

***PENICILLIUM RADICUM*: STUDIES ON THE MECHANISMS OF
GROWTH PROMOTION IN WHEAT**

SIMON ANSTIS

Thesis submitted for the degree of Doctor of Philosophy

in

The University of Adelaide

(Faculty of Sciences)

May 2004.

School of Earth and Environmental Sciences

Waite Campus

The University of Adelaide, Australia

TABLE OF CONTENTS

LIST OF FIGURES	vi
LIST OF TABLES	ix
DECLARATION	xi
ABBREVIATIONS	xii
ACKNOWLEDGEMENTS	xiii
SUMMARY AND SCOPE OF THESIS	xiv
CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW.....	1
1.1 Soil Phosphate.....	1
1.1.1 Soil inorganic P.....	1
1.1.2 Soil organic P.....	2
1.1.3 P fertilisation: the problem	2
1.2 Phosphate solubilising microorganisms	4
1.2.1 Mechanisms of microbial P solubilisation.....	4
1.2.2 Factors affecting the efficacy of P solubilising microorganisms.....	7
1.2.3 Isotopic labeling to determine P solubilisation	8
1.3 Penicillium bilaiae	10
1.3.1 Isolation.....	10
1.3.2 Mechanisms of plant growth promotion	10
1.3.3 Interaction of P. bilaiae PB-50 with N-fixing rhizobia.....	12
1.3.4 Interaction with vesicular-arbuscular mycorrhiza	13
1.4 Penicillium radicum	15
1.4.1 Species description.....	16
1.4.2 The effect of P. radicum on the growth of wheat.....	16
1.4.3 The solubilisation of phosphate in-vitro by P. radicum.	18
1.5 Plant growth regulators	19
1.5.1 Chemical identity of auxin.....	19
1.5.2 Effect of auxin on plant growth.....	20
1.5.3 Effects of auxin on root growth.....	21
1.5.4 Production of auxins by root-associated microorganisms.....	23
1.5.5 Effect of microorganisms on root growth	25
1.5.6 Ethylene.....	26
1.6 Soilborne cereal root diseases.....	28
1.6.1 Incidence and severity of soilborne diseases in the Australian wheat belt.....	29
1.6.2 Management strategies for root disease	30
1.6.2 (i) Take-all caused by Ggt.....	30
1.6.2 (ii) Rhizoctonia root-rot	31
1.6.2 (iii) Pythium root-rot	32
1.6.2 (iv) Fusarium crown-rot	32
1.6.2 (v) Common root rot	32
1.6.2 (vi) Summary of methods to control root diseases.....	33
1.6.3 Use of specific microorganisms for root disease control.....	34

1.6.4 Greenhouse and field trials demonstrating the use of <i>Penicillium spp.</i> in the reduction of plant disease	35
1.7 Mechanisms of root disease suppression by plant beneficial fungi	39
1.7.1 Competition	39
1.7.2 Antibiosis	40
1.7.3 Mycoparasitism	41
1.7.3 (i) Signalling	41
1.7.3 (ii) Hyphal-hyphal interactions	42
1.7.3 (iii) Lytic enzyme complexes	42
1.8 Summary	44
1.9 Background and objectives of thesis	45
CHAPTER 2. GENERAL MATERIALS AND METHODS	48
2.1 Fungal species, culturing and inoculation techniques	48
2.1.1 Fungi used in the research	48
2.1.2 Preparation of seed and ryegrass inoculum	49
2.1.3 Isolation of <i>P. radicum</i> from roots using semi-selective medium	50
2.2 Plant growth and analysis	51
2.2.1 Glasshouse conditions	51
2.2.2 Chemical analysis of plant material	51
2.2.3 Preparation of Long Ashton nutrient solution for plant growth in sand culture	52
2.3 Rock phosphate: preparation and chemical analysis	52
2.4 Statistical Analysis	53
2.5 Chemical and physical analysis of field soils	54
CHAPTER 3. SOLUBILISATION OF ROCK PHOSPHATE BY <i>PENICILLIUM RADICUM</i> AND <i>P. BILAIAE</i> RS7B-SD1 IN LIQUID CULTURE	57
3.1 Introduction	57
3.2 Methods	59
3.2.1 RP solubilisation assay	59
3.2.2 Identification of organic anions by capillary zone electrophoresis (CZE)	60
3.3 Results	61
3.3.1 Capillary zone electrophoresis of RP solubilisation assays.	61
3.3.2 RP solubilisation assay	63
3.3.2 (i) Non-buffered medium	63
3.3.2 (ii) Buffered medium	68
3.3.3 Fungal biomass production	72
3.4 Discussion	73
CHAPTER 4. THE EFFECT OF <i>PENICILLIUM RADICUM</i> ON WHEAT PHOSPHORUS UPTAKE FROM SUBSTRATES OF CONTRASTING SOLUBILITY IN SAND CULTURE	78
4.1 Introduction	78

