Chapter 1 Introduction

1.1 General Introduction

Microbes play a key role in many processes that are important to natural ecosystems (Larrainzar *et al.* 2005). These processes include turnover of organic matter, completion of biogeochemical cycles and promotion of plant health and soil fertility (Larrainzar *et al.* 2005). The rhizosphere is a part of the ecosystem where plant roots continually interact with soil microorganisms. Some soil microorganisms, such as bacteria and fungi, live in plant tissues and are involved in active beneficial relationships with their host plants without causing any pathogenic symptoms (Lynch 1990a, Kloepper 1992, Baldani *et al.* 2000). Certain strains of bacteria that stimulate growth and improve plant health in stressful conditions are referred to as plant growth-promoting rhizobacteria (PGPR) (Lynch 1976). They stimulate plant growth in the rhizosphere by antagonizing the effects of pathogens (Cook 1993, Cook *et al.* 1995). They have also been reported to suppress disease in several agricultural crops (Kloepper 1992, Kloepper and Beauchamp 1992, Ryu *et al.* 2003). This has been achieved by introducing bacteria into soil or on seeds, roots, bulbs or other planting material (Weller 1988, Whipps 2001). Other objectives of bacterial inoculation include enhancement of symbiotic association and degradation of xenobiotic compounds (Whipps 2001).

Rhizobacteria cause disease suppression or growth enhancement of plants by a range of processes such as producing various anti microbial metabolites or enzymes (Cook 1993, Duffy *et al.* 2004, Ryu *et al.* 2004a). It is also believed that host-pathogen signalling may have a potential role in disease suppression (Duffy *et al.* 2004). The study of root-associated bacteria and their antagonistic potential is important to understand their role in disease suppression and interaction with the pathogen. To date, many bacterial genera have been applied as soil inoculants including *Aceinetobacter*, *Agrobacterium*, *Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Bradyrhizobium, Frankia, Pantoea, Pseudomonas, Rhizobium, Serratia, Streptomyces* and *Thiobacillus* (Weller 1988, Van Elsas and Heijnen 1990, Whipps 2001).

This study is focused on a group of three types of beneficial bacteria, *Pantoea agglomerans*, *Exiguobacterium acetylicum* and *Microbacterium* (PEM), which have been isolated from

disease suppressive soil from Avon, South Australia. PEM have been shown to suppress *Rhizoctonia solani* disease on wheat (Barnett *et al.* 2006). The greatest reduction in *Rhizoctonia* disease as noticed when all three groups of bacteria (PEM) were added together in the bioassay (Barnett *et al.* 2006). The beneficial PEM are novel in that they have not previously been associated with suppression of *Rhizoctonia* disease on wheat. These bacteria do not reduce the pathogen levels in the soil but they play a significant role in disease suppression in wheat (Barnett, personal communication).

It was been suggested that *Microbacterium* reduces infection of wheat roots by pathogens while *Pantoea* and *Exiguobacterium* promote the growth of infected plants (Barnett *et al.* 2006). The mechanisms by which infection is reduced or plant growth is enhanced are not known for PEM. This study investigated the interaction of beneficial PEM with wheat and the pathogen *Rhizoctonia*. Using molecular tools to elucidate the potential involvement of genetic factors can provide valuable information in our understanding of the disease suppression ability of PEM.

Within a context of assisting researchers and land mangers to enhance the benefecial effects of PEM against Rhizoctonia, this research sought to:

a) better understand the biology of the PEM consorrtium,
b) quantify the magnitude and conditions affecting PEM efficacy,
c) investigate several machanisms for the benefecial affects of PEM, and
d) determine the localization pattern and colonization ability of PEM on wheat roots.

Chapter 2 Literature review

2.1 Disease history in South Australia

Wheat is an important cereal crop (Brennan and Murray 1988) and South Australia (SA) is the second largest wheat growing region in Australia. Wheat crops are affected by several plant pathogens including bacteria and fungi. Researchers in Adelaide, SA have been working on soil-borne diseases and their impact on wheat crops for several years, especially disease caused by the soil fungus *Rhizoctonia solani* (Mussared 1996). It was reported that *Rhizoctonia* affects wheat crops in Southern New South Wales, Victoria, South Australia and Western Australia but strongly affects the sandy soil regions of South Australia and Western Australia.

A field at Avon, a site near Adelaide, has been shown to be suppressive to *R. solani* bare patch disease (casual agent *Rhizoconia solani* AG-8) (Roget 1995, Barnett *et al.* 2006). This field site was set up to investigate the effect of different cultivation methods, crop rotation and cultural practices (Rovira 1986). The suppression of Rhizoctonia was first reported by Roget in 1995 and demonstrated that there was a decline in the incidence of Rhizoctonia root rot of wheat. Severity of the disease started in 1983 with the patches of poor plant growth accounting for up to 46% of the crop area and was then reported to decline to low levels by 1990. The decline of Rhizoctonia root rot in the field experiment was investigated after adding *R. solani* to soil collected from the experimental site in 1985. It was found that wheat grown in soil from direct drilled plots had significantly lower disease severity compared to soil from cultivated treatments, indicated the possible development of suppression to Rhizoctonia root rot disease (Roget 1995).

The nature of suppression of Rhizoctonia bare patches of wheat was further studied by Wiseman in 1996. The suppressive nature of the soil was investigated after fumigating and reinoculating Avon soil (Wiseman *et al.* 1996). It was found that the natural suppression of the soil could be transferred to an autoclaved or sterile soil by adding 10% (w/w) of the unsterilized soil. There was no suppression detected when non suppressive soil from a nearby site was added to the autoclaved or sterilized soil. Thus it was confirmed that the suppression of this root rotting disease of wheat caused by *R. solani* AG-8 was due to microorganisms

(Wiseman *et al.* 1996). Later, Barnett *et al.*(2006) reported that several microorganisms are involved in *Rhizoctonia* disease suppression. These were *Pantoea agglomerans*, *Exiguobacterium acetylicum* and *Microbacterium* (PEM). These organisms were isolated from Avon soil and have been demonstrated to have disease suppression potential against *R. solani* (Barnett *et al.* 2006).

2.2 Plant pathogen *Rhizoctonia solani*

Rhizoctonia solani, the most widely recognized *Rhizoctonia* species, was originally described on potato by Julius Kühn in 1858. This common soil-borne fungal pathogen has a great diversity of host plants and causes root rot and damping-off diseases. *R. solani* does not produce spores (conidia) and only occasionally produces sexual spores (basidiospores). It exists primarily as vegetative mycelium and/or sclerotia in nature (Adam 1988), and can survive for many years by producing small (1 to 3 mm diameter), irregular-shaped, brown to black structures (sclerotia) in the soil or plant tissues (Agrios 1997, Bockus and Shroyer 1998, Cook 2001).`

As a soil-borne fungal pathogen, *R. solani* develops in both cultured and non-cultured soils and causes disease to different crops, the most serious of which is bare patch disease (Sneh et al. 1991). Rhizoctonia bare patch disease of cereals was first recorded in Australia in the 1920s and subsequently in many other parts of the world (MacNish and Fang 1987). *R. solani* consists of a number of reproductively isolated groups of strains called anastomosis group (AG). These differ in their biology and pathogenicity (Anderson 1982). The disease causes separate patches of stunted root called Rhizoctonia root rot of wheat (MacNish and Fang 1987). This disease is caused by *Rhizoctonia solani* AG-8 which causes major yield losses in wheat worldwide (Rovira 1986) and is a major constraint to the uptake of sustainable agricultural practices such as direct drill or minimal tillage and stubble retention, as Rhizoctonia root rot increased under these practices (Barnett *et al.* 2006). The Rhizoctonia root rot disease has been historically associated with southern Australia (Roget 1995).

R. solani and *R. oryzae* are known to cause root rot disease in wheat (Bockus and Shroyer 1998, Cook 2001). This fungus attacks several plant parts often causing multiple disease symptoms such as delayed maturity, stunting and root rot. Infected seedlings usually display a dark brown root terminus that tapers to a fine point, two to three inches from the crown or seed. Although most Rhizoctonia diseases are initiated by mycelium of the fungus which is

present in the rotted tissues and root stubs near the crown (Dickson 1956), several diseases of beans, sugar beet and tobacco result from basidiophore infection.

The wide host range of *R. solani* does not allow the effective control of this disease through crop rotation (Rovira 1986). Scope for crop rotation is limited because *R. solani* has great diversity and it infects over 500 plant species, making it one of the most common plant pathogens. Severity of the disease can also be greatly reduced by soil disturbance methods such as conventional tillage (Rovira 1986).

2.3 The species *Pantoea agglomerans*

Introduction

P. agglomerans (Beijerinck 1888) is a Gamma Proteobacteria, which belongs to the family Enterobacteriaceae (Manulis and Barash 2003). The species contains strains belonging to the '*Erwinia herbicola-Enterobacter agglomerans* complex' (Gavini *et al.* 1989). The relationship between *P. agglomerans* and *Erwinia herbicola* has long been disputed until resolved by using phenotypic properties and DNA/DNA hybridization (Ewing and Fife 1972). The genus name *Pantoea* was first established by Gavini et al. (1989). On the basis of DNA homology *E. herbicola*, *E. milletiae*, and *Enterobacter agglomerans* were considered to belong the same genus, however, there were some phenotypic distinctions (Gavini *et al.* 1989). Therefore, some strains that were originally assigned to *E. agglomerans* were transferred to *P. agglomerans* and *P. dispersa* species. A number of other strains of *Pantoea* remain unclassified and retained as *E. agglomerans.* The species in the genus *Pantoea* are, therefore, very heterogeneous and can only be identified on the basis of phylogenetic properties (Gavini *et al.* 1989).

Growth and morphology in cultures

Colonies grow well at 30°C on solid agar. Colony morphology is golden yellow, pigmented, circular, smooth and more or less convex with entire margins (Gavini *et al.* 1989).

Physiological and biochemical characteristics of Pantoea

Some important characteristics of *Pantoea* are summarized in Table 2-1 (Iimura and Hosono 1996, Zohar-Perez *et al.* 2002). *Pantoea* are non-capsulated, non-spore forming, straight rods measuring 0.5-1.0 µm in width by 1.0-3.0 µm in length. Most species are motile and have peritrichous flagella. *Pantoea* are Gram-negative, facultative anaerobic, oxidase-negative

bacteria and have around 55.1% to 56.8% G+C content (Gavini *et al.* 1989, Manulis and Barash 2003). In nature, *P. agglomerans* is widely distributed on the surface of different plants, and *Pantoea* spp. have been isolated from seeds, water, humans (wounds, blood, urine, internal organs) and animals (Gavini *et al.* 1989). Isolates of *Pantoea* have also been noted to produce antibiotics (Wright *et al.* 2001) and auxins (Lindow and Brandl 2003). Auxins are known to promote root growth. There are also reports of various strains of *P. agglomerans* that fix nitrogen, solubilise phosphorous and produce exopolysaccharides (EPS) (Amellal *et al.* 1998b). Many of these biochemicals have important roles in plant growth.

Pantoea - plant root associations

The rhizosphere is a complex system which constitutes the principal source of organic material in the soil. It contributes to soil organic matter through the decomposition of roots, and through root exudation which stimulates microbial activity and biomass (Lynch 1990a, Lynch 1990b). Soil water potential influences the availability of nutrients, competition and predation for the survival and activity of microorganisms (Amellal *et al.* 1998a, Amellal *et al.* 1998b). Bacterial EPS protect bacteria from various stresses, assist in water retention in the microbial environment and appeared to be involved in the regulation of metabolites. The EPS-producing *P. agglomerans* strain NAS206 has been reported for its role in regulating the water content of the rhizosphere of wheat by improving soil aggregation (Amellal *et al.* 1998b).

Pantoea have been reported for their endophytic nature (Ruppel *et al.* 1992, Remus *et al.* 2000, Verma *et al.* 2004). Ruppel *et al.* (1992) and Remus *et al*. (2000) showed that *P. agglomerans* colonized wheat endophytically while Verma *et al.* (2004) reported it on rice. Some PGPR strains of *Pantoea* were also shown to have nitrogen fixation ability (Ruppel *et al.* 1992a, Remus *et al.* 2000, Verma *et al.* 2004). The *P. agglomerans* isolated from Avon are root associated, i.e. there is greater frequency of isolation from roots compared to the rhizoplane or rhizosphere soil (Barnett *et al.* 2006). *Pantoea* does not reduce primary infection by *Rhizoctonia,* however, it promotes the growth of infected roots (Barnett *et al.* 2006). They may also multiply on *Rhizoctonia* infected sites reducing carbon substrate availability to *Rhizoctonia* and its ability to grow from the primary infection site to infect new roots (Barnett personal communication).

Table 2-1. Phenotypic characteristics of *Pantoea agglomerans*.

Characteristics	*P. agglomerans*	Reference
Symplasmeta formation	+(strain NO30)	Achouak *et al*. 1996
Metal reduction	+ (strain SP1)	Francis *et al*. 2000
Chitinolytic property	+ (strain IC1270)	Zohar-Perez *et al.* 2002
Phenol production (Anti microbial)	+	Dillon *et al*. 1995
EPS production	+(strain NAS 206)	(Amellal *et al.* 1998a)
Phytohormones	strain D5/23	Bodenfruchtbarkeit *et al*. 1992
Cytokinin	+	
Auxin	+	
Indole acetic acid	+	
Biochemical characteristics	30 strains	Iimura *et al*. 1996
Catalase	+	
KCN	+	
Gealatin Liquifaction	+	
Nitrate reduced to nitrite -phenyl alanine deaminase	+	
β-Galactosidase	+	
L-Arabinose	+	
D-Ribose	+	
D-Xylose	+	
D-Fructose	+	
D-Glucose	+	
D-Mannose	+	
D-Rhamnose	+	
D-Galactose	+	
D-Maltose	+	
D-Sucrose	+	
D-Trehalose	+	
D-Mannitol	+	
Anaerobic growth	+	
Motility at 30ºC	+	
Cytochrome	+	
Oxidase	-	
Urease	-	
Arginine dihydrolase	-	
Lysine	-	
Ornithine decarboxylase	-	
H_2S	-	

+ Positive reaction, - Negative reaction

EPS production

Some beneficial microorganisms produce one or more high molecular mass EPS (Denny et al., 1999). These large polymers may be associated with the bacterial cells as a capsule or released as a fluidal slime and are sometimes present in both forms. The EPSs made by bacteria are usually hetero-polysaccharides, which contain a mixture of sugars precisely arranged in repeating subunits. However, some species also produce homo-polysaccharides, which have only a single type of sugar. The details concerning composition and structure of these EPSs are available in several review articles (Leigh and Coplin 1992, Jumel *et al.* 1997). Bacterial EPS can protect bacteria from various stresses and possibly enhance water retention in the microbial environment and seem to regulate the diffusion of carbon sources (Roberson and Firestone 1992, Chenu 1993, Chenu and Roberson 1996, Amellal *et al.* 1998a, Amellal *et al.* 1998b). The effect of EPS-producing *Pantoea agglomerans* strain NAS206 on the physical properties of wheat rhizosphere has been investigated and identification was confirmed by amplified ribosomal DNA restriction analysis (Amellal *et al.* 1998a, Amellal *et al.* 1998b) Remus,(2000) found EPS like material around *P. agglomerans* cells on wheat roots surfaces using scanning electron microscopy.

Symplasmata formation

Some bacteria form an aggregate structure called symplasmata (Achouak *et al.* 1996, Feng *et al.* 2003). A symplasmata is a multicellular structure in which at least two and up to hundreds of individual cells tightly bind together. However, the function of these structures in the plant system is unknown (Feng *et al.* 2003). In numerous studies, *P. agglomerans* has been reported to form cell aggregates such as the NO30 strain, isolated from rhizosphere of rice. Its colonization pattern was characterized by the formation of symplasmata (Achouak *et al.* 1996). Another study based on the phylogenetic analysis of the strain YS19 demonstrated that symplasmata formed by aggregation of cells (Feng *et al.* 2003, Duan *et al.* 2007).

2.4 The species *Exiguobacterium acetylicum*

Introduction

The isolation of the alkalophilic bacteria of the genus *Exiguobacterium* was first reported by Gee et al., (1980). It resembled coryneform bacteria but its true taxonomic position was not confirmed at that time (Gee *et al.* 1980). The genus *Exiguobacterium* was later described by Collins et al., (1983) and originally consisted of only one species, *E. aurantiacum,* an alkalophilic bacterium isolated from potato processing effluent. Studies using 16S ribosomal

RNA (rRNA) gene sequencing (Farrow *et al.* 1994) revealed considerable heterogeneity within the genus *Exiguobacterium*. On the basis of the results of comparative analyses of almost complete 16S rRNA sequences, it has been suggested (Jones 1975, Collins *et al.* 1983b) that *Brevibacterium acetylicum* may be related to the genus *Exiguobacterium* rather than to *Brevibacterium linens*. However, in 1994, Farrow et al., included the species *B. acetylicum* in the same genus (Funke *et al.* 1997). The genus is closely related to *Bacillus* but does not produce spores (Funke *et al.* 1997, Fruhling *et al.* 2002). *E. aurantiacum* has several common chemotaxonomic features with the group-2 bacilli but the organism was considered to be a sufficiently distinct genus (Farrow *et al.* 1994)

Growth and morphology in cultures

Colony morphology of *Exiguobacterium* species on solid tryptic soy agar (TSA) media is 2-3 mm in diameter after 2 days at 25°C, pale orange or shiny orange in color, circular, smooth, and grows well at 25-37^{o}C (Funke *et al.* 1997, Fruhling *et al.* 2002).

Physiology and biochemistry of Exiguobacterium species

Cells of *Exiguobacterium* species vary in shape and size between rod (3.2 x 1.2 μm) and cocci forms (1.4 x 1.1 μm). The organism is facultative anaerobic, motile (peritrichous flagella), catalase positive and oxidase negative (Gee *et al.* 1980, Collins *et al.* 1983b, Fruhling *et al.* 2002). They are Gram positive, non-spore forming and the cell wall contains a group A type peptidoglycon based on lycine and lipids. The polar lipids consist of diphosphatidyl glycerol, phosphatidyl glycerol and phosphatidyl ethanolamine (Collins *et al.* 1983b, Farrow *et al.* 1994, Fruhling *et al.* 2002). The DNA base composition is around 47% G+C content (Funke *et al.* 1997).

Exiguobacterium - plant root association

There are no reports on *Exiguobacteium* attachment to plant roots but it has been hypothesized that these bacteria are root associated (e.g. greater frequency of isolation on roots compared to rhizoplane or rhizosphere soil) (Barnett *et al.* 2006). They do not reduce primary infection by *Rhizoctonia* but promote the growth of infected roots. This is evident by an increase in secondary roots with more root hairs (Barnett personal communication).

2.5 The genus *Microbacterium*

Introduction

The genus *Microbacterium* proposed by Jensen in 1919, underwent a number of changes until 1983, when the description was amended by Collins *et al* (1983b) on the basis of 5S rRNA nucleotide sequencing and associated chemotaxonomic data. Park *et al.*(1993) proposed the family Microbacteriaceae to accommodate Gram positive bacteria containing G+C rich DNA and all the peptidoglycan group B Actinomycetes (Schleifer and Kandler 1972) genera *Agromyces* (Gledhill and Casida 1979) *Aureobacteium* (Collins *et al.* 1983a), *Clavibacter* (Davis *et al.* 1984)) *Curtobacterium* (Yamada and Komagata 1972) and *Microbacterium* (Orla-Jensen 1919).

The members of the genus *Microbacterium* may be differentiated from other coryneform bacteria by the presence of the diamino acid lysine and the peptidoglycan of the cell wall (Schleifer and Kandler 1972, Keddie and Cure 1978). On the basis of phylogenetic clustering using 16S rDNA sequencing (Stackebrandt *et al.* 1997), six species of *Microbacterium* have been described previously (Yakota *et al.* 1993). The family *Microbacteriaceae* as well as the genera *Agrococcus* (Groth *et al.* 1996), *Agromyces* (Gledhill and Casida 1979), *Aureobacteium* (Collins *et al.* 1983a), *Clavibacter* (Davis *et al.* 1984), *Curtobacterium* (Yamada and Komagata 1972) and *Rathayibacter* (Zgurskaya et al 1993) were proposed. Recently, the new genera *Leucobacter* (Takeuchi and Hatano 1998) and *Cryobacterium* (Suzuki *et al.* 1997) have also been proposed. Currently, a total of nine genera are included in this family (Takeuchi and Hatano 1998).

Growth and morphology

Colony sizes on solid media are ~1-3 mm in diameter and show a grey, white or yellow-orange on colour, depending on the species. The minimum growth temperature is 10°C and maximum is in the range of 36-40°C (Collins *et al.* 1983b).

Physiology and biochemistry Microbacterium genus

The primary chemotaxonomic characteristics of these bacteria are summarized by Collins *et al.* (1983a). They are aerobic but also have been reported to give weak anaerobic growth (Orla-Jensen 1919). *Microbacterium* contains high G+C content around 69%-75% (Guido Funke *et al.* 1997). Some strains of the *Microbacteria* have been reported to produce L-threonine (Debabov 1999).

Microbacterium - plant root association

Reports have been made about the endophytic nature of *Microbacterium testaceum* and its use in biocontrol and other applications seems promising (Zinniel *et al.* 2002). A study conducted on 853 endophytic bacteria from agronomic crops and prairie fields showed that *Microbacteria* exhibit promising levels of colonization and persistence ability. The microbacteria were identified by 16S rRNA gene sequence, fatty acid and carbon source utilization analyses (Zinniel *et al.* 2002).

2.6 Plant microbe interaction

Hiltner described the rhizosphere in 1904 as a most intense area of interactions between microbes and plants (Morrissey *et al.* 2004). The influence of plants on a diverse range of microorganisms in the rhizosphere has important ecological implications for soil function, including chemical and biological processes. Similarly, soil microbes have a tremendous influence on plant health and productivity (Bloemberg and Lugtenberg 2001, Gregory 2006). The interaction between rhizosphere microbes and plants depends on the association between organisms and plants with which they communicate (Walker *et al.* 2003). The formation of these communities is based on activation of some chemical signals or compounds in response to plants that have been shown to play an important role in root-microbe interactions. For example, flavonoids present in the root exudates of legumes triggered the nodulation genes in *Rhizobium meliloti* (Walker *et al.* 2003). Another example of plant-derived signals is that phenolics exuded from plant wounds induce the expression of virulence genes in *Agrobacterium* spp. that influence microbial gene expression (Newton and Fray 2004).

Microorganisms can exhibit a variety of characteristics which benefit the plant by enhancing the plant growth or disease suppression, directly or indirectly (Whipps 2001). Several possible mechanisms of plant-microbe interactions are summarized in Figure 2-1 (Arshad and Frankenberger 1998). Among these, production of biologically active metabolites, particularly the plant growth regulators and better supply and access to nutrients by rhizosphere microbiota affect plant growth (Arshad and Frankenberger 1998). An example of microbial association is the symbiotic relationship between plant roots and nitrogen fixing rhizobacteria which has been well documented for decades (Lodwig *et al.* 2003). There are many more possible mechanisms involved in these plant microbial interactions as described in Figure 2-1 (Arshad and Frankenberger 1998). Beside enhancing the nutrient supply to the plants, some

microbes such as *Pseudomonas*, *Bacillus* and *Trichoderma* has been reported to protect the plant from diseases due to production of a range of metabolites against phytopathogenic fungi (Bloemberg and Lugtenberg 2001, Weller *et al.* 2002). Although various microbial inoculants are already available, their wide spread application is restricted due to limitations and inconsistent performance in the field. A better understanding of plant-microbe interaction using recent molecular techniques with traditional approaches could help to overcome at least some of these problems.

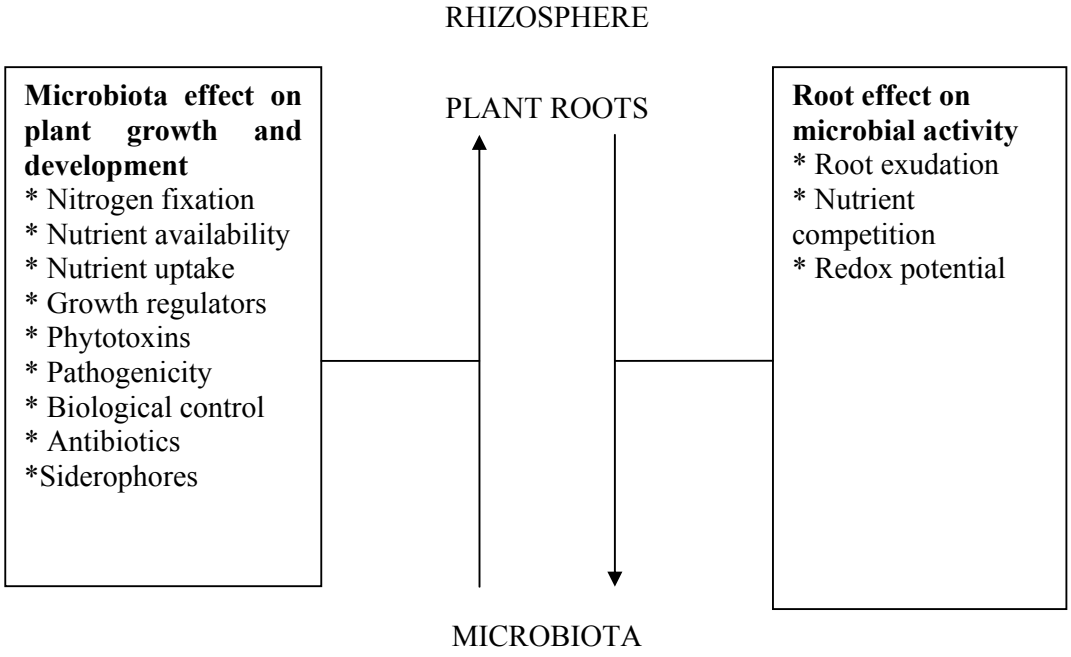


Figure 2-1. Possible plant microbe interactions (adapted from Frankenberger and Arshad, 1995).

Plant growth promotion

Beneficial and associative bacteria found in the rhizosphere are named plant growth-promoting rhizobacteria (PGPR) (Kloepper and Beauchamp 1992). There are several direct or indirect ways by which PGPR can promote plant growth (Figure 2-2) (Glick 1995, Glick *et al.* 2001, Persello-Cartieaux *et al.* 2003).

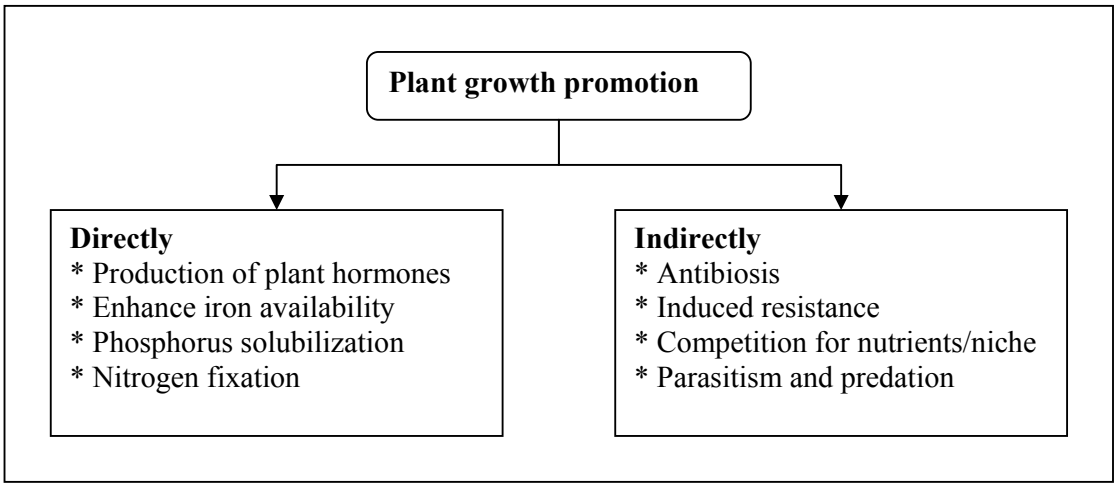


Figure 2-2. Plant growth promoting bacteria affect plant growth directly or indirectly.

Direct growth promotion involves the production of plant growth regulators (PGR) (Zimmer and Bothe 1988), fixation of atmospheric nitrogen (Lima *et al.* 1987), solubilization of mineral nutrients such as phosphorus (Piccini and Azcon 1987, Goldstein and Braverman 1990), production of siderophores that solubilize and sequester iron, and reduced ethylene production in roots (Glick 1995). These process enhance plant growth at different stages of development such as germination and early growth.

Indirect growth promotion may be involved when PGPR promote plant growth by reducing or relieving growth restricting conditions (Glick *et al.* 1999), such as the production of substances that are antagonistic to pathogens or indirectly by inducing resistance to pathogens (Glick, 1995). The production of antimicrobial metabolites, however, does not necessarily mean that they are involved in disease suppression as bacteria need a range of mechanisms for successful disease suppression (Loper *et al.* 1994). A bacterium can affect plant growth by one or more of these mechanisms and may use different abilities for growth promotion at different stages of the plant lifecycle (Glick *et al.* 1999)

Several microorganisms are capable of producing PGR such as auxins, cytokinins, gibberellins, ethylene, or abscisic acid. Auxins are the most common hormones produced by several rhizobacterial genera, e.g. *Azospirillum, Agrobacterium, Pseudomonas* and *Erwinia* (Costacurta and Vanderleyden 1995). Ethylene, a hormone produced in all plants, mediates several responses to developmental and environmental signals in plants (Glick 1995, Glick 2004). Several bacteria including *Azotobacter, Azospirillum*, various species of*, Bacillus, Enterobacter, Klebisiella, Pseudomonas* and *Xanthomonas,* have been reported for different

PGP (plant growth promotion) properties (Costacurta and Vanderleyden 1995, Glick 1995, Arshad and Frankenberger 1998, Lucy *et al.* 2004). Some endophytic bacteria and their beneficial association with plants have also been widely studied (Baldani *et al.* 2000, Elbeltagy *et al.* 2001, Gyaneshwar *et al.* 2001).

IAA production

Indole-3-acetic acid (IAA), the predominant naturally occurring plant auxin, is implicated in many aspect of plant growth and development, including cell division, cell elongation, cell differentiation, tropism, flower development and vascular system patterning. The ability to produce the plant hormone IAA is widespread among soil and plant colonising bacteria (Costacurta and Vanderleyden 1995, Patten and Glick 1996, Brandl and Lindow 1998). In some microorganisms, including *Agrobacterium tumefaciens*, *A. rhizogenes*, *Pseudomonas syringae* pv. *savastanoi*, *P. syringae* pv. *syringae*, *Erwinia herbicola* pv. *gypsophilae* (Ehg), IAA is involved in pathogenesis (Clark *et al.* 1993) while in others, such as members of the genera *Azospirillum, Rhizobium, Enterobacter, Xanthomonas* and other *Pseudomonas* spp., IAA benefited the bacterium by stimulating plant growth and increasing the release of plant metabolites that the bacteria can utilize (Bar and Okon 1993, Glick 1995, Patten and Glick 1996). The high quantity of the IAA synthesis ability in tryptophan supplemented culture is prevalent among strains of *E. herbicola* (Brandl *et al.* 1996). IAA production has been reported in some strains of *P. agglomerans* (Cimmino *et al.* 2006).

Nitrogen fixation

Nitrogen (N) is an essential nutrient required for growth and survival of all organisms as it forms an integral part of proteins, nucleic acids and other important biomolecules (Bøckman 1997). The earth's atmosphere consists of 80% N but it is in a form that is unavailable for plants. It needs to be converted into ammonia and nitrate, a form available for plants and other eukaryotes (Kim and Rees 1994). Several groups of microorganism are capable of using atmospheric nitrogen and transforming it into available biological compounds (Robertson and Kuenen 1988). The process in which bacteria convert di nitrogen (N_2) compounds into simple ammonium compounds in the presence of the nitrogenase enzyme is called biological nitrogen fixation. This process contributes about 60% of the earth's available N which represents an economically beneficial and environmentally friendly alternative to chemical fertilizers (Ladha *et al.* 1997).

Rhizobia are the best known beneficial plant-associated bacteria because of their importance in the nitrogen fixation that occurs during legume *Rhizobium* symbiosis. Inoculation of legumes with rhizobia enhances crop productivity (Garcia *et al.* 2004). PGPR that fix N in non-leguminous plants are diazotrophs that form a non-obligate interaction with the host (Glick *et al.* 1999).

Azospirillum sp. is one of the best studied diazotrophs for which N fixation was first observed by Beijerinck in 1925 in a N free medium (Holguin *et al.* 1999). *Azospirillum* species belong to the facultative endophytic diazotrophs group which colonize the surface and the interior of roots. This association is considered to be the starting point of most ongoing N fixation processes with non-legume plants worldwide (Baldani *et al.* 1997). Members of this genus are capable of fixing atmospheric N and of promoting plant growth. A mixed inoculum of *Staphylococcus* and *Azospirillum* promoted the N fixation activity of *Azospirillum* (Holguin and Bashan 1997). Other N fixing bacteria such as *Enterobacter cloacae*, *Erwinia herbicola*, *Klebsiella pneumoniae*, *Azotobacter vinelandi*, *Paenibacillus polymixa*, *Azospirillum* spp., *Herbaspirillum* spp. and *Gluconacetobacter diazotrophicus* have been reported to colonise the sugarcane plant and its tissues (Olivares *et al.* 1996). Endophytic bacteria that colonize the interior of plant tissues such as roots, stem and leaves and are able to fix N are also found to be beneficial for plant growth (James and Olivares 1997). Some endophytic diazotrophs of rice, maize and sugarcane have also been reported as N fixers when other available sources of N are absent or present at low levels (Dobereiner *et al.* 1995, Kirchhof *et al.* 1997). Involvement of *nif*H genes and nitrogen fixation ability of *Pantoea* and *Microbacterium* have been reported in various studies Ruppel *et al.* (Remus *et al.* 2000, Loiret *et al.* 2004, Verma *et al.* 2004, Zakhia *et al.* 2006).

ACC deaminase

The plant hormone ethylene plays an important role in agricultural production (Glick 2005). Ethylene is one of the simplest organic molecules which is an efficient plant growth regulator at very low concentration. Ethylene is involved in various biological activities (Abeles 1992) including plant development and stress responses (Deikman 1997). Soil microorganisms that produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase are able to promote plant growth by sequestering and cleaving plant-produced ACC, an ethylene precursor and thereby lowering the level of ethylene in the plant. Decreased ethylene levels allows the plant to be more resistant to a wide variety of environmental stresses (Glick 2005).

This enzyme has been identified in soil bacteria and has been proposed to play a key role in microbe-plant associations (Hontzeas *et al.* 2004). Studies performed by Honma and his coworkers showed the different physio-chemical properties and modes of action of ACC deaminase isolated from soil microorganisms (Honma 1985, Honma *et al.* 1993a, Honma *et al.* 1993b, Minami *et al.* 1998).

Disease suppression

The use of rhizobacteria to suppress plant diseases has been reviewed (Weller 1988, Whipps 2001). It has been well established that some rhizobacteria have the capacity to suppress major plant diseases (Weller 1988, Kloepper and Beauchamp 1992, Glick 1995, Nehl *et al.* 1997, Whipps 2001). Potential mechanisms involved in disease reduction and the control of soil borne pathogens include antibiotic and siderophore production, competition with pathogens and production of a variety of enzymes (Weller 1988, Glick 1995, Nehl *et al.* 1997, Whipps 2001).

Most natural soils possess some ability to suppress soil borne pathogens due to the presence and activity of other microorganisms. This phenomena is known as general suppression (Mazzola 2002). General suppression increases with increasing microbial biomass in the soil (Weller *et al.* 2002). Specific suppression involves the activity of an individual or selected group of microorganisms that are antagonistic to a particular pathogen (Mazzola 2002). The role of microorganisms in disease suppression has been investgated for a number of plant diseases, including the disease caused by the bacterium *Streptomyces scabies* (Menzies 1959) and the fungi *Fusarium oxysporum* (Scher and Baker 1980), *Gaeumannomyces graminis* var. *tritici* (Cook and Rovira 1976) and *Rhizoctonia solani* (Henis *et al.* 1979).

PEM have been reported to suppress Rhizoctonia disease on wheat roots (Barnett *et al.* 2006). Among these microorganisms, *Pantoea* has been reported to produce various antimicrobial metabolites that may be involved in disease suppression (Figure 2-2) (Dillon and Charnley 1995, Minogue *et al.* 2002, Giddens and Bean 2007). While not all of the metabolites listed in Figure 2-2 may be important with a particular bacterial pathogen strain, especially where more than one mechanism is involved, the relative importance of individual mechanisms will depend on the plant and pathogen involved (Maurhofer *et al.* 1994). For example, production of chitinolytic (fungal cells wall degrading) enzymes by some biocontrol strains of *P. agglomerans* was shown to be anatagonistic to fungal plant pathogens (Chernin *et al.* 1995).

However little is known about the antibiotics of E and M in disease suppression or plant growth promotion.

Induced systemic resistence

Plants generally have many defense mechanisms against pests and diseases. Disease intensity can be reduced if the defense mechanism could be triggered by a stimulus before the infection by the pathogen starts. Induced systemic resistance (ISR) is a phenomenon whereby disease resistance is systematically induced by local infection or treatment with microbial products. Some of the compounds are directly antimicrobial whereas others prohibit the activity of pathogens by forming barriers (Kuć 2001). While pathogen induced systemic resistance in plants is well established, there exists evidence of nonpathogenic rhizobacteria induced systemic resistance in plants (van Loon *et al.* 1998). There is also evidence that systemic resistance induced by different beneficial organisms is regulated by similar jasmonate dependent and ethylene dependent signaling pathways and is associated with priming for enhanced defense (Van Wees *et al.* 2008).

Ortmann *et al* (2006) showed that growth medium of *P. agglomerans* appears to act as an elicitor of defense reactions. The bacterial induced systemic resistance is often associated with the induction of pathogenesis related proteins (PR) such as chitinases, ß-1, 3 glucanases and proteinase inhibitors which is dependent on bacterial spcies and host plant involvd (Pieterse *et al.* 1998, Whipps 2001). The changes in plant roots due to bacterial-root interactions involving ISR exhibits incresed levels of enzymes such as chitinase, peroxidase, polyphenol oxidase and phenyl alanine ammonia lyase (Whipps 2001). Most of the literature reviewing PGPR includes ISR as one of the possible mechanisms (Whipps 2001) Therefore testing the defense responses of the plant is an important assay to study microbial interaction and biocontrol in the rhizosphere.

2.7 Methods for studying of microorganisms in interaction with plants

Characterization of bacterial communities was studied using microbial cultivation techniques for a long time (Rheims *et al.* 1996).There is some limitation due to the inability to culture some soil microorganisms. To overcome problems associated with these non culturable bacteria and fungi various methods including fatty acid analysis, and numerous DNA and RNA based methods are currently employed to characterize bacterial populations (Kirk *et al.*

2004). These methods are not dependent on the growth of the organisms in the laboratory (Rheims *et al.* 1996) and enabled us to study the abundance, localization and activity of microorganisms *in situ* (Amann and Kühl 1998, Cardon and Gage 2006).

PCR based techniques to identify microorganisms

The polymerase chain reaction (PCR) is the most common method for studying the microbial community because it can be used to amplify specifically targeted DNA sequences (Kennedy and Clipson 2003). Ribosomal RNAs occur in all organisms and are good target molecules for bacterial identification. Sequence analysis of the 16S rRNA gene has become a standard technique for identifying unknown bacteria because this gene is highly conserved with minor variations (Rheims *et al.* 1996, Sessitsch *et al.* 2004). The gene encoding the small subunit rRNA has been sequenced and is useful for defining phylogenies at the genera and higher taxonomic levels (Rossello-Mora and Amann 2001). Specific genes are targeted in PCR through PCR primers, the oligonucleotides used defining the start position on each DNA strand for a DNA polymerase. The phylogenetic breadth of a primer is determined during primer design by choosing oligonucleotides which bind to regions of the gene that are conserved within the particular taxa being targeted but differ from other taxa (Blackwood *et al.* 2005).

