the autoclaved soil-sand mix used for Experiment 1 (i.e., AMF effect on glomalin production) were determined by ALS laboratory (Saskatoon, SK) and were as follows: sandy loam; 4.5 μg NO_3-N g^{-1}; 32.5 μg P g^{-1}; 638.4 μg K g^{-1}; 35.3 μg SO_4-S g^{-1}; 1.1 μg Cu g^{-1}; 109.8 μg Mn g^{-1}; 2.4 μg Zn g^{-1}; 1.3 μg B g^{-1}; 14.6 μg Fe g^{-1}; 29.1 μg Cl g^{-1}; pH 7.4; conductivity 0.5 mS cm^{-1}. The physical and chemical properties of the autoclaved soil-sand mix used for Experiment 2 (i.e., plant effect on glomalin production) were as follows: sandy loam; 2.8 μg NO_3-N g^{-1}; 40.9 μg P g^{-1}; 489.4 μg K g^{-1}; 52.1 μg SO_4-S g^{-1}; 1.3 μg Cu g^{-1}; 145.0 μg Mn g^{-1}; 4.9 μg Zn g^{-1}; 1.1 μg B g^{-1}; 20.2 μg Fe g^{-1}; 29.7 μg Cl g^{-1}; pH 7.2; conductivity 0.5 mS cm^{-1}.

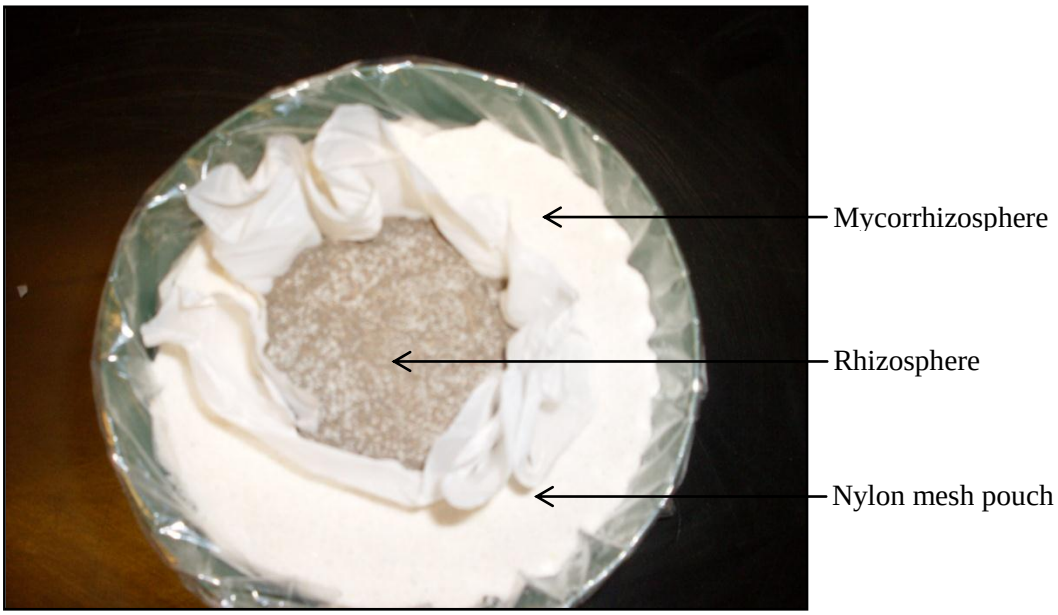

Fig. 3.1. A photograph of the growth medium showing the separation of the rhizosphere (soil-sand mix) from the mycorrhizosphere (sand mix) using a nylon mesh pouch.

The pots were covered with aluminum foil to allow only the plant coleoptile to pass through, and the hole created around the coleoptile was covered with sterile cotton wrapped with aluminum foil to limit airborne contamination. The plants were thinned to two per pot after seedling emergence. These experiments were conducted in a growth chamber under the following conditions: 25˚C, 16 h day and 20˚C, 8 h night, 375-400 µE m^{-2} s^{-1} of irradiance and relative humidity of 60%. Soil moisture was maintained at 70% water holding capacity by regular additions of sterile distilled water.

3.2.3 Experimental design

Two experiments were set up to determine effects of AMF and host plant on GRSP production. The first experiment consisted of the three AMF species (*G. clarum*, *G. intraradices*, and *G. mosseae*) and a control using corn as the host plant. The second experiment was done using corn, pea, and wheat as host plants inoculated with *G. intraradices*. *Glomus intraradices* was selected in part, because of the ease of culturing and retrieving the spores of this species. Control treatments consisted of uninoculated

corn, pea and wheat. The experiments were conducted using a completely randomized design, each replicated four times.

3.2.4 Glomalin extraction

Glomalin-related soil protein extractions were conducted as described by Wright and Upadhyaya (1996). Total glomalin was extracted from each sample using 50 m*M* sodium citrate solution (pH 8.0). The mixture was autoclaved for 60 min at 121˚C, with 1 g of soil in 8 mL extractant. The extraction procedure was repeated until the supernatant was almost colourless. Most soil-sand mix (rhizosphere) samples needed four extractions; glomalin was extracted from the sand mix (mycorrhizosphere) samples twice. Samples were centrifuged at 5,000 × g for 15 min immediately after extraction and the supernatant containing the extracted protein was decanted and stored at 4˚C for analysis.

3.2.5 Glomalin quantification

Glomalin concentration in each extract was determined by the Bradford dye-binding protein assay as BRSP (Wright and Upadhyaya, 1996; Rillig, 2004b). Although the Bradford protein assay may not be specific for glomalin (Rillig and Steinberg, 2002), values obtained from the Bradford and ELISA assays are reportedly correlated (Wright and Upadhyaya, 1996, 1999; Rosier et al., 2006). The Bradford method also is useful in determining GRSP concentrations in low organic matter content soils (Rosier et al., 2006) including autoclaved soil and in sand mixes, such as the sand mix used in these experiments. Thus, the Bradford assay was expected to provide a realistic estimate of GRSP (Rosier et al., 2006) in the experimental samples.

The Bradford assay was carried out using 96-well plates. Protein standards in a range of 1.25 to 5 μg protein per well were prepared using bovine serum albumin (BSA). Extract from each sample was filtered using C-free Quantitative Q2 filter paper (Fisher Scientific, Ottawa, ON), aliquoted, and centrifuged at 10,000 × g for 5 min to remove residual soil particles and other insoluble materials. Three replicate wells of the 96-well assay plate were loaded with 50 μg of protein in 150 μL of phosphate buffer saline (PBS) for the soil-sand mix extract, and 150 μg of protein in 50 μL of PBS for the sand mix extract. Fifty micro-litres of Bio-Rad dye (Bio-Rad, Laboratories, Inc., CA) was added

into each well containing protein in PBS, mixed thoroughly, and the absorbance read at a wavelength of 590 nm (A_{590}) within 5 min of addition. A standard curve was generated by plotting optical density values against protein of known concentration (BSA). Protein concentrations in micrograms per well of GRSP extracts were calculated from the equation describing the standard curve.

3.2.6 Statistical analysis

Effects of AMF and host plant on GRSP production were tested using a one-way analysis of variance (ANOVA); treatment means were compared using the least significant difference (LSD) test at a significance level of 0.05. Statistical analyses were performed using SPSS software version 16.0 for Windows (SPSS Inc., 2008).

3.3 Results

3.3.1 Fungal effects on glomalin-related soil protein

Concentrations of BRSP in the sand-mix (mycorrhizosphere) were below detection limits; therefore, only values obtained from the soil-sand mix (rhizosphere) are reported and discussed. Differences between BRSP concentrations were only significant at p = 0.075. At this level of significance, *G. mosseae* yielded higher BRSP concentrations than soil inoculated with *G. clarum* and *G. intraradices* (Figure 3.2).

3.3.2 Plant effects on glomalin-related soil protein

The mean values of BRSP in the rhizosphere ranged from 1.09 to 1.39 mg g^{-1} soil. The inoculation of corn significantly (p = 0.002) enhanced the production of BRSP (Figure 3.3). No other significant differences in BRSP were detected.

3.4 Discussion

The amount of BRSP extracted ranged from 1.09 to 1.72 mg g^{-1} soil, which falls at the lower end of the range reported by Wright and Upadhyaya (1998). Wright and Upadhyaya (1998) reported BRSP concentrations ranging from 1 to 21 mg g^{-1} soil in thirty-seven soils from five geographical locations. On the other hand, BRSP concentrations in the study by Antibus et al. (2006) ranged from 1 to 5.5 mg g^{-1} soil. Moreover, Lovelock et al. (2004b) reported lower GRSP concentrations for pot cultures.

In this experiment, the presence of BRSP in control soil (Figure 3.2) reflects the previous crop history and associated glomalin.

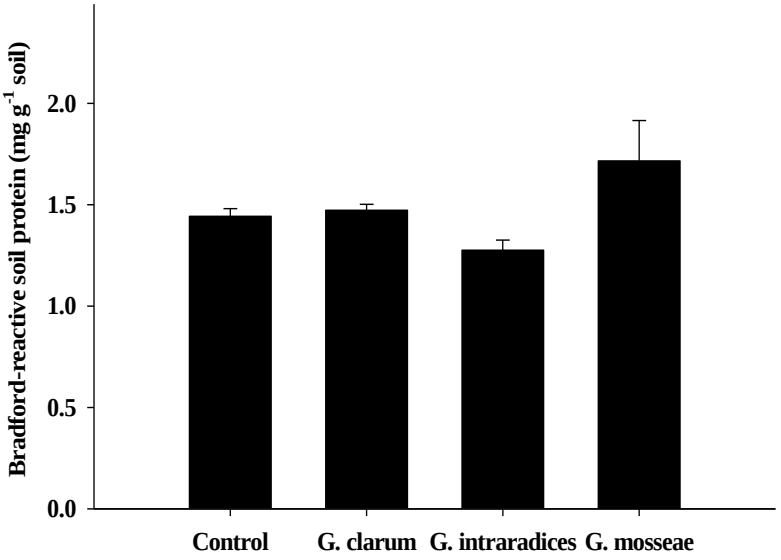

Fig. 3.2. Bradford-reactive soil protein (BRSP) in the rhizosphere of corn inoculated with the arbuscular mycorrhizal fungi (AMF) species 12 weeks after planting (WAP). Error bars are standard errors of the mean (n = 4). No significant differences detected.

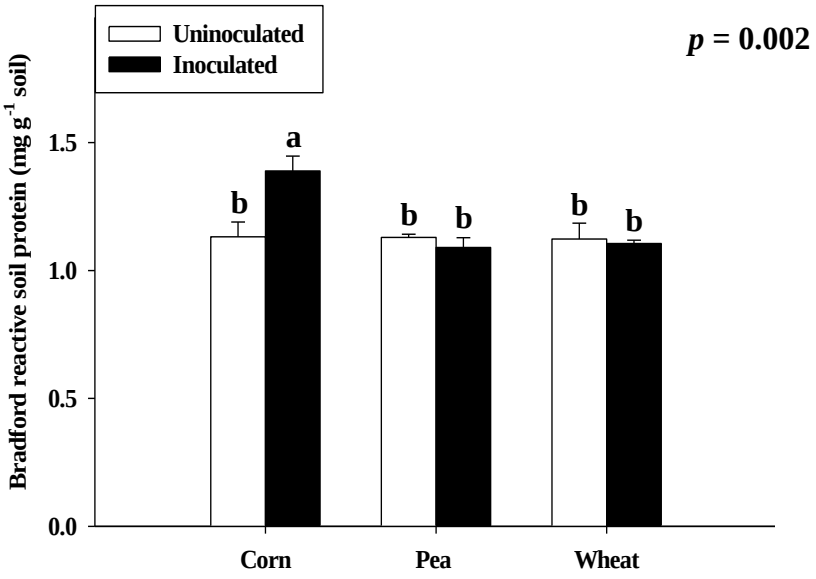

Fig. 3.3. Bradford-reactive soil protein (BRSP) in the rhizosphere of corn, pea, and wheat inoculated with *G. intraradices* 12 weeks after planting (WAP). Corn-C, Pea-C, and Wheat-C denotes the uninoculated Corn, Pea and Wheat, respectively. Means followed by the same letter are not significantly different according to the least significant (LSD) test ($p \leq 0.05$). Error bars are standard errors of the mean (n = 4).

Glomus mosseae tended to produce more BRSP, but results were more variable, than other AMF species; therefore, differences in glomalin production by the three AMF species were not statistically significant. In contrast, Lovelock et al. (2004b) reported that immunoreactive glomalin production varied significantly across AMF species from pot cultures with corn serving as the host plant**.** They found that *A. morrowiae* produced higher GRSP compared with *Gi. rosea*, *G. etunicatum* and *G. intraradices*, and GRSP production by *G. intraradices* was significantly lower than *Gi. rosea* and *G. etunicatum*. Nichols and Wright (2004) also reported that GRSP concentrations varied among five AMF species: *G. etunicatum*, *G. viscosum*, *G. caledonium*, *Gi. rosea*, and *Gi. gigantea*. Glomalin-related soil protein concentrations were lowest in *G. viscosum* and highest in *G. etunicatum*. In fact, they reported differences among isolates of the same species. Furthermore, Violi et al. (2007) found that *Scutellospora heterogama* produced more glomalin in comparison with *G. intraradices*, and observed that production was independent of plant size and the P status of the soil. These differences in glomalin production may be due to differences in fungal activity (Bedini et al., 2007).

Driver et al. (2005) reported that glomalin is a component of the hyphae wall. However, whereas Treseder and Turner (2007) found that hyphal length does not correlate with glomalin production; Lovelock et al. (2004b) noted that diameter is an important determinant of glomalin production. Lovelock and co-workers (2004b) demonstrated that glomalin concentrations may be lower in fine hyphae compared to coarse hyphae, probably a result of greater proportion of cytoplasm to hyphal wall volume in fine hyphae (Steinberg and Rillig, 2003). Therefore, higher production of glomalin by *G. mosseae* may be a result of hyphal characteristics, the inherent ability of the fungus to produce glomalin, or a more favourable response to corn as the host plant. Nonetheless, Nichols and Wright (2004) stated that overall variations observed among isolates, species, and hosts of AMF do not have a similar trend; thus, factors other than fungal activity (Bedini et al., 2007) and host productivity (Violi et al., 2007) must be considered.

Significantly higher BRSP concentrations were observed in the rhizosphere of corn compared with pea and wheat inoculated with *G. intraradices* (Figure 3.3). Nichols and Wright (2004) also reported higher glomalin production by *Gi. gigantea* with corn as the

host plant as compared to clover. Furthermore, there are reports (Schenck and Kinloch, 1980) that *Glomus* species more readily form associations with monocotyledonous crops (bahia grass, corn, sorghum) than dicotyledonous crops (cotton, peanut, soybean), which influenced changes in the occurrence of AMF observed in their study. Jakobsen and Nielsen (1983) noted that the absolute root length colonized by AMF is similar in pea and wheat while relating infection levels and spore numbers to time, soil depth, and root density. Thus, the observation that GRSP did not differ significantly between wheat and pea may be related to absolute root length colonized.

It has been argued that plant species respond differently to AMF colonization because of the variation in plant dependence on AMF (van der Heijden et al., 1998a; Treseder and Allen, 2000; Burleigh et al., 2002). For example, nutrients gained from individual AMF species vary among plant species (Burleigh et al., 2002; Smith et al., 2004). Also, positive and significant effects of plant growth on glomalin production have been observed (Violi et al., 2007; Bedini et al., 2009). Although Bedini et al. (2009) reported that plant effect on GRSP levels was not significant when plant size was separated from mycorrhizal effect; they noted that host plant biomass may be related to GSRP production by AMF. Plant effects on GRSP production have been linked with the amount of C allotted by the plant to the AMF (Treseder and Turner, 2007; Violi et al., 2007). Violi et al. (2007) observed that glomalin production by S. *heterogama* increased with increases in plant C gained by the fungus. This implies that the higher glomalin production by *G. intraradices* with corn as the host (Figure 3.3) may be because corn produces more total biomass, it allotted more C to the fungus compared to pea and wheat, and thus more glomalin was produced by the fungus.

Several studies have reported corn as the best host plant for producing AMF spores such as *G. geosporum* (Talukdar, 1993) and *G. macrocarpum* (Struble and Skipper, 1988) under growth chamber conditions. Recently, Bai et al. (2009) reported a significant and positive correlation between spore density and BRSP. Thus, given that the available evidence indicates that glomalin is located in AMF spore and hyphal walls (Driver et al., 2005; Purin and Rillig, 2007), greater spore production by AMF may indicate higher glomalin production. This would then explain the higher BRSP production observed in the rhizosphere of corn.

Interestingly, BRSP concentrations in the rhizosphere of some uninoculated control plants were similar to levels in the rhizosphere of corn. For example, concentrations of BRSP in the rhizosphere of corn inoculated with *G. intraradices* were 0.16 mg g^{-1} soil lower than the uninoculated plant, although this difference was not statistically significant. Similarly, Rosier et al. (2008) found no significant differences in the levels of Bradford-root protein in plants colonized by *G. intraradices* or *Entrophospora colombiana* and uncolonized control while comparing the levels of Bradford-root protein and immunoreactive-root protein in *Bromus inermis* colonized by different AMF isolates. Also, Kohler et al. (2009a) found no significant differences between mycorrhizal and non-mycorrhizal treatments on GRSP concentrations in rhizosphere (< 2 mm) of lettuce (*Lactuca sativa* L.). The Bradford assay measures total protein, and detects proteins and charged compounds even at low concentrations (Sedmak and Grossberg, 1977, cited by Rosier et al., 2008); therefore, protein produced by rhizosphere organisms other than AMF may be detected (Rosier et al., 2008). Moreover, glomalin extraction procedures do not eliminate all non-glomalin sources (Rosier et al., 2006; Whiffen et al., 2007).

3.5 Conclusion

Although BRSP production was independent of the AMF species, *G. mosseae* tended to produce more BRSP than *G. clarum* and *G. intraradices*. Plant effects, however, were observed. The concentration of BRSP in the rhizosphere of corn was significantly higher than that of either pea or wheat. This observation may be of great importance in selecting AMF-host combinations that may enhance BRSP production, thereby increasing beneficial qualities attributed to glomalin production by AMF.

4. ASSESSMENT OF PLANT GROWTH-PROMOTING RHIZOBACTERIA

4.1 Introduction

Plant growth-promoting rhizobacteria (PGPR) are known for their ability to stimulate growth of many crops using mechanisms such as phosphorus (P) solubilization and production of siderophores, antibiotics, and growth hormones (Kloepper et al., 1988; Glick, 1995; Karlidag et al., 2007; Richardson et al., 2009). Through the production of antibiotics and siderophores, PGPR control phytopathogens such as *Rhizoctonia* and *Fusarium* species (Glick, 1995; Whipps, 2001; Lugtenberg and Kamilova, 2009). Despite reported beneficial qualities of these organisms on their host plants, studies have shown that results are inconsistent (Germida and Walley, 1996; Lucy et al., 2004). For instance, soil fertility, moisture content, and competition between PGPR and other rhizosphere inhabitants may influence plant response to PGPR (Burr et al., 1978; de Freitas and Germida, 1990a, 1992; Lucy et al., 2004; Strigul and Kravchenko, 2006). Because the overall objective of this study was to determine if glomalin production by arbuscular mycorrhizal fungi (AMF) could be enhanced by co-inoculating with PGPR, selection of PGPR with consistent beneficial qualities was required.

In a study to identify PGPR associated with winter wheat (*Triticum aestivum* L. cv. Norstar) in Saskatchewan, de Freitas (1990) reported that some PGPR isolates have the ability to withstand a wide range of temperatures; produce relatively high amounts of an indole-acetic acid (IAA)-like substance(s), which promotes root development; have the ability to inhibit the growth of soil pathogens through the production of siderophores (de Freitas and Germida, 1991); rapidly colonize wheat roots (de Freitas and Germida, 1990b); and promote plant growth under growth chamber conditions (de Freitas and Germida (1990a, 1992). Additionally, some of these isolates increased the harvest index (the percentage ratio of seed mass to seed plus shoot mass) of spring wheat (*Triticum aestivum* L.) (Germida and Walley, 1996), and stimulated the growth of other crops including cabbage (*Brassica oleracea* L.), lettuce (*Latuca sativa* L.), and onion (*Allium cepa* L.) (Germida and de Freitas, 1994). The objective of this set of experiments was to verify that the PGPR isolates initially used in these studies still possess the qualities

reported by de Freitas (1990) after almost two decades of storage, thereby assessing the current state of these bacterial inoculants for future use.

4.2 Materials and Methods

4.2.1 Emergence assay

4.2.1.1 Plant growth-promoting rhizobacteria inoculation

The strains selected (*Pseudomonas cepacia* R55 and R85; *P. aeruginosa* R75; *P. putida* R105; and *P. fluorescence* R111) were tested for purity. Each strain was taken from stock cultures and streaked on trypticase soy agar (TSA) (Difco Laboratories, Inc., Detroit, MI) plates, incubated at 27˚C for 48 h, and checked for contamination. As described by de Freitas (1990), pure strains isolated from the contamination plates were grown on King's B (KB) (Difco Laboratories, Inc., Detroit, MI) medium supplemented with antibiotics (5 mg chloramphenicol, 75 mg cycloheximide, 45 mg novobiocin and 75,000 units penicillin G L^{-1}) (Sands and Rovira, 1970) for 48 h at 27˚C. The pure cultures were then scraped into 20 mL sterile tap water. Seeds of spring wheat were surface sterilized by immersing in 70% (v/v) ethanol for 2 min, transferring to 1.2% (v/v) sodium hypochlorite for 10 min, and rinsing 10 times in sterile distilled water. One hundred surface sterilized seeds were added to each bacterial suspension, and agitated on a rotary shaker (110 rev min^{-1}) for 4 h at 27˚C, yielding approximately 10^7 colony forming units (cfu) per seed, as determined on KB medium.

4.2.1.2 Soil preparation and planting

A low nutrient soil (Calcareous Dark Brown Chernozem), collected from the top 15 cm of a soil from the Bradwell Association, was sieved (2 mm) and mixed with autoclaved silica sand (2:1 w/w). The physical and chemical properties of the soil-sand mix were determined by ALS laboratory (Saskatoon, SK) and were as follows: loamy sand; 9.5 μg NO_3-N g^{-1}; 14.4 μg P g^{-1}; 402.1 μg K g^{-1}; 30.8 μg SO_4-S g^{-1}; pH 7.4; conductivity 0.5 mS cm^{-1}. A low nutrient soil was used because de Freitas and Germida (1992) observed greater plant stimulation by PGPR when a less fertile soil was used while studying the effects of these isolates on wheat. One hundred and seventy grams of soil-sand mix was packed into a cone-tainer (4 cm diameter × 20 cm long) (Stuewe and

Sons Inc., Oregon), and five sterilized wheat seeds inoculated with appropriate bacterial suspension were planted at a depth of approximately 2 cm. Cone-tainers were placed in a growth chamber (photosynthetic irradiance of ~450-500 μE m^{-2} s^{-1}) at 24˚C under a 14 h light / 10 h dark cycle, and watered daily with distilled water. Seedling emergence was evaluated at 5, 10, and 15 days after planting (DAP). The control consisted of wheat seeds inoculated with autoclaved suspensions of R111. The experiment was conducted using a completely randomized design with six replicates.

4.2.2 Total biomass promotion assay

The effect of PGPR on wheat biomass production was assessed as described for the emergence assay (Section 4.2.1) except that the seedlings were thinned to two plants per cone-tainer after emergence, grown for 30 d, and shoot, root, and total biomass determined at harvest. The roots were washed under running tap water until they were free of soil particles, then each plant material was oven dried at 65˚C for 48 h and weighed. The control consisted of wheat seeds inoculated with autoclaved suspensions of R111. The experiment was conducted using a completely randomized design with five replicates.

4.2.3 Fungal inhibition assay

Fungal inhibitory characteristics of the five PGPR strains were examined using three *Fusarim* species (*F. oxysporum, F. sporochoides,* and *F. acuminatum*) on potato dextrose agar (PDA) (Difco Laboratories, Inc., Detroit, MI) and KB agar medium. Two media were used because the ability of *Pseudomonas* to inhibit fungal growth has been shown to depend on growth medium characteristics (de Freitas and Germida, 1990a; Maurhofer et al., 1995).

A rhizobacterium strain was inoculated onto plates containing either PDA or KB agar medium by streaking the appropriate bacterial suspension on each medium at opposite ends near the edge of the plate (Figure 4.1). Plates were incubated at 27˚C for 48 h to allow growth of the bacterium. A 5 mm diameter mycelial plug was obtained from fungal cultures grown on PDA for 7 to 10 d. The fungal plug was then placed in the centre of the PDA and KB agar medium that were previously inoculated with the bacterial strain.

Control plates included a non-inoculated PDA plug. The plates were re-incubated at 27˚C for 10 d. Fungal inhibition was determined by measuring the distance between the edge of bacterial colonies and mycelial plug at 3 and 10 d after inoculation. The experiment was replicated three times, and each replicate consisted of one plate per treatment for each medium.

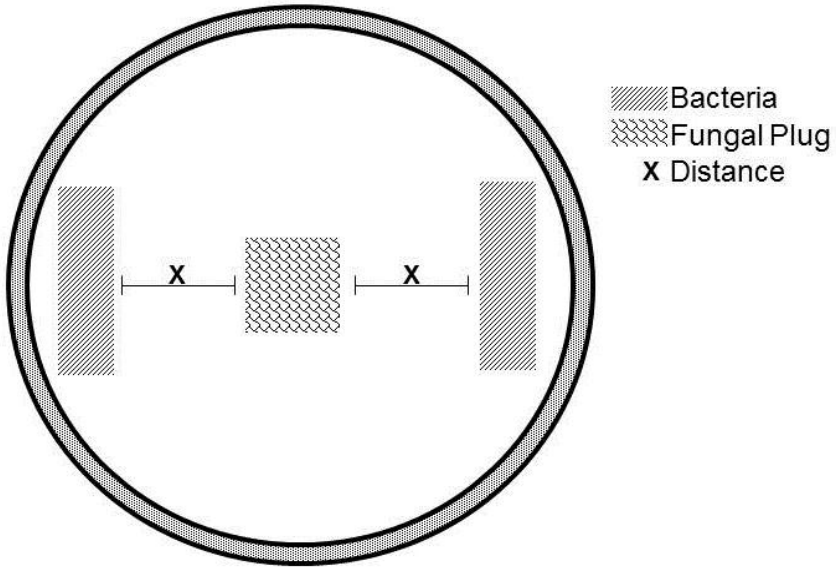

Fig. 4.1. A Schematic drawing (not drawn to scale) for fungal inhibition assay viewed from the top.

