Plant growth-promoting bacteria from Western Australian soils

by

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BSc (Hons) Murdoch

This thesis is presented for the degree of

Doctor of Philosophy

of

Murdoch University

December 2016

I hereby declare that, unless otherwise stated, the work presented in this thesis is my own and has not been submitted for a degree at any other institution.

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Publications arising from this thesis

Rebecca Swift, Liza Parkinson, Thomas J. Edwards, Regina Carr, Jen McComb, Graham W. O'Hara, Giles E. St. John Hardy, Lambert Bräu, John Howieson. 2016. Potential for agricultural production on soils mined for apatite using legumes and beneficial microbes. *Soil* (in review).

This was included as Chapter 4 in this thesis as I was involved with the field work on Christmas Island and the analysis of the results, through the field testing of one of my isolates. Additionally, the paper was largely written by myself.

Acknowledgements

There are so many people to thank who have supported and helped me throughout the long course of my PhD. I would like to start by thanking my wonderful supervisors, Professor Jen McComb, Professor Giles Hardy and Dr Lambert Bräu. Your expertise, support and determination to see this thesis to completion are appreciated more than I can express.

Professor John Howieson and his sons Sam and Scott, Pip Maxwell, Dr Heidi Nistelberger and Dr Ron Yates were all involved with the field trials. Professor Howieson also provided me with the opportunity to participate in the Christmas Island field trials.

Papori Barua, Regina Carr, Dr Daniel Mulas-Garcia, Dr Trudy Paap and Liza Parkinson helped set up and harvest glasshouse trials and Dr Leila Eshraghi advised on the auxin assays. Leila, Liza, Papori, and Trudy also provided support in many other ways, my very sincere thanks to you for everything.

My thanks to Gordon Thomson for help with histology, Professor Mike Calver for assistance with statistical analysis and Professor Graham O'Hara for financial support and many valuable discussions. Also, many thanks to Dr Ravi Tiwari for advice on the molecular components of the project.

I would like to express my thanks to visiting international experts Professor Khalid El Tarabily and Professor Bernard Glick, who also kindly provided the strain, *Pseudomonas* sp. UW4.

My colleagues created a supportive environment both inside and outside of work. These include Aliyu, Amanuel, Bill, Chris, Diane, Domingo, Elmi (Mohammed),

Felipe, George, Jesica, Julie, Laghima, Macarana, Mazvita, Neil, Pilar, Pranjal, Rhiannon, Rob, Rui (Tina), Sarah, Suchi, Stephan, Tim, Tom, Vanessa, Wani and Wayne.

I would also like thank my friends, particularly Julie-Ann, Louise and Wendy, who always encouraged me. Most of all, my thanks to my son Jonathon (Jono) Swift, who was my inspiration and my biggest supporter throughout. I dedicate this thesis to him.

Abstract

Harnessing the abilities of soil microbes to improve plant health and productivity may be an important factor in obtaining food security for the future. In this study, 179 potential plant growth-promoting bacteria (PGPB) were isolated from the rhizosphere of five types of plants from three Western Australian soils. On the basis of *in vitro* plant growth promotion assays, seven isolates were selected for testing in field trials in Western Australia. Two of the PGPB, *Burkholderia caledonica* NCH45 and *Enterobacter soli* ANMK1, improved the yield of wheat by 23% and 9% respectively. The isolate, *Pseudomonas granadensis* PMK4, improved nodulation when co-inoculated with rhizobia on peas by up to 71% and grain yields by 35%. *P. granadensis* PMK4 was shown to inhabit the nodules of the field grown peas using strain specific primers developed in this study from the 16S-23S rRNA ITS1 region of this isolate.

P. granadensis PMK4 was also tested in field trials on Christmas Island on several legume species at three different fertilizer levels (nil, low and high). Significant increases in nodulation and/or plant yields were observed for soybean and mungbean co-inoculated with PMK4 and rhizobia at a low level of applied fertilizer compared with rhizobia only controls. Co-inoculation with PMK4 also significantly increased the copper and phosphorus concentration in the shoots of lablab and soybean at the nil (lablab) and low (lablab and soybean) fertilizer levels. Glasshouse trials using a full phosphorus response curve demonstrated that phosphorus solubilisation is not the mechanism of action by NCH45 and PMK4 in wheat. However, growth pouch assays using the auxin transport inhibitor, 2,3,5-triiodobenzoic acid, indicate that production of indole-3-acetic acid may be at least partly responsible for increasing wheat seedling root

lengths. These results support the further testing of the three promising isolates in field

trials to determine optimal conditions for improving plant productivity.

Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
Amp	Ampicillin
BNF	Biological nitrogen fixation
CAP	Calcium phosphate ($CaHPO_4$)
CAS	Chrome azurol S (agar)
Cm	Chloramphenicol
Cx	Cycloheximide
DF	Dworkin and Foster (medium)
DMF	Dimethylforamide
DRMO	Deleterious rhizosphere microorganisms
GC-MS	Gas chromatography mass spectrometry
GA	Gibberellin
GPB	Glucose peptone broth
Grp E	*Rhizobium leguminosarum* bv. *viciae* SU303
HCN	Hydrogen cyanide
HDTMA	Hexadecyltrimethylammonium bromide
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin layer chromatography
IAA	Indole-3-acetic acid
IAM	Indoleacetamide
IBA	Indole-3-butyric acid
IPyA	Indolepyruvic acid
LB	Luria-Bertani (medium)

L-TRP	L-tryptophan
MDAM	Moeller's decarboxylase agar medium
NA	Nutrient Agar
N-AHSL	N-acyl homoserine lactone
NB	Nutrient Broth
NBRIP	National Botanical Research Institute's phosphate growth medium
NCBI	National Centre for Biotechnology Information
NO	Nitric oxide
PAF	*Pseudomonas* AF medium
PDA	Potato dextrose agar
PGPB	Plant growth-promoting bacteria
PGR	Plant growth regulator
PSB	Phosphorus solubilising bacteria
PSM	Phosphorus solubilising microorganism
PVK	Pikovskaya (medium)
SDW	Shoot dry weight
SNA	Starch nitrate agar
TE	Tris EDTA (medium)
TFB	Transformation buffer
TIBA	2,3,5-triiodobenzoic acid
TSB	Trypticase Soy Broth
TTC	2,3,5-triphenyltetrazolium chloride
TY	Tryptone-yeast medium

Chapter 1:
Literature Review

Pea field at Kojonup

1.1   Agriculture and future food security

It is estimated that the world's population will increase from the 7 billion at the end of 2011 to 9 billion by 2050. To feed this increasing population, without pushing the food prices above what the poorest in the world can afford, staple food production must increase by 60% by 2050 (Fischer et al. 2014).

An important constraint to meeting this increased demand for food is climate change. The predicted increase in global temperature and more frequent and intense drought and flooding events (IPCC 2013) will have different impacts in various agricultural regions, but without changes in agricultural practices, the overall impact will be negative.

The decline in agricultural productivity due to climate change may be partly alleviated by plant breeding programs. Improvement to abiotic stress tolerance has long been a target for breeding programs, however, the complex nature of stress tolerance poses many challenges (Sutton 2009) and for example, some genetically modified wheat varieties with introduced drought tolerance genes showed promise in glasshouse studies but not in the field (Fischer et al. 2014; Saint Pierre et al. 2012).

Other constraints to future food productivity include a decline in soil security through contemporary agricultural practices. Soil security is defined as "the maintenance and improvement of soils worldwide so that they can continue to provide food, fibre and fresh water, contribute to energy and climate sustainability and help to maintain biodiversity and protect ecosystem goods and services" (Koch et al. 2012). History has frequently demonstrated the disastrous consequences for civilizations when soil conservation practices are ignored; when fertile soils washed or blew away (Koch et al. 2013). In recent times, applications of fertilizers and pesticides have had adverse effects on soils through increasing heavy metal concentrations (Atafar et al. 2010;

McLaughlin et al. 2000) and soil acidification (Tian and Niu 2015) as well as contributing to non-point source pollution (Sharpley and Menzel 1987; Sun et al. 2012). Use of these agrichemicals have also uncoupled plants from the beneficial microorganisms that that maintain essential soil processes and ensure effective plant production (Lucero et al. 2014).

Recent advances in molecular techniques such as metagenomics have highlighted the interdependence between plants and the surrounding microbiome (Barrow et al. 2008). Up to 20% of the plant's photosynthetically fixed carbon is released into the rhizosphere, which creates an energy-rich habitat around the roots that hosts a large microbiome (Bisseling et al. 2009; Zamioudis et al. 2013), with one gram of soil typically containing up to 10^9 bacteria (Zamioudis 2013). It is clear that the plant microbiome contains a vast genetic reservoir with enormous potential for plant improvement (Barrow et al. 2008). As microbes have a shorter generation time and a large genetic diversity, they can respond much more rapidly to climate and environmental challenges than plants similarly situated. Lucero et al. (2014) suggest that these microbial communities may be able to help plants adapt to these challenges more rapidly than plant genetic adaption alone, and if this is the case, the microbial community could be considered an easily managed extension as of the plant's genotype (Donn et al. 2015).

Contemporary agricultural practices have resulted in the loss of microbial biodiversity (Lucero et al. 2014) as plant breeding programs, mechanical planting, tilling, cultivation and harvesting, as well as the application of fertilizers and pesticides, have disturbed the beneficial microbial communities which in turn increases the reliance on artificial inputs to maintain productivity (Lucero et al. 2014).

This suggests that restoring microbial diversity to agroecosystems could be fundamental for agricultural sustainability under the challenges to food security towards 2050. For instance, inoculation of soil with *Nostoc muscorum*, without tillage, increased soil aggregate stability and lettuce seedling emergence; while tillage decreased aggregate stability and seedling emergence (Rogers and Burns 1994). Inoculation with this cyanobacterium also increased the soils' indigenous microbial communities. Furthermore, Nie et al. (2014) demonstrated that soil aggregate size mediates soil microbial feedback to multiple climate change factors and they further suggest that soil microorganisms may be able to adjust nutrient acquisition ratios inienn response to climate change.

1.2 The potential of soil microbes to promote plant growth and health

Beneficial bacteria may impact on plant growth and development either indirectly or directly (Penrose and Glick 2003). Indirect promotion of plant growth occurs when bacteria decrease or prevent some of the deleterious effects of phytopathogenic organisms (Penrose and Glick 2003), and the term plant growth-promoting rhizobacteria (PGPR) was coined by Kloepper and Schroth (1978) to describe these bacteria. Some mechanisms of action in these bacteria include antibiotic protection against pathogenic bacteria, suppression of nematodes, reduction of iron available to phytopathogens, synthesis of fungal cell wall-lysing enzymes and competition with detrimental microbes for sites on plant roots (Lucy et al. 2004). For reviews on indirect mechanisms of action by PGPR, please see Whipps (2001), Zehnder et al. (2001), Compant et al. (2005), Beneduzi et al. (2012), and Tailor and Joshi (2014).

Direct promotion of plant growth is the process where plants take up compounds that are synthesised by soil microbes or where microbial action facilitates the uptake of

nutrients from the environment. Bashan and Holguin (1998) suggest that these bacteria be known as plant growth-promoting bacteria (PGPB) and that bacteria with biological control mechanisms (previously PGPR), be known as biocontrol-PGPB, and this terminology is adopted in this thesis.

Examples of direct growth promotion include the synthesis of bioavailable phosphorus that is available for plant uptake, nitrogen fixation, sequestration of iron for plants by siderophores, and production of plant hormones and vitamins that the plants may access (Glick 1995; Glick et al. 1999). This review focuses on the direct modes of action of PGPB on the growth promotion of plants.

1.3   PGPB as biofertilizers

Fertilizer pricing is directly linked to fossil fuel prices and since the spike in 2008, fertilizer prices have remained at high levels to 2015. Additionally, over the last 20 years in some areas, nutrient use efficiency has decreased as increases in fertilizer application have not resulted in corresponding increases in yield (Shen et al. 2012; Zhang et al. 2010). This overuse of fertilizers has occurred because the biological processes in soil nutrient acquisition have either been underestimated and/or ignored (Shen et al. 2012). If PGPB were able to be substituted for even a fraction of the fertilizers currently in use, this would decrease pressure on these non-renewable resources, reduce the environmental impacts of their use and potentially reduce food production costs.

1.3.1   Biological nitrogen fixation

Biological nitrogen fixation (BNF) by rhizobia in symbiosis with legumes is a major input of nitrogen to agricultural soils and the inoculation of crops with rhizobia can be traced back for centuries (Bashan 1998). By the end of the 19th century, mixing soil

from a previous legume crop with soil in which legumes were to be grown was common practice to improve yields (Bashan 1998; Smith 1992). In 1896 an inoculant 'nitragin' that contained rhizobia was marketed as a commercial product (Arshad and Frankenberger 1991) and the practice of legume inoculation with rhizobia became common in the United States (Bashan 1998). The Australian government began distributing rhizobial inoculants to farmers in NSW in 1914 and peat-based carriers for inoculants were adopted by the NSW Department of Agriculture in 1952 (Bullard et al. 2005). It is estimated that global N_2 fixation by legume-rhizobia symbiosis is in the order of 33-46 Tg N annually (Herridge et al. 2008).

The bacterial conversion of atmospheric nitrogen (N_2) into ammonia is catalyzed by the enzyme nitrogenase in the reaction:

$$N_2 + 8H + 8e^- + 16\ ATP \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16\ ADP + 16\ P_i$$
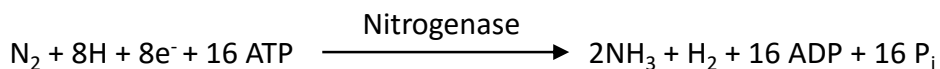
In legumes, this occurs in specialized root organs called nodules and nodulation is initiated by the secretion of molecules, mostly flavonoids, by the plants that are sensed by the rhizobia. The rhizobia synthesize Nod factors in response, which trigger nodule development (Desbrosses and Stougaard 2011). The supply of ammonia by N_2 fixing rhizobia to their legume host plants is well understood (Desbrosses and Stougaard 2011; Gualtieri and Bisseling 2000; Jones et al. 2007; Mylona et al. 1995; Oldroyd et al. 2011; Spaink 2000; Udvardi and Poole 2013). Therefore, this review will focus on biological nitrogen fixation (BNF) in non-legume plants.

Bacteria that colonise the interior of the plant (endophytes) and free-living bacteria in close association with the root (associative) are also able to convert atmospheric N_2 to

ammonia that is of benefit to plants. These bacteria are commonly referred to as diazotrophs (Headey and Fan 2010), and it is speculated that up to 29 Tg N is fixed by these organisms per annum (Herridge et al. 2008). The most studied diazotrophs are *Azospirillum* spp. and in the 1970's, Dobereiner and her collaborators (Dobereiner and Day 1976) "rediscovered" the *Azospirillum* species which had been isolated 50 years previously and then forgotten. While researching dinitrogen fixation in grasses, the *Azospirillum* (previously *Spirillum*) was found to enhance non-legume (tropical grass) plant growth (Dobereiner and Day 1976) by directly affecting plant metabolism (Bashan and Holguin 1997).

Other associative and endophytic N_2 fixing bacteria include *Azoarcus* spp., *Burkholderia* spp., *Enterobacter* spp., *Gluconacetobacter diaztrophicus* (previously *Acetobacter*), *Herbaspirillum* spp., and *Phyllobacterium* spp. (Baldani et al. 2000; Hurek et al. 2002; Mirza et al. 2001; Rojas et al. 2001; Sevilla et al. 2001). However, in contrast to *Rhizobium* spp. living symbiotically within the host plant that excrete nitrogen from their cells (Mylona et al. 1995), diazotrophs do not appear to excrete biologically fixed nitrogen (BNF) (Vessey 2003). Rather the fixed nitrogen is released to the host mainly after death and decay of the bacterial biomass (Rao et al. 1998).

For instance, studies using endophytic wild-type strains, *nifH* reporter strains and mutants unable to fix nitrogen (*nif⁻*) of *Azoarcus* sp. strain BH72 (Egener et al. 1999; Hurek et al. 2002) and *Acetobacter diazotrophicus* (Sevilla et al. 2001) have demonstrated that there is transfer of nitrogen from the diazotroph to the host plant. Although the diazotrophs were fixing N_2 *in planta*, the researchers were unable to determine whether the transfer was direct or indirect after death of the bacteria (Dobbelaere et al. 2003). Likewise, use of the ^{15}N natural abundance technique for

determining N_2 fixation in the field and greenhouse experiments have shown that a substantial percentage of nitrogen in certain crops is derived from BNF (Boddey et al. 2001; Lima et al. 1987; Montañez et al. 2009; Taulé et al. 2012; Yoneyama et al. 1997). However, in these studies, the organisms responsible for BNF were not identified, and there is no evidence that growth stimulation was due to the direct transfer of fixed nitrogen to the plants.

However, White et al. (2012) demonstrated that seedlings of some members of the grass family secreted H_2O_2 that oxidized diazotrophic bacteria and allowed the plants to harvest the nitrogen fixed by the bacteria. H_2O_2 production was only detected in species in which the seeds had adherent paleas and lemmas, which may serve to cultivate and deliver diazotrophic bacteria to the seedlings. Wheat, rye, sorghum and maize do not produce seeds with adherent paleas and lemmas, which have been removed through breeding and cultivation, and the authors suggest that reestablishing symbioses with diazotrophs may reduce the need for nitrogen inputs in these cereals. For instance, using a *nifH* mutant and ^{15}N isotope dilution techniques, Iniguez et al. (2004) demonstrated that wheat plants inoculated with *Klebsiella pneumoniae* 342 derived 41-49% of their nitrogen from BNF 6 weeks after planting.

An example of the successful use of diazotrophs to in broad scale agriculture is the Brazilian sugarcane industry (de Carvalho et al. 2011). The application of N fertilizer to sugarcane in Brazil is less than 60 kg N per hectare and yet the sugarcane crops accumulate between 100 and 200 kg N per hectare per year (Boddey et al. 2003; Reis et al. 2007), meaning Brazil uses up to one tenth less nitrogen fertilizer than other countries (de Carvalho et al. 2011; Lin et al. 2012). While the surplus N in sugarcane in Brazil has been demonstrated to be acquired from BNF (Table 1.1), studies in Australia

(Biggs et al. 2002) and South Africa (Hoefsloot et al. 2005) concluded that BNF was not a major contributor to the nitrogen balance in sugarcane grown in these areas. It has been suggested that Brazilian sugarcane varieties from the initial breeding program were under selection pressure for BNF as soil fertility was low and N fertilizer was not used (Boddey et al. 2003). BNF has also been demonstrated in several other important non-legume species (Table 1.2).

Improved plant growth can also be observed with some diazotrophs when N_2 fixation can be discounted. For instance, the Nif⁻ mutant of *A. brasilense* Cd was able to improve several growth parameters of tomato, to a similar extent as the wild type, even though it was unable to fix N_2 (Bashan et al. 1989). Similarly, the growth promotion of rice inoculated with *Azoarcus* spp. was not diminished with the Nif⁻ mutant (Hurek et al. 1994). Under N-limiting conditions, the Nif⁻ mutant of *G. diazotrophicus* did not improve sugarcane growth, but when N was not limiting, the mutant enhanced sugarcane growth to a level comparative to the wild type (Sevilla et al. 2001). Even in the absence of BNF, several species of rhizobia have been shown to improve N, P and K uptake (Biswas et al. 2000). It is speculated that the mechanisms may include alterations to root system architecture and the production of phytohormones (de Carvalho et al. 2011; Eskin et al. 2014; Vessey 2003).

The challenge for wider application of BNF in non-legume systems is to identify the most effective host-diazotroph partnership(s) for the target edaphic conditions. While endophytes are attractive as diazotrophs, as they can colonise the plant interior in niches where maximal expression of nitrogen fixation can occur, there are limiting factors to their application. For instance, some diazotrophs, such as *G. diazotrophicus*, appear to be obligate endophytes and have a low survival in the soil in the absence of a host plant

(Baldani et al. 1997). Furthermore, some plant cultivars demonstrate specificity for certain diazotrophs (de Carvalho et al. 2011; Yoneyama et al. 1997) and a wide diversity in effectiveness for BNF (Montañez et al. 2009). One of the factors affecting the ability of plant cultivars to exploit BNF is differential sensitivity to fertilizers (Montañez et al. 2009) which can also have an adverse effect on some diazotroph species (Bueno Dos Reis Junior et al. 2000).

For instance, Carvalho et al. (2014) speculate that N status in plants could regulate BNF through regulating bacterial colonization and therefore allow colonization by diazotrophs only in suboptimal N conditions. Clearly, a better understanding of the association of diazotrophs and non-legume hosts is necessary to maximize the benefits of these interactions. Research to optimize BNF for non-legume species promises considerable long-term savings in N fertilizer application and reduction of non-point source pollution.

1.3.2 Phosphate solubilisation

After nitrogen, the mineral nutrient that commonly limits the growth of plants is phosphorus (P) (Vessey 2003). Plants derive their P requirements almost exclusively from the inorganic (P_i) forms (HPO_4^{-2} and $H_2PO_4^{-1}$) contained within the soil solution (Richardson 2001). Although soils may have large reserves of total P, due to the insolubility of P compounds, the amount of P in the soil solution at any given time is usually very low (Holford 1997). Subsequently, the application of soluble P fertilizers such as superphosphate is commonplace, particularly for cereal crops (Whitelaw et al. 1997). Unfortunately, the recovery of P from fertilizer is poor with only 10-20% of applied P being obtained by plants (McLaughlin et al. 1988).

Table 1.1: Evidence for the contribution of biological nitrogen fixation (BNF) to the N balance in sugarcane

Inoculant/inoculation technique	Type of study	Method for determination of BNF	Contribution of BNF	Reference
Natural soil microflora	Pots placed in the field, Brazil	^{15}N-labelled fertilizer	Up to 25 g N per 21 month old plant (40-60%)	Lima et al. (1987)
Natural soil microflora	Concrete tanks, Brazil	^{15}N-labelled soil	Up to 70%	Urquiaga et al. (1992)
Natural soil microflora	Field (Brazil, Japan, Phillipines)	^{15}N abundance	0-72%	Yoneyama et al. (1997)
Natural soil microflora	Field (11 sites), Brazil	^{15}N abundance	0-60%	Boddey et al. (2001)
Acetobacter diazotrophicus PA15	Growth chamber, greenhouse, field, Brazil	^{15}N$_2$ incorporation, *nifH* mutant	-0.4-0.2% over 24 hour period	Sevilla et al. (2001)
Natural soil microflora	Glasshouse and field, Japan	^{15}N isotopic dilution, δ^{15}N abundance	Glasshouse: Stem 12%, leaves 21%, roots 26%; Field 27-38%	Asis et al. (2002)
Natural soil microflora	Field, Australia	δ^{15}N abundance	No significant BNF observed	Biggs et al. (2002)
Five endophytic diazotroph species	Glasshouse, Brazil	^{15}N isotopic dilution	The highest BNF was recorded for a combination of the 5 species (23.7-33.7%)	Oliveira et al. (2002)
Natural soil microflora	Field and glasshouse, South Africa	^{15}N$_2$ incorporation, δ^{15}N abundance	No significant BNF observed	Hoefsloot et al. (2005)
Natural soil microflora	Glasshouse, covering 2 seasons, Japan	^{15}N$_2$ incorporation	18-24% during high temp. season, negligible during low temp. season	Thaweenut et al. (2011)
Natural soil microflora	Long term field trial (1990-2004), Brazil	^{15}N abundance	40 kg N ha^{-1} yr^{-1}	Urquiaga et al. (2012)
Natural soil microflora, some later identifed	Greenhouse, Uruguay	^{15}N-labelled fertilizer	41-59% at 10 mg applied N kg^{-1} and 35-50% at 50 mg applied N kg^{-1}	Taulé et al. (2012)
Enterobacter spp.	Growth chamber, China	^{15}N isotopic dilution	6.7-6.7% 55 days after transplant	Lin et al. (2012)
Burkholderia australis sp. nov.	Growth chamber, Australia	^{15}N$_2$ incorporation	Significant BNF observed	Paungfoo-Lonhienne et al. (2014)

Table 1.2: Evidence for the contribution of biological nitrogen fixation (BNF) to the N balance in rice and maize

Inoculant/inoculation technique	Host	Type of study	Method for determination of BNF	Contribution of BNF	Reference
A. liopferum *A. brasilense* *Herbaspirillum* spp. *Azoarcus* K-1 *Zoogloea* Ky-1 *Pseudomonas* 96-51	Rice	Greenhouse using the Super Basmati and Basmati 385 varieties	^{15}N isotope dilution	20-59 (% Ndfa) 20-47 (% Ndfa) 20-58 (% Ndfa) 11-22 (% Ndfa) 22-47 (% Ndfa) 24-44 (% Ndfa)	Mirza et al. (2000)
H. seropedicae *Burkholderia* spp.	Rice	Glasshouse, 80 days after transplanting	^{15}N isotope dilution	17-19 % leaves plus stem, 4.4 % seeds 11-20 % leaves plus stem, 3.8 % seeds	Baldani et al. (2000)
Herbaspirillum sp. B501	Rice	Controlled temp, 24 hours	^{15}N$_2$ incorporation	381 δ^{15}N (‰)	Elbeltagy et al. (2001)
B. vietnamiensis MGK3 *B. vietnamiensis* LMG10929	Rice	Plants obtained from glasshouse and field, maintained in controlled temp in vermiculite filled tubes	^{15}N isotope dilution	36-40 % pot; 39-42 % field grown 36-37% pot; 37-40% field grown Note, these plants were grown in vermiculite and plants can access N from interstitial pores thus overestimating N$_2$ fixation (Giller et al. 1986)	Govindarajan et al. (2008)
A. amazonense *A. brasilense* (Cd)	Rice	Greenhouse	^{15}N isotope dilution	9.7-28 (% Ndfa) 15 (% Ndfa)	Rodrigues et al. (2008)
Natural soil microbiota	Maize	Growth room, 10 cultivars, 2 N rates	^{15}N isotope dilution	12-27% in 5 mg N kg^{-1} 12-24% in 25 mg N kg^{-1}	Montañez et al. (2009)
H. seropedicae (12 strains) *H. rubisubalbicans* (5 strains) *H. frisingense* (4 strains)	Maize	Greenhouse and field	^{15}N abundance	Up to 37 % (Ndfa) on maize genotype SHS5050 inoculated with *H. seropedicae* ZAE94 in the field	Alves et al. (2015)

Ndfa = nitrogen derived from air

Plants have various strategies for improving the uptake of P in limiting conditions. These generally relate to the capacity of the roots to explore regions of the soil more replete with plant available forms of P (Richardson et al. 2009). P is predominantly found in the top layer of the soil where fertilizer application combines with organic matter to immobilize this nutrient (Lynch and Brown 2001). Plants with shallow root angles and increased branching in the surface soil have a greater ability to exploit P and other nutrients in this region, although this may compromise the ability of the plant to access water at deeper soil depth during drought conditions (Ho et al. 2005). Root hairs are also a major contributor to P acquisition in some plant species. Up to 90% of the total uptake of P can be attributed to root hairs which is partly credited to increasing the root surface area (Föhse et al. 1991). Some plant species form cluster roots which are compact clusters of branch roots (rootlets) or root hairs in a small volume, typically developing in the upper layers of the soil, that increase the root system's surface area in this nutrient rich area (Lambers et al. 2006).

Other traits to improve P acquisition include expression of high-affinity P transporters, hydraulic redistribution, the release of root exudates and interactions with soil microorganisms (Lambers et al. 2006). For instance, mycorrhizal fungi form symbiotic associations with over 80% of land plant species (Wang and Qiu 2006), and this can provide the dominant uptake route for P in these plants (Smith et al. 2003). Soil microbes also play an important role in the cycling of phosphorus in natural ecosystems (Whitelaw et al. 1997). Phosphorus is consumed by plants and is returned to the soil either in plant or animal residues which are in turn broken down by soil microbes (Whitelaw et al. 1997). In fact, 68-78% of the total soil biomass P is contained in soil microbes with plant P representing only a small fraction of the biomass P (Turner et al. 2013). Using oxygen isotopes, Tamburini et al. (2012) demonstrated that the available

fraction of P is channeled through soil microorganisms before being released to plant available pools upon microbial death or cell lysis. Microbial biomass P is rapidly turned over in the soil and is believed to be a significant contribution of P nutrition to plants (Kouno et al. 2002; Turan et al. 2012). In subsistence or organic agriculture, the microbial P flux is the rate limiting step in the supply of nutrients to plants and better management of the microbial biomass may greatly improve low input agricultural systems (Kouno et al. 2002).

Soil microbes mineralize organic P in the soil through the production of phosphatase enzymes. Unlike plants, that only produce acid phosphatases, microbes can also produce alkaline phosphatases and it is suggested that microbially produced phosphatases are the major enzymes in the soil (Dick et al. 1983; Nannipieri et al. 2011; Rodriguez and Fraga 1999). The solubilisation of inorganic P has been demonstrated by a wide range of soil microbes including the bacteria, *Actinomyces*, *Pseudomonas*, *Bacillus* and *Rhizobium* spp. and fungi such as *Aspergillus* and *Penicillium* spp. (Nautiyal et al. 2000; Richardson 2001; Richardson and Simpson 2011; Rodriguez and Fraga 1999). These P-solubilising microorganisms produce organic acids, protons, hydroxyl ions and siderophores which can mobilize mineral P through anion exchange of PO_4^{2-} or chelation of calcium, aluminum or iron ions associated with P (Jones 1998; Khan et al. 2009; Nautiyal et al. 2000; Park et al. 2009; Sharma et al. 2013). However, it is suggested that the production of organic acids by plant roots and their associated microbes is the major route for solubilisation of phosphorus in the rhizosphere (Bashan et al. 2013b). For instance, soybean exudes malate under P deficiency conditions in acid soils (Liang et al. 2013) and malate also selectively recruits beneficial soil bacteria, in particular *Bacillus subtilis* (Rudrappa et al. 2008), which is capable of solubilising rock

phosphate (Hassimi et al. 2013) possibly through gluconic acid production (Tahir et al. 2013).

Glucose is converted to gluconic acid by glucose dehydrogenase (Gcd) and gluconic acid to 2-ketogluconate by gluconate dehydrogenase (Gad) in the periplasmic space of bacteria (de Werra et al. 2009). *P. fluorescens* CHA0 with the mutation Gcd (Δ*gcd*) or Δ*gcd* Δ*gad*, showed a reduction in production of gluconic acid and loss of P solubilisation ability on NBRIP agar containing an insoluble form of P (de Werra et al. 2009). In contrast, the Δ*gad* mutant resulted in an accumulation of gluconic acid and an increase in P solubilisation on the agar plates showing that phosphorus solubilisation by this bacterium is at least partly dependent on its ability to produce gluconic acid.

Despite the large number of phosphorus solubilising bacteria (PSB) isolated from soils and the rhizosphere, many putative PSB prove ineffective for P uptake in plants when tested for phosphorus solubilisation under field conditions (Bashan et al. 2013b; Khan et al. 2009). In the 1930's and 1940's, inoculation with a nonsymbiotic, associative bacterium, *Azotobacter*, was used in a preparation called "Azotogen" and commercialized in the Soviet Union for soil and seed treatments (Arshad and Frankenberger 1991; Bashan 1998). *Bacillus magaterium* var. *phospaticum*, which was thought to enhance phosphorus availability, was also developed in the 1930's as an inoculant by Soviet microbiologists (Arshad and Frankenberger 1991; Bashan 1998; Brown 1974; Cooper 1959). However, both these early nonsymbiotic inoculants were abandoned as they either failed or produced inconclusive results (Bashan 1998).

More recently, Valverde et al. (2006) isolated up to 2×10^4 PSB cells from Spanish agricultural soils using plate assays, and tested the highest P-solubilising isolate, *P. jessenii* further in the glasshouse and field. When co-inoculated with *Mesorhizobium*

ciceri on chickpeas, this isolate improved plant biomass and grain yield but did not improve P uptake by the plants. In contrast, when inoculated with several different PGPB, either singly or as mixed inoculants, improvements to both the yield and P concentration of wheat plants in field studies was observed by Turan et al. (2012). In this study, increases in microbial P concentrations were shown to correspond to the increases in P concentration in various plant organs.

Other field trials that demonstrate increased plant uptake of P mediated by PSB are detailed in Table 1.3. However, the mechanism of increased P uptake by plants inoculated with these bacteria has not been determined and in some cases, only the uptake of P is reported, which may simply be due to an increase in plant biomass.

It is difficult to determine the mechanism of increased P uptake in plant trials as potential P solubilising genes may serve essential functions for the bacteria that would be detrimental to their growth if inactivated. For instance, in the presence of glucose as a sole carbon source, initial growth is delayed in the *gcd* and *gad* mutants of *P. fluorescens* CHA0 (de Werra et al. 2009). As glucose is a major carbon source exuded by plant roots (de Werra et al. 2009), it is possible that this delay may cause the bacteria to be rapidly outcompeted by other microbes in a glucose enriched rhizosphere, leading to inaccuracies in the potential for P solubilisation by these bacteria on certain plants. Furthermore, mechanisms other than P solubilisation by the bacteria are potentially responsible for increased P uptake by plants in the field. For instance, the production of phytohormones by the PSB may increase root surface area, facilitating an increase in P acquisition by the plants (Kumar and Narula 1999).

Table 1.3: Increases in yield or P concentration/uptake in field trials with phosphate solubilising bacteria on various plant species.

Inoculant	Plant species	Findings	Reference
Rhizobium leguminosarum strains, *Serratia* sp. *Pseudomonas* sp. *Rhizopus* sp.	Lettuce and maize	Increases in dry matter were observed for lettuce at the low and highly fertile soil sites, but P concentration was only increased in the highly fertile soil by a strain of *R. leguminosarum*. At the moderately fertile soil site, P concentration was increased with the *R. leguminosarum* strains and *Rhizopus* sp.	Chabot et al. (1996)
Azospirillum, Azotobacter, Mesorhizobium and *Pseudomonas* spp.	Chickpea	All inoculants improved yields and P uptake compared with the controls, especially *Azospirillum* and *Azotobacter* treatments.	Rokhzadi and Toashih (2011)
Bacillus spp.	Wheat	Inoculation with *Bacillus* spp. with ACC deaminase and P solubilisation traits increased biomass and P uptake in wheat, with the highest results obtained from bacteria exhibiting both traits.	Baig et al. (2012)
Pantoea cypripedii and *Pseudomonas plecoglossicida*	Maize	Grain yields and concentration of P in plant tissues is increased in an organic field	Kaur and Reddy (2013)
Pantoea cypripedii and *Pseudomonas plecoglossicida*	Wheat and maize	The PSB increased the P uptake in wheat and maize without fertilizer and when used in conjunction with rock phosphate (RP) compared with uninoculated and RP controls	Kaur and Reddy (2015)
Serratia marcescens and *Pseudomonas* sp.	Maize	Grain yields and P uptake were increased	Hameeda et al. (2008)

Although the exact mechanism(s) by which P-solubilising rhizobacteria promote plant growth is still unclear, it is obvious that mobilization of P by soil microbes is a major contributor to plant P nutrition (de Freitas et al. 1997; Richardson et al. 2011). The combination of biochemical and genomic analyses will facilitate a better understanding of the mechanisms involved in P solubilisation by PSB and may illustrate how these mechanisms can be manipulated for more effective P utilization in agricultural systems.

1.3.3   Iron acquisition

Iron is another essential mineral required by plants that is relatively insoluble in soil solutions (Vessey 2003). In sterilized soils, there is a reduced uptake of Fe and decreased vigor in plants (Jin et al. 2006; Masalha et al. 2000; Rroço et al. 2003). This suggests that microbes play an important role in the mobilization of Fe in the soil, and can potentially be exploited to enhance the Fe status of plants in agriculture.

Ferric (Fe^{2+}) ion is more easily absorbed by plants. In soils that are well aerated, ferric (Fe^{2+}) ion is oxidised to the ferrous (Fe^{3+}) ion which precipitates in the form of iron oxides making Fe^{3+} the most common form found in soil (Salisbury and Ross 1992). Furthermore, at pH > 6, total inorganic Fe drops to approximately 10^{-5} fold lower than that required for mass flow transport (Lindsay 1974).

Two strategies have evolved in plants that facilitate iron acquisition. The first involves the release of phenol–like ligands that chelate Fe^{3+}. The chelated iron is delivered to the root surface where it is reduced to Fe^{2+}, and while still chelated, the reduced Fe^{2+} is absorbed by the plant (Salisbury and Ross 1992). In the second strategy, which appears to be confined to the Gramineae (grasses) (Takagi 1993), the plants respond to iron-deficiency by forming and releasing powerful ligands that chelate Fe^{3+} (phytosiderophores) (Salisbury and Ross 1992). This system also involves a high-

affinity transport system in the root cells for Fe^{3+}-phytosiderophore complexes (Marschner and Romheld 1994). The roots absorb the Fe^{3+} phytosiderophore complex and reduce the iron to Fe^{2+} which is then released and utilised by the plant (Salisbury and Ross 1992) (Figure. 1.1). Plant species that are high producers of phytosiderophores, such as barley, wheat, and rye, are less susceptible to iron deficiency compared to lower producers such as rice and maize (Ahmed and Holmström 2014).
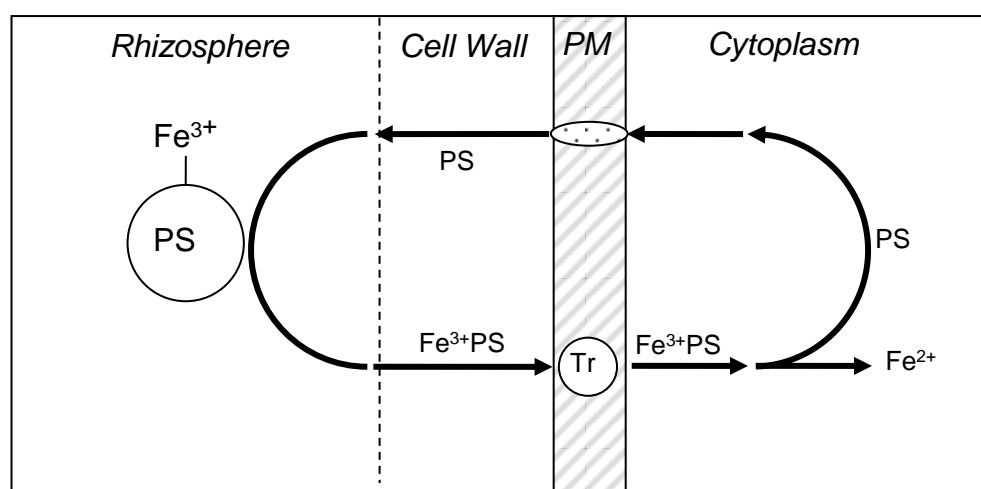
Figure 1.1: **Model for root responses to iron deficiency in Strategy II plants.** **PS = phytosiderophore; Tr = translocator for Fe3+ phytosiderophores in the plasma membrane; PM = plasma membrane. Modified from Marschner and Romheld (1994).**

Rhizosphere microbes may also be involved in supplying Fe to plants as, with few exceptions, aerobic and facultative anaerobic bacteria can form siderophores (Neilands and Leong 1986). Bacterial siderophores demonstrate a higher stability constant than phytosiderophores (Ahmed and Holmström 2014) and the abundance of these bacterial siderophores in soil may increase the mobility of Fe in the soil and rhizosphere, making Fe more available for plants (Dobbelaere et al. 2003). There is also some evidence that plants can absorb Fe^{3+}-siderophore complexes (Bar-Ness et al. 1991), yet the uptake of microbial siderophores by plants is the subject of controversy (Marschner and Romheld 1994). Bar-Ness et al. (1992a) later concluded that the bacterial siderophores were

inefficient as Fe sources for the plant and that siderophore-producing bacteria are in direct competition with plants for Fe (Bar-Ness et al. 1992b). In contrast, Vansuyt et al. (2007) used an enzyme-linked immunosorbent assay and tracing of ^{15}N-pyoverdine to demonstrate that pyoverdine is incorporated into the plant and that iron chelated Fe-pyoverdine is more efficiently assimilated by the plant than Fe-EDTA.

Some researchers suggest a vital role for bacterial siderophores as a source of Fe for plants in alkaline or calcareous soils (Jurkevitch et al. 1988; Masalha et al. 2000; Sharma and Johri 2003), or contaminated soils (Rajkumar et al. (2005); 2006), or to reduce the negative impacts of iron chelates on the environment (Radzki et al. 2013). The evidence for Fe uptake by plants from microbial siderophores is limited in these studies except in the research by Jurkevitch et al. (1988) where Fe-siderophore complexes from *Pseudomonas putida* were the only Fe source for peanut plants. The plants treated with bacterial-siderophore had up to 75% of the chlorophyll content of those treated with FeEDDHA.

Recent research has also demonstrated a role for siderophores in the uptake of other essential metals required for metabolism and to protect the bacteria from metal toxicity. For instance pyochelin, a *Pseudomonas* siderophore with only moderate affinity for iron can strongly chelate divalent cations such as zinc and copper (Brandel et al. 2012). However, even essential metals can become toxic to bacteria and chelation of these metals by siderophores may protect the bacteria from their toxicity (Braud et al. 2010). In *P. aeruginosa* exposed to copper-stress conditions, the genes involved in the production of the siderophore pyoverdine are upregulated, but the genes involved in pyochelin and pyoverdine-Fe transport are downregulated (Schalk et al. 2011; Teitzel et al. 2006). As the siderophore uptake pathways are generally impervious to all the metal

ions except for iron (Braud et al. 2010), there is potential for these siderophore-chelated metals to become available to plants.

It has been proposed that microbially derived siderophores could enhance phytoremediation through improving removal of heavy metals from contaminated soils (Braud et al. 2009; Rajkumar et al. 2009; Rajkumar et al. 2010; Rojas-Tapias et al. 2012; Wang et al. 2011) however, the exact mechanism is yet to be elucidated. Siderophore production by bacteria is also implicated in biological control of plant pathogenic fungi through iron depletion in the target organism (Kloepper et al. 1980; Loper and Ishimaru 1991; Weller 2007), although it has been suggested that this role is only minor (Hamdan et al. 1991; Loper 1988; Ownley et al. 1992).

There are several other possible mechanisms by which soil microbes may enhance Fe acquisition of plants: acidification of the rhizosphere through the generation of protons by the microbes (Jin et al. 2014), or through increased proton release capacity of roots (as demonstrated by *Bacillus subtilis* GB03 in *Arabidopsis* (Zhang et al. 2009)) and regulation of iron uptake through the manipulation of signaling processes in plants. Zhang et al. (2009) demonstrated that GB03 also increased the transcript abundance of the strategy I iron uptake response genes Fe3$^+$-chelate reductase (*FRO2*) and the Fe^{2+} transporter (*IRT1*). The whole-plant iron content of *Arabidopsis* increased by two-fold 14 days after exposure to GB03. Production of plant hormones by soil microbes in modifying Fe deficiency signaling in plants is another possibility under investigation (Jin et al. 2014).

1.3.4 Vitamins

Vitamins are essential factors in a number of metabolic pathways; signaling molecules for biotic and abiotic stress responses; and antioxidants in plants, animals, and bacteria

(Asensi-Fabado and Munné-Bosch 2010; Colinas and Fitzpatrick 2015; Smith et al. 2007; Survase et al. 2006). Organically grown edible plants compared with those grown conventionally have a higher (5.7%) percentage of minerals and vitamins (Hunter et al. 2011) possibly due to the action of bacteria. Metabolites of *Acetobacter aceti* induced the production of vitamins in barley seeds (Yousaf et al. 2015) while in strawberries, mycorrhizal fungi and *Pseudomonas* spp. increased vitamin content both separately and when co-inoculated on the plants (Bona et al. 2015).

However, some plant and bacterial species cannot produce the vitamins that are essential to their metabolism (Palacios et al. 2014). For instance, the putative vitamin, pyrroloquinoline-quinone (PQQ), affects numerous physiological and biochemical processes in bacteria, plants and animals, yet so far its synthesis has only been determined in prokaryotes (Kasahara and Kato 2005; Misra et al. 2012). The human diet requires vitamin B_{12} and microbially produced B_{12} and organic fertilizer have been shown to increase B_{12} content in agronomically important plants which could be useful to ameliorate B_{12} deficiency in the vegetarian diet (Mozafar 1994; Mozafar and Oertli 1992).

Several soil bacterial species are vitamin auxotrophs and can obtain the vitamins they are unable to produce from plant root exudates (some of the vitamins may have initially been derived from other bacterial species (Palacios et al. 2014)). Additionally, certain bacteria, possess salvage pathways for vitamin synthesis rather than de novo synthesis pathways. For instance, *Rhizobium leguminosarum* bv. *viciae* strain 3841 lacks the *thiCOGE* genes essential for the de novo pathway for thiamine synthesis, but contain the *thiMED* genes responsible for a thiamine salvage pathway (Karunakaran et al. 2006). *R. leguminosarum* strains with only the *thiMED* pathway grow poorly on media

lacking thiamine suggesting that bacteria that lack the genes for the synthesis of vitamins may be at a competitive disadvantage in rhizospheres with little root exudate.

Co-inoculation of vitamin auxotrophs with vitamin producing strains may prove beneficial in agricultural systems. For instance, when *P. fluorescens* strain 267.1, which can produce several B group vitamins, was co-inoculated with the thiamine auxotroph, *R. leguminosarum* strain bv. *trifolii* 24.1, nodulation and plant biomass of red clover was increased compared to plants inoculated with the rhizobial strain alone (Marek-Kozaczuk and Skorupska 2001).

It has been proposed that production of vitamins by PGPB, particularly the B group, is a potential mechanism for improving plant growth (Revillas et al. 2000) and this possibility is being explored further (Palacios et al. 2014).

1.4   Production of plant growth regulators

The production of plant growth regulators (PGRs) is one of the most important mechanisms of action through which rhizosphere microbiota affects plant growth (Arshad and Frankenberger 1998). The five main classes of PGR are auxins, gibberellins, cytokinins, ethylene and abscisic acid (Teale et al. 2006) and have recently been supplemented by jasmonates, salicylates, strigolactones, brassinosteroids, polyamines and some peptides (Munné-Bosch and Müller 2013). These hormones interact to coordinate both normal plant growth and responses to stress (Munné-Bosch and Müller 2013). Application of PGPB capable of producing PGRs can enhance the rapidity of a plant's response to stress (Glick 2012). For instance, bacterially produced auxins promote root growth and therefore enable to the plant to better access nutrients and water in the soil under limiting conditions (Lambrecht et al. 2000).

1.4.1   Auxins

Naturally occurring auxins include indole-3-acetic acid (IAA), indole-3-butyric acid and phenoxyacetic acid (Dodd et al. 2010; Patten and Glick 1996) and the effects they have on plants include: gravitropism, thigmotropism and phototropism; apical dominance; vascular tissue differentiation in elongating shoots; promotion of adventitious roots; stimulation of fruit development; stimulation of ethylene synthesis; and inhibition of leaf and fruit abscission (Cassán et al. 2014; Chehab et al. 2009; Raven et al. 1999; Teale et al. 2006).

Soil fungi (Frankenberger and Poth 1987; Nassar and El-Tarabily 2005), bacteria (Sarwar and Kremer 1995), and algae (Moss 1965) are known to produce auxin. Bacteria synthesise auxin via at least five tryptophan-dependent pathways including the indole-3-pyruvate pathway that is a major auxin pathway in plants (Spaepen and Vanderleyden 2011). Indeed the ability of these organisms to produce auxins via several different pathways (Patten and Glick 1996) has confounded attempts to fully elucidate the mechanisms of auxin production and suggests multiple roles for auxins in bacteria (Spaepen and Vanderleyden 2011).

Auxin production is much higher in the rhizosphere soil than the bulk soil (Frankenberger and Arshad 1995; Rossi et al. 1984) with up to 80% of bacteria from the rhizosphere being known to produce IAA (Patten and Glick 1996). This high occurrence of auxin production by rhizosphere bacteria suggests that auxin has a fundamental role in the plant-microbe interaction. For instance, production of auxin may allow bacteria to detoxify excess tryptophan or tryptophan analogs secreted by the plant that are deleterious to bacterial cells (Bar and Okon 1992; Glick et al. 1999). Auxins may also affect root cell permeability and increase in root exudates for bacterial

growth (Bowen and Rovira 1999). Further, bacterial IAA has been shown to stimulate the development of the host plant root system (Patten and Glick 2002). This results in a greater root surface area and increases the available rhizosphere for bacteria while enabling the plant to access more nutrients from the soil (Vessey 2003). More recently, auxin has been demonstrated to be a signaling molecule in bacteria in both pathogenic and phytostimulatory situations, and also in the manipulation of microbial behaviour (Spaepen and Vanderleyden 2011; Van Puyvelde et al. 2011). For instance, in the presence of IAA, the expression of genes involved in the adaptation to unfavourable environmental conditions are induced in *Escherichia coli*, improving the bacteria's response to stress (Bianco et al. 2006).

Auxin production by soil microbes affects the physiology of different plant organs based on the developmental stage of the plant and its endogenous auxin levels (Glick 2012; Pilet and Saugy 1987). Auxin levels above or below the optimum destabilize auxin transport (PIN) proteins at the plasma membrane and induce PIN2 trafficking to the vacuole for degradation (Baster et al. 2013). These PIN2 proteins are essential in the response of the plant roots to gravitropism, and optimal auxin levels are required to stabilize PIN2 proteins for their action in gravitropic responses. Since the optimal levels of auxin in roots are more than five orders lower than for shoots (Glick 2012), it would be reasonable to assume that bacterially produced auxins have a more profound effect on roots than shoots.

As an example, while low concentrations of IAA or low-density inocula of bacteria that produce high levels of IAA can stimulate primary root elongation, high levels of bacterial IAA stimulate the formation of lateral and adventitious roots or may inhibit root elongation altogether (Xie et al. 1996). Additionally, Patten and Glick (2002)

found that canola seedling primary roots were 35-50% longer when treated with wild-type *Pseudomonas putida* GR12-2 than with an IAA-deficient mutant. In their study, the *ipdC* gene was knocked out and hence the indolepyruvic acid pathway inactivated, but low levels of auxin were still being produced via one of the L-tryptophan induced pathways.

Increasing auxin concentrations in the shoot can have a positive effect on shoot dry weights. A number of bacterial strains increased wheat shoot growth parameters and this was highly correlated with auxin production by the bacteria (Ali et al. 2009). Additionally, auxin production by the bacteria was significantly correlated with endogenous IAA content of the plants. All the auxin producing bacteria reduced root lengths but enhanced the number of lateral roots in wheat plants. Some of the effects of IAA produced by bacteria on root morphology may be explained through an upregulation of ethylene synthesis (Xie et al. 1996) (discussed below). For instance, *A. brasilense* FT236 is an IAA producing bacterium that doubled tomato shoot ethylene production and increased root hair development, an effect that was mimicked by exogenous application of ethylene and reduced by application of an ethylene inhibitor (Ribaudo et al. 2006).

It is also suggested that bacterial auxin metabolism may play a role in the beneficial plant-microbe interaction (Dodd et al. 2010). Bacterial IAA degraders can effect the concentration of auxins in the rhizosphere. In work carried out by Leveau and Lindow (2005) an IAA-degrading strain (*P. putida* 1290R) coinoculated with high auxin producing bacteria or IAA, negated the deleterious effects of high levels of IAA on radish roots and in some cases resulted in the stimulation of root development. These

results need to be confirmed using mutants unable to degrade IAA. Empirical studies demonstrating the role of auxin in plant growth promotion are listed in Table 1.4.

1.4.2 Ethylene

Ethylene has been described as distorting seedling growth, inducing fruit ripening and a number of other physiological processes from germination of seeds to senescence of various plant organs (Abeles 1973; Abeles et al. 1992). During stress responses, it inhibits root elongation, nodulation and auxin transport and speeds the plant aging process (Glick et al. 2007).

A diverse group of microflora have been shown to produce ethylene in soils (Arshad and Frankenberger 1989; Lynch 1972; Primrose 1976; Smith and Russell 1969). Ethylene concentrations in soils have been found to be higher than those known to damage plant roots in laboratory studies (Smith and Russell 1969). Ethylene inhibits root elongation at levels as low as 0.1ppm and at these concentrations can also cause a characteristic coiling of some roots (Abeles 1973).

In 1978 Honma and Shimomura purified a microbial enzyme that was able to degrade the ethylene intermediate 1-aminocyclopropane-1-carboxylic acid (ACC) to α-ketobutyric acid from a soil bacterium, *Pseudomonas* sp. (Honma and Shimomura 1978). It was proposed that the introduction of this gene into plants could be useful in limiting ethylene biosynthesis in plants and control the onset of fruit ripening and leaf and flower senescence (Sheehy et al. 1991). Klee et al. (1991) introduced the gene for this enzyme into tomato plants and reported a delay in fruit ripening and an extension of the shelf life of the fruits.

Table 1.4: Selected empirical studies which demonstrate that bacteria can promote plant growth through the production of indole-3-acetic acid (IAA)

PGPB	Host	Results	Reference
Azospirillum brasilense Sp6	Wheat	Inoculation of wheat seedlings with the Nif$^-$/IAA$^-$ mutant loses the ability to increase lateral root number and length compared with the Nif$^-$/IAA$^+$ mutant and the wild-type strain	Barbieri et al. (1986)
Azospirillum brasilense SP6	Wheat	A mutant with 90% reduced IAA biosynthesis is reduced in ability to promote root system development compared with the wild-type.	Barbieri and Galli (1993)
Pseudomonas putida GR12-2	Canola	An auxin overproducing mutant lost the ability to stimulate root elongation in canola compared to the wild-type strain	Xie et al. (1996)
Pseudomonas putida GR12-2	Canola/mung bean	An IAA-deficient mutant constructed by insertional mutagenesis is reduced in its ability to increase root numbers and lengths compared with the wild-type strain	Patten and Glick (2002)
Azospirillum brasilense Sp245	Wheat	An *ipdC* mutant with 90% reduced IAA production was reduced in its ability to increase plant biomass, compared with the wild-type strain, however constitutive or plant inducible *ipdC* mutants increased plant biomass compared to the wild-type strain	Spaepen et al. (2008)
Azospirillum brasilense Sp245	Common bean	The *ipdC* mutant FAJ009, with 90% reduced production of IAA, lost the ability to increase root biomass and nodulation compared with the wild type. Response to inoculation with Sp245 varied between bean genotypes.	Remans et al. (2008)
Bacillus amyloliquefaciens SQR9	Cucumber	Transposon mutants with reduced production of IAA demonstrated reduced biomass compared to the wild-type strain, but still increased biomass compared with the uninoculated control. Growth promotion is therefore attributed to more than one mechanism.	Shao et al. (2015)

Based on this and other work, Glick and his colleagues (Glick et al. 1998; Jacobson et al. 1994) suggested that the enzyme ACC deaminase is one of the mechanisms by which soil bacteria stimulate the growth of plants and proposed the following model for lowering of plant ethylene concentrations by PGPB (Fig 1.2) (Glick 2014). "Stress ethylene" is usually produced in two peaks. The first and smaller peak is derived from the induction of ACC synthase following a biotic or abiotic stress. This enzyme catalyses the first committed step in ethylene biosynthesis where S-adenosylmethionine (SAM) is converted to ACC. The ACC is converted to ethylene by the enzyme ACC oxidase and this small burst of ethylene induces transcription of genes involved in plant protection and/or defense. Additionally, IAA synthesised by a PGPB in response to tryptophan exuded by the seed or root, is taken up by the plant where it may (in conjunction with the endogenous plant IAA) either stimulate plant cell proliferation and/or elongation, or stimulate the activity of the enzyme ACC synthase.

A significant portion of the ACC produced is exuded from the plant and taken up by soil bacteria which acts as a sink for ACC. The ACC is hydrolysed in the bacteria by the enzyme ACC deaminase to ammonia and α-ketobutyric acid, providing a nitrogen source for the bacterium. The ACC levels within the plant are subsequently reduced and as the amount of ethylene produced by the plant decreases, there is a concomitant reduction in the second, deleterious peak of ethylene by 50-90%. Furthermore, the IAA signal transduction of ACC synthase is feedback inhibited by ethylene which limits the activation of ACC synthase by bacterially produced IAA. Therefore, with bacteria that produce both IAA and ACC deaminase, there is less feedback inhibition of IAA signal transduction so the bacterial IAA continues to promote plant growth and the additional ACC is cleaved by bacterial ACC deaminase lowering deleterious levels of ethylene in the plant.
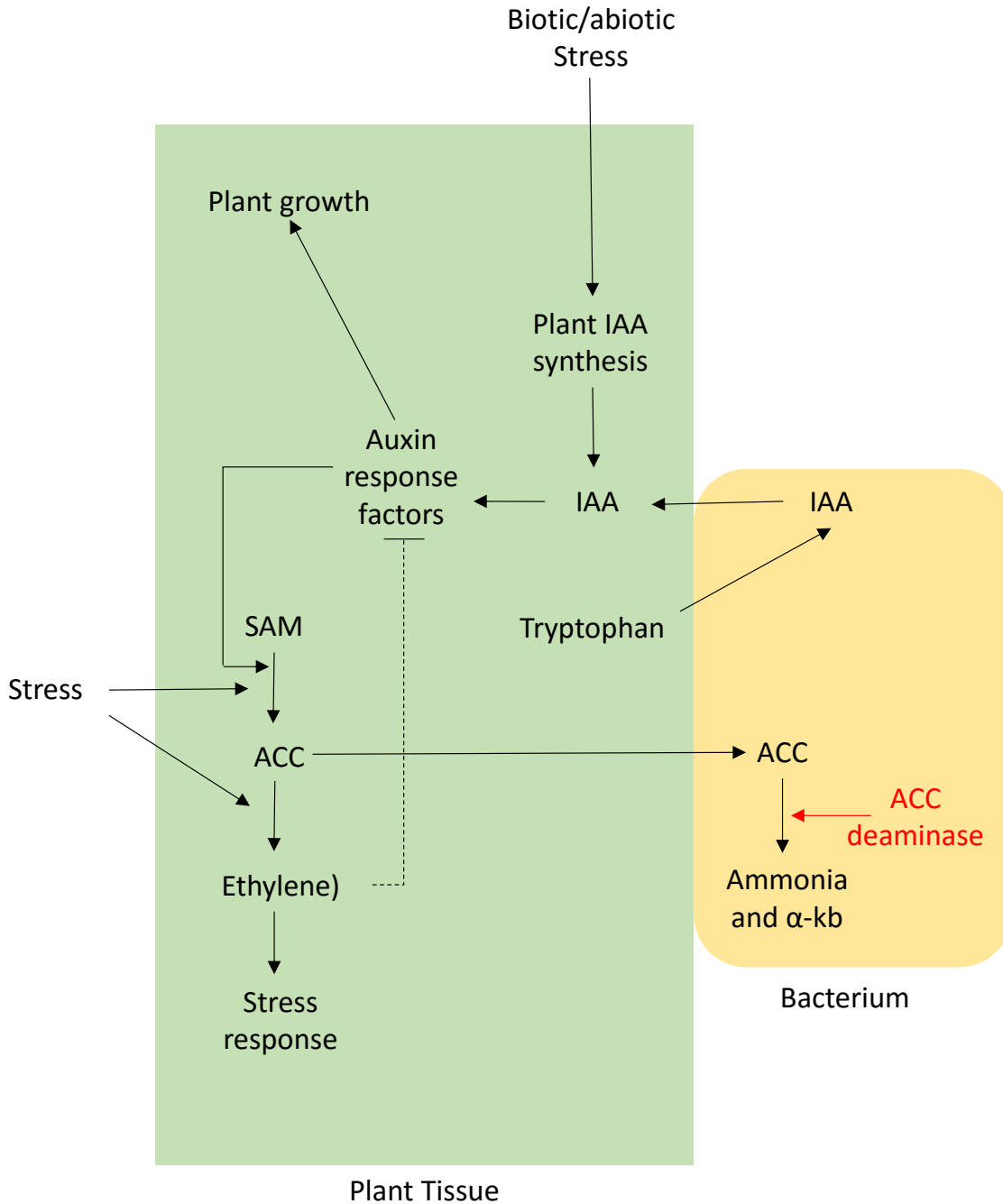
Fig 1.2: Schematic representation of how a PGPB that is able to produce both IAA and ACC deaminase, that is bound to either a seed or plant root, may facilitate plant growth. The arrows indicate a chemical or physical step in the mechanism and dashed line indicates inhibition. ACC deaminase (in red) is the only enzyme shown in this model. Key: IAA, indole-3-acetic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; SAM, S-adenosylmethionine; α-kb, α-ketobutyrate. Modified from Glick (2014)

Pseudomonas sp. UW4 (formerly *Enterobacter cloacae* UW4) is a well-characterised ACC deaminase producing, PGPB. The ACC deaminase produced by this PGPB has been implicated in amelioration of a number of plant stress responses including flooding (Grichko and Glick 2001), salinity (Cheng et al. 2007; 2012) and phytopathogens (Toklikishvili et al. 2010). Additionally, P deficiency in common bean roots increases the amount of ethylene produced in roots (Borch et al. 1999) and ethylene production is implicated in the reduction of nodulation in many species including bean (Lorteau et al. 2001; Nukui et al. 2000; Tamimi and Timko 2003). The reduction in nodulation under low P stress is overcome by co-inoculation with the rhizobia and *Pseudomonas* sp. UW4, but not with the UW4 ACC deaminase minus mutant strain (Remans et al. 2007).