The genetic diversity of complex microbial communities can be determined by denaturing gradient gel electrophoresis (DGGE) of the amplified genes encoding 16S rRNA. DNA fragments of the same length but different base-pairs can be separated by DGGE. This technique has been successfully applied to identifying sequence variation among genes in several different organisms (Muyzer *et al.* 1993, Rheims *et al.* 1996).

Techniques used for the localization of microorganisms

A major problem hindering successful application of introduced microbes in the soil for biological control is to determine the fate of these microbes after inoculation. For example, the bacterial inoculant strain in the field is commonly indistinguishable from the indigenous bacterial populations in the soil. To study the establishment of bacteria, one often needs to find out precisely the location and physiological activity status of the introduced bacteria. It is necessary to have a sensitive and reliable means for specifically detecting and quantifying the bacterial strain in the soil.

Traditional approaches to studying bacterial establishment have employed bioassays using endogenous molecules as markers. For example, rhizobia may be distinguished by patterns of intrinsic (Josey et al. 1979) or induced (Turco et al. 1986) antibiotic resistance. The other traditional class of molecular markers used in bacterial ecology are antigens expressed on the cell surface that can be recognised by specific antisera. This is commonly achieved through the use of ELISA, fluorescently labelled antibodies or immunodiffusion method. In recent years, however new techniques based on molecular markers or PCR have been developed to specifically monitor bacteria in the field (Muyzer *et al.* 1993, Rheims *et al.* 1996, Kennedy and Clipson 2003).

Molecular markers

Over the last few years, the use of marker genes, such as *gusA, lacZ, celB, xylA, luxAB,* have been become an important tool in studies on microbial ecology as they allow the simple visualisation of marked organisms (Sessitsch *et al.* 1996). The primary consideration in choosing a marker gene is, therefore, the availability of assays for its detection. These must be simple, highly sensitive and there should be little or no detectable background activity in either the microbes being studied or other components being assayed, such as soil or plant roots. The *lacZ* gene, encoding β-galactosidase, has been used to study nodule infection by *Rhizobium* (Ba *et al.* 2002) and for root colonization studies in *Azospirillum* (Pereg Gerk *et al.* 2000). The *cel*B gene from *Pyrococcus furiosus,* which encodes a thermostable B-glucosidase, and the *Escherichia coli gus*A marker gene have been used for the detection of *Rhizobium* strains (Sessitsch *et al.* 1996). The marker genes can be introduced into the recipient bacterium as a direct insertion in the genome, through the use of transposable elements (de Lorenzo *et al.* 1990).

Green fluorescent protein

The detection of microorganisms in nature (*in situ*) has also been severely limited due to a lack of visualization methodologies. The application of Green Fluorescent Protein (GFP) has enhanced the ability to monitor microbial population *in situ* (Unge *et al.* 1998) and is considered a useful tool to provide evidence for direct localization of bacterial populations (Gyaneshwar *et al.* 2001). The gene encoding the GFP of *Aequorea victoria* can be used as a reporter for bacterial localization. The GFP protein is a 238 amino acid peptide and has become an important visual marker of gene expression (Chalfie *et al.* 1994). GFP is more sensitive than other reporter genes, requires no special factors for detection (Chalfie *et al.*

1994, Stretton *et al.* 1998) and can be quantified with a spectrofluorimeter (Kremer *et al.* 1995). Interest in GFP has broadened since the demonstration that heterologous expression of the *gfp* cDNA in a variety of species produces a fluorescent protein (Chalfie *et al.* 1994, Inouye and Tsuji 1994, Sengupta and Colbert 1994, Wang *et al.* 2000) The spectral characteristics of GFP, small size and fluorescent activity in the absence of other *A. victoria* proteins make it an attractive candidate as an *in vivo* marker for studies of promoter activity, cellular protein trafficking, gene transfer, and protein-protein interactions (Chalfie *et al.* 1994, Heim *et al.* 1994, Marshall *et al.* 1995).

GFP can be used to tag an endophyte and study its location and life cycle of the endophyte *in planta* (Elbeltagy *et al.* 2001, Singh *et al.* 2004) and also can be used for the observation of individual cells (Lugtenberg *et al.* 2001). The *gfp*-tagged cells can be visualized and tracked by a variety of methods developed for the detection of fluorescent cells, such as epifluorescence microscopy, confocal laser microscopy and/or flow cytometry (Kremer *et al.* 1995, Unge *et al.* 1998).

Quantitative Real-time PCR

The invention of the polymerase chain reaction (PCR) in the 1980s allowed the possibility of rapid and specific detection of the low amount of DNA (Saiki et al., 1985). The limitation of conventional PCR was that the amplified product was observed after PCR completion and gives no reliable quantitative information in the sample (Linz *et al.* 1990). The development of real-time PCR differs from the conventional PCR in that the quantity of PCR product or amplicon is detected as it is amplified. In real time PCR, the accumulation of specific product in the reaction is monitored continuously during the cycle by the addition of fluorescent dye. Fluorescence plays an important role for the detection of PCR product after each PCR cycle (Okubara *et al.* 2005). The most popular fluorescence markers used for the quantification are SYBR green I or TaqMan probes. The SYBR green I dye is a simple and cheap method which fluoresces after binding to double stranded PCR product. TaqMan probes are based on fluorogenic extendable sequence which is labeled with reporter or quencher dye and signals are emitted after 5'exonuclease activity.

The product is determined by identifying the number of cycles at which reporter dye emission intensity rises above the background level known as threshold cycle (Ct). The quantity of target DNA in the sample is inversely proportional to the Ct value.

This technique offers the quantitative amplification of PCR product. Real time or quantitative, polymerase chain reaction (Q-PCR) allows a specific method for rapid and sensitive detection of plant pathogens in soil, water, air or plant samples (Klein 2002). The real-time PCR technique has been used for a wide range of applications such as plant-microbe interactions (Gachon *et al.* 2004, Marchi *et al.* 2006) detection and quantification of bacteria and pathogen populations (Klein 2002, Lees *et al.* 2002, Schaad and Frederick 2002, Salm and Geider 2004b) and quantitative gene expression studies (Annapaula Giulietti 2001, Almeida *et al.* 2004, Radonic *et al.* 2004).

2.8 Aims of the proposed project

This study was designed to generate information about the biology and ecology of three groups of beneficial bacteria. A better understanding of the factors involved in the interaction of disease suppression by PEM in relation to the pathogen may improve their potential as biological control agents against *R. solani* on wheat.

The overall aim of the thesis was to identify potential mechanisms involved in disease suppression. To achieve this aim objectives were set :

- To understand the biology of the PEM consorrtium
- To identify plant growth promotion potential of PEM
- To identify potential mechanism involved in disease suppression of *R. solani*
- To identify spatial arrangement of PEM in relation to wheat roots

This would ultimately contribute to a better understanding of disease management strategy against *Rhizoctonia* by PEM strains.

Chapter 3 Influence of environmental factors and nutritional utilization patterns on disease suppressive consortium PEM

3.1 Introduction

Microbes play a key role in many processes that are important in the function of natural ecosystems (Larrainzar *et al.* 2005). These processes include turnover of organic matter, completion of biogeochemical cycles and promotion of plant health and soil fertility (Larrainzar *et al.* 2005). Microbial antagonists are emerging as a potential alternative to traditional methods to control plant diseases as well as common crop management practices. A major problem hindering the successful and consistent application of microbes for biocontrol is that they are influenced by environmental conditions or ecological parameters that are often difficult to control such as temperature, pH, water availability, substrate utilization profile and their interactions. Thus, to study the establishment of these bacteria, it is necessary to find out precisely the location and physiological activity status of the introduced bacteria.

PEM strains (*Pantoea*, *Exiguobacterium* and *Microbacterium*) have been reported to efficiently suppress wheat root rot caused by *Rhizoctonia solani* (Barnett et al., 2006). However; the impact of ecological conditions on individual strains of PEM has not been characterized in previous studies.

In this chapter, routine microbiological methods were used to characterize the PEM strains for their different physiological and morphological properties. All the PEM strains were assessed on a physiological-biochemical and cultural basis to identify characteristics, such as cell morphology, motility and fermentative abilities. The objectives of this study were i) to determine differences among PEM strains for characteristics such as growth responses to temperature, pH, and water availability which may affect their behaviour in the rhizosphere; and (ii) to determine whether substrate utilization profiles can provide information about rhizospheric interactions or the metabolic potential of PEM.

3.2 Materials and Methods

Bacterial and Fungal strains

The bacteria *Pantoea* (P3) *Exiguobacterium* (E1) and *Microbacterium* (Kma1) strains and pathogen *Rhizoctonia solani* (AG-8) used in this study were originally received from S. Barnett, South Australian Research & Development Institute (SARDI), Adelaide. All the bacterial and fungal strains were stored in 50% glycerol at -70 ºC until needed. Each experiment started with the fresh glycerol stock.

Growth media and inoculation

The survival and continued growth of microorganisms depend upon an adequate supply of nutrients and favorable growth environment. The basic growth medium used for culturing PEM strains in this study was LB broth (Tryptophan 10 g/L, Yeast Extract 5g/L, NaCl 5g/L) adjusted to pH 7.5. This was supplemented with 1.5% agar when a semi-solid medium (LB agar) was required. Loop full cultures of PEM strains were taken from the glycerol stocks and spread onto LB agar plates while working and these were sub cultured weekly onto the fresh LB plates. Stock cultures of *Rhizoctonia solani* AG-8 were maintained on sterile millet seeds and were cultured after placing millet seeds onto the centre of quarter strength potato dextrose agar (¼ PDA) plates. To subculture, a 8 mm agar plug was taken from the edge of the plate and transferred onto a fresh ¼ PDA plate. All the bacterial and fungal cultures were maintained at 4ºC for routine use.

Morphological characterization

Each PEM strain was grown separately on LB agar at 25ºC, 30ºC and 37ºC for 3 days and the optimum growth temperature was determined qualitatively. The growth was assessed after selecting colonies on the plates, well spaced from others, as colony size is affected by competition for nutrients. At least 4-6 colonies per plate on 3 plates of each PEM strain were selected and growth was recorded on a qualitative scale based on the colony diameter (+: 0.5-1 mm, ++: 1-2 mm, +++: 2-3 mm, ++++: 3-4 mm). The colony morphology (shape, color, texture and other distinctive traits) of PEM strains grown on LB agar were examined for 3 days were described on the basis of standard microbiology laboratory methods (Pollack *et al.* 2002). The individual bacterial cells of PEM isolates were observed under compound microscope and morphology was recorded. Smears of each of the PEM strains were prepared and staining was performed for simple (0.5% safranin) and Gram staining using standard laboratory methods described by (Pollack *et al.* 2002). Colony characteristics of PEM strains

on the LB agar plates were used for the identification of PEM strains in subsequent experiments such as selection of transformants and viable cell counts.

Antibiotic resistance/ sensitivity test

Sensitivity to the antibiotics kanamycin (Km), tetracycline (Tc) and ampicillin (Amp) of the PEM strains was determined by adding the antibiotics in both liquid and solid medium to obtain a final concentration of 20 μg/mL (Km) and 5, 10 or 20 μg/mL (Tc) and 100 μg/mL (Amp). Fresh cultures of PEM strains were spread or inoculated into the medium supplemented with antibiotics and incubated at optimum temperature (30ºC for *Pantoea* and *Microbacterium* and 37ºC for *Exiguobacterium*) for 24 hrs. Growth was assessed qualitatively on the solid medium plates and liquid culture amended with antibiotics and compared with PEM strains grown in the absence of antibiotics. Strains were considered sensitive to an antibiotic if they did not show visible growth at one of the concentrations used.

Physiological characteristics of the bacteria that might effect the ecology of roots or soil

Motility test

Motility of the PEM strains was tested by using LB broth supplemented with 0.3% agar. The motility was tested on Petri plates as well as in McCartney bottles containing semi-solid LB medium. The centre of the plates and bottles were inoculated with fresh grown cultures of the bacteria and incubated at optimum temperature. The increased growth was assessed up to four days according to microbiology laboratory protocol (Pollack *et al.* 2002). The increase in colony diameter or spread away from the original inoculated spot was considered as motility.

Aerotolerance test

Cultures of PEM strains were grown overnight in LB broth under optimal growth conditions. Molten LB agar medium at 45ºC was poured into 15 x 150 mm glass test tubes covered with aluminum caps under sterile conditions. Then 1 mL of overnight culture was inoculated into unsolidified LB agar inside the glass tubes. The culture was then immediately mixed by vortexing and cooled on ice to solidify quickly. Tubes were incubated at optimum temperature for 3-6 days. The growth of PEM strains in the tubes was observed and compared with the bacterial growth shown in microbiology laboratory protocol (Pollack *et al.* 2002). The different oxygen requirements for PEM strains were assessed on the basis of standard growth parameters: strict anaerobe, if growth only in the deeper portion of the medium;

facultative anaerobe. if growth throughout the medium; aerobic, if growth only on the top surface; and microaerophilic, if growth found near but below the surface of the medium.

Oxidase test on the basis of cytochrome oxidase activity

Oxidase enzymes are important for electron transport system during aerobic respiration. Cytochrome oxidase catalyzes the oxidation of a reduced cytochrome by molecular oxygen (O_2) and produces H_2O or H_2O_2 (Cappuccino and Sherman 2004). Isolated bacterial colonies of PEM strains were taken from fresh grown cultures and spread onto filter papers with a plastic inoculation loop. The oxidase reagent (1% N,N,N,N-tetramethyl-*p*-phenylenediamine dihydrochloride dissolved in sterile distilled water) was added to the surface of the filter paper on top of the bacterial culture. The bacterial spot on the filter paper was examined for the presence or absence of color changes. The test was considered positive if the spot color turned into blue or purple and negative if there was no change in color.

Catalase test

Some aerobic and facultative anaerobic bacteria are capable of producing the catalase enzyme which degrades hydrogen peroxide into water and oxygen gas (Cappuccino and Sherman 2004). A drop of 3% hydrogen peroxide was added onto the top of an isolated colony of each of the PEM strains and the colony was observed for the presence or absence of a bubbling or foaming reaction. For each of the PEM strain 3-4 isolated well spaced colonies were selected. Cultures producing bubbling were recorded as positive for catalase activity.

Comparison of bacterial characteristics and the ecology of the pathogen

Optimal growth temperature

Bacteria grow within different temperature limits but the optimum temperature is that at which bacterial cells divide most rapidly. The PEM strains were grown overnight and absorbance of the bacterial culture was measured at 600 nm (A_{600}) using a spectrophotometer. The overnight cultures were then diluted to an A_{600} of 0.1 and 0.2 in 50 mL LB broth in 250 mL flasks. Optimal growth temperature of PEM strains was tested by incubating culture flasks at temperatures of 10ºC, 15ºC, 20ºC , 25ºC, 30ºC and 37ºC with shaking at 180 rpm and the absorbance (A_{600}) was measured every hour for up to 4 hours, with the first reading at time zero as per standard microbiology laboratory protocols. The pathogen *Rhizoctonia solani* was grown on quarter strength PDA (¼ PDA). Infested agar plugs (8 mm) were taken from margin of *Rhizoctonia* culture plates and placed in the center of the fresh ¼ PDA plates. The

plates were incubated at temperatures as above for 4-7 days and growth was recorded as the increase in diameter of the colonies.

pH test

Bacteria and fungi require a certain physiological pH inside their cells for survival and changes in pH of the surrounding environment significantly influence the growth and metabolism of the organisms. LB agar medium and ¼ PDA were prepared for various pH range 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5. The pH of the medium was adjusted before autoclaving by adding 1 M HCl or 1 M NaOH and poured into Petri dishes. Each of the PEM strains on LB agar or *Rhizoctonia* on ¼ PDA was uniformly streaked or transferred respectively onto the different pH plates and then incubated at optimum temperature. The plates were assessed for several days and the growth of PEM strains and *Rhizoctonia* was recorded.

Water potential

The effect of water potential on bacteria and pathogen growth was determined by inoculating PEM strains in LB broth and *Rhizoctonia* on ¼ PDA supplemented with different NaCl (1-10%) concentrations. Water potentials of LB and ¼ PDA media were measured using a Wescor Vapro S525 vapor pressure osmometer. The water potential of different concentrations of NaCl (1-10%) supplemented media were determined based on the data reported by Lang (1967). An overnight grown culture of PEM strains was diluted to an A_{600} of between 0.1-0.2 and added to 20 mL of LB-NaCl broth medium in 50 mL flasks. The LB-NaCl broth cultures (time zero) were then incubated at 30ºC with 180 rpm shaking and absorbance (A_{600}) was measured every 40 min, with the first reading at time zero. *Rhizoctonia* infested agar plugs were transferred onto ¼ PDA supplemented with NaCl (1-10%). The plates were incubated at 25ºC and growth in diameter was recorded daily.

Substrate utilization

BIOLOG EcoPlates (BIOLOG Inc, Hayward, California) were designed to assess the potential metabolic diversity of microbial communities. They contain 31 carbon sources that are relevant to soil microbes, and the ability of organisms to use these is indicated by a colour change during incubation. The substrate utilization patterns of each PEM strain individually and in all combinations, as well as that of *Rhizoctonia*, were determined using EcoPlates. Fresh cultures of each bacteria were diluted to achieve 10^5 CFU/mL and then 100 µL culture was inoculated into each well of the microtiter plate according to the manufacturer's

instruction manual. Combinations of bacteria were diluted so that the total concentration was 10^5 CFU/mL, with each bacterium in the combination present at equal concentration. The fresh grown *Rhizoctonia* were scraped from the ¼ PDA plate and resuspended to achieve 10^5 hyphal fragments/mL and counted using the haemocytometer. The plates were incubated at 25ºC as per specified instructions (Oxoid Australia). There were 3 replicates of every treatment. Substrate utilization by the bacteria was assessed quantitatively at 24 h using a microplate reader. The colour reaction did not occur properly in the fungal plates, so growth of *Rhizoctonia* was recorded qualitatively on a 0-3 scale depending on the abundance of mycelium. Substrate utilization patterns were compared using cluster analysis. For comparison of quantitative data Eucledean data was used. The corelation coefficient was used to compare qualitative and quantitaive data. Clustering was done using group-average (UPGMA) clustering in the SPSS stastical package.

3.3 Results

Morphological characterization

All of the PEM bacteria grew at the lowest temperature 25°C. The optimum temperature for *Pantoea* and *Microbacterium* was found to be 30°C whereas the optimum temperature for *Exiguobacterium* was 37°C (Table 3-1). The growth of the bacterial colonies was almost the same for all the replicates used in the experiments.

Colonies of all of the PEM bacteria grown on LB agar at optimum temperature for 3 days had these characteristics (Table 3-1): yellow or shiny orange in colour, 0.5-4 mm in diameter, circular, convex, smooth, with an entire edge and sticky in texture. An odour was strongly present on all plates especially with *Microbacterium*.

In the microscopic examination, *Pantoea* cells were straight rods to slightly curved rods 1.0-2µm in size, and non motile. *Pantoea* cells stained Gram negative and occurred mostly as single cells, but sometimes paired cells with short chains were also seen. *Exiguobacterium* cells were observed as cocci, 0.5-2 µm in size with a non motile characteristic. *Exiguobacterium* cells stained Gram positive and cells occurred as single or sometimes in pairs. Sometimes cells tended to form clump like structures and chains of cells were also observed. *Microbacterium* cells were rods to cocci 0.5-1.5 µm in size, occurred mainly singly or in chains, were non motile and stained as Gram positive.

Table 3-1. Colony morphology of PEM on LB agar.

Characteristic	*Pantoea*	*Exiguobacterium*	*Microbacterium*
Colour	Golden yellow	Pale orange, shiny	Pale yellow
Colony morphology	Circular, smooth, convex	Circular, smooth	Circular, smooth, convex
Growth at temperature			
25 °C	++[A]	+	+
30 °C	+++	++	+++
37 °C	++	+++	++
Antibiotic sensitivity			
Kanamycin	S[B]	S[B]	S[B]
Tetracycline 5μg/mL	S[B]	S[B]	R
Tetracycline 10μg/mL	S[B]	S[B]	S[B]
Ampicillin	R	R	R

[A]Growth, +: 0.5-1mm, ++:1-2mm, +++: 2-3mm, ++++:3-4mm

[B]S sensitive; R resistant

Antibiotics test

PEM strains were sensitive to the antibiotics kanamycin (Km) and tetracycline (Tc). There was no growth observed in the presence of 20 μg/mL Km and 5 μg/mL Tc in the medium for *Pantoea* and *Exiguobacterium*. However, *Microbacterium* was sensitive to only higher concentrations of 10 or 20 μg/mL Tc. Each of the PEM strains was resistant to ampicillin (Amp), as growth was observed in the presence of 100 μg/mL Amp (Table 3-1).

Physiological characteristics

Pantoea and *Exiguobacterium* were shown to be facultative anaerobic, whereas *Microbacterium* appeared to be microaerophilic. Each of the PEM strains was shown to be non motile on the stab culture or on the semisolid medium. All the PEM strains appeared to be negative for the presence of cytochrome oxidase. Only *Pantoea* showed positive results in the catalase tests.

Optimal growth temperature, pH and salt tolerance for optimal growth

All the PEM strains grew in liquid culture at all temperatures from 10-37ºC (Figure 3-1A). The optimum growth temperature in liquid culture for all the PEM strains was 37ºC, which was almost the same as on agar plates. The growth of the pathogen *Rhizoctonia solani* occured over a broad temperature range (10-30ºC), whereas maximum growth was recorded at 20-25ºC

The PEM strains and pathogen showed a broad pH range and grew well between pH 6.5-9.5, with optimal growth at about pH 7.5. No growth occurred at low pH 4.5 for the PEM strains, whereas *Rhizoctonia* grew well at broad pH ranges from 4.5-9.5. The bacteria and pathogen all showed a high growth rate from a range of pH 6.5 to 8.5 (Figure 3-1B).

Growth rate of the PEM strains and *Rhizoctonia* decreased as the salt concentration of LB broth or PDA/4 was increased. Growth rates were reduced by 50% at about -3 Mpa. *Rhizoctonia* stopped growing at a water potential of -3.4 Mpa, while the PEM strains continued to grow at lower water potentials. *Microbacterium* was more tolerant of water potential below -5 Mpa than the other bacteria (Figure 3-1C).

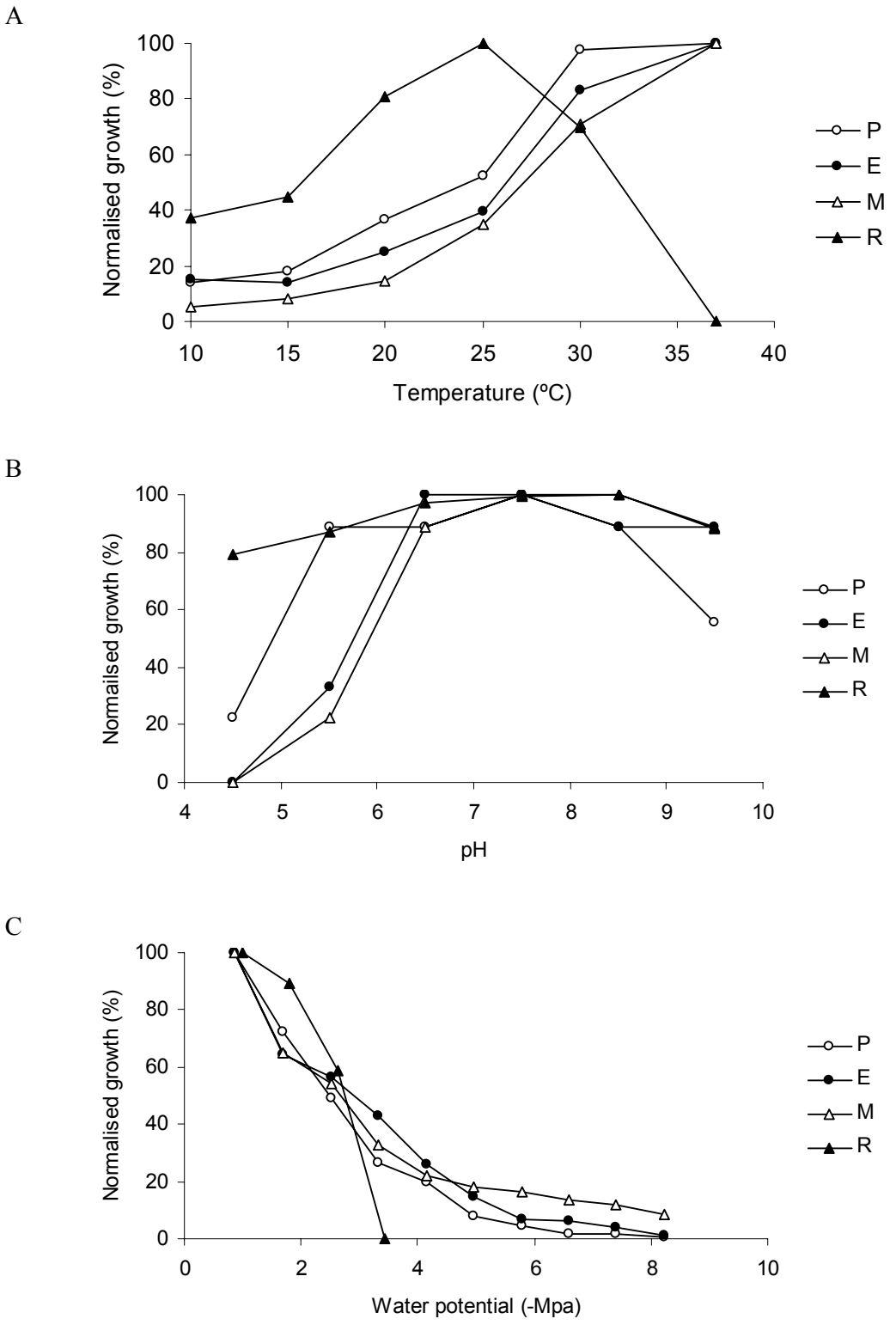


Figure 3-1. Effect of A) temperature, B) pH, C) water potential on relative growth of *Pantoea* (P), *Exiguobacterium* (E), *Microbacterium* (M) and *Rhizoctonia* (R).

Substrate utilization pattern

Substrate utilization for the PEM strains and *Rhizoctonia* each showed heterogeneity in their profiles (Table 3-2). *Pantoea* showed the broadest range of substrate utilization pattern among the bacterial strains. Clustering of BIOLOG EcoPlate data using Euclidean distance as a measure of similarity, and group average (UPGMA) clustering showed that all the inoculations with P in them were very similar to each other, and very different from the E, M and EM inoculations (Figures 3-2 and 3-3). Only ordinal data were available for *Rhizoctonia*, so this was compared with the bacterial inoculations using the correlation coefficient (Figure 3-3). Substrate utilization by *Rhizoctonia* was very different from all of the bacterial strains and consortia. Among substrates used by *Rhizoctonia* but not the bacteria were glycogen, cellobiose and lactose, and among substrates used by most bacteria but poorly or not at all by *Rhizoctonia* were pyruvic acid methyl ester, N-acetyl glucosamine, glycyl glutamate and malic acid (Table 3-2).

Table 3-2. Relative utilization of C sources on BIOLOG Ecoplates by *R. solani* and PEM bacteria. (R = *Rhizoctonia*, P = *Pantoea*, E = *Exiguobacterium*, M = *Microbacterium*.)

Substrate	**Relative utilization**			
	R	**P**	**E**	**M**
β-Methyl-D- glucoside	+++	+++	+++	+
D-Galactonic acid γ-lactone	++	0	0	0
L-Arginine	0	+	0	0
Pyruvic acid methyl ester	0	+++	+++	0
D-Xylose	+++	+++	+++	++
D-Galacturonic acid	++	+++	+++	+
L-Asparagine	+	+++	++	0
Tween 40	++	+	+	+
i-Erythritol	0	0	0	0
2-Hydroxy benzoic acid	0	0	0	0
L-Phenylalanine	0	0	+	0
Tween 80	+++	++	+	+
D-Mannitol	++	+++	+++	++
4-Hydroxy benzoic acid	0	0	0	0
L-Serine	0	++	+	0
α-Cyclodextrin	0	0	0	0
N-Acetyl-D-glucosamine	0	++	++	++
γ-Hydroxybutyric acid	0	0	0	0
L-Threonine	0	0	0	0
Glycogen	+++	0	0	+
D-Glucosaminic acid	+	0	0	0
Itaconic acid	0	0	0	0
Glycyl-L-glutamic acid	0	+++	+	+
D-Cellobiose	+++	0	+	0
Glucose-1-phosphate	+	+++	++	+
α-Ketobutyric acid	0	0	+	0
Phenylethylamine	0	0	0	0
α-D-Lactose	+++	0	0	0
D,L-α-Glycerol phosphate	+	++	+	+
D-Malic acid	0	+++	++	++
Putrescine	0	0	0	0

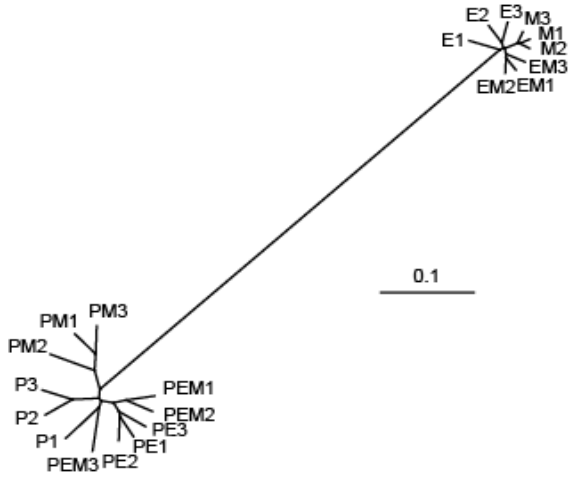


Figure 3-2. Unrooted cluster tree showing the relationship between substrate utilization patterns of PEM strains alone and in combination on BIOLOG EcoPlates after 24 h incubation. Clustering was done using Euclidean distances and pair-group (UPGMA) clustering. P = *Pantoea*, E = *Exiguobacterium*, M *Microbacterium*, PE, EM, etc. are combined inoculations; numbers show replicates.

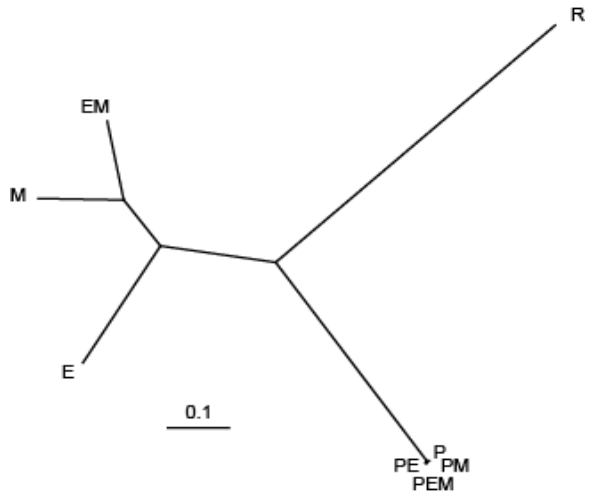


Figure 3-3. Unrooted cluster tree showing the relationship between substrate utilization patterns of *Rhizoctonia* and PEM strains alone and in combination on BIOLOG EcoPlates after 24 h incubation. Clustering was done using correlation coefficients and pair-group (UPGMA) clustering.

3.4 Discussion

The effects of ecological and physiological conditions on bacterial growth provides important information for understanding of the activity of these bacteria under many adverse soil conditions when introducing microbes for increasing plant growth and productivity (van Veen *et al.* 1997). The activity of introduced microorganisms is only effective if ecological conditions are favorable for these microorganisms. Abiotic soil factors such as temperature, pH and osmotic tolerance and substrate utilization need to be critically assessed for PEM strains, as they are important factors determining the survival and activity of these microorganisms in the rhizosphere (Gray 1975).

PEM biology

The distribution of PEM strains in the wheat rhizosphere has been previously assessed on the basis of culturing these bacteria (Barnett *et al.* 2006). This work on colony morphology and the growth of PEM strains confirmed the morphological similarity or culturable ability of PEM strains. The purpose of this work was also to assess the growth of these bacteria on nutrient media under different environmental parameters. The agar medium is considered non selective and is widely used as a general medium for isolation of diverse bacterium populations from natural systems such as root, soil and water samples. The present results confirmed the culturable ability of PEM strains which have consequences for the isolation and application of these strains to use as inoculants.

Motility was tested for the PEM strains to explore whether movement ability might contribute to initiation of colonization. Bacterial motility has been shown to contribute to survival in soil and the initial phase of colonization, where attachment and movement onto the root surface are important (Turnbull *et al.* 2001). Several studies conducted for *Azospirillum*, showed that bacterial motility was an important factor in the initiation of wheat root colonization and establishment of the *Azospirillum*-plant root association (Troch and Vanderleyden, Vande Broek *et al.* 1998). *Azospirillum* strains also have been shown to be attracted towards root exudates and root mucilage (Heinrich and Hess 1985, Mandimba *et al.* 1986). PEM strains showed non motile activity in both solid and liquid medium. Although all the bacterial strains appeared to be non motile in this study however, their possibility of motile activity should be cheacked by other methods. Absence of motility does not mean that they are deficient in colonization activity as there are several other factors which influence the bacterial root colonization. In some studies, when non motile strains of *Pseudomonas* were compared with

motile strains it was suggested that motility might have a role in the movement towards and/or along roots rather than the multiplication on the roots (Ikeda *et al.* 1997).

PEM eco-physiology

Plant microbe interactions are close associations between plants and associative organisms. Temperature dependent variation in the activity of inoculated bacteria during the growth and development of plants has been demonstrated by several authors. For example, it has been shown that bacterial colonization is correlated with the rhizosphere temperature. Rattray *et al.* (1993) reported that wheat inoculated with *Pseudomonas fluorescens* showed better colonization at 8ºC than 22ºC. In another study conducted by Egamberdiyeva and Höflich (2002), *Cellulomonas* sp. showed better colonization of winter wheat and pea roots at 16ºC than 26ºC. Results obtained for PEM showed that the growth temperature of these strains is within the range of other soil bacteria which have demonstrated root colonization activity in the wheat rhizosphere. *Rhizoctonia* grew better at lower temperature (≤ 25ºC) but did not tolerate high temperature, indicating that PEM may be more successful against *Rhizoctonia* in warmer conditions. PEM has shown a broad temperature range growth profile which suggests that the consortia could be used for effective colonization in wheat rhizosphere. The mean temperature during the wheat growing season is 15-25ºC in the suppressive soil in the field at Avon. This is below optimum for bacterial growth and optimum temperature for pathogen growth. However, suppression still occurs (Barnett *et al.* 2006). Further study is needed to find out whether the wheat rhizosphere and growth temperatures correlate with the PEM and pathogen interaction in the rhizosphere**.** The temperature range for PEM strains and *Rhizoctonia* was found to be in agreement with other reports related to soil microorganisms (Kumar *et al.* 1999, Costa *et al.* 2002).

It has been reported that pH affected a group of disease suppressive bacteria in *Fusarium* wilt suppressive soil, with less protection from wilting when soil pH was lowered from 8 to 6 (Scher and Baker 1980). In this study, the tolerance of pH for PEM and *Rhizoctonia* indicates that both the microorganisms can tolerate lower (5.5) and higher (8.5) pH levels. *Pantoea* was active at quite a low pH (5.5), showing that they can grow effectively in mildly acidic environmental conditions. *Rhizoctonia* is more robust to a wide range of pH levels, whereas PEM (especially E and M) tended to have reduced growth under non-neutral conditions. This indicates that PEM is more likely to be effective against *Rhizoctonia* in near neutral soils. P

preferred more acidic conditions (pH 5.5-8.5) while E and M preferred more alkaline conditions (pH 6.5-9.5).

There are wide variations in the reported salt tolerance of different species of rhizobia. A number of strains of *Bradyrhizobium japonicum* were inhibited at less then 100 mM NaCl, whereas rhizobia from *Hedysarum*, *Acacia, Prosopis* and *Leucaena* can tolerate up to 500-800 mM NaCl (Tilak *et al.* 2005). It has also been reported that high salt tolerance can aid in tolerance of high pH and temperature (Kulkarni and Nautiyal 2000). PEM strains and *Rhizoctonia* grew well at a water potential of -2 Mpa in the presence of NaCl. However, growth of PEM was inhibited below -7.5 Mpa. The results indicate that PEM consortia were more tolerant of dry conditions providing a potential advantage over *Rhizoctonia* in terms of growth and possibly survival. The results obtained for *Rhizoctonia* are similar to the published results of Kumar *et al.*(1999). The ability of all three PEM isolates from this study to tolerate high salt concentrations and a broad range of pH levels and temperatures gives them a potential competitive advantage to be active in a broad range of ecological conditions. Overall, soil environmental conditions are unlikely to seriously limit the activity of PEM relative to *Rhizoctonia*.

PEM resource utilization

The adaptability of bacteria has been reported to be affected by nutrient requirements and the utilization of root exudates or interaction with plants (van Veen *et al.* 1997). It has also been reported that microorganisms present in the rhizosphere may compete for carbon or energy sources which provides a basis for biological control. It has been established that the spatial and temporal arrangement of the rhizosphere community is based on the plant species and availability or composition of root exudates (Haas and Defago 2005).

Various substances including amino acids, sugars, organic acids, metal ions and phenol exudates by roots to the rhizosphere have been shown to serve as the basic source of carbon and nitrogen for microorganisms (Patkowska 2002, Pięta and Kęsik 2007). Among these substances, some substances have a stimulatory effect whereas some have an inhibitory effect towards microorganisms (Pięta and Kęsik 2007). The utilization of these compounds by rhizobacteria influence rhizosphere competition (Goddard *et al.* 2001).

The pattern obtained for substrate utilization in the BIOLOG system was developed to assess the similarities between microbial ecosystems in soil and related environments. Substrate utilization patterns were also used previously to assess the nutritional similarity between pathogenic and non pathogenic bacteria and also demonstrated that competition for carbon sources was probably involved in the biological control of disease (Ji and Wilson 2002). In this study, the PEM isolates were analysed for the oxidative utilization pattern for different carbon sources. The observed profile of carbon sources metabolized reflects the catabolic potential of PEM. The pattern obtained for combined PEM was different from some individual treatments. *Pantoea* had a different pattern of utilization from the other bacteria, suggesting that it does not compete with them for nutrients.

In this study, PEM strains and *Rhizoctonia* have been shown to utilize some ecologically significant compounds which have been reported to be exuded by wheat roots (Rovira 1969). Wheat root exudates such as glucose, xylose, asparagine and glutamic acid were utilized by all the PEM strains and *R. solani*. Some exudates like serine were utilized by P and E only whereas malic acid utilization was shown by PEM but not by the pathogen. The results obtained from this study indicate that competetion for nutrients is unlikely to be important as a mechanism for disease suppression by PEM.

The purpose of this study was to establish different physiological and ecological parameters for PEM strains and pathogen *R solani* under laboratory conditions. PEM strains were able to tolerate broad temperature and pH ranges and high water potential and have a broad range of carbon utilization profile. The above findings suggest that they have a broad range of ecologically significant growth parameters in soil thus confering a potential to be effective biological control agents. Therefore, where near neutral pH, high water potential and high temperature, PEM is likely to have advantages or compete more effectively over *R. solani*. Verification of these responses by PEM and *Rhizoctonia* under field soil conditions is recommended in future research.

