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

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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.

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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
Ggt	*Gaeumannomyces graminis* var. *tritici* 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

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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 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 $KH_2{}^{32}PO_4$ 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 µM. 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.

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Soil Phosphate

Phosphorus (P) is an essential nutrient for all organisms. It is present in biologically important molecules such as nucleic acid, phospholipid and is involved in cellular and metabolic processes. Plants meet the majority of their P requirements by absorbing soluble *ortho*-phosphate ions (HPO_4^{2-}, $H_2PO_4^-$) from the soil solution. Typical concentrations of soil solution P are <10 μM while the mean total phosphorus is present at approximately 0.05% of the soil weight (Frossard et al., 1994; Barber, 1995; Sharpley et al., 2000). Therefore, most of the soil P is unavailable to the plant. The majority of soil P is found either adsorbed to soil surfaces, as insoluble minerals or within insoluble organic complexes.

1.1.1 Soil inorganic P

Under acid conditions, inorganic P in soil is mainly associated with Fe and Al compounds. These compounds may form through either precipitation as insoluble Fe-P and Al-P compounds or adsorption to oxy-hydroxide Fe and Al polymers (Sanyal & De Datta, 1991). Adsorption can occur through physical charge associations or chemical adsorption mechanisms. Chemical adsorption generally involves the ligand exchange of phosphate with surface reactive hydroxyl groups (Rajan, 1976; Parfitt, 1978; Goldberg & Sposito, 1985; Sanyal & De Datta, 1991). Under alkaline conditions, P is precipitated as various Ca or Mg complexes or adsorbed to calcium based surfaces (Sanyal & De Datta, 1991; Tunesi et al., 1999; Miyasaka & Habte, 2001).

1.1.2 Soil organic P

The proportion of soil organic P contributing to total P is variable, but it may be considerable, accounting for up to 90% of the total soil P (Harrison, 1987). The amount of organic P present in the soil is influenced by soil type, climate and use. For example, under cultivation the level of organic P declines, while under pasture the amounts of organic P can increase. In coarse textured soils the organic P content is likely to be less than for clay soils. Moreover, in areas of low rainfall the amount of organic P is lower than in areas of higher rainfall. For further information on soil organic P the reader is referred to reviews available on the topic (Dalal, 1978; Anderson, 1980; Harrison, 1987). An important chemical characteristic of soil organic P is its difficulty to be characterized using traditional chemical techniques. The characterisable fraction contains predominantly inositol hexaphosphate and is likely to arise from the microbial resynthesis of plant residues (L'Annunziata, 1975; L'Annunziata et al., 1977). Inositol hexaphosphate residues may account for up to 80% of the organic soil P (Dalal, 1978). The covalent C-O-P bond needs to be hydrolyzed before this source of P is available to plants. Plants have a small capacity to release phytase enzymes into the rhizosphere; if the phytate fraction could be mineralised by root associated microorganisms this would provide a large amount of extra P to plants (Asmar, 1997; Li et al., 1997; Hayes et al., 1999).

1.1.3 P fertilisation: the problem

The alleviation of soil P deficiency is not always readily achieved by the cost-efficient application of P fertiliser. Following application, P fertiliser is rapidly converted to products of lower solubility (Sample et al., 1980; Sanyal & De Datta 1991). In one growing season, it has been estimated that only 10-30% of the fertiliser applied is

utilised by plants (Stevenson & Cole, 1999; Sharpley et al., 2000). The remainder is immobilised within the soil as the previously described unavailable forms of soil P. Repeated application of P fertiliser to agricultural soils has led to the accumulation of large P reserves within arable soils (Stevenson & Cole, 1999; Frossard et al., 2000).

The problems associated with the repeated application of P fertiliser to soil highlight the need for improving plant P supply and hence nutrition. Through wind erosion and water run-off, increased nutrient loading in water bodies increases the incidence of eutrophication and toxic algal blooms (Sharpley et al., 2000). In addition, once reserves of minable P for fertiliser manufacture are depleted (within 100 years), lower grade rock phosphate (RP) will require processing into fertilizer at a significantly higher cost (see references cited within Richardson, 2001). P fertilisers are expensive and account for a large proportion of farm expenditure in developed countries. Hence, increasing P fertilizer efficiency would significantly reduce farm production costs. In non-developed regions, farmers may not have access to P fertilizers and need to find alternative ways to increase the available P to their crops. For these reasons, there exists an urgent need to increase the efficiency of P fertilisation practices.

There are many strategies available to increase P efficiency. One such strategy is the application of microorganisms that through various metabolic activities and root associations increase the amount of soil P available to be taken up by plants. The use of P solubilising microorganisms to increase plant P uptake is the general theme of this thesis.

1.2 Phosphate solubilising microorganisms

The application of microorganisms as seed or soil treatments, to increase plant P uptake has received considerable attention for a number of years. Gerretsen (1948) is often credited with identifying the effect of microorganisms on the uptake of P by plants. In Gerretsen's study, plants grown in non-sterile sand medium had yields and P uptake that were dramatically increased compared with plants that were grown in sterile sand. Since Gerretsen's study a considerable number of reviews and research articles on the effect of P solubilising microorganisms to improve plant growth, soil P uptake (Kucey et al., 1989; Richardson, 1994; Rodriguez & Fraga, 1999; Whitelaw, 2000; Richardson, 2001) and the utilization of crude RP have appeared (Azcon et al., 1976; Asea et al., 1988; Kucey, 1988; Salih et al., 1989; Mba, 1994; Nahas, 1996; Vassilev et al., 1996; Omar, 1998). Despite the large amount of work that has gone into the study of P solubilising microorganisms, there have been very few developments that have led to commercially available P solubilising inoculants that have a consistent plant growth response in soils of varying properties. Similarly, there is no specific management strategy available to farmers to increase the efficiency/function of the indigenous microbial population residing within arable soils.

1.2.1 Mechanisms of microbial P solubilisation

The solubilisation of insoluble P compounds by microorganisms is generally thought to occur by either (1) direct acidification (protonation) or (2) action of low molecular weight organic anions (Kucey et al., 1989; Rodriguez & Fraga, 1999; Whitelaw, 2000; Richardson, 2001; Gyaneshwar et al., 2002). Protons may originate from a variety of sources. These may include proton extrusion following the uptake of cations in order to maintain cell charge neutrality (Roos & Luckner, 1984), or released in association with

a low molecular weight anion. Direct protonation of alkali insoluble compounds such as calcium and magnesium phosphate leads to the dissolution of the mineral lattice.

Organic anions release soluble P via a number of chemical mechanisms. These include (1) complex formation by chelating cations that precipitate P, (2) competition with P for adsorption sites to prevent adsorption and (3) ligand exchange of adsorbed P. The chelating ability of an organic anion is related to its molecular structure. For example, tricarboxylate anions were more effective chelators than dicarboxylate anions that were more effective than mono-carboxylate anions. Organic anions that had an α-hydroxy functional group were more effective than the corresponding aromatic or aliphatic acid (Bolan et al., 1994; Kpomblekou & Tabatabai, 1994).