4.2.4 Root length bioassay

A root length bioassay was conducted using growth pouches (16 cm wide × 18 cm long, Mega International, West St. Paul, MN). Growth pouches were sterilized, and each pouch was filled with 30 mL of sterilized 1/5 strength Hoagland's nutrient solution (Hoagland and Arnon, 1938). Pouches were wrapped with aluminum foil to minimize exposure to light. Wheat seeds were surface sterilized and inoculated with PGPR as described in Section 4.2.1.1. Five inoculated seeds were placed on paper troughs inside each growth pouch. The control consisted of wheat seeds inoculated with autoclaved suspension of R111. The inoculation and planting procedures were conducted under sterile conditions, using a laminar flow hood to limit contamination.

The pouches were placed in a growth chamber (photosynthetic irradiance of ~450-500 μE m^{-2} s^{-1}) at 24˚C under a 14 h light / 10 h dark cycle. After germination and emergence, the seedlings were thinned to two per growth pouch. During the growth period, sterile distilled water was added as required to maintain the moisture content in the pouches and each growth pouch was supplemented with 15 mL sterile 1/5 strength Hoagland's nutrient solution at 15 DAP. The experiment was conducted using a completely randomized design with five replicates.

After 30 d, the plants were harvested, and root length was estimated from the fresh roots of 12 subsamples using a root scanner (Hawlett Packard Scanjet 6100C, Scantastic). The roots were stained with methyl violet stain (1 g methyl violet dissolved in 100 mL of 100% ethyl alcohol, and drops added to the water in which the root samples were immersed) before scanning to make fine roots more visible. The root lengths of these subsamples were regressed with their weight to estimate the actual root length per unit mass.

4.2.5 Statistical analysis

Data were subjected to analysis of variance (ANOVA) to determine any significant effects of PGPR inoculation on wheat seedling emergence, root length, and total biomass production (total dry weight). The treatment means were compared using the least significant difference (LSD) test at a significance level of 0.05. Normality of distributions and homogeneity of variances were assessed before conducting any statistical analysis. Statistical analyses were performed using SPSS software version 16.0 for Windows (SPSS Inc., 2008).

4.3 Results

4.3.1 Seedling emergence

Treatment of wheat seeds with strains R55, R75, R85, R105, and R111 did not significantly ($p \leq 0.05$) affect seedling emergence at 5, 10, and 15 DAP ($p = 0.866$, $p = 0.414$, $p = 0.545$, for each DAP, respectively) (Figure 4.2).

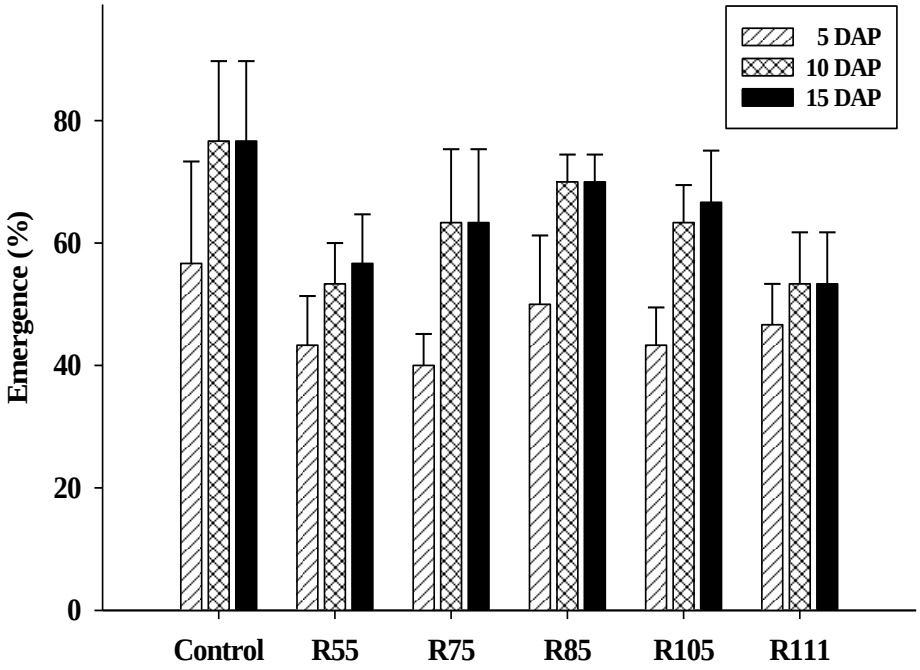

Fig. 4.2. Percent emergence of spring wheat inoculated with plant growth-promoting rhizobacteria (PGPR) strains 5, 10, and 15 days after planting (DAP). Error bars are standard errors of the mean (n = 6). No significant differences detected for each day.

4.3.2 Total biomass and root length

Total biomass was significantly enhanced (p = 0.001) by all PGPR strains, relative to the control, with increases ranging from 23 to 56% (Table 4.1). *Pseudomonas putida* R105 was the most effective strain, enhancing shoot, root, and total dry weight by 48, 65, and 56%, respectively. The beneficial effects of R55 and R111 were comparable to that of R105 and were greater than those of R75 and R85 ($p \leq 0.05$). However, none of the PGPR strains had significant beneficial effects on wheat root length (Table 4.1)

4.3.3 Fungal inhibition

All PGPR strains inhibited the growth of at least one fungus on PDA or KB agar plates (Table. 4.2). Of all strains, R85 was the least inhibitory, inhibiting growth of only one fungus, *F. acuminatum*, on the KB agar plate. *Fusarium acuminatum*, inhibited by R85, was the most susceptible fungal isolate; it was the only isolate that was inhibited by all PGPR strains on PDA and KB agar plates. Furthermore, because PGPR strains

demonstrated antagonism to fungal isolates more on KB agar plate, KB medium was more favourable for fungal inhibition by the PGPR.

Table 4.1. Shoot, root, and total dry weight (n = 6), and root length (n = 5) of wheat inoculated with the plant growth-promoting rhizobacteria (PGPR) strains 30 days after planting (DAP).

Treatment	Shoot	Root	Total	Root length
		Dry weight (mg plant^{-1})		cm plant^{-1}
Control	83c†	60c	143c	541
R55	109ab	83ab	193ab	479
R75	107ab	69bc	176b	507
R85	100bc	77bc	178b	549
R105	123a	99a	223a	571
R111	106ab	87ab	193ab	541
LSD ($p \leq 0.05$)	19	20	31	NS‡

†Means followed by the same letter are not different ($p \leq 0.05$), determined according to the least significant difference test.
‡NS denotes no significant differences.

Table 4.2. Antibiosis of plant growth-promoting rhizobacteria (PGPR) strains against some pathogenic fungi on potato dextrose agar (PDA) and King's B (KB) agar medium. n = 3

Fungal Isolates	Plant growth-promoting rhizobacteria strains									
	R55		R75		R85		R105		R111	
	Growth media									
	PDA	KB	PDA	KB	PDA	KB	PDA	KB	PDA	KB
F. sporochoides	○	○	○	○	○	○	○	○	○	●
F. oxysporum	○	●	○	●	○	○	○	⊗	○	○
F. acuminatum	●	●	●	●	○	●	●	●	⊗	●

○ - Represents no fungal inhibition.
⊗ - Represents fungal inhibition zone ≤ 6 mm from the bacterial colony.
● - Represents fungal inhibition zone at > 6 mm from the bacterial colony.

4.4 Discussion

Results of these experiments are comparable to those of de Freitas and Germida (1990a), indicating that long-term storage had no significant adverse effects on the selected PGPR strains. De Freitas and Germida (1990a) reported that all the strains, with the exception of R55, significantly inhibited seedling emergence in a low nutrient soil. Whereas Kropp et al. (1996) stated that some PGPR strains increase emergence only in the presence of pathogens, the reason why PGPR inhibit seedling emergence is not clear. However, studies have shown that soil type, moisture content (Burr et al., 1978), and fertility (de Freitas and Germida, 1990a, 1992) all influence the expression of PGPR effects. In the present study, seedling emergence was generally lower in the PGPR-inoculated wheat. It is not clear, however, whether this reflects a change in the PGPR themselves or the differences in the soils.

The significant increase in total dry weight observed in this study also was reported by de Freitas and Germida (1990a). Increases in total dry matter yield confirmed that the PGPR strains still possess their growth promoting abilities since there are several mechanisms involved in observed growth promotion by PGPR (Kloepper et al., 1988; Lucy et al., 2004; Richardson et al., 2009). Mechanisms such as P solubilization (Cattelan et al., 1999), and siderophore (Kumar and Dube, 1992; Yasmin et al., 2009) and phytohormone production (Glick et al., 1998) are frequently reported, and de Freitas (1990) related growth promotion by the PGPR strains to these mechanisms.

Although the fungal pathogens used in this study differed from those used by de Freitas and Germida (1990a), all of the PGPR strains demonstrated antagonism against at least one of the three phytopathogens. De Freitas and Germida (1990a) also reported that all five PGPR strains inhibited the growth of at least one of the fungal pathogens used in the study. *Pseudomonas cepacia* R85 caused the least fungal inhibition, and inhibited the growth of only one fungus (*F. acuminatum*) on KB plate. De Freitas and Germida (1990a) similarly reported that R85 inhibited *Leptosphaeria maculans* "Unity", but had no effect on the growth of other fungal isolates.

The overall objective of this study was to determine if interactions between AMF and PGPR could increase glomalin production by AMF, thus the fungistatic activity (i.e., inhibition of fungal growth) of the PGPR may be undesirable. There also are reports that

some of these PGPR strains can alter beneficial associations between plants and native AMF (Germida and Walley, 1996). Whether positive or negative, interactions between PGPR and AMF may affect glomalin production. For example, glomalin has been reported as a homolog of heat shock protein (Hsp) 60 (Purin and Rillig, 2007). Heat shock proteins (Hsps) are conserved proteins produced by eukaryotes and prokaryotes when their cells are exposed to high temperatures (Lindquist and Craig, 1988) or other stresses, such as pH change and starvation (Tereshina, 2005). Some Hsps are chaperones for other cell's proteins and have catalytic activity that allows proteins to fold, thereby preventing them from being denatured or damaged (Lindquist and Craig, 1988; Purin and Rillig, 2007). Because glomalin production may increase with stress, the reported antagonism between PGPR and AMF (Germida and Walley, 1996; Walley and Germida, 1997) may actually serve to enhance glomalin production.

Plant growth-promoting rhizobacteria are known for their inconsistent performance in growth chamber and field studies (Germida and Walley, 1996; Requena et al., 1997; Walley and Germida, 1997; Lucy et al., 2004) and these inconsistencies have been related to the complex interactions between PGPR and other rhizosphere organisms (Germida and Walley, 1996; Requena et al., 1997; Strigul and Kravchenko, 2006). Unfortunately, PGPR growth promotion can only be fully observed when in association with other organisms (Schroth and Weinhold, 1986; Walley, 1993). Requena et al. (1997) reported PGPR that promoted emergence of *Anthylhis cytoisoides* under non-sterile conditions, but reduced the plant's emergence in sterilized soil. As a result, they attributed the observations to the population size of the inoculated PGPR. The authors explained that under non-sterile conditions, other soil microbial inhabitants may compete with the introduced PGPR, thereby reducing the population size of the PGPR to that optimum for plant growth. On the other hand, under sterile systems, the same bacterial population may be high causing the PGPR to become inhibitory to the host.

Apart from microbial effects, other factors also influence plant response to PGPR. Soil type, fertility, nutrient level, and moisture content (de Freitas and Germida, 1990a, 1992; Lucy et al., 2004; Gholami et al., 2009) are important factors that determine plant response to PGPR. Additionally, a high level of specificity has been reported between plant and PGPR genotypes (Bashan, et al. 1989; Dobbelaere et al., 1999). This, to some

extent, explains some of the differences observed between the response of spring and winter wheat to PGPR inoculation (Germida and Walley, 1996).

4.5 Conclusion

The selected strains significantly increased the total dry weight of spring wheat, and demonstrated antibiosis to fungal pathogens. Thus, although they had been in cold (-80˚C) storage for almost twenty years, it is clear that the five PGPR strains evaluated (R55, R75, R85, R105, and R111) retained their beneficial qualities. *Pseudomonas putida* R105 was relatively more consistent than the other strains in improving plant growth parameters (root length, shoot, root, and total dry weight).

5. ISOLATION AND TESTING OF ARBUSCULAR MYCORRHIZAL FUNGI SPORE-ASSOCIATED BACTERIA

5.1 Introduction

A number of studies have demonstrated that arbuscular mycorrhizal fungi (AMF) harbour and support the growth of certain bacterial communities (Bianciotto et al., 1996; Roesti et al., 2005). These bacteria are found on spore walls (Mayo et al., 1986; Walley and Germida, 1996; Xavier and Germida, 2003a), within spore walls (Macdonald and Chandler, 1981; Walley and Germida, 1996), in cytoplasm (Bianciotto et al., 1996; 2003), extraradical hyphae (Toljander et al., 2006), and intraradical mycelia (Schüßler, 2002) of AMF. Because spores are long-term reproductive structures of AMF, they provide a suitable condition for the growth and development of these bacteria (Bianciotto et al., 1996; Roesti et al., 2005; Bharadwaj et al., 2008b). Although spore-associated bacteria (SAB) usually colonize the outer wall layer, and are rarely found in the inner spore layers (Walley and Germida, 1996; Maia and Kimbrough, 1998), the failure to decontaminate AMF spores, even under rigorous treatment conditions has been attributed to the presence of SAB (Walley and Germida, 1996). Genera *Arthrobacter*, *Bacillus*, *Bukholderia*, *Cellulomonas*, *Clavibacter*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Paenibacillus*, and *Pseudomonas* (Mayo et al., 1986; Xavier and Germida, 2003a; Bharadwaj et al., 2008b; Cruz et al., 2008) are among the frequently reported SAB.

Studies have shown that some SAB adversely affect the growth and development of their host spore (Walley and Germida, 1996); nonetheless, there is documented evidence that some SAB improve the performance of their fungal host by enhancing production and germination of spores (Mayo et al., 1986; Xavier and Germida, 2003a), growth of extraradical hyphae (Gryndler, 2000; Hildebrandt et al., 2002), and colonization of plant roots (Garbaye, 1994; Gryndler, 2000; Mamatha et al., 2002). Spore-associated bacteria also enhance the uptake of P by AMF (Cruz et al., 2008), and protect AMF against pathogens (Budi et al., 1999; Cruz et al., 2008). As a result, they have been termed ‘mycorrhization helper bacteria’ (MHB) (Garbaye, 1994). Furthermore, some SAB have beneficial effects on plants (Bharadwaj et al., 2008a). Bharadwaj et al. (2008a) found that

some bacteria isolated from surface-decontaminated spores of *G. intraradices* and *G. mosseae* promoted the growth of potato by increasing nutrient uptake and inhibiting the growth of pathogens. They observed that the mechanisms involved are the same as those used by plant growth-promoting rhizobacteria (PGPR) such as phosphorus (P) solubilization, production of siderophores, and growth regulators like indole-acetic acid (IAA). Having similar attributes as PGPR implies they can be regarded as plant growth-promoting bacteria (PGPB) (Bharadwaj et al., 2008a). The objective of this study was to identify bacteria associated with disinfested spores of *G. intraradices*, *G. mosseae*, and *G. clarum*, and determine the effects of these bacteria on wheat (*Triticum aestivum* L.) yield.

5.2 Materials and Methods

5.2.1 Surface sterilization of arbuscular mycorrhizal fungal spores

Spores were retrieved from cultures using a wet-sieving method (Section 3.2.1) (Dandan and Zhiwei, 2007), and surface disinfested as described by Walley and Germida (1996). Four hundred to six hundred spores were washed in a sterile centrifuge tube by vortexing in 10 mL sterile 0.1 mg mL^{-1} sodium dodecyl sulphate (SDS) (VWR Int., Mississauga, ON) solution for 3 min. The spores were washed to remove loosely bound contaminants. The washed spores were poured into a sterile filter apparatus by rinsing the centrifuge tube twice with 40 mL sterile tap water to minimize spore loss. The sterile filter apparatus was made by cutting the end of a 10 mL autoclavable syringe and annealing a 40 μm pore size monofilament fabric screen (Sefar America, Inc., Chicoutimi, QC) to the open end. The spores were rinsed in 500 mL of 0.1 mg mL^{-1} SDS solution after which they were transferred into a second sterile filter apparatus. The apparatus was placed in a 50 mL centrifuge tube with 20 mL filter sterilized (0.2 μm) 5% chloramine-T (Sigma, Oakville, ON) sterilant solution. The centrifuge tube was vortexed for 30 s, and maintained at 30°C in a water bath after which the apparatus was transferred into new centrifuge tube and sterilant solution in 30 min increments for a total of 90 min. Each centrifuge was vortexed for 30 s before returning to the water bath each time. The filter sterilized sterilant solution was prepared and maintained at 5°C in sterile centrifuge tubes prior to the initiation of the sterilization procedure. It was removed from the

refrigerator 20 or 30 min before transfer. The sterilant was allowed to warm to room temperature (20°C), and 5 min before the transfer, it was placed in a water bath at 30°C. Spores were rinsed with 500 mL 0.1 mg mL^{-1} SDS solution followed by a rinse with 1000 mL sterile reverse osmosis water.

5.2.2 Isolation and identification of spore-associated bacteria

The surface disinfested spores were transferred in a small volume of sterile tap water into a Petri dish with an approximate equal volume of 0.1% (w/v) water agar (Difco Laboratories, Inc., Detroit, MI). The addition of water agar facilitated the suspension of spores in the solution. Each spore, delivered in a 7 µL droplet was placed onto 1/10 trypticase soy agar (TSA) in 100 × 15 mm Petri dishes using an automated microvolume multidispense pipette fitted with a sterile 100 µL Eppendorf pipette tip. Twenty evenly spaced droplets were put on each plate, and replicated 10 times. The plates were inverted, incubated in the dark at 27°C, and checked for microbial growth. Bacterial colonies were picked based on their colony characteristics such as colour, shape, size, edge morphology, surface and pigment production (Budi et al., 1999; Bharadwaj et al., 2008b). The selected SAB were purified and maintained in a sterile mixture of glycerol and trypticase soy broth (TSB) (1:5), and stored at - 80°C.

For identification, cells were cultured on TSA for 24 h at 27°C, and 40 mg of fresh cell mass was used for fatty acids extraction. Fatty acid methyl ester (FAME) profiles of all isolates were generated using an Hewlett Packard 5890 series II gas chromatograph equipped with microbial identification software (TSBA version 4.1). Acceptable identification of bacteria for FAME analysis is a similarity index (SI) greater than 0.3 (MIDI Inc., Delaware, USA); therefore, a SI ≥ 0.3 was chosen for reliable identification of all the isolates.

5.2.3 Assessment of biomass promotion

5.2.3.1 Bacteria inoculation

Nine of the SAB isolates were tested for biomass promotion on wheat. The individual bacteria were inoculated into 40 mL of TSB, and grown on a rotary shaker (110 rev min^{-1}) for 48 h at 27°C. Cultures were harvested by centrifugation (15 min at 5000 × g),

washed two times in sterile phosphate buffered saline (PBS), and resuspended in 40 mL sterile tap water, yielding approximately 10^6-10^8 colony forming units (cfu) mL^{-1} bacterial suspension, as determined on TSA medium.

5.2.3.2 Growth medium preparation and planting

Wheat seeds were surface sterilized by immersing in 70% (v/v) ethanol for 2 min, transferring to 1.2% (v/v) sodium hypochlorite for 10 min, and rinsing 10 times in sterile distilled water. Surface sterilized seeds were aseptically transferred onto 1.5% (w/v) water agar in sterile Petri dishes and allowed to germinate in the dark at 27°C.

A low nutrient growth medium was prepared by mixing sieved (2 mm) loamy soil (Calcareous Dark Brown Chernozem), collected from the top 15 cm of soil from the Bradwell Association, with autoclaved silica sand (2:1 w/w). The physical and chemical properties of the growth media were determined by ALS laboratory (Saskatoon, SK), and were as follows: 35.3 μg NO_3-N g^{-1}; 13.4 μg P g^{-1}; 649.6 μg K g^{-1}; 23.0 μg SO_4-S g^{-1}; 1.1 μg Cu g^{-1}; 35.6 μg Mn g^{-1}; 11.3 μg Zn g^{-1}; 2.1 μg B g^{-1}; 20.2 μg Fe g^{-1}; 13.4 μg Cl g^{-1}; pH 7.3; conductivity 0.5 mS cm^{-1}.

One hundred and fifty grams of soil-sand mix was used to fill a cone-tainer, and two uniform seedlings were transferred into the cone-tainer in the centre. One milliliter of the appropriate bacterial suspension (approximately 10^6 cfu) was pipetted into the transplant hole. The control treatment received 1 mL of autoclaved suspension of *B. licheniformis* S17. Cone-tainers were placed in a growth chamber (photosynthetic irradiance of ~450-500 μE m^{-2} s^{-1}) at 24°C under a 14 h light / 10 h dark cycle, and watered daily with distilled water. The plants were grown for 30 d, and total biomass was determined at harvest. The plants were washed, oven dried at 65°C for 48 h, and the oven-dry weight determined.

5.2.3.3 Experimental design

The experiment was conducted using a completely randomized design with five replications.

5.2.3.4 Statistical analysis

Effects of SAB treatments on shoot, root, and total biomass of wheat were tested using a one-way analysis of variance (ANOVA). Normality of distributions and homogeneity of variances were assessed before conducting any statistical analysis. Statistical analyses were performed using SPSS software version 16.0 for Windows (SPSS Inc., 2008).

5.3 Results

5.3.1 Isolation and identification of spore-associated bacteria

A total of 16 bacteria were isolated from the disinfested AMF spores of which 94% were identified by FAME analysis with SI ≥ 0.3 (Table 5.1). One of the isolates was not identified due to its slow growth. The identified isolates were taxonomically distributed into two phyla (*Actinobacteria* and *Firmicutes*), four genera (*Arthrobacter, Bacillus*, *Micrococcus*, and *Paenibacillus*), and nine species (Table 5.1). Spores of *G. mosseae* produced the highest number of isolates, i.e., approximately 44% of the isolated bacteria. Thirty-eight percent of the isolates were from *G. clarum*, and 19% from *G. intraradices*. A significant number of the SAB were *Bacillus* species, comprising 70% of the total bacterial isolates, and *Bacillus pumilus* was the most dominant (25%) of the bacteria isolates. *Arthrobacter* species were isolated from the spores of *G. clarum* and *G. mosseae* but not from *G. intraradices*. *Paenibacillus polymyxa* was isolated from *G. clarum* spores, and *Micrococcus luteus* was isolated from spores of *G. mosseae*. As determined on TSA, the cfu of the SAB used as inoculant after 48 h of growth ranged from 10^6 to 10^9, with *B. megaterium* isolated from *G. intraradices* exhibiting the lowest growth, and *B. pumilus* from *G. clarum* the highest.

5.3.2 Total biomass promotion

In general, shoot, root, and total dry weight of wheat were reduced by inoculation with SAB (Table 5.2). Shoot dry weight varied from 73-99% of the control; root dry and total weight varied by 75-103% and 75-97%, respectively. However, these inhibitory effects were not significant ($p \leq 0.05$).

Table 5.1 Identification of spore-associated bacteria (SAB) isolates by FAME profiles.

Isolate	Source	FAME	SI†
S20	*Glomus clarum*	*Arthrobacter atrocyaneus*	0.280‡
S15	*G. mosseae*	*A. ilicis*	0.740
S17	*G. mosseae*	*Bacillus licheniformis*	0.621
S6	*G. intraradices*	*B. licheniformis*	0.619
S14	*G. mosseae*	*B. licheniformis*	0.617
S12	*G. mosseae*	*B. marinus*	0.302
S3	*G. clarum*	*B. megaterium*	0.559
S7	*G. intraradices*	*B. megaterium*	0.428
S2	*G. clarum*	*B. pumilus*	0.790
S18	*G. clarum*	*B. pumilus*	0.803
S11	*G. mosseae*	*B. pumilus*	0.612
S16	*G. mosseae*	*B. pumilus*	0.586
S19	*G. clarum*	*B. psychrosacchrolytieus*	0.675
S13	*G. mosseae*	*Micrococcus luteus*	0.646
S1	*G. clarum*	*Paenibacillus polymyxa*	0.847

†SI: similarity index. Similarity index is a value that compares the fatty acid composition of an unknown bacterium (i.e., bacterium to be identified) with the average of fatty acid composition of known bacteria strains in the MIDI library.
‡Although SI for *A. atrocyaneus* was less than 0.3, it was included.

5.4 Discussion

Similar to the findings of others (Mayo et al., 1986; Walley and Germida, 1996; Budi et al., 1999; Xavier and Germida, 2003a; and Bharadwaj et al., 2008b) spores of AMF *G. clarum*, *G. intraradices*, and *G. mosseae* harboured a variety of bacteria. The SAB belonged to the genera of *Arthrobacter, Bacillus, Micrococcus, and Paenibacillus*, all of which are among the frequently reported SAB (Azcon-Aguilar et al., 1986; Budi et al., 1999; Xavier and Germida, 2003a; Bharadwaj et al., 2008b). Although *Bacillus* species were the most common SAB, results showed that different bacterial communities are associated with each AMF. For example, *Arthrobacter* species found on the spores of *G. clarum* and *G. mosseae* were not observed on spores of *G. intraradices*. Furthermore, a

P. polymyxa was isolated only from *G. clarum* and the only *Micrococcu*s species (*M. luteus*) observed was isolated from spores of *G. mosseae.*

Table 5.2. Shoot, root, and total dry weight of wheat inoculated with the spore-associated bacteria (SAB) 30 days after planting (DAP). n = 5.

Isolate	Shoot weight†	Root weight†	Total weight†
		mg plant^{-1}	
Control	78.96	49.84	128.80
A. atrocyaneus S20	77.38	42.61	119.98
A. ilicis S15	57.97	38.13	96.09
B. licheniformis S17	68.83	47.23	116.07
B. marinus S12	68.13	45.59	113.72
B. megaterium S3	70.03	47.00	117.03
B. pumilus S18	73.23	51.29	124.53
B. psychrosacchrolytieus S19	74.33	42.58	116.92
M. luteus S13	66.09	37.33	103.43
P. polymyxa S1	78.55	45.55	124.10

†No significant differences.