Chapter 4 Plant growth promotion potential of PEM

4.1 Introduction

Plant growth-promoting rhizobacteria (PGPR) (Kloepper 1992) colonize plant roots and exert beneficial effects on plant health and development (Ryu *et al.* 2004b). Some PGPR are involved in plant growth promotion by a variety of mechanisms (Glick 1995). Stimulation and enhancement of root hairs and root growth is one of the several methods of plant growth promotion by these PGPR. The beneficial effects of use of PGPR have been well defined and described by several studies (Kapulnik *et al.* 1985, Molla *et al.* 2001, Burdman *et al.* 2002). There are several factors involved such as release of hormones (Costacurta and Vanderleyden 1995) or reduction in the level of growth limiting factors (Wang *et al.* 2000), alteration of mineral nutrition (Bertrand *et al.* 2000) or enhancement of ability of mineral uptake (Goldstein and Braverman 1990) or many more mechanisms which could affect the growth of the plant.

The plant growth promotion effect by PGPR has several changes in terms of fresh and dry mass accumulation, root length and surface area, lateral root number and density (Frommel *et al.* 1991, Sarig *et al.* 1992, Barbieri and Galli 1993, Leinhos and Bergmann 1995, Larcher *et al.* 2003). Whatever mechanisms are involved, the ultimate impact is a quantitative changes in root and/or shoot growth (Larcher *et al.* 2003), increased lateral root number or root hair formation (Tien *et al.* 1979, Jain and Patriquin 1985), water and mineral uptake (Okon and Kapulnik 1986) and nitrogen fixation (Holguin and Bashan 1997).

In this study, the growth promotion potential ability of PEM strains was investigated. In previous studies on the disease suppression ability of PEM (Barnett *et al.* 2006), the shoot growth of wheat plants infected with *R. solani* was increased, but there were no reports on growth promotion of uninfected plants. The work described in this chapter demonstrated that PEM has significant potential to promote root and shoot growth under different conditions.

4.2 Materials and Methods

Bacterial strains

All the bacterial strains, PEM (*Pantoea*, *Exiguobacterium*, *Microbacterium*) were cultured on LB agar or TSA/10 agar and for liquid culture in LB broth or TSA/10 broth medium (appendix 1).

Pathogen

Pathogen strain *Rhizoctonia solani* AG8, isolate 36 was used, which is known to cause disease on wheat. Stock cultures of *R. solani* AG-8 were maintained on agar blocks in sterile distilled water (SDW) and sub-cultured onto quarter strength PDA (potato dextrose agar/4) (appendix 1), and grown for 4-7 days at 25^{o}C.

Infested agar plugs 8 mm in diameter were taken from the edge of *Rhizoctonia* colonies and added to the centre of the experimental pot (wherever applicable). Each block of the experiment was inoculated with the same *Rhizoctonia* culture.

Plant cultivar

The effect of PEM was studied on wheat cv. Yitpi and the wheat seeds were obtained from Dr. Steve Barnett, Plant Pathology division, South Australian Research & Development Institute (SARDI), Adelaide, SA.

Seed sterilization and pre-germination

A standard method of surface sterilization was used for the wheat seeds. Seeds were surface sterilized using 1% sodium hypochlorite for 10 minute and rinsed 3 times with SDW, and with 70% ethanol and SDW briefly, followed by soaking of the seeds in 2% sodium thiosulphate for 5 minutes and rinsed with SDW. Surface sterilized seeds were placed on a 2mm thick pile of filter papers in Petri dishes (12-15 seeds per dish), covered with three moist filter papers and incubated in the dark at 25^{o}C for 2-3 days for pre-germination prior to use. Surface sterilized seeds were also transferred from time to time to hydroponics agar medium supplemented with 1% agar (HMA) plates to check for contamination.

Growth chamber studies in soil

In order to determine the growth promotion ability by PEM, a growth chamber experiment was conducted using Avon Soil. This was done by mixing PEM strains with the soil and

pathogen *Rhizoctonia solani* (AG-8) in the pots, as described by Barnett *et al.* (2006). PEM and pathogen culture conditions and inoculum preparation and sterilization of seeds were performed as described above. The soil were sterilized by autoclaving three times Pots of size 10 cm deep X 13 cm diameter were filled with 160 g sterile soil (15% w/v moisture content, field capacity) and two 8 mm *Rhizoctonia* infested agar plugs were added to the centre of the pot, followed by an additional 160 g of soil (15% w/v moisture content, field capacity). Sterile soil was premixed with P (1.5 x 10^6 cfu/g), E (3.3 x 10^5 cfu/g) and M (6.3 x 10^5 cfu/g) prior to adding soil into the pots and covered with lids to prevent evaporation. There were four treatments in the experiments, healthy control, pathogen control, PEM with and without pathogen. Pots were pre incubated at 15°C for 2 weeks to allow colonisation of the bacteria in the soil (Barnett *et al.* 2006). Seven surface sterilised and pre-germinated wheat seeds were transferred to the pots and covered with an additional 25 g sterile soil (15% w/v moisture content, field capacity). Wheat plants were reduced to 5 plants per pot after emergence. For each treatment there were 4 replicates. The pots were arranged in a randomized manner inside the growth chamber at 15/25ºC temperature, 65% relative humidity and 800 µmol/m^2/s photon flux density with 12 hours day/night cycle. Pots were rewatered 2-3 times in a week to their initial weight. Wheat plants were grown for 4 weeks and then were harvested and the dry weights of roots and shoots determined. The experiment was a factoral design, with the bacaterial inoculation treatment crossed with the fungal inoculation treatment.

Growth promotion studies in hydroponics system

Growth studies on hydroponics agar medium plates

To determine the growth promotion effect on PEM inoculated wheat plants an assay was performed on hydroponics medium plates supplemented with 1% agar (appendix 1). A system was used to grow the wheat plants on the Petri-plates. A hot knife was used to make a 2 cm wide hole on one edge of the plate to allow the shoot to grow out of the plate. Bacterial culture conditions and surface sterilization of seeds were performed as above. Surface sterilised seeds were pre-germinated on HMA plates. Three days old seedlings were dipped into 10^{6-7} cfu/mL bacterial culture of each strain individually, or all three strains together, for 5-10 min and transferred to HMA plates so that the shoot projected out the hole in the edge. The wheat seedlings were well shaken to remove excess bacterial culture prior to transferring them onto HMA plates. All the plates were sealed with a thick layer of Parafilm. Replicates were performed (10 plants per treatment) and experimental plates were incubated flat at 20°C

temperature, 65% relative humidity and 800 µmol/m^2/s photon flux density with 12 hours day/night cycle. Root length and shoot length were recorded every two days for 10 days after bacterial inoculation. Pteri-plates were large enough to accommodate the root of the 10 days old wheat plants. At the end of the incubation period, plants were removed from the Petri plates and root length, branching pattern of the root and root and shoot dry weights were determined.

Hydroponics pot system and growth chamber conditions for plant assay

A hydroponics system was constructed to grow the experimental plants in a controlled environment growth chamber. Plants were grown in a 0.5 L polystyrene container (approximately 10 cm high and 12 cm in diameter). A thick piece of polystyrene foam holding a 2 cm wide hollow plastic tube was placed over the top of the container. The bottom end of the tube was fitted with mesh to support the seedlings. The container was surface sterilised by using sodium hypochlorite (4% W/V) prior to use. Surface sterilization and pre-germination of seeds were performed as described above. The container was filled with 0.5 L hydroponics medium and pre-germinated seedlings (8-10) from moist filter paper were taken and placed onto the net in the tube which was sitting on the top of the pot containing hydroponics medium. The side wall of the hollow tube supported the plant shoot growth and the net at the other end of the tube allowed the roots to easily reach the hydroponics medium due to gravity. PEM strains were cultured freshly and a single fresh colony of the each of the bacterial strain was grown in LB broth medium. The 3-4 days grown wheat seedlings in the hydroponics medium were thinned to six plants per pot and inoculated with PEM strains culture by directly inoculating 100 µl of 10^{6-7} cfu/mL diluted culture into the medium near the roots. All the pots were transferred to the growth chamber at 15/25°C temperature 65% relative humidity and 800 µmol/m^2/s photon flux density with 12 hours day/night cycle for 10-12 days. There were 5 replicates of each treatment. At the end of the incubation period plants were harvested from the pots and separated roots and shoots were dried at 80ºC for 48 hours and weighed.

This experiment was performed twice. In the second repeat, total length of shoots, branching pattern qualitatively in number of lateral roots per cm, total length of branched and unbranched sections of the roots was recorded 8 and 12 days after bacterial inoculation, as well as root and shoot dry weight at 12 days. The length of the branched section was the distance from the base of the largest root to the most distal lateral roots, while the length of

the unbranched section was the distance from this lateral root to the root tip (Hackett and Bartlett 1971).

Plant growth promotion effect by PEM in nitrogen free hydroponics medium and measurement of nitrogen content

In order to determine the growth promotion effect in nitrogen deficient condition and also with the aim to detect if PEM has any nitrogen fixation potential, the above experiment was repeated without any nitrogen source in the hydroponics medium (appendix 1). At the end of the incubation period (12 days) plants were harvested from the pots and dry weights of separated roots and shoots were determined. To assess the total nitrogen uptake by plants, dried root and shoot samples from all the bacterial treatments were ground to a fine powder of <0.05 mm size using a mortar and pestle and analysed for total N content with a Carlo Erba NA1500 solid sample analyser.

Statistical Analysis

Every experiment was performed with replicates and data are presented as the mean of respective replicates. Data were analyzed by analysis of variance (ANOVA) and significance level was calculated at $P < 0.05$. Statistically significant means were separated by Tukey's method. Qualitative data were analysed using the non-parametric Kruskall-Wallis test.

4.3 Results

Growth promotion effect in soil

The pathogen treated plants showed a significantly reduced root dry weight per plant, but there was no difference between bacterial treatments in root dry weight per plant (Figure 4-1), and no significant interaction between pathogen and bacterial treatments. Treatment with bacteria significantly increased shoot dry weight per plant, and treatment with the pathogen significantly decreased shoot dry weight per plant (Figure 4-2). Again, there was no interaction between bacterial and pathogen treatment. This showed that the growth promotion effect of PEM occurred in both the presence and absence of the pathogen, and affected the shoots more than the roots.

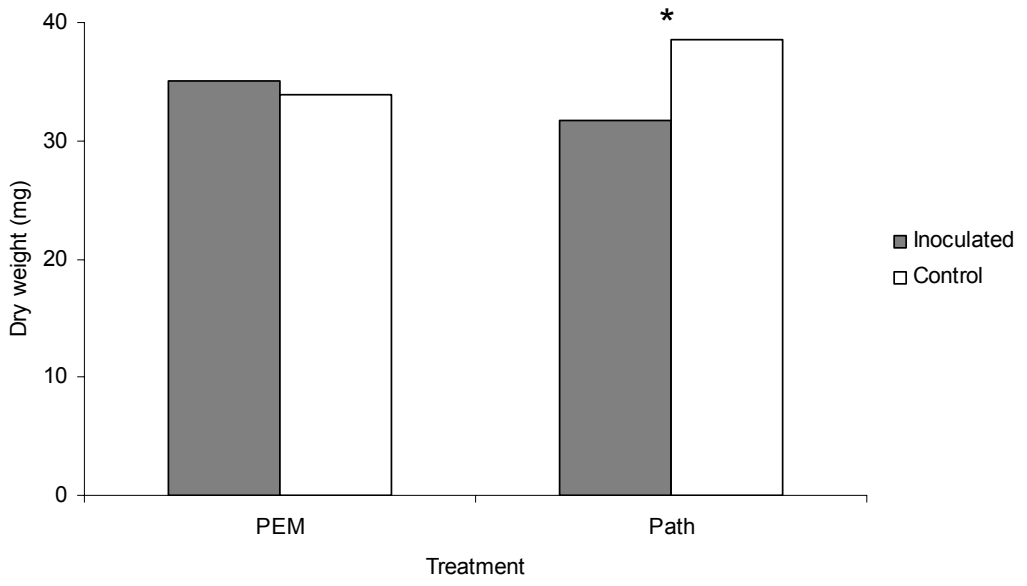


Figure 4-1. Effect of PEM and pathogen (*R. solani*) on dry weight of roots of wheat plants in soil. Significant ($P < 0.05$) comparisons are indicated by *. There were no significant interactions between PEM and pathogen.

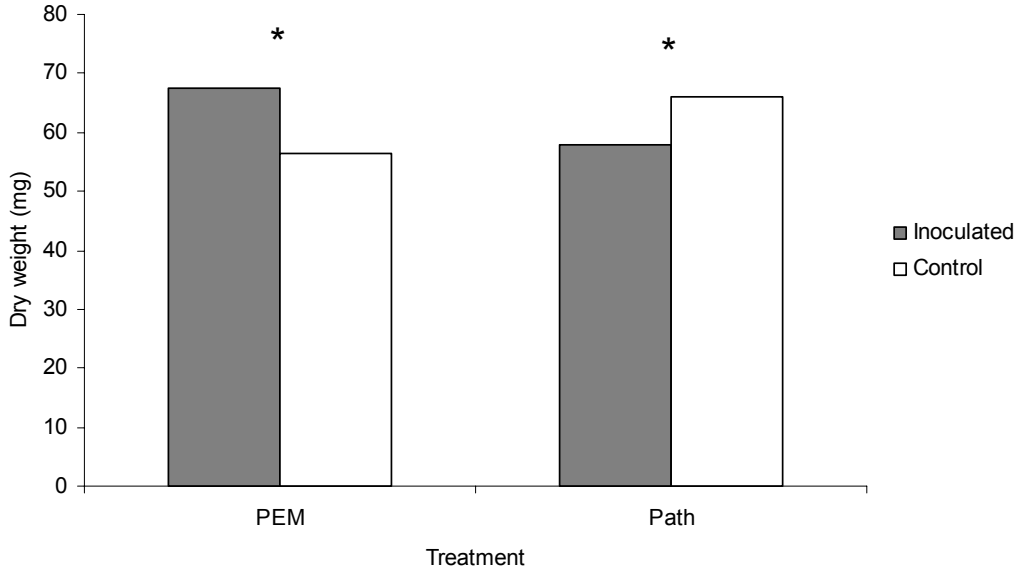


Figure 4-2. Effect of PEM and pathogen (*R. solani*) on dry weight of shoots of wheat plants in soil. Significant ($P < 0.05$) comparisons are indicated by *. There were no significant interactions between PEM and pathogen.

Growth promotion studies on hydroponics agar medium plates

In the Petri plate assay there was a significant increase in shoot dry weight in the combined PEM treatment compared with the uninoculated control (H) (Figure 4-3).

There was a significant effect on rate of root elongation at 4 and 6 days. Plants treated with the bacteria either individually or as a group showed significantly higher root growth rates at 4 days and 6 days than the control (data not shown), however, this effect disappeared by 8 days. Root dry weight and total root showed no significant difference between different treatments.

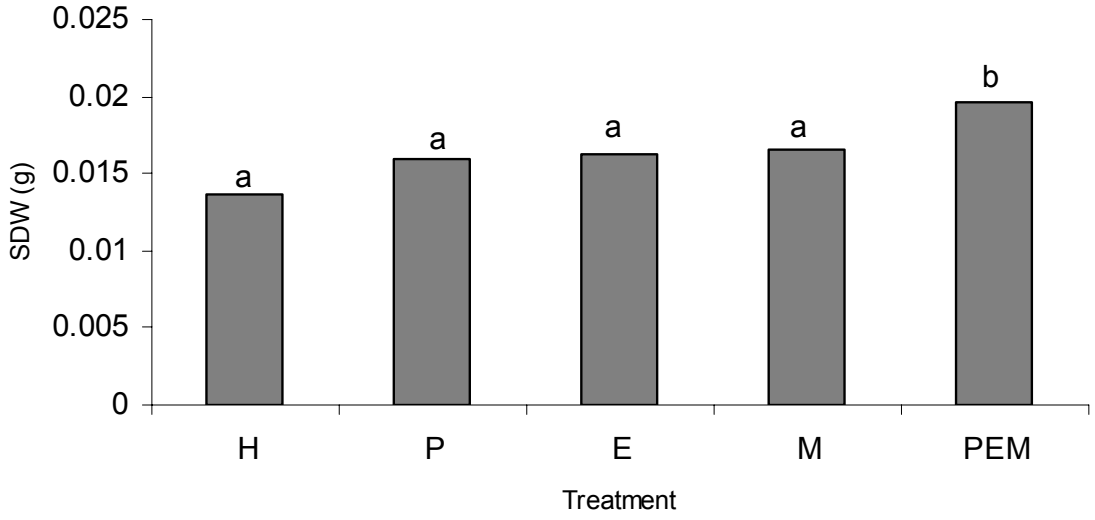


Figure 4-3. Wheat shoot dry weight recorded 10 days after PEM inoculation. PEM are showing increased shoot dry weight compare to H, P, E and M on the Petriplates hydroponics system. Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*, M = *Microbacterium*, PEM = *Pantoea, Exiguobacterium* and *Microbacterium*. Column labelled with same letter are not significantly different at $P < 0.05$ (Tukeys).

Bacterial treatment significantly ($P < 0.001$) affected the degree of branching of root systems, with the combined PEM treatment having the most highly branched roots and the uninoculated control the least branched (Figure 4-4). Because a non-parametric test was used for qualitative data, no post-hoc tests for mean separation could be done. This experiment was repeated with similar results (data not shown).

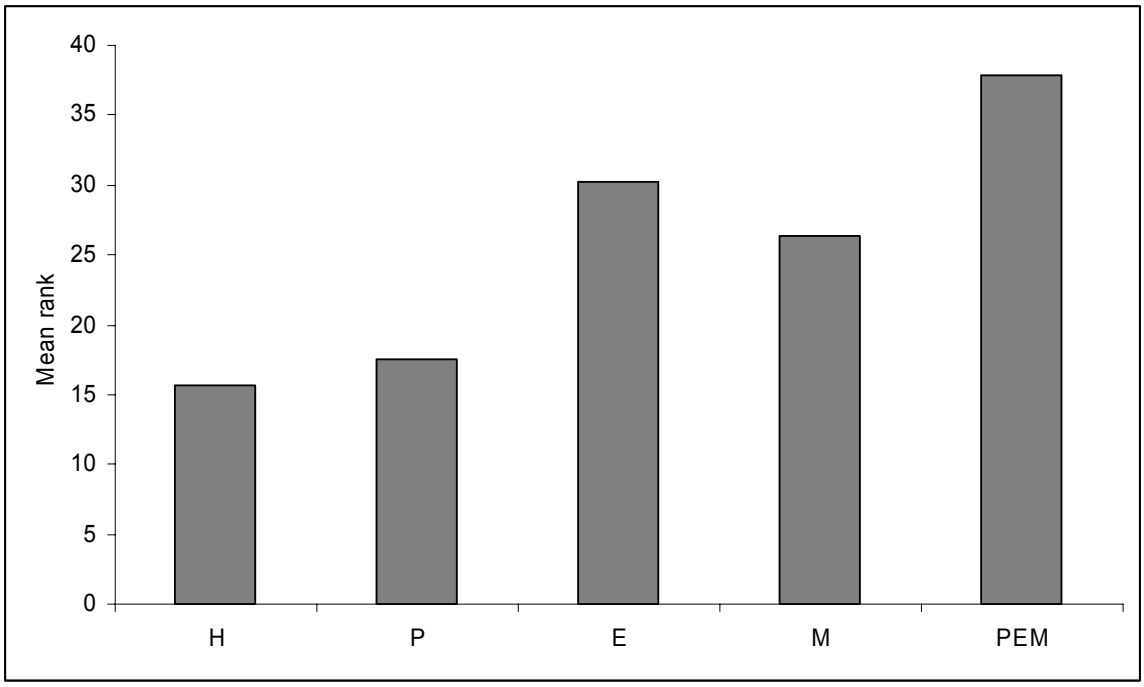


Figure 4-4. Mean rank (out of 50) for degree of branching of wheat seedlings in a Petri dish bioassay. The higher the rank, the more highly branched the root system. Effect of treatment was significant at $P < 0.001$ (Kruskal-Wallis test). Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*, M = *Microbacterium*, PEM = *Pantoea, Exiguobacterium* and *Microbacterium*.

Growth promotion effect in nitrogen added hydroponics medium

In the nitrogen added hydroponics medium there was a significant effect of treatment on root and shoot dry weight, root:shoot ratio and total dry weight of wheat plants. The plants treated with P, E, M, and PEM had increased root dry weights compared to control healthy plants (Figure 4-5). Root dry weight in the PEM treatment was also significantly greater than in the P treatment. Plants inoculated with the PEM combination had significantly increased shoot dry weight compared to control healthy plants (**Figure 4-6**). The root:shoot ratios was based on the weight which showed that the plants inoculated with E and PEM strains together were significantly (P<0.05) increased from H however there was no significant difference from the P and M treatments (Figure 4-7). When total DW of different treatments was analyzed there was a strongly significant difference in bacterial (P, E, M, and PEM) inoculated plants compared to healthy plants (Figure 4-8).

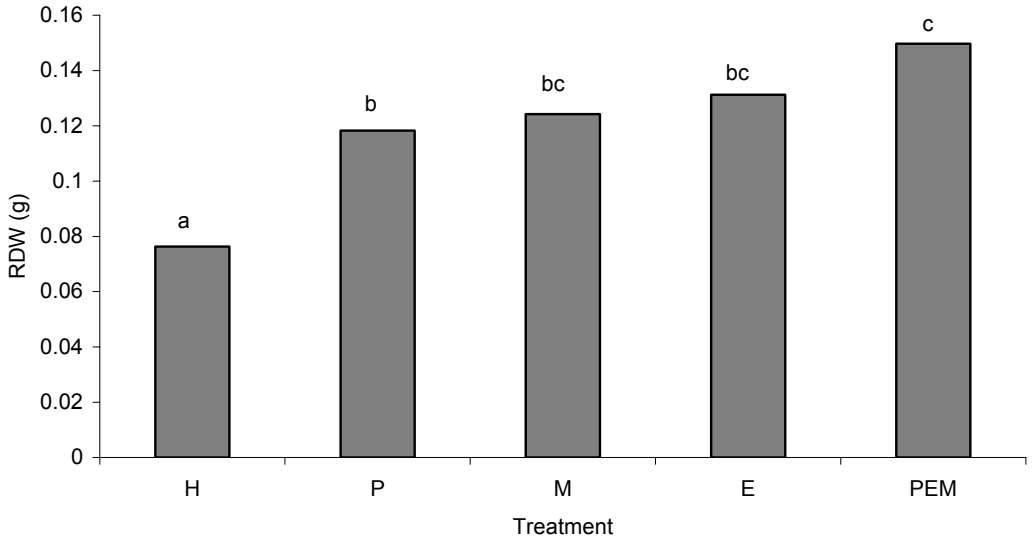


Figure 4-5. Root dry weight (RDW) of wheat plants inoculated with PEM strains in nitrogen added hydroponics medium. Treaments were: H = healthy, P = *Pantoea*, M = *Microbacterium*, E = *Exiguobacterium*, PEM = *Pantoea, Exiguobacterium* and *Microbacterium*. Columns labelled with the same letter are not significantly different at *P* < 0.05 (Tukeys).

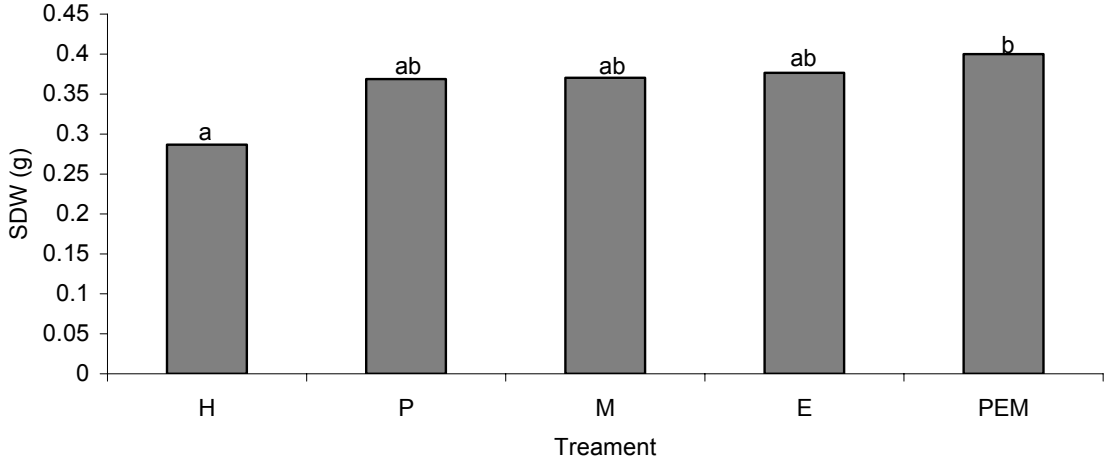


Figure 4-6. Shoot dry weight (SDW) of wheat plants inoculated with PEM strains in nitrogen added hydroponics medium.. Treaments were: H = healthy, P = *Pantoea*, M = *Microbacterium*, E = *Exiguobacterium*, PEM = *Pantoea, Exiguobacterium* and *Microbacterium*. Column labelled with same letter are not significantly different at *P* < 0.05 (Tukeys).

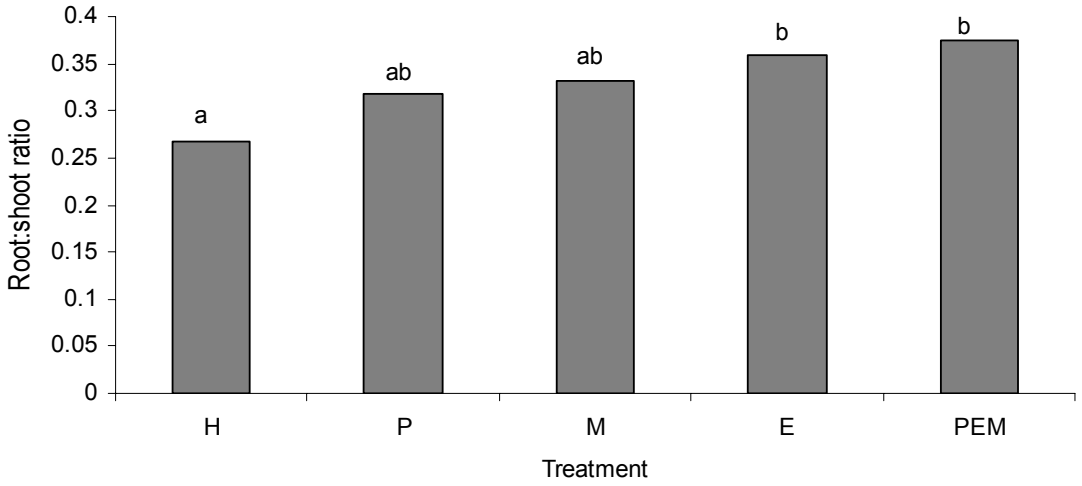


Figure 4-7. Root/shoot (dry weight) ratio of PEM strains inoculated wheat plants. Treaments were: H = healthy, P = *Pantoea*, M = *Microbacterium*, E = *Exiguobacterium*, PEM = *Pantoea, Exiguobacterium* and *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

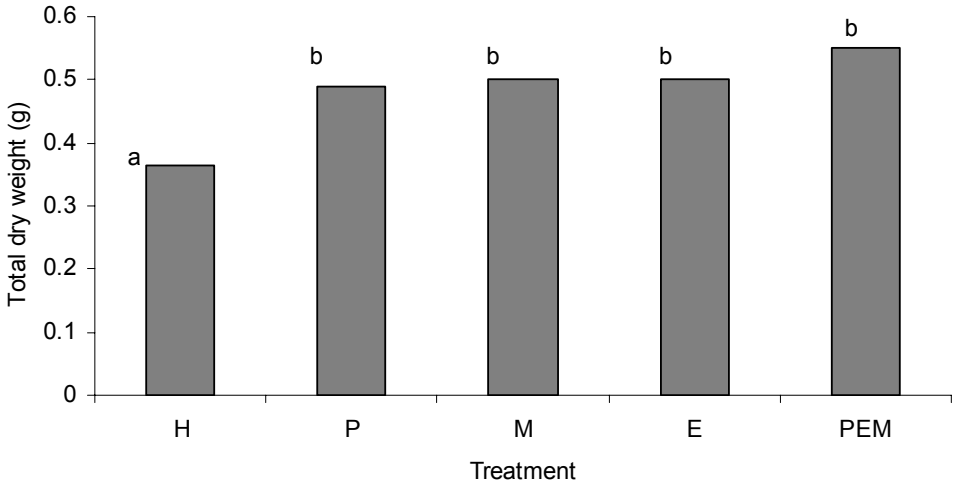


Figure 4-8. Total dry weight (DW) of wheat plants inoculated with PEM strains in nitrogen added hydroponics medium. Treaments were: H = healthy, P = *Pantoea*, M = *Microbacterium*, E = *Exiguobacterium*, PEM = *Pantoea, Exiguobacterium* and *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

Studies in nitrogen free hydroponics medium

Growth promotion effect

When individual PEM strains were inoculated onto plants in nitrogen deficient conditions there was a strong effect on increased shoot growth compared to healthy un-inoculated plants. Bacterial inoculated plants showed significantly increased shoot dry weight and total dry weight, and a significantly lower root:shoot ratio, than uninoculated plants (Figure 4-9 to Figure 4-11). There were no significant differences between the three bacterial treatments for any of these parameters. There was no significant difference in root dry weight between different treatments (Figure 4-12).

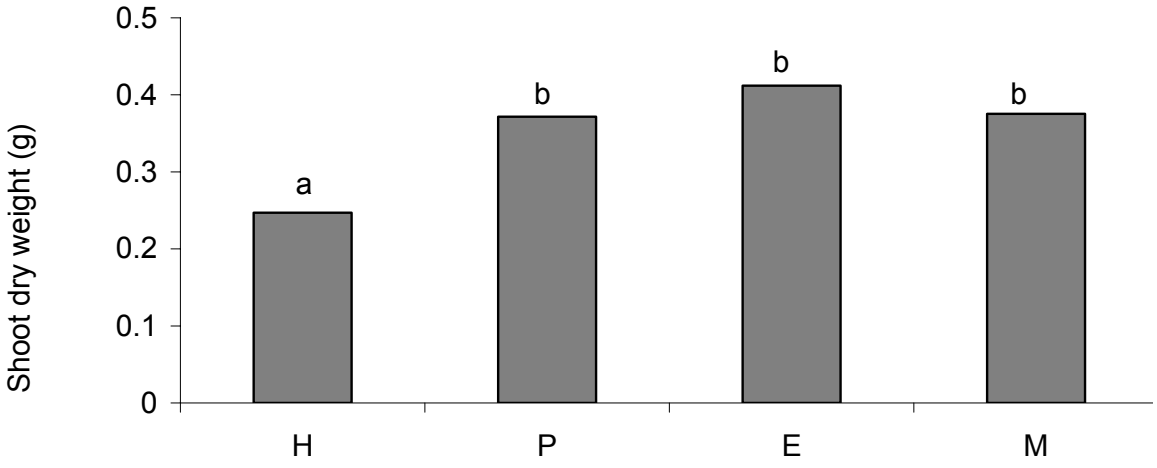


Figure 4-9. Total shoot dry weight of PEM strains inoculated wheat plants in nitrogen-free medium. All bacterial treated plants had significantly increased shoot dry weight. Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*, M = *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

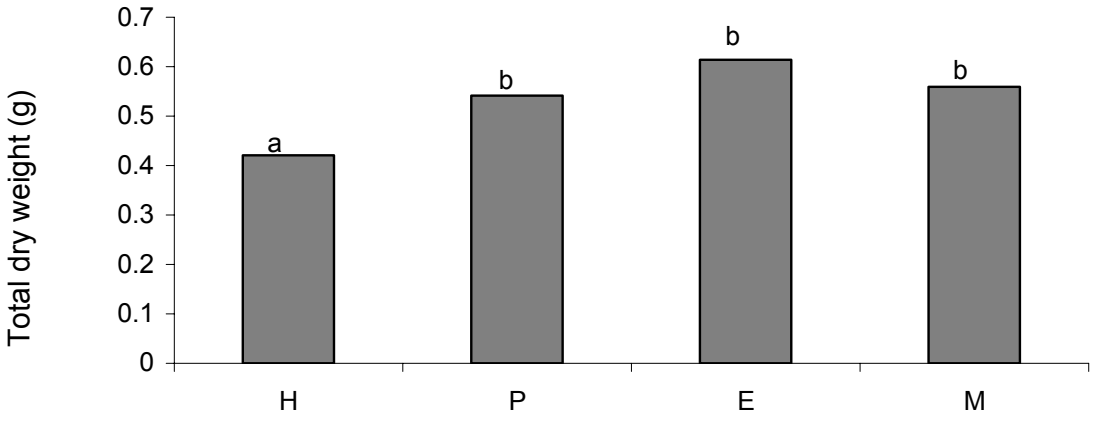


Figure 4-10. Total dry weight (DW) of PEM strains inoculated wheat plants in nitrogen-free medium. All bacterial treated plants have significantly increase total dry weight. Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*, M = *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

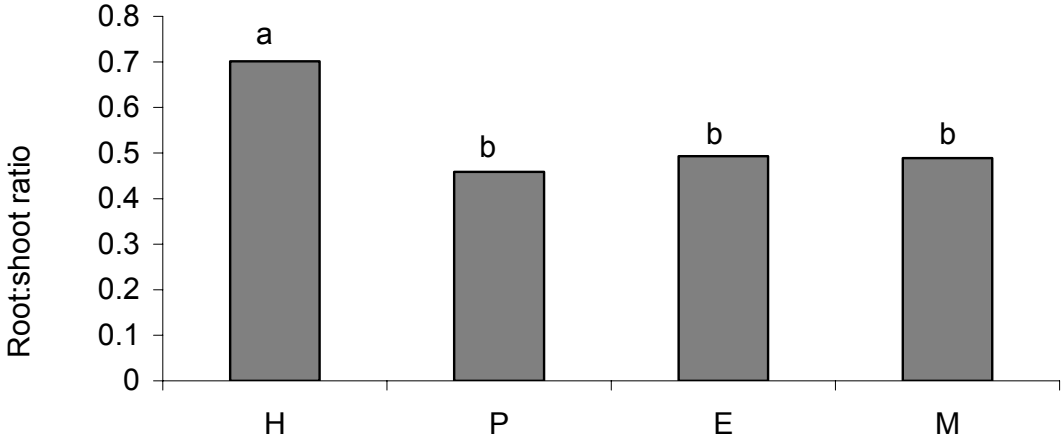


Figure 4-11. Root: shoot (RS) ratio of PEM strains inoculated wheat plants on nitrogen-free medium. All bacterial treated plants have significantly decreased root: shoot ratio. Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*, M = *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

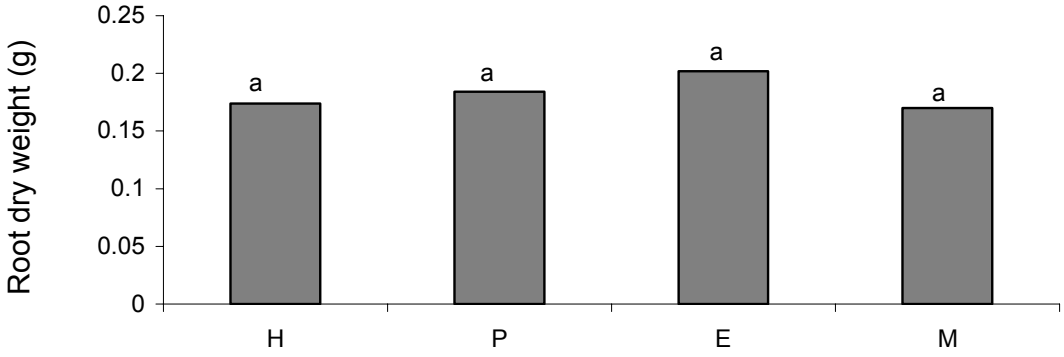


Figure 4-12. Root dry weight (DW) of PEM strains inoculated wheat plants on nitrogen-free medium. The different bacterial (PEM) treatments had no effect on root dry weight. Treaments were: H = healthy, P = *Pantoea*, M = *Microbacterium*, E = *Exiguobacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

Nitrogen uptake of bacterial inoculated plant

Nitrogen percentage analysis of root and shoot tissues showed different results from each other. The nitrogen percentage in root tissue was significantly increased by P and E, but not by M (Figure 4-13A). For shoot tissue, there was no significant difference between treatments (Figure 4-13B).

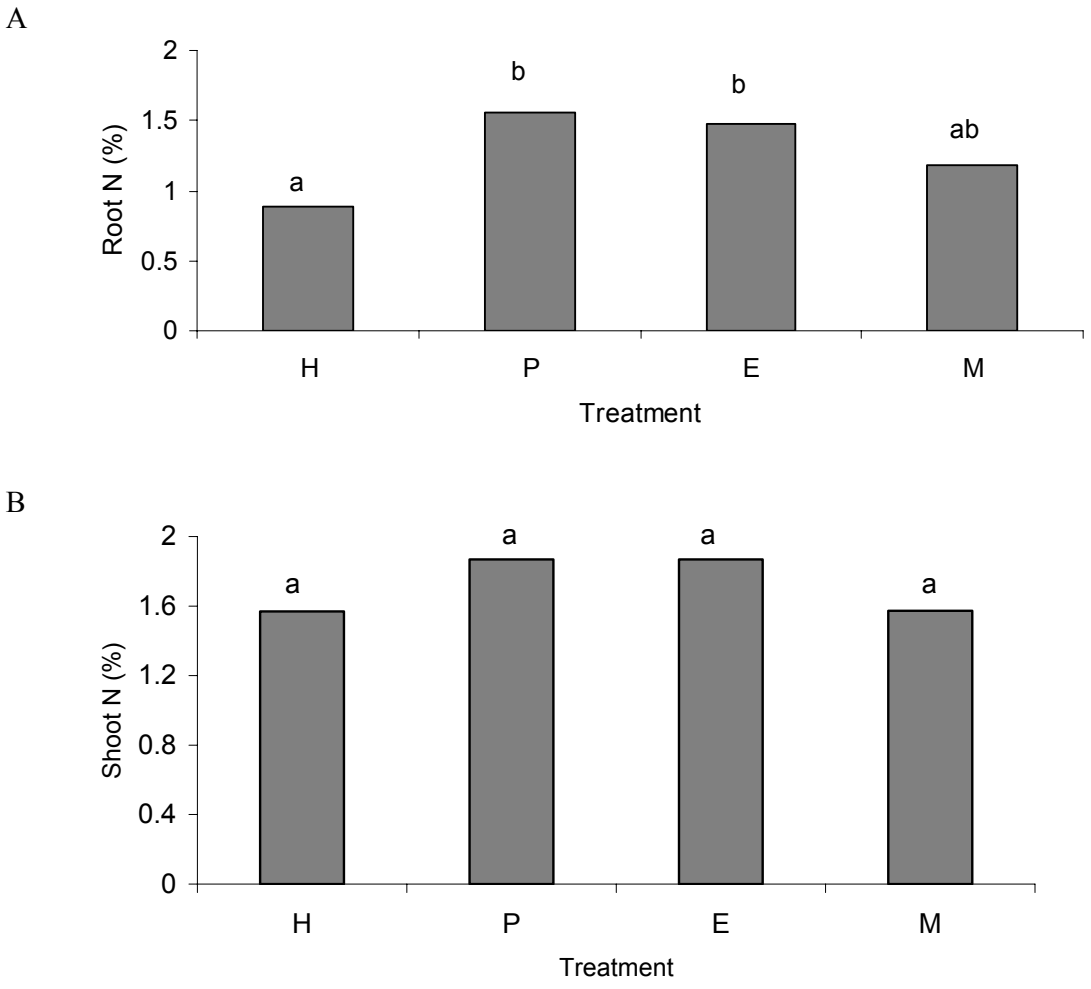


Figure 4-13. Nitrogen percentage in A) root and B) shoot of wheat plants grown in nitrogen free medium. Root tissue showed a significantly increased nitrogen percentage between bacterial treatments. No significant difference detected in shoot tissue. Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*, M = *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

The total nitrogen content detected for root, shoot and total plant tissues showed similar results (Figure 4-14). Inoculation with P and E increased the total amount of N (mg) in the plant tissues, but the M treatment was not significantly different from the control. This was the same for roots, shoots and total.