Stearns et al. (2012) used an *Arabidopsis thaliana* oligonucleotide microarray approach to study canola (*Brassica napus*) transcriptional responses to colonization by a wild-type *Pseudomonas* sp. UW4 and an ACC deaminase minus mutant strain. The upregulation of genes for auxin response factors and downregulation of stress response genes was only observed during inoculation with the wild-type strain suggesting a direct link between ethylene and auxin response. The production of ACC deaminase has also been implicated as a mechanism of plant growth promotion by several other PGPB (Table 1.5).

1.4.3 Cytokinins

There are currently over 30 growth promoting compounds of the cytokinin group (N^6-substituted aminopurines) from both plants and plant-associated microorganisms (Timmusk et al. 1999). Cytokinins stimulate cell division, prolong juvenile stages of development, retard senescence and promote lateral bud development (Abeles et al. 1992) as well as having an effect on root development and root hair formation

(Frankenberger and Arshad 1995). Cytokinins inhibit root growth and this is partly attributed to the stimulation of ethylene production by cytokinins (Cary et al. 1995; Dodd et al. 2010; Lorteau et al. 2001). However, when produced microbially, cytokinins may play an important role in stimulating plant growth and development (Arshad and Frankenberger 1998). This hypothesis is supported by the work of Zahir et al. (2001) who applied a cytokinin (kinetin) and its precursors (adenine + isopentyl alcohol) to rice seedlings and noted increases in yield and the N, P and K content of grains. However, they were unable to determine whether precursors were metabolised into cytokinins outside the roots by rhizosphere microorganisms.

Table 1.5: Selected empirical studies which demonstrate that the promotion of plant growth by plant growth promoting bacteria is due to the bacterially produced 1-aminocyclopropane-1-carboxylic acid (ACC)

Isolate	Host	Reference
Pseudomonas sp. strain 6G5	Tomato	Klee et al. (1991)
Enterobacter cloacae CAL2	Canola	Shah et al. (1998)
Pseudomonas putida GR12-2	Mung bean	Mayak et al. (1999)
Rhizobium leguminosarum bv. viciae 128C54K	Alfalfa	Ma et al. (2004)
Azospirillum brasilense Cd 1843	Carnation	Li et al. (2005)
Rhizobium sp. TAL1145 & *Sinorhizobium* sp. BL3	*Leucaena leucocephala*	Tittabutr et al. (2008)
Variovorax paradoxus 5C-2	Pea	Belimov et al. (2009)
Burkholderia unamae MTI-641[T]	Tomato	Onofre-Lemus et al. (2009)
Burkholderia phytofirmans YS3	Canola	Sun et al. (2009)
Pseudomonas migulae 8R6 & *P. fluorescens* YsS6	Tomato	Ali et al. (2014)

Noel et al. (1996) studied the interaction of *Rhizobium leguminosarum* on non-legumes in which both nitrogen-fixing and non-nitrogen-fixing derivatives of *R. leguminosarum* significantly promoted early seedling root growth of canola and lettuce. As growth promotion did not appear to involve nitrogen fixation, the involvement of PGRs was suspected. Three tryptophan auxotrophs and an adenosine auxotroph mutant were

generated (tryptophan and adenosine being the precursors for IAA and cytokinin biosynthesis, respectively) and the auxotrophic mutants did not promote plant growth to the same extent as the wild-type strain (Noel et al. 1996).

Although a number of studies have shown PGPB organisms to be capable of producing cytokinins (*Pseudomonas fluorescens*, *Paenibacillus polymyxa*, *Bacillus subtilis* and *Axotobacter chroococcum*), these studies did not demonstrate that their ability to promote plant growth was due to the cytokinins *per se* (Arkhipova et al. 2005; Garcia de Salamone et al. 2001; Nieto and Frankenberger 1990; Timmusk et al. 1999). Mutant strains of beneficial bacterial cytokinin producers have not been engineered to date (Dobbelaere et al. 2003), however, the use of *A. thaliana* mutants lacking in several cytokinin receptors demonstrated a complimentary role for these receptors in plant growth promotion by *Bacillus megaterium* (Ortíz-Castro et al. 2008). The genes and enzymes involved in the biosynthesis of cytokinins have been characterised for a few phytopathogens (Arshad and Frankenberger 1998; Stes et al. 2011) and the recent development of mutants of the phytopathogen *Rhodocuccus fascians* with altered cytokinin production (Stes et al. 2013) may be useful in determining a role for cytokinins during beneficial plant-microbe interactions.

1.4.4 Gibberellins

At least 130 gibberellins (GAs) have been discovered (Dodd 2007) with a wide variety of physiological effects including growth promotion, breaking of dormancy and flower and fruit development (Abeles et al. 1992), as well as the promotion of primary root elongation and lateral root growth (El-Antably and Larsen 1974; Yaxley et al. 2001). Gutierrez-Manero et al. (2001) showed that inoculation of alder (*Alnus glutinosa*) with

gibberellin producing *Bacillus pumilus* and *B. licheniformis* reversed the dwarfing effects of the gibberellin biosynthesis inhibitor paclobutrazol.

While the most widely recognised GA is GA_3 (a fungal product), the most active in plants is GA_1 (Arshad and Frankenberger 1998) which is primarily responsible for stem elongation (Davies 1995). Several plant growth-promoting *Azospirillum* spp. have been shown to produce GAs (Bottini et al. 1989; Kucey 1988) as well as rhizobia (Gutierrez-Manero et al. 2001) and other species of bacteria (Bastian et al. 1998). As is the case with cytokinins, the bacterial genetic determinants of GAs have not been identified so far, consequently there have been no mutants developed to provide evidence for GAs playing a role in plant growth promotion (Dobbelaere et al. 2001; Dodd 2007).

1.4.5 Abscisic acid

Abscisic acid (ABA) is generally considered a "stress hormone" as ABA levels increase in the presence of biotic and abiotic stresses, however, ABA is also necessary for non-stress regulatory functions such as seed development and sugar sensing (Schwartz and Zeevaart 2010; Zeevaart and Creelman 1988). During water stress, ABA biosynthesis in plants is stimulated, which maintains elongation of roots, inhibits lateral root formation and partially closes the stomata (Borel and Simonneau 2002; Davies and Zhang 1991; De Smet et al. 2006; Dodd 2007). It is suggested that some of these effects occur through the ABA-mediated inhibition of ethylene synthesis (Sharp and LeNoble 2002).

Several bacterial species produce ABA *in vitro* including the *A. brasilense* strains Az39, Cd and Sp245 (Cohen et al. 2008; Perrig et al. 2007) and *A. lipoferum* strain USA 59b (Cohen et al. 2009). ABA production by Sp245 increased when the osmotic potential of growth media was lowered by addition of 100 mM NaCl, and inoculation of *A. thaliana*

with Sp245 increased the plant's ABA content (Cohen et al. 2008). This strain also improved the drought tolerance of *A. thaliana* (Cohen et al. 2015). In the same study, *A. thaliana aba2-1* mutants that were defective in ABA biosynthesis had only 37% of the ABA measured in the Col-0, but inoculation with Sp245 increased ABA content not only in Col-0, but also in the *aba2-1* mutants. However, it is not clear if the increase in ABA in the plants during inoculation with Sp245 is due to uptake of ABA synthesised by the bacteria or if the bacteria increases plant biosynthesis of ABA. Future work needs to incorporate ABA-deficient bacterial mutants to determine the effects of these bacteria on ABA mediated stress responses in plants.

1.4.6 Other plant growth regulators

A number of other plant growth regulators have demonstrated plant growth promotion when produced by beneficial bacteria. For instance, nitric oxide (NO) has been demonstrated to mediate IAA signaling pathways involved in lateral and adventitious root formation in plants (Correa-Aragunde et al. 2006), and several beneficial bacteria are able to produce NO (Creus et al. 2005). In a nitrate-containing media, an *A. brasilense* mutant with reduced NO production was not able to promote lateral or adventitious root formation compared with the wild-type strain and an IAA-attenuated mutant (Molina-Favero et al. 2008). NO overproducing strains of *A. brasilense* SM improved sorghum root length and lateral root branching, and the authors suggest that the role of NO may be additive to IAA in root development (Koul et al. 2015).

Polyamines are also considered plant growth regulators involved in plant growth and stress responses (Cassán et al. 2009). One of the best documented polyamines is diamine cadaverine (1,5-diaminopentane) (Cassán et al. 2014) which is important for the normal rooting of soybean (Gamarnik and Frydman 1991). Inoculation of rice

seedlings with *A. brasilense* Az39 or exogenous cadaverine application in rice seedlings mitigated osmotic stress (Cassán et al. 2009). Although this indicates a role for bacterially produced cadaverine in the reduction of osmotic stress in plants, the authors suggest that it is probably just one of many factors involved in plant growth promotion as described by the "additive hypothesis" of Bashan et al. (2004).

1.5   Combined modes of action and mixed inoculants

It has become apparent that more than one mechanism may be responsible for increases in plant growth induced by PGPBs, and that the effect of a PGPB may be different at different stages of plant growth. For instance, following germination, production of ACC deaminase by the bacterium may lower ethylene levels and reduce ethylene inhibition on root length. At later stages of plant development, the bacterium may facilitate the supply of nitrogen, iron and phosphorus to the plant via N_2 fixation, siderophore production and phosphate solubilisation, respectively (Glick et al. 1999). Mayak et al. (1999) used the model proposed by Glick et al. (1998), to further test the combined effects of auxin and ACC deaminase produced by bacteria on mung bean plants by using three bacterial strains: wild type *P. putida* GR12-2, *P. putida* GR12-2/*aux*1; and *P. putida* GR12-2/acd36 an ACC deaminase minus mutant. All three bacterial strains produce IAA and *P. putida* GR12-2/*aux*1 overproduces IAA. Compared to the control plants, there were more long roots for the wild-type *P. putida* GR12-2 inoculated plants but the plants inoculated with the two mutant strains had shorter roots compared with the control plants. However, the number of roots per cutting were significantly greater in the wild-type *P. putida* GR12-2 and the ACC deaminase mutant *P. putida* GR12-2/acd36 treated plants compared with the control plants. Levels of ethylene in the roots were highest in the auxin overproducing mutant, and lowest in the wild type strain (Mayak et al. 1999). Thus these experiments showed

that a combination of auxin and ACC deaminase production resulted in a larger increase in root length than auxin production alone.

An increasing number of studies are showing the benefit of combining two or more PGPB species to obtain plant growth promotion effects. These mixtures allow a synergistic interaction between the species which may include providing nutrients or removing inhibitors (Glick et al. 1999). The nitrogen fixing activity of *Azospirillum brasilense* Cd, was increased when grown in culture with *Staphylococcus* sp., a non-N_2-fixing bacterium isolated from mangrove roots (Holguin and Bashan 1996) and mixed cultures of the P solubiliser *Bacillus licheniformis* and N_2 fixing *Phyllobacterium* sp. inoculated on mangroves increased the number and ^{15}N levels of leaves (Rojas et al. 2001). Co-inoculation of leguminous plants with *Azospirillium* and rhizobia resulted in increases in grain yields (Bashan and Holguin 1997; Chibeba et al. 2015; Hungria et al. 2013; 2015), possibly due to the stimulation of nodulation and nodule function. Co-inoculation of mung bean with *Bradyrhizobium* and ACC deaminase producing *Pseudomonas* spp. increased root elongation, total biomass and nodulation and improved tolerance to salt stress (Ahmad et al. 2011; Shaharoona et al. 2006). The P acquisition of plants was enhanced by the co-inoculation of P solubilising bacteria with arbuscular mycorrhizal (AM) fungi (Gamalero et al. 2003; Toro et al. 1998) and co-inoculation of *Acinetobacter* sp. and the AM fungus *Glomus intraradices* ameliorated the effects of a petroleum contaminated soil on oat plants (Xun et al. 2015).

1.6   Future research with PGPB

Exciting possibilities exist for the application of PGPB as inoculants to enhance crop productivity. Examples of commercial products using free-living PGPB for a wide variety of crop species are given in Lucy et al. (2004), Glick et al. (1999) Dobbelaere et

al. (2003) and (Hungria et al. 2010). While these and other PGPB have shown positive effects under specific environmental conditions with particular plant species (Lucy et al. 2004), the mechanism(s) of action of these PGPB on their host species is not well understood. For instance, while free-living, nitrogen-fixing, P-solubilising or siderophore-producing organisms have received much attention in the past, the evidence usually suggests the increases in plant growth are not facilitated by the direct supply of nitrogen, iron and phosphorus by the bacteria to the plant. In many cases, the authors suggest that the true mechanism of action is the manipulation of exogenous/endogenous plant hormone levels by the bacteria and the concomitant modification of plant processes, such as changes in root architecture, that result in increases in root surface area for the absorption of nutrients (Vessey 2003).

1.7 Concluding remarks

While the benefits of the inoculation of leguminous crop species with rhizobia has been recognized for over a century (Bashan 1998), in the last thirty years, there has been increasing interest in the use of other rhizosphere organisms as plant growth enhancing inoculants on non-leguminous crops. Although the mechanisms of action of these plant growth promoting bacteria (PGPB) are not conclusively known, some evidence exists for beneficial nitrogen fixation in sugar cane, rice and maize, and the production of the plant growth regulators indole-3-acetic acid (IAA) and 1-aminocylopropane-1-carboxylic acid deaminase (ACC deaminase), by PGPB in a number of crop species. While many PGPBs are isolated from the rhizosphere of target plants, there is scope for the isolation of these beneficial bacteria from other sources.

1.8   Aims

It was hypothesized that novel plant growth-promoting bacteria could be isolated from the rhizosphere of plants growing in Western Australian soils, to improve the growth of agronomically important crop species. Therefore, the aims of this thesis were to:

1.   isolate bacteria from typical Western Australian soils and screen them for potential plant growth promotion on model cereal and legume species;

2.   assess the effectiveness of the PGPB isolates in the field under a variety of conditions including location, soil type and plant species;

3.   elucidate the mechanism(s) of action of the PGPB.

Chapter 2:
Isolation, identification and characterization of potential plant growth-promoting bacterial (PGPB) isolates from Western Australian soils.

Burkholderia caledonica NCH45
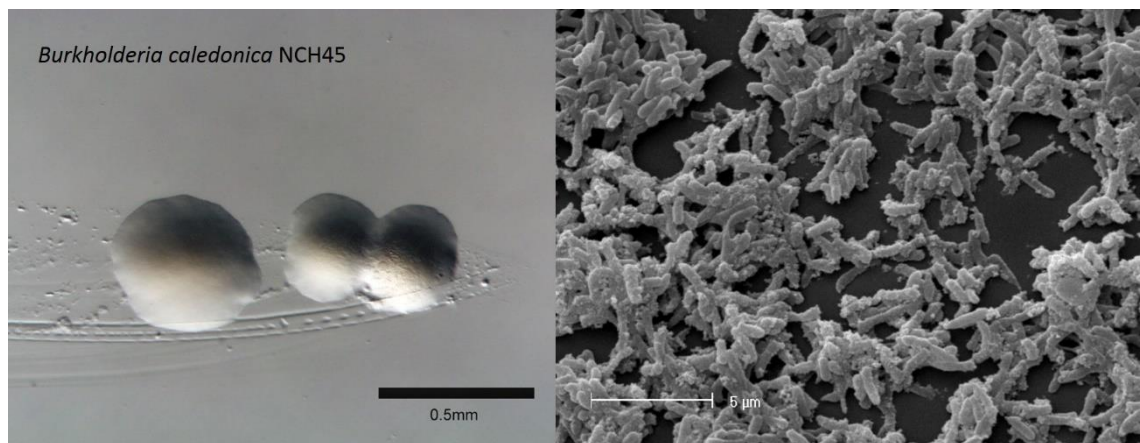
2.1 Introduction

The term 'rhizosphere" was first coined by Hiltner in 1904 and is defined as the volume of soil influenced by the presence of living plant roots. As they push through the soil, the roots of plants release exudates that act directly as a lubricant and indirectly as a nutrient source (Gamalero et al. 2003). The contributions of plant root exudates such as amino acids, proteins, sugars, organic acids and vitamins to the rhizosphere create a nutrient rich environment that stimulates microbial density and activity (Gray and Smith 2005; Somers et al. 2004). As a result, the number and diversity of microorganisms is substantially higher in the rhizosphere than in the bulk soil (Antoun and Prevost 2005; Glick 2014). Rhizosphere organisms generally occur within 50 µm of the root surface and populations within 10 µm of the root surface may be as high as 1.2×10^8 cells cm^{-3} (Gray and Smith 2005). Among the numerous species of microorganisms that flourish in the rhizosphere of plants are the plant growth-promoting bacteria (PGPB) (Vessey 2003).

There is no standard approach for the isolation and selection of PGPB (Asghar et al. 2004). In the past, rhizosphere organisms were screened for their potential as PGPB by inoculating isolates onto seed and/or roots of target plants under anexic conditions and monitoring the effects of inoculation on growth parameters such as root/shoot lengths and weights (Zahir et al. 2004). However, this is a laborious and time consuming process that does not elucidate the mechanism of action of the PGPB (Zahir et al. 2004).

On the other hand, the ability of some rhizosphere microorganisms to utilize unusual carbon or nitrogen sources such as opines, 1-aminocyclopropane-1-carboxylic acid (ACC) or xenobiotic compounds, may be used as a rapid screening method to isolate PGPB from rhizosphere soil (Glick et al. 1995; 1999). For example, ACC deaminase

producing rhizobacteria have been shown to promote plant growth (see Chapter 1) and Glick and colleagues (Glick et al. 1995; Penrose and Glick 2003) have developed selective media to isolate soil bacteria that can use ACC as a sole nitrogen source due to their ability to produce ACC deaminase.

While this method is rapid and quite specific, it precludes the isolation of a large number of potential PGPB isolates as only a relatively small number of rhizobacteria produce ACC deaminase. In the case of the production of auxin (another proposed mechanism of action of PGPB), isolation involves assessment of *in vitro* production of indole-3-acetic acid (IAA) which occurs in an estimated 80% of rhizobacteria (Glick et al. 1999; Khalid et al. 2004a).

There are several methods currently used for the determination of auxin production in bacteria all of which are based on colorimetric methods (Gordon and Weber 1951). In the standard method (Gordon and Weber 1951), auxin present in culture filtrates is reacted with Salkowski reagent to yield a pink product which is measured spectrophotometrically. The microplate method is a modification of the standard method whereby much smaller amounts of culture filtrate react with Salkowski reagent, after which a microplate reader is used to determine colour production. This allows many more samples to be measured concurrently (Sarwar and Kremer 1995). Both methods are somewhat limited as broth cultures must be generated prior to assay. In an attempt to overcome this limitation, the nitrocellulose membrane assay (Bric et al. 1991) utilizes inoculated agar plates that are overlaid with a nitrocellulose membrane, saturated with Salkowski reagent and incubated until a distinct red halo forms around the colonies producing auxin. To obtain quantitative data, potential auxin producers have to be tested using one of the above spectrophotometric methods.

Potential PGPB are generally isolated from the rhizosphere of the target plant species (Cattelan et al. 1999; Gutierrez-Manero et al. 1996; Khalid et al. 2004a; Kishore et al. 2005; Kuklinsky-Sobral et al. 2004). Since the ultimate aim is to reintroduce the PGPB to that species, such isolates are expected to be well adapted to that environment and may survive better than isolates from another source. This does not mean, however, that PGPB can not be sourced from other environments. For instance, beneficial microorganisms that have been lost from agricultural soils through practices that have adverse effects on soil microorganisms (Dick 1992) may still exist in the rhizosphere of native stands untouched by agricultural practices. Therefore, in the search for novel PGPB, it is possible that new and interesting species of PGPB can be isolated not only from target plant species, but also from the rhizosphere of non-target plant species.

Although the methods based on the presence ACC deaminase and IAA production have limitations, they are nonetheless robust and are still the most commonly used tools to screen multiple soil samples relatively rapidly and cost effectively for potential PGPB isolates. Therefore, the aims of this study were to:

- isolate potential PGPB from Western Australian soils from target and non-target plant species on the basis of auxin production and/or ACC deaminase production, utilizing the methods of Penrose & Glick (2003);

- screen the isolates for common PGPB traits, for effect on wheat and clover seedling germination and for their ability to improve plant (wheat) growth in a gnotobiotic assay.

2.2 Materials and Methods

2.2.1 Isolation of bacteria from rhizosphere soil

The rhizosphere soils were obtained from several locations in Western Australia (Table 2.1). Two grams of each soil type was suspended in 20 mL sterile distilled water and shaken for 30 min at 200 rpm at 28°C. Serial dilutions (10^{-2} – 10^{-5}) were made into sterile saline solution (0.89% NaCl w/v) and 0.2 mL aliquots spread with a sterile glass rod over the surface of ½ nutrient agar (NA) (Difco) or starch nitrate agar (SNA) (10 g soluble starch, 2.0 g KNO_3, 1.0 g K_2HPO_4, 0.5 g $MgSO_4$, 0.5 g NaCl, 3.0 g $CaCO_3$, 0.01 g $FeSO_4$, 1 mL trace salt solution (0.1 mg per litre of each of the following salts: $FeSO_4$, $MgCl$, $CuSO_4$, $ZnSO_4$) 20 g agar, 1000 mL deionized water) (El-Tarabily 1997). Cycloheximide (Sigma) (50 mg L^{-1}) and nystatin (Sigma) (50 mg L^{-1}) were added immediately prior to pouring plates to inhibit fungal growth (El-Tarabily et al. 2003). Three plates were used per dilution. Plates were incubated in the dark for 3-7 days and isolates with differing morphologies were selected and re-streaked onto fresh agar (½ NA or SNA) until pure colonies were obtained.

Table 2.1: Rhizosphere soils, location and plant types.

Location	Coordinates	Plant type(s)	Soil type	pH (CaCl$_2$)	Average rainfall (mm)
Meckering	31° 37'S, 117° 00' E	Wheat, lupins, native species	Duplex: sand over gravel	5.3	366
Merredin	31° 29'S, 118° 17' E	Lupins	Deep loamy sand	4.8	326
Chittering	31° 28'S, 116° 04'E	Native species	Duplex: sandy loam over clay	5.8	571

Bacteria were also isolated using methods adapted from Penrose and Glick (2003) for the selection of putative ACC deaminase producing isolates. One gram of the

rhizosphere soil (Table 2.1) was added to 50 mL sterile *Pseudomonas* AF (PAF) medium (10 g proteose peptone, 10 g casein hydrolysate, 1.5 g anhydrous $MgSO_4$, 1.5 g K_2HPO_4, 10 mL glycerol, to 1000 mL with H_2O) in a 250 mL flask and incubated at 28°C on a rotary shaker at 200 rpm. After 24 h a 1 mL aliquot was removed from the flask and was transferred to fresh sterile PAF medium and incubated again for 24 h.

A 1 mL aliquot was removed from the second culture and transferred to a 250 mL flask containing 50 mL sterile DF (Dworkin and Foster 1958) salts minimal medium amended with glucose and gluconic acid and incubated at 28ºC on a rotary shaker at 200 rpm for 24 hours. Nitrogen was supplied to these flasks prior to addition of the 1 mL aliquot as $(NH_4)_2SO_4$ using 0.3 mL filter sterilized stock solution (10.0 g $(NH_4)_2SO_4$ in 30 mL H_2O) providing 2.0 g $(NH_4)_2SO_4$ per 1000 mL. After 24 hours, a 1 mL aliquot was removed and transferred to 50 mL sterile DF salts minimal medium in a 250 mL flask containing 3.0 mM ACC (300 µL flask^{-1}) instead of $(NH_4)_2SO_4$ as the source of nitrogen. A 0.5 M stock solution of ACC was prepared by dissolving 0.5 g ACC into 9.89 mL distilled water and filter sterilizing through a 0.2 µm membrane. The filtrate was collected and aliquots of 1.5 mL was dispensed into microcentrifuge tubes at a volume of 600 µl and frozen at -20ºC. Prior to inoculation, the ACC solution was thawed and a 300 µl aliquot added to 50 mL sterile DF salts. After inoculation, the culture was incubated at 28ºC on a rotary shaker at 200 rpm for 24 hours.

To isolate organisms able to utilize ACC as a nitrogen source, DF salts agar plates were prepared using 1.8% Grade A Agar (BBL) (no nitrogen content) and spread with 0.5 M ACC solution (300 µL plate^{-1}). A 50 µL aliquot of the 24 hour old DF culture with ACC was spread onto the plates which were incubated at 28ºC and examined daily for 5

days. Colonies showing strong growth were subcultured onto NA plates and restreaked until pure colonies were obtained.

All the putative PGPB isolates were maintained in 20% glycerol at -80°C.

2.2.2 Clover and wheat seed germination assays

The isolates recording highest amount of auxin production from the initial assay and all the ACC deaminase isolates were subjected to seedling germination assays using clover and wheat. Selected isolates were inoculated into NB for 48 hours at 28°C on a rotary shaker at 200 rpm. The absorbance of the cultures was measured at 600_{nm} (Hitachi UV-1100 Spectrophotometer) and this measurement was used to adjust the OD of the cultures to 0.5 with sterile saline solution (0.89% NaCl w/v). Clover (*Trifolium subterraneum* cv. York) seeds, which were scarified prior to use, and wheat (*Triticum aestivum* cv. Tincurrin) seeds were sterilized by soaking in 70% ethanol for 1 min, then in 4% NaOCl for 6 min followed by six washes with sterile distilled water. Uniform seeds were placed into sterile Petri dishes and each dish was incubated at room temperature for 1 hour with 8 mL of the appropriate treatment: sterile 0.89% saline solution (used as a negative control) or bacterial suspensions in sterile 0.89% saline solution. Following the incubation period, 30 seeds for the clover or 20 seeds for the wheat were transferred to 1% water agar plates with 3 replicate plates for each bacterium. The plates were sealed with parafilm and incubated in the dark at 28°C. After 3 days incubation, the number of germinated seeds was counted. At this stage, some strains were becoming difficult to subculture and were not used further in this study. During routine culturing, it was observed that APMK2 was a mixed colony and subsequently two different pure cultures were obtained and renamed APMK2W for the white and APMK2Y for the yellow colonies.

2.2.3 Rhizosphere colonization assay

Selected isolates were assessed for their ability to colonise the root system of wheat in a gnotobiotic screening method (Silva et al. 2003). Bacteria were grown in 5 mL NB for 48 hours at 28°C on a rotary shaker at 200 rpm, then washed twice in sterile 0.03 M $MgSO_4$, to ensure that the pellet was free of the bacterial growth medium, and resuspended at an OD_{600} of 0.15 in fresh 0.03 M $MgSO_4$. $MgSO_4$ was chosen in place of saline to avoid deleterious effects the saline may have had on seed germination. Wheat (*Triticum aestivum* cv. Wyalkatchem) seed was surface sterilized as above and uniform seeds were placed into sterile Petri dishes and each dish was incubated at room temperature for 1 hour with 10 mL of either sterile 0.03 M $MgSO_4$ (used as a negative control) or bacterial suspensions in sterile 0.03 M $MgSO_4$. The seeds were then transferred to sterile test tubes containing 0.6% water-agar with 4 replicates per bacterial isolate. Growth of the seedlings and bacteria in the tubes was monitored for 12 days and the presence of bacterial colonies on the roots recorded at 12 days.

In order to improve the assessment of the ability of the PGPB to colonise the root system of wheat seedlings, a sand culture test tube method was developed. The gravimetric soil water capacity of washed river sand was determined by filling four 130 mL free-draining plastic pots with the sand and watering the pots till they dripped through. The pots were covered with plastic and left at room temperature for 24 hours. Approximately 30 g of the soil was then placed in aluminum foil trays in an oven set to 102°C. When the soil had reached a stable weight (2 days), the water holding capacity of the soil was determined to be 16%. Acid (10% HCl) washed test tubes were filled with 42 g river sand, autoclaved twice, then 6.7 mL sterile deionized water was added to each tube. Two of the potential PGPB isolates and an *Escherichia coli* (MUE9) strain and a *Bacillus subtilis* (MUB6) strain from the Murdoch University collection were

inoculated on TY agar or NA for 48 hours. The *E. coli* and *B. subtilis* strains were used in this experiment as they would potentially be considered negative and positive controls respectively for root colonization of wheat. The OD_{600} of the bacteria was adjusted to 0.5 in sterile 0.03 M $MgSO_4$ and surface sterilized wheat (*Triticum aestivum* cv. Wyalkatchem) seed that had been sterilized as described above, was immersed in 10 mL of the culture suspensions for 1 hour. One seed was placed into a river sand filled test tube using aseptic techniques and there were 10 replicate test tubes per treatment. The tubes were placed in racks in a 25°C controlled temperature room under artificial light (16/8 hours). After 7 days plants were taken from the tubes and soil was removed from the excised roots using sterile 0.05 M phosphate buffer (29.25 mL 0.2 M $NaH_2PO_4.2H_2O$ stock; 47.75 mL 0.2 M Na_2HPO_4 stock; 225 mL H_2O) at pH 7.0. Four replicates were placed in TTC dye (1.5 g L^{-1} 2,3,5 triphenyltetrazolium chloride (Merck) and 0.625 g L^{-1} malate (Sigma) in 0.1 M phosphate buffer (pH 7.3)) and incubated at 28°C for 48 hours. The remaining roots were placed onto Petri dishes containing a thin layer of tryptone-yeast (5 g tryptone, 3 g yeast extract, 0.84 g $CaCl_2.2H_2O$, 1.5% agar; TY) agar. The plates were incubated overnight and then an overlay of 1:1 mixture of 2% agar and TTC dye solution was poured over the roots. The plates were incubated 1 hour at 28°C and were assessed for a colour change of the TTC dye solution along the root surface that would indicate root colonization by the bacteria. The plates were returned to 28°C incubation for another 24 hours. To confirm that the TTC dye/agar mixture was suitable for the detection of each of the 4 bacterial isolates, the bacteria were patch plated onto TY agar and incubated for 24 hours at 28°C. One hundred µL of the TTC-1% agar mixture was applied over the top of the bacterial colonies on the TY agar plates or over uninoculated TY agar as a control and the colour change of the TTC dye was monitored.

2.2.4 Wheat seedling growth pouch assays

Isolates selected for further study from the seedling toxicity assays were tested for the ability to promote the growth of wheat seedlings in cyg™ seed germination (growth) pouch (Mega-International) assays. The isolate PMK9 was included in this experiment as wheat seed exposed to this isolate had a consistent 70% germination which was above the criteria (60%) a used for selection purposes. Two isolates that reduced germination of the wheat seed (NCH7 and PMK4) were also used to observe any further deleterious effect on the developing seedlings. Although wheat seedlings inoculated with NCH38 demonstrated 75% germination, this isolate was becoming difficult to sub-culture and was not used in the growth pouch assay at this stage. Due to the limitation in equipment availability, the isolates were tested in 3 batches.

The isolates were inoculated into 10 mL of NB in McCartney bottles and cultured for two days at 28ºC on a rotary shaker at 200 rpm. The cells were harvested and washed twice before being resuspended in 0.03 M $MgSO_4$ to a final OD_{600} of 0.15.

Wheat (*Triticum aestivum* cv. Whyalcatchem) seeds were surface sterilized and uniform seeds were placed into sterile Petri dishes and then incubated at room temperature for 1 hour with 10 mL of either sterile 0.03 M $MgSO_4$ (control) or bacterial suspensions in sterile 0.03 M $MgSO_4$.

Seed growth pouches were filled with 10 mL of ½ strength Hoagland solution, wrapped individually in aluminum foil and autoclaved at 121ºC/20 min in an upright position. Hydroponic boxes and growth pouch racks were surface sterilized using 70% ethanol and placed under UV light for at least 2 hours before use. Following the 1 hour incubation period, 6-8 seeds were aseptically placed in the top groove of each growth pouch using sterilised forceps, and the pouches positioned upright in metal racks, which

were then placed in the hydroponic boxes. Sterile distilled water (100 mL) was added to the box to prevent dehydration. The foil wrapped around the bottom of the pouches ensured the roots were in the dark. Each box had only one treatment (one isolate) to prevent cross-contamination and there were 10 pouches per treatment (i.e. 60-80 seedlings/treatment). Hydroponic boxes were placed in the glasshouse with constant temperature (23ºC ±2ºC) under natural illumination. In the first experiment, the seedlings were thinned to 4 seedlings (after 3 days) and another 5 mL of ½ strength Hoagland solution was added. In subsequent experiments, the seedlings were not thinned and Hoagland solution was added when pouches showed signs of drying out. After 7 days, the total root and shoot length was measured. Percent germination was also determined in non-thinned assays.

2.2.5 *In vitro* assays for PGPB traits

The ability of several promising PGPB to produce auxins, ACC deaminase, and other typical PGPB traits was assessed to elucidate a potential mechanism of their action.

2.2.5.1 Auxin production

Auxin production by the rhizobacterial strains obtained from the initial isolation procedure was determined in the presence or absence of L-tryptophan (L-TRP) by colorimetric analysis using the Salkowski reagent method (Khalid et al. 2004a). Pure bacterial colonies were looped into sterile 5 mL sterile nutrient broth (NB) (Becton Dickinson) in McCartney bottles and shaken on a rotary shaker at 200 rpm and 28ºC for four days.

Cultures at 4 days old were adjusted to an OD_{600} of 0.5 and 1 mL of culture solution was added to 10 mL of glucose peptone broth (GPB) (glucose 10.0g, peptone 5.0g, 1000 mL H_20, pH 7.0) in McCartney bottles giving a final volume of 11.0 mL. To test

for auxin activity in the presence of L-tryptophan (L-TRP), 1 mL of the OD adjusted culture solution was also added to GPB amended with 1 mL of filter sterilized (0.2 µm membrane filter, Schleicher & Schuell) 0.2% L-TRP solution giving a final concentration of 0.167 g/L L-TRP and volume of 12.0 mL. Cultures were incubated at 28°C for 48 hours in the dark at 200 rpm. Non-inoculated controls with and without L-TRP were included for comparison. After incubation, 1.5 mL of the culture medium was centrifuged (5000 g, 10 min) to remove the bacterial cells. A 1 mL aliquot of the supernatant was mixed with 4 mL Salkowski reagent and allowed to stand for 30 min for colour to develop before absorbance at 535_{nm} was measured. Auxin compounds expressed as IAA ($\mu g\ mL^{-1}$) equivalents were determined by comparison with an IAA (Sigma) standard curve over the range 0 to 50 $\mu g\ mL^{-1}$. To 4 mL Salkowski reagent, 1 mL of each IAA standard was added, allowed to stand for 30 min then the absorbance measured at 535_{nm}.

2.2.5.2 ACC deaminase production

To confirm the production of ACC deaminase by the isolates the method of Li et al. (2011) was used. A single colony of selected isolates was looped into 5 mL sterile tryptone-yeast broth (5 g tryptone, 3 g yeast extract, 0.89 g $CaCl_2.2H_2O$, 1000 mL H_2O; TY) in triplicate and incubated overnight at 28°C on a rotary shaker at 200 rpm. The bacterial isolate *Sinorhizobium meliloti* SM1021 does not have ACC deaminase activity (Ma et al. 2003c) and was included as a negative control. Two mL of the overnight cultures were centrifuged at 8000 g for 5 min, then the cell pellet washed twice with 1 mL sterile DF salts solution and finally resuspened in 1 mL fresh DF salts. The 1 mL culture suspension was added to a sterile 30 mL tube containing 1 mL DF salts and to this 12 µL of a 0.5 mM ACC solution was added to give a final concentration of 3.0

mM. Two mL of sterile DF salts amended with 3.0 mM ACC was prepared as a blank. All the tubes were incubated for 24 hours on a shaker at 200 rpm and 28°C.

Just prior to conducting the ACC consumption assay, the ninhydrin reagent was prepared. The ninhydrin reagent consisted of reagent A: (1 M citrate buffer; 1.53 g citric acid, 15.42 g sodium citrate, 60 mL H_2O) combined with reagent B: (500 mg ninhydrin, 15 mg L-ascorbic acid, 60 mL ethylene glycol) to make up a final volume of 120 mL. A standard curve was prepared using DF salts as a diluent for concentrations of ACC ranging from 0.05-0.50 mM.

A 1 mL aliquot of each culture and blank was centrifuged in a 1.5 mL centrifuge tube at 8000 g for 5 min and the supernatant was diluted 1/10 with fresh DF salts. Diluted cultures (60 µL), blanks and standards were added to separate wells in a 96 well chimney top PCR microplate (Axygen) and to each well 120 µL of the ninhydrin reagent was added. The PCR plate was covered with a silicon mat (Axygen) and placed in a thermocycler (Bio-Rad MyCycler Version 1.065) at 100°C for 30 min. The plate was then allowed to cool and the colour to develop for 10-15 min at room temperature. The absorbance was then read at 570_{nm} on a microplate reader (Beckman & Coulter DTX 880 Multimode Detector).

2.2.5.3 *β1,3-glucanse and chitinase production*

The baker's yeast *Saccharomyces cerevisiae* is known to contain both β-1,3-glucans and chitin in their cells walls (Klis et al. 2002) and therefore the putative production of β-1,3-glucanase and chitinase by the PGPB was assessed on medium containing this yeast. The baker's yeast agar contained 4.0 g L^{-1} baker's yeast (Rising in the Yeast, Myaree, Western Australia) and 16 g L^{-1} agar (Grade A, BBL) and was autoclaved at

121°C for 20 min prior to use. A zone of clearing surrounding the colony is considered positive for β-1,3-glucanase and/or chitinase production.

2.2.5.4 Cellulase production

Cellulase production was assessed using the methods of Cattelan et al. (1999) and Gupta et al. (2012). Yeast extract (1.2 g L^{-1}, Beckton, Dickinson and Company), cellulose (10 g L^{-1}, Sigma-Aldrich), 0.2 g L^{-1} Congo red (George T. Gurr Limited) and agar (8 g L^{-1}, Grade A) were added to 800 mL H_2O and autoclaved at 121°C for 20 min. After autoclaving, 200 mL sterile M9 salts (Miller 1974), 2 mL of sterile 1 M $MgSO_4$ and 100 µL of sterile 1 M $CaCl_2$, were added. Twenty µL of a 0.5 OD_{600} culture solution of each PGPB in 0.89 % (w/v) NaCl solution was placed in the centre of a cellulose plate in triplicate. After 7 days of incubation at 28ºC, isolates surrounded by clear halos were considered positive for cellulase production.

2.2.5.5 Pectinase production

Pectinase production was also determined in M9 medium except the cellulose was replaced with pectin (4.8 g L^{-1}, Sigma-Aldrich) and the Congo red omitted. Twenty µL of a 0.5 OD_{600} culture solution in 0.89 % (w/v) NaCl solution was placed in the centre a pectin plate in triplicate. After 7 days of incubation at 28ºC, the plates were flooded with 2 M HCl and isolates surrounded by clear halos were considered positive for pectinase production (Cattelan et al. 1999).

2.2.5.6 Phosphorus solubilisation

The ability of selected isolates to solubilise phosphorus *in vitro* was assessed on several different media. In a method adapted from de Freitas et al. (1997), sterile calcium phosphate precipitate produced from combining 50 mL 10% (w/v) K_2HPO_4 and 100 mL 10% (w/v) $CaCl_2$, was added to 850 mL autoclaved potato dextrose agar (PDA)

(Beckton, Dickinson and Company), while stirring to produce a final volume of 1000 mL. The PGPB isolates were patch inoculated on to the CaHPO$_4$ plates using sterile orange sticks (Technical Service Consultants Ltd) from cultures grown on TY agar at 28°C for 2 days. The inoculated CaHPO$_4$ plates were incubated for 7 days at 28°C and the assay was repeated twice.

Pikovskaya (PVK) medium and the National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal 1999) were also used to determine phosphorus solubilisation in plate assays. PVK and NBRIP media contained 5.0 g tricalcium phosphate (Ca$_3$(PO$_4$)$_2$; KT) as hydroxyapatite (Sigma-Aldrich). Plates were inoculated in triplicate with 20 µL of a 0.5 OD$_{600}$ bacterial culture in sterile NaCl (0.89% w/v) solution and incubated at 28°C.

After 7 days, the colony diameter and zone of clearing was measured for all the plate assays and the solubilisation index (diameter of colony + halo divided by diameter of colony) (Kumar and Narula 1999) determined.

Phosphorus solubilisation was also verified in liquid PVK medium. One hundred µL of an OD$_{600}$ 0.5 culture in sterile deionized water of each PGPB was inoculated into 5 mL sterile PVK. The cultures were incubated, along with uninoculated (100 µL of sterile deionized water) PVK as controls, for 7 days at 28°C on a rotary shaker at 200 rpm. Just prior to the assay, Barton's reagent (Gautam et al. 2012) was prepared. Solution A contained 25 g ammonium molybdate in 400 mL water. Solution B contained 1.25 g ammonium metavanadate dissolved in 300 mL boiling water, which was then allowed to cool before 250 mL concentrated HNO$_3$ (AR grade) was added. Solution A and B were then mixed and made up to 1000 mL. A standard curve was prepared using KH$_2$PO$_4$ from 5 to 100 ppm. One mL of each PGPB culture in PVK was spun down at

7000 g for 5 min and 200 µL of the supernatant was transferred to a test tube containing 1800 µL of deionized water. To this diluted culture solution, 5 mL of Barton's reagent and 3 mL deionized water was added, the solution mixed and the yellow colour was allowed to develop for 10 mins at room temperature. The OD of the standards and cultures was measured at 400_{nm}.

2.2.5.7 Pathogenic fungi inhibition in vitro.

Selected isolates were tested for antagonistic activity against the common wheat pathogenic fungi *Rhizoctonia solani* MU60, *Sclerotinia minor* MU219 and *Sclerotinia sclerotiorum* MU67. Pure cultures of these fungi were initially grown on ½ PDA for 7 days at 20ºC. A 10 mm streak of each bacterial isolate was placed at 4 equidistant sites on separate 90 mm Petri dishes containing ½ PDA. After incubating these plates for 24 hours at 20°C, 5 mm disks were cut from the edge of the active growth of colonies of each fungus with a cork borer. One disk was then transferred to the centre of the plates inoculated with the bacteria. Each treatment had three replicates and the Petri dishes were then incubated at 20ºC for 7 days. After this incubation period, the inhibition zones were measured for each bacterial isolate in relation to the growth of the fungi.

2.2.5.8 Polyamine production

Polyamine production by the bacteria was determined using a modified Moeller's decarboxylase agar medium (MDAM) (Nassar et al. 2003). This media contains 5.0 g peptone, 3.0 g yeast, 0.02 g phenol red (Labchem) 0.03 g $MnSO_4$ 15.0 g agar in 1000 mL deionized water. L-arginine-hydrochloride (1 g L^{-1}) (Sigma) was added to the MDAM prior to autoclaving. The control MDAM did not contain L-arginine. Bacteria were patch plated onto the MDAM plates and incubated in the dark for 24 hours at 28°C. The ability to produce arginine decarboxylase and hence putrescine from the

arginine contained in these agar plates is indicated by a dark red halo beneath the bacterial colony (Nassar et al. 2003).

2.2.5.9 Siderophore production

To detect siderophore production by the isolates, chrome azurol S (CAS) agar plates (Schwyn and Neilands 1987) were prepared using the modified method of Alexander and Zuberer (1991). The PGPB were patched from TY agar onto the CAS agar plates using a sterile orange stick. There were 4 PGPB per CAS agar plate and 3 replicate plates per PGPB. The plates were incubated at 28°C for up to 48 hours and an orange halo produced around the bacterial colonies was considered positive for siderophore production. As hexadecyltrimethylammonium bromide (HDTMA), which is used to complex the CAS and the ferric solution, has antibacterial properties (Alexander and Zuberer 1991), the effect of this cationic detergent was evaluated by preparing 0.1x TSA with 73 mg L^{-1} HDTMA and bacterial growth was compared with bacteria patched onto unamended 0.1x TSA.

2.2.6 PCR analyses

2.2.6.1 16S rRNA sequence identification of PGPB isolates

Based on the results of the growth pouch assays, several isolates were selected for field trials on various plant species in several locations (Chapters 3 and 4). Six of these isolates were identified by PCR using the 16S rRNA gene region in this study, and two (WMK10 and PMK4) were identified in a previous study (Zappia 2008). The six strains identified in this study were grown overnight on TY agar and one colony was suspended in 100 µL sterile PCR grade water (Fisher Biotech). Initially, the 16S rRNA was amplified using 1.0 µM of each of the universal 16S primers 27f and 1492r (Table 2.2) in a reaction mixture containing 1x GoTaq (Promega), 1 µL cell suspension and PCR water to 25 µL. The PCR conditions consisted of an initial denaturation cycle of

95°C for 5 min, then 30 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 5 min before one final cycle of 72°C for 5 min in a Bio-Rad thermocycler (MyCycler Version 1.065). The PCR products were analysed on a 1% (w/v) agarose gel stained with Sybr® Safe electrophoresed at 80 V for 60 min. The bands were visualized on a Bio-Rad Molecular Imager® (Bio-Rad Gel Doc™ XR$^+$). The products were purified by excising the bands into a 200 µL filter tip which had been placed in a 1.5 mL Eppendorf tube and centrifuging at full speed for 1 min. This purified product was used as the template for the sequencing reactions.

The primers used for the sequencing reaction were 27f, 1492r and the internal primers 900f, 1100f, 520r and 820r (Table 2.2). For each primer, a ¼ strength sequencing reaction was prepared using 2.0 µL Big Dye Terminator (version 3.1), 1 µL of 3.2 pmole/µL primer, 2.0 µL purified PCR product, 1x buffer, and PCR water up to a volume of 10 µL per reaction. The sequencing temperature profile consisted of 96°C for 2 min, followed by 25 cycles of 96°C for 10 sec, 5°C for 5 sec, 60°C for 4 min. The sequences were determined by the SABC Sequencing Service (Murdoch University) and were compared to the EzTaxon server (http://www.ezbiocloud.net/eztaxon; Kim et al. 2012) or where no match to species was obtained from this database, to sequences in the NCBI nr/nt database using the BLAST N algorithim (http://blast.ncbi.nlm.nih.gov/) to identify the strains.

Table 2.2: List of primers used in this study.

Name	Sequence (5'-3')	Reference
27f	AGAGTTTGATCCTGGCTCAG	Lane (1991)
1492r	GGTTACCTTGTTACGACTT	Lane (1991)
800f	GTAGTCCACGCCGTAAACGA	Yanagi and Yamasato (1993)
1100f	AAGTCCCGCAACGAGCGCAA	Yanagi and Yamasato (1993)
520r	GCGGCTGCTGGCACGAAGTT	Yanagi and Yamasato (1993)

820r	CATCGTTTACGGCGTGGACT	Yanagi and Yamasato (1993)
DegACCf	GGBGGVAAYAARMYVMGSAAGCTYGA	Hontzeas et al. (2005)
DegACCr	TTDCCHKYRTANACBGGRTC	Hontzeas et al. (2005)

2.2.6.2 PCR amplification ACC deaminase encoding (acdS) genes

Genomic DNA for was extracted using the protocols of Chen and Kuo (1993) and where this was not successful (ie. for APMK2Y), a cell suspension (one colony in 100 µL PCR grade H_2O) was used as a DNA template. Preliminary PCR reactions with concentrated genomic DNA (gDNA) for NCH45 did not return an amplification product and therefore the gDNA was diluted 1/10 and a cell suspension was used as a template for subsequent PCR reactions. The isolate *Burkholderia phymatum* STM 815 was used as a positive control for the *acdS* gene. For the amplification of the ACC deaminase (*acdS*) genes, the DegACCf/DegACCr primer pair are known to be universal (Nikolic et al. 2011) and were used for several of the PGPB to determine if these genes are present. The PCR reactions consisted of 1x GoTaq (Promega), 1.0 µM each primer, 2.0 µL DNA template (cells or gDNA) and PCR H_2O up to a volume of 25 µL. The cycling conditions were as follows: initial denaturation for 3 min at 95°C; then 35 cycles of denaturation at 95°C for 30 sec, an annealing temperature of 46°C, 50°C or 55°C for 1 min, elongation at 72°C for 1 min; and a final elongation at 72°C for 5 min. The PCR products were analysed on a 1% (w/v) agarose gel stained with Sybr® Safe electrophoresed at 80 V for 60 min.

2.2.7   Statistical analysis

Data were subjected to analysis of variance (ANOVA) and if significant, treatment means were compared using Fisher's Least Significant Difference test (LSD) or Tukey's Honest Significant Difference (HSD) if equal variance was assumed or using Games-Howell Test if equal variance was not assumed (where indicated) (IBM SPSS

V22). Differences were considered to be significant at the P<0.05 level and confidence intervals are displayed where meaningful. The size of the effect was calculated as Eta-squared (η^2) and the power (β) is shown.

2.3   Results

2.3.1   Isolation of potential PGPB from Western Australian soils

The initial isolation procedure yielded 166 putative PGPB isolates from 3 soils. Those isolated on SNA and many from ½ NA resembled actinomycetes (based on their substrate mycelium, typical soil odour and production of conidiospores) and did not grow well or at all in either NB or GPB. This meant that they could not be assayed for auxin production at this time. Other organisms also failed to grow in either NB or GPB and were not assayed further.

2.3.1.1 Auxin production

Of the 72 isolates successfully assayed for auxin production 44% (32 isolates) produced less than 1.00 μg mL^{-1} IAA-equivalents, in the absence of L-TRP (Table 2.3). In the presence of L-TRP, production of IAA-equivalents ranged from: low (<5.0 μg mL^{-1}), 27 isolates; medium (5.0-10.0 μg mL^{-1}), 25 isolates; and high (>10.0 μg mL^{-1}), 20 isolates. The two isolates producing the highest amount of IAA-equivalents were PMK4 and NCH7 which in the presence of L-TRP produced IAA-equivalents of 37.0 and 35.0 μg mL^{-1}, respectively.

2.3.1.2 Isolation of potential ACC deaminase PGPB

The soil from the rhizosphere of 5 plant types were screened for potential PGPB capable of using ACC as a sole nitrogen source and 13 pure cultures of putative ACC deaminase producing isolates were obtained (Table 2.4).

Table 2.3: *In vitro* production of auxin compounds expressed as indole-3-acetic acid (IAA) equivalents (µg mL^{-1}) in the presence or absence of L-tryptophan (+/-TRP) by bacteria isolated from four Western Australian rhizosphere soils.

Isolate	-TRP	+TRP	Isolate	-TRP	+TRP
NMK25	1.00	8.50	WMK10	2.50	22.00
NMK19a	<1.00	8.00	WMK17	2.50	16.00
NMK8S	2.50	7.00	WMK2	3.25	15.00
NMK2	<1.00	5.67	WMK21	2.50	15.00
NMK26	1.00	5.00	WMK7	0.80	15.00
NMK8	<1.00	4.83	WMK6	<1.00	14.00
NMK6	<1.00	3.67	WMK6	<1.00	10.00
NMK20	<1.00	2.50	WMK14	<1.00	8.00
NMK24	1.00	2.50	WMK2	2.75	6.75
NMK12a	1.25	2.25	WMK9	2.00	6.50
NMK28	1.00	1.50	WMK22	<1.00	6.00
NMK1	<1.0	1.25	WMK11	<1.00	5.50
NMK4	<1.0	1.00	WMK15	<1.00	4.75
NMK27	2.00	1.00	WMK12	<1.00	4.67
NMK9	<1.00	<1/00	WMK4	<1.00	2.50
NMK16	<1.00	<1.00	WMK19	0.75	1.75
NMK18	0.00	0.00	PMK4	4.00	37.00
NCH7	3.75	35.00	PMK9	2.50	19.00
NCH54	4.75	17.25	PMK16	1.25	19.00
NCH45	2.00	13.25	PMK23	2.00	18.50
NCH46	1.25	10.25	PMK6	0.75	17.00
NCH38	3.00	9.25	PMK19	1.75	14.00
NCH26	2.00	9.00	PMK2	2.50	12.50
NCH48	<1.00	8.00	PMK12A	1.50	12.50
NCH50	<1.00	7.00	PMK27	1.50	11.75
NCH3	2.00	7.00	PMK24	1.25	10.75
NCH16	1.00	6.25	PMK12B	4.75	9.50
NCH53	1.00	6.25	PMK33	<1.00	6.83
NCH31	2.00	6.00	PMK5	<1.00	6.83
NCH14	1.50	5.25	PMK1	1.50	6.00
NCH47	<1.00	5.00	PMK26	<1.00	4.50
NCH17	<1.00	4.75	PMK15	<1.00	2.50
NCH63	<1.00	4.50	PMK3	<1.00	2.33
NCH40	1.00	4.00			
NCH15	<1.00	3.75			
NCH32	<1.00	3.75			
NCH35	<1.00	3.75			
NCH63	1.25	3.50			
NCH30	1.50	0.00			

Isolates beginning with NMK were derived from native plants from Meckering, Western Australia; NCH from native plants from Chittering, Western Australia; WMK from wheat plants from Meckering; and PMK from pea plants from Meckering

Table 2.4: Rhizobacteria from Western Australian soils isolated from plates containing 1-aminocyclopropane-1-carboxylic acid (ACC) as the sole nitrogen source

Location	Plant Type	Name
Chittering	Native Species	ANCH1
Chittering	Native Species	ANCH2
Meckering	Native Species	ANMK1
Meckering	Native Species	ANMK2
Meckering	Native Species	ANMK4
Meckering	Peas	APMK1
Meckering	Peas	APMK2
Meckering	Peas	APMK3
Meckering	Wheat	AWMK1
Meckering	Wheat	AWMK2
Meckering	Wheat	AWMK3
Merredin	Lupins	ALMR1
Merredin	Lupins	ALMR2

2.3.2   Seedling germination assay

The 13 isolates that grew well on the ACC deaminase isolation plates and the 10 isolates that produced the highest amount of auxin in the presence of L-TRP and the 4 isolates that produced the highest amounts in the absence of L-TRP were tested for effect on seedling germination. Control, uninoculated clover seeds had an average germination of 90% and there was a significant ($F_{(27,68)} = 5.871$; P=0.001; $\eta^2 = 7.00$; $\beta = 1.00$) effect of bacterization of the seed with the potential PGPB. The ACC deaminase isolate, AWMK3, was most detrimental (Games Howell, P=0.024) reducing germination to 23% (Figure 2.1).
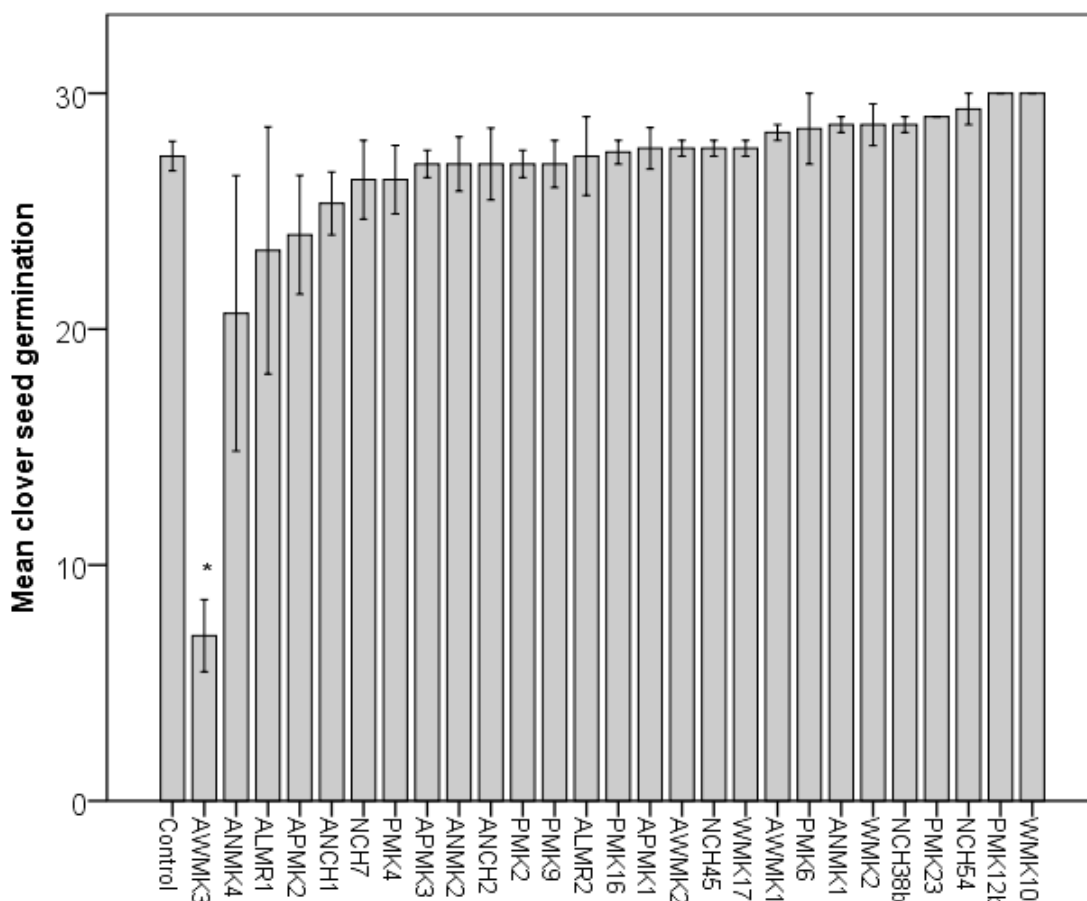
Figure 2.1: The effect of PGPB isolates on the germination of clover seeds. There were 3 replicates of 30 seeds per treatment (PMK6 and PMK16 had 2 replicates only). The bars above the columns depict standard errors and asterisks above the columns indicate a significant (P<0.05) difference from the control according to the Games-Howell Test

More PGPB isolates had a negative effect on wheat seed germination ($F_{(27,71)}$ = 12.997, P=0.000; η^2=0.832; β=1.00). In particular, NCH7 (P=0.000), PMK4 (P=0.001), PMK6 (P=0.001), PMK9 (P=0.001), AWMK1 (P=0.003) and ALMR1 (P=0.045) had a significantly reduced germination compared with the controls according to the Games-Howell Test (Figure 2.2 and 2.3). However, germination of the uninoculated wheat seed was 80% and the best germination of wheat seeds treated with a PGPB isolate was observed for seeds inoculated with AWMK3 (75%).
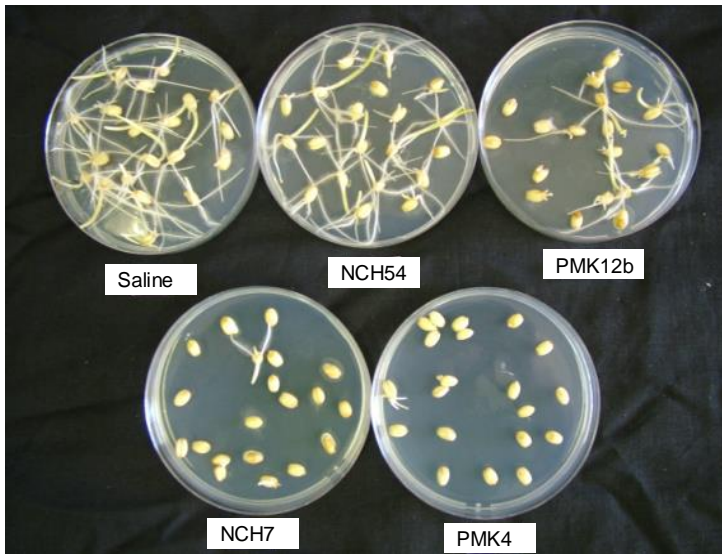
Figure 2.2: Effect of IAA producing isolates on the germination of wheat seeds
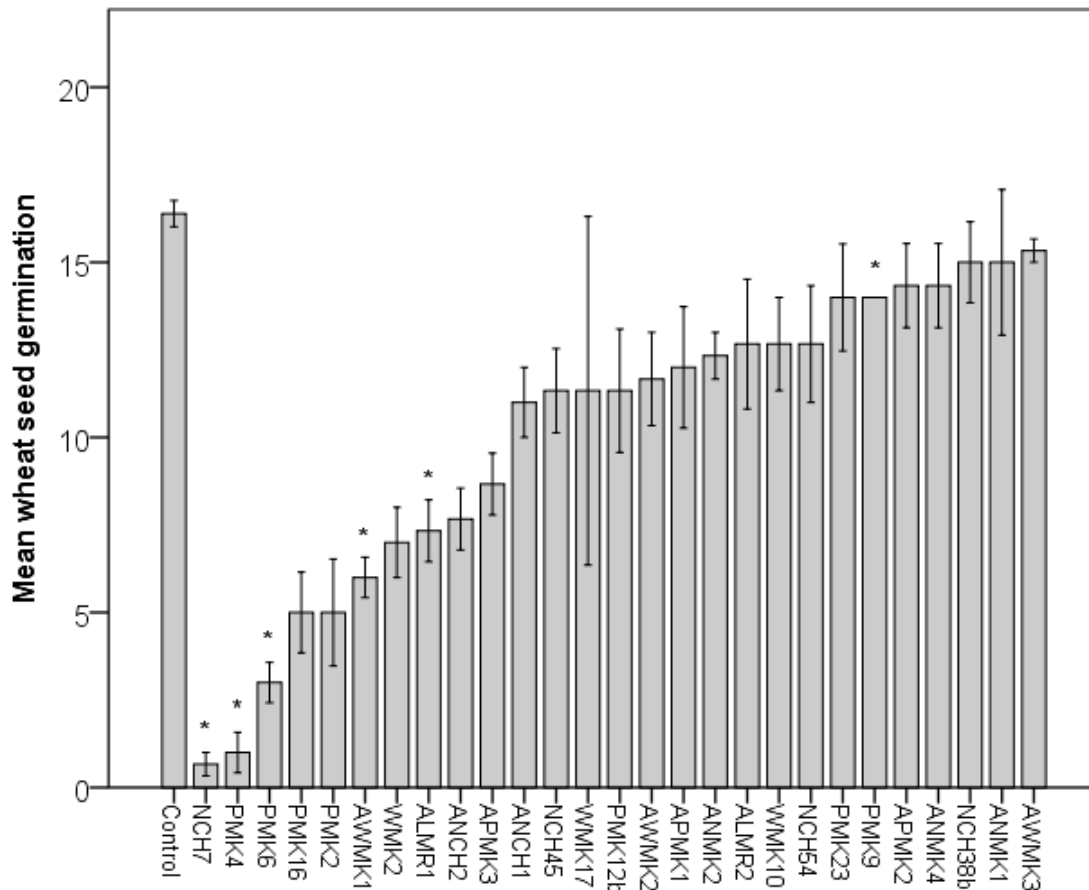

Figure 2.3: The effect of PGPB isolates on the germination of wheat seeds. There were 20 seeds per treatment in 3 replicates. The bars above the columns depict standard errors and asterisks above the columns demonstrate a significant (P<0.05) difference from the control according to the Games-Howell Test

2.3.3 Wheat seedling rhizosphere colonization assay

When bacterized wheat seedlings were grown on 0.6% water agar in test tubes for 12 days, colonization of the roots by APMK2W, APMK2Y, NCH38 and NCH45 could be observed but the colonization was often difficult to see (Figure 2.4). Germination of wheat seeds was poor in this medium (Table 2.5).