4.2 Methods	79
4.2.1 Preparation of the plant growth medium	79
4.2.2 Experiment 1. The effect of <i>P. radicum</i> on plant growth and nutrient uptake from contrasting P substrates	81
4.2.3 Experiment 2. The effect of N source on the plant response to <i>P. radicum</i> inoculation.....	82
4.2.4 Isolation of <i>P. radicum</i> from wheat roots.....	82
4.3 Results	83
4.3.1 Experiment 1	83
4.3.1 (i) Plant growth response to P source.....	83
4.3.1 (ii) Plant growth response to fungal inoculation	83
4.3.1 (iii) Phosphorus nutrition	85
4.3.2 Experiment 2	87
4.3.2 (i) Plant growth.....	87
4.3.2 (ii) Phosphorus nutrition.....	90
4.3.3(iii) Isolation of <i>P. radicum</i> from root sections.....	92
4.4 Discussion	92
CHAPTER 5. EVALUATION OF L-VALUES IN A VARIETY OF FIELD SOILS: DOES P SOLUBILISATION PLAY A ROLE IN THE GROWTH PROMOTION OF WHEAT BY <i>P. RADICUM</i> ?	103
5.1 Introduction	103
5.2 Methods	104
5.2.1 Seed preparation	104
5.2.2 Soils.....	104
5.2.3 L-value determination	104
5.2.3 (i) Soil preparation, plant growth and analysis	104
5.2.3 (ii) Experiment 1	106
5.2.3 (iii) Experiment 2	106
5.2.4 Isolation of <i>P. radicum</i> seed inoculant.....	107
5.3. Results	108
5.3.1 Experiment 1	108
5.3.2 Experiment 2	108
5.3.2 (i) Plant growth.....	108
5.3.2 (ii) P uptake	111
5.3.3 (iii) L-values	113
5.3.4 (iv) Isolation of <i>P. radicum</i> from roots	113
5.4 Discussion	115
CHAPTER 6. EVIDENCE FOR PRODUCTION OF AUXIN BY <i>PENICILLIUM RADICUM</i>	120
6.1 Introduction	120
6.2 Methods	121
6.2.1 <i>P. radicum</i> culture, extraction and thin layer chromatography.....	121
6.2.2 <i>Avena</i> coleoptile straight growth assay	122
6.2.3 Generation of diazomethane	123

6.2.4 Enzyme linked immunosorbent assay (ELISA).....	123
6.3 Results	126
6.3.1 Thin-layer chromatography	126
6.3.2 Avena straight growth assay	129
6.3.2 (i) Response to synthetic indole acetic acid	129
6.3.2 (ii) Application to <i>P. radicum</i> culture extracts.....	130
6.3.3 ELISA	134
6.4 Discussion	134
6.5 Conclusion.....	142
CHAPTER 7. IN-VITRO INDICATORS OF THE DISEASE SUPPRESSING ACTIVITIES OF <i>PENICILLIUM RADICUM</i>	143
7.1 Introduction	143
7.2 Methods	145
7.2.1 Experiment 1. In-vitro inhibition of cereal root pathogens <i>Gaeumannomyces graminis var. tritici</i> , <i>Pythium irregulare</i> and <i>Rhizoctonia solani</i> by <i>P. radicum</i>	145
7.2.2 Experiment 2. Hyphal and colony interactions of <i>P. radicum</i> with cereal root pathogens.	146
7.2.3 Experiment 3. Growth inhibition of cereal root pathogens by diffusible <i>P. radicum exudates</i>	148
7.2.4 Experiment 4. In-vitro examination of the lytic enzyme activity of <i>P. radicum culture</i>	148
7.2.4 (i) Chitinase activity.	148
7.2.4 (ii) β -1,3-glucanase activity.....	150
7.2.4 (iii) β -1,4-glucanase activity	150
7.2.4 (iv) Protease production.	150
7.2.5 Experiment 5. Glucose oxidase activity of <i>P. radicum</i> culture supernatant. ..	151
7.3 Results	152
7.3.1 Experiment 1. In-vitro inhibition of cereal root pathogens <i>Gaeumannomyces graminis var. tritici</i> , <i>Rhizoctonia solani</i> and <i>Pythium irregulare</i> by <i>P. radicum</i> in solid media.	152
7.3.1 (i) Inhibition ratings of cereal root pathogens in dual inoculation with <i>P. radicum</i>	152
7.3.1 (ii) Description of colony interactions between <i>P. radicum</i> and cereal root pathogens.....	153
7.3.2 Experiment 2. Hyphal and colony interactions of <i>P. radicum</i> with cereal root pathogens.	156
7.3.2 (i) Colony interactions on ¼ PDA.....	156
7.3.2 (ii) Colony interactions on 0.2% G agar	162
7.3.2 (iii) Hyphal interactions	166
7.3.3 Experiment 3. Fungal growth inhibition by diffusible <i>P. radicum</i> metabolites.	171
7.3.3 (i) Growth inhibition on ¼ PDA	171
7.3.3 (ii) Growth inhibition on 0.2% G agar	175
7.3.4 Experiment 4. Production of cell wall lysing and glucose oxidase enzymes by <i>P. radicum</i>	177
7.3.4 (i) Chitinase activity	177