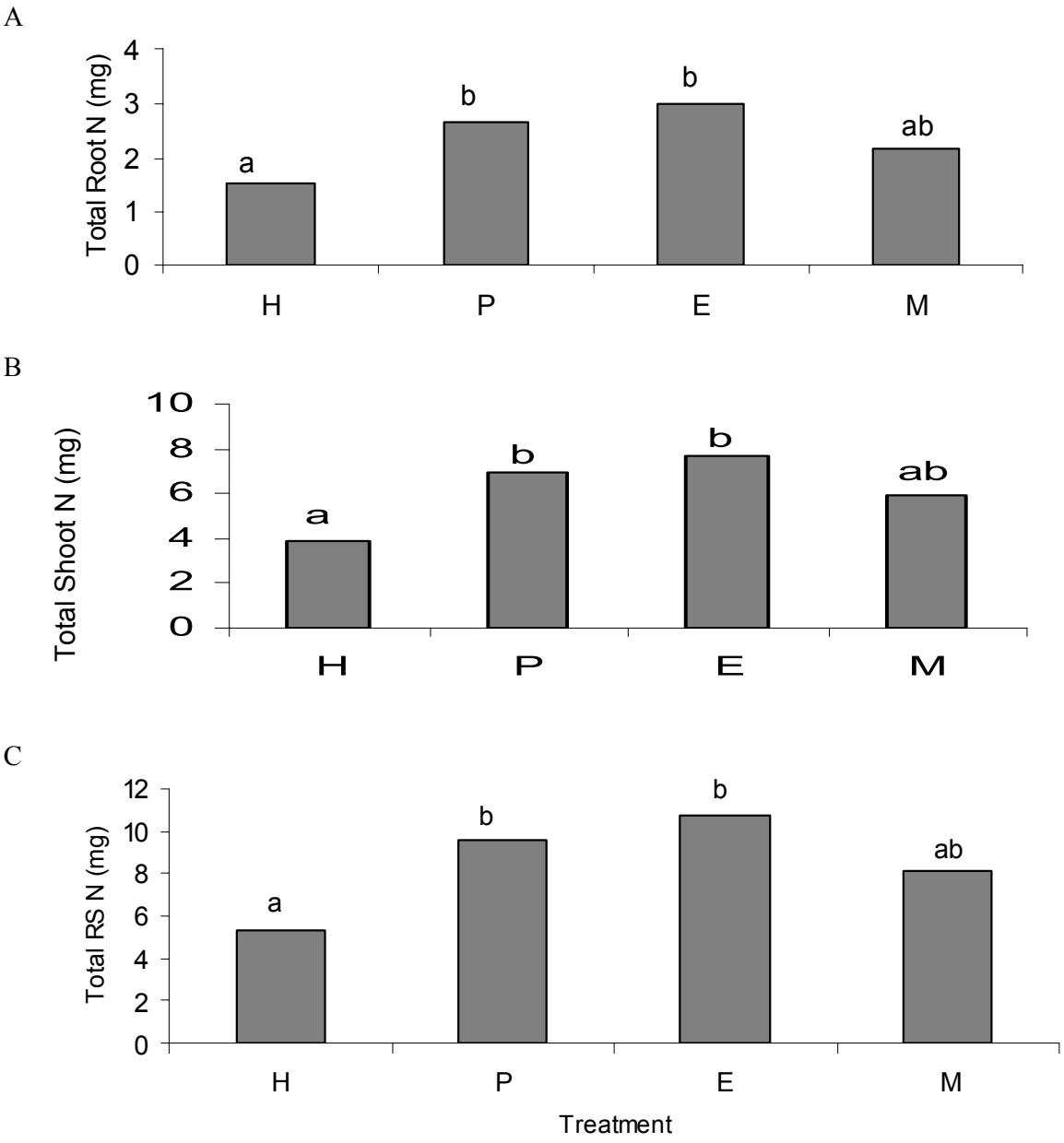


Figure 4-14. Total nitrogen content analysis of A) root, B) shoot and C) root + shoot. PEM strains inoculated plants (E&M) have significantly increased nitrogen content compared to M and un inoculated plants. Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*, M = *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

Root system architecture changes induced by PEM

Several parameters of root and shoot architecture were analyzed for plants in nitrogen-added hydroponics medium inoculated with individual bacterial strains (P, E and M). Statistical analysis of the data revealed that there was a significant effect on shoot length, number of lateral roots and total length of branched or un-branched sections of the plant root.

The total shoot length of P treated plants was significantly greater compared to uninoculated plants at 10 and 14 days after bacterial inoculation (Figure 4-15). However, E and M treatments were not significantly different from H although there appeared to be an increased shoot length in the M treatment.

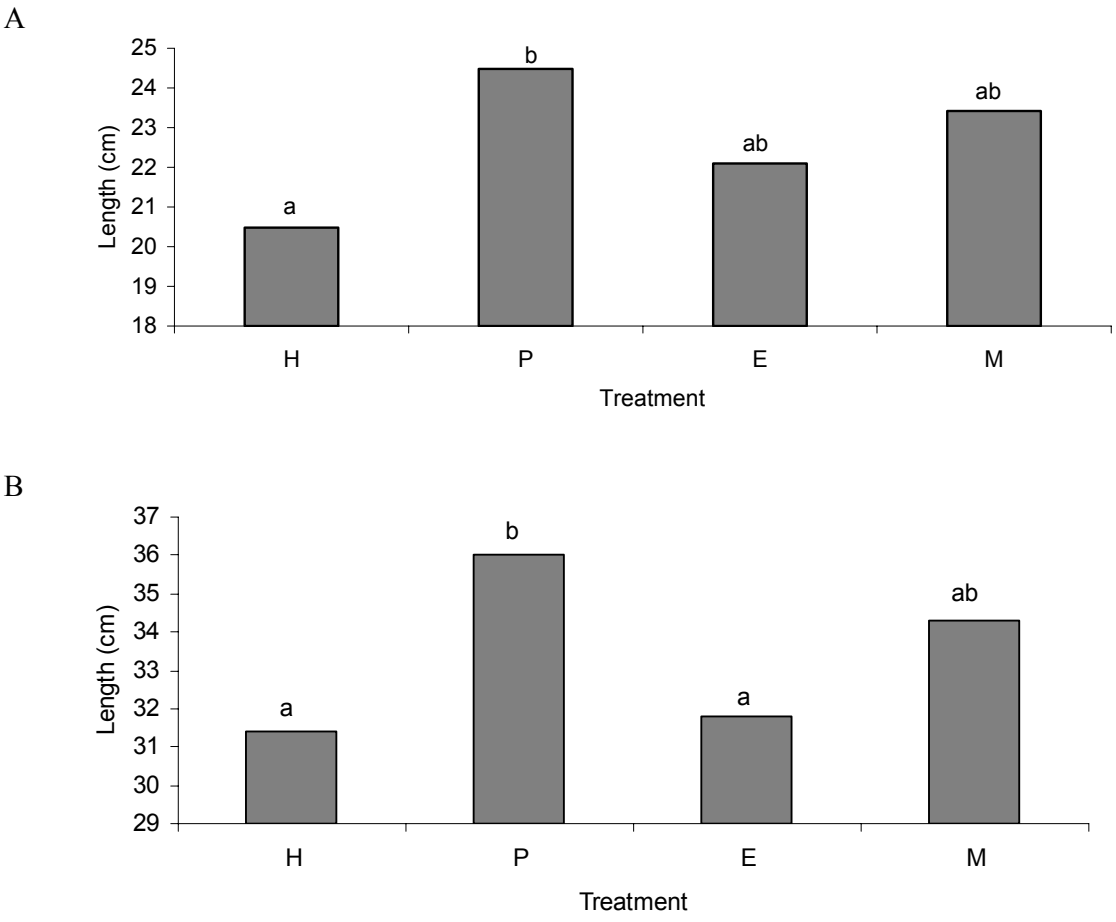


Figure 4-15. Total shoot length of PEM strains inoculated wheat plants at A) 10 days and B) 14 days. Wheat plants inoculated with the bacterial strain P showed increased shoot length compared to H, E & M in nitrogen added hydroponics medium. Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*, M = *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

All bacterial inoculated plants had an increased number of lateral roots compared to uninoculated plants at 10 days (Figure 4-16A). However after 14 days (Figure 4-16B), only P and E treated plant roots had significantly increased number of lateral roots and M was not significantly different from any of the other treatments.

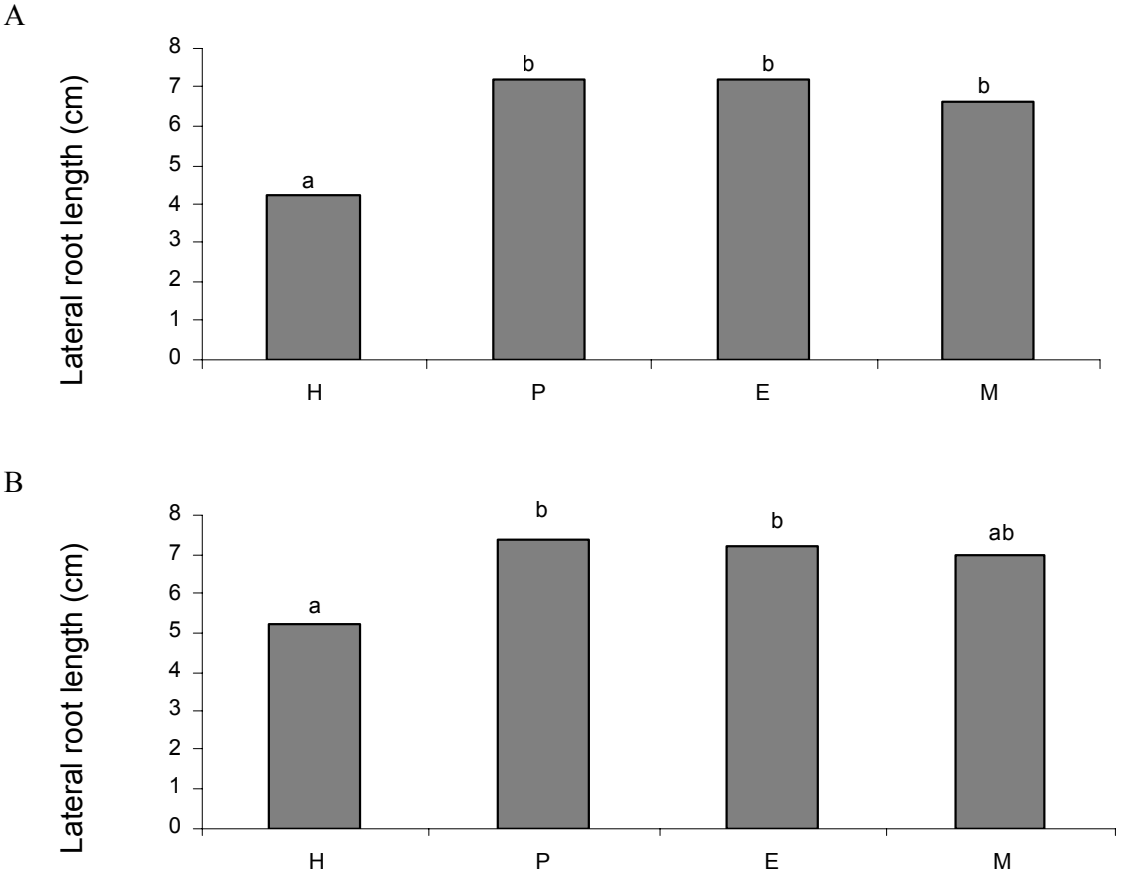


Figure 4-16. Lateral root length of PEM strains inoculated wheat plants at A) 10 days and B) 14 days. Wheat plants inoculated with the bacterial strain P, E M had increased length of lateral root compare to H in nitrogen added hydroponics medium. Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*, M = *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

Analysis of data for the total length of branched or un-branched sections of the roots revealed that there was no significant difference in branched section at 10 days and un-branched section at 14 days. The E treatment plants had significantly shorter un-branched sections than

uninoculated plants at 10 days (Figure 4-17A) and P and E treated plants had a significantly increased branched section compare to controls at 14 days old (Figure 4-17B).

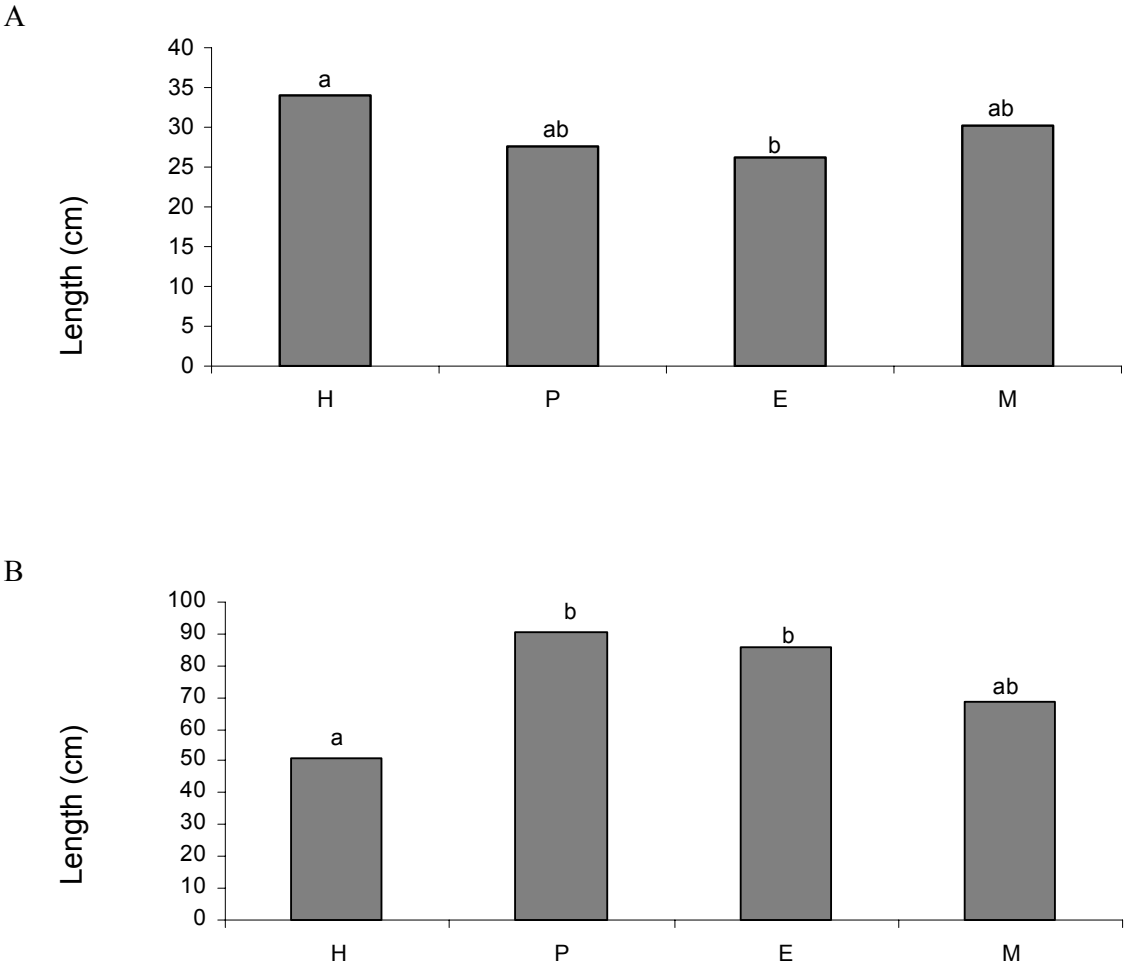


Figure 4-17. Length of A) unbranched section of roots at 10 days and B) branched section of root at 14 days of wheat plants inoculated with PEM bacteria. Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*, M = *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

4.4 Discussion

In this study, the PEM strains were evaluated for their potential to promote plant growth by different methodologies.

The growth promotion effect on the hydroponics medium plates gave some significant indication of growth promotion between different treatments. PEM strains showed a significantly increased shoot dry weight which indicates the plant growth stimulation effect in hydroponics agar system. There was also an increased degree of branching of roots of PEM treated plants. The other growth promotion parameters showed differences in the results obtained between experimental systems. The differences among experiments may be due to the involvement of several factors, such as absence of nutrients related to plant growth in the hydroponics agar medium plates. Another possibility is that inoculated bacteria adhering to the roots after shaking off were not enough or not constant to induce the growth promotion effect. In initial studies, plant growth on the medium plates was doubtful due to lack of space so it was decided that there should be some bigger experimental system which can provide more space for the growth of the plant. The PVC pots system containing hydroponics medium was chosen to grow the plants with the aim to provide more space and enough nutrient medium to the plants.

The data showed that PEM had significant growth promotion effects under hydroponics medium conditions. There was significantly increased root and shoot growth between different treatments when PEM strains were inoculated individually or together in bioassay. Many studies have shown growth promotion effects with bacterial inoculation and also have demonstrated the plant growth promotion as increase in plant biomass in bacterized plants compared with uninoculated controls (Hameeda *et al.* 2006). Results here are in agreement with previous studies of the growth promotion effects after PGPR inoculation to the plant. Barnett *et al.*(2006) showed that PEM has a disease suppression ability after interaction with pathogen however there are no reports of growth promotion potential of PEM in the absence of the pathogen. However, the study conducted by (Barnett *et al.* 2006) demonstrated an effect on shoot growth of diseased plants by PEM. This supports some aspects of findings of Barnett *et al.* (2006) but most of them have been added as a new information on the growth promotion potential effects of PEM.

PGPR have been reported to stimulate root morphogenesis (Larcher *et al.* 2003). This study demonstrated that PEM strains have an ability to change root/shoot system architecture. Many studies have shown that the inoculation of wheat, and other cereals seeds, with bacteria of the genus *Azospirillum* resulted in an increase in both volume and number of roots (Bashan and Levanony 1990, Didonet and Magalhaes 1993, Gouvêa *et al.* 1997). This improved the uptake of nutrients and water (Murty and Ladha 1988). In this study PEM caused an increased in branching pattern such as number of lateral roots, an increased volume and shoot length. This finding is similar to that for *Azospirillum* as mentioned above. It has also been revealed previously that many of the growth promoting bacteria have nitrogen fixation ability. With the aim to determine the nitrogen fixation ability of PEM strains, bacterial inoculated plants were grown in nitrogen free hydroponics medium. The result showed that PEM strains have a strong effect on growth promotion and nitrogen content in nitrogen deficient conditions. It was also observed that bacterial inoculated plants looked much healthier than the un-inoculated control plants.

There was evidence for significant plant nitrogen increases due to inoculation with PEM. The increase in the nitrogen content of inoculated plants was much greater than could be accounted for the nitrogen present in the inoculam. Increased branching pattern could have improved the nutrient uptake in bacterial inoculated plants. The fact that nitrogen was increased in the PEM inoculated plants relative to the control plants indicates that PEM has some nitrogen fixation ability. These results also support the hypothesis of root association and N_2 fixation ability of PEM strains. The increase in total N content by bacterial activity of free-living diazotrophs has been shown by Baldani *et al.* (1997). In this study the increase in nitrogen uptake as a result of inoculation has been proved. Though PEM strains could possess multiple plant growth promotion traits, the increased nitrogen uptake by bacterized plants could be mainly due to nitrogen fixing ability of PEM strains, which could also have stimulated plant growth.

Chapter 5 Mechanism of plant growth promotion by PEM

5.1 Introduction

The concept of plant growth-promoting rhizobacteria (PGPR) represents the beneficial effects of rhizospheric bacteria manifested as improved plant health (Kloepper and Beauchamp 1992). These beneficial effects of the PGPR are attributed to a series of mechanisms or several characteristics which influence or stimulate plant growth and development (Glick 1995, Whipps 2001). Some of the most common traits are the ability to produce various growth regulators such as production of phytohormones (Glick 1995), lowering ethylene levels (Glick 1995, Glick *et al.* 1999), fixation of atmospheric nitrogen (Kennedy *et al.* 1997), antagonism of pathogens by production of siderophores (Elad and Baker 1985), solubilization of inorganic phosphates (Katiyar and Goel 2003), induction of systemic disease resistance, and many more which could be involved in either growth promotion or disease suppression processes. However, a number of studies related to PGPR have shown plant growth promotion effects only in gnobiotic conditions or in potting media where bacteria do not need to compete with normal soil microflora (Tien *et al.* 1979, Polonenko *et al.* 1987, Fuhrmann and Wollum 1989, Shenbagarathai 1993, Glick 1995, Cattelan *et al.* 1999).

Many plant associated bacteria synthesize the phytohormone indole acetic acid (IAA), which has an important role in increased root growth, and increased number of secondary roots and root hairs (Barbieri and Galli 1993, Steenhoudt and Vanderleyden 2000, Patten and Glick 2002). IAA has a role in root initiation, elongation and a number of other processes concerned with the plant life cycle (Arshad and Frankenberger 1998). Several members of bacterial genera such as *Azospirillum, Rhizobium, Enterobacter, Xanthomonas* and *Pseudomonas* have been shown to produce IAA which has a stimulatory effect on plant growth (Bar and Okon 1993, Glick 1995, Patten and Glick 1996).

Some of the earliest studies demonstrated that a number of PGPR, including *Pseudomonas*, produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the immediate precursor of the plant hormone ethylene (Penrose and Glick 2003, Belimov *et al.* 2005). The reduction in ethylene level due to production of the ACC deaminase facilitates growth and development in plants (Glick *et al.* 2007). *Pseudomonas fluorescens* has been reported to stimulate plant growth and vitality and give higher yield

despite pathogen attack due to the ACC deminase effect. ACC deaminase has been screened in rhizobacteria in activity assays and using PCR (Babalola *et al.* 2003, Penrose and Glick 2003).

Plant associated N_2 fixing bacteria have been considered as a possible alternative to inorganic nitrogen fertilizers to promote plant growth and yield (Ladha *et al.* 1997). A number of bacteria, such as *Enterobacter*, *Klebsiella pneumoniae*, *Azotobacter*, *Paenibacillus polymixa*, *Azospirillum, Acetobacter,* and *Pseudomonas* spp., have been reported for their beneficial effects and ability to fix nitrogen (Olivares *et al.* 1996, Minamisawa *et al.* 2004, Tejera *et al.* 2005).

Competition for iron seems to be a major factor in some plant growth promotion and also appears to be involved in other mechanisms of biocontrol of diseases caused by microbial plant pathogens. Plant-growth-promoting activity by siderophore production was described by many workers (Loper and Henkels 1999, Barthakur 2000, Haas and Keel 2003, Siddiqui 2006). Siderophores are low molecular weight Fe(III)-specific ligands produced by microorganisms in order to combat low iron stress (Neilands and Leong 1986). The high-affinity iron Fe^{+++} chelators enhance the acquisition of iron in an iron-deficient environment thereby making iron available to plants (Mori 1999, Deka Boruah and Dileep Kumar 2002). It has been reported that microbial siderophores may be utilized by plants as an iron source and additionally one of the mechanisms to combat pathogens (Duijff *et al.* 1993). The production of siderophores has been demonstrated in several bacteria including genera such as *Pseudomonas*, *Rhizobium, Streptomyces* and *Azotobacter* (Neilands and Leong 1986, Challis and Ravel 2000, Fiedler *et al.* 2001, Haas 2003).

Another mechanism of plant growth by PGPR is an increase in P availability due to solubilization of phosphates. Phosphate solubilizing bacteria are considered to play an important role in mobilizing insoluble inorganic phosphates from the surrounding matrix which is then available or absorbed by plant roots to improve growth and yield (Jones and Darrah 1994, Goldstein and Krishnaraj 2007).

Plants have the ability to acquire an enhanced resistance mechanism to pathogen attack after induction with a variety of organisms or compounds (Pieterse *et al.* 1998, Tuzun 2001). Production of hydrolytic enzymes such as chitinase, glucanase and peroxidase or/and other

gene products related to plant defenses play an important role in disease resistance. The pre-treatment of plants with an inducing organism or compound appears to incite the plant to mount an effective defense response upon subsequent encounters with pathogens (van Loon *et al.* 1998, Tuzun 2001).

The information summarized above provides a clear scenario of plant growth stimulation by inoculated bacteria using a series of potential growth promotion traits. This chapter is based on an investigation to determine the mechanisms involved in the growth promotion or disease suppression effects of PEM. In the previous chapter the growth promotion ability of PEM strains was demonstrated but there is no information about potential mechanisms involved in this process. Assessing the involvement of possible mechanisms could be helpful for better understanding and management of growth promotion or disease suppression ability of PEM.

5.2 Materials and Methods

Bacterial strains and Pathogen

All the bacterial (*Pantoea, Exiguobacterium* and *Microbacterium*) or pathogen (*Rhizoctonia solani*) strains were cultured on LB agar or LB broth or PDA/4 as described in the previous Chapter.

Production of IAA

Production of IAA by PEM strains was assayed based on the method described by Patten and Glick (2002). PEM strains were cultured freshly on LB agar medium plates. Single fresh colonies of individual PEM strains were inoculated into 5 mL of LB broth medium, unamended and supplemented with 1% and 2% tryptophan, and were grown for 48 hours at 30ºC with 180 rpm shaking. The density of the culture was measured at 600 nm and then bacterial cells were harvested by centrifugation at 6000 rpm for 10 min**.** A 1 mL aliquot of the supernatant was mixed with 4 mL of Salkowski's reagent (150 mL of concentrated H_2SO_4, 250 mL of DW, 7.5 mL of 0.5 M $FeCl_3.6H_2O$) and incubated at room temperature for 20 min**.** The absorbance was measured at 535 nm using a Beckman DU-640 spectrophotometer. The experiment was repeated three times with three replicates.

In the next experiment, 29 strains of *Pantoea*, including Q1, Q2, Q3, Q4, Q5, Q6, Q7, Q8, Q9, Q10, W1, W2, W3, W4, W5, W6, W7, W8, W9, W10, H1, H2, H3, H4, H5, H6, H7, H8,

H9 were received from S. Barnett, South Australian Research & Development Institute (SARDI), Adelaide and were also tested for IAA production as described above to determine variability among different isolates. There were two replicates for each of the isolate.

Production of ACC deaminase

The ACC deaminase activity of PEM strains was assayed as described by Penrose and Glick (2003) with some modifications. The PEM strains were freshly cultured on LB agar plates and induction of ACC deaminase for the assay was conducted as described below. Single colonies of the individual bacteria were inoculated in 15 mL LB medium and grown to mid log phase after incubating overnight with shaking at 200 rpm at 30ºC. The overnight grown cells were harvested by centrifugation at 6000 g for 10 min at 4ºC. The supernatant was removed and the cells were washed with 5 mL DF salts minimal medium (Appendix 1). To ensure that cells were washed properly, an additional centrifugation for 10 min at 6000 g at 4ºC was done, and the cells were suspended in 7.5 mL DF salts minimal medium in a fresh culture tube. A 45 µl aliquot from the frozen 0.5 M ACC stock solution was added to the cell suspension to obtain a final ACC concentration of 3.0 mM just prior to incubation. The bacterial cells were returned to incubation with shaking at 200 rpm at 30ºC for 24 h. The grown cells were harvested by centrifugation at 6000 g for 10 min at 4ºC. The supernatant was removed and the cells were washed by suspending the cell pellet in 5 mL of 0.1M Tris–HCl, pH 7 and transferred to a 15 mL centrifuge tube (Sarstedt, Australia). The supernatant from the above step was removed after centrifugation at 12,000 g for 5 min and the pellet was finally re-suspended in 600 µl of 0.1 M Tris–HCl, pH 8.5. A 30 µl aliquot of toluene was added to the above cell suspension and vortexed at the highest setting for 30 s and later stored at 4ºC for assay of ACC deaminase activity.

All sample measurements were carried out in triplicate. 200 µl of the toluenized cells were placed in a fresh 1.5 mL microcentrifuge tube and 20 µl of 0.5 M ACC was added to the suspension, briefly vortexed and then incubated at 30ºC for 15 min. Following the addition of 1 mL of 0.56 M HCl, the mixture was vortexed and centrifuged for 5 min at 12 000 g at room temperature. A 1 mL aliquot of the supernatant was vortexed together with 800 µl of 0.56 M HCl. Then, 300 µl of 0.2% 2, 4-dinitrophenyl hydrazine in 2M HCl was added to the glass tube, the contents vortexed and then incubated for 30 min at 30ºC. Following the addition and mixing of 2 mL of 2 N NaOH, the absorbance was measured at 540 nm.

To calculate the ACC deaminase activity the method described by Penrose and Glick (2003), adapted from Honma (1985), was used. The method measures the amount of α-ketobutyrate released when the enzyme ACC deaminase cleaves the ACC. The number of µmol of α-ketobutyrate produced by this reaction was determined by comparing the absorbance at 540 nm of a sample to standard curve of α-ketobutyrate ranging from 0.1 and 1.0 µmol. A stock solution of 100 mM α-ketobutyrate was prepared in 0.1 M Tris-HCl pH 8.5 and diluted with the same buffer to obtain 10 mM solution from which a standard curve was generated. Each of the diluted series of known α-ketobutyrate concentrations was prepared to a final volume of 200 µl and mixed with 300 µl of the 2,4-dinitrophenyl hydrazine reagent. The mixture was then vortexed and incubated at 30ºC for 30 min. This incubation period allowed the α-ketobutyrate to be derivatized as a phenylhydrazone and following the addition of 2 mL of 2 M NaOH a colour was developed. The optical density of the mixtures was measured at 540 nm.

PCR was used to screen for ACC deaminase genes from PEM strains as described by Hontzeas *et al.* (2005). Genomic DNA of PEM strains was extracted by using the QIAGEN DNeasy Tissue Kit and stored at -20 ^{o}C. The degenerate primer DegACC5'-(5'-GGB GGV AAY AAR MYV MGS AAG CTY GA) and DegACC3'-(5'-TTD CCH KYR TAN ACB GGR TC), where B=G/T/C, V=G/C/A, Y=T/C, R=G/A, M=A/C, S=G/C, D=G/A/T, H=A/C/T, K=G/T, N=A/G/C/T (Hontzeas *et al.* 2005) were synthesized by Geneworks (Thebarton, SA, Australia). These primers were used to amplify the ACC deaminase genes from purified genomic DNA of PEM strains. The PCR reaction mix and cycle protocol were obtained from N. Hontzeas (pers. comm.).

The PCR reactions were done in 25 µl reaction mixture using Taq PCR Core kit (QIAGEN) containing 2.5 µl of 10X Taq DNA Polymerase buffer, 2 µl of 2.5 mM dNTPs, 1 µl of 100 µM of each degenerate primers, 0.5 µL (2.5U) of *Taq* polymerase (Qiagen, Australia) and 40 ng template DNA. The PCR (Palm Cycler, Corbett Research CG1-96, Australia) cycle program consisted of initial denaturation at 95ºC for 3 min; 35 cycles of denaturation at 95ºC for 1 min followed by annealing at 46ºC for 1 min and extension at 72ºC for 1 min; and final extension at 72ºC for 10 min. The PCR amplified products were analyzed on 2% (w/v) agarose gels prepared in TBE buffer and band sizes were checked after staining with ethidium bromide. PCR product was purified using the QIAprep spin miniprep kit (QIAGEN). The sequencing reaction was prepared by adding 10 pmol each primer (DegACC5' and

DegACC3') to PCR purified DNA (~50 ng diluted with sterile milli-Q water) in 16 µL total sequencing reaction mixture. The sequencing reaction mix was sent to SUPAMAC, Sydney for sequencing.

*Amplification of nitrogen fixation (*nifH*) genes*

So far several PGPR that fix nitrogen in non-leguminous plants have been studied and identified (Glick *et al.* 1999). In this study, PEM were screened for the presence of the *nifH* gene, which codes for dinitrogenase reductase, a key enzyme in nitrogen fixation, by using PCR as described by Zakhia *et al.* (2006) and Shrestha *et al.* (2007). To detect the *nifH* gene among PEM strains a nested PCR amplification using three sets of primers was performed with the purified genomic DNA of PEM strains. Two replicates of genomic DNA extracts from each bacterium were tested.

Three primers were taken from Zakhia *et al.* (2006), as originally published by Widmer *et al.* (1999). The primer sequences nifH (forA) 5'-GCI WTI TAY GGN AAR GGN GG-3', nifH (forB) 5' -GGI TGT GAY CCN AAV GCN GA-3'and nifH reverse 5'-GCR TAI ABN GCC ATC ATY TC-3, were synthesized by Gene Works (Thebarton, SA, Australia). The first PCR was carried out with the forward primer *nifH* (for A) and reverse primer *nifH* (rev.). The second (nested) PCR was performed with the forward primer *nifH* (forB) and the same reverse primer *nifH* (rev.). The expected band size was 370 bp. The PCR cycling conditions used for both the reactions were: initial denaturation at 95ºC for 5 min; 35 cycles of denaturation at 94ºC for 30 s followed by annealing at 48ºC for 30 s and extension at 74ºC for 30 s; and final extension cycle at 72ºC for 10 min.

Another set of primers were taken from Shrestha *et al.* (2007) and were synthesized by Gene Works (Thebarton, SA, Australia). The first PCR was performed using the forward primer FGPH19 (5'-TAC GGC AAR GGT GGN ATH G-3') and the reverse primer PoIR (5'-ATS GCC ATC ATY TCR CCG GA-3'). The amplification product for first PCR was 429 bp. Then, the second PCR was performed with the forward primer PoIF-GC (5'-TGC GAY CCS AAR GCB GAC TC-3' with a GC clamp) and the reverse primer AQER (5'-GAC GAT GTA GAT YTC CTG- 3'). The amplification product for the second PCR was 360 bp. The cycling conditions used were: 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min at 55°C for the first and at 48°C for the second PCR, and primer extension at 72°C for 2 min, with a final extension at 72°C for 5 min.

The PCR (Palm Cycler, Corbett Research CG1-96, Australia) was conducted using *Taq* PCR Core kit (QIAGEN) and reactions were set up as a 25 µl reaction volume which contained 2.5 µl of 10X Taq DNA Polymerase buffer, 2 µl of 2.5 mM dNTPs, 1 µl of 100 µM of each degenerate primer, 0.5 µL (2.5U) of *Taq* polymerase (Qiagen, Australia) and 40 ng template DNA for the first reaction. The reaction mixture for the second PCR was the same as the first except the template used for the second PCR was 2 µl of the first PCR product. The PCR product was then subjected to 2% (w/v) agarose gel electrophoresis run at 80 v and staining with ethidium bromide.

Detection of siderophores

Siderophore production by PGPR strains was detected and described earlier by several authors. The assay was performed on King's B medium (Appendix 1) (King et al., 1954) plates and utilized the ternary complex chrome azurol S/iron (III)/hexadecyltrimethylammonium bromide as an indicator (Appendix 1). Colour changes in the dye from blue to orange indicate production of siderophores. PEM strains were spread plated on Kings B medium plates and were incubated at optimum growth temperatures (30°C for *Pantoea* and *Microbacterium* whereas 37°C for *Exiguobacterium*) for 2-6 days. The plates were observed for an orange colour on the Kings B plates which was scored as positive for siderophore production.

Solubilization of inorganic phosphate and acid production

PEM strains were evaluated for their ability to solubilize phosphate and for acid production on agar medium plates. For screening of PEM strains to solubilise $CaHPO_4$ agar medium was used as described by Will and Sylvia (1990) (Appendix 1). Purple medium containing a pH indicator, bromocresol purple, was used to investigate the production of acid by bacteria (Appendix 1). PEM strains were freshly cultured and 50µl of bacterial culture was placed on their respective medium plates to test for phosphate solubilisation or acid production. The plates were incubated at optimum growth temperatures for each of the strains up to 2-6 days and were observed for a visible zone of clearing. A hollow clear zone surrounding the growing bacterial culture, indicating $CaHPO_4$ solubilization and acid production on their respective plates.

Biosurfactant production

Biosurfactants are produced by a variety of microorganisms and are amphipathic molecules with hydrophilic and hydrophobic domains (Kuiper *et al.* 2004). Biosurfactants can accumulate at interfaces, can form micelles, and lower the surface tension which can enhance the solubility of poorly soluble compounds in water (Rosenberg and Ron 1999, Kuiper *et al.* 2004). In one study, *Pseudomonas putida* has been shown to have lipopeptide biosurfactant activity which inhibits biofilm formation and enhances breakdown of existing biofilms (Kuiper *et al.* 2004).

In order to assess whether PEM strains were able to reduce the surface tension between water and hydrophobic surfaces, the ability to collapse a droplet of water was tested (Jain *et al.* 1991, Kuiper *et al.* 2004). All the PEM strains and *Escherichia coli* were cultivated on LB agar and KB medium (Appendix 1) plates for 24-48 hours at 25°C. The grown bacterial cultures were scraped from the plates and resuspended in sterile water. The suspension was vortexed for 1-2 minutes and 25 µl suspension was pipetted as a droplet onto Parafilm M laboratory film (Pechiney Plastic Packaging). The flattening and spreading of the droplet was observed for drop collapse.

Markers of induced systemic resistance

Wheat plants were grown in the hydroponics pot system and inoculated with the PEM strains as per the method described in chapter 4. Treatments were uninoculated control (H), P, E, M and PEM combined. There were 6 plants in each pot and 4 replicates of each treatment. Wheat plants were harvested from the hydroponics system 12 days after bacterial inoculation. Root and shoot samples (1 g fresh weight) from each pot were collected and extracted for the determination of enzyme activities. Root and shoot samples were ground with 2 mL 50 mM potassium acetate buffer pH 5.0, containing 1 mM EDTA, 5 mM reduced glutathione and 1% w/w PVPP. Extracts were centrifuged for 20 min at 9000 g and supernatants were transferred to sterile microcentrifuge tubes and stored at -20°C until used as crude extracts in assays of enzyme activities.

A colorimetric assay was performed to determine chitinase activity in crude extracts as per Dann and Deverall (2000). The assay was based on the utilization of carboxyl-methyl chitin linked with the dye Remazol Brilliant Violet 5R (Wirth and Wolf 1990). After incubation with CM-chitin-RBV (manufactured by Loewe Biochemica, Sauerlach, Germany) together

with the crude extract the reaction was terminated by the addition of 2M HCl which precipitates the non degraded substrate (manufacturers manual). The absorbance of the supernatant containing degraded substrate was plotted as a function of incubation time to measure the enzyme activity.

Sodium acetate buffer (0.4 mL, 0.1 M, pH 5.0) and 0.1 mL of a suitably diluted extract were added to a microcentrifuge tube and allowed to equilibrate at 25°C for 10 min. The reaction was initiated by adding 0.2 mL CM-chitin-RBV (2 mg/mL solution) to the crude extract and was stopped after 10 min by adding 0.3 mL 2M HCl. Tubes were cooled on ice for 10 min. and then centrifuged at 9000 g for 5 min. The absorbance of the supernatant was measured at 550 nm and results were calculated as a change in optical density at 550 nm (ΔOD_{550}) per mg protein/min.

