Enhancing the chickpea-*Mesorhizobium* symbiosis

using beneficial rhizobacteria

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Table of Contents

Publications arising from the thesis 1

Acronyms and abbreviations 2

Acknowledgement 4

Abstract 5

Declaration 10

Chapter 1. Introduction 11

1.1. Aims 13
1.2. Thesis structure 13

Chapter 2 Literature review 18

2.1. Statement of Authorship 18
2.2. Introduction 21
2.3. Legume-rhizobia-PGPR interactions 22
2.4. ACC deaminase producing NER 25
2.4.1. Nodule initiation 26
2.4.2. Rhizobial competitiveness for nodulation 28
2.4.3. Nodule function 29
2.5. Indoleacetic Acid Producing NER 36
2.5.1. Nodule primordium development 36
2.5.2. Rhizobial survival and competitiveness for nodulation 38
2.5.3. N_2 fixation and their associated gene expression 47
2.6. Transcriptional changes in free-living rhizobia 50
2.7. Suggested future research 51
2.8. Conclusions 51
2.9. References 53

Chapter 3 Large-scale screening of rhizobacteria to enhance the chickpea-Mesorhizobium symbiosis using a plant-based bioassay 68

3.1 Statement of Authorship 69
3.2. Abstract 71
3.3. Introduction 72
3.4. Materials and methods 73

3.4.1. Description of sampling sites 73
3.4.2. Collection of rhizosphere soils 74
3.4.2. Isolation of *Pseudomonas*- and *Bacillus*-like rhizobacteria 74
3.4.3. Seedling growth bioassay 75
3.4.4. IAA production 75
3.4.5. ACC deaminase production 75
3.4.6. The sequence of 16S rRNA and phylogenetic analysis 76
3.4.7. Growth pouch experiment 77
3.4.8. Data analysis 77
3.5. Results 78
3.5.1. Plant growth-promoting characteristics 78
3.5.2. Seedling vigour test 78
3.5.3. Phylogenetic diversity 79
3.5.4. Coinoculation experiment 79
3.6. Discussion 81
3.7. Conclusion 83
3.8. References 84
Chapter 4 Soil environment influences plant growth-promoting traits of isolated rhizobacteria 100
4.1. Statement of Authorship 101
4.2. Abstract 103
4.3. Introduction 104
4.4. Material and methods 105
4.4.1. Soil samples and physio climatic conditions of the sampling sites 105
4.4.2. Isolation of rhizobacteria 106
4.4.3. Determination of PGP potential of strains 106
4.4.4. Phylogenetic analysis of *Bacillus*-, *Burkholderia*- and *Pseudomonas*-like bacteria 108
4.4.5. Effect of stress on IAA production and PGPR viability 108
4.4.6. Statistical analysis 109
4.5. Results 110
4.5.1. Correlation between PGP potential of rhizobacteria with the soil environment 110
4.5.2. Correlation between plant growth-promoting traits and soil environment 111

4.5.3. Multivariate coordination....111
4.5.4. Phylogenetic diversity of isolates....111
4.5.5. The response of PGPR strains to metal ions and water stress in vitro....112
4.6. Discussion....114
4.6.1. Association between source environmental conditions and PGPR performance 114
4.6.2. Environmental conditions associated with the capacity of the rhizobacteria to produce IAA....114
4.6.3. Diversity of *Bacillus*, *Burkholderia* and *Pseudomonas* isolates from soils....115
4.7. Conclusion....116
4.8. References....116
Chapter 5 Enrichment and isolation of novel phosphate solubilizing bacteria from rhizosphere soils of chickpea....133
5.1. Statement of Authorship....134
5.2. Abstract....136
5.3. Introduction....137
5.4. Materials and methods....139
5.4.1. Soil sample collection and physico-chemical properties of soils....139
5.4.2. Enumeration of PSB communities in agricultural soils....139
5.4.3. Selection of PSB....140
5.4.4. Plant growth promoting traits....141
5.4.4. Sequencing and phylogenetic analysis of 16S rRNA....142
5.4.5. P solubilisation efficiency in liquid medium....142
5.4.5. Inorganic phosphate determination....143
5.4.6. Carboxylate production....143
5.4.7. Data analysis....143
5.5. Results....144
5.5.1. Abundance and diversity of PSB....144
5.5.2. Enrichment of P solubilizing bacteria....145
5.5.3. P solubilisation efficiency....146
5.6. Discussion....148
5.7. Conclusion....151
5.8. References....153

Chapter 6 Is phosphate solubilising ability in plant growth-promoting rhizobacteria isolated from chickpea linked to their ability to produce ACC deaminase? 169
6.1. Statement of Authorship 170
6.2. Abstract 172
6.3. Introduction 173
6.4. Materials and Methods 174
6.4.1. Soil sampling and analysis 174
6.4.2. Isolation and identification of PSB 175
6.4.3. Quantification of plant growth-promoting traits 176
6.4.4. *acdS* and 16S rDNA sequencing and phylogenetic analysis 176
6.4.5. P solubilisation efficiency 177
6.4.6. Comparative effectiveness of different carboxylates in solubilisation of P 178
6.4.7. Determination of available P 178
6.4.10. Data analysis 179
6.5 Results 179
6.5.1. Identification of ACC deaminase producing bacteria 179
6.5.2. P solubilisation efficacy 179
6.5.3. Relative efficiency of carboxylates in P solubilisation 181
6.6. Discussion 181
6.6.1. Identification of ACC deaminase producing bacteria 181
6.6.2. P solubilisation efficacy 182
6.7. Conclusion 184
6.8. References 185
Chapter 7 Ability to produce indole acetic acid is associated with improved phosphate solubilising activity of rhizobacteria 200
7.1. Statement of Authorship 201
7.2. Abstract 203
7.3. Introduction 204
7.4. Materials and Methods 206
7.4.1. Sources of isolates 206
7.4.2. Determination of IAA production 207
7.4.3. Diversity of IAA producing rhizobacteria 207
7.4.4. Experiment 1 208

7.4.5. Experiment 2 208
7.4.6. Experiment 3 208
7.4.7. P analysis 209
7.4.8. Determination of carboxylate production 209
7.4.9. Statistical analysis 209
7.5. Results 210
7.5.1. Diversity of IAA producing- and P solubilising- rhizobacteria 210
7.5.2. P solubilisation efficacy 211
7.6. Discussion 212
7.7. Conclusion 215
7.8. References 216
Chapter 8. The role of carboxylate concentration, rhizosphere pH, the reactivity of P sources, and phosphate solubilising bacteria in P acquisition by N_2 fixing chickpeas 233
8.1. Statement of Authorship 234
8.2. Abstract 236
8.3. Introduction 237
8.4. Materials and Methods 239
8.4.1. Soil collection 239
8.4.2. Isolation of bacteria 239
8.4.3. Phylogenetic diversity of phosphate solubilising bacteria 239
8.4.4. Identification of phosphate solubilising bacteria 240
8.4.5. Type of rock phosphate 240
8.4.6. Experiment 1. The solubility of rock phosphate in carboxylate solutions 240
8.4.7. Experiment 2. Microbial rock phosphate solubilisation 241
8.4.8. Experiment 3. The solubility of rock phosphates in chickpea root exudates 241
8.4.9. Experiment 4. Plant inoculation experiment 242
8.4.10. Determination of available P in the supernatant 243
8.4.11. Data analysis 243
8.5. Results 244
8.5.1. The release of P from rock phosphate by efficient P solubilisers 244
8.5.2. The solubility of rock phosphate in carboxylates 245
8.5.3. The solubility of rock phosphate in root exudates of chickpea cultivars 246
8.5.4. Effect of PSB inoculation on the Chickpea-*Mesorhizobium* symbiosis 246
8.6. Discussion 248

8.7. Conclusion....252
8.8. References....253
Chapter 9. General discussion....270
9.1. Conclusions and recommendations....273
9.2. Future research....274
9.3. References....276
10. Appendixes....280
11. Media and reagents....290

Publications arising from the thesis

Published peer reviewed articles

1. Alemneh AA, Zhou Y, Ryder MH, Denton MD. 2020. Mechanisms in plant growth promoting rhizobacteria that enhance legume-rhizobia symbiosis. *Journal of Applied Microbiology* 129:1133-1156. http://doi:10.1111/jam.14754.
2. Alemneh AA, Zhou Y, Ryder MH, Denton MD. 2021. Pre-screening of rhizobacteria to enhance the chickpea-*Mesorhizobium* symbiosis using plant based and plant growth-promoting traits. *Rhizosphere* 18:1-11. https://doi.org/10.1016/j.rhisph.2021.100361.
3. Alemneh AA, Zhou Y, Ryder MH, Denton MD. 2021. Is phosphate solubilising ability in plant growth-promoting rhizobacteria linked to their ability to produce ACC deaminase? *Archives of Microbiology* https://doi.org/10.1007/s00203-021-02364-w.
4. Alemneh AA, Cawthray GR, Zhou Y, Ryder MH, Denton MD. 2021. Ability to produce indole acetic acid is associated with improved phosphate solubilising activity of rhizobacteria. *Journal of Applied Microbiology* http://doi:10.1111/jam.15108.

Acronyms and abbreviations

A	Absorbance
ACC	1-aminocyclopropane-1-carboxylate
AGRF	Australian Genome Research Facility
ANOVA	Analysis of variance
ARA	Acetylene reduction assay
BLAST	Basic local alignment search tool
BNF	Biological N_2 fixation
cm	Centimetre
cv.	Cultivar
dS m^{-1}	deciSiemens per metre
DTPA	Diethylene triamine pentaacetic acid
DW	Dry weight
EC	Electrical conductivity
FAOSTAT	Food and Agriculture Organisation Statistics
g	Gram
h	hour
ha	hectare
h	hour
HPLC	High Performance Liquid Chromatography
HSD	Honestly significant difference
ICP	Inductively coupled plasma spectrometry
LSD	Least standard deviation
Kg ha^{-1}	Kilogram per hectare
K_M	Michaelis constant
mM	Millimolar
M	Molar
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram
µg L^{-1}	Microgram per litre
µmol $plant^{-1}$	Micromole per plant
µmol $m^{-2}s^{-1}$	Micromole per square metre per second

Microeinsteins $m^{-2}s^{-1}$	Microeinsteins per square metre per second
µL	Microlitre
µm	Micrometre
min	Minute
mL	Millilitre
mm	Millimetre
mM	millimolar
mmol L^{-1}	Millimole per litre
Mpa	Megapascal
N	Normality
NBRIP	National Botanical Research Institute's Phosphate
NER	Nodule enhancing rhizobacteria
nm	Nanometer
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDA	Photodiode array detector
PEG	Polyethylene glycol
PET	Potential evapotranspiration
PGP	Plant growth promoting
PGPR	Plant growth promoting rhizobacteria
PSB	Phosphate solubilising bacteria
PSI	Phosphate solubilising index
rpm	Revolution per minute
s	Second
SARDI	South Australian Research and Development Institute
Tg	Teragram
TSA	Tryptone soy agar
TSB	Tryptone soy broth
UNEP	United Nations Environment Programme
v/v	Volume per volume
w/w	Weight per weight

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Abstract

Chickpea is a major pulse crop grown in the Australian cropping system. It can fix a substantial amount of nitrogen (N) when it forms a symbiotic association with highly effective *Mesorhizobium* spp. Phosphorus (P) is an important nutrient for efficient chickpea-*Mesorhizobium* symbiosis. Chickpea exudes large amounts of carboxylates that can mobilise P from sparingly soluble P sources. Additionally, a number of bacteria associated with plant roots are capable of solubilising P, and these bacteria are generally called phosphate solubilising bacteria (PSB). Such bacteria that are able to promote plant growth more generally are designated as plant growth-promoting rhizobacteria (PGPR). However, PSB do not always enhance the chickpea-*Mesorhizobium* symbiosis under different P conditions. Additionally, the responsible plant growth-promoting (PGP) mechanisms have not always enhanced P solubilisation. Therefore, this study was investigated whether efficient PSB could enhance the chickpea-*Mesorhizobium* symbiosis in a widely varied P condition. Firstly, this study was tested whether pre-screening methods result in efficient PSB, if selected efficient P solubilisers had the ability of PSB to solubilise P from a wide array of P sources, and if the expression of PGP characteristics can directly and indirectly affect the plant P nutrition. Additionally, plant-related factors that may affect chickpea P nutrition were investigated. Accordingly, this study was hypothesised that the presence of high carboxylate concentrations and acidic pH in the chickpea rhizosphere may affect the efficiency of PSB.

Seventy-four soil samples collected from major agricultural lands across Australia were used in this research. Major soil chemical and physical properties were examined. A total of 743 isolates of *Bacillus*- and *Pseudomonas*-like bacteria were isolated using taxonomically selective methods of extraction. Based on 16S rRNA sequences, these isolates were closely related to diverse species of *Bacillus*, *Pseudomonas* and *Burkholderia* spp. (formerly classified as *Pseudomonas* spp.). Of 743 isolates, 616 (83%) were able to produce IAA in the presence of L-tryptophan. 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity was detected in 57 isolates (7.7%) *in vitro*. All isolates were further tested for their ability to promote seedling growth. Most of these isolates (71%) were able to promote seedling root elongation. The effect of isolates on seedling growth predicted their effect on nodulation and growth of chickpea after dual inoculation in aseptic conditions. The effect of rhizobacteria on seedling root growth and chickpea-*Mesorhizobium* symbiosis was associated with their capacity to produce IAA and ACC deaminase. The synthesis of IAA along with ACC deaminase activity

by rhizobacteria gave an added advantage by promoting the ability of rhizobia to form an efficient chickpea-*Mesorhizobium* symbiosis as measured by biomass production and nodulation. Most of the isolates able to express both PGP traits belonged to the genus *Burkholderia*. Hence, the use of a plant-based first-stage screening strategy in combination with assays for *in vitro* production of IAA and ACC deaminase enabled the identification of efficient PGPR that were able to enhance the legume-rhizobia symbiosis.

The influence of soil, climatic and crop management variables on the occurrence of PGPR and their PGP characteristics was examined. IAA production by isolates and their beneficial effects on chickpea root elongation were associated positively with soil copper, manganese and zinc concentrations and the aridity index, and negatively with soil carbon (C), N, C/N ratio, Ca and P at the sampling sites from which the PGPR were isolated. Additionally, the P solubilisation activity of the isolates was also negatively correlated with C/N ratio, N, P, C and magnesium content of the soils. However, none of the investigated soil environment variables were correlated with the potential of the isolates to express ACC deaminase. A greater proportion of IAA producing PGPR had a greater ability to withstand metal ion toxicity and water stress. Therefore, these findings have potential application in designing a strategy for the development of efficient PGPR that have ecological traits and plant growth-promoting mechanisms that may increase chickpea production.

An additional 98 novel strains of P solubilising bacteria were isolated through enrichment in media with AlO_4P, $Ca_3(PO_4)_2$ or FeO_4P. Following enrichment, the proportion of PSB among total culturable bacteria was significantly increased compared with the PSB population in the original soils. These isolates were assigned into twenty-five bacteria genera based on 16S rRNA sequences. The majority of these isolates belonged to *Burkholderia*, *Variovorax*, *Leifsonia*, *Pantoea* and *Rhizobium*. These isolates had a greater P solubilisation index (PSI) than those obtained using the taxonomically selective method. From both isolation methods, seven isolates, namely *Peribacillus simplex* 37F, *Bacillus megaterium* 8F, *Pseudomonas fluorescens* 27F, *Bacillus pumilus* 98F, *Bacillus cepacia* 126F, *Burkholderia* sp. 12F and *Burkholderia cenocepacia* 127F were proportionally selected, based on their PSI values, to investigate their P solubilisation ability in liquid media. Generally, isolates obtained following enrichment were able to solubilise between 1.2 to 2.8, 1.2 to 3.1 and 1.5 to 4.5-fold Pi from AlO_4P, $Ca_3(PO_4)_2$ or FeO_4P than those obtained using the taxonomically selective method. The P solubilising efficiency was related to citrate and α-ketoglutarate production *in vitro*.

Enriching rhizobacteria by culture in conditions with sparingly soluble P increased the likelihood of isolating elite PSB from bulk soils and plant rhizospheres.

The relationship between the phosphate solubilising ability of plant growth-promoting rhizobacteria and their ability to produce ACC deaminase was analysed. Five isolates, namely *B. megaterium* 8F, *B. pumilus* 98F, *B. cepacia* 126F, *Burkholderia* sp. 12F and *B. cenocepacia* 127F were selected based on their potential for P solubilisation and their ability to express ACC deaminase. Generally, ACC deaminase had no role in AlO_4P and FeO_4P solubilisation. However, the amount of ACC deaminase produced by PSB was significantly associated with the liberation of Pi from $Ca_3(PO_4)_2$ when ACC was the sole N source. $Ca_3(PO_4)_2$ solubilisation was associated with the extent of acidification of the medium. Additionally, α-ketobutyrate by itself was able to solubilise significant amounts of Pi from AlO_4P, $Ca_3(PO_4)_2$ and FeO_4P. Conversely, the P solubilisation potential of PSB was independent of their ability to express ACC deaminase activity when $(NH_4)_2SO_4$ was the sole N source.

The ability of efficient PSB isolates, namely *Burkholderia* sp. 12F, *P. fluorescens* 27F and *B. cenocepacia* 127F, selected based on their ability to solubilise P from different rock phosphates (as above), was investigated. Results showed the highest potential of *Burkholderia* sp. 12F in P solubilisation from Boucraa, Togo, Sechura and Weng Fu rock phosphates. All bacterial isolates poorly solubilised Phalaborwa, Peru or Vietnam rock phosphate. The solubilisation of these P sources by the PSB was not related to the amount of available and total P, nor to the concentration of Al^{3+}, Fe^{2+} and Cd^{3+} in the rock phosphate. Additionally, their solubility in carboxylates was varied and higher solubility was recorded in di- and tri-carboxylates than for mono-carboxylates added separately. The variation in P solubilising activities between *Burkholderia* sp. 12F, *B. cenocepacia* 127F and *P. fluorescens* 27F was not associated with acidification of their culture media. The highest P solubilising activity of *Burkholderia* sp. 12F was related to its ability to produce citrate, malate and maleate during mineral phosphate solubilisation.

The effect of IAA and its precursor L-tryptophan on the P solubilising activity of rhizobacteria, namely *B. pumilus* 98F, *B. cenocepacia* 127F, *Burkholderia* sp. 12F and *P. fluorescens* 27F, was investigated. The ability to produce IAA was related to the improved potential of PSB to solubilise P from rock phosphate. The addition of L-tryptophan to growth media improved the P solubilising activity of PSB that were able to produce IAA. A remarkable effect of this precursor on P solubilisation was observed for *B. cenocepacia* 127F and *Burkholderia* sp. 12F,

that produced 41.9 and 54.3 μg mL^{-1} IAA, respectively. Additionally, the potential of *Burkholderia* sp. 12F to solubilise rock phosphate was increased with increasing IAA concentration in the media. This effect was connected to the reduction of pH and release of high concentrations of carboxylates, comprising α-ketoglutarate, cis-aconitate, citrate, malate and succinate. IAA solution by itself was able to liberate Pi only between 1.45 to 3.00 μg Pi L^{-1} from rock phosphate. Therefore, increased production of organic acids rather than IAA production per se may be the possible mechanism by which IAA ultimately resulted in the improved capacity of PSB in P solubilisation.

Based on the above experiments, *Burkholderia* sp. 12F was selected to examine its P solubilising activity in chickpea root exudates and its effect on the chickpea-*Mesorhizobium* symbiosis. The ability of the root exudates obtained from six chickpea cultivars to mobilise P was tested in the presence and absence of PSB. In particular, the root exudates were able to solubilise Togo rock phosphate but not Peru rock phosphate *in vitro*. In this case, the amount of solubilised P by root exudates was not related to the extent of acidity in the root exudates before and after incubation. The presence of PSB significantly increased the amount of solubilised P in all root exudates from both rock phosphates.

The efficiency of *Burkholderia* sp. 12F to alter the chickpea-*Mesorhziobium* symbiosis was tested using six cultivars. In this experiment, four P sources including Peru and Togo rock phosphate, K_2HPO_4 and the control check (no added P) were used. Inoculation of PSB significantly increased shoot and root biomass production, and nodulation of chickpea cvv. Genesis-863, PBA-Striker and PBA-Slasher but did not significantly affect Ambar, Genesis-079 and Genesis-090. Increased nodulation and growth of chickpea following inoculation of PSB were not always explained by increasing P nutrition. PSB inoculation significantly increased the P concentration in the rhizosphere of plants fertilised with Togo and Peru rock phosphate. In this case, P uptake was associated with P concentration in the rhizosphere extract. Additionally, P uptake by plants fertilised with K_2HPO_4 was increased following inoculation with PSB. In this case, the PSB did not affect the P concentration in the rhizosphere but improved root biomass. An increased P concentration in the rhizosphere following PSB inoculation was not related to the extent of acidity in the rhizosphere. More acidity in rhizosphere was instead associated with more nodulation. This may suggest that more nodulation may have a positive feedback effect on further solubilisation of P, which acidified the rhizosphere.

In conclusion, the selection of PSB following enrichment and selection of rhizosphere isolates from the genera *Bacillus*, *Pseudomonas* and *Burkholderia* provided efficient isolates able to solubilise P from diverse P sources. Plant-based screening of these isolates indicated the possible PGP traits (ACC deaminase and IAA production) that could affect chickpea growth and nodulation. These traits affected the P solubilising activity of efficient PSB *in vitro* as well. Although chickpea releases a large amount of carboxylate and has an acidic rhizosphere pH, inoculation of the PSB *Burkholderia* sp. 12F increased the nodulation, growth and P uptake of some cultivars of chickpea. This PSB isolate did not affect the cultivars that by themselves produce relatively high carboxylate concentrations. Enhancing the chickpea-*Mesorhizobium* symbiosis was mediated by multiple PGP traits, including P solubilisation and possibly IAA and ACC deaminase production. Future research is considered in the final part of this document.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Anteneh Argaw Alemneh

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Chapter 1. Introduction

Chickpea is the third most important cool-season legume after common beans and peas, with an approximate production of 17.2 million tons cultivated over 17.8 million ha (FAOSTAT, 2020). In that same period, about 0.9 million tons of chickpea was produced in Australia over 1.1 million ha of cultivated land. Chickpea is a good source of protein, energy, minerals, vitamins, fiber, and also contains health-beneficial phytochemicals in human diets (Yadav and Chen 2007). Chickpea obtains a significant proportion of its N requirement from symbiotic N_2 fixation, and has a high N-fixing efficiency of symbiosis, fixing between 55 to 72% of total plant N derived from the air (Mefti 2003). In Australia, the percentage of crop N derived from N_2 fixation ranged between 0 to 81% and total N_2 fixed from 0 to 99 kg ha^{-1} with net N balance between -47 to +46 kg ha^{-1} (Schwenke et al. 1998).

The productivity of chickpea and its ability to fix N is affected by abiotic and biotic stresses. Of those stresses, phosphorus (P) is one of the most critical nutrients limiting chickpea productivity in major chickpea growing areas (Srinivasarao et al. 2006; Ahlawat et al. 2007; Korbu et al. 2020). In particular, the legume-rhizobia symbiosis requires more P for maximum N_2 fixation activity because this process requires energy in the form of adenosine triphosphate. P deficiency suppresses N_2 fixation through decreased sucrose and hexose supply and energy status in nodules, total respiration rate and root ATP concentrations (Liu et al. 2018). In the rhizosphere, there are heterogeneous and naturally abundant rhizospheric bacteria that can solubilise forms of P otherwise unavailable ot the plant. These bacteria are known as phosphate solubilising bacteria (PSB). When PSB are able to promote plant growth, they are generally designated as plant growth-promoting rhizobacteria (PGPR) (Swift et al. 2018; Alemneh et al. 2020). Nodulation and yield of chickpea increased following inoculation of PGPR able to solubilise P, although their effect was not always linked with P nutrition (Zaidi et al. 2003; Rudresh et al. 2005; Mittal et al. 2008; Verma et al. 2012; Singh et al. 2014; Verma et al. 2014; Imen et al. 2015; Saxena et al. 2015; Israr et al. 2016; Rajwar et al. 2018). Additionally, PGPR with high potential P solubilising activity *in vitro* reported in the literature have not always enhanced chickpea-*Mesorhizobium* symbiosis under controlled environment and field conditions. Such a discrepancy in effect between *in vitro* and *in vivo* conditions may be due to many factors associated with the host plant and the PSB.

Phosphorus is assimilated by the soil microbial biomass as an essential component for crucial cellular functions. To fulfil this requirement, soil microorganisms solubilise P from sparingly soluble P source to satisfy their needs. PSB are thought to solubilise P over and above their own needs, and then directly provide P to the plant to serve its growth. Recent studies using isotope techniques have indicated that an improved P uptake following PSB inoculation was not related to an increase in P concentration in the rhizosphere (Meyer et al. 2017; Sarabia et al. 2018), suggesting the involvement of PGP traits other than P solubilising activity. A recent review paper has also suggested that P solubilising microorganisms do not solubilise adequate P to change to the crops' nutrient environment under *in vivo* conditions (Raymond et al. 2021). Therefore, the P solubilising activity of PSB to be able to serve plant growth is considered uncertain. This indicates the need for selecting the most efficient PSB capable of expressing PGP traits besides P solubilising activity that can promote plant growth and enhance nodulation.

The relatively large amount of carboxylates in the root exudate in the chickpea rhizosphere compared with other legume plants (Ohwaki and Hirata 1992; Veneklaas et al. 2003; Wouterlood et al. 2005; Pearse et al. 2007; Kabir et al. 2015), enables them to use P from insoluble soil P reserves and other poorly soluble P sources (Veneklaas et al. 2003; Pang et al. 2018; Sharma et al. 2020; Wen et al. 2020). This characteristic of chickpea may affect the ability of the PSB function in plant P nutrition. The purpose of this thesis is, therefore, to address the following questions: (1) does a seedling growth bioassay predict plant growth potential of rhizobacteria in chickpea-*Mesorhizobium* symbiosis? (2) do rhizosphere soils incubated in low P conditions become enriched in efficient PSB? (3) do soil and environmental factors predict the performance of soil bacteria in promotion of plant growth and their ability to produce IAA and ACC deaminase, and to solubilise P? (4) is phosphate solubilising ability in plant growth-promoting rhizobacteria linked to their ability to produce ACC deaminase? (5) does IAA concentration affect the potential for rhizobacteria to solubilise P *in vitro*? and (6) do the amount of carboxylates and rhizosphere pH in various chickpea cultivars and the ability of PSB to express different PGP mechanisms besides P solubilising activity determine the efficiency of PSB in improving P concentration in plants, nodulation and growth of chickpea co-inoculated with rhizobia? The relationship between these research questions is indicated as follows:

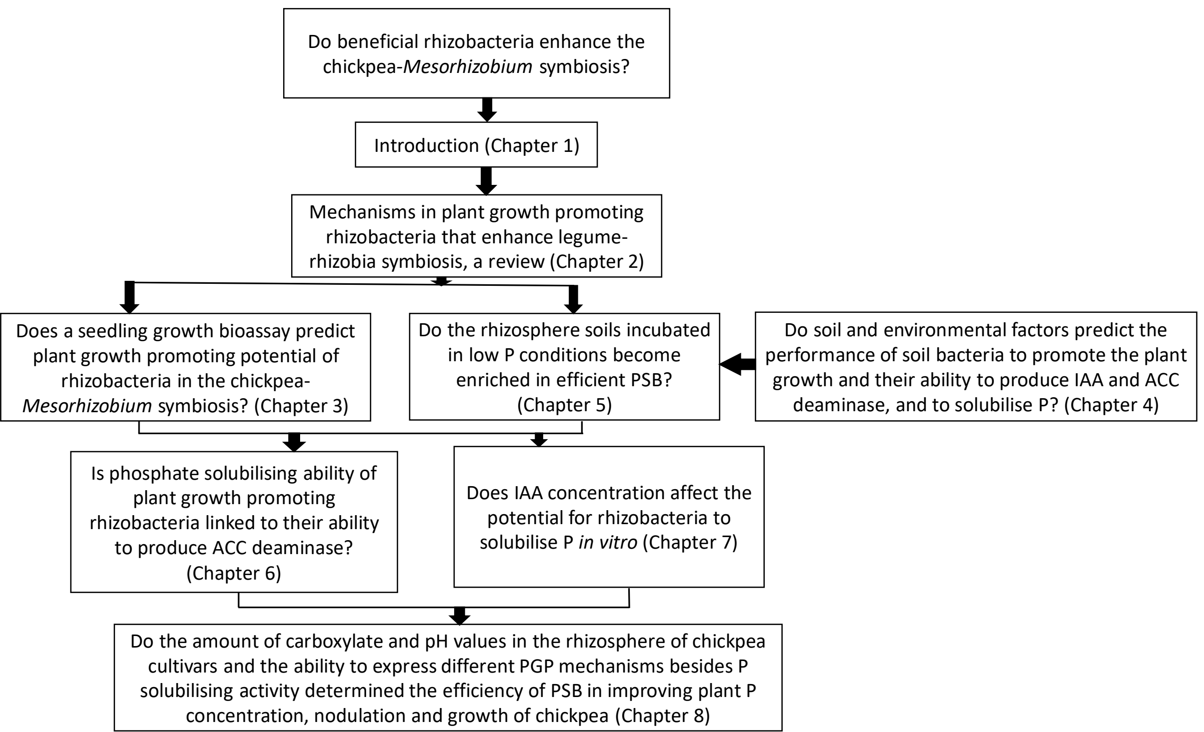

Fig. 1. Thesis structure highlighting the relationship among specific research questions for the experimental chapters

1.1. Aims

The purpose of this study was to investigate if effective PSB could enhance the chickpea-*Mesorhizobium* symbiosis through improved P nutrition. The specific questions formulated to address the overall purposes of the thesis are provided in Fig 1.

This study hypothesised that novel and efficient PSB with IAA and ACC deaminase activities would enhance the chickpea-*Mesorhizobium* symbiosis through improving P nutrition.

1.2. Thesis structure

The thesis is structured according to the questions shown in Fig. 1; each question is addressed in the associated chapter. Chapter 1 provides a general introduction, states the purpose of the research and outlines the structure of the thesis. Chapter 2 reviews pertinent literature about the theoretical background to the research and methodologies of the experiments. Chapter 3 investigates the comparative value of selecting plant growth-promoting rhizobacteria using *in vitro* or plant-based assays. Chapter 4 examines the soil and environmental factors that might predict the capacity of soil and rhizosphere bacteria to promote plant growth. Comparing the

effectiveness of PSB obtained using either enrichment or taxonomically selective methods is presented in Chapter 5. Analysing the relationship between phosphate solubilising ability of plant growth-promoting rhizobacteria and their ability to produce ACC deaminase is presented in Chapter 6. An investigation of the relationship between phosphate solubilising ability of plant growth-promoting rhizobacteria with their ability to produce IAA is presented in Chapter 7. The performance of PSB when inoculated on to chickpea cultivars with contrasting rhizosphere carboxylate concentrations and pH and root biomass is investigated in chapter 8. In Chapter 9, the significance of the results of the experiments conducted in presented in the context of the literature and potential future research activities are suggested.

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Chapter 2 Literature review

2.1. Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Anteneh Argaw Alemneh		
Contribution to the Paper	downloaded articles related to the topic, developed figures and tables, and wrote the manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	26/03/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Matthew Denton		
Contribution to the Paper	Supervised development of the review ideas and edited the manuscript		
Signature		Date	26/03/2021

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Contribution to the Paper	Supervised development of the review ideas and edited the manuscript		
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Contribution to the Paper	Supervised development of the review ideas and edited the manuscript		
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REVIEW ARTICLE

Mechanisms in plant growth-promoting rhizobacteria that enhance legume–rhizobial symbioses

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Keywords
ACC deaminase, IAA, N_2 fixation, nodule-enhancing rhizobacteria (NER), Rhizobia.

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Abstract

Nitrogen fixation is an important biological process in terrestrial ecosystems and for global crop production. Legume nodulation and N_2 fixation have been improved using nodule-enhancing rhizobacteria (NER) under both regular and stressed conditions. The positive effect of NER on legume–rhizobia symbiosis can be facilitated by plant growth-promoting (PGP) mechanisms, some of which remain to be identified. NER that produce aminocyclopropane-1-carboxylic acid deaminase and indole acetic acid enhance the legume–rhizobia symbiosis through (i) enhancing the nodule induction, (ii) improving the competitiveness of rhizobia for nodulation, (iii) prolonging functional nodules by suppressing nodule senescence and (iv) upregulating genes associated with legume–rhizobia symbiosis. The means by which these processes enhance the legume–rhizobia symbiosis is the focus of this review. A better understanding of the mechanisms by which PGP rhizobacteria operate, and how they can be altered, will provide opportunities to enhance legume–rhizobial interactions, to provide new advances in plant growth promotion and N_2 fixation.

2.2. Introduction

Biological N_2 fixation (BNF) is one of the most important microbiological processes in terrestrial ecosystems through which reduced N is introduced into the biosphere. There are three major types of BNF: free-living, associative and symbiotic N_2 fixation. In terrestrial ecosystems, approximately 33 to 46 Tg of N $year^{-1}$ is obtained through the legume-rhizobia symbiosis, which represents about 80% of the total of biologically fixed N (Herridge 2008). N_2 fixation in legume-rhizobia symbioses is facilitated by rhizobia, which are gram-negative, facultative aerobic and motile bacteria. At present, 180 nodulating species of bacteria in 21 genera have been identified as nodule forming endosymbionts (Wang *et al*. 2019). Among them, the major genera of bacteria that can form a symbiotic association with legumes are *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Neorhizobium*, *Pararhizobium* and *Allorhizobium*, collectively known as 'rhizobia'. Moreover, beta-rhizobia belonging to genera *Cupriavidus*, *Paraburkholderia* and *Trinickia* are identified; these bacterial genera form mutual interactions with *Papilionoideae* and *Caesalpinioideae* (LPWG 2017; Sprent *et al*. 2017).

The rhizosphere is an important soil chemical and ecological zone close to the roots of plants where substantial plant-microbe interactions occur (Zhou *et al*. 2020). Some of the bacteria in the rhizosphere are able to promote plant growth and protect plants from biotic and abiotic stresses are known as plant growth-promoting rhizobacteria (PGPR) (Berg 2009). Non-rhizobial bacteria that specifically improve legume-rhizobia interactions can be termed nodulation enhancing rhizobacteria (NER). The beneficial effects of NER are mediated by a variety of mechanisms including the production of diverse metabolites and enzymes from microbes and those indirectly elicited from plants due to NER activity.

Among plant growth-promoting (PGP) metabolites and enzymes, indoleacetic acid (IAA) and/or 1-aminocyclopropane-1-carboxylate (ACC) deaminase have been frequently suggested as important PGPR mechanisms that can promote plant growth (Li *et al*. 2000; Gravel *et al*. 2007; Bal *et al*. 2013). ACC deaminase is an enzyme that can decrease deleterious amounts of ethylene in higher plants. IAA is an important phytohormone that is involved in many plant developmental processes. Consequently, IAA and ACC deaminase are both important in regulating plant growth. However, plant growth inhibition can occur when plants are exposed to a high concentration of IAA in their rhizosphere (Tabatabaei *et al*. 2016). This inhibition effect could be associated with the transcription of ACC synthase (Glick *et al*. 2007) and

increased production of ethylene. This feedback inhibits IAA transport and signal transcription, suppressing the effect of IAA on plant growth. However, the presence of ACC deaminase in the rhizosphere can reduce the ethylene concentration from the plant through hydrolysis of ACC, the immediate precursor for ethylene synthesis, into NH_3 and α-ketobutyrate (Glick *et al*. 1998; Glick 2014). In this way, IAA and ACC deaminase work together and promote greater plant growth, as suggested in Fig. 1. Considerable progress has been made in our understanding of the morphological, molecular and physiological effects of ACC deaminase and IAA on plant growth and development under optimal growth and stressed conditions. However, the detailed mechanisms by which ACC deaminase and IAA, expressed either individually or simultaneously by a PGPR isolate, influence the legume-rhizobia symbiosis, remain elusive. Current knowledge of PGPR, their metabolites, and the effect of inoculation of PGPR on plant growth and development under stressed and standard growth conditions have been summarized in several reviews (Ahemad and Kibret 2014; Pérez-Montaño *et al*. 2014; Zaidi *et al*. 2015; Forni *et al*. 2017). This review focuses specifically on the roles played by IAA production and ACC deaminase in the enhancement of the legume-rhizobia symbiosis by NER and discusses possible modes of action by which these two PGP traits promote nodule induction and nodule function under normal and stressed conditions.

2.3. Legume-rhizobia-PGPR interactions

Nodule formation and the effectiveness of nitrogen fixation symbioses are partially determined by the populations of rhizobia in soils or commercial rhizobia applied at seeding and their effectiveness in N_2 fixation. Soils that have low rhizobial populations result in insufficient nodulation and N_2 fixation that can be increased by inoculation with elite rhizobia (Mapfumo *et al*. 2000; Vargas *et al*. 2000; Furseth *et al*. 2012; Batista *et al*. 2015). Legume seed inoculated with commercial rhizobial isolates need to out-compete less effective resident rhizobia to form nodules (Denton *et al*. 2002). Inoculation with known strains of effective rhizobia can increase rhizobial populations in the rhizosphere and enhance nodulation and N_2 fixation (Brockwell *et al*. 1995; Deaker *et al*. 2004; Denton *et al*. 2013; Denton *et al*. 2017). In situations where resident soil rhizobia populations are above 100 g^{-1} soil, they can cause a competitive barrier that interfere with the nodule formation by the introduced rhizobia (Thies *et al*. 1991; Brockwell *et al*. 1995). One alternative strategy to improve the efficacy of introduced and resident rhizobia can be through the combined use of NER (Tilak *et al*. 2006; Hungria *et al*. 2013).

Nodule enhancing rhizobacteria have the potential to enhance nodule formation and N_2 fixation of legumes when co-inoculated with elite rhizobia. NER improve nodulation and N_2 fixation through different direct and indirect mechanisms, including production of one or more phytohormones, most often IAA, gibberellic acid and cytokinins (Bottini *et al*. 1989; Hayashi *et al*. 2014). In addition, they can increase nutrient (mainly P and Fe) availability in the root zone (Chebotar *et al*. 2001; Argaw 2012), inhibit phytopathogens (Tavares *et al*. 2018) and modify rhizosphere chemicals, such as ethylene (Nascimento *et al*. 2018). The use of NER can significantly increase the ability of rhizobia resident in soil to form nodules, fix N and increase growth in soybean, *Glycine max* (Dashti *et al*. 1998), mung bean (Shaharoona *et al*. 2006), chickpea, *Cicer arietinum* (Abdiev *et al*. 2019), and common bean (Chihaoui *et al*. 2015). Likewise, the use of a NER, *Azospirillum brasilense*, inoculated in combination with elite rhizobial strains can increase nodulation and amount of fixed N derived from symbiosis in soybean (Hungria *et al*. 2015), common bean, *Phaseolus vulgaris* (Remans *et al*. 2008) and lentil, *Lens culinaris* (Tsigie *et al*. 2011). In particular, the co-inoculation of *Bradyrhizobium* in combination with *A. brasilense* on soybean under different soil and climatic conditions in Brazil resulted in yield increase up to 11.2% over the rhizobia inoculation alone (Hungria *et al*. 2015; Okon *et al*. 2015; Galindo *et al*. 2018). Despite this, the mechanisms by which the NER enhance the nodulation potential of inoculant and soil rhizobia remain elusive.

Environmental factors affect the efficacy of rhizobia-NER-host plant interactions by altering the ability of NER and rhizobia to survive and express PGP traits. The level of P in the soils determines the efficacy of the introduced NER in nodulation and plant growth (Remans *et al*. 2007). In that study, the effect of IAA producing *A. brasilense* Sp245 and *Bacillus subtilis* 7135 on nodulation and growth of common bean was more pronounced in soil supplied with sufficient P when inoculated with *R. etli* CNPAF512 but co-inoculation was detrimental to those factors in P deficient soils. Furthermore, soil pH directly affects P dynamics and can determine the impact of inoculating an IAA-producing *Pseudomonas monteilii* on the legume-rhizobia symbiosis (Sánchez *et al*. 2014). In this study, bacterial inoculation had a positive effect on nodulation at neutral soil pH, but the effect of NER on nodulation and plant growth in acidic soils was detrimental. The lethal effect of IAA from NERs under stress conditions is related to high ethylene production as a result of P deficient conditions (Persello-Cartieaux *et al*. 2003; Ona *et al*. 2005) relating to synthesis of more ACC synthase (Xie *et al*. 1996). This result supports the work of Tittabutr *et al*. (2013), in which *Pseudomonas putida* UW4 reduced ethylene based on the production of ACC deaminase (Glick 2005; Ali *et al*. 2014). The ethylene

reduction occurred more strongly under P deficient than P sufficient conditions. Similar effects of ACC deaminase have been observed under a variety of stress conditions including saline soils (Ahmad *et al*. 2012; Chinnaswamy *et al*. 2018), drought (Belimov *et al*. 2019), high temperatures (Tittabutr *et al*. 2013) and in metal contaminated soils (Safronova *et al*. 2012; Ju *et al*. 2019). Salt-tolerant ACC-deaminase producing NER enhanced the nodule formation by salt intolerant rhizobia on fodder galega (Egamberdieva *et al*. 2016). When only rhizobia were inoculated, the ability to colonise the plant rhizosphere under salt stress was impaired, suggesting that NER can be involved in the survival and persistence of inoculated rhizobia in the rhizosphere. A positive influence of ACC deaminase activity under stress can be associated with greater production of the enzyme by the NER (Tittabutr *et al*. 2013) which results in further reduction of ethylene, a negative regulator of nodule formation (Guinel and Geil 2002). Moreover, ACC deaminase effectively stimulated root growth (Noreen *et al*. 2012), which helps the plant withstand a stressed environment by taking up more nutrients in nutrient-deficient soils (Barnawal *et al*. 2014) and can increase water uptake in drought conditions (Belimov *et al*. 2009). The positive influence of NER on nodulation and plant growth under stress are also associated with the expression of genes involved in the stress-tolerance of the host plant (El-Esawi *et al*. 2018). In that study, up-regulation of genes encoding four antioxidant enzymes (APX, CAT, POD, Fe-SOD), increased the potential to eliminate toxic free radicals, and led to the up-regulation of transcripts of six stress-related genes (GmVSP, GmPHD2, GmbZIP62, GmWRKY54, GmOLPb, CHS) when soybean was inoculated with *Serratia liquefaciens* KM4. However, the positive influence of PGPR traits, such as ACC deaminase, are more important in plant growth promotion under stress conditions, and can be impaired when plants are challenged with multiple stresses (Belimov *et al*. 2019). Under these conditions, the use of salt and drought tolerant NER's able to express multiple PGP traits improved the nodulation potential of co-inoculated rhizobia under drought and salt-affected soils compared with rhizobia inoculated alone (Noori *et al*. 2018). This result indicates that the ability of both symbiotic partners to tolerate multiple stresses determines the successful synergy that ultimately results in improvement in nodulation and growth of the plant. The efficacy of NER inoculants in the field has frequently been very low and the literature reviewed suggests that understanding how the environmental factors influence the performance of NER and the expression of associated PGP traits is important to improve the performance and reliability of these beneficial bacteria in field conditions.

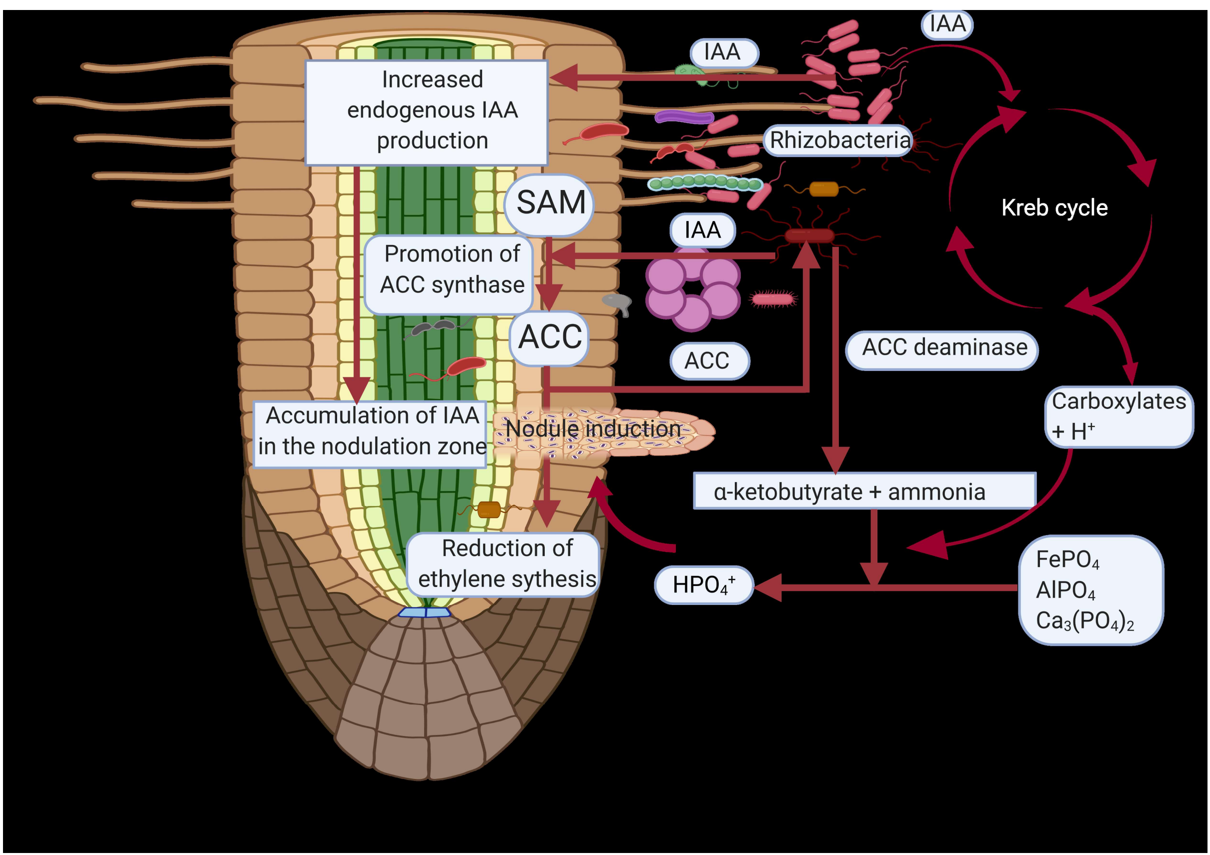

Fig. 1 overall mechnisms of reduction of ethylene synthesis and accumulation of IAA in the nodule induction region by ACC deaminase and IAA producing PGPR. ACC 1-aminpcyclopropane-1-carboxylic acid; SAM, S-adenosylmethionine

2.4. ACC deaminase producing NER

ACC deaminase is a pyridoxal 5-phosphate (PLP)-dependent multimeric enzyme with a molecular mass of 35 - 42 kDa and was first reported by Honma and Shimomura (1978). ACC deaminase was first described as a PGP molecule by Glick *et al*. (1998). This enzyme is localized within the cytoplasm of the microorganisms that produce it (Jacobson *et al*. 1994). The gene that encodes ACC deaminase is the *acd*S gene (Honma and Shimomura, 1978) although some microbial isolates carrying the *acdS* gene did not express the enzyme in the absence of a host plant (Contesto *et al*. 2008). Along with this finding, in endosymbiotic bacteria such as *M. loti*, the *acdS* gene was found in the symbiotic island and its expression depends upon the N_2 fixation regulator *NifA2* (Nukui *et al*. 2006).

A broad range of rhizobacteria expresses ACC deaminase (Minami *et al*. 1998; Shah *et al*. 1998; Sterky *et al*. 1998; Jia *et al*. 2000). When ACC is produced by a plant and exuded, rhizobacteria that express ACC deaminase can take up ACC and degrade it within the bacterial

cytoplasm (Glick *et al*. 1998; Penrose and Glick 2003). ACC deaminase can also degrade substrates other than ACC, such as D-serine (Walsh *et al*. 1981). The enzyme has low Km values for degradation of ACC, varying between 1.5 and 17.5 mM (Honma and Shimomura 1978), that are either constitutively produced or newly synthesised during nodule initiation (Ligero *et al*. 1986; Spaink 1997) and under stressed conditions in plant roots (Saleem *et al*. 2007). The bacteria that take up ACC from the rhizosphere can use it as an energy and/or N source (Glick *et al*. 1998). Because ACC is a precursor of ethylene, its degradation by ACC deaminase does help to lower ethylene levels, particularly in plants exposed to stress.

2.4.1. Nodule initiation

Ethylene is synthesised by the plant in response to biotic and abiotic stresses, and when roots initiate the symbiotic association with rhizobia (Ligero *et al*. 1986; Abeles 1992; Suganuma *et al*. 1995; Spaink 1997). Ethylene inhibits infection of rhizobia and nodulation of various legume species including *Pisum sativum* (Lee and LaRue 1992), *Trifolium repens* (Goodlass and Smith 1979) and *Medicago sativa* (Peters and Crist-Estes 1989). Ethylene production in the legume roots is typically increased after the application of rhizobial cells and this phytohormone acts as an autoregulator for nodule formation and development during symbiosis (Ligero *et al*. 1991; Lohar *et al*. 2009). Ethylene inhibits nodule formation by interfering with root hair deformation, elongation of the infection thread into the inner cortex, calcium spiking and proliferation of rhizobia (Penmetsa and Cook 1997; Oldroyd *et al*. 2001; Tamimi and Timko 2003). In soybean, up to 8 mmol L^{-1} ethylene was evolved between 24-48 hrs after inoculation with *Bradyrhizobium* (Ligero *et al*. 1999). Moreover, nodules accumulated almost twice as much ethylene per unit weight as roots did (Hunter 1993). An ethylene concentration as low as 0.014 mmol L^{-1} inhibited nodulation in *Pisum sativum* (Drennan and Norton 1972). When soybean was inoculated with *Bradyrhizobium* and Ag^{+} (an inhibitor of ethylene action), nodule number was markedly increased due to a reduced ethylene synthesis compared with inoculation alone (Ligero *et al*. 1999).

Nodule reduction at early stage because of ethylene production can be attenuated by NER (Nascimento *et al*. 2016). Reduction of ethylene synthesis by ACC deaminase likely plays an important role in directly assisting nodule formation. The co-inoculation of elite rhizobia and NER with high ACC deaminase activity has enhanced nodulation in chickpea (Shahzad *et al*. 2010) and lentil (Zahir *et al*. 2011) relative to nodulation in the absence of the NER. Further examples of the effects on nodulation of legume plants from ACC deaminase produced either

by the endosymbiont or by the NER are shown in Table 1. Ma *et al*. (2003) delineated the role of ACC deaminase from *Rhizobium leguminosarum* bv. *viciae* 128Sm in the nodulation process; two knockout mutants that did not produce ACC deaminase and one mutant that could overproduce this protein were investigated for their ability to nodulate *P. sativum* L. cv. Sparkle. The number of nodules induced by the ACC deaminase negative mutant of rhizobia was approximately 25% less than for nodulation induced by the parental strain. In addition, an ACC deaminase-producing *Sinorhizobium meliloti* strain had up to 40% greater efficiency in forming nodules in *M. sativa* than did a mutant of the parent strain lacking ACC deaminase activity (Ma *et al*. 2004). The positive influence of this enzyme on the early stage of nodule formation is likely to be achieved through reducing the ethylene concentration in the region between the root epidermis and the outermost cortical cell layer where the infection threads progress towards the site of nodule primordium formation. This is the main site at which ethylene interferes with nodule development (Guinel and Geil 2002). However, a mutant that produced over twice the ACC deaminase (3.24 ± 0.24 µmol of α-ketobutyrate. h^{-1}. mg of $protein^{-1}$) did not significantly improve nodulation compared with the ACC deaminase producing parent strain (1.56 ± 0.23 µmol of α-ketobutyrate. h^{-1}. mg of $protein^{-1}$). On the other hand, inoculation with *S. meliloti* (2.26 µmol of α-ketobutyrate. h^{-1}. mg of $protein^{-1}$) containing exogenous *acdS* as well as co-inoculation with *S. meliloti* and *P. putida* UW4 (3.38 µmol of α-ketobutyrate. h^{-1}. mg of $protein^{-1}$) in *Medicago lupulina*, showed a remarkable increase in nodule number compared with plants inoculated with wild type *S. meliloti* under copper stress (Kong *et al*. 2015). This positive influence of overproduction of ACC deaminase on nodule initiation indicates the need for higher ACC deaminase activities to alleviate the ethylene-associated constraint on nodulation.

ACC deaminase produced by endophytic bacteria improves nodule development besides inducing more nodules. The endophytic *Serratia grimesii* BXF1 overproduced ACC deaminase (12.0 µmol of α-ketobutyrate h^{-1}. mg of $protein^{-1}$) when co-inoculated with *R. tropici* CIAT899 and led not only to increased nodulation when co-inoculated with *Rhizobium*, but also enhanced nodule development, with the nodules appearing larger and with a more pronounced pink tone (indicative of greater N_2 fixation) (Tavares *et al*. 2018). This suggests a role of ACC deaminase in promoting nodule development when the enzyme is present in the nodule. *Leucaena leucocephala* was co-inoculated with a strain of *Rhizobium* sp. TAL1145 and a mutant of the strain *Sinorhizobium* sp. BL3 that, because of the presence of multiple copies of plasmids containing *acdS*, produced more ACC deaminase (5.4 mmol of α-ketobutyrate mg^{-1} protein h^{-}

[1]) than the wild type strain. The co-inoculation resulted in a greater number and size of nodules than on plants inoculated with the wild type *Sinorhizobium* sp. BL3 strain which produced less ACC deaminase (Tittabutr *et al*. 2008). These studies clearly indicate that the expression of ACC deaminase by endosymbiont and endophytic bacteria in the nodule tissue can determine the degree of enhancement of nodulation.

2.4.2. Rhizobial competitiveness for nodulation

NER can improve the competitiveness and nodule occupancy by introduced elite rhizobia relative to the indigenous rhizobia (Tilak *et al*. 2006). Many rhizosphere microbial communities that enhance plant growth and provide protection from stresses are also able to increase the competitiveness of effective rhizobia for nodulation. In this regard, a study on the competitiveness of *Bradyrhizobium diazoefficiens* (formerly *B. japonicum* USDA110) and *B. japonicum* USDA 118 to form nodules on soybean revealed that out of 17 NER that were co-inoculated with these strains, 3 increased nodulation potential of *B. diazoefficiens* USDA 110 (Polonenko *et al*. 1987). This positive effect was not observed with *B. diazoefficiens* USDA 110 inoculated alone. Another study reported an increase in the proportion of nodules occupied by inoculant *Rhizobium* from 50% (with *Rhizobium* alone) to 85% on pigeonpea through co-inoculation with *Pseudomonas putida* (Tilak *et al*. 2006). Greater colonisation of nodules by the introduced *Rhizobium* strain is likely to enhance N_2 fixation. The mechanisms by which NER influence the survival and establishment of introduced rhizobia in the soil, rhizosphere, rhizoplane and endorhizosphere needs further investigation to better exploit these traits for improvement of N fixation.

Rhizobial strains vary in their ability to compete against background rhizobia populations in the soil for nodule formation (Brockwell *et al*. 1995). The ability to compete for nodule formation may in part be associated with the expression of PGP traits, in particular, ACC deaminase with chickpea (Nascimento *et al*. 2012), *Lotus* spp. (Conforte *et al*. 2010), mung bean, *Vigna radiata* (Shaharoona *et al*. 2006) and pea (Ma *et al*. 2003). To identify whether the constitutive expression of ACC deaminase by rhizobia influenced their competitive advantage to induce nodules, two *Mesorhizobium loti* MAFF *acdS* mutants that produced more ACC deaminase than the wild type strain were constructed (Conforte *et al*. 2010). More than 67% of *L. japonicum* and *Lotus tenuis* nodules were induced by the engineered strain at 28 days after inoculation (DAI), even though the engineered strain only represented a quarter of the bacterial suspension in the inoculant. Moreover, the mutant had 50% more viable cells colonizing the

plant's roots than did the wild type strain after 21 days. This study also confirms that rhizobial ACC deaminase production in the free-living state confers a competitive advantage to the mutant bacteria over the wild type strain. This supports a model from Glick *et al*. (1998) who postulated that the greater competitiveness of a modified strain expressing more ACC deaminase can be due to a greater capacity to use ACC from the root exudate as C and N sources. Further studies on the impact of ACC deaminase activity on the expression of genes related to stress tolerance and the physiological response in the free-living condition in the soils and rhizosphere is required for the development of effective multiple strain-based inoculant that can be applied in a wide range of environments including under stress conditions.

2.4.3. Nodule function

Ethylene is strongly involved in nodule senescence and consequently impairs N_2 fixation in nodules (Guinel 2015). The gene *PvACS* encoding ACC synthase, an enzyme involved in ethylene synthesis, was upregulated in senescent nodules (Nascimento *et al*. 2018). The transcription of this gene was specifically increased in the senescent zone of nodules (Serova *et al*. 2017; Serova *et al*. 2018). In the senescent nodules, $PvGS_{(n-1)}$, a gene controlling the transcription of glutamine synthetase, that is crucial for ammonium assimilation (Hungria and Kaschuk 2014), was down-regulated (da Silva *et al*. 2019). The expression of this gene is linked with increased nitrogenase activity and leghaemoglobin (Lara *et al*. 1983). This suggests a negative effect of senescence on ammonia assimilation and N_2 fixation. Moreover, Uricase II, coding an enzyme crucial for exporting the N assimilated from nodules to the shoot, decreased in senescent nodules (Papadopoulou *et al*. 1995; Capote-Mainez and Sánchez 1997). Leghaemoglobin, an important physiological marker for nodule senescence (Loscos *et al*. 2008) and required to protect the oxygen-sensitive nitrogenase (Ott *et al*. 2005), was also degraded in nodules undergoing senescence (da Silva *et al*. 2019).

Beneficial bacteria that are ACC deaminase producers can enhance the amount of N_2 fixation by extending the lifespan of functional nodules. Interestingly, some PGPR can stimulate plant growth through the synthesis and activity of the ACC deaminase that potentially reduces ethylene levels in the plant (López *et al*. 2018). In legume-rhizobia interactions, transcription of the *acdS* gene in *Mesorhizobium loti* occurred in root systems with mature nodules but this was not observed in roots without mature nodules (Nukui *et al*. 2006). The accumulation of ACC deaminase protein in effective nodules occurs in N_2 fixing bacteroids. To investigate the physiological function of ACC deaminase in bacteroids, a transformed *Mesorhizobium*

expressing ACC deaminase and its parental type strain without this activity were inoculated on to chickpea; nitrogenase activity was detected at 31 days in nodules of the mutant but not in in nodules of the wild type rhizobia (Nascimento *et al*. 2012). Moreover, inoculating *Sinorhizobium* able to express ACC deaminase increased nitrogenase activity in mung bean up to 5-fold when compared with plants inoculated with the wild-type strain (Tittabutr *et al*. 2015). However, the highest production of ACC deaminase (16.3 µmol of α-ketobutyrate. h^{-1}. mg of $protein^{-1}$) by a modified strain of rhizobia containing multiple copies of *acdS* did not contribute more to the symbiosis than the wild type rhizobia that produced low level of ACC deaminase (4.5 µmol of α-ketobutyrate. h^{-1}. mg of $protein^{-1}$). On the other hand, a mutant rhizobia unable to express ACC deaminase resulted in nodules entering the senescent phase earlier. Later, at 7 weeks after inoculation, a modified strain of rhizobia lacking ACC deaminase produced nodules with smaller symbiosomes and greater bacteroid mortality than nodules induced by the wild type isolate (Tittabutr *et al*. 2015). This indicates that the loss of ACC deaminase production from rhizobia may accelerate nodule senescence. Taken together, ACC deaminase extended the duration of nitrogenase activity by lowering ethylene around the bacteroids, although the detailed physiological effect of ACC deaminase on bacteroids is not yet clear. So far, studies have revealed that ACC deaminase prolongs the time that nodules are functional and fixing N_2. However, the impact of ACC deaminase on the expression of rhizobial genes directly and indirectly associated with of N_2 fixation in both free-living conditions and in bacteroids requires greater investigation.

Table 1. Effect of rhizobia and/or PGPR with ACC deaminase activity on nodulation and yield of legume plants

Plant species	PGPR species	Rhizobia species	Amount of ACC deaminase activity (μmole α-ketobutyrate mg^{-1} protein h^{-1})	Experimental conditions	Effects/mechanisms	References
Cicer arietinum	-	*Mesorhizobium ciceri* LMS-1(pRKACC) mutant	2,035	Greenhouse, non-sterile soil	Inoculation increased the nodule number and nodule weight up to 180% and 147% respectively, compared with those inoculated with parental strain.	Nascimento et al. (2012)
	Serratia proteamaculans J119*		0.440	Pouch, greenhouse, and field conditions	PGPR inoculation increased root biomass by 83% over uninoculated plants under axenic conditions and inoculation increased grain yield, nodule number and	Shahzad et al. (2010)

					weight by 60%, 75% and 63% over uninoculated, respectively, under field conditions.	
	-	Salt tolerant and salt sensitive *Mesorhizobium* containing *acdS* gene mutant	0.768 - 0.398	Growth chamber	*acdS*-transformed *Mesorhizobium* strains promoted increases in shoot dry weight and nodule number of 54% and 120%, respectively, in plants under salinity stress compared with those inoculated with the wild type strain.	Brígido et al. (2013)
P. sativum	*Arthrobacter protophormiae*		0.241	Greenhouse	ACC deaminase producing PGPR inoculated with *Rhizobium* in *P. sativum* increased the nodule number by 2%, 24% and 67% over plants inoculated with *Rhizobium* alone.	Barnawal et al. (2014)
	-	*R. leguminosarum* with ACC deaminase activity and it's	1060	Growth chamber	The number of nodules induced on plants inoculated with an engineered strain lacking the	Ma et al. (2003)

		mutant lacking this activity			*acdS* gene was 25% less than nodule number on plants inoculated with the parent strain.	
	Pseudomonas putida PSE3	*R. leguminosarum* RP2	625.6	Greenhouse and field conditions	Co-inoculation increased the nodule number and weight up to 16.7% and 9%, respectively, over inoculated with *Rhizobium* alone under greenhouse conditions. Under field conditions, NN and NDW increased due to co-inoculation were 32.5% and 62.8% over those inoculated with *Rhizobium* alone.	Ahmad et al. (2013)
Vigna radiata	-	*Sinorhizobium* sp. containing ACC deaminase	16.34	Leonard jar	Nodule occupancy increased from 34% to 66% due to increasing the ACC deaminase production ability of inoculated rhizobia by 2.6 times over the parental strain.	Tittabutr et al. (2015)

	Pseudomonas putida, *Pseudomonas fluorescens* producing ACC deaminase	*Bradyrhizobium* sp.	207 - 333	Greenhouse in unsterile sandy clay loam soil	Dual inoculation with PGPR and *Bradyrhizobium* resulted in 11-times more nodule number than uninoculated control and 48% than *Bradyrhizobium* alone.	(Shaharoona et al., 2006)
	Pseudomonas sp. containing ACC deaminase activity	*Rhizobium* sp.	-	Growth room, pouch experiment	Co-inoculation increased nodule number by 19% and 62.8% and nodule dry weight by 12% and 12.5% over *Rhizobium* alone under normal and 4 dS m^{-1}, respectively.	Ahmad et al. (2011)
L. japonicum and *L. tenuis*	-	*Mesorhizobium loti* mutant containing ACC deaminase	12	Autoclaved Leonard jars filled with vermiculite	Inoculation with recombinant *Mesorhizobium* increased nodules and nodule occupancy by 37% and 80% over the wild-type strain.	Conforte et al. (2010)
Leucaena leucocephala	-	*Recombinant Rhizobium* sp. strain TAL1145 mutant containing *acdS* gene	0.817	Leonard jar containing sterile sand	Mutant with ACC deaminase production increased nodule number and dry weight 64%	Tittabutr et al. (2008)

					and 60% over inoculation with the wild-type strain.	
Lentil	*Pseudomonas* and *Serratia* containing ACC deaminase activity	*R. leguminosarum*	537	Greenhouse and field conditions	Co-inoculation of *Rhizobium* with ACC deaminase producing bacteria increased nodulation and grain yield up to 98% and 82% over uninoculated, respectively, under field conditions.	Zahir et al. (2011)
Medicago lupulina	*Pseudomonas putida* mutant	Genetically engineered *S. meliloti* strain overproducing ACC deaminase	3.38	Greenhouse experiment in sterile growth medium	Engineered rhizobia increased nodule number and nitrogenase activity up to 18.2% and 10.7% under copper stressed conditions and 0% and up to 6% under normal conditions over those inoculated with wild-type strain.	Kong et al. (2015)
M. sativa	-	Engineered ACC deaminase producing insertion derivative of *S. meliloti*	6.5	Growth chamber in Leonard jar in vermiculite	ACC deaminase-producing *Sinorhizobium* sp. increased nodulation up to 40% over the wild-type strain.	Ma et al. (2004)

*- Opportunistic human pathogen

2.5. Indoleacetic Acid Producing NER

Auxins are well-recognized plant growth hormones that affect all aspects of plant growth and development. Besides the plant itself, more than 80% of rhizosphere bacteria, including rhizobia, are able to produce the auxin indole acetic acid (IAA) (Badenoch-Jones *et al*. 1984; Glick *et al*. 1998; Khalid *et al*. 2004; Ahemad 2015). So far, five different pathways of IAA synthesis have been identified in bacteria. Of these, the indole-3-pyruvate and indole-3-acetamide pathways are the two major IAA biosynthesis pathways (Khalid *et al*. 2004). IAA production by different rhizobacteria including rhizobial species can lead to beneficial effects, both in free-living bacteria, host plants, and during their symbiotic interaction.