In addition to complex formation, organic anions may displace P from adsorption sites in soil or prevent the re-adsorption of P by occupying adsorption sites (Jones, 1998; Hinsinger, 2001). However, the effectiveness of these processes as a mechanism that contributes significant quantities of P for plant uptake is questionable. The surface area of adsorption sites is so high that the concentration of organic acids required for effective mobilisation would be beyond the metabolic capacities of microorganisms under the competitive stress of the rhizosphere (Jones & Darrah, 1994).

Some studies have not detected the presence of any organic acids in the culture filtrates of phosphate solubilising microorganisms (Illmer & Schinner, 1992; Illmer et al., 1995; Illmer & Schinner 1995), and it was suggested that protons excreted following the uptake of NH_4^+ were the principal agents of P solubilisation (Illmer & Schinner, 1995). Hence, for a large range of microorganisms, the mechanism of P solubilisation was

dependent on the supply of N. In most cases, the supply of NH_4^+ leads to greater rates of P dissolution compared to microbial growth with NO_3^- as the sole source of N. However, when the microorganism produced anions that effectively chelate cations that precipitate P, the presence of NO_3^- led to higher rates of P dissolution (Cunningham & Kuiack, 1992; Gharieb, 2000).

The interaction of P solubilising microorganisms with plants is complex and is most likely to involve many components. The use of P solubilising organisms may increase plant growth via a multiple component mechanism. For example, the production of plant growth regulators, suppression of disease, improved micronutrient utilisation and greater seedling establishment in addition to P solubilisation may also be associated with the use of P solubilising inoculants (Barea, 1976; Sattar & Gaur, 1987; Kucey et al., 1989; Chabot et al., 1993; Leinhos & Vacek, 1994; Richardson, 2001; Gyaneshwar et al., 2002).

It is outside the scope of this thesis to discuss the effects of microbial inoculants on micronutrient availability. The production of plant growth regulators is discussed in Section 1.5 and disease suppression is discussed in section 1.6.

Despite the intensive research efforts on P solubilising microorganisms, there have been few developments of commercially successful P solubilising inoculants. This is predominantly due to the lack of a reliable plant growth response associated with the use of these organisms (Katznelson, 1965; Richardson, 1994; Richardson, 2001).

1.2.2 Factors affecting the efficacy of P solubilising microorganisms

In order to increase the efficacy of P solubilising microorganisms, factors influencing their ability to improve plant growth need to be identified. Many reasons for low efficacy have been suggested and relate to incompatibility with the soil type and unfavourable environmental conditions for growth of the inoculant. It has also been suggested that ineffectiveness of phosphate solubilising microorganisms is due to a lack of available carbon and nitrogen in the soil (Kucey et al., 1989; Gyaneshwar et al., 1998). In this respect, rhizosphere competence and root colonisation may increase the supply of nutrients to the inoculant. Thus the ability of the microorganism to colonize the roots would be an important characteristic of a successful inoculant. Richardson (2001) suggested that a lack of association between the roots of the host plant and the inoculant may account for variable responses to inoculation with some P solubilising microorganisms.

The effectiveness of an introduced inoculant may also be related to the indigenous soil microbial population. The indigenous soil microbial community may be better adapted to the soil and environmental conditions where the inoculant is being introduced. As a result these communities may readily outcompete the inoculant for nutrient resources and colonisation sites in the soil environment (Kucey et al., 1989; Salih et al., 1989).

Tinker (1984) raised concerns that microbial acidification is unlikely to occur to any significant extent in buffered soil conditions and that microorganisms are unlikely to mobilize significant quantities of soil P that are surplus to their own requirements under these conditions. In some cases, categorising a microorganism as a P solubiliser may be

inappropriate as the P solubilising phenotype determined in laboratory assays may not necessarily reflect function under soil conditions (Richardson, 2001).

1.2.3 Isotopic labeling to determine P solubilisation

Proof that microbial P solubilisation measured under laboratory conditions increases the amount of soil P available for plant uptake could be determined by assessment of consistent differences in the *L*-value between plants inoculated and not inoculated with P solubilising microorganisms (Larsen, 1952; Larsen, 1967). The *L*-value is defined as the ratio of the amount of ^{32}P added to the soil to the specific activity of plant tissue. As microorganisms solubilise soil P, non-exchangeable insoluble forms of P become available to the plant and the ratio of ^{32}P to ^{31}P absorbed by the plant (specific activity) is reduced.

Determination of *L*-values has been performed for different plant species to identify the effectiveness of plant roots to solubilise P for uptake. Different plant species usually yielded similar values indicating that they drew their P from sources of similar solubility (Nye & Foster, 1958). However, when white lupin (*Lupinus albus*) was compared to soybean, narrow-leafed lupin, pigeon pea, canola, sunflower and wheat, values for the specific activity of ^{32}P found in the shoots of white lupin were less and *L*-values were greater compared to the other species (Braum & Helmke 1995; Hocking et al., 1997). This indicated that white lupin obtained P from a 'pool' or 'type' of soil P that was not available to these other species of plants. The ability of white lupin to acquire P from low fertility soils has been linked to a number of plant traits. These include the secretion of phosphatase enzymes that mineralise organic P (Tadano et al., 1993) and the release of large quantities of H^+ and citrate ions (Dinkelaker et al., 1989)

that through processes of rhizosphere acidification, reduction, chelation and ligand exchange extensively mobilise bound soil P (Gardner et al., 1983; Dinkelaker et al., 1995).

Isotopic labeling has also been used to show that the fraction of soil P that vesicular-arbuscular mycorrhizae (VAM) absorb and transfer to plants is the same as that available to the plant without a VAM association. The specific activity of ^{32}P in shoots of mycorrhizal and non-mycorrhizal plants has generally been observed to be the same, indicating that the source/solubility of P for mycorrhizal and non-mycorrhizal plants is the same (Bolan, 1984; Bolan, 1991). The increase in P uptake by mycorrhizal plants has been related to hyphal length and the ability of the hyphae to explore a greater volume in soil. However, when mycorrhizal plants were inoculated with P solubilising bacteria, the specific activity of the plants was reduced, indicating that the P solubilising bacteria were able to release P from insoluble sources (Toro et al., 1997).

Isotopic labeling has been used to identify the effectiveness of P solubilising microorganisms. *Bacillus megatherium* var. *phosphaticum* was originally claimed by various researchers in the USSR to increase the P availability to plants. However, in soils labeled with ^{32}P, *B. megatherium* var. *phosphaticum* did not increase the specific activity in plants and therefore doubts were cast on its ability to increase the P availability to plants (Smith et al., 1961). While the use of ^{32}P labeling has been used to verify the P solubilising activity of *P. bilaiae* (Asea et al., 1988; Chambers & Yeomans, 1991) this approach has not been applied to determine the P solubilising activity of *P. radicum* and the ability of this fungus to increase the available pool of P to plants has not been evaluated.