An assay for β-1,3 glucanase was performed accordng to the method of Dann and Deverall (2000). Azurine-crosslinked pachyman (AZCL-Pachyman. Megazyme, Ireland) was suspended at 0.03 mg/mL in double deionized water and mixed properly to make a homogeneous suspension. Sodium acetate buffer (0.4 mL, 0.1 M, pH 5.0) and 0.1 mL of a suitably diluted enzyme extract were added to a microcentrifuge tube and allowed to equilibrate at 25° C for 10 min. The reaction was initiated by adding 0.1 mL substrate suspension and was terminated after 10 min by adding 0.7 mL 20% w/v Tris. The tubes were vortexed to mix properly and incubated at room temperature for 10 min for the color development. The tubes were then centrifuged for 2 min. at 9000 g and the supernatants were transferred to 1 mL cuvettes to measure the absorbance at 610 nm. Results were calculated as a change in optical density at 610 nm (ΔOD_{610}) per mg protein/min.

The peroxidise activity of crude extract was determined by the method of Dann and Deverall (2000). The assay was based on the development of a pink/brown colour with the oxidation of guaiacol in the presence of hydrogen peroxide. 0.1 mL of crude extract was mixed with guaiacol (50 µl, 0.02 M), H_2O_2 (0.5 mL, 0.38 M) and sodium phosphate buffer (2 mL, 0.2 M, pH 5.8). The mixture was then transferred to a 3 mL cuvette and enzyme kinetics was measured at 470 nm by using a Cary UV spectrophotometer up to 60 s. Results were recorded as change in optical density at 470 nm (ΔOD_{470}) per mg protein/min.

The total root and shoot protein concentration was measured by using the Bradford dye-binding protein assay. The protein reagent was prepared by adding Coomassie Blue G250 (100 mg) in 95% ethanol (50 mL) and the mixture was then added to 85% phosphoric acid (100 mL) and made up to 1 L with distilled water. The reagent was then filtered through Whatman no. 1 filter paper and stored in an amber coloured bottle at room temperature. A 0.1 mL aliquot of 10 fold diluted crude extract was mixed with 5 mL of protein reagent and optical density was measured at 595 nm. Bovine serum albumin (BSA) was used to make a standard curve for protein concentration.

In vitro *antagonism of PEM strains against* R. solani

The dual-culture plate test was performed for screening the *in vitro* antagonistic effect of the PEM strains against the pathogen *Rhizoctonia solani* according to method described by Thomashow and Weller (1988). *Rhizoctonia solani* was cultured on PDA/4. A 0.5 cm-diameter agar plug was taken from the margin of the colony and was placed on the center of LB agar, YMM medium plates (Yeast extract-Malt extract –Glucose-Agar medium plates) and DF salt minimal medium plates (appendix 1). After two days of incubation at 25°C PEM strains overnight cultures (50 µl) were inoculated as spots onto the same pathogen inoculated LB, YMM and DF minimal medium plates at two locations, approximately 3 cm from the centre. All the PEM strains were inoculated separately and with the combination of two or three on the medium plates. A control plate with water (without PEM strains) was also inoculated. All the plates were incubated at 25°C and were observed for the zone of inhibition by measuring the distance between edges of fungal and bacterial colony up to 2-6 days.

Another experiment was conducted to test if there is volatile mediated antagonism between PEM strain and *R. solani* using the method described by Fernando *et al.* (2005). PEM strains were streaked onto one side of divided Petri plates containing LB and YMM medium. All the plates were wrapped with Parafilm and grown for 36 hrs at 30ºC and then a 8 mm *Rhizoctonia* agar plug was taken from the fresh PDA/4 plates and transferred to the other side of the plate. Plates streaked with the bacteria only or with the pathogen only were used as controls. The plates were observed for growth inhibition effect and growth of the fungus was compared with the control plate up to 2-6 days.

Statistical analysis

For experiments giving quantitative data, the effect of treatments was tested by ANOVA. Significantly different means were separated using Tukey's method.

5.3 Results

Production of IAA

PEM strains were assayed for their ability to produce IAA in the presence of the precursor tryptophan. Among all the PEM strains only *Pantoea* produced a red colour after adding Salkowski reagent in the assay, which indicated the positive reaction for IAA production. The absorbance at 540 nm of the reaction mixture also increased in the presence of tryptophan for *Exiguobacterium* and *Microbacterium* (Figure 5-1) but the reactions were brown rather than red in colour, indicating the presence of other indole compounds. Therefore IAA production could not be detected in *Exiguobacterium* and *Microbacterium.*

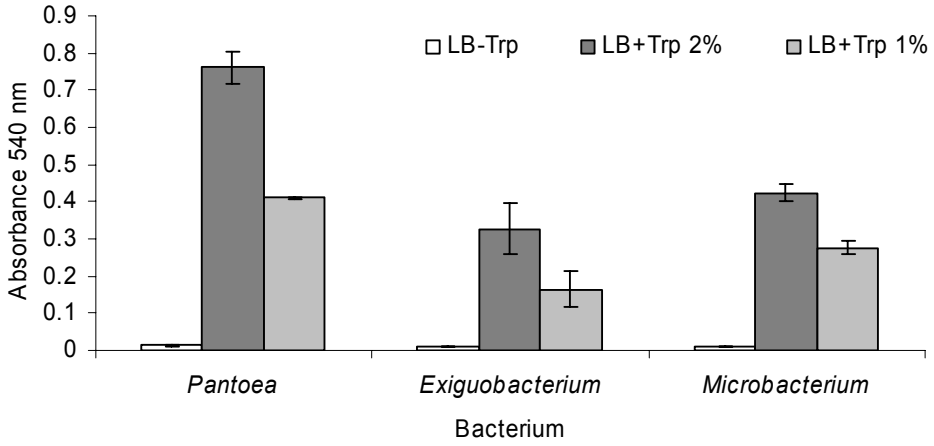


Figure 5-1. Production of IAA by PEM strains after induction with 0, 1 and 2% tryptophan. Error bars show standard errors (n = 3).

A total of 30 isolates of *Pantoea* from various sources were screened for their ability to produce IAA in the presence of tryptophan. In the presence of tryptophan, all but 2 isolates gave significantly higher production of IAA than isolate P3 without tryptophan, which was used as the negative control (Figure 5-2).

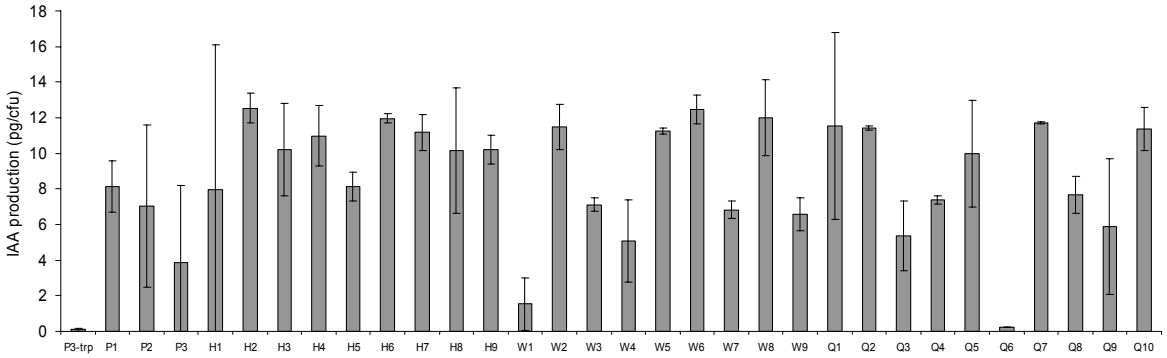


Figure 5-2. Production of IAA by different strains of *Pantoea* after induction with 300 µg /mL tryptophan. Error bars show standard deviation. Treaments were: P3-trp = *Pantoea* without tryptophan; Other codes= *Pantoea* isolates with tryptophan.

ACC deaminase activity

There were significant differences between PEM strains in ACC deaminase activity (Figure 5-3). *Pantoea* (23.1 nmol/mg/h) and *Microbacterium* (22.6 nmol/mg/h) each had significantly higher ACC deaminase activity compared to *Exiguobacterium* in two out of three repeats of the assay. As suggested by Penrose and Glick (2003), ACC deminase activity ≥ 20 n mol/mg/h is sufficient to permit a bacterium to grow on ACC and to act as a PGPR. The P and M strains in this study had ACC deaminase activity in this range.

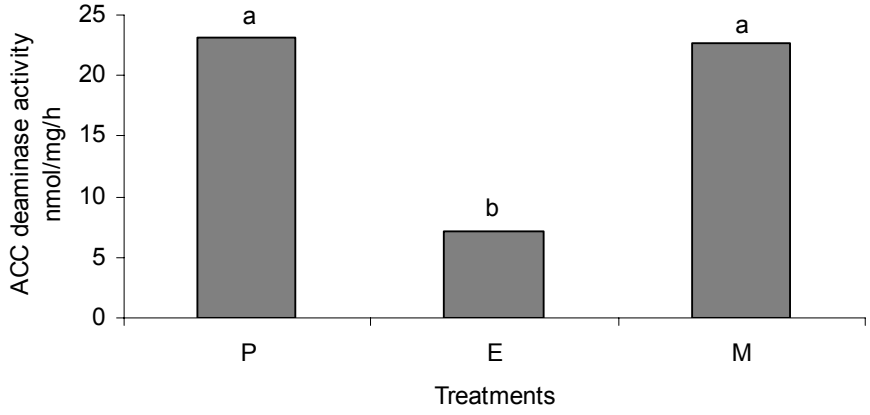


Figure 5-3. ACC deaminase activity of PEM strains. Treaments were: P = *Pantoea*, E = *Exiguobacterium*,M = *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

Amplification and sequencing of ACC deaminase genes

ACC deaminase genes of PEM strains were amplified using degenerate PCR primers. Among the bacteria only *Pantoea* strains showed the expected product of ~750bp (Figure 5-4). By using degenerate primers for the ACC deaminase gene amplified product, partial sequences were obtained after sequencing. The obtained sequences for the *Pantoea* strain showed most similar match with the ACC deaminase gene (A7604544) from *Enterobacter aerogenes* deposited by Hontzeas *et al.* (2005).

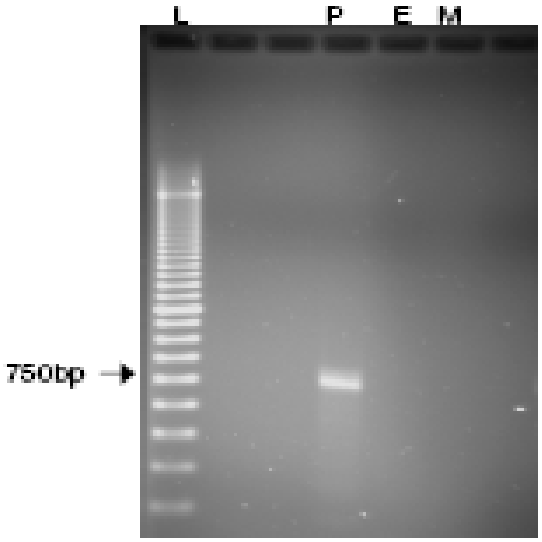


Figure 5-4. PCR amplified product of ACC deaminase gene in *Pantoea.* Lanes are; L: 1kb ladder; P: *Pantoea*; E: *Exiguobacterium*; M: *Microbacterium*

Nitrogen fixation (nifH) genes amplification

All the PEM strains were tested for amplification of *nifH* genes. The method of Zakhia *et al.* (2006) was performed with the *nifH* PCR primers *nifH* (for A) and *nifH* (rev.) and showed multiple amplification bands. The nested PCR amplification performed with the *nifH* (for B) and *nifH* (rev.) also showed several bands and none of them were the expected *nifH* fragment size. The second set of primers as described by Shrestha *et al.*(2007) showed several bands in the first PCR amplification but only one prominent band for the second PCR reaction which was the expected size of ~360bp. *Pantoea*, *Exiguobacterium*, *Microbacterium* and the positive control *Azospirillum* showed strong positive amplification for the *nifH* gene (Figure 5-5). Amplification of *nifH* was not detected in one of the two replicate extracts of

Microbacterium. I attempted to sequence the *nifH* gene but was unable to do this due to degenerate primers.

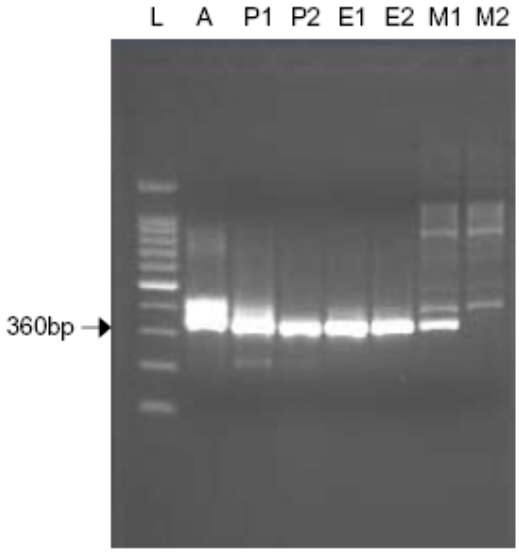


Figure 5-6. Nested PCR amplification of *nifH* genes from PEM strains and Azosprillum (positive control). Amplified product on agarose gel showing lanes are; L: 1kb ladder; P1, P2: *Pantoea*; E1, E2: *Exiguobacterium*; M1, M2: *Microbacterium*; A: *Azospirillum.*

Production of siderophores, acid production and ability to solubilise phosphate

There was no indication of phosphate solubilisation or siderophore production by PEM strains, as no strains showed zones of clearing around the colonies on their respective medium plates. PEM strains showed an indication of acid production on the purple medium which was higher in *Pantoea* compared to E and M (Table 5-1).

Table 5-1. Production of siderophores, acid and phosphate solubilisation

Metabolite Test	Medium	Activity		
		Pantoea	*Exiguobacterium*	*Microbacteria*
Phosphate solubilization	$CaHPO_4$ agar	No	No	No
Acid production	Bromocresol purple	++++	+	++
Siderophore production	Kings B	No	No	No

Collapse activity

Droplets of PEM strains did not show any flattening or spreading activity on Parafilm and were similar to those of *E. coli* or sterile KB medium used as a control. Therefore, no reduction of the surface tension was seen compared to the control. This revealed that compounds secreted by the bacteria were not able to reduce the surface tension of LB or KB culture medium.

Chitinase, peroxidase and β-1-3 glucanase activity

Chitinase, peroxidase and β-1-3 glucanase activity were measured in wheat seedling tissues as a marker for induced systemic resistance. There were no significant differences between treatments in chitinase activity in roots or shoots (Figure 5-7). Differences in glucanase activity could not detected in roots however, there was a significant effect of inoculation on glucanase activity in shoots. The glucanase activity was found to be significantly higher in *Pantoea* treated plants than the un-inoculated or combined PEM inoculated treatments (Figure 5-7). There were no significant differences in peroxidase activity of roots between treatments (Figure 5-7). Peroxidase activity in shoots of plants treated with *Pantoea* was significantly higher than the other bacterial inoculations.

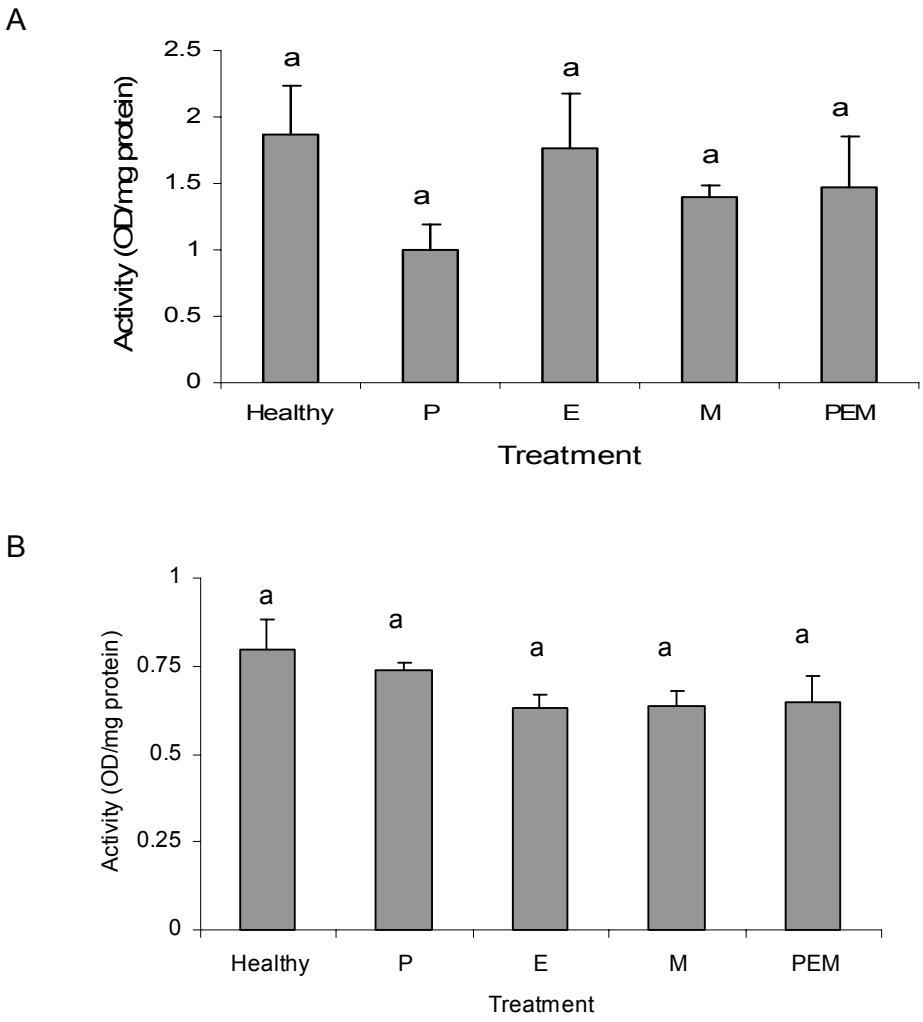


Figure 5-7. Chitinase activity in A) roots B) shoots of wheat plants grown hydroponically and inoculated with bacteria. Error bars show standard errors (n = 4). Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*,M = *Microbacterium*, PEM = *Pantoea, Exiguobacterium* and *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

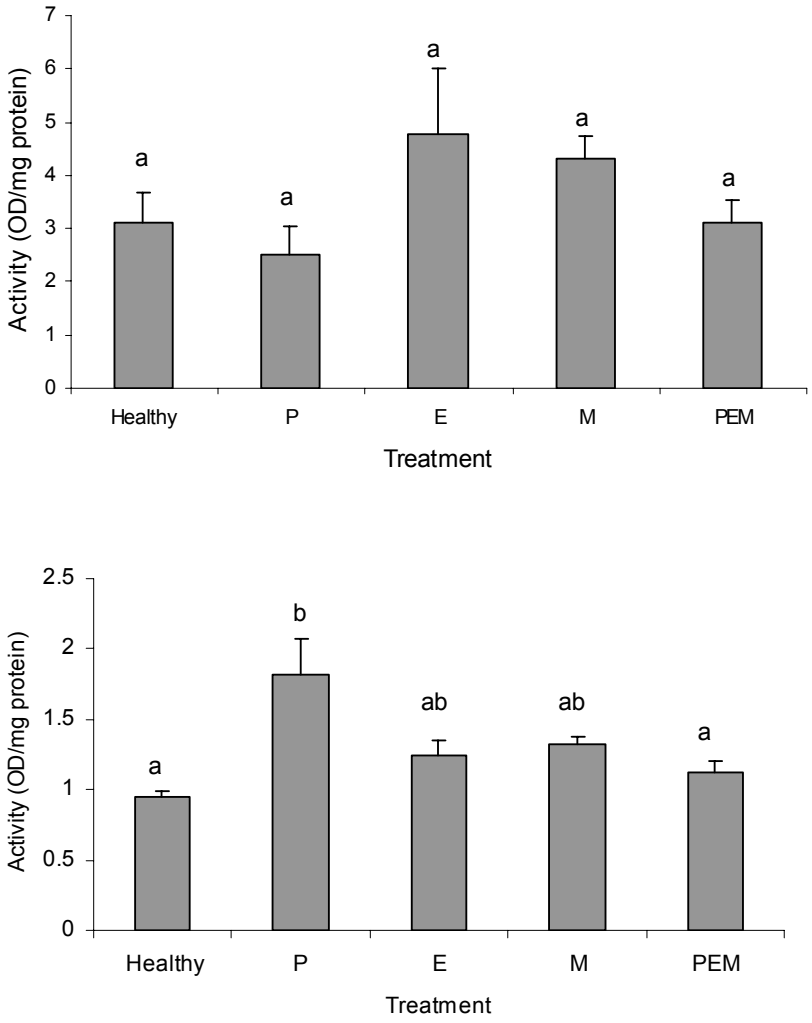


Figure 5-8. Glucanase activity in A) roots B) shoots of wheat plants grown hydroponically and inoculated with bacteria. Error bars show standard errors (n = 4). Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*,M = *Microbacterium*, PEM = *Pantoea, Exiguobacterium* and *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

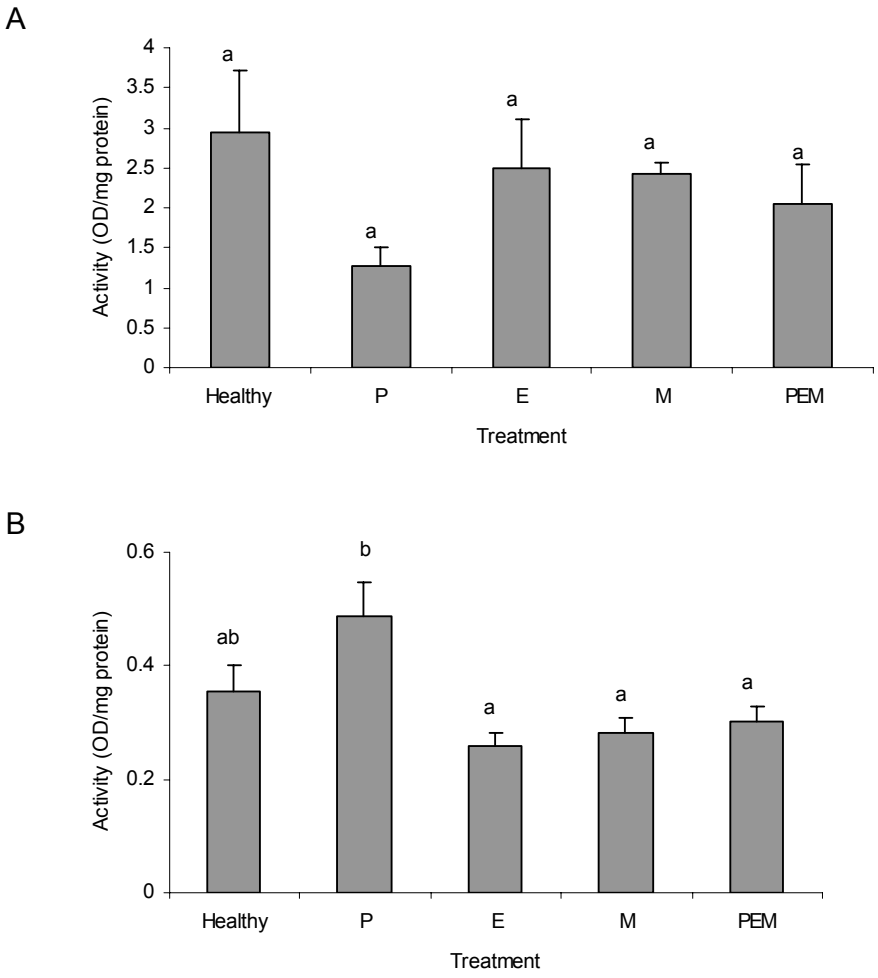


Figure 5-9 Peroxidase activity in A) roots B) shoots of wheat plants grown hydroponically and inoculated with bacteria. Error bars show standard errors (n = 4). Treaments were: H = healthy, P = *Pantoea*, E = *Exiguobacterium*,M = *Microbacterium*, PEM = *Pantoea, Exiguobacterium* and *Microbacterium*. Columns labelled with the same letter are not significantly different at $P < 0.05$ (Tukeys).

In vitro Antagonism among PEM strains and Rhizoctonia solani

PEM strains and *Rhizoctonia solani* both grew well on the media used for this study However, there were no signs of inhibition of fungal or bacterial growth on the YMM or LB or DF minimal medium plates. *Rhizoctonia solani* grew towards PEM strains and overlapped the growth of bacteria. In the experiment where volatile mediated antagonism was tested there was no inhibition of growth of either pathogen or PEM strains by volatiles produced by PEM.

5.4 Discussion

Microorganisms exhibit a variety of characteristics responsible for plant growth promotion or disease suppression which may be involved directly or indirectly (Whipps 2001). However, a bacterium can affect plant growth by one or more of these mechanisms and also can use different abilities for growth promotion at the different stages of the plant lifecycle. In the present investigation, PEM strains were evaluated for their possible possession of different PGPR traits with the aim to support the previous finding related to plant growth promotion.

IAA production has been reported for several PGPR in the family *Enterobacteriacae* including *Pantoea agglomerans* (Zimmer *et al.* 1994). The findings related to IAA production by P are in agreement with previous published studies. The activity assay was consistent in all the repeats and only *Pantoea* was shown to be positive for IAA production. Production of IAA has been reported to increase root growth, increase number of secondary roots and also to increase root hairs (Barbieri and Galli 1993, Steenhoudt and Vanderleyden 2000, Patten and Glick 2002). In the previous chapter, *Pantoea* was shown to have a significant effect on increased plant growth, with more root branching which could be explained by the above finding of IAA production. IAA might have contributed to the enhancd growth of P inoculated plants. Similar observations where PGPR enhanced plant growth was associated with the production of IAA have been extensively reported (Steenhoudt and Vanderleyden 2000, Verma *et al.* 2001) (Xie *et al.* 1996, Pal *et al.* 2001).

ACC deaminase has been reported in various plant growth promoting bacteria as well as some yeasts and fungi (Shah *et al.* 1998, Jia *et al.* 2000, Ma *et al.* 2003). Rhizobacteria attached to the surface of plant roots or seeds are able to utilize the ACC from plant exudates through the ACC deaminase action (Glick *et al.* 1998). Glick and his research group have studied the effect and activity of ACC deaminase for a long time and demonstrated the methodology to test for the presence of ACC deaminase in bacteria using PCR as well as by activity assay (Glick *et al.* 1998, Penrose and Glick 2003). For our study, initially we used the PCR method using degenerate primers to screen for the ACC deaminase gene in the PEM strains. Among PEM only P showed the expected size amplicon for the ACC deaminase gene whereas other two bacteria E and M did not give PCR amplification. When PCR product of the P strain was sequenced, it appeared that the sequence was homologous with the sequences of known ACC deaminase in the databases (Hontzeas *et al.* 2005).

Later, the activity assay method was followed to test for the presence of ACC deaminase among PEM strains. The assay was done according to the method described by the Glick group. Strains of P and M were shown to have an indication of ACC deaminase production in the activity assay. The experiment was repeated three times using the conditions published by Penrose and Glick (2003) but the results were inconsistent due to the lack of proper information in the published methodology. The Glick group is the only group which has done most of the ACC deaminase related work. Most of the experimental conditions are copied exactly in all the published reports, which are very confusing, incomplete and not easy to repeat. However, the results for the presence of ACC deaminase in P and M are in agreement with the previous finding related to plant growth promotion, where both strains were shown to have a significantly increased effect on root and shoot growth as well as increased root branching pattern (as demonstrated in the previous chapter). The failure to amplify the ACC deaminase gene in M may have been because the primers did not match the sequence in this species.

Another factor contributing to the growth promotion effect could be nitrogen fixation which has been demonstrated among several PGPR bacteria (Olivares *et al.* 1996, Minamisawa *et al.* 2004, Tejera *et al.* 2005). In the previous chapter plants treated with PEM demonstrated significantly increased growth under nitrogen deficient conditions and also showed an increased nitrogen uptake by PEM inoculated plants. The above finding suggested the possibility of nitrogen fixing ability by PEM strains, thereby increasing total nitrogen in PEM inoculated plants. To screen for nitrogen fixation ability of PEM, all the strains were tested by nested PCR using degenerate primers for *nif*H genes. The *nif*H amplification results obtained for P, E and M was another confirmation of our hypothesis related to nitrogen fixation. Nitrogen fixation is known for several strains of *P. agglomerans*. (Loiret *et al.* 2004, Verma *et al.* 2004) and has also been reported in *Microbacterium* (Zakhia *et al.* 2006) as well as species in the family *Bacillaceae* (Ding *et al.* 2005) to which *Exiguobacterium* belongs. The results obtained for nitrogen fixation ability of the PEM strains are in agreement with the previous published results. The PCR amplified product for *nif*H gene of the PEM strains were attempted to sequence but results were unsuccessful due to degenerate primers. Nitrogen fixation by PEM strains could be further confirmed by the acetylene reduction assay.

PEM strains were negative for some PGPR traits such as siderophore production or phosphate solubilisation in this study. The absence of these PGPR traits does not necessarily mean that

PEM are less effective as anatagonists if such traits are absent. It has already been discussed by several researcher that PGPR possesses a range of mechanisms to act as a successful biocontrol agents and require only one or two of these mechanism to work effectively.

The induction of systemic resistance was tested for by assaying activity of chitinase, glucanase and peroxidase in plants inoculated with the bacteria. The enzymatic investigation for the plants treated with PEM strains had no significant effect except for *Pantoea* treated plants which showed significantly increased ß-1,3-glucanase and peroxidase activity in the shoots. The absence of a response in the roots suggests that induced resistance is not likely to be an important component of the suppression of disease caused by *R. solani* in this study.

PEM had no antifungal activity against *Rhizoctonia solani* in dual culture. However, inhibitory effects are medium dependent factors which vary with nutrient composition of the medium. It has been reported that nutrient composition of the medium plays an important role in influencing the production of particular antifungal metabolites (Hebbar *et al.* 1992). It is therefore possible that some antagonism does occur in the rhizosphere, but that could not be detected by the methods used here.

In this chapter, some possible mechanisms for the growth promotion effect of PEM strains were investigated. The *Pantoea* strain demonstrated positive results for some of the PGPR traits such as IAA production, ACC deminase production, nitrogen fixation (*nif*H) etc, which have been discussed above separately. The presence of these characteristics for the P strain are in agreement with the findings of significantly increased root/ shoot growth, root branching pattern and significantly increased nitrogen content which has been demonstrated in the previous chapter. Plant inoculated with the other two bacteria E and M strain were shown to have an increased nitrogen uptake (as descibed in the previous chapter) and were detected for nitrogen fixation (*nif*H) ability. The P and M strains showed ACC deaminase activity. It has already been discussed (Penrose and Glick 2003) that organisms having potential to produce ACC deminase can have beneficial effect and act as PGPR. Further investigations are necessary to determine the ecological significance and some more biological control activity of PEM strains.

Chapter 6 Scanning Electron Microscopy of PEM on wheat roots

6.1 Introduction

Direct observation of fungi, protozoa, bacteria and other microorganisms has been studied for a long time by using different microscopic techniques. Use of the scanning electron microscope (SEM) for viewing bacterial cells and their attachment to surfaces such as plant roots or biofilm formation has been demonstrated often (Gray 1967, Dart 1971, Bowen and Rovira 1976, Foster and Rovira 1976, Hong *et al.* 1991, Fratesi *et al.* 2004). Interactions between bacteria and plants can be divided into different steps, most importantly are initial attraction, attachment, proliferation, and colonization e.g. of roots, leaves, stem and flowers (Bloemberg 2007). SEM can provide excellent resolution and therefore permits the localization of microorganisms in relation to root structure (Foster and Rovira 1976).

SEM has been widely used for bacterial localization especially to identify morphological features and colonization pattern on the plant roots. Gray (1967) used SEM to view soil organisms in situ and also demonstrated that the SEM technique is superior to other previous techniques due to greater magnification and less distortion of the specimen. Dart (1971) demonstrated the *Rhizobium* distribution on clover roots by using this technique. Colonization of tomato rhizosphere by the biocontrol agent *P. fluorescens* visualized by SEM was reported by Chin-A-Woeng *et al* (1998). Hong *et al.*(1991) showed the binding pattern of *Pseudomonas putida* GR12-2 to canola seeds, which stimulates root elongation under gnotobiotic conditions by using SEM. The presence of two endophytic strains *Bacillus pumilus* and *Corynebacterium* on rice plants has been observed and identified by SEM (Bacilio-Jiménez *et al.* 2001). A study conducted by Ruppel *et al.* (Ruppel *et al.* 1992) demonstrated the colonization ability of *Pantoea agglomerans* on various regions and tissues of the wheat plant using transmission electron microscopy.

The aim of this study was to investigate the localization of three bacteria (PEM) in relation to wheat roots by SEM. The beneficial effect of PEM can not be fully achieved unless we have information related to the fate and ecology of these microorganisms. The efficient exploitation of PEM in agriculture requires knowledge of traits including localization of these bacteria in

relation to roots that enhances ecological performance in the rhizosphere. Use of scanning electron microscopy is a powerful tool to study the interaction and localization of microorganisms in the rhizosphere. SEM was used to determine the ability of PEM bacterial cells to grow and survive on the surface of the plant tissue and also response of the bacterial cells to the plant.

6.2 Materials and Methods

Growing seedlings and bacterial inoculation

Surface sterilization of wheat seeds, bacterial culture condition and inoculation of bacterial cultures were performed as described in chapter 4. Surface sterilised seeds were pre-germinated on HMA plates. Three days old seedlings were dipped into 10^{6-7} cfu/mL bacterial culture (PEM strains separately) and transferred onto fresh HMA plates. The wheat seedlings were shaken to remove excess bacterial culture prior to transferring them onto HMA plates. All the experimental plates were incubated at 20°C temperature with 12 hours day/night cycle. Plants were harvested 10 days after PEM inoculation and roots were excised and processed for scanning electron microscopy.

Sample preparation and SEM of PEM inoculated wheat roots

The wheat roots were divided into three parts : branched, unbranched and root tip regions and each part was cut into small sections (2-3 cm). A standard glutaraldehyde fixation method was used for sample preparation. The root pieces were immersed in 2 % glutaraldehyde in sodium phosphate buffer 25 mM, pH 6.8. The root pieces were infiltrated with fixative in a vacuum desiccator using a water aspirator and then fixed overnight at room temperature. The root pieces were rinsed in two 10 min changes of 25 mM, pH 6.8 sodium phosphate buffer. Roots were then subjected to dehydration with acetone or alcohol and finally critical point dried before gold coating at 2.2 kv. Dehydration was done in a series of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and two changes of 100 % ethanol at 4ºC for 30 minutes each change. The prepared specimens were observed under the scanning electron microscope at the electron microscopy facility, UNE.

6.3 Results

Examination of wheat roots after inoculation with PEM strains revealed that there were not many differences in the structure and appearance of these strains on the root tissue. PEM

strains were detected all over the surface of the root tissue. Most importantly, all the strains were detected on the main root and lateral roots at the part of emergence of lateral roots and also a few cells were seen on the root tip region.

Appearance and distribution of the bacterial cells on the root surface

Pantoea cells appeared to adhere together (Figure 6-1A and B) and often had what looked like dried mucilage connecting them (Figure 6-1C). The *Pantoea* cells could be individual or form larger groups or clumps (Figure 6-1D).

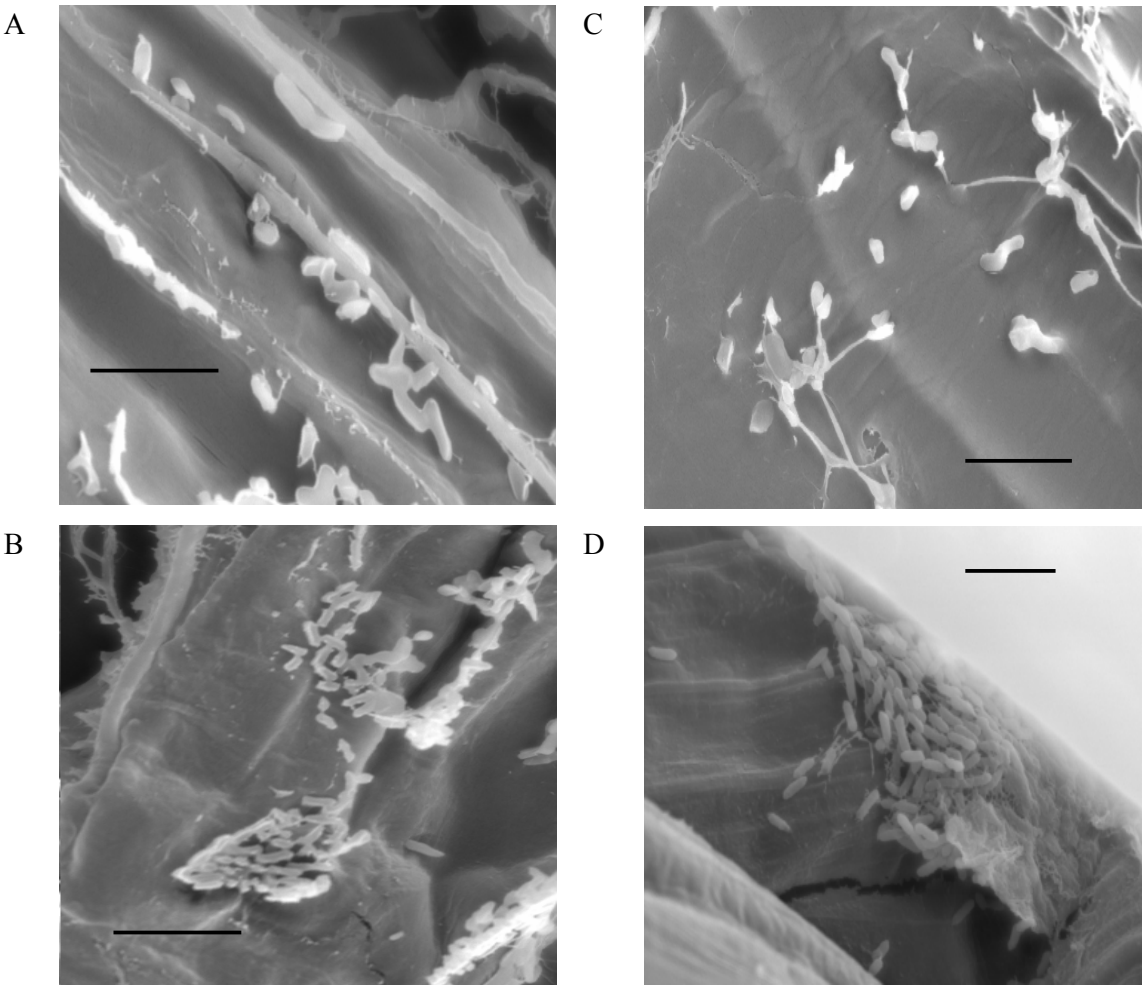


Figure 6-1. Scanning electron micrograph of *Pantoea* on wheat root. Scale bars = 5 µm.

Exiguobacterium cells appeared to be embedded in a mucilage or extracellular polysaccharide (Figure 6-2). These bacteria often occurred in clusters.