2.5.1. Nodule primordium development

Nodule initiation and development requires high levels of IAA to initiate cell division and establish the nodule primordium. The effects of IAA produced either by the rhizobia or by the NER on the nodulation of legume plants are shown in Table 2. To investigate the influence of IAA on nodulation, an experiment was conducted using a *B. elkanii* strain able to synthesise and secrete IAA in culture, and a mutant derivative, lacking IAA production (Fukuhara *et al*. 1994). The IAA negative mutant induced fewer nodules in soybean compared with inoculation by the parent strain. Exogenous IAA application restored the number of nodules formed on soybean inoculated with the modified strain to the original level. This positive influence of IAA was associated with facilitating root cortical cell division that is important for nodule primordium development (Benková *et al*. 2003; Roudier *et al*. 2003; Suzaki *et al*. 2012). Active root cortical cell division as a result of IAA was evidenced by a 61.5% higher nodule dry weight 7 weeks after infection, and an enlarged and more active nodule meristem induced by a modified strain of *Rhizobium leguminosarum* bv. *viciae* LPR1105 contained an IAA biosynthetic pathway not present in the wild-type parental strain (Camerini *et al*. 2008). The role of the IAA produced by the modified strain in this study was evidenced by the levels of IAA in the nodule being up to 60-fold higher than in nodules initiated by the parent strain. Likewise, IAA concentrations in roots with nodules induced by an IAA over-producing mutant of rhizobia were more than threefold higher than in those inoculated with wild type strains (Defez *et al*. 2019). Increased IAA during symbiosis was positively correlated with the higher expression of IAA biosynthesis genes under free living conditions (Bianco *et al*. 2014).

Elevated IAA in nodules derived either from the above-ground plant parts through polar transport (Fedorova *et al*. 2005) and/or directly from the prokaryotic microsymbiont

(Lambrecht *et al*. 2000) induces the development of more nodules (Pii *et al*. 2007). When the plant root is exposed to increased IAA levels from NER, endogenous IAA concentrations can be elevated (Figueiredo *et al*. 2008). The endogenous IAA synthesised in the shoot and transported to nodules and roots is facilitated by auxin efflux transporters such as those encoded by the PUN gene (Prayitno *et al*. 2006). To investigate the role of accumulated endogenous IAA in the nodulation region on nodule development, a mutant line of *L. japonicus* containing the auxin-reporter GH3 fused to β-glucuronidase (GUS) was generated (Takanashi *et al*. 2011). When rhizobia were inoculated, the GUS signal was detected in actively dividing outer cortical cells, during initiation of nodule primordia and was later detected in the vascular tissues of mature nodules. Moreover, such elevated endogenous IAA induced a few pseudonodules in common bean in the absence of any *Rhizobium* (Srinivasan *et al*. 1997). Such spontaneous nodules in the presence of IAA devoid of *Rhizobium* has been reported in alfalfa (Caetano-Anollés *et al*. 1990) and white clover (*Trifolium repens*) (Blauenfeldt *et al*. 1994). However, a *Rhizobium* mutant lacking IAA production did not abolish nodule formation (Hirsch and Fang 1994). Another study also reported that IAA was not involved in root hair curling, an important early stage of nodule initiation (Mulligan and Long 1985). Moreover, in indeterminate nodules, such as those in *L. japonicus*, an IAA inhibitor did not alter the process of nodule development but interfered with the formation of lenticels on the nodule surface (Takanashi *et al*. 2011).

IAA enhances the formation of more nodules by altering the physiology and expression of genes associated with rhizobia and those involved in nodule initiation in plant cells. The transcription of *noe*A and *noe*B genes involved in the early nodule initiation process in *S. meliloti* were also up-regulated in the engineered strain RD64 producing IAA (Defez *et al*. 2016). Moreover, transcription of the *Glyma17g07330* gene, encoding MYB transcription factor functions, was upregulated 7 days after inoculating an IAA-expressing NER in soybean but was strongly disrupted at later stages of plant growth. This suggests that the main role of this gene is in developing nodules rather than mature nodules. The role of this gene in rhizobial infection or early steps of nodule development was also observed by Libault *et al*. (2009). During nodule induction, the production of short fatty acids, such as caproic acid, which facilitates plant cell membrane fluidity and is essential for rhizobia colonization on the plant surface, was also increased when IAA producing NER were used for co-inoculation (Brechenmacher *et al*. 2010; Cagide *et al*. 2018).

2.5.2. Rhizobial survival and competitiveness for nodulation

Successful nodulation of legumes by inoculant rhizobia is a complex process that depends on many factors associated with the environment, the host plant and the soil microbiomes. Among the factors associated with soil microorganisms, the ability for the rhizobia or their nearby associated bacteria to produce active biomolecules affects their ability to induce nodules (Menéndez et al. 2017). IAA is one biomolecule that can affect the legume-rhizobia interaction. A reduction in nodule occupancy by a mutant of *B. elkanii* that lacked the capacity to express IAA as compared to the parent strain (Fukuhara *et al.* 1994) was observed. Moreover, nodulation was reduced by up to 60% due to lack of IAA production by a modified strain of *Rhizobium tropici* relative to inoculation with the parent strain (Martinez *et al.* 1993). These studies suggested that IAA production by rhizobia assists in inducing and sustaining meristematic activity, which in turn enhances nodulation.

The ability of inoculant rhizobia to withstand stress conditions that reduce their survival in the root zone could be one factor determining their competitiveness to induce nodules in the competition with the background rhizobia (Howieson and Ballard 2004). For example, introduced rhizobia that are sensitive to salinity *in vitro* had low competitiveness for inducing nodulation compared with the natural rhizobial population in a saline soil (Ventorino *et al.* 2012). Adaptation to environmental stresses is a complex phenomenon involving many biochemical and physiological processes that are accompanied by changes in regulation and expression of genes and genetic pathways (Djordjevic *et al.* 2003; Wei *et al.* 2004). There is evidence that IAA production by *E. coli* is able to coordinate bacterial behaviour to enable protection against damage by stresses (Bianco *et al.* 2006) and that IAA induces the coordinated expression of genes related to survival under stress conditions. Moreover, with a modified strain of *S. meliloti* RD64 that overproduced IAA, the level of gene expression associated with stress tolerance was upregulated to a greater extent than that of its wild derivative (Defez *et al.* 2016). In that study, transcript levels of *rpoH1*, coding for one of the two alternative s32 factors, which operate under heat shock and oxidative stress, and proline biosynthesis genes (*proB2*, and *SMc03253*), required for effective nitrogen-fixing symbiosis were significantly increased in a modified strain (Mitsui et al. 2004). Furthermore, many genes induced during a stress response (*ibpA*, *htpG*, *clpB*, *groES5*, *groEL*, *dnaK*) and belonging to the *rpoH* regulon in different rhizobia (Mitsui et al. 2004; Martínez-Salazar et al. 2009; de Lucena et al. 2010) were upregulated. Another gene, *ocd,* that converts L-ornithine to L-proline, in many bacteria (Soto *et al.* 1994; Sleator and Hill 2002) was upregulated during

stress. The presence of transcriptional products of the aforementioned genes in the modified strain, but not in its parent strain, is evidence for the role of IAA in better survival and persistence of bacteria in stressed environments (Defez 2006). This could, in turn, result in long-time survival and persistence in the soil of introduced rhizobia positively associated with competent nodule induction (Narożna et al. 2015).

Table 2. Effect of IAA production by PGPR on the nodulation and N_2 fixation of different legumes

Plant species	Source of IAA	Bacterial species	IAA concentrations	Experimental condition	Effects/mechanisms	References
Vicia sativa spp. *nigra*	rhizobacteia	*Azospirillum brasilense* and a mutant derivative lacking IAA production		Greenhouse in sterile vermiculite	Increased secretion of nod-gene-inducing flavonoid species in a wild *Azospirillum* compared to a mutant of the same strain lacking IAA resulted in more nodule formation	(Star et al. 2012)
M. sativa L.	rhizobacteria	*Micromonospora*	22.5 µg ml^{-1}	Greenhouse in sterile soil	Selected endophytic *Micromonospora* significantly increased the nodulation of alfalfa by *Ensifer meliloti* 1021.	(Martínez-Hidalgo et al. 2014)
M. truncatula	rhizobia	IAA overproducing *S. meliloti* mutant	0.068 µg ml^{-1}	Greenhouse	IAA over-producing *S. meliloti* mutant increased nitrogenase activity per nodule and stem dry weight of six week old plants by 35% and 28%, respectively, over the value obtained from plants inoculated with the parental strain at 6 weeks after planting.	Imperlini et al. (2009)

	rhizobia	IAA overproducing *S. meliloti* mutant	0.068 µg ml^{-1}	Greenhouse	Higher nodule and lateral root system growth were found in plants inoculated with IAA overproducing modified strain than those inoculated with the wild-type strain.	Bianco and Defez (2009)
Glycine max	rhizobia	*B. japonicum* IAA producing mutant	1. 60 µg ml^{-1} in the presence of 8 mg ml^{-1} of L-tryptophan 2. 5.5 µg ml^{-1} in the absence of L-tryptophan	Pouch experiment	High IAA producing *B. japonicum* reduced nodule mass and N fixed per gram of nodule by 81.8% and 160% over the plants inoculated with the wild-type strain.	Hunter (1987)
	rhizobia	*B. elkanii* USDA 31 mutant lacking IAA synthesis	The mutant produced half the IAA produced by the parent strain	Greenhouse	Plants inoculated with mutant produced 157% less nodules compared with those inoculated with the wild-type strain.	Fukuhara et al. (1994)
Vetch	rhizobia	IAA overproducing *R. leguminosarum* bv.	0.963 $\pm$ 0.122 and 13.314 $\pm$ 0.7 µg ml^{-1},	Greenhouse, in sterile soils	The mutant increased the IAA level in the nodule 60-fold compared with plants inoculated	Camerini et al. (2008)

		viciae LPR1105 mutant	respectively, for the wild-type strain and the engineered strains		with wild-type parent. The mutant also increased the nodule weight by 61.5% over that obtained from plants inoculated with the parent strain.	
V. radiata L.	Exogenous IAA		10^{-6} M IAA	Greenhouse in non-sterile soil-farm yard manure	Nodule number, nodule dry weight, leghemoglobin content and nitrogenase activity increased by 18.2%, 19.05%, 13.4% and 15.8%, in plants treated with IAA compared to untreated plants.	Ali et al. (2008)
M. sativa	rhizobia	*S. meliloti* IAA overproducing strains	9.987-11.036 µg IAA ml^{-1}	Greenhouse	Plants nodulated by IAA overproducing strain upregulated the nitrogenase gene, *nifH* and increased number of nodules by 26% compared with plants nodulated by the wild-type strain.	Bianco et al. (2014)

	rhizobacteria	*Azospirillum brasilense* strain Cd		Large petri dishes on sterile agar	Combined inoculation of rhizobia and *A. brasilense* Cd increased the number of nodules but reduction of nodules was observed at higher concentration (>10^4 CFU/mL) of *Azospirillum* sp.	Schmidt et al. (1988)
P. sativum	synthetic	Exogenous IAA application,	-	Petri dish assay	Functional nodules which fix N had higher IAA accumulation than nodules that could not fix N	Badenoch-Jones et al. (1983)
C. arietinum	rhizobacteria	IAA producing *Pseudomonas sp.*	Up to 40 µg IAA ml^{-1}	Chillum jar, sterile sand culture	Co-inoculation of *Mesorhizobium* with PGPR increased the nodule number and dry weight and nitrogenase activity by 140%, 260% and 45% over those inoculated with *Mesorhizobium* alone	Malik and Sindhu (2011)
Peanut	rhizobacteria	IAA producing *Bacillus muralis*		Pot experiments	Increase in root length, plant fresh weight, and height of peanuts, by 186%, 3%, and 13% respectively, due to inoculation	Jiang et al. (2016)

					of *B. muralis*, compared with non-inoculated control	
Phaseolus vulgaris L.	rhizobacteria	IAA producing *A. brasilense* Sp245 ACC deaminase producing *Pseudomonas putida* UW4	4 µg ml^{-1} in the presence of 200 µg ml^{-1}	Pots containing perlite-matrix	Under low P conditions, IAA producing bacteria reduced NN by 75% while ACC deaminase producing bacteria enhanced NN by 12.5%. Under high P conditions, both ACC deaminase and IAA producing PGPR enhanced NN by 12% and 8%, respectively.	Remans et al. (2007)
	Exogenous IAA application	-	-		Low concentration IAA (below 15 nM IAA) enhanced nodules while high IAA (beyond 15 nM IAA) inhibited nodulation, regardless of P in the growing medium. At 100 nM IAA, the NN in low P conditions was reduced by 42% compared with nil IAA but	

					did not reduce NN in high P conditions.	
	rhizobacteria	*A. brasilense* Cd (ATCC 29729)		Pots containing volcanic gravel	Co-inoculation of rhizobia with *A. brasilense* increased the number of nodules up to 29.5% over the *Rhizobium* inoculated alone	Burdman et al. (1996)
	rhizobacteria	*Bacillus spp.*	0.40-4.8 µg ml^{-1} in the presence of L-tryptophan (100 pg L^{-1}) and 0.3-0.89 in the absence of L-tryptophan	1:1 v/v of sterile planting medium of industrial sand and Turface™	Co-inoculation of *R. etli* TAL 182 with *Bacillus* spp. producing 4.88 µg/ml promoted the nodule number (NN), nodule fresh weight (NFW), leghemoglobin and acetylene reduction assay (ARA) by 87%, 83%, 136% and 91%, respectively, compared with *Rhizobium* single inoculation. α -methyltryptophan-resistant mutants *Bacillus* producing 13 µg/ml of IAA inoculated with	Srinivasan et al. (1996)

					rhizobia significantly reduced NN, NFW, and ARA.	
white clover	Exogenous α-napthaleneacetic acid (NAA), a synthetic IAA	-	-	Rapid-plate-screening method	Nodule formation was inhibited at 10^{-4} and 10^{-5} M NAA while nodulation was enhanced at 10^{-7} to 10^{-10} M NAA	Plazinski and Rolfe (1985)
	Co-inoculaion of rhizobia and rhizobacteria	*Rhizobium trifolii* ANU794 and ANU1030 and *A. brasilense* SP245	4 µg ml^{-1} IAA in the presence of 200 µg ml^{-1} L-tryptophan		2000 to 1 ratio of *Azospirillum*-*Rhizobium trifolii* mixed culture resulted in complete inhibition of nodule formation	

Key: NN- nodule number; NFW- Nodule fresh weight, ARA- acetylene reduction assay

2.5.3. N_2 fixation and their associated gene expression

IAA can enhance N_2 fixation through increasing the activities of enzymes and metabolites directly involved in bacteroid activity in the nodules. Increases in nitrogenase and leghaemoglobin content up to 32% and 45% were observed at 45 days after sowing when plants were treated with 10^{-8} M exogenous IAA as compared with untreated plants (Ali *et al*. 2008). Greater nitrogenase and leghaemoglobin production in the nodules resulted in higher N_2 fixation as measured by acetylene reduction activity in nodules induced by IAA overproducing rhizobia compared with the wild type strain (Kaneshiro and Kwolek 1985). Likewise, nitrogenase activity and leghaemoglobin synthesis in nodules that undertake N_2 fixation were increased when the plants were inoculated with rhizobia able to produce IAA and when co-inoculated with NER also producing IAA (Yahalom *et al*. 1990; Chakrabarti *et al*. 2010; Egamberdieva *et al*. 2010). Furthermore, a engineered strain of rhizobia that overproduced IAA showed a significant increase in acetylene reduction activity compared to those nodulated by the parental strain (Defez *et al*. 2019).

IAA enhances effective nodule formation and promotes a delay in nodule senescence by increasing the viability of bacteroids inside nodules. Effective nodules have been increased when inoculated with IAA-producing rhizobia in *Cajanus cajan* (Ghosh *et al*. 2013), *Glycine max* (Hunter 1987) and *Lupinus luteus* (Kretovich *et al*. 1972). Higher IAA in the nodules directly related to IAA production *in vitro* by inoculated rhizobia suggested that much of the IAA produced in the nodules can be derived from the transformed rhizobia (bacteroids) in the nodules (Hunter 1987). Furthermore, this association is also strengthened by the presence of a higher IAA in effective nodules than in ineffective nodules (Badenoch-Jones *et al*. 1983). Effective nodules induced by IAA-producing rhizobia had higher IAA concentration in the bacteroids, larger nodules, higher nitrogenase activity and delayed nodule senescence (Hunter 1989; Camerini *et al*. 2004). Nodules induced by the modified strain of rhizobia able to produce IAA had an extended nitrogen-fixing zone and a reduction of the senescent zone in 45-day old nodules, as compared with plants nodulated by a wild type strain (Defez *et al*. 2016). The actively dividing cells in the meristematic region of nodules induced by IAA producing rhizobia were observed at a late stage of plant growth 52 days after infection (Camerini *et al*. 2008). In this study, active bacteroids were present in the nitrogen-fixing region of nodules but were not observed in senescent parts of nodules, suggesting the role of IAA in enhancing N_2 fixation through delaying early nodule senescence.

One mechanism by which IAA can delay nodule senescence is via the formation of poly-3-hydroxybutyrate (PHB) granules in bacteroids inside root nodules. PHB granules can act as a reserve source of energy and carbon in free-living rhizobia, particularly under carbon-starved conditions, and can improve bacteroid fitness, multiplication and survival inside the senescing nodules and in soils (Wong and Evans 1971; Prakamhang *et al*. 2014; Cagide *et al*. 2018). Moreover, PHB accumulation in the cells can function as a carbon sink for the TCA cycle operating in the microaerobic conditions required for N_2 fixation in nodules (Dunn 1998). This prolongs nodule functioning and can provide more fixed N to the host plant, which in turn can supply suitable carbon sources to be used as energy for bacteroids in the nodules.

In legume-rhizobia interactions, IAA production either by the rhizobia or by NER triggers the expression of genes closely associated with the symbiosis. The expression of N_2 fixation genes and the activity of the nitrogenase enzyme was significantly increased in nodules induced by the modified strain of *Ensifer* (*Sinorhizobium*) *meliloti* RD64 which was able to overproduce IAA as compared with the wild type strain (Defez *et al*. 2017; 2019). Among the N_2 fixation genes upregulated within mutant induced nodules, *fixJ*, which codes for the response regulator that turns on N_2 fixation genes during plant development, reached the highest level of transcription at 42 DAI, when the plant fixed the highest amount of N. A similar trend in transcription was observed for the genes *nifA*, *fixK1* and *fixK2*, which are directly regulated by the FixJ protein, and for *nifH*, which codes for the nitrogenase enzyme. The gene *fixA*, which is part of the *fixABCX* transcriptional unit involved in the electron transport to nitrogenase (Fischer 1994) and *fdxB*, coding for the ferredoxin, which acts as an electron donor for nitrogenase reductase (Barnett et al. 2001), were highly upregulated in nodules induced by strain RD64, a mutant of *Ensifer meliloti* (Defez et al. 2019). Likewise, in legume-rhizobia-NER interactions in mature nodules where N_2 fixation occurs, *nif*H was upregulated in plants co-inoculated with *Bradyrhizobium* and IAA producing NER compared with those inoculated with *Bradyrhizobium* alone (Prakamhang *et al*. 2014).

IAA can enhance N_2 fixation by altering the expression of genes that indirectly influence the efficacy of N_2 fixation by bacteroids in the nodules. In nodules induced by IAA producing rhizobia, the $fixNOQP_{1,2}$ operon genes, coding for haem-copper cbb3-type oxidases that have high affinity for oxygen, was increased and reached a maximum induction at 40 DAI (Defez et al. 2019). In this study, the expression of *gltA*, *icd* and *sucA*, coding for the main TCA cycle enzymes, was significantly upregulated in bacteroids. Another gene, *otsA*, encoding trehalose 6-phosphate synthase, which facilitate nodule formation and nitrogenase production under

stress (Suárez *et al*. 2008) and also has a role in nodule respiration (Bargaz *et al*. 2013) was upregulated when soybean was inoculated with IAA-producing NER. Furthermore, soybean co-inoculated with *Bradyrhizobium* and IAA-producing NER resulted in increased transcription of the *dctA* gene, encoding the dicarboxylate transport protein DctA, a transporter of carbon sources from the host plant to the bacteroids to sustain their functions, as compared with plants inoculated with *Bradyrhizobium* alone (Batista *et al*. 2009). For all the genes described above, IAA can improve the efficacy of legume-rhizobia interactions in fixing more N through upregulation of genes involved in carbon transport to the nodules and metabolism by bacteroids.

IAA increases the amount of N_2 fixation in the legume-rhizobia interaction by increasing the amount of photosynthetic products provided to bacteroids in root nodules (Tsikou *et al*. 2013; Erice *et al*. 2014). To confirm the role of IAA in photosynthetic activity of the plant and consequently on the N_2 fixation potential of bacteroids in nodules, a wild rhizobial strain that produced very low levels of IAA and its derivative overproducing IAA were used to inoculate *M. sativa* plants (Defez et al. 2019). At 42 DAI, the leaves of plants inoculated with the overproducing mutant had an increase of 37% in chlorophyll a (the major pigment) and of 84% in chlorophyll b (the accessory pigment), which allows the plants to more efficiently use the light spectrum (Porra 1997). Moreover, plants inoculated with a modified strain had higher concentration of carotenoid (light-harvesting pigment). Plants inoculated with a mutant had a higher quantity of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase), the enzyme widely considered as the rate-limiting step in photosynthetic carbon fixation (Carmo-Silva *et al*. 2015). Although the stress conditions reduced the Rubisco content in the plants, the level of this molecule in stressed plants inoculated with the IAA-overproducing rhizobial strain was still 25% higher than in the non-stressed plants inoculated with the wild strain (Defez et al. 2017). Other metabolites including the free amino acids (valine, alanine, aspartic acid and glutamic acids) significantly increased in plants inoculated with a modified strain compared with those inoculated with the wild type strain under normal conditions (Defez *et al*. 2019). Greater production of amino acids in the plants indicates the presence of greater N_2 fixation that is associated with the presence of more energy sources for the bacteroids from improved photosynthesis (Vauclare *et al*. 2010). Therefore, increased amino acids indicate a connection between high levels of Rubisco, increased photosynthetic products and enhanced nitrogenase activity in the nodules. In addition to the above, the production of organic acids, mainly malate and citrate, was higher in plants inoculated with modified strains than those inoculated with

the wild type strain. An increase of malate, a primary source of carbon for bacteroids metabolism to generate ATP (Schulze *et al*. 2002), indicate that IAA also increases the usable form of carbon for bacteroids in the root nodules. Moreover, an increase in total carbon and organic acids in plants was associated with a higher PHB content, which accumulated in the Interzone II-III of their root nodules (Imperlini *et al*. 2009), the region in which the majority of N_2 fixation occurs. This is one of the mechanisms by which IAA production in the nodules extends Interzone II-III and prolongs its ability to fix N (Oldroyd and Downie 2008; Defez *et al*. 2019).

2.6. Transcriptional changes in free-living rhizobia

Some key genes associated with N_2 fixation in rhizobia are induced by IAA production under free-living conditions that resemble those that occur in nodule bacteroids. The overproduction of IAA by *S. meliloti* mutant (RD64) in the free-living state positively affect the expression of the *nifA* and *fixK* genes that encode the two main intermediate regulators and that in turn induce the expression of *nif* and *fix* structural genes involved in respiration and N_2 fixation (Defez *et al*. 2016). Furthermore, IAA induced the expression of the major regulator of N_2 fixation, *fixJ* and the intermediate regulators *nifA* and *fixK* in *S. meliloti* mutant (RD64). In the same modified strain, higher expression of N_2 fixation genes (*fixK1*, *fixK2*, and *fixT2*) was determined than in the wild type strain producing very low levels of IAA (Defez *et al*. 2016). Moreover, in the modified strain the *fixNOQP* operon genes encoding the bb3-type cytochrome c oxidase, which has a high affinity for O_2 and is required to support bacteroid respiration in root nodules (Fischer 1994; Dixon and Kahn 2004), were highly upregulated. Enhanced expression of genes having high affinity for O_2 may lead to low O_2 concentration around the cells, thus allowing the transcription of aforementioned N_2 fixation genes (Reyrat *et al*. 1993). Moreover, genes related to denitrification (*nosD*, *nosF*, *nosY* and *nosZ*) and which prevent early nodule senescence and maintain an efficient symbiosis, were also upregulated (Bobik et al. 2006; Cabeza et al. 2014). In *S. meliloti* mutant (RD64), genes encoding components involved in motility and flagellar biosynthesis (*flhA* and *flaD*) were repressed. A reduction in motility of rhizobia near the root surface increases its ability to invade the root cells and induce nodules (Vercruysse *et al*. 2011; Li *et al*. 2013). When an analysis of gene expression was carried out in nodules, a down-regulation of flagella and chemotaxis genes was also observed. The suppression of the aforementioned two genes consequently affect the formation of effective nodules (Barnett *et al*. 2001; Capela *et al*. 2006). Taking into account the overall findings, the

overproduction of IAA by free living *S. meliloti* cells induced many of the transcriptional changes that normally occur in N_2 fixing root nodules.

2.7. Suggested future research

Despite research on PGPR over the last few decades, PGPR inoculants have not been commercialised efficiently. Both basic and applied research is needed to unlock the potential of PGPR. The following are some key research areas that need to be investigated to identify efficient NERs to improve the legume-rhizobia symbiosis under field conditions.

1. Determine the level of ACC deaminase expression and IAA production and its effect on the survival of the NER and co-inoculated rhizobia in diverse environments and soils.
2. Examine the expression of the aforementioned PGP traits by NER, either endophytic bacteria or transformed rhizobia (bacteroids) in the rhizosphere soil and inside the nodule environment, and determine the associated effect on the legume-rhizobia symbiosis.
3. Define the concentrations at which ACC deaminase expression and IAA production, alone and in combination, result in the highest beneficial effect on the legume-rhizobia symbiosis. Moreover, identify the major interactions among multiple traits including those mentioned above and others such as the potential for phosphate solubilisation, and the net outcome of these interactions on the legume-rhizobia symbiosis.
4. Identify the underlying molecular mechanisms relating to how these two traits affect the ability of rhizobia to induce nodules, to compete with the background rhizobia for nodule induction, and to increase the effectiveness in N_2 fixation.

2.8. Conclusions

The amount of N derived from symbiotic N_2 fixation can potentially be improved by the additional use of non-rhizobial PGPR. PGPR, and, specifically, NER, could improve nodulation and N_2 fixation by enhancing the effectiveness of both inoculant and soil rhizobia. We suggest that NER increase nodulation and N_2 fixation through the following mechanisms: 1) upregulating the production of root exudate components involved in the legume-rhizobia symbiosis; 2) enriching beneficial rhizosphere microbial communities around the root zone; 3) inducing the transcription of genes in the plant; 4) directly stimulating nodules to increase symbiotic efficiency. The effect of NER on the symbiosis is determined by the prevailing

environmental conditions that affect survival and competitiveness in the microbial communities in the root zone and influence the contribution of various PGP traits for nodule promotion. Among several identified PGP traits of NER, ACC deaminase and IAA production are two main mechanisms that enhance the legume-rhizobia symbiosis. These traits enhance the legume-rhizobia symbiosis through increasing nodulation and nodule function, and the competitiveness of the rhizobial inoculant. When both traits are expressed simultaneously by NER, ACC deaminase can reduce the deleterious effect of very high IAA and thus improve nodulation and the potential N_2 fixation. In general, the use of rhizobial inoculants containing multiple NER can be developed by investigating the legume plant-rhizobia-NER-environmental conditions that can enhance the effectiveness of inoculated rhizobia across a wide range of environments. Understanding the molecular and physiological mechanisms of NER production of either IAA and/or ACC deaminase can influence the entire process of the legume-rhizobia association, and therefore warrants further consideration. Therefore, the use of rhizobial inoculants containing NER, with IAA and ACC deaminase (among other mechanisms), may provide the greatest benefits for legume-rhizobia symbiosis in diverse environments, even in soils containing an abundance of naturalised rhizobia.

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Chapter 3 Large-scale screening of rhizobacteria to enhance the chickpea-Mesorhizobium symbiosis using a plant-based bioassay

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Contribution to the Paper	Planned the study conducted all experiments, analysed and interpreted data and wrote the manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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3.2. Abstract

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Large-scale screening of rhizobacteria to enhance the chickpea-*Mesorhizobium* symbiosis using a plant-based strategy

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ABSTRACT

Rhizobacteria able to express multiple plant growth-promoting (PGP) traits has been frequently failed to enhance the chickpea-*Mesorhizobium* symbiosis possibly because a relatively large amount of organic acids exudated by chickpea root to form an acid rhizosphere reduced the survival and colonisation of plant growth-promoting rhizobacteria (PGPR). Therefore, this study was initiated to screen rhizobacteria that would enhance the chickpea-*Mesorhizobium* symbiosis using a plant-basedstrategyA total of 743 *Bacillus*-, *Burkholderia*- and *Pseudomonas*-like bacteria were isolated from 74 agricultural soil samples collected across Australia. Only 167 isolates were selected, based upon their individual performance in a seedling growth bioassay, to test their effect on nodulation and growth of chickpea in sterile growth pouches. Also, their ability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase and indole acetic acid (IAA) was investigated in relation to their mode of action. The diversity of the 167 isolates was investigated using 16S rDNA sequencing. Of 743 isolates, 697 (83%) were able to produce IAA in the presence of L-tryptophan. ACC deaminase production was detected in 57 isolates (7.7%). Commercial inoculant strain *Mesorhizobium ciceri* CC1192 produced 7 μg mL^{-1} IAA and lacked ACC deaminase activity. In further investigation, the promotion of seedling root elongation by rhizobacteria predicted their effect on nodulation and growth of chickpea after dual inoculation in aseptic conditions. The effect of rhizobacteria on seedling root growth as well as shoot growth and nodulation was associated with the capacity of the isolates to produce IAA and ACC deaminase. These isolates were superior in plant growth promotion than those PGPR unable to produce PGP traits. The synthesis of IAA along with ACC deaminase activity by rhizobacteria gave an added advantage in promoting the ability of rhizobia to form an efficient chickpea-*Mesorhizobium* symbiosis as measured by biomass production and nodulation. Most of these rhizobacteria belonged to the genus *Burkholderia*. The use of a plant-based first-stage screening strategy in combination with assays for *in vitro* production of IAA and ACC deaminase enabled the identification of efficient PGPR that were able to enhance the legume-rhizobia symbiosis.

3.3. Introduction

The rhizosphere is the soil habitat close to the plant root with intensified biological and chemical properties relative to bulk soil and a major focus of sustainable agricultural research (Young, 1998). In the rhizosphere, there is enormous interaction between plant and microbe facilitated by plant root exudation (McCully, 1999; Read et al., 2003). Microbes associated with plants can be harmful, beneficial or neutral to the plant. Beneficial bacteria that can promote plant growth by colonizing plant roots are generally called plant growth-promoting rhizobacteria (PGPR) (Lugtenberg and Kamilova, 2009). In particular, genera closely related to *Bacillus* and *Pseudomonas* are the bacteria most commonly studied and exploited as PGPR (Santoyo et al., 2012; Gouda et al. 2018; Saxena et al., 2019). These PGPR express various plant growth-promoting (PGP) properties that benefit the plant either directly or indirectly, resulting in increased plant growth (Swift et al., 2018).

The use of PGPR inoculation in chickpea has been frequently failed to enhance the growth and nodulation under controlled and field conditions (Wani et al., 2007; Hynes et al., 2008; Patel et al., 2012; Verma et al., 2012; Baliyan et al., 2018; Mahmood Aulakh et al., 2020). Most of these isolates have been selected based on their ability to express multiple plant growth-promoting (PGP) traits *in vitro* conditions. Additionally, their deleterious and beneficial effect of rhizobacteria on plant growth has been unrelated to their ability to express various types and concentrations of PGP traits *in vitro* conditions. For instance, rhizobacteria able to express multiple beneficial PGP traits occasionally performed poorly as compared to other isolates inefficient *in vitro* condition (Wani et al., 2007; Baliyan et al. 2018). In this regard, the isolate able to express, IAA, ACC deaminase, P solubilisation, N_2 fixation and biocontrol activities *in vitro* was inferior in plant growth promotion as compared to those with IAA and P solubilisation (Oteino et al. 2013). However, the later isolates were most efficient in root colonisation than the former ones. The main reason behind the inconsistent results between laboratory and *in planta* can be related to the ability of the isolates to colonisation and persistence in the rhizosphere. Recently, investigation of microbial root colonisation related traits, including formation of biofilm and flagella, resistance to hydrogen peroxide and utilisation of carbon substrates, could assist the selection of more competent and best root coloniser strains to be tested *in planta* (Amaya-Gómez et al. 2020). However, the identification and selection of PGPR based on PGP and root colonisation related traits and their metabolic activities can be time-consuming, laborious, costly and frequently unreliable. Therefore, the

pre-screening strategy that ultimately results in the most reliable rhizobacteria in plant growth promotion would be extremely important.

With the consideration of high amount of carboxylates and more acidic pH in the exudate in chickpea rhizosphere compared to other legume plants (Ohwaki and Hirata, 1992; Veneklaas et al., 2003; Wouterlood et al., 2005; Pearse et al., 2007; Kabir et al., 2015), we hypothesised that plant-based pre-screening may predict the efficiency of rhizobacteria in plant growth promotion in N_2 fixing chickpea. Hence, the objective of this study was to screen rhizobacteria that would enhance the chickpea-*Mesorhizobium* symbiosis using a plant-based bioassay.

3.4. Materials and methods

3.4.1. Description of sampling sites

Seventy-four soil samples were collected across the major agricultural lands of Australia (Appendix Fig. 1). Sampling sites were selected based on their cropping history. Crop rotation was the main cropping system at all the sampling sites. Wheat was cultivated in rotation with legumes (chickpea, faba bean, field pea, lentil, lupin or vetch) and other non-leguminous crops (barley, canola, oats or sorghum) at the majority of the sampling sites. Of the 74 sites, forty one had a legume grown in the previous 5 years. The remaining sites had no history of legume cultivation. Eleven sampling sites had a chickpea cropping history within the last five years.

The long-term average rainfall at the study sites ranged from 289 to 695 mm (Bureau of Meteorology 2019). The potential evapotranspiration (PET) varied within the range 1,168 to 1,358 mm. The aridity index was calculated using P/PET, where P is the average annual precipitation (UNEP 1992). Aridity index values of the study sites varied between 0.23 and 0.53.

The physicochemical properties of soils were analysed following the methods described in Rayment and Lyons (2011). Soil C was determined following the Walkley Black method. A modified Kjeldahl method was used to determine total soil N. Total P in the soil was analysed by a colorimetric method after extracting elemental P by perchloric acid digestion. Soil pH in $CaCl_2$ was determined from a water suspension containing a 1:2.5 of soil to $CaCl_2$ mixture. The electrical conductivity of the water extract from a 1: 2.5 soil: water suspension was determined. The textural composition of the soils was analysed by dispersal and hydrometric readings. Ammonium acetate extractable Ca, K and Mg in soils were determined using atomic absorption spectrophotometry. Diethylene triamine pentaacetic acid (DTPA) extractable

concentrations of Cu, Mn and Zn in soils was determined. The physico-chemical properties of soils are presented in Appendix Table 1.

3.4.2. Collection of rhizosphere soils

One hundred g of soil from each sample was placed in pots (4.5 cm diameter, 10 cm high) to grow chickpea cv. HatTrick in a growth chamber with light supplied by fluorescent and incandescent lamps with an intensity of 1000 µmol $m^{-2}s^{-1}$ for a 16 h photoperiod, with a constant temperature of 20 ºC and relative humidity at 70%. The soil closely adhering to the root system (approximately 1 - 5 mm from the root surface) was considered to be 'rhizosphere soil' and was carefully collected from chickpea seedlings four weeks after germination in sterile specimen containers and immediately kept at 4 ºC. The samples were processed within 24 h of collection.

3.4.2. Isolation of *Pseudomonas*- and *Bacillus*-like rhizobacteria

Pseudomonas and *Bacillus* are relatively easily cultured *in vitro* and can be preliminary dentified using phenotypic characteristics. Additionally, they are commonly used in commercial products. In the present study, *Pseudomonas* and *Bacillus* were selectively isolated from soils because most of PGPR belong to the same genera (Swift et al., 2018, Alemneh et al. 2020). Each rhizosphere soil was serially diluted from 10^{-1} - 10^{-6} in sterile water. One g of rhizosphere soil was placed in a test tube containing 9 mL of sterile MilliQ water. A 100 µL soil suspension from 10^{-5} or 10^{-6} was taken and spread in duplicate onto Petri dishes containing 15 mL of 10% tryptone soya agar (TSA) (OXOID LTD, Basingstoke, Hampshire, England), supplemented with 50 µg mL^{-1} of ampicillin (99%, Sigma Aldrich Chemie GmbH, Steinheim, Germany), 12.5 µg mL^{-1} of chloramphenicol (99%, Sigma Aldrich) and 75 µg mL^{-1} of cycloheximide (Sigma Aldrich) to inhibit non-*Pseudomonas* and fungal growth (Simon and Ridge, 1974). The remaining aliquots from 10^{-5} and 10^{-6} were incubated (15 min, 80 ºC) in a water bath to kill the non-endospore-forming bacterial cells. Then, 100 µL of the diluted suspension was spread onto TSA media to obtain aerobic endospore-inducing bacteria (*Bacillus*). Plates were incubated for 5 days at 28 ºC. Bacterial colonies were selected according to their morphological characters such as colony size, colony edge appearance, and colony colour. The selected colonies were purified through subsequent sub-culture on fresh TSA medium. A total of 743 purified isolates was kept at 4 ºC on slant cultures for immediate use and additionally at -80 ºC for long-term storage.

3.4.3. Seedling growth bioassay

A plant growth experiment with chickpea cv. HatTrick inoculated with bacterial isolates was carried out in a growth chamber in the aforementioned conditions. A pure colony of each isolate was inoculated into 10 mL of tryptone soya broth (TSB). Seeds of chickpea were surface-disinfected by washing in 1% hypochlorite for 3 min followed by thorough washing with sterile MilliQ water. The treated seeds were germinated in a sterile Petri dish containing moistened filter paper and kept in an incubator until the radicle was approximately 5 mm long. The germinating seeds were transferred into a large Petri dish (10 cm diameter) containing 1% water agar and inoculated with 1 mL suspension of a 48-h bacterial culture. Each Petri dish contained ten seedlings. A control (non-inoculated) treatment was included and treated with a sterilised broth. Treatments were laid out in a Complete Randomized Design with two replications. After 7 days, root length was recorded.

3.4.4. IAA production

The potential of isolates to produce IAA in the presence and absence of L-tryptophan. IAA production was determined using colorimetric analysis (Patten and Glick, 2002). Rhizobacteria were grown in 10 mL of DF-minimal medium (Dworkin and Foster, 1958) containing (L^{-1}) 4.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 2.0 g glucose, 2.0 g gluconic acid and 2.0 g citric acid with trace elements: 1mg $FeSO_4.7H_2O$, 10 mg H_3BO_3, 11.19 mg $MnSO_4.H_2O$, 124.6 mg $ZnSO_4.7H_2O$, 78.22 mg $CuSO_4.5H_2O$, 10 mg MoO_3, pH=7.0 supplemented with L-tryptophan (500 µg mL^{-1}) in 20 mL test tubes. This culture solution was incubated on a shaker (48 h, 28 ºC, 160 rpm). A non-inoculated treatment was included for comparison. All test was done in triplicate. After incubation, a subsample of 5 mL of culture solution was centrifuged (10 min, 10,000 ×*g*). One mL of the supernatant was mixed with 2 mL of Salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M $FeCl_3$ solution) and the mixture was kept for 1 h in the dark. The development of pink colour in the mixture indicated that the isolate was positive for IAA production and the intensity of the colour was measured at 520 nm using a spectrophotometer (Implen GmbH, München, Germany). The concentration of IAA was interpolated using a standard curve. Isolates unable to produce pink colour in the mixed solution were considered to be negative for IAA production.

3.4.5. ACC deaminase production

Qualitatively, bacteria able to utilise ACC as sole N source were identified based on their growth in DF minimal medium supplemented with 3 mM ACC (TCE, Tokyo Chemical

Industry Co. LTD, Toshima, Kita-Ku, Tokyo). ACC was added to sterile DF-minimal medium after filter-sterilisation through a 0.22 μm pore size membrane. Bacterial isolates unable to grow on this medium were considered to be negative for ACC deaminase activity. Those isolates able to grow on this medium (72 rhizobacteria) were further investigated for their ability to express ACC deaminase activity in DF minimal medium containing 3 mM ACC as the sole N source. A pure colony was inoculated into the broth and the culture was incubated on a shaker (3 h, 28 °C, 160 rpm). The supernatant was separated from the culture solution by centrifugation (10 min, 10,000 ×*g*). The determination of ACC deaminase activity was carried out following the procedure described by Penrose and Glick (2003). All ACC deaminase determinations were made in triplicate.

Besides the *in vitro* identification of ACC deaminase activity, the presence of this capability in the PGPR was confirmed by sequencing of the *acdS* gene using consensus-degenerate hybrid oligonucleotide primers (CODEHOP) (Li et al., 2015). The partial sequences of *acdS* genes with an expected amplicon size of ~760 bp was amplified using acdSf3 (ATCGGCGGCATCCAGWSNAAYCANAC) (Sigma-Aldrich) and acdSr3 (GTGCATCGACTTGCCCTCRTANACNGGRT) (Sigma-Aldrich). Amplification reactions contained 25 μL containing 1 μL of each of 10× diluted reverse and forward primer, 12.5 μL of 2x master mix (Sigma-Aldrich) and 10.5 μL MilliQ water. The PCR conditions were as follows: an initial denaturation at 95 °C for 15 s, followed by 35 cycles of 95 °C for 30 s, annealing at 53 °C for 1 min and extension at 72 °C for 1 min and a final extension at 72 °C for 5 min.

3.4.6. The sequence of 16S rRNA and phylogenetic analysis

Isolates that represented a cross-section of the best, moderate and poor performing isolates in seeding vigor test were selected to examine their molecular diceisty using 16S rRNA sequence. To amplify the 16S rRNA, the reverse primer rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) (Sigma-Aldrich) and forward primer fD1(5′-AGAGTTTGATCCTGGCTCAG-3′) (Sigma-Aldrich) were used (Weisburg et al., 1991). Amplification was performed with the following PCR conditions: initial denaturation at 95 °C for 30 s, 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. Purified PCR products were sequenced by Sanger sequencing (Australian Genome Research Facility, Adelaide, Australia) with an expected amplicon size of ~1500 bp. The purity of PCR products was checked by gel electrophoresis. Sequences homologous to 16S rRNA

were retrieved from the National Centre for Biotechnology Information (NCBI) GenBank database using the BLASTn tool. The sequences were submitted to NCBI and assigned accession number (MW692202 - MW692329). The phylogenetic analysis was conducted using the neighbour-joining method and the tree was generated using molecular evolutionary genetics analysis (MEGA) software version 7 (Institute of Molecular Evolutionary Genetics, Pennsylvania State University, University Park, PA 16802, USA). The stability of the clades in the phylogenetic tree was assessed using a bootstrap value with 1000 replications.

3.4.7. Growth pouch experiment

Based on the seedling growth bioassay, 167 isolates that represented a cross-section of the best, moderate and poor performing isolates were selected to examine their effect on the chickpea-*Mesorhizobium* symbiosis. Moistened CYG germination pouch bags (Mega International of Minneapolis 1800 Kolff St. Newport, MN; 55055 USA), each 36 X 16.5 cm, were used after sterilisation (20 min, 121 ºC). Surface-sterilised chickpea cv. HatTrick seeds were germinated in Petri dishes containing moist filter papers. Forty-eight-h germinating seeds were transferred to the sterile and moistened growth pouch. *M. ciceri* strain CC1192 and the rhizobacteria were grown in TSB medium on a shaker (48 h, 28 ºC, 160 rpm). One mL of culture solution containing approximately 10^8 colony-forming unit (CFU) mL^{-1} was added per seedling. Two control treatments, non-inoculated and plants inoculated only with strain CC1192, were included. All treatments were replicated three times. Pouches were placed upright in a rack. Ten mL of McKnight N free nutrient solution per growth pouch was supplied every week (McKnight, 1949). The experiment was harvested seven weeks after bacterial inoculation. Nodule and shoot dry weight were determined.

3.4.8. Data analysis

The regression analyses and box plots were prepared using SigmaPlot ver. 14. The correlation analysis among the plant growth and nodulation parameters was analysed using Genstat ver. 18. Data were also subjected to analysis of variance and the difference between treatment means were determined by Fisher's protected least significant difference ($P < 0.05$). Before analysis, the normal distribution of the data was checked using the Shapiro-Wilk test (Shapiro and Wilk, 1965). Homogeneity of variances was evaluated using Bartlett's tests (Bartlett and Kendall, 1946).

3.5. Results

3.5.1. Plant growth-promoting characteristics

The rhizobacteria were found to produce a wide range of ACC deaminase activities and IAA concentrations. Out of 743 isolates, 616 (83%) secreted IAA in the presence of L-tryptophan and the amount varied between 4.1 and 67.2 µg mL^{-1}. One *Bacillus* isolate produced 22.2 µg mL^{-1} of IAA in the absence of its precursor.

Although 94 isolates (12.6%) were qualitatively identified as ACC utilising bacteria, the quantitative investigation identified that only 57 isolates were found to produce ACC deaminase, with activities between 0.335 and 8.12 µmol α-ketobutyrate h^{-1}. The *acdS* gene was also detected in these isolates. Among 37 isolates unable to express ACC deaminase activity, only 15 were able to grow in N-free media. A total of 35 isolates possessed both PGP traits, i.e. IAA and ACC deaminase production. *M. ciceri* CC1192 also produced 7 µg mL^{-1} of IAA but did not show ACC deaminase activity.

3.5.2. Seedling vigour test

A total of 743 rhizobacteria isolated from the chickpea root zone were investigated for their efficacy in seedling growth promotion in aseptic conditions. A majority of isolates (525) significantly increased seedling root length compared to the control check. Plants inoculated with the remaining (218) isolates had shorter roots than non-inoculated plants. Based on these results, 167 isolates that represented a cross-section of the best (55), moderate (56) and poor (55) performing isolates were selected for further investigation.

Regression analysis between seedling root elongation and ACC deaminase activity or IAA production by inoculated isolates was carried out to identify the underlying mechanisms in seedling growth promotion. A significant and polynomial relationship between seedling root length and IAA production was observed in plants inoculated with isolates regardless of their ability to produce ACC deaminase (Fig 1a and b). A similar association was observed between root length and ACC deaminase production in plants inoculated with PGPR that were unable to produce IAA (Fig. 1c). However, none of the ACC deaminase-producing bacteria suppressed root elongation (Fig. 1a and c). In addition, seventeen isolates able to produce IAA greater than 30 µg mL^{-1} and below 10 µg mL^{-1} suppressed seedling root growth as compared to non-inoculated plants. These isolates were unable to produce ACC deaminase. The association

between ACC deaminase activity and seedling root length was non-significant in plants inoculated with PGPR possessing both PGP traits (Fig. 1d).

3.5.3. Phylogenetic diversity

Phylogenetic analysis of the selected isolates identified diverse species of bacteria belonging to *Bacillus, Burkholderia* and *Pseudomonas* (Fig. 2). Interestingly, *Burkholderia* (previously classified as *Pseudomonas*)-like bacteria were obtained using a medium semi-selective for *Pseudomonas*. The dominant isolates were closely related to *Paraburkhoderia phenoliruptrix, Paraburkholderia terricola, Burkholderia caledonica, Burkholderia cepacia*, and *Burkholderia cenocepacia.* Additionally, isolates with high similarity to *Pseudomonas corrugata, Pseudomonas fluorescens, Pseudomonas frederiksbergensis, Pseudomonas geniculata, Pseudomonas jessenii* and *Pseudomonas putida* were identified. The majority of the isolates (98%) of these genera were able to express ACC deaminase and/or IAA *in vitro* (Appendix Table 2). Of these, 41.7% were able to express both mechanisms.

Fig. 2b shows that nineteen species of *Bacillus* were identified. The dominant species were *Bacillus megaterium, Bacillus flexus, Bacillus amyloliquefaciens*, *Bacillus toyonensis, Bacillus niacini and Peribacillus simplex.* Additionally, few isolates closely related to *Bacillus cereus, Bacillus cucumis, Bacillus bataviensis, Bacillus pumilus, Bacillus subtilis, Bacillus atrophaeus, Bacillus tequilensis, Bacillus mycoides* and *Bacillus thuringiensis* were identified. An isolate that showed 97% similarity to *Bacillus endophyticus* NM3E6 was also identified. The majority of these isolates (85.4%) produced IAA. About 11% of tested isolates lacked both mechanisms (Appendix Table 2). The remaining isolates expressed ACC deaminase activity alone.

3.5.4. Coinoculation experiment

Plants inoculated with *M. ciceri* strain CC1192 in combination with PGPR had the highest nodulation and growth, compared to those plants inoculated with either PGPR or CC1192 separately (Fig. 3a, b, c and d). In this case, the effect on nodulation was associated with the capacity of PGP isolates to produce ACC deaminase and IAA. The highest response to coinoculation was observed in plants inoculated with PGPR able to produce both ACC deaminase and IAA. Plants inoculated with PGPR able to produce ACC deaminase had greater nodulation and biomass production than plants inoculated with PGPR producing IAA. The lowest nodulation and plant growth was obtained in plants coinoculated with PGPR producing

neither IAA nor ACC deaminase. However, these plants had better nodulation and growth than control non-inoculated plants.

The mean values of plant growth and nodulation were plotted against the *in vitro* production of IAA and ACC deaminase by inoculated PGPR to investigate their mechanisms of action on the chickpea-*Mesorhizobium* symbiosis. Regardless of their ability to produce ACC deaminase, the amount of IAA production across all rhizobacteria was significantly and non-linearly associated with the effect on nodule dry weight (NDW) and shoot dry weight (SDW) of chickpea (Fig. 4a, b, c and d). No further promotion of the NDW and SDW of chickpea was observed with increasing IAA concentration beyond 20 $\mu g\ mL^{-1}$ produced by ACC deaminase negative rhizobacteria. Beyond 35 $\mu g\ mL^{-1}$ IAA, nodulation and biomass production was lower than for non-inoculated plants. Both plant parameters were also significantly and positively correlated with ACC deaminase activity produced by PGPR that lacked IAA production (Fig. 4e and f). However, these associations were not observed in plants inoculated with isolates able to produce IAA (Fig. 5g and h).

We further analysed the results to determine whether the seedling growth bioassay test was the best predictor of the PGPR effect on the chickpea-*Mesorhizobium* symbiosis. Seedling root length was linearly and significantly associated with SDW (r = 0.59, P < 0.01) and NDW (r = 0.81, P < 0.01) (Figures 5a and b). In this association, inoculated PGPR possessed the capability to produce IAA and ACC deaminase. Likewise, seedling root length promotion was directly and significantly associated with SDW (r = 0.82, P < 0.01) and NDW (r = 0.82, P < 0.01) in plants inoculated with IAA-producing PGPR (Figures 5c and d). In plants inoculated with PGPR producing only ACC deaminase activity, seedling root length promotion was weakly associated with SDW (r = 0.17, P < 0.01) and NDW (r = 0.27, P < 0.01) (Fig. 5e and f). When plants inoculated with PGPR unable to express either beneficial PGP trait, the association between seedling root growth and NDW (r = 0.30, P < 0.01) was significant but the association between seedling root growth and SDW was non-significant (Fig. 5g and h).

We did further analysis to determine whether plant growth promotion following inoculation of PGPR was associated with enhanced nodulation. A significant and linear association was observed between SDW and NDW when plants were co-inoculated with isolates able to produce IAA and/or ACC deaminase (Fig 6a, b and c). However, plant growth promotion was not linked with enhanced nodulation in plants inoculated with isolates that lacked both beneficial PGP traits (Fig. 6d).

3.6. Discussion

The performance of rhizobacteria in stimulating seedling root elongation, together with their ability to produce IAA and ACC deaminase *in vitro* predicted their efficacy in enhancing the chickpea-*Mesorhizobium* symbiosis. Most of the PGPR that resulted in better growth and nodulation of chickpea belonged to the genus *Burkholderia*.

Seedling vigour test predict the potential effect of rhizobacteria in chickpea-*Mesorhizobium* symbiosis

Plant-related factors in the rhizosphere, particularly the production of organic compounds in root exudates, could affect the survival and colonisation of PGPR and their ability to express various PGP properties (Drogue et al., 2012). Taking this into consideration, we used a plant-based strategy for PGPR development. Rhizobacterial isolates significantly differed in their potential to promote seedling root elongation. Rhizobacteria which promoted seedling root elongation also enhanced nodulation and growth of chickpea. Therefore, the results of the seedling root elongation test predicted the potential effect of rhizobacteria on the nodulation and growth of chickpea seedlings in aseptic conditions.

Plant growth promotion and nodulation increase following inoculation of PGPR solely or in combination with rhizobia have been shown in several studies (Molla et al., 2001; Vessey, 2003; Dey et al., 2004; Malik and Sindhu, 2011; Nascimento et al., 2012; Zaheer et al., 2016; Korir et al., 2017). However, the underlying mechanisms that triggered the growth-promoting effects of rhizobacteria in these studies remained unexplored. In the present study, the positive influence of rhizobacteria in chickpea-*Mesorhizobium* symbiosis was related to their ability to express ACC deaminase and IAA. ACC deaminase might act to stimulate plant growth and nodulation in particular by hydrolysing ACC released from germinating seed during nodule initiation (Glick, 2014; Guinel, 2015). This would decrease the ACC concentration and consequently the ethylene production in the rhizosphere (Nascimento et al., 2018). However, if the level of ethylene remains high in the rhizosphere after seed germination and nodule induction, legume root growth and nodulation are inhibited (Guinel, 2015). This might also be the reason for no further increase in nodulation and growth of plants inoculated with isolates that produced IAA above 20 $\mu g\ mL^{-1}$ in this study; this is attributed to the stimulation of ethylene synthesis by IAA (Glick, 2014).

Rhizobacteria able to produce IAA and ACC deaminase *in vitro* resulted in greater growth and nodulation of chickpea than isolates positive for either of the two traits individually. Inhibition

of plant growth and nodulation following inoculation of rhizobacteria producing IAA greater than 40 µg mL^{-1} were attenuated by the presence of ACC deaminase activity. This may be mediated by the decreasing the ethylene synthesis, which permits IAA activity that can continue to stimulate plant growth (Glick, 2014; Kang et al., 2019). Consequently, ACC deaminase appears to facilitate plant growth stimulation by IAA. Most of the isolates expressing both PGP traits in the present study were closely related to *Burkholderia* spp. We can, therefore, confirm that IAA along with ACC deaminase produced by rhizobacteria gives an added advantage in promoting the ability of rhizobia to form an efficient chickpea-*Mesorhizobium* symbiosis, as determined by nodule weight.

Association between plant growth and nodulation was dependent on the ability of inoculated rhizobacteria to express ACC deaminase and/or IAA

Rhizobacteria improve the growth of various legume plants but this effect is not always associated with enhancing nodulation (Molla et al., 2001; Dey et al., 2004; Korir et al., 2017). The association between plant growth and nodulation in the present study was significant when plant inoculated with rhizobacteria able to express ACC deaminase and/or IAA *in vitro*. This may indicate the importance of ACC deaminase and IAA to enhance nodulation *per se* that ultimately results in plant growth promotion. Recently, we reviewed that ACC deaminase and IAA can enhance legume-rhizobia symbiosis by enhancing nodule induction, prolonging functional nodules and upregulating genes associated with N_2 fixation (Alemneh et al., 2020).

Although isolates were unable to express ACC deaminase and IAA, most isolates belonging to *Bacillus* spp. improved the growth and nodulation of chickpea. In this case, the plant growth promotion was not related nodulation. Therefore, the effect of *Bacillus* in plant growth might be related to their ability to express PGP traits other than IAA and ACC deaminase.

Unidentified PGP mechanism determined the potential of few PGPR

Some rhizobacteria, which did not possess any of the PGP traits tested *in vitro* increased seedling root elongation as well as the growth and nodulation of chickpea. Why these isolates are also a PGPR is unclear. The efficacy of these isolates may be associated with some other PGP activities for which we did not test. However, these isolates poorly performed in plant growth promotion as compared to other PGPR that were able to produce ACC deaminase and IAA. Other studies have also found PGPR or deleterious rhizobacteria capable of expressing a wide array of PGP traits, without ACC deaminase or IAA production (Cattelan et al., 1999). This effect may be related to the ability of rhizobacteria to express other PGP properties

including cytokinin, gibberellic acids, and volatile compounds (Lorteau et al., 2001; Mirza et al., 2007), but we did not carry out any assays to confirm this. Most of these deleterious isolates were closely related to *Bacillus* spp. few rhizobacteria, which showed the closest sequence similarity with *B. thuringiensis*, *B. cucumis*, *P. simplex*, *B. muralis* and *P. frederiksbergensis* in this study inhibited the nodulation and growth of chickpea. The deleterious effect of rhizobacteria on nodulation and plant growth has been previously reported (Cattelan et al. 1999; Mirza et al. 2007; Valverde et al. 2007; Malik and Sindhu 2011; Smyth et al. 2011). However, the underlying mechanisms that cause harm in these studies have not been investigated. Antibiotics, siderophore, hydrogen cyanide and other inhibitory substances produced by inoculated rhizobacteria can negatively affect the survival of rhizobia in the root zone and also interfere initial infection process during nodule induction (Li and Alexander, 1990; Berggren et al., 2001; Camacho et al., 2001; Chebotar et al., 2001; Gowtham et al., 2017).

***Burkholderia* were more efficient plant growth promotion than Bacillus and Pseudomonas**

Rhizobacteria belonging to *Pseudomonas* and *Bacillus* are known to be efficient in plant growth promotion (Baliyan et al., 2018; Pandey et al., 2019). However, *Burkholderia* spp. in the present study were identified as the most efficient PGPR in enhancing chickpea-*Mesorhizobium* symbiosis as compared to *Bacillus* spp. Most of *Burkholderia* spp. were able to express ACC deaminase and IAA *in vitro*. Therefore, PGP potential of *Burkholderia* spp. can be related to their ability to produce ACC deaminase and IAA. Most of *Burkholderia* spp. including *P. phenoliruptrix, P. terricola, B. caledonica, B. cepacia*, and *B. cenocepacia* have been endophytic bacteria in different plant species including chickpea and recognised as effective symbiont associate (Lu et al., 2012; Rasolomampianina et al., 2005; Tapia-García et al., 2020). Endophytic *Burkholderia* spp. facilitated infection of plant root through deforming root hair, and increasing nodules and their efficiency in N_2 fixation (Compant et al., 2008). Based on their ability to colonise the nodules and roots of different plants (Dong et al., 2019; Kim et al., 2019; Putrie et al., 2020), further study is required to identify whether the *Burkholderia* in the present study can colonise internal nodules and subsequently their role in nodule induction and functioning.

3.7. Conclusion

The results of the seedling root elongation test predicted the potential effect of rhizobacteria on the nodulation and growth of PGPR and rhizobia co-inoculated chickpea in aseptic conditions.

PGPR may have more than one mechanism for enhancing nodulation and plant growth and the experimental evidence indicated that increased nodulation and plant growth were the net results of ACC deaminase and IAA production by PGPR. The best performing PGPR were closely related to *Burkholderia*. Hence, the use of a seedling growth bioassay as a first stage screening strategy for PGPR development appeared to integrate the impact of IAA and ACC deaminase activities on growth and nodulation. Rhizobacteria able to produce high concentrations of IAA in combination with ACC deaminase activity are suitable PGPR candidates for inclusion in a second stage screening program using non-sterile soil in the greenhouse and/or field conditions.

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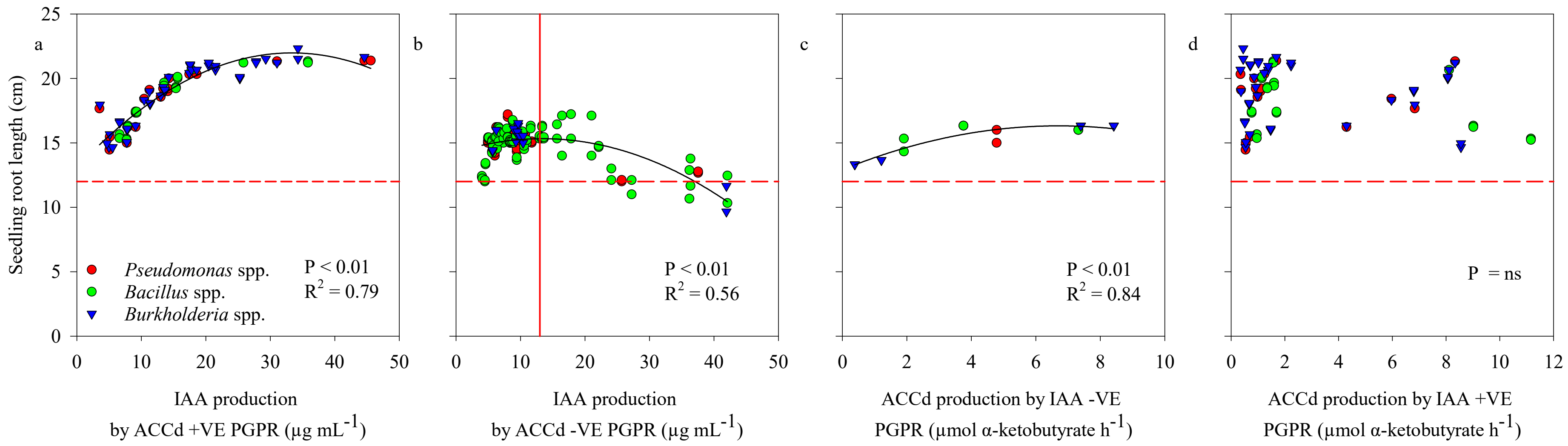

Fig. 1. The association between seedling root length promotion (at 7 days after germination) following inoculation of PGPR and their capacity to produce IAA and ACC deaminase *in vitro*. Data from three replications were combined after confirming homogeneity of variances with Bartlett's test. Each point represents an average of three replications within an experiment. The continuous line within each frame is a best-fit regression line with the respective significance level and strength of association. The vertical red line in Fig 1b indicates the critical maximum level of IAA production for stimulation of seedling root elongation. Beyond this point, no further increase in seedling root elongation was observed. The broken red horizontal line indicates seedling root length with no inoculation of rhizobacteria. ACCd: ACC deaminase, +VE: Positive and –VE: negative. $P < 0.01$- highly significant, P = ns- non-significant.

IAA
ACCd
a
28F (MW692301)
Paraburkholderia phenoliruptrix 08BF07TK (KX146477)
87F (MW692317)
86F (MW692316)
84F (MW692314)
7F (MW692294)
85F (MW692315)
108F (MW692321)
13F (MW692296)
Burkholderia rhynchosiae WSM3930 (EU219864)
35F (MW692303)
Paraburkholderia terricola R 8118 (NR 029044)
73F (MW692313)
21F (MW692299)
30F (MW692302)
Burkholderia graminis (U96939)
53F (MW692308)
94F (MW692318)
52F (MW692307)
18F (MW692297)
12F (MW692295)
120F (MW692325)
20F (MW692298)
Burkholderia sp. WSM4671 (MF949042)
55F (MW692309)
101F (MW692319)
Paraburkholderia phytofirmans PsJN 16S (NR 042931)
Burkholderia caledonica B5 (JX010968)
41F (MW692304)
132F (MW692329)
Burkholderia caryophylli (AB021423)
127F (MW692327)
Burkholderia cenocepacia 262 (KP974656)
Burkholderia vietnamiensis LMG 10929 (NR 041720)
126F (MW692326)
Burkholderia cepacia KSB-32 (MK280757)
68F (MW692312)
130F (MW692328)
Pseudomonas geniculata ATCC 19374 = JCM 13324 (NR 024708)
Pseudomonas thivervalensis PE32 (KJ420530)
Pseudomonas corrugata Slade 939/1 (NR 037135)
102F (MW692320)
Pseudomonas fragi ATCC 4973 (NR 024946)
Pseudomonas azotoformans KS 0034 (NR 037092)
Pseudomonas extremorientalis KMM 3447 (NR 025174)
Pseudomonas syringae NCPPB 281 (NR 043716)
117F (MW692324)
Pseudomonas frederiksbergensis (MN865449)
114F (MW692322)
116F (MW692323)
Pseudomonas aeruginosa DSM 50071 (NR 026078)
Pseudomonas stutzeri ATCC 17588 = LMG 11199 (NR 103934)
45F (MW692305)
Pseudomonas putida (AY456706)
Pseudomonas monteilii CIP 104883 (NR 024910)
Pseudomonas monteilii WPm-Del (MG462704)
Pseudomonas alcaligenes IAM 12411 (NR 043419)
27F (MW692300)
Pseudomonas fluorescens CREA-C16 (CP017951)
Pseudomonas koreensis Ps 9-14 (NR 025228)
46F (MW692306)
Pseudomonas sp. AP3 22 (MF554631)
62F (MW692310)
64F (MW692311)
0.020

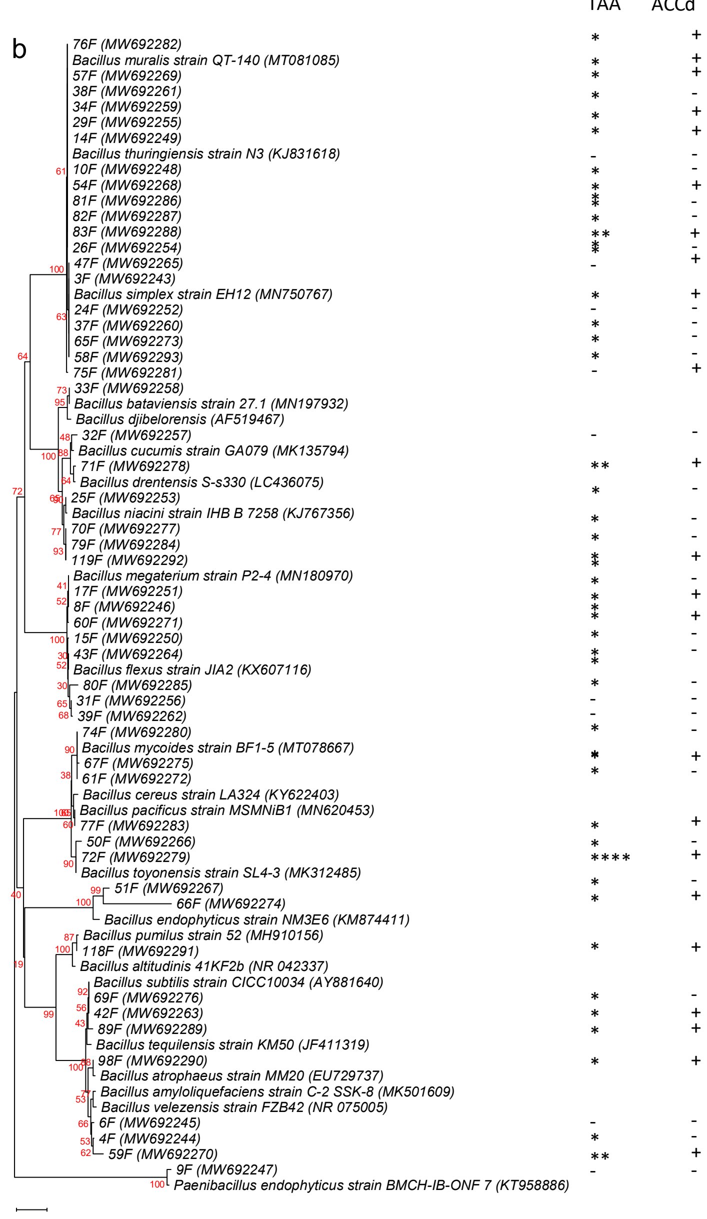

Fig. 2. Dendrogram showing the genetic relationships among the 16S rRNA gene sequences (1500 bp) of strains of (a) *Burkholderia* and *Pseudomonas*, and (b) *Bacillus* isolated from chickpea rhizospheric soils and their related type strains. The tree was created by the neighbour-joining method. The numbers on the tree indicate the percentages of bootstrap sampling derived from 1000 replications. PSI: phosphate solubilisation index, *- PSI value <2, **- 2-3, ***>3-4, ****>4.0. IAA ($\mu g\ mL^{-1}$) concentration * < 10, ** 10 - 20, *** 20 – 30, ****30 – 40, ***** 40 – 50, ****** 50 – 60 and ******* 60 – 70. ACCd: ACC deaminase, '+' isolates positive for ACC deaminase activity and '-' isolates negative for ACC deaminase activity.