1.3 Penicillium bilaiae

The most successful inoculant that has been reported to consistently increase growth and P uptake of a range of crops is *Penicillium bilaiae* PB-50. Further information and studies on the plant growth promotion and mode of action of this fungus are described in following sections.

1.3.1 Isolation

The Canadian isolate of *P. bilaiae* PB-50 was selected following a screening program of soils from southern Alberta, Canada (Kucey, 1983). The fungus *P. bilaiae* PB-50 was selected as it was the fungal isolate that had the greatest ability to solubilise insoluble forms of P, such as rock phosphate (RP), in liquid assay and plate assay under a range of conditions. Since then the ability of *P. bilaiae* PB-50 to promote the growth of a variety of crops in field trials and glasshouse experiments has been demonstrated. A spore preparation of the fungus has been commercialised as an inoculant sold under the trade name "Jumpstart" (formerly "Provide PB-50") and sold by Philom Bios Pty Ltd, Saskatoon, Canada. There is now considerable evidence that *P. bilaiae* PB-50 increases the growth and P nutrition in a range of cereal and pulse crops (Kucey, 1987; Kucey, 1988; Asea et al., 1988; Beckie et al., 1997; Gleddie, 1992; Goos et al., 1994).

1.3.2 Mechanisms of plant growth promotion

A number of studies have provided information on the mechanisms of plant growth promotion by *P. bilaiae* PB-50. Generally, the greatest response is seen early in the plant's growing period and diminishes as the crop matures. *P. bilaiae* PB-50 does not tend to promote growth in P sufficient soils and benefit is removed by the application of soluble P fertiliser. This suggests that P solubilisation is an important plant growth

promotion mechanism used by this fungus. Cunningham et al., (1990) identified citric and oxalic acids as two predominant organic acids produced under liquid culture conditions. Oxalic acid was produced as a secondary metabolite under C limiting conditions, while citric acid was produced under N-limiting conditions. The chelating activities of the anions suggest that Ca chelation accounts for the effectiveness of the fungus under alkaline conditions. A further mechanism of P solubilisation was identified by Asea et al., (1988) who demonstrated that proton export following NH_4^+ assimilation led to increased RP solubilisation.

In addition to acidification and chelation effects, the effect of *P. bilaiae* PB-50 inoculation on root growth may also explain increased P uptake. Plants grown under P deficient conditions and inoculated with *P. bilaiae* PB-50 tend to have root systems of a greater mass and increased length (Vessey & Heisinger, 2001; Philom Bios, 2002). This would enable exploration of a greater volume of soil for P acquisition. P uptake could also be increased by *P. bilaiae* PB-50 effects on root hair growth. Gulden & Vessey (2000) reported that *P. bilaiae* PB-50 increased the frequency and length of root hairs of *Phaseolus vulgaris*. While there is no published report on the production of plant growth regulators by *P. bilaiae* PB-50, the modifying influence of *P. bilaiae* PB-50 on root architecture and morphology suggest that this is worth investigating.

Asea et al. (1988) hypothesized that if P solubilisation proceeds through a mechanism of Ca chelation, it should be possible to show that other relatively in soluble minerals could also be solubilised by *P. bilaiae* PB-50. Kucey (1988) showed that *P. bilaiae* PB-50 solubilised 25-54% of CuO, 95% of Zn metal and 2% of insoluble Fe compounds in liquid culture. By comparison with the addition of EDTA to sterile growth media at a

similar pH to growing cultures of *P. bilaiae* PB-50, it was concluded that the major portion of mineral solubilisation occurred by chelation mechanisms (Kucey 1988).

The micronutrient solubilising activity of *P. bilaiae* PB-50 in relation to plant growth was studied under glasshouse and field conditions (Kucey 1988). Under glasshouse conditions, *P. bilaiae* PB-50 significantly increased the plant yield and uptake of P, Cu and Zn. However, there was no consistent correlation between growth promotion and increased plant uptake of micronutrients. Under field conditions, *P. bilaiae* PB-50 significantly increased Zn uptake while Cu and Fe were unaffected. This limited study suggested that improved Zn and Cu uptake could be implicated in plant growth responses following *P. bilaiae* PB-50 inoculation.

1.3.3 Interaction of P. *bilaiae PB-50 with N-fixing* rhizobia

A number of studies have investigated the effect of a dual *P. bilaiae* PB-50 - *Rhizobium* (N-fixing) inoculant on plant growth. The addition of *Rhizobium* and *P. bilaiae* PB-50 in a combined inoculation had an additive effect on one or more plant growth variables such as dry matter production, P and/or N uptake (Downey & van Kessel, 1990; Gleddie, 1992; Rice et al., 2000). The ability of *P. bilaiae* PB-50 to increase the amount of N fixed may have been due to its ability to alter root hair development (Gulden & Vessey, 2000). If *P. bilaiae* PB-50 increases the number of root hairs, this may increase the number available for nodulation and thus increase the efficiency of the *Rhizobium* inoculation. In addition, the increased P availability may also increase the frequency of nodulation.

Under field conditions it appears that the main benefit of including *P. bilaiae* PB-50 in an inoculum mix with *Rhizobium* relates to increased plant P nutrition. Correlation of soil analysis with field responsiveness of a dual inoculant suggests that additive effects of a combined inoculation are only beneficial in soils where plant growth responds to P fertiliser addition (Rice et al., 2000; Philom Bios, 2002, Saskatoon, Canada, unpublished data).

1.3.4 Interaction with vesicular-arbuscular mycorrhiza

In some instances, particularly for soils low in P availability, the co-inoculation of both P solubilising microorganisms (fungi and bacteria) and vesicular-arbuscular mycorrhizae (VAM) gave greater plant growth and or P response in comparison to inoculation with either organism alone (Azcon et al., 1976; Kucey 1983; Toro et al., 1996; Toro et al., 1997; Barea et al., 2000; Vassilev et al., 2001). For example, *P. bilaiae* PB-50 inoculation had no significant effect on shoot dry weight or P uptake of fully mature wheat. However, when *P. bilaiae* PB-50 was added with insoluble RP (at a rate of 45 mg P kg soil^{-1}) in a co-inoculation with VAM, there was a significant increase in shoot dry weight and shoot P content by 21% and 37%, respectively (Kucey, 1983). This response was greater than the addition of VAM alone and was just as effective (measured as shoot dry weight) or better (measured as plant P uptake) than the addition of triple superphosphate at a rate of 15 mg P kg soil^{-1}. Considering that the test soil had a pH of 7.2, and the agronomic effectiveness of RP fertilisation is generally low in alkaline soil conditions, this result suggested that the interaction of *P. bilaiae* PB-50 with VAM may offer a means of increasing the P fertilisation efficiency of applying RP under alkaline conditions. As tracers (^{32}P) were not used in this study, it

was not possible to determine if the co-inoculation had increased utilisation of the RP or solubilised forms of P present within the soil.