7.3.4 (ii) β -1,3-glucanase activity	178
7.3.4 (iii) β -1,4-glucanase production	178
7.3.4 (iv) Protease activity.....	179
7.3.5 Experiment 5. Production of glucose oxidase activity.....	179
7.4 Discussion	180
CHAPTER 8. THE EFFECT OF <i>PENICILLIUM RADICUM</i> ON ROOT DISEASE CAUSED BY <i>GAEUMANNOMYCES GRAMINIS</i> VAR. <i>TRITICI</i> IN A WHEAT SEEDLING BIOASSAY	189
8.1 Introduction	189
8.2 Methods	190
8.2.1 Experiment 1. Effect of <i>P. radicum</i> on take-all severity in a wheat seedling bioassay using a sand/soil medium.	190
8.2.2 Experiment 2. Effect of <i>P. radicum</i> on take-all severity in a field soil.....	192
8.2.3 Root disease analysis	193
8. 3 Results	194
8.3.1 Experiment 1. The effect of <i>P. radicum</i> on take-all severity in a wheat seedling bioassay.....	194
8.3.1 (i) Plant growth and water consumption	194
8.3.1 (ii) P nutrition	194
8.3.1 (iii) Root disease.....	195
8.3.2 Experiment 2. The effect of <i>P. radicum</i> on take-all severity in a field soil.....	197
8.3.2 (i) Plant growth.....	197
8.3.2 (ii) P nutrition	197
8.3.2 (iii) Root disease.....	198
8.3.2 (iv) Seedling and disease development after 8 weeks growth	198
8.4 Discussion	201
CHAPTER 9. GENERAL DISCUSSION	208
9.1 Introduction: Project aims & background	208
9.2 P solubilisation.....	208
9.3 Production of auxin.....	211
9.4 Interaction of <i>P. radicum</i> with soilborne pathogens.....	213
9.5 Further mechanisms to account for plant growth promotion by <i>P. radicum</i>. ...	216
9.6 Future Research	219
9.7 Conclusion.....	222
REFERENCES.....	223