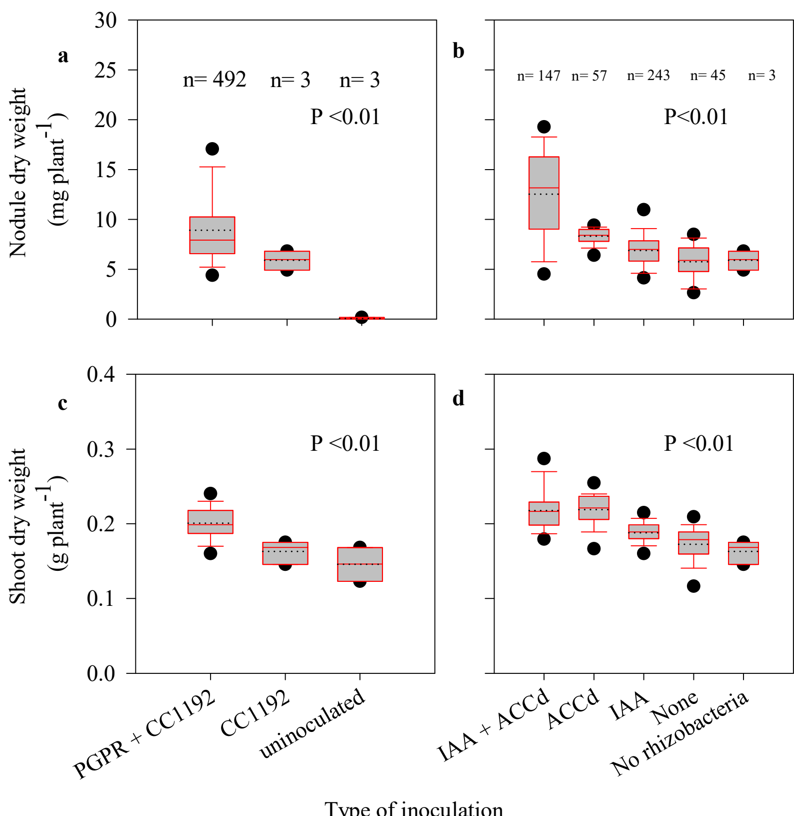

Fig. 3. Box plots showing the overall effect of PGPR inoculation on shoot dry weight and nodule dry weight based on the number of isolates inoculated (**PGPR + CC1192**: plants co-inoculated with PGPR and *Mesorhizobium ciceri* CC1192, **CC1192**: plants inoculated only with *Mesorhizobium* sp. and **uninoculated**: plants not inoculated. **IAA + ACCd**: plant co-inoculated with ACC deaminase and IAA producing PGPR and CC1192, **ACCd**: plants inoculated with ACC deaminase-producing PGPR + CC1192, **IAA**: plants inoculated with IAA-producing PGPR + CC1192, **None**: plants co-inoculated with PGPR lacking both traits + CC1192 and **No rhizobacteria**: plants inoculated with only CC1192. Each treatment was replicated three times. Data from the three replications were combined after confirming the homogeneity of variances with Bartlett's test. The dots on each side of the box plot represent the 95% confidence interval of the data distribution. The broken line in the box plot indicates the mean value. PGPR: Plant growth-promoting rhizobacteria, ACCd: ACC deaminase, CC1192: *M. ciceri* strain CC1192. n = number of isolates. $P < 0.01$- highly significant.

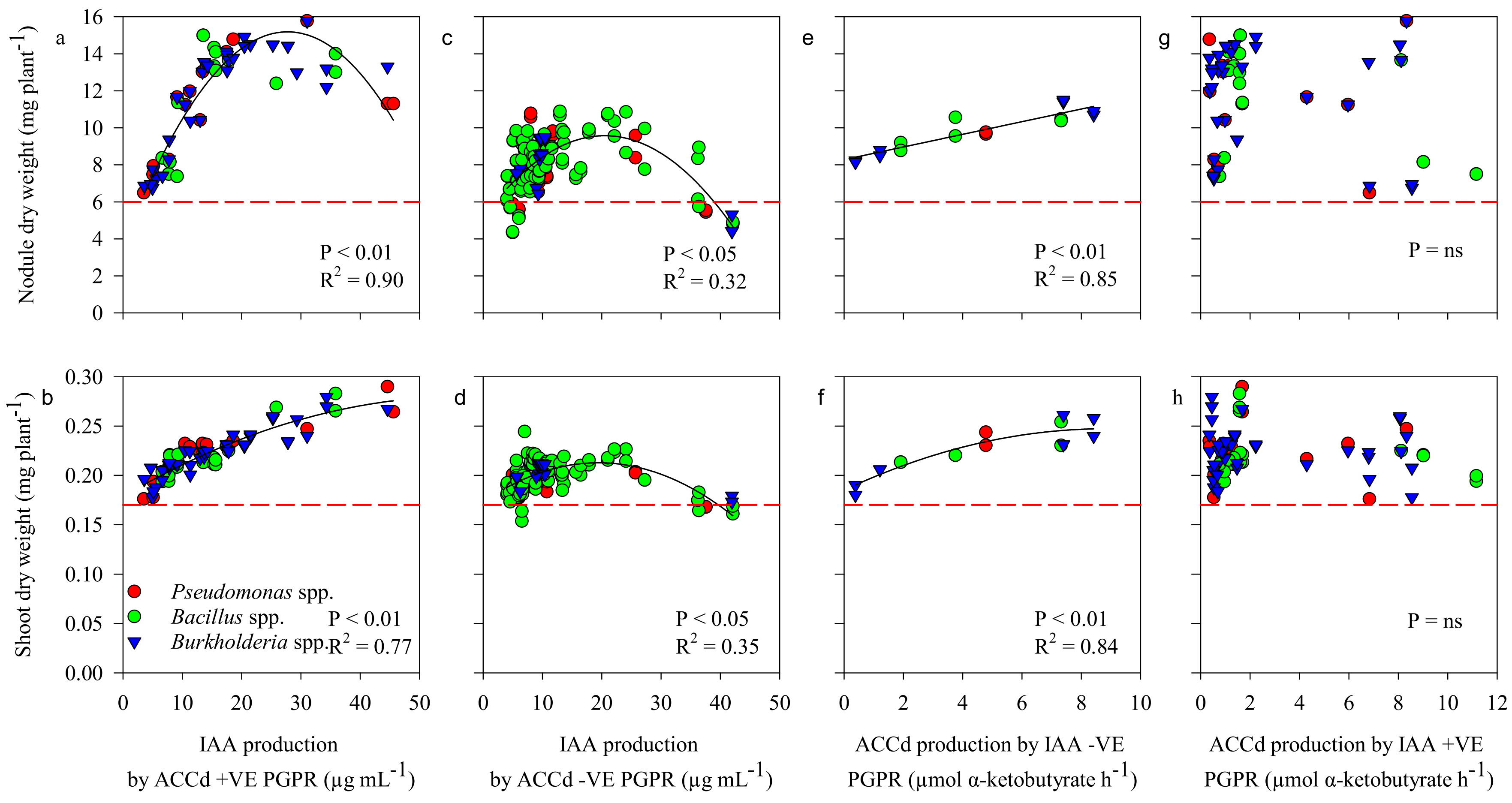

Fig. 4. Relationship (regression analysis) between plant parameters (nodule and shoot dry weight) and the level of IAA production by ACCd positive PGPR (a and b), level of IAA produced by ACC deaminase-negative PGPR (c and d), ACC deaminase activity expressed by IAA-negative PGPR (e and f) and ACC deaminase production by IAA positive PGPR (g and h). All seedlings were co-inoculated with *M. ciceri* CC1192. Data from the three replications were combined after confirming the homogeneity of variances with Bartlett's test. Each point represents an average of

three replications within an experiment. The continuous line within each frame is a best-fitted regression line with the respective significance level and strength of association. The red line indicates the shoot and nodule dry weight obtained from plants without rhizobacteria inoculation. -VE: negative and +VE: positive. $P < 0.01$- highly significant, $P < 0.05$- significant, P = ns- non-significant.

ACCd: ACC deaminase, PGPR: Plant growth promoting rhizobacteria

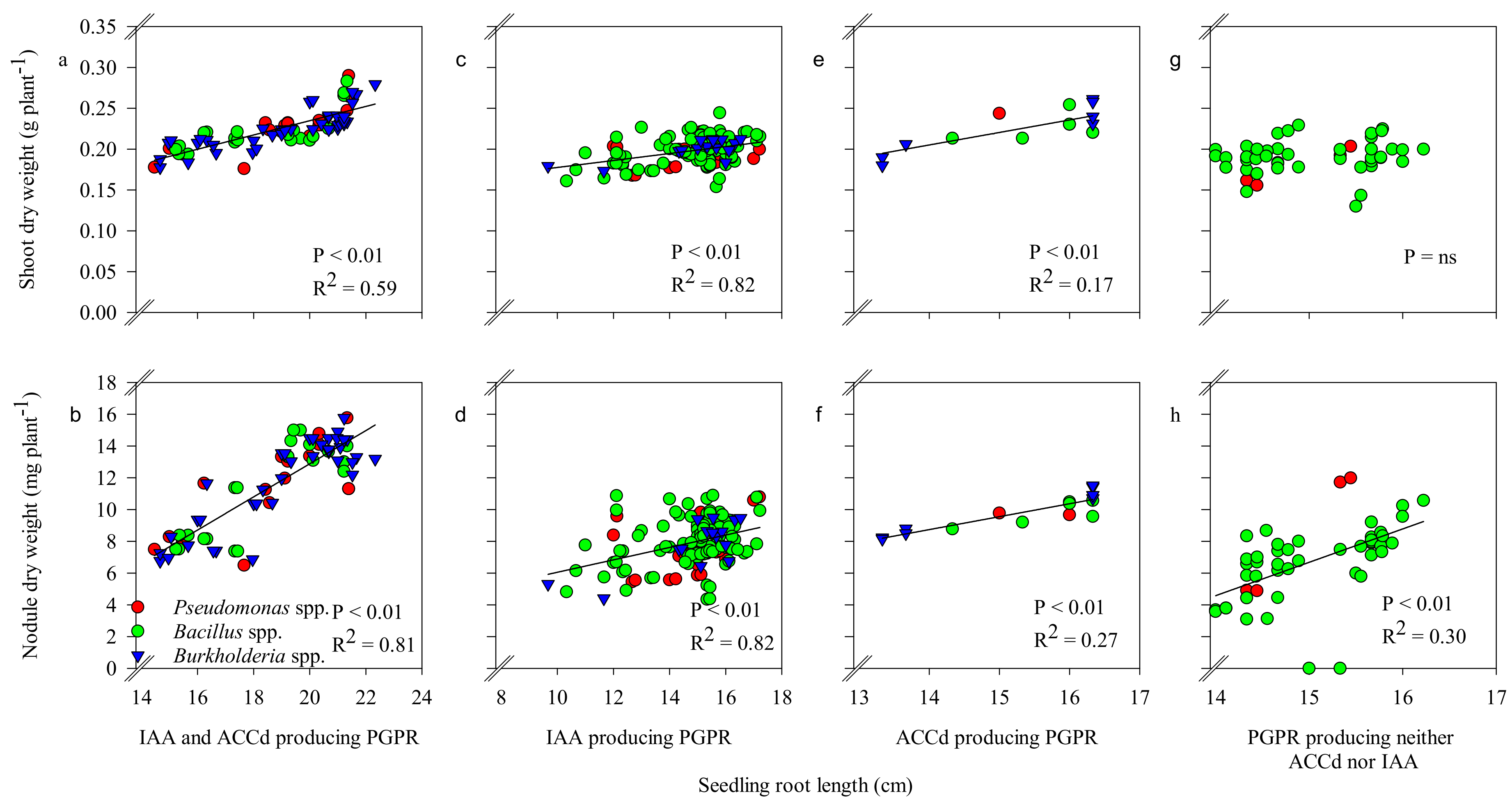

Fig. 5. Association between seedling root elongation after 7 days in aseptic conditions and shoot and nodule dry weight in plants co-inoculated with *M. ciceri* CC1192 and IAA- and ACC deaminase-producing PGPR (a and b); IAA producing PGPR (c and d), ACC deaminase producing

PGPR in the presence of L-tryptophan (e and f) and PGPR with neither capability (g and h). Each treatment was replicated three times. Data from the three replications were combined after confirming the homogeneity of variances with Bartlett's test. Each point represents an average of three replications within an experiment. The continuous line within each frame is a best-fitted regression line with the respective significance level and strength of association. ACCd: ACC deaminase, PGPR: Plant growth promoting rhizobacteria. $P < 0.01$- highly significant, P = ns- non-significant.

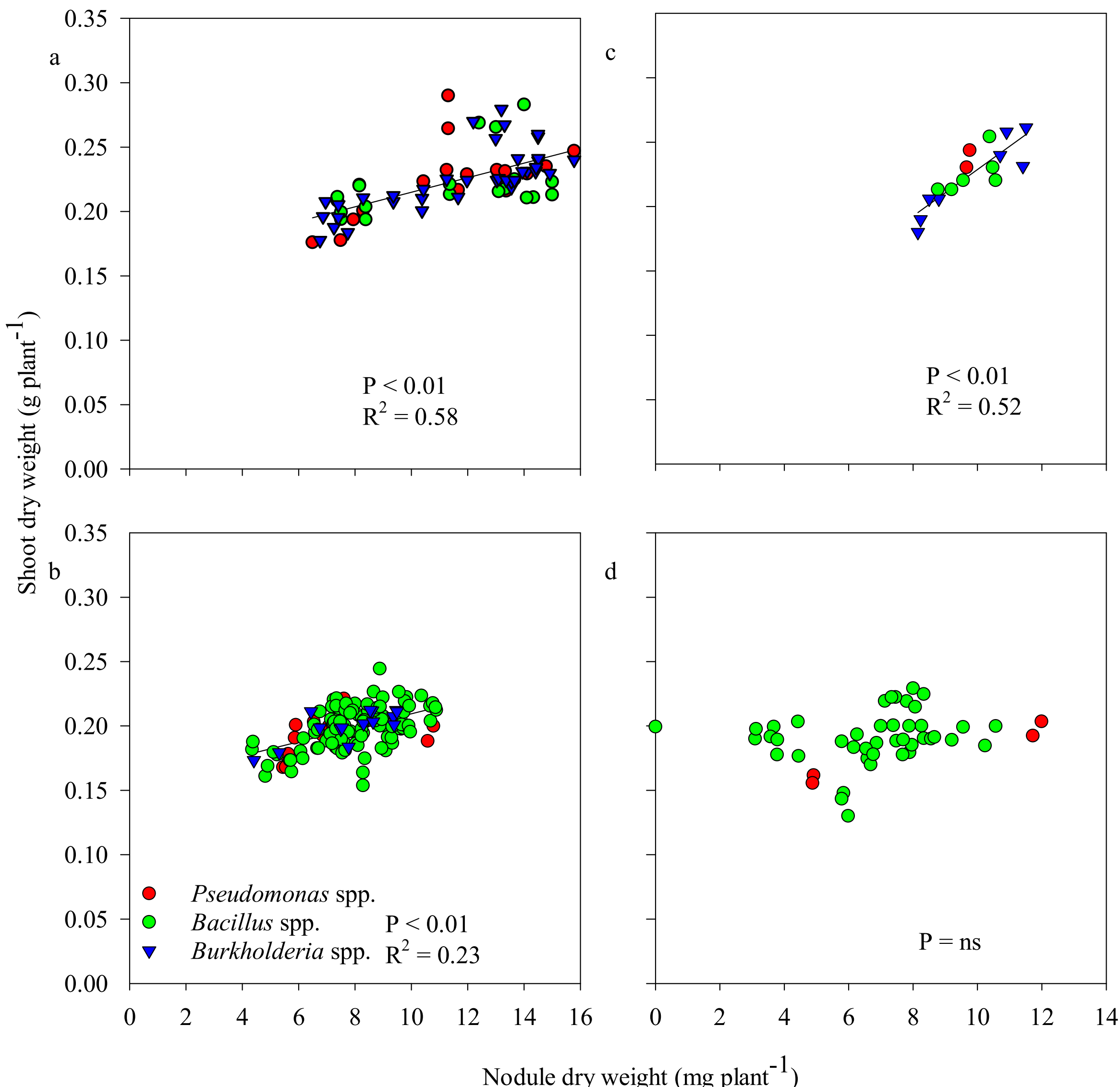

Fig. 6. The association between shoot dry and nodule dry weight of chickpea inoculated with PGPR able to produce (a) ACC deaminase and IAA, (b) IAA alone, (c) ACC deaminase alone and (d) neither of the two traits. Data from three replications were combined after confirming the homogeneity of variances with Bartlett's test. Each point represents an average of three replications within an experiment. The continuous line within each frame is a best-fitted regression line with the respective significance level and strength of their association. $P <$ 0.01- highly significant, P = ns- non-significant.

Chapter 4 Soil environment influences plant growth-promoting traits of isolated rhizobacteria

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4.1. Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Anteneh Argaw		
Contribution to the Paper	Planned the study conducted all experiments, analysed and interpreted data and wrote the manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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1 ## 4.2. Abstract

2 Plant growth-promoting rhizobacteria (PGPR) occur naturally in the rhizosphere, but still little
3 is understood regarding the soil and climatic variables that influence the occurrence of
4 beneficial bacteria. Here, we examined the effects of environmental factors on the occurrence
5 of PGPR in the chickpea rhizosphere soils. A total of 743 rhizobacteria were isolated from the
6 chickpea rhizosphere collected from 74 sampling sites across Australia. Results revealed that
7 the ability of isolates to produce indole acetic acid (IAA) and 1-aminocyclopropane-1-
8 carboxylate (ACC) deaminase were positively correlated with the potential of rhizobacteria to
9 promote root growth. The amount of IAA production was positively correlated with soil
10 copper, manganese and zinc concentrations, electric conductivity and the aridity index, but
11 negatively correlated with soil carbon (C), nitrogen (N) and phosphorus (P) at the sampling
12 sites from which the PGPR were isolated. Additionally, the ability of PGPR to tolerate metal
13 ion toxicity and water stress was related to their ability to express IAA. The potential of isolates
14 to solubilise P was negatively correlated with the C/N ratio, N, P, C and magnesium content of
15 the soils but none of the soil environmental factors was correlated with the ACC deaminase
16 activity of the isolates. In a principal component analysis, these environmental variables
17 explained 53% of the variation in the ability of the rhizobacteria to produce IAA and ACC
18 deaminase and/or to solubilise P. These findings could be applied to the design of an efficient
19 PGPR development strategy that incorporates ecological traits and plant growth-promoting
20 mechanisms to increase chickpea production.

21 **Keywords**: Aridity index, chickpea, cropping history, IAA, metal ions, plant growth-
22 promoting rhizobacteria

4.3. Introduction

Soils contain diverse communities of microorganisms that interact with plants, and respond to specific physical and chemical characteristics of soils. These microbial communities contribute to essential ecosystem services in soils, including soil organic matter decomposition, nutrient cycling, disease control and plant nutrition (Van Elsas et al., 2006). Soil microbial communities are not randomly distributed but exhibit spatial aggregation (Bardgett 2005; Martiny et al., 2006; Katsalirou et al., 2010; Oh et al., 2012; Zhou et al., 2020). Aggregation of soil microbial communities in particular microenvironments can develop as a result of long-term stress, through adaptive evolution (Wu and Hahn, 2006; Feingersch et al., 2012; Gubry-Rangin et al., 2015; Zhao et al., 2016) that leads to the selection of soil microbes with distinct genetic and metabolic features (Chase, 2005; Bursy et al., 2008; Oh et al., 2012). This adaptive evolution mediates biodiversity in soils (Gómez and Buckling, 2013). Soil microbial communities can express diversity in a wide variety of traits including light-, temperature-, and pH-tolerance, nutrient assimilation, carbon substrate usage, biofilm formation and antibiotic resistance (Larkin and Martiny, 2017).

Plant growth-promoting rhizobacteria (PGPR), a subset of the soil microbial community, can be isolated from soils, cultured and used to improve plant growth in a wide range of climatic conditions. Rhizobacteria belonging to the genera *Bacillus*, *Burkholderia* and *Pseudomonas* are very well-known PGPR (Alves et al., 2016; Hu et al., 2017; Batista et al., 2018; Cardoso et al., 2018; Tagele et al., 2018; Sharma et al., 2019). PGPR promote plant growth through direct and indirect plant growth-promoting (PGP) mechanisms (Swift et al., 2019). Of these, indole acetic acid (IAA) production, ACC deaminase activity and P solubilisation are desirable traits that are often suggested as mechanisms to promote plant growth by effective PGPR (Alemneh et al., 2020). ACC deaminase activity can improve plant growth by reducing the concentration of ethylene that is synthesised by plants under stress conditions (Saleem et al., 2018; Singh et al., 2015). ACC deaminase and IAA can promote root growth, thereby potentially improving nutrient and water uptake in soils of low fertility and low moisture content (Bal et al., 2013; Chandra et al., 2018; Mpanga et al., 2018). P solubilising activity can improve P availability for plants growing in soils of low fertility (Mpanga et al., 2018; Panhwar et al., 2014).

Since the aforementioned PGP traits can play a role in host plant ability to withstand stress conditions, we hypothesised that the environmental conditions at the sampling sites would be associated with the types and expression levels of different PGP traits of the isolated bacteria,

55 and influence their potential to promote plant growth. Accordingly, we addressed the following
56 question: how are the performance and plant growth promoting mechanisms of rhizobacteria
57 associated with the environmental conditions of their source soils?.

58 4.4. Material and methods

59 4.4.1. Soil samples and physio climatic conditions of the sampling sites

60 Soil samples were collected from 74 sites across major agricultural lands of Australia during
61 the cropping season in 2017 (Supplementary Fig. 1). Sampling sites were selected based on
62 their cropping history in South Australia, Victoria, New south Wales, Western Australia and
63 Queensland. The latitude and longitude coordinate of the sampling sites were ranged between
64 (-23.17'46.6" to -36.18'58.2") and (115.17'26.3" to 151.29'42.3"), respectively.

65 Crop rotation is the main cropping system in the sampling sites. Wheat was cultivated in
66 rotation with legumes (chickpea, faba bean, field pea, lentil, lupin and vetch) and other non-
67 leguminous crops (barley, canola, oat and sorghum) at the majority of the sampling sites.

68 In this study, seven sites were excluded due to lack of cropping history. Of 67 sites, thirty-
69 seven had the legume cultivation history within the last five years. Among them, chickpea was
70 cultivated at eleven sampling sites. The remaining locations had no history of legume growth.

71 The long-term average rainfall at the study sites ranged from 289 to 695 mm (Bureau of
72 Meteorology, 2019). The potential evapotranspiration (PET) varied within the range 1,168 to
73 1,358 mm. The aridity index was calculated using P/PET, where P is the average annual
74 precipitation (UNEP, 1992). Aridity index values of the study sites varied between 0.23 and
75 0.53.

76 Soil samples were taken from 0 to 15 cm depth and were kept in the refrigerator at 4 ºC until
77 analysed. Samples were air-dried and passed through a 2 mm sieve. The physicochemical
78 properties of soils were analysed following the methods described in Rayment and Lyons
79 (2011). Soil C was determined following the Walkley Black method. A modified Kjeldahl
80 method was used to determine total soil N. Total P in the soil was analysed by a colorimetric
81 method after extracting elemental P by perchloric acid digestion. Soil pH in $CaCl_2$ was
82 determined from a water suspension containing a 1:2.5 of soil to $CaCl_2$ mixture. The electrical
83 conductivity (EC) of the water extract from a 1: 2.5 soil: water suspension was determined.
84 The textural composition of the soils was analysed by dispersal and hydrometric readings.

85 Ammonium acetate extractable Ca, K and Mg in soils were determined using atomic absorption
86 spectrophotometry. Diethylene triamine pentaacetic acid (DTPA) extractable concentrations of
87 Cu, Mn and Zn in soils was determined. The physicochemical properties of soils are presented
88 in Supplementary Table 1.

89 **4.4.2. Isolation of rhizobacteria**

90 Chickpea seedlings were grown in pots containing soil collected from various sampling sites
91 to obtain rhizosphere soils. Seedlings were harvested four weeks after germination. The soils
92 adhering strongly to the roots of chickpea seedlings were collected. The rhizosphere soil was
93 serially diluted up to 10^{-6} in sterile MilliQ water. A 0.1 mL aliquot from 10^{-5} and 10^{-6} was taken
94 and spread on semi-selective tryptone soya agar (TSA) for isolation of *Pseudomonas*-like
95 bacteria. The semi-selective medium contained 50 µg ml^{-1} of ampicillin (99%, Sigma-Aldrich,
96 Darmstadt, Germany), 12.5 µg ml^{-1} of chloramphenicol (99%, Sigma-Aldrich,) and 75 µg mL^{-}
97 ^{1} of cycloheximide (Sigma-Aldrich) to inhibit the growth of non-*Pseudomonas* bacteria and
98 fungi, respectively (Simon and Ridge, 1974). This medium also recovered bacteria of the
99 closely related genus *Burkholderia* (formerly classified as *Pseudomonas*). The above-
100 mentioned aliquots, diluted to 10^{-5} and 10^{-6}, were heated for 10 min at 80 ºC to promote the
101 selection of aerobic endospore-producing bacteria (*Bacillus* spp.) (Travers et al. 1987). Heat-
102 treated aliquots were spread on TSA. Plates were incubated for 5 days at 28 ºC. Based on the
103 colony morphologies, 7 to 12 colonies were transferred to the same medium for further
104 purification of the strains. Pure colonies were preserved on slant cultures at 4 ºC and in 20%
105 glycerol at -80 ºC for further study.

106 **4.4.3. Determination of PGP potential of strains**

107 The effect of strain inoculation on seedling vigour in large Petri dishes (20 cm diameter) was
108 investigated. Seeds of chickpea cv. HatTrick was surface sterilised in 70% ethyl alcohol for 3
109 min and 5% sodium hypochlorite for 3 min, followed by five rinses with sterile MilliQ water.
110 Surface sterilised seeds were germinated in Petri dishes (10 cm diameter) containing moistened
111 sterile sand. After 48 h, ten healthy seedlings were transferred into disinfected 20-cm Petri
112 dishes containing 1% water agar. Liquid inoculant was prepared using a single colony of each
113 strain in a 10 mL test tube containing tryptone soya broth (TSB). The cultures were incubated
114 at 28 ºC on a shaker agitated at 180 rpm for 48 h. An aliquot (1 mL) of the culture was pelleted
115 by centrifugation at 10,000 ×g for 10 min. The pellet was suspended in 1 mL of sterile water.
116 The number of viable cells in the culture suspension (10^{8} cells mL^{-1}) was optimised based on

117 their optical density estimated using a spectrophotometer (Implen GmbH, Schatzbogen,
118 München, Germany). Then, 0.1 mL of cell suspension was inoculated per seedling. The
119 experiment included three replicates. Seven days after inoculation, root length was measured.
120 Rhizobacteria were further investigated for their potential to produce IAA and ACC deaminase,
121 and to solubilise P.

122 The potential of isolates to produce IAA and ACC deaminase and to solubilise mineral
123 phosphate were investigated *in vitro*. The control treatment with an uninoculated medium was
124 processed in the same way to account for the effect of any residual nutrients from the medium.
125 After a 48-h incubation in a DF-minimal medium containing 0.5 g L^{-1} of L-tryptophan, the
126 culture solution was centrifuged at 10,000 ×*g* for 10 min. DF-minimal medium (Patten and
127 Glick, 2002). One mL of supernatant and 4 mL of Salkowski's reagent were mixed and kept in
128 the dark at room temperature for 30 min before measuring absorbance at 535 nm using
129 spectrophotometer. The IAA concentration in the supernatant was calculated relative to a
130 standard curve of IAA (Glentham LIFE SCIENCES, Ingoldmells Court Corsham, United
131 Kingdom) ranging from 1 to 100 µg mL^{-1}.

132 Preliminary screening of bacterial strains for ACC deaminase production was performed based
133 on their ability to utilise ACC as a sole N source in DF minimal medium (Penrose and Glick
134 2003). A filter-sterilised solution of ACC (3 mM) was added to the autoclaved DF agar medium
135 instead of $(NH_4)_2SO_4$. Each strain was streaked on a DF-minimal agar medium and incubated
136 for 5 days at 28 ºC. Strains able to grow on this medium were further assessed for their
137 quantitative production of ACC deaminase in liquid DF-minimal medium in the presence of
138 ACC. Quantitatively, ACC deaminase activity was determined following the procedure
139 indicated in Penrose and Glick (2003). A 48-h culture was inoculated into DF-medium and the
140 solution was incubated in a shaker incubator (3 h, 28 ºC, 160 rpm). The culture solution was
141 centrifuged at 10,000 ×g for 10 min. The ACC deaminase activity was estimated from the
142 supernatant.

143 The ability to dissolve sparingly soluble inorganic phosphate [AlO_4P, $Ca_3(PO_4)_2$ and
144 $FeO_4P.2H_2O$] was investigated using the National Botanical Research Institute's phosphate
145 growth medium (NBRIP) (Nautiyal 1999). In this medium, 5 g L^{-1} of either $Ca_3(PO_4)_2$ (>96%,
146 Sigma-Aldrich), $FeO_4P.2H_2O$ (Sigma-Aldrich) or AlO_4P (Sigma-Aldrich) was supplied. A 48-
147 h culture was streaked onto NBRIP medium and incubated at 28 ºC for 7 days. The colony
148 diameter and total diameter of halo (clearing) zone plus colony diameter were measured from

149 randomly selected colonies. Solubilisation index was calculated following the method
150 described in Nguyen et al. (1992).

151 4.4.4. Phylogenetic analysis of *Bacillus*-, *Burkholderia*- and *Pseudomonas*-like
152 bacteria

153 Bacterial isolates (167) that represented a cross-section of the best (55), moderate (56) and poor
154 (56) performing isolates in seedling vigour test were selected for taxonomic identification using
155 16S rRNA. A standard PCR methodology was used for presumed taxonomic identification of
156 rhizobacteria. In brief, amplification of 16S rRNA was performed from 48-h-old pure colonies,
157 using universal reverse primer rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) (Sigma-Aldrich)
158 and forward primer fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) (Sigma-Aldrich) as
159 described by Weisburg et al. (1991). The 25 μL PCR reaction comprised: 12.5 μL of 2× Taq
160 polymerase MiFi mix (Sigma-Aldrich), 10.5 μL of water and 1 μL of each primer. The PCR
161 cycling conditions were as follows: 1 min at 95 ºC followed by 35 rounds of thermal cycling
162 conditions [95 ºC for 30 s, 57 ºC for 30 s and 72 ºC for 1 min] and a final extension at 72 ºC
163 for 5 min. The PCR products were visualised in a 1.5% agarose gel under a UV
164 transilluminator. PCR products were sequenced by Sanger sequencing (Australian Genome
165 Research Facility, Adelaide, SA, Australia). Homologous sequences in the National Centre for
166 Biotechnology Information GenBank databases were identified from BLAST searches. A
167 phylogenetic tree was constructed using the neighbour-joining method. Tree topologies were
168 evaluated by performing bootstrap analysis of 1,000 resamplings using the latest version of
169 Molecular Evolutionary Genetics Analysis (MEGA-7) (Institute of Molecular Evolutionary
170 Genetics, The Pennsylvania State University, University Park, PA, USA) (Kumar et al., 2016).

171 4.4.5. Effect of stress on IAA production and PGPR viability

172 The effect of metal ion concentration and water stress on cell viability and IAA production
173 were assayed. For this experiment, four PGPR efficient at promoting chickpea seedling growth,
174 putatively identified as *Bacillus pumilus* 98F*, Burkholderia cenocepacia* 127F*, Burkholderia*
175 sp. 12F and *Pseudomonas fluorescens* 27F were selected (Alemneh et al. 2021a). These isolates
176 were able to solubilise phosphate from various inorganic P sources including rock phosphate.
177 All these strains also produced IAA in the presence of L-tryptophan as well as ACC deaminase
178 *in vitro* (Supplementary Table 2). However, they were unable to produce IAA in the absence
179 of L-tryptophan (Alemneh et al. 2021a). Strains were purified on TSA. Plates were incubated
180 at 28 ºC for 72 h. A pure colony was transferred into TSB and the culture solution was

181 incubated at 28 °C for 48 h. For each isolate, the optical density (OD) of cell suspension was
182 measured using spectrophotometer at 2h time interval. From the same cell suspension, the
183 number of viable cells were counted by culturing the solution on TSA medium after 48h
184 incubation at 28 °C. Later, the relationship between the OD and number of viable cells was
185 determined in linear regression analysis. Based on regression equation, number of viable cells
186 in the cell suspension were determined and adjusted to 10^8 per mL for next experiment.

187 The cell viability and potential for IAA production by the four selected rhizobacteria in TSB
188 containing different levels of Cu, Mn and Zn were investigated. The metal ions were supplied
189 in the form of $CuSO_4.7H_2O$, $MnSO_4$ and $ZnSO_4.5H_2O$ after filter sterilisation using a 0.22-µm
190 microfilter, at four concentrations (0, 0.5, 1 and 2 mM) in DF-minimal medium containing 500
191 µg L^{-1} of L-tryptophan (AppliChem GmbH, Darmstadt, Germany). The concentration was
192 designed based on metal ion concentrations present in soils from the sampling sites and data
193 reported in the literature (Smolders et al., 2004; Oorts et al., 2006; Carlos et al., 2016). The
194 literature reported that these concentrations can suppress the growth and activity of bacteria in
195 soils. Additionally, the effect of water potential gradient on cell viability and IAA production
196 was investigated by supplying PEG-6000 to simulate drought in the medium (Michel and
197 Kaufmann, 1973). Five PEG-6000 concentrations (0, 10, 20, 30 and 45%) inducing water
198 potentials of 0, -0.07, -0.17, -0.28 and -0.49 megapascals (Mpa), respectively, in growing
199 media were used. Treatments were replicated three times. The culture solution without
200 rhizobacteria and metal ions was used as a control. After incubating for 48 h at 28 °C in a
201 shaker incubator, 0.1 mL of culture solution was serially diluted in 0.9 mL of sterile MilliQ
202 water to 10^{-5}. Twenty µL from the 10^{-4} to 10^{-5} dilutions were spread on plates containing TSA
203 and incubated for 72 h at 28 °C. District colonies were counted and the number of colonies
204 were converted into number of viable cells per unit mL. From the same cell suspension, IAA
205 and ACC deaminase were analysed as described above.

206 **4.4.6. Statistical analysis**

207 All statistical analyses were performed using Genstat 18th edition statistical software (VSN
208 International Limited, Hertfordshire, United Kingdom). Data were analysed by two-way
209 analysis of variance, followed by Tukey HSD ($P < 0.05$). The association between the effect
210 of rhizobacteria on seedling root elongation and the edaphic and climatic conditions at the soil
211 sampling sites from which the PGPR were isolated was tested using linear regression analysis.
212 Principal Component analysis (PCA) was performed to examine how combined soil and

213 environmental characteristics influenced the biological attributes of the isolates, and to
214 determine which inter-related parameters most influenced the PGP potential of the isolates.
215 Sixteen environmental and soil characteristics were used as explanatory variables, and PGP
216 traits (ACC deaminase, IAA and P solubilisation) and seedling root length were the response
217 variables. Pearson's correlation between the explanatory variables was done and the variables
218 with high autocorrelation were removed from the PCA analysis. Accordingly, C/N ratio, Ca
219 and Zn were excluded from PCA. The normality of the data was evaluated using Shapiro-
220 Wilk's test. Bartlett's test was used to test the homogeneity of variances.

221 ## 4.5. Results

222 A total of 743 isolates was obtained from the rhizosphere of chickpea grown in soils collected
223 from 74 sampling locations across major agricultural lands in Australia (Supplementary Table
224 1). The efficacy of these isolates in seedling root elongation *in vitro* condition was investigated.
225 The association between the performance of isolates in seedling root elongation and the
226 environmental and soil conditions of their sources were also investigated.

227 ### 4.5.1. Correlation between PGP potential of rhizobacteria with the soil
228 ### environment

229 The association between the performance of rhizobacteria in seedling root growth promotion
230 and the environmental conditions prevailing at the soil sampling sites from which the strains
231 were isolated, are presented in Table 1. Root growth promotion by rhizobacteria was
232 significantly and positively correlated with the aridity index (r = 0.35, P < 0.01), potential
233 evapotranspiration (r = 0.56, P < 0.01), EC (r = 0.35, P < 0.01) and the concentration of Cu (r
234 = 0.39, P < 0.01), Mn (r = 0.35, P < 0.05) and Zn (r = 0.34, P < 0.01) in the soils. Conversely,
235 the effect of rhizobacteria on root length was negatively and significantly correlated with total
236 soil N (r = -0.22, P < 0.05), P (r = -0.21, P < 0.05), Ca (r = -0.39, P < 0.01), organic C (r = -
237 0.30, P < 0.01) and the C/N ratio (r = -0.21, P < 0.05).

238 Correlation between root growth promotion and the PGPR strains' ability to express PGP traits
239 was tested. The ability of isolates to promote root length was positively correlated with their
240 potential to express IAA and ACC deaminase activity. Their potential to solubilise P was not
241 correlated with root growth promotion.

242 #### 4.5.2. Correlation between plant growth-promoting traits and soil environment

243 Correlation between the ability of isolates to (a) produce IAA and ACC deaminase *in vitro* or
244 (b) to solubilise phosphate on NBRIP medium and selected environmental conditions
245 prevailing at the locations from which soils were sourced was investigated. The capacity of
246 IAA production by PGPR was positively correlated with aridity index ($r = 0.24$, $P < 0.01$) and
247 the concentration of Cu ($r = 0.12$, $P < 0.05$), Mn ($r = 0.17$, $P < 0.05$) and Zn ($r = 0.1$, $P < 0.05$)
248 in the soils but negatively correlated with soil N ($r = -0.13$, $P < 0.05$), C ($r = -0.13$, $P < 0.05$)
249 and Ca ($r = -0.14$, $P < 0.05$) (Supplementary Table 2). Likewise, the potential of rhizobacteria
250 to solubilise P was negatively correlated with soil pH ($r = -0.40$, $P < 0.01$), C ($r = -0.40$, $P <$
251 0.01), C/N ratio ($r = -0.48$, $P < 0.01$) and Mg ($r = -0.46$, $P < 0.01$), but positively correlated
252 with Ca ($r = 0.42$, $P < 0.01$). The soil environmental variables tested showed no correlation
253 with the ability of isolates to express ACC deaminase.

254 #### 4.5.3. Multivariate coordination

255 The association between the ability of rhizobacteria to express PGP traits and to promote
256 seedling growth, and soil and environmental variables were predicated by PCA (Table 2, Fig.
257 1). There were 6 PC with eigenvalues > 1 (Fig 1b). Environmental and soil properties
258 explained the 53% of the total variation of rhizobacteria to produce IAA alone and in
259 combination with ACC deaminase and P solubilisation (Fig 1a and c). Of which, 31.1% of the
260 variability was accounted for primarily by Cu, Mn, EC, aridity index, and clay. These
261 characteristics were positively correlated with the ability of rhizobacteria to promote root
262 growth. Soil Cu content was significantly correlated with Zn ($r = 0.81$, $P < 0.01$) (Table 1).
263 The remaining 21.8% of the variance was primarily explained by soil C, N and P. These
264 variables were negatively correlated with IAA production and the potential of rhizobacteria to
265 promote plant growth. Soil C content was were significantly correlated with the C/N ratio ($r =$
266 0.77, $P < 0.01$) and Ca content ($r = 0.93$, $P < 0.01$) (Table 1). The PCA showed that the isolates
267 able to express IAA, ACC deaminase and P solubilisation were loaded on the PC1 (Fig 1b).
268 Most isolates that could not express the three PGP traits were clustered in the PC2.

269 #### 4.5.4. Phylogenetic diversity of isolates

270 One hundred and sixty-seven out of 743 that represented a cross-section of the best, moderate
271 and poor performing isolates were selected for phylogenetic analysis (Fig. 2a and b).
272 Phylogenetic analysis was based on a ~1500 bp sequence of the 16S rRNA gene to identify the

273 diversity of bacteria belonging to the genera *Bacillus*, *Burkholderia* and *Pseudomonas*. Among
274 *Bacillus*-related bacteria, five phylogenetic groups related to distinct genera and species of
275 *Bacillus* spp. were identified. The dominant PGPR belonging to *Bacillus* were *Bacillus muralis*
276 followed by *Peribacillus simplex*, *Bacillus flexus* and *Bacillus niacini*. A few isolates belonging
277 to species of *Bacillus* affiliated to *Bacillus bataviensis*, *Bacillus cucumis*, *Bacillus*
278 *endophyticus*, *Bacillus pumilus*, *Bacillus altitudinis*, *Bacillus amyloliquefaciens*, *Bacillus*
279 *atrophaesus*, *Bacillus mycoides*, *Bacillus cereus*, *Bacillus pacificus*, *Bacillus toyonensis* and
280 *Paenibacillus endophyticus* were also identified.

281 The PGPR among *Burkholderia*- and *Pseudomonas*-like bacteria were predominantly assigned
282 to *Burkholderia* spp., *Burkholderia graminis, Paraburkholderia phenoliruptrix, Burkholderia*
283 *cepacia* and *Pseudomonas frederiksbergensis*. In this study, promising PGPR assigned to
284 *Burkholderia caledonica, Pseudomonas putida*, *P. fluorescens* and *P. corrugata* were also
285 identified. The results showed that the most effective *Burkholderia* spp. in promoting seedling
286 growth and the chickpea-*Mesorhizobium* symbiosis were isolated from dry environments with
287 aridity index > 0.35 (Supplementary Table 2). These isolates were efficient in P solubilisation
288 and produced high concentrations of IAA *in vitro*.

289 Taxonomic position for the genera *Pseudomonas* and *Bacillus* was not associated with the
290 environmental conditions of their source. Compared to the *Burkholderia* spp., *Bacillus* isolates
291 produced lower concentrations of IAA.

292 **4.5.5. The response of PGPR strains to metal ions and water stress in vitro**

293 Four efficient PGPR strains were selected to study their response to metal ions and water stress
294 *in vitro*. Results showed the differences in the performance of strains in IAA production and
295 their viability when exposed to metal ions and water stress (Fig. 3). A significant reduction in
296 IAA production with *B. pumilus* 98F and *P. fluorescens* 27F subjected to increasing levels of
297 Cu was observed (Fig. 3a and d). The presence of Zn did not affect the IAA production of *B.*
298 *pumilus* 98F but at 1 mM caused a significant increase in IAA production from *P. fluorescens*
299 27F. In the case of *Burkholderia* sp. 12F, IAA production increased with Cu and Zn
300 concentration up to 1 mM (Fig. 3b and c). Regardless of the PGPR used, Mn enhanced their
301 IAA production but slightly reduced IAA production at 1 mM from *B. pumilus* 98F.

302 The addition of L-tryptophan to the culture medium improved cell viability. In the presence of
303 L-tryptophan, cell viability of selected PGPR showed varying responses to metal ion

304 concentration (Fig. 4a-d). A significant reduction in the viable cell population was found for
305 *B. pumilus* 98F and *P. fluorescens* 27F in the presence of Cu beyond 0.5 mM. A slight increase
306 in cell viability of these strains was observed at 0.5 mM Mn compared with the control
307 treatment. However, the cell viability was significantly reduced at 2 mM Zn. In the case of
308 *Burkholderia* sp. 12F and *B. cenocepacia* 127F*,* viability were increased up to 1 mM of all
309 metal ions, excluding Cu, for which viable cells were increased up to 0.5 mM. At 2 mM, their
310 viability was suppressed. Increased viability of *Burkholderia* sp. 12F was observed at 0.5 mM
311 Cu compared to the control treatment.

312 The four tested isolates did not produce IAA in the absence of L-tryptophan supply in the
313 medium. When L-tryptophan was supplied, cell viability of the selected strains was
314 significantly reduced beyond 0.5 mM metal ion concentration compared with the control
315 treatment (Fig. 4e-h). At 0.5 mM Zn and Mn, cell viability was not affected.

316 The osmotic stress induced by PEG-6000 in the culture medium significantly influenced IAA
317 production (Fig. 5a - d). There was a significant increase in IAA production by *Burkholderia*
318 sp. 12F at -0.07 Mpa. Compared with the control treatment, a lower amount of IAA production
319 was recorded at -0.07 Mpa from *P. fluorescens* 27F. Beyond -0.07 Mpa osmotic stress, the IAA
320 production ability of this strain was strongly inhibited. In the case of *B. pumilus* 98F, IAA
321 production was not significantly affected by water potential up to -0.17 Mpa as compared to
322 the control check treatment. Beyond this level, IAA production was significantly ($P < 0.01$)
323 reduced.

324 Cell viability was improved in the presence of L-tryptophan under water stress conditions (Fig.
325 6). In the presence of L-tryptophan, there was a significant variation in cell proliferation among
326 tested strains along water potential gradients (Fig. 6a). The viable cell populations for *B.*
327 *pumilus* 98F and *P. fluorescens* 27F were not significantly affected by water potential up to -
328 0.17 Mpa. Beyond this level, the cell viability was significantly reduced compared to the
329 control check. The viability of *B. cenocepacia* 127F and *Burkholderia* sp. 12F was slightly
330 increased up to -0.17 Mpa. In the absence of L-tryptophan, cell viability declined significantly
331 in the presence of osmotic stress (Fig. 6b).

4.6. Discussion

4.6.1. Association between source environmental conditions and PGPR performance

Soil organic C, P, Ca and N are important nutritional factors affecting soil microbial abundance (Navarro-Noya et al., 2013) and activities (Lori et al., 2017). In the present study, rhizobacteria isolated from high C and N containing soils performed poorly in root length promotion as compared to strains isolated from soils with lower levels of C and N. Additionally, most isolates incapable of producing IAA or ACC deaminase and solubilising P clustered in the direction of soil C, N, P, Mg, pH and Ca in PCA. The correlation of these soil properties was also highly significant at $P < 0.01$ (Table 1). These results suggest that isolates that were obtained from fertile soils with high soil C, N, P, and cations, may be less efficient in expressing IAA and solubilising P. The PGP traits are important to the plant growth, particularly the plant growing in areas with low soil fertility and low water availability. Therefore, the bacteria were able to develop adaptive traits that could improve the plant's fitness under biotic and abiotic stresses when bacteria coevolved with plant roots in harsh environments over many millions of years (Denison 2012). Additionally, P solubilising activity, ACC deaminase and IAA production may also important for bacteria themselves to adapt to environmental stress. When a plant is exposed to stress, it releases relatively high ACC concentration (Singh et al., 2015). This substrate can act as an alternative N source for the bacteria under low fertile soils if isolates are able to express ACC deaminase (Alemneh et al., 2021b). In low fertile soils, the bacteria can easily acquire their P needs through P solubilisation. In the present study, the effect of IAA in bacteria stress tolerance was investigated in detail *in vitro* conditions here below.

4.6.2. Environmental conditions associated with the capacity of the rhizobacteria to produce IAA

Among three PGP traits, the capacity of PGPR to produce IAA was positively correlated with metal ion concentration and aridity index but negatively correlated with C, N, P and Ca contents and C/N ratio of the soils and sampling sites from which the strains were isolated. This result suggests the occurrence of high IAA-producing bacteria in soils of low fertility and with higher metal ion concentrations and aridity index. However, this result alone did not demonstrate whether the ability of isolates to produce IAA would assist the bacteria themselves to withstand the stress conditions. In a further experiment, an increase in the production of IAA was observed in the presence of 1.0 mM Mn and Zn with *Burkholderia* sp. 12F and *B. cenocepacia*

363 127F. However, viable cell populations of these strains were slightly reduced. Consistent with
364 these findings, nitrogen starvation that suppresses cell viability can increase IAA production
365 by the same bacterial strain *in vitro* (Malhotra and Srivastava, 2009). However, IAA production
366 and cell viability of these isolates in the presence of the same concentration of Cu as used in
367 this study were reduced. This result could be related to the highly toxic effect of Cu on
368 microbial cell viability and expression of metabolites, as compared with Zn and Mn (Biswas
369 et al., 2017; Cai et al., 2019).

370 The potential of rhizobacteria to produce IAA was related to their ability to withstand stressful
371 conditions (metal ions and osmotic stress). We found greater tolerance to the high level of
372 metal ion concentrations and simulated drought conditions for *Burkholderia* sp. 12F and *B.*
373 *cenocepacia* 127F than for *P. fluorescens* 27F and *B. pumilus* 98F. These different responses
374 to stress may be related to the nature of the environments from which they were isolated. In
375 this regard, *Burkholderia* sp. 12F and *B. cenocepacia* 127F were isolated from sites that had
376 higher levels of metal ions in the soil and a higher aridity index. *Burkholderia* sp. 12F and *B.*
377 *cenocepacia* 127F were able to produce 54.3 and 41.9 µg mL^{-1} IAA in the presence of L-
378 tryptophan *in vitro*, respectively. Based on these results and those presented in Fig 1, we
379 suggest that water stress and low soil fertility with higher levels of Cu, Mn and Zn at the site
380 of the source can result in the selection of rhizobacteria with higher IAA production potential.
381 The exogenous supply of IAA in *Bradyrhizobium* E109 *in vitro* induced greater production of
382 exopolysaccharides that are important for rhizobacterial protection from environmental stress
383 (Naseem et al., 2018; Torres et al., 2018). Therefore, the ability to express IAA may also help
384 isolates to tolerate stress conditions

385 **4.6.3. Diversity of *Bacillus*, *Burkholderia* and *Pseudomonas* isolates from soils**

386 Diverse species of bacteria belonging to *Bacillus*, *Burkholderia* and *Pseudomonas* isolated
387 from rhizospheric soil of chickpea were identified in this study. *Bacillus* was represented by
388 nineteen distinct species, *Burkholderia* by eight species and *Pseudomonas* by seven species.
389 Numerous studies have reported that chickpea is capable of supporting beneficial species of
390 rhizobacteria (Hynes et al., 2008; Patel et al., 2012; Dubey et al., 2013; Yadav and Verma,
391 2014; Benjelloun et al. 2019; Alok et al., 2020). Recently, the types of PGPR identified in this
392 study were also among the prominent genera identified as endophytic bacteria isolated from
393 nodules and roots of chickpea (Alok et al., 2020). The dominant species identified in the present
394 study have been shown to promote plant growth and yield in greenhouse and field conditions

395 (Xu et al., 2014; Zeng et al., 2017; Shahid and Khan, 2018; Bhatt and Maheshwari. 2020; You
396 et al., 2020).

397 The taxonomic position of PGPR in the phylogenetic tree was partly associated with
398 environmental conditions at the soil sampling sites. Specifically, *Burkholderia* spp. were
399 predominantly isolated from dry environments (Supplementary Table 1 and 2). Most
400 *Burkholderia* spp. identified in the present sampling sites have been frequently reported as
401 symbiotic N_2 fixing bacteria associated with plants grown under arid conditions (Garau et al.,
402 2009; Sprent and Gehlot, 2010). However, the taxonomic position of bacterial species
403 belonging to the genera *Pseudomonas* and *Bacillus* was not associated with the environmental
404 conditions of their sources. This is possibly indicated that *Pseudomonas* and *Bacillus* is widely
405 spread in sampling sites.

406 **4.7. Conclusion**

407 Our results demonstrate that high aridity index, higher concentrations of metal ions and low C,
408 N, P and Ca in soils appear to exert selective pressure on rhizobacterial phenotypes for IAA
409 production and ACC deaminase capacity. Likewise, soils with low fertility, alkaline pH and
410 high Ca and Mg contents harbour PGPR with high potential for P solubilisation. IAA
411 production could enable the bacteria to withstand stress conditions. IAA and ACC deaminase
412 production ability of PGPR were the main mechanisms of plant growth promotion *in vitro*.
413 However, the ability to solubilise P had no role in plant growth promotion at the seedling stage.
414 In general, these studies suggest possible targets for isolation of efficient PGPR with ecological
415 traits and PGP mechanisms that may enhance chickpea production and eventually reduce the
416 time and budget required for inoculant development.

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Table 1. Pearson's correlation matrix for 18 soil and environmental characteristics and PGP attributes and potential of plant growth promotion of 743 rhizobacteria isolated from 74 soil samples. Trait abbreviations: C/N, soil carbon to nitrogen ratio; Ca, soil calcium content; C, soil carbon content; N, soil nitrogen content; P, soil phosphorus; pH, the soil pH in $CaCl_2$; K, soil potassium content; Mg, magnesium; Zn, soil zinc content; Cu, soil copper content; AI, Aridity index; Clay, soil clay content; Mn, soil manganese content; Etp, Evapotranspiration; EC, soil electrical conductivity; S, soil sulphur content; sand, soil sand content; ACCd, ACC deaminase production; RL, root length, PS, P solubilisation.

	ACCd	IAA	RL	EC	pH	Clay	Sand	N	C	C/N	P	Zn	Mg	Mn	K	Cu	Ca	S	Etp	AI
PS	-0.029	0.057	0.011	-0.039	-0.403	0.074	-0.05	0.015	-0.399	-0.483	-0.261	0.049	-0.46	0.007	0.103	0.035	0.423	0.237	-0.106	-0.028
ACCd		-0.075	0.201	0.053	0.009	0.046	-0.046	-0.088	-0.08	-0.049	0.005	0.018	0.05	0.074	-0.05	0.025	-0.062	0.014	0.076	0.099
IAA			0.441	-0.014	-0.015	0.046	-0.02	-0.131	-0.128	-0.098	-0.079	0.1	-0.02	0.168	-0.006	0.122	-0.136	-0.09	0.395	0.244
RL				0.352	0.107	0.582	-0.49	-0.223	-0.296	-0.208	-0.209	0.342	0.177	0.35	0.113	0.39	-0.217	-0.15	0.56	0.352
EC					0.021	0.415	-0.475	0.14	-0.001	-0.108	-0.031	0.176	0.056	0.32	0.018	0.123	-0.02	0.001	0.395	0.329
pH						0.064	0.014	-0.008	0.272	0.35	0.196	0.347	0.573	0.127	0.432	0.135	0.467	-0.087	0.183	-0.124
Clay							-0.927	-0.075	-0.301	-0.317	-0.027	0.734	0.308	0.7	0.318	0.684	-0.282	-0.06	0.638	0.704
Sand								-0.119	0.194	0.32	-0.133	-0.722	-0.299	-0.672	-0.402	-0.698	0.223	0.029	-0.507	-0.741
N									0.593	0.04	0.651	0.105	0.18	-0.171	0.545	-0.058	0.38	0.148	-0.443	0.189
C										0.771	0.75	0.014	0.494	-0.229	0.356	-0.163	0.928	0.033	-0.253	-0.172
C/N											0.433	-0.074	0.426	-0.194	-0.022	-0.184	0.849	-0.079	-0.021	-0.296
P												0.293	0.537	-0.133	0.657	0.247	0.631	0.197	-0.277	0.079
Zn													0.574	0.551	0.579	0.805	0.096	-0.074	0.446	0.581
Mg														0.184	0.501	0.465	0.558	-0.078	0.32	0.257
Mn															0.023	0.522	-0.176	-0.033	0.646	0.592
K																0.417	0.33	0.071	-0.161	0.174
Cu																	-0.129	-0.003	0.476	0.569
Ca																		-0.018	-0.125	-0.246
S																			-0.074	-0.105
rain																			0.611	0.98
Etp																				0.446

631 Table 2. Percentage contribution, cumulative variances and eigenvectors of the first six
632 principal components (PC1-6) for 18 soil and environmental characteristics and the ability of
633 the rhizobacteria isolated from those soils to produce IAA and ACC deaminase and to promote
634 root growth.

Parameter	PC1	PC2	PC3	PC4	PC 5	PC6
Eigenvalue	7.16	5.015	2.3	2.004	1.357	1.049
%variance	31.13	21.81	10	8.71	5.9	4.56
Cumulative(%)	31.13	52.937	62.936	71.648	77.548	82.109
Character			**Eigenvalue**			
Root growth promotion	**0.599**	-0.112	0.164	0.290	-0.047	-0.278
Electrical conductivity	0.371	-0.038	0.679	0.107	0.399	-0.107
Soil pH	0.061	**0.508**	0.084	0.487	-0.430	-0.179
Soil clay	**0.923**	0.028	0.163	0.002	-0.020	-0.069
Soil sand	**-0.886**	-0.126	-0.214	0.175	-0.076	0.078
Soil N	-0.115	**0.549**	0.317	-0.637	0.208	-0.089
Soil C	-0.377	**0.839**	0.198	0.045	0.230	0.123
Soil C/N	-0.388	**0.620**	0.003	0.512	0.192	0.228
Soil P	-0.063	**0.832**	0.170	-0.337	-0.067	0.156
Soil Zn	**0.769**	0.449	-0.032	-0.014	-0.221	-0.088
Soil Mg	0.338	**0.757**	0.005	0.277	-0.169	0.099
Soil Mn	**0.764**	-0.001	-0.026	0.181	0.171	0.146
Soil K	0.250	**0.671**	0.152	-0.368	-0.418	-0.294
Soil Cu	**0.785**	0.229	-0.011	-0.055	-0.303	0.074
Soil Ca	-0.349	**0.847**	0.117	0.291	0.127	0.069
Soil S	-0.090	-0.005	0.239	-0.322	-0.257	0.689
Evapotranspiration	**0.707**	-0.107	0.113	0.569	0.070	0.221
Aridity index	**0.823**	0.099	-0.069	-0.251	0.377	0.039

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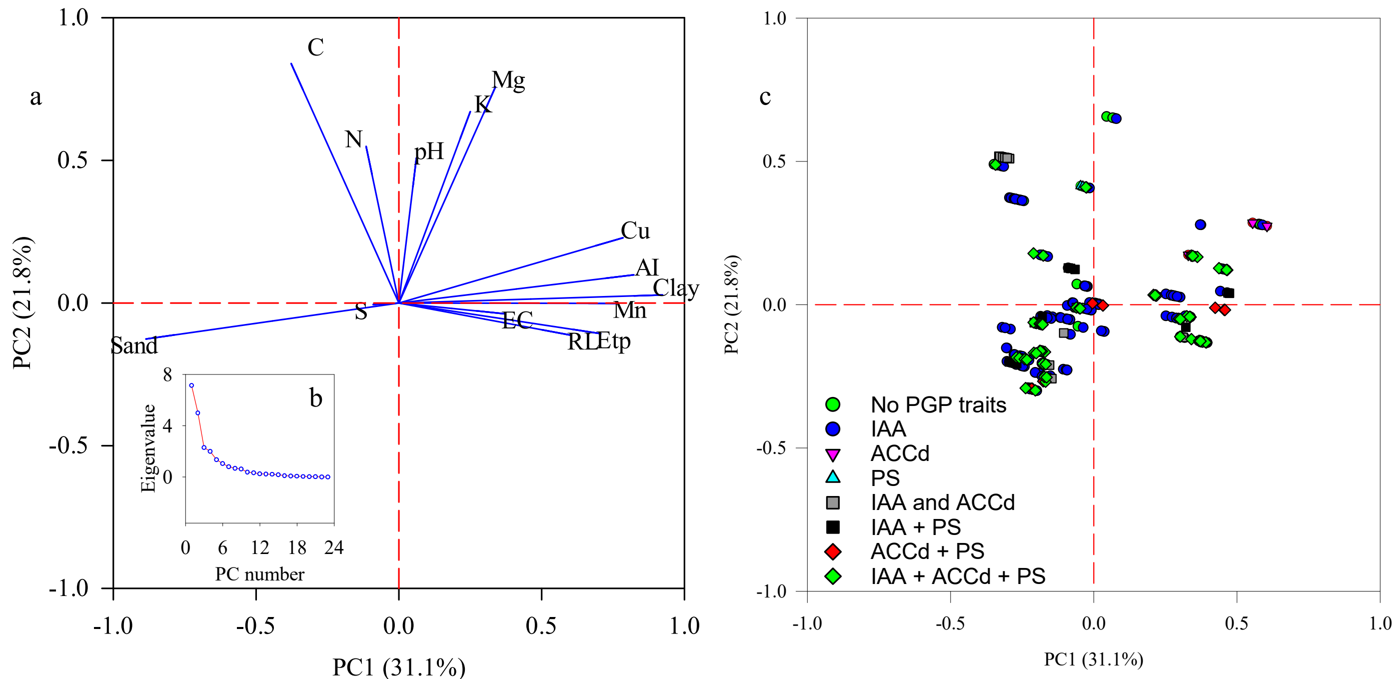

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640 Fig. 1. Principal Component analysis (PCA) biplot combining thirteen soil and environmental characteristics (a) to determine which inter-related
641 parameters most influenced the PGP potential of the isolates, (b) 6 principal components (PC) with eigenvalues > 1 and (c) to examine their effect
642 on the biological attributes of PGPR isolates. Trait abbreviations: C, soil carbon content; N, soil nitrogen content; P, phosphorus; pH, soil pH; K,
643 soil potassium content; Mg, magnesium; Cu, soil copper content; AI, Aridity index; Clay, soil clay content; Mn, soil manganese content; Etp,
644 Evapotranspiration; EC, soil electrical conductivity; S, soil sulphur content; sand, soil sand content; RL, root length; IAA, IAA production,
645 ACCd, ACC deaminase production; PS, P solubilisation.