A B

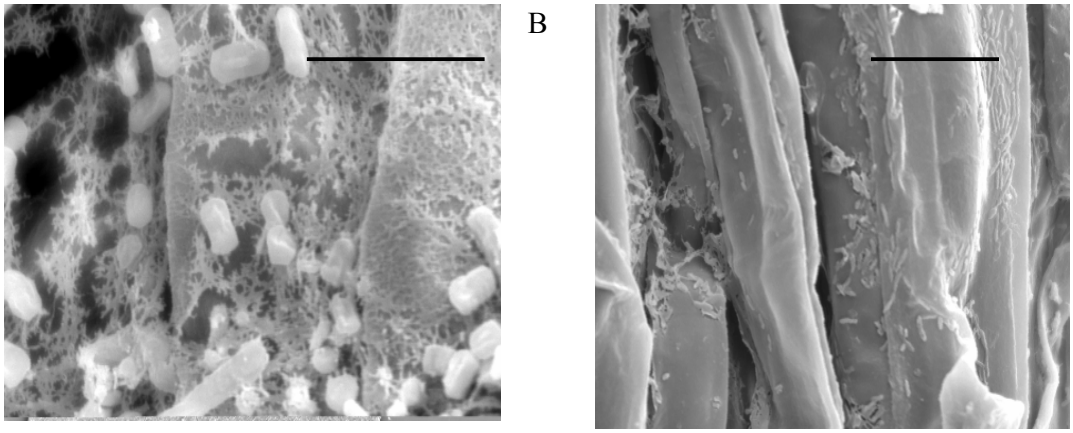


Figure 6-2. Scanning electron micrograph of *Exiguobacterium* on wheat root. Scale bars (A) = 5 µm; (B) = 10 µm.

Microbacterium cells appeared to be separate from each other and were not closely attached to the root surface (Figure 6-3).

A B

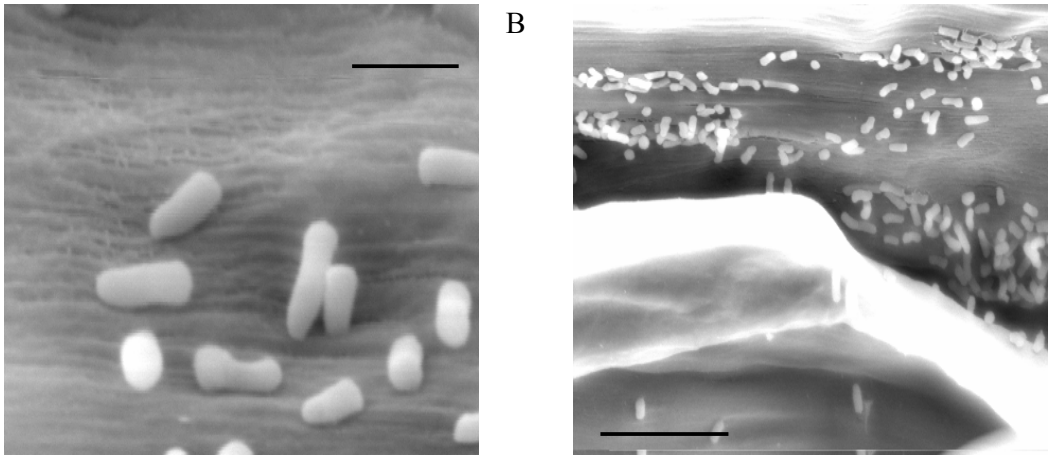


Figure 6-3. Scanning electron micrograph of *Microbacterium* on wheat root. Scale bars (A) = 1 µm; (B) = 5 µm.

6.4 Discussion

The aim of this study was to determine the location of PEM on the wheat roots. The study was important in a number of aspects. There are no previous studies of localization or attachment of this consortium of bacteria on wheat roots and only little is known about the interaction of PEM on the plant roots. Scanning electron microscopy (SEM) was used for the localization of PEM strains on the wheat roots. The result obtained from this study indicate that bacterial cells are present all over the surface of the different parts of the root. PEM strains were shown to have colonization ability as all the roots were grown up to 10 days after inoculation but all the bacteria were able to colonize the lateral branches as well as the main root axis. All the bacteria were found on all parts of the roots although they were lower in number near the root tip. Therefore, all of them were able to occur at potential sites of infection. However this artificial system condition could be different to soil. This investigation was also limited as it was not able to explain the interaction between bacteria because they can not be reliably distinguished from each other in SEM.

The bacterial strains *Pantoea*, and especially *Exiguobacterium* seem to produce EPS on the roots that may have an involvement in attachment. Remus *et al* (2000) saw EPS like material around *P. agglomerans* cells on wheat roots surfaces in SEM which are in agreement with the results obtained for *Pantoea* in this study. Barnett *et al.* (2006) also reported that *Pantoea* and *Exiguobacterium* were root associated, whereas *Microbacterium* was associated with the rhizosphere. There is evidence for EPS production by other PGPR strains of *Pantoea* (Amellal *et al.* 1998b). A strain of *Pantoea agglomerans* was detected on the surface of wheat roots using TEM (transmission electron microscopy) and appeared to be embedded in a gel on the root surface (Ruppel *et al.* 1992). No reports could be found for EPS production by *E. acetylicum*.

The advantage of the SEM was that this test allows examination of bacterial cells on the root tissue and also investigation of bacterial attachment on the root which is an important trait of naturally occurring bacterial strains in agricultural ecosystems. The presence of bacterial strains on the different parts of roots such as unbranched sections, lateral roots or root tips has been shown to have several beneficial effects on the plant as described by several authors (Wenzel *et al.* 1994, Mantelin and Touraine 2004). The present results that PEM strains can survive and can be detected on the root surface indicates that these strains are able to develop a close association with the plant tissue and may be able to multiply their population. This

offers the possibility of using these strains for successful plant-microbe interaction and biopreparations. As shown in the previous chapter of this thesis PEM strains have the potential to promote plant growth. Plant growth promotion and phytohormonal activity by *P. agglomerans* on wheat plants in relation to colonization ability has been already discussed by Ruppel *et al.* (1992) and Scholz-Seidel and Ruppel (1992). It can be concluded from the above results that the presence of PEM strains on the roots indicates the beneficial association between bacteria and the plant and this host-microbe relationship makes PEM a strong potential candidate for a biocontrol agent.

SEM can be further used for more detailed studies of the structure and spatial relationships of PEM strains in relation to roots. However, it has limitations. It is difficult to distinguish between bacteria in mixed inoculations, and it is difficult to quantify bacteria on the roots. Alternative methods to do this are explored in the next two chapters.

Chapter 7 Localization and colonization ability of *Pantoea* on wheat roots

7.1 Introduction

The microbial ecology of PGPR is important for understanding their interaction with plant species and for the development of biopreparations to apply in agriculture (Remus *et al.* 2000). Most of the beneficial PGPR are only effective in agriculture when they successfully colonize and persist in the plant rhizosphere (Lugtenberg *et al.* 2001). The ability to colonize the root surfaces and the closely adhering soil interface in the rhizosphere influences the growth promotion or disease suppression ability of these bacteria (Compant *et al.* 2005).

The detection of microorganisms in nature, particularly soil bacteria, has been severely limited due to the inability to culture many of these organisms and lack of visualization methodologies. Over the last few years, the use of marker genes, such as *gusA, lacZ, celB, xylA, luxAB,* has been become an important tool in studies on microbial ecology as they allow the simple visualisation of marked organisms (Sessitsch *et al.* 1996). The primary consideration in choosing a marker gene is the availability of assays for its detection. These must be simple, highly sensitive and, additionally, there should be little or no detectable background activity in either the microbes being studied or other components being assayed, such as soil or plant roots.

The *lacZ* gene, encoding β-galactosidase, has been used to study nodule infection by *Rhizobium* (Ba *et al.* 2002) and for root colonization studies in *Azospirillum* (Pereg Gerk *et al.* 2000). The *cel*B gene from *Pyrococcus furiosus,* which encodes a thermostable B-glucosidase, and the *Escherichia coli gus* A marker gene have been used for the detection of *Rhizobium* strains (Sessitsch *et al.* 1996). The marker genes can be introduced into the recipient bacterium as a direct insertion in the genome, through the use of transposable elements (de Lorenzo *et al.* 1990).

The application of Green Fluorescent Protein (GFP) technology has enhanced the ability to monitor microbial populations in natural environments, such as in biofilms or on plant roots (Unge *et al.* 1998) and is considered a useful tool to provide evidence for direct localization of bacterial populations (Gyaneshwar *et al.* 2001). The GFP protein is a 238 amino acid peptide

and has become an important visual marker of gene expression. The gene cassette encoding the GFP of *Aequorea victoria* can be used as a reporter for bacterial expression or localization (Chalfie *et al.* 1994). GFP is more sensitive than other reporter genes, requires no special factors for detection and also samples need not be disturbed by techniques such as fixing, washing, hybridization or staining (Chalfie *et al.* 1994, Stretton *et al.* 1998). GFP tagged cells can be visualized and tracked by a variety of methods developed for the detection of fluorescent cells, such as epifluorescence microscopy, confocal laser scanning microscopy (CLSM) or flow cytometry (Kremer *et al.* 1995, Unge *et al.* 1998). GFP with CLSM has become a very popular technique to study plant microbe interactions as samples can be easily analyzed without disturbing the plant structure (Chalfie *et al.* 1994). In several studies, GFP has been used to tag endophytes and study the location and life cycle of the endophytes *in planta* (Elbeltagy *et al.* 2001, Singh *et al.* 2004).

In this study, the *Pantoea* strain was labelled with a *lacZ* construct which allowed the examination of wheat root colonization after X-Gal staining. The *gfp* gene was also used as a fluorescent marker gene encoding the green fluorescent protein (GFP) to visualize *Pantoea* species. The expression of *Panoea* cells carrying the *gfp* cassette was investigated using fluorescence and confocal laser scanning microscopy and was found to be stable. Further, GFP-tagged *Pantoea* was investigated for its colonization ability and arrangement on the root surface.

7.2 Materials and Methods

Bacterial strains and plasmids

Rifampicin resistant PEM (PEMr) strains were cultured as described in chapter 3. A *kanamycin* resistant pLA *lacZ* plasmid which was maintained in *E. coli* S17-1 was received from Lily Pereg Gerk, UNE, Armidale. The pLA *lacZ* plasmid was used to tag the *Pantoea*. A *gfp* gene containing plasmid pDSK519np+2gfp which was maintained in *E. coli*-DH5α strain was used for the transformation of PEM strains to generate green fluorescent protein expressing strains. The npt2Po promoter was stably maintained in the plasmid pDSK519np+2gfp. It contains a *kanamycin resistant* gene for selection. Plasmid pDSK519np+2gfp and the helper *E. coli* strain pNJ5000 we kindly provided by Serina Stretton from the research group of Prof. Amanda Goodman, School of Biological Sciences, Flinders University, Adelaide, Australia.

Growth media and culture conditions

PEMr strains were streaked on LB agar plates from the glycerol stocks. Single colonies were picked and inoculated into 100 mL LB broth supplemented with rifampicin (5 μg/mL) and incubated at 30ºC over night. The *E. coli* S17-1 strain containing pLA *lac* Z was cultured in LB broth supplemented with kanamycin (20 μg/mL) or ampicillin (100 μg/mL) and incubated over night at 37ºC. The *E. coli* cells containing plasmid pDSK519np+2gfp which contains the *gfp* gene were grown at 37ºC on LB broth supplemented with kanamycin (20 μg/mL) and/or ampicillin (100 μg/mL). *E. coli* helper strain pNJ5000 was grown in LB broth at 37ºC supplemented with tetracycline (5 μg/mL).

General conjugation procedure

Conjugation is a method of gene transfer between donor and recipient strain. The donor strain has the capacity to mobilize the plasmid into the recipient strain. Helper strains were needed in transformation processes where *E. coli* strains lacked the transfer gene. Single fresh colonies of recipient and donor strains were grown in LB broth medium with suitable antibiotics overnight at optimum temperature and 180 rpm shaking. 1 mL of overnight grown culture was taken and centrifuged at 12000 rpm for 5 minutes and resuspended in 1 mL of fresh LB broth medium. Equal volumes of donor and recipient cells were mixed and placed onto the top of fresh LB agar medium in Petri dishes. Strains grown in media supplemented with antibiotics required an extra wash step with plain LB before the above step. A scrape of transconjugants culture grown on Petri dishes was taken and resuspended into 1 mL fresh LB broth and serially diluted cultures were plated on selective medium plates containing suitable antibiotics.

Transfer of lacZ *gene present in pLA* lacZ *plasmid into* Pantoea *strain*

Pantoea cells were labeled with the *lac*Z cassette by transforming with pLA *lac*Z plasmid maintained in *E. coli* S17.1. This is a stable plasmid which constitutively expresses the *lac*Z gene encoding for the β-galactosidase that can be effectively used to monitor *Pantoea* interactions with the wheat roots. β-galactosidase activity produces a blue color in the presence of a specific substrate. The recipient strain (*Pantoea*) and donor strain (*E. coli* S17.1 containing pLA *lac*Z) were grown in LB media containing suitable antibiotics and transformation was performed by using the general conjugation protocol as described above. The *Pantoea* transformants containing the *lac*Z cassette were selected on LB agar plates supplemented with kanamycin (20 μg/mL) and tetracycline (5 μg/mL). The selection of

transformants was also done on the basis of differences between *Pantoea* strains and the plasmid strains with respect to growth characteristics in pure culture on the medium plates.

The selected transconjugants were tested for the presence of the *lac*Z marker gene by cultivating on the LB medium supplemented with Tc, Km, X-Gal (40 mg/mL) and IPTG (10 mg/mL). Production of the blue colonies indicated the expression of the *lac*Z gene in selected transformants.

Detection of lacZ tagged Pantoea *on the wheat roots*

Wheat plants were grown in a hydroponics test tubes system. Sterilized wheat seeds were pre-germinated and were transferred to sterile 20 x 150 mm glass plant tubes containing 15 mL hydroponics solution as described by Zeman *et al* (1992). Three days old seedlings were inoculated with 0.1 mL of *lac*Z labeled *Pantoea* culture (~10^6 bacterial cells) pipetted into hydroponics solution and incubated for 10 days in controlled chamber at 25°C. The control plants were inoculated with untransformed wild type *Pantoea* strain.

To observe the colonization of the bacteria on the wheat roots, plants were removed from the hydroponics system 10 days after bacterial inoculation. The roots were cut into small sections of 1-2 cm length and placed into vials containing 2% glutaraldehyde in Z buffer (Na_2HPO_4 70 mM, Na_2HPO_4 30 mM, KCl 10 mM, $MgSO_4$ 1 mM pH 7.4). The vials containing the segments of the roots were placed under vacuum for 30 minutes and then incubated for another 60 minutes without vacuum at room temperature. The glutaraldehyde solution was discarded and samples were washed twice with Z buffer for 15 minutes. Each of the samples was then covered with the X-Gal solution (20 mg/mL X-Gal in dimethyl-formamide, 100 mM $K_4[Fe(CN)_6]$, 100 mM $K_3[Fe(CN)_6]$, 400µl Z buffer) and incubated for 24 hr at room temperature under dark conditions. The root samples were finally washed three times in Z buffer and twice in sterile distilled water for 5 minutes and examined under a light microscope.

Green Fluorescent Protein (GFP) was used as a reporter to label Pantoea

The plasmid p519ngfp containing the *gfp* gene under npt2 promoter was transformed into *Pantoea* rifampicin resistent strain using the general conjugation protocol as described above. *Pantoea* strain, *E. coli* cells containing p519ngfp and pNJ5000 helper *E. coli* strain were inoculated in liquid LB medium with suitable antibiotics kanamycin (20 µg/mL) and/or

ampicillin (100 μg/mL) and grown overnight at 30°C and 180 rpm. 1 mL of overnight culture was centrifuged at 12000 rpm and supernatant was removed. The pellets were washed with 1 mL of fresh LB and finally resuspended in 1 mL fresh LB without antibiotics. 50 μl of the p519ngfp culture were inoculated as a spot on the centre of the LB agar medium plate containing no antibiotics. After this dried, 50 μl of *Pantoea* culture was inoculated on the top of the p519ngfp spot and after this second culture dried 50 μl culture of helper strain pNJ5000 was inoculated onto the spot and plates were incubated overnight at 30°C. Transconjugants were plated on LB medium plates containing kanamycin (20 μg/mL) and rifampicin (5 μg/mL) Transformants were selected for their ability to grow on selective antibiotics medium plates.

Visualization of GFP tagged Pantoea

Epi-fluorescence detection of fluorescent colonies

GFP labeled *Pantoea* (P-GFP) were screened for the expression of green fluorescent protein. Single fresh isolated colonies of the transformed strain of *Pantoea* and wild type strain were grown in 5 mL LB broth supplemented with kanamycin (20 μg/mL) and rifampcin (5 μg/mL) respectively, after incubating overnight at 30ºC with shaking at 180 rpm. A drop of overnight liquid culture was placed on a microscope slide and spread on the slide. GFP expression was detected using an Olympus BH-2 fluorescent microscope, equipped with a blue excitation filter set (IF-490 excitation filter and O-515 barrier filter) at 10-40x objectives. Digital images were obtained by using a Nikon DS-5M camera fitted to the epifluoroscent microscope.

Investigation of the attachment of GFP labeled Pantoea *to the wheat roots*

Wheat plants were grown in a Petri dish hydroponics system as described in Chapter 3. The P-GFP strain was cultured in LB broth with suitable antibiotics and shaking (180 rpm) at 30ºC. 1 mL of overnight culture was inoculated in fresh LB medium and grown up to A_{600} ~0.8. The bacterial concentration was adjusted to $1x10^7$ cells/mL by dilution and inoculated to the wheat plants on the hydroponics medium plates. Wheat plants inoculated with water (uninoculated healthy) and with wild type *Pantoea* strain were used as controls. The wheat plant growing system was transferred to controlled environment under 12 hrs of light/dark cycle at 25ºC. Roots were excised from the plants 10 days after inoculation and visualized under the epifluorescent microscope at suitable magnification. The structure of root tissue was also visualized for changes under epifluorescence microscope by their autoflorescence. P-GFP

expression on the wheat root was evaluated with epifluorescence microscope and pictures were recorded by using a Nikon digital microscope camera.

Examination of the interaction of PEM with the wheat roots in relation to pathogen

The GFP labeled *Pantoea* were used to study the interaction with other bacterium EM and pathogen on the wheat roots. The root colonization by PEM on wheat roots was studied using the hydroponics Petri plates as described above. The P3-GFP and EM strains were cultured at 30ºC with 180 rpm shaking and were directly inoculated to wheat roots to examine the interaction with the plant. Pathogen *R. solani* culture from the PDA/4 plate was sliced into long and thin sections of 5 mm by 1 mm and one slice was placed to the hydroponics Petri plates medium, adjacent to roots wherever pathogen was involved in the treatment. The roots were excised 10 days after the bacterial inoculation. Epifluorescence microscopy was used to obtain images of the colonization patterns.

Use of DAPI stain for microscopic observation of EM strains

The DAPI (4',6-diamidino-2-phenylindole) stain was used to investigate the unlabeled strains *Exiguobacterium* and *Microbacterium* attachment to the root surface (Takanashi *et al.* 2006). To study the root colonization by EM strains on the wheat roots, hydroponics Petri plates method were used as described above. The EM bacteria inoculated wheat roots were excised 10 days after bacterial inoculation and cut into small sections (2-3 cm). Each root sample was then dipped into 1 mL DAPI solution (2 μg/mL) for 5 min. and observed under the microscope. The DAPI counter stain was also directly pippetted to the pure bacterial culture and plant tissue as a control treatment to observe them under the fluorescent microscope.

Epi-fluorescence and Confocal laser scanning microscopy

The excised root samples of 6-9 plants were taken for the localization of PEM under the microscope. Roots were visualized under the epi-fluorescent microscope (Olympus BH-2) at different magnification and fluorescence was detected using 480nm excitation filter and 515nm long pass emission filter. Digital images were obtained using a Nikon CCD camera fitted to the epi-fluorescence microscope.

A Nikon Z-C1 confocal laser scanning microscope equipped with three laser sources (excitation wavelength 408, 488 and 514 nm) was used to obtain images of P-GFP on the wheat roots. Different location of roots were scanned and analyzed. The bacterial attachment

on the surface and inside the root junctions were investigated using 408 and 488nm wavelength.

Detection of Pantoea fluorescence in soil system

A preliminary experiment was designed to determine the soil auto-fluorescence and the number of *Pantoea* cells required to detect or to differentiate the bacterial fluorescence under the microscope. P-GFP culture was grown in liquid LB medium and 2 g of sterile Avon soil (50% soil + 50% sand) was inoculated with 1 mL of diluted bacterial culture 10^5, 10^6, 10^7, 10^8 or 10^9 cfu/mL. The soil was incubated at room temperature for 2-4 hours. The soil was then suspended in 2 mL of sterile water and mixed properly by vortexing. A drop of the supernatant was placed onto a microscopic slide and examined under the epi-fluorescent microscope and the auto fluorescent particles were visualized and compared with the GFP expressing bacteria.

7.3 Results

Labeling of Pantoea with lac*Z to study the colonization pattern of wheat roots*

A standard conjugation method was used to label *Pantoea* with the *lacZ* marker. The expression of *lac* Z was confirmed by X-Gal blue white colony screening. Blue colonies were selected as positive for X-Gal expression and treated as successful transformants. The white colonies were treated as unsuccessful transformants for *lacZ* expression. Plant roots were observed under the microscope in order to localize the colonization by *Pantoea* on the root surface. All the roots became blue after X-Gal staining. The distribution of *Pantoea* population on the root was observed under the microscope and color blue was very intense suggesting a high level of colonization. *Pantoea* was observed to be colonized all over the surface of wheat root and root hairs. In Figure 7-2, *Pantoea* colonization can be seen. There was no color detected on the plants inoculated with wild type *Pantoea*.

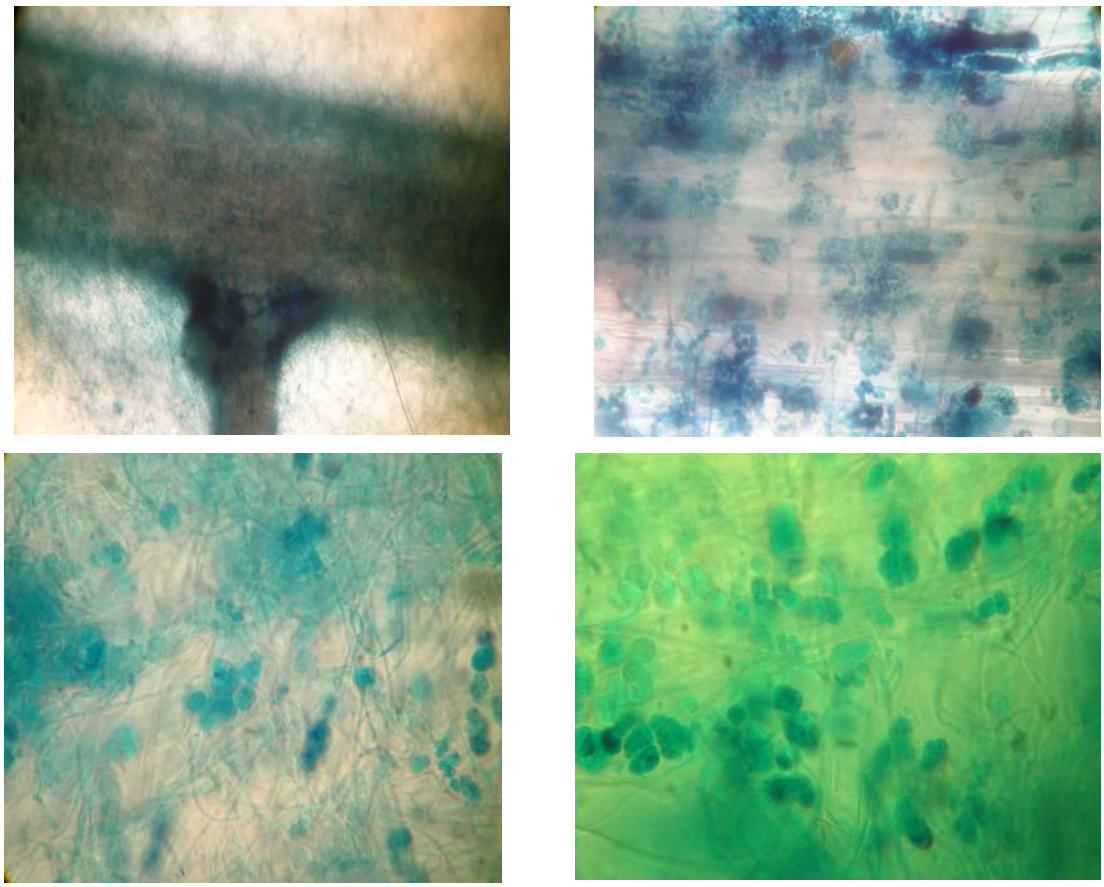


Figure 7-1. Colonization of wheat roots with *lac*Z labelled *Pantoea* observed with microscope. The blue colonies indicated the colonization on root surface and root hairs.

Visualization of GFP tagged Pantoea

Pantoea was successfully transformed with the GFP expressing gene, whereas Gram positive strains *Exiguobacterium* and *Microbacterium* were not. Transformation protocols were tried to transform both the Gram positive strains but results were unsuccessful (Appendix 2). The *Exiguobacterium* and *Microbacterium* bacteria could not be transformed with the protocol used for *Pantoea,* which was a standard protocol for Gram negative bacteria. Moreover, the promoter placed in the plasmid is only suitable for Gram negative bacteria (*Pantoea*) and it may not work in Gram positive bacteria such as *Exiguobacterium* and *Microbacterium*.

Epi fluorescence detection of fluorescent colonies

The GFP-transformed *Pantoea* strain showed strong GFP expression when visualized under a epifluorescence microscope in comparison to the control wild type *Pantoea* strain, which displayed no fluorescence. *Pantoea* cells carrying the *gfp*-expression cassette showed stable expression of the GFP fluorescence (Figure 7-2). The *gfp*-expression of *Pantoea* cells allowed visualization of the localization and interactions of *Pantoea* in the rhizosphere.

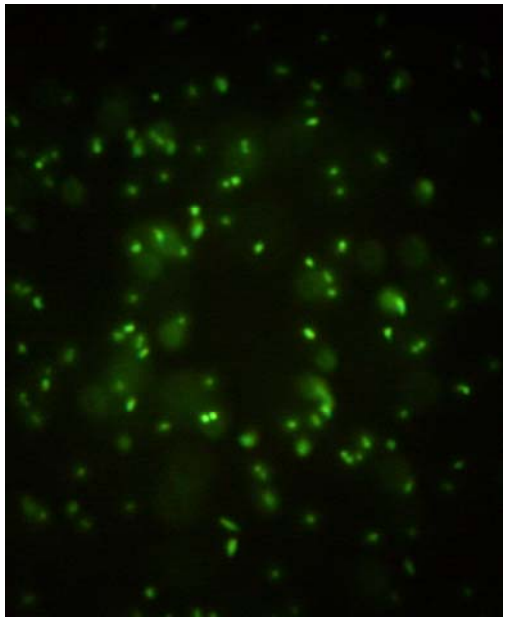


Figure 7-2. Gfp tagged *Pantoea* cells fluoresces green fluorescent colonies under epiflorescence microscope excited at 488nm and emission at 512nm.

Pantoea *root colonization studies*

Epi-fluorescent microscopy

The wheat root tissue showed yellow auto fluorescence (Figure 7-3) however, green florescence from GFP-tagged *Pantoea* cells was easily detected on the root under epifluorescence microscopy. The presence of *gfp*-tagged *Pantoea* colonization was detected all over the surface of wheat roots. *Pantoea* cells were often present on the junction of the cell wall. The GFP labeled *Pantoea* were found all over the surface of the root however, distribution of *Pantoea* cells varied along the root surface (Figure 7-4).

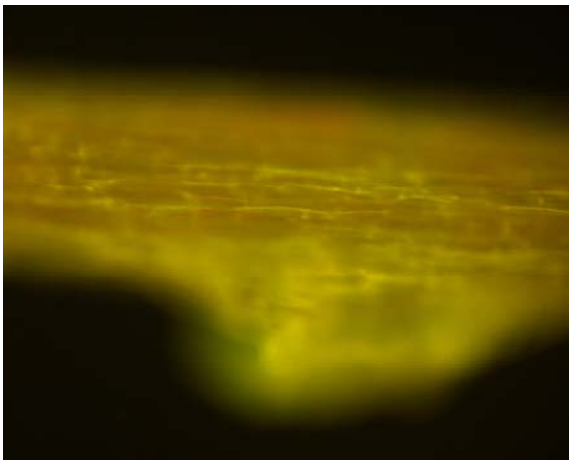


Figure 7-3. Wheat auto-fluorescence under epi-fluorescent microscope

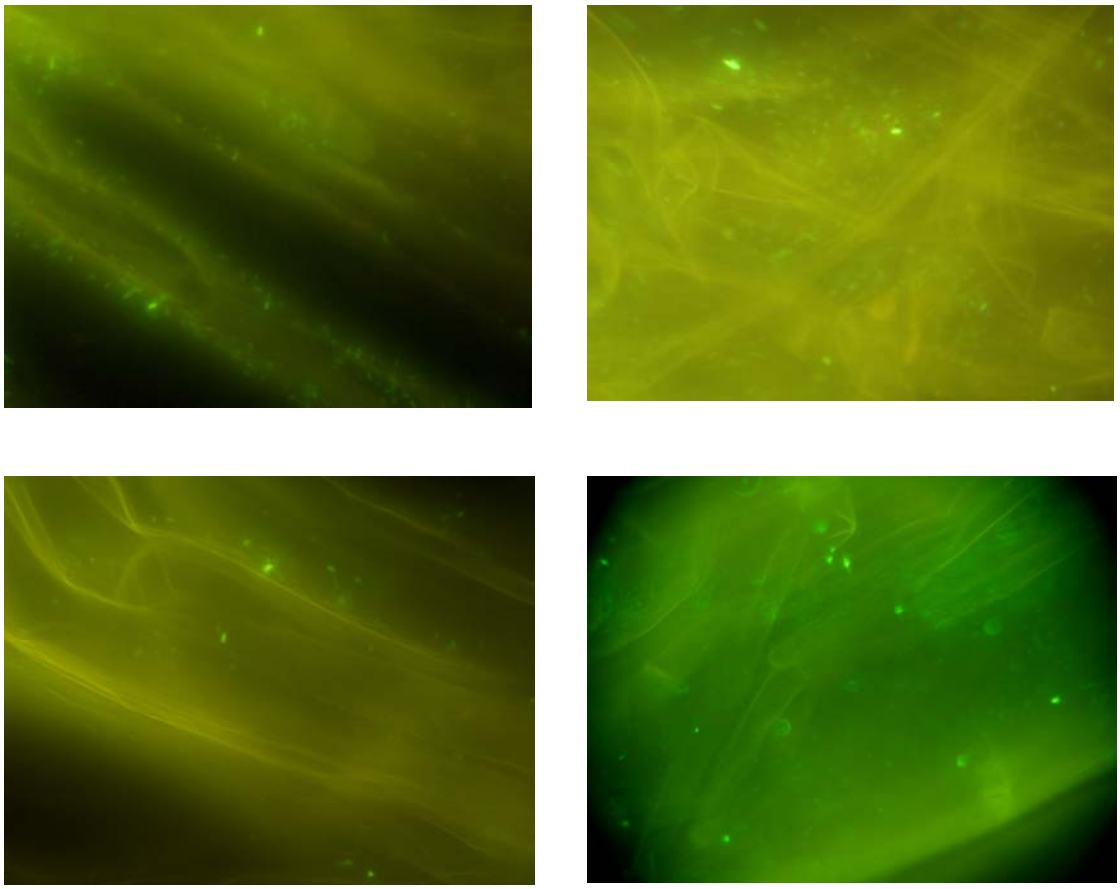


Figure 7-4. GFP tagged *Pantoea* cells detected by epi-flouroscence microscope and were present all over the root surface.

Confocal laser scanning microscopy of p-GFP

The distribution of *Pantoea* population on the washed roots was observed under the microscope and it was observed that cells colonized all over the surface of wheat root and also on the junctions of the root cells (Figure 7-5)

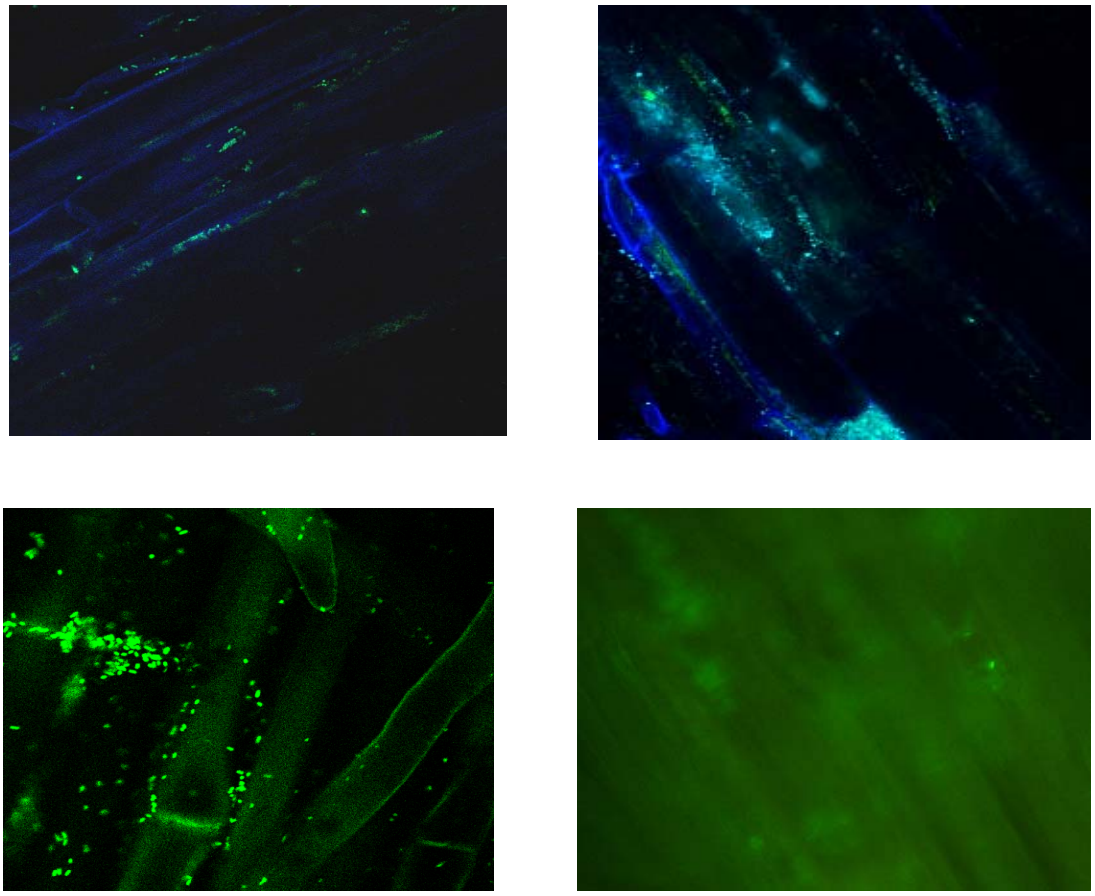


Figure 7-5. GFP tagged *Pantoea* cells detected by CLSM and *Pantoea* were present all over the root surface especially on wall junctions.

Interaction of gfp-tagged Pantoea *in presence of EM and Pathogen*

GFP labeled *Pantoea* (P-GFP) inoculated with EM and/or pathogen did not show any significant difference in localization from the P-GFP alone. Only P-GFP was detected on the wheat roots inoculated with P-GFP and EM, whereas the other two bacteria (E and M) could not detected under the epi-fluorescence microscope. The colonization pattern of *Pantoea* was the same as described above. The plants inoculated with PEM and pathogen also did not show any differences in the interaction or colonization behavior of P-GFP strain.

Localization of EM on the wheat roots

The nucleic acid based stain DAPI was used to counter stain the *Exiguobacterium* and *Microbacterium*. Both bacterial strains were detectable in pure culture using DAPI but blue background fluoroscence on the root made it difficult to localize them on the root surface (Figure 7-6).

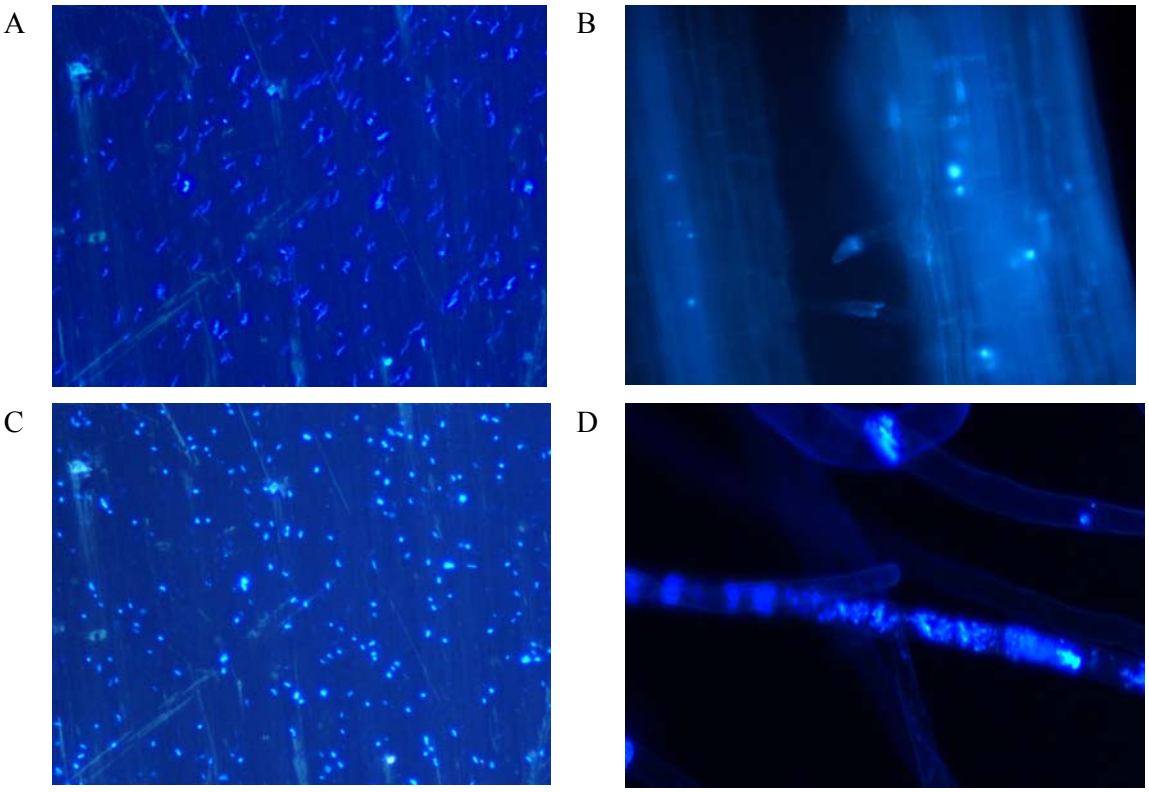


Figure 7-6. DAPI stained bacteria. A) *Exiguobacterium* in culture B) *Exiguobacterium* on the wheat root, C) *Microbacterium* in culture D) *Microbacterium* on the wheat root

Fluorescence of GFP labeled Pantoea in soil

The soil has auto-florescence but GFP-tagged *Pantoea* were easily detected in soil extracts by epi-fluorescence microscopy. It was found that 10^6 -10^5 cfu/mL bacterial cells could be easily detected and distinguished from the soil. Bacterial and soil fluorescence was easily differentiated from each other in the soil mixture used (Figure 7-7). Hence the GFP expressing bacteria could be used to estimate bacterial plant interaction in soil as the GFP fluorescence can be distinguished from the soil background fluorescence.