LIST OF FIGURES

- Figure 1.1** Schematic representation of the growth responses of root, buds and stems when exposed to an auxin concentration range (Russell, 1978).21
- Figure 3.1** Representative electropherograms of culture filtrates of *P. radicum* and *P. bilaiae* RS7B-SD1. These samples were taken after 168 hours incubation in cultures that were supplied with either NO_3^- or NH_4^+ as the sole source of N. G = gluconate, U = main organic anion produced by *P. bilaiae* RS7B-SD1 when cultured with NO_3^- . Y axis = absorbance (210 nm), X axis = migration time (minutes).62
- Figure 3.2** Changes in gluconate concentration, soluble P and pH in cultures of *P. radicum* (Figure 3.2a) and *P. bilaiae* RS7B-SD1 (Figure 3.2b) during a 168 hour incubation in non-buffered medium with NO_3^- as the sole source of N. Error bars indicate s.e (n=3). It should be noted there are differences in scale for gluconate and soluble PO_4 concentration.66
- Figure 3.3** Changes in gluconate concentration, soluble P and pH in cultures of *P. radicum* (Figure 3.3a) and *P. bilaiae* RS7B-SD1 (Figure 3.3b) during a 168 hour incubation in non-buffered medium with NH_4^+ as the sole source of N. Error bars indicate s.e (n=3). It should be noted that there are differences in scale for gluconate and soluble PO_4 concentration.67
- Figure 3.4** Changes in gluconate concentration, soluble P and pH in cultures of *P. radicum* (Figure 3.3a) and *P. bilaiae* RS7B-SD1 (Figure 3.3b) during a 168 hour incubation in medium buffered using 100 mM Tris-HCl pH 7.8 with NO_3^- as the sole source of N. Error bars indicate s.e (n=3). It should be noted that there are differences in scale of gluconate and soluble PO_4 concentration.70
- Figure 3.5** Changes in gluconate concentration, soluble P and pH in cultures of *P. radicum* (Figure 3.5a) and *P. bilaiae* RS7B-SD1 (Figure 3.5b) during a 168 hour incubation in a medium buffered using 100 mM Tris-HCl pH 7.8 with NO_3^- as the sole source of N. Error bars indicate s.e. (n=3). It should be noted that there are differences in scale of gluconate and soluble PO_4 production.71
- Figure 4.1** The effect of *P. radicum* on plant dry weight in sand culture supplying P substrates of varying degrees of solubility (Experiment 1). P sources were: Ca-P = CaHPO_4 ; Fe-P = FePO_4 , RP = rock phosphate and; P_o = phytic acid. Values in parentheses indicate total dry weight pot^{-1} (g), where l.s.d = 0.24 g. Error bars on shoot weights indicate s.e (n=5) and root weights indicate l.s.d (α 0.05). 84
- Figure 4.2** The effect of *P. radicum* on the P nutrition of plants grown in sand culture that supplied P substrates of varying solubility. P sources were: Ca-P = CaHPO_4 ; Fe-P = FePO_4 , RP = rock phosphate and; P_o = phytic acid. Error bars indicate 3-way l.s.d (α 0.05).86
- Figure 4.3** Shoot height (cm) after 4 weeks growth of wheat plants supplied with either RP (rock phosphate) or Ca-P (CaHPO_4) as the P source and supplied with either NH_4^+ or NO_3^- as the sole source of N. Error bars indicate 3-way l.s.d (α 0.05). ... 87
- Figure 4.4** The effect of *P. radicum* dry shoot weight, Figure 4.4(a), and dry root weight, Figure 4.4(b), in sand culture supplying either NH_4^+ or NO_3^- as the sole

- source of N. RP and Ca-P indicate P sources rock phosphate and CaHPO_4 , respectively. Error bars indicate 3-way l.s.d (α 0.05). 89
- Figure 4.5** The effect of *P. radicum* on the P nutrition of plants grown with either RP (rock phosphate) or Ca-P (CaHPO_4) as the P source and supplied with either NH_4^+ or NO_3^- as the source of N. Error bars indicate 3-way l.s.d (α 0.05). 91
- Figure 5.1** The effect of *P. radicum* inoculation on dry shoot weight of wheat seedlings grown in four γ -irradiated soils with or without re-inoculation with 10% non-sterile soil. Error bars indicate s.e. (n=5). 110
- Figure 5.2** The effect of *P. radicum* inoculation on dry root weight of wheat seedlings grown in four γ -irradiated soils with or without re-inoculation with 10% non-sterile soil. Error bars indicate s.e. (n=5). 110
- Figure 5.3** The effect of *P. radicum* on shoot P concentration of wheat seedlings grown in γ -irradiated soil with or without re-inoculation with 10% non-sterile soil to re-introduce indigenous microflora. Error bars indicate s.e. (n=5). 112
- Figure 5.4** The effect of *P. radicum* on shoot P uptake of wheat seedlings grown in γ -irradiated soil with or without re-inoculation with 10% non-sterile soil to re-introduce indigenous microflora. Error bars indicate s.e. (n=5). 112
- Figure 5.5** Mean *L*-values of wheat seed treated with *P. radicum* and grown in γ -irradiated soils with or without re-inoculation with 10% non-sterile soil. Error bars indicate s.e. (n=5) 113
- Figure 6.1** (opposite page) Thin layer chromatogram of Salkowski reactive compounds in acidified diethyl ether extracts taken from *P. radicum* cultured in Malt Extract Broth over a five day incubation period. The R_f of reactive zones were compared to standard compounds tryptophan (TRP), tryptamine (TAM), indole acetic acid (IAA) and tryptophol (TOL). Salkowski reactive bands that were too faint to appear in the photograph have been outlined. After 5 days incubation in MEB that was amended 30 mg L^{-1} TRP, *P. radicum* produced bands that were similar to IAA in R_f and Salkowski reaction (6.1a). No IAA like substances were detected in extracts of *P. radicum* in MEB with no TRP amendment (6.1b). 127
- Figure 6.2** Activity curve of coleoptile extension for the *Avena* straight growth assay using standard indole-3-acetic acid (IAA) over a concentration range of 6.4×10^{-7} M to 0.01 M. The horizontal line marked with an asterisk (*) indicates extension of coleoptiles in 4% sucrose citrate phosphate buffer. Error bars show s.e (n=5). .. 130
- Figure 6.3** Auxin-like activity in cultures of *P. radicum* (MEB) as determined by the *Avena* straight growth assay. Fungal culture was sampled following 1, 2, 3, 4 and 5 days of incubation. The samples were acidified (pH 4), extracted using diethyl ether and developed on silica gel plates (CHCl_3 :EtOH, 80:20). Areas marked as squares (▣) at the appropriate R_f values were Salkowski reactive. The R_f of IAA for the solvent system is marked by a double ended arrow (↔). The line marked with an asterisk (*) represents the % coleoptile extension of coleoptiles grown in sterile 4% SCP. Error bars indicate s.e. (n=5). 132
- Figure 6.4** Auxin-like activity in cultures of *P. radicum* (MEB + 30 mg L^{-1} TRP) as determined by the *Avena* straight growth assay. Fungal culture was sampled following 1, 2, 3, 4 and 5 days of *P. radicum* incubation and extracted using