A subsequent study (Asea et al., 1988) did not find any benefit of a VAM-*P. bilaiae* PB-50 co-inoculation in comparison with a single inoculation of either organism alone. This result is consistent with other studies that indicate not all combinations of VAM and P solubilising microorganisms result in plant growth or nutrition improvements (Toro et al., 1996).

Kucey (1983) suggested that mycorrhizae are a necessary part of a rhizosphere P solubilising/absorbing system that includes P solubilising microorganisms. When present the mycorrhizae are likely to increase the effectiveness of the rhizosphere solubilising system by increasing the ability of the root to absorb P. In addition, the mycorrhizal hyphae may absorb P mobilised by the P solubilising microbes before it is re-immobilised within the soil. As *P. bilaiae* PB-50 and a range of other P solubilising microorganisms have required VAM presence to achieve the maximum benefit to plant growth, it is also possible that *P. radicum* needs VAM to significantly increase plant growth and/or P uptake. To date there is no published data on the interaction of *P. radicum* with VAM or the requirement of VAM by *P. radicum* to significantly influence plant growth. As the interaction of *P. radicum* with other rhizosphere organisms was considered to be outside the scope of this thesis, VAM and their relationship with *P. radicum* was not considered in the experiments reported within this thesis.

The effectiveness of *P. bilaiae* PB-50 described in these reports suggests that there is a potential for use as a P solubilising inoculant in Australian agriculture. However, in field trials and glasshouse trials the effects of *P. bilaiae* PB-50 under Australian conditions was shown to be low and/or inconsistent (Bio-Care Technology Pty Ltd unpublished results; CSIRO Land and Water, unpublished results; Whitelaw, 1998). The lack of effects associated with the use of *P. bilaiae* PB-50 are probably directly related to differences in climatic and soil conditions between Australia and Canada. Hence, a P solubilising inoculant intended for use under Australian conditions may have an improved efficacy if it was isolated and hence adapted to Australian soils. *Penicillium radicum* is an example of one such inoculant. The species history and the effects on plant growth are described in the section below.

1.4 Penicillium radicum

A screening program was conducted to identify potential growth promoting rhizosphere microorganisms (Whitelaw, 1998). The healthiest plants from a wheat crop grown in Wagga Wagga, NSW, were selected. Microorganisms isolated from the rhizosphere of each plant using semi-selective *Penicillium* growth medium and a general microbial growth medium were screened for their ability to produce a zone of clearance in $CaHPO_4$ agar indicating their ability to solubilise this source of P. Phosphate solubilising isolates that were also capable of colonising wheat roots in a gnotobiotic vermiculite assay were then assessed for their ability to promote the growth of wheat in a vermiculite-sand mix with soluble *ortho* PO_4^{3-} under glasshouse conditions. *P. radicum* was the only isolate that increased the growth of wheat in this experiment and hence chosen for further trials (Whitelaw, 1998).

1.4.1 Species description

Following its isolation and characterization as a fungus promoting the growth of wheat in glasshouse trials, *P. radicum* A.D. Hocking & Whitelaw was classified as a new species. According to Hocking et al. (1998), *P. radicum* can be placed in the subgenus *Biverticillium*, Section *Simplicum*, Series *Islandica*. Closely related species that belong to the *Biverticillium* subgenus are *P. variabile*, *Talaromyces wortmannii*, *P. zacinthae* and *P. allahabadense*. These fungi can be distinguished from *P. radicum* by morphological characteristics, secondary metabolites and DNA profiles. *P. radicum* is also clearly distinct from the P solubilising *P. bilaiae* PB-50. The two fungi can be visually discriminated on the basis of colony and conidiophore morphology. As *P. bilaiae* PB-50 develops a monoverticilliate conidiophore it belongs to the subgenus *Aspergilloides* which is separate to the *Biverticillium* subgenus.

1.4.2 The effect of P. radicum *on the growth of wheat*

To date most of the information pertaining to *P. radicum* performance in the field may only be found in sources that have not been subjected to scientific review. Hence, most of the data used to summarise *P. radicum* performance on the growth of wheat has been taken from unpublished sources. A large number of field trials have been conducted by the company formerly known as Bio-Care Technology Pty Ltd (greater than 200) in order to identify particular regions or conditions where *P. radicum* may have an economically significant benefit. While no common factor was identified in the yield response to *P. radicum*, when the data of these field trials was averaged, the mean yield increase to *P. radicum* inoculation was found to be approximately 8% (formerly Bio-Care Technology Pty Ltd, unpublished results).

Particular areas where plant growth has been responsive to *P. radicum* were in red-brown earths of south-central areas of the wheat belt in NSW and yellow acidic sands of the wheat belt in WA that were low in available P (Whitelaw et al., 1997; Mingenew-Irwin Group, WA, unpublished results). There is generally nil to marginal effects in alkaline soils (Eyre Peninsula Farming Systems 2002 Summary, Minnipa Agricultural Centre, SARDI; formerly Bio-Care Technology Pty Ltd, unpublished results). While most of the field trials have been conducted on wheat, *P. radicum* has also shown potential to improve the growth of barley, canola and faba bean (formerly Bio-Care Technology Pty Ltd, unpublished results).

One of the highest reported responses to *P. radicum* inoculation occurred in the Eradu sandplains of Western Australia. Field grown wheat (cv Carnamah) that was inoculated with *P. radicum* had an increase in yield from 1.92 t ha^{-1} to 2.34 t ha^{-1} (Mingenew-Irwin Group WA, unpublished results). However, field trials conducted in the following season did not yield significant responses to *P. radicum* inoculation. This suggests that the plant response to *P. radicum* may vary from year to year and may also be dependent on the plant cultivar (Bio-Care Technology Pty Ltd unpublished results; Peter Grieve, personal communication; Steven Wakelin CSIRO Land and Water, personal communication).

Whitelaw et al. (1997) studied the ability of *P. radicum* to improve the growth of wheat under greenhouse and field conditions in acidic red-brown earth (pH 4.6) treated with five levels (0, 5, 10, 15, 20 kg P ha^{-1}) of P applied as single superphosphate. In the field trial, *P. radicum*, increased the yield by an average 14%. Under glasshouse conditions, the mean increase in yield was 9% and in P uptake, 10%. For the nil P treatment, *P.*

radicum inoculation resulted in a 17% increase in yield. A negative interaction between *P. radicum* inoculation and P level led the authors to suggest that P solubilisation may have been partially responsible for growth promotion. Under field conditions, however, there was no interaction between P application and *P. radicum.* This indicates that other plant growth promotion mechanisms were likely to be involved.