Control   NCH45

Figure 2.4 Rhizosphere colonization of PGPB isolate NCH45 on wheat roots in test tubes containing 0.6% water agar. Left, uninoculated roots (control) showing distinct root hairs. Right, inoculated roots with a turbid zone around roots

When roots from wheat seedlings grown in river sand were placed in the TTC solution, the root tips of both control and bacterized roots turned red in 24 hours while after 48 hours colour developed on older sections of the root system of the bacteria treated seedlings (Table 2.6, Figure 2.5). In contrast, when the TTC dye/agar overlay was poured over the root systems that had been incubated overnight on TY agar, colour development on all the bacteria treated root systems was rapid (less than 60 minutes) and there was no colour development on the root system of the controls. After a further

24 hours incubation, the bacteria had swarmed over the plates and the red dye development had also intensified. The germination of the wheat seeds in this system was between 80-100%, higher than when growing the seedlings in the test tubes with 0.6% water agar. The *E. coli* and *B. subtilis* strains, both were also able to successfully colonise the roots of the seedlings.

Table 2.5: Wheat seed germination and root colonization by PGPB in 0.6% water-agar

Treatment	No of germinated seeds (out of 4)	Root colonization observed
Control	2	X
ANMK1	1	X
ANMK4	3	X
APMK1	2	X
APMK2W	3	√
APMK2Y	4	√
AWMK3	1	X
NCH38	2	√
NCH45	3	√
PMK4	1	X
PMK9	2	X
WMK10	1	X

√, trait observed; X, trait not observed

Table 2.6: Detection of bacteria on wheat seedling roots systems using a 2,3,5 triphenyltetrazolium chloride (TTC) dye solution in test tubes (tubes) or as a TTC dye/agar overlay on root systems plated overnight on TY agar (plates)

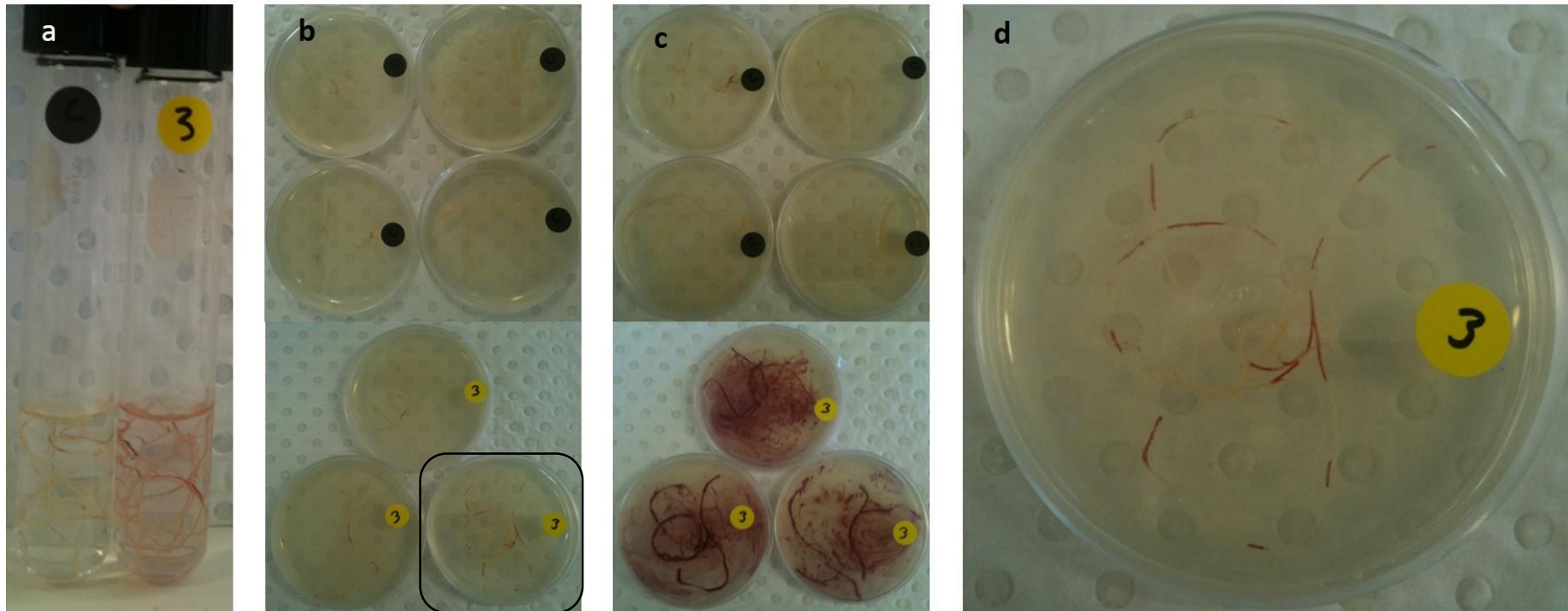| Treatment	Germination (%)	Dye development on root in 1 hour		Dye development on roots in 24 hours		Dye development on roots in 48 hours
		Tubes	Plates	Tubes	Plates	Tubes
Control	80	Nil	Nil	root tips	Nil	root tips
E. coli	80	Nil	Good	root tips	Strong	Good
B. subtilis	90	Nil	Good	root tips	Strong	Good
NCH45	100	Nil	Good	root tips	Strong	Good
PMK4	90	Nil	Good	root tips	Strong	Good

Figure 2.5: Roots of wheat seedlings depicting root colonization by the PGPB isolate NCH45 using TTC dye solutions. a) Uninoculated root system (left) and root system inoculated with NCH45 (right) in TTC solution in test tubes after 48 hours incubation; b) uninoculated root systems (top) and root systems inoculated with NCH45 (below) on TY agar plates overlaid with TTC/agar 1 hour incubation after pouring the overlay; c) uninoculated root systems (top) and root systems inoculated with NCH45 (below) on TY agar plates overlaid with TTC/agar 24 hours incubation after pouring the overlay; d) root system inoculated with NCH45 on TY agar overlaid with TTC/agar 1 hour incubation after pouring the TTC/agar overlay showing detailed colour development of the TTC dye depicting root colonization by the bacteria from the plate indicated by the square in Fig 2.5b

2.3.4   Wheat seedling growth pouch assays

Isolate PMK4 reduced germination of the wheat seed to 65% in the growth pouch assay, but seedling root length was significantly (P=0.001) increased compared with the control (Table 2.7). The putative ACC deaminase isolate AWMK3 also resulted in a significant (P=0.000) increase in root lengths but had no effect on germination percentage. Seedling root lengths were also significantly increased when seed was inoculated with WMK10 (P=0.002) and PMK9 (P=0.000). A significant (P=0.041) reduction in root length was observed for the IAA producing isolate NCH54. This isolate also significantly (P=0.000) reduced leaf lengths, as did NCH7 (P=0.009). A significant increase in leaf length was observed in seedlings treated with AWMK3 (P=0.004) and APMK2W (P=0.033).

2.3.5   Production of ACC deaminase by PGPB isolates

2.3.5.1 ACC consumption assay

The isolate that utilized the greatest (LSD, P=0.000) amount of ACC in this assay was NCH45, from the initial isolation procedure (Figure 2.6 and 2.7). Isolate APMK2Y was the only PGPB isolated on ACC-DF salts medium that was able to utilize ACC (LSD, P=0.000). In the culture supernatant of the remaining isolates, 0.4 mM ACC and above was detected.

2.3.5.2 Amplification of the acdS gene

Non-specific bands were observed when the annealing temperature of the PCR protocol for the DegACCf/DegACCr primers was 46°C as specified by (Nikolic et al. 2011) (data not shown). The annealing temperature was increased to 50°C to increase specificity of primer annealing in the PCR reaction. A product of the expected size (~750 bp) was amplified for several isolates at an annealing temperature of 50°C for the isolate ANMK1, NCH45 (both gDNA and cell suspension), PMK4, WMK10 and the

positive control *B. phymatum* STM 815 (Figure 2.8). However, non-specific bands were still observed and the annealing temperature was increased to 55°C. At this temperature, distinct bands of the expected size were observed for NCH45 (cell suspension) and STM815 only.

Table 2.7: The effect of plant growth promoting bacteria on root length and leaf length of wheat seedlings in growth pouches. Data are presented as mean root or leaf length with standard errors in brackets. Data with the same letter in the column for each experiment do not differ significantly at the P>0.05

		Total root length (mm)	Leaf length (mm)	Germination (% control)
Experiment 1[*]	Control	459 (17)^{ab}	67 (3)^{a}	
	NCH7	421 (18)^{bc}	58 (2)^{b}	ND
	NCH45	485 (13)^{a}	68 (1)^{a}	ND
	NCH54	404 (10)^{c}	55 (1)^{b}	ND
	ANOVA	$F(3, 147)=6.483$; $P=0.000$; $\eta^2=0.117$; $\beta=0.967$	$F(3, 147)=11.68$; $P=0.000$; $\eta^2=0.195$; $\beta=1.00$	
Experiment 2[*]	Control	235 (24)^{b}	35 (4)^{a}	
	NCH45	305 (26)^{ab}	42 (3)^{a}	100
	PMK4	413 (41)^{a}	47 (5)^{a}	65
	WMK10	387 (29)^{a}	51 (4)^{a}	80
	ANOVA	$F(3, 119)=7.281$; $P=0.000$; $\eta^2=0.155$; $\beta=0.981$	$F(3, 119)=2.710$; $P=0.048$; $\eta^2=0.065$; $\beta=0.646$	
Experiment 3[#]	Control	122 (6)^{cd}	44 (1)^{b}	
	ANMK1	111 (5)^{d}	44 (1)^{b}	91
	APMK2W	131 (6)^{bc}	49 (1)^{a}	100
	AWMK3	220 (7)^{a}	49 (1)^{a}	100
	PMK9	178 (11)^{b}	49 (1)^{b}	96
	ANOVA	$F(4, 384)=37.658$; $P=0.000$; $\eta^2=0.282$; $\beta=1.00$	$F(3, 147)=4.391$; $P=0.002$; $\eta^2=0.044$; $\beta=0.934$	

ND, not determined: [*], means compared using Tukey's HSD Test (P<0.05); [#], means compared using Games-Howell Test (P<0.05)
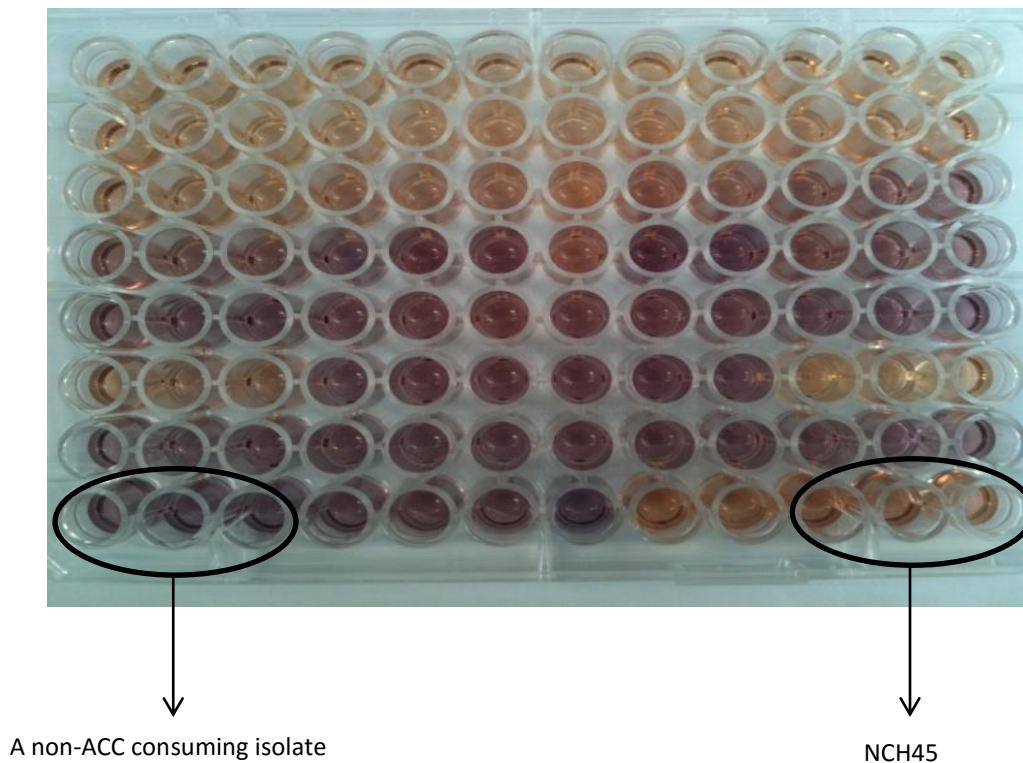
A non-ACC consuming isolate

NCH45

Figure 2.6: Colour development of the ninhydrin reagent when incubated with the supernatant of PGPB isolates grown in DF medium containing ACC as a nitrogen source. The dark red colour on the bottom left depicts a non-ACC deaminase producing isolate. The orange colour to the bottom right depicts colour development for an isolate (NCH45) that produces ACC deaminase in the medium

2.3.6 Production of siderophores and polyamines by PGPB isolates

With the exception of PMK9, all the PGPB grew on media containing HTDMA (Table 2.8). Subsequently, PMK9 grew on only one replicate plate of the CAS agar (which contained HDTMA) and the orange halo indicating siderophore production was observed in this replicate (Figure 2.9). On the other hand, APMK2Y did grow well on agar containing HDTMA, and also grew well on the CAS agar, but did not produce an orange halo, therefore it appears that this isolate does not produce siderophores that can be detected by this medium. All the other PGPB isolates produced an orange halo indicating that they are able to produce siderophores. The only two isolates that

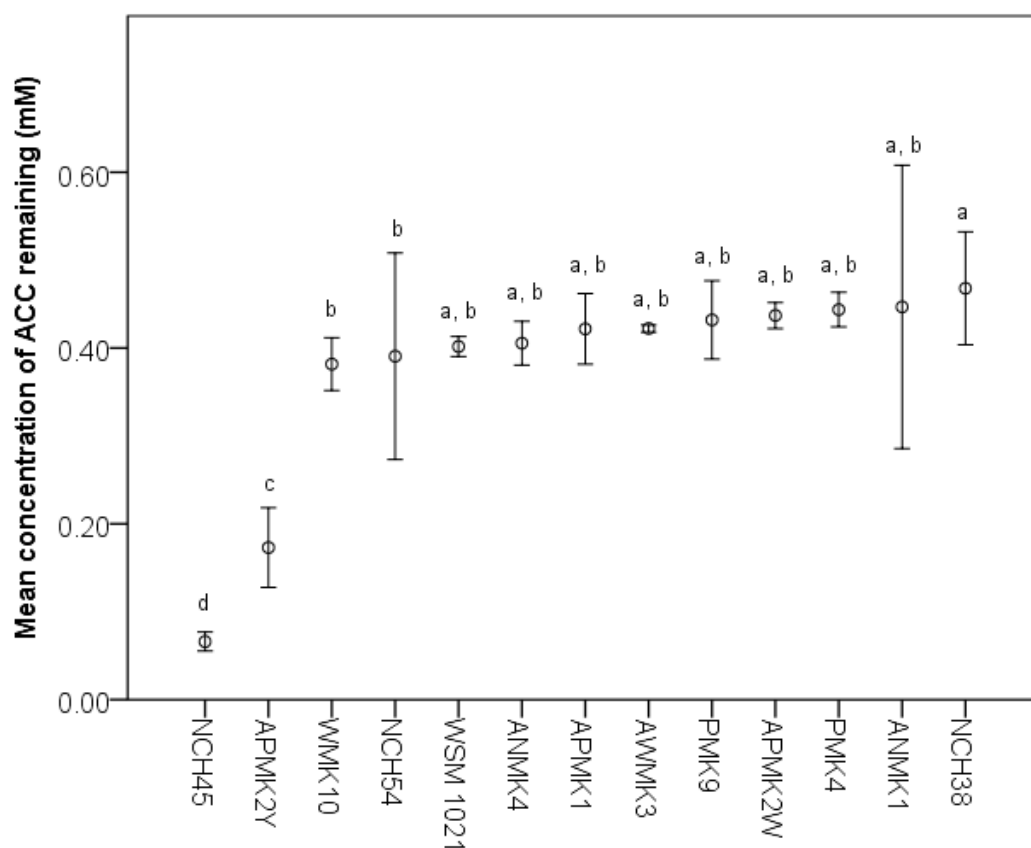produced halos on the polyamine agar and not the L-arginine non amended controls, were PMK4 and WMK10.

Figure 2. 7: Mean concentration of 1-aminocyclopropane-1-carboxylic acid (ACC) (mM) remaining in the DF medium amended with ACC (3.0 mM) after incubation with the PGPB for 24 hours. Bars above the means depict 95% confidence intervals and means with the same letters are not significantly (P>0.05) different according to Tukey's HSD Test

2.3.7 Antifungal activity and production of cellulase, pectinase, chitinase and β1,3-glucanase by PGPB isolates

There was no evidence of antifungal activity by the bacteria against any of the fungal species tested except for an inhibition of *R. solani* on ½ PDA by PMK9 which as not evident after 7 days. Furthermore, with the exception of the halo produced by APMK2Y on baker's yeast agar, the bacteria did not produce halos on the test plates for baker's yeast, cellulose or pectinase.
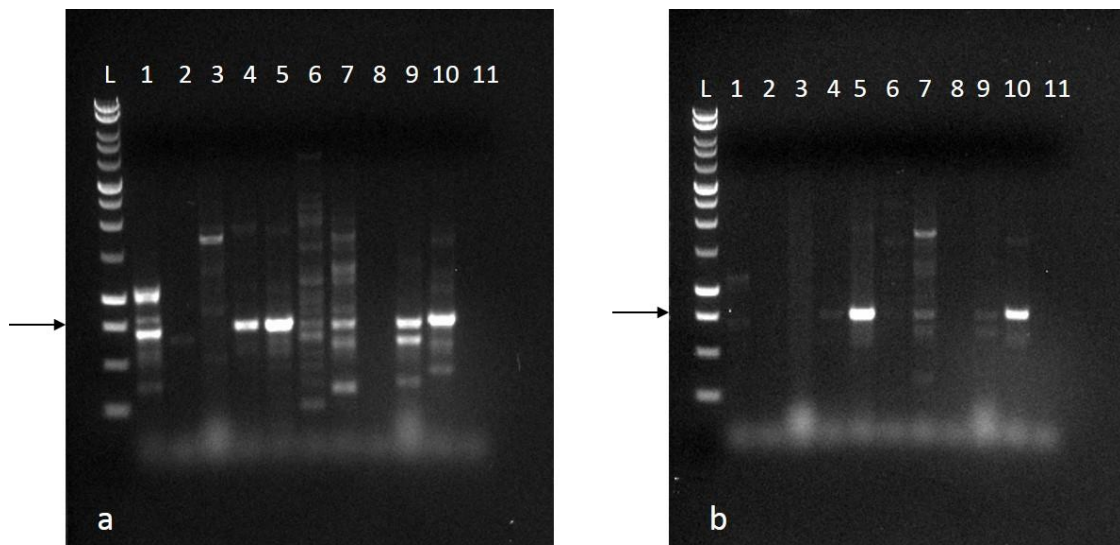
Figure 2.8: Amplification of the *acdS* gene using the DegACCf/DegACCr primers at 50°C annealing temperature (a) or 55°C annealing temperature (b). The isolates were: ANMK1 (lane 1), APMK2W (lane 2), APMK2Y (lane 3), NCH45 gDNA (lane 4) NCH45 cells (lane 5), NCH54 (lane 6), PMK4 (lane 7), PMK9 (lane 8), WMK10 (lane 9) and the positive control *B. phymatum* STM 815 (lane 10). Water (lane 11) was used as a negative control. The product was approximately 750 bp in size as indicated by the arrows next to the 1 kb ladder (L)
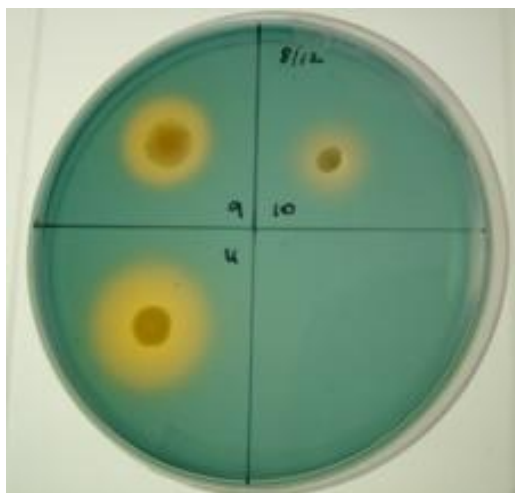
Figure 2.9: Siderophore production by PGPB isolates on blue CAS agar plates The orange halo surrounding the bacterial colonies demonstrate the removal of the ferric ion from the CAS-HDMA complex by the production of a strong chelator (siderophores) by the bacteria. The isolates are PMK4 (9), PMK9 (10) and WMK10 (11) with a control (blank) in the last quarter of the plate

2.3.8   Phosphate solubilisation by PGPB isolates

The ability of the PGPB to solubilise sparingly soluble phosphate was assessed on 3 different types of solid media. The isolates APMK2Y and PMK9 did not produce a halo, indicating no phosphate solubilisation, on any of the solid media (Table 2.9).

ANMK1 produced a solubilisation halo on CAP medium but not on PVK or NBRIP media. The remaining isolates produced solubilisation halos of varying intensity on all the solid media (Figure 2.10).

When the isolates were assessed for phosphorus solubilisation in liquid PVK medium, there was a significant ($F_{(7, 15)}$ = 26.666; P=0.000; η^2=0.926; β=1.00) difference in the solubilisation of the hydroxyapatite by the PGPB. Isolate NCH45 demonstrated the highest level of phosphate solubilised (452 ppm) (Figure 2.11). ANMK1 which only produced a clear solubilisation halo on CAP plates, was able to solubilise the hydroxyapatite contained in the PVK medium (220 ppm). The isolate PMK9 which did not produce a halo on any of the solid media, was able to solubilise a substantial amount of phosphate in the PVK medium (262 ppm). Isolate APMK2Y did not produce a solubilisation halo on any of the solid media nor did it solubilise an appreciable amount of TCP in liquid medium (26 ppm).

2.3.9 16S rRNA identification of PGPB isolates

Isolates that warranted further testing for their ability to promote plant growth in the field were identified on the basis of the 16S rRNA gene sequence and the closest match (to species level) for each isolate is depicted in Table 2.10. The isolate APMK2Y was most phylogenetically related to *Stenotrophomonas maltophilia* and as it difficult to make a distinction between the plant beneficial strains and the opportunistic human pathogens (Berg and Martinez 2015) this strain was not included in the field trials. Three of the isolates closely related to *Pseudomonas* species; *P. granadensis* (PMK4), *P. brassicacearum* (WMK10) and a *Pseudomonas* sp. (NCH54) that does not closely match with a related species. Only one Gram positive isolate was identified, PMK9, which is most phylogenetically related to *Bacillus megatarium*.

Table 2.8: Assessment of PGPB traits and the ability to grow on hexadecyltrimethylammonium bromide (HDTMA) (used in CAS agar) in plate assays. The PGPB were inoculated on each type of agar plate in triplicate and incubated at 28°C

Treatment	Growth on HDTMA		Indication of PGPB trait on selected media					
	0.1X TSA +HDTMA	0.1X TSA -HDTMA	Siderophores (CAS agar)	Polyamines (MDAM)	Antifungal activity (various fungal pathogens)	β1,3-glucanase/ chitinase (Baker's yeast agar)	Cellulase	Pectinase
ANMK1	√	√	√	X	X	X	X	X
APMK2W	√	√	√	X	X	X	X	X
APMK2Y	√	√	X	X	X	√	X	X
NCH45	√	√	√	X	X	X	X	X
NCH54	√	√	√	X	X	X	X	X
PMK4	√	√	√	√	X	X	X	X
PMK9	X	√	*	X	X	X	X	X
WMK10	√	√	√	√	X	X	X	X

√, trait observed on all three replicate plates; X, trait not observed; *, growth of PMK9 and an orange halo was observed on CAS agar on one replicate plate only

Table 2.9 Solubilisation of calcium phosphate on solid media by PGPB. Phosphorus solubilisation is presented as the solubilisation index (diameter of colony and solubilisation halo divided by diameter of colony) of the sparingly soluble phosphorus sources $CaHPO_4$ (MCP) or $Ca_3(HPO_4)_2$ (TCP) in various media. Standard errors are shown in parenthesis

	Phosphorus solubilisation index		
Treatment	CAP plates (MCP)	PVK Plates (TCP)	NBPRIP Plates (TCP)
ANMK1	1.05 (0.03)	0	0
APMK2W	2.90 (0.11)	1.61 (0.06)	1.38 (0.10)
APMK2Y	0	0	0
NCH45	1.30 (0.12)	1.41 (0.06)	1.62 (0.05)
NCH54	1.25 (0.12)	1.48 (0.12)	1.14 (0.00)
PMK4	1.86 (0.40)	1.22 (0.02)	1.56 (0.10)
PMK9	0	0	0
WMK10	2.31	1.78 (0.10)	1.49 (0.05)
ANOVA	$F_{(7, 34)} = 33.488$; $P=0.000$; $\eta^2=0.873$; $\beta=1.00$	$F_{(7, 16)} = 162.752$; $P=0.000$; $\eta^2=0.986$; $\beta=1.00$	$F_{(7, 16)} = 200.885$; $P=0.000$; $\eta^2=0.989$; $\beta=1.00$

ANMK1 on NBRIP medium   NCH45 on NBRIP medium   NCH54 on NBRIP medium

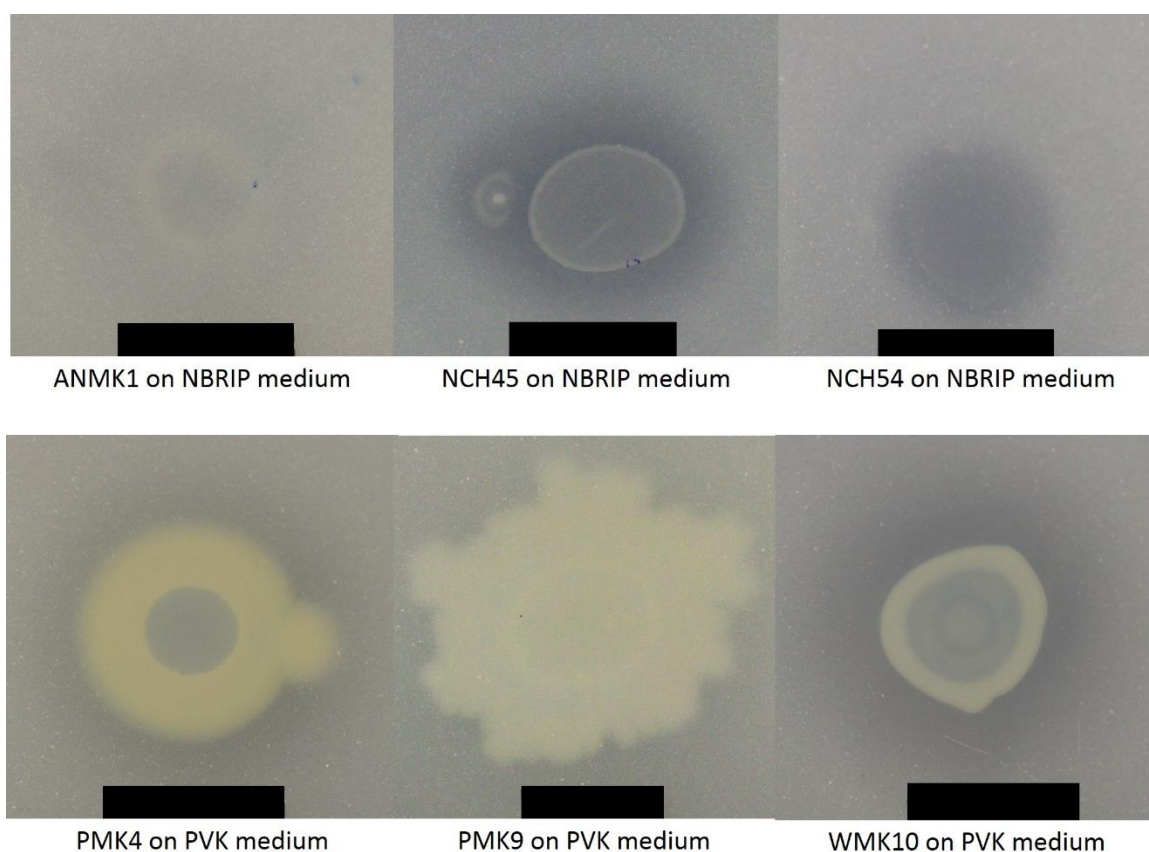PMK4 on PVK medium   PMK9 on PVK medium   WMK10 on PVK medium

Figure 2.10: The presence or absence of a solubilisation halo surrounding the colonies of PGPB isolates on agar media. The isolates NCH45, NCH54 and PMK4 demonstrate clear phosphate solubilisation halos around the colonies. Isolates ANMK1 and PMK9 do not demonstrate a clear halo. The black bar represents 1 cm
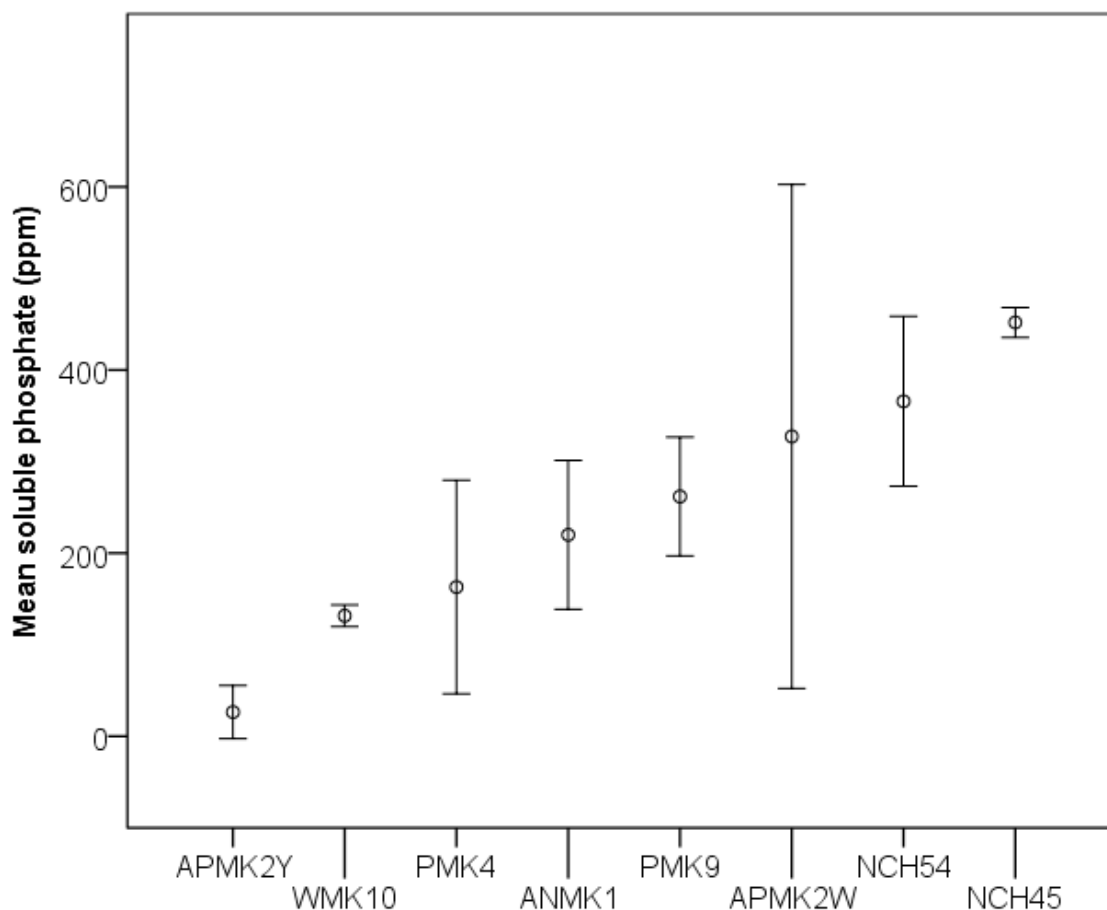
Figure 2.11: Solubilisation of tricalcium phosphate in liquid Pikovskaya medium by selected PGPB. Bars on the means depict 95% confidence intervals

Table 2.10: Identification of the PGPB strains isolated in the current study using 16S rRNA PCR techniques

Treatment	Closest Match (Accession no. in brackets)	Database	No. bp	% sequence identity
ANMK1	*Enterobacter soli* (CP003026)	EzTaxon	1485	99.1
APMK2W	*Acinetobacter calcoaceticus* (AIEC01000170)	EzTaxon	1478	100.0
APMK2Y	*Stenotrophomonas maltophilia* (FJ707375)	NCBI	1503	99.7
NCH45	*Burkholderia caledonica* (BAYE01000050)	ExTaxon	1477	99.7
NCH54	*Pseudomonas* sp. (EU680989)	NCBI	1479	98.9
PMK4	*Pseudomonas granadensis* (HG764746)	EzTaxon	1399	99.9
PMK9	*Bacillus megaterium* (JJMH01000057)	EzTaxon	1495	100.0
WMK10	*Pseudomonas brassicacearum* (EU391388)	EzTaxon	1439	99.7

2.4   Discussion

A total of 166 rhizosphere isolates were obtained by the preliminary isolation technique. However, this does not represent the total culturable proportion of rhizosphere organisms in these soils. The culturable fraction of soil microorganisms is generally found to be less than 5% of the total microscopically countable cells (Sait et al. 2002) which is attributed to the intrinsic selectivity of any medium utilized as well as the incubation conditions (Stevenson et al. 2004). Furthermore, in the current study, the true population of bacteria in the soils may be underrepresented due to loss of viability of some bacteria during drying of the rhizosphere soils before plating onto the agar media as well as the selection of only fast growing colonies on the ½ NA agar.

Previous studies have shown that around 80% of rhizosphere organisms are able to produce auxin (Glick et al. 1999; Patten and Glick 1996), and in the current study 97% of the organisms tested produced detectable levels of auxin. A greater number of high auxin producing (>10.0 μg mL^{-1} in presence of L-TRP) isolates were obtained from the rhizosphere of crop species than from native species. For instance, 16 high auxin producing isolates were obtained from the rhizosphere soils of peas and wheat at Meckering compared with four from the rhizosphere soil of native species at Chittering. The two highest auxin producing isolates were obtained from peas at Meckering (*P. granadensis* PMK4) and from native species at Chittering (NCH7).

Auxin production by the rhizobacterial isolates was substantially increased with the addition of L-TRP to the culture medium. The number of isolates capable of producing >1.0 μg mL^{-1} IAA equivalents increased by 50% with the addition of L-TRP to the medium. These results complement those of other workers on the increases in microbial production of IAA upon the addition of L-TRP to culture media and soils (Asghar et al.

2004; Khalid et al. 2001; 2004b; Sarwar et al. 1992), and indicates that L-TRP serves as a physiological precursor for the biosynthesis of auxins in microbes.

Root exudates are a natural source of the amino acid tryptophan for rhizosphere microflora in soil (Khalid et al. 2001). Martens and Frankenberger (1994) found detectable amounts of tryptophan in the root exudates of some, but not all varietes of wheat, and it is suggested, therefore, that not all plants release tryptophan in quantities adequate for the microbial production of auxin (Khalid et al. 2001). However, Prinsen et al. (1993) showed that *Azospirillum brasilense* has a tryptophan independent auxin biosynthesis pathway that accounts for 90% of the auxin produced by this bacterium in the absence of exogenously applied L-TRP.

It has frequently been demonstrated that formulations of the Salkowski reagent show varying specificity for auxin and indolic compounds. For instance, Glickman and Dessaux (1995) examined the specificity of three versions of the Salkowski reagent and the three formulations also reacted with indolepyruvic acid (IPyA) and indoleacetamide (IAM), which are intermediates in the IAA biosynthesis pathway. As a consequence, Glickman and Dessaux (1995) suggest the production of IPyA and IAM by bacterial isolates may overestimate IAA production using these techniques. In support of this, using reverse phase high performance liquid chromatography (RP-HPLC) Szkop et al. (2012) detected high levels of indole-3-lactic acid, which is detected by some forms of the Salkowski reagent, as well as IAA and IAM in the culture supernatant of a *P. putida* strain. Furthermore, Goswami et al. (2015) determined IAA production by *Pseudomonas aeruginosa* strain OG by the spectrophotometric (Salkowski) method as 28.83 μg mL^{-1} whereas using high performance thin layer chromatography (HPTLC), production of IAA was 1.60 μg mL^{-1}. This indicates that IAA intermediate compounds

or other metabolites are being detected by the spectrophotometric method and this needs to be borne in mind when interpreting results from these assays. Therefore, the accurate identification and quantification of the auxins produced by the bacteria in the current study needs to be confirmed by one of the more specific methods detailed above.

In comparison to isolating PGPB based on IAA production, isolating PGPB based on production of ACC deaminase was a much more rapid and straightforward method. The PAF medium is selective for *Pseudomonas* species and thus a lower number of isolates was obtained than in the IAA methods, however, this method could easily be altered to select other common PGPB such as *Azospirillum*, *Azotobacter* and *Bacillus* as well as Actinomycetes by altering the isolation medium.

The ability of selected isolates to utilize ACC was confirmed using the rapid method by Li et al. (2011) which demonstrated that only one of six PGPB (*Stenotrophomonas maltophilia* APMK2Y) isolated on the medium containing ACC as a sole nitrogen source, was capable of utilizing ACC in the DF salts medium. The isolate that utilized the highest amount of ACC in the DF medium was *Burkholderia caledonica* NCH45, and the presence of the gene for ACC deaminase production was confirmed using the DegACCf/DegACCr primer set designed to amplify the *acdS* (ACC deaminase) gene. Using these primers, a 750 bp fragment of the expected size was also amplified for the isolates *Enterobacter soli* ANMK1, *P. granadensis* PMK4 and *P. brassicacearum* WMK10 using the annealing temperature of 50°C in the PCR protocol, although non-specific banding was also observed for these isolates at this temperature. This suggests that these isolates may contain the *acdS* gene and sequencing of these bands will confirm if this gene is present in these bacteria. Identification of the ACC deaminase genes in bacteria could also be improved using consensus-degenerate hybrid

oligonucleotide primers based on differentiating the key residues in ACC deaminases from those of homologs developed by Li et al. (2015).

In order to rapidly screen a large number of isolates at a reduced cost, transferring the products of the ninhydrin reaction to microplates and determining the absorbances can be omitted (Li et al. 2011). This is because the colour depth of the media inoculated with isolates capable of degrading ACC deaminase is greatly reduced compared with media inoculated with an isolate incapable of degrading ACC (Figure 2.6) and therefore, the ACC deaminase producing isolates can simply be visually determined.

The production of ACC deaminase by the PGPB isolates can also be confirmed and quantified using the methods outlined in Penrose and Glick (2003) based on an assay that measures the production of α-ketobutyrate that is produced when the enzyme cleaves ACC. However, these assays are not suitable for all species and there is potential for a false negative response for some ACC deaminase producing strains. For instance, in *Mesorhizobia* species, the *acdS* gene is under the transcriptional control of the *nifA* promoter, which activates transcription of the nitrogen fixation genes (Brígido et al. 2015; Nascimento et al. 2012a; Nukui et al. 2006; Uchiumi et al. 2004). These mesorhizobial species have been demonstrated to produce ACC deaminase only in the bacteriod stage (during nitrogen fixation) therefore under free-living conditions, the ability of these strains to produce ACC deaminase is not detected using biochemical assays.

Based on the above, the ability of microorganisms to produce IAA or ACC deaminase does not immediately imply that these organisms will act as PGPB on target plants. It is possible that some of the microorganisms isolated will be deleterious. However, germination of clover seeds was not significantly affected by bacterization with the

PGPB, except for isolate AWMK3 which consistently reduced clover germination. Contrary to the effects of the isolates on clover seeds, the response of the wheat seeds to the PGPB was varied. The two highest IAA equivalent producing organisms *P. granadensis* PMK4 and NCH7 elicited the lowest germination of wheat seeds and several of the PGPB also significantly lowered germination in comparison to the controls.

The results for the wheat seed assays compliment the findings in the literature that rhizobacteria that produce high levels of IAA can have a deleterious effect on plant growth (Loper and Schroth 1986; Xie et al. 1996). However, the apparent toxic effects of the isolates on the seeds used in this trial are not necessarily due to the high production of IAA by these organisms. Non-pathogenic rhizosphere organisms commonly known as deleterious rhizosphere microorganisms (DRMOs) have been shown to be detrimental to plant growth not only via the production of phytohormones in high concentrations but also by the production of phytotoxins, hydrogen cyanide (HCN) and the siderophore-mediated competition for iron (Bowen and Rovira 1999; Kremer and Souissi 2001). For instance, a non-fluorescent *Pseudomonas* sp. isolated from winter wheat was found to produce an unidentified water soluble toxin that inhibited the growth of both winter wheat and several different microorganisms (Bolton and Elliott 1989; Bolton et al. 1989). Further, Kremer and Souissi (2001) isolated rhizobacteria from the roots of *Euphorbia* spp. and reported that production of HCN at rates greater than 5 nmoles mg cellular protein^{-1} from these organisms contributed to growth inhibition of selected weeds. Therefore, there may be scope for the use of some of the isolates that demonstrate deleterious effects on only the target weed species as biocontrol agents (Kremer and Kennedy 1996).

Isolate AWMK3 demonstrated the largest increase in wheat root lengths (81% increase) in the growth pouch assays. Unfortunately, this isolate became difficult to subculture and was not analysed further at this stage. Other PGPB that significantly increased wheat root lengths in the growth pouch assays included the isolates *P. granadensis* PMK4, *Bacillus megaterium* PMK9, and *P. brassicacearum* WMK10 and although the increases in root length were not significant, *B. caledonica* NCH45 demonstrated a trend towards increasing root lengths.

To be considered a true plant-growth promoting rhizobacteria, the bacteria needs to demonstrate its ability to colonise the root system of the target host plant (Chin-A-Woeng et al. 2000; Lugtenberg et al. 2001; Misaghi 1990; Simons et al. 1996). In this study, two methods were utilized to determine the ability of the PGPB to colonise wheat roots. The first method using 0.6% water agar in tubes (Silva et al. 2003) was not suitable for growth of wheat seedlings and therefore rhizosphere colonization was difficult to determine. The wheat seedling germination and growth was more normal using sand in tubes and the TTC agar overlay method allowed clear detection of the bacteria on the roots. This method showed that the PGPB isolates NCH45 and PMK4 were able to colonise the root system of the wheat seedlings well, along with the *E. coli* and *B. subtilis* strains. *B. subtilis* has previously demonstrated the ability to colonise wheat roots (Dijkstra et al. 1987) and is currently a commercial PGPB inoculant (Glick 2012). Morales et al. (1996) demonstrated that an *E. coli* strain was able to persist but not grow in the wheat rhizosphere after 7 days. However, in contrast to this, an *E. coli* strain used by Schloter and Hartmann (1998), was not able to colonise wheat seedling roots. The ability of *E. coli* and the PGPB to colonise and persist in the wheat rhizosphere is likely dependent on factors such as exopolysaccharide production and nutritional diversity of the bacterial strains (Morales et al. 1996) as well as the plant

variety. However, the ability of bacteria to colonise the host root in sterile conditions (rhizosphere colonization) does not often correlate with their ability to persist in the presence of indigenous soil bacteria (rhizosphere competence). *Pseudomonas* spp. that are rhizosphere competent display traits including siderophore-mediated iron acquisition, expansive substrate utilization, and (total) denitrification as well as ability to produce antibiotics, phenazines and N-AHSLs (Ghirardi et al. 2012). Factors reducing the ability of the bacteria to survive in the rhizosphere include predation by protozoa (Ramirez and Alexander 1980) although several *Pseudomonas* spp. have been shown to produce antibiotics effective against protozoa (Pedersen et al. 2010). A method to detect the ability of *P. granadensis* PMK4 to successfully colonise the wheat rhizosphere and legume nodules in sterile and non-sterile conditions is described in Chapter 7.

To determine the potential mechanism(s) of action of the PGPB, several plate assays were performed for traits commonly associated with beneficial bacteria. Most of the isolates were able to produce siderophores with the exception of APMK2Y and to some extent PMK9. However, Alexander and Zuberer (1991) found that several strains of bacteria that produced siderophores in liquid media did not produce a halo on CAS agar. A more effective method to detect siderophore production in isolates like *B. megaterium* PMK9 and other Gram positive bacteria and fungi that are sensitive to HDTMA, is the CAS overlay method (O-CAS) described by (Pérez-Miranda et al. 2007).

Two of the *Pseudomonas* species WMK10 and PMK4 produced polyamines and these isolates also consistently produced halos on the solid calcium phosphate media, as did *A. calcoaceticus* APMK2W, *B. caledonica* NCH45 and *Pseudomonas* sp. NCH54. The ability of *B. megaterium* PMK9 and *E. soli* ANMK1 to solubilise the hydroxyapatite in

the liquid but not solid media is similar to results observed by Gupta et al. (1994) Pérez et al. (2007) and Marra et al. (2011). However, the use of calcium phosphate is not as applicable to iron rich and acidic soils where the dominant forms of insoluble phosphate include Fe-P and Al-P complexes (Pérez et al. 2007). In these target soils, the use of $FePO_4$ and $AlPO_4$ instead of calcium phosphate may prove more useful in the isolation of potential PSM.

In conclusion, 85 potential PGPB were isolated from the rhizosphere of agricultural and native plant species based on their potential ability to produce IAA (72 isolates) and ACC deaminase (13 isolates). These isolates were screened in plant assays to select the most suitable isolates for legume and cereal plant species and eight isolates including seven Gram negative and one Gram positive bacterial species were selected. These bacteria were assayed for potential PGPB traits additional to IAA and ACC deaminase production. None demonstrated antifungal activity, but most had the ability to produce siderophores (7 isolates) and solubilise P (7 isolates) in either plate or liquid assays. These isolates were taken to field trials to ascertain their ability to promote plant growth in conventional agricultural settings (Chapter 3).

Chapter 3:
The effect of potential plant growth-promoting bacteria on wheat and peas in Western Australian field soils

The emergence of wheat at the Kojonup field trial in Western Australia

3.1   Introduction

As pointed out by Moore (2001) the basic resource for almost all of agriculture is the soil. Plants rely on the soil to support and anchor them as they grow, to supply water and nutrients and to exchange atmospheric gases. Soils that can meet these requirements are generally non-limiting to plant growth. However, in Western Australia much of the landscape is highly weathered and the absence of tectonic, volcanic and glacial activity has resulted in minimal soil renewal, making these soils a non-renewable resource. The major agricultural soils in Western Australia are shallow, sandy duplex soils and sands which would be considered non-arable elsewhere. Most of these soils have physical and/or chemical properties that make them limiting to plant growth and require substantial management to ensure adequate crop yields. The main reason agriculture is possible on these soils is due to the Mediterranean climate with its relatively reliable rainfall.

One of the major factors in the development of millions of hectares of farmlands in Western Australia in the late 19th century was the import and application of superphosphate (Burvill 1979a). During the early 20th century, when it was discovered that plants also require trace elements for good growth, vast areas of Western Australian soils were found to be deficient in copper, zinc, manganese and molybdenum as well as nitrogen (Burvill 1979b; c). Post World War II, improvements to fertilizers were accompanied by a new range of chemicals to control insect pests, weeds and fungal and bacterial pathogens (Burvill 1979b). Yet, despite these improvements, there are several problems still facing agriculture in Western Australia. These include, soil salinity (Nulsen 2004), soil acidification (Schoknecht 2004), soil structure decline (Hamza and Penny 2005) weeds, pests and the leaching of agrichemicals through the soil profile (Lantzke 2005; Russell and Calder 1998).

Furthermore, there has been increasing public concern about the effects of agrichemical residues on humans, animals and the environment (Campbell 1980). In addition to this, the sustainability of fertilizer production and their rising costs is a potential constraint to agriculture in the future and alternatives must be identified (Bockman 1997; Cordell and White 2010; White et al. 2010).

One approach to these problems is the use of biological agents such as plant growth promoting bacteria (PGPB). However, *in vitro* and glasshouse assessments of the ability of potential PGPB organisms to act as plant growth promoters must progress to field trials to determine their capacity to survive and multiply in the soil and to enhance plant growth under field conditions (Bowen and Rovira 1999). There is often inconsistency between results obtained under the controlled conditions of the laboratory or glasshouse and those obtained in the field (Lucy et al. 2004). The factors that may affect the viability of PGPB in the field include soil characteristics, such as soil type, nutrition, moisture, temperature, and pH; plant species, even genotypes within species; and competition from other rhizosphere microorganisms (Kloepper et al. 1989; Zahir et al. 2004). Nonetheless, several field studies have indicated that PGPB can improve the yield of various agricultural crop species with and without biotic abiotic stresses (Díaz-Zorita and Fernández-Canigia 2009; Hungria et al. 2010; 2013).

The aim of the work reported here was to test the effectiveness of seven PGPB isolated from Western Australian soils, under a variety of field conditions on wheat and peas as model cereal and legume species respectively. Five separate locations were selected in Western Australia that encompassed soils of different types and pH. The isolates' ability to improve the growth and yield of two wheat varieties was tested at Kojunup, Boyup Brook and Wongan Hills (2 locations). Their ability to enhance the nodulation

and yield of peas when co-inoculated with a commercial *Rhizobium* strain was tested at Kojonup and Brookton.

3.2 Materials and Methods

3.2.1 Inoculum

The identification and characterization of the plant growth promoting bacteria (PGPB) isolates used in these field trials are described in Table 3.1 and the field experimental conditions are detailed in Table 3.2. The PGPB isolates were delivered in the field using the clay based inoculum technology Alosca™ which was prepared as per manufacturer's instructions (confidential) or as a peat based inoculum (Brookton). The wheat trials all included control uninoculated plots. At the pea trials at Kojonup and Brookton, seeds were co-inoculated with the PGPB and Group E rhizobia (Grp E) (*Rhizobium leguminosarum* bv. *viciae* SU303) that was supplied by the Centre for *Rhizobium* Studies at Murdoch University. The control pea plots were inoculated with only Group E rhizobia in either Alosca™ or peat.

3.2.2 Kojonup field trial - wheat

3.2.2.1 Site description

Kojonup (33º50´S, 117º09´E) is located 265 km southwest of Perth, Western Australia and the soil at this site was a gravelly loam, duplex soil with clay at a depth of approximately 25 cm. The pH of the top soil was 4.7, dropping to pH 4.6 at the clay layer (Table 3.2). The rainfall for the growing season was 585.9 mm. Crop nutrition and protection was applied as in Table 3.3.

Table 3.1: Identification and characterization of plant growth promoting bacteria used in the field trials in this study

Isolate	Closest 16S rDNA match	IAA	ACC deaminase production	Production of siderophores	Production of polyamines	Phosphorus solubilisation
ANMK1	*Enterobacter soli*	√	#	√	x	√
APMK2W	*Acinetobacter calcoaceticus*	√	x	√	x	√
NCH45	*Burkholderia caledonica*	√	√	√	x	√
NCH54	*Pseudomonas* sp.	√	x	√	x	√
PMK4	*P. granadensis*	√	x	√	√	√
PMK9	*Bacillus megaterium*	√	x	*	x	√
WMK10	*P. brassicacearum*	√	x	√	√	√

* - siderophore production seen on one replicate plate only; #- .ACC deaminase (*acdS*) gene potentially present

Table 3.2: Experimental conditions and strains of plant growth promoting rhizobacteria used in the field trials

Site	Kojonup	Kojonup	Boyup Brook	Wongan Hills Heavy Soil	Wongan Hills Light Soil	Brookton
Plant/Cultivar	Peas cv. Parafield	Wheat cv. Tincurrin	Wheat cv. Wyalcatchem	Wheat cv. Wyalcatchem	Wheat cv. Wyalcatchem	Peas cv. Kaspa
Soil Type	Duplex	Duplex	Duplex	Clay loam	Sandy loam	Duplex
pH (CaCl$_2$)	4.6-4.7	4.6-4.7	4.8	6.5-7.5	5.5-6.2	4.7-5.1
Rainfall (mm)[#]	585.9	585.9	345.4	230	230	184.9
Plot size	40 m^2	40 m^2	40 m^2	44 m^2	44 m^2	18 m^2
Number of reps	3	3	4	3	3	4
Inoculum type	Alosca™ granules	Alosca™ granules	Alosca™ granules	Alosca™ granules	Alosca™ granules	Peat
PGPB inoculation	PMK4 + Grp E	PMK4	PMK4	PMK4	PMK4	PMK4 + Grp E
	NCH54 + Grp E	NCH54	ANMK1	NCH54	NCH54	
	WMK10 + Grp E	WMK10	NCH45	WMK10	WMK10	
	PMK9 + Grp E	PMK9	APMK2W	PMK9	PMK9	
	NCH45 + Grp E		PMK4 & NCH45	NCH45	NCH45	
Controls	Grp E	uninoculated	uninoculated	uninoculated	uninoculated	Grp E & uninoculated

[#] rainfall data are for the actual growing season for the field trials (May/June-November).

Table 3.3: Crop nutrition and protection applied to wheat plots at the Kojonup field trial

Date	Treatment	Type of Treatment	Dosage
06/05/2005	Roundup®	Non selective herbicide	1.0 L ha^{-1}
20/05/2005	Superphosphate	Phosphatic fertilizer	10 kg ha^{-1}
20/05/2005	SpraySeed®	Knockdown herbicide	1.6 L ha^{-1}
20/05/2005	Trifluramax® 480	Selective herbicide	1.6 L ha^{-1}
20/05/2005	Talstar®	Pesticide	260 mL ha^{-1}
27/05/2005	Supercharge®	Surfactant	375 mL ha^{-1}
27/07/2005	Achieve®	Selective herbicide	380 g ha^{-1}
04/08/2005	Ally	Pesticide	3.0 g ha^{-1}
04/08/2005	Giant	Selective broadleaf herbicide	600 mL ha^{-1}
19/09/2005	Tilt®	Fungicide	500 mL ha^{-1}
19/09/2005	Fastac®	Pesticide	150 mL ha^{-1}

3.2.2.2 Experimental design

The wheat (*Triticum aestivum* cv. Tincurrin) seed was inoculated with four of the PGPB isolates (Table 3.2) applied as Alosca™ granules prepared according to manufacturer's instructions. The control was uninoculated seed (Nil). The Alosca™ treatments were applied to the seed 24 hours prior to sowing. The inoculated seeds (300 g plot^{-1}) and the controls were sown on 20th May 2005 by an airseeder in 2 m x 20 m plots in rows separated by 1 m buffers in a complete randomized block design with three replicates.

3.2.3 Boyup Brook field trial - wheat

3.2.3.1 Site description

Boyup Brook (33º84´S, 116º42´E) is located 263 km southwest of Perth, Western Australia and the soil at this site was a gravelly loam, duplex soil. The pH of the top soil was 4.8 (Table 3.2). The rainfall for the growing season was 345.4 mm. Crop protection and nutrition were applied as detailed for the Kojonup Field Trial (Table 3.3).

3.2.3.2 Experimental design

The wheat (*Triticum aestivum* cv. Wyalcatchem) seed was inoculated with four of the PGPB isolates (Table 3.2), as well as a mixed inocula of PMK4 and NCH45, applied as Alosca™ granules prepared according to manufacturer's instructions. The control was uninoculated seed (Nil). The Alosca™ treatments and were applied to the seed 24 hours prior to sowing. The inoculated seeds (300 g plot^{-1}) and the controls were sown in May 2006 by an airseeder in 2 m x 20 m plots in rows separated by 1 m buffers in a complete randomized block design with four replicate plots.

3.2.4 Wongan Hills heavy soil site - wheat

3.2.4.1 Site description

Wongan Hills (30º55´S, 116º45´E) is located 270 km northeast of Perth and the soil at the heavy soil site was a clay loam with pH that ranged from 6.5 to 7.5. This site had cropped wheat for the 3 years prior to this experiment and the rainfall for the growing season was 230 mm. Crop nutrition and protection was applied as shown in Table 3.4.

Table 3.4: Crop nutrition and protection applied to wheat plots at the Wongan Hills heavy soil site field trial

Date	Treatment	Type of Treatment	Dosage
14/06/05	MAPSZC®[#]	Compound fertilizer	120 kg ha^{-1}
14/06/05	SpraySeed®	Knockdown herbicide	2 L ha^{-1}
14/06/05	Trifluralin	Selective pre-emergence herbicide	1 L ha^{-1}
14/06/05	Chlorpyifos	Organophosphate insecticide	1 L ha^{-1}
14/06/05	BS1000®	Wetting Agent	0.1 %v/v
27/07/05	Tigrex®	Selective broadleaf herbicide	600 mL ha^{-1}
27/07/05	Lontrel®	Broadleaf herbicide	150 mL ha^{-1}
12/08/05	Urea	Fertilizer (46% N)	100 kg ha^{-1}
4/10/05	Tilt®	Fungicide	250 mL ha^{-1}
4/10/05	Logran®	Knockdown pre-emergent herbicide	10 g ha^{-1}
4/10/05	BS1000®	Wetting Agent	0.1 %v/v

[#] (%) = N – 10.6, P – 21.4, S – 8.0, Cu – 0.3, Zn – 0.3, Mn – 0.02; Mo - 0.0008.