Similar results have been reported by Roesti et al. (2005) and Bharadwaj et al. (2008b). In a study to assess the bacteria associated with the spores of *G. geosporum* and *G. constrictum*, Roesti et al. (2005) observed a *Pseudomonas* species on spores of *G. constricum*, but this species was absent on spores of *G. geosporum*. In addition, 84% of the SAB isolated from *G. geosporum* were biopolymer-degrading genera (i.e., bacteria capable of hydrolysing biopolymers, including proteins and chitin) while only 73% of the isolates from *G. constrictum* were biopolymer-degraders when both were cultured with *Hieracium pilosella.* This implies that some biopolymer-degraders found on spores of *G. geosporum* were absent on *G. connstrictum* spores. Roesti et al. (2005) attributed these differences to the composition of spore wall, or exudates released by the AMF itself. In addition, Bharadwaj et al. (2008b) observed *Acidovorax delafieldii* on spores of *G. mosseae*, but not on spores of *G. intraradices*. Spore traits, such as wall thickness, were

reported as one of the key factors that determine the occurrence and abundance of SAB (Bharadwaj et al., 2008b).

Effects of host plant on the number and species of SAB also have been reported (Roesti et al., 2005; Long et al., 2008; Bharadwaj et al., 2008b). Although Roesti et al. (2005) and Bharadwaj et al. (2008b) found that the influence of AMF species was greater on SAB than host plant species, Long et al. (2008) reported that host plant effect was equally important. Furthermore, Long and co-workers (2008) observed the influence of culture substrate (growth medium) on SAB. They explained that growth of different bacterial populations may occur as a result of differences in physical and chemical conditions of growth medium. The variations in spore type, host plants, and growth conditions may explain why different studies found different SAB to associate with AMF spores. Roesti et al. (2005), for instance, reported the genera of *Cellvibrio*, *Chondromyces*, *Flexibacter*, *Lysobacter*, and *Pseudomonas* to associate with the disinfested spores of *G. geosporum* and *G. constricum*, but were not detected in this study, or the study of Long et al. (2008).

Treatment of the spores with 5% chloramine-T solution may have preferentially eliminated Gram negative bacteria. All the SAB observed in this study were Gram positive, while Gram negative bacteria such as *Agrobacterium, Burkholderia*, *Flavobacterium*, and *Pseudomonas* species that commonly associate with AMF (Mayo et al., 1986; Xavier and Germida, 2003a; Bharadwaj et al., 2008b) were not detected. Similarly, using this method of decontamination, Xavier and Germida (2003a) found that all the bacteria isolated from the decontaminated *G. clarum* spores were Gram positive, whereas both Gram positive and Gram negative bacteria were isolated from the non-decontaminated spores. Nonetheless, Bharadwaj et al. (2008b) found both Gram negative and Gram positive bacteria on surface decontaminated *G. intraradices* and *G. mosseae* spores. They used PBS, a buffer solution, for surface decontamination. This implies that the method of decontamination influences the number and type of SAB. Additionally, the successful elimination of some bacteria using chloramine-T solution may clarify why about 70% of the SAB in this study were *Bacillus* species. It is possible that *Bacillus* species are those resistant to decontamination with chloramine-T solution even under harsh treatment conditions (Walley and Germida, 1996; Xavier and Germida, 2003a).

Xavier and Germida (2003a) also reported about 80% of SAB isolated from the disinfested spores of *G. clarum* NT4 were *Bacillus* species.

Total biomass assay of inoculated wheat revealed that the SAB isolates failed to enhance growth of spring wheat and thus are not considered PGPB. In fact, all isolates inhibited the growth of the plant. This is contrary to the observation made by Budi et al. (1999). Budi and co-workers (1999) found that a *Paenibacillus* species isolated from the mycorrhizosphere of *Sorghum bicolor* increased the shoot and root weights of mycorrhizal tomato plants. The growth stimulation was related to the ability of the bacteria and its metabolites to inhibit radial growth of fungal pathogens such as *Phytophthora parasitica*, *Fusarium oxysporum*, and *Rhizoctonia solani*. Bharadwaj et al. (2008a) found that some SAB increased the number of primary and lateral roots of potato and its shoot and root length, but had no significant effect on the plants shoot, root, and total fresh weight.

In their efforts to assess the effect of SAB isolated from disinfested spores of *G. clarum* NT4 on pea-AMF symbiosis, Xavier and Germida (2003a) reported that the two SAB isolates *B. pabuli* LA3 and *B. chitinosporus* LA6a studied had no significant effect on shoot and root dry weight of pea (*Pisum sativum* L.) compared with the uninoculated control. In fact, *B. pabuli* LA3 reduced plant root biomass, though not significantly. Interestingly, plants inoculated with *B. pabuli* LA3 had a significantly higher shoot nitrogen (N) and P content compared with plants inoculated with the other isolate *B. chitinosporus* LA6a, and the uninoculated control. Furthermore, *B. pabuli* LA3 promoted the hyphal growth of the disinfested spores. These beneficial effects of *B. pabuli* LA3 on hyphal growth were related to stimulatory non-volatile compounds produced by the SAB. Their findings imply that the stimulatory compounds produced by these organisms may not be involved in growth promotion of their host plant; moreover, *B. pabuli* LA3 had a stimulatory effect on the spores but had no beneficial effect on the root and shoot dry weight of pea.

Andrade et al. (1995) also reported a resemblance of *B. simplex* isolated from surface-sterilized *G. mosseae* spores (Azcon-Aguilar et al., 1986) that had no significant effect on the total biomass production by pea. It is clear that some SAB perform functions not associated with growth promotion which suggest that the role, if any, of SAB is likely

limited to association with AMF spores directly, and is not linked to any direct effects on the host plant. Future studies are required to understand how SAB affect AMF performances and to determine their roles as inhabitants of the AMF spores.

5.5 Conclusion

Disinfested spores of *G. clarum*, *G. intraradices*, and *G. mosseae* harboured certain bacteria species, and these bacteria varied with AMF species. *Bacillus* species were the most common SAB, associating with AMF spores irrespective of AMF identity. Although this may be an artefact of the decontamination procedure, the possibility exists that these *Bacillus* species play a biologically significant role in the AMF symbioses. None of the SAB had beneficial effects on wheat growth; they all inhibited the total biomass of the plant. Further studies may clarify the observed inhibitory effect and identify the functions of these organisms on disinfested spores of the studied AMF species.

6. EFFECTS OF ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT GROWTH-PROMOTING RHIZOBACTERIA ON GLOMALIN PRODUCTION UNDER GNOTOBIOTIC CONDITIONS

6.1 Introduction

The effects of any soil microorganism on ecosystem functioning and productivity are determined by complex interactions with other soil inhabitants (Johnson et al., 1997; Requena et al., 1997; Biró et al., 2000). Thus, to fully understand the contributions of arbuscular mycorrhizal fungi (AMF) (van der Heijden et al., 1998b), especially in terms of glomalin production (Nichols and Wright, 2006), it is vital to consider their interactions with other soil microorganisms (Purin and Rillig, 2008). Although a number of studies have identified several microbial interactions with AMF (Hodge, 2000; Barea et al., 2002), interactions between AMF and plant growth-promoting rhizobacteria (PGPR) are among the most important (Andrade et al., 1995; Walley and Germida, 1997; Barea et al., 2002; Roesti et al., 2006). Arbuscular mycorrhizal fungi govern photosynthate deposition into the mycorrhizosphere, which increases bacterial population in the root zone (Hodge, 2000; Treseder and Allen, 2000). Also, AMF enhance the activities of phosphate solubilizing rhizobacteria by acting as a bridge between phosphate solubilized by PGPR and plant roots (Toro et al., 1997; Barea et al., 2002). Likewise, PGPR stimulate mycorrhizal symbioses by increasing the number of vesicles (Azcòn, 1987) and promoting AMF colonization (Toro et al., 1997; Requena et al., 1997; Dwivedi et al., 2009). Importantly, these studies showed that beneficial AMF and PGPR interactions promote plant growth and development.

Nonetheless, AMF and PGPR interaction effects cannot be generalized. Vazquez et al. (2000) found that the inoculation of *Glomus deserticola* decreased the population of fluorescent pseudomonads in corn (*Zea mays* L.) rhizosphere, which they attributed to a reduction in rhizosphere carbon (C) levels caused by the AM fungus. Walley and Germida (1997) reported a PGPR strain that reduced spore germination and root colonization by *G. clarum*. Recently, Dwivedi et al. (2009) also demonstrated that a phenazine producing strain of PGPR reduced AMF colonization. Also, there are reports that the interactions between some AMF and PGPR species reduce plant growth and

nutrient uptake (Requena et al., 1997; Walley and Germida, 1997). The interaction between *G. coronatum* and an exotic or a native PGPR strain decreased shoot nitrogen (N) content of *Anthyllis cytisoides* relative to the individual effects of the rhizobacteria (Requena et al., 1997). Walley and Germida (1997) reported interactions between *G. clarum* NT4 and *Pseudomonas cepacia* R85 that reduced the beneficial effect on R85 on wheat (*Triticum aestivum* L.) growth. These studies show that functional incompatibilities exist between some AMF and PGPR species.

Because the beneficial interactions between compatible AMF and *Rhizobium* species resulted in growth and yield increases of pea (*Pisum sativum* L.), Xavier and Germida (2003b) suggested that, through careful selection, it may be possible to identify compatible microsymbionts. This implies that there may be potential to select AMF and PGPR combinations that enhance glomalin production by AMF. The aim of this study was to examine AMF and PGPR interactions that could increase glomalin production in the rhizosphere of pea. Since the rhizosphere is an interaction-rich environment, effects of bacteria and AMF should first be observed under sterile conditions, where other microbial effects are excluded (Purin and Rillig, 2008). Thus, this study was conducted using a growth medium described by Wright and Upadhyaya (1999) that eliminates other soil organisms and reduces contamination. Additionally, using this approach helps in determining glomalin-related soil protein (GRSP) concentrations in the rhizosphere and mycorrhizosphere of the host plant, and those deposited on horticultural film, which was used to trap glomalin (Wright and Upadhyaya, 1999).

6.2 Materials and Methods

6.2.1 Plant growth-promoting rhizobacteria

Plant growth-promoting rhizobacteria *Pseudomonas cepacia* R55 and R85, *P. aeruginosa* R75, *P. putida* R105, and *P. fluorescence* R111 were selected based on the findings of de Freitas and Germida (1990a). In addition, results of growth chamber and laboratory experiments (Chapter 4) showed that, after about two decades of storage, these strains still possess the desirable attributes reported by de Freitas and Germida (1990a). Although the PGPR strains inhibited wheat seedling emergence, they all increased total biomass of the plant compared with the uninoculated control ($p = 0.001$).

Plant growth-promoting rhizobacteria isolates were inoculated into 40 mL of King's B (KB) medium and grown on a rotary shaker (110 rev min^{-1}) for 48 h at 27°C. Bacterial suspensions were centrifuged (15 min at 5000 × g), and the KB medium was decanted. Cultures were washed two times in phosphate buffered saline (PBS), and then suspended in 40 mL sterile tap water. One milliliter of bacterial suspension contained approximately 10^6 to 10^8 colony forming units (cfu) determined on KB medium supplemented with antibiotics (5 mg chloramphenicol, 75 mg cycloheximide, 45 mg novobiocin and 75,000 units penicillin G L^{-1}) (Sands and Rovira, 1970).

6.2.2 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi *G. clarum*, *G. intraradices*, and *G. mosseae* were selected based on preliminary findings that indicated that there were no significant differences among these AMF species in respect to glomalin production. Spores of the three AMF species were extracted from 20 g samples of root-soil mixtures in which they were propagated. The propagation and extraction procedure were described in Section 3.2.1.

6.2.3 Growth medium preparation, inoculation, and planting

Growth medium was prepared using the method developed by Wright and Upadhyaya (1999), described in Section 3.2.2. However, three strips (8 × 3 cm) of horticultural film (UV-treated polyethylene with "Microfunnels®" that permits air and water flux through the fabric; WeedBlock, Easy Gardner, Waco, Tex., USA) were inserted in the mycorrhizosphere. Two strips were vertically inserted at opposite sides along the outer wall of nylon mesh pouch positioned at the centre of the sand mix; the third strip was placed in the bottom of each pot below the sand mix (Figures 6.1). Planting and inoculation were modified as follows: surface-sterilized pea seeds were aseptically germinated on 1.5% water agar for 5 d and four uniform sterile seedlings were aseptically placed at the centre of the enclosed autoclaved medium. The PGPR treatment was applied by pipetting 1 mL of the appropriate bacterial suspension (approximately 10^6 cfu) into the transplant hole. For the AMF treatment, 100 spores of appropriate AMF were placed in the transplant hole. Arbuscular mycorrhizal fungi and PGPR treatments were applied by

pipetting 1 mL of the PGPR suspension into the transplant hole along with 100 spores of AMF.

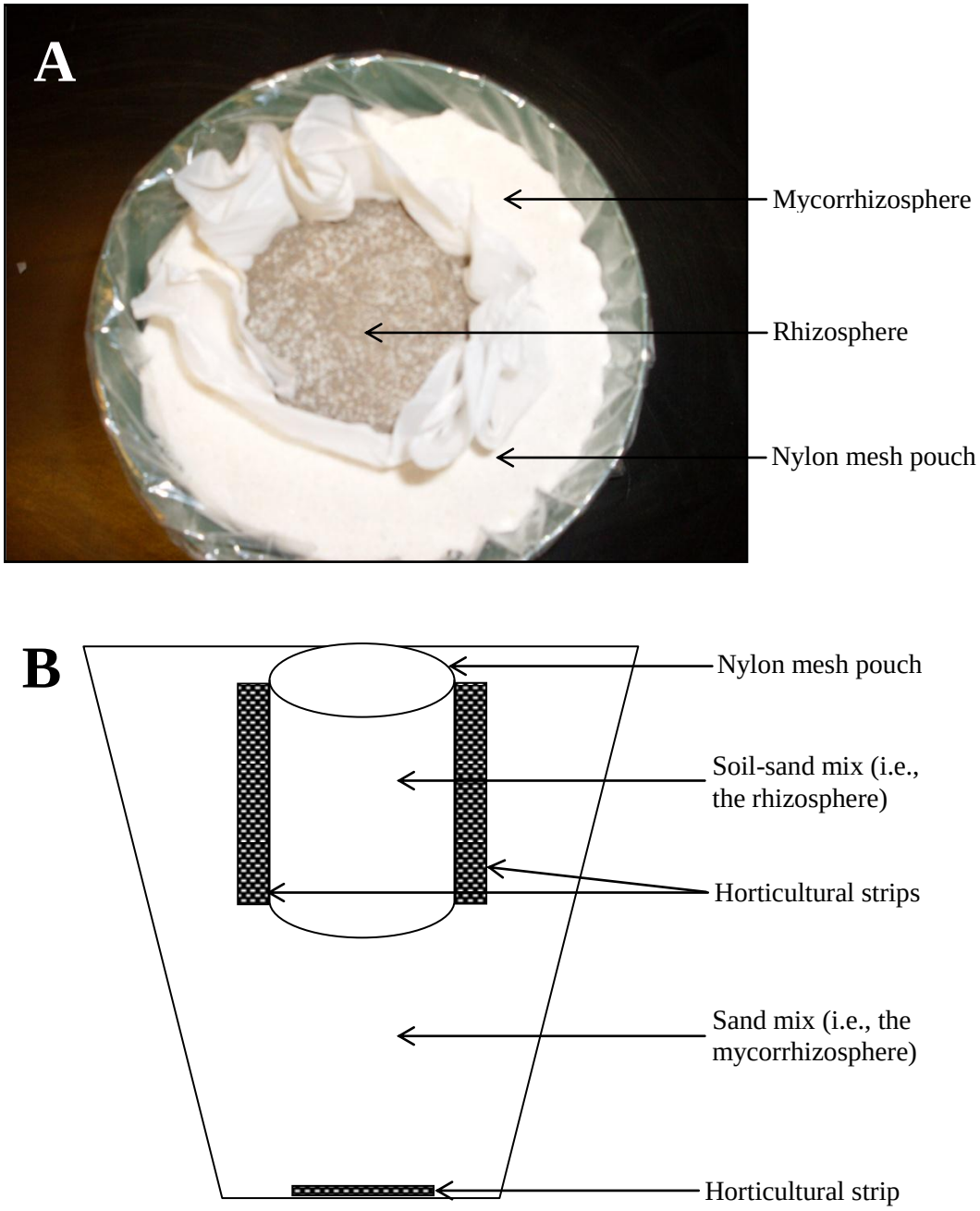

Fig. 6.1. A photograph (A) and schematic drawing (B) of the growth medium showing the separation of the rhizosphere (soil-sand mix) from the mycorrhizosphere (sand mix) using a nylon mesh pouch. The schematic drawing (not drawn to scale) shows the placement of strips of horticultural film in the mycorrhizosphere.

The plants were thinned to two per pot after seedling emergence. One hundred milliliters of a low phosphorus (P) Hoagland's nutrient solution (Millner and Kitt, 1992) was supplied to each pot every week to replenish soil nutrients. The physical and chemical properties of the growth medium were determined by ALS laboratory (Saskatoon, SK) and were as follows: sandy loam; 30.8 μg NO_3-N g^{-1}; 39.2 μg P g^{-1}; 565.5 μg K g^{-1}; 53.2 μg SO_4-S g^{-1}; 1.5 μg Cu g^{-1}; 227.4 μg Mn g^{-1}; 3.8 μg Zn g^{-1}; 2.1 μg B g^{-1}; 16.2 μg Fe g^{-1}; 31.4 μg Cl g^{-1}; pH 7.2; conductivity 0.7 mS cm^{-1}. This experiment was conducted in a growth chamber under the following conditions: 25˚C, 16 h day and 20˚C, 8 h night, 375-400 μE m^{-2} s^{-1} of irradiance and relative humidity of 60%. Soil moisture was maintained at 60% water holding capacity.

6.2.4 Experimental design

The experiment was initially designed as a three by five factorial design with AMF (n = 3) as the first factor and PGPR (n = 5) as the second factor. However, combinations of R75 and R85 with *G. mosseae* were not included because of an insufficient supply of *G. mosseae* spores. Control treatments were uninoculated pea and pea inoculated with the autoclaved suspension of R111. The experiment was set up in a completely randomized design with four replicates, for a total of 92 pots.

6.2.5 Plant analysis

Twelve weeks after planting (WAP), plants were harvested and the effects of the microbial inoculants were observed on shoot biomass and N and P content. Shoot biomass was determined after washing and oven-drying at 65˚C for 48 h. To analyze shoot N, shoot material was ground with a ball mill, and shoot N measured using a LECO CNS 2000 automated combustion analyzer (LECO Instruments Ltd., St. Joseph, MI). For shoot P analysis, the plant material was digested in sulphuric acid (Thomas et al., 1967), and P content measured using an Auto Analyzer II Technicon® system (Technicon Industrial Systems, Tarrytown, USA).

6.2.6 Glomalin extraction

The rhizosphere (soil-sand mix) was separated from mycorrhizosphere (sand mix), and the sand particles on the horticultural strips removed using a sterile spatula. Easily extractable glomalin-related soil protein (EE-GRSP) was obtained from the soil-sand mix in 20 m*M* sodium citrate (pH 7.0) and the mixture autoclaved at 121˚C for 30 min (Wright and Upadhyaya, 1996; Rillig, 2004b). Glomalin-related soil protein was extracted from the soil-sand and sand mixes using 50 m*M* sodium citrate solution (pH 8.0) and autoclaved at 121˚C for 60 min. To obtain GRSP from the soil-sand mix, the extraction procedure was repeated until the supernatant was straw-coloured, which needed five extraction cycles. Glomalin-related soil protein extracted from the sand mix also involved five extraction cycles each, though the extracts were colourless. All the extractions were performed with 1 g of sample in 8 mL extractant. Samples were centrifuged at 5,000 × g for 15 min immediately after extraction. Glomalin-related protein (GRP) deposited on the horticultural strips was extracted in 6 mL of 20 m*M* sodium citrate solution (pH 7.0) followed by autoclaving for 60 min at 121˚C. Strips were first cut into small pieces and placed in glass vials before the extraction procedure (Wright and Upadhyaya, 1999). The supernatant containing the extracted protein was decanted and stored at 4˚C for analysis.

6.2.7 Glomalin quantification

As described by Wright and Upadhyaya (1996), EE-GRSP and GRSP concentrations were determined by the Bradford dye-binding protein assay, and these are reported as easily extractable Bradford-reactive soil protein (EE-BRSP) and Bradford-reactive soil protein (BRSP), respectively (Rillig, 2004b). Glomalin-related protein from the horticultural strip was quantified as Bradford-reactive protein (BRP). The assay was performed using 96-well plates. Protein standards in a range of 1.25 to 5 μg protein per well were prepared using bovine serum albumin (BSA). An extract from each sample was pooled and centrifuged at 10,000 × g for 5 min to remove residual soil particles and other insoluble materials. Duplicate wells of the 96-well assay plate were loaded with 25 μL of EE-GRSP and 50 μL of GRSP extract obtained from the soil-sand mix. For the sand mix and strip extracts, wells were loaded with 150 μL of the GRSP and GRP extracts,

respectively. Appropriate volumes of PBS were added to each well to achieve a total of 200 μL of protein-PBS mix per well. Fifty microlitres of Bio-Rad dye (Bio-Rad, Laboratories, Inc., CA) was then added to each well, mixed thoroughly, and the absorbance read at a wavelength of 590 nm (A_{590}) within 5 min of addition. A standard curve was generated by plotting optical density values against protein of known concentration (BSA). Protein concentrations in micrograms per well of GRSP and GRP extracts were calculated from the equation of the line generated from the curve. Prior to the Bradford assay, strips and sand-mix extracts were concentrated to detect low glomalin levels (Wright and Upadhyaya, 1999). Extracts were concentrated using a PIERCE Reati-Therm-III TM heating module evaporator. Because preliminary observations showed that heating may increase estimates of glomalin concentrations, extracts were evaporated by blowing N_2 gas over the extracts without heating.

6.2.8 Statistical analysis

Analysis of variance (ANOVA) was performed using SPSS software version 16.0 for Windows (SPSS Inc., 2008) to determine any significant microbial effect on shoot biomass, N and P content, BRSP concentrations in the rhizosphere and mycorrhizosphere, and BRP on horticultural strips inserted into the mycorrhizosphere. The treatment means were compared using the least significant difference (LSD) test at a significance level of 0.05.

Where interactions between AMF and PGPR were not significant, data are presented separately (i.e., AMF treatments and PGPR treatments are presented in separate figures); if interactions were significant, all data are presented in a single figure.

6.3 Results

6.3.1 The effects of inoculants on plant growth

The inoculation of pea with AMF and PGPR, both singly and in combination, affected shoot and seed weight of pea (Table 6.1; a detailed summary of the results is presented in Appendix D.1); however, interaction effects were not significant. *Glomus mosseae* significantly ($p < 0.001$) increased shoot weight of pea compared with other AMF species and non-AMF control treatments (i.e., control + non-AMF PGPR treatments) (Figure

6.2A). The PGPR strains also influenced shoot dry weight of pea significantly ($p = 0.014$) (Figure 6.2B). Inoculation with R55 increased the shoot biomass of the plant compared to R85 and R105, although no significant increases relative to the control was detected (Figure 6.2B). Treatment of pea with R85 caused a decrease in the shoot dry biomass of pea relative to the control. Generally, the highest shoot biomass was found in the *G. mosseae* treatment and lowest was observed from plants inoculated with R85.

Table 6.1. Statistical analysis (probability) and significance for shoot and seed dry weight of pea inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Source of Variation	df	Shoot	Seed
		Probability	
Total	91		
AMF	3	0.000**	0.000**
PGPR	5	0.014*	0.111
AMF * PGPR	13	0.339	0.482
Error	70		

*, **Significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

There was also a significant mycorrhizal effect on seed weight of pea, with *G. mosseae* significantly ($p < 0.001$) increasing seed weight of the plant compared with the other AMF species and the control (Figure 6.2A and Table 6.1). Effects of the PGPR and their interactions with the AMF species did not significantly affect seed weight of pea (Table 6.1). These observations indicate that of all the microbial inoculants, only *G. mosseae* had significant beneficial effects on pea growth.