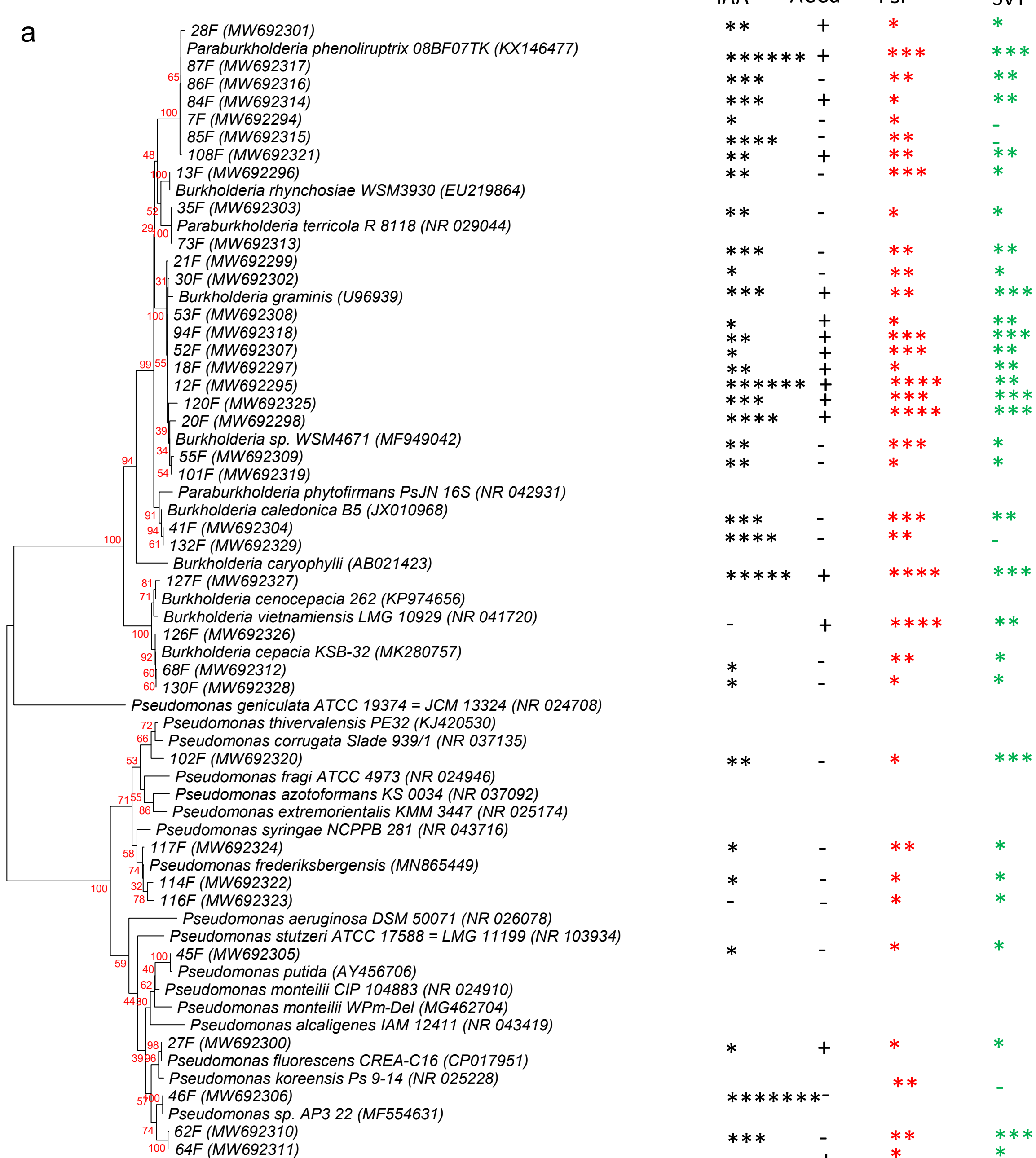

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Fig. 2. Dendrogram showing the genetic relationship among the 16S rDNA gene sequences (1500 bp) of strains of (a) *Burkholderia* and *Pseudomonas*, and (b) *Bacillus* isolated from chickpea rhizospheric soils and their related type strains. The tree was created by the neighbour-joining method. The numbers on the tree indicate the percentages of bootstrap sampling derived from 1000 replications. PSI: phosphate solubilisation index, *- PSI value <2, **- 2-3, ***>3-4, ****>4.0. IAA concentration (µg mL^{-1}) * < 10, ** 10 - 20, *** 20 – 30, ****30 – 40, ***** 40 – 50, ****** 50 – 60 and ******* 60 – 70. ACCd: ACC deaminase, '+' isolates positive for ACC deaminase activity and '-' isolates negative for ACC deaminase activity. SVT: Seeding vigour test, '-', '*', '**', and '***' - no effect on seedling promotion, up to 25%, 25-50% and >50% seedling root length promotion,

656 respectively. Seedling root length promotion = Root length (inoculated) – Root length (control)/ Root length
657 (inoculated)

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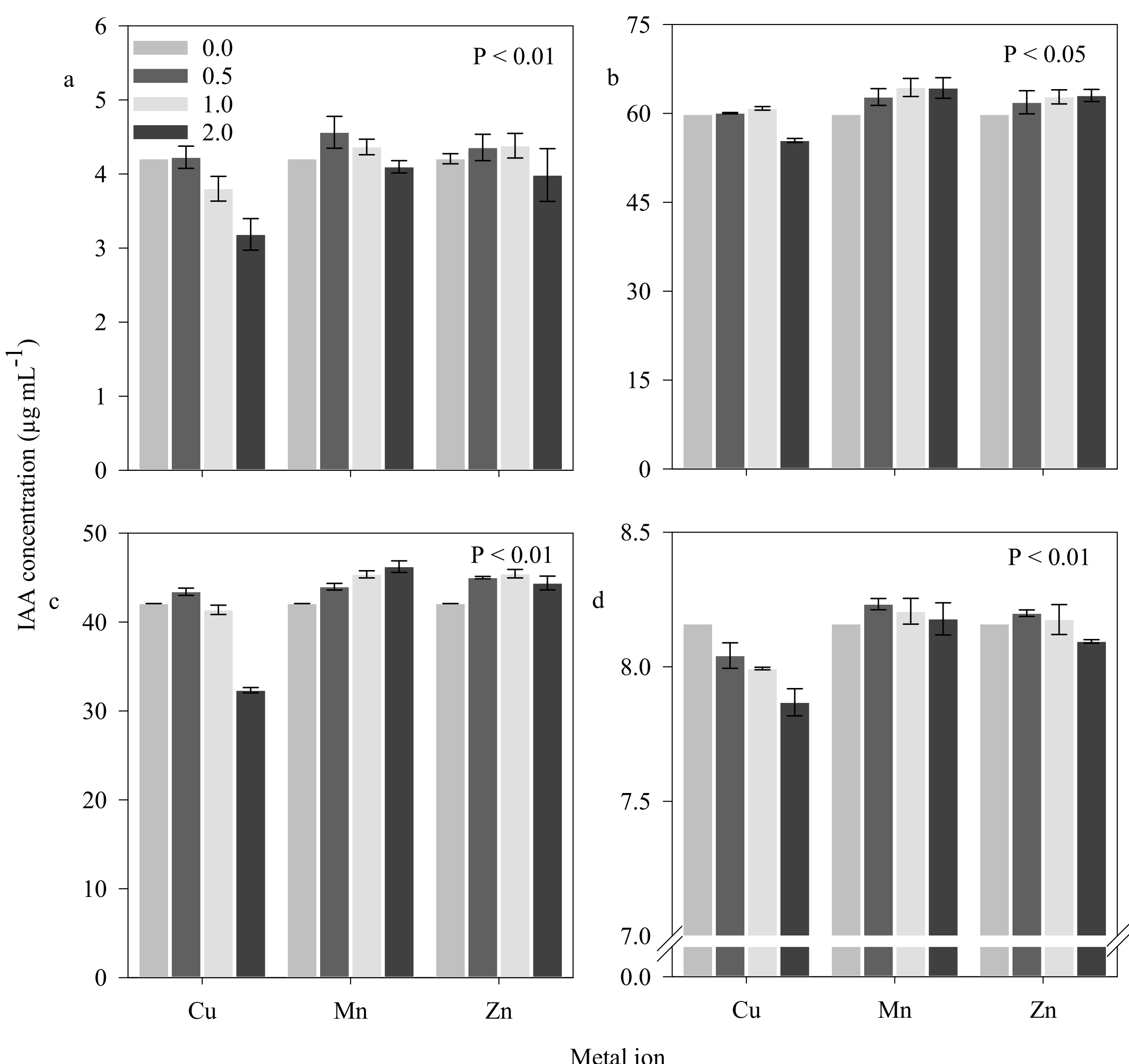

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662 Fig. **3** Effect of metal ion, copper (Cu), manganese (Mn) and zinc (Zn), on the production of
663 IAA by (a) *Bacillus pumilus* 98F, (b) *Burkholderia* sp. 12F, (c) *Burkholderia cenocepacia* 127F
664 and (d) *Pseudomonas fluorescens* 27F isolated from chickpea rhizosphere soils. The culture
665 solution was incubated in shaker incubator (28 ºC, 160 rpm) for 48 h. Experiments were
666 performed in triplicate and the values are presented as means. Error bars show standard error
667 (n = 3)

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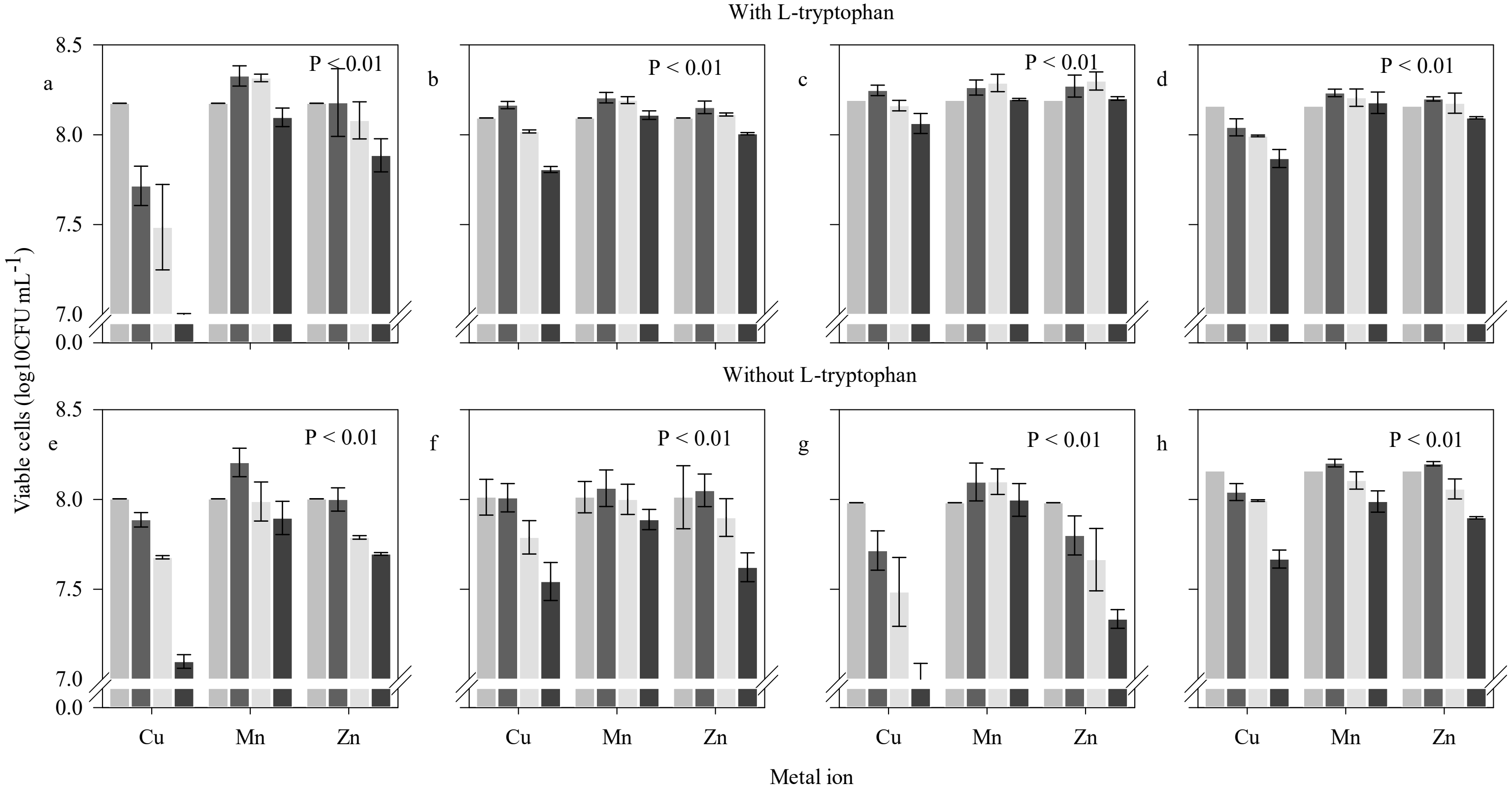

670 **Fig. 4** The cell viability of selected strains of rhizobacteria isolated from chickpea rhizospheric soils with increasing concentration metal ions (Cu,
671 n and Zn) in liquid medium in the presence and absence of L-tryptophan (500 µg mL^{-1}) for *Bacillus pumilus* 98F (a and e), *Burkholderia* sp. 12F
672 (b and f), *Burkholderia cenocepacia* 127F (c and g) and *Pseudomonas fluorescens* 27F (d and h). The culture solution was incubated in shaker
673 incubator (28 ºC, 160 rpm) for 48 h. Experiments were performed in triplicate and the values are presented as means. Vertical bars show standard
674 error (n = 3)

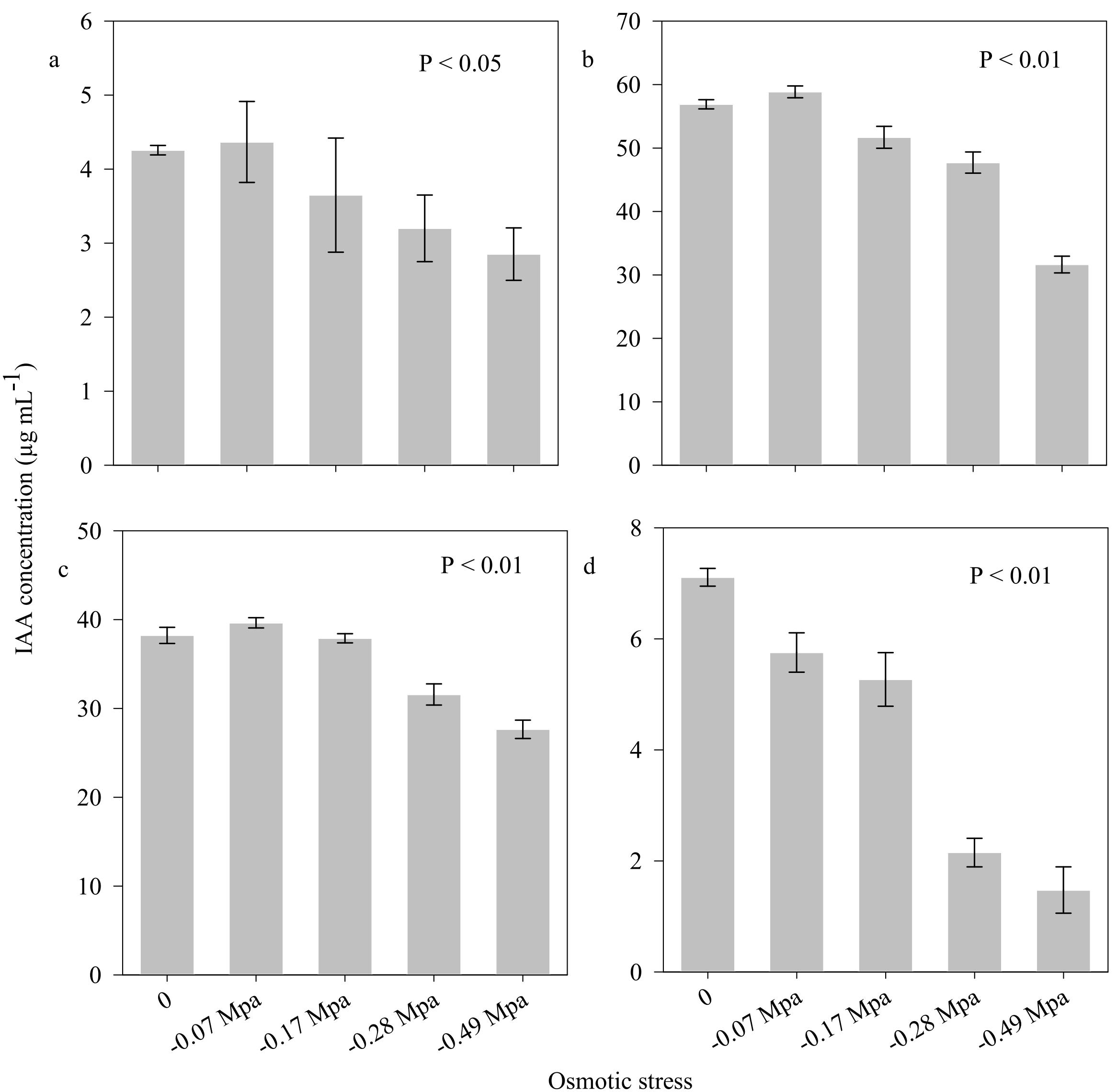

Fig. 5 Effect of osmotic stress induced by PEG-6000 on IAA production by selected PGPR such as (a) *Bacillus pumilus* 98F, (b) *Burkholderia* sp. 12F, (c) *Burkholderia cenocepacia* 127F and (d) *Pseudomonas fluorescens* 27F isolated from chickpea rhizosphere soils. Five PEG-6000 concentration (0, 10, 20, 30 and 45%) correspond to 0, -0.07, -0.17, -0.28 and -0.49 Mpa, respectively. The culture solution was incubated in shaker incubator (28 ºC, 160 rpm) for 48 h. Experiments were performed in triplicate and the values are represented as means. Error bars show standard error (n = 3). PEG-6000: Polyethylene glycol 6000

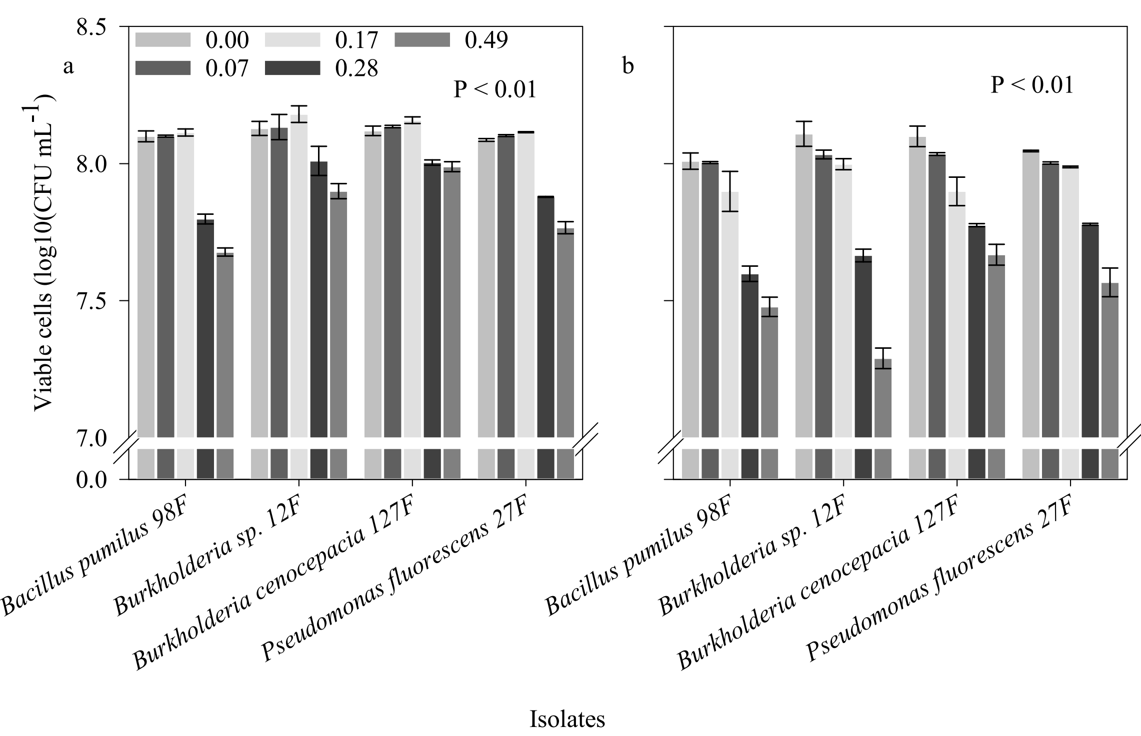

Fig. 6 Viable cell populations exposed to five PEG-6000 concentration (0, 10, 20, 30 and 45%) corresponding to 0, -0.07, -0.17, -0.28 and -0.49 Mpa, respectively, in the presence of the IAA (a) and with no IAA (b). The culture solution was incubated in shaker incubator (28 ºC, 160 rpm) for 48 h. Experiments were performed in triplicate and the values are presented as means. Error bars show standard error (n = 3). PEG-6000: Polyethylene glycol 6000

Chapter 5 Enrichment and isolation of novel phosphate solubilizing bacteria from rhizosphere soils of chickpea

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5.1. Statement of Authorship

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Contribution to the Paper	Planned the study conducted all experiments, analysed and interpreted data and wrote the manuscript		
Overall percentage (%)			
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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5.2. Abstract

Phosphate solubilising bacteria (PSB) that are screened for $Ca_3(PO_4)_2$ solubilisation ability *in vitro* are not always efficient solubilisers in soils that contain diverse forms of phosphorus (P). Therefore, we investigated P solubilisation from a variety of P sources. PSB isolated through enrichment in media with AlO_4P, $Ca_3(PO_4)_2$ or FeO_4P were compared with *Pseudomonas*- and *Bacillus*-like bacteria able to solubilise P. Furthermore, we investigated the diversity of culturable PSB by sequencing 16S rRNA. There was a positive and highly significant (P <0.05) association between the number of culturable PSB in chickpea rhizosphere soils and calcium (r = 0.41), magnesium (r = 0.33), potassium (r = 0.49), total nitrogen (r = 0.60), P (r = 0.58), carbon (r = 0.57), electrical conductivity (r = 0.40) and silt (%) (r = 0.56) content of soils from which the PSB originated. However, this association was inverse to the sand content in soil (r= 0.33). Following enrichment, the prevalence of PSB among total culturable bacteria was significantly increased. The most prominent PSB belonged to *Burkholderia*, *Variovorax*, *Leifsonia*, *Pantoea* and *Rhizobium*. Additionally, rhizobacteria belonging to *Curtobacterium*, *Microbacterium*, *Cellulomonas*, *Mycolibacterium*, *Rhodococcus*, *Inquilinus*, *Phyllobacterium, Dyella* and *Mucilaginibacter* were identified. P solubilisers belonging to *Inquilinus*, *Dyella* and *Mucilaginibacter* were newly identified. Results further showed greater P solubilising isolates enriched in the presence of FeO_4P than AlO_4P and $Ca_3(PO_4)_2$. Isolates enriched with FeO_4P were able to produce predominantly tri- and di-carboxylates. Enriching rhizobacteria in culture conditions with sparingly soluble P increased the likelihood of isolating elite PSB from bulk soils and plant rhizospheres.

Keywords *Burkholderia* sp., Carboxylate, Rhizobacteria, Sparingly soluble P

5.3. Introduction

Phosphorus (P) is an essential nutrient for all living organisms. Because of its chemically reactive nature, it is often bound as sparingly soluble mineral phosphates in soil (Holford 1997). As an example, in soils with 400 to 1200 mg kg^{-1} of P, only ~1 mg kg^{-1} was dissolved P in the soil solution (Rodríguez and Fraga, 1999). For plants to use insoluble soil P reserves, it is necessary to convert them into primary and secondary orthophosphate ions ($H_2PO_4^-$ and HPO_4^{2-}) within the typical pH range (4.0 - 9.0) of most soils (Pierzynski et al., 2005). Plant and soil microorganisms may improve plant P use efficiency through three main strategies, including root foraging, P mining and improvement of plant P utilisation efficiency (Richardson et al., 2011). Of these strategies, P mining through dissolution or mineralisation of P from sparingly available sources by action of root exudate and soil microorganisms, is the main mechanism to improve P nutrition for plants.

Plant associated bacteria can benefit plants by growth promotion via direct and indirect mechanisms, including P solubilisation (Swift et al., 2018; Alemneh et al., 2020). The dissolution of soil P into plant available forms is demonstrated by numerous soil bacteria *in vitro* (Richardson et al., 2009). These types of beneficial bacteria have been termed 'phosphate solubilising bacteria' (PSB) and among them are strains that belong to *Bacillus*, *Burkholderia*, *Pseudomonas*, *Serratia*, *Alcaligenes*, *Arthrobacter*, *Enterobacter*, *Pantoea*, *Acinetobacter, Stenotrophomonas*, *Cupriavidus*, *Agrobacterium*, *Acinetobacter*, *Rhodococcus* and *Exiguobacterium* (Chung et al., 2005; Collavino et al., 2010; Yu et al., 2011; Azziz et al., 2012). The diversity and abundance of culturable PSB communities in soils varies with location, and are affected by environmental (Mander et al., 2012) and management (Azziz et al., 2012; Zheng et al., 2017) practices. A diversity of microsites in impose strong selective constraints and thereby lead to highly diverse PSB (Fernández et al., 2015). In some instances, PSB belonging to the genera *Burkholderia* (Estrada et al., 2013), *Herbaspirillum* (Estrada et al., 2013), *Entrobacter* spp. (Jorquera et al., 2008; Collavino et al., 2010), *Streptomyces* and *Micromonospora* (Hamdali et al., 2008) and *Pantoea* (Jorquera et al., 2008) have been identified in different locations from various plant species. However, information about the dominant genera of soil bacteria that are efficient in P solubilisation is scant. Understanding the effect of environmental and agricultural management practices on soil communities of PSB and their diversity is important to inform strategies that enhance agroecosystems productivity through increasing plant available P.

Among the aforementioned genera of PSB, rhizosphere isolates of *Pseudomonas* and *Bacillus* have been described as effective phosphate solubilisers (Igual et al., 2001; Browne et al., 2009; Meyer et al., 2011; Yadav et al., 2011; Pei-Xiang et al., 2012). In one investigation of the diversity of PSB, a phylogenetic tree showed that 85% of superior PSB were linked to one lineage within *Pseudomonas fluorescens* (Browne et al., 2009). Focussing on particular organisms may be one strategy to enhance soil inorganic P mobilisation and P utilisation efficiency by plants. Hence, identification of species and genera of bacteria directly affiliated with P solubilisation is important for the development of efficient PSB that can enhance plant growth through increasing the P nutrient supply to associated host plants.

Although soils contain a variety of chemically diverse phosphate forms, a large proportion of efficient PSB have been selected from rhizosphere soils using *in vitro* culture media containing insoluble $Ca_3(PO_4)_2$ (Karagöz et al., 2012; Liu et al., 2015; Manzoor et al., 2017; Bautista-Cruz et al., 2019; Solans et al., 2019). None of the PSB in these studies showed efficient P solubilisation from iron phosphate ($FePO_4$) and aluminium phosphate ($AlPO_4$) which limits their usefulness in acidic soils, where P is often associated with iron or aluminium. Therefore, there is a need for developing isolation strategy that result in an efficient PSB capable of solubilising P concentration well beyond their own needs (Raymond et al., 2021).

Dissolution of P from insoluble P forms by rhizobacteria can be mediated by the production of organic and inorganic acids, the release of protons (H^+), siderophores, hydroxyl (OH^-) ions and CO_2 (Illmer et al., 1995; Rodríguez and Fraga, 1999; Vassilev et al., 2006; Sharma et al., 2013). A significant amount of P from $Ca_3(PO_4)_2$ can be released through acidification of the media (Jones and Oburger 2011). However, the production of chelators, including carboxylates, siderophores and other organic derivatives, is essential for efficient solubilisation of P from sparingly soluble P sources, including $FePO_4$ and $AlPO_4$ (Walpola et al., 2012). Because of this, $Ca_3(PO_4)_2$ is an inappropriate compound to use in universal selection for PSB adapted to diverse agricultural soils. Therefore, we focused on the development of P solubilisers that can efficiently access P from a variety of P sources and in this study, PSB were isolated through enrichment in media containing AlP, CaP or FeP as sources of sparingly soluble mineral phosphates. These organisms were compared with PSB isolated from rhizosphere soils without enrichment. Moreover, we investigated the diversity of culturable PSB communities across a range of sampling sites. The objectives of this study were therefore (1) to determine the abundance and proportion of PSB in a variety of agricultural soils, (2) to investigate the

phylogenetic diversity of PSB, and (3) to develop an effective selection strategy for isolation of efficient PSB.

5.4. Materials and methods

5.4.1. Soil sample collection and physico-chemical properties of soils

Soil samples (0 to 10 cm depth) were collected from 74 cropping sites in Australia (Appendix Fig. 1; Appendix Table 1) and kept at 4 ºC. Soils were prepared for analysis by air-drying and sieving (2 mm). The physico-chemical properties of the soils were analysed following the methods described in Rayment and Lyons, (2011). Soil carbon was determined with the Walkley Black method. Total N was determined following a modified Kjeldahl method. Total soil P was determined following a colorimetric method after extracting elemental P by perchloric acid digestion. Soil pH was determined from a suspension containing a 1:2.5 soil: $CaCl_2$ (0.01 M). The electrical conductivity (EC) of a water extract (1: 2.5 soil: water suspension) was determined. The textural composition of the soils was analysed by dispersal and hydrometric readings. Ammonium acetate-extractable Ca, K and Mg in soils were determined using atomic absorption spectrophotometry. Plant available Cu, Zn and Mn in soils were determined using diethylenetriaminepentaacetic acid (DTPA) micronutrient extraction method.

5.4.2. Enumeration of PSB communities in agricultural soils

Chickpea cv. HatTrick seedlings were grown in pots (4.5 cm × 4.5 cm ×10 cm) containing soil collected from a wide variety of sampling sites across major agricultural lands in Australia to obtain rhizosphere soils. The pots were kept in a growth chamber with lighting (1000 µmol) programmed for a 16 h photoperiod, with a constant temperature at 20 ºC, CO_2 concentration at 2890 ppm and 70% relative humidity. Seedlings were harvested four weeks after germination. The soils adhering strongly to the roots of each chickpea seedling were collected and kept at 4 ºC. A total of 74 chickpea rhizosphere soils were collected. One gram of each soil was suspended in 9 mL of sterile MilliQ water. Then, tenfold serial dilutions were prepared to a factor of 10^{-6}. From the dilution suspensions, the total number of heterotrophic bacteria and PSB were enumerated. A 100 µL aliquot from the 10^{-4} and 10^{-5} dilutions in triplicate was inoculated on to National Botanical Research Institute's phosphate (NBRIP) growth media supplemented separately with 5 g of either AlO_4P (Sigma-Aldrich, CHEMIE GmbH, Steinheim, Germany), $Ca_3(PO_4)_2$ (>96%, Sigma-Aldrich) or $FeO_4P.2H_2O$ (Sigma-Aldrich) and

with 75 µg mL^{-1} of cycloheximide. The acidic pH of media containing $FeO_4P.2H_2O$ and AlO_4P was adjusted to 7.00 before autoclaving and confirmed after sterilisation. The experiment had three replications. Inoculated plates were incubated at 28 ºC for 10 days. Distinct colonies with clearing (halo) zones around them were identified as PSB. The population of PSB as a proportion of the total culturable heterotrophic rhizobacteria was calculated based on colony forming units (CFU).

5.4.3. Selection of PSB

We used two methods to isolate PSB from rhizosphere soils collected from chickpea. The first method was the selective isolation of PSB favouring two bacterial taxa: *Pseudomonas* and *Bacillus*, that have been identified as two genera of efficient PSB associated with various plant species (Yu et al. 2011; Liu et al. 2015; Mukhtar et al. 2020). These genera are commonly used in commercial products and relatively easily cultured *in vitro*. The second method was based on a previous finding of the present work that showed an inverse association between P solubilising capacity of soil bacteria *in vitro* and available soil P content (Chapter 4). Accordingly, we hypothesised that incubating the soils in media containing insoluble P sources may enrich selectively P solubilising bacteria but discourage the growth of non-P solubilising bacteria. Therefore, the second method was designed to selectively enrich for efficient PSB using media containing various sparingly soluble P sources [AlO_4P, $Ca_3(PO_4)_2$ and FeO_4P].

Method 1: Taxonomically based selection of PSB

From the above-mentioned dilution suspension, a 100 µL aliquot from the 10^{-5} and 10^{-6} dilutions was aseptically spread onto a semi-selective medium for *Pseudomonas*-like bacteria, consisting of 1/10 strength Tryptone Soya Agar (TSA) (OXOID LTD, Basingstoke, Hampshire, England) supplemented with 50 µg mL^{-1} of ampicillin (99%, Sigma-Aldrich, CHEMIE GmbH, Steinheim, Germany), 12.5 µg mL^{-1} of chloramphenicol (99%, Sigma-Aldrich) and 75 µg mL^{-1} of cycloheximide (Simon and Ridge, 1974). As an alternative strategy, *Bacillus*-like rhizobacteria were isolated after heat treatment (15 min, 80 ºC) followed by streaking on TSA and incubation (5 days, 28 ºC) (Travers et al. 1987). According to the morphological appearance of colonies, 540 *Bacillus*-like and 303 *Pseudomonas*-like rhizobacteria were purified on the same medium and maintained in slant culture at 4 ºC and in 20% glycerol at -80 ºC. To detect the presence of P solubilisation properties among the collected bacteria, isolates were streaked on NBRIP media (Nautiyal, 1999), containing the following ingredients (L^{-1}): glucose, 10 g; $MgCl_2.6H_2O$, 5 g; $MgSO_4.H_2O$, 0.25 g; KCl, 0.2 g,

and $(NH_4)_2SO_4$, 0.1 g supplemented with 5 g of $Ca_3(PO_4)_2$ (>96%, Sigma-Aldrich). After ten days of incubation at 28 ºC, isolates that created clearing zones around their colonies were considered to be PSB.

Method 2: Selective enrichment of PSB

PSB were enriched and isolated from chickpea rhizosphere soils that varied in pH and P content. One gram of rhizosphere soil was added to sterile bottles containing 100 mL of NBRIP broth supplemented separately with one sparingly soluble form of P: AlO_4P (Sigma-Aldrich), $Ca_3(PO_4)_2$ or $FeO_4P.2H_2O$ (Sigma-Aldrich) as the sole P source. The bottles were shaken at 28 ºC at 160 rpm for one week. One mL of the enriched culture was successively transferred into fresh NBRIP (100 mL) containing the same type of insoluble P for a second round of enrichment. After seven days of incubation, 1 mL of culture solution was transferred into a fresh medium for third round enrichment of PSB enrichment in the media.

From every round of enrichment, samples were taken and the total population of aerobic culturable bacteria, the population of bacteria that produced a P clearing zone, the concentration of soluble inorganic P (Pi) and pH of the culture were determined. One mL of culture suspension was transferred into 9 mL of sterile MilliQ water and a dilution series was made to 10^{-6}. A 100 µL sample from the 10^{-5} and 10^{-6} dilutions was spread on the NBRIP agar medium supplemented separately with 5 g L^{-1} of AlO_4P, $Ca_3(PO_4)_2$ or $FeO_4P.2H_2O$. Plates were incubated at 28 ºC for 10 days.

The size of the solubilisation zone and colony diameter of P solubilizing isolates were determined. Based on these measurements, the phosphate solubilisation index (PSI) was calculated by applying the formula described by Nguyen et al. (1992).

$$\mathrm{PSI} = \frac{\mathrm{SD}}{\mathrm{CD}}$$

Where SD- Solubilisation (clearing zone) diameter and CD- Colony diameter

5.4.4. Plant growth promoting traits

The amounts of indole acetic acid (IAA) produced in the presence and absence of L-tryptophan were determined using Dworkin Foster (DF)-minimal broth (Patten and Glick, 2002). Aminocyclopropane carboxylic acid (ACC) deaminase-producing rhizobacteria were identified using DF-minimal medium containing ACC as the sole N source. ACC deaminase-

positive strains were further investigated for quantitative ACC deaminase production in the same media. Cultures were incubated at 28 ºC and shaking at 150 rpm for 6 h before the cultures were centrifuged to obtain the supernatant. When a bacterium utilises ACC, it produces NH_4^+ and α-ketobutyrate. In this experiment, we measured the concentration of NH_4^+ in the supernatant using a colorimetric method (Harwood and Kühn, 1970). The concentration of NH_4^+ was converted into ACC deaminase activity expressed as nmol α-ketobutyrate produced h^{-1}.

5.4.4. Sequencing and phylogenetic analysis of 16S rRNA

Phosphate solubilising bacteria with PSI greater than 2 were selected for phylogenetic analysis using the 16S rRNA gene. An approximately 1500 bp fragment of 16S ribosomal DNA from 167 strains was amplified using universal forward primer: fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal reverse primer: rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) (Weisburg et al. 1991). Amplification reactions were performed in 25 µL containing 1 µL of each of 10× diluted reverse and forward primer, 12.5 µL of 2× master mix and 10.5 µL MilliQ water. The PCR cycle conditions were as follows: initial denaturation at 95 ºC for 1 min followed by 35 amplification cycles comprising denaturation at 95 ºC for 15 s, annealing at 57 ºC for 30 s, extension at 72 ºC for 1 min and a final extension at 72 ºC for 5 min. All amplified PCR products were confirmed by electrophoresis on 1.5% agarose gels in 1x TAE buffer containing gel red nucleic acid stain (3 µL L^{-1}), using gene ruler 2 Kb DNA ladder. DNA sequencing was performed on a direct sequence of PCR products by AGRF Sanger Service (Plant functional genomes, University of Adelaide, South Australia).

DNA sequences obtained were compared with those deposited in the Genebank database from the National Centre for Biotechnology Information (NCBI) (http//www.ncbi.nim.nih.gov/BLAST) for taxonomic assignment. Phylogenetic analyses of the 16S rRNA gene sequences were performed by the Maximum Likelihood approach, based on the Tamura 3 parameter model using MEGA version 7. Phylogenetic tree topology, based on 1000 times the neighbour-joining data set, was evaluated by bootstrap analysis.

5.4.5. P solubilisation efficiency in liquid medium

Efficient PSB were selected proportionally from both isolation methods, based on PSI values. Five isolates, namely *Bacillus simplex* 37F, *Bacillus megaterium* 8F, *P. fluorescens* 27F,

Bacillus pumilus 98F and *Burkholderia cepacia* 126F were selected from those obtained directly from rhizosphere soils. The remaining two isolates: *Burkholderia sp.* 12F and *Burkholderia cenocepacia* 127F were selected from those obtained after enrichment. The P solubilisation potential of these strains was investigated in liquid NBRIP medium containing sparingly soluble P sources [AlO_4P, $Ca_3(PO_4)_2$ or $FeO_4P.2H_2O$]. Each treatment was replicated three times. Every two days for three weeks of incubation, 5 mL of culture solution was taken and centrifuged (10,000 ×*g* for 10 min). The supernatant was analysed for Pi. One mL of culture was also taken for enumeration of viable cell populations. Cultures were serially diluted using sterile MilliQ water and 100 µL samples were plated on TSA and incubated at 28 ºC for seven days.

5.4.5. Inorganic phosphate determination

Soluble phosphate was determined using the phospho-molybdate method (Murphy and Riley, 1962). Briefly, after centrifugation 0.4 mL of supernatant and 2 mL of mixed reagent were transferred into test tubes. The absorbance of the mixed solution was measured by Nanophotometer (Implen GmbH, Schatzbogen, Münichen, Germany) at 882 nm against a set of standards spanning 4.567 x to y (µg mL^{-1}) of Pi. X and y represent the spectrophotometer reading and Pi (µg mL^{-1}) in the culture solution, respectively. The net Pi in the aliquot was calculated by deducting the Pi in a non-inoculated solution from that of the respective inoculated solution.

5.4.6. Carboxylate production

Carboxylate production during P solubilisation was analysed by high-performance liquid chromatography of culture supernatant using HPLC (600E pump, 717plus autoinjector, 996 Photodiode array detector, Waters, Milford, MA, USA) and an Alltima C-18 reverse-phase column (Cawthray, 2003). The culture solution was centrifuged at 10,000 ×*g* for 10 min. The supernatant was further filtered through a 0.22 µm filter and the pH was adjusted to <4.0 using H_3PO_4. Authentic standards included gluconic, pyruvic, malic, *iso*-citric, malonic, shikimic, lactic, acetic, α-ketobutyric, α-ketoglutaric, maleic, citric, succinic, fumaric, *cis*-aconitic, and *trans*-aconitic acids.

5.4.7. Data analysis

All experiments followed a completely randomised design with three replications. Data for Pi and pH at day 14 of incubation were subjected to ANOVA and an LSD test, with a significance

level of P <0.05. A correlation analysis between soil characteristics and PSB population and the proportion of PSB in chickpea rhizospheres at the sampling sites was carried out. Data were analysed using Genstat 18th edition. Before analysis, normal distribution of the data was checked using the Shapiro-Wilk test. Homogeneity of variances was evaluated using Bartlett's tests. Figures were constructed using SigmaPlot ver. 14.0.

5.5. Results

5.5.1. Abundance and diversity of PSB

The total population of culturable aerobic bacteria and the percentage of PSB in rhizospheres of chickpea grown in soils from the various study sites were enumerated. The PSB populations varied between 4.3 to 4.95 $\log_{10}$ (CFU g^{-1} DW of rhizosphere soil). The correlation between the soil and environmental variables and the populations of PSB are presented in Table 1. The abundance of PSB in rhizosphere soils had a positive and highly significant (P <0.05) association with soil Ca^{2+} (r = 0.410), Mg^{2+} (r = 0.33), K^{+} (r = 0.49), total N (r = 0.60), total P (r = 0.58), total carbon (r = 0.57), EC (r = 0.40) and silt (%) (r = 0.56). In contrast, there was a significant and inverse association between the PSB population in the rhizosphere and sand content (r = 0.33).

A total of 743 rhizobacteria were isolated selectively as *Bacillus*- and *Pseudomonas*-like bacteria. Besides this, 98 rhizobacteria able to solubilise phosphate from different forms of P were enriched in the presence of either AlO_4P, $Ca_3(PO_4)_2$ or $FeO_4P.2H_2O$. Out of the 841 isolates, 683 (81.3%) were able to produce IAA in the presence of L-tryptophan. ACC deaminase activity was detected in 90 isolates (10.7%). Of 743 *Bacillus*- and *Pseudomonas*-like bacteria, only 72 (9.7%) were able to solubilise $Ca_3(PO_4)_2$ in a plate assay.

Diverse species of PSB belonging to the genus *Bacillus* were identified (Fig.1). Among the dominant species, *B. simplex*, *Bacillus muralis, B. megaterium*, *Bacillus niacini*, *Bacillus cucumis* and *Bacillus amyloliquefaciens* were identified. One strain each belonging to *Bacillus subtilis, Bacillus endophyticus*, *Bacillus atrophaeus*, *Bacillus pacificus*, *Bacillus pumilus, Bacillus mycoides*, *Bacillus toyonensis* and *Paenibacillus endophyticus* were identified. On the basis of the percentage similarity of isolates with the reference strains, our isolates were categorised into nine clades. Clustering was made at 99.94% of similarity, which was the highest percentage similarity of our isolates with reference strains. Between clustering, the average value of PSI was varied. Bacteria in the clade containing *B. pumilus* 52 scored the

highest average PSI value (4.70). Following this clade, PSB that were closely related to *B. megaterium* had an average PSI of 2.68. Although the majority of P solubilisers among the *Bacillus* isolates were assigned to the group containing *B. simplex* EH12, these isolates scored the lowest average PSI value (1.46). None of the isolates closely related to *B. simplex* EH12 had PSI greater than 3.00.

Pseudomonas-like bacteria were isolated using a semi-selective medium which was also able to recover *Burkholderia*-like bacteria (formerly included in *Pseudomonas*). Hence, we identified a number of phosphate solubilising *Pseudomonas* and *Burkholderia* isolates with PSI >2.00 (Fig. 2). The dominant species were assigned to *Burkholderia* sp., *Paraburkholderia phenoliruptix* followed by *B. cepacia, Pseudomonas frederiksbergensis* and *Pseudomonas jessenii*. One strain was assigned to each of *Burkholderia territorii*, *Burkholderia rhynchosiae*, *Burkholderia caledonica*, *Pseudomonas corrugate*, *P. fluorescens*, *Pseudomonas putida* and *Pseudomonas* sp. Compared with the strains belonging to *Bacillus* and *Pseudomonas*, a large proportion of *Burkholderia* isolates were found to be somewhat more efficient P solubilisers *in vitro*. On the basis of similarity percentage, the strains were categorised into four groups having varied PSI values. The highest average PSI value (3.58) was observed for *B. cenocepacia* 262- and *B. territorii* KBB5-like strains, followed by strains that were closely related to *Burkholderia* spp. from the database. Among seven *Pseudomonas* strains in the present study, only one isolate had a PSI value greater than 3.00.

5.5.2. Enrichment of P solubilizing bacteria

Phosphate-solubilising bacteria were isolated from enriched consortia of rhizobacteria cultured in the presence of sparingly soluble P as the sole P source. We selected PSB that had PSI >2.00 for phylogenetic analysis. We recovered 16 genera of efficient PSB (Fig. 3). The majority of P solubilising strains belonged to *Burkholderia*, *Variovorax*, *Leifsonia, Rhizobium* and *Pantoea*. Also, a few isolates belonging to *Microbacterium*, *Mycolicibacterium*, *Rhodococcus*, *Caulobacter* and *Phyllobacterium* were recovered. Of the newly isolated bacteria, one strain belonged to each of the following genera: *Cellulomonas*, *Inquilinus*, *Sphingomonas*, *Dyella* and *Mucilaginibacter*. One strain showed less than 97% similarity with *Bacillus endophyticus* NM3E6 in the database. On the basis of average PSI values, isolates were categorised into eleven groups. The group of PSB that was most similar to *Burkholderia* from the database scored the highest average PSI value (4.06). This value that was associated with *Burkholderia*

was higher than the average value of PSI (3.29) for *Burkholderia* isolated from non-enriched soils.

The concentration of Pi (Fig. 4a), solution pH (Fig 4b), PSB population (Fig. 4c) and their proportion out of the total culturable bacteria (Fig. 4d) varied significantly along the enrichment process. The highest PSB population in enriched consortia occurred with $Ca_3(PO_4)_2$ as the sole P source, followed by $FeO_4P.2H_2O$ and AlO_4P. However, the highest proportion of PSB out of the total culturable bacteria used $FeO_4P.2H_2O$ and $Ca_3(PO_4)_2$ in the last enrichment stage. During enrichment, the culturable bacteria, presumably unable to release Pi, declined (Fig.4e). Although a large number of P solubilisers was enriched in the presence of $Ca_3(PO_4)_2$, most of the isolates from $Ca_3(PO_4)_2$ enrichment medium had low P solubilisation efficiency. Efficient PSB with PSI greater than 2.00 were obtained from bacterial consortia enriched in the presence of $FeO_4P.2H_2O$ and AlO_4P (Fig. 3).

5.5.3. P solubilisation efficiency

Greater PSI values were observed in PSB isolated through selective enrichment methods than for those strains isolated using the selective taxonomic method (Fig. 5a). PSB enriched in the presence of $FeO_4P.2H_2O$ had significantly higher average PSI values, followed by those enriched in AlO_4P and then those enriched in $Ca_3(PO_4)_2$ (Fig. 5b). On the basis of high PSI values, two strains from enriched consortia and five strains isolated from non-enriched rhizosphere soils were selected for further investigation of P solubilisation efficiency in liquid media. Phosphate solubilisation potential of the seven selected strains in liquid medium differed at $P < 0.05$. The highest Pi concentration occurred after 14 days of incubation and later declined. For further investigation of the performance of PSB in P solubilisation, we measured Pi after 14 days of incubation. Accordingly, Pi solubilisation ranged between 16.8 to 36.7 µg mL^{-1}, 61.3 to 184.3 µg mL^{-1} and 13.7 to 43.6 µg mL^{-1} from AlO_4P, $Ca_3(PO_4)_2$, and $FeO_4P.2H_2O$, respectively (Fig. 6a, b and c). The highest Pi from AlO_4P (36.5 and 36.7 µg mL^{-1}), $Ca_3(PO_4)_2$ (184.3 and 147.5 µg mL^{-1}), and $FeO_4P.2H_2O$ (43.6 and 41.3 µg mL^{-1}) were recorded with *B. cenocepacia* 127F and *Burkholderia* sp. 12F, respectively. These strains solubilized 1.8 to 2.2, 1.4 to 2.9 and 2.2 to 3.3-fold higher Pi from $Ca_3(PO_4)_2$, AlO_4P and $FeO_4P.2H_2O$, respectively than other strains obtained using the taxonomic selection method. Among five strains isolated from rhizosphere soils, *P. fluorescens* 27F produced the highest Pi from $Ca_3(PO_4)_2$ and AlO_4P while *B. megaterium* 8F mobilised the highest Pi from AlO_4P.

Quantitative and qualitative differences in carboxylate production were observed between efficient P solubilisers during the P solubilisation process (Table 2). Thirteen types of carboxylates were detected in culture media containing sparingly soluble P sources. Most strains produced lactic and fumaric acids. *B. cenocepacia* 127F followed by *B. simplex* 37F and *B. megaterium* 8F produced the highest total concentrations of carboxylates (5,313, 2,383 and 1,122 μM) during $Ca_3(PO_4)_2$ solubilisation, respectively. An efficient P solubiliser, *B. cenocepacia* 127F, produced predominantly citrate (213 μM), fumarate (144 μM), α-ketoglutarate (2,756 μM), pyruvate (1,814 μM), malate (1,333 μM), acetate (966 μM) and succinate (663 μM) accounting for 3.1%, 2.1%, 40.0%, 26.2%, 19.3%, 14.0% and 9.6%, of total carboxylates, respectively. Acetate, lactate, and malate and a very low amount of fumarate were produced by *B. simplex* 37F. *Burkholderia* sp. 12F produced lactate (132 μM) and fumarate (0.1 μM) during P solubilisation. This strain also produced the tricarboxylates *cis*-aconitate, α-ketoglutarate, citrate and *trans*-aconitate. Among the P sources used, the highest total carboxylate production and more types of carboxylates were recorded when $Ca_3(PO_4)_2$ was the sole P source.

During P solubilisation, the pH of the medium decreased significantly (Fig. 6d, e and f). The pH value in the non-inoculated treatment with $Ca_3(PO_4)_2$ was 6.89. However, slightly acidic pH (5.82 and 5.44) was observed in AlO_4P and $FeO_4P.2H_2O$ containing media, respectively. These pH values were further acidified to 3.00 and 3.02 in the presence of *B. simplex* 37F. At these pH values, viable cells for *B. simplex* 37F were not detected after 14 days of incubation. Moreover, these media without inoculation had a pH below 4.7 after shaking for 14 days. When $Ca_3(PO_4)_2$ was solubilised, *Burkholderia* sp. 12F induced the lowest pH (3.7) in the culture solution.

5.6. Discussion

Bacteria have a major role in P cycling in the terrestrial ecosystem (Acevedo et al. 2014; Azziz et al. 2012; Narsian and Patel 2009). Accordingly, we investigated the populations of PSB in a wide range of agricultural soils collected from different agricultural cropping regions across southern and eastern Australia. Most soils had very low populations of culturable PSB (10^2-10^4 CFU g^{-1} of DW soil) compared with those reported in the reported previously. However, our results demonstrated up to 15.7% of culturable aerobic rhizobacteria could solubilise P from $Ca_3(PO_4)_2$. The proportion of PSB in some soils in the present study was higher than those reported in soils from in other reports (Alireza Fallah and Ave, 2006; Chatli et al., 2008; Van Der Heijden et al., 2008; Zhou et al., 2020). In these studies, the percentage of PSB in populations of aerobic soil bacteria varied between 4.0% and 14.4%. The highest proportion of PSB in the present study was in soil with a pH of 8.2 and high aridity index. In a previous study, a significantly higher percentage of PSB was found in a harsh environment with high aridity and alkaline soil pH (Timmusk et al., 2011).

Environmental conditions and agricultural practices affect the populations and P solubilisation activity of rhizobacteria (Fernández et al., 2015). Soil characteristics, rather than agricultural management, can have a greater impact on microbial community structure and catabolic function (Wakelin et al., 2008). Accordingly, we identified soil factors that influenced the population and proportion of PSB in cropped soils collected from the southern and eastern agricultural environments of Australia. There was a positive association between the abundance and proportion of culturable PSB in the sampling sites and soil carbon, N, P, total cation (Ca^{2+}, Mg^{2+}, and K^+) and silt content. However, the association between PSB count and proportion, and sand content was negative, suggesting an effect of soil type, as they were high in loamy soil and low in soils with a greater proportion of sand (Xia et al., 2020). Previous reports also found a direct correlation between culturable bacteria that can solubilise P and soil carbon, as well as the concentration of cationic nutrients (Ca^{2+} and Mg^{2+}) (Kim et al., 1997; Ndung'u-Magiroi et al., 2012). PSBs are heterotrophic bacteria that require external carbon sources to solubilise P. Incorporation of organic residues has enhanced the abundance of PSB (Perez et al., 2007; Panda et al., 2016; Wei et al., 2017). An increase in the proportion of PSB with increasing Ca^{2+}, Mg^{2+} and K^+ concentration in soils could also indicate the influence of these cations on the prevalence of heterotrophic bacteria that can utilise P from Ca/Mg-PO_4 complexes, which are the dominant forms of P in soils of neutral and alkaline pH.

Plant growth-promoting rhizobacteria belonging to *Bacillus*, *Pseudomonas* and *Burkholderia* have been frequently reported as the most powerful P solubilisers present in chickpea rhizosphere soils when tested *in vitro* (Yadav and Verma, 2014; Midekssa et al., 2016; Laabas et al., 2017; Brígido et al., 2019; Pandey et al., 2019). Isolates belonging to these bacterial genera have improved plant growth through various mechanisms including phosphorus solubilisation (Laabas et al., 2017; Pandey et al., 2019; Zaheer et al., 2019). Accordingly, we performed semi-selective isolation methods to obtain isolates of the three above-mentioned genera of bacteria that commonly solubilise $Ca_3(PO_4)_2$ *in vitro*. We identified 25 species of phosphate solubilising *Bacillus,* the predominant species being *Bacillus* spp.: *B. simplex*, *B. muralis*, *B. thuringiensis*, *B. megaterium*, *B. flexus, B. niacini, B. amyloliquefaciens* and *B. subtilis*. Selected isolates from most of these *Bacillus* species have been previously identified as efficient PSB from chickpea rhizosphere soils (Singh et al., 2014; Yadav and Verma, 2014; Sharma et al., 2019), as have *Paraburkholderia phenoliruptrix*, *B. graminis*, and *P. frederiksbergensis* (Coenye et al., 2004; Barriuso et al., 2008; De Meyer et al., 2013) identified here. We also identified an efficient P solubilising isolate of *B. rhynchosiae* that was previously described from root nodules of the South African legume *Rhynchosia ferulifolia* (De Meyer et al., 2013). In addition, *P. putida* and *P. fluorescens* have been frequently reported as potent P solubilisers (Vyas and Gulati, 2009; Zabihi et al., 2011; Patel et al., 2012), and were also isolated from the rhizospheres of chickpea grown in soils from a diverse collection of agricultural soils.

Among forty isolates of P solubilising bacteria from an enriched consortia, we identified 17 main genera including *Leifsonia*, *Pantoea*, *Variovorax, Rhizobium* and *Burkholderia*. *Burkholderia* has been identified in several studies as a dominant P solubiliser isolated from different plant species including chickpea. *Burkholderia* spp. are also known to express multiple plant growth-promoting traits (Park et al., 2010; Ambrosini et al., 2012; Arruda et al., 2013; Batista et al., 2018). Besides the above-mentioned genera, P solubilising isolates in the present study were observed from the genera *Curtobacterium*, *Microbacterium*, *Cellulomonas*, *Mycolibacterium*, *Rhodococcus*, *Inquilinus*, *Phyllobacterium, Dyella* and *Mucilaginibacter*. In previous studies these genera identified here, excluding *Inquilinus*, *Dyella* and *Mucilaginibacter*, have been isolated from chickpea rhizosphere soils and could solubilise P (Hynes et al., 2008; Oves et al., 2013; Brígido et al., 2019). Selected isolates belonging to most of these genera can promote plant growth through multiple mechanisms including increasing available P concentration in soils and enhancing plant P uptake (Rahi et al., 2010; Khan et al.,

2013). The bacterial genera *Inquilinus*, *Dyella* and *Mucilaginibacter* have been rarely reported in association with chickpea rhizosphere soils but were recovered here as efficient PSB following enrichment. These genera have been identified as plant growth-promoting endophytic and rhizosphere bacteria in association with other plant species (Bent and Chanway, 1998; Afzal et al., 2017; Proença et al., 2017).

Our results demonstrate that PSB isolated after enrichment were more efficient in solubilising P than PSB isolated directly from rhizosphere soils. In particular, *B. cenocepacia* 127F produced 1.2 to 4.2-fold higher P concentrations in liquid culture than other strains. Most of the efficient PSB identified in the present study were recovered from enriched consortia in the presence of AlO_4P or $FeO_4P.2H_2O$ as a sole P source. The amount of Pi from all three P sources was not associated with the capacity of strains to induce acidic pH conditions in the culture solution. Although the direct correlation of medium acidification directly correlated with Pi in culture media has been reported in certain cases (Barroso et al., 2006; de Oliveira Mendes et al., 2014), in other instances, the degree of solubilisation was always proportional to the decline in pH (Mehta and Bhide, 1970; Wani and PV, 1979; Goldstein, 1986; Asea et al., 1988).

In the present study, the tested strains produced a diverse type and quantity of carboxylates during P solubilisation. The types of carboxylate production by the tested strains explained the P dissolution potential. It has been observed previously that the type of carboxylate (tri- di- or mono-carboxylate) is more important for P-solubilisation than the total amount of carboxylates produced by PSB (Cunningham and Kuiack, 1992; Scervino et al., 2010). In our experiments, the highest P dissolution was recorded by *Burkholderia* sp. 12F and *B. cenocepacia* 127F, which produced predominantly tri- and di-carboxylates. Conversely, monocarboxylates were the dominant type of carboxylate produced by *B. simplex* 37F, which performed poorly in P solubilisation. In line with our findings, others have reported that the ability to produce citric acid, a tricarboxylate, determined the potential of PSB to solubilise P from AlO_4P and FeO_4P (de Oliveira Mendes et al., 2014). Carboxylates increase P availability mainly through decreased adsorption of P and increased solubilisation of phosphates. P solubilisation by carboxylates is facilitated through acidification, anion exchange of PO_4^- by acid anion or chelation of metal ions associated with phosphate (Omar, 1997; He and Zhu, 1998; Henri et al. 2008).

The main mechanism involved in efficient P solubilisers in this study was associated with the type of carboxylates produced, particularly citrate and α-ketoglutarate. The effect of particular

carboxylates on P solubilisation can be associated with their chemical nature (Jones and Oburger, 2011), including their concentration, the position of the -COOH and -OH functional groups, the stability of the metal ion-organic acid complex, the amount and types of metal ions in solution and the pH (Pohlman and Mc Coll, 1986; Bolan et al., 1994; Kpomblekou-a and Tabatabai, 1994; Whitelaw, 1999). In particular, carboxyl and hydroxyl groups are the main chemical constituents affecting the amount of cation mobilisation by carboxylates (Sagoe et al., 1998). Carboxyl groups facilitate the chelation of the cation bound in sparingly soluble phosphates leading to the release of Pi (Kpomblekou-a and Tabatabai, 1994). Chelating metal ions that may be associated with complexed forms of P facilitate the release of adsorbed P (Bolan et al., 1994). The P desorption ability of different carboxylates decreases with reduction in stability constants of Fe- and Al-carboxylate complexes in the order: citrate > oxalate > malonate/malate > tartrate > lactate > gluconate > acetate > formate (Ryan et al., 2001). The ability of carboxylates to chelate metals is greatly affected by their molecular structure and number of carboxyl groups (Khan et al., 2009). The aforementioned carboxylates produced by efficient PSB in the presence study contain α-carboxyl and β-hydroxyl groups that are important in forming a stable organometallic six-member ring structure (Pohlman and Mc Coll, 1986). Through the formation of this structure, tri-carboxylates could increase the concentration of Pi from AlO_4P and $FeO_4P.2H_2O$ and improve the P availability to plants. Increased P uptake from rock phosphate was observed in plants supplied with di- and tri-carboxylates previously (Bolan et al., 1994).

5.7. Conclusion

This research has shown a high diversity of PSB belonging to 16 genera isolated from rhizosphere soils of chickpea through a selective enrichment process. This enrichment method of PSB isolation improved the selection of efficient P solubilising bacteria from rhizosphere soils. In particular, rhizobacteria enriched in the presence of $FeO_4P.2H_2O$ were efficient P solubilisers. Moreover, the efficiency of P solubilising bacteria was associated with their capacity to produce di-and tri-carboxylic acids. The majority of efficient PSB that were obtained from the rhizosphere of chickpea grown in soils sampled from a wide survey of Australia's agricultural regions were assigned to the genus *Burkholderia*. Newly isolated *Burkholderia* strains that were obtained from enriched consortia had 1.2- to 4.2- fold greater efficiency in P solubilisation than those of the same genus isolated from rhizosphere soils without enrichment. This study shows that enriching rhizobacteria in culture conditions with

sparingly soluble P increased the likelihood of encountering elite PSB from bulk soils and rhizospheres.

5.8. References

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Table 1. Correlation between soil chemical and physical properties and the presence and abundance of PSB in rhizospheric soil obtained from chickpea seedlings grown in greenhouse

Soil properties	Population of PSB	% of PSB
Soil pH	0.03	0.09
EC	0.40**	0.33**
Calcium (mg kg^{-1})	0.41***	0.36**
Magnesium(mg kg^{-1})	0.332**	0.38**
Potassium (mg kg^{-1})	0.49***	0.45***
Carbon (%)	0.57***	0.48***
Nitrogen (%)	0.60***	0.51***
Phosphorus (mg kg^{-1})	0.58***	0.58***
Sulfur (mg kg^{-1})	0.20	0.19
Copper (mg kg^{-1})	-0.02	0.11
Manganese (mg kg^{-1})	-0.12	-0.09
Zinc (mg kg^{-1})	0.16	0.20
Clay (%)	0.06	0.11
Sand (%)	-0.33***	-0.34**
Silt (%)	0.56***	0.52***

PSB - Phosphate solubilising bacteria; %PSB is the proportion of culturable bacteria able to solubilise $Ca_3(PO_4)_2$ in NBRIP medium relative to total culturable aerobic bacteria assessed by growth on TSA medium. Concentration of Ca, Mg and K measured after ammonium acetate extraction. Diethylenetriaminepentaacetic acid (DTPA) extractable concentration of Cu, Mn and Zn are reported. Phosphorus reported here is total P after perchloric acid digestion followed by extraction of total elemental P.