3.2.4.2 Experimental design

Wheat (*Triticum aestivum* cv. Wyalcatchem) seeds were inoculated with the five PGPB (Table 3.2) treatments applied as Alosca™ granules prepared as described above (3.2.2.2). Uninoculated seed (Nil) treatments were also included. The inoculated seed (330 g plot^{-1}) and the controls were sown on 14th June 2005 by breeder seeder in 2.2 m x 20 m plots in complete randomized block design with three replicates.

3.2.5 Wongan Hills light soil site - wheat

3.2.5.1 Site description

The light soil site at Wongan Hills was approximately 6.2 km from the heavy soils site. This soil was a sandy loam with a pH that ranged from 5.5 to 6.2. This site had been in pasture for the 2 years prior to this experiment and had cropped wheat in the year prior to pasture.

3.2.5.2 Experimental design

Wheat (*Triticum aestivum* cv. Wyalcatchem) seeds were sown on 15th June and treatments, field management and sampling procedures were the same as those at the heavy soil site with the exception that crop protection and nutrients were applied as shown in Table 3.5

Table 3.5: Crop nutrition and protection applied to wheat plots at the Wongan Hills light soil site field trial

Date	Treatment	Type of Treatment	Dosage
15/06/05	K-Gold®$^{\#}$	Compound fertilizer	120 kg ha^{-1}
15/06/05	SpraySeed®	Knockdown herbicide	2 L ha^{-1}
15/06/05	Trifluralin	Selective pre-emergence herbicide	1 L ha^{-1}
15/06/05	Logran	Knockdown pre-emergent herbicide	35 g ha^{-1}
15/06/05	DC-Trate	Wetting Agent	0.1 % v/v
27/07/05	Tigrex®	Selective broadleaf herbicide	600 mL ha^{-1}
27/07/05	Lontrel®	Broadleaf herbicide	150 mL ha^{-1}

12/08/05	Urea	Fertilizer (46% N)	100 kg ha^{-1}
4/10/05	Tilt®	Fungicide	250 mL ha^{-1}
4/10/05	Logran®	Knockdown pre-emergent herbicide	10 g ha^{-1}
4/10/05	BS1000®	Wetting Agent	0.1 %v/v

[#] (%) = N – 7.4, P – 12.1, K – 10.2, S – 8.8, Ca – 3.6, Cu – 0.17, Zn – 0.2.

3.2.6 Kojonup field trial - Peas

3.2.6.1 Site description

The pea trial at Kojonup was adjacent to the wheat trial and crop protection and nutrients were applied as shown in Table 3.6.

Table 3.6: Crop nutrition and protection applied to pea plots at the Kojonup field trial

Date	Treatment	Type of Treatment	Dosage
06/05/2005	Roundup®	Non selective herbicide	1.0 L ha^{-1}
20/05/2005	Superphosphate	Phosphatic fertilizer	10 kg ha^{-1}
20/05/2005	SpraySeed®	Knockdown herbicide	1.6 L ha^{-1}
20/05/2005	Trifluramax® 480	Selective herbicide	1.6 L ha^{-1}
20/05/2005	Talstar	Pesticide	260 mL ha^{-1}
19/10/2005	Fastac®	Pesticide	200 mL ha^{-1}

3.2.6.2 Experimental design

The pea (*Pisum sativum* var. Parafield) seed was co-inoculated with Group E rhizobia (*Rhizobium leguminosarum* bv. *viciae*) and the PGPB (Table 3.2), applied with the clay-based carrier, Alosca™ granules (10 kg ha^{-1} = 36 g plot^{-1}), prepared as per manufacturer's instructions. The control was Group E rhizobia in Alosca™ granules. Seeds were mixed with the carrier 24 hours prior to sowing. The inoculated seeds (500 g plot^{-1}) and the controls were then sown on 20th May 2005 by an airseeder in 2 m x 20 m plots in rows separated by 1 m buffers in a complete randomized block design with three replicates per treatment. Plots were sampled 7 weeks after sowing by digging a subset of plants from the plots (~18 plants/plot). The nodulation of these plants was

rated (Table 3.7) and shoot dry weights were determined after drying at 60°C for 5 days. A sub-sample of pea plants in the buffers at either end of the plot were included in the nodulation rating to ensure that there was no rhizobial cross-contamination from other trials being conducted at this site.

Table 3.7: Nodule scoring system used for scoring pulse legumes. Adapted from Howieson and Dilworth (2016) (Appendix I)

Nodule Score	Description
0	No nodules
1	1-5 small nodules or 1 large nodule
2	6-10 small nodules or 2 large nodules
3	11-15 small nodules or 3 large nodules
4	16-20 small nodules or 4 large nodules
5	21-25 small nodules or 5 large nodules
6	26-30 small nodules or >5 large nodules
7	Crown nodulation incomplete or >30 nodules
8	Crown nodulation $<1cm^3$
9	Crown nodulation $>1cm^3$
10	>1 crown nodule $>1cm^3$

3.2.7 Brookton field trial - Peas

3.2.7.1 Site description

Brookton (32.37°S, 117.01°E) is located 140 km southeast of Perth, Western Australia and the soil at this site was a grey sandy loam duplex soil with gravelly clay at a depth of approximately 25-50 cm. The site was limed (1 tonne ha^{-1}) in February 2015 and was measured at a pH ($CaCl_2$) of 5.1 at 0-10 cm and pH 4.7 at 10-20 cm. The rainfall for the growing season was 184.9 mm.

3.2.7.2 Experimental design

This trial was implemented by Ron Yates of the Department of Agriculture and Food, Western Australia. The pea seed (*Pisum sativum* cv. Kaspa) was co-inoculated with

Group E rhizobia (*Rhizobium leguminosarum* bv. *viciae*) and the PGPB isolate PMK4 (Table 3.1), applied as a peat formulation at a rate of 250 g peat to 100 kg seed. The controls were Group E rhizobia in peat and uninoculated seed. Inoculated seed treatments were mixed with the carrier 21 hours prior to sowing. The inoculated seeds (200 g plot^{-1}) and the controls were then sown on 5th June 2015 by an experimental 6 row cone seeder at 15 m lengths (1.2 m wide) into a drying soil. The trial was a complete randomized block design with four replicates per treatment but separated into two banks containing 2 replicates divided by a 10 m buffer. Fertilizer was applied as superphosphate:potash (3:1) at a rate of 120 kg ha^{-1}. Plots were sampled 12 weeks after sowing by digging a subset of plants from the plots (10 plants plot^{-1}). The nodulation of these plants was rated (Table 3.7) and shoot dry weights were determined after drying at 60°C for 5 days. Additionally, later in the growing season, at late anthesis/early pod fill, mixed plant leaf samples from each plot were gathered, dried and evaluated for %Ndfa. Wild radish plants were taken as reference plants to assist in the calculation of the $^{N}15$ natural abundance technique. Samples were sent to the mass spectrometer housed at the Botany Department at University of Western Australia to be processed.

3.2.8 Harvesting

Wheat from the heavy and light soil sites at Wongan Hills were harvested in November and the yields per plot (kg plot^{-1}) determined. The seed was analyzed for quality data including: % protein content; moisture content; specific weight; and screenings (Agritech Crop Research Pty Ltd). Peas and wheat from the Kojonup sites were harvested in December and the yields per plot determined (kg plot^{-1}) and the % nitrogen content of the seed analysed (CSBP Soil & Plant Analysis Service, Bibra Lake, WA). The Boyup Brook site was harvested in November and the grain yields per plot (kg plot^{-1}) determined. At the Brookton site, grain was harvested for each plot in November

by removing and combining senesced material from two random 1 m^2 quadrat cuts within the plots. Samples were threshed to release pea seed, cleaned and the weight measured to estimate kg ha^{-1}.

3.2.9   Statistical analysis

In order to ascertain the sample size necessary to ensure that type 2 errors (false negative) are minimized, power (the probability that the null hypothesis will be rejected when it is false) was calculated for a one-way ANOVA on grain yields from the Kojonup wheat field trial (Statisica V12, Statsoft Inc. 2013). This power analysis determined that a minimum of 10 replicate field plots would be necessary to retain an alpha of 0.05 at a power of 0.90 (Figure 3.1). As these were preliminary trials to test several different PGPB for effectiveness in the field, and the area allocated to these field trials was limited, replication had to be kept to a minimum. Using the Kojonup wheat field trial, a power calculation was conducted using hypothetical 3 replicates/treatment and 4 replicates/treatment (Figure 3.2). In order to retain the highest power possible without subjecting the data to potential type 1 (false positive) errors, the alpha for 3 replicates was determined to be 0.2 with a corresponding power of 0.60 and for 4 replicates the alpha is 0.10 in order to achieve a power of 0.60. An analysis of the effect of using an alpha of 0.05 or 0.10 determined that the size of the effect is very similar in each case (Figure 3.3). Using the results from this analysis, the alpha was relaxed in subsequent trials to 0.10 and a priori contrasts comparing each treatment to the control were specified for experiments including controls. The Brookton field trial was analysed using a general linear model and where significant, data were compared using Fischer's Least Significant Difference (LSD) test. All analyses were conducted using IBM SPSS Statistics 21.
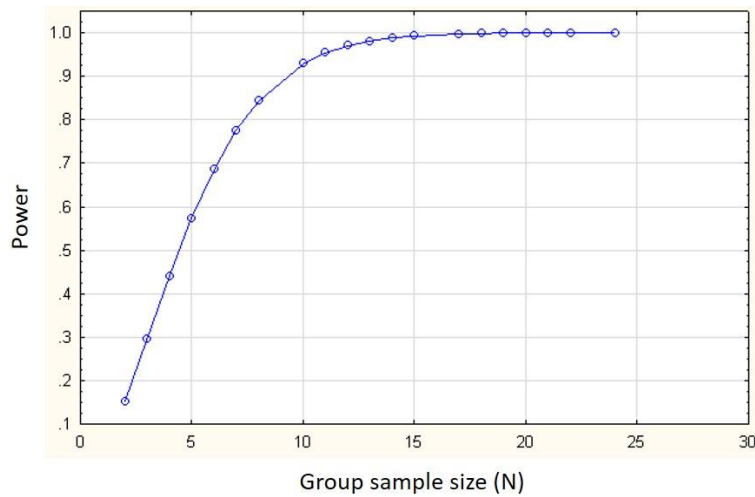
Figure 3.1: Power analysis of wheat field trial at Konjonup using the 5 treatments (4 PGPB plus uninoculated control) with the alpha set to 0.05
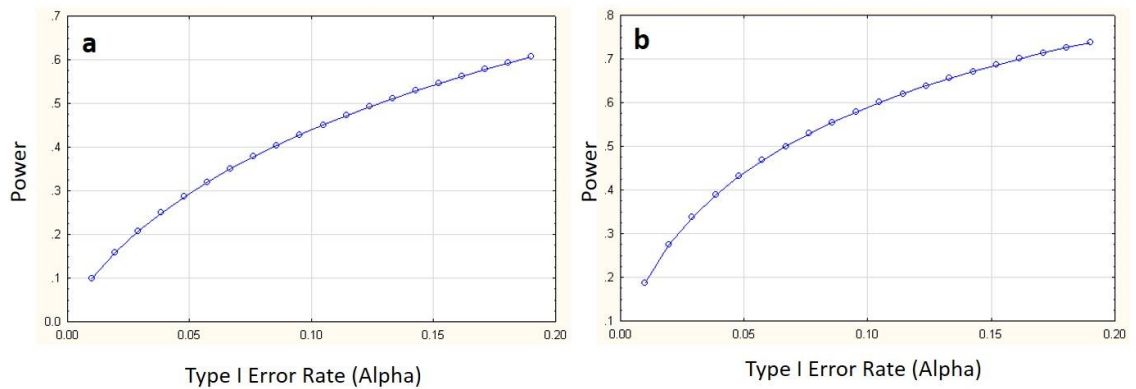

Figure 3.2: Power analysis of wheat field trial at Konjonup using the 5 treatments (4 PGPB plus uninoculated control) with the replicate plot number set to 3 (a) or 4 (b)
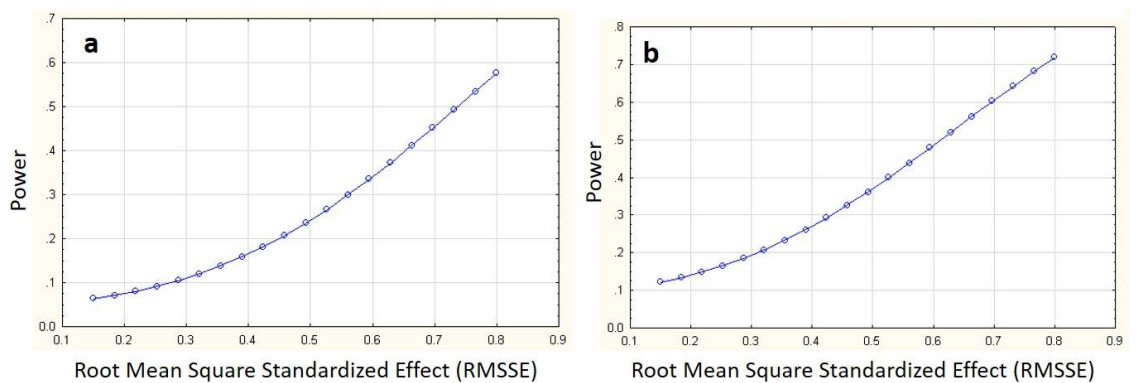

Figure 3.3: A comparison of an effect analysis of the wheat field trial at Konjonup using the 5 treatments (4 PGPB plus uninoculated control) with the replicate plot number set to 4 for an alpha set to 0.05 (a) or 0.10 (b)

3.3   Results

3.3.1   Kojonup field trials – wheat

The average grain yield for wheat at Kojonup was 9 kg plot^{-1} (2.25 tonne ha^{-1}) and inoculation of the wheat with PGPB at this site did not significantly (P>0.10) affect the yields (Figure 3.4). Furthermore, there was no significant (P>0.10) difference in % seed nitrogen contents across the treatments (data not shown).
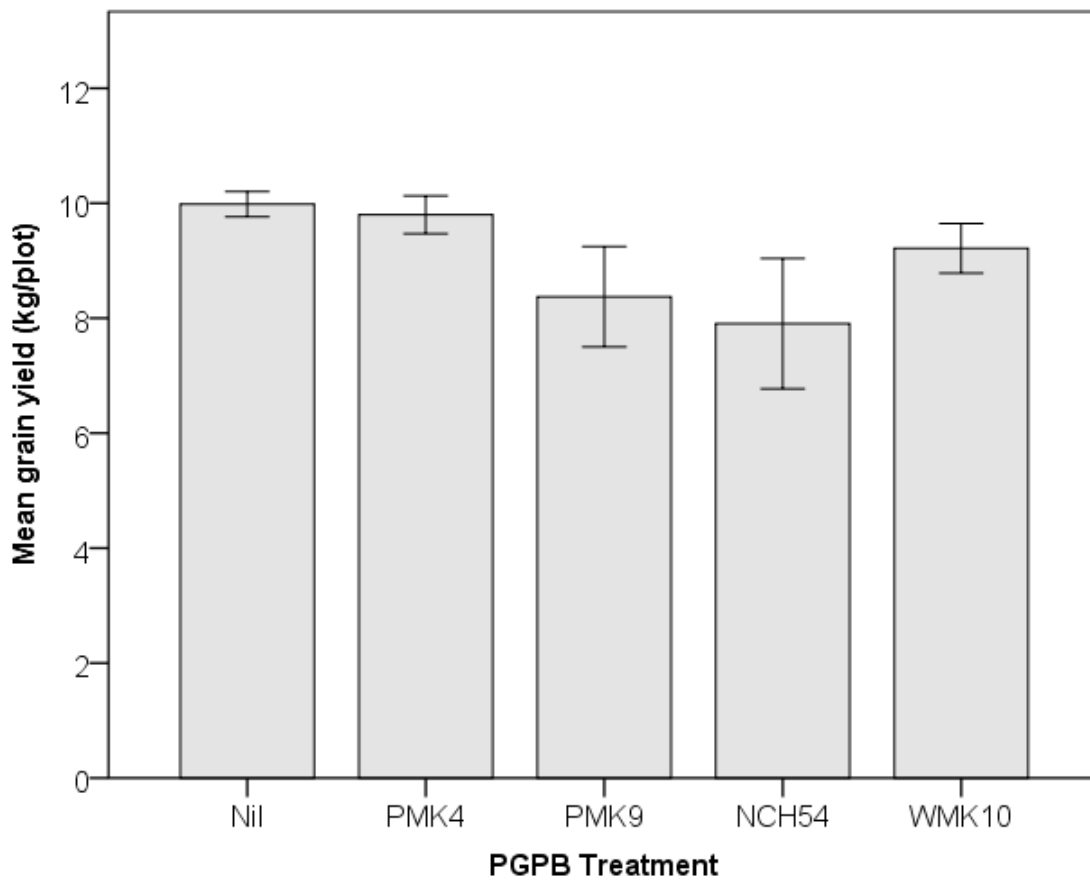

Figure 3.4: The effect of plant growth promoting bacteria (PGPB) on yield of wheat at the Kojonup field site at harvest. Data are represented as mean grain yield (kg plot^{-1}) and bars represent standard error of the mean

3.3.2 Boyup Brook field trial - wheat

All of the PGPB isolates increased the grain yield of wheat compared with the controls at the Boyup Brook Field site, ranging from a 2-9% increase (Figure 3.5). However, the increase in grain yield was only significant ($P<0.10$) for the isolate ANMK1 (8.3 kg plot^{-1}, 2.1 tonne ha^{-1}) compared with the control (7.6 kg plot^{-1}, 1.9 tonne ha^{-1}) (Table 3.8).
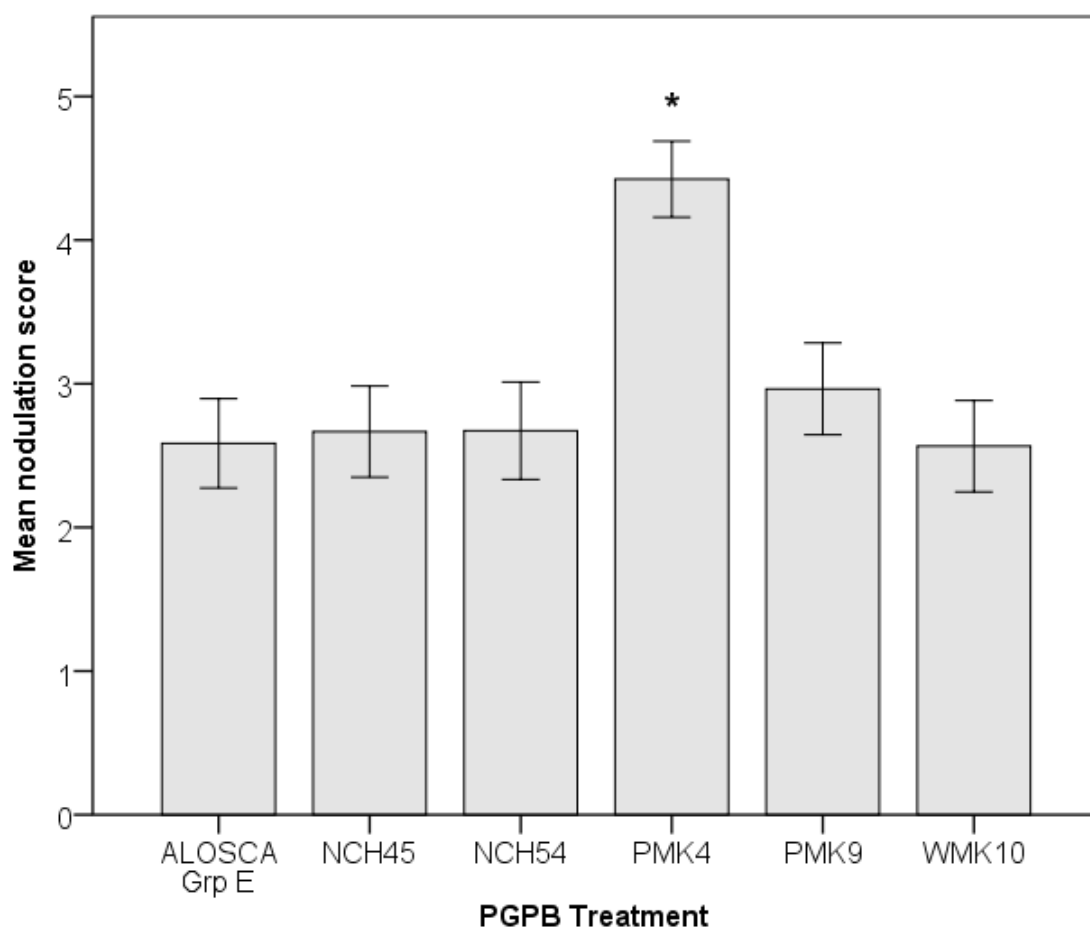

Figure 3.5: The effect of plant growth promoting bacteria (PGPB) on grain yield of wheat at the Boyup Brook field site at harvest. Data are represented as mean grain yield (kg plot^{-1}) and asterisks above columns denote significant ($P<0.10$) difference from the control (Nil). Bars represent standard error of the mean

Table 3.8: Statistical analysis of the effect of plant growth promoting bacteria inoculation compared with the controls on wheat yields at Boyup Brook at harvest

Contrast	Value of Contrast	Std. Error	t	df	Sig. (2-tailed)
Nil vs ANMK1	-.733	.375	-1.954	20	.065
Nil vs APMK2W	-.258	.375	-.688	20	.499
Nil vs NCH45	-.177	.342	-.517	20	.611
Nil vs PMK4	-.406	.375	-1.083	20	.292
Nil vs PMK4 & NCH45	-.309	.375	-.824	20	.420

3.3.3   Wongan Hills heavy soil site - wheat

Prior to harvest, two plots had sustained damage, probably caused by a hail storm. The damaged plots were one plot each for the uninoculated (Nil) and NCH45 treatments. The yields from these damaged plots were not included in the mean yield measurements. At harvest, the largest increase in mean yields was seen in treatments with the isolate NCH45 (11.0 kg plot^{-1}, 2.5 tonne ha^{-1}), which was a significant (P=0.02) increase in yield of 23% compared with Nil treatments (9.0 kg plot^{-1}, 2.0 tonne ha^{-1}) (Figure 3.6, Table 3.9).

The seed was processed for quality post harvest and included the plots mentioned above that had been damaged. There were no significant (P>0.10) differences in any of the quality parameters (% protein, % moisture, % screenings, specific weight) in any of the treatments (Table 3.10).
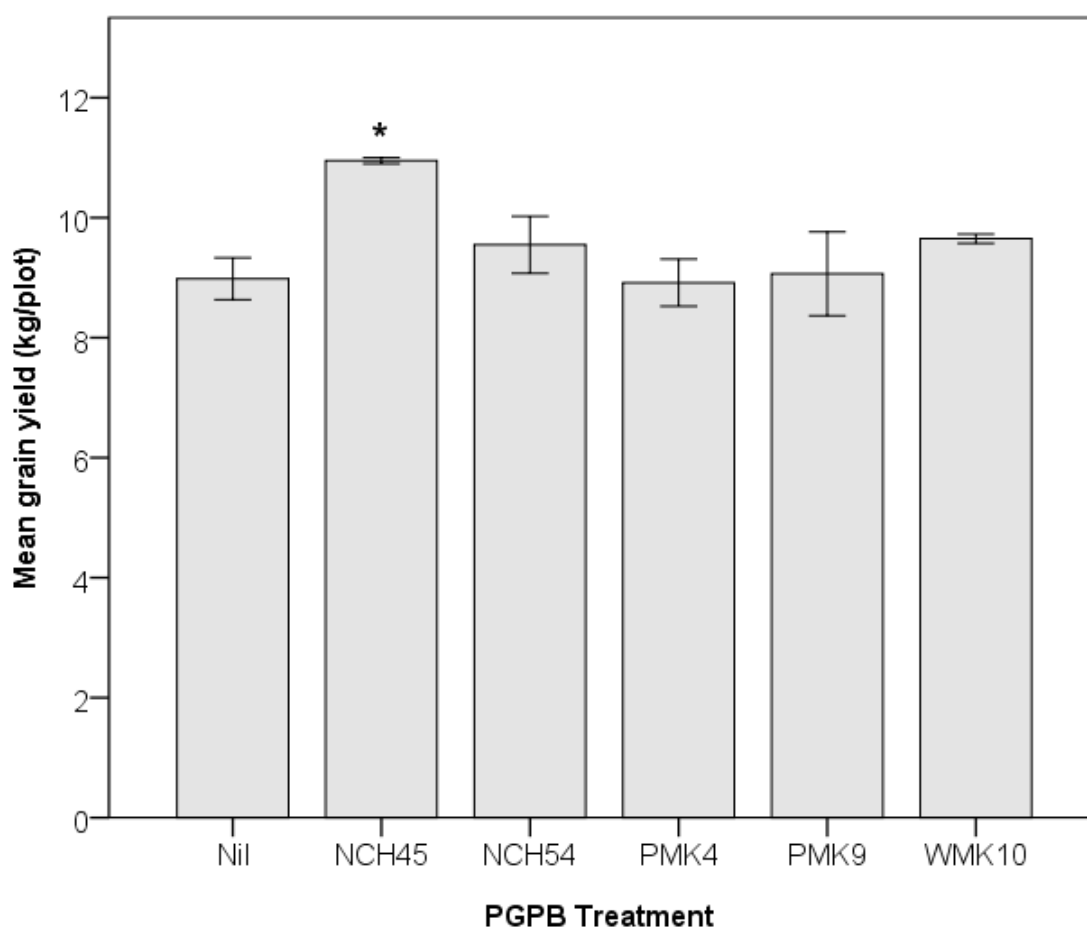
Figure 3.6: The effect of plant growth promoting bacteria (PGPB) on grain yield of wheat at the Wongan Hills heavy soil site at harvest. Data are presented as mean yield (kg plot^{-1}) and asterisks above the columns depict significant (P<0.05) differences from the control. Bars represent standard error of the mean

Table 3.9: Statistical analysis of the effect of plant growth promoting bacteria inoculation compared with the controls on wheat yields at the Wongan Hills heavy soil site at harvest

Contrast	Value of Contrast	Std. Error	t	df	Sig. (2-tailed)
NIL vs NCH45	-2.075	.768	-2.700	10	.022
NIL vs NCH54	-.675	.701	-.962	10	.359
NIL vs PMK4	-.042	.701	-.059	10	.954
NIL vs PMK9	-.1917	.701	-.273	10	.790
NIL vs WMK10	-.775	.701	-1.105	10	.295

Table 3.10: Seed quality data for wheat grain inoculated with plant growth promoting bacteria and harvested from the Wongan Hills heavy soil site.

Treatment	Specific Weight (kg/hL)	Std Error	% Protein	Std Error	% Screenings	Std Error	% Moisture	Std Error
Nil	84.93	1.76	10.47	0.77	2.50	0.53	10.07	0.06
NCH45	83.20	1.47	11.00	0.31	2.47	0.20	10.00	0.00
NCH54	82.60	0.72	11.30	0.31	2.47	0.32	10.00	0.10
PMK4	84.93	1.07	10.93	0.24	2.33	0.17	10.03	0.06
PMK9	83.87	1.17	10.87	0.55	2.63	0.32	10.00	0.10
WMK10	85.00	1.29	10.70	0.40	2.13	0.33	10.00	0.00

3.3.4 Wongan Hills light soil site - wheat

At harvest, the average grain yield was 7.1 kg plot^{-1} (1.6 tonne ha^{-1}) and there were no significant (P>0.10) differences in grain yield (Figure 3.7) or seed quality parameters between treatments at this site (Table 3.11).
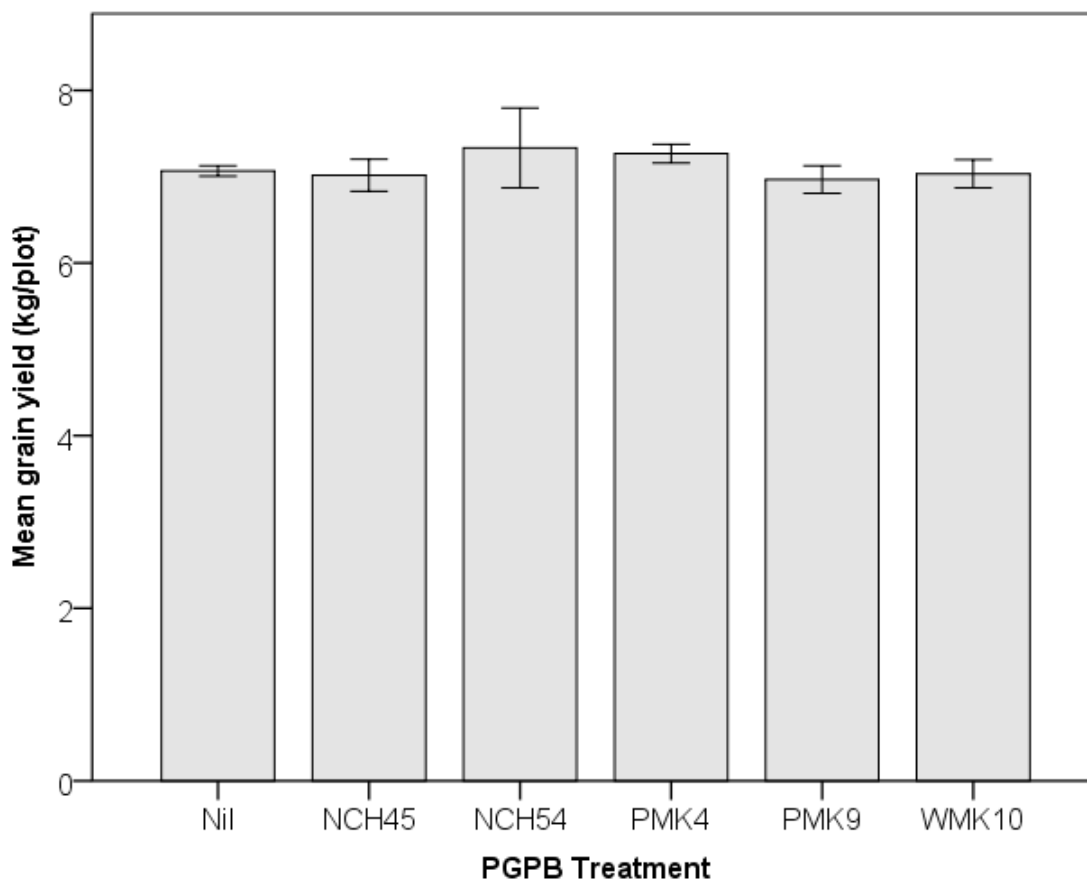

Figure 3.7: The effect of plant growth promoting bacteria (PGPB) on grain yield of wheat at the Wongan Hills light soil site at harvest. Data are presented as mean yield (kg plot^{-1}) and bars represent standard error of the mean

Table 3.11: Seed quality data for wheat grain inoculated with plant growth promoting bacteria and harvested from the Wongan Hills light soil site.

Treatment	Specific Weight (kg/hL)	Std Error	% Protein	Std Error	% Screenings	Std Error	% Moisture	Std Error
Nil	80.07	1.05	9.67	0.03	1.10	0.00	10.47	0.03
NCH45	79.20	1.15	9.77	0.07	1.10	0.06	10.53	0.03
NCH54	79.80	0.70	9.73	0.09	1.23	0.03	10.57	0.12
PMK4	79.53	0.77	9.73	0.07	1.07	0.03	10.73	0.12
PMK9	79.27	0.93	9.67	0.03	1.10	0.06	10.50	0.00
WMK10	80.93	2.01	9.77	0.07	1.03	0.19	10.80	0.35

3.3.5   Kojonup Field trials - peas

Seven weeks after sowing there was no nodulation in the uninoculated plots while nodulation was significantly ($P=0.00$) increased (71%) in the PMK4 treatments compared with the Alosca[TM] Group E controls (Figure 3.8, Table 3.12). There was no significant ($P>0.10$) difference in shoot dry weights for any of the treatments at this time (Figure 3.9).

At harvest, grain yields were above 5 kg plot^{-1} (average 1.5 tonne ha^{-1}) for all treatments, however there was no significant ($P>0.10$) improvement in yields when peas were co-inoculated with the PGPB as well as Group E rhizobia (Figure 3.10). Furthermore, there was no significant ($P>0.05$) difference in % seed nitrogen (data not shown). Late in the growing season, the peas were infected with black spot, a disease complex comprising up to four fungi (*Mycosphaerella pinodes*, *Phoma medicagini*s var *pinodella*, *P. koolunga*, *Ascochyta pisi*, *Macrophomina phaseolina*) (Hawthorne et al. 2012).
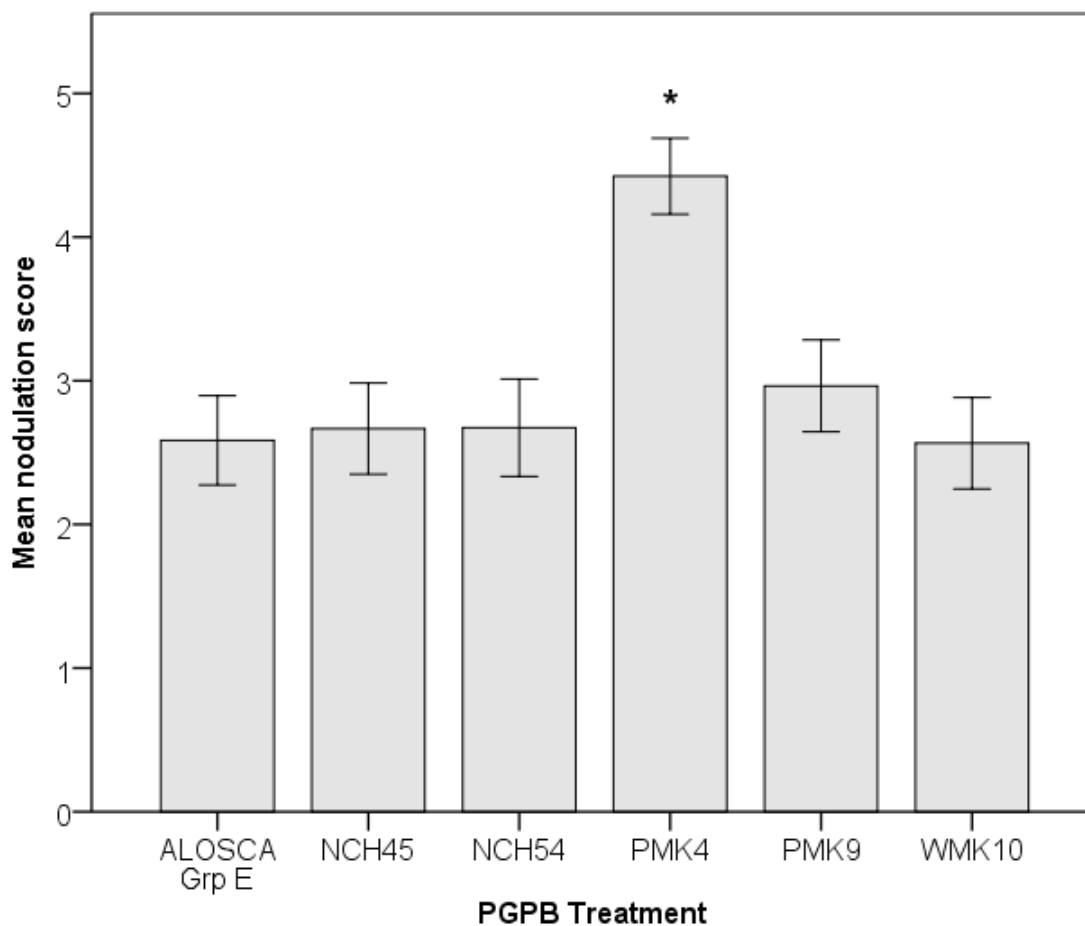
Figure 3.8: Average nodulation rating of peas co-inoculated with plant growth-promoting bacteria (PGPB) and rhizobia (Alosca Grp E) at the Kojonup site 7 weeks after sowing. Data are presented as mean nodulation score and asterisks above the columns represent significant (P<0.05) differences from the control. Bars represent standard error of the mean

Table 3.12: Statistical analysis of the effect of co- inoculation of plant growth-promoting bacteria and rhizobia compared with the Alosca Group E controls on nodulation of peas at 7 weeks after sowing

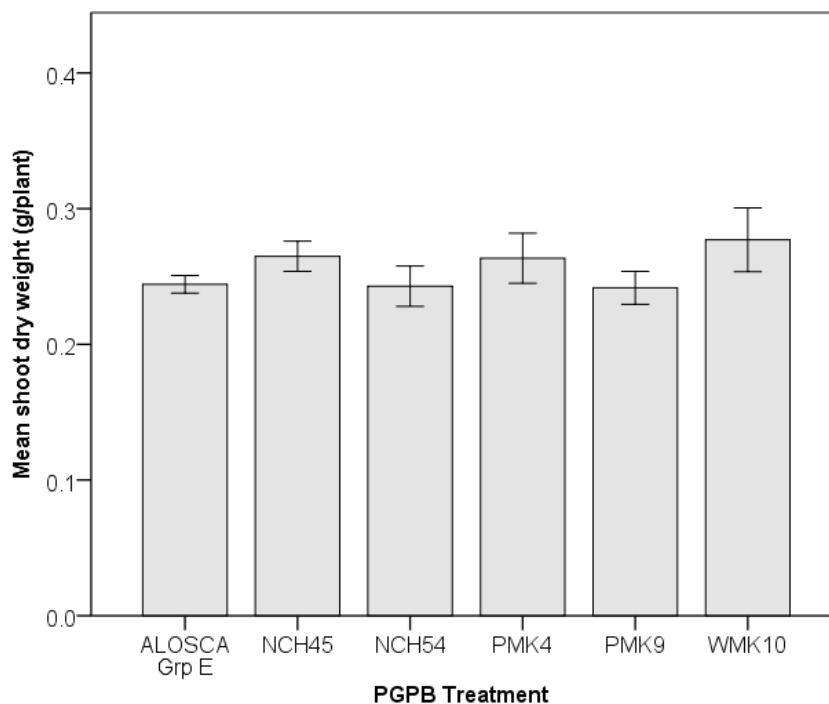| Contrast	Value of Contrast	Std. Error	t	df	Sig. (2-tailed)
Control vs NCH45	-.082	.463	-.177	315	.860
Control vs NCH54	-.088	.440	-.200	315	.842
Control vs PMK4	-1.839	.432	-4.255	315	.000
Control vs PMK9	-.380	.438	-.867	315	.387
Control vs WMK10	.019	.444	.043	315	.966

Figure 3.9: Average shoot dry weight (g plant^{-1}) of peas co-inoculated with plant growth-promoting bacteria (PGPB) and rhizobia (ALOSCA Grp E) at the Kojonup site 7 weeks after sowing. Data are presented as mean nodulation score and bars represent standard error of the mean
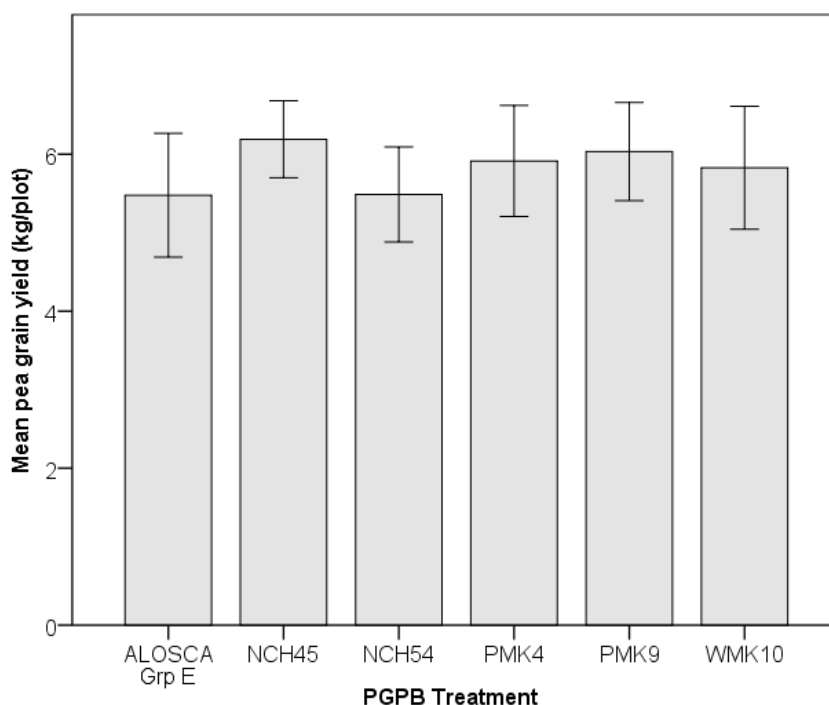

Figure 3.10: The effect of plant growth promoting bacteria (PGPB) on yield of peas at the Kojonup field site at harvest. Data are presented as mean yield (kg plot^{-1}) and bars represent standard error of the mean

3.3.6   Brookton Field trials - peas

At sampling 12 weeks after sowing, some nodulation was observed in one of the uninoculated plots (Figure 3.12). Nodulation was significantly (P=0.00, df=116) increased (34%) in the Group E and PMK4 co-inoculated treatments compared with the Group E single inoculation treatments (Figure 3.11 & 3.12). The shoot dry weights for the Group E only and the Group E and PMK4 co-inoculated treatments were significantly (P=0.01, df=11) different from the uninoculated controls (Figure 3.13). At harvest, co-inoculation with PMK4 and Group E significantly (P=0.00, df=11) increased pea grain yields by 120% compared with uninoculated and by 35% compared with Group E only controls. At the time of writing, the [N]15 natural abundance results were not available.

Figure 3.11: Nodulation in peas from the Brookton (2015) field site in plants co-inoculated with *P. granadensis* PMK4 and *Rhizobium leguminosarum* bv. *viciae* SU303 (Group E rhizobia) (left) or inoculated only with SU303 (right) (Photo Ron Yates)
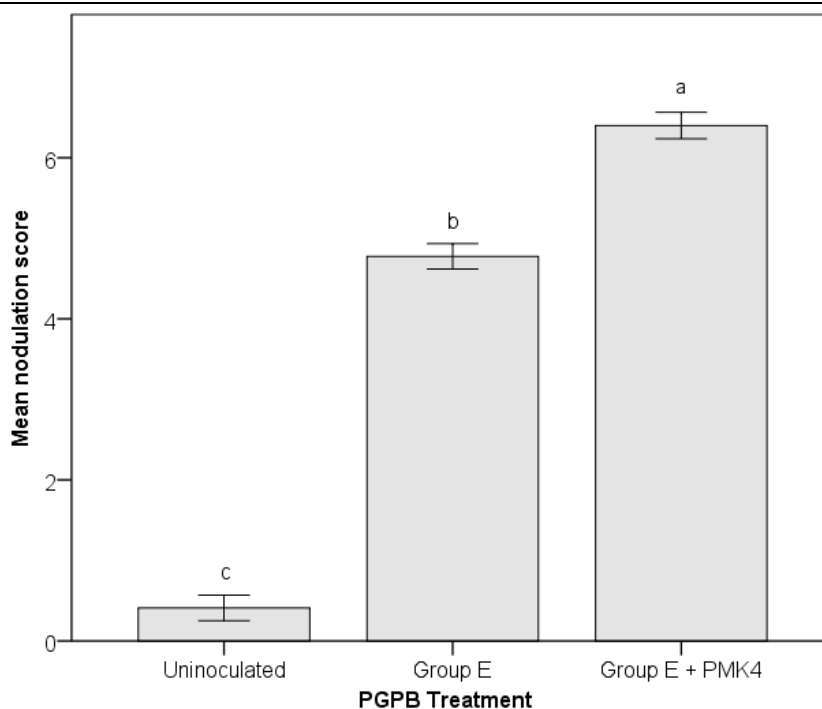
Figure 3.12: Average nodulation rating of peas co-inoculated with PMK4 and Group E (SU303) rhizobia at the Brookton site 12 weeks after sowing. Data are presented as mean nodulation score and the same letter above the columns represent data that do not differ significantly (P>0.05). Bars represent standard error of the mean
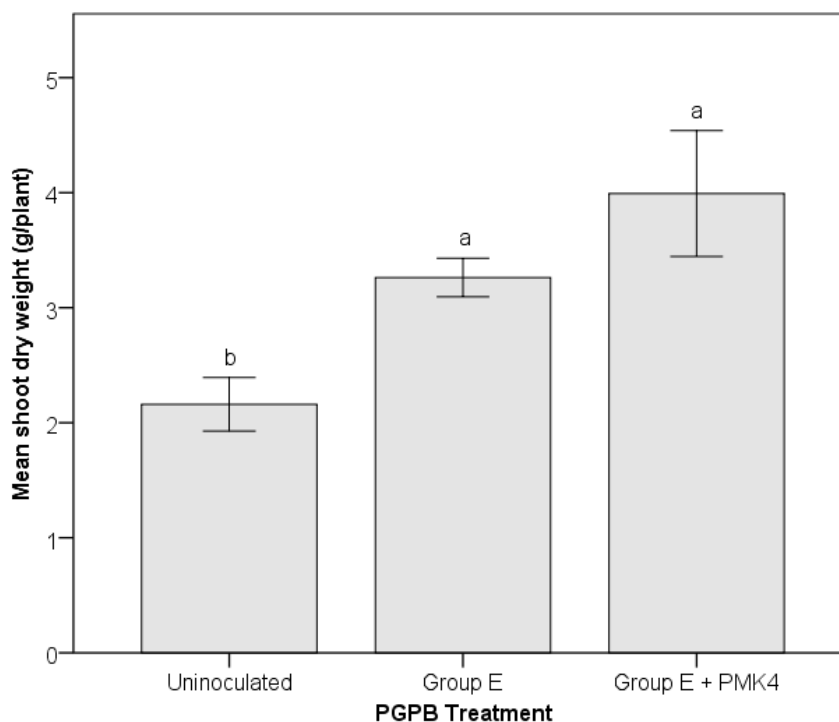

Figure 3.13: Mean shoot dry weight (g plant[-1]) of peas inoculated with PMK4 and Group E (SU303) rhizobia at the Brookton site 12 weeks after sowing. The same letter above the columns represent data that do not differ significantly (P>0.05) and bars represent standard error of the mean

Figure 3.14: Mean grain yields (tonne ha^{-1}) of peas inoculated with PMK4 and Group E (SU303) rhizobia at the Brookton site at harvest. The same letter above the columns represent data that do not differ significantly (P>0.05) and bars represent standard error of the mean

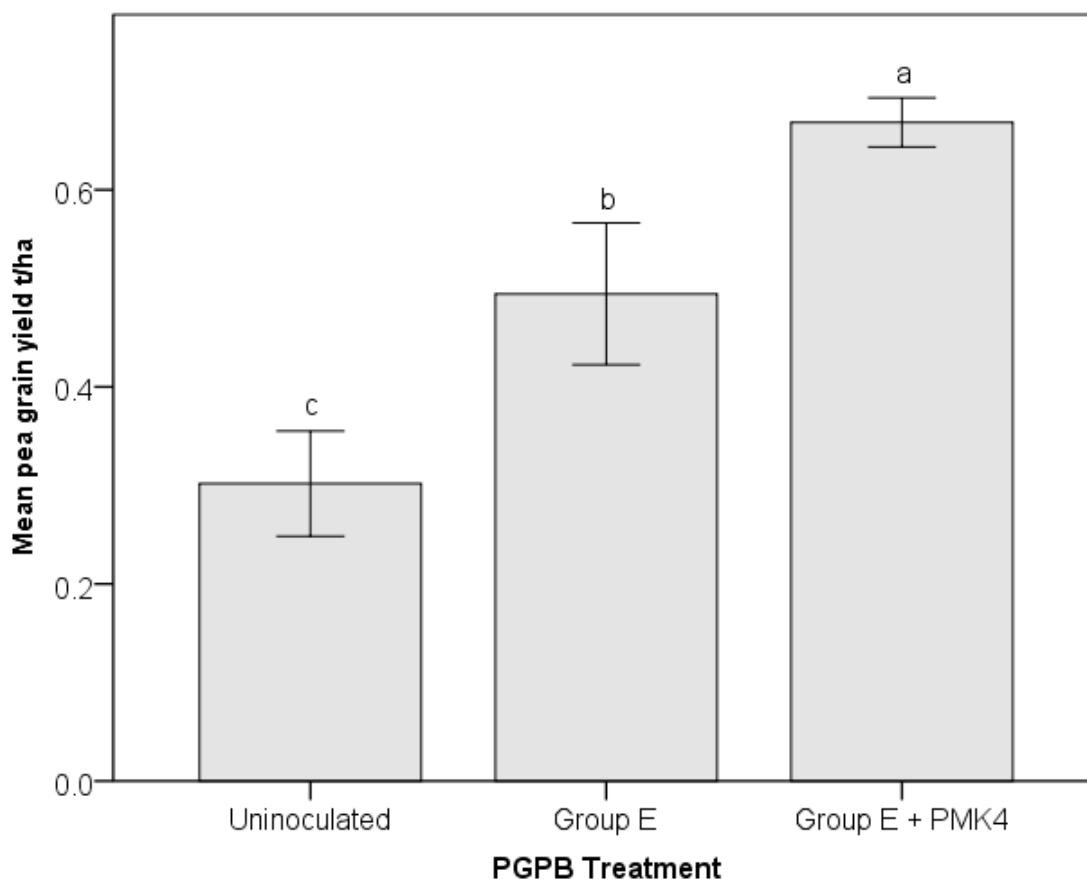## 3.4   Discussion

Three of the PGPB were able to improve plant growth and yields in the field in the current study.   This includes the isolate *P. granadensis* PMK4 which increased nodulation of the peas at the Kojonup field site by 71% and at the Brookton site by 34% compared with the rhizobia only controls. Shoot dry weights (SDW) and grain yields were not significantly increased upon co-inoculation with PMK4 and the rhizobia at the Kojonup site, possibly due to the black spot infection which may have reduced the yields at this site, although they were higher than the average for Western Australia (1.35 tonne ha^{-1}) in 2005 (Slatter et al. 2006). Grain yields were low overall at the

Brookton site due to the reduced rainfall (41% less than the average) in the growing season, however, there were significant increases in grain yields in peas co-inoculated with PMK4 and Group E compared with the controls. Field pea yields in Western Australia can exceed 2.0 tonne ha^{-1} under favourable growing conditions particularly with the newer varieties (Pulse Australia 2011). Therefore, co-inoculation with PMK4 needs to be investigated in the field with other varieties of pea to determine if nodulation and yields are improved in these varieties with this PGPB.

At the Wongan Hills heavy soil site, the isolate *B. caledonica* NCH45 increased the yield of wheat by 23% compared with the controls to 2.5 tonne ha^{-1} without compromising the grain quality (Table 3.10). The average wheat yield for the Wongan Hills/Ballidu district in 2005 was 2.56 tonne ha^{-1} (T. Scanlan, pers. comm.[1]). This isolate demonstrates several of the common traits associated with beneficial plant-microbe interactions, including production of the auxin IAA, production of ACC deaminase, siderophore production, as well as the solubilisation of sparingly soluble phosphates in both solid and liquid media (Table 3.2). Due to the high level of phosphates solubilised in liquid media, the potential for this trait as the mechanism of action of growth promotion by this bacteria was investigated further in glasshouse trials in Chapter 5.

At the other two sites where NCH45 was inoculated on wheat (Wongan Hills light soil and Boyup Brook), this bacterium did not improve wheat yields. The reasons for the lack of effect are not understood at this stage but may be due to a reduction in cell viability in the carrier medium prior to sowing. Certain *Burkholderia* species have

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[1] These figures are compiled from combined CBH (Co-operative Bulk Handling) Group and Australian Bureau of Statistics data. Timothy Scanlon is from the Department of Agriculture and Food, Western Australia. As at 18 February 2015.

previously been shown to be susceptible to desiccation in bentonite media as well as on polyethylene beads (Howieson et al. 2013) and as a *Burkholderia* sp., the isolate NCH45 may have also been susceptible to desiccation in this study. The Alosca^{TM} inoculum was produced in different batches and bacterial cell viability of the separate batches was not performed. This highlights that future work will need to determine the desiccation tolerance of NCH45 and ensure that quality controls are in place before sowing. Also, a lack of effect on crop yield by potential PGPB can be attributed to poor adaptation to the target soil or competition from native soil bacteria (Okon and Labandera-Gonzalez 1994). Although the ability of NCH45 to colonise the wheat root system in aseptic conditions has been demonstrated in Chapter 2, the ability of this isolate to survive and compete with other microorganisms in the field in the current study was not assessed. Techniques to determine the persistence of the respective PGPB in the rhizosphere in the field need to be developed (see Chapter 7) to allow a better match between soil, host and PGPB.

At the Boyup Brook site, the PGPB isolate *E. soli* ANMK1 improved yields by 9% to 2.1 tonne ha^{-1}. The control treatments averaged 1.9 tonne ha^{-1} which was very similar to the region average for the year of 1.85 tonne ha^{-1} (T. Scanlan, pers. comm.). Rainfall for the growing season at this site was over 200 mm less than the average rainfall and grain yields for the region were reduced by 36% compared with yields from the previous year (2.92 tonne ha^{-1} in 2005). The isolate ANMK1 was originally isolated on an ACC deaminase selective medium but was later found not to consume ACC using the ninhyrin-ACC assay even though the *acdS* (ACC deaminase) gene may be present (Chapter 2, Table 3.2). Further research into the effects of bacterially produced plant hormones on wheat is required and investigations of this isolate should also focus on its potential ability to improve yields under drought conditions.

At the Wongan Hills light soil site, inoculation with the PGPB had no effect on grain yields which were substantially less (26%) at this site compared with the heavy soil site. It is possible that the constraints to wheat growth at this site were also impacting the ability of the bacteria to persist in the rhizosphere and hence there was a lack of response to inoculation of the wheat with the PGPB.

At the Kojonup site, inoculation with the PGPB did not improve wheat yields. Average yields for the region were 2.92 tonne ha^{-1} (T. Scanlan, pers. comm.) which represents a reduction at the Kojonup site of 23%. However, the variety used at the Kojonup site was Tincurrin, which can yield 3% less than the Wyalkatchem variety in some agricultural zones (Zaicou et al. 2008). Furthermore, different plant cultivars can respond differently to inoculation with PGPB (Shirinzadeh et al. 2013; Vargas et al. 2012). For instance, one wheat genotype (CD 120) responded positively to inoculation with *Herbaspirillum seropedicae,* particularly for grain yield, while other genotypes either showed no response, or responded negatively (CD 104) to inoculation with *H. seropedicae* (Neiverth et al. 2014). These authors suggested that the different responses could be due to improvements in cultivars like CD 104 for acquisition of nutrients, such as nitrogen, and in the fertile soils used in their glasshouse experiments, the response to the PGPB was nullified. This seems reasonable since this cultivar did not respond to the application of nitrogen.

Since the field trials in the current study were conducted, several new varieties of wheat have been released in Western Australia. For instance, 59.1% of the total area sown to wheat in Western Australia was the Mace variety for the 2014/15 year while the area sown to the variety Wyalkatchem has declined from 32.7 % in 2009/10 to 5.0%. in 2014/15 (Trainor et al. 2015). Therefore, the PGPB used in the current study need to be

assessed on these emerging varieties on a range of soil types to determine their effectiveness.

With rising populations putting pressure on farming communities to increase global food production, combined with predictions for escalating global water scarcity and higher temperatures, increasing attention needs to be paid to research into crop production in drought prone environments (Chaves and Davis 2010). In Western Australia in particular, there has been a shift to consistently drier winter conditions with annual rainfall declining in south-west Western Australia by about 10% since the 1970s (Hope and Foster 2005). Using plant biological-based technologies along with PGPB with proven plant drought tolerance inducing properties, may improve water use efficiency in a variety of crop species, providing food security for the growing global population (Chaves and Davis 2010).

This study demonstrates that there is potential for PGPB to improve the yields of wheat in Western Australian soils. However, this appears to be dependent on several factors including the carrier medium, soil type, climatic conditions, indigenous microflora and/or the variety of the wheat. Additionally, the PGPB isolate PMK4 improved nodulation and grain yields in field peas. The next step with these PGPB is the large scale implementation of field studies using the most promising isolates, PMK4, ANMK1 and NCH45. This should include a range of wheat and pea varieties, over a range of soil types and climatic conditions, in order to determine the most effective use of these PGPB in Western Australian agronomy.

Chapter 4:
Potential for agricultural production on remnant soils mined for apatite using legumes and beneficial microbes

Phosphorus Resources Limited executives and board members with the Murdoch University research team at a field site at Christmas Island (2013). The author is in the front in the red shirt.

4.1   Introduction

When phosphate deposits are exhausted and mining ceases, there may be severe impacts on local economies. During the mining process, the natural vegetation and soil have frequently been removed to pinnacles of bedrock, and in many instances the replacement soils are low in some nutrients and organic matter but often high in heavy metals. In various countries there have also been problems in water availability and erosion, weed invasion and fire. In arid areas such as north west Queensland, restoration to natural vegetation is the goal (Gillespie and Erskine 2012) but in many areas restoration to natural vegetation is not possible. In more ambient environments such as Florida, phosphate mines have been returned to wetlands, natural forest or grazing land (Brown 2005; Hanlon et al. 1996). In Morocco, phosphate mined land has been successfully planted with citrus and olives as well as forest species (United Nations Environment Program and International Fertiliser Industry Association 2001), but rehabilitation to agricultural cropping is uncommon.

Post-mining reclamation of the land needs to consider not only if the land is suitable for restoration to its natural vegetation or to be developed for agriculture, but also the economic requirements of local residents. When phosphorus reserves are depleted, the loss of income can have serious effects on the local economy. For instance, 80% of the island of Nauru, located in the central Pacific, was mined for phosphorus until 2000. The mined land is largely unsuitable for crops or grazing and although efforts are continuing to develop agricultural enterprises (Fa'anunu 2012) this small nation at present has crippling economic and health issues (Gowdy and McDaniel 1999). Similarly on the small island of Banaba, restoration of the phosphate mined land to productivity appears very difficult resulting in relocation of the population.

Actions are now being taken to prevent Christmas Island experiencing similar problems to Nauru when rock phosphate mining operations cease in 2034 (King and Snowdon 2013). Christmas Island is a remote tropical island in the Indian Ocean, 380 km south of Jakarta (Indonesia) (Beeton et al. 2010; National Archives of Australia 2013). The island crowns an ancient volcano that rises 5000 m above the sea floor (Director of National Parks 2012) with a cap consisting of alternating carbonic and volcanic rocks extending to a height of 361 m above sea level (Trueman 1965). The climate is tropical with a wet season from November to May and an average annual rainfall of 2034 mm (Bureau of Meteorology 2013). The rainforest is conserved in a National Park which covers 63% of the island, and is supported by a levy (currently $1.2m pa) from Phosphorus Resources Limited. On the 22% (3000 ha) of the island that has been mined for apatite, if soil quality can be improved, the rehabilitated mined areas will provide an opportunity to develop alternative industries to replace mining for the economic future of the island. Unlike Nauru, Christmas Island was uninhabited before the discovery of phosphate so there is no tradition of self-sustaining food production. Most of the food for Christmas Island is imported and expensive, and large scale commercial agricultural cropping has never been attempted.

Christmas Island soils are currently a constraint to agriculture. Most soils on the island are phosphatic, derived from limestone or basalt with a pH 7.0-8.0 (Director of National Parks 2012). Removal of the soil during mining has resulted in the return to limestone base rock in some areas (Beeton et al. 2010). Additionally, soil fertility is poor as soils are deficient in N, K and S, but extremely high in P (CSBP Report 7QS12022-12038, unpublished) and would be unsuitable for most agricultural crops without heavy inputs of fertilizers.

As has been shown in other degraded areas (Duponnois et al. 2013; Skujinš and Allen 1986; Thrall et al. 2007) reintroduction of beneficial symbionts such as rhizobia and mycorrhizal fungi, usually absent from disturbed soils, can aid in the remediation of degraded soils. Legumes are valuable for revegetation as their symbiotic associations with bacteria and mycorrhizal fungi aids plant growth through nitrogen fixation and P solubilisation, respectively (Herrera et al. 1993).