6.3.2 The effects of inoculants on plant nitrogen and phosphorus

The individual effect of AMF and the PGPR on N uptake by pea was significant, although their interaction was not (Table 6.2; see Appendix D.3 for data summary). *Glomus mosseae* significantly ($p < 0.001$) increased N uptake by pea compared with other AMF species and the non-AMF control treatments (Figure 6.3A). *Pseudomonas*

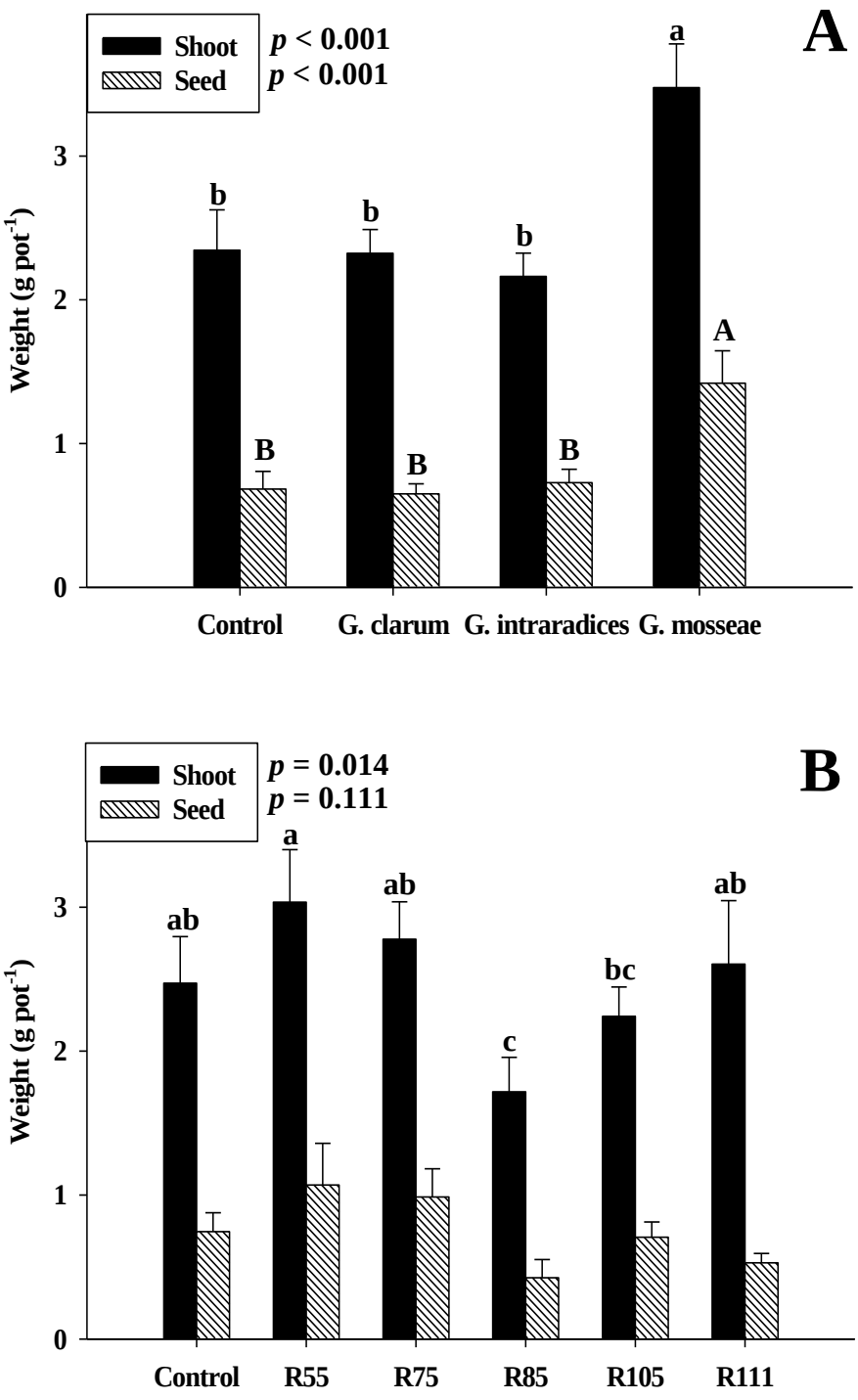

Fig. 6.2. Main effects of arbuscular mycorrhizal fungi (AMF) (A) and plant growth-promoting rhizobacteria (PGPR) (B) on shoot and seed dry weight of pea 12 weeks after planting (WAP). Error bars are standard errors of the mean (n = 4). Means followed by the same letter are not significantly different according to the least significant difference (LSD) test ($p \leq 0.05$). The *p*-value is for the analysis of variance (ANOVA). Lower case denotes comparison between shoot. Upper case denotes comparison between seed.

cepacia R55 significantly (p = 0.05) enhanced N uptake by pea compared with R85, R105, and the non-PGPR control treatments (Figure 6.3B). However, the effect of R55 was similar to R75 and R111, and there were no significant differences between R85, R105, and the control in their effect on N uptake.

Only the mycorrhizal treatment had a significant (p = 0.006) effect on N concentration in the plant tissue (Figure 6.4 and Table 6.2). Shoot N concentration decreased in response to inoculation with AMF, but was significant only for *G. clarum* (Figure 6.4). In contrast, all the PGPR strains increased N concentration of pea shoot compared with the non-PGPR treatments although significant differences were only detected at p = 0.076.

Table 6.2. Statistical analysis (probability) and significance for shoot nitrogen (N) and phosphorus (P) concentrations in pea tissue and N and P uptake by pea inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Source of Variation	df	Shoot N uptake	Shoot N concentration	Shoot P uptake	Shoot P concentration§
		Probability			
Total	91†				
AMF	3	0.000**	0.006**	0.001**	0.523
PGPR	5	0.050*	0.076	0.141	0.371
AMF * PGPR	13	0.783	0.500	0.175	0.488
Error	70‡				

†The total degree of freedom for shoot N and shoot N uptake is 82 due to missing values.
‡The error degree of freedom for shoot N and shoot N uptake is 61, due to missing values.
§There were not statistically significant effects detected.
*, **Significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Arbuscular mycorrhizal fungi also had a significant (p = 0.001) effect on P uptake by pea (Table 6.2). *Glomus mosseae* significantly enhanced P uptake compared with the other AMF and the non-AMF control treatments (Figure 6.5). Although the effect of *G. clarum* on P uptake was not significantly different from the non-AMF treatments, it was high compared with *G. intraradices*. However, the PGPR and their interaction with the

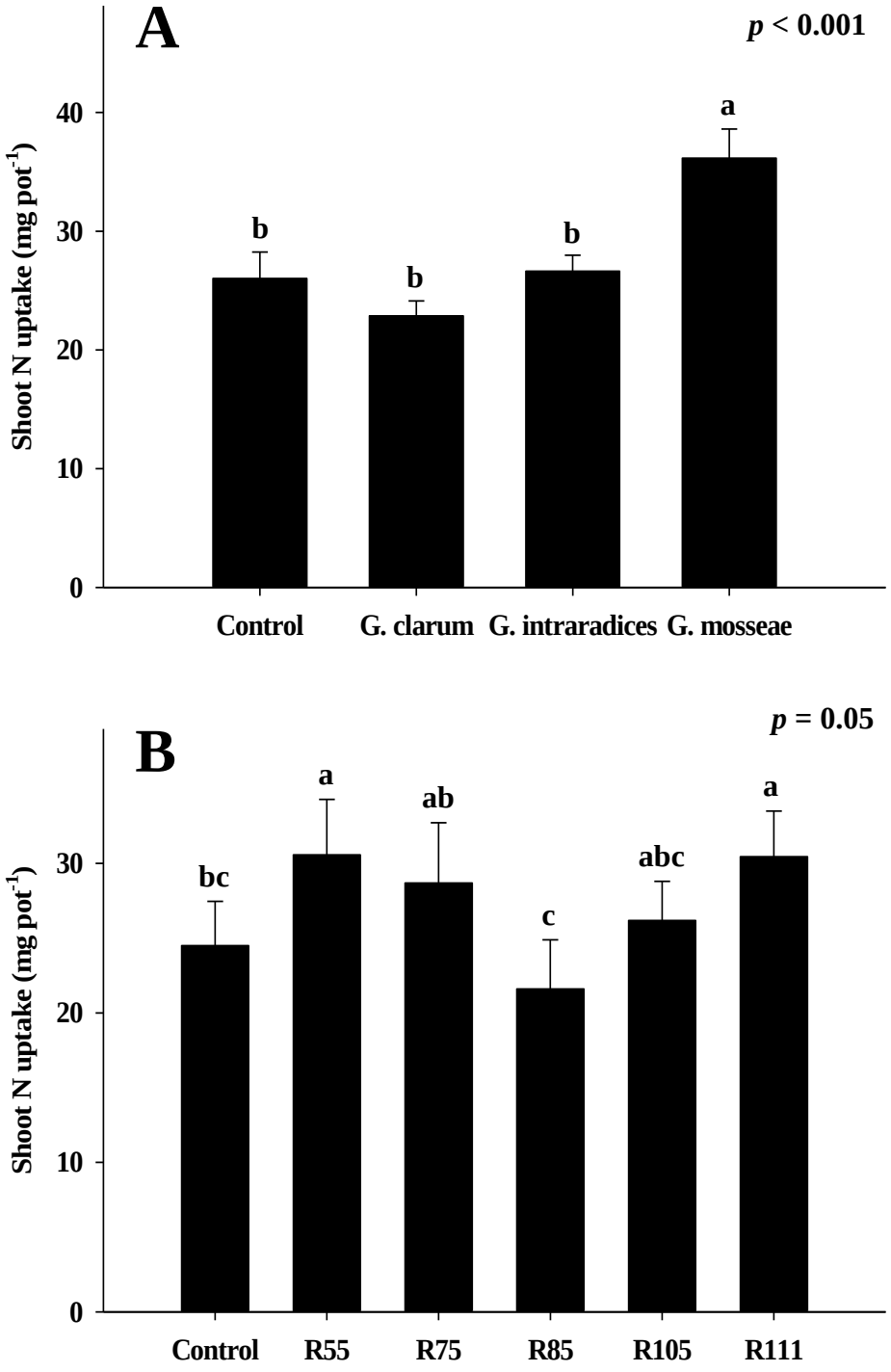

Fig. 6.3. Main effect of arbuscular mycorrhizal fungi (AMF) (A) and plant growth-promoting rhizobacteria (PGPR) (B) on shoot nitrogen (N) uptake by pea 12 weeks after planting (WAP). Error bars are standard errors of the mean (n = 4). Means followed by the same letter are not significantly different according to the least significant difference (LSD) test ($p \leq 0.05$). The *p*-value is for the analysis of variance (ANOVA).

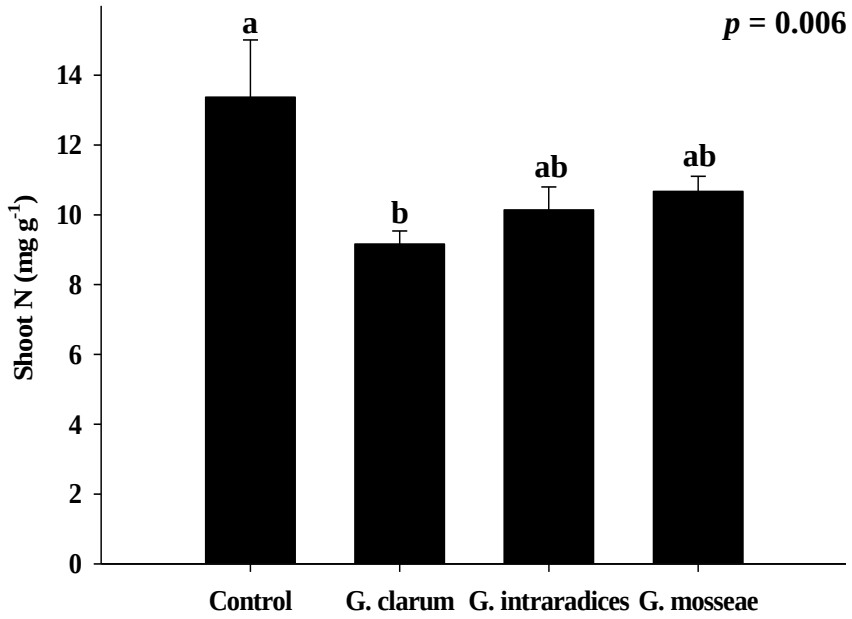

Fig. 6.4. Main effect of arbuscular mycorrhizal fungi (AMF) on shoot nitrogen (N) concentration of pea 12 weeks after planting (WAP). Error bars are standard errors of the mean (n = 4). Means followed by the same letter are not significantly different according to the least significant difference (LSD) test ($p \leq 0.05$). The *p*-value is for the analysis of variance (ANOVA).

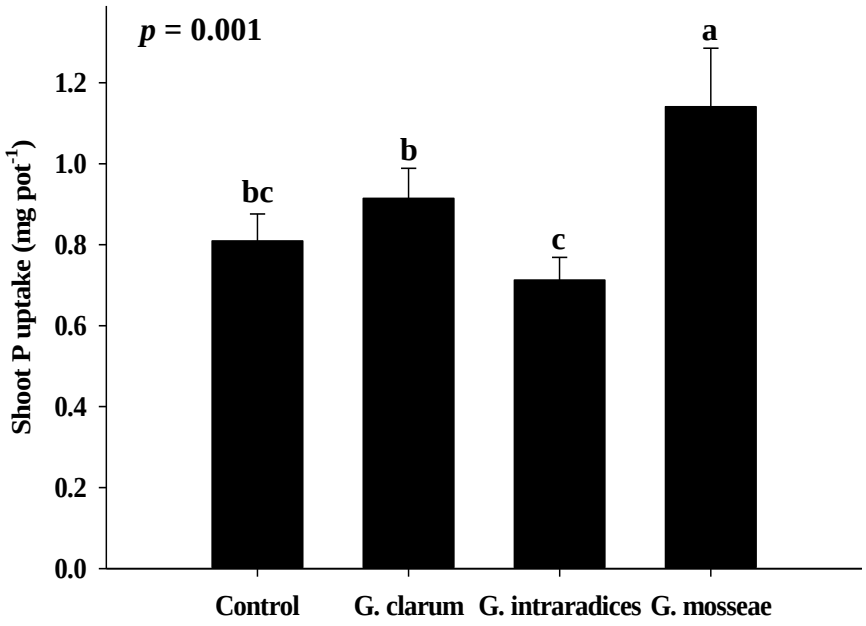

Fig. 6.5. Main effect of arbuscular mycorrhizal fungi (AMF) on shoot phosphorus (P) uptake by pea 12 weeks after planting (WAP). Error bars are standard errors of the mean (n = 4). Means followed by the same letter are not significantly different according to the least significant difference (LSD) test ($p \leq 0.05$). The *p*-value is for the analysis of variance (ANOVA).

AMF species did not significantly affect P uptake by the plant. None of the microbial inoculants applied singly or in combination significantly affected P concentration in pea shoot (Table 6.2).

6.3.3 The effects of inoculants on Bradford-reactive soil protein in the rhizosphere

Both the mycorrhizal and bacterial treatments significantly ($p < 0.001$) affected EE-BRSP concentration, but the amount of EE-BRSP was not significantly ($p = 0.061$) affected by the interactions between the AMF species and the PGPR strains (Table 6.3; a detailed summary of the results is presented in Appendix D.5). Interestingly, the amount of EE-BRSP was greater in the non-AMF treatments ($p < 0.001$) compared with the AMF treatments (Figure 6.6A). *Pseudomonas fluorescence* R111 had the greatest effect on EE-BRSP relative to the other PGPR strains and the non-PGPR treatment controls (Figure 6.6B).

Table 6.3. Statistical analysis (probability) and significance for concentrations of easily extractable Bradford-reactive soil protein (EE-BRSP) and Bradford-reactive soil protein (BRSP) in the rhizosphere, and BRSP and Bradford-reactive protein (BRP) deposited on strips inserted into the mycorrhizosphere inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Source of Variation	df	EE-BRSP	BRSP	BRP	BRSP
		Rhizosphere		Mycorrhizosphere	
		Probability			
Total	91				
AMF	3	0.000**	0.000**	0.046*	0.000**
PGPR	5	0.000**	0.000**	0.076	0.003**
AMF * PGPR	13	0.061	0.001**	0.084	0.000**
Error	70				

*, **Significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

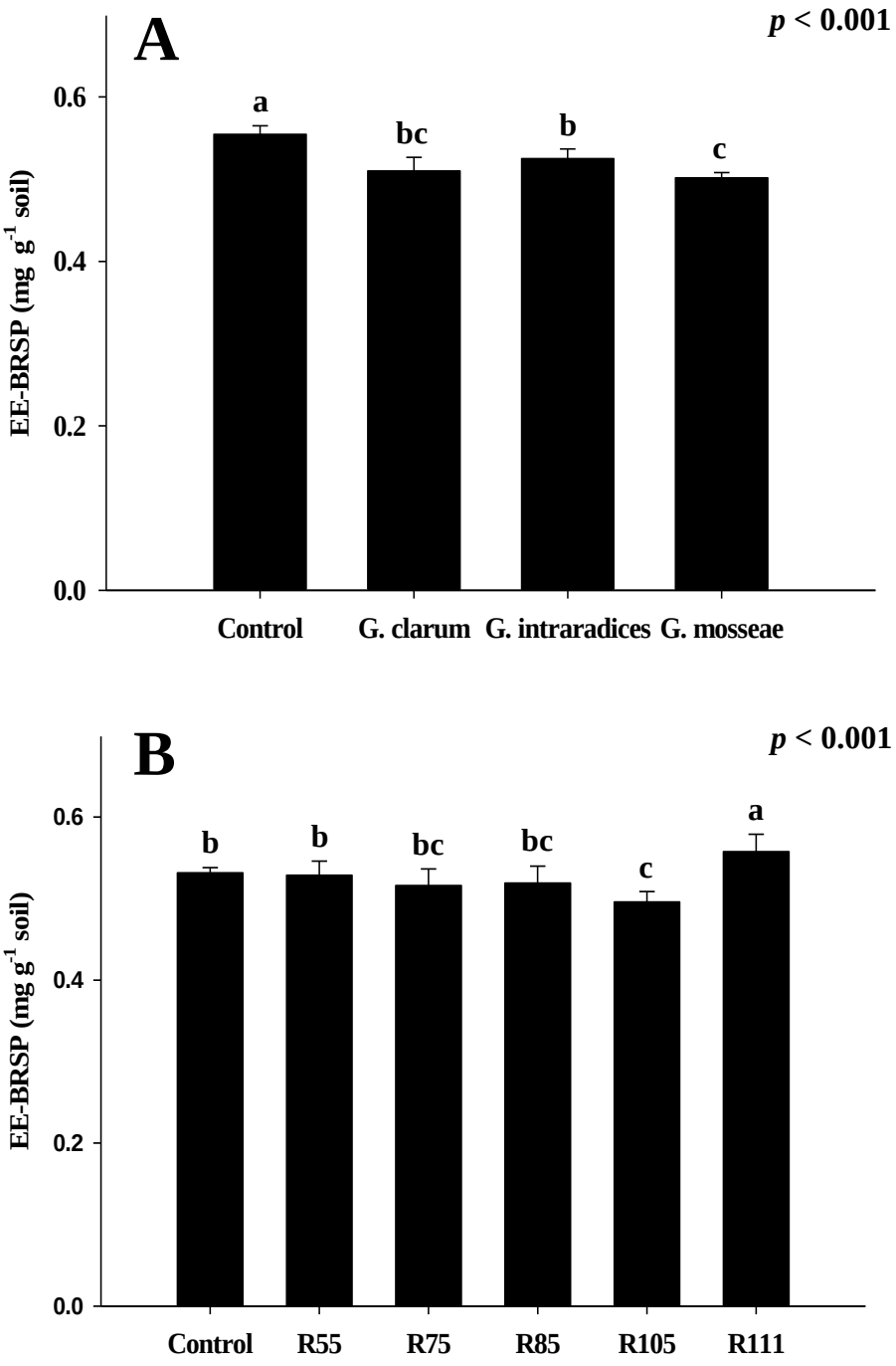

Fig. 6.6. Main effect of arbuscular mycorrhizal fungi (AMF) (A) plant growth-promoting rhizobacteria (PGPR) (B) on easily extractable Bradford-reactive soil protein in the rhizosphere of pea 12 weeks after planting (12 WAP). Error bars are standard errors of the mean (n = 4). Means followed by the same letter are not significantly different according to the least significant difference (LSD) test ($p \leq 0.05$). The *p*-value is for the analysis of variance (ANOVA).

Concentrations of BRSP also were significantly (p < 0.001) influenced by the microbial inoculants (Figure 6.7 and Table 6.3). There were significant (p = 0.001) interactions between the AMF species and the PGPR strains on BRSP levels. For example, the combination of *G. mosseae* and R105 or R111 significantly enhanced the total protein concentrations (32 and 36%, respectively) in the pea rhizosphere relative to single inoculation of *G. mosseae*. Conversely, all combinations of the PGPR strains and *G. clarum* produced lower BRSP concentrations when compared to *G. clarum* alone, though the reductions were not significant. Additionally, it is worth noting that, the interaction between R55 and the three AMF species were similar. The co-inoculation of R55 and *G. clarum*, *G. intraradices*, or *G. mosseae* yielded the lowest BRSP concentrations compared with other treatment combinations.

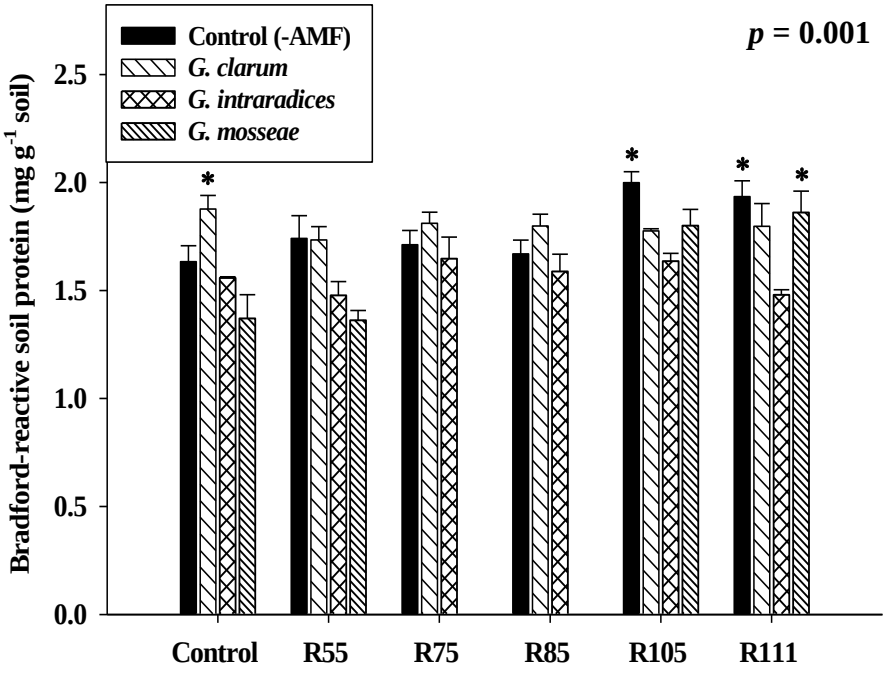

Fig. 6.7. Bradford-reactive soil protein (BRSP) in the rhizosphere of pea inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). Error bars are standard errors of the mean (n = 4). The *p*-value is for the analysis of variance (ANOVA). Asterisk (∗) denotes significantly different from the uninoculated control (i.e., -AMF and -PGPR) according to the least significant difference (LSD) test ($p \leq 0.05$).

6.3.4 The effects of inoculants on Bradford-reactive protein and Bradford-reactive soil protein in the mycorrhizosphere

Bradford-reactive protein is the estimate obtained from the horticultural strips inserted into the mycorrhizosphere (sand mix), whereas BRSP is the estimate from the sand mix itself. After concentrating these protein fractions they were readily detectable in all samples; however, values of BRP obtained from the horticultural strips were low compared with BRSP from the sand fraction. Only the main effect of the mycorrhizal treatments was significant (p = 0.046) with respect to glomalin deposited on the horticultural strips (Table 6.3; see Appendix D.5 for data summary). *Glomus intraradices* significantly increased BRP obtained from the strips compared with *G. clarum* (Figure 6.8). Nevertheless, this beneficial effect was not evident relative to other treatments, including the non-mycorrhizal control treatments.

The main effect of the AMF was significant (p < 0.001) on BRSP levels in the mycorrhizosphere of pea (Table 6.3). *Glomus intraradices* significantly enhanced BRSP compared with *G. clarum* and *G. mosseae*. The effect of *G. clarum* was higher than *G. mosseae*, though similar to non-AMF treatment controls. *Glomus mosseae* inoculation had no beneficial effect on BRSP concentrations in the mycorrhizosphere. It is interesting to note that the main effect of the PGPR also was significant on BRSP concentrations in the mycorrhizosphere (p = 0.003). *Pseudomonas cepacia* R85 increased the levels of BRSP relative to other treatments, with the exception of R75.

Similarly, there were significant (p < 0.001) effects of AMF and PGPR interactions on BRSP obtained from the mycorrhizosphere (Figure 6.9 and Table 6.3). The co-inoculation of *G. intraradices* with R75, R85, or R105 significantly increased BRSP concentrations in the mycorrhizosphere compared with the individual inoculation of *G. intraradices*. The differences among *G. intraradices* and other mycorrhizal treatments on BRSP concentrations in pea mycorrhizosphere increased as a result of R75, R85, and R105 inoculations; R85 having the highest effect. Hence, the best interaction effect on sand BRSP was that of R85 and *G. intraradices*. However, pairing *G. mosseae* with R105 or R111 significantly reduced BRSP in pea mycorrhizosphere compared with when *G. mosseae* was applied alone.

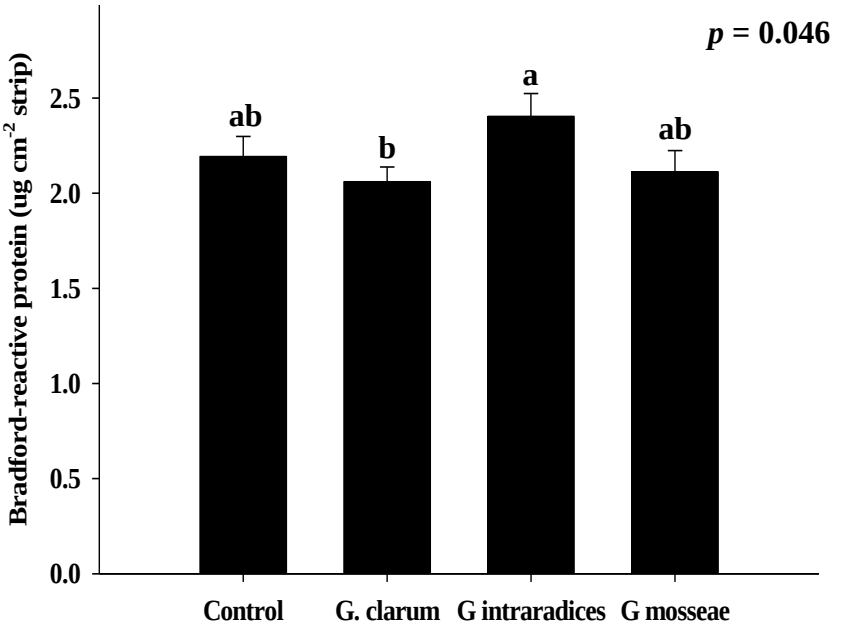

Fig. 6.8. Main effect of arbuscular mycorrhizal fungi (AMF) on Bradford-reactive protein (BRP) on horticultural film from the mycorrhizosphere of AMF inoculated pea, 12 weeks after planting. Error bars are standard errors of the mean (n = 4). Means followed by the same letter are not significantly different according to the least significant difference (LSD) test ($p \leq 0.05$). The *p*-value is for the analysis of variance (ANOVA).