Table 2. Types and amounts (µM) of organic acids produced by selected PSB grown in NBYM liquid medium supplemented with $Ca_3(PO_4)_2$, AlO_4P or $FeO_4P.2H_2O$ after seven days of incubation at 28 ^oC

Bacterial Strains	Monocarboxylate				Dicarboxylate				Tricarboxylate				Total organic acid
	Acetate	Lactate	Shikimate	Pyruvate	Malate	Succinate	Fumarate	Maleate	cis-aconitate	α-ketoglutarate	Citrate	Trans-aconitate	
$FeO_4P.2H_2O$													
B. megaterium	443.9	345.0	0.31	n.d.	n.d.	39.3	59.48	55.12	0.07	n.d.	n.d.	n.d.	943.1
B. pumilus	n.d.	21.0	n.d.	n.d.	n.d.	n.d.	0.11	n.d.	n.d.	n.d.	n.d.	n.d.	21.1
B. simplex	321.0	234.0	n.d.	n.d.	321.0	n.d.	2.70	n.d.	3.56	n.d.	n.d.	n.d.	557.7
B. cepacia	n.d.	n.d.	n.d.	9.0	n.d.	n.d.	0.17	n.d.	n.d.	n.d.	n.d.	n.d.	9.2
P. fluorescens	n.d.	19.7	n.d.	592.6	n.d.	n.d.	0.08	n.d.	n.d.	n.d.	n.d.	n.d.	612.4
Burkholderia sp.	n.d.	34.0	n.d.	n.d.	n.d.	n.d.	0.02	n.d.	2.37	310.7	5.7	n.d.	352.8
B. cenocepacia	234.0	n.d.	n.d.	287.0	24.2	n.d.	5.14	n.d.	n.d.	301.8	17.6	2.1	871.7
AlO_4P													
B. megaterium	478.0	378.0	0.46	n.d.	n.d.	56.0	61.00	0.05	0.14	n.d.	n.d.	n.d.	973.7
B. pumilus	n.d.	27.9	n.d.	n.d.	n.d.	n.d.	0.13	n.d.	0.12	n.d.	n.d.	n.d.	28.2
B. simplex	345.0	363.1	n.d.	n.d.	469.2	n.d.	3.50	n.d.	n.d.	n.d.	n.d.	n.d.	1180.8
B. cepacia	n.d.	n.d.	n.d.	18.8	n.d.	n.d.	0.33	n.d.	0.27	n.d.	n.d.	n.d.	19.4
P. fluorescens	n.d.	56.9	n.d.	244.2	0.5	n.d.	0.06	n.d.	0.09	n.d.	n.d.	n.d.	301.7
Burkholderia sp.	n.d.	40.6	n.d.	n.d.	n.d.	n.d.	0.03	n.d.	n.d.	321.7	24.7	1.7	388.6
B. cenocepacia	301.3	n.d.	n.d.	341.0	456.0	176.8	65.00	n.d.	1.16	362.1	43.0	4.7	1750.9
$Ca_3(PO_4)_2$													
B. megaterium	564.0	410.0	0.59	n.d.	n.d.	72.0	76.00	0.05	0.09	n.d.	n.d.	n.d.	1122.7
B. pumilus	284.6	148.3	n.d.	n.d.	n.d.	n.d.	0.09	0.03	0.20	n.d.	n.d.	n.d.	433.2
B. simplex	613.6	1223.0	n.d.	n.d.	546.0	n.d.	0.13	n.d.	n.d.	n.d.	n.d.	n.d.	2382.7
B. cepacia	n.d.	n.d.	n.d.	28.0	n.d.	n.d.	0.17	n.d.	0.33	n.d.	n.d.	n.d.	28.5
P. fluorescens	n.d.	67.0	0.46	306.4	363.1	n.d.	0.07	n.d.	0.14	n.d.	n.d.	n.d.	737.1
Burkholderia sp.	202.9	57.0	n.d.	n.d.	55.1	39.3	0.06	n.d.	4.56	364.0	n.d.	11.0	733.9
B. cenocepacia	431.0	n.d.	n.d.	1186.0	852.9	486.9	74.27	n.d.	9.06	2092.1	152.5	29.0	5313.7

Key: n.d.: Not detected. *Media inoculated with B. megaterium* 8F*, 2: B. pumilus* 98F, *3: B. simplex* 37F*, 4: B. cepacia* 126F, *5: P. fluorescens* 27F*, 6: Burkholderia* sp. 12F*,* and *7: B. cenocepacia* 127F

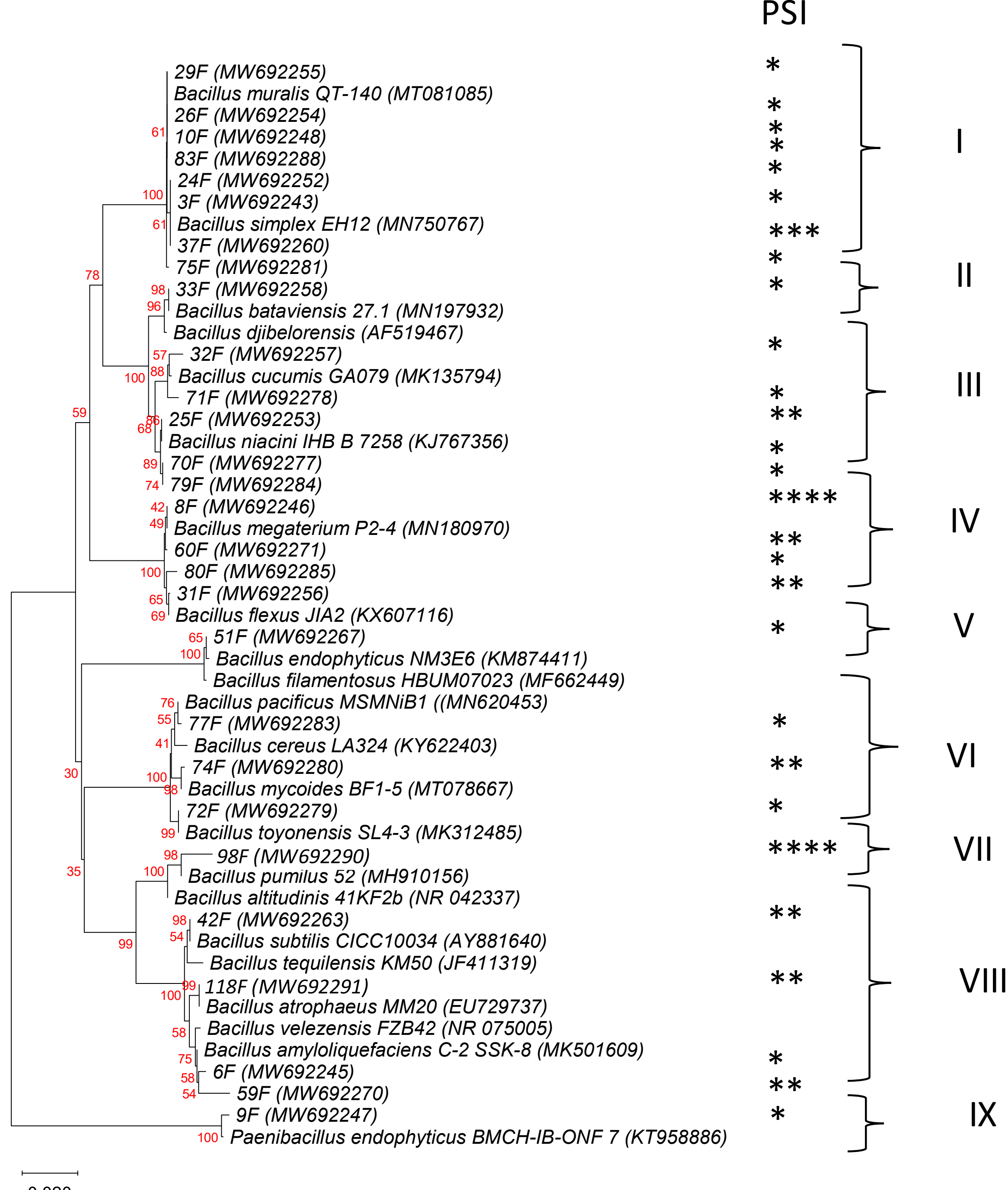

Fig. 1 Neighbour–joining phylogenetic tree based on the 16S rRNA gene sequences of culturable phosphate solubilising *Bacillus* spp. isolated from chickpea rhizospheric soils. Genetic distances were constructed using Kimura's 3-parameter model. PSI: phosphate solubilisation index, *- PSI value <2, **- 2-3, ***- >3-4, ****- >4.0

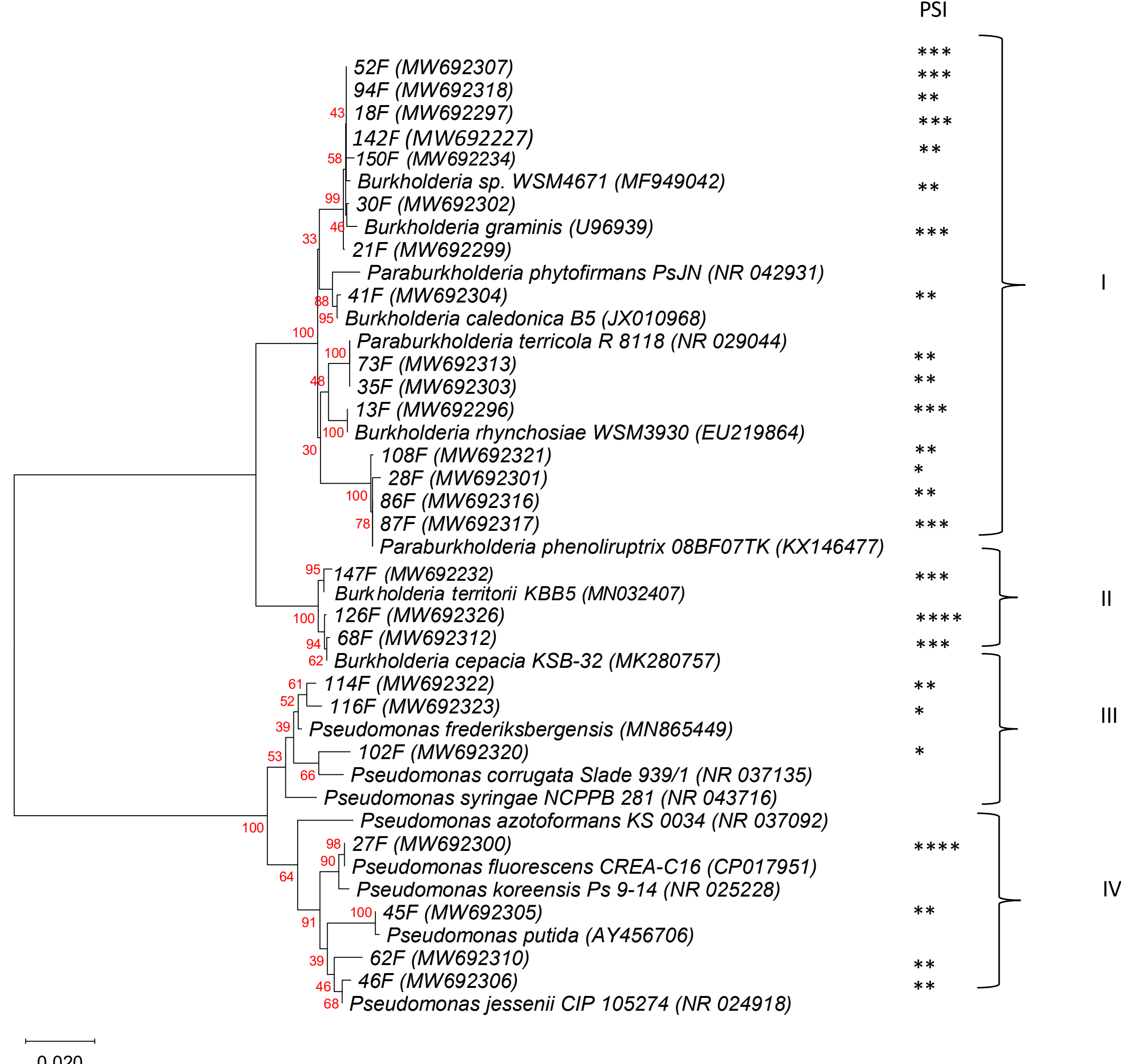

Fig. 2 Neighbour–joining phylogenetic tree based on a comparison of the 16S rRNA gene sequences of phosphate solubilising *Pseudomonas*- and *Burkholderia*-like bacteria isolated from chickpea rhizospheric soils using a semi-selective medium. Genetic distances were constructed using Kimura's 3-parameter model. PSI: phosphate solubilisation index, *- PSI value <2, **- 2-3, ***- >3-4, ****- >4.0

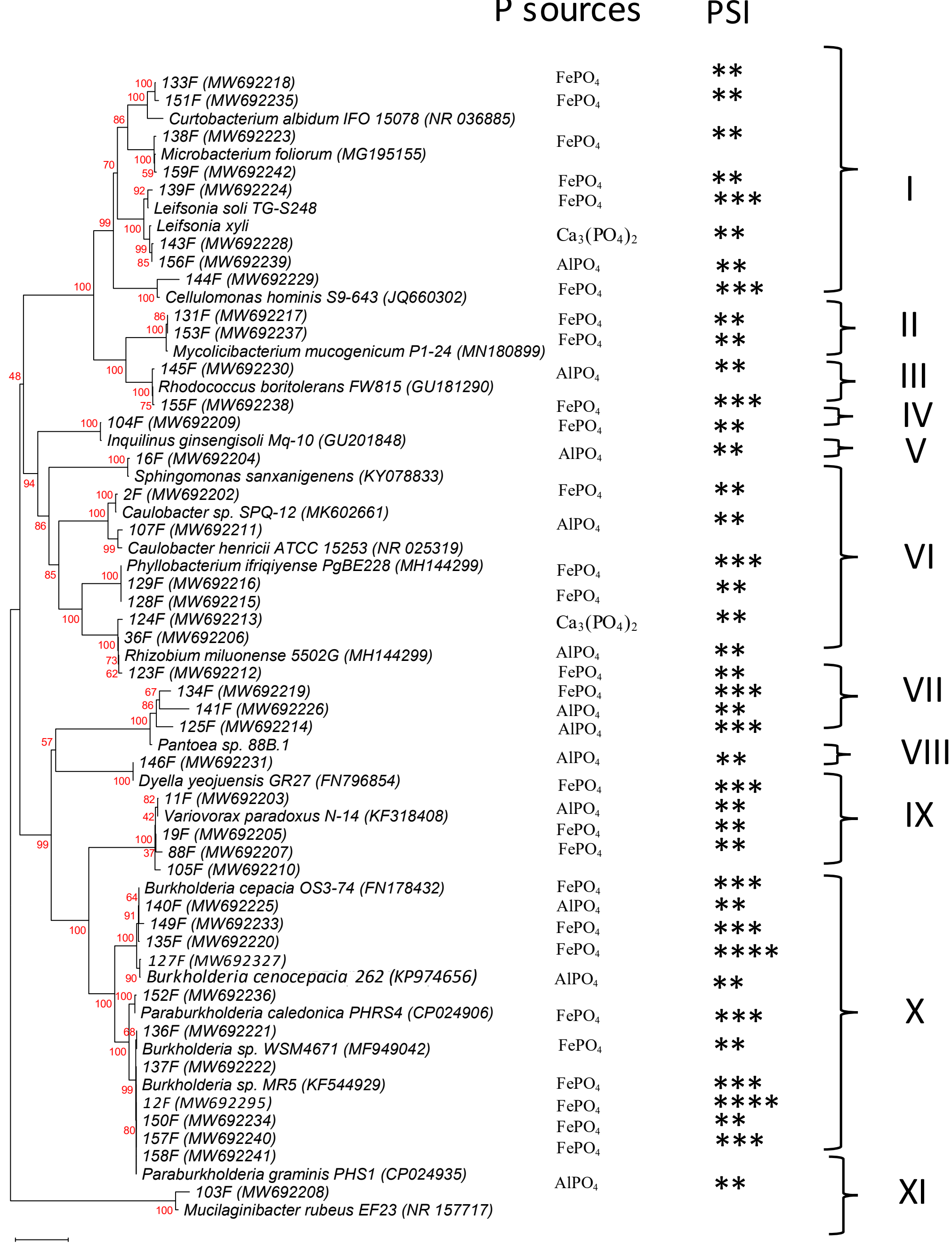

Fig. 3 Phylogenetic tree of 16S rRNA gene sequences showing the relationships among bacterial strains isolated after repeated culture for the enrichment of PSB from rhizospheric soils in different P sources mentioned in the figure. The data of type strains of related species were obtained from the GenBank database. Genetic distances were constructed using Kimura's 3-parameter model. PSI: phosphate solubilisation index, *- PSI value <2, **- 2-3, ***- >3-4, ****- >4.0

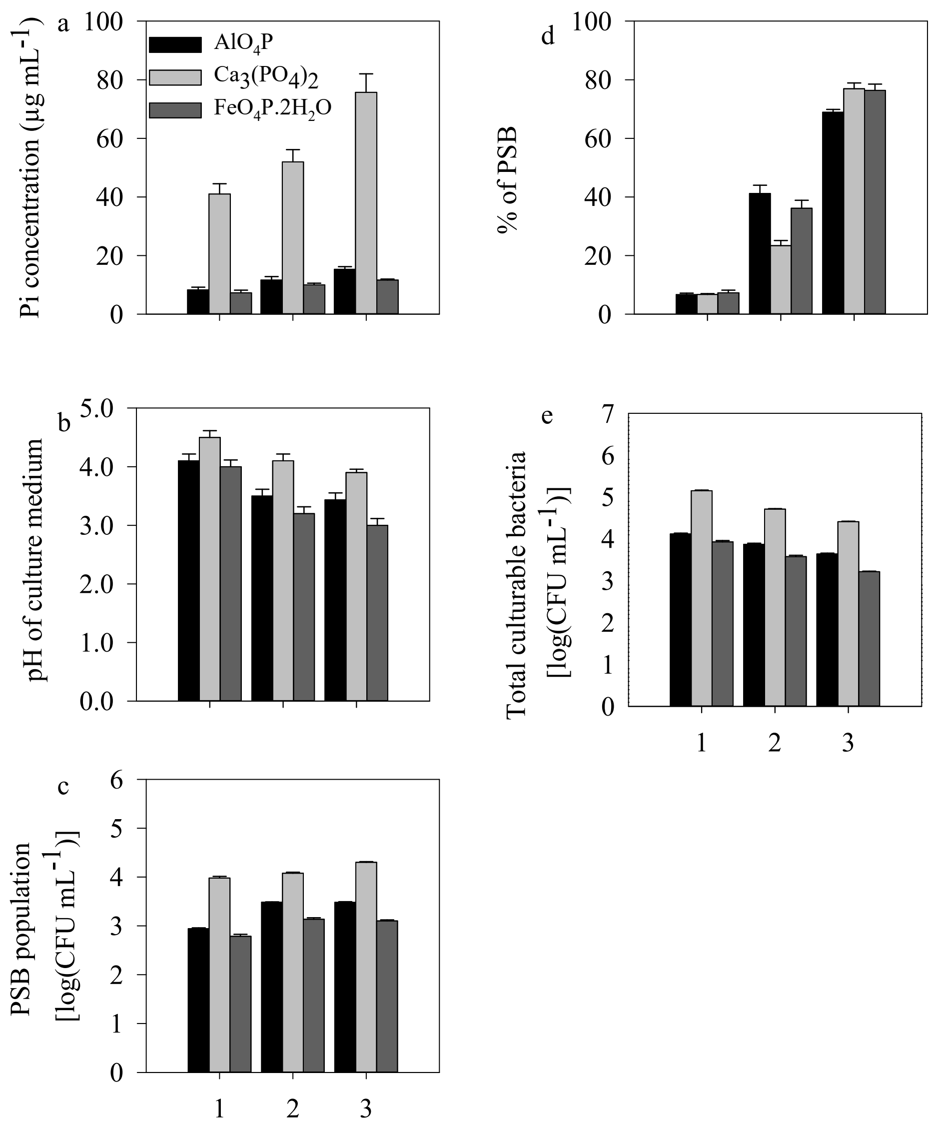

Fig. 4 Enrichment for PSB on three different P sources. Mean of three replicates ±SE of (a) Soluble P, (b) pH of enriched culture medium, (c) the population of PSB, (d) % of PSB and (e) the population of total culturable bacteria in each of three rounds of enrichment of rhizospheric soils in NBYM liquid media that contained $Ca_3(PO_4)_2$, AlO_4P or $FeO_4P.2H_2O$. Each enrichment round took seven days of incubation at 28 ºC and agitated at 160 rpm. Sub-samples were taken at the end of each enrichment stage. The population of PSB was screened in NBRIP medium containing $Ca_3(PO_4)_2$.

Key: % of PSB = cell density of total phosphate solubilising bacteria $\log_{10}$(CFU mL^{-1}) X 100 / cell density of total of culturable bacteria $\log_{10}$(CFU mL^{-1})

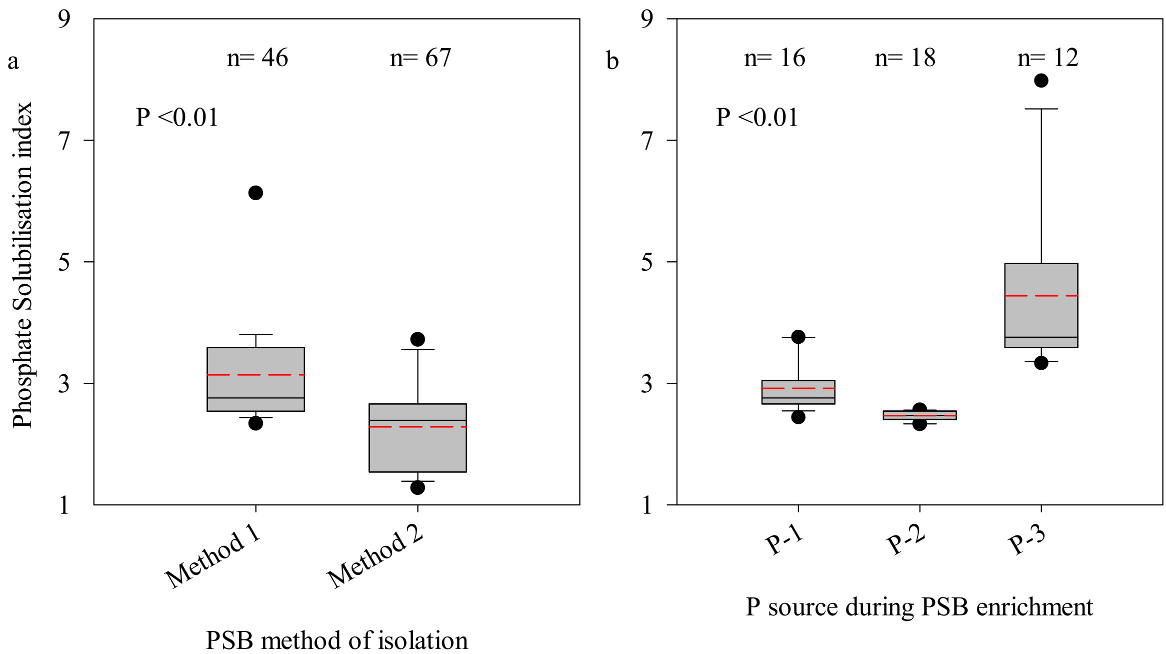

Fig. 5 Average phosphate solubilisation index (PSI) of PSB obtained using method-1: Enrichment method and method-2: Taxonomic method and the PSI of P solubilisers supplying three sparingly soluble P: P-1: AlO_4P, P-2: $Ca_3(PO_4)_2$ and P-3: $FeO_4P.2H_2O$. The black line within the box indicates the median. The red broken line in the box shows the mean value. The lines outside the box boundaries indicate the upper and lower quartiles. Dots outside the box indicate $5^{th}/95^{th}$ percentiles.

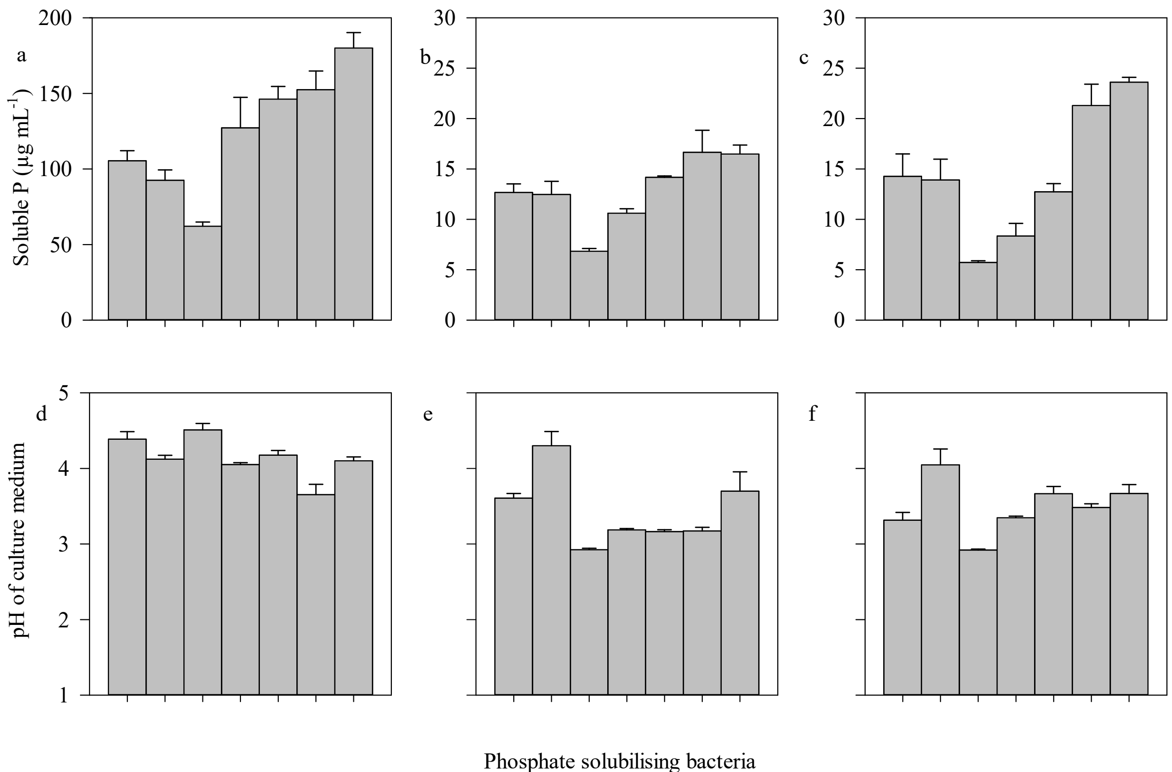

Fig. 6 Mean of three replicates ±SE of dissolved P concentration and pH in liquid NBRIP medium supplemented with three P sources: $Ca_3(PO_4)_2$ (a and d), AlO_4P (b and e) and $FeO_4P.2H_2O$ (c and f), respectively, after 14 days of incubation in shaker incubator (28 ºC, 160 rpm). Bar indicates the mean values of three replications. Error bar indicates the standard error. PSB included 1: *B. megaterium* 8F, 2: *B. pumilus* 98F, 3: *B. simplex* 37F, 4: *B. cepacia* 126F, 5: *P. fluorescens* 27F, 6: *Burkholderia* sp. 12F and 7: *B. cenocepacia* 127F

Chapter 6 Is phosphate solubilising ability in plant growth-promoting rhizobacteria isolated from chickpea linked to their ability to produce ACC deaminase?

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6.1. Statement of Authorship

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Contribution to the Paper	Planned the study conducted all experiments, analysed and interpreted data and wrote the manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	26/03/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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6.2. Abstract

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ORIGINAL ARTICLE

Is phosphate solubilizing ability in plant growth-promoting rhizobacteria isolated from chickpea linked to their ability to produce ACC deaminase?

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Abstract

Aims: Since most phosphate solubilizing bacteria (PSB) also produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, we investigated if there was an association between these two plant growth-promoting properties under *in vitro* conditions.

Methods and Results: A total of 841 bacterial isolates were obtained using selective and enrichment isolation methods. ACC deaminase was investigated using *in vitro* methods and by sequencing the *acdS* gene. The effect of ACC deaminase on P solubilization was investigated further using five efficient PSB. ACC deaminase production ability was found amongst a wide range of bacteria belonging to the genera *Bacillus*, *Burkholderia*, *Pseudomonas* and *Variovorax*. The amount of ACC deaminase produced by PSB was significantly associated with the liberation of Pi from Ca-P when ACC was the sole N source. Ca-P solubilization was associated with the degree of acidification of the medium. Additionally, the P solubilization potential of PSB with $(NH_4)_2SO_4$ was determined by the type of carboxylates produced. An *in-planta* experiment was conducted using *Burkholderia* sp. 12F on chickpea cv. Genesis-863 in sand : vermiculite (1 : 1 v/v) amended with rock phosphate and inoculation of this efficient PSB significantly increased growth, nodulation and P uptake of chickpea fertilized with rock phosphate.

6.3. Introduction

Soil microorganisms can increase the bioavailability of P through the mineralization of organic P and the solubilisation of bound inorganic P (Richardson *et al*. 2009). Microorganisms that solubilise P are referred to as phosphate solubilising bacteria (PSB) (Bashan *et al*. 2013). These bacteria facilitate P solubilisation through secretion of low molecular weight carboxylates and protons (H^+) that ultimately result in localised acidification (Khan *et al*. 2013). PSB that enhance plant growth through P solubilisation are referred to as plant growth-promoting rhizobacteria (PGPR) (Swift *et al*. 2018; Alemneh *et al*. 2020). In some instances, PGPR unable to solubilise P nevertheless can increase P concentration in various plant species (Barnawal *et al*. 2012; 2014; Chandra *et al*. 2019), through expression of 1-aminocyclopropane-1-carboxylate (ACC) deaminase.

ACC deaminase is the principal mechanism of plant growth-promotion expressed by most beneficial bacteria belonging to several genera (Marques *et al*. 2010; Etesami *et al*. 2014; Qin *et al*. 2014; Gontia-Mishra *et al*. 2017; Misra *et al*. 2017; Stromberger *et al*. 2017). The dominant ACC deaminase-producing bacterial genera are: *Achromobacter*, *Agrobacterium, Azospirillum, Bacillus, Bradyrhizobium*, *Chryseobacterium, Enterobacter, Herbaspirillum, Mesorhizobium, Phyllobacterium, Pseudomonas, Ralstonia*, *Serratia, Sinorhizobium, Sphingobacterium* and *Variovorax,* although ACC deaminase activities vary under in vitro conditions (Shah *et al*. 1998; Ma *et al*. 2003b; Hontzeas *et al*. 2005; Blaha *et al*. 2006; Caballero-Mellado *et al*. 2007; Duan *et al*. 2009; Onofre-Lemus *et al*. 2009; Nikolic *et al*. 2011; Nascimento *et al*. 2014; Li *et al*. 2015; Bouffaud *et al*. 2018). ACC deaminase as low as 0.02 µmol is sufficient to enable a bacterium to promote plant growth. Plant growth-promotion by expression of ACC deaminase activity is facilitated through the reduction of a lethal concentration of ethylene that is often produced when plants are exposed to stresses (Glick 2014). ACC deaminase-producing rhizobacteria can also enhance nodulation and plant growth under sub-lethal production of ethylene in plants (Ma *et al*. 2003a; Shaharoona *et al*. 2006; Zahir *et al*. 2011; Barnawal *et al*. 2012; 2014). In these studies, inoculation of PGPR also improved plant P concentration. An increased P content was observed through stimulation of root growth and/or enhanced P concentration in the rhizosphere through P solubilisation (Safronova *et al*. 2006; Shahzad *et al*. 2013).

ACC deaminase catalyses the conversion of ACC to α-ketobutyrate and ammonia (Glick *et al*. 1998). α-ketobutyrate is occasionally produced by rhizobacteria during mineral P

solubilisation, (Gaur 1990; Vazquez *et al*. 2000; Pal *et al*. 2001). In these reports, rhizosphere microorganisms that produce α-ketobutyrate during P solubilisation include strains of *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus atrophaeus*, *Paenibacillus macerans*, *Pseudomonas striata, Pseudomonas fluorescens* and *Xanthobacter agilis*. Ammonia is another product of ACC utilisation and it is present as ammonium in soils. Ammonium can be a source of H^+ via microbial assimilation (Illmer and Schinner 1995). Therefore, the assimilation of ACC via the action of ACC deaminase might affect the P solubilising activity of microorganisms.

ACC deaminase-producing bacteria are typically isolated on DF-minimal media containing ACC as the sole N source (Penrose and Glick 2003). However, growth on this medium is not confirmative of ACC deaminase activity (Ma et al. 2003b; Blaha et al. 2006; McDonnell et al. 2009; Li et al. 2011; Nascimento et al. 2012; Nascimento et al. 2014) and mis-identification of ACC deaminase activity causes misunderstanding of microbial function. Detection of the *acdS* gene is important for the identification of ACC deaminase-producing bacteria and for predicting ACC deaminase activity. The use of consensus-degenerate hybrid oligonucleotide primers (CODEHOP) that can differentiate authentic *acdS* genes from their homologs can be used to amplify these genes from a wide range of bacteria (Li *et al*. 2015).

In our experiments, most ACC deaminase producing bacteria were able to release P from relatively insoluble Ca-P. Taking into consideration the role of NH_4^+ and α-ketobutyrate in P solubilisation and observations from our preliminary experiments, we hypothesised that the capability to produce ACC deaminase would improve the mineral P solubilisation potential of PSB when ACC is the sole N source. The objectives of this study were to screen and identify bacteria containing ACC deaminase using *in vitro* methods and to sequence *acdS* genes to examine whether ACC deaminase influenced the P solubilisation potential of PSB.

6.4. Materials and Methods

6.4.1. Soil sampling and analysis

Soil samples were collected from 74 sampling sites in major agricultural cropping lands across Australia (Appwndix Fig. 1). The collection sites have a diverse range of rainfall and evapotranspiration. The soil physico-chemical properties of the sampling sites are also very diverse (Appendix Table 1). Soil pH (H_2O) varied between 5.82 and 8.2 and EC from 40 to 271 µS cm^{-1}. The Ca^{2+}, K^+ and Mg^{2+} concentrations in the soils varied between 0.276 to 100 g

kg^{-1}, 308 to 7,500 mg kg^{-1} and 0.173 to 18.8 g kg^{-1}, respectively. The concentration of Cu, Mn and Zn in soils varied from 2 to 29 mg kg^{-1}, 21 to 1,600 mg kg^{-1}, and 3.3 to 61.0 mg kg^{-1}, respectively. The soil total carbon, N and P contents ranged from 0.23 to 8.85%, 0.009 to 0.208% and 88 to 596 ppm, respectively. The sand textural component in soils varied between 18.8 and 86.3% across sampling sites.

One hundred g of soil from each sample was taken and placed in small pots sown to chickpea cv. HatTrick which was grown in a growth chamber with lighting intensity of 1000 µmol m^{-2} s^{-1} with a 16 h photoperiod and a constant temperature at 20 ºC, with relative humidity between 60% and 70%. The soil strongly adhering to roots was considered to be 'rhizosphere soil' and was collected from chickpea seedlings four weeks after germination and kept at 4 ºC until further work.

6.4.2. Isolation and identification of PSB

One gram of rhizosphere soil was added to 9 mL of sterile MilliQ water and diluted with tenfold serial dilution up to 10^{-6}. Aliquots of 100 µL from 10^{-5} and 10^{-6} were taken and spread on semi-selective 1/10 strength tryptone soya agar (TSA) (Oxoid LTD, Basingstoke, Hampshire, England) supplemented with 50 µg mL^{-1} of ampicillin (99%, Sigma-Aldrich, Chemie GmbH, Steinheim, Germany), 12.5 µg mL^{-1} of chloramphenicol (Sigma-Aldrich, 99%) and 75 µg mL^{-1} of cycloheximide (Sigma-Aldrich) for isolation of *Pseudomonas*-like bacteria (Simon and Ridge 1974). The remaining aliquots were incubated in a water bath for 10 min at 80 ºC to selectively enrich aerobic endospore inducing bacteria such as *Bacillus*. A 100 µL of aliquot was spread on 1/10 strength TSA media and plates were incubated at 28 ºC for 5 days. Thereafter, bacterial colonies were transferred to fresh TSA media incubated in the same conditions. Well-developed colonies were selected based on their colony morphologies and further purified in TSA media. Purified isolates were further evaluated for P solubilisation potential, based on the National Botanical Research Institute's phosphate (NBRIP) growth media (Nautiyal 1999) containing the following ingredients (L^{-1}): glucose, 10 g; $MgCl_2.6H_2O$, 5 g; $MgSO_4.H_2O$, 0.25 g; KCl, 0.2 g, and $(NH_4)_2SO_4$, 0.1 g supplemented with 5 g of $Ca_3(PO_4)_2$ (Ca-P, >96%, Sigma-Aldrich). Besides this protocol, novel PSB were isolated using an enrichment method in NBRIP media containing 5 g of insoluble P sources, including AlO_4P (Al-P, Sigma-Aldrich), Ca-P or $FeO_4P.2H_2O$ (Fe-P, Sigma-Aldrich) supplemented with 75µg mL^{-1} of cycloheximide (Sigma-Aldrich), to inhibit fungal growth. Novel PSB were isolated using NBRIP agar media. Plates were incubated at 28 ºC for 10 days. Distinct colonies

surrounded by solubilisation zones were selected and re-streaked on fresh NBRIP agar media for further purification. Purified colonies were selected based on the phosphate solubilisation index, calculated using the formula described in (Nguyen *et al*. 1992b). Prior to preserving these bacterial strains, the ability to grow at 37 ºC were checked to exclude isolates that were potential human pathogens. Culture incapable of growing at this temperature were maintained in 10 % glycerol at -80 ºC and also on slant culture at 4 ºC. The screening procedure for PSB capable of expressing ACC deaminase is indicated in Appendix Fig. 2.

6.4.3. Quantification of plant growth-promoting traits

A total of 841 rhizobacteria were obtained using the aforementioned two isolation methods. The P solubilisation and ACC deaminase production ability of isolates were assayed. Bacterial isolates able to solubilise inorganic phosphate were further investigated for ACC deaminase production. Qualitatively, ACC deaminase activity was screened in a plate assay on DF-minimal medium containing 3 m mmol l^{-1} of ACC (TCE, Tokyo Chemical Industry Co. LTD, Toshima, Kita-Ku, Tokyo) instead of $NH_4(SO_4)_2$ (Dworkin and Foster 1958). Isolates able to grow in this medium were further characterized for their quantitative production of ACC deaminase (Penrose and Glick 2003). IAA production in the presence and absence of its precursor tryptophan was also investigated in DF-minimal liquid medium (Patten and Glick 2002). ACC deaminase-positive bacteria were further screened for their ability to grow on Jensen's N-free medium by streak inoculation of individual cultures and incubation at 28 ºC for 5 days. All tests were replicated three times to check the repeatability of the result.

6.4.4. *acdS* and 16S rDNA sequencing and phylogenetic analysis

The genetic diversity of 48 bacterial isolates able to solubilise P and express ACC deaminase activity was investigated by sequencing the ACC deaminase structural gene (*acdS*). The CODEHOP such as acdSf3 (ATCGGCGGCATCCAGWSNAAYCANAC) and acdSr3 (GTGCATCGACTTGCCCTCRTANACNGGRT) were used to amplify partial sequences of *acdS* genes with an expected amplicon size of ~760 bp (Li *et al*. 2015). *Rhizobium rhizogenes* (formerly *Agrobacterium radiobacter*) K84, a known biocontrol agent containing the *acdS* gene, was used as a positive control. The PCR cycle was as follows: an initial denaturation at 95 ºC for 15 s, followed by 35 cycles of 95 ºC for 30 s, annealing at 53 ºC for 1 min and extension at 72 ºC for 1 min and a final extension at 72 ºC for 5 min.

Additionally, the universal forward primer fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer rP2 (5′-ACGGCTACCTTGTTACGACTT-3′) were used to amplify 16S rDNA with an approximate size of 1500 bp (Weisburg *et al*. 1991). PCR assays were performed using 25 µL reaction mixture with 2*x* concentrated *Taq* polymerase under the following conditions: initial denaturation at 94 ºC for 5 min, 35 amplification cycles of denaturation at 95 ºC for 15 s, annealing at 57 ºC for 30 s, extension at 72 ºC for 1 min and a final extension at 72 ºC for 5 min.

Amplification of the target gene was confirmed by electrophoresis of PCR products on a 1 % agarose gel. The PCR products were sequenced by the Australian Genome Research Facility, Sanger Service (University of Adelaide, South Australia). Phylogenetic analysis of the 16S rDNA sequences was carried out. PCR products were separated by electrophoresis and visualised on a 1% agarose gel. Related sequences were obtained from the GenBank database, National Center for Biotechnology Information (NCBI) (http//www.ncbi.nim.nih.gov/BLAST). The tree topology was inferred by the neighbour-joining method using the latest version of Molecular Evolutionary Genetics Analysis (MEGA-7) (Institute of Molecular Evolutionary Genetics, Pennsylvania State University, University Park, PA 16802, USA) (Kumar *et al*. 2016b). A distance matrix in the phylogeny was generated following Kimura's 3 parameter distance model.

6.4.5. P solubilisation efficiency

Forty-eight PSB able to grow on DF-minimal medium containing ACC as sole N source were used. A pre-screening of their P solubilisation capacity from Ca-P was assessed on NBRIP agar media (Nautiyal 1999) using one of two N sources, ACC or $(NH_4)_2SO_4$. Streaked plates were incubated for 14 days at 28 ºC. The colony diameter and halo clearing zone were measured every two days. The phosphate solubilisation index (PSI) was calculated following the formula described in Nguyen *et al*. (1992a).

The effect of ACC deaminase on P dissolution from Al-P, Ca-P or Fe-P was further investigated in the presence of ACC as sole N source. Five bacterial isolates, namely *Bacillus megaterium* 8F, *Bacillus pumilus* 98F, 3: *Burkholderia cepacia* 126F, 4: *Burkholderia* sp. 12F and 5: *Burkholderia cenocepacia* 127F were efficient in P solubilisation from diverse P sources (Chapter 4) and were used in this study. These PSB were efficient P solubilisers in the presence of $(NH_4)_2SO_4$ and able to produce a range of concentrations of ACC deaminase. The NBRIP medium was prepared, the pH was adjusted to 7.00 and ACC equivalent to the amount of N in

0.1 g of $(NH_4)_2SO_4$ was filter sterilised and mixed with the sterile medium. Fifty ml of medium was transferred into a glass bottle (200 mL capacity) and autoclaved (20 min, 121 ºC). A pure colony of each isolate was inoculated into DF-minimal medium and the pre-culture was incubated for 48 h at 28 ºC. Then, 100 µL of liquid culture was inoculated into bottles containing the sterile NBRIP media and cultures were incubated on a shaker (28 ºC, 160 rpm) for 21 days. All treatments were replicated three times. Every two days, 5 mL of culture solution were drawn from each bottle. The amount of soluble P (Pi) and the pH of culture media were determined.

6.4.6. Comparative effectiveness of different carboxylates in solubilisation of P

Alpha-ketobutyrate together with the dominant carboxylates (acetate, citrate, gluconate, malate, maleate, α-ketoglutarate, oxalate, and succinate) produced by efficient P solubilisers were investigated for their relative efficiency in P solubilisation from sparingly soluble P sources *in vitro*. Inorganic acids (HCl and H_2SO_4) and H_2O were included as controls. The assay was performed in 150 mL bottles containing 50 mL of 10 mmol L^{-1} carboxylate solution, which is the approximate total carboxylate concentration produced in liquid culture by an efficient PSB (Yi *et al.* 2008). Five g of each insoluble mineral phosphate was added to the carboxylate solution. The solution was sterilised (20 min, 121 ºC) by autoclaving. To avoid the thermal degradation of carboxylate during sterilisation, a solution of each carboxylate or inorganic acid was prepared and sterilised by filtration using a 0.22 µm microfilter. All bottles were kept in a shaker incubator at room temperature and agitated at 160 rpm for three days. Quantitative estimations of Pi and pH were made.

6.4.7. Determination of available P

The Pi concentration in sample solutions was determined following the method described by Murphy and Riley (1962). The mixed reagent comprised the following: 5 N sulphuric acid, 20 g ammonium molybdate dissolved 100 mL water, 1.32 g of ascorbic acid dissolved in 75 mL of water and 0.2743 g of potassium antimonyl tartrate in 100 mL water. Samples were centrifuged for 10 min at 10,000 ×*g*. A 2 mL sample of the supernatant and 0.4 mL of the mixed reagent was placed in a 10 mL test tube. The mixture was kept for 30 min and absorbance at 882 nm was determined using a spectrophotometer. The absorbance values were converted into Pi concentration using a standard calibration curve.

6.4.10. Data analysis

All experiments were conducted using a completely randomized design and all treatments were replicated three times. Data were analysed by ANOVA using GENSTAT 18th edition statistical software. Differences between treatments were compared with LSD at $P < 0.05$. Normal distribution of the data was determined using Shapiro-Wilk's test. Bartlett's test was used to determine the homogeneity of variances.

6.5 Results

6.5.1. Identification of ACC deaminase producing bacteria

A total of 841 isolates of rhizobacteria was obtained from 74 chickpea rhizosphere soils using taxonomically selective and enrichment isolation protocols. Of these isolates, 743 *Bacillus*- and *Pseudomonas*-like bacteria were obtained using taxonomically selective methods of isolation. Only 72 (9.7%) isolates were able to solubilise Ca-P in a plate assay. Besides this, 98 efficient P solubilising bacteria closely related to *Burkholderia*, *Caulobacter, Cellulomonas*, *Dyella, Inquilinus*, *Leifsonia, Microbacterium*, *Mycolicibacterium*, *Mucilaginibacter, Pantoea*, *Phyllobacterium*, *Rhodococcus*, *Rhizobium, Sphingomonas* and *Variovorax* were enriched from the rhizosphere soils (Chapter 4). Of the 170 isolates of PSB, only 48 belonging to the genera *Bacillus*, *Burkholderia*, *Pseudomonas* and *Variovorax* were found to grow in DF-minimal medium containing ACC as sole N source (Fig. 1). Of the 48 isolates, 44 were found to produce ACC deaminase in the liquid bioassay.

The ability of PSB to produce ACC deaminase was further confirmed by detection of *acdS* gene sequences. Of the 48 isolates able to utilise ACC, the *acdS* gene was amplified from 25 isolates. The *acdS* gene was not detected in 23 isolates. Of those, nineteen isolates were closely related to *Bacillus* spp. However, these isolates expressed ACC deaminase activity. The remaining four isolates belonged to *Burkholderia* and *Pseudomonas.* These isolates were able to grow on the DF-minimal medium in the presence and absence of ACC as N source but did not display ACC deaminase activity (Table 1).

6.5.2. P solubilisation efficacy

Out of the forty-eight PSB isolates which grew on DF-minimal medium with ACC as sole N source, thirty-two were able to solubilise Ca-P when the N source in NBRIP medium was $(NH_4)_2SO_4$ (Table 1). In general, PSI values were lower with ACC than with $(NH_4)_2SO_4$,

excluding the PSB isolates able to express ACC deaminase at > 2.50 µmol α-ketobutyrate h^{-1}. An isolate closely related to *Bacillus subtilis* CICC10034, which was able to express ACC deaminase at 7.32 µmol α-ketobutyrate h^{-1}, exhibited comparable PSI values in media containing ACC and $(NH_4)_2SO_4$. Few isolates unable to solubilise P with $(NH_4)_2SO_4$ displayed very weak solubilisation capacity (PSI < 2) when N was supplied as ACC (Table 1). These isolates produced ACC deaminase activity greater than 4.24 µmole α-ketobutyrate h^{-1}.

In a further P solubilisation assay, a significant and positive relationship was observed between ACC deaminase activity and the ability to release Pi from Ca-P when the sole N source was ACC (Fig. 2a). This association was non-significant with Al-P or Fe-P as P source (Fig. 2b and c). In these latter cases, the potential to solubilise Pi from Al-P and Fe-P was not related to the ability of tested isolates to produce ACC deaminase.

An inverse and significant association between ACC deaminase production and pH of the culture medium was found with all P sources (Fig. 2d, e and f). The most acidic pH was recorded when the inoculated bacteria produced the highest ACC deaminase activity.

The relative P solubilisation efficiency of ACC deaminase-producing isolates in the presence of ACC, as compared to $(NH_4)_2SO_4$, was investigated in liquid media. Three out of five isolates produced lower Pi and degree of acidification with ACC than with $(NH_4)_2SO_4$ (Fig. 3a, b, c, d, e and f). The remaining two isolates, *B. pumilus* 98F and *B. cepacia* 126F, which expressed ACC deaminase at 6.80 and 8.42 µmol α-ketobutyrate h^{-1} in DF-minimal medium, were able to release significantly higher Pi from Ca-P with ACC than with $(NH_4)_2SO_4$. These isolates were also able to induce a higher degree of acidity with ACC than with $(NH_4)_2SO_4$. However, all isolates solubilised P poorly from Al-P and Fe-P with ACC as compared to $(NH_4)_2SO_4$ as N source.

P solubilising activity of the tested isolates with $(NH_4)_2SO_4$ was related to the types of carboxylates produced (Chapter 4, Table 2). The highly efficient P solubilisers, including isolates of *B. megaterium*, *Burkholderia* sp. and *B. cenocepacia*, produced predominantly di- and tri-carboxylates. In contrast, the isolates that were poor in P solubilisation produced predominantly mono-carboxylates.

In the presence of ACC as the sole N source, the amount of Pi released from Ca-P was inversely and significantly associated with the pH of the culture medium (Fig. 4a). However, this association was non-significant when Pi was obtained from Al-P and Fe-P (Fig. 4b and c).

6.5.3. Relative efficiency of carboxylates in P solubilisation

The relative efficiency of α-ketobutyrate in P solubilisation compared with that of carboxylates commonly produced by efficient PSB was investigated (Table 2). The addition of carboxylate decreased the mean value of Pi liberated from Ca-P in the following order: malate > succinate > oxalate > DL-maleate > citrate > α-ketoglutarate > pyruvate > α-ketobutyrate > acetate > gluconate. α-ketobutyrate solubilised significantly more Pi (43.2 µg mL^{-1}) from Ca-P than gluconate (28.5 µg mL^{-1}) and acetate (37.4 µg mL^{-1}). α-ketobutyrate was also able to release 29.7% more Pi from Al-P than acetate. With Fe-P as P source, Pi solubilised by α-ketobutyrate was 25.3% higher than for gluconate.

Tests of P solubilisation in the presence of carboxylates showed that there was no clear relationship between the degree of acidification due to carboxylates and their ability to release Pi (Table 2 and 3). In this regard, α-ketobutyrate, which produced the most acidic pH, released the lowest Pi as compared with the pH obtained in solutions of DL-maleate, α-ketoglutarate, malate, pyruvate and succinate.

The efficacy of carboxylates in P solubilisation varied with P source. Malate was the most efficient carboxylate in the dissolution of P from Al-P and Ca-P. Conversely, the highest Pi concentration (47.3 µg L^{-1}) from Fe-P was obtained in succinate. Malate and succinate resulted in higher P solubilisation than HCl.

6.6. Discussion

In this study, potential PSB were isolated from wide array of agricultural lands across Australia. Using P solubilisation and ACC deaminase bioassays, we identified that ACC deaminase activity of PSB was associated with their ability to solubilise P from Ca-P when ACC was the sole N source in the growth medium. Previous studies have demonstrated the role of other plant growth promoting (PGP) traits such as siderophore and exopolysaccharide production in mineral P solubilisation through their ability to form complexes with metals that can precipitate soluble P (Altomare *et al*. 1999; Yi *et al*. 2008). Our findings suggest the need to consider other PGP characteristics including the ability of rhizobacteria to produce ACC deaminase.

6.6.1. Identification of ACC deaminase producing bacteria

We used *in vitro* methods and sequencing of the *acdS* gene to identify ACC deaminase-positive PSB belonging to *Burkholderia*, *Caulobacter, Cellulomonas*, *Dyella, Inquilinus*, *Leifsonia,*

Microbacterium, *Mucilaginibacter,* *Mycolicibacterium*, *Pantoea*, *Phyllobacterium*, *Rhizobium, Rhodococcus*, *Sphingomonas*, and *Variovorax*. The *acdS* gene sequences were amplified using primers designed to detect homologous genes of *Agrobacterium*, *Azospirillium*, *Burkholderia*, *Mesorhizobium*, *Phyllobacterium*, *Pseudomonas*, *Rhizobium*, *Ralstonia*, *Sinorhizobium*, *Variovorax*, *Penicillium* and *Trichoderma* (Li *et al*. 2015). In the present study, some isolates that were closely related to *Bacillus*, *Burkholderia*, *Pseudomonas* and *Variovorax* spp. were able to grow on DF-minimal medium containing ACC as the sole N source. Most isolates belonging to these genera were able to express ACC deaminase activity. We also amplified the *acdS* gene sequences from ACC deaminase expressing bacteria. Bacterial strains belonging to *Bacillus*, *Burkholderia*, *Pseudomonas* and *Variovorax* spp. have been previously characterised as known PGPR able to express ACC deaminase activity and solubilise P from mineral phosphates isolated from the chickpea rhizosphere (Kumar *et al*. 2016a; Shahid and Khan 2018; Pandey *et al*. 2019). However, the *acdS* gene was not amplified from isolates closely related to *Burkholderia* sp., *Paraburkholderia caledonica* and *Pseudomonas putida* that were able to grow on ACC-containing media. Besides the aforementioned isolates, we were unable to amplify the *acdS* gene from *Bacillus* spp. that were capable of expressing ACC deaminase activity. Previously, *acdS* genes have not been amplified from ACC deaminase-expressing *Bacillus* and *Paenibacillus* spp. isolated from soil and plant roots using different degenerate primer combinations (Nascimento *et al*. 2014; Li *et al*. 2015). The discrepancy between ACC deaminase *in vitro* results and the sequencing of the *acdS* gene may be due to: (*i*) the isolates may have another putative *acdS* gene that is unrelated to the well-known *acdS* gene sequences (Glick *et al*. 2007; Nascimento *et al*. 2014), and (*ii*) the primers may not amplify the *acdS* gene from all bacterial genera (Blaha *et al*. 2006; Caballero-Mellado *et al*. 2007; Govindasamy *et al*. 2008; Onofre-Lemus *et al*. 2009).

6.6.2. P solubilisation efficacy

In the present study, most of the ACC deaminase-producing bacteria (76%) were able to solubilise P *in vitro*. ACC deaminase-producing bacteria are typically able to solubilise P efficiently from mineral phosphates (Safronova *et al*. 2006; Siddikee *et al*. 2011; Zabihi *et al*. 2011; Shahzad *et al*. 2013; Çakmakçı 2016; Gontia-Mishra *et al*. 2017). Our results further showed the potential for ACC deaminase in PSB to solubilise Ca-P when ACC was the sole N source in the growth medium.

An increased ACC deaminase produced by PSB in the present study was associated with more acidity in the media. It is well documented that acidification of media is an effective solubilisation mechanism, particularly from Ca-P sources (Illmer and Schinner 1992). ACC deaminase-expressing bacteria produce ammonia and α-ketobutyrate through cleaving ACC (Glick *et al*. 1995). Bacteria expressing greater ACC deaminase activity could produce more NH_4^+ from ACC. NH_4^+-N is an important N source for PSB to release Pi from insoluble mineral phosphate (Asea *et al*. 1988; Halder *et al*. 1992) since H^+ secretion, generated by the assimilation of NH_4^+, is one of the mechanisms for solubilising phosphate (Illmer and Schinner 1995). Therefore, greater NH_4^+ uptake in exchange for H^+ production could result in a high degree of media acidification (Jones and Oburger 2011) and lead to more mineral P solubilisation.

During Al-P and Fe-P solubilisation in the present study, ACC deaminase activity was inversely, and significantly, associated with the pH of the culture solution. However, the ability of PSB to express ACC deaminase activity was not associated with the potential to mobilise P from these sources. These results suggest that proton extrusion as a result of NH_4^+ assimilation is probably of less importance for P solubilisation from Al-P and Fe-P; atlternatively another mechanism is involved in P solubilisation.

The type of carboxylates produced by the PSB determined their potential to solubilise P from Al-P, Ca-P and Fe-P. Di- and tri-carboxylates were the main carboxylates produced by PSB capable of solubilising the highest amounts of P. Several studies have attributed the solubilisation of insoluble P to the production and release of carboxylates (Gulati *et al*. 2010; Scervino *et al*. 2010; Wei *et al*. 2018; Khan *et al*. 2013). In these studies, PSB are able to produce a wide range of low molecular weight organic acids, mainly acetic, citric, formic, gluconic, 2-keto gluconic, lactic, malic, oxalic and succinic acids. PSB have been known to produce various di- and tri-carboxylates that impact both Al-P and Fe-P solubilisation (de Oliveira Mendes *et al*. 2014; Jiang *et al*. 2020).

The carboxylate α-ketobutyrate can be produced by PSB in NBRIP medium containing glucose and $(NH_4)_2SO_4$ as the sole carbon and N sources (Gaur 1990; Vazquez *et al*. 2000; Pal *et al*. 2001). It is also produced by ACC deaminase-producing bacteria during ACC utilisation (Glick *et al*. 1995). In the present study, a solution of α-ketobutyrate alone was able to release a statistically comparable amount of Pi from mineral inorganic phosphate as compared to that produced by acetate and gluconate. However, Pi concentration in α-ketobutyrate was lower

than that obtained with DL-maleate, α-ketoglutarate, malate, oxalate or succinate. The potential of carboxylates to solubilise P is typically dependent on their affinities for chelation of different metal ions released from mineral phosphates and the contact time with the organo-mineral complex (Kpomblekou-a and Tabatabai 2003). Complex formation depends on the types and numbers of functional groups, such as carboxyl and hydroxyl, present in the carboxylates (Pohlman and Mc Coll 1986; Kpomblekou-a and Tabatabai 1994; Sagoe *et al.* 1998). α-carboxyl groups in di- and tri-carboxylates are important in the formation of stable organo-mineral complexes with Fe and Al. These groups can be responsible for the high efficacy of tri-carboxylates, particularly citrate, in the solubilisation of P from rock phosphate, Al-P and Fe-P compared with di- and mono-carboxylates (e.g. α-ketobutyrate, gluconate and acetate) (Kpomblekou-a and Tabatabai 1994; de Oliveira Mendes *et al.* 2020).

6.7. Conclusion

The results presented in this work indicate the presence of a diverse species of ACC deaminase producing bacteria, most of them solubilised mineral phosphates. The extent of ACC deaminase activity was associated with the capacity of PSB to solubilise P from Ca-P, but only if N was supplied as ACC. In this case, ACC deaminase could mediate P solubilisation, most likely through modulating the production of NH_4^+, which is an effective source of media acidification. Additionally, α-ketobutyrate (produced from ACC via ACC deaminase activity) can facilitate P solubilisation through organic-metal complex formation. On the other hand, Fe-P and Al-P solubilisation in culture media containing ACC as sole N source was not associated with the ability of PSB to express ACC deaminase activity. In addition, the amount of ACC deaminase activity was not associated with the P solubilisation potential of rhizobacteria when $(NH_4)_2SO_4$ rather than ACC was the sole N source. The P solubilising activity of PSB with $(NH_4)_2SO_4$ was rather associated with the type of carboxylates produced.

6.8. References

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Table 1. Plant growth-promoting traits and taxonomic identity of ACC deaminase-producing bacteria on the basis of 16S rDNA sequences

Strain	**Accession No.**	**Putative species**	****% identity**	**IAA production (µg mL^{-1})**	**Growth in DF-medium with ACC**	***ACCd (µmole †α-KB h^{-1})**	**‡PSI with $(NH_4)_2SO_4$**	**PSI with ¶ ACC**	**Growth in N free media**
59F	MW692270	*Bacillus amyloliquefaciens*	98.9	10.3	+	0.96	§**	*	+
33F	MW692258	*Bacillus dijibelorensis*	99.8	n.d.	+	1.24	§**	*	n.d.
77F	MW692283	*Bacillus cereus*	98.9	8.5	+	6.83	**	*	n.d.
8F	MW692246	*Bacillus megaterium*	99.4	9.3	+	0.35	§****	*	n.d.
71F	MW692278	*Bacillus cucumis*	99	12.9	+	0.96	*	*	n.d.
60F	MW692271	*Bacillus megaterium*	99.8	6.2	+	1.14	**	*	+
38F	MW692261	*Bacillus simplex*	98.5	11	+	3.76	n.d.	*	+
14F	MW692249	*Bacillus simplex*	99.1	4.1	+	4.79	n.d.	*	n.d.
57F	MW692269	*Bacillus simplex*	99.4	8	+	7.32	n.d.	*	n.d.
98F	MW692290	*Bacillus pumilus*	99.7	5.7	+	6.8	****	****	+
3F	MW692243	*Bacillus simplex*	99.3	n.d.	+	0.45	**	*	+
24F	MW692252	*Bacillus simplex*	99.4	5.1	+	1.38	**	*	+
26F	MW692254	*Bacillus simplex*	99.6	8	+	2.23	**	*	+
42F	MW692263	*Bacillus subtilis*	98.7	5.7	+	7.32	**	**	n.d.
29F	MW692255	*Bacillus simplex*	99.3	5.6	+	0.55	***	*	n.d.
72F	MW692279	*Bacillus toyonensis*	99.5	n.d.	+	1.14	**	*	n.d.

127F	MW692327	*Burkholderia cenocepacia*	99.3	41.9	+	0.54	****	**	+
18F	MW692297	*Burkholderia* sp.	98.5	18.7	+	n.d.	*	n.d.	+
137F	MW692222	*Burkholderia* sp.	99.8	5.5	+	0.51	**	*	n.d.
150F	MW692234	*Burkholderia* sp.	99.3	5.6	+	0.55	**	*	n.d.
52F	MW692307	*Burkholderia* sp.	98.5	9.5	+	0.92	***	*	+
87F	MW692317	*Paraburkholderia phenolirutrix*	98.8	n.d.	+	1.02	***	*	+
12F	MW692295	*Burkholderia* sp.	98.3	54.3	+	1.16	****	*	n.d.
157F	MW692240	*Burkholderia* sp.	99.8	n.d.	+	1.24	**	*	n.d.
30F	MW692302	*Burkholderia* sp.	99.5	10.4	+	1.35	***	*	n.d.
108F	MW692321	*Paraburkholderia phenolirutrix*	99.4	13.5	+	1.6	**	*	+
28F	MW692301	*Paraburkholderia phenolirutrix*	98.8	9.4	+	1.69	*	*	n.d.
120F	MW692325	*Burkholderia* sp.	99.2	10.4	+	1.91	***	*	n.d.
136F	MW692221	*Burkholderia* sp.	99.6	8	+	2.23	***	*	+
94F	MW692318	*Burkholderia* sp.	98.5	6.2	+	4.3	***	**	+
20F	MW692298	*Burkholderia* sp.	99.3	24.9	+	5.97	***	***	+
84F	MW692314	*Paraburkholderia phenolirutrix*	100	n.d.	+	6.8	**	***	n.d.
126F	MW692326	*Burkholderia cepacia*	99.3	7.4	+	8.42	****	****	+
9F	MW692247	*Paenibacillus endophyticus*	97.7	4.3	+	0.55	**	n.d.	n.d.
41F	MW692304	*Paraburkholderia caledonica*	99.5	10.4	+	n.d.	**	*	+
142F	MW692227	*Paraburkholderia caledonica*	98.8	9.4	+	n.d.	***	*	+
27F	MW692300	*Pseudomonas fluorescens*	99.8	5.5	+	0.51	***	*	n.d.

45F	MW692305	*Pseudomonas putida*	99.3	8.5	+	n.d.	*	n.d.	+
62F	MW692310	*Pseudomonas putida*	98.7	29.3	+	0.66	**	n.d.	n.d.
46F	MW692306	*Pseudomonas* sp.	99.2	15.3	+	5.22	**	*	n.d.
19F	MW692205	*Variovorax paradoxus*	99.3	n.d.	+	1.28	**	*	n.d.
105F	MW692210	*Variovorax paradoxus*	99.4	5.1	+	1.38	**	*	+
88F	MW692207	*Variovorax paradoxus*	98.8	9.4	+	2.56	**	*	n.d.

*ACCd- ACC deaminase,

†α-KB- α-ketobutyrate, n.d. not detected,

‡PSI: phosphate solubilisation index

§PSI value <2 - *, 2 – 3 - **, 3 – 4 - **, >4.0 - ****

¶ ACC: 1-aminocyclopropane-1-carboxylic acid

** The percentage similarity values are based upon comparisons with the Genbank database using the BLASTN program. PSI was calculated based on the ratio of total colony diameter to the total diameter of halo zone. Results are means of three experiments conducted separately under identical conditions.

Table 2. The concentration of soluble P in suspensions supplied with various sources of P and 10 mmol L^{-1} of the respective carboxylate after agitation at 150 rpm for 24 h.

Carboxylate	*Al-P	†Ca-P	‡Fe-P	Mean
Acetate	29.7p	37.4k-p	33.4nop	33.5
Citrate	46.5g-l	48.8e-j	45.8h-m	47.0
DL-Maleate	43.7i-n	57.2a-f	43.0i-n	48.0
Gluconate	32.5op	28.6p	35.4m-p	32.2
α- ketobutyrate	36.2l-p	43.2i-n	41.9i-o	40.4
α-ketoglutarate	45.5h-m	51.2c-i	38.8j-p	45.2
Malate	59.7a-d	61.8ab	46.4g-l	56.0
Oxalate	45.7h-m	58.0a-e	45.6h-m	49.8
Pyruvate	43.1i-n	51.9b-i	38.7j-p	44.5
Succinate	44.7i-m	61.3abc	47.3f-k	51.1
H_2SO_4§	62.8a	64.3a	50.1d-i	59.1
HCl¶	55.7a-h	56.8a-g	42.2i-o	51.5
LSD (*P* <0.05) **				
P†† * Carboxylate	10.454			
CV%	6.88			

*$AlPO_4$ = Al-P

†$Ca_3(PO_4)_2$ = Ca-P

‡$FePO_4$ = Fe-P

§H_2SO_4 = sulphuric acid

¶HCl = hydrochloric acid

**LSD = Values follow by the same letter are not significant different at $P < 0.05$

††P = Phosphorus

Table 3. The pH of suspensions containing various sources of P and 10 mmol L^{-1} of respective carboxylate after agitating at 150 rpm for 24 h.

Carboxylate	***Al-P**	**†Ca-P**	**‡Fe-P**	**Mean**
Acetate	3.09d-g	4.33c	3.43d	3.62
Citrate	2.56ghi	4.33c	2.58f-i	3.16
DL-maleate	2.75e-i	4.52c	2.77e-i	3.35
Gluconate	5.51b	7.03a	4.54c	5.70
α-ketobutyrate	2.31hij	3.48d	2.27hij	2.68
α-ketoglutarate	2.44hij	4.49c	2.35hij	3.09
Malate	2.33hij	4.61c	2.28hij	3.07
Oxalate	2.52ghi	4.26c	2.49hi	3.09
Pyruvate	2.34hij	4.21c	2.23ij	2.93
Succinate	3.13def	4.36c	3.22de	3.57
H_2SO_4§	1.88jk	3.43d	1.52k	2.28
HCl¶	2.39hij	5.34b	2.81e-h	3.51
LSD (*P* <0.05) **				
P†† * Carboxylate	0.57			
cv%	5.25			

*$AlPO_4$ = Al-P

†$Ca_3(PO_4)_2$ = Ca-P

‡$FePO_4$ = Fe-P

§H_2SO_4 = sulphuric acid

¶HCl = hydrochloric acid

**LSD = Values follow by the same letter are not significant different at $P < 0.05$

††P = Phosphorus

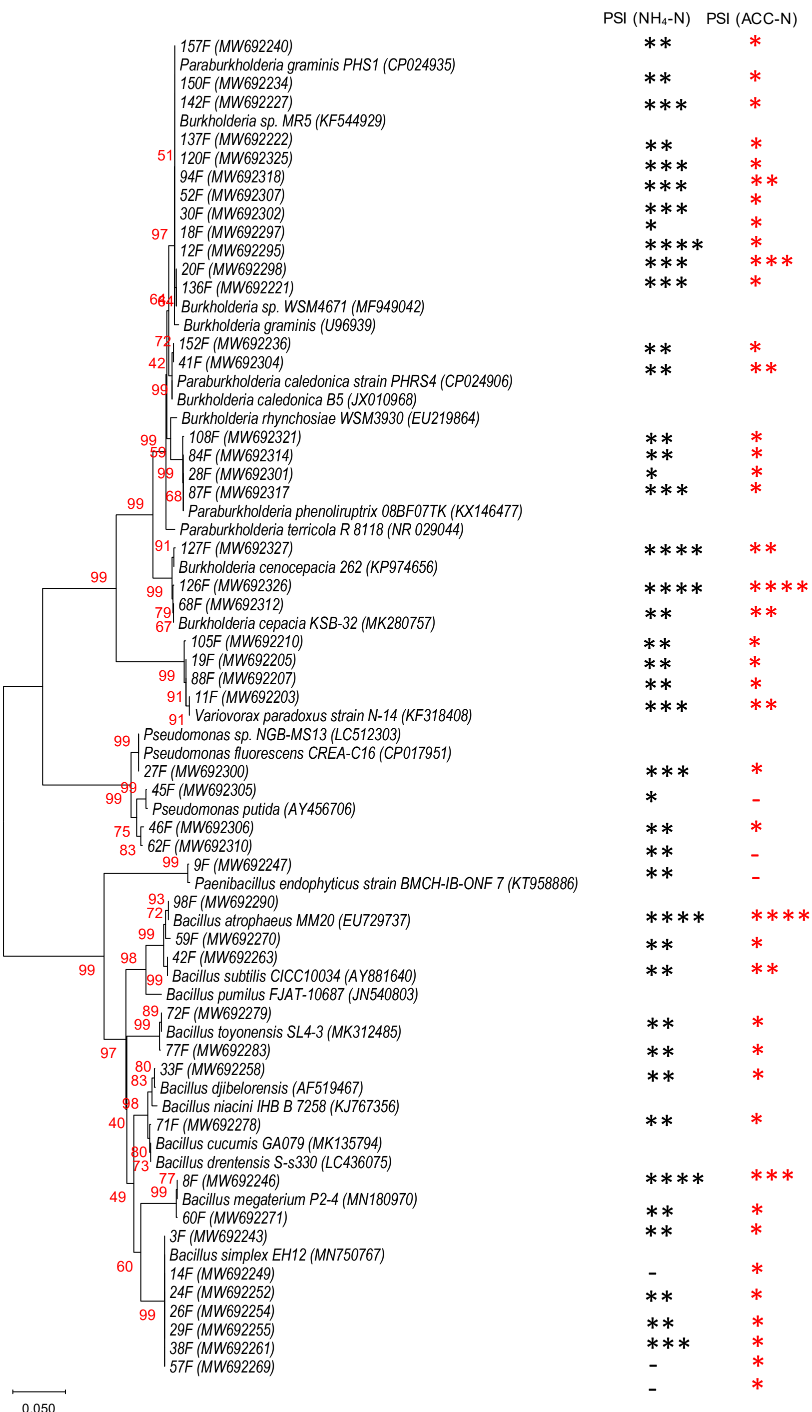

Figure 1. Neighbour–joining phylogenetic tree based on the 16S rRNA gene sequences of culturable phosphate solubilising bacteria containing *acdS* gene encoding ACC deaminase isolated from chickpea rhizosphere soils. Genetic distances were constructed using Kimura's 3-parameter model. PSI: phosphate solubilisation index, *- PSI value <2, **- 2-3, ***->3-4, ****->4.0 and '-' no P solubilising activity. Black and red asterisks represent the PSI with $(NH_4)_2SO_4$ and ACC, respectively.