A B

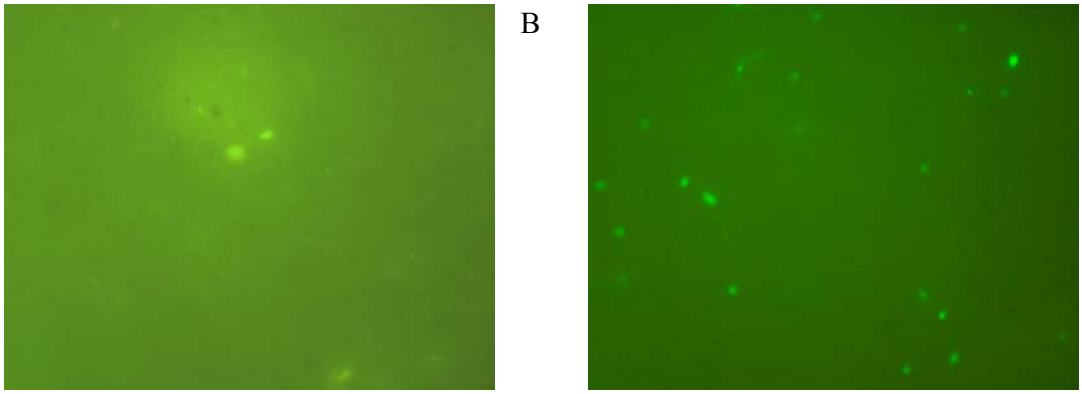


Figure 7-7. A) Soil auto fluorescence and B) *Pantoea* cells fluorescing in soil mixture.

7.4 Discussion

As reviewed by Bloemberg (2007), microscopic analysis to detect the PGPR in their natural environment and in specific interactions with the host plant is important for elucidation of their functioning and also to use as a successful bio-inoculants. There are no reports about the colonization pattern or location of the PEM strains on the wheat roots. Therefore, it is important to know which part of the roots PEM strains are attaching to for effective disease suppression ability against *Rhizoctonia solani*. This study investigated the colonization behavior of PEM strains on the wheat roots to determine their behavior in the plant rhizosphere by detection of PEM strains using electron microscopy (previous chapter), followed by the use of methods based on reporter genes, such as *lac*Z and GFP.

Pantoea colonization on the wheat roots was monitored using molecular markers and qualitative detection. The Gram positive EM strains were not successfully labeled with molecular markers. This failure could be explained by the fact that E and M are Gram positive bacteria, whereas the transformation protocol and plasmid used in this study were designed for Gram negative bacteria. However the Gram negative *Pantoea* strain was successfully labeled with *lac* Z and the GFP marker. Alternatively, the nucleic acid based stain DAPI was used to counter stain for *Exiguobacterium* and *Microbacterium*.

In the present study, *Pantoea* was labeled with the *lac*Z gene, encoding β-galactosidase and root colonization was studied. The *lac*Z gene has been used to study nodule infection by *Rhizobium* (Bovin et al., 1990) and for studies of root colonization in *Azospirillum* (Katupitiya *et al.* 1995). By using the labeled *Pantoea*, which is constitutively expressing the

*lac*Z genes, the *Pantoea* associations with plants roots were investigated. The *lac*Z labeled *Pantoea* inoculated in hydroponics solution showed colonization on the root surface, especially in the root elongation area and also on the base of the lateral roots. This observation was easily detected by X-gal blue staining of bacteria containing a constitutively expressing *lac*Z fusion. A similar study, using *lacZ* were conducted for *Azospirillum* by (Kapulnik *et al.* 1987). Although *lacZ* demonstrated the colonization ability of *Pantoea* on the wheat roots it has some limitations such as samples needing large amounts of time for fixing and staining and the need for extra substrate for visualization.

GFP is a useful tool for monitoring gene expression and protein localization (Chalfie *et al.* 1994), bacterial localization on the plant root (Coombs and Franco 2003) and virus movement in plant systems (Baulcombe and Cruz 1995). The advantages of using GFP as a marker include easy detection and no exogenous substrate or energy source or processing of cells is required to monitor individual cells (Lawrence *et al.* 1991). Light and electron microscopy requires fixing or staining which can dehydrate or agitate biological samples or can produce artifacts during sample preparation. In this study, *Pantoea* was successfully transformed with a plasmid which constitutively expresses GFP. *Pantoea* was not easily discerned with conventional light microscopy, and other microscopic methods which involve fixation or staining to resolve the bacteria. The ability to easily visualize individual *Pantoea* cells by using GFP made it reasonably easy to localize the attachment on the root surface. The result clearly indicated the colonization of *Pantoea* all over the surface of wheat roots. It also showed that *Pantoea* cells were present on the junctions of the cell wall. A similar study was conducted by Verma *et al.* (2004), where a *Pantoea* strain showed endophytic colonization ability in deep water rice. The use of GFP and related proteins to study bacterium-plant interactions summarises the results of several studies by demonstrating that PGPR preferentially colonize the junctions between cells on the root surface (Bloemberg 2007). The results obtained from this study will assist in the successful application of PEM strains to enhance crop productivity and also to assess the fate of these bacteria in the field, since the released strains are mostly not distinguishable from the indigenous bacterial populations in soil. The results for *Pantoea* in this study are in agreement with other PGPR colonization studies. However, good evidence for endophytic nature of *Pantoea* was not obtained and it could not be detected on internal junctions of root tissue. The reason may be explained due to technical problems. Cross-sections of roots may need to be examined to detect endophytic growth.

The colonization ability of *Pantoea* on wheat roots was also investigated by confocal laser scanning microscopy (CLSM). The advantage of CLSM is that it allows study of the colonization patterns on different segments of the roots without any additional need for staining and sectioning (Bloemberg *et al.* 1997, Verma *et al.* 2004). The epi-fluorescence is simple and easy to detect the bacterial fluorescence. However, CLSM provides higher resolution and sharper pictures of the object. In this study, *Pantoea* attachment were investigated using both microscopes and it was found that CLSM has some advantages over epi- fluorescence.

The interaction between PEM strains in the presence or absence of pathogen was investigated by using the nucleic acid based stain DAPI, which allows the EM strains to be detectable under the microscope. The cells adhering to the surface of the root into presence and absence of the pathogen were observed but a problem occurred with DAPI in that there was a strong blue background color detected on the roots. The results were not convincing enough to interpret results for bacterial interaction and did not allow visualization of the EM populations present on the root surface.

In general, this study demonstrates, for the first time, the visualization of GFP tagged *Pantoea* on the wheat roots. Bacterial colonization of the outer surface of sugar beet and cotton seeds has been studies by using scanning electron microscopy, conventional microscopy and confocal microscopy with conventional staining techniques (Fukui *et al.* 1994, Hood *et al.* 1998). The attachment of PEM strains to the roots shown by scanning electron microscopy (in previous chapter) was also confirmed by molecular based marker *lacZ* and GFP. For *Pantoea*, this study shows that GFP and *lac Z* marker can be successfully used to assess the colonization ability of *Pantoea*.

Further work is needed to label the other two bacteria (*Exiguobacterium* and *Microbacterium*) and localize them on the roots. It may be necessary to assess the interaction of disease suppressive consortium PEM using fluorescent labeled EM strains. The labeling of Gram positive strains (*Exiguobacterium* and *Microbacterium*) with the fluorescent marker by finding a suitable method will provide important information related to bacterial interaction. It is recommended that use of different color markers such as RFP (Red fluorescent protein) or YFP (Yellow fluorescent protein) will be useful. Another suggestion is to use the FISH

(Fluorescent *In Situ* Hybridization) method based on species specific probes which could be an useful technique to investigate the interaction. Watt *et al.* (2006), used the FISH method and demonstrated that root caps are most heavily colonised by bacteria, and elongation zones (just behind the root cap) least colonised. Some of these PGPR can also enter the interior part of the root and establish endophytic colonization which reflects the ability of bacteria to selectively adapt to these specific ecological niches (2008). Therefore, it would be worthwhile investigating the endophytic colonization ability of PEM strains after labeling them with fluorescent markers.

As suggested by Watt *et al.* (2006), the high spatial variability of bacterial colonization makes FISH and other microscopic methods of limited use for studying larger effects of treatments on the rhizosphere or root system. They suggested that the limitations of microscopic methods allow only detection of a very small section of the root. GFP could be very useful for studying small-scale features of interactions. However, for larger-scale effects on bacterial populations there is need to use different approaches for quantification of bacterial population such as use of real-time PCR amplification.

This chapter provides new evidence of the colonization pattern of *Pantoea* on wheat roots. The results indicate that *Pantoea* can readily colonize the most of the root surface and also on the junctions between cells, making it a suitable candidate for development as a bio-control agent. Further work is needed for *Exiguobacterium* and *Microbacterium* to confirm their localization and interaction with wheat roots.

Chapter 8 Quantification of PEM populations on roots

8.1 Introduction

Successful application of plant beneficial bacteria as biocontrol agents requires knowledge about their fate and activity in the rhizosphere (Gamalero *et al.* 2003). Several microbiological and molecular methods allow the detection and quantification of bacterial populations from different parts of the plant such as roots or leaves. The traditional method was based on culturing to enumerate bacterial populations. Later, serological tests such as ELISA and immunofluorescence were developed for the identification and detection of bacteria. These techniques for the enumeration of bacterial populations were popular for a long time but they have certain limitations, are time consuming and have chances for false positive reactions (Lin and Stahl 1997) (Gamalero *et al.* 2003, Salm and Geider 2004a)

Current approaches using PCR and real-time PCR methods based on species-specific primers are sensitive and accurate for the identification and detection of microorganisms from different samples. Use of conventional PCR is very popular for the detection and screening of microorganisms However, it can give only qualitative data. Real-time PCR using species-specific primers is an advanced and effective method for quantification and detection purposes. Real-time PCR is a highly sensitive method, which has been used for quantification and enumeration of bacterial species from environmental samples based on the 16S rDNA gene as a target or using functional markers (Gruntzig *et al.* 2001, Hermansson and Lindgren 2001, Stults *et al.* 2001, Becker *et al.* 2002, Stubner 2002, Qiu *et al.* 2004).

PEM vary in their population distribution after interaction with the pathogen, as shown by (Barnett *et al.* 2006). *Pantoea* and *Exiguobacterium* are known to promote the growth of infected plants (Barnett *et al.* 2005, Barnett *et al.* 2006). Part of this is much reduced secondary infection from the primary infection site possibly by competition for plant exudates from the infection site, preventing *Rhizoctonia* producing secondary infections (Barnett *et al.* 2005, Barnett *et al.* 2006). Root lesions may be an important niche for suppression of Rhizoctonia disease on wheat by PEM bacteria. It is not known if PEM colonise infection sites or are preferentially selected for on infection sites.

In this study, populations of PEM strains on roots inoculated with the pathogen *R. solani* were compared with those on healthy roots. PEM strains were targeted and distinguished from each other by traditional culture techniques and molecular based real-time PCR method using species-specific primers. Real time PCR using SYBR Green together with the DNA extracted from plant samples provides useful tools for detection and quantification of PEM populations.

8.2 Materials and methods

Bacterial strains, media and DNA extraction

Spontaneous rifampicin resistant strains of PEM (P3r3, E1r1 and KMa1r1) were cultured on LB agar or TSA/10 agar, and for liquid culture in LB broth or TSA/10 broth medium (appendix 1). The selective media for P, E and M were TCVr, MMrc-fe and TCV/10rn (appendix 1) respectively. Bacterial genomic DNA and total plant DNA was extracted by using QIAGEN DNeasy Tissue Kit and DNeasy Plant mini kit respectively.

16S rRNA gene amplification and sequence analysis

Genomic DNA of PEM strains was extracted by using the QIAGEN DNeasy Tissue Kit (QIAGEN) according to the manufacturer's instructions and stored at -20^{o}C. The 16S rDNA genes from the genomic DNA of PEM strains were amplified by polymerase chain reaction (PCR) using primer 27F [GAGCTCAGAGTTTGATCMTGGCTCAG] and 1492R [CACGYTACCTTGTTACGACTT], which give a product approximately 1.5 kb in size (Valinsky *et al.* 2002). The primers used for sequencing anneal to positions 27 for the forward reaction (27F) and 1492 for the reverse reaction (1492R).

The PCR amplification of 16S rRNA gene was carried out using the Taq PCR Core Kit (QIAGEN, Australia). The reaction volume of 25 µL for each reaction contained 2.5 µL of PCR Buffer (10x), 0.5 µL of dNTP mix (10mM each), 1 µL of 0.5µM primer 27F, 1 µL of 0.5 µM primer 1492R, 2 µL of genomic DNA template, 0.5 µL (2.5U) of *Taq* polymerase (Qiagen, Australia), and 17.5 µL of sterile milli-Q water. A reaction with no template was used as a negative control in all the PCR reactions. The DNA amplification was performed in a PCR thermal cycler (Palm Cycler, Corbett Research, Australia or PTC-100, MJ Research Inc., USA) using the following program: 94ºC for 3 minutes initial denaturation; 35 cycles of 94ºC for 30 s followed by annealing at 48ºC for 30 s, and extension at 72ºC for 1minute; and final extension at 72ºC for 10 minutes. The PCR primers and cycle protocol were obtained from Scott Godwin, UNE, Armidale.

Agarose gel electrophoresis was done to check the size of the PCR amplified 16S rDNA. The amplified DNA samples were mixed with gel-loading dye and were run on a 1% agarose gel in TBE buffer (1x TBE buffer: 89 mM Tris-HCl, pH 7.6, 89 mM boric acid, 2 mM EDTA). Electrophoresis was performed at 100 volts. To determine the band size, 1 kb DNA ladders (New England Biolabs) were run on the same gel and gels were stained with ethidium bromide (1.5-2 μg/mL) solution. Stained gels were visualized by exposure to UV light and photographed with a digital system (Gel Doc, Bio-Rad, Australia) or software Grab-It (version 2.5; Synoptics Ltd., UK). The expected 1.5 kb size bands were cut from the gel and purified using the QIAprep spin miniprep kit (QIAGEN, Australia).

The sequencing reaction was prepared by adding 10 pmol each primer (27F and 1492R) to PCR purified DNA (~50 ng diluted with sterile milli-Q water) in 16 μL total sequencing reaction mixture. The sequencing reaction mix was sent to SUPAMAC, Sydney for sequencing. Sequences obtained from SUPAMAC were subjected to a BLAST (Basic Local Alignment Search Tool) analysis available on the GeneBank database of the National Centre for Biology Information database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to check the possible homology.

Development of species specific real-time PCR primers to detect the PEM strains

Species specific PCR primers were designed from the 16S rDNA of the PEM strains to detect the individual bacterial populations. The 16S rDNA sequences obtained from this study were aligned with the sequences obtained from S. Barnett, South Australian Research & Development Institute (SARDI), Adelaide. T-Coffee multiple alignment software tools (http://www.ebi.ac.uk/Tools/t-coffee/index.htmL) were used to align the PEM strain sequences. The variable regions for each of the PEM strains were selected to find unique sequences different from each other. For each of the PEM strains, both the forward and reverse primers were designed from the selected variable unique regions using the software Primer3 program (http://fokker.wi.mit.edu/primer3/input.htm). The most conserved regions were excluded from the data prior to using the Primer 3 software. Primers were designed to have a small product size (100-200 bp) suitable for SYBR Green quantification. All the primers were synthesized by Gene Works (Thebarton, SA, Australia). The species specific primers set for *Pantoea* were APPF (forward) and APPR (reverse), for *Exiguobacterium*,

APEF (forward) and APER (reverse) and for *Microbacterium,* APMF (forward) and APMR (reverse). The PCR primer sequences for all the PEM strains and their respective properties are described in Table 8-1.

Table 8-1. PEM species specific primer for PCR amplification.

Primer	Sequence	Tm (ºC)	Binding position	Product size(bp)	Target strain
APPF	CGGACGGGTGAGTAATGTCT	54	92	153	*Pantoea*
APPR	ATAGTGAGAGGCCCGAAGGT	54	210		
APEF	AGGTGTTGGGGGGTTTCCGC	58	840	194	*Exiguobacterium*
APER	TGATCTCTCAAGCGGTCAAG	52	1034		
APMF	GAACTTGCTGTGTGGGATCA	52	71	118	*Microbacterium*
APMR	CCCATCCCCAACCAATAAAT	50	224		

Conventional PCR and real time PCR assay with SYBR green for primer specificity and optimization

Conventional PCR (Palm Cycler, Corbett Research, Australia) was conducted using Taq PCR Core kit (QIAGEN), to check the primer specificity and to optimize reactions. A 25 µl reaction volume was set up for PCR which contained 2.5 µl of 10x PCR buffer, 0.5 µl of 10 mM dNTPs mix (10 mM each), 1 µl of 0.5 µM of each set of the primers for individual strains and 1 µl of bacterial genomic DNA (~40-60 ng) as a template, 0.5 µL (2.5U) of *Taq* polymerase (Qiagen, Australia), and 17.5 µl of sterile milli-Q water. A reaction with no template was used as a negative control in all the PCR reactions. All the primers were tested for their respective strain as well as for other strains to check for cross reaction between PEM strains and also to confirm that specific primers amplified a single band of expected size for the respective strain The primers were also optimized for annealing temperature in a range of 50-55ºC using temperature gradient cycler programs. The PCR product was then subjected to 2% (w/v) agarose gel electrophoresis run at 100 v and staining with ethidium bromide as described above.

Quantitative real-time PCR (Q-PCR) was optimized in a Corbett Rotor-Gene 6000 Cycler (Corbett, Australia) using the software Version 1.7. The QuantiTect SYBR Green PCR Kit (QIAGEN, Australia) was used as a fluorophore to detect the signal. PCR reactions were performed in 0.1 mL tube (Corbett, Australia) in a 15 µl reaction volume which contained 7.5 µl QuantiTect SYBR Green PCR master mix (2X), 1 µl of each of the forward and reverse primers (0.5 µM), 1 µl DNA template DNA (~1 µg) and 4.5 µl RNase free water. A reaction

with no template was used as a negative control. The concentration of total genomic DNA of all extracts was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, USA). The cycling program included 15 min initial denaturation at 95ºC followed by 42 cycles of denaturation at 95ºC for 15 s, annealing at 53-55ºC for 30 s and extension at 72ºC for 30 s. The specificity of amplification was also checked by melting curve analysis.

PEM population study: Interaction of PEM strains with the wheat roots and pathogen

Plant assay in soil and recovery of bacterial populations from root

A plant assay was conducted using Avon soil to determine the spatial variation of PEM populations on the different parts of the roots, after interaction with the pathogen *R. solani*. This growth chamber interaction study was conducted by mixing PEM strains with the soil and pathogen *R. solani* (AG-8) in pots, a method used by Barnett *et al.* (2006). PEM and pathogen culture conditions and inoculum preparation and sterilization of seeds were performed as described in sections 4.2.1, 4.2.2 and 4.2.4. Pots of size 10 cms X 13 cms in diameter were filled with 160 g non sterile soil (15% w/v moisture content, field capacity) and 2 x 8 mm *Rhizoctonia* infested agar plugs were added to the centre of the pot, followed by an additional 160 g of soil. Non sterile soil was premixed with P (1.5 x 10^6 cfu/g), E (3.3 x 10^5 cfu/g) and M (6.3 x 10^5 cfu/g) prior to adding soil into the pots and covered with lids to prevent evaporation. The inoculam concentrations were similar to the natural paopulations of each bacterium in soil (Barnett *et al.* 2006). Pots were pre incubated at 15°C for 2 weeks to allow colonisation of the pathogen in the soil as described by Barnett *et al.* (2006). Seven surface sterilised and pre-germinated wheat seeds (Section 4.2) were transferred to the pots which were then filled with an additional 25 g sterile soil (15% w/v moisture content, field capacity). Wheat plants were reduced to 5 plants per pot after emergence, and polyethylene beads (25 g) were added to the soil surface to reduce evaporation. For each treatment 4 replicate pots were set up. The pots were arranged in a randomized manner inside a growth chamber at 15/20ºC night/day with 12 hours photoperiod. Pots were watered and maintained with the same weight by monitoring during growth. Wheat plants were grown in the controlled chamber for 4 weeks and then harvested.

Roots were excised from the plants and assessed for *Rhizoctonia solani* infection and compared to control plants. Each plant root was sectioned and grouped into diseased section

(lesion part of the root) and non diseased section (non-lesion part of the root). A total of 25 cm of lesion or non-lesion sections of root material was selected from each plant and was washed with sterile water (SDW) to remove soil. The root was then blotted dry and macerated using 3 mL of PBS (appendix 1). Ten fold dilution series for root extracts were prepared using PBS and 20 µl aliquots were spread onto four selective media to identify PEM strains by incubating at 25ºC for 5-6 days. TCVr medium was used for the selection of the Gram negative bacterium *Pantoea*, whereas MMrc and TCV/10rn were used for *Exiguobacterium* and *Microbacterium* respectively. Diluted root extract was also plated on non selective medium TSA/10 to enumerate total aerobic bacteria. The colonies were counted from individual plates after 2-5 days and mean value of the number of the plates were analysed by ANOVA after log10 transformation.

Plant assay in test tubes hydroponics system and recovery of PEM populations from root sample

Wheat plants were grown in test tubes hydroponics system as described in chapter 7.2. The pathogen (*Rhizoctonia solani*) was cultured on PDA/4 as described in chapter 3. P-GFP and EM strains were grown in LB broth medium supplemented with antibiotics kanamycin (20 µg/mL) and rifampicin (5 µg/mL.) respectively. Bacterial cultures were grown at 30ºC and 180 rpm shaking until A_{600} reached ~0.8. The bacterial concentration was adjusted to $1x10^7$ cells/mL and 100 µl culture was inoculated in hydroponics solution containing 3 days old wheat seedlings with and without pathogen treatment. The control plants were inoculated with only P-GFP or only with pathogen. Pathogen *R. solani* culture from the PDA/4 plate was sliced into long and thin sections of 5 mm by 1 mm and one slice inoculated into the hydroponics medium, adjacent to roots wherever pathogen was involved in the treatment. There were six replicates of each treatment. The wheat plant-growing system was transferred to a controlled environment with 12 hr light/dark cycling at 25°C. Roots were excised from the plants 10 days after inoculation for analysis. Six plants from each treatment and control were harvested and roots were detached from the plant. Roots were washed gently with SDW to remove hydroponics solution. Each root sample was weighed and ground in 0.5-0.7 mL LB broth medium. The numbers of viable cells on the root sample for each of the PEM strains were counted by plating serial tenfold dilution of the suspension. Diluted sample suspensions (100 µl) were plated on LB medium and antibiotic Km (20 µg/mL) supplemented medium for the recovery of PEM strains.

Interaction of PEM and pathogen on hydroponics agar medium plates:

Wheat seeds were surface sterilized with 1% mercuric chloride and pre-germinated as described in Chapter 4. Bacterial culture conditions, surface sterilization of seeds and inoculation of bacterial cultures were performed (Section 4.2). Surface sterilised seeds were pre-germinated on HMA plates. Three days old seedlings were dipped into 10^7 cfu/mL of each strain of all bacteria diluted into PBS and then transferred to HMA plates. The wheat seedlings were well shaken to remove excess bacterial culture before transferring them onto HMA plates. Pathogen cultures were established on the HMA plates prior to bacterial inoculation of the wheat seedlings by transferring an 8 mm *Rhizoctonia* infested agar plug from a fresh culture on PDA to the HMA plates. Replicates were performed (10 plants per treatment, one plant per Petri plate) and experimental plates were incubated at 20°C temperature with 12 hours day/night cycle. The development of root lesions was observed under the dissecting microscope in pathogen inoculated plants. Plants were harvested 7 days after PEM inoculation and lesion (diseased), non lesion (non diseased) and healthy (uninoculated) parts of the roots were selected from each replicate for every treatment. The root tissues were weighed into 2 mL microcentrifuge tubes (~0.1 g fresh weight). The tubes were then transferred to a freeze dryer and dry weights of samples were measured after drying overnight.

Genomic DNA extraction of root tissue

Stainless steel balls, 5 mm diameter, were added to each tube. The tubes were then placed in a QIAGEN tissue lyser for two periods of 30 s at 30 Hz to grind the root tissue to fine powder. The positions of the tubes were reversed between each period of beating to ensure uniformity. The total genomic DNA from the fine root tissue powder was extracted using the DNeasy Plant mini kit (QIAGEN) as per the instruction manual.

Quantification of DNA extracts from root tissue using real-time PCR and conventional PCR amplification

A preliminary multiplex conventional PCR was conducted to detect the amplification of extracted genomic DNA of PEM treated root tissue samples. A 25 µl PCR reaction volume and primers for *Pantoea* and *Microbacterium* were used as described above except that 2 µl genomic DNA was added to the PCR reaction mix. The PCR reaction profile was the same as above except that annealing was done at the determined optimum temperature of 53ºC.

In real time PCR, the extracted genomic DNA of three treatments (roots inoculated with PEM only, and lesioned and non-lesioned sections of roots inoculated with both PEM and *R. solani*) were subjected to real time PCR quantification using each set of species specific primers for PEM strains separately. Multiplex PCR was not possible using SYBR Green. Each of the species specific primers set for P, E and M were used to amplify the extracted genomic DNA using QuantiTect SYBR Green PCR Kit (Qiagen, Australia) in 15 µl reaction volume as described above. Reactions with no template, and genomic DNA of uninoculated wheat roots, were used as negative controls. There were 6 replicate extracts (from individual plants) for each treatment, and each reaction was done twice. The cycling program included 15 min initial denaturation at 95ºC followed by 42 cycles of denaturation at 95ºC for 15 s, annealing at 53º C for 30 s and extension at 72ºC for 30 s. For each primer pair, relative quantification was done using the log of the threshold cycle (C_T). This was expressed as a praportion of the sample with the highest target DNA content. The relative DNA concentration of each sample was then adjusted for the dry weight of the original root sample. The data were subjected to ANOVA with mean separation by Tukey's method, using SPSS.

8.3 Results

16S rDNA amplification and sequence analysis

The PCR amplification showed a band of approximately 1.5kb in size on the agarose gel for each of the PEM strains with the 27F and 1492 primers (Figure 8-1). The partial sequences obtained were matched with the NCBI data base. The 16S rDNA of P, E and M strains showed 96-99% sequence identity with corresponding gene sequences obtained for PEM strains. The matching sequence accession number and their homology is summarized in Table 8-2.

Table 8-2. PEM strains and their best matching sequence from NCBI database.

	Best matching sequences			
Strain	**Accession no.**	**Identities**	**Similarity (%)**	**Organism**
Pantoea	AM293680	908/937	96	*Pantoea agglomerans*
Exigubacterium	AJ297437	885/898	98	*Exiguobacterium acetylicum*
Microbacterium	AB330407	482/486	99	*Microbacterium* sp.

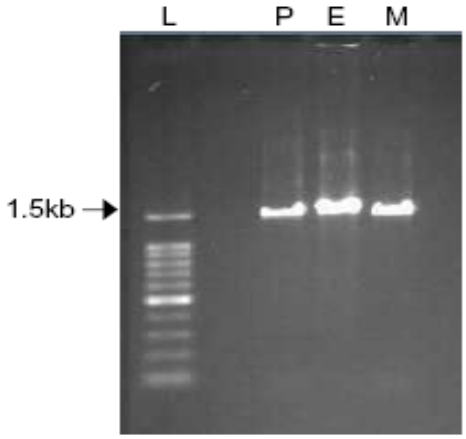


Figure 8-1. The result of 16srDNA amplified product of PEM strains showing 1.5kb band on agarose gel. Lanes are; L: 100bp ladder; P: *Pantoea*; E: *Exiguobacterium*; M: *Microbacterium*.

Conventional PCR and real-time PCR assay with SYBR green for primer specificity and optimization

The species specific primers for P, E and M showed amplification in the PCR reaction of the genomic DNA extracted from pure culture of the respective PEM strains. Each primer set showed specificity to their respective strain only and there was no cross reaction to different strains (Figure 8-2). The expected product size were 153bp for *Pantoea*, 194bp for *Exiguobacetrium* and 118bp for *Microbacterium* was detected. The annealing temperature for all the primers was found optimum for 30s at 53ºC.

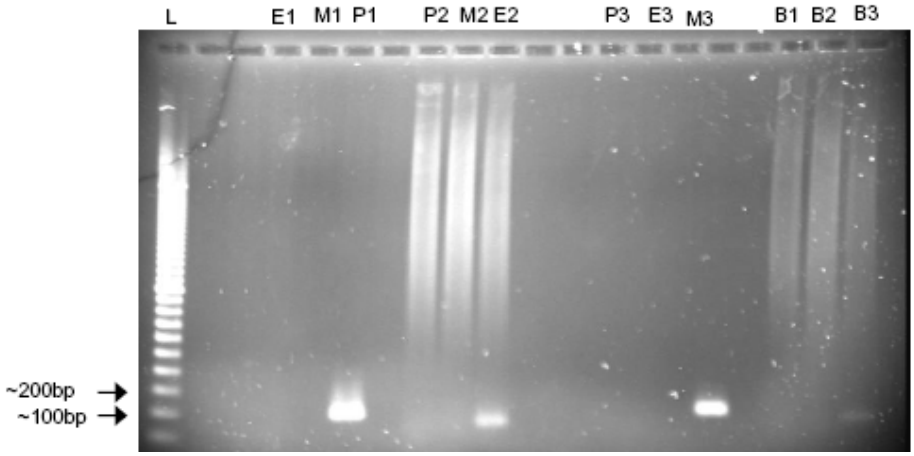
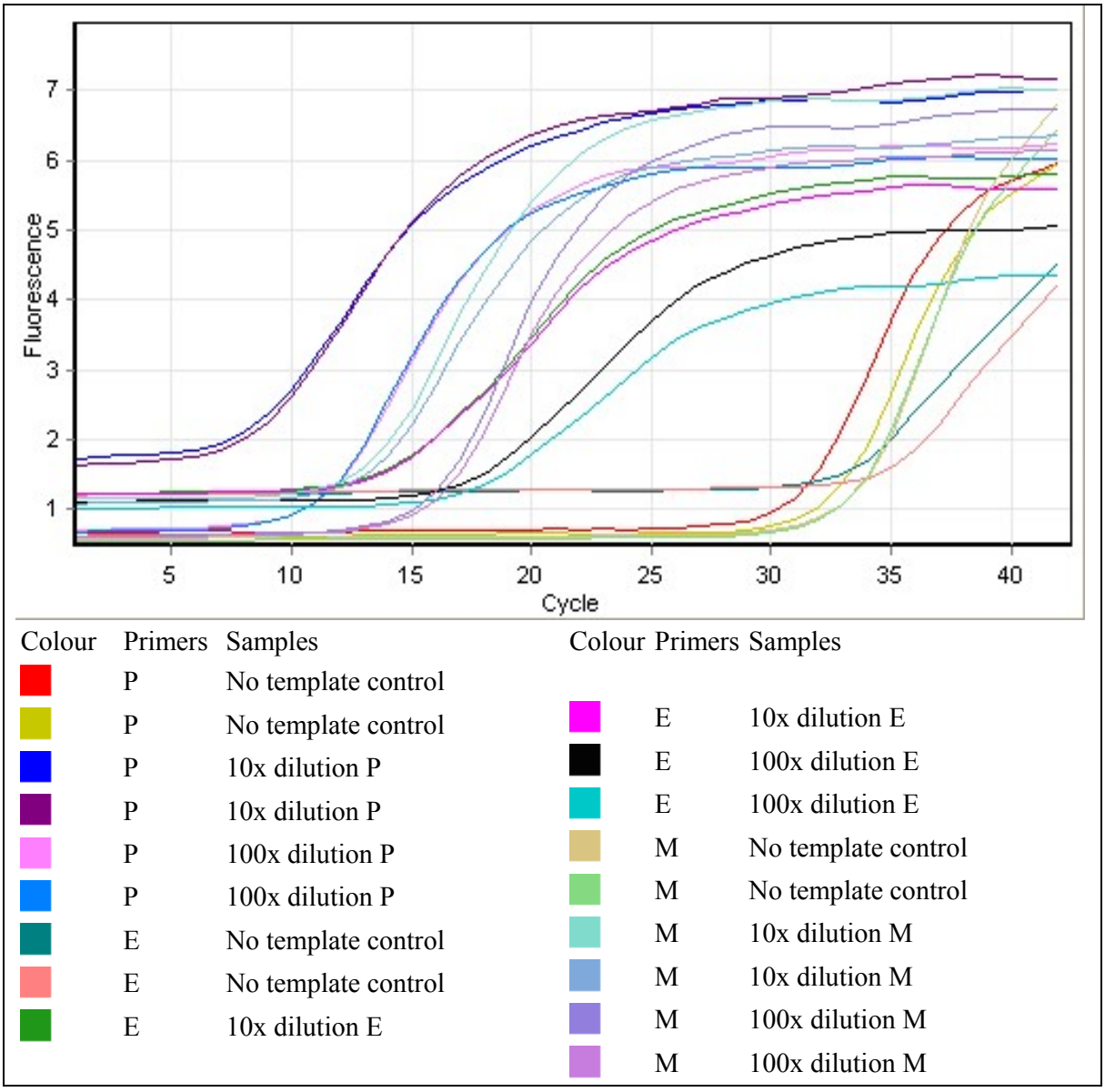


Figure 8-2. The result of PCR amplification using P, E and M specific primers of the PEM genomic DNA. Lanes are; L: 1kb ladder; 1: *Pantoea* primer ; 2: *Exiguobacterium* primer; 3: *Microbacterium* primer; P: *Pantoea*; E: *Exiguobacterium*; M: *Microbacterium*; B: No template control.

Real-time PCR was also conducted to test the ability of the primers to quantify pure genomic DNA of the individual PEM strains. The optimum annealing temperature for each primer was found to be 30s at 53ºC and the rest of the conditions were optimized as described in the methods section. There were increments of 3-4 in the threshold reaction cycle with the every 10-fold dilution of genomic DNA (Figure 8-3).



Colour	Primers	Samples
	P	No template control
	P	No template control
	P	10x dilution P
	P	10x dilution P
	P	100x dilution P
	P	100x dilution P
	E	No template control
	E	No template control
	E	10x dilution E

Colour	Primers	Samples
	E	10x dilution E
	E	100x dilution E
	E	100x dilution E
	M	No template control
	M	No template control
	M	10x dilution M
	M	10x dilution M
	M	100x dilution M
	M	100x dilution M

Figure 8-3. Test of real-time PCR quantification of PEM genomic DNA from pure culture using SYBR Green.

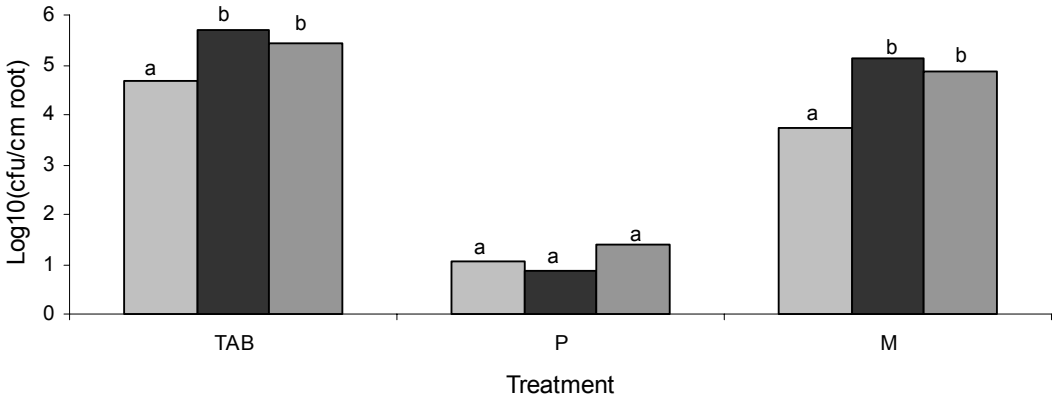
Spatial variation of PEM population on the root tissue

In the *Rhizoctonia* pot bio-assay, roots without PEM, where only the pathogen was inoculated showed severe infection (60-100%) by *Rhizoctonia solani* compared with the control healthy plants. The effect of root infection caused by *Rhizoctonia solani* was reduced to one third level in PEMr inoculated plants. To assess the interaction between bacterial groups in disease suppression, the PEMr isolates population was counted on selective media plates. The PEMr

population in the roots was shown to be related to changes observed in root growth and root infection of plants (Figure 8-4).

In this study, *Exiguobacterium* was not detected. However, *Pantoea* and *Microbacterium* were present, with a higher population of *Microbacterium* than of *Pantoea* (Figure 8-4). *Microbacterium* was increased on the root lesions caused by *Rhizoctonia* and this was confirmed by comparing the populations of *Microbacteria* on lesioned and non-lesioned sections of the same roots. Populations of total aerobic bacteria (TAB) were also higher in the roots of diseased plants than healthy part of the roots.

A



B

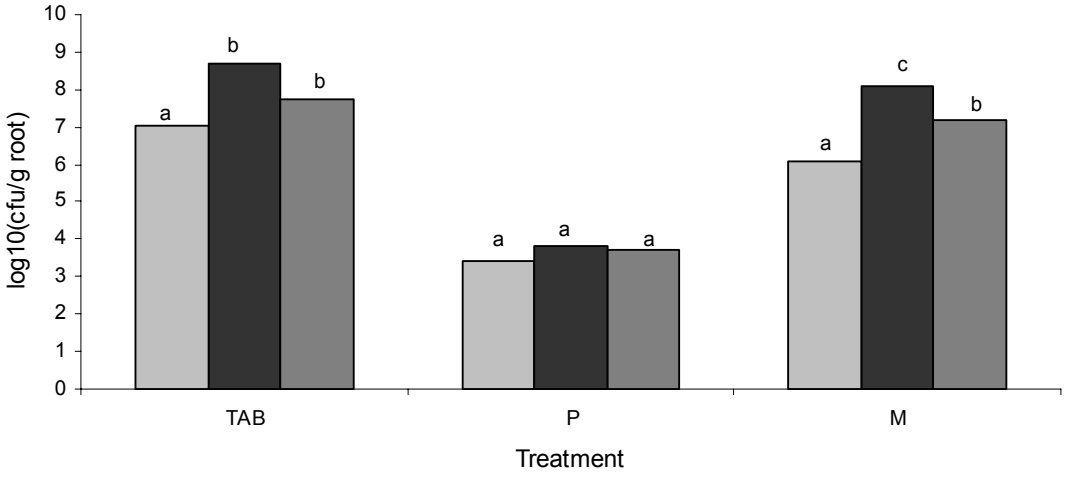


Figure 8-4. PEM recovered from healthy ( ), diseased ( ) and non diseased ( ) sections of the roots: A) per cm of root and B) per gram of root. TAB-total aerobic bacteria; P-*Pantoea*; M-*Microbacterium*. Columns labelled with the same letter for each bacterium are not significantly different.

Interaction of PEM and pathogen in test tube hydroponics system

A culture dependent method was used for the quantification of bacteria on wheat roots in a test tube hydroponics system. The bacterial counts on medium with or without antibiotics (Km, 20 μg/ml) showed that there were no significant differences between treatments in the populations of *Pantoea* or *Microbacterium*, and *Exiguobacterium* could not detected in the treatments. There were no significant differences in *Pantoea* counts on LB medium with or without antibiotics. There was some indication that *Microbacterium* populations were higher in the presence of the pathogen but this was not significant ($P = 0.11$) (Figure 8-4).