1.4.3 The solubilisation of phosphate in-vitro *by* P. radicum.

The ability of *P. radicum* to solubilise insoluble phosphates was studied *in-vitro* using P compounds that were representative of those occurring in alkaline soils: calcium monohydrate phosphate ($CaHPO_4$) and calcium orthophosphate ($Ca_3(PO_4)_2$). In addition, insoluble P compounds that represent the products of P precipitation in acid soils were also used: crystalline ferric phosphate; crystalline aluminium phosphate; colloidal Al-P and Fe-P (Whitelaw et al., 1999). Of the total amounts added to the liquid growth medium, up to 47.5% $CaHPO_4$, 36% $Ca_3(PO_4)_2$ and 21% of the colloidal Al-P was liberated as soluble PO_4. *P. radicum* did not solubilise significant quantities of Fe-P compounds. The main mechanism leading to P solubilisation was decreased pH. Solubilisation was higher when NH_4^+ was used as the sole source of N compared to NO_3^-. For example when supplied with NH_4^+, *P. radicum* solubilised up to 36% of $Ca_3(PO_4)_2$ whereas only 21% of the $Ca_3(PO_4)_2$ was solubilised in liquid culture that supplied NO_3^- as the sole N source. By comparison with abiotic cultures using gluconate and HCl to liberate P it was suggested that chelation of Al by gluconic acid may have been a factor in the solubilisation of colloidal Al-P by *P. radicum* (Whitelaw et al., 1999). However, further studies are required to investigate the ability of the fungus to solubilise these aforementioned phosphate minerals and to use acidification mechanisms in the rhizosphere of plants.

1.5 Plant growth regulators

Plant growth regulators (PGR) have been generally defined as: *organic compounds synthesised in one part of a plant and translocated to another part of the plant where they have a physiological effect* (Frankenberger & Arshad, 1995). The concentration at which PGR's can affect plant physiology (i.e. in the nano to micro-molar range) is generally far lower than for nutrients, vitamins or other plant metabolites. While microorganisms have been found to produce compounds that belong to the five classes of plant growth regulators: auxin, gibberellin, cytokinin, abscisic acid and ethylene (Frankenberger & Arshad, 1995), there are a variety of other microbial metabolites with hormone-like activity that do not fit into these categories (Cutler, 1988; Kimura et al., 1992; Kusano et al., 1997; Kimura et al., 2000). Since PGR's dramatically affect plant growth, and root associated microbes produce PGR's, it is possible that rhizosphere microorganisms promote the growth of plants via PGR production. An extensive literature review to cover the function and mechanism of action of all classes of PGR is beyond the scope of this thesis. This review deals with well defined effects of auxin on plant growth, in particular root growth, as this is how auxin producing soil microorganisms may affect plant growth.

1.5.1 Chemical identity of auxin

Auxins are a well defined group of PGR. Compounds with auxin activity can be grouped into five categories: chlorophenoxy acids; napthalene acids; benzoic acids, picolinic acid and indole acid derivatives. Aside from certain indole and benzoic acid derivatives, most of these auxin compounds are not produced by plants and microorganisms. The naturally occurring compound indole-3-acetic acid (IAA), has the greatest physiological activity and is considered to be the most important auxin for

plant growth. Hence, the other synthetic categories of auxins will not considered any further in this thesis.

1.5.2 Effect of auxin on plant growth

Auxin is a generic term for compounds that typically stimulate cell elongation, but auxins have a complex set of functions that includes regulating fruit development and regulating the phototropic, gravitropic and apical dominance habits of plant growth. In stems, buds and roots, auxin normally promotes cell elongation but the response is dependent on auxin concentration. The typical growth response to auxin is summarised is Figure 1. At low concentration (10^{-7} M to 10^{-13} M), auxins stimulate root elongation, at a higher concentration, root elongation is inhibited (Scott, 1972; Torrey, 1976; Salisbury & Ross, 1978; Taiz & Zeiger, 1991). Roots are less sensitive to auxin concentration than bud or shoot tissue and require higher concentrations of auxin for growth inhibition (Salisbury & Ross, 1978; Taiz & Zeiger, 1991).

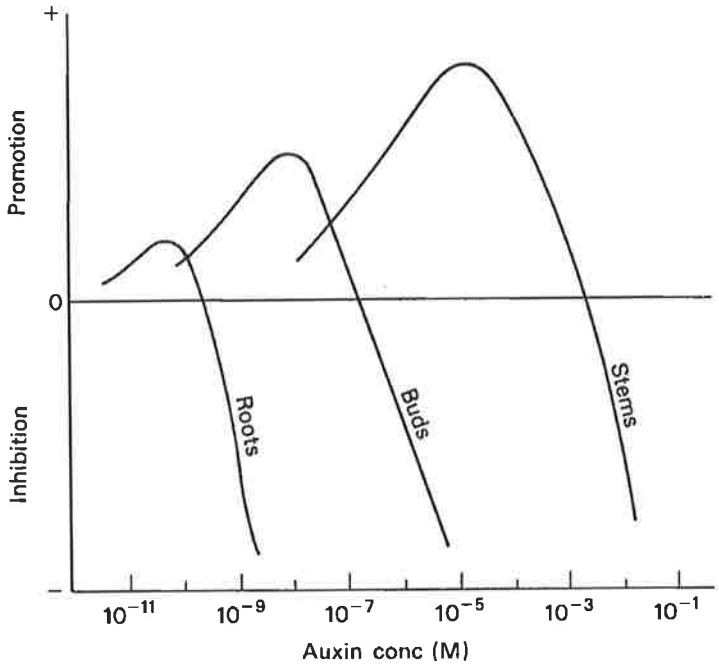


Figure 1.1 Schematic representation of the growth responses of root, buds and stems when exposed to an auxin concentration range (Russell, 1978).

1.5.3 Effects of auxin on root growth

Roots are sensitive to the presence of auxins which cause significant alterations in root morphology and architecture. In addition to influencing root elongation (mentioned previously), auxin is involved in the formation of lateral roots. This has been demonstrated by studies where the addition of auxin to root systems, typically at concentrations that were inhibitory to root elongation, stimulated the formation of lateral root primordia from the pericycle (Torrey, 1976; Salisbury & Ross, 1978; Taiz & Seger, 1991). Under conditions of P deficiency, decreased root elongation and a higher incidence of root branching would be of benefit to the plant as the increased branching of the roots in the upper soil horizon, that generally contains higher levels of soluble P compared to lower horizons, would allow a greater exploration of soil for patches rich in P (Nielsen, 1999; Lynch & Brown, 2001).

There is also evidence that auxin is associated with promoting root hair production. This has been measured as either an increase in root hair distributed over the root, increased root hair density (number of root hairs per mm of root) and/or increased root hair length. When applied to intact root systems it has been shown that auxin increased these parameters of root hair production (Jackson, 1960; Bates & Lynch, 1996; Pitts et al., 1998; Schmidt & Schikora, 2001; Rahman et al., 2002; Schikora & Schmidt, 2002; Lopez-Bucio et al., 2002).