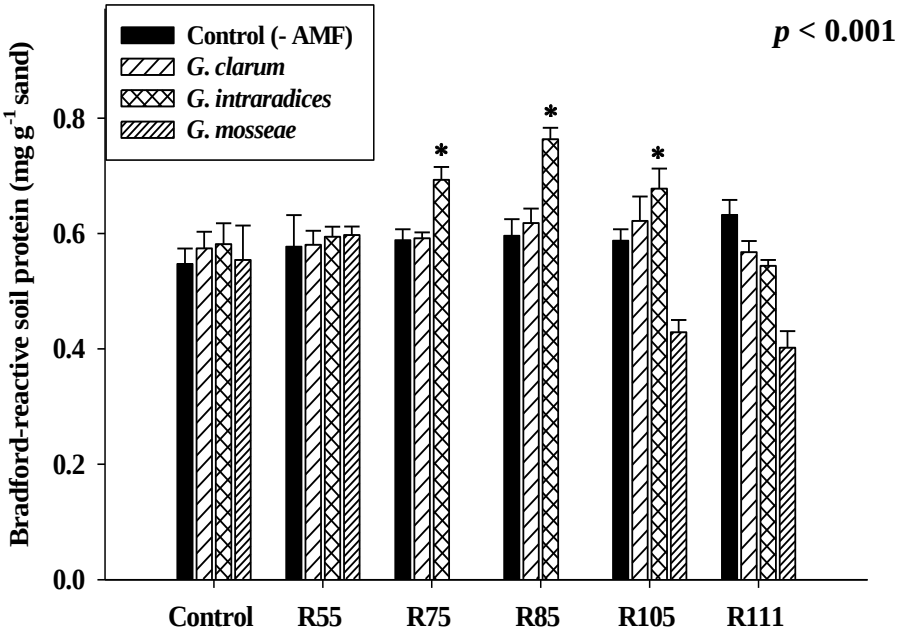

Fig. 6.9. Bradford-reactive soil protein (BRSP) in the mycorrhizosphere of pea inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). Error bars are standard errors of the mean (n = 4). The *p*-value is for the analysis of variance (ANOVA). Asterisk (*) denotes significantly different from the uninoculated control (i.e., -AMF and -PGPR) according to the least significant difference (LSD) test ($p \leq 0.05$).

6.4 Discussion

6.4.1 The effects of inoculants on plant growth

Mycorrhizal effects were evident on pea shoot growth as *G. mosseae* significantly enhanced shoot biomass of the plant relative to the other AMF species and the non-AMF treatments. This beneficial effect also extended to seed weight. Requena et al. (1997) reported a similar observation while exploring the interaction effects of some rhizosphere organisms on *A. cytisoides*. They found that inoculation of *A. cytisoides* with *G. intraradices* increased shoot weight of the plant compared with the non-inoculated control. Arbuscular mycorrhizal fungi are known for their beneficial effect on growth of their host (George et al., 1995; Richardson et al., 2009). They utilize several mechanisms to enhance their symbiotic association with the host plant. Mechanisms such as nutrient acquisition, biocontrol, and alleviation of cultural stress including drought, are well documented (George et al., 1995; Koide and Mosse, 2004; Richardson et al., 2009). Because this current study was conducted under a controlled environment, growth increases are likely only attributable to enhanced nutrient uptake by the AMF species.

Similar to the findings of Requena et al. (1997), significant differences were observed in the ability of the AMF species to stimulate plant growth. *Glomus mosseae* had greater effect on enhancing shoot weight than *G. clarum* and *G. intraradices*. Violi et al. (2007) also found significant differences in plant response to AMF inoculation using a sterile buffer-sand mixture. They observed that *Persea americana* inoculated with *G. intraradices* grew significantly faster relative to the uninoculated control, whereas those inoculated with *Scutellospora heterogama* were not different from the control. Differences in AMF effect on plant growth parameters may be related to their hyphae capacity (George et al., 1995). In their review on N and P uptake by AMF, George et al. (1995) pointed out that AMF hyphae have different capacities to transport nutrients to the host perhaps because hyphae differ in size, distribution patterns, and tensile strength (Rillig and Mummey, 2006).

The reasons why the PGPR strains had neutral or detrimental effects on pea growth is not clear because all the PGPR strains were selected based on their growth promoting abilities demonstrated in previous studies conducted by de Freitas and Germida (1990a) who used winter wheat (*Triticum aestivum* L. cv. Norstar) as the test crop. A series of

preliminary studies were carried out to determine if long-term storage had affected the growth-promoting characteristics of these strains. It was affirmed that the strains were still effective. Furthermore, the preliminary studies showed that the PGPR strains were effective for both spring and winter wheat. Nonetheless, beneficial traits attributed to PGPR may be species dependent. Some PGPR are cultivar and species specific (Bashan et al., 1989; Germida and Walley, 1996). Cultivar specificity, although not observed on wheat growth in preliminary studies, was previously reported by Germida and Walley (1996) under field conditions as some of these strains did not promote growth of spring wheat. Alternatively, some growth promoting traits of PGPR are only evident under non-sterile conditions where the PGPR interact with a diverse group of microorganisms. For example, some PGPR enhance the productivity of the host plant only by inhibiting growth of pathogens (Kropp et al., 1996; Requena et al., 1997).

Contrary to the findings of Kim et al. (1998) and Biró et al. (2000), the interaction effects of AMF and PGPR in this study did not significantly affect shoot weight of pea. Biró et al. (2000) reported significant increases in shoot, root, and total weight of alfalfa (*Medicago sativa* L.) by pairing *G. fasciculatum* and *Azospirillum brasilense* as compared to the uninoculated control. Nonetheless, interaction effects of AMF and PGPR on plant biomass reported by others include those that are neutral or detrimental (Requena et al., 1997; Walley and Germida, 1997). Using sterilized soil, Requena et al. (1997) found some positive and synergistic interaction effects in the rhizosphere of *A. cytisoides*, however, the combination of a rhizobacterium "E", identified as *Bacillus* species, and *G. coronatum* reduced the root weight of the plant significantly. Similarly, under sterile conditions, Walley and Germida (1997) observed that when combined with *G. clarum* NT4, the beneficial effect of R85 on shoot and root weight of spring wheat was not as evident as when the PGPR strain was applied alone. Generally, in their study none of the interaction effects was greater than that obtained from inoculation of the PGPR strains alone. These studies confirm that, even under sterile conditions, some AMF and PGPR interactions may have negative or no effects on plant productivity as was observed in the present study.

6.4.2 The effects of inoculants on plant nitrogen and phosphorus

Glomus mosseae significantly enhanced total uptake of N and P by pea compared to other treatments. Several studies also have reported positive effects of AMF on plant nutrient uptake (George et al., 1995; Koide and Mosse, 2004; Richardson et al., 2009). Hyphae of AMF explore regions that cannot be accessed by the roots or the root hairs; therefore, hyphae are able to translocate nutrients from soil regions inaccessible to the plant (Koide and Mosse, 2004; Richardson et al., 2009). In addition, mycorrhizal symbioses allow plants to access organic and inorganic N and P which are usually unavailable for plant uptake (George et al., 1995; Koide and Kabir, 2000; Hodge et al. 2001; Richardson et al., 2009). Nevertheless, because AMF differ in their characteristics, their contributions to plant nutrition vary (George et al., 1995; van der Heijden et al., 2006; Rillig and Mummey, 2006). The observations by these authors may clarify why the AMF species differed in their abilities to increase N and P uptake by pea in the present study.

Although N and P uptake were affected by inoculation of pea with the AMF species, the inoculants had no significant beneficial effect on N and P concentrations in the plant tissue. Conversely, Roesti and co-workers (2006) observed a positive response of N, P, and potassium (K) content in wheat grain due to mycorrhizal treatment. Similarly, Biró et al. (2000) observed that *G. fasciculatum* increased N, P, and K content of alfalfa shoots. However, Rodríguez-Romero et al. (2005) found no significant effect of *G. manihotis* on N and P content of banana leaf compared with the uninoculated control. The reason why *G. mosseae* significantly increased N and P uptake of pea, but had no effect on plant tissue nutrient concentration may be attributed to a nutrient dilution effect, i.e., reduction in nutrient concentration in plant tissue due to increased plant biomass production (Bagayoko et al., 2000). Kim et al. (1998) also reported a similar response of tomato (*Lycopersicon esculentum* MILL.) to mycorrhizal treatment. Kim and co-workers (1998) found a significant effect of *G. etunicatum* on total N and P uptake of tomato, but the fungus did not influence N and P concentration of the plant. They related their observation to the dilution effect.

Plant growth-promoting rhizobacteria often do not contribute substantially to N nutrition of plants (Glick, 1995; Dobbelaere et al., 2002; Richardson et al., 2009).

Dobbelaere et al. (2002) reported inoculations of corn and wheat with *A. brasilense* or *A. irakense* that failed to increase N content of the plants. In a growth pouch experiment, de Freitas et al. (1993) observed that *P. putida* R105 had no effect on acetylene reduction activity in pea nodules (i.e., a measure of N_2 fixation), although nodulation increased. In another study, de Freitas et al. (1997) reported that *P. cepacia* R85 increased rock phosphate solubilization in liquid cultures, but did not affect P uptake of canola. Furthermore, they found no relationship between phosphate solubilizing activity of the PGPR strain and its growth promoting ability. They concluded that other mechanisms such as hormone production may have contributed to growth promotion by the PGPR. The latter study, however, was conducted under non-sterile conditions where other organisms may influence PGPR activities. In this study, R55 and R111 significantly enhanced N uptake by pea compared with the non-PGPR treatments; however, none of the PGPR strains significantly affected N concentration in the plant tissue. Also, none of the PGPR strains had an effect on P uptake and P concentration in pea tissue. These observations are in line with what has been previously reported. Because of the inconsistencies in PGPR activities, it is difficult to generalize their growth promoting abilities (Germida and Walley, 1996; Lucy et al., 2004).

In combination, the AMF species and PGPR strains observed in this study had no significant effect on N and P uptake by pea or N and P concentrations in the plant tissue, although nutrient uptake was enhanced by some microorganisms when applied alone. Under sterile conditions, previous studies have reported varying effects of microbial inoculation on plant nutrient content (Requena et al., 1997; Walley and Germida, 1997; Biró et al., 2000). For example, Biró et al. (2000) found that the co-inoculation of *G. fasciculatum* and *A. brasilense* significantly increased N, P, and K concentrations in alfalfa shoots. In contrast, Requena et al. (1997) reported a negative interaction between rhizobacterium "E" and *G. coronatum* on shoot N and P of *A. cytisoides*, whereas Walley and Germida (1997) observed non-significant interaction effects of some *Pseudomonas* species with *G. clarum* NT4 on N and P content of spring wheat. Theoretically, phosphate solubilizers such as R85 (de Freitas et al., 1997) should enhance P acquisition by a mycorrhizal plant (Toro et al., 1997; Barea et al., 2002), but that is not always the case (Walley and Germida, 1997). It is possible that some PGPR strains may not be as

effective (de Freitas et al., 1997) or are just not compatible with certain AMF (Requena et al., 1997). Further studies are necessary to shed light on non-beneficial interaction effects on plant nutrient concentrations.

6.4.3 The effects of inoculants on Bradford-reactive soil protein in the rhizosphere

Concentrations of GRSP in this study were within the lower range of values reported by Wright and Upadhyaya (1998). Wright and co-worker (1998) reported GRSP in a range of 2 to 14 mg g^{-1} soil in 14 temperate soils, and here, GRSP levels were slightly below 2 mg g^{-1} soil. Except for a few speculations of biotic influences on glomalin production by AMF (Purin and Rillig, 2007, 2008), little is known of how other microorganisms may affect glomalin production. Recently, while determining the influence of drought on soil aggregate formation, Kohler et al. (2009b) reported no significant interaction effects of *G. intraradices* and *P. mendocina* on EE-BRSP levels in the rhizosphere of lettuce. However, significant increases where observed when *G. mosseae* was inoculated with the same PGPR. In contrast, in this study, none of the AMF and PGPR interaction effects significantly affected EE-BRSP. The lack of significant interaction effects indicate either that no interactions existed or, alternatively, the EE-BRSP fraction may not be suitable if the goal is to select AMF and PGPR combinations that could enhance glomalin production.

Contrary to the observation made on the EE-BRSP, the co-inoculation of the PGPR with the AMF species significantly affected the quantity of BRSP in the pea rhizosphere. The combination of *G. mosseae* with R105 or R111 enhanced the total protein concentrations in the plant rhizosphere compared to the control or to the application of *G. mosseae* alone. In contrast, the co-inoculation of *G. clarum* with any of the PGPR strains reduced BRSP levels in the soil compared to inoculation with *G. clarum* alone. In general, mycorrhizal treatment effects on BRSP were not significant. In fact, when applied singly, effects of the PGPR strains on BRSP were greater than the individual effects of *G. intraradices* and *G. mosseae*. Also, it is worth noting that the significant interaction effects observed between these PGPR strains and *G. mosseae* is more likely a result of PGPR influences than the interaction between the PGPR and AMF. For example, in the absence of AMF, R105 and R111 had the most impact on total protein

concentrations in pea rhizosphere compared with other microbial inoculants, including those applied in combination.

Although by definition bacteria do not produce glomalin (Wright et al., 1996; Wright and Upadhyaya, 1996), both microorganisms and plant roots secrete some proteinaceous substances, such as amino acids (Jones et al., 1994; Deakin and Broughton, 2009), which may be co-extracted with glomalin and detected by the Bradford assay (Rosier et al., 2006, 2008; Schindler et al., 2007). De Freitas and Germida (1990b) reported that some of the PGPR strains significantly enhanced root hair formation; it follows that these PGPR may similarly stimulate the exudation of proteinaceous substances by pea. Another possibility is that phytohormones such as IAA-like substances produced by these PGPR strains (de Freitas 1990; de Freitas et al., 1997) may enhance root development, and thus root exudation (Wu et al., 2005). The influence of PGPR on plant roots may clarify why higher BRSP was detected in the rhizosphere of some non-mycorrhizal plants. Moreover, in the present study, roots were concentrated in the rhizosphere by separating the hyphae from the root and root hairs using a 40 µm nylon mesh. Thus, it is likely that the interaction effects observed were basically plant effects on rhizosphere proteins.

Furthermore, it is important to be cognizant that AMF may reduce total protein concentration in pea rhizosphere by decreasing protein losses from the plant root. Hamel et al. (1991) reported the likelihood that AMF reduces root exudation while determining the roles of AMF in N transfer between soybean and corn. They linked the observation to the mechanism by which AMF enhance the recovery of N lost by host plant. In that case, the non-AMF treatments may have more proteinaceous compounds in their root zone which were determined by the Bradford total protein assay as GRSP. Also AMF are capable of remobilizing exuded substances such as GRSP into their hyphae (Jones et al., 2004). The reports by Jones and co-workers (2004), however, need to be investigated as it is now generally thought that glomalin is only released after the death of AMF (Driver et al., 2005) and not exuded as was once speculated (Wright and Upadhyaya, 1996).

Despite the reported correlations between enzyme-linked immunosorbent assay (ELISA) with Bradford values (Wright et al., 1996; Wright and Upadhyaya, 1996, 1999), Bradford assay is a total protein assay, and less specific for glomalin (Wright et al., 1999). The Bradford total protein assay involves the use of Coomassie dye that binds

with almost all protein (Rosier et al., 2006; Whiffen et al., 2007). This may be problematic as glomalin extraction does not eliminate all non-glomalin sources (Schindler et al., 2007; Whiffen et al., 2007). For example, the procedure co-extracts humic and tannic acids (Nichols and Wright, 2005, 2006; Whiffen et al., 2007). Plant-derived proteins such as dehydrins and heat shock proteins (Wisniewski et al., 1996) may also survive the extraction procedure (Rosier et al., 2006). Rosier et al. (2008) recently showed that protein produced by other organisms may be measured as Bradford-root protein and immunoreactive-root protein. These findings imply that BRSP is not completely related to AMF and needs thorough investigation (Whiffen et al., 2007). Although ELISA may not be consistently precise (Rosier et al., 2008), the analysis is specific for glomalin (Rillig and Steinberg, 2002), and hence may clarify these observations.

6.4.4 The effects of inoculants on Bradford-reactive protein and Bradford-reactive soil protein in the mycorrhizosphere

Unlike Wright and Upadhyaya (1999), that reported horticultural strips as a good trap of glomalin, the low concentration of BRP and lack of significant interaction effects of this fraction, observed in the current study, indicate that the horticultural strip is ineffective for detecting the best AMF and PGPR interaction effects on glomalin production. Nevertheless, AMF and PGPR interactions influenced the total protein concentration in the mycorrhizosphere (sand mix). The co-inoculation of *G. intraradices* and R75, R85, or R105 increased BRSP levels in the mycorrhizosphere of pea by approximately 27, 40, and 24%, respectively, compared with the uninoculated control (no AMF and PGPR). The interaction effects of *G. intraradices* and R75, R85, or R105 also were higher than the effect of *G. intraradices* alone and accounted for increases of approximately 20, 30, and 17%, respectively. Thus, the interactions between *G. intraradices* and R75, R85, or R105 were beneficial for increasing BRSP concentrations. The possibility that they have the potential to increase the concentrations of this glycoprotein in the presence of other soil organisms will be discussed in the next study.

Interestingly, R85, which had the greatest beneficial influence on BRSP levels when inoculated with *G. intraradices*, has been reported to inhibit the germination of *G. clarum*

NT4 spores, and reduce AMF colonization and colonized root length of spring wheat (Walley and Germida, 1997). The inhibitory effect was attributed to the antagonistic trait of the PGPR strains reported by de Freitas and Germida (1990a; 1991). Purin and Rillig (2007) reported glomalin as a homolog of heat shock protein 60, which are conserved proteins produced by eukaryotes and prokaryotes when their cells are stressed. Consequently, glomalin production (and hence, BRSP) may increase with stress. Therefore, the antagonistic effect of the PGPR, especially R85 might have enhanced glomalin production by *G. intraradices*.

Levels of BRSP in the mycorrhizosphere of pea were low when *G. mosseae* was paired with R105 or R111 relative to the uninoculated control. This observation also may suggest negative interactions between these organisms. As biocontrol agents, some PGPR have non-target effects on AMF which may reduce AMF fitness and efficiency (Walley and Germida, 1997; Purin and Rillig, 2008). Because a reduction in AMF fitness and efficiency will decrease nutrient uptake and translocation, their host plant also could be negatively affected. The negative influence some AMF and PGPR interactions have on their host plant may reduce C allocation to AMF. For example, any disruption of P flux may decrease the levels of C allotted to AMF by its host. Alternatively, AMF can become a C drain to its host as a result of its parasitic association with PGPR. Since a considerable fraction of C obtained by AMF from the host is invested in glomalin production (Treseder and Turner, 2007), detrimental associations between PGPR and mycorrhizal plants may affect glomalin production by AMF. Partly, observations by these authors may explain why the interactions between *G. mosseae* and R105 or R111 reduced glomalin concentration in the mycorrhizosphere of pea compared with the uninoculated control, though their interaction effects on pea growth and nutrient uptake were not significant.

In comparison, BRSP in the sand mix (mycorrhizosphere) showed a different trend compared to BRSP obtained form the soil-sand mix (rhizosphere) (Figures 6.7 and 6.9). For example, the BRSP fraction in the mycorrhizosphere increased with the co-inoculation of *G. intraradices* and R75, R85, or R105, while the interaction effects were not different from the uninoculated control (without AMF and PGPR) on BRSP fraction in the rhizosphere. As a result, BRSP obtained from the mycorrhizosphere may better

explain the effects of microbial inoculation on total protein concentrations. Moreover, the mycorrhizosphere is the soil region directly under the influence of hyphae. In addition, the influence of root and organic matter on BRSP in the mycorrhizosphere has been eliminated through the physical separation of the rhizosphere from the mycorrhizosphere using a nylon mesh. Because some AMF and PGPR interaction effects significantly increased BRSP concentrations in the mycorrhizosphere, it is possible to select AMF and PGPR combinations that may enhance glomalin production by AMF.

6.5 Conclusion

Positive to negative effects of AMF and PGPR were found on shoot biomass of pea, N and P uptake by pea, and BRSP concentrations in rhizosphere and mycorrhizosphere of pea. Because of the non-significant interaction between AMF and PGPR on plant growth and nutrient content, it was difficult to relate microbial effects on growth parameters to glomalin production. Generally, it was evident that some AMF and PGPR interactions may influence BRSP concentrations determined in the rhizosphere and mycorrhizosphere under sterile conditions. Both increases and decreases in BRSP concentration may be attributed to the reported antagonism between certain AMF species and PGPR strains. Nevertheless, we cannot rule out the occurrence of other proteins in the glomalin extracts as some non-mycorrhizal treatments had a greater influence on BRSP concentration than the mycorrhizal treatments.

7. EFFECTS OF ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT GROWTH-PROMOTING RHIZOBACTERIA ON GLOMALIN PRODUCTION, SOIL CARBON AND NITROGEN STORAGE, AND PEA GROWTH UNDER NON-STERILE CONDITIONS

7.1 Introduction

Examining microbial effects on plant growth parameters under gnotobiotic conditions is crucial because it serves as a foundation for what could be expected under non-sterile or field conditions (Burr et al., 1978). Nonetheless, for any significant microbial effect observed under gnotobiotic conditions to be applicable, it should be evident under non-sterile conditions in the midst of other soil inhabitants (Schroth and Weinhold, 1986; Walley, 1993). For example, Biró et al. (2000) reported significant interactions between *Glomus fasciculatum* and *Rhizobium meliloti* affecting nitrogen (N), phosphorus (P), and potassium (K) content of alfalfa (*Medicago sativa* L.) in gamma-sterilized soils. However, the beneficial interactions were not evident in non-sterile soil. Additionally, these authors reported that *Azospirillum brasilense* reduced root colonization by *G. fasciculatum* in non-sterile soils, but the detrimental effect was not observed under gnotobiotic conditions. In the previous study (Chapter 6), while screening for the best AMF (arbuscular mycorrhizal fungi) and PGPR (plant growth-promoting rhizobacteria) interaction effects on glomalin production under gnotobiotic conditions, the influence of other microorganisms on the inoculants were eliminated. Therefore, there is a need to examine these interactions in non-sterile soil. Although little is known of the biotic effect on glomalin production by AMF, several microorganisms may affect AMF establishment and activities (Fitter and Garbaye, 1994; Hodge, 2000; Purin and Rillig, 2008), and hence glomalin production.

Some rhizobacteria may compete with AMF by reducing spore germination and inhibiting the growth and spread of hyphae (Fitter and Garbaye, 1994; Walley, and Germida, 1997). Extraradical hyphae of AMF are even more susceptible to predators including macroorganisms (Fitter and Garbaye, 1994; Schreiner and Bethlenfalvay, 2003), and any negative effect of these organisms on AMF may reduce glomalin production directly since glomalin is mainly found in AMF hyphae. Indirect effects could

result from the disruption of nutrient flow, especially P from AMF to the host plant (Fitter and Garbaye, 1994), thereby influencing carbon (C) allotted to AMF for glomalin production. Thus, the effect of AMF species and their interactions with the PGPR strains on glomalin-related soil protein (GRSP) levels observed under gnotobiotic systems (as in the previous study) should also be studied under non-sterile conditions in the presence of other soil organisms.

Carbon storage, necessitated by the increasing atmospheric concentration of carbon dioxide (CO_2), can be maximized by manipulating soil C pools such as GRSP (Nichols and Wright, 2006). Although it is generally assumed that plants govern C sequestration as primary producers, microbes are equally important because of their influence on C mineralization and immobilization (Zhu and Miller, 2003). Of importance is the contribution of AMF to C storage (Treseder and Allen, 2000; Rillig et al., 2001; Zhu and Miller, 2003). By facilitating nutrient uptake, AMF increase plant growth and net C gain by the plant (Zhu and Miller, 2003). Arbuscular mycorrhizal fungi also enhance C storage by promoting aggregate formation and stabilization through their hyphae and the production of glomalin (Rillig and Mummey, 2006; Wilson et al., 2009). Glomalin is inherently stable and hydrophobic, and contributes to C storage in soil aggregates (Wright and Upadhyaya, 1998; Wright and Anderson, 2000; Rillig et al. 2002). Additionally, glomalin enhances soil C and N pools because it contains 36 to 59% C and 3 to 5% N in its structure (Lovelock et al., 2004a; Schindler et al., 2007). In fact, Nichols and Wright (2006) observed that glomalin was the largest pool of soil N and organic C compared to other soil pools; namely, humic acid, fluvic acid, and particulate organic matter. Hence, the objective of this study was to examine the effect of AMF and PGPR interactions on pea (*Pisum sativum* L.) growth, glomalin production, and C and N storage in pea rhizosphere under non-sterile conditions.

7.2 Materials and Methods

7.2.1 Plant growth-promoting rhizobacteria

Plant growth-promoting rhizobacteria *Pseudomonas aeruginosa* R75, *P. cepacia* R85, and *P. putida* R105 were selected based on their beneficial interaction with *G. intraradices*. In combination, these organisms enhanced Bradford-reactive soil protein

(BRSP) concentration in the mycorrhizosphere (sand mix) of pea under gnotobiotic conditions (Chapter 6). The strains were cultured as described in Section 4.2.1.

7.2.2 Arbuscular mycorrhizal fungi

Glomus intraradices was selected due to its interaction with the PGPR strains on BSRP in the mycorrhizosphere of pea under gnotobiotic conditions (Chapter 6). Also, *G. clarum* was selected with the assumption that its interaction with the PGPR strains could be beneficial. Spores of these AMF were used as an inoculum. They were extracted and isolated as described in Section 3.2.1.

7.2.3 Soil preparation

A low P Elbow soil (Calcareous Dark Brown Chernozem), collected from the top 15 cm of a soil from the Bradwell Association, was sieved (2 mm) and mixed with silica sand (1:1 w/w). Fifty milliliters of modified Hoagland's nutrient solution (minus P) per kilogram of soil-sand mix was added and thoroughly mixed with the soil-sand mix. Two kilograms of the soil-sand mix were placed in 15 cm diameter pots. Following nutrient addition, the physical and chemical properties of the soil-sand mix were determined by ALS laboratory (Saskatoon, SK) and were as follows: sandy loam; 14.6 μg NO_3-N g^{-1}; 12.3 μg P g^{-1}; 604.8 μg K g^{-1}; 28.6 μg SO_4-S g^{-1}; 3.5 μg Cu g^{-1}; 48.1 μg Mn g^{-1}; 5.6 μg Zn g^{-1}; 1.4 μg B g^{-1}; 22.4 μg Fe g^{-1}; 8.4 μg Cl g^{-1}; pH 7.5; conductivity 0.2 mS cm^{-1}.