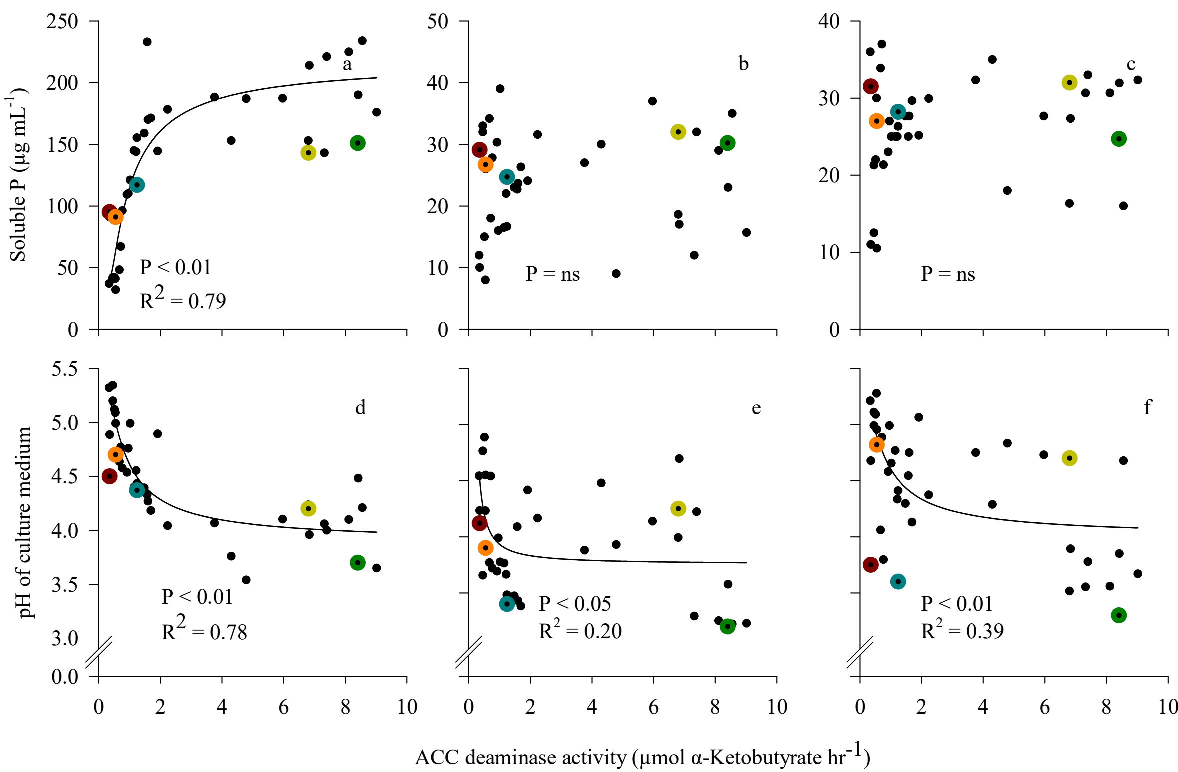

Figure 2. Regression analysis between amount of soluble P and pH in NBRIP liquid medium containing ACC as sole N source with $Ca_2(PO_4)_3$ (**a** and **d**), AlO_4P (**b** and **e**) and $FeO_4P.2H_2O$ (**c** and **f**) with ACC deaminase produced by PSB in DF minimal medium containing ACC as sole source of N. Data points represent the data obtained from individual bacterial isolates. Data points identified by colours indicate the results obtained from isolates efficient in P solubilisation. Symbols represented as: (•) PSB; (●) *B. cenocepacia* 127F; (●) *B. cepacia* 126F; (●) *B. pumilus* 98F; (●) *B. megaterium* 8F and (●) *Burkholderia* sp. 12F. Nonlinear regressions in "a", "d", "e" and "f" are polynomial, inverse second order functions, $Y_0=(a/x)+(b/x^2)$. *P* = ns- non-significant, *P* < 0.05- significant, *P* < 0.01- highly significant.

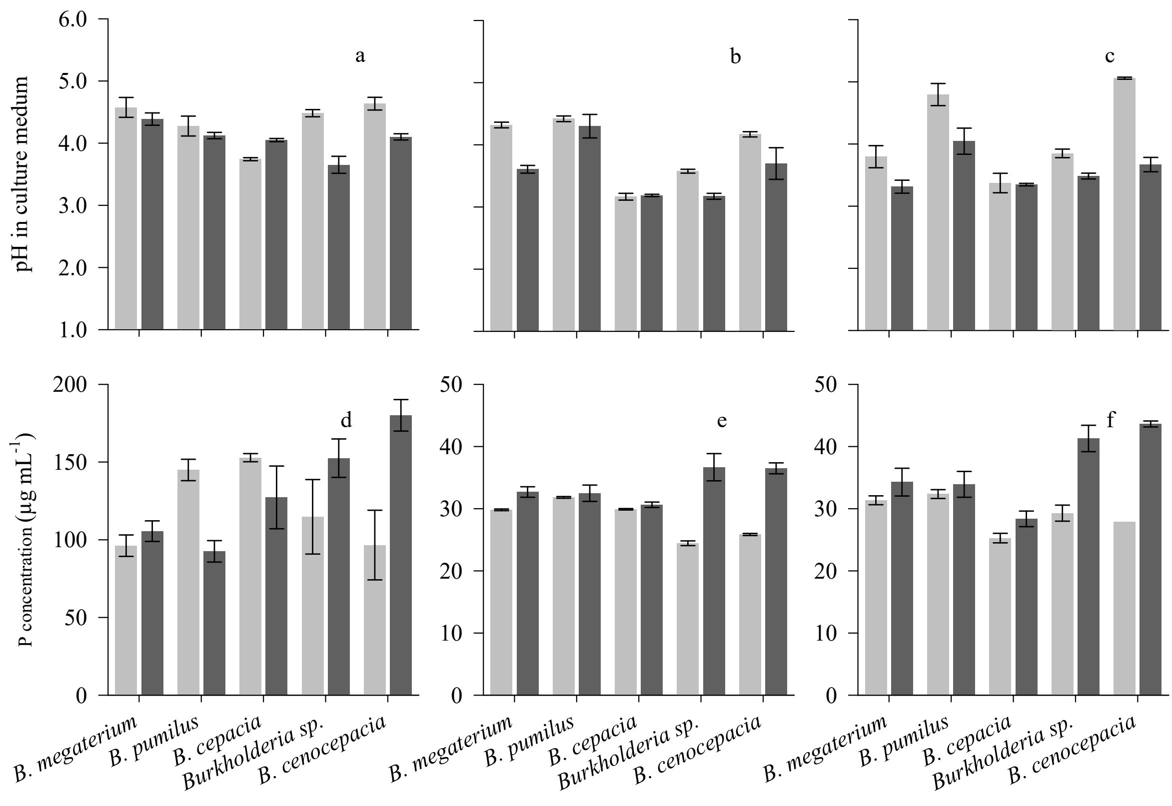

Figure 3. Dissolved P and pH of NBRIP medium supplemented with two types of N source [ACC and $(NH_4)_2SO_4$] and three P sources including $Ca_3(PO_4)_2$ (**a** and **d**), AlO_4P (**b** and **e**) and $FeO_4P.2H_2O$ (**c** and **f**), respectively. PSB included 1: *B. megaterium* 8F, 2: *B. pumilus* 98F, 3: *B. cepacia* 126F, 4: *Burkholderia* sp. 12F and 5: *B. cenocepacia* 127F. The bar graphs indicate the mean values of three replications. The lighter (▬) and darker (▬) bars represent the P and pH values in NBRIP containing ACC and $(NH_4)_2SO_4$ as sole N source, respectively. Error bars represent Fisher's $LSD_{0.05}$ of differences in each section.

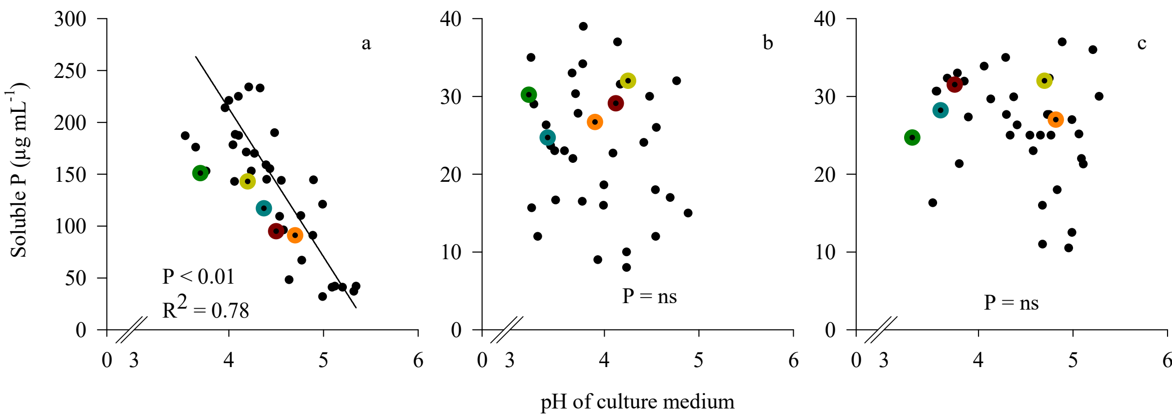

Figure 4. Regression analysis between soluble P and pH of the culture medium determined at 14 days after inoculation of NBRIP liquid medium containing ACC as sole N source with (**a**) $Ca_3(PO_4)_2$, (**b**) AlO_4P and (**c**) FeO_4P. Data points represent the data obtained from individual bacterial isolates. Data points identified by colours indicate the results obtained from isolates efficient in P solubilisation. Symbols represented as: (•) PSB; (●) *B. cenocepacia* 127F; (●) *B. cepacia* 126F; (●) *B. pumilus* 98F; (●) *B. megaterium* 8F and (●) *Burkholderia* sp. 12F Regression line in "a" is linear, Y_0= a +bX. *P* = ns- non-significant, *P* < 0.01- highly significant.

Chapter 7 Ability to produce indole acetic acid is associated with improved phosphate solubilising activity of rhizobacteria

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7.1. Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Anteneh Argaw		
Contribution to the Paper	Planned the study conducted all experiments, analysed and interpreted data and wrote the manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	26/03/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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7.2. Abstract

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ORIGINAL PAPER

Ability to produce indole acetic acid is associated with improved phosphate solubilising activity of rhizobacteria

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Abstract
Indole acetic acid (IAA) can upregulate genes encoding enzymes responsible for the synthesis of carboxylates involved in phosphorus (P) solubilisation. Here, we investigated whether IAA and its precursor affect the P-solubilising activity of rhizobacteria. A total of 841 rhizobacteria were obtained using taxonomically selective and enrichment isolation methods. Phylogenetic analysis revealed 15 genera of phosphate solubilising bacteria (PSB) capable of producing a wide range of IAA concentrations between 4.1 and 67.2 μg mL^{-1} in vitro. Addition of L-tryptophan to growth media improved the P-solubilising activity of PSB that were able to produce IAA greater than 20 μg mL^{-1}. This effect was connected to the drop of pH and release of a high concentration of carboxylates, comprising α-ketoglutarate, *cis*-aconitate, citrate, malate and succinate. An increase in production of organic acids rather than IAA production per se appears to result in the improved P solubilisation in PSB.

Keywords Acidification · IAA · L-tryptophan · Rhizobacteria · Rock phosphate

7.3. Introduction

Phosphorus (P) is the second most limiting plant nutrient in the soil after nitrogen (N). The amount of plant-available P in most agricultural soils is below 1 µg/g soil dry weight (DW), which is insufficient for normal plant growth (Rodríguez and Fraga 1999) and impairs agricultural productivity and threatens food security (Runge-Metzger 1995). Phosphate fertilizer application is commonly practiced to stimulate crop yield in P deficient soils and is predominantly based upon application of mineral fertilizer derived from mined phosphate rock, a finite resource which is steadily declining (Reijnders 2014). However, this input may cause environmental pollution where phosphate fertilizer has been used in large quantities (Molina et al. 2009). A proportion of added inorganic fertilizers (up to 90%) can also be converted to sparingly soluble P forms, predominantly iron and aluminium phosphates in acidic soils, and calcium phosphate in alkaline soils (Chacon et al. 2006) which are poorly available to most plants (Sashidhar and Podile 2010; Zhang et al. 2017).

The application of rock phosphate, a naturally occurring and cheap P source, is a promising approach that might reduce dependence on soluble phosphate fertilisers (Van Straaten 2006). However, the P in this input is not readily available to plants and is an agronomically ineffective P source when it is applied directly to the soil (Biswas and Narayanasamy 2006). The use of phosphate solubilising bacteria (PSB) may increase the plants efficiency in P utilization from rock phosphate (Yin et al. 2015; do Carmo et al. 2019; Ghosh and Mandal 2020). PSB have been shown to increase plant available P from rock phosphate in many studies (Gupta et al. 2007; de Oliveira Mendes et al. 2014; da Costa et al. 2015; Kaleem Abbasi and Manzoor 2018; Ben Zineb et al. 2020).

Plant growth promotion following inoculation of beneficial bacteria is the net result of multiple mechanisms that may be activated simultaneously (Martínez-Viveros et al. 2010). Previous studies have demonstrated a wide range of genera of rhizobacteria able to express multiple PGP characteristics in the chickpea root zone (Singh et al. 2014; Midekssa et al. 2016). In particular, phosphate solubilising bacteria with the ability to produce IAA are frequently identified in the rhizosphere (Ghosh et al. 2013; Masciarelli et al. 2014; Tariq et al. 2014). Hence, investigating the diversity and potential of rhizobacteria in IAA production and P solubilisation is important because these PGP mechanisms can be synergistically involved in plant growth promotion (Kumar and Narula 1999; Prashanth and Mathivanan 2010; Mehta et al. 2014).

Plant growth promotion following inoculation with plant growth promoting rhizobacteria (PGPR) capable of both producing IAA and solubilising P are frequently associated with increasing P concentration in the rhizosphere and in plant tissues (Sattar and Gaur 1987; Lippmann et al. 1995; De Freitas et al. 1997; Kumar and Narula 1999; Bianco and Defez 2010a). IAA-producing and P-solubilising rhizobacteria can enhance P nutrition through two possible ways: (*i*) IAA can promote root growth and the active root system is able to explore more soil to encounter phosphate liberated from insoluble P sources (Egamberdiyeva 2007; Fierro-Coronado et al. 2014), and (*ii*) plant-associated bacteria enhance nutrient availability through direct mineralisation and solubilisation of the unavailable nutrient, particularly P, from soils (Wani et al. 2007; Charana Walpola and Yoon 2013). PSB were more effective as inoculants when they were able to produce IAA (Bianco and Defez 2010b; Vitorino et al. 2012). An IAA-overproducing engineered bacterium was also more efficient in P dissolution from rock phosphate than its wild derivative that was unable to express IAA (Bianco and Defez 2010a). However, previous studies did not investigate the role of IAA in P solubilisation and whether the amount of IAA produced by PSB would predict their potential to solubilise phosphate.

A diverse array of bacteria including species of *Acinetobacter*, *Achromobacter*, *Arthrobacter*, *Azotobacter*, *Bacillus*, *Burkholderia,* *Klebsiella*, *Microbacterium*, *Ochrobactrum*, *Pseudomonas*, *Pantoea*, *Serratia, Staphylococcus* and *Yokenella* have been shown to produce IAA and solubilise phosphate (Patten and Glick 2002; Ahmad et al. 2005; Sergeeva et al. 2007; Upadhyay et al. 2009; Dutta and Thakur 2017; Govindasamy et al. 2017). Rhizobacteria can produce a wide range of IAA concentrations *in vitro* between nil and 142.5 $\mu g\ mL^{-1}$ (Wilson 1999; Arruda et al. 2013; Ghosh et al. 2013). The ability of rhizobacteria to express PGP activities, including IAA and P solubilisation, and their diversity, can be influenced by characteristics of the host plant including root exudates, management practices and local microenvironmental conditions (Petchey and Gaston 2002; Martínez et al. 2011; Chaparro et al. 2012; Arruda et al. 2013; Louca et al. 2016; Malhotra et al. 2017; Vives-Peris et al. 2020). Thus, the objectives of this study were (*i*) to investigate the occurrence of potential culturable IAA-producing and P-solubilising rhizobacteria obtained using taxonomically selective and enrichment methods, and (*ii*) to examine the effect of IAA concentration on the potential of PSB to solubilise P *in vitro*.

7.4. Materials and Methods

7.4.1. Sources of isolates

Soil samples were collected from 74 agricultural fields across Australia. A sub-sample of soil (100 g) was taken and placed in a small pot (4.5 cm × 4.5 cm × 10 cm) to grow chickpea cv. HatTrick. The seedlings were grown in a growth chamber with light supplied by fluorescent and incandescent lamps with an intensity of 1000 µmol m^{-2} s^{-1} for a 16 h photoperiod and a constant temperature at 20 ºC, with relative humidity at 70%. The rhizosphere soil (soil strongly adhering to the chickpea root) was collected from four-week-old seedlings.

Rhizobacteria were isolated using selective and enrichment methods to obtain efficient phosphate solubilisers. Rhizosphere soil (1 g) was transferred to a test tube containing 9 mL of sterile MilliQ water and the aliquot was serially diluted 10 fold up to 10^{-6}. Then, 100 µL of the 10^{-5} and 10^{-6} dilutions were spread on semi-selective 1/10 strength tryptone soya agar (TSA) (OXOID LTD, Basingstoke, Hampshire, England) supplemented with 50 µg mL^{-1} ampicillin (99%, Sigma Aldrich Chemie GmbH, Steinheim, Germany), 12.5 µg mL^{-1} chloramphenicol (99%, Sigma-Aldrich) and 75 µg mL^{-1} cycloheximide (Sigma-Aldrich) to obtain *Pseudomonas* like bacteria (Simon and Ridge 1974). The remaining aliquots from 10^{-5} and 10^{-6} dilutions were incubated in a water bath (10 min, 80 ºC) and *Bacillus* like bacteria were isolated using 1/10 strength TSA. Plates were incubated at 28 ºC for five days. Based on the colony morphology, distinct colonies were selected and further streaked on TSA for purification. A total of 743 isolates was purified. The P solubilisation ability of these isolates was tested using National Botanical Research Institute's phosphate growth (NBRIP) medium containing the following ingredients (L^{-1}): glucose, 10 g; $MgCl_2.6H_2O$, 5 g; $MgSO_4.H_2O$, 0.25 g; KCl, 0.2 g; and $(NH_4)_2SO_4$, 0.1 g and containing 5 g of $Ca_3(PO_4)_2$ (Sigma-Aldrich) (Nautiyal 1999). A pure colony of each isolate was inoculated into DF-minimal media and incubated in a shaker incubator (48 h, 28 ºC, 160 rpm). Bacterial suspensions of 100 µL were streaked on NBRIP medium. Plates were incubated for 10 days at 28 ºC and the P solubilisation index (PSI) value was calculated (Nguyen et al. 1992).

Additionally, efficient phosphate solubilising heterotrophic bacteria were enriched from rhizospheric soils in NBRIP medium containing insoluble P sources such as $Ca_3(PO_4)_2$, AlO_4P (Sigma-Aldrich) or FeO_4P (Sigma-Aldrich). Plates were incubated for 10 days at 28 ºC. PSB with PSI > 2.00 were selected for further study.

7.4.2. Determination of IAA production

Phosphate solubilising bacteria were further investigated for their ability to produce IAA in the absence and presence of 500 µg mL^{-1} of L-tryptophan (BioChemica) in DF-minimal medium. The culture was placed in a shaker incubator (48 h, 28 ºC, 160 rpm). The culture was then centrifuged (10 min, 10,000 x *g*). The concentration of IAA in the supernatant was quantified by colorimetric methods using the Salkowski reagent (40 mM $FeCl_3$, 7.9 M H_2SO_4) (Patten and Glick 2002). A mixture containing 1 mL of supernatant and 4 mL Salkowski reagent was kept in the dark for 30 min and absorbance at 530 nm was determined by a spectrophotometer (Implen GmbH, Schatzbogen, Münichen, Germany). IAA concentration was calculated based on a standard curve developed using pure IAA (0, 2, 4, 8, 10, 15, 20, 25 and 30 µg mL^{-1}).

7.4.3. Diversity of IAA producing rhizobacteria

The genetic diversity of IAA-producing bacteria was investigated based on the sequence of 16S rDNA. The universal primer: fD1:5′-AGAGTTTGATCCTGGCTCAG-3′ (Sigma-Aldrich) and rP2: 5′-ACGGCTACCTTGTTACGACTT-3′ (Sigma-Aldrich) were used to amplify the 16S rDNA with an approximate product size of 1500 bp (Weisburg et al. 1991). The PCR mixture contained 10.5 µL sterile MilliQ water, 12.5 µL of 2× MyFi™ Mix (Sigma-Aldrich) and 1 µL of 10× diluted each primer. MyFi™ Mix contained MyFi DNA polymerase, dNTPs, $MgCl_2$ and enhancers at optimal concentrations. PCR amplification was performed using a thermocycler (Bioer Version 1.10, GeneWorks, Hindmarsh, South Australia), with the following conditions: 1 min initial denaturation step at 95 ºC followed by 35 amplification cycles consisting of 15 s denaturation step at 95 ºC, 30 s annealing step at 57 ºC, 1 min extension at 72 ºC and a final extension at 72 ºC for 5 min. DNA sequencing of PCR products was performed by the Australian Genome Research Facility, Sanger Service (University of Adelaide, Australia).

Homologous 16S rDNA sequences were obtained from databases of the National Centre for Biotechnology Information (NCBI) (http//www.ncbi.nim.nih.gov/BLAST) for taxonomic assignment. The sequences were submitted to NCBI and assigned accession number (MW692202 - MW692329). The phylogenetic tree was generated using Molecular Evolutionary Genetics Analysis (MEGA-7) (Institute of Molecular Evolutionary Genetics, The Pennsylvania State University, University Park, PA 16802, USA) with Kimura's 3 substitution model (Kumar et al. 2016). The neighbour-joining method was applied to the distance matrix to construct the phylogenetic tree.

7.4.4. Experiment 1

P solubilisation was investigated in NBRIP medium supplemented with L-tryptophan (Biochemica) at 500 µg mL^{-1}. Efficient PSB able to produce IAA in the presence of L-tryptophan were selected for this experiment. One hundred µL of 48 h culture solution was spot inoculated at four places at equal distance on the NBRIP agar medium containing 5 g of $Ca_3(PO_4)_2$. After 10 days of incubation, the colony and clearing zone plus colony diameter were measured and PSI values were calculated.

7.4.5. Experiment 2

The effect of IAA (Glentham Life Sciences Ltd, Corsham, United Kingdom) and L-tryptophan on P solubilisation potential of PSB were investigated in liquid NBRIP medium. Four strains, namely *Bacillus pumilus* 98F, *Burkholderia cenocepacia* 127F, *Burkholderia* sp. 12F and *Pseudomonas fluorescens* 27F, were used. These strains mobilised the highest concentrations of dissolved P (Pi) (92 to 189 µg L^{-1}) from $Ca_3(PO_4)_2$ (Chapter 4). The strains also displayed a wide range of IAA production, from 4.1 to 54.3 µg mL^{-1} in the presence of its precursor but did not synthesise IAA in the absence of its precursor.

Two types of rock phosphate, Peru rock phosphate (low reactive) and Togo rock phosphate (moderately reactive) were used. The rock phosphate was ground, passed through a 500 µm mesh sieve, and sterilised in autoclave at 121 °C for 30 min.

The treatments consisted of a factorial combination of the abovementioned four PSB, two types of rock phosphate and three types of supplement (IAA: 100 µg mL^{-1}, L-tryptophan: 500 µg mL^{-1} and control, no addition). All treatments were set up in triplicate. The media was incubated in shaker incubator (28 °C, 160 rpm). The Pi concentration and pH of the cultures were determined over a 21-day incubation period.

7.4.6. Experiment 3

The effect of IAA concentration on P solubilisation potential of an efficient P solubiliser (*Burkholderia* sp. 12F) was tested in liquid NBRIP medium. This strain produced 54.3 µg mL^{-1} IAA in the presence of its precursor. Four IAA concentrations (0, 25, 50 and 100 µg mL^{-1}) were used as supplements to the NBRIP broth medium. Each treatment was replicated three times. The culture solution was incubated in shaker incubator (28 °C, 160 rpm) for seven days.

The Pi concentration, pH and carboxylate production were determined in culture solution taken different incubation time.

7.4.7. P analysis

The Pi concentration in the sample solution (5 mL) obtained from experiments 2 and 3 at the 3rd, 7th, 14th and 21st day of incubation were determined. Samples were centrifuged (10,000 × *g*, 10 min) and supernatants were used for P analysis. The concentration of Pi was estimated using the phospho-molybdate blue colour method (Murphy and Riley 1962). The mixed reagent was prepared from the following solutions: (*i*) 125 mL of sulphuric acid (5 *N*), (*ii*) 37.5 mL of 20 g of ammonium molybdate dissolved in 75 mL of water, (*iii*) 75 mL of ascorbic acid (0.1 M), and (*iv*) 12.5 mL of 0.2743 g of potassium antimonyl tartrate dissolved in 100 mL of water. Two mL of the supernatant was mixed with 0.4 mL of the mixed reagent and the solution was kept under the dark condition for 30 min. The absorbance at 882 nm was determined by spectrophotometry (Implen GmbH, Schatzbogen, Münichen, Germany) and fitted to a standard calibration curve.

7.4.8. Determination of carboxylate production

The effect of IAA concentration on the ability of an efficient PSB, *Burkholderia* sp. 12F, to produce carboxylates was investigated. The carboxylate analysis was done using HPLC (600E pump, 717plus autoinjector, 996 Photodiode array detector (PDA), Waters, Milford, MA) by adapting the method described in (Cawthray 2003). The culture extract was separated by centrifugation (10 min, 10,000 × g) and then by filtration using a 0.22 µm microfilter. The filtrate was acidified to pH <4.00 using concentrated H_3PO_4 before HPLC, using a sample injection volume of 100 µL. The working standards included authentic gluconate, pyruvate, malate, iso-citrate, malonate, shikimate, lactate, acetate, α-ketobutyrate, α-ketoglutarate, maleate, citrate, succinate, fumarate, cis-aconitate, and trans-aconitate. Detection and quantification of carboxylates were done at 210 nm, from absorbance data collected between 195 to 400 nm for spectrum matching and PDA peak spectral analysis. Data acquisition and processing were done with Empower3 (Waters) chromatography software.

7.4.9. Statistical analysis

Data were analysed by multifactorial analysis of variance using Genstat 18th version. Treatments were considered to be significantly different at the P <0.05 level. The normality of

the data was checked using Shapiro-Wilk's test. Bartlett's test was used to determine the homogeneity of variances.

7.5. Results

7.5.1. Diversity of IAA producing- and P solubilising- rhizobacteria

A total of 841 bacterial isolates was obtained from rhizosphere soils of chickpea grown in 74 soils collected from major agricultural lands across Australia using three isolation methods. These isolation methods were used as a selection strategy to obtain a variety of putative PSB isolates able to promote plant growth. Of 841 isolates, 683 were found to produce IAA (ranging from 4.1 to 67.2 $\mu g\ mL^{-1}$) in the presence of L-tryptophan. Among IAA producers, 112 were found to solubilise P from $Ca_3(PO_4)_2$ with PSI values > 2.00.

Of the 841 isolates, 743 isolates closely related to *Bacillus*, *Burkholderia* (formerly classified as *Pseudomonas*) and *Pseudomonas* were obtained (Chapter 3). Among the *Bacillus*-like isolates, eleven distinct species of *Bacillus* were identified. A large number of *Bacillus*-like-isolates (33%) were closely related to *Peribacillus simplex* followed by *Bacillus megaterium* (4 isolates) and *Bacillus niacini* (4 isolates). The remaining eight species were each represented by fewer than 3 isolates. Thirty-three isolates were identified as *Burkholderia* species. Of these isolates, 20 were closely related to *Burkholderia* sp., 5 isolates were closely related to *Burkholderia cepacia*. The remaining six isolates were closely related to *Paraburkholderia phenoliruptrix*. Other dominant genera identified were *Pseudomonas* and *Variovorax*, represented by seven and four isolates, respectively.

The remaining 98 isolates were efficient PSB obtained separately using the enrichment isolation method in NBRIP medium containing insoluble P. Analysis of diversity, based on 16S rDNA sequence, classified these isolates into 15 different genera (Fig. 1). Bacterial isolates were assigned to the genera *Burkholderia*, *Bacillus*, *Caulobacter*, *Cellulomonas*, *Curtobacterium*, *Dyella*, *Lefsonia,* *Microbacterium*, *Mycolicibacterium,* *Pantoea*, *Phyllobacterium*, *Pseudomonas*, *Rhizobium*, *Sphingomonas* and *Variovorax*. The majority of isolates with great capacity in IAA production and P solubilisation were closely related to *Burkholderia* spp.

7.5.2. P solubilisation efficacy

The PSI level of selected PSB was affected by the supply of L-tryptophan, the metabolic precursor of IAA, in the NBRIP medium (Fig. 2 and 3). In this investigation, all isolates were incapable of IAA production in the absence of L-tryptophan. In the absence of L-tryptophan, isolates exhibited P solubilising ability that was unrelated to their potential to produce IAA. In contrast, the ability to produce IAA and PSI was positively and significantly related (Fig. 2). Additionally, isolates which were able to produce IAA above 40 µg mL^{-1} had greater PSI with L-tryptophan than with no supplement (Fig. 1; Fig. 3a-d). Few isolates that were incapable of solubilising P also became low-level P solubilisers when supplied with L-tryptophan (example, Fig. 3c and d). These isolates produced IAA greater than 40 µg mL^{-1}. On the contrary, P solubilisation potential of isolates was not affected by L-tryptophan (Fig. 1; Fig 3e and f). In this case, the isolates produced IAA less than 20 µg mL^{-1}.

Quantitatively, the P solubilising activity of selected isolates was significantly influenced by IAA and L-tryptophan supplements in liquid NBRIP medium. The effect of L-tryptophan was dependent on the ability of isolates to synthesise IAA in the presence of its precursor. L-tryptophan did not affect the P solubilising capacity of *B. pumilus* 98F and *P. fluorescens* 27F that were able to produce 5.7 and 5.5 µg mL^{-1} IAA, respectively (Fig. 4a and b). However, the ability of these isolates to solubilise P was improved by the supply of IAA (100 µg mL^{-1}) in the medium. Addition of L-tryptophan to the culture medium significantly improved the P solubilisation potential of *B. cenocepacia* 127F and *Burkholderia* sp. 12F, that produced 41.9 and 54.3 µg mL^{-1} IAA, respectively (Fig. 4c and d).

The effect of L-tryptophan on the pH of the culture medium was dependent on the type of isolate inoculated. All isolates tested were able to induce a higher acidity in culture media containing L-tryptophan than the control uninoculated check treatment. *B. pumilus* 98F and *P. fluorescens* 27F caused lower pH in media supplemented with IAA than with L-tryptophan (Fig. 4e and f). The effect of IAA and L-tryptophan supplement on pH was non-significant in the case of *Burkholderia* sp. 12F (Fig. 4g). Conversely, *B. cenocepacia* 127F caused more acid production in media containing L-tryptophan than with IAA (Fig. 4h).

In a further study, amendment with IAA significantly increased the ability of *Burkholderia* sp. 12F to solubilise P from Peru rock phosphate. Solubilisation of P increased with increasing IAA concentration. The highest P solubilising activity of PSB with a large amount of solubilised P (24.1 µg L^{-1}) after 21 days' incubation was obtained in media containing 100 µg

mL^{-1} IAA (Fig. 5a). Additionally, the IAA solution was also able to liberate Pi between 1.45 to 2.68 µg mL^{-1} from rock phosphates (Fig. 5b). However, Pi concentration in IAA solution declined after incubation beyond 5 days.

To identify the mode of action of IAA on the potential of PSB to solubilise P, we measured the extent of acidity and organic acid production in media. Addition of IAA to a culture of *Burkholderia* sp. 12F significantly reduced the pH of the culture medium (Fig. 6a). The most acidic pH was obtained in media containing the highest IAA (100 µg mL^{-1}) at 21 days of incubation. One hundred µg mL^{-1} IAA by itself resulted in acidic pH down to 5.6 before agitating the solution. The pH value of uninoculated culture solution gradually increased, reaching 8.2 after prolonged incubation beyond the fifth day (Fig. 6b).

Addition of IAA affected the type and total concentration of carboxylates produced by *Burkholderia* sp. 12F in the culture medium (Table 1). In the absence of IAA in the media, this PSB was able to produce acetate, cis-aconitate, fumarate, maleate, malate, pyruvate and lactate. When the strain was treated with 25 µg mL^{-1} IAA, citrate was additionally produced and the concentrations of malate, pyruvate and cis-aconitate were increased. At 50 µg mL^{-1} IAA, the isolate produced α-ketoglutarate and the concentrations of fumarate, lactate, malate were increased. When the cells were treated with 100 µg mL^{-1} IAA, α-ketoglutarate, cis-aconitate, lactate, citrate, malate and succinate were detected, and the highest amount of citrate, malate and α-ketoglutarate were measured. In this culture solution, we measured the highest total carboxylate concentration.

7.6. Discussion

A high proportion of IAA producing PSB belonging to *Bacillus*, *Burkholderia* and *Pseudomonas* identified in the rhizosphere of chickpea grown in agricultural field soils

The proportion of rhizobacteria able to solubilise P and produce IAA vary between not detectable to approximately 80% of the culturable bacteria among different locations (Lottmann et al. 1999; Vestergård et al. 2007; Bal et al. 2013; Hariprasad et al. 2014; Hussein and Joo 2015; Verma et al. 2015; Gontia-Mishra et al. 2017; Brígido et al. 2019). In soils from our sampling sites, we detected a relatively high proportion of IAA-producing bacteria (83.4%) among isolates of *Burkholderia*, *Pseudomonas* and *Bacillus*. These genera have been frequently identified as IAA producing bacteria (Castro-González et al. 2011; Wahyudi et al. 2011; Castanheira et al. 2016; Li et al. 2017; Yarzábal et al. 2018).

Additionally, the prevalence of IAA-producing and P-solubilising bacteria has been higher in water-limited and nutrient-poor soils than in soils with more favourable conditions (Martínez et al. 2011; Timmusk et al. 2011; Shim et al. 2015; Malhotra et al. 2017). In the present study, the most of the efficient isolates in P solubilisation and IAA production were obtained from sites with high aridity index and low soil fertility. These isolates were closely related to *Burkholderia* spp. Microorganisms that produce IAA have previously been reported to be able to withstand stress conditions in the rhizosphere (Donati et al. 2013; Ouyang et al. 2017; Molina et al. 2018), indicating the significant ecological role of IAA. IAA can also increase the bacteria's ability to tolerate several stress conditions through upregulating the production of trehalose in the cells and enhancing production of lipopolysaccharide, exopolysaccharide and increase biofilm formation (Bianco et al. 2006; Donati et al. 2013; Ouyang et al. 2017).

IAA improved the ability of PSB to solubilise P

L-tryptophan is not only important for IAA production in the root zone (Kravchenko et al. 2004; Karnwal 2009) but as in the present study, amendment of media with this amino acid resulted in an increased ability of PSB to mobilise P from rock phosphate. The effect of L-tryptophan was associated with the ability of PSB to produce IAA. Increased P solubilisation efficiency of PSB with increasing IAA concentration in NBRIP medium was also observed. These results suggest that the production of IAA in the plant root zone may not only improve root growth in the plant-microbe interaction (Kravchenko et al. 2004; Naveed et al. 2015), but also enhance P solubilisation by PSB in the root zone. Additionally, bacterial isolates incapable of P solubilisation but able to produce IAA greater than 20 $\mu g\ mL^{-1}$ were able to develop a P-solubilising zone around their colonies in media containing L-tryptophan. This result suggests that the pre-screening for P solubilisation using NBRIP or Pikovskaya media *in vitro* may not indicate their efficiency of P solubilisation in the rhizosphere zone. Bacteria that have a low ability to solubilise P and those lacking this activity *in vitro* might nevertheless be efficient P mobilising bacteria in the rhizosphere as part of a plant-microbe association in which L-tryptophan may be produced by the root. This suggests the need to consider the addition of organic compounds, e.g. L-tryptophan, during first stage screening for PGPR, to enhance P nutrition.

IAA amendment increased the capacity of PSB to produce carboxylates

The positive influence of IAA and its precursor on the P solubilisation activity of particular bacterial strains was associated with the extent of acidity in the culture media solution. Here

we report that an increased IAA concentration in culture solution was also accompanied by an increased acidity. The lowest pH was recorded in bacteria cultures amended with 100 µg mL^{-1} IAA. Media acidification through H^+ excretion and organic acid production are the principal mechanisms for phosphate solubilisation (Illmer and Schinner 1992). Organic acids not only acidify the medium, but the deprotonated forms of these compounds also generate negatively charged carboxyl groups. These carboxyl groups chelate metal ions that can increase the P dissolution rate (Jones 1998; Whitelaw et al. 1999; Gyaneshwar et al. 2002).

IAA amendment of a cultre of *Burkholderia* sp. 12F affected the concentration and type of carboxylates synthesised by this PSB. Acetate and pyruvate were predominantly produced by this PSB in media with no added IAA. When the cells were treated with 100 µg mL^{-1} IAA, the bacteria produced a higher concentration of carboxylates, comprising α-ketoglutarate, malate, citrate and succinate. In previous studies, IAA triggered the production of enzymes involved in the tricarboxylic acid cycle in *Escherchia coli* (Bianco et al. 2006) and *Sinorhizobium meliloti* (Imperlini et al. 2009). In particular, the production of citrate synthase, isocitrate lyase, succinyl-CoA synthase and malate synthase were upregulated in *Escherichia coli* cells when treated with IAA (Bianco et al. 2006; Imperlini et al. 2009). These enzymes are responsible for the synthesis of carboxylates that were detected in culture media amended with IAA above 25 µg mL^{-1} in the present study. Therefore, a considerable increase in P solubilising activity of PSB in the presence of IAA can be associated with their ability to synthesise a high concentration of carboxylates, predominantly di- and tri-carboxylates. These types of carboxylates are efficient in solubilising P from relatively insoluble rock phosphates (Chen et al. 2006; Yi et al. 2008; Shahid et al. 2012; de Oliveira Mendes et al. 2014).

IAA was a poor mobiliser of phosphate from low reactive rock phosphate

IAA by itself was able to release small concentrations of Pi from rock phosphate. P solubilisation in IAA solution was associated with the extent of solution acidification. The production of H^+ and indole-3-acetate during the dissociation of IAA could be the possible source of acidic pH in the solution. Indole-3-acetate has a bidentate ligand that can chelate metal ions via its side-chain carboxyl (Kamnev et al. 2001). This characteristic of indole-3-acetate could facilitate P solubilisation through chelation of metal ions, to release precipitated Pi. However, the amount of Pi solubilised in IAA solutions was less than 3 µg L^{-1}, indicating a poor P solubilising ability of IAA by itself. The inability of IAA to release a significant amount Pi could be due to its ability to induce a lower acidity (pH > 5.5). Additionally, indole-

3-acetate belongs to the mono-carboxylate group and is therefore likely have a low P solubilising ability (Kpomblekou-a and Tabatabai 1994). Hence, the induction of an increased ability to produce organic acids rather than production of IAA *per se* could be the main mode of action of IAA in enhancing the ability of PSB to solubilise P.

7.7. Conclusion

We report PSB from fifteen genera that were able to produce a wide range of IAA concentrations *in vitro*, isolated from rhizospheres of chickpea grown in soils from diverse soil types and environmental conditions of cropping lands of Australia. In general, the presence of IAA or its precursor in culture media enhanced the isolates' ability to solubilise P from sparingly soluble rock phosphate *in vitro*. Addition of L-tryptophan caused a substantial positive effect on P solubilisation by PSB able to produce IAA *in vitro* (more than or equal to 20 µg mL^{-1}). The extent of improvement in P solubilising activity of selected PSB was linked to an increasing concentration of IAA in the culture medium. In this case, greater P solubilisation by selected PSB was linked to lowered pH of the medium and the production of carboxylates, predominantly di- and tri-carboxylates. Potential agricultural inoculants that can both produce IAA and solubilise P may offer an opportunity to maximise the provision of bioavailable P for improved crop growth in soils with low levels of available P.

7.8. References

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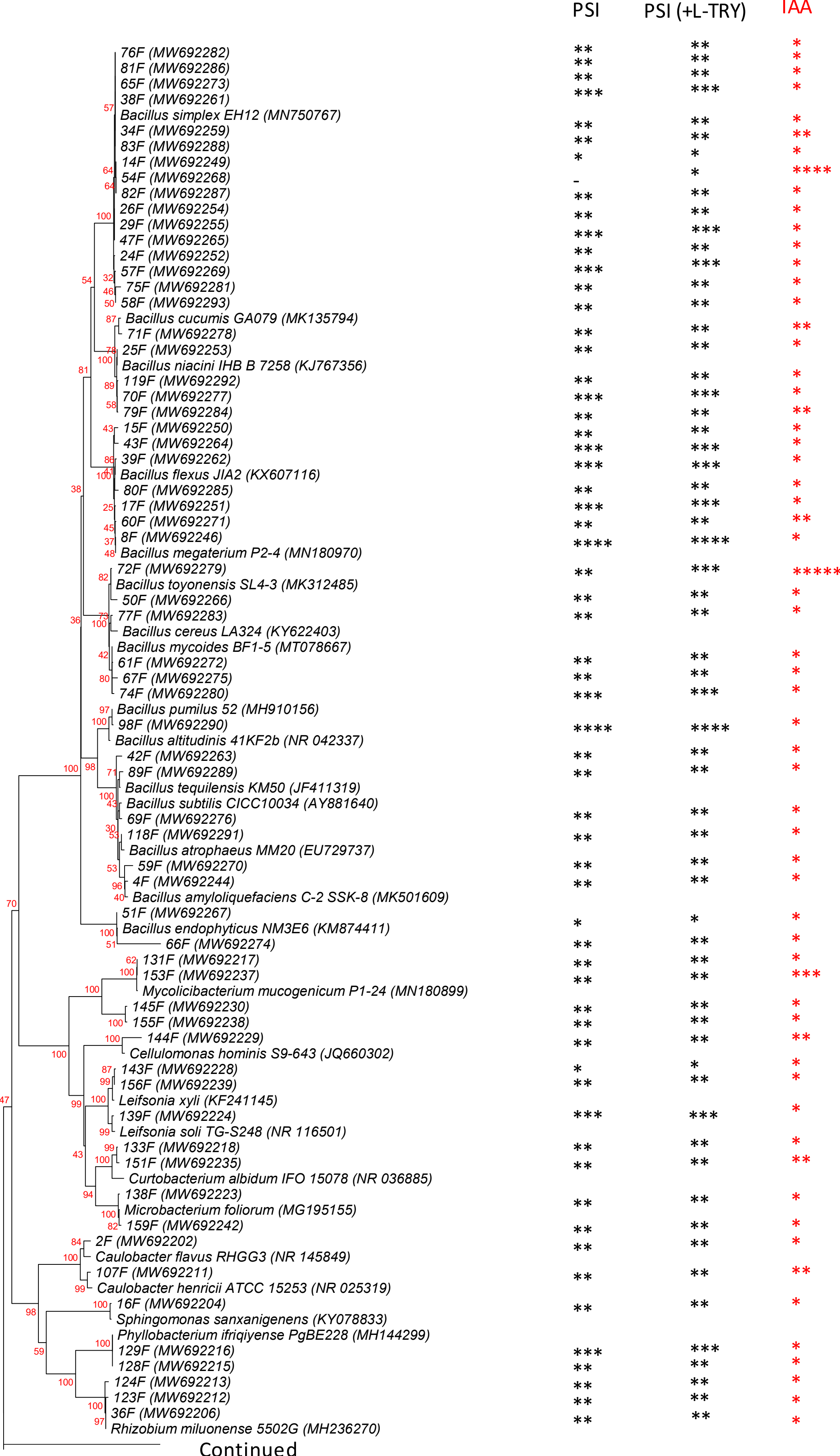
Table 1. Amounts of carboxylate (mM) produced by *Burkholderia* sp. 12F during the solubilisation of Peru rock phosphate obtained from mixed solution (three replications) after 7 days of incubation (28 ºC, 160 rpm).

Carboxylates	Limit of detection (mM)	Concentration of added IAA (µg mL^{-1})			
		control	25	50	100
Acetate	24	443.9	301.3	n.d.	n.d.
α-ketobutyrate	5.0	n.d.	n.d.	n.d.	n.d.
α-ketoglutarate	4.0	n.d.	n.d.	166.6	362.1
Cis-aconitate	0.1	0.193	8.643	0.250	1.158
Citrate	5.0	n.d.	15.76	n.d.	42.95
Fumarate	0.06	0.129	0.077	3.47	n.d.
Gluconate	8.0	n.d.	n.d.	n.d.	n.d.
Lactate	13	56.93	19.67	363.1	27.92
Maleate	0.05	0.057	n.d.	n.d.	n.d.
Malate	7.0	197.5	156.2	469.2	543.5
Malonate	8.0	n.d.	n.d.	n.d.	n.d.
Pyruvate	5.0	244.2	592.6	n.d	n.d.
Succinate	15	n.d.	n.d.	n.d.	486.9
Trans-aconitate	0.1	n.d.	n.d.	n.d.	n.d.
Total concentration		942.9	1094.3	1002.6	1464.5

n.d. not detected



PSI
PSI (+L-TRY)
IAA
76F (MW692282)
81F (MW692286)
65F (MW692273)
38F (MW692261)
Bacillus simplex EH12 (MN750767)
34F (MW692259)
83F (MW692288)
14F (MW692249)
54F (MW692268)
82F (MW692287)
26F (MW692254)
29F (MW692255)
47F (MW692265)
24F (MW692252)
57F (MW692269)
75F (MW692281)
58F (MW692293)
Bacillus cucumis GA079 (MK135794)
71F (MW692278)
25F (MW692253)
Bacillus niacini IHB B 7258 (KJ767356)
119F (MW692292)
70F (MW692277)
79F (MW692284)
15F (MW692250)
43F (MW692264)
39F (MW692262)
Bacillus flexus JIA2 (KX607116)
80F (MW692285)
17F (MW692251)
60F (MW692271)
8F (MW692246)
Bacillus megaterium P2-4 (MN180970)
72F (MW692279)
Bacillus toyonensis SL4-3 (MK312485)
50F (MW692266)
77F (MW692283)
Bacillus cereus LA324 (KY622403)
Bacillus mycoides BF1-5 (MT078667)
61F (MW692272)
67F (MW692275)
74F (MW692280)
Bacillus pumilus 52 (MH910156)
98F (MW692290)
Bacillus altitudinis 41KF2b (NR 042337)
42F (MW692263)
89F (MW692289)
Bacillus tequilensis KM50 (JF411319)
Bacillus subtilis CICC10034 (AY881640)
69F (MW692276)
118F (MW692291)
Bacillus atrophaeus MM20 (EU729737)
59F (MW692270)
4F (MW692244)
Bacillus amyloliquefaciens C-2 SSK-8 (MK501609)
51F (MW692267)
Bacillus endophyticus NM3E6 (KM874411)
66F (MW692274)
131F (MW692217)
153F (MW692237)
Mycolicibacterium mucogenicum P1-24 (MN180899)
145F (MW692230)
155F (MW692238)
144F (MW692229)
Cellulomonas hominis S9-643 (JQ660302)
143F (MW692228)
156F (MW692239)
Leifsonia xyli (KF241145)
139F (MW692224)
Leifsonia soli TG-S248 (NR 116501)
133F (MW692218)
151F (MW692235)
Curtobacterium albidum IFO 15078 (NR 036885)
138F (MW692223)
Microbacterium foliorum (MG195155)
159F (MW692242)
2F (MW692202)
Caulobacter flavus RHGG3 (NR 145849)
107F (MW692211)
Caulobacter henricii ATCC 15253 (NR 025319)
16F (MW692204)
Sphingomonas sanxanigenens (KY078833)
Phyllobacterium ifriqiyense PgBE228 (MH144299)
129F (MW692216)
128F (MW692215)
124F (MW692213)
123F (MW692212)
36F (MW692206)
Rhizobium miluonense 5502G (MH236270)
Continued

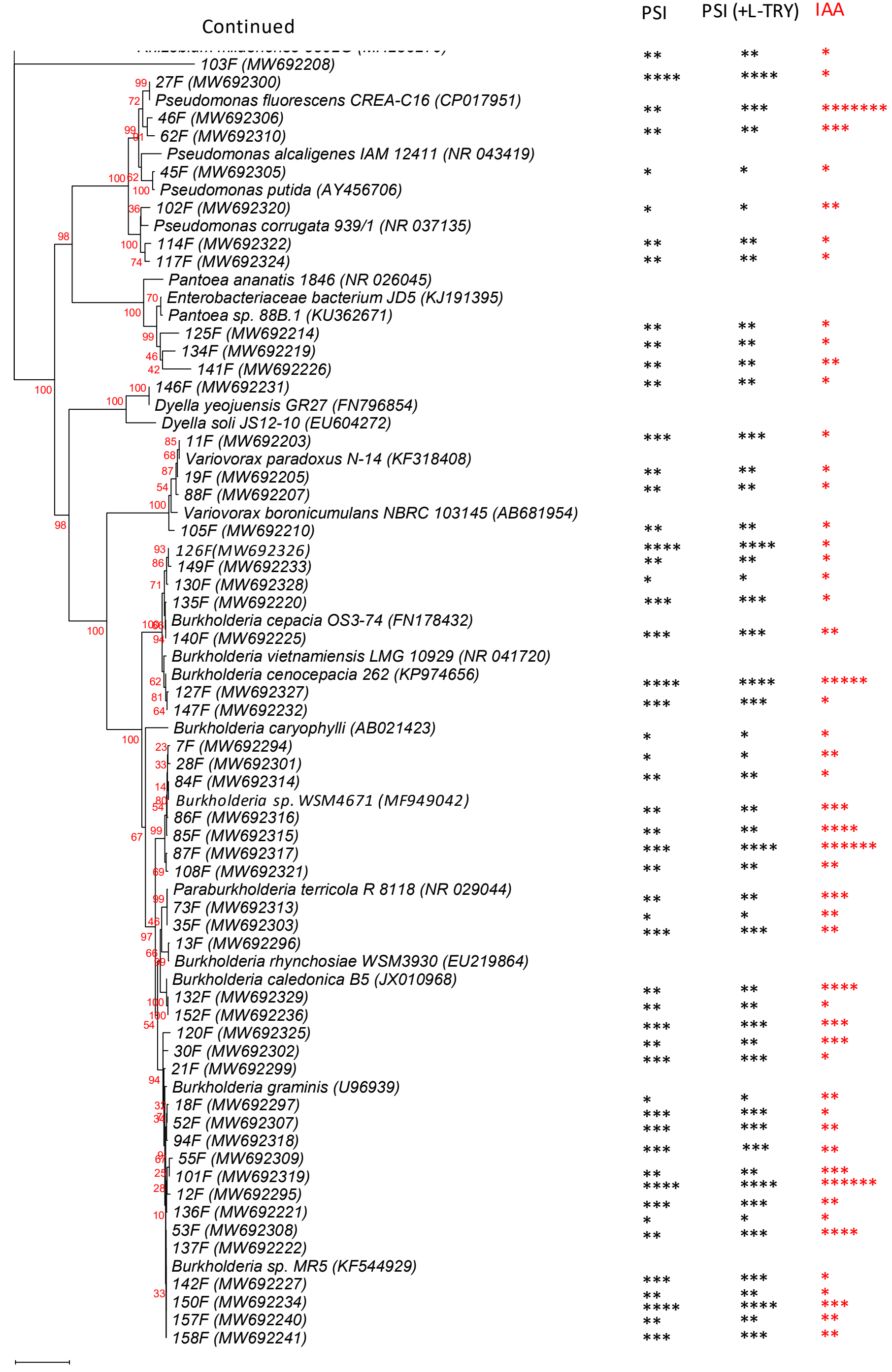


Fig. 1 Neighbour-joining phylogenetic tree showing the relationship between the isolates and their related type strains based on 16S rDNA sequences. Bar, 0.02 substitutions per nucleotide position. PSI: phosphate solubilisation index, *- PSI value <2, **- 2-3, ***>3-4, ****>4.0. IAA (µg mL^{-1}) concentration *- < 10, **- 10 - 20, ***- 20 – 30, ****-30 – 40, *****- 40 – 50, ******- 50 – 60 and *******- 60 – 70. Black and red asterisks represent PSI value and IAA concentration, respectively.

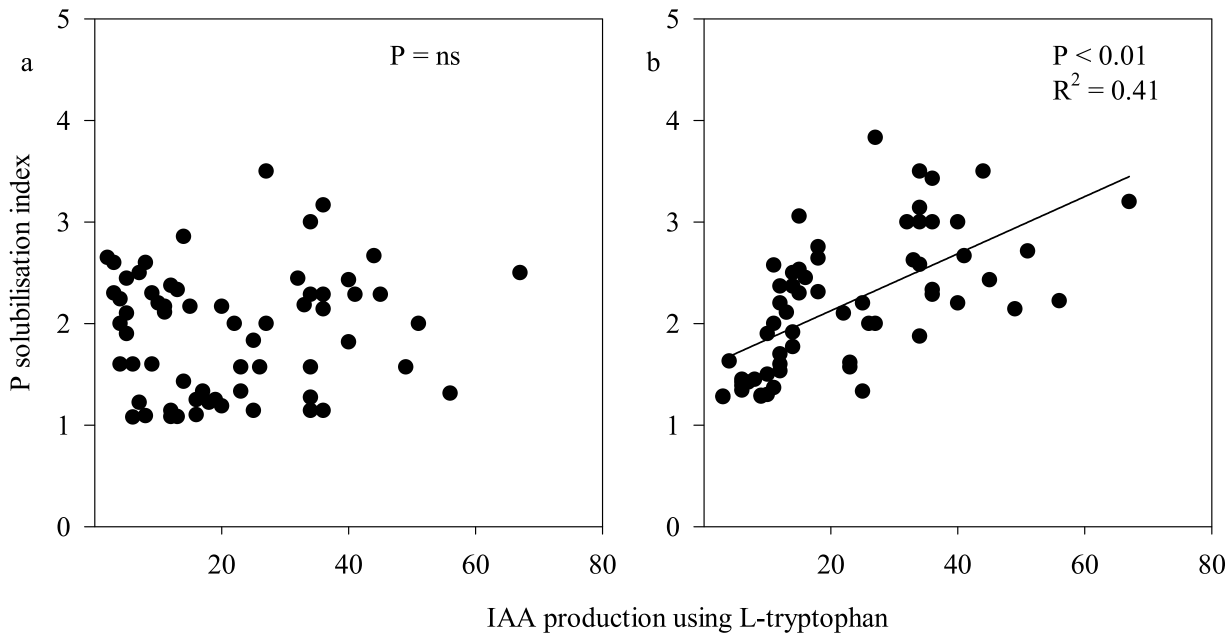


Fig. 2 The relationship between solubilisation index of 98 isolates of P solubilising bacteria in National Botanical Research Institute's Phosphate growth (NBRIP) medium with (a) no supplement of L-tryptophan and (b) L-tryptophan (500 µg mL^{-1}), after 10 days of incubation and the ability of rhizobacteria to produce IAA (µg mL^{-1}) in DF-minimal media using L-tryptophan. *P* = ns- non-significant, *P* < 0.01- highly significant.

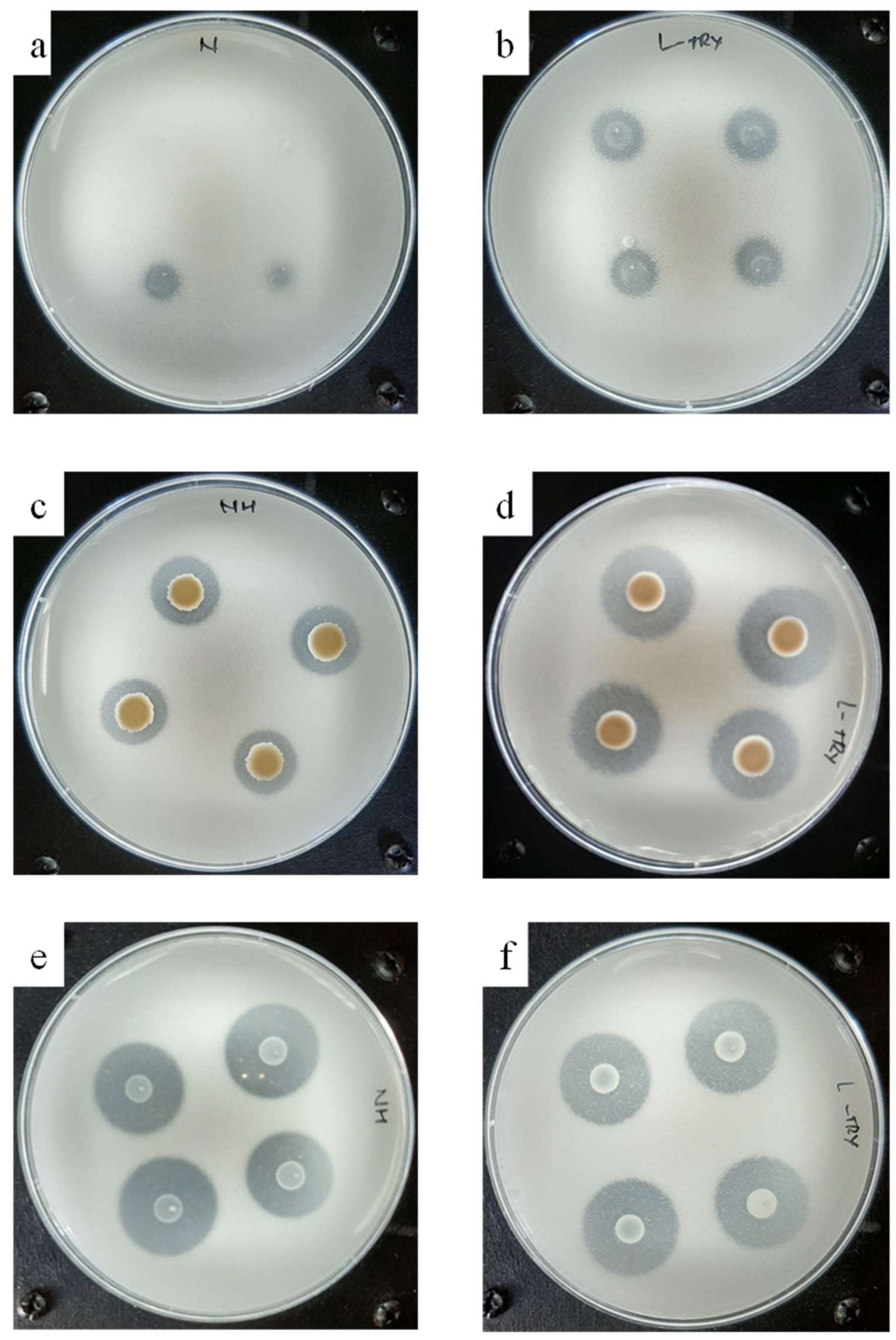


Fig. 3 P solubilisation zone for *Peribacillus simplex* 54F (a and b), *Burkholderia* sp. 12F (c and d) and *Burkholderia cenocepacia* 127F (e and f) on National Botanical Research Institute's Phosphate growth (NBRIP) medium containing $Ca_3(PO_4)_2$ as sole P source. The medium was supplemented with L-tryptophan (b, d and f) or not supplemented (a, c and e).

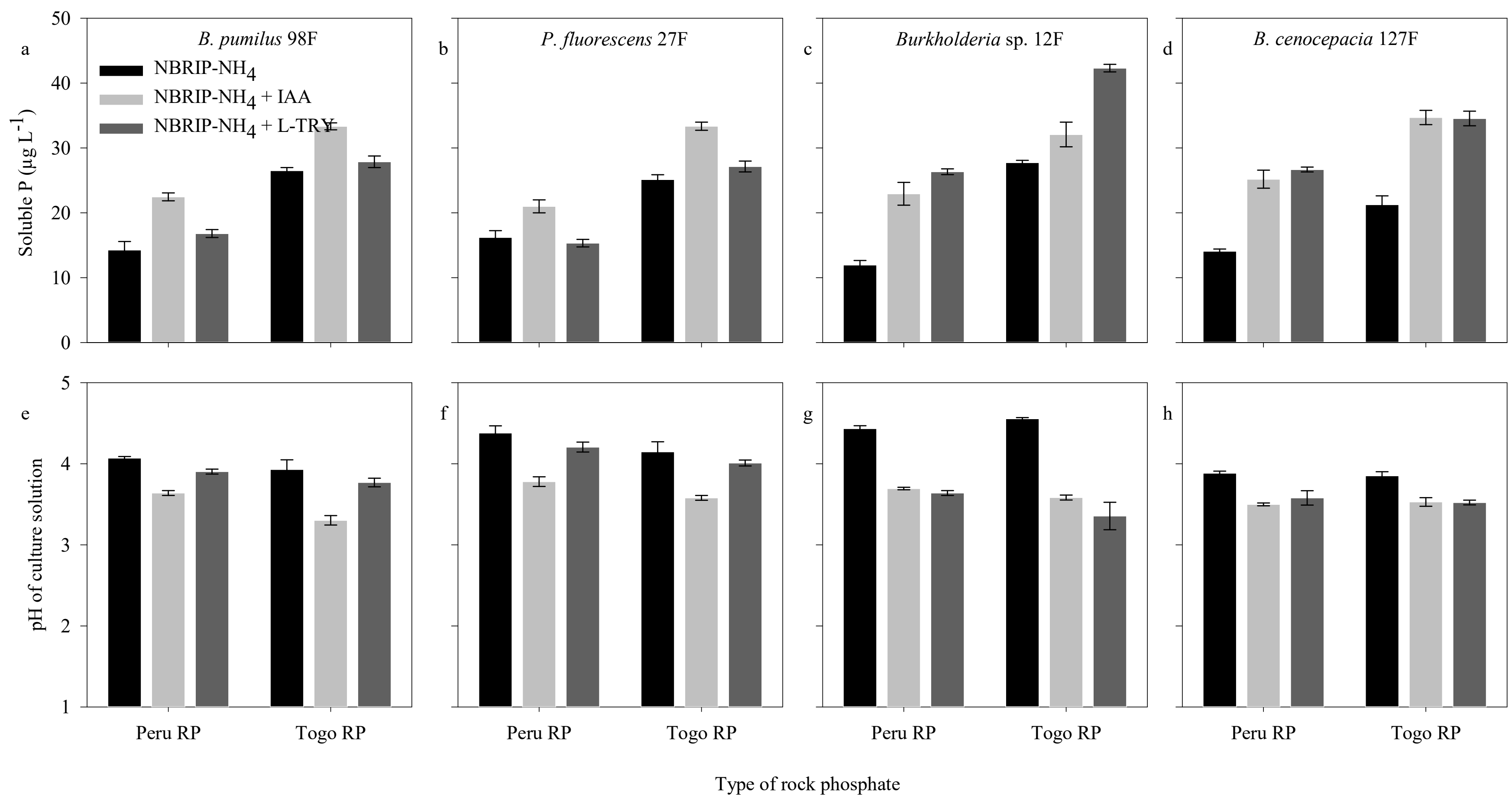


Fig. 4 Ability of *Bacillus pumilus* 98F (a and e), *Pseudomonas fluorescens* 27F (b and f), *Bukholderia* sp. 12F (c and g) and *Burkholderia cenocepacia* 127F (d and h) to solubilise P and alter the pH of National Botanical Research Institute's Phosphate growth (NBRIP) medium containing 5 g per litre of Peru or Togo rock phosphate with no supplement, IAA (100 µg mL^{-1}) and L-tryptophan (500 µg mL^{-1}). Cultures were incubated on a shaker (28 ºC, 160 rpm) for 14 days. Results represent the mean of three replicates ±SE. TRY: L-tryptophan, RP: Rock phosphate.