acidified (pH 4) diethyl ether and developed on silica gel plates. Plates were visualised by spraying with Salkowski reagent. Zones that developed are marked as squares (▨) at the appropriate R_f values. The R_f of IAA standard for the solvent system is marked by a double ended arrow (↔). The line marked with an asterix (*) represents the mean percent coleoptile extension of coleoptiles grown in sterile 4% SCP. Error bars indicate s.e. (n = 5).....	133
Figure 7.1 Schematic diagram of dual inoculated Petri dish indicating the direction of measurements r_1 and r_2 for equation given in Section 7.2.1.....	146
Figure 7.2 Growth and interaction of <i>P. radicum</i> and cereal root pathogens on MEA at 25 °C.....	155
Figure 7.3 Typical growth and colony interaction of <i>P. radicum</i> and soilborne pathogens on ¼ PDA at 25 °C. Incubation time was 5 days unless otherwise stated.....	160
Figure 7.4 Growth and interactions of <i>P. radicum</i> with cereal root pathogens on 0.2% G agar at 25 °C	164
Figure 7.5 Hyphal interactions between <i>P. radicum</i> and other fungi in dual inoculation on 0.2% G agar at 25 °C.....	168
Figure 7.6 Growth inhibition of cereal root pathogens by diffusible <i>P. radicum</i> exudates when tested on ¼ PDA agar at 25 °C. Black arrow indicates the position of test agar plugs.....	173
Figure 7.7 Growth inhibition of cereal root pathogens by diffusible exudates of <i>P. radicum</i> when tested on 0.2% G agar at 25 °C. Black arrows indicate position of agar plugs.	176
Figure 8.1. Banding procedure used to fill the pots in Experiment 1. In the first band both <i>P. radicum</i> and <i>Ggt</i> were added, for the second medium only <i>P. radicum</i> was added. Seven pregerminated seeds, thinned to five, were added on top of the second band. In the third band, used to cover the seeds, the soil mix was not inoculated. Plastic beads were added as the final layer to reduce moisture loss by evaporation	192

LIST OF TABLES

Table 1.1 The distribution and relative importance of diseases of the crown and root caused by fungi in Australian wheat production.....	30
Table 1.2 Use of <i>Penicillium</i> spp. in the protection of plants against soilborne diseases.	36
Table 2.1 Fungal isolates used in this thesis.....	49
Table 3.1 Characteristics of RP solubilisation in cultures of <i>P. radicum</i> and <i>P. bilaiae</i> RS7B-SD1 culture were recorded as: the pH at maximum concentration of soluble P detected during the entire incubation; the maximum soluble P recorded for the entire incubation; the highest detected gluconate anion concentration and; the ratio of biomass : soluble P produced after 168 hours of incubation. Data given is the mean of three replicates per treatment. An asterisk indicates level of significance based on degrees of freedom (n=8) where **** = 0.001, *** = 0.005, ** = 0.02 and * = 0.05.....	65
Table 3.2 Production of biomass by <i>P. bilaiae</i> RS7B-SD1 and <i>P. radicum</i> in liquid culture after 168 hours of incubation. The growth medium supplied either NO ₃ ⁻ or NH ₄ ⁺ as the sole source of N. Data given is the mean of three replicates per treatment, l.s.d = least significant difference.....	72
Table 4.1 P composition of sand growth media used in the two sand culture assay experiments.	81
Table 5.1 The effect of <i>P. radicum</i> on dry shoot weight, dry shoot P concentration and L-value of wheat seedlings in three field soils after five weeks growth. Data given are the mean of five experimental replicates followed by s.e (n=5).	108
Table 5.2 Isolation of <i>P. radicum</i> from the root sections of wheat seedlings (0-2 cm from seed) after four weeks growth in γ -irradiated soil or soil that had been re-inoculated with 10% non-sterile soil. s.e indicates standard error of the mean with four replicates.	114
Table 6.1. Use of ELISA to determine the production of IAA by <i>P. radicum</i> in Malt Extract Broth (MEB) and Glucose minimal salts (GMS) either with or without TRP addition. Errors (\pm) indicate s.e (n=3).....	134
Table 7.1 The effect of <i>P. radicum</i> incubation on the % inhibition rating (% I.R.) of pathogen colony growth when tested on two media of contrasting nutrient availability, MEA and 0.2% G agar. Errors (\pm) indicate s.e (n=3).	153
Table 7.2 Inhibition rating (% I.R.) and antibiosis colony interactions between <i>P. radicum</i> and cereal root pathogens in dual inoculation on 0.2% G agar and ¼ PDA. Errors (\pm) refer to s.e. of three experimental replicates.....	158
Table 7.3. % Inhibition rating of cereal root pathogen colony growth on either ¼ PDA or 0.2% G agar by diffusible <i>P. radicum</i> exudates after 3 days incubation at 25 °C. Errors (\pm) indicate s.e of four experimental replicates.....	171
Table 7.4 The production of cell wall degrading enzymes by <i>P. radicum</i>	177