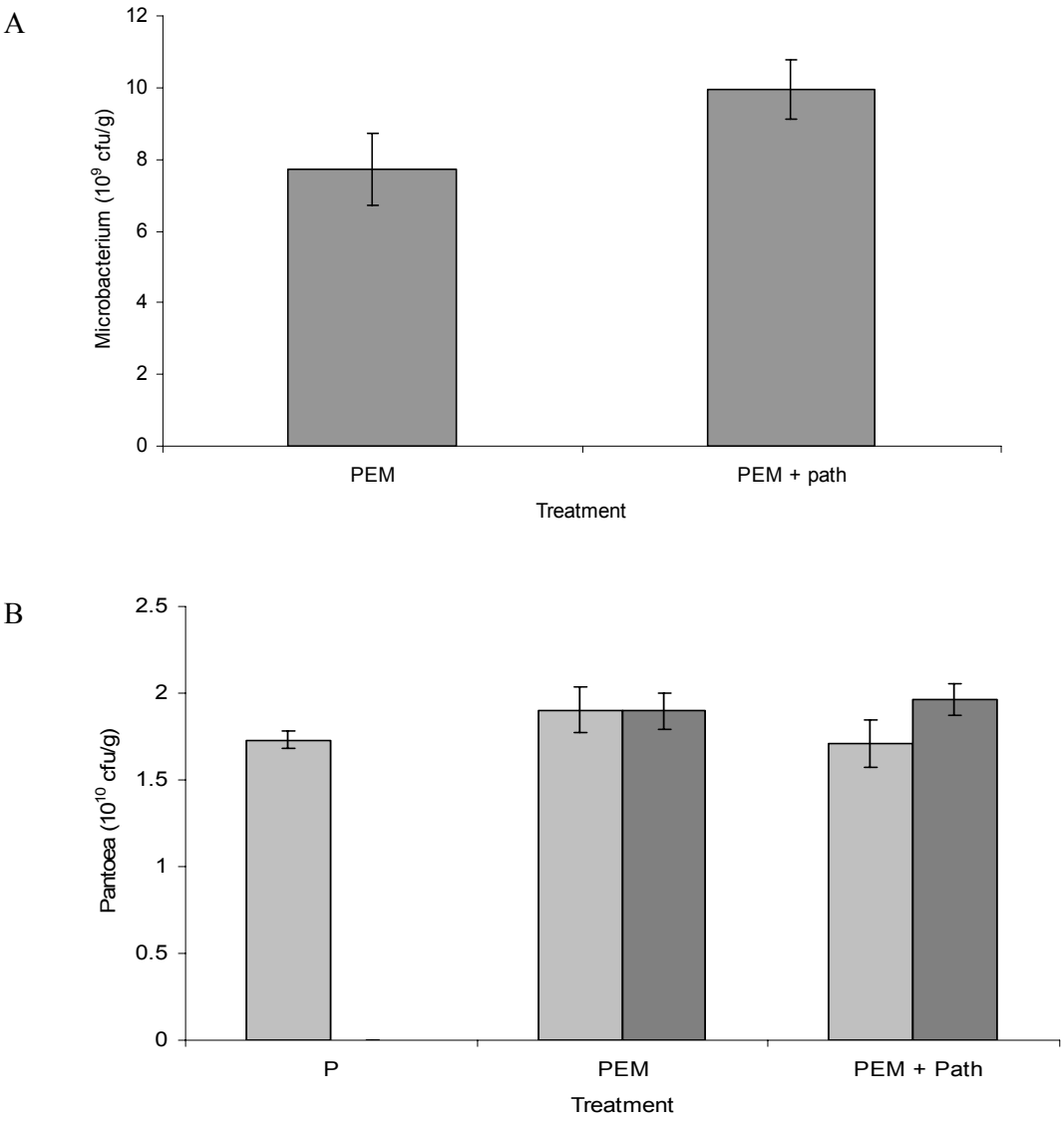


Figure 8-5. Recovery of A) *Microbacterium* and B) *Pantoea* after interaction between PEM populations and pathogen on wheat roots in a test tubes hydroponics system. P inoculated with *Pantoea* only; PEM; inoculated with all three bacteria; PEM+Path; inoculated with three bacteria and pathogen *Rhizoctonia solani*. *Exiguobacterium* could not be detected.

= without antibiotic and = with antibiotics. Error bars shown standard errors (n=6).

Detection and quantification of PEM population using the DNA extracts from root tissue by Real Time PCR and conventional PCR amplification

The PCR primers APPF, APPR, APMF, APMR were tested in conventional PCR against genomic DNA extracted from the three treatments (roots inoculated with PEM only and lesion and non-lesioned sections of roots inoculated with PEM and *R. solani*). Both primer pairs showed amplification of the expected size bands in multiplex PCR (Figure 8-6). Amplification of the expected size band was detected in all PEM inoculated root samples with or without the pathogen. In the lesion (diseased) and non lesion (non diseased) parts where the pathogen was inoculated, there were two clearly amplified bands. The two fragments detected on the agarose gel showed a smaller size band for *Microbacterium* (118bp) and higher size band for *Pantoea* (153bp). However, without the pathogen there were less intense bands compared to diseased or non diseased sections of the root. The result obtained on the agarose gel qualitatively indicated differences among treatments.

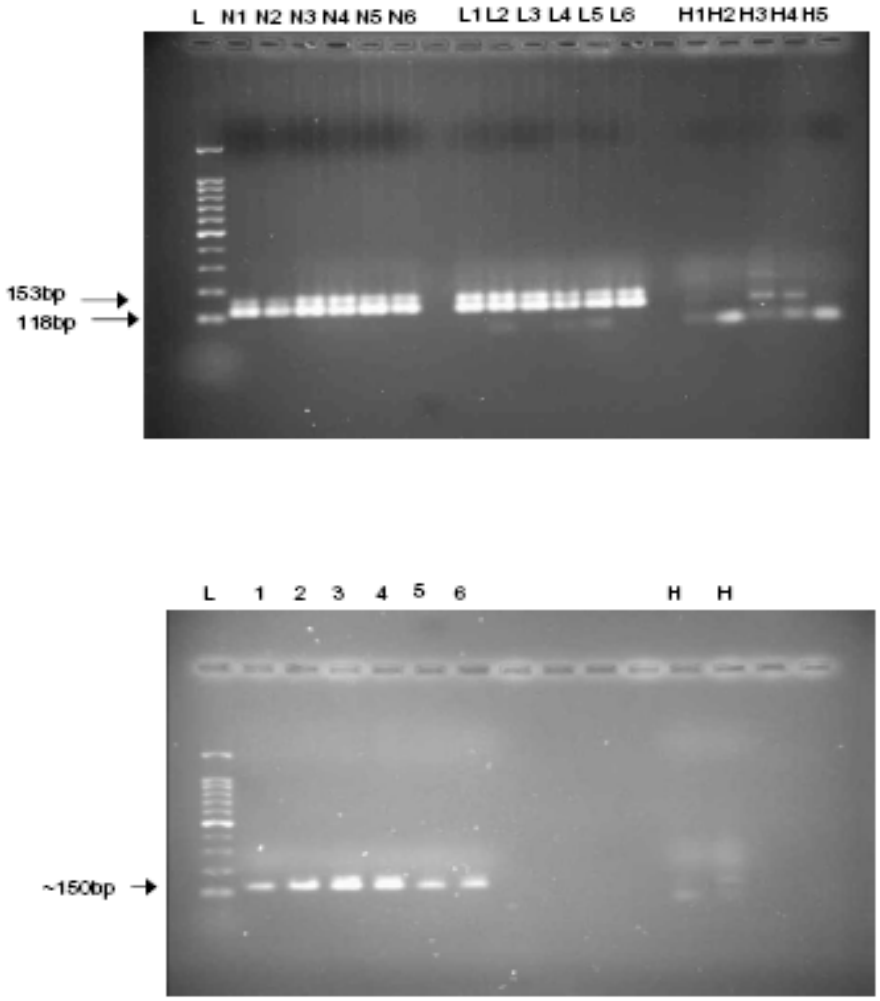


Figure 8-6. PCR amplification using P and M specific primers to the healthy (H,H1,2,3,4,5), PEM inoculated (1,2,3,4,5,6) diseased (L1,2,3,4,5,6) and non diseased (N1,2,3,4,5,6) sections of the root. "L" alone refers to the ladder.

The populations of P, E and M strains were detected with the real-time PCR quantification assay using species-specific primers pairs. Similar to conventional PCR, there was variation detected in the presence of PEM populations among different treatments. The real-time PCR quantification of *Pantoea* showed that there was a marginally significantly (P = 0.053) increased population of *Pantoea* in lesioned parts of the root compared to the healthy un-inoculated part. However, there were no significant differences between populations on the non-lesioned parts of *R. solani*-inoculated roots and either of the other two treatments (Figure 8-7).

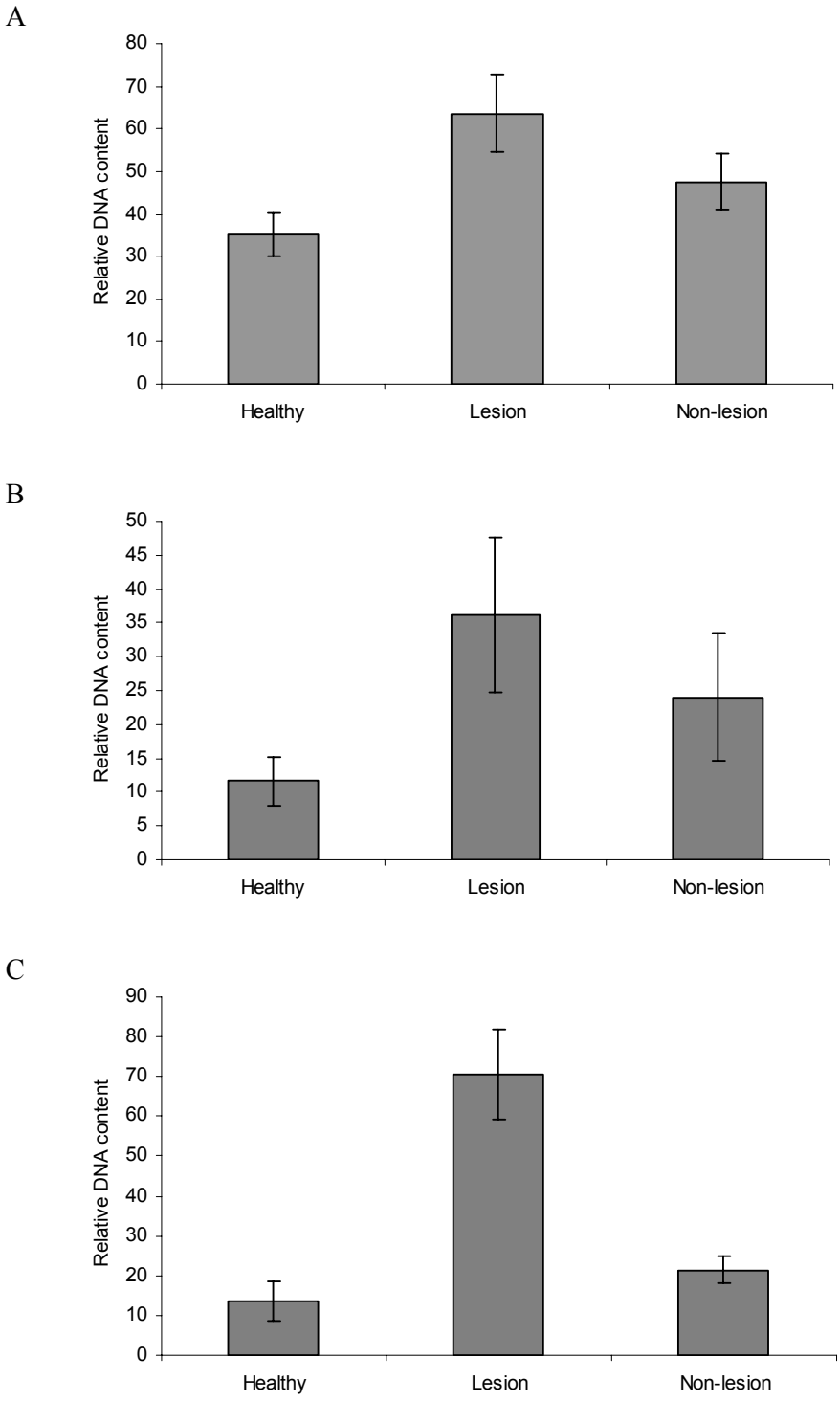


Figure 8-7. Relative Real-time PCR quantification of A) *Pantoea*, B) *Exiguobacterium* and C) *Microbacterium* for the healthy, diseased and non diseased sections of the roots. Error bars are standard errors (n=6). The y-axis scale is relative to an arbitrarily chosen sample.

The *Exiguobacterium* amplification was detected in both the lesion and non lesion parts of roots. However, there was no significant difference between the treatments.

The real-time amplification for *Microbacterium* showed similar results to those suggested by conventional PCR. There was a significant increase in *Microbacterium* populations in the lesion part of the root compared with non lesion and healthy un-inoculated plants.

8.4 Discussion

From past research it is known that PEM causes disease suppression after interaction with pathogens on wheat plants (Barnett *et al.* 2006). However, it is not known if PEM populations are preferentially increased on the diseased or non diseased parts of the roots. It is already established that microbial communities vary depending upon their location in relation to roots (Barnett *et al.* 1999, Mazzola 2004). This study was conducted with the aim to determine if the disease suppressive consortium PEM preferentially occurs on *Rhizoctonia* affected root lesions in wheat. This was suggested by a study published on the effect of *Pseudomonas corrugata* 2140 on take all diseased roots compared with healthy root systems (Barnett *et al.* 1999). That study demonstrated that pseudomonads preferentially select the Ggt (*Gaeumannomyces graminis* var *tritici*) infected sites and had increased colonization on the diseased sections of the roots compared to healthy or non diseased sections of the roots (Weller 1983, Barnett *et al.* 1999).

To assess the interaction between PEM and pathogen *R. solani* on wheat, various methods were adopted to detect the bacterial population in relation to the root or pathogen. The initial study was based on the soil system using a pot bioassay and the bacterial populations were identified using a range of selective medium as described by Barnett *et al.* (2006). In a pot experiment using soil from Avon (South Australia) supplemented with PEM, populations of *Pantoea* were the same on diseased and healthy roots, as assessed by dilution plating. Populations of *Microbacterium* were significantly higher on lesions than on non-lesion parts of diseased roots or on healthy roots, while *Exiguobacterium* could not be detected. This experiment indicated that the population of *Microbacterium* is increased on the root lesion part of the root compared to healthy and non diseased part of the roots.

In order to reduce the complexity of the soil system, a hydroponic system based on a test tube liquid system was used, where roots floated in the hydroponics medium and bacteria and

pathogen were inoculated into the medium near the roots. This experiment was the first known attempt to study the colonization pattern under such controlled conditions. The traditional plating method was used for the recovery of PEM strains. Results obtained in response to the treatments were similar to the soil experiment, but the order of magnitude of bacterial counts was higher (10X). There was some indication that *Microbacterium* numbers were higher in the presence of the pathogen but this was not significant. The lack of positive results may be due to bacterial and pathogen cultures floating in the liquid medium not having consistent physical and chemical interaction between microorganisms. It was therefore decided that the liquid hydroponics system may not be a suitable system and that solid media might be better.

From the first two experiments, it was concluded that *Microbacterium* populations were enriched on diseased sections of the roots caused by *R. solani*. The hydroponics agar medium plates (HMA) method was then used to confirm these findings. The use of HMA plates made it easier to observe the bacterial and pathogen interaction under the microscope and also to select the diseased and non diseased sections of the roots. The traditional plating method is a reliable method for the culturing and screening the microorganisms however, it has some limitations. Hence, some recent molecular based methods were used to for quantify of PEM populations on wheat roots.

The PCR assay was able to amplify the 16S rDNA of the PEM strains. However, the normal PCR reaction is only useful for the detection or screening of the presence or absence of bacteria. The 16S rDNA based species specific primers are useful in that they allow detection of only bacteria belonging to their own species. The use of real-time PCR with specific primers and SYBR Green has been shown to be a good method for quantification purposes. Quantitative real-time PCR is a sensitive technique for quantification purposes as it can detect smaller amounts of bacterial DNA.

Real-time PCR was used to confirm the above findings in a gnotobiotic system. The quantity of *Microbacterium* DNA was 4-fold higher on lesions than on non-lesion parts of roots. There was an indication that *Pantoea* and *Exiguobacterium* were also enriched on lesions, but the differences were not significant for these bacteria. Real-time PCR with SYBR Green was therefore more reliable for the quantification of PEM strainsthan any other traditional counting methods. The relative size of the effect varied between plate counts and real time

PCR but the overall results in both experiments confirmed the finding that *Microbacterium* selects the *Rhizoctonia* infected site on the roots.

Pantoea and *Microbacterium* strains recovered on the medium plates showed that they were present in viable form. However *Exiguobacterium* mey be present in a viable but not cultivated form in the presence of the pathogen. The results with populations of *Exiguobacterium* were consistent and they could not be detected by the selective medium plating technique. However, they were detected by real-time PCR. The real-time PCR using species specific primers for *Exiguobacterium* was a sensitive detection technique and was able to detect the species even at low numbers of bacterial populations.

This study was focused on the interaction between PEM and the pathogen *R. solani* in the rhizosphere. Root lesions may be an important niche for the suppression of Rhizoctonia disease on wheat by PEM strains. It can be concluded that PEM isolates attach to both the disease lesion and non lesion part of the root and that populations of one bacterium, *Microbacterium*, increased in numbers on the diseased parts of the root. These results are in agreement with a study (Barnett *et al.* 1999) related to variation in the population levels of *Pseudomonas* on Ggt affected diseased or non diseased section of root. Real-time PCR gave greater specificity, and was more sensitive, than culture-based methods. The increase in bacterial populations of associated with lesions has implications for the mechanisms of biological control. These findings could be further utilized for PEM population studies *in vitro* or in the field to develop disease management strategies against *R.solani*.

Chapter 9 General discussion

9.1 Key findings

The aim of this thesis was to identify potential mechanisms involved in the suppression of *Rhizoctonia* root rot of wheat by a consortium of *Pantoea agglomerans*, *Exiguobacterium acetylicum* and *Microbacterium* species (PEM). The PEM strains were known for their disease suppression ability prior to this work (Barnett et al. 2006). The research into the mechanisms of plant growth promotion by PEM strains has provided a greater understanding about the factors affecting their role in plant growth enhancement ability in the presence and absence of the pathogen.

The growth of PEM strains under different environmental conditions provides important information for understanding the activity of these bacteria under adverse soil conditions. PEM strains were able to tolerate broad temperature and pH ranges and low water potential. This confers the potential to be effective biological control agents over a wide range of soil conditions. In particular, under near neutral pH, low water potential and high temperature, PEM is likely to have advantages over *R. solani*. Verification of these responses by PEM and *Rhizoctonia* under field soil conditions is recommended in future research.

PEM strains and *Rhizoctonia* were shown to utilize some ecologically significant compounds which have been reported to be exuded by wheat roots (Rovira 1969). Wheat root exudates such as glucose, xylose, asparagine, and glutamic acid were utilized by all the PEM strains and by *R. solani*. Some exudates like serine were utilized by P and E only whereas malic acid utilization was shown by PEM but not by the pathogen. The results obtained from this study indicate that there are ecologically significant compounds that overlap in the bacteria and pathogen in terms of utilization patterns.

The PEM strains gave significant growth promotion effects under hydroponic conditions in the absence of the pathogen. There was significantly increased root and shoot growth when PEM strains were inoculated individually or together in bioassays. Results here are in agreement with previous studies of the growth promotion effects after PGPR inoculation to the plant. Barnett *et al.* (2006) showed that PEM had a growth promotion effect on diseased

plants. However there were no reports of growth promotion potential of PEM in the absence of the pathogen. This supports some aspects of findings of Barnett *et al.* (2006) but most of them have been added as a new information on the growth promotion potential effects of PEM.

This study demonstrated that PEM strains have an ability to change the root system architecture. PGPR have been reported to stimulate root morphogenesis (Larcher *et al.* 2003). Many studies have shown that the inoculation of wheat, and other cereal seeds, with bacteria of the genus *Azospirillum* resulted in an increase in both volume and number of roots (Bashan and Levanony 1990, Didonet and Magalhaes 1993, Gouvêa *et al.* 1997). This improved the uptake of nutrients and water (Murty and Ladha 1988). In this study PEM caused an increase in branching of roots, and increased root mass and shoot length. This finding is similar to that for *Azospirillum* as mentioned above.

Changes in root morphology of PEM-inoculated plants could be due to hormonal effects. IAA production has been reported for several PGPR in the family *Enterobacteriacae* including *Pantoea agglomerans* (Zimmer *et al.* 1994). In this study only *Pantoea* was shown to be positive for IAA production. Production of IAA has been reported to increase root growth, increase number of secondary roots and also to increase root hairs (Barbieri and Galli 1993, Steenhoudt and Vanderleyden 2000, Patten and Glick 2002). *Pantoea* was shown to have a significant effect on all of these, and IAA might have significantly contributed to the enhanced growth of P inoculated plants.

ACC deaminase has been reported in various plant growth promoting bacteria, and acts by reducing the levels of the plant hormone ethylene. In this study, PCR with degenerate primers was used to screen for the ACC deaminase gene in the PEM strains. Among PEM only P showed the expected size amplicon for the ACC deaminase gene. Strains of P and M were shown to have an indication of ACC deaminase production in the activity assay. The experiment was repeated three times using the conditions published by Penrose and Glick (2003) but the results were inconsistent due to the lack of proper information in the published methodology. However, the results for the presence of ACC deaminase in P and M are in agreement with the previous findings related to plant growth promotion, where both strains were shown to have a significantly increased effect on root and shoot growth as well as increased root branching pattern.

It was found that the PEM strains had a strong effect on growth promotion and nitrogen content in nitrogen deficient conditions. It was observed that bacterial inoculated plants looked much healthier than the un-inoculated control plants. There was evidence for significant plant nitrogen increases due to inoculation with PEM. Increased branching pattern could have improved the nutrient uptake in bacterial inoculated plants. However, the fact that nitrogen content was increased in the PEM inoculated plants relative to the control plants under nitrogen deficient conditions indicates that PEM has some nitrogen fixation ability. These results also support the hypothesis of root association and N_2 fixation ability of PEM strains. The increase in total N content by bacterial activity of free-living diazotrophs has been shown by Baldani *et al.* (1997). Though PEM strains could possess multiple plant growth promotion traits, the increased nitrogen uptake by bacterized plants could be mainly due to nitrogen fixing ability of PEM strains, which could also have stimulated plant growth. To screen for nitrogen fixation ability of PEM, all the strains were tested by nested PCR using degenerate primers for *nif*H genes. The positive *nif*H amplification results obtained for P, E and M was another confirmation of the hypothesis of nitrogen fixation. Nitrogen fixation by PEM strains could be further confirmed by the acetylene reduction assay.

An important aspect of biocontrol is the location of PEM on the wheat roots. There were no previous studies of localization or attachment of this consortium of bacteria on wheat roots and only little was known about the interaction of PEM on the plant roots. Barnett *et al.*(2006) had reported that *Pantoea* and *Exiguobacterium* were root associated, whereas *Microbacterium* was associated with the rhizosphere. There were a number of reports of the localization of other strains of *Pantoea agglomerans* on wheat and rice roots, but these would not necessarily behave in the same way as in the PEM consortium. Scanning electron microscopy (SEM) was used for the localization of PEM strains on the wheat roots. The results indicated that bacterial cells were present all over the surface of the different parts of the root. PEM strains were shown to have colonization ability as all the roots were grown up to 10 days after inoculation but all the bacteria were able to colonize the lateral branches as well as the main root axis. Therefore, all bacteria were able to occur at potential sites of infection.

The advantage of the SEM was that it allowed examination of bacterial cells on the root tissue and also investigation of bacterial attachment on the root which is an important trait of

naturally occurring bacterial strains in agricultural ecosystems. For example, *Pantoea* and *Exiguobacterium* appeared to produce EPS on the roots that may have an involvement in attachment. However, SEM has limitations. It is difficult to distinguish between bacteria in mixed inoculations, and it is difficult to quantify bacteria on the roots. Therefore microscopic methods which distinguished between the bacteria were explored.

The approach used was a method where bacterial colonization on the wheat roots was monitored using molecular markers and qualitative detection. The Gram positive EM strains were not successfully labeled with molecular markers. However the Gram negative *Pantoea* strain was successfully labeled with *lac* Z and the GFP marker. The use of DAPI as a counterstain for *Exiguobacterium* and *Microbacterium* was unsuccessful, so only *Pantoea* could be localized.

By using the labeled *Pantoea* which was constitutively expressing the *lac*Z genes, the *Pantoea* association with plants roots was investigated. The *lac*Z labeled *Pantoea* inoculated in hydroponics solution showed colonization on the root surface, especially in the root elongation area and also on the base of the lateral roots. This was similar to the results of a study using *lacZ* labelled *Azospirillum* by Kapulnik *et al.*(1987). Although *lacZ* demonstrated the colonization ability of *Pantoea* on the wheat roots it required a time-consuming and complex process for visualization.

GFP was used as a marker because of easy detection and no need for exogenous substrates or processing of cells for visualization (Lawrence *et al.* 1991). In this study, *Pantoea* was successfully transformed with a plasmid which constitutively expresses GFP. The result clearly indicated the colonization of *Pantoea* all over the surface of wheat roots. It also showed that *Pantoea* cells were present on the junctions of the cell wall. The use of GFP and similar markers to study bacterium-plant interactions in several studies has shown that in general, PGPR preferentially colonize the junctions between cells on the root surface (Bloemberg 2007).

A similar study was conducted by Verma *et al.* (2004), where a *Pantoea* strain showed endophytic colonization ability in deep water rice. *Pantoea agglomerans* has also been reported to colonize winter wheat roots endophytically (Ruppel *et al.* 1992). However, no good evidence for endophytic colonization of *Pantoea* was obtained in this study and it could

not be detected on internal junctions of root tissue. Further experiments, using embedded and sectioned material, would be worthwhile to test for endophytic growth by PEM strains.

From past research it was known that PEM causes disease suppression after interaction with pathogens on wheat plants (Barnett *et al.* 2006). However, it was not known if PEM populations are preferentially increased on the diseased or non diseased parts of the roots. It is already established that microbial communities vary depending upon their location in relation to roots (Barnett *et al.* 1999, Mazzola 2004). To assess the interaction between PEM strains with the pathogen *R. solani* on wheat, various methods were adopted to detect the bacterial population in relation to the root or pathogen. The initial study was based on the soil system using a pot bioassay and the bacterial populations were identified using a range of selective medium as described by (Barnett *et al.* 2006). Later it was decided to try a hydroponics system as elucidation of the microorganisms from the complex soil microflora is difficult. Doing an assay in hydroponics medium or on agar plates is much easier in relation to control of growth and more repeatable than in soil. The first hydroponics system used the traditional plating method for the recovery of PEM strains, while the second system used quantitative real-time PCR.

Real-time PCR and dilution plating consistently showed that *Microbacterium* populations were higher on lesions than on non-lesion parts of roots. There was an indication that *Pantoea* and *Exiguobacterium* were also enriched on lesions, but the differences were not significant for these bacteria. The magnitude of the effect varied between plate counts and real time PCR but the overall results obtained in both experiments had the same finding that *Microbacterium* selects the *Rhizoctonia* infected site on the roots.

Pantoea and *Microbacterium* strains were recovered on the agar medium after dilution plating but *Exiguobacterium* could not be detected by the selective medium plating technique. However, it was detected by real-time PCR. The real-time PCR using species specific primers for *Exiguobacterium* was a sensitive detection technique and was able to detect even low number of bacterial populations.

The study was focused on the interaction between PEM and the pathogen *R. solani* in the rhizosphere. Root lesions may be an important niche for suppression of Rhizoctonia disease on wheat by PEM strains. It can be concluded that PEM isolates attach to the disease lesion

and non lesion part of the root. Populations of one bacterium, *Microbacterium* increased in their number on diseased parts of the root. These results are in agreement with a published study (Barnett *et al.* 1999) related to variation in the population of *Pseudomonas* on Ggt affected diseased or non diseased section of the roots. This phenomenon does not appear to have been explored by other workers. The increase in populations of bacteria associated with lesions has implications for the mechanisms of biological control. The real-time PCR technique could be further utilized for PEM population studies *in vitro* or in the field to develop the disease management strategy.

PEM strains indicated some typical PGPR charecteristics such as a wide range of physiological characteristics, direct effects on plant growth, production of different plant growth regulators, nitrogen fixation etc. It was clearly demonstrated that PEM colonized the surface of plant roots which indicates their potential to directly influence pH, growth, and microbial ecology at the root : soil interface. It was revealed from past research that PEM strains work best together in a bioassay as a consortium which is in agreement with the results obtained for significant bacterial growth in the plant assays.

PEM and *Rhizoctonia* have common resource utilizatation and habitat requirments. PEM demonstrated N fixing and root modification effects on plants. Good colonization occurred along the root surface, maximizing the potential for release of PGR and nutrients by PEM to benefit the plant. These factors indicate that PEM is potentially a good candidate as a biocontrol agent against Rhizoctonia.

9.2 Future research recommendations

Further attempts should be made to label the other two bacteria (*Exiguobacterium* and *Microbacterium*) and localize them on the roots. For further research, it will be necessary to assess the interactions of the disease suppressive consortium PEM using fluorescent labeled EM strains. The labeling of Gram positive strains (*Exiguobacterium* and *Microbacterium*) with fluorescent markers will provide important information related to bacterial interaction. GFP is not the only marker available to investigate the interaction. It is recommended that use of different colored markers such as RFP (Red fluorescent protein) or YFP (Yellow fluorescent protein) will be useful. Another suggestion is to use FISH (Fluorescent *In Situ* Hybridization) method based on species specific probes which could be an interesting technique to investigate the interaction. Watt et al. (2006) used the FISH method and

demonstrated that root caps are most heavily colonised by bacteria, and elongation zones (just behind the root cap) least colonised.

As reviewed by Gray and Smith (2005) and more recently by Compant *et al.* (2005), some PGPR can enter the interior part of the root and establish endophytic colonization which reflects the ability of bacteria to selectively adapt to these specific ecological niches. Therefore, it would be useful to investigate the endophytic colonization ability of PEM strains after labeling them with the fluorescent marker.

For future research, the effect of bacteria on each other should be examined to see if they do interact with each other in any way. Studies conducted on three way interactions of bacteria, plant and pathogen indicated that there is a large number of protein factors which are associated with these multi-player interactions (Marra *et al.* 2006). Two dimensional electrophoresis to separate the proteome from each, single, two or three partner interaction will have important implications to understand the interaction of PEM consortium with each other and with the pathogen. Discovery of the individual bacterial components may improve PEM strains as potential biocontrol agents which would ultimately contribute to novel stratgies of disease control.

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Appendices

Appendix 1. Media and solutions

Luria Bertani

	1000ml
Bacto Tryptone	10g
NaCl	5g
Yeast Extract	5g

-For agar add 16g (1.6%)

-Make up to 1000mL with dH_2O and sterilise by autoclaving 120^oC for 20 min

TSA/10 Broth

	1000mL
Tryoptic soy broth	3g

-For agar add 16g (1.6%)

-Make up to 1000mL with dH_2O and sterilise by autoclaving 120^oC for 20 min

½ Potato Dextrose Agar (2.2%)

	500ml
Potato Dextrose Agar	10g
Agar	7.5g

-Make up to 500mL with dH_2O and sterilise by autoclaving 120^oC for 20 min

Yeast manitol medium (YMM)

	100ml
Sucrose	0.4 g
Yeast extract	0.4 g
Malt extract	0.4 g
Agar	1.6 g

-Make up to 100mL with dH_2O and sterilise by autoclaving 120^oC for 20 min

Kings B medium KB

	1000mL
Proteose peptone	20g
K_2HPO_4	1.5g
$MgSO_4.7H_2O$	1.5g
Agar	15g
Glycerol	10g

-Make up to 1000mL with dH_2O and sterilise by autoclaving 120^oC for 20 min

Phosphate-Buffered saline (PBS)

	1000mL
$Na_2HPO_4.2H_2O$	0.40g
$Na_2HPO_4.12H_2O$	1.15g
NaCl	8g

-Make up to 1000mL with dH_2O, adjust the pH 7.4 and sterilise by autoclaving 120^oC for 20 min

Hydroponics medium (HM)

Compound	**Amount in stock solution g/L**	**Nitrogen added (mL)**	**Nitrogen free (mL)**
Macronutrients			
$Ca(NO_3)_2.4H_2O$	236	3	
KNO_3	101.10	2	
$NH_4H_2PO_4$	115.00	2	
$MgSO_4.7H_2O$	184.80	1	1
KCl	149.00	1	1
KH_2PO_4	136.00		2
$Ca(CH_3COO)_2$	158.20		3
Micronutrients			
Fe Sequestrene	64.360	1	1
$MnCl_2.4H_2O$	2.969	1	1
$ZnCl_2$	.0.204	1	1
$CuCl_2$	0.134	1	1
H_3BO_3	0.031	1	1
$(NH_4)_6 Mo_7 O_{24}.4HO$	0.012	1	1

-For hydroponics medium agar (HMA) add 1g agar(1% agar) (HMA)

DF salts (Dworkin and Foster 1958)

	1000mL
KH_2PO_4……	. 4 g
6.0 g Na_2HPO_4.....	6 g
$MgSO_4.7H_2O$….	2 g
Glucose……..	. 2 g
Gluconic acid…	.. 2 g
Citric acid	2 g
Trace elements:	
$FeSO_4.7H_2O$	1mg
H3BO3	10mg
$MnSO_4.H_2O$	11.19 mg
$ZnSO_4.7H_2O$	124.6 mg
$CuSO_4.5H_2O$	78.22 mg
MoO_3	10 mg
$(NH_4)_2SO_4$	2.0 g

TCV medium for isolation of Pantoea agglomerans

Ingrediant	**1L**
Tryoptic soy broth (g)	3
Difco Bacto Agar (g)	15
NaCl (g)	25
Autoclave 121oC, 15 min	
After autoclaving add	ml
Cycloheximide (100mg/ml)	1
Crystal (genetian) violet (5 mg/ml)	1

Plate out 10^2, 10^3, dilutions from macerated root extracts

Count TCV14 colonies (2-3 mm, flat, translucent, circular, brown-yellow) after 4-6 days at 25°C

MMc (Mannitol media) for isolation of *Exiguobacterium acetylicum* (based on Weaver *et al.* 1975)

Ingredient	**1L**
Mannitol (g)	10
yeast extract (mg)	500
Agar (g)	15
NaCl (g)	25
water (ml)	935
Super salts (20x) (ml)	50
P buffer B (ml)	15
Mix and adjust to pH 7.0	
Autoclave at 110oC for 30 min	
After autoclaving add	Ml
Cycloheximide (100mg/ml)	1
Colistin (10 mg/ml)	1

Plate out 10^2, 10^3, dilutions from macerated root extracts

Count CM18 colonies (1-3 mm, convex, opaque, circular, pale orange) after 5-6 days at 25°C

Stock solutions for MMc and CM media

Super Salts	**1L**
MgSO4.7H20 (g)	2
CaCl2.2H20 (g)	2
FeSO4.7H20 (g)	0.44
EDTA (g)	0.4
Solution A (ml)	20
Solution A (ml)	
ZnSO4.7H2O (g)	0.43
MnSO4.H2O (g)	1.3
NaMo.H2O (g)	0.75
H3BO3 (g)	2.8
CuSO4.5H2O (mg)	26
CoSO4.7H2O (mg)	70

P Buffer B

K2HPO4 (g)	60
KH2PO4 (g)	40

Autoclave super salts P Buffer B and Solution A at 121°C for 15-20 min

CMnp (3 carbon media) for isolation of *Microbacterium* (based on Weaver *et al*. 1975)

Ingredient	**1L**
Glucose (g)	5
Mannitol (g)	5
Starch (g)	4.5
yeast extract (mg)	300
Agar (g)	15
NaCl (g)	5
water (ml)	935
Super salts (20x) (ml)	50
P buffer B (ml)	15

Mix and adjust to pH 7.0

Autoclave at 110oC for 30 min

After autoclaving add	ml
Cycloheximide (100mg/ml)	1

For CMnp media for isolation of Microbacteria

Nalidixic acid (10mg/ml)	1
PolymyxinB (32mg/ml)	1

Plate out 10^2, 10^3, 10^4 dilutions from soil extracts

Count CM6 colonies (0.5-2 mm, flat-convex, translucent circular, yellow) after 7 days at 25°C

For general growth of isolates in the lab and for inoculation of seeds or soil

Pantoea and *Exiguobacterium*: Grow on 1/10 strength Tryptic soy agar (TSA/10)

Microbacteria: Grow on 3CM medium without NaCl or antibiotics

Purple medium(Schleifer et al 1981) Indicator media

	g/L	g/100ml
Maltose	10	1.00
Proteose Peptone no.3	10	1.00
Beef extract	1	0.10
NaCl	5	0.50
Bacto Agar	15	1.50
1.5% Bromocresol Purple (ml)	1.4	0.14

Used to measure growth and acid production and compared to controls

Appendix 2. Transformation protocol

(Pajunen *et al.* 2005)

Preparation of electro competent cells

- Overnight grown bacterial culture (PEM strains) were diluted to 1:500 in a total volume of 200 ml and were incubated at 37ºC with shaking.
- Cells were collected by centrigugation at 4 ºC with 3000 rpm for 15 min.
- Cells washed with 0.5 M Sucrose (20 mL) and centrifuged as above.
- Cells washed with 0.5 M sucrose followed by 10%(v/v) glycerol and were centrifuged as above.
- Cells were resuspended in 0.5 mL of 0.5 M sucrose and 10% (v/v) glycerol.

Electroporation

- The 50 µl competent cells were thawed and mixed with the 10 µl pDNA and transfred to pre-chilled cuvette.
- The cuvette was then subjected to electroporation with a range of different pulse conditions 1.8-2.3kV, 100-200 Ω, 15-25 µF.
- LB medium supplemnted with 0.5M sucrose were then added to the cuvette and incubated at 37°C for 90 min.
- The cells were then diluted and plated onto the LB medium supplemented with appropriarte antibiotics.

Appendix 3. Pantoea ACC deaminase sequence

Raw sequence

CNCNNANCCNTGCNTTNANNNNNGNNNTCGCCGNNNNNNGNCGAANAGNANTNANNNNNANGGTANTCGGNGNCT
TAGCCTGNNNTTCNNNAGNCTCCCCCAGCGACTGACGCAGCCGCTCCACCAGCGGCAACTGCGCCTCAACCTGGC
GTGAAACGGTGACGCCCACCAGCTCGGTCTCCGGCAGCAGATGCTCCAGACCCACCGCCAGTCCGGCATGGGTAC
CAGCACTGCCCGAGGCCACGACCACGGCGGCGAAATCGACCACGCCTTCGCTCTGATGAGCAATCTCCTGCGCGC
ACTCTACATAGCCTAGTGCACCCAGCGCATTTGAGCCGCCGACCGGCACGATGTAAGGACGATAACCCTGGGCTT
CCAGACNCGTGGCCTGNTCAGCCAGCTGNNNCCGCANGNNTATTCAGCNCATCCNCCNTGATCNNNTNNNCGTCC
NTCNNNNNNNGCNANNNANNGNNGNCGTTGNNNCNNGNAANTTTNNANCNNGGGNNNCNANCNGGNTTNNCNNNA
ATGNNNNNCNNNTNNNGNCNAGCCNGGNCNNNACTGNCNNCCNNNNGANNCNNATGANTGNANTGGANAGCNNCN
NNNNNCANCANNNNATNGGNNCCNTNA

High quality partial sequence

CTCCCCCAGCGACTGACGCAGCCGCTCCACCAGCGGCAACTGCGCCTCAACCTGGCGTGAAACGGTGACGCCCAC
CAGCTCGGTCTCCGGCAGCAGATGCTCCAGACCCACCGCCAGTCCGGCATGGGTACCAGCACTGCCCGAGGCCAC
GACCACGGCGGCGAAATCGACCACGCCTTCGCTCTGATGAGCAATCTCCTGCGCGCACTCTACATAGCCTAGTGC
ACCCAGCGCATTTGAGCCGCCGACCGGCACGATGTAAGGACGATAACCCTGGGCTTCCAGAC

Translation of high quality sequence

LEAQGYRPYIVPVGGSNALGALGYVECAQEIAHQSEGVVDFAAVVVASGSAGTHAGLAVGLEHLLPETELVGVTV
SRQVEAQLPLVERLRQSLGE