7.2.4 Inoculation and planting

Surface-sterilized pea seeds were aseptically germinated on 1.5% water agar for 5 d. Two pre-germinated pea seedlings were transplanted into each pot. For the AMF treatment, 100 spores of the appropriate AMF were placed in the transplant hole. The PGPR treatment was applied by pipetting 1 mL of the appropriate bacterial suspension (approximately 10^6 colony forming units) into the transplant hole. In combination, AMF and PGPR inocula were applied by placing 100 spores of AMF and pipetting 1 mL of the appropriate suspension into the transplant hole. Autoclaved polypropylene beads were applied on the soil surface to prevent cross contamination and excessive moisture loss. One hundred milliliters of modified Hoagland's nutrient solution (Millner and Kitt, 1992)

was supplied to each pot every week to replenish soil nutrients. Plants were grown in a growth chamber under the following conditions: 25˚C, 16 h day and 20˚C, 8 h night, 375 to 400 µE m^{-2} s^{-1} of irradiance and relative humidity of 60%. Soil moisture was maintained at 60% water holding capacity by regular additions of distilled water.

7.2.5 Experimental design

The experiment was set up using a two by three factorial design. The first factor (AMF) consisted of *G. clarum* and *G. intraradices*; and the second factor (PGPR) consisted of R75, R85, and R105. Thus, there were six combinations of these organisms. Control treatments were uninoculated pea. Treatments were replicated four times, making a total of 48 pots. Pots were completely randomized and repositioned thrice during the growth period.

7.2.6 Plant analysis

Plants were grown for 12 weeks, and total biomass was determined at harvest. Shoot biomass was determined by separating the shoot from the root at the stem base, followed by washing and oven-drying at 65˚C for 48 h, after which oven-dried weight was determined. Roots were extracted from experimental soil by placing the root ball on a screen that restricted the plant roots but allowed soil particles to pass through. The root balls were massaged to loosen soil attached to the roots. Roots were washed under running tap water until they were free of soil particles, and then rinsed with distilled water. Root weight was measured after oven-drying at 65˚C for 48 h. Pea pods were threshed manually to separate seeds from the shoot (above ground) biomass, and seed weight was determined. The shoot and seeds were ground separately prior to nutrient analyses. Shoot and seed N were measured using CNS 2000 automated combustion analyzer (LECO Instruments Ltd., St. Joseph, MI). Concentration of P in the seed, shoot, and root tissue was determined by digesting the plant materials in sulphuric acid (Thomas et al., 1967), and P was measured using an Auto Analyzer II Technicon® system (Technicon Industrial Systems, Tarrytown, N.Y., U.S.A).

7.2.7 Percent arbuscular mycorrhizal fungi colonization

Percentage of AMF colonization was determined as described by Vierheilig et al. (1998). Subsamples of oven-dried roots were hydrated overnight, transferred into a cassette (VWR Int., Mississauga, ON), and cleared by inserting the cassette into almost boiling 10% potassium hydroxide solution for 25 min. Cleared roots were rinsed thoroughly in tap water, then placed in boiling 5% Sheaffer ink-vinegar stain solution for 3 min. Stained roots were rinsed in tap water and destained in tap water containing a few drops of vinegar solution for 5 d. Roots were then transferred into 50% glycerol solution and stored at 4˚C until percent AMF colonization was determined using a modification of gridline intersect method (Giovannetti and Moss, 1980) described by Walley (1993). Briefly, root samples were placed on a Petri dish marked with 0.5 cm gridlines, and observed for AMF structures (hyphae, vesicles, arbuscules, or appressoria) with a microscope (100 × magnification). Presence of any of these structures was marked as positive. The total number of positive observations out of 100 observations gave the percentage of AMF colonization.

7.2.8 Glomalin extraction

Glomalin-related soil protein was extracted from subsamples of experimental soil collected from the root balls prior to washing. Soil samples were air dried for 4 d and sieved using a 2 mm sieve to remove roots and organic debris. Easily extractable glomalin-related soil protein (EE-GRSP) and GRSP were extracted from each sample (Wright and Upadhyaya, 1996; Rillig, 2004b). Easily extractable glomalin-related soil protein was extracted in 20 m*M* sodium citrate solution (pH 7) and the mixture autoclaved at 121˚C for 30 min. Glomalin-related soil protein was extracted using 50 m*M* sodium citrate solution (pH 8.0) and the mixture was autoclaved at 121˚C for 60 min. Both fractions were extracted from separate 1-g soil samples in 8 mL extractant. For GRSP, the extraction procedure was repeated until the supernatant was almost colourless, which required five extraction cycles. Samples were centrifuged at 5,000 × g for 15 min immediately after extraction, and the supernatant containing the extracted protein was decanted and stored at 4˚C for analysis.

7.2.9 Glomalin quantification

As described by Wright and Upadhyaya (1996), EE-GRSP and GRSP concentrations were determined using the Bradford dye-binding protein assay as easily extractable Bradford-reactive soil protein (EE-BRSP) and BRSP, respectively (Rillig, 2004b). The assay was performed using 96-well plates. Protein standards in a range of 1.25 to 5 μg protein per well were prepared using bovine serum albumin (BSA). Extract from each sample was pooled and centrifuged at 10,000 × g for 5 min to remove residual soil particles and other insoluble materials. Duplicate wells of the 96-well assay plate were loaded with 25 μg of protein solution in 175 μL of phosphate buffer saline (PBS) for EE-GRSP extract, and 50 μg of protein solution in 150 μL of PBS for GRSP extract. Fifty microlitres of Bio-Rad dye (Bio-Rad, Laboratories, Inc., CA) was added into each well containing protein in PBS, mixed thoroughly, and the absorbance read at a wavelength of 590 nm (A_{590}) within 5 min of addition. A standard curve was generated by plotting optical density values against protein of known concentrations (BSA). Protein concentrations, in microgram per well of glomalin extract, were calculated from the equation of the line generated from the curve.

Immunoreactive fractions of EE-GRSP and GRSP i.e., the easily extractable immunoreactive soil protein (EE-IRSP) and the immunoreactive soil protein (IRSP) were measured using an indirect enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody MAb32B11 developed against crushed spores of *G. intraradices* (Wright and Upadhyaya, 1996, Rillig, 2004b). In total, there were four soil glomalin fractions (BRSP, EE-BRSP, IRSP and EE-IRSP) for each soil sample.

7.2.10 Determination of soil carbon and nitrogen

Soil organic carbon (SOC) and soil total nitrogen (STN) were determined using a LECO CR-12 Carbon System (781-600) (LECO Corporation, St. Joseph, MI) and LECO CNS 2000 automated combustion analyzer (LECO Instruments Ltd., St. Joseph, MI), respectively. Prior to the analyses, subsamples of experimental soil were ground in a ball mill to ensure homogeneity of the samples.

7.2.11 Statistical analysis

Data were subjected to analysis of variance (ANOVA) to determine any significant effects of the microbial inoculants on plant weight, N and P, GRSP concentrations, and organic C and N storage in the rhizosphere. The treatment means were compared using the least significant difference (LSD) test at a significance level of 0.05. Correlations between soil GRSP concentrations, percent AMF colonization, and organic C and N storage were determined by calculating Pearson correlation coefficients. Normality of distributions and homogeneity of variances were assessed before conducting any statistical analysis. Statistical analyses were performed using SPSS software version 16.0 for Windows (SPSS Inc., 2008).

7.3 Results

7.3.1 The effects of inoculants on plant growth

Inoculation of pea with AMF and PGPR, alone and in combination, had no effect on seed, shoot, or root weight (Table 7.1). However, it is worth noting that the co-inoculation of *G. intraradices* with each of the three PGPR strains showed beneficial tendencies with regard to shoot, root, and total biomass production when compared to inoculation with *G. intraradices* alone (Table 7.2).

Table 7.1. Statistical analysis (probability) for seed, shoot, root, and total weight of pea inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Source of Variation	df	Seed	Shoot	Root	Total
		Probability			
Total	47				
AMF	2	0.994	0.839	0.868	0.863
PGPR	3	0.868	0.612	0.393	0.545
AMF * PGPR	6	0.575	0.759	0.577	0.708
Error	36				

Table 7.2. Seed, shoot, root, and total dry weight of pea inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Treatment	Seed†	Shoot†	Root†	Total†
	Dry weight (g pot^{-1})			
Control (-AMF and -PGPR)	2.31	7.25	0.51	7.75
R75	2.16	6.95	0.39	7.33
R85	2.26	7.55	0.69	8.23
R105	1.75	7.22	0.54	7.76
G. clarum	2.12	7.13	0.48	7.60
R75 + *G. clarum*	1.64	6.86	0.52	7.38
R85 + *G. clarum*	1.83	6.63	0.46	7.09
R105 + *G. clarum*	3.00	8.03	0.54	8.56
G. intraradices	2.23	6.28	0.39	6.67
R75 + *G. intraradices*	2.13	7.08	0.55	7.63
R85 + *G. intraradices*	2.18	7.29	0.59	7.87
R105 + *G. intraradices*	2.08	7.29	0.59	7.89

†No significant differences.

7.3.2 The effects of inoculants on plant nitrogen and phosphorus

There were no significant main effects of the AMF and the PGPR on N and P concentrations in the seed, shoot, and root tissue of pea; however, the fungal species tended to increase N and P concentrations in the shoot and root tissue, whereas the PGPR decreased N and P concentrations in the plant tissue (Tables 7.3 and 7.4). Also, the interaction effects of these organisms on N and P concentrations in seed, shoot, and root tissue of pea were not significant (Tables 7.3 and 7.4).

7.3.3 The effects of inoculants on percent arbuscular mycorrhizal fungi colonization

The main effect of the AMF on percent AMF colonization was significant ($p = 0.005$) (Table 7.5; a detailed summary of the results is presented in Appendix E.3). *Glomus clarum* inoculation significantly increased the percentage of AMF colonization compared with *G. intraradices* or the non-AMF control treatments. Although the main effect of the

PGPR did not significantly (p = 0.099) affect percent AMF colonization, significant (p = 0.04) interactions existed between the PGPR and AMF on (Figure 7.1 and Table 7.5). For example, percent AMF colonization by *G. clarum* decreased from an average of 58 to 41% when inoculated with R85. Also, the dual inoculation of *G. clarum* with R105 increased percentage of AMF colonization by 15 and 25% over the individual inoculation of *G. clarum* and R105, respectively. The co-inoculation of R105 and *G. clarum* resulted in the highest percentage AMF colonization, while the lowest interaction effect was that of *G. intraradices* and R85. The co-inoculation of R85 with *G. intraradices* reduced percent AMF colonization by 30% below that observed for R105 and *G. clarum* when applied in combination.

Table 7.3. Statistical analysis (probability) and significance for nitrogen (N) and phosphorus (P) concentrations in pea tissue inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Source of Variation	df	Seed	Shoot	Seed	Shoot	Root
		Nitrogen[§]		Phosphorus		
		Probability				
Total	47[†]					
AMF	2	0.962	0.744	0.630	0.769	0.798
PGPR	3	0.816	0.903	0.997	0.338	0.897
AMF * PGPR	6	0.187	0.797	0.270	0.657	0.305
Error	36[‡]					

†The total degree of freedom for seed N and root P is 46 and 44, respectively, due to missing values.
‡The error degree of freedom for seed N and root P is 35 and 33, respectively, due to missing values.
§There were no statistically significant effects detected.

Association of *G. clarum* with the indigenous AMF species promoted pea root colonization, whereas in the presence *G. intraradices*, root colonization was reduced by almost 17% (Figure 7.1 and Table 7.5). Percent colonization by the native AMF species decreased with inoculation of the PGPR strains. *Pseudomonas aeruginosa* R75 had the greatest impact on these native fungi, reducing colonization by 22%.

Table 7.4. Nitrogen (N) and phosphorus (P) concentrations in seed, shoot, and root tissue of pea inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Treatment	Seed†	Shoot†	Seed†	Shoot†	Root†
	Nitrogen (mg g^{-1})		Phosphorus (mg g^{-1})		
Control (-AMF and -PGPR)	37.13	9.52	1.87	0.39	0.74
R75	33.40	8.70	1.82	0.37	0.72
R85	35.15	9.44	1.79	0.37	0.83
R105	38.00	10.63	2.04	0.46	0.88
G. clarum	35.68	12.58	1.95	0.54	0.89
R75 + *G. clarum*	37.27	10.40	1.90	0.37	0.93
R85 + *G. clarum*	37.50	11.42	2.11	0.46	0.81
R105 + *G. clarum*	34.43	8.44	1.83	0.35	0.72
G. intraradices	36.33	10.76	1.93	0.53	0.89
R75 + *G. intraradices*	36.15	8.85	2.08	0.37	0.82
R85 + *G. intraradices*	34.40	9.40	1.85	0.35	0.71
R105 + *G. intraradices*	37.28	10.20	1.90	0.50	0.81

†No significant differences.

7.3.4 The effects of inoculants on glomalin-related soil protein concentration

Easily extractable Bradford-reactive soil protein concentration in the pea rhizosphere was significantly (p = 0.001) influenced by inoculating with AMF (Table 7.5; see Appendix E.3 for data summary). *Glomus clarum* enhanced EE-BRSP concentration significantly compared to *G. intraradices* or the non-mycorrhizal control treatments (Figure 7.2A). Similarly, the main effect of the PGPR was significant (p = 0.052) on EE-BRSP as R85 and R105 significantly reduced the amount of EE-BRSP in the pea rhizosphere compared with the non-PGPR control treatments (Figure 7.2B and Table 7.5). The interaction effects of AMF and PGPR, however, were not significant on EE-BRSP levels in the pea rhizosphere (Table 7.5).

Table 7.5. Statistical analysis (probability) for percent arbuscular mycorrhizal fungi (AMF) colonization of pea, easily extractable Bradford-reactive soil protein (EE-BRSP) and immunoreactive soil protein (EE-IRSP), Bradford-reactive soil protein (BRSP), and immunoreactive soil protein (IRSP) concentrations in rhizosphere of pea inoculated with AMF and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

		% AMF colonization	EE-BRSP	EE-IRSP	BRSP	IRSP[§]
Source of Variation	df	Probability				
Total	47[†]					
AMF	2	0.005**	0.001**	0.000**	0.021*	0.071
PGPR	3	0.099	0.052*	0.812	0.009**	0.672
AMF * PGPR	6	0.040*	0.143	0.651	0.029*	0.709
Error	36[‡]					

†The total degree of freedom for EE-BRSP, EE-IRSP, BRSP, and IRSP is 46, 45, 46, and 45, respectively, due to missing values.
‡The error degree of freedom for EE-BRSP, EE-IRSP, BRSP, and IRSP is 35, 34, 35, and 34, respectively, due to missing values.
§There were no statistically significant effects detected.
*, **Significant at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Concentrations of EE-IRSP were significantly ($p < 0.001$) affected by the AMF species as observed on EE-BRSP (Table 7.5). The two AMF species showed significant positive effects on the levels of EE-IRSP compared with the non-AMF control treatments (Figure 7.2A). In contrast, no significant PGPR effect was found on the EE-IRSP (Figure 7.2b and Table 7.5). Additionally, interactions between the AMF and PGPR had no significant effect on EE-IRSP concentrations (Table 7.3).

Both the AMF ($p = 0.021$) and the PGPR ($p = 0.009$) had significant effects on BRSP concentration (Table 7.5). Similarly, concentrations of BRSP were significantly ($p = 0.029$) influenced by the interaction effects of the organisms (Figure 7.3 and Table 7.5). Bradford-reactive soil protein concentration increased by inoculating R75 and R85 with *G. intraradices*. In contrast, inoculation of *G. clarum* with R85 and R105 significantly reduced the levels of BRSP in the pea rhizosphere compared with when *G. clarum* was applied alone. Without the PGPR strains, *G. clarum* had a higher influence on BRSP

concentration than *G. intraradices*. Thus, PGPR inoculation seemed to be detrimental to glomalin production by *G. clarum*, but desirable for *G. intraradices*.

Although the single and the dual inoculations with AMF and PGPR affected BRSP significantly, neither the main nor interaction effects of the inoculants were significant on IRSP (Table 7.5).

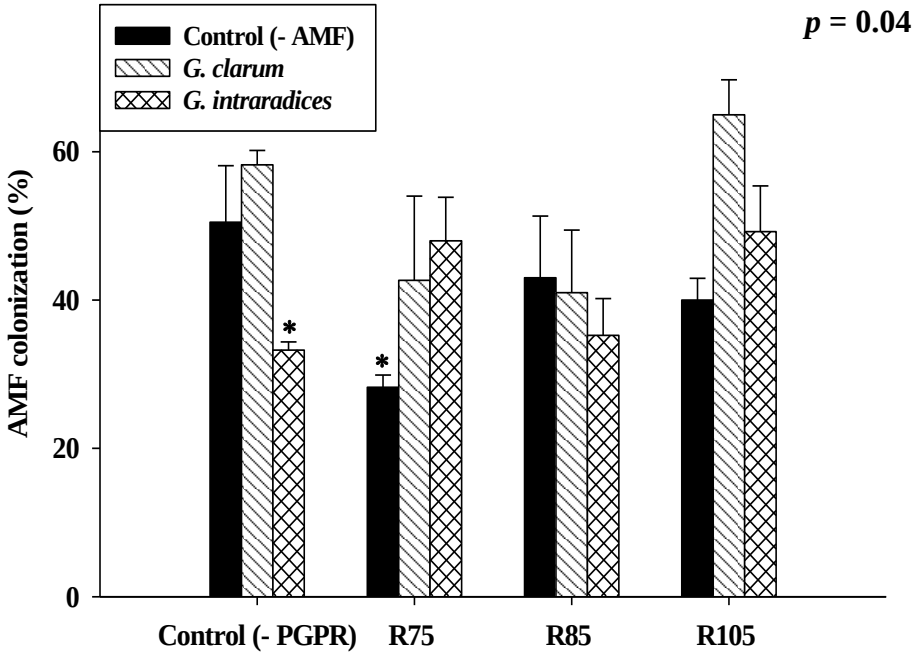

Fig. 7.1. Arbuscular mycorrhizal fungi (AMF) colonization of pea inoculated with AMF species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). Error bars are standard errors of the mean (n = 4). The *p*-value is for the analysis of variance (ANOVA). Asterisk (∗) denotes significantly different from the uninoculated control (i.e., -AMF and -PGPR) according to the least significant difference (LSD) test ($p \leq 0.05$).

7.3.5 The effects of inoculants on soil organic carbon and total nitrogen

Inoculation with AMF or PGPR had no significant effect on SOC and STN (Tables 7.6 and 7.7). Likewise, the interactions between the AMF and PGPR did not significantly affect SOC or STN (Tables 7.6 and 7.7).

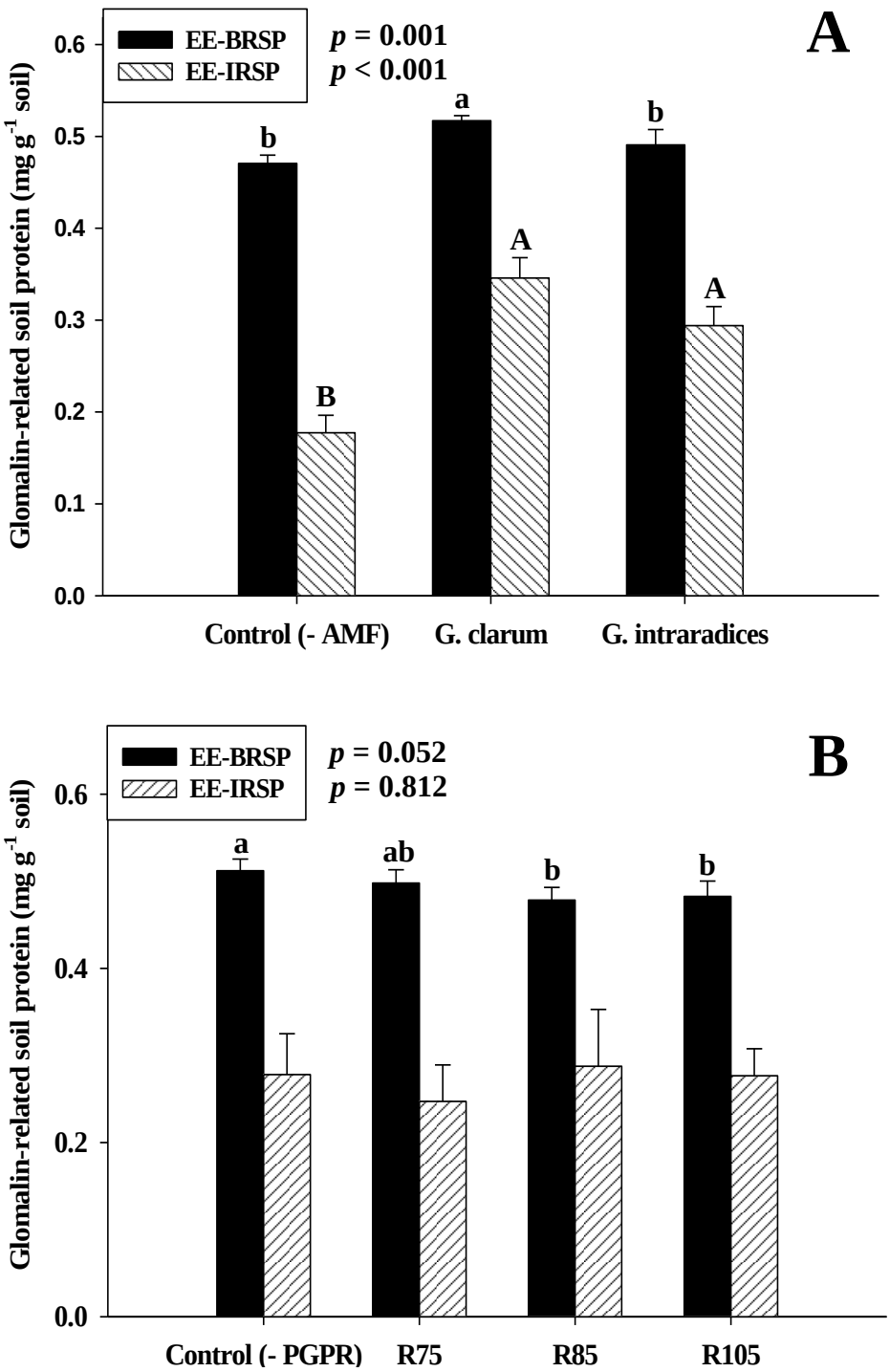

Fig. 7.2. Main effect of arbuscular mycorrhizal fungi (AMF) (A) and plant growth-promoting rhizobacteria (PGPR) (B) on easily extractable Bradford-reactive soil protein (EE-BRSP) and immunoreactive soil protein (EE-IRSP) in the rhizosphere of pea 12 weeks after planting (WAP). Error bars are standard errors of the mean (n = 4). Means followed by the same letter are not significantly different according to the least significant difference (LSD) test ($p \leq 0.05$). The *p*-value is for the analysis of variance (ANOVA). Lower case denotes comparison between shoot. Upper case denotes comparison between seed.

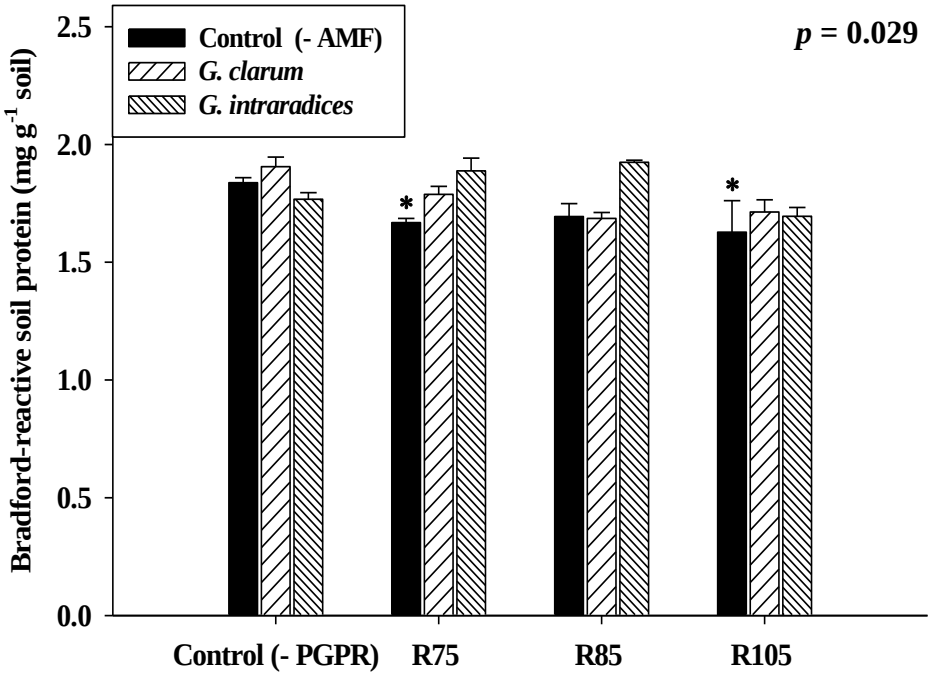

Fig. 7.3. Bradford-reactive soil protein (BRSP) in the rhizosphere of pea inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). Error bars are standard errors of the mean (n = 4). The *p*-value is for the analysis of variance (ANOVA). Asterisk (∗) denotes significantly different from the uninoculated control (i.e., -AMF and -PGPR) according to the least significant difference (LSD) test ($p \leq 0.05$).

Table 7.6. Statistical analysis (probability) for organic carbon (C) and total nitrogen (N) of soil inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Source of Variation	df	SOC†	Soil N†
		Probability	
Total	47		
AMF	2	0.947	0.744
PGPR	3	0.191	0.930
AMF * PGPR	6	0.374	0.797
Error	36		

† No significant differences.

Table 7.7. Organic carbon (C) and total nitrogen (N) of soil inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Treatment	SOC†	Soil N†
	(mg g $^{-1}$)	
Control (-AMF and -PGPR)	6.02	0.91
R75	5.57	0.91
R85	5.10	0.95
R105	6.24	0.92
G. clarum	5.31	0.93
R75 + *G. clarum*	5.81	0.88
R85 + *G. clarum*	5.59	0.87
R105 + *G. clarum*	5.89	0.93
G. intraradices	6.29	0.89
R75 + *G. intraradices*	5.24	0.91
R85 + *G. intraradices*	5.43	0.95
R105 + *G. intraradices*	5.74	0.91

†No significant differences.

7.3.6 Correlations between GRSP, AMF colonization, plant growth and nutrient concentration, and soil carbon and nitrogen storage

Among the four GRSP fractions, EE-BRSP correlated with EE-IRSP (r = 0.312, $p \leq 0.05$) (Table 7.5). However, none of the GRSP fraction correlated with pea growth, nutrient concentrations in the plant tissue, or soil organic C and N content, with the exception of a significant negative correlation between pea root weight and EE-BRSP ($p \leq 0.01$). Although percent AMF colonization did not correlate with the four GRSP fractions and soil parameters, significant ($p \leq 0.05$) positive correlations were found between percent AMF colonization and shoot (r = 0.29) or total (r = 0.31) weight of pea.