In many agricultural situations inoculation using elite strains of rhizobia make addition of nitrogenous fertilizers unnecessary for the current and subsequent crops. In Brazil, the fixation of up to 300 kg N ha^{-1} by soybeans (*Glycine max*) and their bradyrhizobial symbiont, together with the release of 20-30 kg N ha^{-1} into the soils for the following crop saves ~ US\$7 billion a year in nitrogenous fertilizer (Hungria et al. 2013). Soybean represents 50% of the global crop legume area and 77% of the N fixed by crop legumes (Herridge et al. 2008). Use of rhizobial inoculants with legumes rather than commercial nitrogenous fertilizers also contributes to a reduction in greenhouse gas emissions (Hungria et al. 2013).

Microorganisms that enhance plant growth are collectively known as plant growth-promoting (PGP) microorganisms, and include symbiotic bacteria, free-living bacteria and mycorrhizal fungi. The beneficial characteristics of PGP microorganisms can include the ability to fix N (Dobbelaere et al. 2003), suppression of plant pathogens and making available the poorly soluble plant nutrients (Vessey 2003). Many free-living PGP bacteria (PGPB) and fungi are able to cycle the P in the soil from various pools to plant available forms (Richardson et al. 2009). For instance, Panhwar et al. (2011) found that use of two phosphorus solubilising bacteria (PSB) on aerobic rice

significantly improved the phosphorus uptake from Christmas Island rock phosphate resulting in an increase in dry matter yield.

The current study aimed to establish the scientific basis upon which agriculture can effectively be developed on Christmas Island mining leases during rehabilitation. A research program was developed using legume agronomy, with the aid of beneficial microbes, to improve soil fertility; to test the production of high value pulse seeds for human consumption; to improve the soils for future agricultural pursuits; and to underpin potential future animal feedlot operations, aquaculture or aquaponic operations. The specific aims of the work attached to this PhD study were to determine the effect of co-inoculation of the PGPB, *Pseduomonas* sp. PMK4, with commercial rhizobial isolates on the growth and nutrient acquisition of pulse legumes grown on Christmas Island.

4.2   Methods

The experiment investigated the response of six different legume species (Table 4.1) to different levels of fertilizer, and to inoculation with rhizobia or co-inoculation with rhizobia and PGPB.

4.2.1   Site preparation

The site (2 ha) had been previously mined for apatite and after mining, the residual soil was returned to this site. The soil was analysed by CSBP Limited (Bibra Lake, Western Australia) (Table 4.2). As the site has a 5-10 degree slope, three terraces were constructed to control water run-off. The site surface was scarified with the tynes of a grader and then leveled with the seeder to produce a surface suitable for sowing machinery.

4.2.2   Sowing

The rhizobial strains were supplied as commercial peat formulations (Alosca Technologies Pty Ltd) (Table 4.1).   The PGPB (*Pseudomonas granadensis* PMK4) inoculant was prepared by injecting 50 ml of a two day broth culture into a sterile peat similar to the rhizobial inoculants.   Eight legume varieties (Table 4.1) were sown in triplicate and were inoculated with the peat formulations as rhizobia alone or rhizobia plus the PGPB at three different fertilizer rates (Table 4.3) in a factorial split-plot design.   There were 144 machine sown plots and each plot measured 2.5 x 20 m.   An additional block (non-replicated) of uninoculated legumes was machine sown on the extreme south-eastern end of the site to allow an assessment of the background rhizobia i.e., nodulation achieved by rhizobia already in the soil. This block received the high level of fertilizer.   The soil was dry at the time of sowing (5-9 February 2013), but 20 mm of rain fell within 24 hours and seedling emergence was excellent.   Total rainfall during the growing season (Feb-June 2013) was 2004 mm.

Table 4.1: Legume species used in these experiments and their commercial inoculant (Drew et al. 2012).

Legume	Species name	Rhizobial Strain	Sowing Rate (kg/ha)
Soybean	*Glycine max* cv A6785	*Bradyrhizobium diazoefficiens* CB1809[#]	*60*
Mungbean	*Vigna radiata*	*Bradyrhizobium* sp.CB1015	*30*
Navybean	*Phaseolus vulgaris*	*Rhizobium phaseoli* CC511[*]	70
Pigeon pea	*Cajanus cajan*	*Bradyrhizobium* sp.CB1024	*30*
Lablab	*Lablab purpureus* cv Highworth	*Bradyrhizobium* sp. CB1024	*30*
Lablab	*Lablab purpureus* cv Rongai	*Bradyrhizobium* sp. CB1024	*30*
Cowpea	*Vigna unguiculata* cv Caloona	*Bradyrhizobium* sp.CB1015	*30*
Cowpea	*Vigna unguiculata* cv Ebony	*Bradyrhizobium* sp.CB1015	*30*

[#] CB = The CB *Rhizobium* Collection, Australia; [*] CC = CSIRO Canbera *Rhizobium* Collection, Australia

Table 4.2: Soil analysis for the experimental site.

NH$_4^+$ N mg/kg	NO$_3$ N mg/kg	Colwell P mg/k	Colwell K mg/kg	S mg/kg	C %	pH level CaCl2	DTPA Fe mg/kg	DTPA Mn mg/kg	DTPA Zn mg/kg
1	1	299	<15	2.7	0.12	7.0	8.17	12.98	9.04

Table 4.3: Fertilizer application rates

Fertilizer Rate	Fertilizer Applied[#]
Nil	No fertilizer
Low	Potassium sulphate @10 kg ha^{-1}
	Ferrous sulphate Hepta @5.0 kg ha^{-1}
	Superphosphate [^]@10 kg ha^{-1}
	TEK Phos 2:1 [*]@15 kg ha^{-1}
High	Five times the low fertilizer rate

[#] Fertilizers supplied by CSBP Limited; [^] P (w/w%) = 9; [*] TEK Phos components (w/w%): P = 6.0, K = 16.3, S = 6.8, Ca = 12.7, Cu = 0.40, Zn = 0.20, Mo = 0.040

4.2.3   Assessment

The plants were sampled twice, in April 2013 and in June 2013. At the first sample collection (9 weeks after sowing), ten random plants from each plot (including the uninoculated plots) were dug out ensuring most of the root system remained intact and the nodulation of the roots was assessed using a nodulation rating system (Table 4.4). Ten nodules from each treatment were removed and stored in 5 mL vials with silica gel as a dehydration agent until further use (Chapter 7). The nodulation data were collected by Liza Parkinson who permitted the inclusion of the data in this chapter for clarification purposes.

Water erosion damage was observed in many plots but particularly the PGPB plots. The damaged areas were also sampled but these plots were removed from statistical analysis. Only one variety each was collected for lablab (Highworth) and cowpea

(Ebony) at this assessment. The top biomass was removed and dried at 60°C for 2 days and the biomass dry weights determined. At this growth stage, soybean and mungbean had set seed, however, lablab was still in the vegetative stage. Nutrient concentrations in dry matter for the three representative species soybean, mungbean and lablab, were analysed by CSBP Limited (Bibra Lake, Western Australia) and the shoot nutrient concentrations were compared with those reported in Reuter and Robinson (1997) at the closest comparable growth stage for each species.

Table 4.4: Nodule scoring system used for scoring pulse legumes. Adequate nodulation for most pulse legumes can be observed at a nodulation score of 5 and above. Adapted from Howieson and Dilworth (2016) (Appendix I)

Nodule Score	Description
0	No nodules
1	1-5 small nodules or 1 large nodule
2	6-10 small nodules or 2 large nodules
3	11-15 small nodules or 3 large nodules
4	16-20 small nodules or 4 large nodules
5	21-25 small nodules or 5 large nodules
6	26-30 small nodules or >5 large nodules
7	Crown nodulation incomplete or >30 nodules
8	Crown nodulation $<1cm^3$
9	Crown nodulation $>1cm^3$
10	>1 crown nodule $>1cm^3$

At the second sampling (18 weeks after sowing) the terrace encompassing replicate 3 was completely waterlogged and the biomass was not collected. In the remaining two replicates, the above ground biomass from 1 m^2 quadrats were cut by hand and the fresh weights determined. Representative (10% total wet biomass) samples were then dried as above to determine dry weights.

4.2.4   Statistical analysis

Univariate analysis of variance (ANOVA) of the means was performed using IBM SPSS 21 and where significant (P<0.05), means were compared using Fischer's Least Significant Difference (LSD) test if variance was evenly distributed or Games-Howell analysis if variance was not evenly distributed.

4.3   Results

4.3.1   Cowpea

The application of fertilizer had a significant (P=0.003) positive effect on cowpea (var. Ebony) shoot biomass at nine weeks after sowing (Table 4.5, Figure 4.1a). At the low level, fertilizer increased biomass and at the high level there was a further increase compared to the nil fertilizer, in the rhizobia only treatments. At this time, cowpea was nodulated at all the fertilizer levels and in the uninoculated plots, and there was no significant (P>0.05) effect of fertilizer or PMK4 co-inoculation on nodulation (Table 4.6 and 4.7).

At 18 weeks after sowing, results for the two cowpea varieties were very similar, therefore the data were pooled for analysis (Table 4.8, Figure 4.1b). Again, there was a significant (P=0.000) increase in biomass when fertilizer was applied. Co-inoculation with PMK4 did not have a significant (P>0.05) effect on plant biomass for cowpea at either of the sampling periods when compared with plants inoculated only with rhizobia.

4.3.2   Lablab

At nine weeks after sowing, the application of fertilizer significantly (P=0.024) increased shoot biomass of lablab (var. Highworth) at both fertilizer levels in rhizobia only treatments (Table 4.5, Figure 4.1a). Nodulation of lablab was significantly

(P=0.000) increased at the low fertilizer level for the rhizobia co-inoculated with PMK4 treatments (Table 4.6) and nodulation score was lower in the uninoculated plots (Table 4.7).

Comparison of the nutrient analyses of lablab shoots at nine weeks with data from Reuter and Robinson (1997) suggests that Cu, K (Figure 4.2) and S (data not shown) were below adequate levels even at the highest rate of fertilizer application. Boron levels were below the adequate concentration in plants not co-inoculated with the PGPB. Co-inoculation with PMK4 significantly (P=0.009) increased the P concentration in shoots at the nil and low fertilizer applications.

The results for the two lablab varieties were very similar at 18 weeks after sowing, therefore the data were pooled (Table 4.8, Figure 4.1b). Fertilizer again had a positive (P=0.000) effect on the shoot biomass from the nil to low fertilizer rates for the rhizobia only treatments, however, there was no further increase at the high rate. Co-inoculation with the rhizobia and PMK4 significantly (P=0.036) decreased lablab biomass at the low fertilizer rate compared with the rhizobia only treatment.

4.3.3 Mungbean

Fertilizer application significantly (P=0.000) increased the biomass of mungbean 9 weeks after sowing (Table 4.5, Figure 4.1a). Nodulation of mungbean was significantly increased when rhizobia and fertilizer were applied together (P=0.000) and the PGPB provided no further advantage (Table 4.6). Co-inoculation of the mungbean with rhizobia and PMK4 had no effect (P>0.05) on biomass or shoot nutrient levels at this growth stage. Potassium levels in leaves were well below adequate at all fertilizer levels (Figure 4.2d).

At 18 weeks after sowing, coinciding with the end of biomass production, mungbean produced the highest biomass of any of the legume species (Table 4.8, Figure 4.1b). An average biomass of 589 g dry shoot per m^2 was obtained from plants given low fertilizer rate and co-inoculated with rhizobia and PMK4. The high level of applied fertilizer decreased shoot biomass in the PMK4 treatments as did rhizobia alone treatments, but not significantly (P>0.05).

4.3.4   Navybean

The navybean failed to thrive in all treatments and was not collected for shoot analysis at either 9 weeks or 18 weeks after sowing. However, plants were harvested from the uninoculated plots at 9 weeks after sowing to assess nodulation. These plants were as stunted and necrotic as the plants in the inoculated plots, however they were nodulated and received the highest nodule score (4.5) of the legume species sampled from the uninoculated plots (Table 4.7).

4.3.5   Pigeon Pea

Pigeon pea biomass at 9 weeks after sowing was the lowest for all the legume species sampled and fertilizer application did not significantly (P>0.05) increase yields (Table 4.5, Figure 4.1a). Co-inoculation with PMK4 had no effect and nodulation was relatively low (less than 15 nodules plant^{-1}) and not significant (P>0.05) between the treatments (Table 4.6). Nodulation was also low in the uninoculated plots (Table 4.7).

By 18 weeks after sowing, the biomass of pigeon pea was comparable to the other legume species (Table 4.8, Figure 4.1b). Fertilizer application had a significant (P=0.006) effect on shoot biomass but this was decreased in the PMK4 treatments.

4.3.6 Soybean

Both application of fertilizer and PMK4 inoculation had significant (P=0.00) positive effects on the shoot biomass of soybean at 9 weeks after sowing (Table 4.5, Figure 4.1a). From the nil to low fertilizer rate, there was a significant (P<0.05) increase in biomass and a further increase at the high fertilizer level. At the low fertilizer level, PMK4 co-inoculation increased (P=0.012) biomass above that gained by fertilizer alone, but no further increase was obtained with PMK4 co-inoculation using the high fertilizer rate.

Nodulation of soybean was increased (P=0.03) following inoculation with PMK4 when fertilizer was not applied (Table 4.6). Application of fertilizer at the low level also increased (P<0.05) nodulation both with and without PMK4 co-inoculation. The uninoculated soybean did not thrive and only two of the collected plants contained nodules (Table 4.7).

Fertilizer application significantly (P<0.05) increased nutrient concentrations of Ca (not shown), and K in soybean shoots however K levels were still well below the adequate levels determined in Reuter and Robinson (1997) (Figure 4.2). Co-inoculation of soybean with rhizobia and PMK4 generally increased nutrient concentrations in shoots, however, this was only significant (P<0.05) for Zn (not shown), P and Cu. In fact, Cu was below critical deficiency limits without PMK4 co-inoculation at the low fertilizer rate.

Eighteen weeks after sowing, soybean had dropped most of its leaves and pods however the available biomass was harvested and data included. The low rate of fertilizer had a significant (P<0.05) positive effect on biomass, but there was no further increase

obtained with the high fertilizer rate or co-inoculation with PMK4 (Table 4.8, Figure 4.1b).

4.4   Discussion

All the legumes (with the exception of navybean) grew on Christmas Island despite the fact that the soil was highly disturbed, low in nitrogen and no nitrogen was included with the fertilizer. The application of fertilizer was essential for maximum biomass yields but the response varied among the legume species. In some cases, application of fertilizer at the low level was sufficient to obtain maximum yields. For instance, the highest biomass was from mungbean at low applied fertilizer levels when co-inoculated with *P. granadensis* PMK4.

The fertilizer composition used in this study was deliberately broad in order to cater to the requirements of the different legume varieties and the unknown response of the soils to the fertilizer application. However, several essential plant nutrients were below adequate levels in plant tissues of soybean, mungbean and lablab indicating that the fertilizer blend applied to these species, needs to be optimised. The soil is highly disturbed due to the mining activities and is lacking in humic matter and clay and therefore its cation exchange capacity (CEC) is quite low. This was evidenced by potassium deficiencies in the plants despite the application of 50 kg/ha potassium sulphate. The continual cropping of these legume species will eventually increase the humic matter of the soil, thereby increasing the CEC of the soil, making the exchangeable cations available to subsequent crops. However, until this increase in humic matter is achieved, it is advisable that minerals such as potassium be applied at regular intervals as a single application may be lost from the soil during the frequent heavy rainfall events.

Table 4.5: Statistical analysis of dry legume biomass at 9 weeks after sowing

Legume	Source	Type III sum of squares	df	Mean square	F	Sig.
Cowpea	PMK4	6.052	1	6.052	0.203	0.661
	Fertilizer Rate	**611.953**	**2**	**305.976**	**10.252**	**0.003**
	PMK4 * Fertilizer	10.263	2	5.131	0.172	0.844
Lablab	PMK4	5.891	1	5.891	0.447	0.516
	Fertilizer Rate	**136.53**	**2**	**68.265**	**5.182**	**0.024**
	PMK4 * Fertilizer	16.308	2	8.154	0.619	0.555
Mungbean	PMK4	3.479	1	3.479	0.479	0.502
	Fertilizer Rate	**271.596**	**2**	**135.798**	**18.686**	**0.000**
	PMK4 * Fertilizer	1.187	2	0.594	0.082	0.922
Pigeon Pea	PMK4	1.044	1	1.044	0.417	0.530
	Fertilizer Rate	17.69	2	8.845	3.536	0.062
	PMK4 * Fertilizer	2.407	2	1.203	0.481	0.630
Soybean	**PMK4**	**0.141**	**1**	**0.141**	**21.756**	**0.002**
	Fertilizer	**1.09**	**2**	**0.545**	**83.832**	**0.000**
	PMK4 * Fertilizer	0.029	2	0.014	2.209	0.172

a. Soybean analysis performed on Log_{10} transformed data; b. Factors in bold are significant at the P<0.05 level.

Table 4.6: The mean nodulation rating of legume species at 9 weeks after sowing

	Rhizobia only			Rhizobia plus PMK4		
	Nil Fertilizer	Low Fertilizer	High Fertilizer	Nil Fertilizer	Low Fertilizer	High Fertilizer
Cowpea	2.8[a]	3.9[a]	3.4[a]	3.7[a]	3.1 [a]	4.5 [a]
Lablab	3.7[a]	5.0[a]	4.0[a]	3.8[a]	6.1[b]	4.0[a]
Mungbean	3.4[a]	5.7[b]	5.3[b]	4.1[ab]	4.4[ab]	4.8[ab]
Pigeon pea	2.1 [a]	2.3 [a]	2.6 [a]	2.6 [a]	2.5 [a]	2.5 [a]
Soybean	5.8[a]	8.4[bc]	7.5[abc]	7.2[bc]	8.8[b]	8.5[bc]

a. Letters within rows that are the same indicate data that do not differ significantly (P>0.05) for the plant species.

Table 4.7: The nodulation score of legume species at 9 weeks after sowing in the non-replicated uninoculated plots

Legume	Nodule score
Cowpea	2.4
Lablab	1.6
Mungbean	3.4
Navybean	4.5
Pigeon Pea	1.4
Soybean	0.2

Table 4.8: Statistical analysis of dry legume biomass at 18 weeks after sowing

Legume	Source	Type III sum of squares	df	Mean square	F	Sig.
	PMK4	3839.53	1	3839.53	2.41	0.138
Cowpea	**Fertilizer Rate**	**112079.85**	**2**	**56039.93**	**35.18**	**0.000**
	PMK4 * Fertilizer	1609.53	2	804.77	0.51	0.612
	PMK4	**20617.240**	**1**	**20617.240**	**5.156**	**.036**
Lablab	**Fertilizer Rate**	**114100.712**	**2**	**57050.356**	**14.267**	**.000**
	PMK4 * Fertilizer	14976.99	2	7488.50	1.87	0.182
	PMK4	**38286.66**	**1**	**38286.66**	**7.81**	**0.031**
Mungbean	**Fertilizer Rate**	**306153.26**	**2**	**153076.63**	**31.20**	**0.001**
	PMK4 * Fertilizer	**53614.16**	**2**	**26807.08**	**5.47**	**0.045**
	PMK4	**13551.55**	**1**	**13551.55**	**9.20**	**0.023**
Pigeon Pea	**Fertilizer Rate**	**38843.40**	**2**	**19421.70**	**13.19**	**0.006**
	PMK4 * Fertilizer	2514.01	2	1257.01	0.85	0.472
	PMK4	1795.61	1	1795.61	1.38	0.285
Soybean	**Fertilizer**	**21838.83**	**2**	**10919.41**	**8.36**	**0.018**
	PMK4 * Fertilizer	3031.59	2	1515.79	1.16	0.375

a. Factors in bold are significant at the P<0.05 level.
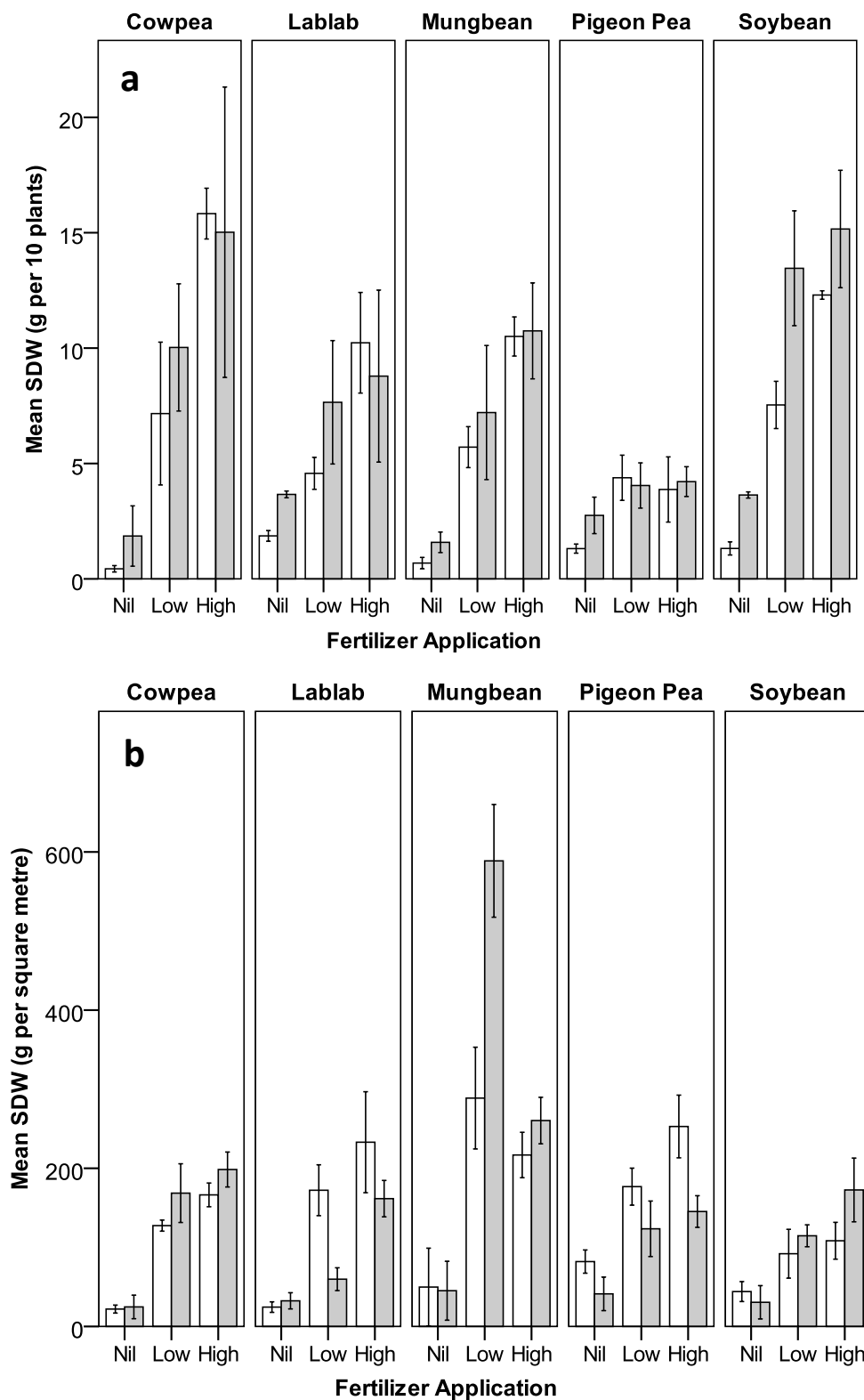
Figure 4.1: The effect of fertilizer and *P. granadensis* PMK4 on all legume species at (a) 9 weeks after sowing and (b) 18 weeks after sowing. Data are presented as mean shoot dry weight (SDW) and the bars above the columns represent standard error. White columns indicate plants inoculated with rhizobia only, grey columns indicate plants inoculated with rhizobia and PMK4
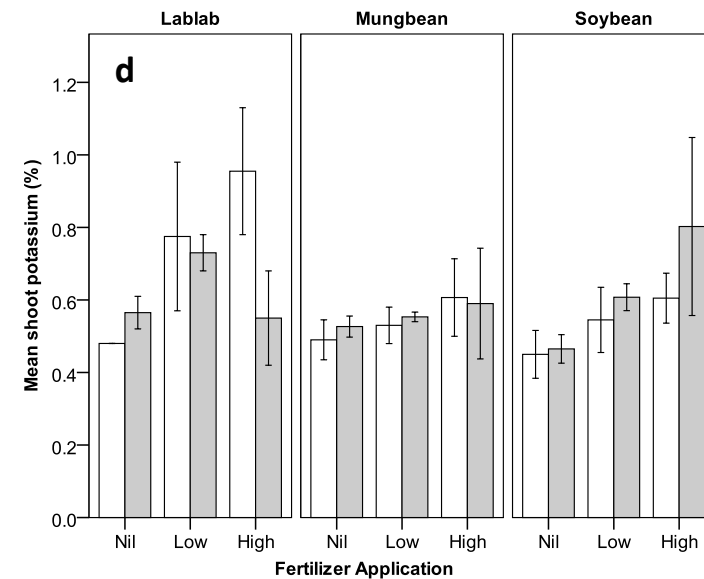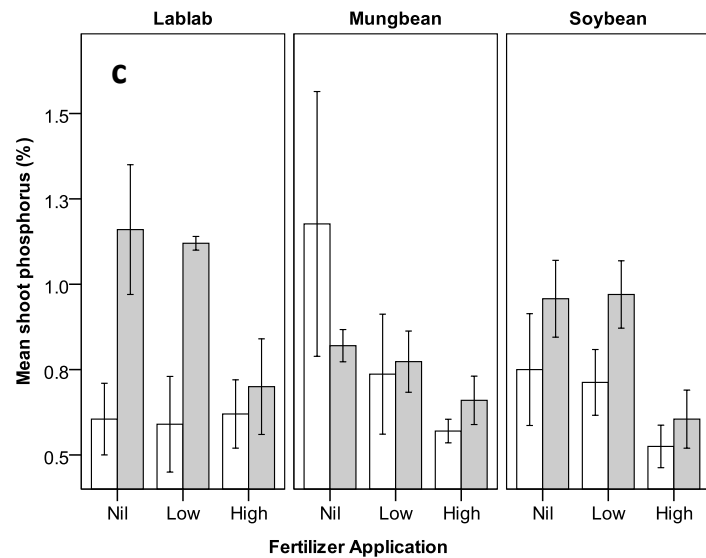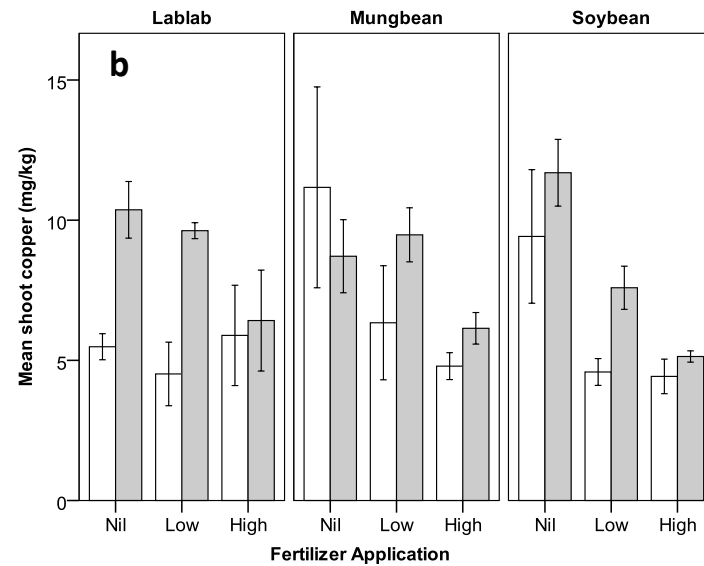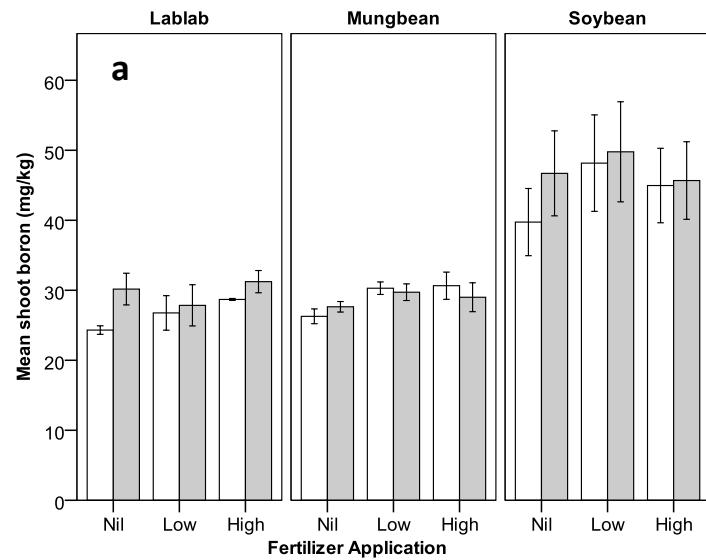
Figure 4.2: The concentration of (a) boron (mg kg^{-1}) (b) copper (mg kg^{-1}), (c) phosphorus (%), and (d) potassium (%) in soybean, mungbean and lablab shoots at the nil, low and high rates of fertilizer application, 9 weeks after planting. The white columns indicate treatments inoculated with *Bradyrhizobium diazoefficiens* CB1809 only and the grey columns indicate treatments co-inoculated with *B. diazoefficiens* CB1809 and the *P. granadensis* PMK4. Bars above the columns indicate standard errors

It is essential that fertilizer use is minimised as the soil on Christmas Island is very porous, with rain water reaching the ground water within about six weeks (SGS Economics and Planning Pty. Ltd. & Trust Nature Pty. Ltd. 2010).

Legumes have different requirements for P and lablab is better able to utilise P than other legumes leading to lower P requirements (Sanginga et al. 1996; Shehu et al. 2001). Lablab is common to Africa (Murphy and Colucci 1999) where low P soils are widespread (Sanginga et al. 1996) and the inclusion of P in the fertilizer blends used in the present study appears to be unnecessary given the high level of plant available phosphorus in the Christmas Island soil. In fact, for the three plant species analysed at nine weeks, phosphorus was in excess in the plant tissues for all treatments. In particular, the lablab treatment (low level fertilizer plus co-inoculated with PMK4) with highest levels of P in shoots had significantly reduced shoot biomass at eighteen weeks. This suggests that phosphorus toxicity and possibly P-induced-Zn deficiency were evident (Hafeez et al. 2013).

Inoculation of legumes with an effective rhizobial strain is vital as demonstrated by the failure of soybean to nodulate with the endemic rhizobia in the uninoculated plots. Conversely, the other legume species were nodulated in the uninoculated plots, including the navybean. The failure of navybean in both the inoculated and uninoculated plots at this site may be due to competition for the commercial inoculant with endemic rhizobia. This plant species is highly promiscuous in terms of its ability to form symbioses with a wide range of rhizobia, which in many cases are ineffective and detrimental to the plant host (Martínez-Romero 2003).

Although no nitrogenous fertilizer was applied and the soil had low N levels, plant tissue showed adequate N. Thus for the crop legumes evaluate in this study, inoculation

with the commercial strains of rhizobia provided sufficient nitrogen for growth and there is no requirement for expensive nitrogenous fertilizers. This is especially relevant in the environmentally sensitive environs of Christmas Island.

Soybean and mungbean showed increased growth when co-inoculated with PGPB and rhizobia, and in soybean the *P. granadensis* PMK4 treatments significantly increased the shoot biomass as well as copper, zinc and phosphorus levels in plant tissue. The PGPB used in this study is able to produce siderophores (Chapter 2) which are known to facilitate Fe acquisition by bacteria and to chelate other metals such as Zn and Cu (Braud et al. 2010; Neilands and Leong 1986). Bacterial siderophores have been shown to effectively provide Fe to plants (Radzki et al. 2013) and it is possible that siderophores produced by the PGPB used in the present study were able to provide adequate Fe, Cu and Zn to the plants.

Alternatively, the mechanism by which inoculation with *P. granadensis* PMK4 improves nutrient acquisition in plants could be attributed to alterations in root system architecture, mediated by the synthesis of plant hormones such as auxins and cytokinins by the PGPB, rather than as a direct consequence of nutrient mobilisation in soils (Richardson et al. 2009; Richardson and Simpson 2011). Zamioudis et al. (2013) demonstrated the role of a PGPB in changing the root system architecture of *Arabidopsis.* The use of several mutants in signaling pathways implicated the production of molecules with auxin activity as an effector in the alteration of the root system. Therefore, it is also reasonable to suggest that by this mechanism the PGPB used in the present study improved some of the legumes' access to nutrients and water subsequently improving the yields of these plants. Further investigation is required to determine the mechanism(s) of action of this PGPB on these plant species.

The biomass yields achieved in this study were promising for most of the legume species. Selecting cultivars better suited to the seasonal and edaphic conditions on Christmas Island can further increase yields. For instance, the soybean cultivar tested flowered and set seed by April, which was too early to exploit all the rainfall and a higher yield might be obtained using slower maturing varieties. The dominant abiotic factors influencing soybean phenology are temperature and day length (Setiyono et al. 2007) and the cultivar used in this study flowered and set seed within 8 weeks of sowing. However, with early maturing varieties, it may be possible to achieve two crops per year, potentially increasing productivity even further (Tasma et al. 2011).

As the area of land available for agriculture exceeds that needed for food production for Christmas Island, potential export markets for commercial agriculture to near neighbours such as Malaysia and Indonesia should be considered. For example, in Indonesia there has been a significant decrease in soybean production from 1.87 million tons in 1992 to 0.776 million tons in 2008 (Tasma et al. 2011) primarily due to drought (Arumingtyas et al. 2013). Consumption in Indonesia is 2.02 million tons and the deficit is met through imports (Tasma et al. 2011). Cash crops might also include those for fibre, perfumery or medical products.

The farming system developed on Christmas Island must be rain fed. Using ground water during the dry season will not be possible as there is already a heavy draw down on aquifers and salination is a danger (SGS Economics and Planning Pty. Ltd. & Trust Nature Pty. Ltd. 2010) and there are only two wetlands on the island – the Dales and Hosnies Spring. These have high conservation value being recognised under the Ramsar Convention on wetlands.

During the wet season, erosion and temporary waterlogging must be controlled as these caused significant problems in this study. Improving site preparation and runoff control is essential for optimal productivity. On these post-mining sites, the mining exit phases should include consideration of landscaping for subsequent agricultural enterprises.

Christmas Island's unique rainforest ecosystems are particularly vulnerable to weed invasion (Director of National Parks 2010). Past introductions for horticulture or mine site rehabilitation have included some species that have become serious weeds (SGS Economics and Planning Pty. Ltd. & Trust Nature Pty. Ltd. 2010). Undesirable introductions have also caused problems during rehabilitation of other phosphate mines such as in Florida (Brown 2005). On Christmas Island new plant introductions are strictly controlled by quarantine and several otherwise suitable horticultural species will be impossible to import for this reason.

The sub-tropical pulse legumes lablab, cowpea, mungbean, pigeon pea and soybean, grew in the environment on Christmas Island. The selection of appropriate legume cultivars and improvements to site preparation are required to optimise productivity. An appropriate rhizobial inoculant for each legume species is essential and the addition of *P. granadensis* PMK4 also increased plant growth. Further studies are needed to determine their ability to persist in these soils and colonise subsequent crops. A fertilizer blend needs to be developed to overcome the deficiencies of K, S and Cu in the soils and take into account the abundance of P.

Chapter 5:
Do the isolates *Burkholderia caledonica* NCH45 and *P. granadensis* PMK4 facilitate plant growth promotion in wheat through the solubilisation of inorganic phosphorus *in vivo*?
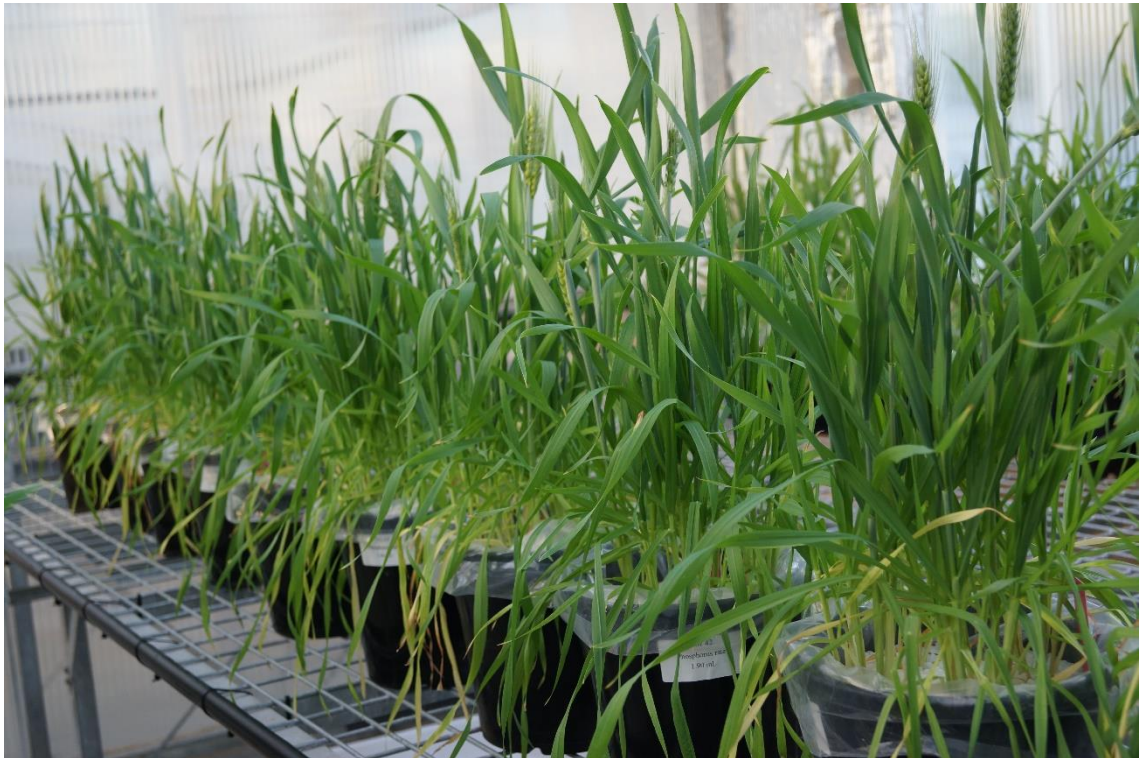
Wheat in glasshouse trials evaluating inoculation with PMK4 and NCH45.

5.1   Introduction

Most soils are highly deficient in plant available phosphorus (P) (Richardson 2001) and in Western Australia, P is one of the most limiting nutrients in agriculture (Quinlan and Wherett 2014). Newly developed land may require up to 400 kg superphosphate ha^{-1} to achieve adequate yields and typical maintenance rates for P on these soils can be between 100-200 kg ha^{-1} (Allen and Jeffrey 1990; Reuter et al. 1995). However, only 5-30% of the applied P is taken up by the plant in the year of application and the remainder is lost through soil erosion and leaching or sorption to various components of the soil (Allen and Jeffrey 1990; McLaughlin et al. 1988; Quinlan and Wherett 2014). Furthermore, a recent investigation of the phosphorus balance efficiency of a number of soils from southern and southeastern Australia demonstrated that P applied in excess of removal (P recovered in harvested materials) ranged from 6.1 to 18.1 kg P ha^{-1} year^{-1} in different farming systems (Weaver and Wong 2011).

Since the unprecedented price spike in 2008 which saw phosphate rock and other fertilizer commodities rise by 800%, the estimated Australian fertilizer bill at the farm gate now exceeds AU$10 billion (Cordell and White 2010; White et al. 2010). This indicates that there is potential to improve financial returns to farmers as well as environmental outcomes by the adoption of improved phosphorus management practices (Weaver and Wong 2011). There are several strategies that can improve phosphorus use efficiency in farming systems and these are reviewed in Simpson et al. (2011). One of these strategies is through the manipulation of soil microorganisms to maximize P mobilization in the soil in synchronization with plant P requirements (Richardson 2001).

Several PGPB from the current study were shown to improve plant growth in the field in Western Australia (Chapter 3) and on Christmas Island (Chapter 4). Amongst the traits that these organisms display, is the ability to solubilise inorganic P *in vitro* (Chapter 2). Therefore, it is possible that these bacteria improve plant growth by increasing uptake of P by the plant. However, while *in vitro* assays using tricalcium phosphates (TCP) as a selection factor can isolate thousands of potential phosphorus solubilising microbes (PSM), this does not mean that the bacteria can make P more accessible to the plant (Bashan et al. 2013b). When tested further for their ability to alleviate P deficiency in plants, authors have reported that only a small fraction of these isolates are true PSM (Bashan et al. 2013a; Sharma et al. 2013). For instance, none of the 13 bacteria with the ability to solubilise TCP in solid and liquid media increased P uptake by soybean plants when applied to soil (Fernández et al. 2007). These authors as well as Bashan et al. (2013b) stress the importance of using a combination of *in vitro* assays to isolate potential PSM. Then they referred to assessing the putative PSM in soil with the host plant as *the ultimate test* for phosphorus solubilisation by these organisms.

As highlighted by Singh et al. (2014), PGPB isolated as PSM are frequently tested on plants at only one or a few P levels. In these cases, when increases in P uptake in the plants is observed it cannot be concluded that the increased concentration of P in the plant is through phosphorus solubilisation and not some other mechanism, such as bacterially produced auxin-mediated alteration in root architecture, enabling the plant greater access to P reserves (Hayat et al. 2010; Richardson et al. 2009; Richardson and Simpson 2011).

Therefore, to determine if phosphorus solubilisation is the mechanism of action by a putative PSM, plant growth should be assessed in the presence or absence of the PSM over a range of phosphorus levels to give a complete phosphorus response curve (Abbott and Robson 1984; Richardson et al. 2009). This will enable the distinction between the three classes of interaction between inoculation with a PSM and P application proposed by Whitelaw et al. (1997). In the first class, inoculation with a PSM relieves P deficiency in the plant and there is no further response to inoculation at the sufficient rate of applied P or beyond this point. This they rather confusingly refer to as a 'negative interaction'. In the second class, the inoculant does not relieve P deficiency but promotes plant growth by another mechanism and the response is not eliminated by the application of P. Thirdly, if the inoculant is effecting plant growth promotion by P solubilisation plus one or more other mechanism(s), a negative interaction between P and inoculation will be observed, as for the first response class. However, growth promotion will still be observed at P rates higher than a rate sufficient to eliminate P deficiency.

The aim of this study was to determine the ability of the PGPB isolates NCH45 and PMK4 to solubilise P for the alleviation of P deficiency. This was performed in the glasshouse using a phosphorus response curve with wheat as a model plant, on three soil types representing typical Western Australian soils containing a range of background P.

5.2   Methods

5.2.1   General glasshouse methods

Three experiments were conducted each using three different soil types: a Yalanbee soil, which represents a virgin Western Australian soil; and two soils from the Wongan Hills research station which represent conventional Western Australian agricultural soils that have had repeated applications of fertilizers over a number of years. To each soil type, the same 10 rates of phosphorus were applied with and without the two putative phosphorus solubilising bacteria *Pseudomonas granadensis* PMK4 and *Burkholderia caledonica*. NCH45. These experiments were conducted in order to determine if the PGPB were able to improve the solubility of a relatively insoluble form of P (as used in the *in vitro* assays in Chapter 2) in soils with a low background of P (Yalanbee soil) and/or if these bacteria are able to solubilise the residual P in soils with a high background of P from repeated fertilizer application (Wongan Hills soils).

5.2.1.1 Soil

Yalanbee soil

The top 0-10 cm of a gravelly loamy sand, classified as Yalanbee soil landscape unit (Fulton and Lantzke 1993) was collected from a site of remnant native vegetation at the Allendale Research Station (The University of Western Australia) at Bakers Hill (31.76° S, 116.48° E) situated 80 km east of Perth. The Yalanbee soil unit is formed from deeply weathered laterite and the surface consists of dark, grayish brown loamy sand and ironstone gravel. The subsurface soil is yellowish brown sandy loam that contains ironstone gravel and some clay at depth (Pritchard 2005) and was not collected. This soil was used in Glasshouse Experiment 1.

Wongan Hills Soil Type A

For the Glasshouse Experiment 2, the top 10 cm of a grey loamy sand classified as Elphin series (Western Australian Dept. of Agriculture 1971) was collected from a fallow paddock (2HDE) (30.49'15"S, 116.42'19"E) at the Wongan Hills Research Station. The research station is situated approximately 190 km North East of Perth, Western Australia and has been operating as an experimental farm since 1924. This soil represents the light textured, infertile soils that are scattered throughout nearly every district of the Western Australian 'Wheatbelt'. Almost the whole area of the station was covered with ironstone gravel or laterite and the weathering of the laterite profile has produced a variety of soils on the station with low inherent fertility. The application of superphosphate, trace elements and cultivation of subterranean clover have significantly improved the fertility of these soils.

Wongan Hills Soil Type B

The soil used in Glasshouse Experiment 3 was from a second collection of soil adjacent (100 m) to paddock 2HDE at the Wongan Hills Research Station.

5.2.1.2 Pots and basal nutrients

Gravel and debris were removed from all three soils by sieving through a 5 mm stainless steel sieve (Kimseed International Pty Ltd) and the soils were stored in 60 L plastic bins until required. Table 5.1 lists the characteristics of these soils.

Polypropylene pots (19 cm high and 19 cm internal diameter) were lined with plastic bags and filled with an air dried mix of 1:1 Yalanbee sand or Wongan Hills Soil Type A or B and river sand and the prepared pots were steamed at 80°C for 2 hours. The soil surface area was 254 cm^2.

Table 5.1: Characteristics of the top 10 cm of the Yalanbee and two Wongan Hills soils

Property	Yalanbee soil[a]	Wongan Hills Type A[b]	Wongan Hills Type B[c]
Texture	Sand	Sand/loam	Loam
Nitrate nitrogen (mg kg^{-1})	1.0	<1.0	1.0
Ammonium nitrogen (mg kg^{-1})	4.0	5.0	3.0
Colwell Phosphorus (mg kg^{-1})	3.0	58.0	40.0
Potassium (mg kg^{-1})	65.0	82.0	305.0
Sulphur (mg kg^{-1})	65.0	5.5	6.76
Organic Carbon (%)	1.71	1.89	1.74
Conductivity (EC) (dS m^{-1})	0.058	0.086	0.073
pH (CaCl$_2$)	5.3	7.3	6.1
pH (H$_2$O)	6.3	8.1	6.9
Phosphorus retention index	ND	0.78	6.6
Total Phosphorus (mg/kg)	ND	216.4	170.6

[a] = Analysis carried out by CSBP Limited: Report ZRS09 007-012
[b] =Analysis carried out by CSBP Limited: Report 3ZS12112
[c] =Analysis carried out by CSBP Limited: Report A2S09008
ND= not determined
pH (CaCl$_2$) in 0.01M CaCl$_2$.
Mg kg^{-1} = milligrams per kilogram, dS/m = deciSiemens per metre

To determine the gravimetric soil water capacity (GSWC) of the soil, 3 polypropylene free-draining pots (as above) were filled with the soil mix and watered until saturated. The pots were then covered with plastic to prevent evaporation. After 24 hours, the top 5 cm of the soil was collected and dried for 48 h at 105°C to determine the moisture content. The GSWC for Yalanbee, Wongan A and Wongan B were 24%, 17% and 16%, respectively.

For each PGPB (NCH45 and PMK4) or the uninoculated control treatment, ten sets of pots were prepared, with increasing amounts of calcium phosphate equivalent to: 0, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 60.0, 100.0 mg kg^{-1} soil. P was applied as sterile calcium phosphate precipitate produced from combining 50 mL 10% (w/v) K$_2$HPO$_4$ and 100 mL

10% (w/v) $CaCl_2$. Two sterile nutrient solutions were applied to all the pots: 15 mL sterile Basal A solution (K_2SO_4: 102 g; $MgSO_4.7H_2O$: 21.4 g; $CuSO_4.5H_2O$: 6.0 g; $ZnSO_4.7H_2O$: 6.0 g; $MnSO_4.H_2O$: 6.0 g and $CoCl_2.6H_2O$: 0.05 g; 2000 mL H_2O) and 15 mL sterile Basal B solution ($CaCl_2.2H_2O$: 102.8 g; $(NH_4)_6Mo_7O_{24}.4H_2O$: 0.05 g; H_3BO_3: 0.43 g; 2000 mL H_2O). All the above nutrients were mixed evenly through the soil. Two mL nitrogen was applied to all pots as NH_4NO_3 (40 mg N mL^{-1}) immediately prior to planting and then every 2 weeks until harvest.

5.2.1.3 Preparation of inocula and sowing

Wheat (*Triticum aestivum* L. cv. Wyalcatchem) seeds were surface sterilized by immersion in 70% ethanol for 1 min then 4% bleach for 3 min followed by 6 washes in sterile distilled water. Ten cavities in the soil in each pot were created to 5 cm depth using a sterile glass rod, and a wheat seed was placed in each cavity. The seed was covered with soil once the bacteria had been applied to the seed (described below).

Two PGPB isolates, PMK4 and NCH45 were grown in 50 mL NB (Beckton Dickinson) in 250 mL flasks with shaking (200 rpm) at 28°C. After 48 hours the entire contents of the flasks were washed twice in sterile 0.3 M $MgSO_4$ and each PGPB isolate was adjusted to an OD_{600} of 0.075 (10^7 cells mL^{-1}) and a volume of 60 mL. In order to verify cell numbers, prior to inoculation, 100 µL of each prepared inoculum was serially diluted and plated onto Miles and Misra plates prepared with ½ strength Nutrient Agar (½NA) (Beckton Dickinson). One mL of the culture suspension for each PGPB was then inoculated onto each seed in the pots for the respective PGPB treatments and 1 mL of sterile 0.3 M $MgSO_4$ was applied to each seed in the uninoculated control treatments. The pots were then covered with sterile plastic high density beads (Universal Polymer Supplies) in order to minimize water loss and to reduce contamination. The pots were

watered to GSWC every day using boiled deionised water and the plants were thinned to 5 plants per pot at the 2.5 leaf stage. There were 4 replicates in Experiment 1 and 5 replicates in Experiments 2 and 3 for each treatment (2 PGPB strains + uninoculated control, 10 rates of P) which were arranged in a randomised complete block design. Every second day the position of the pots was rotated anticlockwise and each week the entire replicate block was rotated anticlockwise to minimize positional effects on plant growth. All three experiments were conducted in a naturally-lit glasshouse with constant temperature (23°C ± 2°C) at Murdoch University, Perth (32°04′S, 115°50′E). Due to the large size of these experiments, they were not conducted concurrently. Experiment 1 was conducted in November-December, Experiment 2 in September-October and Experiment 3 in June-July over three successive years.

5.2.1.4 Plant harvest

Plants were harvested 48 days after sowing in full by cutting the shoots at ground level then drying them at 70°C for 48 hours or until they reached a constant weight. Shoot dry weights were then measured.

5.2.2 Experiment 1 – Yalanbee Soil

5.2.2.1 Preparation of pots

Pots were prepared as described above using 3.4 kg of an air dried mix of 1:1 Yalanbee and river sand.

5.2.3 Experiment 2 – Wongan A Soil

5.2.3.1 Preparation of pots

Pots were prepared as described above using 4.6 kg of an air dried mix of 1:1 Wongan A soil and river sand.

5.2.3.2 Preparation of inocula and sowing

The two PGPB inocula and pots were prepared as described in Section 5.2.1.3 except that the OD_{600} of the inocula was 0.15 (10^7 cells mL^{-1}).

5.2.3.3 Plant harvest

Roots from selected replicates were carefully washed in sterile water, sectioned into top, middle and lower root sections and stored in sterile tubes at -80°C until use for detection of presence of the bacterium (Chapter 7).

5.2.4 Experiment 3 – Wongan B Soil

5.2.4.1 Preparation of pots

Pots were prepared as described above using 4.6 kg of an air dried mix of 1:1 Wongan B soil and river sand.

5.2.4.2 Preparation of inocula and sowing

The two PGPB inocula and pots were prepared as described in Section 5.2.1.3 except that the OD_{600} of the inocula was 0.15 (10^7 cells mL^{-1}).

5.2.4.3 Plant harvest

Soil closely adhering to the roots was collected at harvest and stored in plastic bags until use for the detection of the bacterium (Chapter 7). After final weighing, the shoots from each pot were bulked and 3 replicates from each treatment were analysed for N, P, K, Bo, Ca, Cl, Mg, Na, S, Fe, Mn, Zn and Cu by Inductively Coupled Plasma Spectrophotometry (ICP) at CSBP Bibra Lake.

5.2.5 Statistical analysis of data

Data were subjected to univariate analysis using a general linear model or analysis of variance (ANOVA) and if significant, treatment means were compared using Fisher's

Protected LSD Test at P<0.05, unless otherwise stated. To ensure the homogeneity of variances, the data were log-transformed where necessary. Statistical analyses were conducted by IBM SPSS Statistics 21.0.

5.3   Results

5.3.1   Shoot development and dry matter production

5.3.1.1 Experiment 1

The wheat plants grown in the Yalanbee soil responded to increasing applied phosphorus in a typical dose-response manner (Figure 5.1). The relationship between applied phosphorus and shoot dry weight (SDW) was significantly (P=0.000) positive and the SDW increased substantially at each applied phosphorus level. Inoculation of the wheat plants with the PGPB stimulated growth in the same way as the applied P and was there was no relationship (P>0.05) between phosphorus and inoculation with the PGPB (Table 5.2).

5.3.1.2 Experiment 2

In the Wongan A soil, the wheat did not respond to application of P or to inoculation with PMK4 (Figure 5.1 and 5.2). However, there was an overall significant (P<0.05) reduction in shoot biomass in the NCH45 treatments compared with the controls and PMK4 treatments (Figure 5.2).

5.3.1.3 Experiment 3

Inoculation with the PGPB and application of P had significant (P=0.00) effects on the shoot biomass of the wheat plants in the Wongan B soil particularly when the data for all P levels were averaged (Figure 5.1 and 5.2). The biomass of the plants inoculated with the PGPB was slightly but not significantly (P>0.05) greater than the controls at the lower rates of applied P. However, at 7.5 mg kg^{-1} applied P, NCH45 significantly

(P=0.043) increased the shoot biomass compared with the control at this P rate. Unfortunately, at this P rate (due to incomplete mixing of the initial applied water through the soil) several of the PMK4 treatments had become waterlogged and these replicates were removed from data analysis. At 10 mg kg^{-1}, there was no difference in shoot biomass between the control and the PGPBs and this appears to be the rate at which applied P eliminates P deficiency in the plants. As P rates increased past this apparent optimum, the biomass of the control treatments began to decrease and the reduction in the controls at the highest P rate was significant (P=0.04) compared with controls at the 10 mg kg^{-1} P rate. In contrast, the shoot biomass of the PGPB treatments increased significantly (P<0.05) at 20 mg kg^{-1} (PMK4) and 60 mg kg^{-1} (NCH45).

Table 5.2: Statistical analysis of the effects applied phosphorus and inoculation with PGPB on the shoot dry weights of wheat plants in glasshouse experiments in Yalanbee (Experiment 1), Wongan Hills Type A (Experiment 2), and Wongan Hills Type B (Experiment 3) soils. Subjects in bold denote significant (P<0.05) effects

| Experiment	Source	Type III sum of squares	df	Mean square	F	Sig.
	PGPB	.006	2	.003	.489	.615
Experiment 1	**Phosphorus Rate**	**21.632**	**9**	**2.404**	**377.398**	**.000**
	PGPB * P Rate	.136	18	.008	1.185	.290
	PGPB	**1.171**	**2**	**0.585**	**5.726**	**0.004**
Experiment 2	Phosphorus Rate	1.627	9	0.181	1.768	0.081
	PGPB * P Rate	1.459	18	0.81	0.793	0.705
	PGPB	**.276**	**2**	**.138**	**6.442**	**.002.**
Experiment 3	**Phosphorus Rate**	**.957**	**9**	**.106**	**4.956**	**000**
	PGPB * P Rate	.240	18	.013	.622	.876
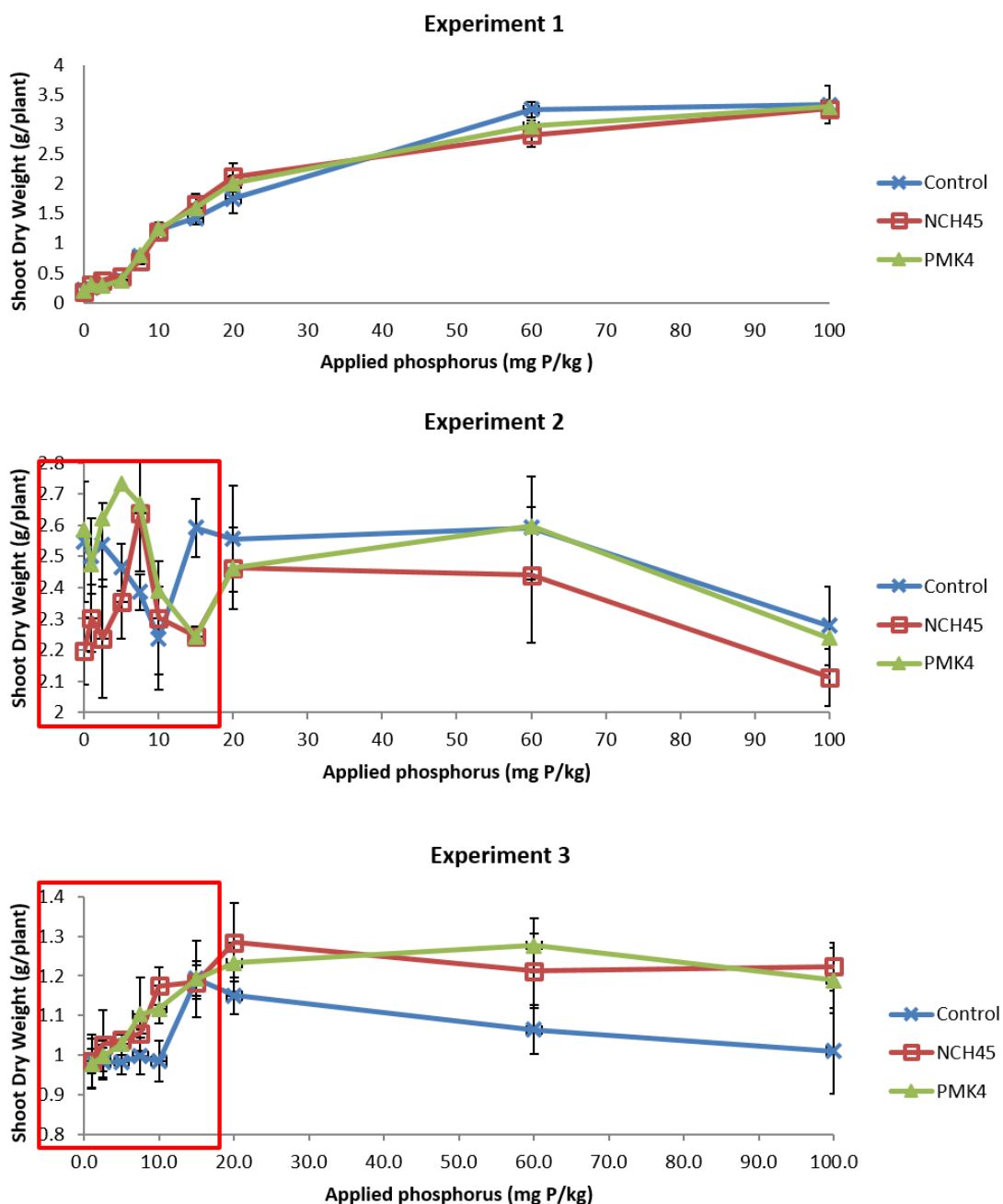
Figure 5.1. Mean shoot dry weights of wheat plants at 48 days after sowing at a range of applied phosphorus levels plus inoculation with PGPB in three Western Australian soils: Yalanbee (Experiment 1) Wongan Hills Type A (Experiment 2) and Wongan Hills Type B (Experiment 3). The Y axis has been adjusted in each graph. Sections of the graphs in boxes are shown in detail in Figure 5.2.
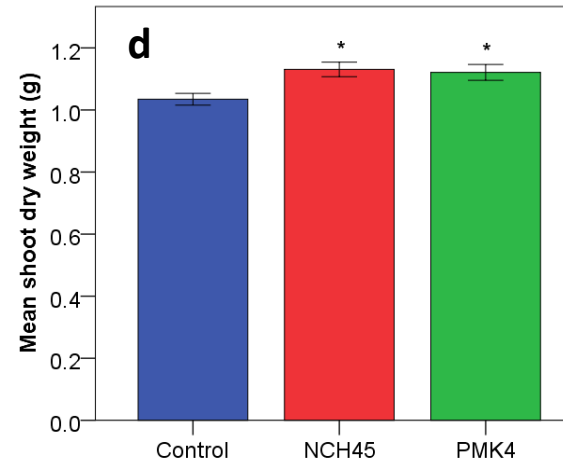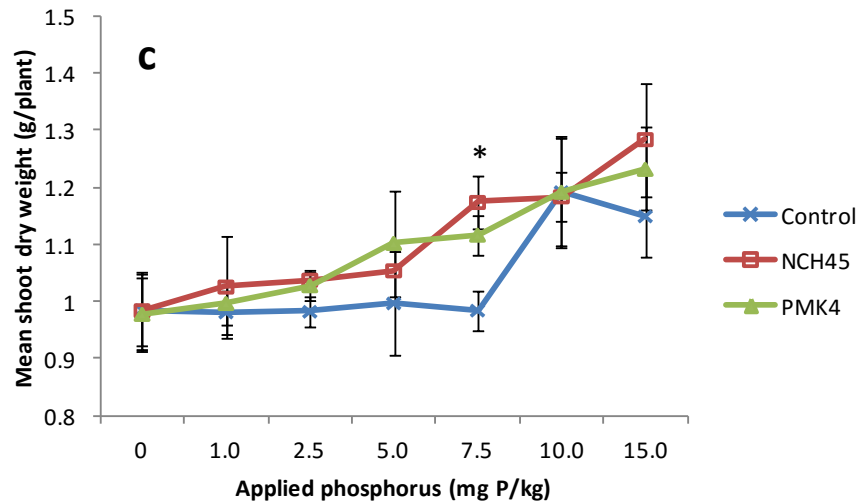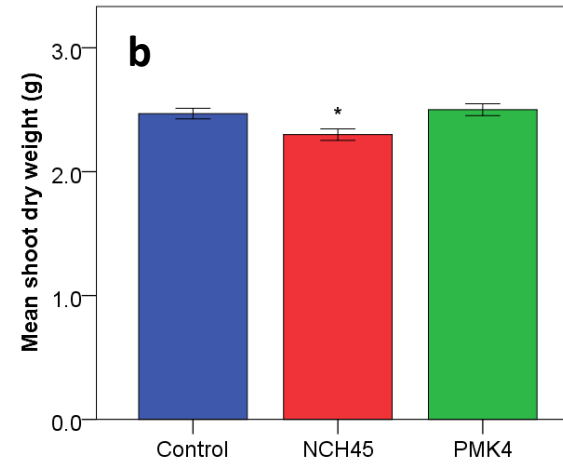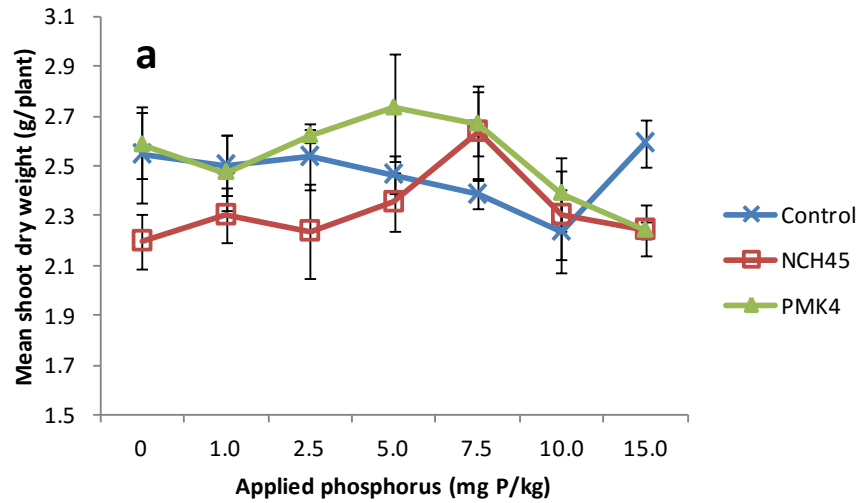
Figure 5.2: The mean shoot dry weights of wheat at 48 days after sowing at a range of applied phosphorus levels, plus/minus inoculation with two PGPB and grown in two different Western Australian soils. The x axis has been truncated in order to display the effect of the lower level of applied phosphorus on the plants in detail for Experiment 2 (a) and Experiment 3 (c). The data were averaged for all 10 P rates for Experiment 2 (b) and Experiment 3 (d). The bars above the lines or columns indicate standard error and the asterisks indicate a significant (P<0.05) difference from the control

5.3.2 Nutrient analyses for Glasshouse Experiment 3

With the exception of boron and magnesium, the application of P had a significant ($P<0.05$) effect on nutrient concentrations in the wheat plants grown in the Wongan B soil (Table 5.3, Figure 5.3-5.5, Appendix II). In particular, as the P rate increased so did the concentration of P in the wheat plants from 0.32% in the nil applied P treatments to 0.69% in the 100 mg kg^{-1} treatments. Large increases in concentrations of calcium (48%), chloride (46%), copper (23%) and manganese (47%) were observed when applied P was increased from nil to 100 mg kg^{-1}.