Table 7.5. Glucose oxidase activity in <i>P. radicum</i> culture supernatant compared with reference <i>Penicillium</i> and <i>Aspergillus</i> strains reported by Petruccioli et al., (1993).	180
Table 8.1 The effect of <i>P. radicum</i> on wheat seedling growth and take-all disease severity in a sand : soil (70 : 30) based seedling bioassay with artificially added populations of <i>Ggt</i> (summary of results from Experiment 1). <i>P. radicum</i> was added as infested ryegrass propagules to a soil-sand mix at five rates. Data is the mean of four replicates.	196
Table 8.2. The effect of <i>P. radicum</i> on wheat seedling growth and take-all disease severity (summary of results from Experiment 2) in an artificially infested field soil at two harvest times.	200

DECLARATION

I declare that the work does not contain material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I consent to this copy of my thesis, when deposited in the University Library being made available for loan or photocopying.

December 2004.

Signed,

Simon Anstis

ABBREVIATIONS

l.s.d	least significant difference
Ca-P	dibasic calcium phosphate, monetite
DRBC	dichloran rose bengal chloramphenicol agar
EtOH	ethanol
Fe-P	crystalline iron phosphate, FePO ₄
IAA	indole-3-acetic acid
<i>Ggt</i>	<i>Gaeumannomyces graminis</i> var. <i>tritici</i> isolate #8
MEA	malt extract agar
MEB	malt extract broth
MeOH	methanol
RP	rock phosphate
s.e	standard error
P _o	phytate, inositol hexaphosphate
PDA	potato dextrose agar
R _f	retention factor
SCP	4% sucrose citrate-phosphate buffer, pH 5
Tris	Tris[hydroxymethyl]aminomethane
TRP	tryptophan
WS	water soluble

ACKNOWLEDGEMENTS

Throughout the duration of this project I received the help and support of many people but most importantly, I would like to thank Bio-Care Technology, Wodonga, NSW (formerly Australian Seed Inoculants Pty Ltd) for financial support of this project and my supervisors Sally Smith and Maarten Ryder for their work in assisting me with the production of the thesis and guidance through the project.

I would also like to thank the following people for their involvement in the project:

Steve Wakelin, for research collaboration and the many long discussions on *P. radicum*. I would also like to thank the people who I shared the laboratory with Rosemary Warren, Paul Harvey and Bruce Hawke for their time, patience and friendly manner during my stay at the laboratory. I would also like to thank Mike McLaughlin, Enzo Lombi and Caroline Johnston (CSIRO) for their assistance with the isotopic component of this project.

I would also like to acknowledge the following people: Stuart McClure (CSIRO) for his advice and technical assistance with scanning electron microscopy; Mark Fritz (CSIRO) for assistance with the XRF fluorescence analysis of rock phosphate; Gareth Lewis (Adelaide University) for assistance with the generation of diazomethane and gas chromatography mass spectrometry; Zuliang Chen (CSIRO) for assistance with the use of capillary electrophoresis and Tony Rathjen for the kind donation of wheat seed used in these experiments.

Chapter 8 was performed in conjunction with CSIRO researchers Rosemary Warren and Steven Wakelin. Rosemary is thanked for her assistance with preparation in Experiment 1 and Stevo is thanked for his assistance with Experiment 2.