Table 7.8. Pearson correlation coefficients for glomalin-related soil protein (GRSP), percent arbuscular mycorrhizal fungi (AMF) colonization, plant weight, nitrogen (N) and phosphorus (P) concentrations in plant tissue, soil organic carbon (SOC), and soil total nitrogen (STN) content.

Variable	EE-BRSP	BRSP	EE-IRSP	IRSP	% AMF coloniza-tion	Shoot weight	Root weight	Total weight	Shoot N	Shoot P	Root P	SOC	STN
EE-BRSP	1.00												
BRSP	0.16	1.00											
EE-IRSP	**0.31***	0.25	1.00										
IRSP	-0.23	-0.08	-0.21	1.00									
% AMF Colonization	0.23	0.12	0.05	-0.14	1.00								
Shoot weight	-0.11	-0.13	0.04	0.06	**0.29***	1.00							
Root weight	**-0.41****	0.15	0.05	-0.09	0.27	**0.43****	1.00						
Total weight	-0.17	-0.17	0.05	0.04	**0.31***	**0.99****	**0.55****	1.00					
Shoot N	0.19	0.02	0.13	-0.01	-0.13	-0.27	**-0.61****	**-0.35***	1.00				
Shoot P	0.18	0.02	0.07	-0.13	-0.07	**-0.38****	**-0.59****	**-0.44****	**0.91****	1.00			
Root P	0.01	0.08	-0.06	0.04	0.28	0.08	**0.31***	0.12	-0.23	0.00	1.00		
SOC	0.24	-0.15	-0.09	0.18	0.01	0.03	-0.11	0.02	-0.06	-0.08	0.02	1.00	
STN	0.02	0.19	-0.07	0.19	0.09	-0.01	0.14	0.01	-0.07	0.02	0.10	-0.11	1.00

**Correlation significant at $p \leq 0.01$ *Correlation significant at the $p \leq 0.05$.

7.4 Discussion

7.4.1 The effects of inoculants on plant growth

Although inoculation with *Glomus intraradices* resulted in slightly smaller plants, the differences in shoot, root, and total biomass were not significant compared with the non-AMF control treatment. While exploring the effects of *G. intraradices* and *P. mendocina* on structural stability of a semiarid agricultural soil, Kohler et al. (2009a) found that the only time *G. intraradices* had no beneficial effect on biomass production by lettuce was under water stress conditions, otherwise, the AMF improved biomass of the plant significantly. Arbuscular mycorrhizal fungi are mutualistic endosymbionts (Smith and Read, 1997) known for their growth promoting characteristics even under stress conditions (Adesemoye et al., 2008). Nevertheless, they are not always beneficial; they could be ineffective and parasitic to their host (Johnson et al., 1997).

Several organisms that co-inhabit the rhizosphere with AMF may reduce their establishment as early as spore germination (Fitter and Garbaye, 1994; Walley and Germida, 1997). There are possibilities that some volatile and non-volatile diffusible substances produced by certain rhizobacteria, including PGPR could reduce AMF spore germination (Walley and Germida, 1997; Xavier and Germida, 2003a). Additionally, some of these organisms compete with AMF within the root by inhibiting the growth and spread of intraradical and extraradical hyphae, consequently reducing AMF roles as mutualists (Fitter and Garbaye, 1994). Since AMF hyphae are vehicles for nutrient transfer to plants, any factor that inhibits nutrient uptake and transfer by the mycelium will reduce plant fitness (Fitter and Garbaye, 1994; Purin and Rillig, 2008). In fact, the activities of these beneficial organisms may be altered to the extent that they become 'cheaters' to their host plant (Johnson et al., 1997). A cheater in a mutualistic association receives, but refuses to give, or gives in an amount significantly below what has been offered by its partner (Johnson et al., 1997). As a result, some AMF become parasitic to their host plant. Although commonly found, most authors avoid using the word to describe an AMF association (Johnson et al., 1997).

Contrary to the AMF effect, the PGPR showed tendencies to enhance pea growth as R85 and R105 increased shoot and root weight of the plant. Growth promotion by PGPR has been observed from seedling stage to maturity both in greenhouse and field studies

(Vessey, 2003; Lucy et al., 2004; Nelson, 2004). Plant growth-promoting rhizobacteria increase seedling emergence, plant vigour, and resistance to diseases, and thus enhance plant productivity (Glick, 1995; Vessey, 2003; Lucy et al., 2004). The non-significant beneficial effects observed in this present study may result from several factors such as the initial PGPR population introduced into the rhizosphere (Dobbelaere et al., 2002; Lucy et al., 2004), physical growth conditions (Walley and Germida, 1997), host plant (Germida and Walley, 1996), plant growth stage at which the beneficial traits were assessed (Roesti et al., 2006; Adesemoye et al., 2008), and the more complex biotic factors including inoculants interaction with other organisms (Requena et al., 1997; Walley and Germida, 1997; Adesemoye et al., 2008).

Considerable time and effort has been devoted to understanding the relationships between AMF and PGPR since the realization that their interactions could enhance plant growth and development (Meyer and Linderman, 1986; Toro et al., 1997). It has been observed that not all associations between these organisms are favourable as their interaction effects could be neutral or detrimental on the host plant (Walley and Germida, 1997; Adesemoye and Kloepper, 2009; Dwivedi et al., 2009). In the present study, the AMF and PGPR interaction effects did not significantly affect the growth parameters examined. However, co-inoculation of *G. intraradices* with the three PGPR strains showed beneficial tendencies. For instance, they all increased shoot, root, and total weight of pea compared with the uninoculated control or when *G. intraradices* was applied alone. Kim et al. (1998) also reported the interaction between a phosphate solubilizing rhizobacterium, *Enterobacter agglomerans*, and *G. etunicatum* which increased the shoot and root weight of tomato, but in the study the interaction effect was significant compared with the non-inoculated control.

7.4.2 The effects of inoculants on plant nitrogen and phosphorus

The AMF species showed tendencies of increasing N and P concentrations in the pea tissue; however, the beneficial effects were not significant. Several studies also have reported non-significant effects of certain AMF species on nutrient concentrations in plant tissue (Walley and Germida, 1997; Rodríguez-Romero et al., 2005; Dwivedi et al., 2009). For example, Rodríguez-Romero et al. (2005) reported that *G. manihotis* increased

N and P content of banana, but not significantly. Dwivedi et al. (2009) also noted non-significant mycorrhizal effects on N content of green gram. The lack of significance was related to N_2 fixation by *Rhizobium* species that associate with leguminous plants. It is possible that the N_2 fixing bacteria increased N content of all the studied plants, and that the non-inoculated control was not different from other treatments (Dwivedi et al., 2009). Biró et al. (2000), however, reported *G. fasciculatum* reduced N, P, and K contents of alfalfa shoot, and the negative effect was significant on P content of the plant. The authors expressed the likelihood that other soil microbiota may reduce the beneficial attributes of *G. fasciculatum* on alfalfa as they found that the AMF enhanced N and P content of the plant in gamma-sterilized soils. They concluded that the introduced AMF species was not competent enough to increase plant growth. Nevertheless, the non-significant beneficial effects of *G. clarum* and *G. intraradices* in the present study cannot be related to the influence of other soil microorganisms; neither AMF stimulated plant nutrient acquisition, even under sterile conditions (as discussed in Chapter 6). These observations imply that *G. clarum* and *G. intraradices* may not be effective at enhancing growth and nutrient uptake by pea.

As found under sterile conditions, none of the PGPR strains significantly influenced N and P concentrations in the pea tissue. Using non-sterile soils, de Freitas and Germida (1992) reported that at 70 days after planting (DAP), R85 and R105 increased weight and nutrient content of winter wheat (*Triticum aestivum* L. cv. Norstar) in a low-fertility soil, but had less effect in a more fertile soil. However, they observed that the significant effects of the strains found in the less fertile soil were not evident at harvest (170 DAP). Interestingly, beneficial effects of some of the strains were significant on growth and N content of wheat in the more fertile soil at harvest. Furthermore, de Freitas et al. (1997) demonstrated that R85 solubilized P; however, it had no effect on N and P uptake of canola. Although it may be argued that PGPR strains are cultivar specific, effectiveness of these rhizobacteria on plant growth and nutrients is not clear; their performances are dependent on soil fertility, and could be transient. Several studies have attributed inconsistencies in PGPR effect to soil and environmental factors (Burr et al., 1978; de Freitas and Germida, 1990a, 1992; Lucy et al., 2004; Adesemoye and Kloepper, 2009), and a number of these studies pointed out that these unpredictable traits of PGPR limits

their use as commercial inoculants (Germida and Walley, 1996; Requena et al., 1997; Nelson et al., 2004).

Even though the co-inoculation of the AMF with the PGPR did not significantly affect N and P concentrations in the pea tissue, the PGPR strains tended to reduce the beneficial effects of the AMF. Reductions in the AMF effect when co-inoculated with PGPR also were observed by Walley and Germida (1997). Often the observations are related to the adverse effects of some PGPR strains on AMF establishments and functions (Germida and Walley, 1996; Walley and Germida, 1997). To share the view of Germida and Walley (1996), the practical application of PGPR is challenging as the growth promoting abilities of PGPR are complex. It is difficult to identify a rhizobacterium that will consistently enhance plant productivity; the rhizobacterium is either influenced by other soil organisms or the rhizobacterium itself influences the beneficial effects of other organisms. Nonetheless, there are possibilities of manipulating PGPR to increase plant productivity (Germida and Walley, 1996).

7.4.3 The effects of inoculants on percent arbuscular mycorrhizal fungi colonization

The percentage of root colonization by the indigenous and introduced AMF with or without the PGPR strains ranged from 28 to 65%. Germida and Walley (1996) found up to 52 and 78% AMF colonization in two different sites. In a separate study, Walley and Germida (1997) reported 0 to 38% colonization, and Kim et al. (1998) observed a range of 29 to 57%. Results of these studies indicate that AMF colonization by native and exotic AMF species range between 0 and 78% in the presence of exotic or indigenous PGPR. The highest colonization observed in this study resulted from the dual inoculation of R105 and *G. clarum*. Because the interaction effect was not significantly greater than the effect of the uninoculated control or when *G. clarum* was applied singly, the high colonization may not be related to the inoculation of R105 with the AMF. Moreover, R105 reduced colonization by the native AMF species, although not significantly. In contrast, Toro et al. (1997) related increases in onion root colonization by *G. intraradices* to the inoculation of *B. subtilis* with the AMF. The lowest interaction effect of the inoculants on AMF colonization in this present study was observed when *G. intraradices* was paired with R85. Interestingly, Walley and Germida (1997) also found that the same

strain significantly decreased AMF colonization of spring wheat (*Triticum aestivum* L.) by *G. clarum* NT4, which was related to the antifungal characteristics of the PGPR strain. Because in the absence of the PGPR percent root colonized by *G. intraradices* was low in the current study, low percent AMF colonization also may be related to the effects of the native organisms.

7.4.4 The effects of inoculants on glomalin-related soil protein concentration

The concentrations of all GRSP fractions examined in this study were slightly below what others have observed in agricultural soils. For example, Nichols and Wright (2006) reported an average of 2.53 mg g^{-1} soil for six soils from different locations, but in the present study the highest GRSP value was 1.92 mg g^{-1} soil. Other studies have reported GRSP concentrations has high as 100 mg g^{-1} soil in forest soils (Rillig et al., 2001) and 414 mg g^{-1} soil in peat soils (Schindler et al., 2007). However, values lower than 1 mg g^{-1} soil were found in desert soils of China (Bai et al., 2009). These variations in GRSP levels confirm its dependence on numerous biotic and biotic factors (Rillig et al., 2001). Of importance is the role of the plant in glomalin production and decomposition. In fact, plant effects may be more crucial than the influence of the AMF itself (Treseder and Turner, 2007). Plants are the primary producers; they assimilate CO_2 for their metabolism through photosynthesis, and then transfer a percentage of the photosynthates to their AMF partner. Because a large percentage of the photosynthates is allotted to glomalin production by AMF (Treseder and Turner, 2007), it is conceivable that plant growth is a major determinant of GRSP levels. This explanation may clarify why GRSP concentrations are usually low in pot experiments relative to field studies (Lovelock et al., 2004b).

Other factors that may influence the amount of GRSP include soil organic matter and iron (Fe) content (Wright and Upadhyaya, 1998; Rillig et al., 2001), cropping systems, and soil management practices (Wright and Anderson, 2000; Rillig et al., 2002; Preger et al., 2007), such as fertilizer application (Lovelock et al., 2004a). For example, Wright and Upadhyaya (1998) related low GRSP concentrations in calcareous Texas soils to low Fe content of the soil. In addition, they observed that low organic matter soil may be low in GRSP. While the reason for the former observation is yet to be identified, the explanation

to the latter is that glomalin accounts for a considerable amount of recalcitrant SOM content (Wright and Upadhyaya, 1998). Schindler and co-workers (2007) also related high GRSP levels in peat soil to SOC content. Although the influence of these factors on GRSP may not be detected in a pot experiment, they could affect the initial, inherent concentration of the glycoprotein in the soil samples used in a pot experiment i.e., prior to collection. Furthermore, Wright and Upadhyaya (1998) previously reported that GRSP concentration may be higher in a clayey soil than sandy textured soil, which could be related to the protection of GRSP from microbial degradation by clay particles (Treseder and Turner, 2007). Also, when a sandy loam soil was mixed with calcinated clay in the study of Bedini et al. (2009), BRSP concentration was less that 2.0 mg g^{-1} soil and EE-BRSP less than 0.50 mg g^{-1} soil, as was observed in the present study. Thus, the low levels of glomalin observed may partly be due to the sandy loam texture of the growth medium.

Both EE-BRSP and EE-IRSP concentrations were influenced by the main effects of the AMF species (Figure 7.2). *Glomus clarum* significantly enhanced EE-BRSP and EE-IRSP levels in the pea rhizosphere compared with the non-mycorrhizal control treatments. Also, *G. intraradices* increased the EE-GRSP fractions, but the effect was only significant on EE-IRSP. A number of studies have observed an effect of AMF inoculation on EE-GRSP concentrations (Bedini et al., 2009; Subramanian et al., 2009). For example, Bedini et al. (2009) reported significant increases in EE-BRSP concentrations in the rhizosphere on *Medicago sativa* when inoculated with *G. intraradices* or *G. mosseae*. Similarly, the PGPR strains affected EE-BRSP concentration in the pea rhizosphere as R85 and R105 significantly reduced the amounts of EE-BRSP in the plant rhizosphere compared with the non-PGPR control treatments. This observation is contrary to the findings of Kohler et al. (2009a). In their study, neither AMF nor PGPR affected EE-BRSP concentrations in the lettuce rhizosphere, except under water stress conditions. However, in another study, Kohler et al. (2009b) showed that the interaction effects of *P. mendocina* and *G. mosseae* significantly increased EE-GRSP levels relative to the control or when the inoculants were applied alone. In contrast, in the present study the AMF and PGPR interactions did not significantly influence EE-BRSP concentrations.

Only a few studies have examined the influence of bacterial inoculation on GRSP levels (Kohler et al., 2009a, b). The major reason may be attributed to the report that apart from AMF, no other microorganism produces glomalin (Wright et al., 1996; Wright and Upadhyaya, 1996). Based on the results of the previous study conducted under a gnotobiotic system (Chapter 6), it was observed that some PGPR strains significantly increased GRSP concentrations as reported by Kohler et al. (2009a, b). The reason for this was not clear, but may be related to PGPR effect on plant root exudates (Wu et al., 2005), and that other soil proteins are detected by the extraction and quantification techniques for GRSP (Rillig, 2004b; Schindler et al., 2007; Whiffen et al., 2007; Rosier et al., 2006, 2008). For example, PGPR may enhance GRSP levels indirectly by enhancing the production of proteinaceous substances by plant roots or stimulating exudates production by root through their influence on root growth (de Freitas and Germida, 1990b; Vessey, 2003). Although in the current study the non-PGPR treatments had higher effect on EE-BRSP levels compared with R85 and R105, their effect was similar to that of R75. Interestingly, no significant differences were observed among the treatments in their effect on the immunoreactive fraction of EE-BRSP (i.e., EE-IRSP) that was expected to separate mycorrhizal and non-mycorrhizal influences on GRSP. Future studies should focus on examining the possible bacterial and plant effects on the four glomalin fractions, especially the immunoreactive fractions.

In the study conducted under gnotobiotic systems, interactions between *G. intraradices* and R75, R85, or R105 increased BRSP concentrations in the mycorrhizosphere of pea compared with when *G. intraradices* was applied singly. The observation of interactions between *G. intraradices* and the three PGPR strains served as the basis of the current study. Apparently, the interactions were beneficial on glomalin production even under non-sterile conditions where the inoculants may be influenced by other soil organisms. *Pseudomonas aeruginosa* R75 and *P. cepacia* R85 increased BRSP concentration when inoculated with *G. intraradices* compared with when *G. intraradices* was applied alone. This observation may be specific as the interaction effect was not found in *G. clarum* inoculated soils or the uninoculated control containing the native AMF. In fact, the presence of the PGPR strains significantly reduced BRSP concentration in the uninoculated soil (Figure 7.3). Even though the beneficial interaction effects found

between *G. intraradices* and R75 or R85 on BRSP concentrations were not significantly different from *G. intraradices* and the non-inoculated control, they should be further examined under field conditions.

7.4.5 The effects of inoculants on soil organic carbon and total nitrogen

Because of the increasing recognition that AMF play important roles in soil C and N storage (Zhu and Miller, 2003; Wilson et al., 2009), a number of studies have focused on manipulating these organisms to store more C and N (Rillig, 2004a; Wilson et al., 2009). Increasing soil C and N pools will enhance productivity, and importantly, reduce soil emissions of CO_2 and nitrous oxide (N_2O) into the atmosphere (Janzen et al., 1998; Lal, 2004; Nichols and Wright, 2006). When examining the influence of AMF on SOC, most studies explore their indirect roles in promoting plant growth and in the formation of water stable aggregates (Jastrow and Miller, 1997). As a consequence, little is known of the direct contributions of the AMF themselves (Rillig et al., 2001). Extraradical hyphae of AMF are an important carbon sink (Zhu and Miller, 2003). Also, AMF contribute to SOC by releasing organic substances, such as glomalin, into the mycorrhizosphere (Rillig et al., 2001; Wright et al., 2007; Wilson et al., 2009). Nevertheless, in this study, the roles of AMF in C and N storage were not evident, as none of the AMF species affected SOC or total N content.

A study by Wilson et al. (2009) showed that the suppression of AMF symbioses through fungicide applications led to C losses in macroaggregates. Furthermore, they found that changes in biomass production influenced SOC and N storage. Interestingly, in the study, biomass production was not affected by AMF suppression, thus plant effect on C storage was independent of AMF symbioses. Therefore, it can be assumed that the non-significant effect of the AMF species on SOC and N in this current study is not necessarily related to their inability to stimulate pea growth. Additionally, the lack of significance cannot be attributed to GRSP levels because the two AMF species significantly increased EE-GRSP concentrations compared with the non-AMF control.

7.4.6 Correlations between GRSP, AMF colonization, plant growth and nutrient concentration, and soil carbon and nitrogen storage

Because one of the major objectives of this study was to examine the potential of GRSP for increasing soil C and N storage, correlation analysis was performed to assess the relationship between GRSP, plant growth, and soil C and N storage. None of the GRSP fractions correlated with plant growth parameters or SOC and N content, except the negative correlation found between root weight and EE-BRSP. This is contrary to previous observations (Wright and Upadhyaya, 1996, 1998; Wright and Anderson, 2000; Preger et al., 2007; Bedini et al., 2007; Wilson et al., 2009). Wright and Upadhyaya (1998) and Wilson et al. (2009) are among several authors that have reported relationships between GRSP and soil C and N content. Mostly, the correlations observed between GRSP fractions and C and N storage are linked with the inherent characteristics of glomalin. Glomalin is a stable N-linked glycoprotein and reportedly hydrophobic in its native state. As a result, it binds with soil particles and facilitates aggregate formation and stabilization (Wright and Upadhyaya, 1998; Wright et al., 2007). By virtue of its role in stable aggregate formation, glomalin protects C stored in aggregates, thereby enhancing soil C storage (Rillig, 2004a, b; Wright et al., 2007; Wilson et al., 2009). The reason for the lack of correlations between BRSP and SOC and N content in this study may be related to the duration of the study. The experiment was a pot study conducted over a period of three months. Consequently, changes in C and N content may not be as evident as compared to field studies.

Knorr et al. (2003) conducted a field study to relate GRSP concentrations in forest soils with fire frequency and landscape position. They reported positive correlations between EE-IRSP or IRSP and soil N concentration, but found no significant changes on the GRSP concentrations over a six-year period. The authors explained that rate of glomalin production may be similar to the decomposition rate, so there was no net change in the concentration of the glycoprotein. Besides, Knorr and co-workers (2003) found no significant effect of fire on GRSP levels, and related the observation to the measurement of the static pool size of the glycoprotein. Therefore, increases and decreases in glomalin production and decomposition as a result of fire may not be detected. Even though this present study was conducted over a short period, it is possible that only the net effect of

the microbial inoculants on GRSP concentrations and organic C and N storage were examined. Thus, some changes might have occurred but were not detected.

Studies have shown that percent AMF colonization and other AMF parameters such as hyphae length may not relate with GRSP concentrations (Lutgen et al., 2003; Steinberg and Rillig, 2003; Bai et al., 2009), partly because of the differential decomposition of these AMF variables (Steinberg and Rillig, 2003). For example, Steinberg and Rillig, (2003) found that under laboratory conditions, AMF hyphae decomposed faster than GRSP. However, Bedini et al. (2007) found a linear correlation between AMF spore bio-volume and levels of GRSP. They suggested that volume of AMF spores could be a better indicator of GRSP concentration. Furthermore, no correlation existed between percent AMF colonization and SOC and N content. However, percent AMF colonization correlated positively with shoot and total weight of pea, an observation similar to Nehl et al. (1996). Nehl and co-workers (1996) reported a positive correlation between AMF colonization and shoot weight of cotton under field conditions. Nonetheless, in the current study, only a small proportion of the variability was explained by the correlations found between percent AMF colonization, shoot and total weight of pea.

7.5 Conclusion

The most important observation made in this study is that associations between *G. intraradices* and *P. aeruginosa* R75 or *P. cepacia* R85 have the potential to increase BRSP concentrations even under non-sterile conditions. Because most of the microbial effects were not evident on pea weight and nutrient concentrations in the plant tissue, the effect of these organisms on GRSP cannot be related to plant growth parameters. Additionally, percent AMF colonization did not explain why some of the inoculants influenced the amount of GRSP as no correlations were found between AMF colonization and the four GRSP fractions studied. Soil C and N content were unaffected by the microbial inoculants, and no relationship existed between GRSP concentrations and soil organic C and N content.

8. SUMMARY AND CONCLUSIONS

The overall goal of this study was to examine the potential for enhancing glomalin production by AMF via dual inoculation with other beneficial rhizosphere organisms i.e., PGPR. The combinations of these organisms that could enhance plant growth and the storage of C and N in the rhizosphere also were examined. Before addressing these objectives, a series of growth chamber and laboratory experiments were conducted to determine the influence of AMF species on glomalin production by comparing the amounts of glomalin produced by *G. clarum*, *G. intraradices*, and *G. mosseae* in association with corn. The effect of plant species on the glycoprotein also was investigated by determining the influence of corn, pea, and wheat on glomalin production by *G. intraradices*. Mycorrhizal effects were not evident as the three AMF species were statistically similar in terms of glomalin production. Plant effects, however, significantly affected BRSP concentrations in the rhizosphere; higher BRSP levels were detected in the corn rhizosphere compared with pea and wheat.

Long-term storage effects were examined on the growth promoting abilities of the selected PGPR strains (*Pseudomonas cepacia* R55 and R85, *P. aeruginosa* R75, *P. putida* R105, and *P. fluorescence* R111) reported by de Freitas and Germida (1990a). Apparently, the PGPR strains were not affected by long-term storage (ca. twenty years at -80˚C) as they all increased total biomass of spring wheat significantly and showed antagonistic activity against the plant pathogenic fungi, *Fusarium* species.

Reports that SAB influence the function and activity of AMF (Walley and Germida, 1996; Xavier and Germida, 2003a; Cruz et al., 2008) spurred an interest to explore SAB effects on glomalin production by first determining their effect on wheat growth. Sixteen bacteria were isolated from disinfested spores of AMF, as described by Walley and Germida (1996). Based on FAME analysis, the majority of the SAB were classified as *Bacillus* species; an observation similar to the findings of Xavier and Germida, (2003a). Nonetheless, the bacteria had no growth promoting abilities as they all reduced the growth of spring wheat. It is possible that these SAB served other functions not related to plant growth promotion. Future studies may clarify this observation by investigating the actual functions of the SAB.

With these preliminary findings, we selected the three AMF species and five PGPR strains to test the hypothesis that AMF and PGPR interaction enhances plant growth and glomalin production under gnotobiotic conditions using pea as the host plant. Pea was chosen as the host plant because it is an important crop in Saskatchewan and known to be highly mycorrhizal. Moreover, there were concerns that corn may increase background BRSP concentrations (i.e., unrelated to glomalin), and thus slight changes in glomalin production due to microbial effects may be undetected.

The AMF had a significant effect on shoot biomass of pea. *Glomus mosseae* significantly increased shoot weight of pea compared with other AMF species and non-AMF treatments. A similar trend was found on pea seed weight. *Glomus mosseae* had greater impact on the seed weight relative to the other treatments. Other studies have shown beneficial effects of AMF on plant growth under sterile conditions (e.g., Biró et al., 2000). Usually, these desirable attributes are linked with nutrient acquisition and transfer by AMF to the host plant (reviewed by Richardson et al., 2009). Nevertheless, AMF could be specific in their activities; their abilities to explore and acquire nutrients from soil differ among species and they interact with host plants differently (George et al., 1995; Requena et al., 1997).

The main effect of the PGPR also was evident on shoot biomass of pea. Inoculation with R85 significantly reduced the shoot biomass of pea compared to R55, R75, R111, and non-PGPR control. Effects of all the PGPR strains, however, were not evident on the seed weight of pea. Studies have shown that growth promotion by PGPR may be dependent on the host plant (Germida and Walley, 1996; Enebak et al. 1998; Lucy et al., 2004), which may explain why the PGPR strains enhanced growth of wheat (de Freitas and Germida, 1990a; Chapter 4), but did not promote pea growth.