Conversely, inoculation of the wheat plants with the PGPB significantly decreased the concentration of all the nutrients except for boron, manganese, nitrate and total nitrogen (N) (Fig 5.3-5.5). The total shoot uptake of P and N per pot were analysed and the PGPB have opposing effects on the uptake of these nutrients. Both NCH45 and PMK4 increase the total N content (Fig 5.6) in the shoots while PMK4 decreased the amount of P in the shoots (Fig 5.7).

5.4 Discussion

The utilization of the full phosphorus response curve has demonstrated that the two PGPB used under the experimental conditions of this study, are promoting plant growth via a mechanism that is not through the alleviation of P deficiency in the wheat plants. When wheat plants were inoculated with NCH45 or PMK4 and grown at a range of P levels, there was no interaction between the PGPB and P application for shoot dry weight. This indicates that an affect other than P solubilisation is applicable here, or a class two response as described by Whitelaw et al. (1997). As observed in the nutrient analyses of the shoot dry matter (for the third glasshouse experiment), these PGPB did not increase the concentration of P in the shoot, instead a decrease in the concentration

Table 5.3: Statistical analyses of the concentration of selected nutrients in wheat plants grown in a Wongan Hills Type B soil. Figures in bold denote significant (P<0.05) effects

| Nutrient	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Boron	Phosphorus rate	29.503	9	3.278	.942	.496
	PBPB	6.683	2	3.342	.961	.388
	P rate * PGPB	67.638	18	3.758	1.080	.392
Calcium	**Phosphorus rate**	**.232**	**9**	**.026**	**10.086**	**.000**
	PBPB	**.036**	**2**	**.018**	**7.076**	**.002**
	P rate * PGPB	.063	18	.004	1.376	.177
Chloride	**Phosphorus rate**	**12.722**	**9**	**1.414**	**17.194**	**.000**
	PBPB	**.534**	**2**	**.267**	**3.247**	**.046**
	P rate * PGPB	1.803	18	.100	1.218	.276
Copper	**Phosphorus rate**	**31.479**	**9**	**3.498**	**5.674**	**.000**
	PBPB	**10.313**	**2**	**5.157**	**8.365**	**.001**
	P rate * PGPB	**29.968**	**18**	**1.665**	**2.701**	**.002**
Iron	**Phosphorus rate**	**5483.181**	**9**	**609.242**	**2.545**	**.015**
	PBPB	**2377.549**	**2**	**1188.774**	**4.966**	**.010**
	P rate * PGPB	6572.246	18	365.125	1.525	.112
Magnesium	Phosphorus rate	.003	9	.000	.762	.651
	PBPB	**.005**	**2**	**.002**	**6.464**	**.003**
	P rate * PGPB	.009	18	.001	1.342	.195
Manganese	**Phosphorus rate**	**3244.534**	**9**	**360.504**	**8.068**	**.000**
	PBPB	91.028	2	45.514	1.019	.367
	P rate * PGPB	**1938.619**	**18**	**107.701**	**2.410**	**.006**
Nitrate	**Phosphorus rate**	**28758479.392**	**9**	**3195386.599**	**6.954**	**.000**
	PBPB	1952975.987	2	976487.993	2.125	.128
	P rate * PGPB	11517364.250	18	639853.569	1.393	.168
Phosphorus	**Phosphorus rate**	**1.110**	**9**	**.123**	**42.007**	**.000**
	PBPB	**.071**	**2**	**.035**	**12.064**	**.000**
	P rate * PGPB	**.157**	**18**	**.009**	**2.969**	**.001**
Potassium	**Phosphorus rate**	**4.892**	**9**	**.544**	**2.038**	**.050**
	PBPB	**2.905**	**2**	**1.452**	**5.446**	**.007**
	P rate * PGPB	5.115	18	.284	1.066	.407
Sodium	**Phosphorus rate**	**.001**	**9**	**9.606E-05**	**3.384**	**.002**
	PBPB	**.000**	**2**	**.000**	**8.009**	**.001**
	P rate * PGPB	.001	18	4.652E-05	1.639	.080
Sulphur	**Phosphorus rate**	**.037**	**9**	**.004**	**5.601**	**.000**
	PBPB	**.013**	**2**	**.007**	**8.949**	**.000**
	P rate * PGPB	**.027**	**18**	**.001**	**2.015**	**.022**
Total Nitrogen	**Phosphorus rate**	**3.536**	**9**	**.393**	**6.461**	**.000**
	PBPB	.112	2	.056	.920	.404
	P rate * PGPB	**2.285**	**18**	**.127**	**2.087**	**.017**
Zinc	**Phosphorus rate**	**992.842**	**9**	**110.316**	**2.255**	**.030**
	PBPB	**373.185**	**2**	**186.593**	**3.814**	**.028**
	P rate * PGPB	912.379	18	50.688	1.036	.436
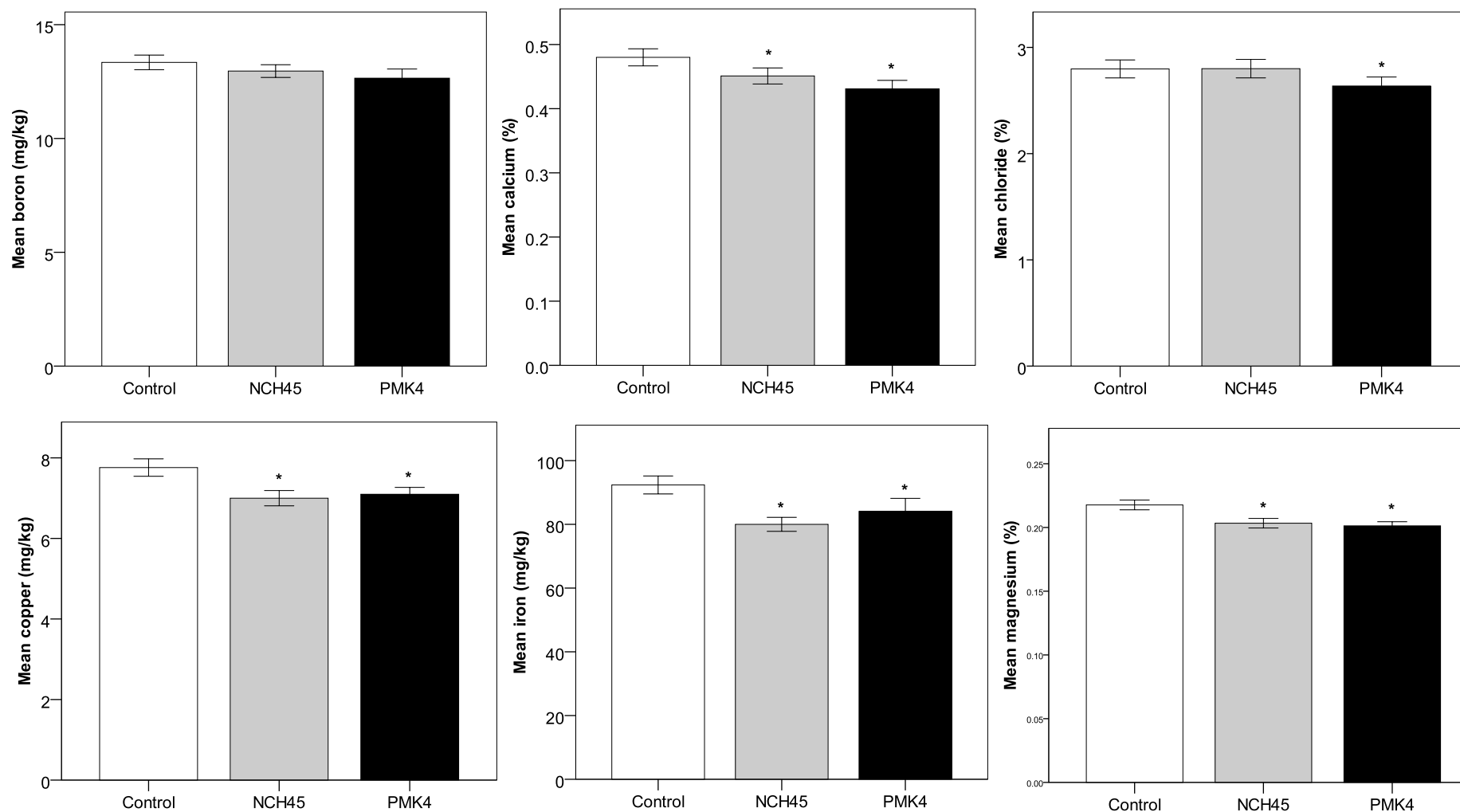
Figure 5.3: The effect of inoculation of wheat plants with PGPB in the Wongan Hills Type B soil on shoot nutrient concentrations of boron, calcium, chloride, copper, iron and magnesium. Bars above the columns represent standard errors of the mean and the asterisks indicate significant (P<0.05) differences from the control
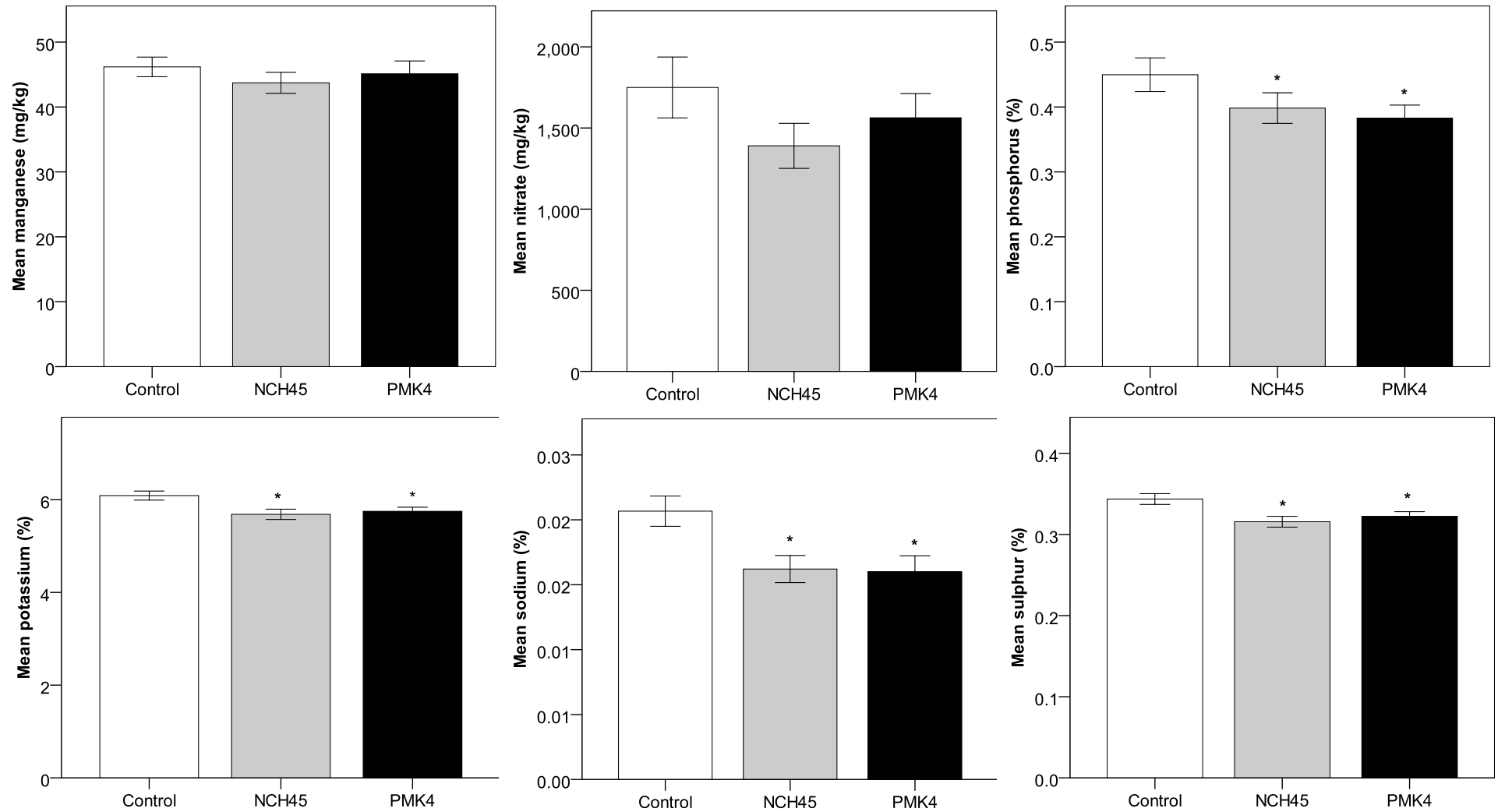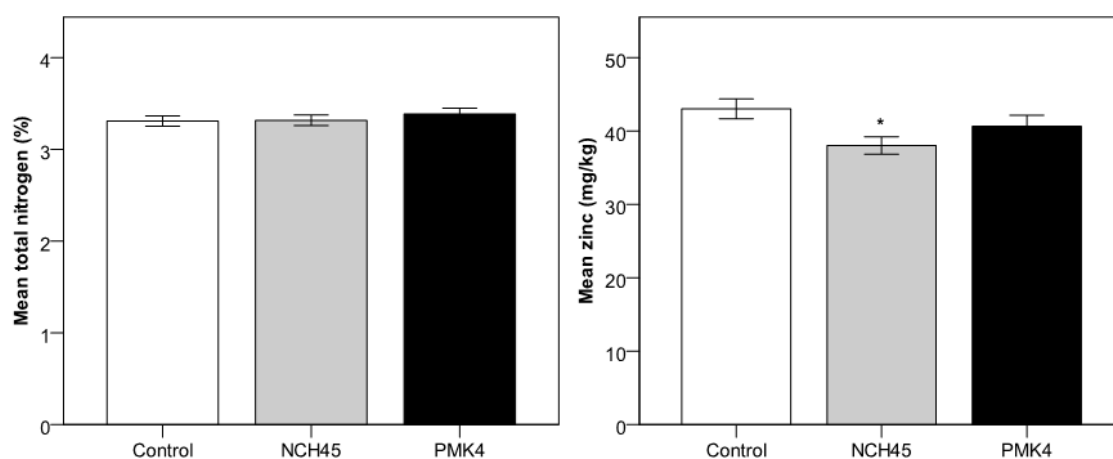
Figure 5.4: The effect of inoculation of wheat plants with PGPB in the Wongan Hills Type B soil on shoot nutrient concentrations of manganese, nitrate, phosphorus, potassium, sodium and sulphur. Bars above the columns represent standard errors of the mean and the asterisks indicate significant (P<0.05) differences from the control

Figure 5.5: The effect of inoculation of wheat plants with PGPB in the Wongan Hills Type B soil on shoot nutrient concentrations of total nitrogen and zinc. Bars above the columns represent standard errors of the mean and the asterisks indicate significant (P<0.05) differences from the control

of P in the shoots was observed in plants inoculated with PMK4. This is probably a dilution effect resulting from the increases in shoot biomass facilitated by inoculation with these PGPB and this dilution effect was also observed for the other nutrients assayed except for boron, manganese, nitrate and total nitrogen.

When the total nitrogen uptake in the shoots was determined, both PMK4 and NCH45 increased the uptake of N by 11% over the controls. In the PGPB treatments, the average total uptake of N in the shoots across all 10 P levels was 200 mg per pot compared to 180 mg in the controls. Since the background level of NO_3/NH_4^+ was very low in the Wongan Hills B soil (approximately 4 mg/kg) and the total N applied as KNO_3 over the trial was 360 mg, this indicates that almost all of the available N was taken up by these plants (assuming an equivalent uptake in the roots). Officer et al. (2009) found that total N uptake was positively related to shoot growth in wheat and it is possible that the growth promotion of the wheat plants observed in the current study is through a mechanism that resulted in improved uptake of N in the plants.
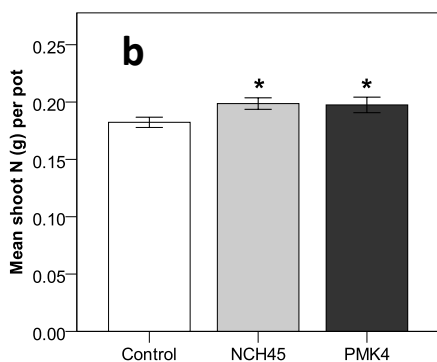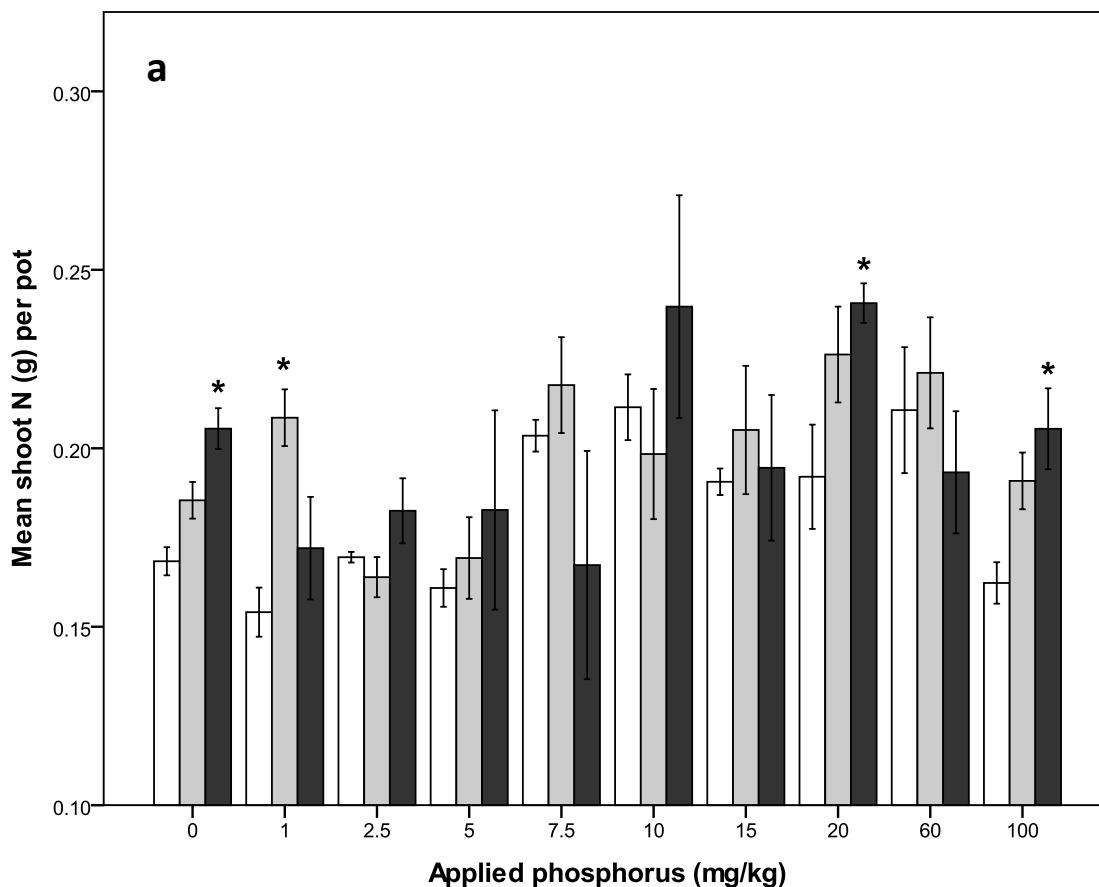
Figure 5.6: **(a) the mean total nitrogen (N) content of the shoots at all applied phosphorus rates (g pot^{-1}); (b) the mean N content of the shoots averaged across all the phosphorus levels.** White columns indicate the control, grey columns indicate NCH45 treatment and black columns indicate PMK4 treatments. Bars above the columns represent standard errors and asterisks above the PGPB treatments represent significant (P<0.05) differences from the non-inoculated control either at each P rate (a) or overall (b)

While the plant growth promotion of rhizobia with their legume hosts is through the symbiotic fixation of N, the contribution of N_2 fixation by non-rhizobial root associated bacteria to the N status of plants is still widely disputed and is not generally credited as the mechanism for the promotion of plant growth by these bacteria (Dobbelaere et al. 2003; Vacheron et al. 2013). It is possible that the increased nitrogen content of plants

co-cultivated with PGPB is related to the ability of these bacteria to increase the NO_3^-

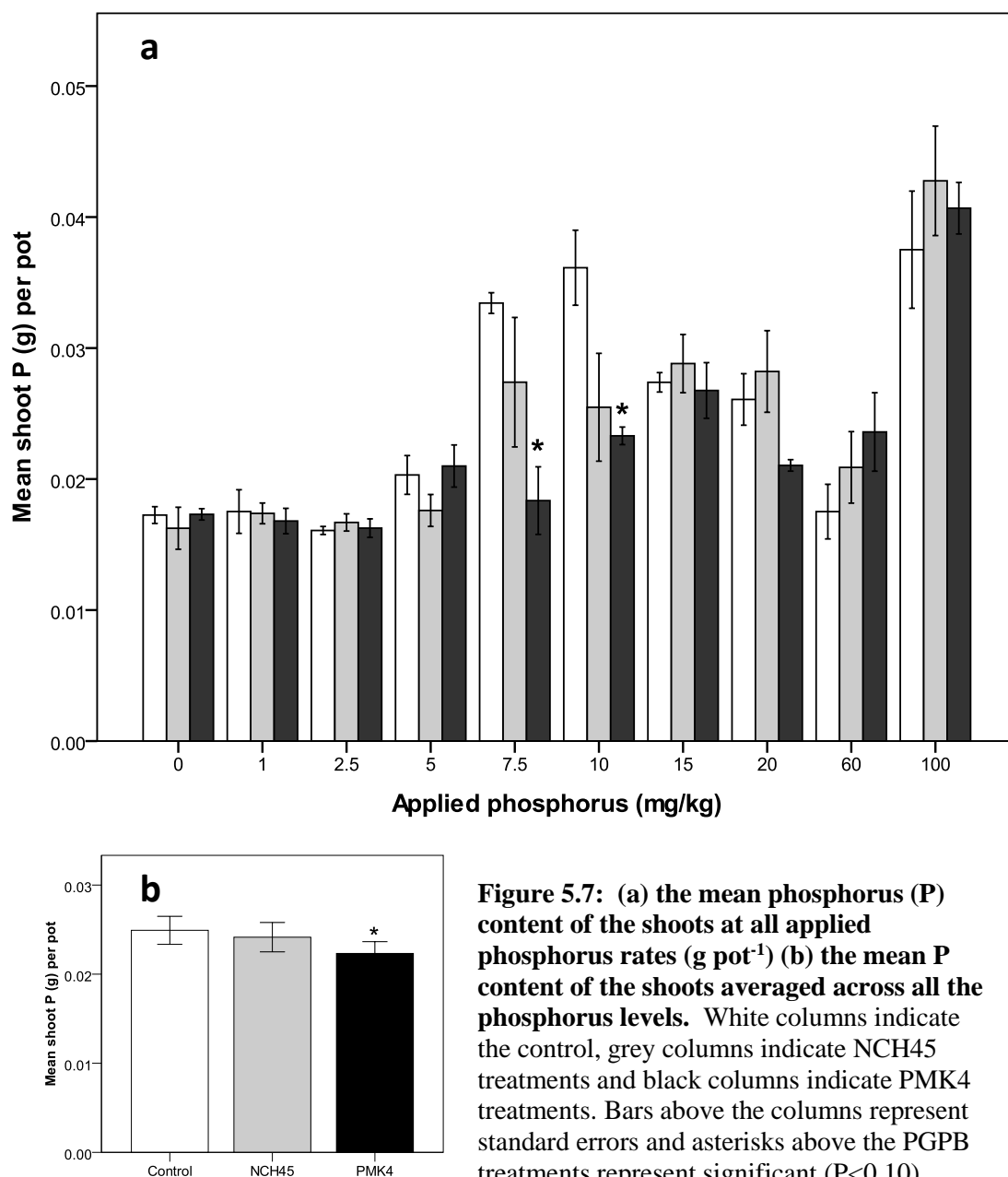uptake capacity and assimilation in the plants (Parra-Cota et al. 2014).

Figure 5.7: **(a) the mean phosphorus (P) content of the shoots at all applied phosphorus rates (g pot^{-1}) (b) the mean P content of the shoots averaged across all the phosphorus levels.** White columns indicate the control, grey columns indicate NCH45 treatments and black columns indicate PMK4 treatments. Bars above the columns represent standard errors and asterisks above the PGPB treatments represent significant (P<0.10) differences from the non-inoculated control either at each P rate (a) or overall (b)

When the root system encounters nitrate-rich patches of soil, localized responses are

stimulated such as the up-regulation of root branching and lateral root elongation (Forde

and Lorenzo 2001; Mantelin et al. 2006). In *Arabidopsis* plants, external NO_3^- induced a gene product, ANR1, that encodes a MADS-box family of transcription factors and is a key regulator of lateral root growth (Gan et al. 2005). The AXR4 gene product that is required for the localization of AUX1, an auxin influx facilitator protein, was also induced (Dharmasiri et al. 2006), suggesting a role for auxin in this pathway (Mantelin et al. 2006). High levels of nitrate in leaves induces a systemic response including down-regulation of the uptake of NO_3^- and reduction in lateral root elongation (Mantelin et al. 2006; Zhang and Forde 1998).

Auxin derived from the shoots is crucial in the development of lateral roots in the early stages of plant development (Zhang et al. 2007). This enables a balance between carbon and nitrogen metabolism to be established via the shoot derived coordination of root development which facilitates the extraction of nutrients and water from the soil (Zhang et al. 2007). Co-cultivation with *Phyllobacterium brassicacearum* STM196 improved the plant nitrogen status and reduced the carbon/nitrogen ratio of *Arabidopsis* which was independent of the external NO_3^- concentration (Mantelin et al. 2006). Concomitantly, the reduction in lateral root number and elongation by high levels of exogenous nitrate were overcome by inoculation of these plants with the bacteria. This physiological expression of the deregulation of NO_3^- uptake was also observed at a molecular level through changes in expression of N transporter genes in inoculated plants.

Contesto et al. (2010) demonstrated that *P. brassicacearum* STM196 effected changes in root system architecture through auxin transport and signaling pathways, even though this bacterium produced very low levels of IAA. However, the ability to produce auxin by a bacterium can be dependent on the type of growth media (Belimov et al. 2015) and

auxin production by the STM196 strain was determined by the colorimetric method of Sarwar et al. (1992). A different result may have been obtained using several different media and a more sensitive method such as GC-MS or HPLC (Iqbal and Hasnain 2013; Kim et al. 2006; Liu et al. 2012).

The ability to regulate auxin homeostasis in plants independently of IAA production is a feature of the PGPB strain *Bacillus subtilis* GB03 (Zhang et al. 2007). This bacterium produces a volatile compound that is capable of inducing differential expression of plant genes involved in auxin signaling, synthesis and transport as well as genes involved in regulation of cell wall loosening enzymes while simultaneously improving the growth of *Arabidopsis* plants. Therefore, the ability of NCH45 and PMK4 to produce auxin (Chapter 2), may have had an effect on auxin signaling and transport in the plants thereby altering root system architecture and by this mechanism improving the uptake of N in the plants.

Alternatively, *Pseudomonas* sp. G62 induced genes in *Arabidopsis* typically associated with carbohydrate starvation responses but the plants were not depleted of soluble sugars (Schwachtje et al. 2011). In fact, in 5-week-old plants treated with *Pseudomonas* sp. G62, sucrose levels in roots were increased by 34%. The authors speculated that induction of the sugar starvation responses in the plant would be beneficial to the bacterium through an increase in the carbohydrates at the rhizoplane (Schwachtje et al. 2011). Similarly, Zhang et al. (2008) were able to demonstrate that *B. subtilis* GB03 modulated sugar/ABA signaling which induced elevated photosynthesis in *Arabidopsis*.

Increases in sucrose levels in the root may induce nitrate uptake as this sugar behaves as a signal for nitrate uptake in both *Arabidopsis* and wheat (Li et al. 2013; Zhou et al. 2009). Thus the PGPB used in the current study may have increased photosynthate

translocation to the roots in the plants grown in the Wongan B soil due to effects on carbon metabolism and thereby indirectly facilitated an increase in nitrogen uptake in the plants through sucrose signaling.

Plants also accumulate sugars and starch under P starvation which increases loading of sucrose in the phloem and this functions to relocate carbon resources to the roots and may also initiate sugar signaling cascades that alter the expression of P starvation genes (Hammond and White 2011). Therefore, it is possible that increased uptake of P by plants inoculated with PSM in other studies is actually through an increase in production of photosynthates, facilitating increased root density and thereby enhanced uptake of P. For instance, Panhwar et al. (2011) found a strong correlation between chlorophyll content, (which significantly increased with PSM inoculation on aerobic rice), and root lengths. Panhwar et al. (2011) used only two rates of rock phosphorus as a P source and a full P response curve would help to clarify if improved uptake of P by the plants is through P solubilisation by these bacteria or through another mechanism such as increased photosynthesis resulting in increased transport of photosynthates to the root system.

Root hairs are the major source of P acquisition by roots and can account for up to 90% of total P uptake (Föhse et al. 1991). Many PGPB have been shown to drive developmental plasticity of roots by promoting lateral root and root hair formation (Zamioudis et al. 2013) and these alterations to root system architecture may facilitate an increased uptake in P. Unfortunately, in the current study the high level of clay in some of the soils and the number of plants in the pots made collection of root data impractical. If the two isolates NCH45 and PMK4 did alter the wheat root systems, there was no benefit in terms of phosphorus uptake in these plants and the wheat plants

appear to have accessed only a small fraction of the plant available P in the soils. For instance, the base level of Colwell P in the Wongan B soil totaled 108 mg in the pots, yet at the nil applied P level, only an average of 17 mg (0.30%) was taken up by the plants. Furthermore, the Yalanbee soil is P limited, as indicated by the sharp increase in shoot biomass with increasing P application, and there was no response to inoculation with the PGPB in this soil.

However, the ability of the PGPB to survive and colonise the roots in all these soils has not been established. While the promotion of growth by the PGPB in the Wongan B soil suggests that the isolates maintained a close association with the wheat plants, PMK4 was not detected in the rhizosphere soil of these plant using strain specific primers, possibly due to desiccation of the bacteria over time. The soil was stored at room temperature for several years before analysis was performed (Chapter 7). Conversely, PMK4 was detected in the root crush of plants grown in Wongan A soil when plated on to agar (Chapter 7). The survival of the PGPB in the Yalanbee soil wheat rhizosphere has not been explored.

Plant available phosphorus in the Wongan A soil was high, even before the application of P and the phosphorus retention index is low (Table 5.1), therefore the solubilisation of P by the PGPB would probably be unnecessary in this soil type. There was no response to inoculation by PMK4 in this soil and the reason for this is not clear. However, the most striking difference between Wongan A and Yalanbee soils compared with the Wongan B soil is the background level of potassium (K) in these soils. While the Wongan B soil contains 305 mg kg^{-1} K, the Yalanbee and Wongan A soils contain 65 and 82 mg kg^{-1}, respectively. Even though K was applied in the nutrient solution (765 mg pot^{-1}) this may have been inadequate for the wheat plants' needs in the latter

two soils. The optimal K requirement for plant growth is 20-50 g kg^{-1} (Ma et al. 2013) and in the Yalanbee soil, at the highest rate of applied P, the average dry shoot biomass is 16.5 g pot^{-1}. This means that the optimal K in the leaves alone could be up to 800 mg per pot and therefore it is possible that K is limiting in this soil.

There are two potential impacts on the plant-microbe interaction of a K deficiency in the soils. Firstly, electroneutrality is maintained in plants during the process of ion uptake by a number of mechanisms which is dependent on plant species. This can include H$^+$ or OH$^-$ (HCO$_3^-$) excretion from the roots or the parallel uptake of cations like K$^+$, Ca^{2+}, Mg^{2+} and Na$^+$ (Kirkby and Knight 1977). Wheat plants, in particular, absorb and accumulate more nitrate (and more K) when treated with K, than Na or Ca (Blevins et al. 1978). When originally cleared for agriculture, most of the sandy soils of Western Australia contained adequate K for agriculture, but have now become deficient for wheat production (Brennan et al. 2004; Wong et al. 2000) and there is an increasing requirement for K when N is applied to wheat in Western Australian soils to achieve 90% of yields (Brennan and Bolland 2009). In terms of the PGPB-wheat interaction in the current study, this could mean that a potential increase in NO$_3^-$ uptake by wheat plants facilitated by NCH45 or PMK4 is obstructed by an insufficient supply of K to the roots in the Wongan A and Yalanbee soils.

Alternatively, photosynthates accumulate in the shoots of K deficient plants (Gerardeaux et al. 2009) and roots of these plants typically have lower sucrose and starch levels than K-replete plants (Cakmak et al. 1994a; b) (with the exception of *Arabidopsis* in Armengaud et al. (2009)), possibly due to the absence of K or an active K channel in the phloem restricting the loading of sucrose to the phloem (Hammond and White 2011; Hermans et al. 2006). This in turn may reduce the delivery of sucrose to

the roots and thereby inhibit sucrose-dependent alterations to the root system (Hermans et al. 2006). Therefore, if inoculation with PMK4 and NCH45 were modulating photosynthesis and sugar signaling in the wheat plants as described earlier, but K is in limiting supply, then the surplus sucrose may not have been able to be translocated to the roots. In this event, the sucrose induced nitrate uptake may have been reduced or the decreased sucrose supply to the roots may have been insufficient to facilitate an enhancement in root growth by the PGPB.

The inability of *Burkholderia* sp. NCH45 to improve plant growth in the Wongan A soil may be due to the alkalinity of this soil (Table 5.1). Although *Burkholderia* species were able to grow in plate assays up to a pH of 11.0 (Estrada-de los Santos et al. 2011), in soils there was a linear decrease in abundance from 0.15-0.23% at pH 4.5 to 0.008-0.01% at pH 7.5 (Stopnisek et al. 2014). The predominant species that Estrada-de los Santos et al. (2011) isolated from Mexican soils was from the genus *Cupriavidus* with only a few species of *Burkholderia* detected and the authors suggested that alkalinity limited the presence of *Burkholderia* spp.

The present study confirms that phosphorus solubilisation in plate assays is not a good indicator of a bacterium being a true PSM and that soil type is an important consideration for choosing a PGPB for a host plant. This study indicates that the mechanism of action in the bacteria may include bacterial synthesis of plant hormones that may alter carbohydrate signaling or root architecture in plants. However, the effects of the bacteria on the wheat root system could not be determined in these experiments. Further investigations of the potential effect of auxin production by the bacteria on wheat roots are described in Chapter 6.

A confounding issue that has not been investigated here (or by many other authors) is the possibility that phosphate-solubilising bacteria may lose this ability after serial subculturing (Sperber 1958). This is in contrast to fungi which retain their phosphorus solubilising activity even after serial subculturing for a number of years (Kucey 1983). In the future, immediately prior to inoculation of host plants in field or glasshouse studies, PSM must be reassessed for their ability to solubilise P. Furthermore, it is necessary to have techniques to detect the PSM in the rhizosphere and confirm they survive and colonise in vivo (see Chapter 7).

Chapter 6:
Do bacterially produced plant growth hormones regulate wheat root system architecture?
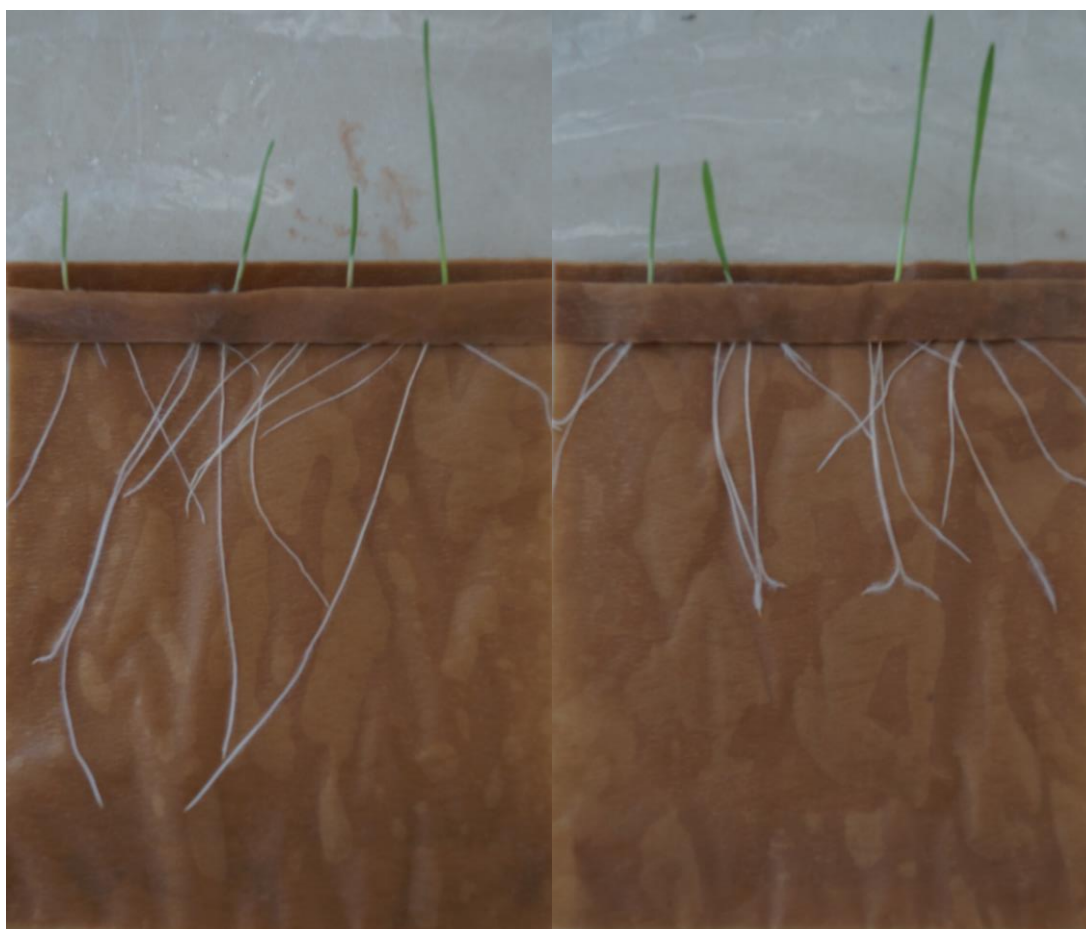
Wheat growing in growth pouches

6.1 Introduction

One of the fundamental mechanisms by which beneficial bacteria and fungi improve the plant host's nutrition is by altering the root system architecture and therefore the plant's capacity to mine the soil for essential minerals and nutrients (Richardson et al. 2009; Zamioudis et al. 2013). In the case of plant beneficial fungi such as mycorrhiza, the surface area of the plant's root system is extended by colonization of the root cortex by the fungi and development of external hyphae into the surrounding soil (Richardson et al. 2009).

Beneficial bacteria effect increases in the plant's root system by an alteration on one or more of the three core components of the root system architecture: the main root(s); the lateral roots; and the root hairs (Zamioudis et al. 2013). These modifications are typically attributed to phytohormone production, especially auxins, by these bacteria (Patten and Glick 2002; Remans et al. 2008). For instance, *Azospirillum brasilense* mutants with indole-3-acetic acid (IAA) production reduced by 70% have decreased ability to promote wheat root system development compared with the wild type strain, particularly in lateral root and root hair development (Barbieri and Galli 1993). The decrease in IAA production was later attributed to interruption of the indole-3-pyruvate decarboxylase gene (*ipdC*) (Costacurta et al. 1994), that codes for a key enzyme of the indole-3-pyruvic acid pathway of IAA biosynthesis in *Azospirillum brasilense* (Malhotra and Srivastava 2008).

At low concentrations, typically 10^{-9} to 10^{-12} M, IAA enhances primary root growth (Elliott 1982; Fallik et al. 1989; Meuwly and Pilet 1991; Patten and Glick 2002; Pilet and Saugy 1987). However, higher concentrations can decrease primary root lengths

but stimulate lateral and adventitious roots and root hair development (Alarcón et al. 2014; Eliasson et al. 1989; Patten and Glick 2002; Vacheron et al. 2013).

The auxin IBA is very similar to IAA and carries a four-carbon side chain compared with a two-carbon side chain carried by IAA (Strader and Bartel 2011) (Figure 6.1). Up to 30% of auxin in *Arabidopsis* may be IBA (Ludwig-Müller et al. 1993; Tognetti et al. 2012) and although some of the auxin activity of IBA results from its conversion to IAA by peroxisomal β-oxidation (Woodward and Bartel 2005), IBA can also act as an auxin without conversion to IAA (Campanella et al. 2004; Chhun et al. 2003; Ludwig-Müller et al. 1995; Tognetti et al. 2010). In fact, IAA can be converted to IBA by IBA synthetase and the production of IBA is enhanced under several biotic and abiotic stresses such as drought and osmotic stress (Ludwig-Müller 2000; Ludwig-Müller et al. 1995). IBA synthesis and levels of free IBA are increased in arbuscular mycorrhizal (AM) inoculated maize roots and Ludwig-Müller (2000) suggests that IBA and not IAA is important in early AM colonization of maize roots.

In maize roots inoculated with *A. brasilense* ATCC 29729 at 10^7 CFU/mL and exposed for 24 h to [2-^{14}C]IAA, the auxin compounds IAA and IBA were only found in the inoculated plants compared with uninoculated controls. Subsequently, Martínez-Morales et al. (2003) detected the production of IBA in *A. brasilense* UAP 154 culture medium. The compound was purified and applied
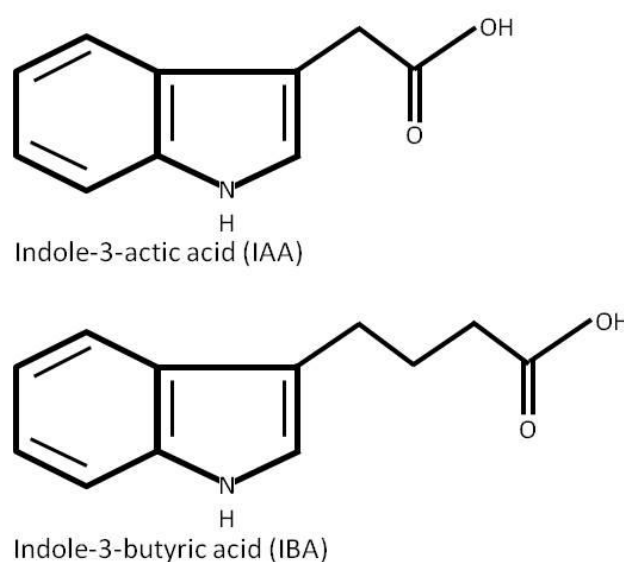

Figure 6.1: The structure of IAA and IBA.

to maize seedlings and elicited the same alterations to root morphology as the IBA standard, promoting lateral root formation and reducing root elongation. However, the dry root biomass was significantly greater in the plants treated with IBA derived from *A. brasilense* UAP 154 than with the standard IBA.

Auxin flow throughout the plant can occur via two main systems: long distance via the vascular system; and in a (mostly) polar cell-to-cell mechanism through multiple auxin transport proteins (Zažímalová et al. 2010). Auxin is a weak acid and in the acidic (~pH 5.5) extracellular apoplastic space around 15% of the IAA exists in the proton-associated IAA (IAAH) form which can enter the cells by lipophilic diffusion (passive movement) (Kerr and Bennett 2007; Zažímalová et al. 2010). Auxin can also enter the cell via influx carriers from the AUX1/LAX family of plasma membrane permeases (Bennett et al. 1996; Swarup et al. 2008). Once inside the cell, the pH of the cytoplasm is approximately 7 and the uncharged IAAH will dissociate almost entirely to the anionic, dissociated form (IAA$^-$) (Kerr and Bennett 2007; Zažímalová et al. 2010). This effectively traps the IAA$^-$ within the cell as anionic auxin cannot diffuse across the plasma membrane (Kerr and Bennett 2007; Zažímalová et al. 2010). The IAA$^-$ can be exported from the cell by efflux proteins from one of the two protein families of auxin exporters; the PIN (PIN-FORMED) family or the ATP-binding cassette (ABC) superfamily of transporters (Zažímalová et al. 2010).

One method widely used to establish the role of auxins in plant development is the application of compounds known as auxin transport inhibitors or anti-auxins (Fujita and Syono 1996; Geldner et al. 2001; Katekar and Geissler 1980; Meng et al. 2013). Auxin transport inhibitors appear to interfere with membrane protein trafficking and in particular, the polar auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) affects

PIN localization at the plasma membrane (Geldner et al. 2001). In the presence of these inhibitors, the transport of auxin is disrupted which results in a number of defects in plant development including root morphology and geotropic responses (Geldner et al. 2001; Katekar and Geissler 1980; Meng et al. 2013; Niemi et al. 2002). Meng et al. (2013), using TIBA and other anti-auxins, were able to demonstrate the direct role of root-derived IAA in the formation of cluster roots under conditions of P deficiency. However, the transport inhibitors naphthylphthalamic acid (NPA) and TIBA block the polar transport of [^{3}H]IAA but not [3H]IBA (Rashotte et al. 2003) making these inhibitors are useful in the discrimination of the effects of IAA and IBA on plant development.

The aim of this study was to determine if auxin production by *Burkholderia caledonica* NCH45 and *Pseudomonas granadensis* PMK4 has an affect on wheat seedlings root system development. Therefore the bacteria were co-cultivated on wheat with and without the auxin transport inhibitor TIBA in growth pouch assays. These plants were compared with those treated with the auxins IAA and IBA to determine if alterations in plant development by either of these auxins were mimicked by exposure to the bacteria.

6.2   Materials and Methods

6.2.1   Growth Pouch Assays

An initial experiment ascertained the appropriate volume of solution, using water in this instance, required to support the growth of wheat in growth pouches for one week's growth. It was concluded that an initial volume of 10 mL was required followed by a further 5 mL 3-4 days later.

6.2.1.1 The effect of plant growth-promoting bacteria (PGPB), auxins and TIBA on wheat seedlings

Stock solutions

IAA and IBA (1 mg mL^{-1}) (Phytotechnology Laboratories-Austratec) were diluted in sterile distilled water to obtain stock concentrations of 50 µM. These stock concentrations were then diluted further to obtain working concentrations of 0.005, 0.05, 0.5, 5.0 and 50 µM IAA and IBA. TIBA (2,3,5-triiodobenzoic acid) (Sigma) (0.049 g) was dissolved in 5 mL 95% ethanol then sterile distilled water was added slowly while stirring, with a few drops of 1M NaOH added if precipitation occurred, to reach a final volume of 100 mL. This 1 mM stock solution was then diluted with sterile distilled water to obtain concentrations of 5, 10, 15 and 20 µM TIBA. A combined IBA/TIBA or IAA/TIBA stock solution was prepared from the 50 uM stock solution of IBA or IAA and 1 mM TIBA to obtain concentrations of 10 µM TIBA in 0, 0.005, 0.05, 0.5, 5.0 and 50 µM IBA or IAA.

Bacterial culture preparation

NCH45 and PMK4 were grown in TY broth for 2 days at 28°C at 200 rpm with or without 10 µM TIBA. The cells grown without TIBA were harvested by centrifugation at 3214 *g* for 5 min and resuspended in sterile water. The bacterial cultures were then diluted to an OD$_{600}$ of 0.15 (approximately 10^7 CFU mL^{-1}). Serial dilutions of the final suspensions and the cells grown in the presence of TIBA were plated out on Miles and Misra plates (TY agar) to confirm bacterial numbers and to determine if TIBA (10 µM) had an effect on bacterial growth.

Growth Pouch Assays

Growth pouches (mega-international.com) were placed into brown paper bags and autoclaved at 121°C for 20 min. Wheat seeds (*Triticum aestivum* cv. Wyalcatchem)

were surface sterilized by immersion in 70% ethanol for 1 min then 4% bleach, containing 2 drops of Tween 20, for 3 min, followed by six washes in sterile distilled water. To prevent any potential effects on germination by the auxins and TIBA, the wheat seeds were pre-germinated for two days on sterile paper towel wet with sterile water in sterilized plastic boxes (sprayed with 70% ethanol then placed under UV for 20 min). Four wheat seedlings with 1-3 roots no longer than 5 mm were placed into each of the growth pouches using sterile forceps and the respective solution applied promptly. The pouches were placed in racks in surface sterilized hydroponic boxes (sterilized as for the germination boxes) in the glasshouse under constant temperature (23°C ±2°C) under natural illumination or in a controlled temperature room (25°C) (12h/12h light/dark cycles) with 50 mL sterile distilled water in the bottom of the box to maintain humidity in the boxes (Figure 6.2). A further 5 mL of the respective solution was applied after 3-4 days.

All seedlings were harvested from all treatments when the roots of any one of the treatments had reached the bottom of the pouches (this was either 7 or 8 days). The total root length and length of the first true leaf for each plant were measured by hand. The number of seminal (seed) roots and lateral roots were also counted.

The experiments were repeated at least twice except for the TIBA only experiment and a figure showing one representative experiment is presented in the results section.

6.2.2 Statistical analysis

Univariate analysis of variance (ANOVA) of the means was performed using IBM SPSS 21 and where significant ($P<0.05$), means were compared using Fischer's Least Significant Difference (LSD) test.
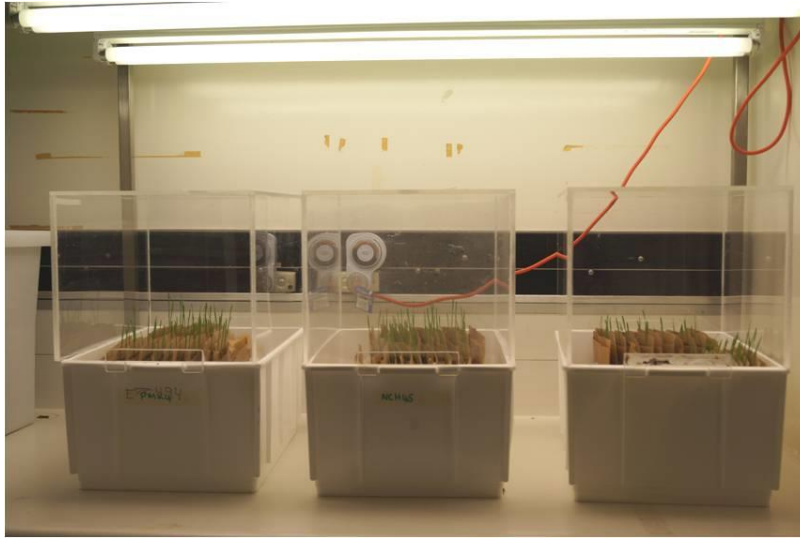
Figure 6.2: Hydroponic boxes containing growth pouches, with wheat seedlings grown under artificial lighting (12/12 hours) at 25°C

6.3 Results

6.3.1 The effect of IAA on wheat seedlings

Concentrations of IAA 5.0 µM and above significantly (P<0.05) reduced the mean root lengths of the wheat seedlings (Figure 6.3A & 6.5). Lateral roots were mostly absent until a concentration of 50.0 µM and at this level the number of seminal roots significantly (P<0.05) increased in the first experiment from a mean of approximately 3.0 to 5.0 (Table 6.1) but was not significant (P>0.05) in the second experiment (data not shown). Leaf lengths were significantly (P=0.001) increased compared with the controls only at 5.0 µM IAA (Figure 6.3B).

6.3.2 The effect of IBA on wheat seedlings

Exogenously applied IBA had no effect on total wheat root lengths until the concentration reached 50 µM (Figure 6.4A & 6.5) at which concentration there was a significant (P=0.042) reduction in lengths compared to the control. Leaf lengths were significantly (P<0.05) shorter than the control at 0.5 µM and 50 µM applied IBA

(Figure 6.4B). The number of seminal roots was not affected by application of IBA, however, at levels of 0.5 µM IBA and above, the number of lateral roots increased significantly (P<0.05) from the controls and lower levels of applied IBA (Table 6.2).