SUMMARY AND SCOPE OF THESIS

The aims of this study were to investigate the P solubilising activity of *Penicillium radicum* and identify other possible mechanisms of plant growth promotion that were not related to P solubilisation. The plant chosen for the studies was wheat, the largest cereal commodity produced by Australian agriculture. Given the large area that is planted annually to wheat, this crop represents a large potential market for *P. radicum*-based inoculants. However, it is unlikely that *P. radicum* will be effective in all wheat growing regions and all environmental conditions seen in these areas. Research on the modes of action may help to identify the conditions where *P. radicum* has a good chance of being effective.

The P solubilising activity of *P. radicum* was assessed by examining the amount of P released from rock phosphate (RP) *in-vitro*. The effectiveness of *P. radicum* to solubilise RP was compared to that of another P solubilising fungus, the isolate *P. bilaiae* RS7B-SD1. Both fungi were cultured in a liquid medium that contained either NO_3^- or NH_4^+ as the sole source of N. Changes in culture pH, soluble P and gluconic acid concentration were determined for both fungi in a 168 hour incubation in both buffered (100 mM Tris-HCl pH 7.8) and non-buffered medium. For *P. radicum*, the maximum concentration of soluble P was 6.8 fold higher in the presence of NH_4^+ compared to NO_3^- . In contrast, for *P. bilaiae* RS7B-SD1 the highest concentration of soluble P measured in the fungal culture was not significantly affected by N-source. In buffered medium, *P. radicum* did not appear to solubilise RP and levels of soluble P were generally $<1 \text{ mg L}^{-1}$. In contrast, the RP solubilising activity of *P. bilaiae* RS7B-SD1 was not affected by buffering. Increased RP solubilisation with NH_4^+ as the N

source and lack of RP solubilisation in buffered medium suggested that acidification was the main mechanism of P solubilisation by *P. radicum*. RP solubilisation by *P. bilaiae* RS7B-SD1 was similar over the range of culture conditions tested and mechanisms of RP solubilisation are likely to be a combination of mechanisms that relate to both acidification and the production of organic anions.

The effect of inoculation with *P. radicum* on plant growth and P nutrition was studied under glasshouse conditions using a sand culture assay that supplied defined sources of P. The plant growth and P response to *P. radicum* inoculation were determined in two separate experiments. In Experiment 1, plants were grown to pre-heading stage and supplied with either dibasic calcium phosphate (Ca-P), crystalline iron phosphate (Fe-P), rock phosphate (RP) or phytate (P_o) as the source of P. In Experiment 2, plants were harvested after 8 weeks and supplied with either NO_3^- or NH_4^+ as the sole source of N and the P sources were either Ca-P or RP. In Experiment 1, the plant P response (defined as higher shoot P concentration and P uptake) to inoculation was dependent on the P source. The greatest plant P response to inoculation was observed for Ca-P and no significant P response was measured in plants that were supplied with Fe-P, RP or P_o . In pots that supplied Fe-P as the P source, there was an increase in shoot dry matter in response to *P. radicum* inoculation but this occurred without a concomitant plant P response. In Experiment 2, the plant P response to inoculation was dependent on the N source. In the presence of NH_4^+ , *P. radicum* significantly increased the availability of P sources RP and Ca-P. While there was no significant plant P response under NO_3^- supply, there was a significant increase in dry matter production due to *P. radicum* inoculation. When the data of Experiments 1 & 2 are taken together, results suggest that *P. radicum* possesses at least two mechanisms of plant growth promotion, (1) P

solubilisation and (2) general growth promotion that is independent of P solubilisation. In agreement with P solubilisation in solution cultures, the P solubilisation mechanism of *P. radicum* in sand culture required NH_4^+ . The ability of *P. radicum* to increase plant growth independently of a plant P response gave further evidence of general growth promoting abilities of the fungus. While sand culture is a useful tool to elucidate the fungal mechanisms of plant growth promotion, this approach cannot fully reflect the complexity of the rhizosphere in non-sterile soil. Hence, a subsequent experiment was done to determine the effect of *P. radicum* on plant growth and P nutrition in a number of field soils.