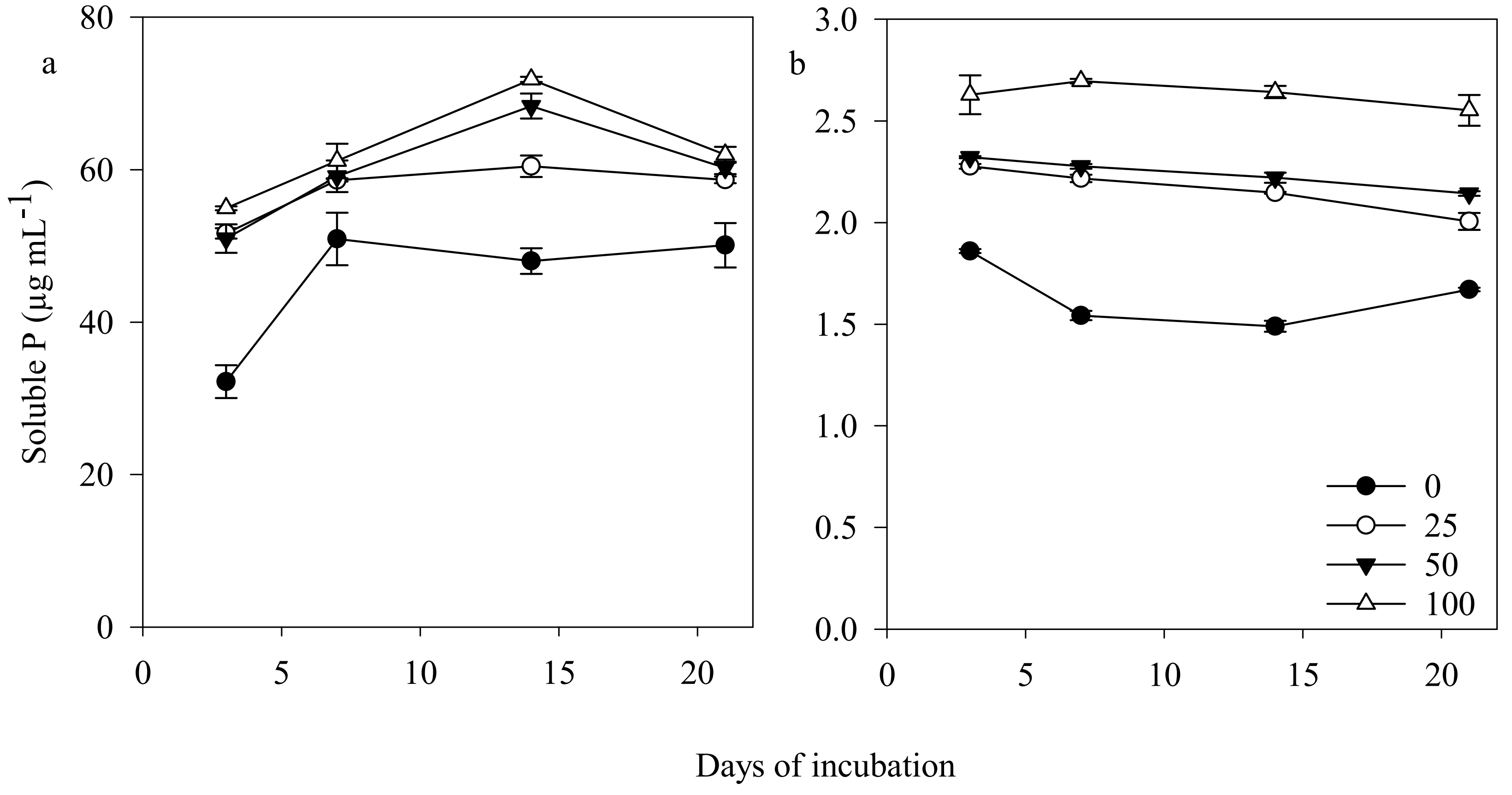


Fig. 5 Amount of Pi solubilised from rock phosphate in National Botanical Research Institute's Phosphate growth (NBRIP) medium containing different IAA concentrations (μg mL^{-1}) (a) inoculated with *Burkholderia* sp. 12F and (b) with no inoculation. Cultures solutions were incubated on a shaker (28 ºC, 160 rpm) for 21 days. Results represent the mean of three replicates ± SE.

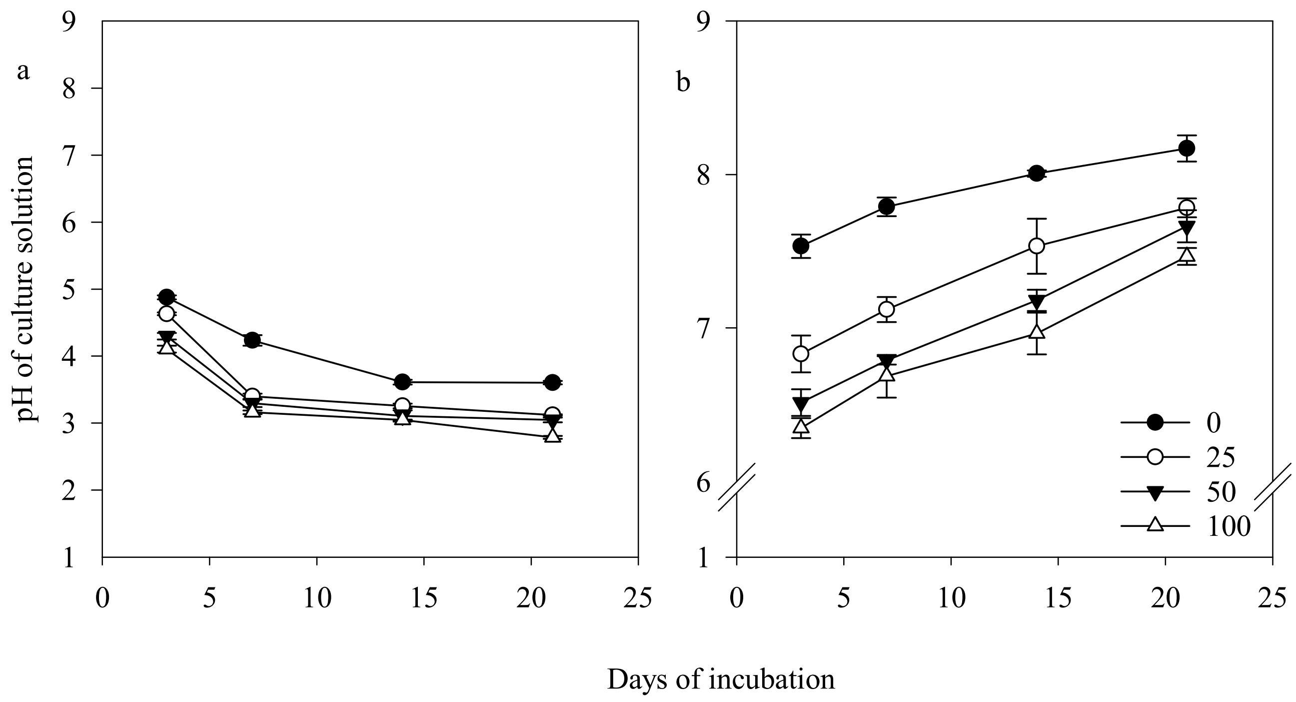


Fig. 6 Change in pH in National Botanical Research Institute's Phosphate growth (NBRIP) medium containing IAA at nil, 25, 50 and 100 µg mL^{-1} with (a) *Burkholderia* sp. 12F and (b) no inoculation. Results presented from the culture solution incubated in shaker incubator (28 ºC, 160 rpm) for 21 days. Results represent the mean of three replicate ±SE.

Chapter 8. The role of carboxylate concentration, rhizosphere pH, the reactivity of P sources, and phosphate solubilising bacteria in P acquisition by N_2 fixing chickpeas

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8.1. Statement of Authorship

Title of Paper	The role of carboxylate concentration, rhizosphere pH, the reactivity of P sources, and phosphate solubilising bacteria in P acquisition by N2 fixing chickpeas
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Principal Author

Name of Principal Author (Candidate)	Anteneh Argaw		
Contribution to the Paper	Planned the study conducted all experiments, analysed and interpreted data and wrote the manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate’s stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate’s stated contribution.

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8.2. Abstract

Chickpea roots exude a large amount of carboxylates that can mobilise P from sparingly soluble P sources but the role of carboxylate concentration, pH, reactivity of P sources and activity of phosphate solubilising bacteria (PSB) in improving P acquisition abilities of chickpea cultivars is poorly understood. Six rock phosphates with different chemical characteristics were used to test the potential for P release when PSB were inoculated. The activity of PSB and carboxylates in the solubilisation of these rock phosphates was determined. The effect of PSB on the chickpea-*Mesorhizobium* symbiosis under different P conditions was investigated using six cultivars with contrasting rhizosphere carboxylate concentrations and pH. The PSB strain *Burkholderia* sp. 12F showed the highest potential solubilisation of P from Boucraa, Togo, Sechura and Weng Fu rock phosphates. None of the bacterial isolates tested were capable of solubilising P from Phalaborwa, Peru and Vietnam rock phosphates. Additionally, chickpea root exudates solubilised Togo rock phosphate but not Peru rock phosphate under *in vitro* conditions. The presence of PSB significantly increased the amount of solubilised P in the root exudates. Inoculation of PSB significantly increased shoot and root biomass production, and nodulation of chickpea cv. Genesis-863, PBA-Striker and PBA-Slasher but did not significantly affect other cultivars (Genesis-090, Genesis-079 and Ambar). The P solubilising activity of PSB was able to explain the growth promotion of responsive cultivars when fertilised with Togo or Peru rock phosphate. P solubilising activity in these cases following inoculation with PSB was not related to the extent of acidification of the rhizosphere. Under no or low P conditions or with the addition of P as KH_2PO_4, growth promotion was observed but was unrelated to the P solubilising activity of PSB, suggesting that other plant growth-promoting (PGP) mechanisms such as indole acetic acid (IAA) and/or 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity may be involved. The amount of carboxylate produced in the rhizosphere may partly explain the efficacy of PSB in promoting chickpea growth.

Keyword(s): Carboxylate, *Burkholderia*, *Pseudomonas,* Rock phosphate

8.3. Introduction

Phosphorus (P) is a major yield-limiting nutrient in most agricultural soils. P deficiency prevails in most soil types, particularly in soils with extremely acidic or alkaline pH and results in the build-up of plant unavailable P, predominantly $FePO_4$ and $AlPO_4$ at low pH, and $Ca_2(PO_4)_3$ at high pH (Whitelaw 1999; Gyaneshwar et al. 2002). Consequently, the concentration of plant-available P in soil solution is frequently very low (0.01 to 10 μM), and can be insufficient for optimal plant growth (Hinsinger 2001). This calls for the use of synthetic chemical phosphate fertilizer in combination with other measures to make fertiliser use more efficient (Arcand and Schneider 2006). Because of its high and unaffordable price in developing countries and the high risk of over-fertilization of P on the environment, the use of rock phosphate is increasing due to increases in organic agriculture worldwide (Straaten 2007; Arcand and Schneider 2006). Rock phosphate is the cheapest means to apply chemical phosphatic fertilizers for plants (Chien et al. 2010). However, the solubilisation of rock phosphate in soils is generally too slow to release inorganic P at rates necessary to satisfy the agronomic requirements of plant growth (Zapata and Zaharah 2002). The low solubility of phosphate rock has discouraged its use as a sole P source for crops.

The use of microbes able to solubilise phosphate is gaining more attention as a way to improve the efficient use of rock phosphates (Gyaneshwar et al. 2002; Vassilev et al. 2006; Swift et al. 2018; Alemneh et al. 2020). The use of efficient phosphate solubilising bacteria (PSB) can improve plant P nutrition from rock phosphate applied to soil (Manzoor et al. 2017; da Costa et al. 2015; Kaleem Abbasi and Manzoor 2018;). Acidification of the growing medium and production of low molecular weight carboxylates have been identified as the main mechanisms involved in microbial rock phosphate solubilisation (Illmer and Schinner 1995). In a successful PSB-root interaction, P nutrition for plants has been improved through increasing root growth and/or P solubilisation in the rhizosphere (Rezakhani et al. 2019; Elhaissoufi et al. 2020).

Chickpea is a cool season pulse crop that can fix N through the formation of a symbiotic association with *Mesorhizobium* spp. An adequate supply of P is required to optimise the process of N_2 fixation. Like some PSB, chickpea can exude large amounts of low molecular-weight carboxylates from the root system (Veneklaas et al. 2003). These are mainly malonate, citrate and malate, which can mobilise a significant amount of P from sparingly soluble P sources and fixed P in soils (Ohwaki and Hirata 1992; Veneklaas et al. 2003; Wouterlood et al. 2004b). Additionally, trace amounts of lactate, maleate, cis-aconitate, fumarate, acetate,

succinate or trans-aconitate have been detected in chickpea root exudates (Neumann and Römheld 1999; Pearse et al. 2006). The amount of carboxylates in chickpea root exudates can be 11 and 24 times more than in pigeon pea and soybean, respectively (Ohwaki and Hirata 1992). A study conducted by Pang et al. (2018) using chickpea genotypes with wide variation in carboxylate production investigated the ability to access P from $FePO_4$. In this study, the P concentration in mature leaves was positively correlated with total carboxylates in the rhizosphere and negatively with rhizosphere pH.

Increases in nodulation and growth promotion following inoculation of PSB on to chickpea is not fully explained by P concentration in the rhizosphere and plant P uptake (Zaidi et al. 2003; Rudresh et al. 2005; Mittal et al. 2008; Verma et al. 2012; Singh et al. 2014; Verma et al. 2014; Imen et al. 2015; Saxena et al. 2015; Israr et al. 2016; Rajwar et al. 2018). These studies were conducted using cultivars that had different rhizosphere carboxylate concentrations, rhizosphere pH and root biomass, in different P conditions, using PSB able to express various beneficial plant growth-promoting (PGP) mechanisms and with different P amendments. A considerable variation in root traits and carboxylate concentration in the root exudates has been found in tests of a wide range of chickpea germplasm (Chen et al. 2017; Pang et al. 2018). The chickpea genotypes with high amount of carboxylate tended to have relatively thinner roots, with lower costs of root construction (Wen et al. 2020). Consequently, there is a need for mechanistic studies to investigate factors, such as rhizosphere pH and root biomass production, that could affect PSB function in chickpea cultivars with contrasting rhizosphere carboxylate concentrations when supplied with different P sources. To examine this, we conducted *in vitro* experiments to investigate the solubilisation of different rock phosphates by efficient PSB and by carboxylates that were produced by tested isolates. Accordingly, we selected two rock phosphates, of moderate and of low reactivity. Six cultivars that produced contrasting concentrations of carboxylates were selected. The objective of this study was to investigate how the PSB benefited the chickpea-*Mesorhizobium* symbiosis under different P sources, which is a test for possible coordination among root biomass production, rhizosphere pH and the effects of PSB inoculation in improving P nutrition and as a result enhancing the chickpea-*Mesorhizobium* symbiosis.

8.4. Materials and Methods

8.4.1. Soil collection

Soil samples were collected from 74 agricultural cropping lands in southern and eastern Australia (Appendix Fig. 1 and Appendix Table 1) and kept at 4ºC until processed further. A 100 g sample was taken from each soil to grow chickpea cv. HatTrick in small pots, which were kept in a growth chamber with fluorescent lighting (400 microeinsteins m^{-1} s^{-1}), programmed for a 16 h photoperiod, with a constant temperature at 17 ºC and 70% relative humidity. After four weeks of growth, the soil strongly adhering to the root surface was collected and kept at 4 ºC before carrying out the microbial experiments.

8.4.2. Isolation of bacteria

Rhizospheric bacteria were obtained using three methods of selective isolation. One g of rhizospheric soil was suspended in 9 mL of sterile MilliQ water and this soil suspension was serially diluted (10^{-2} - 10^{-6}). An aliquot (100 µL) of solution from 10^{-5} and 10^{-6} dilutions was aseptically spread on semi-selective 1/10 strength tryptone soya agar (TSA) (OXOID LTD, Basingstoke, Hampshire, England) supplemented with 50 µg mL^{-1} of ampicillin (99%, Sigma-Aldrich, CHEMIE GmbH, Steinheim, Germany), 12.5 µg mL^{-1} of chloramphenicol (Sigma-Aldrich, 99%) and cycloheximide (75 µg mL^{-1}) to isolate *Pseudomonas*-like bacteria (Simon and Ridge 1974). Aerobic endospore-producing bacteria were isolated using 1/10 TSA after the soil suspensions (10^{-5} and 10^{-6}) were treated at 80 ºC for 10 min. Five days post-incubation at 28 ºC, distinct colonies were further purified by re-streaking on fresh TSA. Additionally, efficient phosphate solubilising bacteria from rhizospheric soil were enriched in National Botanical Research Institute's Phosphate growth medium (NBRIP) supplemented with 5 g of $Ca_3(PO_4)_2$ (Sigma-Aldrich), AlO_4P (Sigma-Aldrich) or $FeO_4P.2H_2O$ (Sigma-Aldrich) (Nautiyal 1999). A 100 µL aliquot of enriched bacterial consortia was aseptically spread on NBRIP agar and incubated at 28 ºC for ten days. Colonies with distinct clearing zones were further purified by streaking on NBRIP agar. Pure colonies were preserved at 4 ºC for immediate use and -80 ºC for long-term storage.

8.4.3. Phylogenetic diversity of phosphate solubilising bacteria

Purified colonies of phosphate solubilising bacteria were grown on TSA medium for 48 h at 28 ºC and used for genetic analysis. The 16S rDNA was amplified using universal primers fD1:5'-AGAGTTTGATCCTGGCTCAG-3' (Sigma-Aldrich) and rP2: 5'-

ACGGCTACCTTGTTACGACTT-3’ (Sigma-Aldrich) (Weisburg et al. 1991). Amplification was carried out in 25 µL containing 12.5 µL MiFi^{TM} Mix (Sigma-Aldrich), 1 µL of each primer and 10.5 µL sterile MilliQ water. Thermocycling conditions were 1 min initial denaturation at 95 ºC followed by 35 amplification cycles consisting of a 15 s denaturation step at 95 ºC, 30 s annealing step at 57 ºC, 1 min extension at 72 ºC and a final extension cycle at 72 ºC for 5 min. Amplicons were confirmed by 1% agarose gel electrophoresis. Sequences closely related to the isolates used in this study were retrieved from the National Centre for Biotechnology Information GenBank BLAST program (www.ncbi-nlm-nih.gov). Sequencing reactions were performed as a direct sequence of PCR products by AGRF (Australian Genome Research Facility) Sanger Service (University of Adelaide, South Australia). Phylogenetic trees were constructed by the neighbour-joining method using MEGA 7.0 software.

8.4.4. Identification of phosphate solubilising bacteria

The phosphate solubilisation characteristics of 841 bacterial isolates were investigated qualitatively by applying 10 µL of bacterial inoculum at four places on NBRIP agar containing $Ca_3(PO_4)_2$. Plates were incubated for 10 days at 28 ºC. The P solubilisation zone and colony diameter were measured. The phosphate solubilisation index (PSI) was calculated using the formula described by Nguyen et al. (1992). Based on PSI values, isolates were selected for a study of quantitative P solubilisation from AlO_4P, $Ca_3(PO_4)_2$ or $FeO_4P.2H_2O$. Based on these preliminary results, three isolates, namely *Burkholderia cenocepacia* 127F, *Pseudomonas fluorescens* 27F and *Burkholderia* sp. 12F exhibiting the highest P solubilisation efficiency were selected (Chapter 4) and used in subsequent experiments. All experiments were carried out in triplicate.

8.4.5. Type of rock phosphate

The mineral composition of the rock phosphates is presented in Appendix Table 3. Seven rock phosphates, namely Boucraa, Vietnam, Weng Fu, Togo, Phalaborwa, Sechura and Peru phosphate rock were used. Rock phosphates were ground and sieved through 500 µm size mesh. Five g of each rock phosphate was used in subsequent experiments.

8.4.6. Experiment 1. The solubility of rock phosphate in carboxylate solutions

Seven rock phosphate with contrasting chemical properties were used to examine their solubility in different carboxylates under sterile condition. The carboxylates comprised acetate, citrate, gluconate, α-ketoglutarate, lactate, maleate, malate, oxalate and succinate. These

carboxylates were produced by efficient PSB during $Ca_3(PO_4)_2$, $FeO_4P.2H_2O$ and AlO_4P solubilisation (Chapter 4). The rock phosphate samples were mixed with 50 mL of solution in 100 mL bottles containing one of the aforementioned carboxylates or one inorganic acid such as H_2SO_4. Both organic and inorganic acids were commercial products of reagent grade. The concentration of organic and inorganic acids was 10 mM, which is an approximate concentration of total carboxylate produced by the chickpea rhizosphere (Wouterlood et al. 2005). Rock phosphates suspended in MilliQ water were used as control check treatments. The solutions were kept in a shaker incubator (3 days, 28 ºC, 160 rpm). After three days of incubation, samples were collected for soluble P (Pi) and pH determination.

8.4.7. Experiment 2. Microbial rock phosphate solubilisation

B. cenocepacia 127F, *P. fluorescens* 27F and *Burkholderia* sp. 12F were further investigated for their potential to solubilise P from different rock phosphates. One mL of active culture solution was transferred into bottles containing sterile 50 ml of NBRIP medium containing 5 g of rock phosphate. The inoculated solutions were incubated in the same conditions as described for Experiment 1. After fourteen days of incubation, five mL of culture suspension were taken every 2 days for pH, carboxylate determination and Pi analysis.

8.4.8. Experiment 3. The solubility of rock phosphates in chickpea root exudates

The ability of chickpea root exudates to solubilise rock phosphate was tested. Six chickpea cultivars with contrasting rhizosphere carboxylate concentrations, rhizosphere pH and root biomass were selected (Pang et al. 2018). Cultivars used were Ambar, Genesis -090, Genesis-079, Genesis-836, PBA-Slasher and PBA-Striker. Genesis-09, Ambar and Genesis 079 had relatively large amounts of carboxylate (13 - 27 µmol $plant^{-1}$) in their rhizosphere. While Genesis-863, PBA-Striker and PBA-Slasher had relatively small amounts (8 - 14 µmol $plant^{-1}$).

One seedling per pot was grown in a growth chamber. Root exudates were collected from each pot separately and kept at -20 ºC for further analysis. The solubility of two types of rock phosphate (Peru and Togo rock phosphates) in root exudate, each collected per seedling, in the presence and absence of PSB was investigated. The rock phosphate was ground and sterilised (121 ºC, 30 min). Root exudate was filter sterilised (0.2 µm). Five g of sterilised rock phosphate was added to 50 mL of filter sterilised root exudate. After 7 days of agitation in a shaker incubator at 160 rpm, pH and Pi were determined.

8.4.9. Experiment 4. Plant inoculation experiment

This experiment was a factorial randomised design with three factors (six chickpea genotypes, + and - PSB inoculation and four P treatments). Seeds were soaked in 5% hypochlorite solution for 3 min and rinsed five times with sterilised MilliQ. This pot experiment was conducted in a growth chamber with fluorescent lighting (400 microeinsteins m^{-1} s^{-1}), programmed for a 16 h photoperiod, with a constant temperature at 17 ºC and 70% relative humidity.

Plastic pots (11 cm × 7 cm × 8 cm) were filled with 1 kg of sand: vermiculite mix (1:1 v/v), which contained 3 µg P kg^{-1} and the mix was sterilised (121 ºC, 1 h). Sparingly soluble P sources were applied at 40 µg P g^{-1} of growing medium. The P treatments comprised K_2HPO_4, Peru and Togo rock phosphate, and control unfertilised treatment. Four surface-sterilised seeds were placed in each pot and these were thinned to two seedlings seven days after sowing and covered by a layer of plastic beads to minimise microbial contamination. The PSB treatment included inoculation of *Burkholderia* sp. 12F and control uninoculated chickpea. This PSB isolate was selected based its ability to solubilise P from rock phosphate *in vitro* in the present study. Colonies of the PSB (*Burkholderia* sp. 12F) and of *Mesorhizobium ciceri* CC1192 were inoculated into yeast-mannitol and tryptone soya broth, respectively. The cultures were incubated in a shaker incubator (28 ºC, 48 h). One mL of culture solution of *Burkholderia* sp. 12F was inoculated per seedling according to the treatment layout. All treatments received 1 mL of *M. ciceri* CC1192 inoculant. All treatments were replicated three times. McKnight's N free nutrient solution containing all essential nutrients was prepared according to McKnight (1949). The growing mix was initially moistened with N-free nutrient solution to approximately 80% of field capacity, which was later maintained during the experiment by watering pots with sterilised MilliQ water.

Plants were harvested seven weeks after inoculation. Shoots were separated from roots. The sand was carefully tipped out of the pots and root systems were gently shaken to remove non-rhizospheric sand. The sand remaining attached to the root was defined as rhizospheric sand. To measure the pH and P concentration in the rhizosphere region, 1 g of sand from the rhizosphere was placed into a vial in 5 mL of MilliQ water. The solution was separated from sand after centrifugation (10,000 ×*g*, 10 min). The supernatant was kept at 4 ºC for further analysis. Soluble P concentration in the supernatant was determined using the molybdate-blue method.

After taking the rhizosphere samples, the roots were washed and nodules were carefully detached. The nodule number and nodule colour rating were recorded. The chlorophyll content of five randomly selected leaves was measured using a SPAD meter. The nodules, roots and shoots were dried at 70 ºC for two days and weighed. The root mass ratio was calculated as the ratio of root dry weight to total shoot dry weight. The dried shoot biomass was ground separately and the shoot P content was determined via acid digestion and inductively coupled plasma spectrometry (Zarcinas et al. 1996).

8.4.10. Determination of available P in the supernatant

The amount Pi in samples collected from the above experiments was determined following the molybdate-blue method (Murphy and Riley 1962). Samples were centrifuged (10,000 ×*g*, 10 min). A 2 mL aliquot of the filtrate and 0.4 mL of reagent containing 5 N of sulphuric acid, 0.1 M of ascorbic acid, 1 mg of potassium antimonyl tartrate were mixed. The mixture was allowed to stand for 30 min and absorbance (A) of the mixed solution was determined at 882 nm using a spectrophotometer.

8.4.11. Data analysis

Before implementing the analysis of results obtained from *in vitro* experiments, values obtained with the uninoculated control check treatment were always subtracted from their respective treatments. All statistical analysis was performed using Genstat ver.15. To investigate P solubilising activities of efficient PSB from chemically diverse rock phosphate, a two-way analysis of variance (ANOVA) was performed to investigate rock phosphate solubilisation by different PSB isolates.

To understand the possible mechanisms of P solubilisation, the solubility of rock phosphates in different carboxylates that were produced by tested PSB was investigated using two-way ANOVA with rock phosphate and carboxylates as factors. The solubility of Peru and Togo rock phosphate in root exudates obtained from six cultivars of chickpea and the effect of PSB were compared using two-way ANOVA. The pooled means are presented as the bar graphs and box plots.

To investigate the role of carboxylate concentration in the chickpea rhizosphere, PSB inoculation and solubilisation of P source for P acquisition strategies of chickpea, the effect of PSB, cultivars and P sources and their interaction on growth, nodulation, P concentration and pH in rhizosphere extract and plants P uptakes were estimated by fitting the following model:

Y = *Cultivar* + *PSB* + *P* + *Cultivar**PSB* + *Cultivar**P* + *P*PSB* + *Cultivar*PSB*P* + *Rep* + *e-*

Where, *Y* is the response variable, as determined by the main effects of *cultivar*, *PSB*, *P* and their interaction. *Rep* and *e* are a random replicate effect and residual error, respectively. Significant differences between pooled means were separated by Fisher's protected LSD at a significance level of $P < 0.05$. The pooled means are presented in bar graphs with error bars denoting standard errors of n = 3 treatment replicates.

The regression analysis between plant parameters, P uptake, P concentration and pH in rhizosphere extract was performed using SigmaPlot ver. 14. Principal component analysis (PCA) was performed using SigmaPlot ver 14 to determine the multivariate coordination of ten plant traits for six cultivars of chickpea fertilised with different P sources under contrasting PSB inoculation treatments.

8.5. Results

A diverse range of species of PSB belonging to *Burkholderia*, *Bacillus*, *Pseudomonas*, *Variovorax*, *Dyella*, *Pantoea*, *Sphingomonas*, *Caulobacter*, *Phyllobacterium*, *Rhizobium*, *Curtobacterium*, *Microbacterium*, *Cellulomonas* and *Mycolibacterium* were obtained from 74 soil samples collected across Australia (Chapter 4) using enrichment and taxonomically selective methods. Among these, bacterial isolates which were able to solubilise a significant amount of Pi from $Ca_3(PO_4)_2$, AlO_4P and $FeO_4P.2H_2O$ were selected to investigate their ability to solubilise P from different types of rock phosphate in the NBRIP medium and in root exudate extracts of six cultivars of chickpea *in vitro*. Their effectiveness in enhancing the chickpea-*Mesorhizobium* symbiosis was also investigated.

8.5.1. The release of P from rock phosphate by efficient P solubilisers

The total P concentration and purity of rock phosphates from different locations worldwide are presented in Appendix Table 1. The amount of total P varied between 12.6 and 16.0 %. Phalaborwa phosphate rock had the highest total P (16.0%) while the lowest P contents were found in Vietnam phosphate rock (12.6%) followed by Peru phosphate rock (14.7%). The concentrations of Al^{3+}, Cd^{2+} and Fe^{3+} varied widely between the rock phosphate samples. Boucraa rock phosphate had the highest concentration of Al^{3+} and Fe^{3+}. The highest concentration Cd^{+2} was present in Togo rock phosphate followed by Boucraa rock phosphate.

The efficiency of PSB in phosphate solubilisation *in vitro* varied widely across the rock phosphate samples. *Burkholderia* sp. 12F produced significantly higher Pi from Boucraa, Sechura and Weng Fu rock phosphate (Fig. 1a, d and g). On the contrary, none of the isolates was able to liberate a significant amount of Pi from Phalaborwa, Peru and Vietnam rock phosphate (Fig. 1b, c and e). The highest Pi concentration released from Sechura rock phosphate by *Burkholderia* sp. 12F was not always associated with the extent of acidification of the culture solutions (Fig. 2a-g). All tested isolates were able to produce diverse carboxylates, predominantly di- and tri-carboxylates during mineral phosphate solubilisation (Chapter 4). The carboxylates included acetate, citrate, gluconate, α-ketoglutarate, lactate, maleate, malate, oxalate and succinate.

8.5.2. The solubility of rock phosphate in carboxylates

To understand the possible mechanisms governing their efficiency to solubilise rock phosphate, the P solubilising potential of the aforementioned carboxylates was investigated *in vitro*. The relative efficiency of these carboxylates in the dissolution of rock phosphate varied widely (Table 1). The lowest Pi concentration released from all rock phosphates was recorded in MilliQ water (negative control) while the highest concentration of Pi released was obtained in dilute H_2SO_4 (10 mM) followed by oxalate, maleate and citrate. Among the carboxylates investigated, the average rate of dissolution of rock phosphates by carboxylates (10 mM) decreased as follows: oxalate > maleate > citrate > succinate > malate > α-ketoglutarate > lactate > acetate > gluconate.

The carboxylates induced varying degrees of acidification of the solutions containing 7 different rock phosphates (Table 2). The low solubility of rock phosphates in the presence of acetate or gluconate was also associated with higher pH. The lowest pH was induced by oxalate and citrate. The remaining carboxylates induced pH ranging between 3.00 and 4.00.

The solubility of the different rock phosphates in carboxylate solutions varied widely: in acetate from 0.48 to 7.16 $\mu g\ mL^{-1}$ Pi, in citrate from 8.6 to 59.9 $\mu g\ mL^{-1}$, in gluconate from 1.0 to 2.6 $\mu g\ mL^{-1}$, in lactate from 8.0 to 19.3 $\mu g\ mL^{-1}$ and water (control) from 1.02 to 2.69 $\mu g\ mL^{-1}$. The lowest amount of Pi was liberated in acetate, citrate or succinate solutions from Phalaborwa rock phosphate. Vietnam rock phosphate released the lowest Pi concentration in lactate, α-ketoglutarate, maleate, malate or oxalate solution. Our results also indicated some variation in solubility among the rock phosphates tested, as measured by Pi released in H_2SO_4 (51.6 to 56.7 $\mu g\ mL^{-1}$). However, the amount of P solubilised varied widely in α-ketoglutarate

(17.5 to 31.5 μg mL^{-1}), maleate (36.0 to 49.6 μg mL^{-1}), malate (17.0 to 36.1 μg mL^{-1}) and oxalate **(**38.8 to 51.6 μg mL^{-1}).

8.5.3. The solubility of rock phosphate in root exudates of chickpea cultivars

The ability of root exudates, collected from six cultivars of chickpea, to solubilise P was investigated *in vitro*. The pH of the exudates varied between 3.2 and 3.6. After seven days of incubation, the pH of the solutions was increased to between 5.25 to 5.7 and 6.45 to 8.22 in the presence and absence of PSB, respectively (Fig. 3a and b). In the absence of PSB, exudates obtained from Genesis-090, Genesis-079, Ambar and PBA-Slasher were slightly more acidic than those from Genesis-863 and PBA-Striker incubated with Togo rock phosphate (Fig. 3c). This variation was not observed when P source was Peru rock phosphate (Fig. 3d).

The amount of solubilised P from Togo and Peru rock phosphate varied between 0.5 and 3.6μg L^{-1} and 12.3 to 21.7 μg L^{-1}, respectively (Fig. 3c and d). The highest concentrations of Pi from Togo and Peru rock phosphates were obtained with the exudate of Genesis-090 followed by exudates of Ambar and PBA-Striker. The lowest Pi concentrations were obtained with the exudate of Genesis-863. In these solutions, the association between the concentration of Pi released from both rock phosphates and the pH of the exudate before and after incubation was non-significant (Fig. 3g, h, i and j).

Addition of PSB to the exudate significantly increased rock phosphate solubilisation (Fig 3e and f). PSB solubilised the highest concentrations of Pi from Togo rock phosphate in exudates of Genesis-090 and PBA-Striker, followed by Ambar (Fig 3c). The highest concentration of Pi from Peru rock phosphate was mobilised by incubation in exudate of Genesis-863 followed by PBA-Striker (Fig. 3d). PSB increased Pi release from Peru and Togo rock phosphate during incubation in exudates from Genesis-863, PBA-Striker and PBA-Slasher by 0.7 to 0.9 and 2.2 to 2.1 times, respectively, compared to uninoculated controls. PSB increased Pi release from Peru and Togo rock phosphate between 0.14 to 0.32 and 0.3 to 0.5 times, respectively, relative to uninoculated controls, when incubated with exudates of cv. Ambar, Genesis-090 and Genesis-079. In these culture media, the amount of Pi released and the extent of acidification were significantly and inversely correlated (Fig. 3k and l).

8.5.4. Effect of PSB inoculation on the Chickpea-*Mesorhizobium* symbiosis

Three-way interactions between chickpea cultivar × PSB × P source impacted the shoot ($P < 0.026$) and root dry weight ($P < 0.05$) (Table 3). Looking at individual P source, the effect of

PSB (*Burkholderia* sp. 12F) on plant growth varied with different cultivars of chickpea. Regardless of the P source supplied, the shoot dry weight (SDW) and root dry weight (RDW) of PBA-Striker were significantly increased by PSB inoculation (Fig. 4a-h). Additionally, PSB increased these plant growth parameters when inoculated on to cv. Genesis-836 and PBA-Slasher when fertilised with Togo or Peru rock phosphate. However, PSB inoculation did not significantly affect biomass production of the remaining cultivars.

Interactions between cultivar, PSB and P source affected nodule number per plant (NN, $P < 0.001$) but not nodule dry weight (NDW, $P = 0.601$) and nodule colour rating (NCR, $P = 0.192$) (Table 3). Nodule dry weight was significantly improved by PSB inoculation for Genesis-863, PBA-Striker and PBA-Slasher with no P application and with Peru rock phosphate (Fig. 4i-l). PSB increased the NDW of PBA-Striker and Genesis-863 when fertilised with Togo rock phosphate and K_2HPO_4, respectively.

Two-way interactions between PSB and P source influenced the rhizosphere P concentration ($P < 0.021$) and pH ($P < 0.001$) (Table 3). The lowest pH (5.1 and 5.3) was induced in the rhizosphere of cv. Genesis-079 and Ambar when fertilised by KH_2PO_4 (Fig. 5a-d). A less acidic pH (6.2 and 6.0) was observed in the rhizosphere of Genesis-863 and PBA-Striker, respectively fertilised, when with Peru rock phosphate. PSB significantly reduced the pH in the rhizosphere of Genesis-090 fertilised with Togo rock phosphate. The rhizosphere pH for the remaining cultivars was not affected by inoculation.

The effect of chickpea cultivar, PSB and P, and their two-way and three-way interactions impacted the P concentration in the rhizosphere. The rhizosphere P concentration in Genesis-863, PBA-Striker and PBA-Slasher with no P application or KH_2PO_4 was significantly reduced up to 40% following inoculation of PSB (Fig. 5e-h). Inoculation increased the P concentration up to 53.6% when plants were supplied with either Togo or Peru rock phosphate.

Two ways interactions between cultivar and P source ($P < 0.001$) and PSB and P source ($P < 0.001$) affected plant P uptake (Table 3). PSB did not influence P uptake where no P was applied and with KH_2PO_4 addition (Fig. 5i-l). Conversely, PSB improved plant P uptake by cvv. Genesis-863, PBA-Striker and PBA-Slasher with both rock phosphates.

Plant P uptake was significantly and positively associated with root growth when plants were grown with no P application ($r = 0.54$, $P < 0.01$; $r = 0.43$, $P < 0.01$), Peru rock phosphate ($r = 0.29$, $P < 0.05$; $r = 0.39$, $P < 0.01$), Togo rock phosphate ($r = 0.21$, $P < 0.05$; $r = 0.37$, $P < 0.01$) and KH_2PO_4 ($r = 0.25$, $P < 0.05$; $r = 0.35$, $P < 0.01$) with and without PSB, respectively (Fig.

6a-d). Additionally, plant P uptake was significantly and positively related to P concentration of all cultivars fertilised with Peru rock phosphate ($r = 0.37$, $P < 0.01$; $r = 0.38$, $P < 0.01$) and Togo rock phosphate ($r = 0.31$, $P < 0.01$; $R^2 = 0.67$, $P < 0.01$) with and without PSB, respectively. (Fig. 7a-d). However, this association was non-significant when plants were supplied with no P or with KH_2PO_4.

The effect of PSB on cultivars with contrasting carboxylate concentrations in their root exudates were differentiated by PCA according to variables that defined the growth, nodulation and P acquisition abilities from different P sources. The cultivars did not cluster in particular PCA compartments based on the total carboxylates produced per plant, rather the clustering of cultivars was observed based on +/- PSB inoculation (Fig. 8a, b, c and d).

With no P application, PC1 explained 28.6% of the variability and the cultivars inoculated with PSB were clustered with SDW and pH of the rhizosphere (Fig. 8a). With the sparingly soluble P sources, PC1 accounted for 36.2% of the variability and the majority of cultivars inoculated with PSB were clustered with rhizosphere P concentration, RDW and plant P uptake (Fig. 8b). With the moderately soluble P sources, PC1 explained 36% of the total variation and cultivars inoculated with PSB were distinguished from uninoculated ones by increased RDW, SDW, plant P uptake and P concentration in the rhizosphere (Fig. 8c). In treatments with KH_2PO_4, regardless of PSB inoculation, the majority of cultivars clustered with NN, RDW and SDW (Fig. 8d).

8.6. Discussion

Total P content or Al^{3+}, Fe^{2+} and Cd^{2+} concentration of rock phosphates did not predict their solubilisation by rhizobacteria

Although the quality and value of rock phosphates is generally based on their P content, solubilisation by PSB in the present study was independent of the total P content and available P concentration of the P source. For instance, we found a very low concentration of Pi (<8 µg L^{-1}) in a suspension of Phalaborwa phosphate rock, which contained the highest total P (16%) compared to the other rock phosphates. In contrast, we measured up to 50 µg L^{-1} of Pi in solution from other rock phosphates, which contained < 16% total P. Apart from total and water-soluble P, the mineralogical and chemical composition of rock phosphate, including the concentration of different cations, mainly Al, Ca and Fe, affect the ability of bacteria to solubilise rock phosphate (Leon et al. 1986; Sagoe et al. 1998). Besides precipitation of Pi in

culture solution by metal ions, potentially lethal concentrations of metal ions released during rock phosphate solubilisation might suppress microbial rock phosphate solubilisation by impairing the growth of the microbes (Hartley et al. 1997; Fomina et al. 2004). In the present study, the concentrations of Al (0.2-15.5% w/w), Cd (329.6 mg Cd kg^{-1} P) and Fe (0.7-5.3% w/w) found in the rock phosphate samples could potentially inhibit microbial growth during the biologically-mediated P solubilisation process (Gadd 2010). However, we observed the liberation of Pi from rock phosphate via microbial solubilisation, irrespective of the concentration of Al, Cd and Fe in the rock phosphates. For instance, the highest liberation of Pi was obtained from Boucraa and Togo rock phosphates in which the concentration of Cd was relatively high (161 and 329 mg Cd kg^{-1} P). Two possible explanations can be given for this. One would be the reduction of free metal ion concentration by metal complexing agents, mainly low molecular weight organic compounds produced by PSB during P solubilisation (Gadd 1994; Krantz-Rülcker et al. 1996; Teng et al. 2019). Alternatively, the PSB used here may have a higher ability to tolerate these metal ions (Fomina et al. 2004). However, this would not be so much a problem in field soil, where the metal ions could be gradually diluted in soil solution and washed down the soil profile with rainfall or move away from the P granule in the soil solution (Wood et al. 2016).

PSB exhibited different P solubilising activity when supplied with rock phosphates from a variety of sources

The ability of three selected PSB to solubilise P from rock phosphates that differ in their chemical properties varied. A PSB isolate that was efficient at solubilising one type of rock phosphate was not necessarily efficient at solubilising other rock phosphates. For instance, *Burkholderia* sp. 12F were able to dissolve relatively high amounts of Pi (39.5 to 51.6 µg L^{-1}) from Boucraa, Sechura, Togo and Weng-Fu rock phosphates. On the other hand, none of the three bacterial isolates was able to solubilise a significant amount of Pi from Phalaborwa, Peru and Vietnam rock phosphates. The P solubilising activity of the tested isolates was not always completely explained by the extent of acidification of the culture medium. These results suggest that other P solubilisation mechanisms may also explain the variation in performance of the PSB (Scervino et al. 2010; Scervino et al. 2011).

In the present study, the PSB produced widely diverse types of carboxylates *in vitro*, including acetate, citrate, gluconate, α-ketoglutarate, lactate, maleate, malate, oxalate and succinate. authentic oxalate and maleate followed by malate mobilised a large amount of Pi from all rock

phosphates. While succinate, citrate followed by lactate was able to release a substantial amount of Pi from all rock phosphate, except for Phalaborwa and Vietnam. Accordingly, the high potential of *Burkholderia* sp. 12F to solubilise P may be related to their ability to produce carboxylates, particularly maleate, malate, citrate, succinate, α-ketoglutarate and lactate, most of which are efficient in P solubilisation.

The response of chickpea cultivars to PSB inoculation was partly explained by their ability to produce carboxylates in the rhizosphere

The chickpea-*Mesorhizobium* symbiosis was improved by addition of rock phosphate fertiliser compared with the unfertilised control. This effect was more pronounced when the plants were fertilised with moderately soluble rock phosphate compared with a poorly soluble rock phosphate. This result indicates the ability of chickpea root exudates to mobilise Pi from rock phosphate in *in vitro* conditions. Previous studies have been shown the various capacity of chickpea genotypes to obtain Pi from sparingly soluble P sources (Wen et al. 2020; Pang et al. 2018). Accordingly, they found the highest plant P uptake in genotypes able to produce the largest amounts of carboxylate. These results were also observed in the present study, in *in vitro* conditions.

Although chickpea seedlings were able to obtain Pi from rock phosphate, inoculation with isolate *Burkholderia* sp.12F further enhanced the chickpea-*Mesorhizobium* symbiosis. This effect was only observed with three of the six cultivars tested (Genesis-863, PBA-Striker and PBA-Slasher). These cultivars were previously reported to produce relatively low carboxylate (Pang et al. 2018). Conversely, 'poorly responsive cultivars' that did not significantly respond to PSB inoculation were able to produce relatively higher rhizosphere carboxylate concentrations than cultivars that responded to PSB inoculation. Therefore, the carboxylate concentration in the chickpea rhizosphere may partly explain the different responses of cultivars to PSB inoculation. The relationship between the positive effects of carboxylate release on P uptake in plants fertilised with sparingly soluble P has been previously reported (Gerke et al. 2000; Pearse et al. 2006; Pang et al. 2018; Zhang et al. 2021). In plants with relatively high carboxylates in the rhizosphere, the plant may mobilise higher Pi from rock phosphate that is possibly have feedback inhibition of the P solubilising activity of the inoculated PSB (Pearse et al. 2006; Zhao et al. 2016; Liu et al. 2020). This isolate also promotes chickpea root elongation that via mechanisms related to IAA production and ACC deaminase

activity (Chapter 3). These mechanisms may be also involved in promoting the growth of the responsive cultivars.

The solubility of the P source affected the type of plant growth-promoting mechanisms of PSB that enhance the chickpea-*Mesorhizobium* symbiosis

The type of P source used affected the PGP mechanism involved in enhancing the chickpea-*Mesorhizobium* symbiosis for responsive chickpea cultivars. Inoculation with PSB strain *Burkholderia* sp. 12F increased the growth and nodulation of plants fertilised with Peru and Togo rock phosphates. This positive effect of PSB was associated with increased P nutrition as determined by P concentration in the rhizosphere and plant total P content. This indicates an important role for the P solubilising activity of this PSB strain in growth promotion. *Burkholderia* sp. 12F increased P solubilisation from rock phosphates in chickpea root exudates *in vitro* (Fig. 1). Similarly, other PSB have increased P uptake in wheat (Lekfeldt et al. 2016), soybean (Fernández et al. 2007), barley (Raymond et al. 2019) and pea (Belimov et al. 2020), where the concentration of carboxylates in the root zone is very low compared with chickpea (Wen et al. 2019). The ability of chickpea itself to release high levels of carboxylates may be insufficient to mobilise the required amount of P for the plant, leading to P deficiency. Additionally, the present study showed a correlation between increased P uptake and root growth promotion, with greater root biomass and high P uptake in plants inoculated with *Burkholderia* sp. 12F. This may indicate the role of IAA and ACC deaminase involved in root growth promotion

In plants with no P application or with added KH_2PO_4, the positive effect of *Burkholderia* sp. 12F on plant growth promotion was not explained by P nutrition *per se*. Results showed that an increased P uptake occurred regardless of the P concentration in rhizosphere extract. In this case, the P solubilising activity of PSB does not appear to lead to growth promotion. Rather, plant P uptake was positively related to root biomass promotion. A previous study has shown the important role of greater root growth of chickpea to improve P acquisition ability when grown in low P conditions (Gahoonia et al. 2007) as well as high P conditions (Wen et al. 2019). Such an association suggests the role of other PGP mechanisms, possibly IAA production and ACC deaminase activity, in the mediation of root growth promotion. The positive role of these PGP traits on growth, nodulation and P uptake of chickpea has been previously reported (Malik and Sindhu 2011; Fierro-Coronado et al. 2014; Yadav and Verma

2014; Zaheer et al. 2016; Ahmad et al. 2019) (Nascimento et al. 2012; Kumar et al. 2016; Ditta et al. 2018).

The extent of rhizosphere acidification was not affected by PSB but was associated with nodulation

Rhizosphere acidification is said to be a prerequisite for the release of solubilised P from inorganic insoluble P sources in the root zone (Hinsinger 2001). However, there was no apparent link between Pi concentration and the extent of acidification in the chickpea rhizosphere in the present study. This result suggests that other mechanisms, particularly carboxylate production after inoculation, may also be involved in explaining Pi release. However, more acidic rhizosphere pH was demonstrated in plants fertilised with K_2HPO_4, followed by Togo rock phosphate, although soluble P has been reported to have a suppressive effect on organic acid production by PSB (Pearse et al. 2006; Zhao et al. 2016; Liu et al. 2020). An increased P availability in the root zone can lead to more effective nodulation, which in turn leads to greater symbiotic N_2 fixation, a biological process that releases a net excess of protons (Dakora and Phillips 2002). These protons can result in reducing rhizosphere pH. Such an effect may lead to further P solubilisation from sparingly soluble P sources. In particular, acidification may accelerate the solubilisation kinetics of sparingly soluble forms of $Ca_3(PO_4)_2$ (Andersson et al. 2015).

8.7. Conclusion

The type of carboxylate produced and the ability to acidify the surrounding medium explained the efficiency of PSB strains to solubilise P from diverse sparingly soluble P sources. The performance of the efficient PSB (*Burkholderia* sp. 12F), in stimulating the chickpea-*Mesorhizobium* symbiosis was partly affected by the ability of the plants to produce carboxylates in their root exudates. PSB strain *Burkholderia* sp. P-81 enhanced the *Mesorhizobium* symbiosis of three chickpea cultivars which produced relatively low amounts of carboxylate in their root exudates. On the other hand, this PSB did not significantly increase nodulation or promote the growth of three other chickpea cultivars, which produced relatively high amounts of carboxylates in their root exudates. The presence of a high concentration of carboxylates in the rhizosphere, whether from plant or PSB origin, may affect the performance of PSB to enhance the chickpea-*Mesorhizobium* symbiosis.

8.8. References

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Table 1 Inorganic P released from various rock phosphates incubated in nine carboxylates (10 mM) and H_2SO_4 (10 mM) in solution for 3 days (mean of three replicates)

RP	Acetate	Citrate	Gluconate	α-Keto-glutarate	Lactate	Maleate	Malate	Oxalate	Succinate	H_2SO_4	Water
Boucraa RP	7.14a	42.14bc	1.55c	30.58ab	19.28a	46.95ab	35.29ab	51.60a	46.67c	56.68ab	1.77bc
Phalaborwa RP	0.48c	8.63d	1.51c	22.57cd	13.25c	46.99ab	27.19b	46.60ab	4.97b	54.76bcd	2.52ab
Peru RP	5.33b	36.93c	2.63b	24.43c	14.78bc	42.78b	31.92ab	51.60a	41.06b	55.68abc	2.65a
Sechura RP	6.92a	59.93a	1.58c	26.16bc	16.96ab	44.81ab	37.47a	50.53ab	42.65b	59.45a	2.67a
Togo RP	5.65b	53.11ab	1.04c	17.72d	15.14bc	40.89bc	34.25ab	50.15ab	42.93c	51.58d	2.69a
Vietnam RP	0.68c	19.06d	1.55c	17.50d	8.03d	35.96c	16.98c	38.77c	5.15a	52.04cd	1.02c
Weng Fu RP	7.16a	32.63c	5.14a	31.45a	17.58ab	49.57a	36.10a	46.00b	46.29c	54.32bcd	1.25c
LSD	0.61	11.12	0.12	5.16	3.22	6.50	8.16	5.04	8.08	3.82	0.86

Key: RP: rock phosphate

Table 2 pH of solutions containing various rock phosphates treated with nine carboxylates (10 mM) and H_2SO_4 (10 mM) and incubated for 3 days (mean of three replicates).

Rock phosphate	Acetate	Citrate	Gluconate	α-Keto-glutarate	Lactate	Maleate	Malate	Oxalate	Succinate	H_2SO_4	Water
Boucraa RP	4.11d	3.46a	6.82c	3.50b	3.48c	3.40d	3.59cd	3.37d	3.88c	3.10c	8.07c
Phalaborwa RP	4.66a	3.36b	7.58a	3.22c	3.34d	3.07e	3.55cd	3.18ef	4.26b	2.38f	8.86a
Peru RP	4.29c	3.36b	7.16b	3.79a	3.57c	3.62c	3.74b	3.50c	4.19b	3.21b	8.31b
Sechura RP	4.47b	3.36b	6.66d	3.79a	3.75b	3.84b	3.97a	3.65b	4.28b	3.40a	7.32d
Togo RP	4.13d	3.36b	6.61d	3.52b	3.47c	3.39d	3.67bc	3.24e	4.01c	2.71d	7.40d
Vietnam RP	4.59ab	3.36b	7.10b	3.93a	5.36a	4.70a	3.99a	3.84a	4.96a	2.49e	3.59f
Weng Fu RP	4.18cd	3.36b	6.57d	3.37bc	3.32d	3.37d	3.49d	3.09f	3.93c	2.69d	6.87e
LSD	0.12	0.06	0.56	0.16	0.13	0.12	0.14	0.12	0.16	0.09	0.11

Key: RP: rock phosphate

Table 3 Three-way ANOVA for growth-, nodulation- and phosphorus-related traits among six cultivars of chickpea with four P sources and +/- PSB strain *Burkholderia* sp. 12F inoculation.

parameters	Cultivar (C)		PSB		P source (P)		C × PSB		C × P		PSB × P		C × PSB ×P	
	F	*P* value	F	*P* value	F	*P* value	F	*P* value	F	*P* value	F	*P* value	F	*P* value
SDW	21.32	0.001	89.97	0.001	439.28	0.001	5.68	0.001	6.53	0.001	5.05	0.003	1.96	0.026
RDW	35.89	0.001	43.46	0.001	150.80	0.001	2.42	0.041	5.25	0.001	2.54	0.061	1.77	0.050
NN	45.82	0.001	77.37	0.001	338.54	0.001	2.03	0.082	13.04	0.001	0.30	0.826	3.61	0.001
NDW	13.78	0.001	24.83	0.001	129.16	0.001	3.21	0.01	3.04	0.001	6.04	0.001	1.23	0.261
NCR	36.25	0.001	54.00	0.001	658.72	0.001	2.04	0.079	1.52	0.115	6.54	0.001	1.34	0.192
RPC	19.89	0.001	27.86	0.001	2041.98	0.001	2.32	0.049	13.08	0.001	8.14	0.001	2.12	0.015
RpH	1.4	0.230	0.08	0.772	13.25	0.001	1.62	0.163	2.02	0.021	4.12	0.009	1.06	0.407
P uptake	18.83	0.001	34.54	0.001	883.85	0.001	1.12	0.353	7.15	0.001	7.70	0.001	0.86	0.608
SPAD	5.38	0.001	57.85	0.001	2559.62	0.001	1.48	0.205	3.62	0.001	7.22	0.001	0.61	0.860

SDW: Shoot dry weight (g plant^{-1}); RDW: Root dry weight (g plant^{-1}); NN: Nodule number per plant; NDW: Nodule dry weight (mg plant^{-1}); NCR: Nodule colour rating; RpH: Rhizosphere pH; RPC: Rhizosphere P concentration (µg P kg^{-1}); SPAD: Chlorophyll content (SPAD reading); PSB: phosphate solubilising bacteria; C: Cultivar; P: P source.

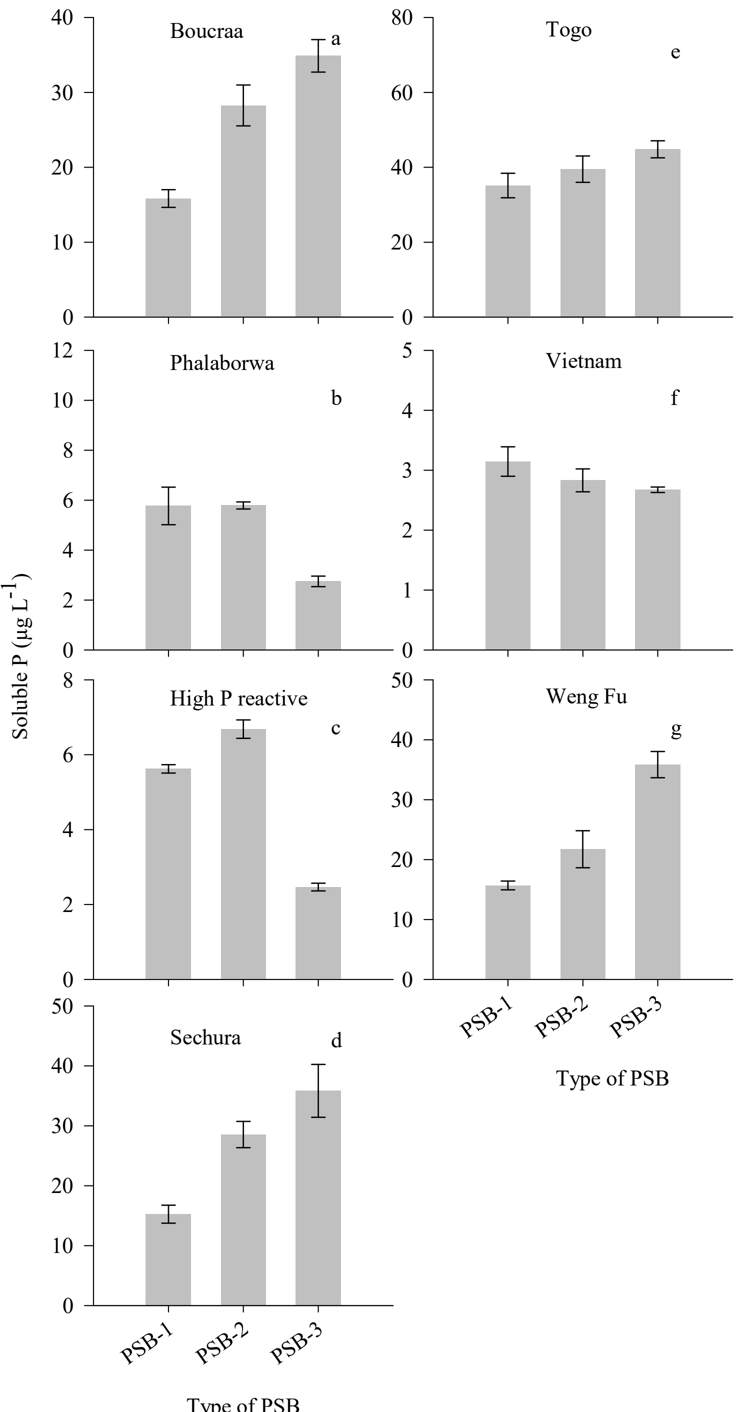

Fig. 1 Pi concentration of culture solutions inoculated with three PSB strains in the presence of 7 different sources of rock phosphate (note differences in Y axis scales). PSB-1= *Burkholderia cenocepacia* 127F, PSB-2 = *Pseudomonas fluorescens* 27F and PSB-3 = *Burkholderia* sp. 12F. The experiment was conducted in NBRIP medium supplemented with 5 g L^{-1} of rock phosphates from different sources, namely Boucraa, Vietnam, Weng Fu Fine, Togo, Phalaborwa, Sechura or Peru phosphate rock. Cultures were incubated on a shaker (28 ºC,160 rpm) for 14 days. Values are mean (±SE) of three replicates. RP – Rock phosphate.

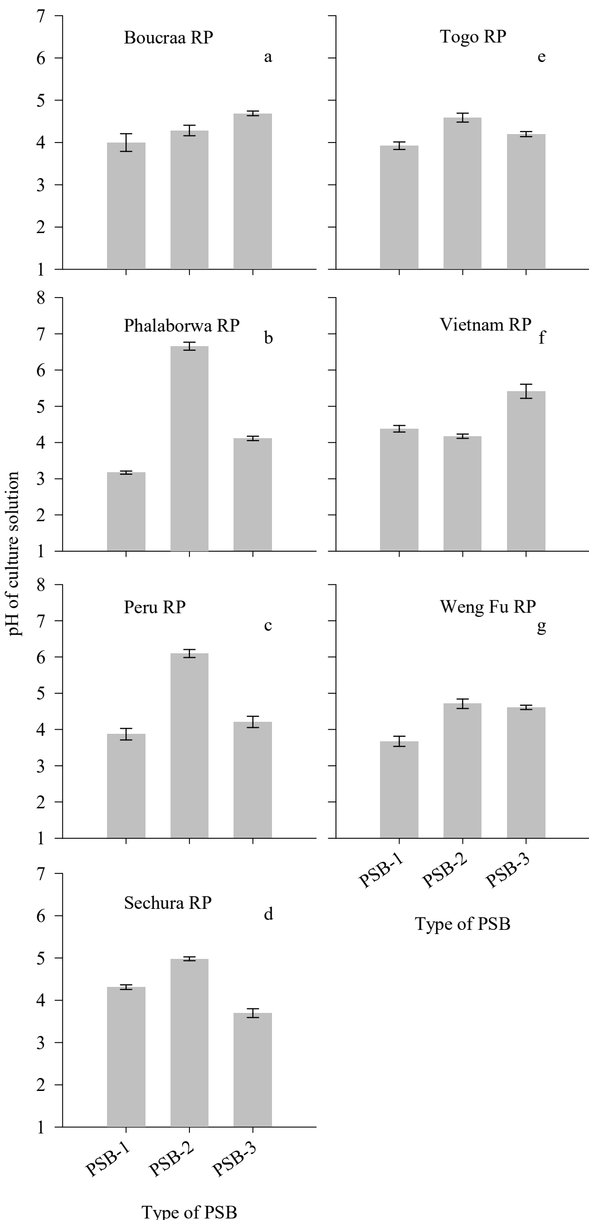

Fig. 2 pH of culture media inoculated with three PSB strains and supplied with different sources of rock phosphate. PSB-1 = *Burkholderia cenocepacia* 127F; PSB-2 = *Pseudomonas fluorescens* 27F and PSB-3 = *Burkholderia* sp. 12F. The experiment was conducted in NBRIP medium supplemented with 5 g L^{-1} of the following rock phosphates: Boucraa, Vietnam, Weng Fu, Togo, Phalaborwa, Sechura or Peru phosphate rock. Cultures were grown in a shaker incubator (28°C, 160 rpm) for 14 days. Values are mean (± standard error) of three replicates. RP – Rock phosphate.

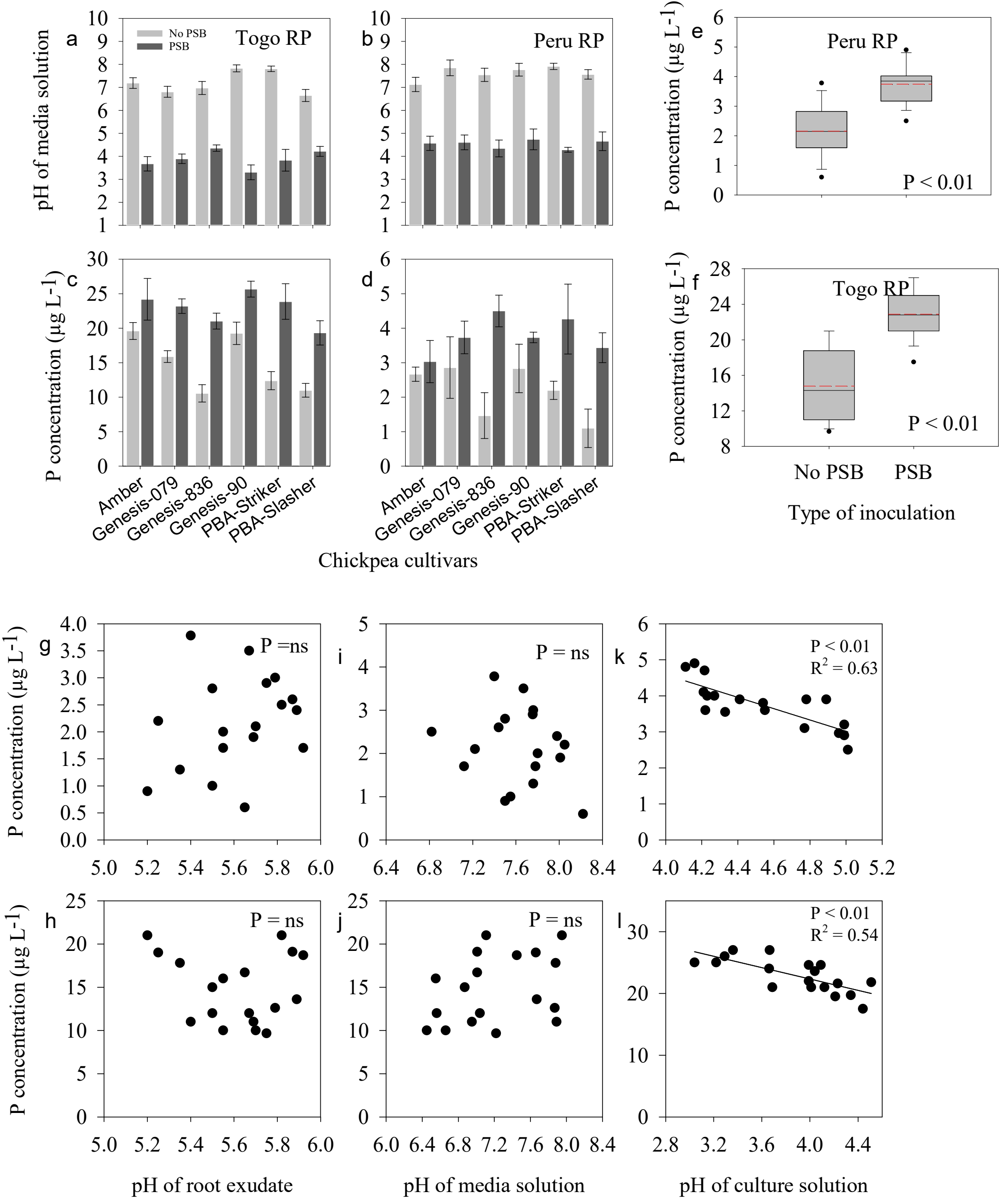

Fig. 3 Variation in P concentration (a and b) and pH (e and f) of root exudates obtained from six chickpea cultivars in the presence and absence of PSB with Peru and Togo rock phosphate. Error bars are standard error (n = 3) to compare means of two-way interactions between PSB × cultivar. In c and d, symbols indicated red lines as mean values and dots as 95% confidence interval. The association between the P concentration and pH of root exudates (g and h), pH of media solution (i and j) and pH of culture solution (k and l) with Peru and Togo rock phosphate. P = ns- non-significant, $P < 0.01$- highly significant.