Any effect that a microorganism has on the production of root hairs is likely to have an impact on the ability of the plant to absorb nutrients including P. There is now a large body of evidence showing that root hairs are important in P acquisition. Gahoonia et al. (1997) measured the length of root hairs in liquid culture of different wheat and barley genotypes. When the length of root hairs of the tested cereal genotypes was related to P acquisition, there was a direct correlation found between root hair length and the depletion of P from rhizosphere soils of different wheat cultivars. These effects also appeared to be transferred to field conditions. Increased P uptake by field grown wheat and barley was found in genotypes with longer root hairs (Gahoonia et al., 1999). Direct evidence of the ability of root hairs to absorb P came from studies of rye grown in PVC cores covered with mesh that only root hairs could permeate and grow into ^{32}P labeled soil (Gahoonia & Nielsen, 1998). When 70 percent of root hairs grew into the labeled soil, they contributed to 63% of the total P uptake, indicating the importance of root hairs in plant P uptake.

Root hairs increase the effectiveness of the root to absorb P and increase the competitive advantage of plants especially under low P conditions (Bates & Lynch, 1996; Bates & Lynch, 2001). As the rate of diffusion of P in soil is very low, P absorbed by the root has to be within close proximity to the root. Increasing the number and/or length of root hairs present on the root results in a greater root surface area exposed to the soil. Hence, the volume of soil that can be exploited for P absorption by the root is greater (Barber, 1995).

1.5.4 Production of auxins by root-associated microorganisms

Root associated microbes can act as a secondary source of auxin for plants (Scott, 1972; Arshad & Frankenberger, 1998). This has been demonstrated by the microbial production of IAA in defined media under laboratory conditions. While some microorganisms produce IAA without specific addition of tryptophan (TRP) as a component of the growth medium, most microorganisms require additions of TRP to produce detectable quantities of IAA. *Penicillium* spp. have been identified as one of the many groups of soil microorganisms that can produce auxin when supplied with the precursor TRP (Dvornikova et al., 1970; Kampert & Strzelczyk, 1975; Sattar & Gaur, 1987).

While a large number of microorganisms produce IAA from TRP, there may not be a simple relationship between microbial IAA production and plant growth promotion. Other factors will influence the plant-microbe interaction. In this regard, if rhizosphere microorganisms such as *P. radicum* colonise roots, then root colonising hyphae may release IAA that would be available for direct uptake and less likely subject to

degradation by other soil rhizosphere organisms if it were released in the exterior root environment.

If microorganisms have the ability to produce auxin in the presence of TRP, this is consistent with a plant-microbe interaction where plant roots exude TRP that is converted to IAA that acts as an exogenous pool available to plants. TRP and other indole containing substrates have been detected in the root exudates of various plants including cereals such as wheat, indicating that these substrates may be available to rhizosphere colonising microorganisms (Scott, 1972; Martens & Frankenberger, 1994; Jaeger et al., 1999). In addition, there is evidence that roots actively absorb exogenous indole compounds, this probably occurs via a specialised set of auxin influx proteins (Scott, 1972; Salisbury & Ross, 1978; Parry et al., 2001).

Previous research has identified that microbes influence the auxin levels of plants. For example, Libbert, (1970) showed that plants in the presence of epiphytic bacteria had higher endogenous auxin than sterile plants. However, it was difficult to elucidate if the source of extra auxin was exogenous (microbial) or endogenous, i.e a response of the plant to the presence of microbes. Martens & Frankenberger (1994) resolved this problem through the use of radiolabelled compounds. In sterile culture, the addition of 2'-^{14}C-IAA to the root zone of wheat seedlings resulted in uptake of 2'-^{14}C-IAA, predominantly to the shoots such that approximately 7% of the original labelled IAA was present in the shoots. In contrast, sterile wheat seedlings absorbed considerably less of the IAA precursor 3'-^{14}C-TRP that was added to the roots. Furthermore, when dilute solutions of TRP were added to the root zone of plants grown in non-sterile soil, 2 out of 3 wheat varieties showed increased growth. This suggests a mechanism exists

whereby plants release TRP that is converted to IAA by root associated microorganisms. Exogenous IAA is then available to influence plant growth.

1.5.5 Effect of microorganisms on root growth

It has been shown that auxin influences root morphology and that rhizosphere microorganisms produce auxin. Hence, it would seem logical to assume auxin producing rhizosphere microorganisms would influence root growth and therefore nutrient uptake. The ability of microorganisms to affect root growth has long been recognised (Rovira, 1965; Brown, 1974). Ridge & Rovira, (1971) in a liquid culture system found that an unidentified *Penicillium* sp. increased root hair length of wheat seedlings. Vessey and co-workers (2001) identified that *P. bilaiae* PB-50 affected root architecture and increased root hair production of bean (see Section 1.3.2).

Some studies have attempted to link alterations in root morphology with the production of IAA by root associated organisms. For example, Persello-Cartiaeux et al. (2001) studied the effect of *Pseudomonas thivervalensis* on *Arabidopsis thaliana.* Both *in-vitro* and *in-vivo*, seed bacterisation led to a more highly branched but shorter root system. *A. thaliana* mutants that did not respond to exogenous auxin were also resistant to root morphological changes induced by *P. thivervalensis* colonisation. *A. thaliana* mutants that were insensitive to other classes of PGR (such as cytokinin, gibberellin, jasmonic acid, abscisic acid) still had changes to the root morphology following *P. thivervalensis* inoculation. The lack of a response in the auxin insensitive mutant only, suggested that the ability of *P. thivervalensis* to alter root morphology was related to auxin and not other PGRs. Further evidence of the role of auxin in altering root growth was provided by analysis of indole compounds produced by *P. thivervalensis.* Asghar et al., (2002)

found that there was a high correlation between IAA production of bacterial rhizosphere isolates of *Brassica juncea* L. and the ability of these isolates to increase root length.

IAA production is also likely to be involved in the alteration of root growth following seed inoculation with *Azospirillum brasilense.* Typical changes are increased root branching and root hair production (see Frankenberger & Arshad, 1995 for review). The addition of synthetic IAA to sterile seeds mimicked the effect of *A. brasilense* inoculation (Tien et al., 1979; Kapulnik et al., 1985; Dobbelaere et al., 1999). This suggests that IAA was involved in the alteration of root morphology. Further evidence of IAA in the root-*Azospirillum* interaction was provided when Dobbelaere et al. (1999) found that inoculation with *A. brasilense* mutants impaired in a quantitatively important pathway for the biosynthesis of IAA, were unable to affect root elongation and root hair production in the same manner as the wild type *A. brasilense.*

While IAA production has been widely studied among rhizosphere microorganisms there has been no previous study on the ability of *P. radicum* to produce IAA. If *P. radicum* produces IAA, this may account for the ability of the fungus to increase P uptake and/or promote plant growth in general.