The P solubilising activity of *P. radicum* was determined in four Australian field soils using isotopic dilution. Three soils were chosen on the basis of their chemistry of P retention: (1) Minnipa soil from South Australia was chosen due to P retention associated with the highly alkaline calcareous nature of this soil; (2) Innisfail Queensland, in this soil P retention was dominated by reaction with Fe oxides; and (3) Mt Schank South Australia, a volcanic soil where P retention was predominantly associated with Al oxides. The fourth soil, from Mingenew Western Australia, was chosen due to previous reports that *P. radicum* inoculation increased the yield of field grown wheat (Bio-Care Technology, unpublished data). The four field soils were each labelled with $\text{KH}_2^{32}\text{PO}_4$ and the specific activity (^{32}P) of the wheat seedling tissue was measured after four weeks growth. When the data was averaged across all four soil types, inoculation with *P. radicum* caused a significant 11.7% increase in the shoot dry weight of these seedlings. However, *P. radicum* did not cause any consistent significant difference in the specific activity (^{32}P) of plants when compared to uninoculated control plants. These results suggested that *P. radicum* did not have a strong ability to

solubilise P from the test soils, and mechanisms other than P solubilisation were in operation to stimulate plant growth.

The production of plant growth regulators (PGR) was considered as a mechanism of plant growth promotion not related to P solubilisation. To further explore the hypothesis that the production of PGR acts as a mechanism of plant growth promotion, the ability of *P. radicum* to produce the auxin, indole-3-acetic acid (IAA) was investigated. Examination with thin-layer chromatography and the *Avena* coleoptile straight growth assay indicated that fractions of *P. radicum* culture medium with chemical characteristics similar to IAA (i.e. similar reaction to the Salkowski reagent and R_f as IAA) also possessed auxin-like activity. Using competitive enzyme linked immunosorbent assay (ELISA) it was found that in liquid culture amended with the precursor tryptophan, *P. radicum* produced IAA at concentrations up to 0.406 μ M. These studies show that *P. radicum* can produce IAA under laboratory culture conditions. To determine the significance of IAA as a mechanism of plant growth promotion, further studies need to link effects on plant growth and development to the production of IAA by *P. radicum*.

The ability of *P. radicum* to antagonise root pathogens and control root disease was considered as a further mechanism of growth promotion. Under *in-vitro* conditions, *P. radicum* produced hyphal growth patterns and enzymes (protease, β -1,3- and β -1,4-glucanase activity) that were indicative of hyperparasitism. Hyperparasitic growth was seen as hyphal coiling and branching of *P. radicum* against host hyphae of *Rhizoctonia solani*, *Fusarium pseudograminearum* and *Pythium irregulare* when these soilborne pathogens were studied in dual culture with *P. radicum*.

The effect of *P. radicum* on the fungal root disease severity of take-all was studied using a seedling bioassay under glasshouse conditions. The ability of *P. radicum* to suppress take-all disease appeared to be related to the timing of *P. radicum* infection of wheat seedling roots and placement of the *Ggt* inoculum in relation to the roots. Compared to soils where *Ggt* inoculum was only distributed at distances >1 cm below the seed, uniform mixing of the *Ggt* inoculum throughout the soil negated the beneficial effect of *P. radicum* on plant growth and its ability to reduce take-all root lesion size. Conversely, early infection of wheat roots by *P. radicum* gave wheat seedlings some protection against take-all disease. Where treatment with *P. radicum* was effective, increasing the inoculum dose significantly reduced take-all lesion size. While *P. radicum* exhibited antagonism towards *F. pseudograminearum*, *Py. irregulare*, *Bipolaris sorokiniana* and *R. solani* cereal root pathogens *in-vitro*, further studies under non-sterile soil conditions are needed to evaluate the potential for *P. radicum* to reduce root disease caused by these fungi.

In conclusion, it is unlikely that one single mechanism explains the beneficial effect of *P. radicum* on wheat growth. *In-vitro* studies showed that *P. radicum* has a number of attributes that could function as mechanisms of plant growth promotion. These attributes were, (1) P solubilisation, (2) production of IAA and (3) the ability to antagonise soilborne pathogens *in-vitro* and reduce the lesion size of the take-all disease in a seedling bioassay. Sand culture assays revealed that at least two plant growth mechanisms were in operation, (1) P solubilisation and (2) a general growth promotion mechanism that was independent of P solubilisation. In agreement with Whitelaw et al. (1999), the P solubilisation mechanism of *P. radicum* operates via an

acidification mechanism. The effectiveness of this mechanism may be limited by the availability of NH_4^+ in the rhizosphere. Since NH_4^+ appears to be required for P solubilisation there may exist an interaction between *P. radicum* and ammoniacal fertilisers. This will have implications for its effectiveness in the field.

In-vitro studies suggest that the general mechanism of growth promotion may be related to the production of PGRs such as IAA. In this aspect the known colonisation of the interior of wheat roots by *P. radicum* would ensure that IAA produced by the fungus is taken up by the root cell and less subject to chemical degradation and/or degradation by other soil microorganisms. Further studies are required to identify the effect of IAA production on plant growth and the effect of *P. radicum* inoculation on root disease severity in non-sterile soil.