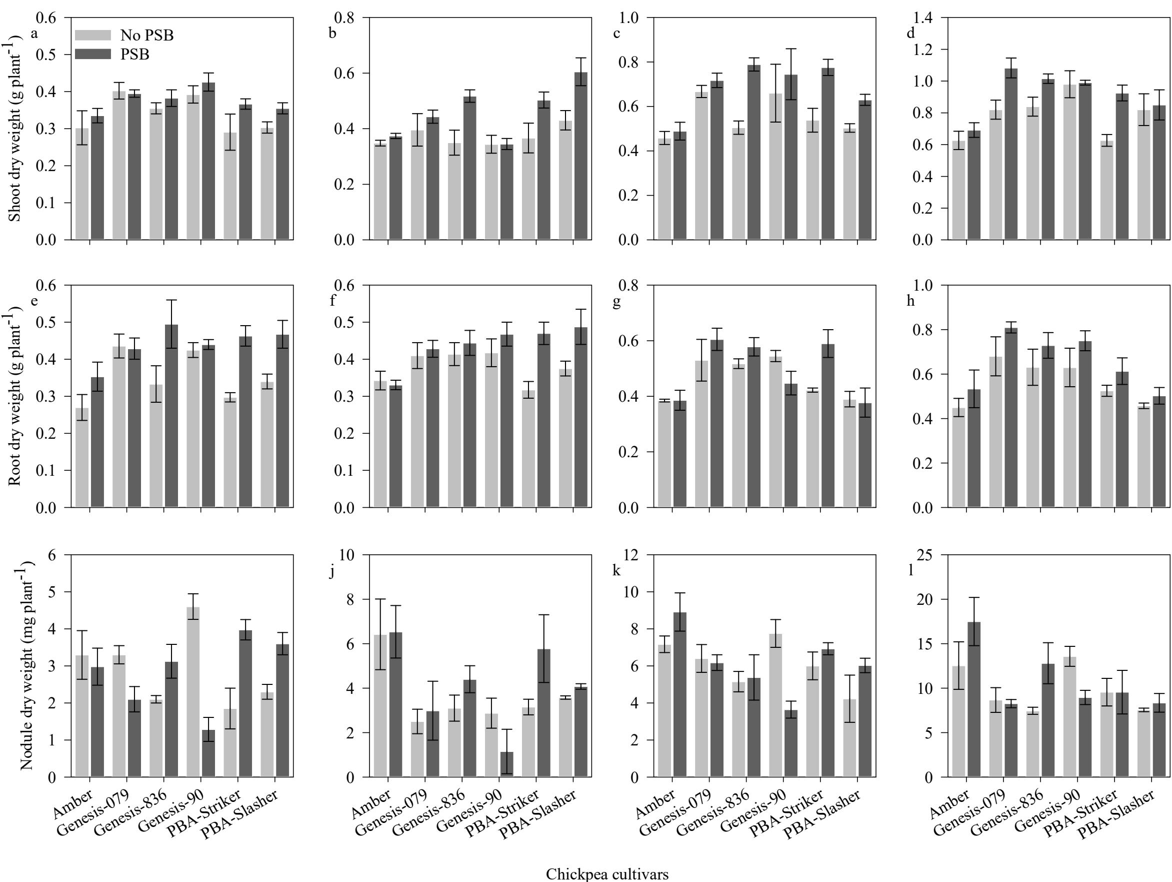

Fig. 4 The responses of shoot dry weight, root dry weight, and nodule dry weight per plant to inoculation of *Burkholderia* sp. 12F in six chickpea cultivars with (a, e, i) no P application, (b, f, j) Peru rock phosphate, (c, g, k) Togo rock phosphate and (d, h, l) K_2HPO_4. Error bars are standard error (n = 3) to compare means of two-way interactions between PSB × cultivar.

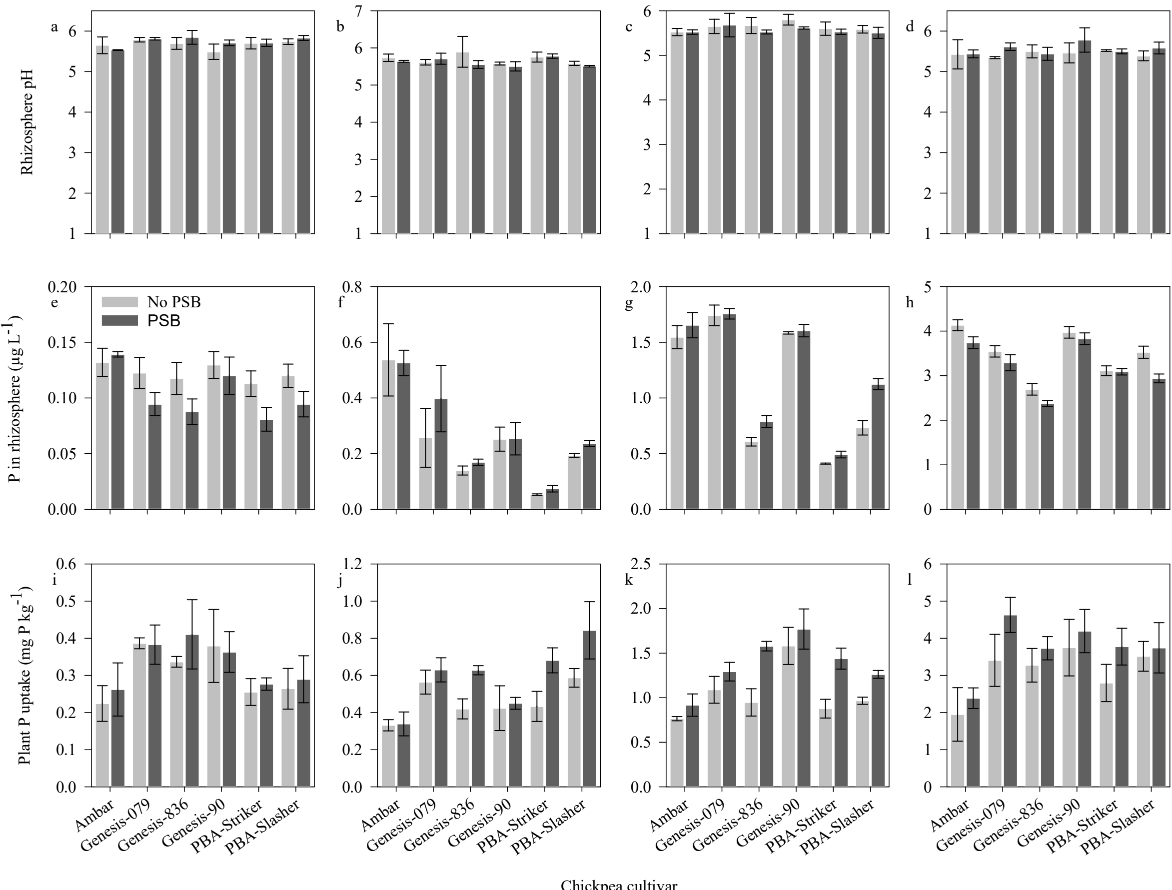

Fig. 5 The responses of rhizosphere pH (a, b, c, and d), P in the rhizosphere (e, f, g and h) and plant P uptake (I, j, k and l) to PSB inoculation of six chickpea cultivars with (a, e, i) no P application, (b, f and j) Peru rock phosphate, (c, g and k) Togo rock phosphate and (d, h and l) KH_2PO_4, respectively. Error bars are standard error (n = 3) to compare means of two-way interactions between PSB × cultivar.

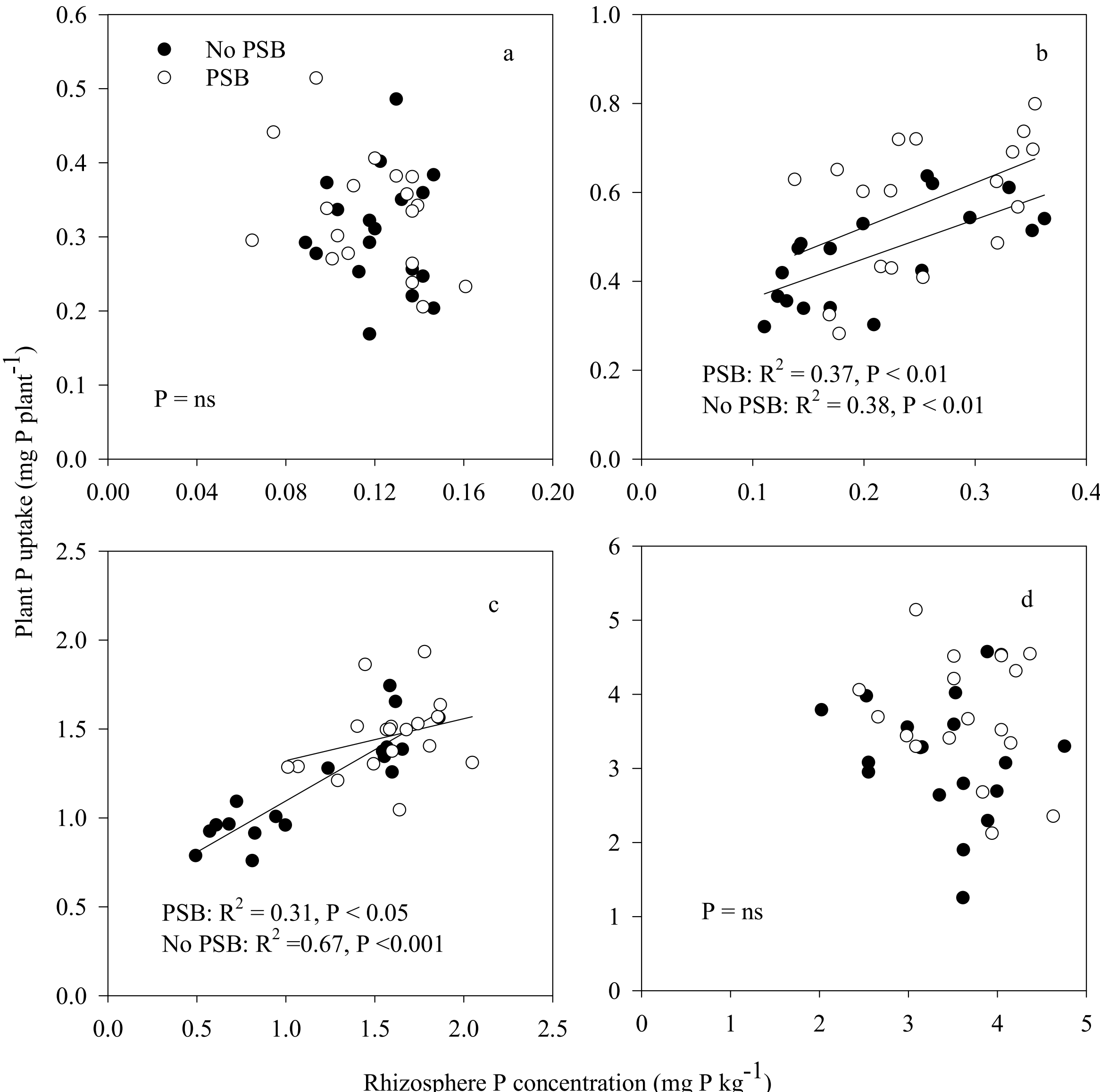

Fig. 6 Relationship between rhizosphere P concentration and plant P uptake of six chickpea cultivars in the presence or absence of PSB strain *Burkholderia* sp. 12F with (a) no P application, (b) Peru rock phosphate, (c) Togo rock phosphate and (d) KH_2PO_4. Lines are least square linear regressions. P = ns- non-significant, $P < 0.05$- significant, $P < 0.01$- highly significant.

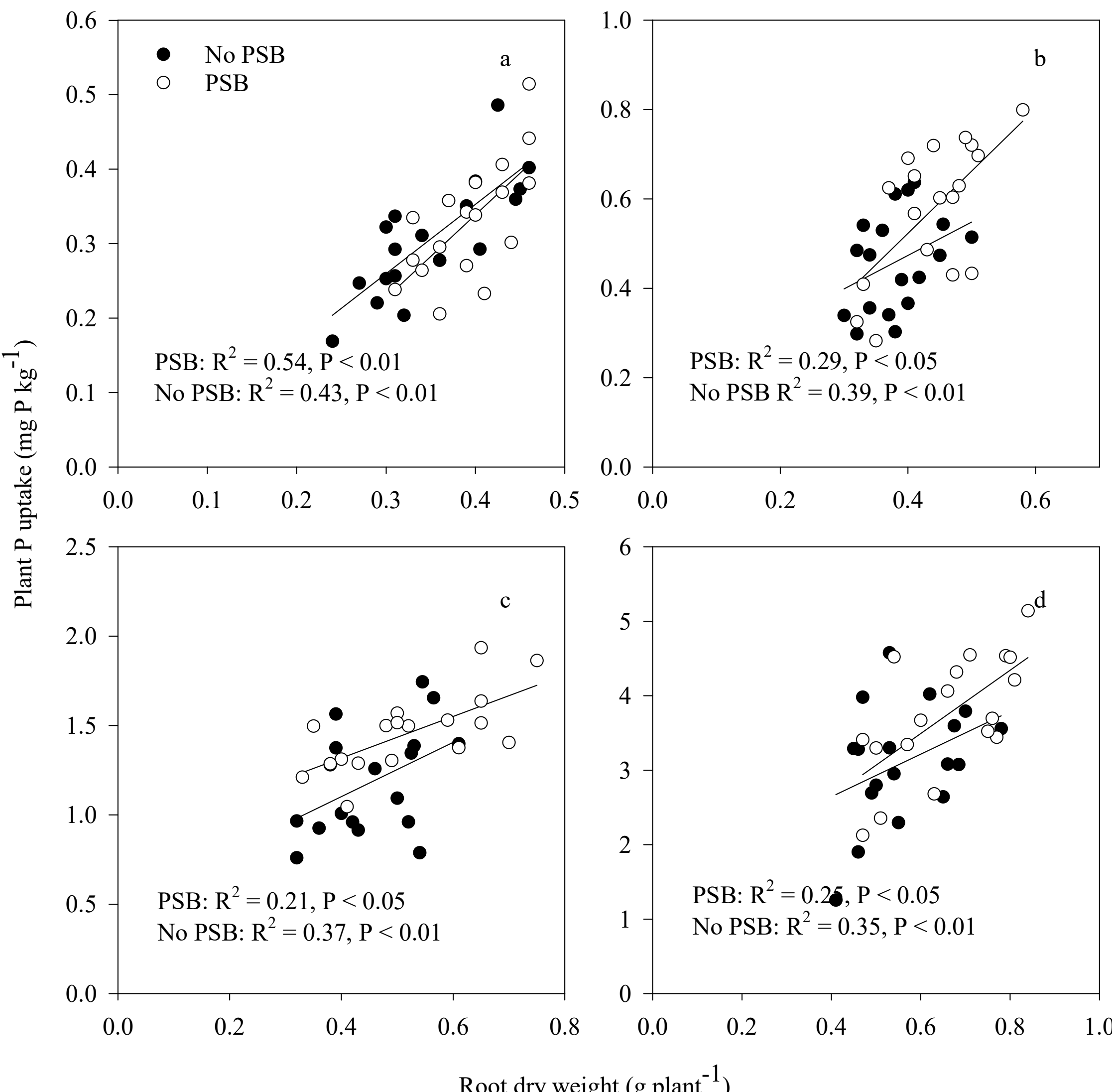

Fig. 7 Relationship between root dry weight and plant P uptake of six chickpea cultivars in the presence or absence of PSB strain *Burkholderia* sp. 12F with (a) no P application, (b) Peru rock phosphate, (c) Togo rock phosphate and (d) KH_2PO_4. Lines are least square linear regressions. $P < 0.05$- significant, $P < 0.01$- highly significant.

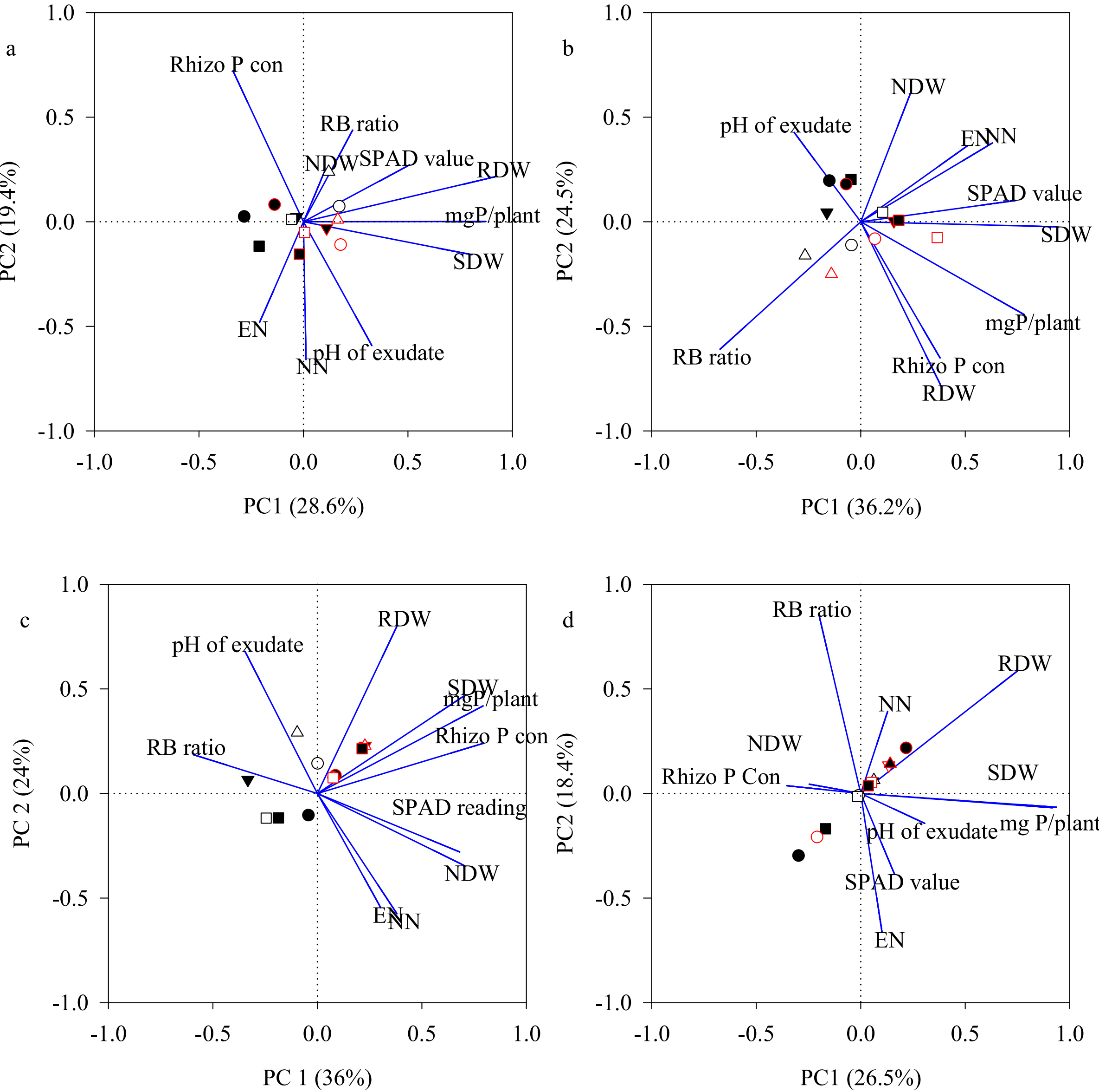

Fig. 8 Principal component analysis (PCA) biplot combining ten variables that contributed to growth, nodulation and P acquisition strategies for six cultivars in response to PSB under (a) no P application, (b) Peru rock phosphate, (c) Togo rock phosphate and (d)KH_2PO_4. Cultivars represented as: ● Ambar; ○ Genesis 079, ▼ Genesis-863, Δ Genesis-090, ■ PBA-Striker and □ PBA-Slasher. Symbols with red edge represent cultivars inoculated with PSB and with black edge represent no PSB addition. Trait abbreviations: RB ratio, root biomass ratio; pH of exudate, the pH of exudates obtained from rhizosphere; RDW, root dry weight; SDW, shoot dry weight; NN, nodule number per plant; NDW, nodule dry weight per plant; Rhizo P con, rhizosphere P concentration; mg P/plant, plant uptake P; SPAD value, chlorophyll reading; EN, nodule colour rating

Chapter 9. General discussion

Increased nodulation and yield of chickpea following inoculation of PGPR able to solubilise P have been observed, although their effect was not always linked with P nutrition *per se* (Zaidi et al. 2003; Rudresh et al. 2005; Mittal et al. 2008; Verma et al. 2012; Singh et al. 2014; Verma et al. 2014; Imen et al. 2015; Saxena et al. 2015; Israr et al. 2016; Rajwar et al. 2018). Additionally, PGPR with high P solubilising activity *in vitro* reported in the literature have not always enhanced chickpea-*Mesorhizobium* symbiosis under controlled and field conditions. Such inconsistent effects may be due to many factors associated with the host plant and the PSB. In this study, we investigated the efficiency of PSB in P solubilisation and their occurrence in the soil environment, the role of PGP characteristics other than P solubilisation, that enhanced P nutrition, and whether the presence of high carboxylate concentrations in the chickpea rhizosphere would affect the efficiency of PSB.

The first stage screening of PSB typically uses differential media, mainly Pikovskaya's and NBRIP supplied with $Ca_3(PO_4)_2$ (Pikovskaya 1948; Nautiyal 1999; Mehta and Nautiyal 2001). However, the use of this P source alone may not indicate the actual P solubilising potential of the PSB in soil (Bashan et al. 2013). Therefore, considering the ability to solubilise P sources other than $Ca_3(PO_4)_2$, including AlO_4P and FeO_4P, that are often found in acidic soils and in rock phosphate is important to obtain broadly efficient PSB at the pre-screening stage. In this experiment, two isolation methods, including taxonomically selective and enrichment methods were used (Chapter 4). Using the taxonomically selective methods, *Pseudomonas*- and *Bacillus*-like bacteria were isolated; these genera are frequently reported as efficient PGPR with a high ability to solubilise P (Ndung'u-Magiroi et al. 2012; Khan et al. 2013; Alaylar et al. 2019). Additionally, isolates with high P solubilising activity were isolated through enrichment in low P conditions (Chapter 4). Results showed that a diverse range of bacteria belonging to *Pseudomonas*, *Burkholderia* and *Bacillus* able to solubilise diverse P sources was obtained. Sixteen genera of bacteria, predominantly *Burkholderia*, *Variovorax*, *Leifsonia*, *Pantoea* and *Rhizobium*, were isolated. These genera have been previously reported as efficient PGPR with P solubilising activity from the rhizosphere of chickpea (Hynes et al. 2008; Shahid and Khan 2018). These isolates were more efficient in P solubilisation than those obtained using the taxonomically selective method. Most of the efficient P solubilisers were enriched in FeO_4P- and AlO_4P- containing media. For example, *Burkholderia* sp. 12F and *B. cenocepacia* 127F, that were obtained following enrichment in media containing $FeO_4P.2H_2O$, were able to produce diverse carboxylates, predominantly di-and tri-carboxylates. These carboxylates by

themselves were previously confirmed as efficient P solubilisers compared with mono-carboxylates (Bolan et al. 1994; Kpomblekou-a and Tabatabai 1994). In particular, their chelation ability enables them to efficiently solubilise moderately reactive rock phosphate as well. *Burkholderia* was the most dominant genus of bacteria that were efficient in P solubilisation in this study. In particular, *B. cenocepacia*, *B. cepacia* and *B. caledonica* have been previously characterised as potent P solubilisers *in vitro* and potential PGPR as well (Suárez-Moreno et al. 2012; Zhao et al. 2014; You et al. 2020).

In the present study, IAA and ACC deaminase production was related to the potential of the bacterial isolates to promote seedling root growth in the plant-based first-stage screening bioassay (Chapter 3). Additionally, the ability of PGPR to produce ACC deaminase enhanced the ability of PSB to solubilise P from $Ca_3(PO_4)_2$ when ACC was the sole N source (Chapter 6). Such an effect was associated with the presence of a more acidic pH. Likewise, the presence of IAA and its precursor L-tryptophan increased the P solubilising potential of PSB from moderate- and low-reactive rock phosphate (Chapter 7). IAA increased P solubilisation through inducing a more acidic pH and the production of more carboxylates, predominantly di- and tri-carboxylates by PSB.

Biofertilizer development typically requires the collection of a large number of microbial isolates from a wide range of agricultural soils. This process requires time and resources to obtain an elite PGPR that can increase the growth and productivity of plants. This led us to investigate the soil and environmental factors that could affect the occurrence of PGPR and their potential to promote plant growth. In the present study, the potential of isolates to promote seedling root elongation in the first stage screening and their ability to produce IAA was positively correlated with soil copper, manganese and zinc concentrations, electrical conductivity and the aridity index of the sampling environment but was negatively correlated with soil carbon (C), nitrogen (N) and phosphorus (P) (Chapter 5). Additionally, the ability to produce IAA and ACC deaminase was related to their ability to promote seedling root growth (Chapter 3 and chapter 5). Results further showed that isolates with multiple PGP traits and with a high ability to solubilise P and produce IAA came from sampling sites with low soil fertility, a high aridity index and high levels of metal ions (Cu, Mn and Zn). Production of ACC deaminase and IAA, and P solubilisation, can be important traits for plant growth promotion in soils of low fertility and at sites with low water availability, through a reduction in stress-induced ethylene synthesis, increasing the nutrient availability and the capacity of the plant to acquire nutrients and water (Bal et al. 2013; Chandra et al. 2018; Mpanga et al. 2018; Saleem

et al. 2018). When bacteria coevolved with plant roots in harsh environments over millions of years, the bacteria acquired the ability to produce ACC deaminase and IAA, and to solubilise P that can now lead to associations with plants that improve plant fitness under biotic and abiotic conditions (Denison 2012). Additionally, IAA production improved the bacteria's ability to withstand the water-stressed and metal toxicity (Chapter 5). This effect of IAA may be related to its ability to induce the synthesis of high amounts of exopolysaccharides that are important for rhizobacteria protection from environmental stress (Naseem et al. 2018; Torres et al. 2018).

Phosphate solubilising bacteria are saprophytically competent in a wide range of environmental conditions and soil types (Raymond et al. 2021). The abundance of PSB and their potential to solubilise P was correlated with soil properties. An increased soil C, N, P, Ca^{2+} and Mg^{2+} content resulted in greater abundance of PSB (Chapter 4). However, their potential to solubilise P in the present study was negatively correlated with soil pH, soil P, soil C, C/N ration and Mg (Chapter 5). High pH soils with high exchangeable cations decrease the available P through the formation of Mg-P and Ca-P complexes (Zheng et al. 2019). This environment may increase the occurrence of soil microbial communities that are capable of solubilising P from sparingly soluble P sources to fulfil their P needs. Soil microbial communities may be evolved to acquire the ability to solubilise P from sparingly soluble P source (Denison 2012). Therefore, these findings have potential application in designing a strategy for the development of efficient PGPR that have ecological traits and plant growth-promoting mechanisms that may increase production of important crops such as chickpea.

An efficient PSB isolate (*Burkholderia* sp. 12F), able to produce IAA and ACC deaminase, was investigated for its effect on the chickpea-*Mesorhizobium* symbiosis using six chickpea cultivars with contrasting carboxylate concentrations in their rhizospheres under different P fertiliser amendments (Chapter 8). The PSB did not enhance the nodulation and growth of all chickpea cultivars. Of the six cultivars, inoculation of PSB increased the nodulation and growth of chickpea cvv. PBA-Striker, PBA-Slasher and Genesis 863. This effect was not significant in the remaining three cultivars (Genesis-090, Genesis-079 and Ambar). The responsive cultivars released relatively less carboxylates than the less-responsive cultivars. These various responses of cultivars to inoculation with PSB strain *Burkholderia* sp. 12F could be due to several reasons include: (i) relatively low pH (down to 5.1) in the rhizosphere of less responsive cultivars may have negatively affected PSB survival, (ii) such a low pH may also suppress the potential of the isolate to express the beneficial traits including P solubilising activity and ACC

deaminase and IAA production (Chaiharn and Lumyong 2009), (iii) the less responsive cultivars may fulfil most of their P needs by themselves, via production of high amounts of carboxylates in the rhizosphere, as has been observed in other plant species (Chien et al. 2003; Sepehr et al. 2012) and (iv) the presence of low/no detectable L-tryptophan in the rhizosphere may suppress the isolate's ability to promote the plant growth v IAA production (Kravchenko et al. 2004).

Additionally, *Burkholderia* sp. 12F improved the growth and nodulation of the highly responsive cultivars directly, via PGP traits other than P solubilising activity when the plants were fertilised with highly soluble P source and under low P conditions. This indicates the ability of PSB to promote plant growth under both optimum P conditions and low P conditions. In this case, an increased P uptake was correlated with root growth promotion, which was possibly mediated by ACC deaminase and IAA production (Chapter 3). Therefore, the use of inoculants with multiple PGP characteristics that may be expressed simultaneously could promote plant growth in diverse conditions.

9.1. Conclusions and recommendations

The results of the seedling root elongation test predicted the effect of rhizobacteria on the nodulation and growth of co-inoculated chickpea in aseptic conditions. Increased nodulation and plant growth were the net results of ACC deaminase and IAA production by PGPR. Rhizobacteria able to produce high concentrations of IAA in combination with ACC deaminase activity are suitable PGPR candidates for further investigation in a second stage screening program using non-sterile soil in the greenhouse and/or field conditions. Accordingly, we recommend the seedling growth bioassay for first-stage screening of PGPR rather than simply relying on their ability to express multiple PGP traits. Additionally, we demonstrate that the soil and environmental variables at the sampling sites a have relevant role in both the occurrence and performance of culturable rhizobacteria that have traits beneficial for plant growth. This influence should be considered when microbial inoculants are developed. Accordingly, PGPR able to efficiently solubilise P were enriched from rhizosphere soil under conditions of sparingly soluble P supply ($FeO_4P.2H_2O$ and AlO_4P). Selected isolates from the enrichment process efficiently solubilised P from chemically varied rock phosphates. *Burkholderia* was the most efficient genus in P solubilisation. This suggests the need for the identification and targeting of genera that are potentially efficient in plant growth promotion associated with particular plant species and sampling sites.

The potential of efficient PSB to solubilise P was related to their ability to produce carboxylates, predominantly di- and tri-carboxylates. Additionally, the potential of PSB to solubilise P *in vitro* was influenced by their ability to produce IAA and ACC deaminase. High ACC deaminase-producing PSB exhibited higher P solubilising activity than those that produced less when the sole N source was ACC. The supply of IAA and its precursor L-tryptophan also increased the P solubilising activity of IAA-producing bacteria This suggests the need to consider the addition of organic compounds, e.g. L-tryptophan, during first stage screening for PGPR that can enhance P nutrition.

In the *in-planta* experiment, an efficient PSB isolate (*Burkholderia* sp. 12F) improved the nodulation and growth of chickpea cultivars with relatively low carboxylates their root exudates. The positive influence of PSB inoculation was mediated by P solubilisation when plants were fertilised with rock phosphates. Increased growth and nodulation under conditions of low P and highly soluble P supply was mediated by PGP traits other than P solubilising activities, suggesting that the plant growth promotion is the net result of multiple PGP traits that may be activated simultaneously. In general, this study indicates the need for considering plant-associated factors, mainly the composition of rhizosphere exudates that may affect the performance of PGPR. Additionally, PGPR with multiple activities, that may directly and indirectly increase P availability and the plant's capacity to acquire P, can stimulate increased plant growth under different and contrasting conditions of P nutrition.

9.2. Future research

PSB enhanced the nodulation and growth of chickpea through increasing P concentration in the rhizosphere as well as plant P uptake. Although rhizosphere acidification is one of the mechanisms responsible for P solubilisation (Neumann and Römheld 2012), increased P in the rhizosphere in the present study was not related to the extent of acidification of the rhizosphere. In chickpea, the rhizosphere contains a large amount of root exudates that comprise a wide array of organic compounds (Ohwaki and Hirata 1992) that can be the source of C for the PSB. The PSB can ultimately produce organic derivatives that might be involved in P solubilisation. Therefore, further research is required to investigate the types and concentrations of different carbon derivatives produced after inoculating with PSB.

Additionally, *Burkholderia* 12F increased the growth and nodulation of chickpea cvv. PBA-Slasher, PBA-Striker and Genesis-863 with no significant effects on the P concentration in their rhizosphere. Root growth promotion, in this case, was explained by an increased plant P

uptake. The root growth promotion might be mediated by PGP mechanisms other than P solubilising activity, such as production of ACC deaminase and IAA. To confirm the role of these PGP traits, mechanistic studies are required to elucidate a potential mechanism, such as by developing mutants with no ACC deaminase activity, IAA production and lacking both of these PGP traits that would allow a distinction to be made between the direct and indirect effects of the PSB on increased plant P uptake.

Our results showed that inoculation of PSB and rhizosphere carboxylate concentration affected the P acquisition strategy of chickpea. Recently, Wen et al. (2019) reported that the amount of carboxylate in the root exudates of chickpea affected colonisation by arbuscular mycorrhizae and the characteristics of root functional traits (root diameter, number of root hairs, length of root hair, root surface area, root biomass density, total root length). In this study, relatively high carboxylate-producing chickpea cultivars tended to have relatively thinner roots with lower cost of root construction, while allocating more resources to carboxylate exudation and potential mycorrhizae colonisation. PSB inoculation can alter the characteristics of root functional traits (Fierro-Coronado et al. 2014) and can possibly affect the composition of carboxylates and P concentration in the rhizosphere. Therefore, a more detailed understanding of chickpea-*M. ciceri* rhizobia-PSB and mycorrhizal interaction in chickpea cultivars with contrasting rhizosphere carboxylate concentrations and under different P conditions is required to fully comprehend the P acquisition strategies of this pulse crop.

Most studies reported in the literature have indicated positive effects of PSB on crop growth under controlled conditions, whereas field experiments have more frequently failed to demonstrate a positive response (Karamanos et al. 2010; Gómez-Muñoz et al. 2017; Meyer et al. 2019; Raymond et al. 2019). Additionally, Raymond et al. (2021) suggested that PSB could not release extra soluble P from sparingly soluble P sources beyond their own needs under field conditions. The positive influence of *Burkholderia* sp.12F on the chickpea-*Mesorhizobium* symbiosis under different P conditions in the present study also warrants further testing in field trials. Accordingly, investigation of the interaction between chickpea cultivars, *M. ciceri* CC1192 and *Burkholderia* sp. 12F under field conditions over a wide range of phosphorus levels that includes both sub- and super-optimal levels of phosphorus is a prerequisite before the isolate can be considered as an inoculant to be used in farming systems. The effectiveness of *Burkholderia* sp. 12F on other plant species or cultivars in field conditions also needs to be assessed. There is also a need for commercial optimization of inoculant formulations containing multiple effective and complementary strains such as rhizobia plus a PGPR.

9.3. References

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10. Appendixes

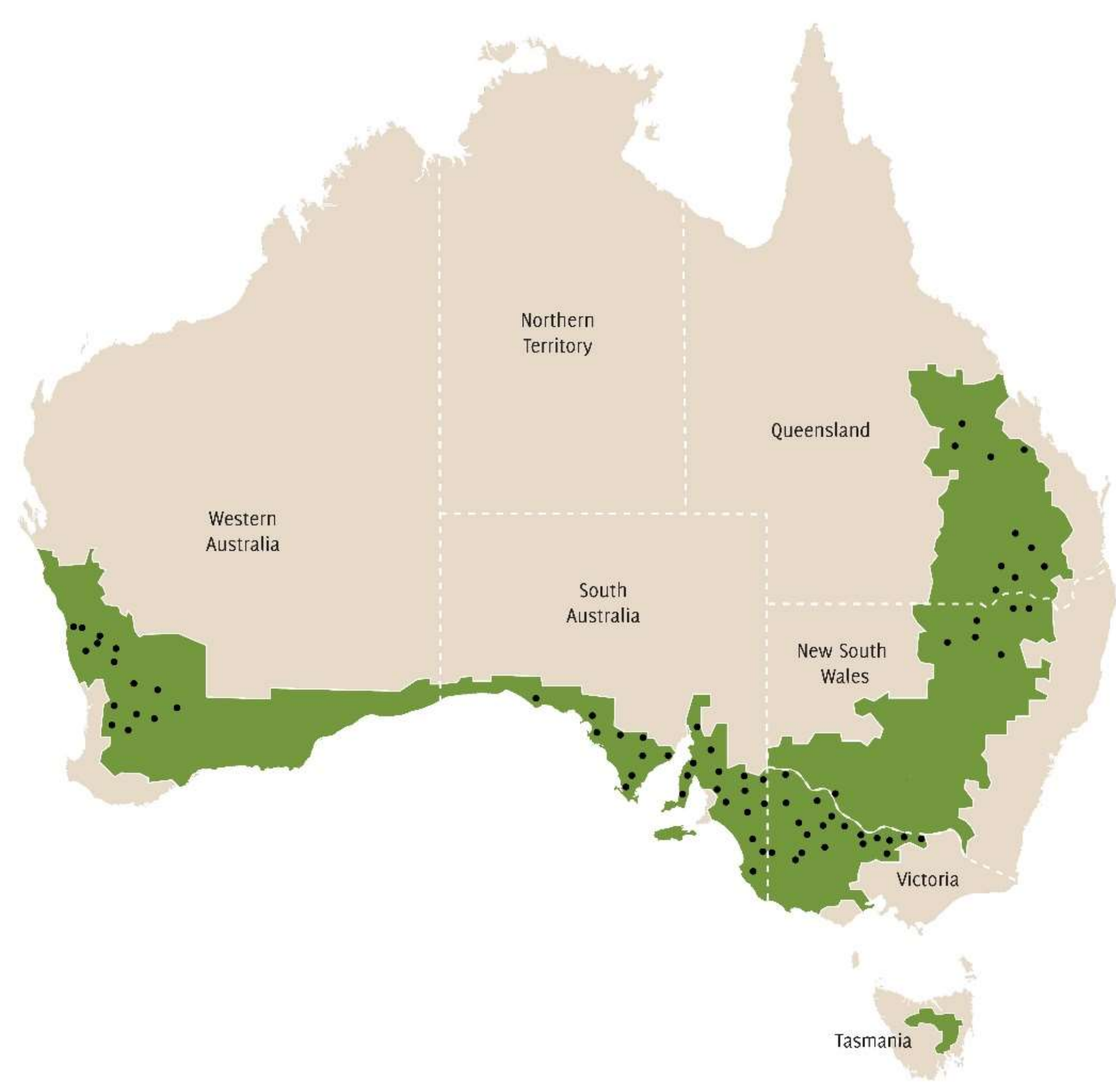

Fig. 1 Sampling sites of soils from major grain cropping regions in Australia. Black dots indicate sampling points

Table 1. The soil physico-chemical properties and the average annual rainfall at the sampling sites

Sampling sites	EC (µS cm^{-1})	pH	Clay	Sand	Total N (%)	Soil C (%)	P (mg kg^{-1})	Zn (mg kg^{-1})	Mg (mg kg^{-1})	Mn (mg kg^{-1})	K (mg kg^{-1})	Cu (mg kg^{-1})	Ca (mg kg^{-1})	Rainfall (mm)	ETP (mm)	Aridity index
1	73.8	7.1	21.5	27.5	0.18	1.78	452.0	20.0	1250.0	1200.0	3580.0	8.9	1960.0	582.9	1173.2	0.50
2	326.2	6.6	16.5	37.5	0.19	2.32	384.0	19.0	1350.0	200.0	3330.0	6.4	3580.0	471.0	1172.2	0.40
5	168.7	6.5	31.5	35.0	0.12	1.72	485.0	35.0	1590.0	710.0	4010.0	29.0	2300.0	548.7	1167.9	0.47
6	181.4	6.4	26.5	22.5	0.15	1.76	400.0	28.0	1510.0	440.0	3690.0	9.7	1640.0	425.8	1168.4	0.36
7	138.2	6.5	26.5	27.5	0.12	1.45	292.0	22.0	1410.0	410.0	3660.0	9.2	1240.0	425.8	1171.1	0.36
8	98.2	7.4	24.0	52.5	0.09	1.33	238.0	24.0	4000.0	190.0	5440.0	5.8	8360.0	383.0	1169.5	0.33
9	113.5	8.2	21.5	61.3	0.07	2.29	330.0	27.0	4710.0	170.0	5200.0	6.5	38400.0	301.3	1182.4	0.25
10	81.4	7.4	9.0	83.8	0.02	0.40	91.0	11.0	603.0	50.0	1300.0	2.0	516.0	323.4	1190.6	0.27
11	81.5	7.2	5.3	86.3			130.0	13.0	1400.0	98.0	2270.0	2.9	1330.0	329.6	1188.9	0.28
12	74.9	7.0	9.0	85.0	0.01	0.23	82.0	8.0	673.0	56.0	1120.0	2.0	597.0	330.7	1187.5	0.28
14	84.8	6.5	11.5	85.0	0.04	0.64	98.0	13.0	687.0	45.0	1300.0	2.0	581.0	296.0	1178.0	0.25
15	104.2	7.1	9.0	82.5	0.01	0.27	88.0	11.0	823.0	48.0	1250.0	2.0	661.0	383.0	1177.2	0.33
16	103.2	7.6	24.0	62.5	0.06	0.86	185.0	19.0	3070.0	160.0	4610.0	3.6	2030.0	390.4	1173.7	0.33
17	106.3	7.5	31.5	45.0	0.13	1.88	297.0	34.0	3570.0	360.0	6560.0	9.8	3090.0	390.4	1166.0	0.33
21	112.0	7.5	36.5	32.5	0.44	6.88	551.0	47.0	7690.0	100.0	9480.0	8.6	72300.0	600.1	1154.9	0.52
22	278.0	7.4	36.5	45.0	0.15	1.79	242.0	23.0	2850.0	100.0	4280.0	3.8	6390.0	460.5	1167.1	0.39

23	151.3	6.9	29.0	57.5	0.16	2.17	248.0	21.0	2010.0	110.0	3080.0	3.0	2640.0	338.4	1167.5	0.29
24	168.5	7.1	14.0	60.0	0.15	1.79	289.0	14.0	3870.0	150.0	2920.0	3.4	4040.0	460.5	1171.5	0.39
25	108.9	6.9	38.0	42.0	0.13	1.66	274.0	40.0	4090.0	440.0	7510.0	9.4	3310.0	366.0	1169.5	0.31
26	52.8	6.9	8.0	84.5	0.03	0.46	142.0	12.0	976.0	75.0	1490.0	2.8	734.0	304.5	1192.0	0.26
27	54.1	8.0	5.5	89.5	0.01	0.16	68.0	5.2	481.0	32.0	853.0	2.0	660.0	272.2	1193.3	0.23
28	83.4	8.1	5.5	89.5	0.02	0.40	134.0	12.0	1380.0	74.0	2050.0	2.1	3650.0	272.2	1198.5	0.23
29	37.0	6.7	8.0	87.0	0.00	0.10	79.0	10.0	956.0	62.0	1850.0	2.0	638.0	266.0	1197.9	0.22
30	66.5	7.5	15.5	72.0	0.05	0.69	183.0	15.0	2510.0	85.0	3160.0	2.7	2640.0	310.8	1186.5	0.26
31	102.7	6.1	5.5	89.5	0.05	0.81	137.0	7.7	452.0	26.0	921.0	2.0	365.0	381.5	1183.6	0.32
33	220.2	5.6	28.0	32.0	0.14	1.96	338.0	38.0	4350.0	500.0	4960.0	16.0	1260.0	542.1	1208.2	0.45
34	158.1	6.0	18.0	52.0	0.08	1.09	251.0	28.0	1480.0	200.0	3710.0	6.7	905.0	474.3	1214.6	0.39
35	105.6	7.9	15.5	59.5	0.10	1.78	343.0	35.0	4160.0	220.0	5410.0	9.0	11500.0	364.4	1225.0	0.30
36	130.2	6.9	11.5	82.5	0.06	0.90	217.0	16.0	1360.0	160.0	2010.0	4.3	3270.0	371.8	1210.7	0.31
37	108.5	6.9	26.5	52.5	0.13	1.61	303.0	28.0	3510.0	310.0	5510.0	8.8	2870.0	329.3	1204.8	0.27
38	243.5	7.7	14.0	58.8	0.21	4.69	596.0	36.0	5370.0	220.0	7500.0	8.6	64000.0	505.2	1197.6	0.42
40	108.4	7.4	38.0	49.5	0.11	2.13	354.0	18.0	3220.0	73.0	5770.0	4.1	21100.0	411.1	1200.4	0.34
41	185.4	7.0	13.0	77.0	0.09	1.54	191.0	57.0	1900.0	69.0	3470.0	4.0	5780.0	402.4	1212.0	0.33
42	83.1	8.0	14.0	70.0	0.07	1.20	179.0	26.0	3070.0	150.0	5000.0	10.0	5610.0	358.2	1212.0	0.30
43	102.7	7.8	11.5	80.8	0.10	1.91	253.0	23.0	2370.0	87.0	3800.0	5.0	10800.0	346.2	1219.0	0.28
44	107.5	7.7	6.5	82.3	0.09	1.63	247.0	17.0	3590.0	280.0	3220.0	4.9	16000.0	311.8	1218.0	0.26
46	101.2	7.7	9.0	79.8	0.07	7.30	526.0	20.0	9800.0	130.0	2940.0	6.1	100000.0	377.4	1231.3	0.31

47	120.9	8.3	6.5	77.3	0.07	4.68	279.0	35.0	7250.0	260.0	3590.0	5.7	100000.0	300.5	1238.4	0.24
48	140.5	7.2	6.5	72.3	0.17	8.85	516.0	16.0	10500.0	200.0	5290.0	4.4	100000.0	282.7	1224.4	0.23
49	271.0	7.4	51.5	23.8	0.03	0.48	143.0	36.0	4250.0	1100.0	2530.0	13.0	4340.0	555.5	1355.4	0.41
50	123.4	8.1	46.5	31.3	0.05	1.07	310.0	51.0	18800.0	720.0	4410.0	26.0	16700.0	659.5	1358.3	0.49
52	92.9	7.8	64.0	15.0	0.10	1.31	696.0	74.0	5360.0	2200.0	6010.0	28.0	9600.0	807.7	1353.5	0.60
53	72.8	8.2	34.0	50.8	0.04	0.98	142.0	28.0	2280.0	1600.0	1050.0	23.0	7110.0	659.6	1347.3	0.49
54	134.8	7.5	59.0	32.3	0.04	1.01	177.0	53.0	5550.0	880.0	2130.0	13.0	4280.0	596.2	1309.4	0.46
56	70.6	7.7	44.0	35.0	0.04	0.69	155.0	36.0	2400.0	3300.0	1840.0	12.0	4190.0	647.3	1309.3	0.49
57	69.7	7.0	31.5	32.5	0.04	0.64	179.0	36.0	2380.0	1700.0	2250.0	11.0	2420.0	574.1	1299.6	0.44
58	88.4	7.2	31.5	42.3	0.07	1.08	185.0	39.0	3130.0	2100.0	2840.0	12.0	4320.0	574.1	1295.5	0.44
59	88.9	7.5	59.0	21.3	0.06	0.44	168.0	44.0	3840.0	1600.0	3380.0	12.0	4050.0	502.7	1289.0	0.39
60	109.2	7.5	36.5	36.3	0.03	0.50	284.0	48.0	4620.0	560.0	6920.0	20.0	5510.0	502.7	1284.6	0.39
61	40.6	6.9	61.5	18.8	0.03	0.46	349.0	60.0	5620.0	620.0	6770.0	23.0	4930.0	502.7	1274.0	0.39
62	66.4	7.9	46.5	31.3	0.02	0.23	252.0	44.0	8470.0	580.0	6140.0	20.0	4500.0	472.9	1267.2	0.37
63	107.7	8.2	39.0	40.0	0.04	0.57	514.0	61.0	11700.0	710.0	4090.0	18.0	5440.0	550.7	1267.6	0.43
66	40.0	6.5	29.0	47.3	0.05	0.90	160.0	36.0	3190.0	740.0	790.0	9.8	2820.0	694.6	1299.4	0.53
67	44.5	6.2	6.3	79.0	0.07	1.36	242.0	6.2	319.0	34.0	199.0	4.0	1050.0	409.4	1240.5	0.33
68	142.4	7.4	7.5	80.3	0.04	0.84	136.0	4.1	223.0	55.0	234.0	2.1	1580.0	308.6	1243.5	0.25
69	134.6	6.7	6.3	80.3	0.03	0.50	124.0	5.7	258.0	38.0	324.0	3.5	1390.0	328.3	1241.7	0.26
70	127.4	6.8	26.3	54.0	0.05	0.76	118.0	11.0	1060.0	160.0	2020.0	6.9	758.0	325.5	1244.7	0.26
71	71.8	7.4	14.0	79.8	0.02	0.35	270.0	15.0	1530.0	370.0	2930.0	8.1	1330.0	307.1	1246.2	0.25

72	91.3	6.3	15.0	66.5	0.04	0.61	136.0	8.5	672.0	180.0	965.0	4.9	624.0	314.2	1256.6	0.25
74	42.8	6.9	15.3	74.8	0.02	0.61	217.0	8.7	902.0	85.0	2020.0	4.4	621.0	325.8	1251.3	0.26
75	81.1	6.9	11.5	74.8	0.06	0.99	261.0	48.0	1490.0	390.0	1420.0	32.0	4210.0	366.4	1247.4	0.29
76	206.2	6.2	16.5	74.8	0.05	0.97	207.0	5.7	323.0	41.0	623.0	2.9	588.0	359.3	1256.4	0.29
77	65.0	6.9	8.8	80.3	0.02	0.51	106.0	3.3	173.0	29.0	308.0	2.0	276.0	359.3	1258.3	0.29
79	145.1	5.8	16.3	71.5	0.05	0.86	210.0	4.9	235.0	21.0	493.0	2.3	381.0	291.9	1261.8	0.23
80	165.7	7.6	12.5	79.0	0.02	0.50	115.0	4.0	212.0	18.0	353.0	2.5	802.0	291.9	1264.3	0.23
81	104.3	7.2	11.3	76.5	0.01	0.40	150.0	4.9	322.0	56.0	663.0	3.6	1050.0	377.7	1270.2	0.30
82	190.0	6.8	6.5	84.8	0.05	0.90	58.0	1.9	133.0	26.0	144.0	2.0	1180.0	533.6	1273.0	0.42

N.B. ETP: Evapotranspiration, EC: Electrical conductivity

Table 2. Taxonomic affiliation of rhizospheric bacteria isolated from chickpea, metal ion concentration and Aridity index at their sampling sites and their capacity to produce IAA and ACC deaminase *in vitro*.

Strains	**Species**	**% identity**	**PSI**	**IAA (μg L^{-1})**	**ACCd (nmole α-KB h^{-1})**	**Zn (mg kg^{-1})**	**Mn (mg kg^{-1})**	**Cu (mg kg^{-1})**	**Aridity index**
7F	*Paraburkholderia phenoliruptrix*	98.28	1.23	7.38	1574.7	35.0	710.0	29.0	0.47
12F	*Burkholderia* sp. MR5	98.29	5.45	54.3	1158.9	35.0	710.0	29.0	0.47
13F	*Burkholderia rhynchosiae* WSM3930	98.89	3.54	15.71	n.d.	24.0	190.0	5.8	0.33
18F	*Burkholderia* sp. HB1	98.52	1.66	18.65	386.2	11.0	48.0	2.0	0.33
20F	*Burkholderia* sp. WSM4671	99.32	2.45	57.00	5966.4	14.0	150.0	3.4	0.39
21F	*Burkholderia* sp. HB1	98.47	2.44	8.08	n.d.	14.0	150.0	3.4	0.39
27F	*Pseudomonas fluorescens* PfAR1	99.79	4.44	5.54	512.6	10.7	26.0	2.0	0.32
28F	*Paraburkholderia phenoliruptrix*	98.77	1.55	19.41	1692.8	14.0	150.0	3.4	0.39
30F	*Burkholderia* sp. MR5	99.45	2.27	27.40	1347.2	5.2	32.0	2.0	0.23
35F	*Paraburkholderia terricola*	98.99	1.76	19.63	n.d.	18.0	73.0	4.1	0.34
41F	*Burkholderia caledonica* B5	99.15	3.45	24.93	n.d.	41.0	120.0	14.0	0.44
45F	*Pseudomonas putida* E46	99.29	1.39	8.52	n.d.	17.0	280.0	4.9	0.26
46F	*Pseudomonas* sp. AP3_22	99.2	2.69	67.58	n.d.	17.0	280.0	4.9	0.26
52F	*Burkholderia* sp. MR5	98.51	3.56	9.51	917.3	16.0	200.0	4.4	0.23
53F	*Burkholderia* sp. HB1	99.19	1.41	7.00	715.0	36.0	1100.0	13.0	0.41
55F	*Burkholderia* sp. WSM4671	97.98	3.77	14.78	n.d.	36.0	1100.0	13.0	0.41
62F	*Pseudomonas putida* GM6	98.69	2.39	26.11	n.d.	36.0	1100.0	13.0	0.41
64F	*Pseudomonas* sp. ICMP 22245	98.06	1.65	n.d.	1094.3	36.0	1100.0	13.0	0.41
68F	*Burkholderia* sp. NA11029	99.66	2.56	9.29	n.d.	36.0	1100.0	13.0	0.41

73F	*Paraburkholderia terricola*	99.49	2.54	29.89	n.d.	36.0	1100.0	13.0	0.41
84F	*Paraburkholderia phenoliruptrix*	100	2.33	4.74	6800.9	51.0	720.0	26.0	0.49
85F	*Paraburkholderia phenoliruptrix*	98.38	2.41	37.55	n.d.	36.0	3300.0	12.0	0.49
86F	*Paraburkholderia phenoliruptrix*	98.99	2.65	23.00	n.d.	44.0	1600.0	12.0	0.39
87F	*Paraburkholderia phenoliruptrix*	98.79	3.54	54.29	1018.4	42.0	1600..0	13.0	0.53
94F	*Burkholderia* sp. MR5	98.5	3.55	16.24	4297.4	60.0	620.0	23.0	0.39
101F	*Burkholderia* sp. WSM4671	99.89	1.59	13.48	n.d.	44.0	580.0	20.0	0.37
102F	*Pseudomonas* sp. 7.3	98.76	1.38	13.26	n.d.	61.0	710.0	18.0	0.43
108F	*Paraburkholderia phenoliruptrix*	99.37	2.71	13.45	1600.0	36.0	740.0	9.8	0.53
114F	*Pseudomonas frederiksbergensis*	98.88	2.36	5.73	n.d.	15.0	370.0	8.1	0.25
116F	*Pseudomonas frederiksbergensis*	98.66	1.45	n.d.	n.d.	15.0	370.0	8.1	0.25
117F	*Pseudomonas frederiksbergensis*	99.46	2.49	4.97	n.d.	15.0	370.0	8.1	0.25
120F	*Burkholderia* sp. HB1	99.23	3.65	23.43	1911.9	8.7	85.0	4.4	0.26
126F	*Burkholderia cepacia* KSB-32	99.26	4.67	n.d.	453.6	36.0	1100.0	13.0	0.41
127F	*Burkholderia cenocepacia* FL-5-3-30-S1-D7	99.3	4.56	41.9	2232.2	36.0	3300.0	12.0	0.49
130F	*Burkholderia cepacia* KSB-32	99.26	1.23	7.25	n.d.	4.9	56.0	3.6	0.30
132F	*Burkholderia caledonica* B5	99.4	2.39	36.33	n.d.	4.9	56.0	3.6	0.30
3F	*Bacillus simplex* EH12	99.3	3.58	n.d.	453.6	28.0	440.0	9.7	0.36
4F	*Bacillus amyloliquefaciens*	99.15	2.67	8.84	n.d.	28.0	440.0	9.7	0.36
6F	*Bacillus amyloliquefaciens* C-2 SSK-8	99.14	2.39	n.d.	n.d.	22.0	410.0	9.2	0.36
8F	*Bacillus megaterium* GBRS04	99.41	4.43	9.32	352.5	22.0	410.0	9.2	0.36
9F	*Paenibacillus endophyticus* BMCH-IB-ONF 7	97.73	2.49	n.d.	n.d.	22.0	410.0	9.2	0.36
10F	*Bacillus thuringiensis* N3	99.5	1.66	n.d.	n.d.	27.0	170.0	6.5	0.25
14F	*Bacillus simplex*	99.1	1.23	4.08	4786.3	13.0	98.0	2.9	0.28
15F	*Bacillus megaterium* 17-Y5	98.65	2.45	4.55	n.d.	13.0	98.0	2.9	0.28
17F	*Bacillus megaterium* GBRS04	99.4	3.76	13.35	n.d.	13.0	45.0	2.0	0.25

24F	*Bacillus simplex* EH12	99.37	2.31	5.06	1380.9	11.0	48.0	2.0	0.33
25F	*Bacillus niacini* IHB B 7258	98.96	2.45	8.36	n.d.	11.0	48.0	2.0	0.33
26F	*Bacillus simplex* G27	99.6	2.55	7.98	2232.2	19.0	160.0	3.6	0.33
29F	*Bacillus simplex*	99.3	2.29	5.57	546.4	47.0	100.0	8.6	0.52
31F	*Bacillus megaterium* J8R4LARS	99.49	2.47	n.d.	n.d.	47.0	100.0	8.6	0.52
32F	*Bacillus cucumis* J20BS1	99.1	3.55	n.d.	n.d.	23.0	100.0	3.8	0.39
33F	*Bacillus bataviensis* 27.1	99.79	5.66	n.d.	1237.6	23.0	100.0	3.8	0.39
34F	*Peribacillus simplex* 136-CR12	99.5	2.41	4.3	n.d.	23.0	100.0	3.8	0.39
37F	*Peribacillus simplex* EH12	99.09	2.47	n.d.	n.d.	23.0	100.0	3.8	0.39
38F	*Peribacillus simplex*	98.45	3.33	9.97	3757.9	21.0	110.0	3.0	0.29
39F	*Bacillus megaterium* J8R4LARS	99.69	3.44	6.01	n.d.	14.0	150.0	3.4	0.39
40F	*Bacillus cereus* WYLW1-7	99.44	2.45	15.64	n.d.	7.7	26.0	2.0	0.32
42F	*Bacillus subtilis* CICC10034	98.73	2.88	5.70	7323.5	36.0	220.0	8.6	0.42
43F	*Bacillus megaterium*	99.06	3.54	5.60	n.d.	36.0	220.0	8.6	0.42
47F	*Peribacillus simplex* EH12	99.7	3.44	5.60	n.d.	36.0	220.0	8.6	0.42
50F	*Bacillus toyonensis*	99.05	2.33	7.47	n.d.	18.0	73.0	4.1	0.34
51F	*Bacillus endophyticus* NM3E6	99.68	1.44	7.89	n.d.	57.0	69.0	4.0	0.33
57F	*Bacillus simplex* QT-140	99.36	3.66	8.01	7324.5	26.0	150.0	10.0	0.30
59F	*Bacillus amyloliquefaciens* SDSB22	98.86	2.59	10.30	959.4	23.0	87.0	5.0	0.28
60F	*Bacillus megaterium* VITAKB20	99.79	2.57	6.20	1144.8	23.0	87.0	5.0	0.28
61F	*Bacillus mycoides*	99.69	2.66	4.46	n.d.	23.0	87.0	5.0	0.28
65F	*Peribacillus simplex* EH12	99.71	2.5	7.35	n.d.	23.0	87.0	5.0	0.28
66F	*Bacillus endophyticus* NM3E6	94.72	2.66	5.60	1684.3	17.0	280.0	4.9	0.26
67F	*Bacillus mycoides*	99.55	2.54	6.27	984.7	20.0	130.0	6.1	0.31
69F	*Bacillus subtilis* CICC10034	99.4	2.44	9.35	n.d.	20.0	130.0	6.1	0.31
70F	*Bacillus niacini*	99.89	3.47	8.40	n.d.	35.0	260.0	5.7	0.24

71F	*Bacillus cucumis*	99	2.56	12.94	959.4	36.0	1100.0	13.0	0.41
72F	*Bacillus toyonensis* SL4-3	99.49	2.44	35.93	1144.8	36.0	1100.0	13.0	0.41
74F	*Bacillus mycoides* BF1-5	99.28	3.33	10.78	n.d.	36.0	1100.0	13.0	0.41
75F	*Bacillus simplex* FC1781	99.26	2.55	8.62	n.d.	36.0	1100.0	13.0	0.41
76F	*Bacillus simplex*	99.61	2.76	5.73	537.9	53.0	880.0	13.0	0.46
77F	*Bacillus cereus* 103	98.93	2.88	8.52	6834.6	53.0	880.0	13.0	0.46
78F	*Bacillus cereus* LA324	99.59	3.87	9.41	n.d.	36.0	3300.0	12.0	0.49
79F	*Bacillus niacini*	99.58	2.76	16.43	n.d.	60.0	620.0	23.0	0.39
80F	*Bacillus megaterium* S188	98.74	2.34	6.36	n.d.	44.0	580.0	20.0	0.37
81F	*Bacillus simplex*	100	2.66	7.56	1212.3	44.0	580.0	20.0	0.37
82F	*Bacillus simplex*	99.7	2.87	6.22	n.d.	44.0	580.0	20.0	0.37
83F	*Bacillus simplex*	99.89	2.66	12.09	n.d.	36.0	740.0	9.8	0.53
89F	*Bacillus subtilis* mmb14	98.66	2.44	6.62	512.6	6.2	34.0	4.0	0.33
98F	*Bacillus pumilus* 315	99.66	4.76	6.52	672.8	6.2	34.0	16.0	0.23
100F	*Bacillus megaterium* S2	97.71	2.76	24.9	5972.3	48.0	390.0	32.0	0.29
111F	*Bacillus pumilus* 52	99.2	3.55	10.68	n.d.	3.3	29.0	2.0	0.29
118F	*Bacillus atrophaeus*	99.67	2.33	6.59	n.d.	4.9	21.0	2.3	0.23
119F	*Bacillus niacini*	99.7	2.77	8.36	335.1	4.9	56.0	3.6	0.30
58F	*Bacillus simplex* EH12	99.79	2.69	4.97	672.8	4.9	56.0	3.6	0.30

ACCd- ACC deaminase, α-KB- α-ketobutyrate, n.d. not detected

Table 3. The chemical characteristics of rock phosphates

Chemical properties	**Boucraa RP**	**Vietnam RP**	**Weng Fu RP**	**Togo RP**	**Phalaborwa RP**	**Peru RP**	**Sechura RP**
Source	Western Sahara	Vietnam	China	West Africa	South Africa	Peru	Peru
Total P (%)	15.6	12.6	14.5	15.2	16.0	14.7	13.7%
Cadmium (mg Cd/kg P)	161	6	15	329	Not available	151	-
BD (T/m^3)	1.6	1.69	-	1.5	-	1.6	-
Iron (%)	5.3	0.88	0.70	1.4	0.7	-	-
Aluminium (%)	15.5	0.47	0.53	0.6	0.2	-	-

Key: BD = bulk density; RP = Rock phosphate

11. Media and reagents

DF minimal medium (Dworkin and Foster, 1958)

Chemical	Quantity (g L^{-1})
KH_2PO_4	4.0
Na_2HPO_4	6.0
$MgSO_4.7H_2O$	0.2
glucose	2.0
gluconic acid	2.0
citric acid	2.0
$FeSO_4.7H_2O$	0.001
H_3BO_3	0.01
$MnSO_4.H_2O$	0.01119
$ZnSO_4.7H_2O$	0.1246
$CuSO_4.5H_2O$	0.07822
MoO_3	0.01
$(NH_4)_2SO_4$	0.1

pH is adjusted to 7.0 and autoclaved at 121 ºC for 20 min.

YEMB medium

Chemical	Quantity (g L^{-1})
Yeast extract	0.5
Mannitol	2.5
L-glutamic acid Na	0.5
$Na_2HPO_4.2H_2O$	0.36
$MgSO_4.7H_2O$	0.1
$CaCl_2$	0.04
$FeCl_3$	0.004

Congo red: 10 mL (0.0025 g/L)

The pH is adjusted to 7.0 and autoclaved for 20 min at 121 ºC. YEMA 15 g L^{-1} is added to YEMB prior to autoclaving.

McKnight's nutrient solution (McKnight et al. 1949)

Chemical	Quantity (per 0.25 L)
$CaSO_4.2H_2O$	6.75 g
$MgSO_4.7H_2O$	1 g
KH_2PO_4	1 g
KCl	1.5 g
A-Z trace elements*	5 mL
D solution**	5 mL
Distilled water	250 mL

*A – Z trace elements 2.86g H_3BO_3, 2.08 g $MnSO_4.7H_2O$, 0.22 g $ZnSO_4.7H_2O$, 0.079 g $CuSO_4.5H_2O$, 0.09 g $H_2MoO_4.H_2O$ L^{-1}.

**D solution is 10 g $FeCl_3$ L^{-1}.

NBRIP medium (Nautiyal 1999)

Chemicals	Quantity (g L^{-1})
glucose	10
$MgCl_2.6H_2O$	5
$MgSO_4.H_2O$	0.25
KCl	0.2
$(NH_4)_2SO_4$	0.1
$Ca_3(PO_4)_2$	5