1.5.6 Ethylene

The activity of ethylene (C_2H_4) as a plant growth regulator has been well established (Torrey, 1976; Frankenberger & Arshad, 1995; Taiz & Zeiger, 1991; Arshad & Frankenberger, 1998). While ethylene can induce senescence and inhibit plant growth, at lower concentration ethylene can stimulate root growth and initiate lateral root

formation. This was demonstrated by Graham & Linderman (1981) who found that the exogenous application of ethylene to the roots of *Pseudotsuga menzesii* grown in soil (0.01-0.05 mg kg^{-1}) stimulated lateral root formation while at higher root concentration (>0.15 mg kg^{-1}), lateral root formation was inhibited. Exogenous application of ethylene (0.54 mg kg^{-1}) increased the dry root weight by 20% compared to non-treated controls. Subsequent studies have suggested that the stimulation of root growth by ethylene is related to induction of IAA production within the roots (Scagel & Linderman, 1998, Scagel & Linderman, 2001).

Scagel & Linderman, (1998) grouped ectomycorrhiza according to their *in-vitro* IAA or ethylene production capacity and inoculated seedlings of either *Pseudotsuga menzesii*, *Picea englemanii* or *Pinus contorta* with ectomycorrhiza of these different groups. Two months after inoculation, it was found that the *in-vitro* IAA fungal production was not always correlated with endogeneous root IAA. However, there was a significant positive correlation between *in-vitro* fungal ethylene production capacity and endogenous root IAA across all three tree species. This was suggested by the authors to be clear indirect evidence that ethylene production by fungal symbionts stimulates IAA production in the roots. The increased IAA present in the roots may then under certain conditions account for stimulated root growth (see Section 1.5.2).

Further evidence for the relationship between ethylene, induction of root IAA and the stimulation of root growth was presented by Scagel and Linderman (2001). In this study, it was found that the application of ethylene releasing compounds to the roots of *Ps. menzesii*, *Pic. englemanii* and *Pin. contorta* at the time of planting into a field, had increased free IAA and conjugated IAA two weeks after planting. Where the

application of ethylene releasing treatments had increased seedling survival and root growth, there was also an increase in the concentration of root IAA. This provides further evidence that ethylene gas stimulates root growth by inducing IAA within the roots.

If *P. radicum* promotes plant growth by stimulating root growth it may do so via the production of ethylene that increases IAA within the root. While ethylene production has been extensively studied amongst *Penicillium* fungi, the species that have been most intensively investigated (e.g *P. digitatum, P. expansum*) are economically significant as the causal pathogens of the post-harvest rot of various fruits and vegetables (for review see Arshad & Frankenberger, 1995). For *Penicillium* spp., ethylene production may have a role in pathogenicity on post-harvest fruit and vegetables (Arshad & Frankenberger, 1995). However, given that *Penicillium* fungi possess the genes for ethylene biosynthesis, it is possible that *P. radicum* may contribute to ethylene in the soil that is available to stimulate root growth. Ethylene was not considered any further in this thesis as there was no readily available analytical equipment capable of directly quantifying ethylene. Without directly quantifying ethylene production by the fungus, it would be difficult to study it as a mechanism of plant growth promotion.

1.6 Soilborne cereal root diseases

Root diseases cause significant loss to cereal production worldwide. The majority of economically important cereal root diseases are caused by soilborne fungi. Trends toward cropping practices that involve no-tillage, continuous cropping and stubble retention increase the root disease threat by improving the survival of fungal root

pathogens (Cook, 2001). Farmers need to seek economically viable and less environmentally damaging methods of controlling root disease. *Penicillium* spp. have previously shown potential as components of the soil microflora that have both inhibited the growth of fungal pathogens and/or improved plant growth in the presence of root pathogens. Knowledge of the interaction of plant beneficial fungi, such as *Penicillium* spp., with disease causing fungi should assist farmers in the development of less environmentally damaging and cost effective management practices for disease control.

1.6.1 Incidence and severity of soilborne diseases in the Australian wheat belt

Five important cereal root diseases that affect Australian (and international cereal production) are Take-all, Fusarium crown rot, Common root rot, Rhizoctonia bare patch and Pythium root rot. The severity of the impact of these diseases differs for each zone of the Australian wheat belt (Table 1.1). With all known control measures in place the estimated losses caused by all of these diseases to Australian cereal production have been estimated to exceed $150 million (Murray & Brennan, 1998). While losses due to Pythium root rot have been estimated at a much lower value in comparison to the other root diseases, there is limited knowledge on the severity of this fungus and estimated losses caused by this disease may be substantially lower than actual values (Paul Harvey, CSIRO Land and Water, personal communication).

Table 1.1 The distribution and relative importance of diseases of the crown and root caused by fungi in Australian wheat production.

Data taken from Murray & Brennen, 1998.

Common name of disease	Incitant	Loss[1]	Relative importance in cereal growing region[2]			
			North	*South-East*	*South-Central*	*West*
Take-all	*Gaeumannomyces graminis* (Sacc.) von Arx & Olivier var. *tritici*	$ 52 m	0.6	4.8	4.4	2.3
Common Root Rot	*Bipolaris sorokiniana* (Sacc. ex Sorok.)	$ 22 m	3.9	2.3	3.6	0.3
Foot and Crown Rot	*Fusarium pseudograminearum* (Aoki et O'Donnell)	$ 56 m	5.2	3.1	4.3	0.6
Rhizoctonia root rot (bare patch)	*Rhizoctonia solani* Kühn AG 8	$ 35 m	0.3	1.5	3.8	1.0
Pythium root rot	*Pythium spp.*	$ 5 m	1.4	1.5	1.0	0

Scale = 1 to 2 low, 2 to 5 moderate, and above 5 very high.
[1] loss to national Australian cereal production with standard control measures in place.
[2] regions:
North – Queensland and Northern NSW; South East – southern NSW and N.E Victoria; South-central – South Australia and the Wimmera and Mallee districts of Victoria; West – Western Australia.

1.6.2 Management strategies for root disease

1.6.2 (i) Take-all caused by Ggt

Ggt has a specialised root parasitic ability such that it invades the root cortical cells and eventually penetrates the stele where it causes necrosis and the roots develop characteristic black lesions. However, the ability of the fungus to parasitise roots is almost exclusive to Gramineae. As the fungus has a poor saprophytic ability, inoculum potential is rapidly reduced in the absence of a host. Hence, break crops are the key management option for control of the pathogen (Scott, 1981).

The supply of adequate nutrition particularly P, N and Mn assist in the reduction of take-all disease severity. The availability of the seed dressing fungicide, fluquinconazole (Jockey^{TM}) in the Australian marketplace has provided growers with an extra management option. A take-all prediction model made possible by the estimation of initial inoculum levels by DNA testing (Herdina & Roget, 2000) has the potential to prevent further losses from this disease (Roget, 2001). Application of the model can be used to provide farmers with better information to assess the potential risk of disease and implement appropriate management decisions.