No significant AMF and PGPR interaction effects were observed on pea growth. Similarly, a number of studies have shown that the effects of AMF and PGPR interactions are not always beneficial and could be detrimental on their host plant (Germida and Walley, 1996; Requena et al., 1997; Walley and Germida, 1997). Factors such as population of introduced bacteria (Requena et al., 1997) and the antifungal activities of the bacteria may affect the relationship between AMF, PGPR, and their host plant (Walley and Germida, 1997).

Nitrogen and P uptake by pea was influenced significantly by the mycorrhizal treatments. *Glomus mosseae* significantly enhanced N and P uptake by pea relative to other AMF species and the non-AMF control treatments. The main contributions of AMF to plant physiology are to modify the plant root to explore the soil for nutrients that are otherwise unavailable for plant uptake (Smith and Read, 1997; Barea et al., 2005). Nevertheless, AMF do not have equal capabilities to perform these beneficial roles (George et al., 1995).

The bacterial treatments also affected N and P uptake by pea. *Pseudomonas cepacia* R55 significantly increased N uptake by pea compared with other treatments, with the exception of R75 and R111. In contrast, none of the PGPR affected P uptake by the plant. A major challenge in the application of PGPR for growth increases is that they are unpredictable. For example, a N_2 fixing PGPR may not contribute to N nutrition of its host (Dobbelaere et al., 2002; Adesemoye and Kloepper, 2009), and a P solubilizing rhizobacteria does not necessarily increase P uptake by the host plant (de Freitas et al., 1997). To date, reasons for these observations are not clear.

As observed on pea growth, interaction effects of the AMF species and the PGPR strains were not significant with regards to N and P uptake by pea. Furthermore, none of the inoculants applied, either singly or in combination, influenced N and P concentrations in the plant tissue, except for a significant reduction in shoot N of plants inoculated with *G. clarum*. The latter observation is attributable to the dilution effect (Bagayoko et al., 2000) for the *G. mosseae* treatment because the AMF increased plant weight and nutrient uptake by pea. However, other inoculants may not be capable of enhancing nutrient concentrations in the pea tissue.

Only the main effects of AMF and PGPR were observed on EE-BRSP; however, both the main and interaction effects of these organisms were found on BRSP concentration in rhizosphere and mycorrhizosphere of pea. The main and interaction effects observed were variable; some were positive while others were negative. Because the Bradford assay measures total protein, and glomalin extraction procedures do not exclude all non-glomalin soil proteins (Rillig, 2004b; Schindler et al., 2007; Whiffen et al., 2007; Rosier et al., 2006, 2008), it is possible that the glomalin extracts contained some bacterial (Rosier et al., 2008) and plant (Rosier et al., 2006) proteins. Thus, it is understandable

that some non-mycorrhizal treatments may have higher influence on total protein concentrations, especially in the rhizosphere. Nevertheless, we were able to select *G. intraradices* and its combination with R75, R85, and R105 as having most potential to enhance glomalin production. Dual inoculation of the AMF and PGPR enhanced BSRP concentrations in the mycorrhizosphere, the region directly influenced by AMF hyphae, perhaps with lower concentrations of non-glomalin-related soil proteins.

Beneficial microbial effects found under sterile conditions should be observed under non-sterile conditions where introduced organisms could be influenced by other soil inhabitants (Walley, 1993). Interaction effects of *G. intraradices* and R75, R85, and R105 were examined on glomalin production in the pea rhizosphere under non-sterile conditions. The effect of the inoculants on pea growth, nutrient content, percent AMF colonization, and C and N storage also were determined. Additionally, *G. clarum* interaction effects with the PGPR strains were observed based on the possibilities that they may influence the parameters of interests. As found under gnotobiotic conditions, *G. intraradices* had no influence on pea biomass, nutrient uptake, and concentrations in pea tissue. Nevertheless, a significant reduction in shoot N caused by *G. clarum* under sterile conditions was not evident under non-sterile conditions. Similarly, the performances of the PGPR strains on pea biomass nutrient uptake and concentrations were not different from the non-PGPR treatments as was observed under sterile conditions. Also, the interactions between the AMF species and PGPR strains did not significantly affect the growth parameters. The lack of significant microbial effects is not surprising because the inoculants were not selected based on growth promoting qualities; they were chosen because of their interaction effects on BSRP concentration in the pea mycorrhizosphere.

Arbuscular mycorrhizal fungi and PGPR interactions had a significant effect on percent AMF colonization. The interaction effects ranged from positive to negative. The interaction between *G. clarum* and R105 resulted in the highest AMF colonization while *G. intraradices* and R85 interaction was the most detrimental on AMF colonization by the introduced AMF species. The detrimental interaction between *G. intraradices* and R85 was related to the antagonistic characteristics of the PGPR (de Freitas and Germida, 1991; Walley and Germida, 1997). It is interesting to note that this same PGPR had the highest influence on glomalin production by *G. intraradices*; an observation similar to

that observed in pea mycorrhizosphere under sterile conditions. Even though R85 did not significantly increase BRSP under non-sterile conditions, the PGPR strain may possess some attributes that influence the functions of *G. intraradices* and glomalin production by the AMF. Moreover, there is accumulating evidence that glomalin is a stress induced protein that is produced in the hyphae of AMF, particularly under unfavourable conditions (Rillig and Steinberg, 2002; Driver et al., 2005; Gadkar and Rillig, 2006; Purin and Rillig, 2007). Thus, it is possible that R85 stimulates glomalin as a stress response mechanism.

The AMF and PGPR interaction effects observed on GRSP concentrations in this study are among the few reports of biotic influences on glomalin production by AMF. Even though it may be challenging to examine these interaction effects under field conditions, it should spur further interest in examining microbial effects on glomalin production by AMF. It is possible that some of the adverse effects of AMF on their host are driven by their detrimental interactions with other soil organisms (Germida and Walley, 1996; Walley and Germida, 1997). In this context, an AMF may be allocating a large fraction of its C and N to the production of the glycoprotein that is serving a protective function. Unfortunately, this defensive mechanism of AMF may drain the host plant of its C, thereby inducing reductions in the plant growth. These are mere speculations that should be investigated.

Nevertheless, to appreciate PGPR performances in influencing glomalin production by AMF, interested researchers need to be cognizant that quantification of GRSP using the Bradford assay and ELISA technique is problematic. The Bradford assay measures the total protein and the glomalin extracts may contain non-glomalin proteins. The challenge of using the ELISA technique was due to its sensitivity. Although the ELISA technique seemed to be more specific for glomalin, the precision was low (i.e., relatively high coefficient of variation) compared with the Bradford assay. Additionally, ELISA is more technical and expensive relative to the Bradford assay.

Importantly, there may be a need to develop a quick strategy of examining beneficial microbial interactions on glomalin production that will eliminate the culturing and planting aspects of the examinations. A modification of split-dish *in vitro* carrot mycorrhizal system developed by St Arnaud et al. (1996) may be suitable. The *in vitro*

system will reduce contamination and the influence of other microorganisms, and the compartmentalization of the system will permit the separation of fungal and root effects. This approach will save time and cost while allowing the selection of best interaction effects for more detailed study under growth chamber or field conditions.

None of the microbial treatments affected organic C and total N content in the pea rhizosphere, and there were no correlations between pea weight, nutrient uptake and concentration, percent AMF colonization, GRSP concentration, and organic C and N content. The lack of correlations between pea growth parameters and GRSP concentrations showed that there were no detectable relationships between glomalin production and pea growth. This is understandable because of the likelihood that a fungus that demands more C from its host for glomalin production may function less as a mutualist, and can be a parasite to the plant (Johnson et al., 1997). If the former is the case, glomalin production may not relate to plant growth; however, for the latter case, the relationship between the glycoprotein and plant growth becomes negative.

Furthermore, it was not surprising that AMF colonization did not correlate with any of the GRSP fractions; percent AMF colonization is not a good index of GRSP concentrations (Lutgen et al., 2003; Bai et al., 2009). However, the reason for the lack of correlations between GRSP and SOC and N content may be related to the duration of the study. Because the experiment was a pot study conducted over a period of three months, changes in C and N content may not be evident. For example, Janzen (2004) highlighted the difficulty in detecting significant change in soil C content within a short period. This implies that long-term studies may detect the relationships between GRSP concentrations and SOC and N storage using the combination of *G. intraradices* and R75 or R85.

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10. APPENDICES

APPENDIX A. DETERMINATION OF ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT EFFECTS ON GLOMALIN PRODUCTION

Table A.1. Analysis of variance (ANOVA) for Bradford-reactive soil protein (BRSP) in the rhizosphere of corn inoculated with *G. clarum*, *G. intraradices*, and *G. mosseae* 12 weeks after planting (WAP). n = 4.

Source of Variation	df	Mean square	F-ratio	Probability
Total	15			
Between groups	3	0.132	2.962	0.075
Within groups	12	0.044		

Table A.2. Analysis of variance (ANOVA) for Bradford-reactive soil protein (BRSP) in the rhizosphere of corn, pea, and wheat inoculated with *G. intraradices* 12 weeks after planting (WAP). n = 4.

Source of Variation	df	Mean square	F-ratio	Probability
Total	23			
Between groups	5	0.051	6.206	0.002
Within groups	18	0.008		

APPENDIX B. ASSESSMENT OF PLANT GROWTH-PROMOTING RHIZOBACTERIA QUALITIES

Table B.1. Analysis of variance (ANOVA) for percent seedling emergence of spring wheat inoculated with *Pseudomonas cepacia* R55 and R85, *P. aeruginosa* R75, *P. putida* R105, and *P. fluorescence* R111 5, 10, and 15 days after planting (DAP). n = 6.

Source of Variation	df	Mean square	F-ratio	Probability
Seedling emergence				
5 DAP				
Total	35	0.533	0.369	0.866
Between groups	5	1.444		
Within groups	30			
10 DAP				
Total	35			
Between groups	5	1.267	1.036	0.414
Within groups	30	1.222		
15 DAP				
Total	35			
Between groups	5	1.111	0.820	0.545
Within groups	30	1.356		

Table B.2. Analysis of variance (ANOVA) for shoot, root, and total dry weight (n = 6), and root length (n = 5) of spring wheat inoculated with *Pseudomonas cepacia* R55 and R85; *P. aeruginosa* R75; *P. putida* R105; and *P. fluorescence* R111 30 days after planting (DAP).

Source of Variation	df	Mean square	F-ratio	Probability
Shoot weight				
Total	29			
Between groups	24	860.273	4.270	0.006
Within groups	5	201.450		
Root weight				
Total	29			
Between groups	24	967.713	4.122	0.008
Within groups	5	234.783		
Total weight				
Total	29			
Between groups	24	3449.333	6.318	0.001
Within groups	5	545.967		
Root length				
Total	29			
Between groups	24	5483.556	0.451	0.808
Within groups	5	12155.833		

APPENDIX C. ISOLATION AND TESTING OF ARBUSCULAR MYCORRHIZAL FUNGI SPORE-ASSOCIATED BACTERIA

Table C.1 Analysis of variance (ANOVA) for shoot, root, and total weight of spring wheat inoculated with spore-associated bacteria isolated from the disinfested spores of *G. clarum*, *G. intraradices*, and *G. mosseae* 30 days after planting (DAP). n = 6.

Source of Variation	df	Mean square	F-ratio	Probability
Shoot weight				
Total	59			
Between groups	50	255.804	1.028	0.431
Within groups	9	248.861		
Root weight				
Total	59	126.309	1.033	0.428
Between groups	50	122.321		
Within groups	9			
Total weight				
Total	59	586.365	0.950	0.492
Between groups	50	617.419		
Within groups	9			

APPENDIX D. EFFECTS OF ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT GROWTH-PROMOTING RHIZOBACTERIA ON GLOMALIN PRODUCTION UNDER GNOTOBIOTIC SYSTEM

Table D.1. Shoot and seed dry weight of pea inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) 12 weeks after planting (WAP). n = 4.

Treatment	Shoot	Seed
	Dry weight (g pot^{-1})	
Control (-AMF and -PGPR)	2.80	0.81
R55	2.78	0.75
R75	3.28	1.24
R85	1.30*	0.27
R105	1.81	0.50
R111	1.68	0.39
G. clarum	1.95	0.47
R55 + *G. clarum*	2.74	0.79
R75 + *G. clarum*	2.42	0.60
R85 + *G. clarum*	1.73	0.58
R105 + *G. clarum*	2.44	0.93
R111 + *G. clarum*	2.68	0.55
G. intraradices	1.52*	0.47
R55 + *G. intraradices*	2.52	0.80
R75 + *G. intraradices*	2.63	1.12
R85 + *G. intraradices*	2.13	0.55
R105 + *G. intraradices*	2.02	0.70
R111 + *G. intraradices*	2.29	0.65
G. mosseae	3.31	1.17
R55 + *G. mosseae*	4.12*	1.94*
R105 + *G. mosseae*	2.71	0.93
R11 + *G. mosseae*	3.77	1.64*
LSD ($p \leq 0.05$)	1.27	0.79

∗Significantly different from the control (-AMF and -PGPR).

Table D.2. Analysis of variance for shoot and seed dry weight of pea inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP) n = 4.

Source of Variation	df	Mean square	F-ratio	Probability
Shoot weight				
Total	91			
AMF	3	5.999	7.389	0.000
PGPR	5	2.505	3.085	0.014
AMF * PGPR	13	0.929	1.145	0.339
Error	70	0.812		
Seed weight				
Total	91			
AMF	3	2.418	7.786	0.000
PGPR	5	0.580	1.869	0.111
AMF * PGPR	13	0.304	0.978	0.482
Error	70	0.311		

Table D.3. Shoot nitrogen (N) and phosphorus (P) concentrations in shoot tissue of pea and N and P uptake by pea inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Treatment	Shoot N uptake	Shoot N concentration	Shoot P uptake	Shoot P concentration
	mg pot^{-1}	mg g^{-1}	mg pot^{-1}	mg g^{-1}
Control (-AMF and -PGPR)	23.64	8.45	0.89	0.32
R55	30.69	11.27	0.79	0.30
R75	32.26	9.67	0.86	0.25
R85	16.96	16.45*	0.54	0.58
R105	25.37	17.03*	1.03	0.74
R111	27.15	17.34*	0.75	0.78
G. clarum	20.63	8.28	0.84	0.70
R55 + *G. clarum*	25.04	9.13	1.05	0.39
R75 + *G. clarum*	24.27	10.03	0.99	0.41
R85 + *G. clarum*	20.96	9.30	0.76	1.05
R105 + *G. clarum*	19.15	7.97	0.69	0.30
R111 + *G. clarum*	27.15	10.28	1.16	0.45
G. intraradices	20.58	7.32	0.46	0.79
R55 + *G. intraradices*	25.56	10.19	0.81	0.32
R75 + *G. intraradices*	29.52	9.62	0.82	0.32
R85 + *G. intraradices*	26.89	10.61	0.75	0.43
R105 + *G. intraradices*	29.33	11.00	0.65	0.88
R111 + *G. intraradices*	27.88	12.10	0.79	0.35
G. mosseae	33.13	9.95	1.14	0.34
R55 + *G. mosseae*	41.00*	10.19	1.55*	0.37
R105 + *G. mosseae*	30.87	11.90	0.89	0.34
R111 + *G. mosseae*	39.60*	10.65	0.99	0.26
LSD ($p \leq 0.05$)	10.57	5.69	0.44	NS†

∗Significantly different from the control (-AMF and -PGPR).
†NS denotes no significant differences.

Table D.4. Analysis of variance for shoot nitrogen (N) and phosphorus (P) concentrations in pea tissue and nitrogen and phosphorus uptake by pea inoculated with the AMF species and/or PGPR strains 12 weeks after planting (WAP). n = 4.

Source of Variation	df	Mean square	F-ratio	Probability
Shoot N uptake				
Total	82			
AMF	3	528.104	9.448	0.000
PGPR	5	131.962	2.361	0.050
AMF * PGPR	13	37.486	0.671	0.783
Error	61	55.898		
Shoot N concentration				
Total	82			
AMF	3	72.736	4.499	0.006
PGPR	5	34.110	2.110	0.076
AMF * PGPR	13	15.513	0.960	0.500
Error	61	16.167		
Shoot P uptake				
Total	91			
AMF	3	0.566	5.790	0.001
PGPR	5	0.168	1.720	0.141
AMF * PGPR	13	0.138	1.415	0.175
Error	70	0.098		
Shoot P concentration				
Total	91			
AMF	3	0.173	0.755	0.523
PGPR	5	0.250	1.095	0.371
AMF * PGPR	13	0.222	0.971	0.488
Error	70	0.228		

Table D.5. Concentrations of easily extractable Bradford-reactive soil protein (EE-BRSP) and Bradford-reactive soil protein (BRSP) in the rhizosphere, and BRSP and Bradford-reactive protein (BRP) deposited on strips inserted into the mycorrhizosphere inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Treatment	EE-BRSP	BRSP	BRSP	BRP
	Rhizosphere		Mycorrhizosphere	
	mg g^{-1} soil		mg g^{-1} sand	μg cm^{-2} strip
Control (-AMF and -PGPR)	0.52	1.63	0.55	2.32
R55	0.56*	1.74	0.58	2.07
R75	0.55	1.71	0.59	2.29
R85	0.55	1.67	0.60	1.72
R105	0.53	2.00*	0.59	2.28
R111	0.60*	1.93*	0.63	2.35
G. clarum	0.52	1.88*	0.57	2.01
R55 + *G. clarum*	0.52	1.73	0.58	2.46
R75 + *G. clarum*	0.48*	1.81	0.59	2.10
R85 + *G. clarum*	0.48*	1.80	0.62	1.70
R105 + *G. clarum*	0.48*	1.78	0.62	1.86
R111 + *G. clarum*	0.58*	1.80	0.57	2.23
G. intraradices	0.55	1.56	0.58	3.18*
R55 + *G. intraradices*	0.55	1.48	0.59	2.14
R75 + *G. intraradices*	0.52	1.65	0.69*	2.24
R85 + *G. intraradices*	0.53	1.59	0.76*	2.18
R105 + *G. intraradices*	0.48*	1.64	0.68*	2.20
R111 + *G. intraradices*	0.54	1.48	0.54	2.48
G. mosseae	0.52	1.37*	0.55	2.05
R55 + *G. mosseae*	0.48*	1.36*	0.60	2.26
R105 + *G. mosseae*	0.50	1.80	0.43*	2.45
R111 + *G. mosseae*	0.51	1.86*	0.40*	1.69
LSD ($p \leq 0.05$)	0.04	0.21	0.09	0.67

∗Significantly different from the control (-AMF and -PGPR).

Table D.6. Analysis of variance for concentrations of easily extractable Bradford-reactive soil protein (EE-BRSP) and Bradford-reactive soil protein (BRSP) in the rhizosphere, and BRSP and Bradford-reactive protein (BRP) deposited on strips inserted into the mycorrhizosphere of pea inoculated with the AMF species and/or PGPR strains 12 weeks after planting (WAP). n = 4.

Source of Variation	df	Mean square	F-ratio	Probability
Rhizosphere EE-BRSP				
Total	91			
AMF	3	0.014	10.755	0.000
PGPR	5	0.007	5.731	0.000
AMF * PGPR	13	0.002	1.794	0.061
Error	70	0.001		
Rhizosphere BRSP				
Total	91			
AMF	3	0.339	15.340	0.000
PGPR	5	0.125	5.570	0.000
AMF * PGPR	13	0.067	3.027	0.001
Error	70	0.022		
Mycorrhizosphere BRP				
Total	91			
AMF	3	0.630	2.807	0.046
PGPR	5	0.470	2.093	0.076
AMF * PGPR	13	0.377	1.682	0.084
Error	70	0.224		
Mycorrhizosphere BRSP				
Total	91			
AMF	3	0.051	13.597	0.000
PGPR	5	0.015	3.995	0.003
AMF * PGPR	13	0.015	4.104	0.000
Error	70	0.004		

APPENDIX E. EFFECTS OF ARBUSCULAR MYCORRHIZAL FUNGI AND PLANT GROWTH-PROMOTING RHIZOBACTERIA ON GLOMALIN PRODUCTION, SOIL CARBON AND NITROGEN STORAGE, AND PEA GROWTH UNDER NON-STERILE CONDITIONS

Table E.1. Analysis of variance (ANOVA) for seed, shoot, root, and total weight of pea inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Source of Variation	df	Mean square	F-ratio	Probability
Seed weight				
Total	47			
AMF	2	0.005	0.006	0.994
PGPR	3	0.224	0.239	0.868
AMF * PGPR	6	0.750	0.802	0.575
Error	36	0.935		
Shoot weight				
Total	47			
AMF	2	0.272	0.177	0.839
PGPR	3	0.941	0.612	0.612
AMF * PGPR	6	0.861	0.560	0.759
Error	36	1.537		
Root weight				
Total	47			
AMF	2	0.006	0.142	0.868
PGPR	3	0.040	1.024	0.393
AMF * PGPR	6	0.031	0.799	0.577
Error	36	0.039		
Total weight				
Total	47			
AMF	2	0.262	0.148	0.863
PGPR	3	1.276	0.723	0.545
AMF * PGPR	6	1.107	0.627	0.708
Error	36	1.766		

Table E.2. Analysis of variance (ANOVA) for nitrogen (N) and phosphorus (P) concentrations in pea tissue inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Source of Variation	df	Mean square	F-ratio	Probability
Seed nitrogen				
Total	46			
AMF	2	0.344	0.039	0.962
PGPR	3	2.733	0.312	0.816
AMF * PGPR	6	13.683	1.563	0.187
Error	35	8.753		
Shoot nitrogen				
Total	47			
AMF	2	0.002	0.299	0.744
PGPR	3	0.001	0.190	0.903
AMF * PGPR	6	0.003	0.510	0.797
Error	36	0.007		
Seed phosphorus				
Total	47			
AMF	2	0.024	0.467	0.630
PGPR	3	0.001	0.016	0.997
AMF * PGPR	6	0.070	1.329	0.270
Error	36	0.052		
Shoot phosphorus				
Total	47			
AMF	2	0.007	0.265	0.769
PGPR	3	0.031	1.162	0.338
AMF * PGPR	6	0.018	0.693	0.657
Error	36	0.026		

Table E.2. (continued)

Source of Variation	df	Mean square	F-ratio	Probability
Root phosphorus				
Total	44			
AMF	2	0.006	0.228	0.798
PGPR	3	0.005	0.197	0.897
AMF * PGPR	6	0.031	1.253	0.305
Error	33	0.024		

Table E.3. Percent arbuscular mycorrhizal fungi (AMF) colonization of pea, easily extractable Bradford-reactive soil protein (EE-BRSP) and immunoreactive soil protein (EE-IRSP), Bradford-reactive soil protein (BRSP), and immunoreactive soil protein concentrations (IRSP) in rhizosphere of pea inoculated with AMF and/or plant growth-promoting rhizobacteria (PGPR) 12 weeks after planting (WAP). n = 4.

Treatment	AMF colonization	EE-BRSP	EE-IRSP	BRSP	IRSP
	%	mg g^{-1} soil			
Control (-AMF and -PGPR)	50.50	0.49	0.20	1.84	1.19
R75	28.25*	0.47	0.16	1.67*	1.12
R85	43.00	0.44*	0.13	1.69*	1.42
R105	40.00	0.48	0.22	1.63*	0.99
G. clarum	58.25	0.52	0.36*	1.91	1.04
R75 + *G. clarum*	42.67	0.53	0.33	1.79	1.06
R85 + *G. clarum*	41.00	0.50	0.39*	1.69*	0.93
R105 + *G. clarum*	65.00	0.52	0.29	1.71	0.93
G. intraradices	33.25*	0.53	0.27	1.77	0.99
R75 + *G. intraradices*	48.00	0.49	0.25	1.89	0.98
R85 + *G. intraradices*	35.25	0.49	0.33	1.92	0.88
R105 + *G. intraradices*	49.25	0.45	0.32	1.70	0.91
LSD ($p \leq 0.05$)	17.20	0.05	0.15	0.15	NS†

*Significantly different from the control (-AMF and -PGPR).
†NS denotes no significant differences.

Table E.4. Analysis of variance (ANOVA) for percent arbuscular mycorrhizal fungi (AMF) colonization of pea, easily extractable Bradford-reactive soil protein (EE-BRSP) and immunoreactive soil protein (EE-IRSP), Bradford-reactive soil protein (BRSP), and immunoreactive soil protein (IRSP) concentrations in rhizosphere of pea inoculated with AMF and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Source of Variation	df	Mean square	F-ratio	Probability
% AMF colonization				
Total	47			
AMF	2	879.521	6.111	0.005
PGPR	3	324.472	2.254	0.099
AMF * PGPR	6	360.660	2.506	0.040
Error	36	143.931		
EE-BRSP				
Total	46			
AMF	2	0.009	8.855	0.001
PGPR	3	0.003	2.840	0.052
AMF * PGPR	6	0.002	1.730	0.143
Error	35	0.001		
EE-IRSP				
Total	45			
AMF	2	0.113	9.847	0.000
PGPR	3	0.004	0.319	0.812
AMF * PGPR	6	0.008	0.701	0.651
Error	34	0.011		
BRSP				
Total	46			
AMF	2	0.049	4.313	0.021
PGPR	3	0.051	4.545	0.009
AMF * PGPR	6	0.031	2.711	0.029
Error	35	0.011		

Table E.4. (continued)

Source of Variation	df	Mean square	F-ratio	Probability
IRSP				
Total	45			
AMF	2	0.254	2.863	0.071
PGPR	3	0.046	0.520	0.672
AMF * PGPR	6	0.055	0.625	0.709
Error	34	0.089		

Table E.5. Analysis of variance (ANOVA) for organic carbon (C) and total nitrogen (N) of soil inoculated with the arbuscular mycorrhizal fungi (AMF) species and/or plant growth-promoting rhizobacteria (PGPR) strains 12 weeks after planting (WAP). n = 4.

Source of Variation	df	Mean square	F-ratio	Probability
SOC				
Total	47			
AMF	2	0.030	0.055	0.947
PGPR	3	0.916	1.669	0.191
AMF * PGPR	6	0.612	1.114	0.374
Error	36	0.549		
Soil total N				
Total	47			
AMF	2	0.002	0.299	0.744
PGPR	3	0.001	0.190	0.930
AMF * PGPR	6	0.003	0.510	0.797
Error	36	0.007		