Figure 6.3: Mean total root lengths (A) and mean leaf lengths (B) of wheat seedlings in growth pouches with applied IAA harvested after 7 days. Results depicted are from the first experiment and values with different letters are significantly (P<0.05) different. Bars above the columns represent standard error

IAA (μM)	No. lateral roots	No. seminal roots
0	0.3 (0.3)[b]	3.3 (0.3)[c]
0.005	0.3 (0.2)[b]	3.3 (0.3)[c]
0.05	0.2 (0.2)[b]	3.4 (0.3)[c]
0.5	0.2 (0.2)[b]	3.6 (0.2)[bc]
5	0.0 (0.0)[b]	4.4 (0.3)[ab]
50	1.4 (0.5)[a]	5.0 (0.4)[a]

Table 6.1: The number of lateral roots and seminal roots of wheat seedlings in growth pouches with applied IAA harvested after 7 days. Results are from the first experiment and are depicted as means with standard errors in brackets. Data in columns with different letters are significantly ($P<0.05$) different
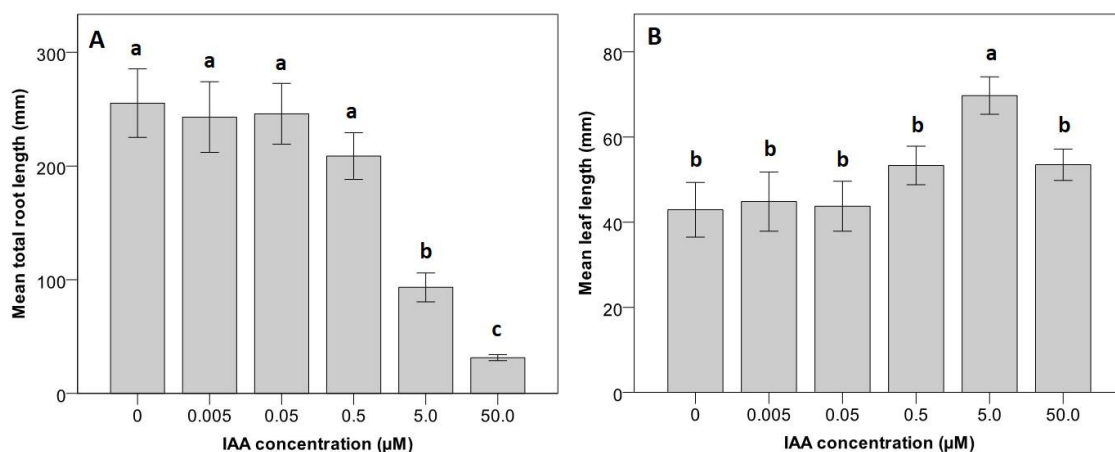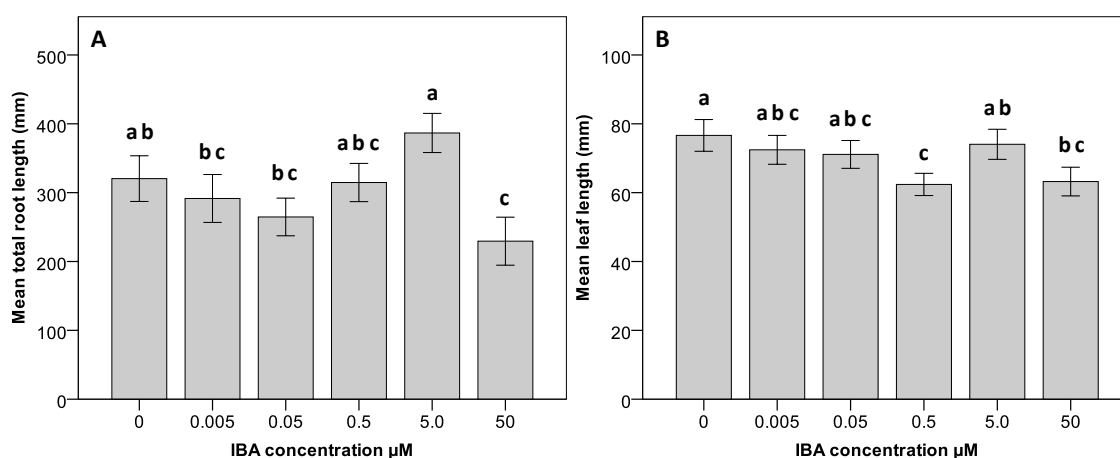

Figure 6.4: Mean total root lengths (A) and mean leaf lengths (B) of wheat seedlings in growth pouches with applied IBA harvested after 7 days. Results depicted are from the second experiment and values with different letters are significantly ($P<0.05$) different. Bars above the columns represent standard error

IBA (μM)	No. lateral roots	No. seminal roots
0	0.6 (0.2)[c]	3.8 (0.3)[a]
0.005	1.4 (0.5)[bc]	4.2 (0.3)[a]
0.05	1.3 (0.6)[bc]	3.8 (0.2)[a]
0.5	5.4 (2.3)[a]	4.1 (0.3)[a]
5	3.6 (1.2)[abc]	3.8 (0.2)[a]
50	4.6 (1.5)[ab]	4.2 (0.2)[a]

Table 6.2: The number of lateral roots and seminal roots of wheat seedlings in growth pouches with applied IBA harvested after 7 days. Results are from the second experiment and are depicted as means with standard errors in brackets. Data in columns with different letters are significantly ($P<0.05$) different

6.3.3   The effect of TIBA on wheat seedlings

At a concentration of 5.0 µM, TIBA significantly ($P<0.05$) increased root lengths compared with the other TIBA treatments (Figure 6.6A) but not from the control. Mean root lengths given the TIBA treatments 10, 15 µM did not significantly ($P>0.05$) differ from each other or the control and therefore, the 10 µM TIBA treatment was used in subsequent assays. The application of 5.0 µM TIBA significantly ($P<0.05$) increased leaf lengths compared with the control (Figure 6.6B), however, application of TIBA had no effect on number of seminal roots or lateral roots (Table 6.3).
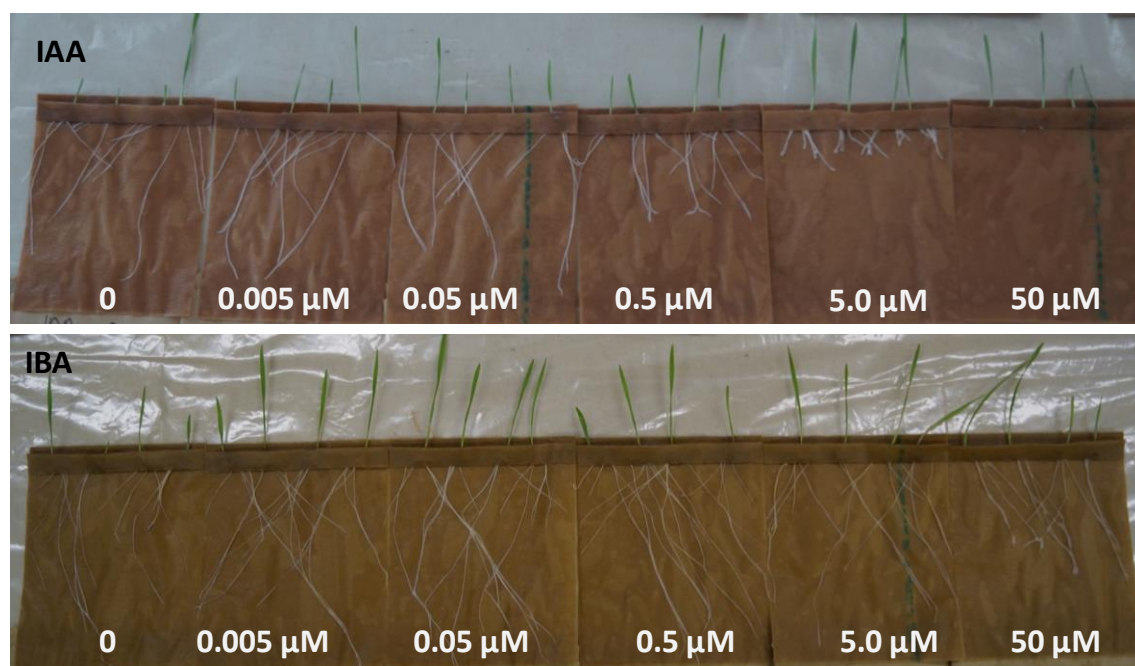

Figure 6.5:   Comparisons of the root systems of wheat seedlings inoculated with a range of concentrations of IAA (top) and IBA (bottom)
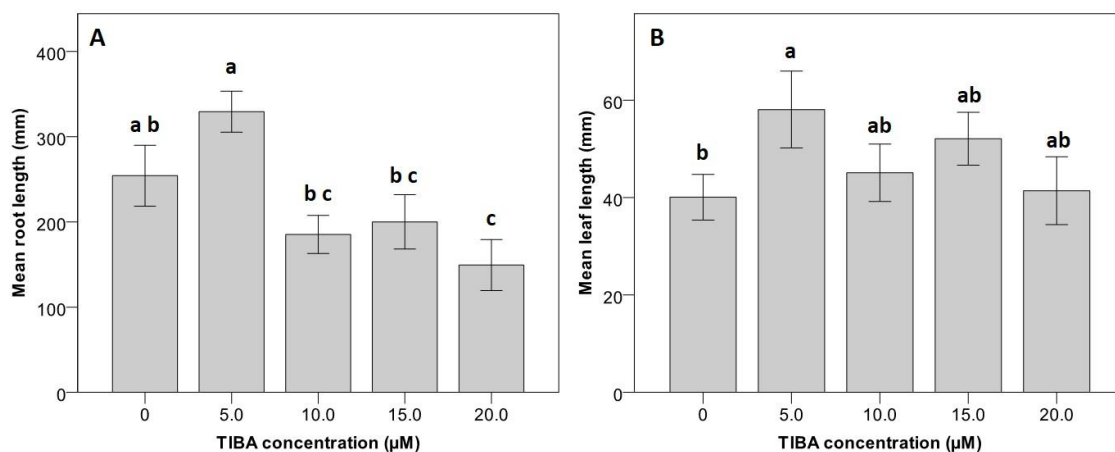
Figure 6.6: Mean root lengths (A) and mean leaf lengths (B) of wheat seedlings in growth pouches with TIBA harvested after 7 days. Values with different letters are significantly (P<0.05) different. Bars above the columns represent standard error

TIBA (µM)	No. lateral roots	No. seminal roots
0	0.2 (0.2)[a]	3.5 (0.4)[a]
5	0.9 (0.3)[a]	4.5 (0.2)[a]
10	1.0 (0.5)[a]	3.9 (0.3)[a]
15	0.7 (0.3)[a]	4.2 (0.4)[a]
20	0.0 (0.0)[a]	3.4 (0.5)[a]

Table 6.3: The number of lateral roots and seminal roots of wheat seedlings in growth pouches with applied TIBA harvested after 7 days. Results are depicted as means with standard errors in brackets. Data in columns with different letters are significantly (P<0.05) different

6.3.4   The effect of TIBA (10 µM) and various concentrations of IAA or IBA on wheat seedlings

The co-application of TIBA with IAA significantly (P<0.05) reduced root lengths at all

IAA concentrations except for 0.05 and 0.5 µM IAA which were comparable to the

controls (Figure 6.7A). Leaf lengths were not affected either by the application of

TIBA or co-application of TIBA and IAA (Figure 6.7B). Low levels of IAA (0.05 µM)

co-applied with TIBA significantly (P<0.05) increased lateral roots although seminal

roots were not affected by any of the treatments (Table 6.4).

The co-application of 10 µM TIBA and IBA had no effect on root lengths until the

concentration of IBA reached 50 µM and at this concentration wheat seedling root

lengths were significantly (P=0.000) reduced (Figure 6.8). The application of TIBA in the presence of the range of concentrations of IBA did not affect leaf lengths or the number of lateral roots. However the number of seminal roots was significantly (P=0.000) increased at IBA 50 µM and TIBA 10 µM concentrations (Table 6.5).

Figure 6.7: Mean root lengths (A) and mean leaf lengths (B) of wheat seedlings in growth pouches applied with 10 µM TIBA and various rates of IAA, harvested after 8 days.
Results depicted are from the second experiment and values with different letters are significantly (P<0.05) different. Bars above the columns represent standard error

Concentration of IAA (µM) applied with TIBA 10 µM	No. lateral roots	No. seminal roots
Water control	0.6 (0.2)[b]	3.8 (0.3)[a]
TIBA 10 µM	1.2 (0.5)[b]	3.8 (0.3)[a]
0.005	2.2 (0.9)[ab]	4.2 (0.3)[a]
0.05	3.2 (1.1)[a]	4.5 (0.2)[a]
0.5	0.8 (0.5)[b]	4.0 (0.3)[a]
5.0	1.5 (0.8)[ab]	4.7 (0.3)[a]
50.0	0.3 (0.6)[b]	4.6 (0.4)[a]

Table 6.4: The number of lateral roots and seminal roots of wheat seedlings in growth pouches with applied TIBA and various rates of IAA harvested after 8 days. Results are depicted as means with standard errors in brackets. Data in columns with different letters are significantly (P<0.05) different
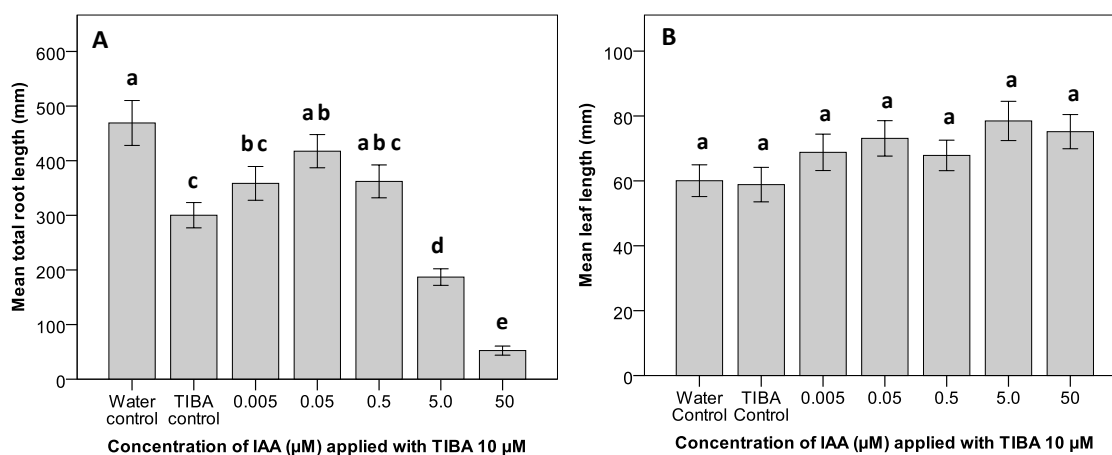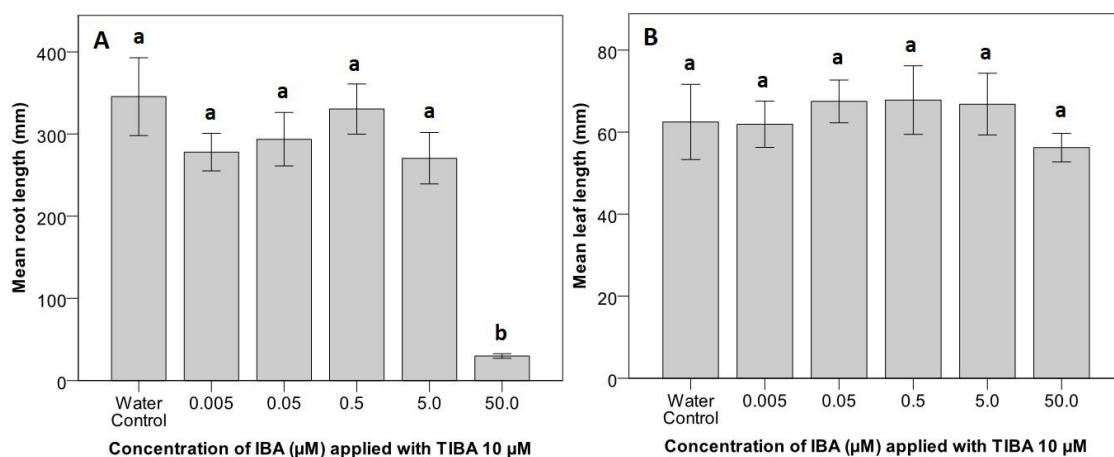
Figure 6.8: Mean root lengths (A) and mean leaf lengths (B) of wheat seedlings in growth pouches applied with 10 μM TIBA and various rates of IBA, harvested after 8 days. Results depicted are from the second experiment and values with different letters are significantly (P<0.05) different. Bars above the columns represent standard error

Concentration of IBA (μM) applied with TIBA 10 μM	No. lateral roots	No. seminal roots
Water control	1.8 (1.2)[a]	4.1(0.3)[b]
0.005	0.7 (0.3)[a]	4.3 (0.3)[b]
0.05	0.5 (0.3)[a]	4.2 (0.4)[b]
0.5	1.3 (0.7)[a]	4.3 (0.3)[b]
5.0	11 (9.5)[a]	4.2 (0.2)[b]
50.0	2.0 (0.7)[a]	6.2 (0.4)[a]

Table 6.5: The number of lateral roots and seminal roots of wheat seedlings in growth pouches with applied TIBA and various rates of IBA harvested after 8 days. Results are depicted as means with standard errors in brackets. Data in columns with different letters are significantly (P<0.05) different

6.3.5 The effect of NCH45 and PMK4 with and without TIBA (10 μM) on wheat seedlings

The TIBA (10 μM) treatments used in this experiment, did not have an effect on cell numbers of either PGPB as determined in the Miles and Misra counts (data not shown). In both experiments, NCH45 on its own significantly increased (P=0.001), while 10 uM TIBA reduced (P=0.005) root lengths over the water control (Figure 6.9A). The roots of wheat seedlings co-cultivated with NCH45 and TIBA (10 μM) were similar to the water controls but significantly (P=0.003) longer than the TIBA control. The root lengths for PMK4 treatments with and without TIBA were not significantly (P>0.05)

different from the water control. However the PMK4 treatment without TIBA was significantly (P=0.000) greater than the TIBA control. Both NCH45 and PMK4 significantly (P<0.05) increased the leaf lengths both in the presence and absence of TIBA compared with the water and TIBA controls (Figure 6.9B). The number of lateral roots was significantly (P<0.05) increased in the presence of both NCH45 and PMK4 when TIBA was not applied in the first experiment, but there are no significant (P>0.05) differences in lateral roots in the second experiment (Table 6.6). There was no significant (P>0.05) difference in the number of roots in either experiment.
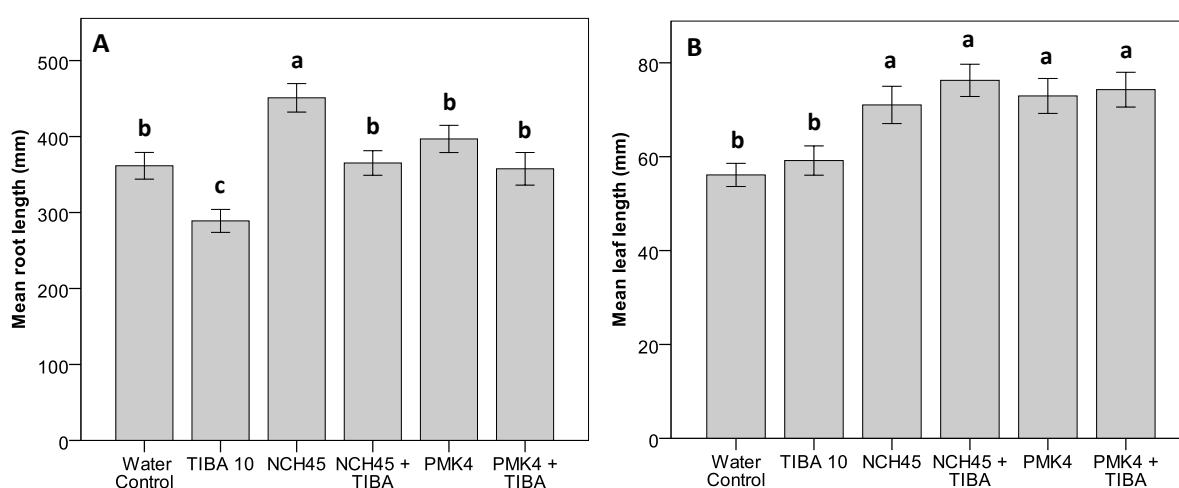

Figure 6.9: Mean root lengths (A) and mean leaf lengths (B) of wheat seedlings in growth pouches inoculated with the PGPBs NCH45 and PMK4 in the presence and absence of TIBA (10 µM) harvested after 7 days. The results depicted are from the second experiment and values with different letters are significantly (P<0.05) different. Bars above the columns represent standard error

Table 6.6: The number of lateral roots and seminal roots of wheat seedlings in growth pouches with the plant growth promoting isolates NCH45 and PMK4 applied with and without TIBA harvested after 7 days. Results are depicted as means with standard errors in brackets. Data in columns with different letters are significantly (P<0.05) different

First Experiment			Second Experiment		
Treatment	No. lateral roots	No. seminal roots	Treatment	No. lateral roots	No. seminal roots
Control	4.9 (1.5)[c]	4.6 (0.2)[a]	Control	0.6 (0.4)[a]	4.7 (0.1)[a]
TIBA (10 µM)	3.6 (1.1)[d]	4.8 (0.1)[a]	TIBA (10µM)	1.5 (0.9)[a]	4.7(0.1)[a]
NCH45	12.9 (2.0)[ab]	4.7 (0.2)[a]	NCH45	2.0 (0.7)[a]	4.7 (0.1)[a]
NCH45 + TIBA	7.7 (2.0)[bcd]	4.7 (0.2)[a]	NCH45 + TIBA	1.5 (0.3)[a]	4.9 (0.1)[a]
PMK4	13 (2.0)[a]	4.8 (0.2)[a]	PMK4	3.2 (0.8)[a]	4.7 (0.1)[a]
PMK4 + TIBA	9.0 (1.7)[abc]	4.6 (0.3)[a]	PMK4 + TIBA	1.9 (0.6)[a]	4.9 (0.1)[a]

6.4   Discussion

The inhibition of root lengths of wheat seedlings co-cultivated with IAA was observed at 5.0 µM (63% reduction) and increased to 88% reduction at 50 µM IAA. In contrast to this, root lengths of wheat seedlings co-cultivated with IBA were not affected until the concentration reached 50 µM and the root lengths decreased (compared with the controls) only by 28% at this concentration. These results are similar to those obtained by Zolman et al. (2000) in which inhibition of root elongation of *Arabidopsis* required a 100 fold higher concentration of IBA compared with IAA.

In studies with a lateral rootless mutant (*Lrt1*) of rice (*Oryza sativa*), only IBA but not IAA was able to restore lateral root formation (Chhun et al. 2003). Furthermore, IBA has been shown to promote lateral root formation independently of IAA in *Arabidopsis* (Strader et al. 2011; Zolman et al. 2000) and in the present study, the increase in the number of lateral roots of the wheat seedlings was observed at lower levels of applied IBA (0.5 µM) compared with IAA (50 µM). It has been proposed that the conversion of IBA to IAA in the peroxisomes promotes the coordinated release of nitric oxide as well

as IAA, and it is the production of these two metabolites that form the basis for the promotion of lateral root formation by IBA (Schlicht et al. 2013).

The increase in root lengths at low (5.0 µM) concentrations of TIBA are similar to those obtained by Eliasson (1960) who demonstrated that some antiauxins, which included TIBA, stimulate root growth at low concentrations. In fact, TIBA in conjunction with $AgNO_3$ is able to enhance the development of wheat plantlets post-regeneration from callus on auxin/cytokinin media (Yu et al. 2012). This is attributed to the inhibition of both auxin transport (TIBA) and ethylene ($AgNO_3$) that may retard further embryo differentiation and development.

When TIBA was co-applied with IAA, a reduction in the root lengths is observed at low levels of applied IAA however, at 0.05-0.5 µM IAA, the root lengths appear to overcome this inhibition by TIBA. This may be due to the auxin being transported via diffusion into the cell that does not require functioning PIN proteins or perhaps by the ABC transport proteins that are not affected by TIBA application (Cho et al. 2012). At higher levels of IAA co-applied with TIBA, the reductions in root lengths resembled the application of IAA alone at these rates. This demonstrates that at low levels of exogenous auxin, TIBA potentially has an effect on auxin transport, altering plant root architecture. However, higher levels of exogenously applied IAA may be able to overcome this inhibitory effect on root lengths. Co-application of TIBA and IBA had no effect on wheat seedling root lengths until a concentration of 50 µM IBA. This confirms that TIBA does not have an effect on exogenously applied IBA as has been demonstrated by other authors (Rashotte et al. 2003).

The root lengths for seedlings co-cultivated with 10 µM TIBA were not significantly different from the controls in the TIBA assays (Figure 6.6). However, in subsequent

assays, the root lengths were significantly reduced in treatments with 10 µM TIBA (Figure 6.7 and 6.9) and the difference is potentially due to the reduced number of replicates (3) in the TIBA assay. Nonetheless, the same trend was observed as for the subsequent assays, and an increase in replicates may provide verification.

The PGPB isolate NCH45 consistently increased root lengths compared with the controls. However, the root lengths were reduced in the presence of NCH45 co-cultivated on wheat seedlings with TIBA suggesting increases in root lengths upon inoculation with this bacterium are at least partially mediated by IAA. Nevertheless, the root lengths in these treatments were reduced only to lengths comparable to the water controls. The stability of the wheat root architecture when inoculated with this bacterium may be due to a reduction in ethylene synthesis in the roots. NCH45 produces ACC deaminase *in vitro* (Chapter 2), and production of this enzyme by bacteria is frequently proposed to be the mechanism of action of plant growth promotion (Grichko and Glick 2001; Hao et al. 2007; Li et al. 2000; Ma et al. 2003a; Nascimento et al. 2012b). The enzyme cleaves the ethylene precursor ACC into α-ketobutyrate and ammonia preventing the build-up of ethylene and its inhibitory effects on plant growth (Cassán et al. 2014). Ethylene production in roots is increased under phosphorus deficiency and root morphology modifications in low P conditions, such as decreased lateral root density, is reversed by the ethylene inhibitor AVG (Borch et al. 1999). This suggests an involvement for ethylene in phosphorus mediated alterations in root morphology (Borch et al. 1999) which may be partly alleviated by ACC deaminase producing bacteria. Similarly, free-IAA concentrations are higher in P-deficient plants and alterations in root morphology are suppressed by auxin transport inhibitors (Meng et al. 2013). Furthermore, the effects of IAA and ethylene on root inhibition have been

demonstrated to be by separate mechanisms that are independent of each other (Eliasson et al. 1989).

NCH45 also produces IAA *in vitro* (Chapter 2) and when plants are inoculated with bacteria that produce both auxins and ACC deaminase, ethylene levels in plants are not elevated to the same extent as when co-cultivated with bacteria that only produce auxin (Glick 2014). Knockout mutants in either or both auxin and ACC deaminase production will clarify if these traits are the mechanism(s) of plant growth promotion by this bacterium.

The isolate PMK4 did not increase root lengths compared with the water control in these experiments. This differs with the results from previous experiments (Chapter 2) where PMK4 was bacterized on the wheat seed prior to being placed in the growth pouch to germinate. In these experiments, significant increases in wheat seedling root lengths were observed on plants co-cultivated with PMK4. This is comparable to results obtained by Parra-Cota et al. (2014) in which soaking seeds of the grain amaranth (*Amaranthus cruentus* and *A. hypochondriacus*) with growth promoting *Burkholderia* spp. achieved better results than when the seedlings were drenched with the bacteria.

When co-applied with TIBA, no reduction in root lengths was observed for PMK4 treatments. This suggests that the metabolite produced by PMK4 that is responsible for the previously observed increases in root lengths is not IAA and could perhaps be another type of auxin, such as IBA or phenylacetic acid which is not transported by a plasma membrane carrier (Johnson and Morris 1987) and is probably insensitive to TIBA. Alternatively, the IAA produced by this bacterium may be in amounts in the

rhizosphere sufficient to overcome the inhibitory effects of applied TIBA as observed at the higher levels of exogenous auxin application in the present study.

Co-cultivation with *P. fluorescens* WCS417 decreased *Arabidopsis* primary root lengths but increased the number and length of lateral roots and root hairs (Zamiodis et al. 2013). The authors found that the number of *Arabidopsis* root meristematic cells was increased upon co-cultivation with WCS417, but the length of root epidermal cells in the elongation and differentiation zones was significantly reduced compared with mock-treated plants. In a series of tests using genetic and pharmacological approaches, Zamioudis et al. (2013) were able to determine, that although auxin production in culture medium is not detected for WCS417, auxin signaling and transport is fundamental in WCS417 co-cultivated plants and that these pathways are distinct from ethylene and jasmonic acid signaling pathways. Some of the responses are due to the production of volatile organic compounds, lateral root formation in particular, leading the authors to conclude that there are multiple determinants affecting processes in root development by this bacteria.

There were very few lateral roots observed in the wheat seedlings grown in the growth pouches in the present study compared with wheat seedlings grown in soil in pot experiments (typically 7 day old plants grown in pots have 5 seminal and 26 lateral roots). In general, the root systems of well-watered plants display reduced root elongation and plants under osmotic stress maintain root elongation (Dodd et al. 2010). Both these responses to water potential are mediated in the plant by abscisic acid (ABA) (Dodd et al. 2010). ABA is stimulated in the plant by water stress (Dodd 2007), and ABA accumulation enhances auxin transport in the root apex (Xu et al. 2013). The increased auxin transport at the root tip activates plasma membrane H^+-ATPase and the

subsequent stimulation of proton secretion maintains primary root elongation under moderate water stress (Xu et al. 2013). Furthermore, exogenous application of ABA at a rate as low as 0.1 µM is sufficient to inhibit lateral root development in *Arabidopsis* (De Smet et al. 2003). Therefore, it is possible that a reduced osmotic potential occurs in the growth pouches which increases ABA accumulation in the wheat seedlings and reduced the lateral root development as observed in these experiments compared with wheat seedlings grown in soil.

The exception to this, in the current study, was the first experiment with the PGPB. In this experiment, it is possible that the growth pouches did not dry out as quickly as in the other experiments since the number of lateral roots in the first PGPB experiment was more comparable to those found in pot experiments also harvested at seven days after planting (data not shown). In the first PGPB experiment, both the PGPB significantly increased the number of lateral roots compared with the water and TIBA controls. However, the number of lateral roots was reduced when the PGPB was co-cultivated with TIBA. It is possible that the difference was due to the first experiment receiving water a day earlier and therefore did not dry out as much as the second experiment.

IBA has also been implicated in plant responses to water stresses. Ghodrat et al. (2012) applied different levels of IBA to wheat roots under increasing salt stress and found that application of IBA increased germination and plant growth parameters over the control and that IBA increased the root lengths significantly compared with the controls. Increased water stress tolerance through the manipulation of IBA homeostasis by reactive oxygen species is independent of ABA homeostasis in *Arabidopsis* (Tognetti

2010) and could facilitate a new avenue in research in crop protection from osmotic stress.

While it cannot be concluded that auxin is the only mechanism of action in enhancing root elongation in wheat seedlings in this study, the results indicate that bacterially produced auxins may indeed be one factor. These results merit the further testing of the potential effects of auxin and/or ACC deaminase production by the bacteria on plant development. This can be accomplished by gene knock-out technologies which could be performed in conjunction with *Arabidopsis* plant mutants in auxin signaling and response (Li et al. 2000; Patten and Glick 2002; Xie et al. 1996; Zamioudis et al. 2013). This will enable the empirical determination of whether auxin or ACC deaminase production are responsible for the plant growth promotion observed by these bacteria in this study.

Chapter 7:
Design and evaluation of 16S-23S internal transcribed spacer region (ITS) strain specific primers for the detection of the *P. granadensis* PMK4 in plant matter and soil

Pseudomonas granadensis PMK4
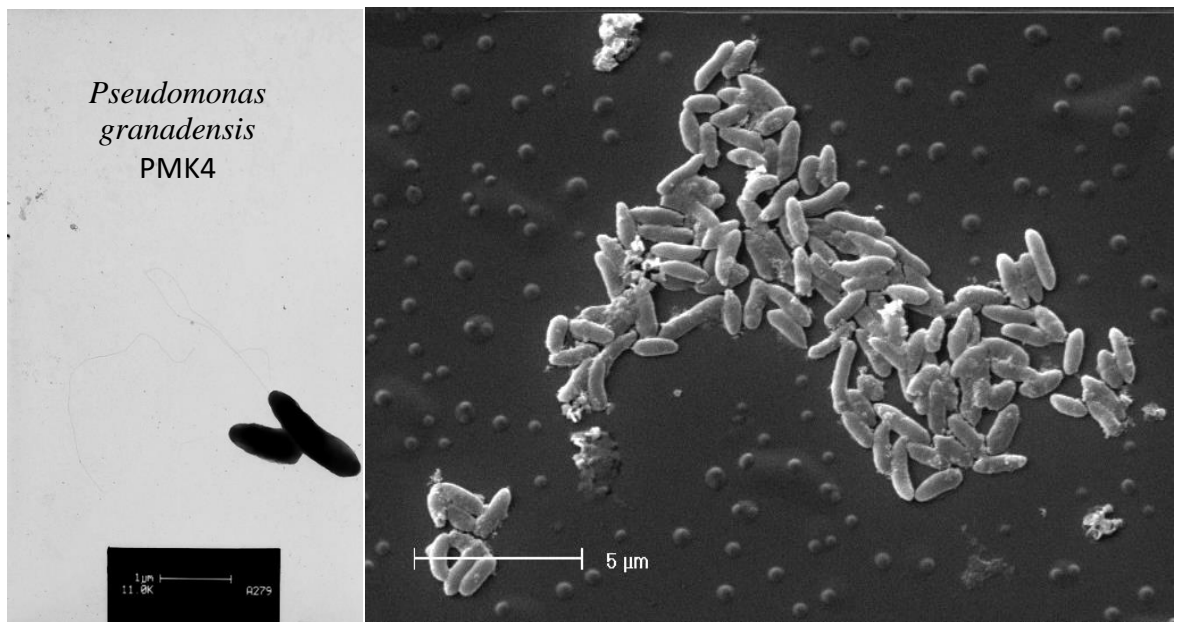
7.1   Introduction

To determine whether PGPB inoculants are able to colonise the root system of a host plant and to survive in the glasshouse and field, requires the development of suitable PGPB detection methods. Baudoin et al. (2010) described four main strategies to evaluate the presence and survival of PGPB inoculants: the introduction of genetic markers such as antibiotic resistance or fluorescent proteins; using spontaneous antibiotic-resistant mutants; the detection of antigenic properties by specific antibodies; and the use of strain-specific DNA sequences. There are several drawbacks to the first three strategies. The release of genetically modified strains is highly regulated, and it is rarely possible to use them in the field (Viebahn et al. 2003). Cell properties can also be affected by the introduction of genetic markers and spontaneous mutation and requires careful monitoring of candidate mutants (Baudoin et al. 2010; Mahaffee et al. 1997). Detection of bacteria using specific antibodies is time-consuming and the detection limit can be high (Baudoin et al. 2010). For instance, the detection limit for the primary antiserum for *Pseudomonas protogens* CHAO-Rif is 10^5 cells g^{-1} dry soil (Mascher et al. 2014).

Another strategy commonly used in rhizobial detection is the use of primers directed at conserved repetitive DNA sequences (rep-PCR) to generate a "fingerprint" profile. Although the rhizobia can be detected directly from crushed nodules from plants inoculated with the target rhizobia in aseptic conditions, use of these primers on field grown nodules generally requires serial sub-culturing to obtain pure colonies before the techniques can be applied (Menna et al. 2009; Richardson et al. 1995; Wilson 1995; Yates et al. 2005).

Identification to species level can be achieved directly from nodules using MALDI-TOF Mass Spectrometry (Ziegler et al. 2012; 2015), however, there are also some limitations to these techniques. To distinguish between nodule occupants, a database of potential isolates must be constructed prior to the assay and at present this technique cannot distinguish between strains of the same species (Ziegler et al. 2015). Given these constraints, the current study focused on developing a detection method based on the fourth strategy (Baudoin et al. 2010) using the DNA sequences of the internal transcribed spacer (ITS1) region of the rRNA genes.

The rRNA genes are organized in an operon (*rrn*) comprised of the 16S (*rrs*), 23S (*rrl*) and 5S (*rrf*) rRNA genes with the ITS regions separating the 16S-23S region (ITS1) and 23S-5S region (ITS2) (Tambong et al. 2009; Tan et al. 2001). The 16S rRNA gene is widely used for the study of bacterial evolution and ecology as it is highly conserved and allows for the analysis of phylogenetic relationships among distant taxa (Větrovský and Baldrian 2013). However, there can be a high similarity of this gene sequence among different organisms so that identification to strain/species level may not be achieved (Tu et al. 2014). Additionally, bacterial genomes often contain multiple non-identical copies of the *rrn* operon, and this may be problematic in molecular systematics and population genetics (Tambong et al. 2009). For instance, *Pseudomonas* species can contain between 4 and 7 *rrn* operons (Bodilis et al. 2012; Duan et al. 2013; Nelson et al. 2002) and may contain up to 4 different types of ITS1 within one strain (Milyutina et al. 2004).

The variation in the ITS1 region can be quite high within species as this region is less conserved than the *rrs* region, and can enable differentiation of strains within a species (Baudoin et al. 2010; Liguori et al. 2011; Saeki et al. 2009; Tan et al. 2001; van Berkum

and Fuhrmann 2000). For instance, strain specific primers based on the 16S-23S ITS region have been developed for a number of species for the detection of strains of interest in the rhizosphere and soil (Baudoin et al. 2010; Norini et al. 2013; Tan et al. 2001) and detection limits using these techniques can be as low as 10^2 CFU g^{-1} soil (Baudoin et al. 2010).

The aim of this study was to develop and evaluate strain-specific primer sets from the 16S-23S ITS1 region of *P. granadensis* PMK4 to allow identification of this specific PGPB from a plant host or the rhizosphere soil using PCR-based methods.

7.2   Methods

7.2.1   Bacterial strains

The bacterial strains used are listed in Table 7.1. The bacteria were grown overnight with shaking at 28°C in liquid TY (tryptone, 5.0 g; yeast extract, 3.0 g; $CaCl_2.2H_2O$, 0.89 g; 1000 mL H_2O) or Trypticase Soy Broth (Beckton, Dickinson and Company; TSB) or in Luria-Bertani (tryptone, 10.0 g; yeast extract, 5.0 g; NaCl 5.0 g; 1000 mL H_2O; LB) at 37°C. The bacterial isolate, *Pseudomonas* sp. UW4, was kindly provided by B. Glick (University of Waterloo).

7.2.2   Antibiotic profile of *P. granadensis* PMK4

PMK4 was grown in triplicate overnight in TY broth then washed in sterile saline (0.89% NaCl w/v) twice and diluted to an OD_{600} of 1.0. A sterile swab was dipped into the culture suspension and streaked onto a TY agar plate. Commercial antibiotic discs (Oxoid) for ampicillin (25 µg), chloramphenicol (30 µg), gentamycin (30 µg), kanamycin (30 µg), penicillin (10 µg), streptomycin (25 µg) and tetracyline (30 µg) plus sterile (5 mm) filter discs soaked with a 50 µg mL^{-1} solution of rifampicin or 100 µg mL^{-1} spectinomycin, were placed onto the culture plates for a total of 9 different

antibiotic discs per plate. Growth of the bacteria on the plate was observed over 5 days. Zones of clearing around the discs were measured to determine sensitivity or resistance to the particular antibiotic.

A suite of agar plates were prepared with a range of antibiotic concentrations (ug mL^{-1}): ampicillin (Amp) (Sigma), 50; Amp 100; chloramphenicol (Cm) (Sigma) 20; Cm 30; and Amp 100 + Cm 20 with and without cycloheximide (Cx) 50. Dilutions (100 µL) of 10^{-5} and 10^{-6} of each bacterial culture was plated onto the respective antibiotic agar or TY control plates. Growth was monitored over 5 days.

Table 7.1: List of bacterial strains used

Strain	Reference/Source
Bradyrhizobium diazoefficiens CB1809	Centre for *Rhizobium* Studies[1]
Burkholderia caledonica NCH45	This study
Eschericia coli DH5α	Invitrogen
P. brassicacearum WMK10	This study
P. granadensis PMK4	This study
Pseudomonas sp. UW4	Duan et al. (2013)
P. fluorescens MUR003	M. Buttery[1]
P. fluorescens WSM3457	Flores-Vargas and O'Hara (2006)

[1] School of Veterinary and Life Sciences, Murdoch University

7.2.3 Growth of plants and collection of soil and plant material

Soybean (*Glycine max*) seeds were surface sterilized by placing them in 70% ethanol for 1 min, then 3 min in 4% bleach, followed by 6 washes in sterile water. The soybean seeds were sown in plastic pots containing a sterile 50:50 lawn sand/brickies sand mix (Soils aint Soils, Canning Vale) and 6 surface sterilized seeds were placed in each pot. Each seed was inoculated with either 1 mL (OD$_{600}$ 0.5) *Bradyrhizobium diazoefficiens* CB1809 culture or 1.0 mL of a culture solution containing OD$_{600}$ 0.5 of each *B.*

diazoefficiens CB1809 and PMK4. The control pots were inoculated with 1.0 mL of sterile water. Each treatment contained 3 replicate pots and the plants were grown in the glasshouse under natural illumination at 23°C (±2 °C).

After seedling emergence, sterile plastic high-density beads (Universal Polymer Supplies, Malaga) were placed in the pots to minimize water loss and to reduce contamination. A sterile watering tube was also placed in the pots and the plants were watered frequently with nutrient solution (Broughton and Dilworth 1971). After two weeks, plants were thinned to 2 per pot and after 4 weeks, the nodules from the inoculated plants were harvested and the controls (uninoculated) were checked for nodulation as a measure of contamination (Table 7.2).

To determine if PMK4 is able to colonise the nodules of field grown soybeans, nodules were collected 9 weeks after sowing from soybean that had been either inoculated with *B. diazoefficiens* CB1809 alone or co-inoculated with CB1809 and PMK4 at the time of sowing in the field in Christmas Island (Chapter 4). The nodules were placed in 5 mL vials containing silica gel as a desiccant for transport back to the laboratory. Prior to use, the nodules were rehydrated for 3 hours in sterile deionized water.

Nodules were collected from peas (*Pisum sativum* cv. Kaspa) grown in field trials using conventional methods in Brookton, Western Australia (Chapter 3). The treatments included peas that were co-inoculated with PMK4 and the commercial pea islolate, *Rhizobium leguminosarum* bv. *viciae* SU303 and pea plants that were inoculated with the rhizobial isolate SU303 alone. Four plants were removed from 3 of the replicate plots for each of the treatments 14 weeks after sowing. The roots were washed in tap water and the nodules excised from the roots and stored at -80°C. Prior to use, the nodules were thawed then surface sterilized as described below.

Surface sterilization of the soybean and pea nodules collected from glasshouse and field trials was performed by immersing the nodule(s) for 30 seconds in 70% ethanol then 60 seconds in 4% bleach, followed by 6 washes in sterile water.

Table 7.2: Source of soybean nodules used in this study

Type of nodule	Type of study	Bacterial inoculant(s)	Source
Fresh	Glasshouse	*Bradyrhizobium diazoefficiens* (CB1809)	This study and L. Parkinson[1]
Fresh	Glasshouse	PMK4 and CB1809	This study and L. Parkinson[1]
Fresh	Glasshouse	*Bradyrhizobium* sp.	M. Chiduwa[1]
Desiccated	Field, CI[2]	PMK4 and CB1809	This study (Chapter 5)
Desiccated	Field,CI[2]	CB1809	This study (Chapter 5)

[1] School of Life and Veterinary Sciences, Murdoch University; [2] Christmas Island

To determine the survival of PMK4 on wheat, sterilized wheat seed was either inoculated with PMK4 or sterile 0.3 M MgSO$_4$ (control) at the time of sowing, in pots filled with pasteurized field soil obtained from the Wongan Hills Research Station in Western Australia and grown in the glasshouse (Glasshouse Experiment 2, Chapter 5). Seven weeks after planting, roots from selected replicates were carefully washed in sterile water, sectioned into top, middle and lower root sections and stored in sterile tubes at -80°C until further use. A similar experiment was conducted using a different soil type collected from the research station and after 7 weeks the rhizosphere soil closely adhering the wheat roots was brushed from the roots and collected in plastic bags till further use (Glasshouse Experiment 3, Chapter 5).

7.2.4   DNA template preparation

Genomic DNA of PMK4 for initial 16S-23S rRNA PCR amplification was extracted using the method of Chen and Kuo (1993). To amplify the 16S-23S rRNA region (for isolates other than PMK4) or to assess the PMK4-ITS1 primers, bacterial strains (Table

7.2) were grown on TY or TSB agar plates and incubated for 48 hours. A large single colony was suspended in 100 µL of sterile PCR water, vortexed and the cell suspension stored at -20°C till further use. For PCR reactions, 1 µL of thawed cell suspension was used as a DNA template.

Sterile nodules collected from glasshouse and field trials were crushed individually and aseptically in 1.5 mL Eppendorf tubes containing 100 µL of PCR grade water, using an orange stick. The tubes were spun briefly to deposit large debris and 1 µL of nodule crush supernatant was used as a DNA template.

DNA was extracted from rhizosphere soil from wheat plants which were grown in Glasshouse Experiment 3 (Chapter 5) using the FavorPrep^{TM} Soil DNA isolation kit (Favorgen®) following manufacturer's instructions. The same soil was suspended in sterile H_2O and 100 uL aliquots were spread onto TY agar containing 100 ug mL^{-1} Amp, 20 ug mL^{-1} Cm, and 50 ug mL^{-1} Cx. The plates were incubated at 28°C for 7 days.

Roots of wheat plants grown in Glasshouse Experiment 2 (Chapter 5) were removed from -80°C storage and placed under sterile conditions in a 2 mL centrifuge tube with a 7.9 mm stainless steel ball and 500 uL sterile PCR water. The tubes were placed in a cell lyser (TissueLyser, Qiagen) and lysed at 25.0 1/s for 8 minutes. This did not pulverize the roots, but left the majority of the roots intact. The tubes were spun briefly and a 1 uL aliquot was used as DNA template. One hundred uL of the root suspensions was also plated out onto TY agar containing 100 ug mL^{-1} Amp, 20 ug mL^{-1} Cm and 50 ug mL^{-1} Cx. The plates were incubated for 2 days at 28°C then the bacterial cells were scraped from the plates into 500 uL sterile PCR water and mixed thoroughly. One uL of the cell suspension was used as a template for a PCR reaction

7.2.5   PCR analyses

7.2.5.1 PCR amplification and sequencing of the ITS1 region

The 16S-23S rRNA internal transcribed spacer (ITS1) region was amplified for the isolates *P. granadensis* PMK4, *P. brassicacearum* WMK10, *P. fluorescens* MUR003, *P. fluorescens* WMS3457 and *Pseudomonas* sp. UW4 (Table 7.1) by the fPs16S and rPs23S primers (Table 7.3) (Locatelli et al. 2002). The reaction mix contained 1x GoTaq Green Master Mix (Promega), 0.25 µM each primer, 1.0 uL template DNA to a total volume of 25 uL. The reactions were performed in a Mycycler (Bio-Rad) with an initial denaturation of 3 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 30 sec at 55 °C, 1 min at 74 °C and a final extension step at 74 °C for 5 min. The PCR products were visualised by electrophoresis in 1.0% agarose gel (Fisher Biotec Australia) after staining with Sybr® Safe. The products were purified either by the desired bands being excised (WMK10, MUR003 and WSM3457) into a filter tip placed in a 1.5 mL Eppendorf tube and centrifuged at full speed for 1 min or using the QIAquick PCR Purification Kit (QIAGEN) (PMK4), following the manufacturer's instructions.

7.2.5.2 PCR amplification with the PMK4-ITS1 primers

The primers PMK4-ITS1 F1-R and F2-R (Table 7.3) were designed for the specific detection of PMK4. Amplification of the target region was performed with reaction mixtures containing 1x GoTaq Green Master Mix (Promega), 0.50 µM each primer and 1.0 uL thawed cell suspension, nodule crush, genomic DNA (from soil), or root crush to a total 20 uL volume. The reactions were performed in a Mycycler (Bio-Rad) with an initial denaturation of 5 min at 98°C, followed by 35 cycles of 10 sec at 98 °C, 30 sec at 55 °C, 30 sec at 72 °C and a final extension step at 72 °C for 5 min.

A multiplex PCR reaction was performed using Universal 16S primers (27f, 1492r) as well as the PMK4-ITS1 F2-R primer set (Table 7.3). The reaction mix contained 1x

GoTaq Green Master Mix (Promega), 0.50 µM each primer (4 primers) and 1.0 uL

nodule crush as the DNA template. The PCR reaction was as described above.

To determine if a more successful amplification of the PMK4-ITS1 region would be

achieved with a high fidelity polymerase, two kits were evaluated. Each reaction

contained 1 unit of either Phusion® High Fidelity DNA polymerase or TEGO™ Direct

PCR DNA polymerase and 1 x their respective buffers, 0.5 µM of each primer (x 4

primers) and 1 uL nodule crush to a 20 uL volume. The cycling conditions were as

described above and only nodules co-inoculated with PMK4 were assessed.

The PCR products were visualised by electrophoresis in 1.0% agarose gel (Fisher

Biotec Australia) after staining with Sybr® Safe.

Table 7.3: List of primers used in this study

Name	Sequence (5'-3')	Reference
fPs16S	ACTGACACTGAGGTGCGAAAGCG	Locatelli et al. (2002)
rPs23S	ACCGTATGCGCTTCTTCACTTGACC	Locatelli et al. (2002)
27f	AGAGTTTGATCCTGGCTCAG	(Lane 1991)
1492r	GGTTACCTTGTTACGACTT	(Lane 1991)
M13pUCF	CCCAGTCACGACGTTGTAAAACG	(Nishikawa et al. 1999)
M13pUCR	AGCGGATAACAATTTCACACAGG	(Nishikawa et al. 1999)
PMK4-ITS1 F1	TCCACCACTACTGCTTCTCT	This study
PMK4-ITS1 F2	AAATGAGCATTCCATCAGTTT	This study
PMK4-ITS1 R	CCGAAAATTCGAATTTCTCAAC	This study

7.2.6   Cloning of the 16S-23S rDNA ITS region of PMK4

7.2.6.1 *Ligation of the PMK4 ITS1 PCR product with pGEM-T*

The PMK4 PCR product amplified by the fPs16S and rPs23S primers was ligated into

the pGEM-T vector as per manufacturer's instructions (Promega). Briefly, to a 1.5 mL

centrifuge tube the following were added: 2x ligation buffer, 5 uL; pGEM-T vector (50

ng), 1 uL; purified PMK4-ITS1 PCR product, 3 uL; T4 DNA ligase (3 Weiss units μl^{-1}), 1 uL. The reaction tubes were incubated overnight at 4 °C, followed by 1 hour at room temperature and 1 hour on ice just prior to transformation into competent cells.

7.2.6.2 Competent cell preparation

Thirty uL of DH5α *Escherichia coli* cells were inoculated into 50 mL LB and incubated overnight at 200 rpm at 37°C. The next morning 5 mL of the culture was transferred to 50 mL fresh LB until the culture reached an OD_{600} of 0.5. The cells were spun down at 1700 *g* for 8 min and the supernatant was removed. The cells were resuspended in 8 mL of ice cold sterile transformation buffer (TFB) (100 mM KCl, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 3 mM Hexamine $CoCl_3$, 0.01M MES buffer) by vortexing and spun down again at 1700 *g* for 8 min. The cells were resuspended in 2 mL ice cold sterile TFB and 1 mL of the cell culture was placed into 2 x 1.5 mL centrifuge tubes. To each tube, 70 uL dimethylforamide (DMF) was added and the tubes incubated on ice for 5 min. Then 70 uL of 0.7 M β-mercaptoethanol was added to each tube and the tubes incubated on ice for a further 10 min. Then another 70 uL of DMF was added to each tube and the tubes re-incubated on ice for 5 min.

7.2.6.3 Transformation of DH5α competent cells with pGEM-T-PMK4-ITS1 ligation

Two 1.5 mL centrifuge tubes were placed on ice for 5-10 minutes. To the tubes, 5 uL of the pGEM-T-PMK4-ITS1 ligation (as described above) and 200 uL of the competent cells were added. The tubes were incubated on ice for 30 min and then were transferred to a 42°C water bath for exactly 90 sec. The tubes were then immediately placed back on ice for another 5 min and then 800 uL of SOC broth (Hanahan 1983) was added. The tubes were incubated at 37 °C at 200 rpm for 1 hour. Three volumes (25 uL, 150 uL and remainder of culture) were spread onto LB agar containing 100 ug mL^{-1} Amp

and 50 ug mL^{-1} X-gal. The plates were incubated at 37°C overnight. Ten white colonies were inoculated into 5 mL LB broth containing 100 ug mL^{-1} Amp and incubated overnight at 37° C at 200 rpm. Three mL of each culture was spun down at top speed for 1 min, the supernatant removed and the pellet resuspended in 100 uL TE (1 M Tris 8.0, 0.1M EDTA, diluted 1/100 for working concentration). The remaining approximately 1.5 mL was spun down at 2700 *g* for 5 min then resuspended in 15% glycerol and stored at -80°C until further use. The plasmid DNA was purified from 100 uL of the TE resuspended culture using alkaline lysis (Sambrook et al. 1989) and digested with *Pst*I to confirm the presence of the PMK4-ITS1 inserts. Plasmid DNA was prepared for sequencing with the QIAprep® Spin Miniprep Kit using instructions provided by the manufacturer (QIAGEN).

7.2.7   Sequencing and analysis of the ITS1 region

The ITS1 PCR products or plasmid DNA were sequenced as follows. A ¼ strength sequencing reaction mixture contained 1x sequencing buffer (1 uL), Big Dye® Terminator (2.0 uL), the primers fPs16S or rPs23S (WMK10 and MUR003) or M13pUCF or M13pUCR (PMK4) (Table 7.1) (3.2 pmol), purified DNA (2 uL), and PCR water up to a total volume of 10 uL. The sequencing temperature profile was performed on the Mycycler (Bio-Rad) thermal cycler and consisted of 96 °C for 2 min, followed by 25 cycles of 96 °C for 10 sec, 5 °C for 5 sec, 60 °C for 4 min. The sequences were determined by the SABC Sequencing Service (Murdoch University). The gene sequences were subjected to a nucleotide (nr) blast in the NCBI database to find the related species or genera and complete ITS1 regions for *Pseudomonas* strains and common soil bacteria were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) database for comparison purposes. All sequences from this study and the comparison species were aligned using the

MEGA 6 software and phylogenetic trees were constructed using the Maximum Likelihood method based on the Kimura 2-parameter model as described by Hall (2013).

7.3 Results

7.3.1 Design and theoretical evaluation of PMK4-ITS1 primers

To design strain specific primers from the ITS1 region of PMK4, the ITS1 region in PMK4 as well as the isolates WMK10, WSM3457, MUR003 and *Pseudomonas* sp. UW4 was amplified by PCR. The PCR produced a product for all strains that was of the expected size, approximately 1300 bp in length (Figure 7.1). When sequenced, an adequate sequence was only obtained for PMK4, WMK10 and WSM3457.
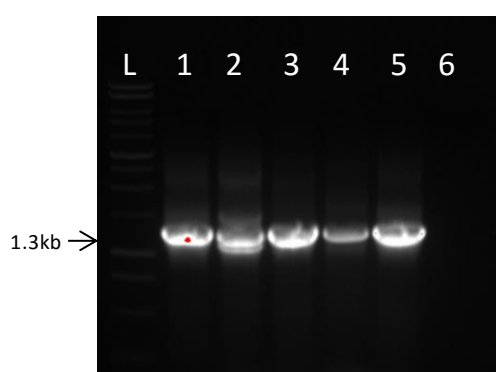
Figure 7.1: Amplification of the ITS1 region of *Pseudomonas* sp. using the fPs16S-rPs23S primers. The isolates were: PMK4 (lane 1), WMK10 (lane 2), UW4 (lane 3), MUR003 (lane 4) and WMS3457 (lane 5). Water (lane 6) was used as a negative control. The product was approximately 1300 bp in size as depicted by the 1 kb ladder (L)

To improve the quality of the sequence produced by these primers for PMK4, the fPs16S-rPs23S PCR product was cloned into pGEM-T, and the 16S-23S region sequenced by the M13pUCF/R primers. The PMK4-ITS1 sequences were aligned using Geneious Alignment and a consensus sequence specific to PMK4 was developed. Two different species of ITS1 from the PMK4 isolate were obtained and both of these ITS1 species were 518 bp in length and only differed by 2 nucleotides.

Complete ITS1 regions for *Pseudomonas* strains and several plant associated soil microorganisms, including several rhizobacteria, were retrieved from Genebank and

aligned with the PMK4 ITS1 sequence to assess the similarity in the ITS1 region between these strains and PMK4. Over 300 ITS1 sequences were retrieved and included several strains of the species *Burkholderia*, *Bradyrhizobium*, *Mesorhizobium*, *Ensifer (Sinorhizobium)* and *Rhizobium* plus 32 *Pseudomonas* strains. Phylogenetic trees using the ITS1 region from the soil microorganisms, *Pseudomonas* spp and the PMK4-ITS1 sequences were constructed using MEGA 6. There is great deal of similarity between *Pseudomonas* species based on the ITS region (Figure 7.2), however although PMK4 and WMK10 were isolated from the same agricultural field, they cluster in separate groups based on this gene region. When compared with the ITS1 region of other soil bacteria (Figure 7.3), the ITS region of PMK4 is very distinct from the other soil species, but is more distinctly separate from the *Burkholderia* species than the rhizobial species.

The target region for the PMK4-ITS1 primers began at approximately 305 bp from the beginning of the *Pseudomonas* consensus ITS1 region (Figure 7.4 and Appendix III). This region displayed the most variability for the *Pseudomonas* species in the ITS1 region and three different primers for the PMK4-ITS1 region were designed in such a way that the 3' end of each primer were specific to PMK4 only. The two forward and 1 reverse primer sequences are detailed in Table 7.1. The melting temperatures (°C) for the F2 and R primers were 54.0 and 54.7, respectively and 57.7 for the F1 primer. The two forward and one reverse primer designed from this region were assessed in the NCBI Primer-BLAST program which confirmed that these primers were highly specific for PMK4.
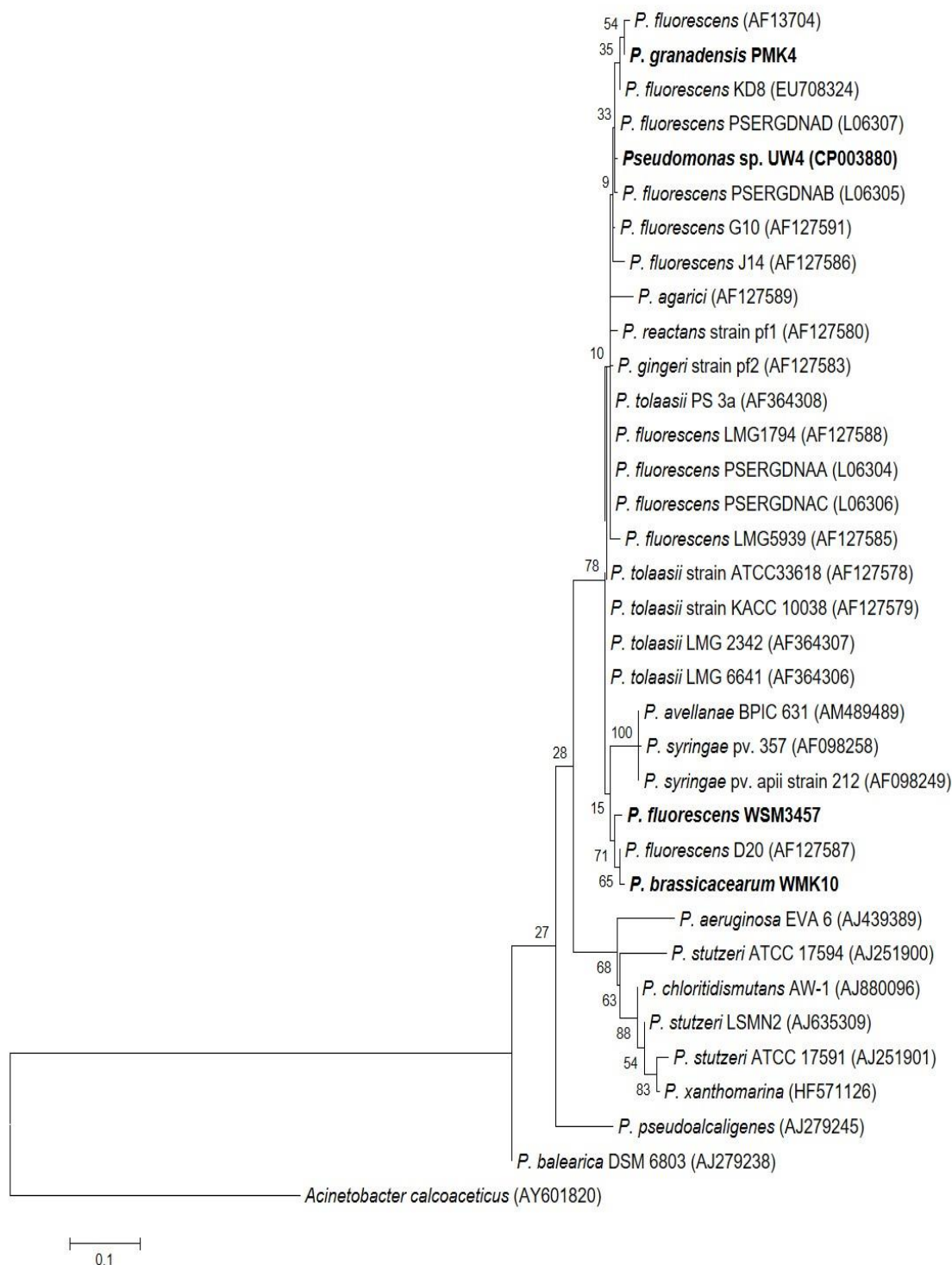
Figure 7.2: Maximum Likelihood tree based on the internal transcribed spacer (ITS1) region of *Pseudomonas* spp. Where available, the strain identification is included and the accession numbers are in brackets. The tree was rooted on *Acinetobacter calcoaceticus*. Bootstrap values are from 1000 replications and are given at the nodes and the scale bar indicates the number of substitutions per site. The isolates used for the experimental evaluation of the PMK4-ITS1 primers are highlighted in bold
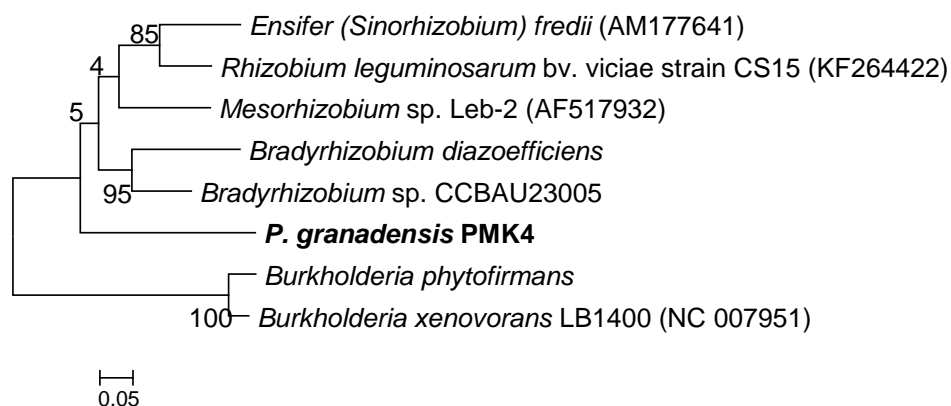
Figure 7.3: Maximum Likelihood tree based on the internal transcribed spacer (ITS1) region of plant associated soil microorganisms. Where available, the strain identification is included and the accession numbers are in brackets. The tree was unrooted and bootstrap values are from 1000 replications and are given at the nodes. The scale bar indicates the number of substitutions per site

7.3.2 Experimental evaluation of PMK4-ITS1 primers

To determine if the PMK4-ITS1 primers designed in this study are specific for PMK4, cell suspensions of several *Pseudomonas* strains and *Burkholderia caledonica*. NCH45 were used as a template for amplification of the PMK4-ITS1 region. In the reactions with the PMK4-ITS1 primers, the F1-R primer set was highly specific for PMK4 (Figure 7.5); however, the F2-R primer set amplified both *P. brassicacearum* WMK10 and *Pseudomonas* sp. UW4 DNA (Table 7.4). The universal 16S (27F/1492r) primers were included in a separate PCR reaction in order to confirm that the correct PCR conditions had been chosen to amplify the DNA of the respective bacteria. A product of approximately 1500 bp was generated by these primers for each of the bacteria (not shown)
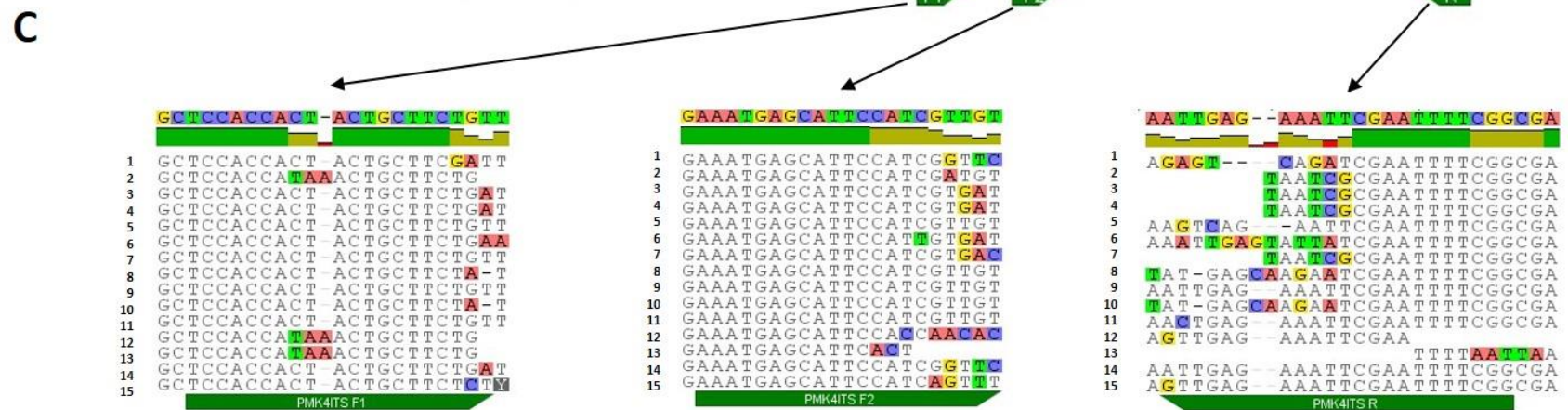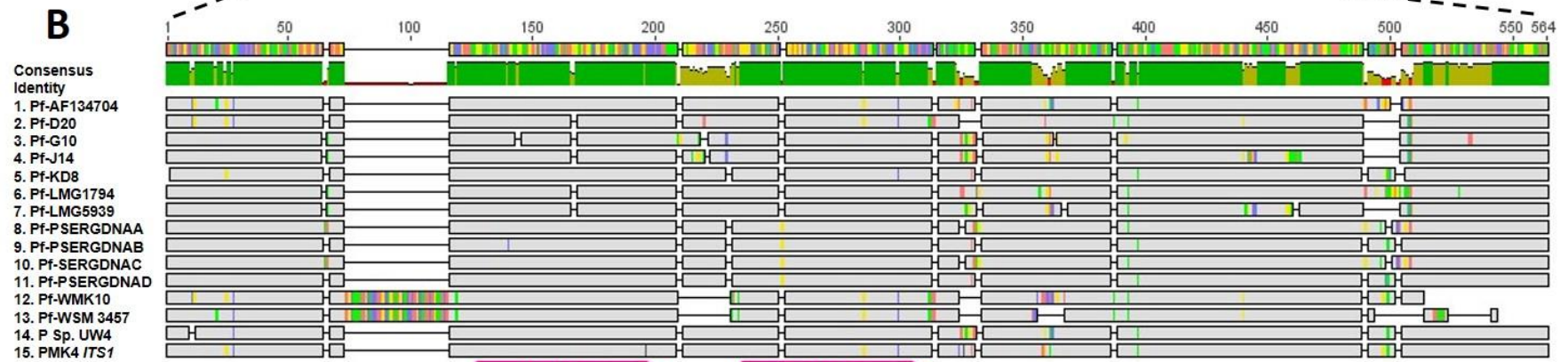
Figure 7.4 (previous page): Genomic layout of the *Pseudomonas fluorescens* internal transcribed spacer (ITS1) region and alignment of the PMK4-ITS1 primers. Panel A shows a diagram of the rRNA operon in *Pseudomonas* species and depicts the location of the 16S-23S ITS1. Panel B shows the low resolution multiple sequence alignment of the ITS1 region generated from known *P. fluorescens* sequences compared with the ITS1 region of PMK4. This includes *P. brassicacearum* WMK10 that was formerly identified as *P. fluorescens*. The location of the PMK4-ITS1 primers are annotated in green and the two *tRNA* species are annotated in pink. The sequence labels include either Pf for *P. fluorescens* or P Sp for *Pseudomonas* sp. and their Accession numbers or strain identification. Panel C shows the detail of the nucleotide sequences used to determine the PMK4-ITS1 primers. The images for panels B and C were created in Geneious version 8.1
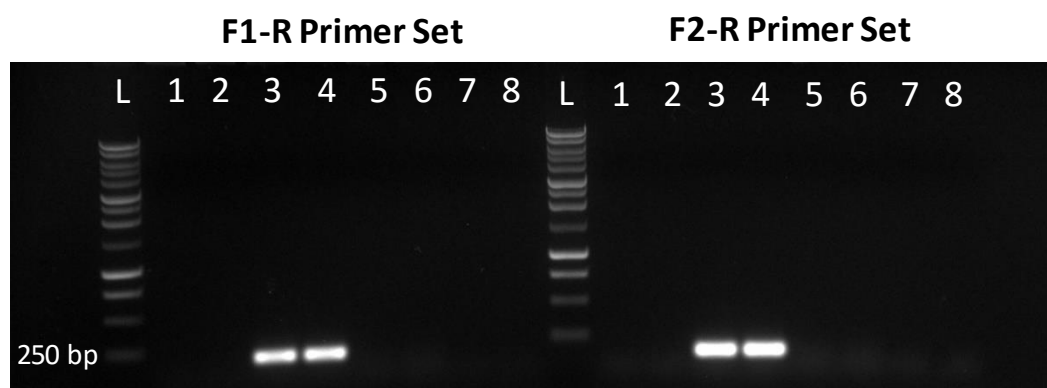
Figure 7.5: PCR amplification of DNA using the strain specific PMK4-ITS1 primer sets F1-R and F2-R for the strains *Burkholderia* sp. NCH45 (Lanes 1 & 2), *P. granadensis* PMK4 (Lanes 3 & 4) and *P. fluorescens* WSM 3457 (Lanes 5 & 6). Water (Lanes 7 & 8) was used as a control and two independent PCR reactions for each strain and control are depicted.

Table 7.4: Amplification of the ITS1 region with PMK4-ITS F1-R and F2-R primer sets compared with the universal 16S primers (27f/1472r)

Strain	F1 primer set[*]	F2 primer set[*]	Universal 16S primers[*]
P. granadensis PMK4	+	+	+
P. brassicacearum WMK10	-	+	+
P. fluorescens WSM 3457	-	-	+
P. fluorescens MUR003	-	-	+
Pseudomonas sp. UW4	-	+	+
Burkholderia sp. NCH45	-	-	+

[*] + indicates generation of a PCR product and – indicates that no product was obtained

When all three PMK4-ITS1 primers (2 forward, 1 reverse) were included in one PCR reaction with PMK4 cell suspension as a template, two bands were observed, one at approximately 450 bp and one at approximately 210 bp (not shown). The 450 bp band is larger than expected for either of the 2 PMK4-ITS1 primer sets so attempts were made to optimize the PCR condition. However, further optimization of the PCR reactions containing all three PMK4-ITS1 primers was not able to obtain bands for the two primer sets of the expected size.

7.3.3 Detection of PMK4 in plant and soil material using PMK4-ITS1 strain specific primers

7.3.3.1 Determining the ability of PMK4 to inhabit nodules

Nodules were collected from soybean grown in the glasshouse that had either been inoculated with *B. diazoefficiens* CB1809 alone or co-inoculated with PMK4 at the time of sowing. Three nodules per plant were sterilized and crushed and this nodule crush template was used to amplify DNA using the PMK4-ITS1 primers. One uL of sterile CB1809 nodule crush spiked with PMK4 cells (by dipping a pipette tip in a PMK4 cell suspension) was used as a positive control to ensure that plant material from the nodule did not inhibit the PCR reaction. The evaluation of the PMK4-ITS1 primers was repeated with nodules sourced from similar glasshouse experiments (Table 7.2). Sixty percent of the fresh glasshouse grown soybean nodules that had been co-inoculated with PMK4 returned bands of the appropriate size for the PMK4-ITS1 primers (Figure 7.6). However, the nodules from the plants that were inoculated only with *B. diazoefficiens* CB1809 did not produce an amplification product with these primers.

Nodules from field grown peas were sterilized, crushed and 1 µL of the nodule crush was used as a DNA template for the PMK4-ITS1 F1 primer set. A product of the expected size for PMK4 was obtained from 58% of the nodules co-inoculated with

PMK4 and the commercial pea isolate *Rhizobium leguminosarum* bv. *viciae* SU303 (Figure 7.7). An amplification product was not obtained for PMK4 nodules inoculated only with SU303 (not shown).

The desiccated nodules from the Christmas Island collection did not produce an amplification product with either of the PMK4-ITS1 primers but did produce a product with the universal 16S primers (27f/1492r). The TEGO™ Direct PCR gave a better amplification product when used in a multiplex PCR reaction and were henceforth used for multiplex PCR reactions.

To determine if the sterilization process destroys surface DNA, soybean nodules that had not been in contract with PMK4 were collected from a glasshouse trial (Table 7.3). Six nodules were soaked in 1 mL sterile H_2O for 1 hour and 6 nodules were soaked in a 1 mL PMK4 cell suspension for 1 hour. Three of the water soaked nodules and 3 of the PMK4 soaked nodules were surface sterilized prior to being crushed for DNA template. A product for the PMK4-ITS1 was only amplified from the non-sterilized nodules pre-soaked with PMK4 cells indicating that surface sterilization was effective in destroying the DNA of this isolate from the surface of the nodules (Table 7.5).

7.3.3.2 Detection of PMK4 in wheat rhizosphere soil and roots

Antibiotic profile of P. granadensis *PMK4*

PMK4 demonstrated resistance to ampicillin, chloramphenicol and penicillin, but did not grow well on the TY agar supplemented with Cm 30 µg mL^{-1} so the concentration was decreased to 20 µg mL^{-1} and good growth was observed. Both PMK4 and NCH45 grew well on plates containing Amp 100 µg mL^{-1} and Cm 20 µg mL^{-1} but the *E. coli* DH5α strain was sensitive to these antibiotics.

Table 7.5: Evaluation of the effect of sterilization of soybean (*Glycine max*) nodules inoculated with *Bradyrhizobium* sp. on amplification of *P. granadensis* PMK4 DNA using the PMK4-ITS1 primers. PCR products were visualised following gel electrophoresis from nodules coated with and without PMK4 (+ indicates generation of a PCR product, - indicates no PCR product)

Experimental conditions	ITS1 bands
Nodules soaked in H_2O then surface sterilized	-
Nodules soaked in PMK4 cells, then surface sterilized	-
Nodules soaked in H_2O then spiked with PMK4 cells	+
Non-sterile nodules soaked in H_2O	-
Non-sterile nodules soaked in PMK4 cells	+

PCR and selective agar detection of P. granadensis *PMK4 from soil and plant materials of glasshouse grown wheat*

For PCR, genomic DNA extracted from rhizosphere soils or root homogenate (crushes) from glasshouse grown wheat plants (Chapter 5) were used as templates. Additionally, viable PMK4 cells were recovered from these rhizosphere soils and root crush by spreading the samples onto PMK4-selective antibiotic media. The PMK4-ITS1 was not amplified in any of the rhizosphere soils. However, a band of the expected size was amplified for the universal 16S rRNA primers in these soils (data not shown). There was no amplification of either the 16S rRNA or the PMK4-ITS1 regions when root crushes were used as a DNA template (not shown).

Cell suspensions scraped from PMK4-selective antibiotic plates inoculated with root crush generated the correct fragment with the PMK4-ITS1 primers for all the root sections that had been inoculated with PMK4 except one for one plate (Figure 7.8). The plated root crush from plants that were not inoculated with PMK4 did not amplify a product with the PMK4-ITS1 primers. When rhizosphere soil dilutions were spread on the PMK4-selective antibiotic media there was no bacterial growth until 7 days and

these isolates did not resemble PMK4, therefore bacterial suspensions from these plates

were not used for PCR reactions.
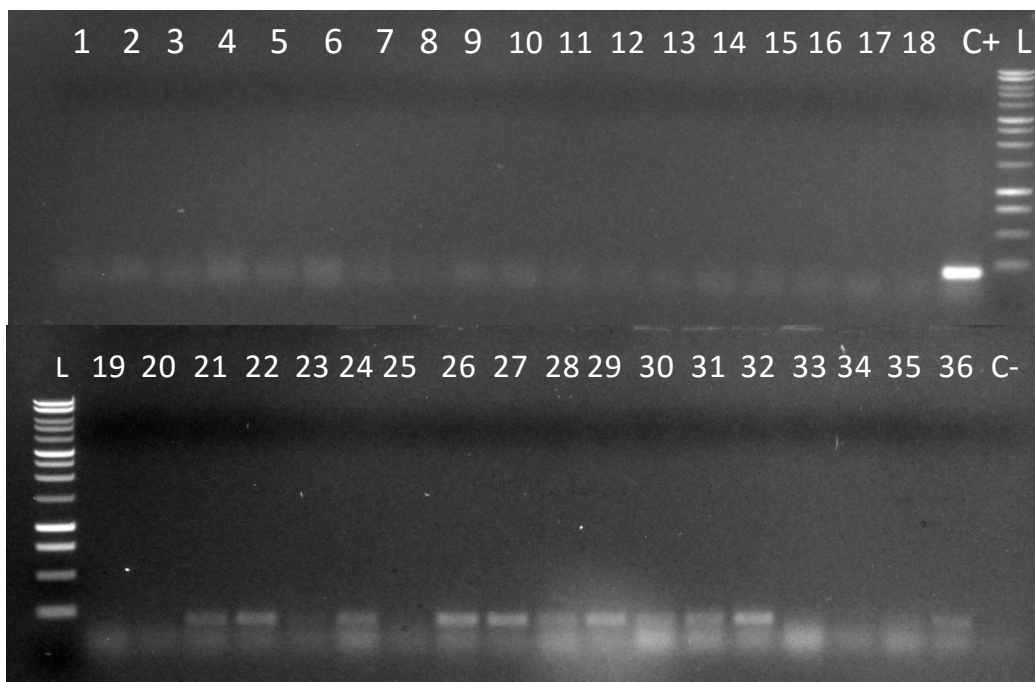
Figure 7.6: The amplification of the ITS1 region from glasshouse grown soybean nodules, co-inoculated with and without *P. granadensis* PMK4 using the strain specific PMK4-ITS1 F1-R primers. Wells 1-18: nodules from plants inoculated with *Bradyrhizobium diazoefficiens* CB1809 only; wells 19-36 nodules from plants co-inoculated with *Bradyrhizobium diazoefficiens* CB1809 and PMK4; C+: positive control (PMK4 cells); C-: negative control (water); L: 1 kb ladder.
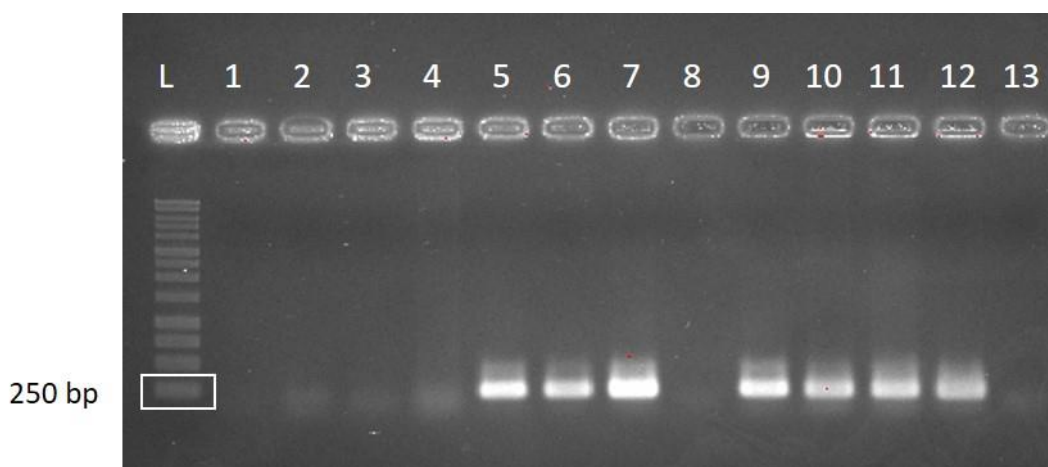
Figure 7.7: The amplification of the PMK4 ITS1 region using the strain specific PMK4-ITS1 F1-R primer set on nodules obtained from field grown pea (*Pisum sativum* cv. Kaspa)

(Chapter 3). Wells 1-12 contained DNA amplified from: nodules that were co-inoculated with *Rhizobium leguminosarum* bv. *viciae* SU303 and and *P. granadensis PMK4* The water control is in well 13. L = 1 kb ladder

Wheat roots **not** inoculated with PMK4

Wheat roots inoculated with PMK4

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

1= uninoculated, zero P, top root section
2= uninoculated, zero P, middle root section
3= uninoculated, zero P, lower root section
4= uninoculated, 1 mg P/kg, top root section
5= uninoculated, 1 mg P/kg , middle root section
6= uninoculated, 1 mg P/kg , lower root section
7= uninoculated, 2.5 mg P/kg , top root section
8= uninoculated, 2.5 mg P/kg , middle root section
9= uninoculated, 2.5 mg P/kg , lower root section

10= inoculated with PMK4, zero P, top root section
11= inoculated with PMK4, zero P, middle root section
12= inoculated with PMK4, zero P, lower root section
13= inoculated with PMK4, 1 mg P/kg, top root section
14= inoculated with PMK4, 1 mg P/kg , middle root section
15= inoculated with PMK4, 1 mg P/kg , lower root section
16= inoculated with PMK4, 2.5 mg P/kg , top root section
17= inoculated with PMK4, 2.5 mg P/kg , middle root section
18= inoculated with PMK4, 2.5 mg P/kg , lower root section
19= PMK4 cells
20= Water control

Figure 7.8: Amplification of the PMK4-ITS1 region from wheat roots inoculated or not inoculated with PMK4 (Glasshouse Experiment 2, Chapter 5) using the PMK4-ITS1 F1-R strain specific primers (209 bp) designed in this study

7.4   Discussion

The strain-specific primers designed from the 16S-23S rRNA ITS1 region for *P. granadensis* PMK4 were highly specific for this isolate. This was demonstrated in silico using Primer-BLAST (NCBI) and confirmed *in vitro* using several strains of *Pseudomonas* spp. as well as other bacterial species. Furthermore, using the PMK4-ITS1 specific primers has confirmed that PMK4 is capable of colonising the root system of wheat as well as the nodules of soybean grown in the glasshouse and the nodules of field-grown peas.

The absence of PMK4 in the desiccated soybean nodules from the Christmas Island field trial (Chapter 4) suggests that PMK4 was either unable to colonise the nodules of field-grown soybeans or that the isolate did not survive desiccation after plant harvest.

However, Fox et al. (2011) observed colonization of nodules by the PGPB isolate *P. fluorescens* WSM3457 in only 40% of *Medicago truncatula* nodules. Therefore, if colonization of nodules by PGPB is potentially this low, it is possible the sample size was too small to detect PMK4. PMK4 was also not detected in rhizosphere soil collected from the glasshouse trial (Chapter 5) and this soil had been air dried before testing for the presence of PMK4 with the PMK4-ITS1 primers. Pseudomonads are susceptible to desiccation (Normander et al. 1999; Roberson and Firestone 1992) and it is possible that during desiccation, the bacteria were more vulnerable to predatory bacteria such as *Micavibrio aeruginosavorus*, or other bacterial predators such as protozoa and bacteriophages (Kadouri et al. 2007), since DNA was not detected. However, Baudoin et al. (2010) were also unable to amplify the *Azospirillum lipoferum* strain CRT1 using primers designed from the ITS1 region of this bacteria directly on soil DNA extracts. They subsequently performed a nested PCR reaction starting with the universal 16S-23S primers FGPS1490-72/FGPL132-38 on the same soil and then obtained a band corresponding to that of strain CRT1 with the fCRT1/rCRT1 primers. Using a similar nested PCR method with the fPs16S/rPs23S primers with the PMK4-ITS1 primers may improve amplification of PMK4 from desiccated sources by amplifying low copy DNA in the first step. Alternatively, the accurate detection of PMK4 may rely on an adequate storage condition for samples destined for detection of this bacterium using strain-specific primers.

PMK4 survives long term storage on plant samples at -80°C. Wheat root samples obtained from the glasshouse trial (Chapter 5), and field pea nodules (Chapter 3) that had been washed and stored at -80°C, were subsequently crushed, and PMK4 was detected using the PMK4-ITS1 primers. Live PMK4 bacteria were cultivated from the wheat root crush by plating it out on the TY antibiotic agar plates after several months

storage at -80°C. The recovery of PMK4 at all three sections (top, middle, bottom) of the root system implies that PMK4 is able to colonise the entire length of the root system of the wheat plants under the conditions of the glasshouse study. Since the soil used in the glasshouse trial was pasteurized rather than sterilized, a mixed bacterial population was obtained on the antibiotic amended TY agar plates for both the PMK4 inoculated and uninoculated roots. Therefore the cultures used as a template for the PMK4-ITS1 primer contained several different types of bacteria and demonstrated that PMK4 can be specifically detected from a mixed bacterial sample. However, the PMK4-ITS1 primers were not effective when the root crush was used as a direct template for the PMK4-ITS1 primers. Soil can inhibit PCR reactions (Braid et al. 2003; Tsai and Olson 1992), and residual soil still adhering to the roots after the washing process or on agar plates (well 13 in Figure 7.8) may have inhibited the PCR reactions in the current study.

While a technique or commercial kit specifically designed for the extraction of DNA from soil or plant samples may overcome the inhibition of PCR reactions by soil particles, it may not be clear if the DNA obtained from these samples is from live or dead cells. However, plating out of the root crush onto the agar plates demonstrated the presence of viable PMK4. Using the strain-specific primers designed in this study eliminates the need to sequentially subculture individual colonies from mixed samples to obtain pure cultures before using molecular techniques (such as 16S rRNA PCR) for bacterial identification.

The sterilization procedure using 4% bleach was shown to remove the PMK4 DNA from the surface of the soybean nodules and demonstrates that PMK4 detection using the PMK4-ITS1 primers was from the interior of the nodules. Several PGPB have

previously been shown to inhabit the nodules of legumes, however, the location of the bacteria within the nodule is often not clear (Fox et al. 2011; Mhamdi et al. 2005). Techniques such as GUS or GFP labeling of the bacterium (Bloemberg 2007; Buddrus-Schiemann et al. 2010; Fan et al. 2011; Mhamdi et al. 2005) would enable a more definitive location of the bacteria within the nodules and on the plant roots.

The PMK4-ITS1 primers would also be useful for the quantitative detection of this bacterium in planta. A real-time PCR method has proven effective for the quantification of the PGPB *Herbaspirillum seropdedicae* SmR1 in the rhizosphere of maize (Pereira et al. 2014), for *Azospirillum lipoferum* in maize (Couillerot et al. 2010a) and for *A. brasilense* in maize (Couillerot et al. 2010b) and wheat (Stets et al. 2015) and this technique could be applied with the PMK4-ITS1 primers.

Furthermore, the detection limit of the PMK4-ITS1 primer set has not been ascertained in this study. This could be determined using a similar method to Baudoin et al. (2010) which involves inoculating soil microcosms with various levels of a particular isolate then performing PCR assays using DNA extracted from the soil. This technique would also enable the refinement of the PCR conditions, such as using a nested PCR reaction, to optimize detection of the isolate from field sources.

To conclude, the PMK4-ITS F1 primer set was highly specific for the PGPB isolate *P. granadensis* PMK4. This primer set facilitated the detection of PMK4 from the nodules of legumes grown in the glasshouse and field as well as the root system of glasshouse grown wheat. To optimize the detection of PMK4 from rhizosphere soil, a nested PCR approach needs to be explored. Furthermore, development of a real-time PCR method using these primers will allow the quantitative monitoring of PMK4 inoculated on a variety of host plants in the field over time.

Chapter 8:
General Discussion

Peas at the Brookton (2015) field site. Left: Uninoculated, Centre: PMK4 and rhizobia co-inoculated, Right: rhizobia only, right. (Photo Tom Edwards).

8.1 Key Findings

This study demonstrated that plant growth-promoting bacteria (PGPB) can be successfully isolated from the target plant species growing in the field. For instance, the bacterium *P. granadensis* PMK4 was isolated from the rhizosphere of peas at Meckering in Western Australia and was selected as a potential isolate due to the high level of indole-3-acetic acid (IAA) equivalents it produced in culture media. When subsequently inoculated on peas at two different agricultural field sites in Western Australia, this PGPB increased nodulation when co-inoculated with the current commercial rhizobial inoculant for peas at both sites and increased grain yields at the Brookton site (Chapter 3). In Australia, there is an increasing problem of agricultural soils becoming more acidic (Gazey and Davies 2009; Scott et al. 2000) and the rhizobial inoculant used in the current study, *Rhizobium leguminosarum* bv. *viciae* SU303, has demonstrated poor efficiency for N_2 fixation and poor saprophytic competence in acidic soils (Evans 2005). The results of this study suggest that PMK4 is able to ameliorate the effects of soil acidity on SU303 nodulation and potentially nitrogen fixation in peas. Shoot matter was collected from the field experiment with peas in Brookton, Western Australia (Chapter 3) to analyse ^{15}N natural abundance. This will demonstrate if nitrogen fixation was enhanced in the peas during co-inoculation with PMK4 and SU303 and the results will be available in early 2016.

Additionally, PMK4 was able to improve the yield of soybean and mung bean in the challenging environment on Christmas Island (Chapter 4), possibly through improving nutrient uptake in these plants. Therefore, PMK4 appears to be effective on a number of legume species on different soil types under widely different climatic conditions. This means that there is potential for this isolate to improve legume yields beyond

Western Australia and may prove beneficial to soybean in Africa where crop production is often limited due to the cost of fertilizer (Sinclair et al. 2014).

In many areas, wheat or other cereals are rotated with legumes and therefore it is important to establish that a PGPB treatment that is effective on a legume species is not detrimental to the following crop. When PMK4 was bacterized *in vitro* on sterile wheat seed at a high level of inoculum (OD_{600} 0.5), germination was significantly reduced compared with the controls (Chapter 2). However, at a lower inoculum level (OD_{600} 0.15), a significant increase in wheat seedling root lengths was observed when compared with the controls. Furthermore, PMK4 was tested in four field sites on two different wheat cultivars and did not affect grain yields at any of these sites. This suggests that PMK4 would be a suitable inoculant for legumes in a rotation system.

This study also demonstrated that isolates from non-target plant species may prove beneficial to plant productivity. The bacterium *Enterobacter soli* ANMK1 was isolated from the rhizosphere of native plants at Meckering on the basis of the ability to grow on media containing 1-aminocyclopropane-1-carboxylic acid (ACC) as a sole nitrogen source (Chapter 2). When inoculated on wheat in the Boyup Brook field trial (Chapter 3), it improved grain yields by 9% compared with the controls. Additionally, the bacterium *Burkholderia caledonica* NCH45, that was originally isolated from native plant species, improved the yield of wheat at the Wongan Hills heavy soil site (23% increase), but not at the Boyup Brook site or the Wongan Hills light soil site. It is possible that this isolate was not successful at all the field sites due to the carrier (Alosca^{TM}) being potentially unsuitable for this isolate. The alkalinity of this clay based inoculant, may be unsuitable for *Burkholderia* spp. (Stopnisek et al. 2014).

Inoculant technologies that may be more suitable for this organism include liquid formulations such as culture media or water; organic formulations including peat, compost, coir, sawdust; bran and manures; perlite; vermiculite; lyophilized and oil dried cultures; and alginate beads to name a few (Bashan et al. 2014). Choosing the right inoculant technology for this and other promising strains isolated in the current study will not only focus on the ability to maintain viable cells numbers over time and under a variety of storage conditions, but will also take into account the target soil conditions, ease of use and the appropriate seeding equipment (Bashan et al. 2014).

Soils from the rhizosphere of five different types of plants were screened for isolates capable of using ACC as a sole nitrogen source in Dworkin and Foster (DF) agar and 13 isolates were obtained. However, only *Stenotrophomonas maltophilia* APMK2Y, was able to utilize ACC in DF salts medium using the ACC consumption assay (Li et al. 2011). Several of the isolates selected based on auxin production were also assessed for ACC consumption in liquid DF medium and the only isolate that utilized ACC was *B. caledonica* NCH45. As some isolates do not demonstrate ACC deaminase activity in free-living states (Ma et al. 2003b; Nascimento et al. 2012a; Nukui et al. 2006), the isolates were analysed for the presence of the *acdS* (ACC deaminase) gene and the presence of this gene was detected in *B. caledonica* NCH45 using the DegACCf/DegACCr primer set (Hontzeas et al. 2005). However, bands of the expected size, as well as some non-specific banding, were also observed in some of the other isolates using these primers. At the time of writing, the amplified gene(s) were not sequenced, and this primer set has recently been shown to additionally amplify *acdS* homologs (Li et al. 2015). None-the-less, the ACC consumption assay proved to be a rapid screening technique for putative ACC deaminase isolates which can be confirmed

using the consensus-degenerate hybrid oligonucleotide primers developed by Li et al. (2015) that demonstrate specificity for the *acdS* gene but not their homologs.

The ability to colonise the root system of the host plant is possibly the most important trait to identify in a potential PGPB isolate. The isolates PMK4 and NCH45 were able to colonise the rhizosphere of wheat in a novel root colonization assay developed in this study (Chapter 2). The use of sand culture tube method improved the germination and growth of the wheat seedlings compared with the agar-tube method and the 2,3,5-triphenyltetrazolium chloride dye (TTC) agar overlay improved visualization of the bacteria on the roots. This is a rapid method for the screening of potential PGPB isolates for rhizosphere colonization on the target plant species and is more accurate for the detection of bacteria on wheat seedlings than methods such as the agar-tube method of Silva et al. (2003).

A molecular method to detect the presence of PMK4 was developed using primers based on the 16S-23S rRNA internal transcribed spacer (ITS1) in the current study (Chapter 7), and enabled the rapid and specific detection of PMK4 in the nodules of glasshouse-grown soybean and field-grown peas. This was performed using DNA direct from crushed, surface sterilized nodules, which eliminates the need for costly DNA extraction methods or successive sub-culturing on agar media to obtain pure colonies. Although other researchers have developed strain specific primers using the ITS region (Baudoin et al. 2010; Norini et al. 2013; Tan et al. 2001), to our knowledge, this is the first instance where a PGPB has been detected from the interior of a field grown nodule using strain-specific primers. These primers also specifically detected PMK4 in a mixed culture of bacteria obtained from the crushed roots of wheat grown in

the glasshouse. These results demonstrate that PMK4 successfully colonised both the nodules of selected legume species and the wheat system of roots.

Agricultural production in Western Australia is frequently limited by phosphorus availability (Quinlan and Wherett 2014). As phosphorus solubilisation was demonstrated *in vitro* by NCH45 and PMK4 (Chapter 2), glasshouse trials were conducted to determine if the growth promotion induced by these bacteria is through this mechanism. To draw conclusions, it is essential that the effect of the bacteria is assessed over a complete range of phosphorus levels that includes both sub and super-optimal levels of phosphorus. In these glasshouse trials, NCH45 increased shoot biomass in the Wongan Hills Type B soil compared with the controls at sub-optimal applied P and PMK4 and NCH45 increased shoot biomass at different super-optimal P levels (Chapter 5). The immediate assumption would be that the PGPB are solubilising P and improving biomass through enhanced P uptake in the plants, however, shoot nutrient analysis did not support this. Additionally, the increased biomass facilitated by the PGPB at the higher rate of P implies a mechanism other than P solubilisation is responsible, as P is not limiting at these levels. This demonstrates that in order to determine if P solubilisation is the mechanism of action, a full P response curve, as utilized in the current study, needs to be performed. Many studies with putative P-solubilising PGPB do not perform this type of glasshouse study, and therefore, the increased uptake of P that is demonstrated in these studies may be due to another mechanism such as improved root system architecture rather than P solubilisation.

The concentration of most of the nutrients analysed in the glasshouse trial, including P, were reduced in PGPB inoculated plants, suggesting that these nutrients were diluted when plant biomass increased under PGPB inoculation. Exceptions to this were boron,

manganese, nitrate and total N. In fact, total N uptake was significantly increased when wheat was inoculated with PGPB although it is not clear how this is effected by the bacteria. However, recent research on the effect of PGPB on nitrogen uptake in various plants may provide some clues.

For instance, when co-cultivated with *Burkholderia* sp., the nitrogen status of grain amaranth was enhanced and this coincided with the upregulation of genes involved in nitrogen transport (*AhNRT.1.1*) as well as those involved in sugar transport and metabolism (*AhSUT1*, *AhBAMY1*) (Parra-Cota et al. 2014). Similarly, the *NRT2.5* and *NRT2.6* (from the *NRT2* family of nitrate transporters) genes are essential for *P. brassicacearum* STM196 induced plant growth promotion of *Arabidopsis* however *NRT2.5* plays an essential role in adult plants under N starvation, along with other members of the *NRT2* family of genes, to ensure the efficient uptake of NO^{3-} (Lezhneva et al. 2014). The fact that these genes are up-regulated in the shoots of plants challenged with STM196, even under conditions of adequate NO^{3-} supply, indicates that the genes are responding to a systemic signal between the roots and shoots (Kechid et al. 2013). The authors postulate that STM196 may affect IAA distribution, but another possibility is that sucrose is the signaling molecule responsible for these responses as sucrose appears to fine tune auxin sensitivity and distribution during plant development (Stokes et al. 2013).

Although a complete wheat reference genome is not currently available (Talboys et al. 2014), several putative genes involved in nitrate transport been identified (Buchner and Hawkesford 2014; Melino et al. 2015). These may help identify if the PGPB in the current study are inducing the upregulation of nitrate transporters in the wheat plants. An assessment of nitrate influx, as described by Melino et al. (2015), may further

confirm that increased N uptake is a potential mechanism of action by these bacteria. Furthermore, recent studies have described various members of the *AUX1/IAA* and *PIN* gene families that are involved in auxin signal transduction and transport respectively in wheat (Singla et al. 2006; Talboys et al. 2014). Expression studies using these gene families may help identify if the effect of the PGPB on wheat root systems is through manipulation of auxin transport or signaling.

As these methods were not available to the author at the time, a simple method was developed to determine if there is a role for auxin production by the bacteria in altering the wheat root system. In growth pouch assays, the root length of wheat plants co-cultivated with NCH45 and the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) were reduced compared with plants inoculated with NCH45 alone. This suggests that there is a role for auxin production by NCH45 in the manipulation of wheat root systems, and knock-out mutants in auxin production in conjunction with the expression studies in the *AUX1/IAA* and *PIN* gene families in wheat may resolve this. Mutants in ACC deaminase production by this bacterium may also elucidate if this enzyme plays a role in plant growth promotion by this bacterium.

Combined application of PMK4 and TIBA had no effect on wheat root lengths compared with plants inoculated only with PMK4. It is possible that PMK4 produced a level of IAA that was sufficient to overcome inhibition of root lengths as demonstrated in the experiments with TIBA (10 µM) applied at increasing levels of IAA. Alternatively, the high level of IAA-equivalents measured in the colorimetric auxin assay may be due to another auxin such as IBA, that is not subject to the same transport system as IAA and therefore is not affected by the application of TIBA (Rashotte et al.

2003). This supports that the hormones produced by these two bacteria needs to be confirmed by HPLC or GCMS/MS techniques (Liu et al. 2012; Szkop et al. 2012).

8.2 Future directions

The results in the current study strongly support the further testing in field trials of PMK4 over a range of soil pH to determine if co-inoculation with PMK4 improves performance of SU303 on pea in acid soils or if this effect is pH independent. There are a number of other rhizobial inoculants for peas (Evans 2005) as well as new pea cultivars and PMK4 needs to be tested with these to determine if it can improve nodulation and yields with these inoculants and cultivars. The effectiveness of PMK4 on other legume species in replicated field trials also needs to be assessed. Furthermore, it would also be valuable to determine if PMK4 is saprophytically competent in the target soils by co-inoculating peas or another legume species with PMK4 one year and not the next. The strain-specific primers developed for PMK4 in this current study (Chapter 7), can be used directly on sterile nodule crushes from field trials to determine if PMK4 remains viable in the soil and is able to invade the nodule in successive years.

The mechanism of interaction between PMK4, the rhizobium and the legume needs to be elucidated. The soybean genome has recently been sequenced (Schmutz et al. 2010), and the auxin-inducible marker gene constructs *DR5:tdT* and *DR5:GUS* may be useful to determine if PMK4 has an effect on nodulation of soybean through modifying auxin activity. Labelling this bacterium using GUS or GFP (Bloemberg 2007; Buddrus-Schiemann et al. 2010; Fan et al. 2011; Mhamdi et al. 2005) may allow elucidation of the mode of entry and the final location of PMK4 inside the soybean and pea root and nodules and may help elucidate a potential mechanism.

The isolates that increased the yield of wheat in this study also warrant further testing in field trials. As with PMK4, these isolates need to be tested over a range of soil pH and other edaphic conditions to determine the environments in which they increase plant productivity. They also need to be applied in a variety of inoculant formulations to optimize delivery of these bacteria to the host plant(s), which may include cereals additional to wheat as well as legumes.

Additional valuable PGPB traits were observed in these isolates, such as polyamine synthesis and siderophore production, but could not be investigated here and should be explored in future research. Determining the mechanism of action through a variety of molecular, pharmacological and physiological techniques (as described above) will facilitate the appropriate connection of PGPB to the host plant species under the edaphic stresses present in their growing environment.

8.3   Conclusions

This research supports the hypothesis that harnessing the abilities of specific soil microbes may be an important contribution to plant health and productivity in the future. Current projections suggest that the human population will reach 9 billion by 2050 and combined with increasing global warming, climate change and a number of other environmental factors, sustainable food security is an issue that needs our immediate attention. Traditional research areas of plant breeding, fertilizer requirements, weed and pest control will, of course, remain very important. However, as many potentially plant beneficial microbes have been lost due to conventional agricultural practices, the hunt for plant probiotics needs to be scaled up, particularly at the local level where adaptation to the local conditions will favour the plant-microbe interaction.

In conclusion, the techniques used in this study were successful for the isolation of PGPB from Western Australian soils. Several isolates demonstrated growth promotion in different plants species, using *in vitro* assays, glasshouse and field trials. In particular isolates ANMK1 and NCH45 demonstrated significant increases in wheat yield in Western Australian field trials. Additionally, PMK4, when co-inoculated with appropriate rhizobia, improved nodulation and/or grain yields in peas, soybean and mungbean in Western Australian and Christmas Island field trials. Phosphorus solubilisation was not shown to be a mechanism of action by PMK4 and NCH45 using wheat in glasshouse trials. However, growth pouch assays suggest that enhancement of the wheat root system may be facilitated by auxin production by the bacteria and the actual mechanism needs to be elucidated in future work. The results of this study support the further testing of three promising isolates in field trials to determine the optimal environmental conditions and plant species or variety to improve agricultural crop productivity using these beneficial bacteria.

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Appendix

Appendix I – Nodulation scoring system

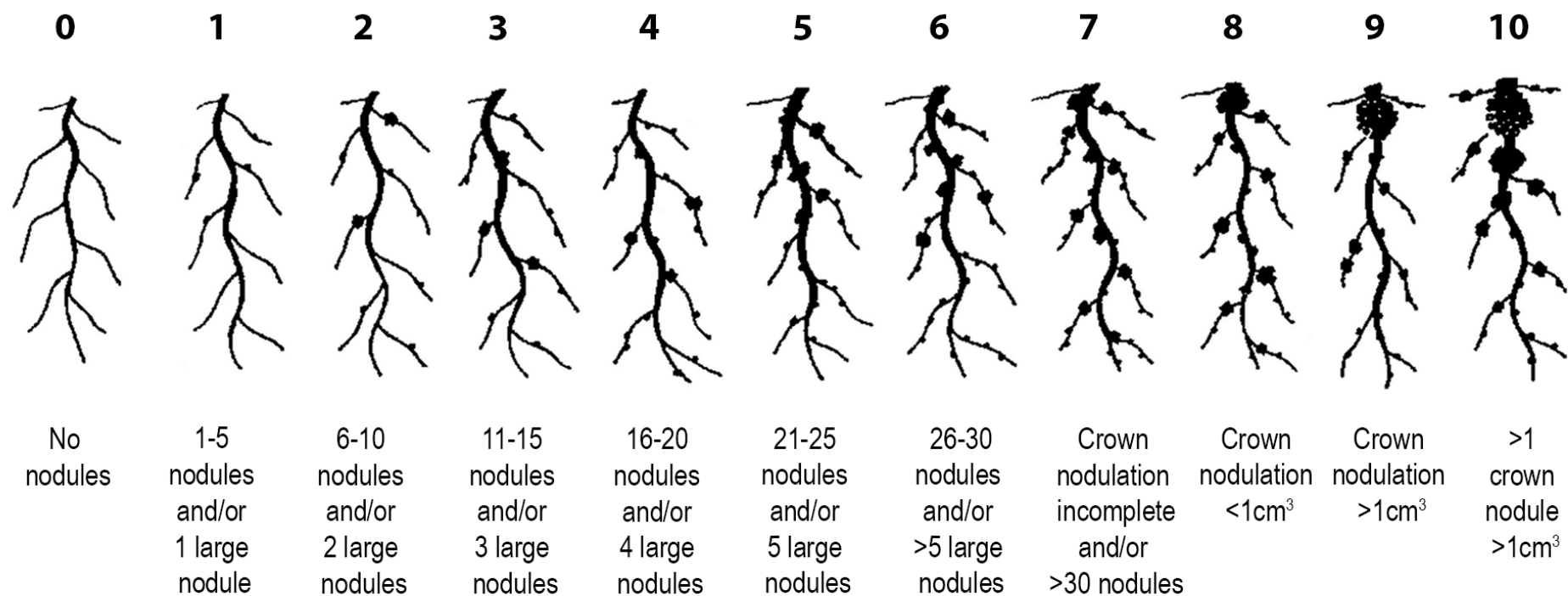| 0	1	2	3	4	5	6	7	8	9	10
No nodules	1-5 nodules and/or 1 large nodule	6-10 nodules and/or 2 large nodules	11-15 nodules and/or 3 large nodules	16-20 nodules and/or 4 large nodules	21-25 nodules and/or 5 large nodules	26-30 nodules and/or >5 large nodules	Crown nodulation incomplete and/or >30 nodules	Crown nodulation <1cm^3	Crown nodulation >1cm^3	>1 crown nodule >1cm^3

Figure 1: Nodulation scoring system used for pulse legumes (Chapters 3 & 4) (Adapted from Howieson and Dilworth 2016)

Appendix II – Nutrient analyses for wheat shoots

Table 1: Nutrient analyses for wheat shoots from Glasshouse Experiment 3 (Chapter 5). Controls are uninoculated plants, PGPB treatments include *Burkholderia caledonica* NCH45 and *P. granadensis PMK4*. Figures in brackets are standard error of the mean of 3 replicates and asterisks denote a significant ($P<0.05$) difference from the control

Applied P (mg/kg)	Boron mg/kg			Calcium %			Chloride %		
	Control	NCH45	PMK4	Control	NCH45	PMK4	Control	NCH45	PMK4
0	14.18 (0.37)	12.89 (0.73)	15.45 (2.06)	0.43 (0.02)	0.38 (0.02)	0.38 (0.02)	2.35 (0.14)	2.58 (0.32)	2.36 (0.36)
1.0	13.60 (1.16)	13.04 (0.34)	11.69 (0.65)	0.45 (0.04)	0.41 (0.02)	0.42 (0.03)	2.81 (0.23)	2.23 (0.20)	2.64 (0.07)
2.5	13.97 (0.91)	11.29 (0.81)	12.45 (0.93)	0.46 (0.02)	0.41 (0.01)	0.38 (0.02)	2.59 (0.21)	2.67 (0.19)	2.25 (0.11)
5.0	13.25 (0.77)	13.07 (0.30)	12.54 (1.25)	0.42 (0.02)	0.42 (0.02)	0.40 (0.02)	3.17 (0.12)	3.17 (0.21)	2.82 (0.02)
7.5	12.23 (0.61)	14.00 (0.96)	13.56 (2.07)	0.55 (0.01)	*0.42 (0.02)	*0.46 (0.01)	2.98 (0.15)	3.14 (0.03)	2.71 (0.11)
10.0	15.24 (1.26)	13.67 (0.77)	12.42 (0.36)	0.55 (0.04)	0.50 (0.05)	0.45 (0.03)	2.64 (0.14)	2.68 (0.13)	2.33 (0.01)
15.0	11.88 (1.25)	13.42 (1.20)	11.74 (1.39)	0.48 (0.01)	0.48 (0.03)	0.42 (0.04)	3.05 (0.05)	3.12 (0.19)	3.00 (0.25)
20.0	12.70 (1.50)	13.72 (1.15)	10.38 (0.40)	0.47 (0.05)	0.47 (0.03)	0.36 (0.03)	2.35 (0.11)	2.34 (0.10)	1.99 (0.18)
60.0	12.88 (1.03)	12.75 (1.43)	12.98 (1.49)	0.40 (0.02)	0.45 (0.04)	0.46 (0.04)	2.38 (0.16)	2.53 (0.11)	2.82 (0.17)
100.0	13.50 (1.03)	13.60 (0.72)	11.76 (0.50)	0.58 (0.04)	0.58 (0.02)	0.57 (0.02)	3.66 (0.14)	3.44 (0.13)	3.55 (0.16)

Applied P (mg/kg)	Copper mg/kg			Iron mg/kg			Magnesium %		
	Control	NCH45	PMK4	Control	NCH45	PMK4	Control	NCH45	PMK4
0	7.97 (0.37)	*6.61 (0.19)	6.74 (0.43)	96.33 (4.51)	77.29 (5.34)	78.45 (6.41)	0.23 (0.003)	*0.19 (0.009)	0.20 (0.009)
1.0	7.55 (0.17)	*6.30 (0.40)	7.17 (0.14)	87.02 (5.57)	78.50 (1.32)	83.13 (3.06)	0.22 (0.012)	0.20 (0.003)	0.21 (0.005)
2.5	6.95 (0.11)	6.22 (0.40)	6.77 (0.17)	83.28 (1.99)	77.77 (0.44)	74.92 (2.98)	0.23 (0.006)	0.20 (0.010)	0.20 (0.012)
5.0	6.95 (0.47)	6.55 (0.54)	6.85 (0.43)	81.15 (4.94)	78.67 (5.51)	83.57 (3.85)	0.21 (0.006)	0.21 (0.006)	0.20 (0.009)
7.5	8.77 (0.29)	*6.55 (0.49)	*6.90 (0.24)	80.74 (6.39)	68.82 (3.89)	78.84 (5.61)	0.22 (0.003)	*0.17 (0.003)	0.21 (0.012)
10.0	8.72 (0.39)	7.57 (0.60)	6.98 (0.13)	104.33 (6.84)	79.21 (4.47)	122.28 (38.6)	0.23 (0.015)	0.21 (0.012)	0.20 (0.007)
15.0	7.68 (0.20)	7.41 (0.34)	6.91 (0.41)	84.66 (3.44)	77.59 (3.70)	72.16 (3.72)	0.20 (0.003)	0.20 (0.010)	0.18 (0.012)
20.0	8.07 (1.09)	8.04 (0.77)	5.76 (0.27)	120.43 (5.11)	*89.78 (10.3)	*77.20 (2.47)	0.22 (0.032)	0.22 (0.019)	0.19 (0.012)
60.0	5.77 (0.44)	6.58 (0.85)	7.88 (0.76)	78.76 (7.03)	91.85 (16.6)	90.84 (4.19)	0.20 (0.006)	0.22 (0.017)	0.21 (0.015)
100.0	9.16 (0.17)	8.98 (0.12)	8.16 (0.43)	106.87 (4.23)	*79.72 (1.85)	*80.51 (4.28)	0.21 (0.009)	0.22 (0.009)	0.21 (0.003)

Table 1: (cont)

Applied P (mg/kg)	Manganese mg/kg			Nitrate mg/kg			Phosphorus %		
	Control	NCH45	PMK4	Control	NCH45	PMK4	Control	NCH45	PMK4
0	48.53 (5.03)	*32.87 (0.56)	40.23 (10.87)	3321 (151)	1908 (622)	2398 (793)	0.34 (0.012)	0.30 (0.012)	0.32 (0.012)
1.0	43.66 (2.80)	41.75 (0.67)	45.77 (4.15)	2042 (678)	2616 (353)	2071 (124)	0.34 (0.015)	0.30 (0.015)	0.33 (0.008)
2.5	41.08 (3.24)	38.44 (4.19)	35.42 (0.16)	2448 (255)	1469 (499)	2431 (71)	0.31 (0.009)	0.32 (0.009)	0.31 (0.009)
5.0	42.07 (2.14)	39.95 (0.91)	42.08 (1.50)	967 (285)	1548 (311)	1471 (54)	0.38 (0.026)	0.33 (0.021)	0.35 (0.020)
7.5	54.36 (3.46)	43.19 (4.59)	40.23 (0.90)	1777 (279)	822 (236)	1357 (398)	0.63 (0.017)	*0.44 (0.067)	*0.40 (0.010)
10.0	53.35 (4.12)	48.13 (4.04)	53.42 (2.16)	1482 (291)	1051 (231)	1265 (602)	0.58 (0.061)	0.45 (0.071)	*0.36 (0.026)
15.0	40.65 (4.32)	45.18 (1.98)	38.53 (1.57)	605 (193)	840 (338)	359 (43.8)	0.45 (0.018)	0.41 (0.023)	0.40 (0.030)
20.0	48.64 (6.24)	43.61 (3.26)	34.04 (2.81)	1300 (622)	1113 (381)	1891 (487)	0.46 (0.033)	0.42 (0.023)	*0.31 (0.015)
60.0	37.11 (3.48)	43.61 (7.82)	55.12 (8.92)	2557 (692)	1659 (169)	*928 (169)	0.31 (0.027)	0.32 (0.035)	0.38 (0.032)
100.0	52.34 (4.45)	66.16 (2.67)	60.48 (3.90)	999 (430)	1289 (160)	875 (233)	0.69 (0.041)	0.69 (0.021)	0.69 (0.064)

Applied P (mg/kg)	Potassium %			Sodium %			Sulfur %		
	Control	NCH45	PMK4	Control	NCH45	PMK4	Control	NCH45	PMK4
0	6.84 (0.12)	*5.73 (0.08)	*6.11 (0.21)	0.02 (0.003)	0.01 (0.003)	0.01 (0.003)	0.41 (0.012)	*0.32 (0.013)	0.37 (0.019)
1.0	6.18 (0.15)	5.84 (0.08)	6.11 (0.18)	0.03 (0.000)	0.02 (0.003)	0.02 (0.005)	0.37 (0.003)	*0.32 (0.012)	0.34 (0.006)
2.5	6.22 (0.16)	5.57 (0.33)	5.88 (0.23)	0.02 (0.003)	0.02 (0.003)	0.02 (0.003)	0.35 (0.013)	0.31 (0.017)	0.33 (0.009)
5.0	6.07 (0.32)	6.03 (0.40)	5.99 (0.16)	0.02 (0.003)	0.02 (0.000)	*0.03 (0.003)	0.33 (0.015)	0.32 (0.009)	0.32 (0.006)
7.5	6.00 (0.34)	4.83 (0.25)	5.44 (0.34)	0.01 (0.000)	0.01 (0.000)	0.01 (0.000)	0.34 (0.013)	*0.26 (0.010)	0.32 (0.007)
10.0	6.26 (0.23)	5.88 (0.56)	5.55 (0.31)	0.02 (0.003)	0.01 (0.003)	*0.01 (0.000)	0.36 (0.012)	0.35 (0.012)	0.32 (0.013)
15.0	5.67 (0.07)	5.64 (0.40)	5.19 (0.38)	0.02 (0.000)	0.01 (0.003)	0.01 (0.003)	0.31 (0.003)	0.30 (0.015)	0.28 (0.015)
20.0	6.21 (0.58)	5.92 (0.53)	5.33 (0.10)	0.02 (0.003)	0.02 (0.003)	0.02 (0.003)	0.35 (0.036)	0.35 (0.032)	0.30 (0.020)
60.0	5.40 (0.21)	5.72 (0.47)	6.01 (0.31)	0.02 (0.003)	0.02 (0.006)	0.01 (0.003)	0.31 (0.006)	0.33 (0.032)	0.35 (0.020)
100.0	6.02 (0.14)	5.77 (0.07)	5.68 (0.03)	0.02 (0.000)	0.02 (0.000)	0.02 (0.000)	0.32 (0.003)	0.30 (0.009)	0.30 (0.012)

Table 1: (cont)

Applied P (mg/kg)	Total Nitrogen %			Zinc mg/kg		
	Control	NCH45	PMK4	Control	NCH45	PMK4
0	3.29 (0.14)	3.49 (0.16)	*3.84 (0.12)	53.01 (4.36)	36.31 (1.11)	50.07 (10.7)
1.0	3.04 (0.08)	3.59 (0.06)	3.35 (0.17)	45.96 (4.82)	41.92 (3.24)	42.49 (2.69)
2.5	3.30 (0.07)	3.11 (0.13)	3.44 (0.14)	42.09 (2.51)	46.18 (4.70)	39.32 (0.29)
5.0	3.01 (0.09)	3.17 (0.20)	2.97 (0.20)	37.52 (3.70)	37.95 (0.75)	40.46 (3.15)
7.5	3.83 (0.65)	3.49 (0.14)	3.59 (0.17)	45.94 (2.93)	*32.09 (2.87)	*35.15 (1.94)
10.0	3.39 (0.02)	3.46 (0.22)	3.63 (0.15)	40.80 (3.94)	36.14 (2.54)	44.17 (0.84)
15.0	3.15 (0.08)	2.94 (0.17)	2.89 (0.21)	37.32 (2.60)	34.23 (0.60)	32.42 (0.80)
20.0	3.35 (0.18)	3.42 (0.16)	3.54 (0.03)	40.84 (4.71)	40.35 (5.50)	35.02 (3.61)
60.0	3.69 (0.16)	3.40 (0.20)	3.15 (0.16)	41.72 (6.50)	33.23 (4.77)	42.73 (7.52)
100.0	3.03 (0.13)	*3.48 (0.03)	3.10 (0.08)	45.17 (2.74)	44.25 (1.59)	42.10 (3.46)

Appendix III – Genomic layout of the *Pseudomonas* sp. ITS1 region
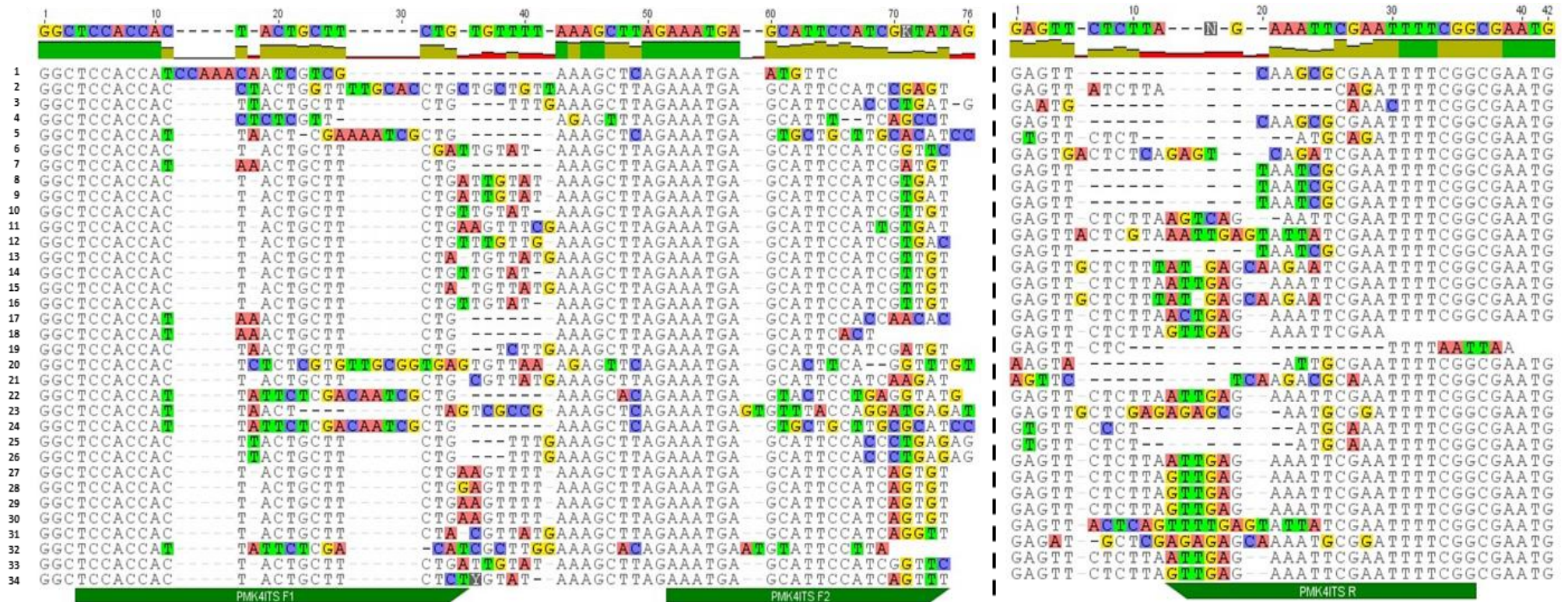

Figure 2: Genomic layout of the *Pseudomonas* species internal transcribed spacer (ITS1) region showing the detail of the nucleotide sequences to determine the PMK4-ITS1 primers (Chapter 7). The location of the PMK4-ITS1 primers are annotated in green. A region containing approximately 160 bp between the PMK4-ITS1 F2 and the R primers has been removed in order to display detail of all three primers, indicated by the dashed line. The image was created in Geneious 7.1. Sequence identities: 1. *P. aeruginosa* EVA6; 2. *P. agarici*; 3. *P. avellanae* PBIC 631; 4. *P. balearica* DSM 6803; 5. *P. chloritdismutans* AW-1; 6. *Pf* AF13704, 7. *Pf* D20; 8. *Pf* G10; 9. *Pf* J14; 10. *Pf* KD8; 11. *Pf* LMG1794; 12. *Pf* LMG5939; 13. *Pf* PSERGDNAA; 14. *Pf* PSERGDNAB; 15. *Pf* PSERGDNAC; 16. *Pf* PSERGDNAD; 17. *P. brassicacearum* WMK10; 18. *Pf* WSM 3457; 19. *P. gingeri* pf2; 20. *P. pseudoalcaligenes* ATCC17440; 21. *P. reactans* pf1; 22. *P. stutzer*i ATCC 17591; 23. *P. stutzeri* ATCC 17594; 24. *P. stutzeri* LSMN2; 25. *P syringae* 357; 26. *P. syringae* pv apii strain 212; 27. *P. tolaasii* ATCC33618; 28. *P. tolaasii* KACC10038; 29. *P. tolaasii* LMG 2342; 30. *P. tolaasii* LMG 6641; 31. *P. tolaasii* Ps 3a; 32. *P. xanthomarina* CCUG 46543T; 33. *Pseudomonas* sp. UW4; 34. *P. granadensis* PMK4 16S-23S ITS1. *Pf* = *Pseudomans fluorescens*