1.6.2 (ii) Rhizoctonia root-rot

Rhizoctonia solani is a broad host range root pathogen and hence no break crop can be used to effectively control root disease caused by this pathogen. A break period from this disease may come from a short-term fallow implemented before seeding and may reduce disease severity by the removal of volunteer autumn weeds that act as a host to the pathogen (Rovira, 1986). Under field conditions fungicides were not shown to be useful. Hence, other strategies are needed for the control of this disease (Cotterill, 1991).

The severity of *Rhizoctonia* root-rot tends to be higher in no-tillage cropping systems. The disturbance of the mycelial network in the soil reduces the inoculum potential (Gill et al., 2002). Hence, cultivation is an option for root-rot control. However, complete control is not obtained by cultivation and the onset of disease may only be delayed. Adequate crop nutrition should also assist in minimising losses due to this disease. In

particular, adequate Zn nutrition is a major factor influencing the severity of this disease (Thongbai et al., 1993).

1.6.2 (iii) Pythium root-rot

As with *Rhizoctonia*, *Pythium* is a non-specific root pathogen that attacks juvenile roots of a wide range of crops. As there is a non-specific host-pathogen relationship, little is known on the effect of crop rotation on *Pythium* induced root rot. Fungicides are available as a management option. Ensuring adequate plant nutrition is a major part of minimising *Pythium* losses.

1.6.2 (iv) Fusarium crown-rot

The main strategy used to control crown-rot is the use of resistant cereal cultivars. However, there are no known varieties of durum wheat that have a high tolerance to crown rot. As the cultivation of durum wheat expands, it is expected that the incidence of this disease will increase (Williams et al., 2002). Disease-causing propagules survive in the soil on organic debris. Hence, disease is higher when stubble is retained and is also higher under no tillage (Wildermuth et al., 1997). Where high levels of infected stubble are retained, even resistant cultivars may become diseased. Hence, there is a need to develop further control measures.

1.6.2 (v) Common root rot

The fungal pathogen causing common root rot (*Bipolaris sorokininana*) mainly survives in the soil as spores. Control of this disease is achieved through practices such as cultivation, burning and adequate plant mineral nutrition (Wildermuth et al., 1997).

1.6.2 (vi) Summary of methods to control root diseases.

None of the above mentioned control methods is a completely satisfactory root disease management strategy for either of two reasons: they do not reduce disease losses to an economically acceptable level or they are environmentally damaging. For example, while cultivation has a major effect beneficial effect on root disease losses, the benefits do not come without cost; cultivation leads to topsoil erosion, reduction in organic material and increased loss of soil moisture. As no-tillage cereal production increases soil organic matter, reduces soil erosion and increases water retention, it is likely that the adoption of no-tillage operations will become more widespread. Direct drilling is already a common practice in many parts of Australia, the Pacific northwest and the North American prairies (Cook, 2001).

While fungicides may provide some control of root diseases, over-reliance on the use of chemical fungicides may be financially costly, lead to fungicide resistance and leave residues in the soil. Fungicides have an additional disadvantage that they may be non-selective and target beneficial microbes.

As crop rotation, use of resistant varieties, cultivation and fungicides do not always provide complete control of root disease, there is a need for new technologies and cropping practices to reduce the loss of cereal root diseases.

The use of soil microorganisms that improve plant growth in the presence of root diseases is a further approach to root disease management. While the introduction of soil microorganisms is unlikely to yield a complete solution for root disease, it may

form an important component of an integrated disease management system where a combination of management practices reduce root disease severity.

1.6.3 Use of specific microorganisms for root disease control

Use of microorganisms in the reduction of root disease can take two approaches: the use of resident competitors, mycoparasites and antibiotic producing microorganisms, or the introduction of specific microorganisms.

Under long term monoculture, the biological phenomenon of disease-suppressive soils develops where the resident microbial communities reduce the severity of the root disease. This phenomenon has been reported to occur and be responsible for the decline in take-all, *Rhizoctonia* and *Fusarium* diseases in field soils (Cook, 1990; Weller, 2002). However, this response does not always occur and many farmers cannot afford to endure low yields before the suppressive nature of the soil develops.

Introduction of specific microorganisms may form a strategy of biological control of root disease. *Penicillium* spp. are common soil fungi that represent a component of soil microflora that suppress disease caused by fungal root pathogens. If populations are naturally low or agricultural practices have altered the balance so that active numbers of *Penicillium* are low, the introduction of *Penicillium* into the rhizosphere may form part of an integrated control strategy for root diseases. The section below discusses examples on the use of *Penicillium* spp. to reduce root disease severity.

1.6.4 Greenhouse and field trials demonstrating the use of Penicillium *spp. in the reduction of plant disease*

The role of *Penicillium* in the protection of plant roots against diseases has been seen for a number of different plant hosts against a number of different plant pathogens. Table 1.2 lists examples where *Penicillium* spp. has improved the growth of plants in the presence of root disease. The diversity of applications of *Penicillium* spp. in the protection against root disease is a reflection of the opportunities that *Penicillium* spp. may have in reducing root disease in cereal cropping. Despite the wide range of *Penicillium* spp. that have been examined for their ability to increase growth in the presence of root diseases, there is no published study on the ability of *P. radicum* to reduce the effects of root diseases. Given the potential of *Penicillium* fungi to suppress disease, further studies should investigate the effect that *P. radicum* has on root disease.

Table 1.2 Use of *Penicillium* spp. in the protection of plants against soilborne diseases.

Antagonist	Disease/pathogen	Host	Description	Effectiveness[A]	Reference:
	Take-all	*Triticum aestivum*	Glasshouse		Sivasithamparam & Parker, 1980
P. funiculosum			Non-sterile yellow sand	83[1]	
P. janthinellum				90	
P. patulum				116	
P. patulum			Non-sterile Gabalong soil	211	
P. fuscum	Take-all	*T. aestivum*	Sterile soil, glasshouse	212[1]	Dewan & Sivasithamparam, 1988
P. nigricans				233	
P. griseofulvum				79	
P. fuscum			Non-sterile soil,	51	
P. nigricans				41	
P. griseofulvum				26	
P. clavariaeform	Rhizoctonia damping off	*Lactuca sativa*	Glasshouse		Wood, 1950
			sterile sand	61[1]	
			sterile soil	123	
			non-sterile soil	not effective	
P. janczewskii	Rhizoctonia damping off	*Gossypium barbadense* L.	Glasshouse	49[3]	Madi & Katan, 1999
		Cucumis melo		39	
P. oxalicum	Pythium root rot	*Cicer arietinum*	Field	10 – 68[2]	Kaiser, 1984
P. raistrickii	Pythium root rot	*Beta vulgaris*	Glasshouse	37[2]	Dodd & Stewart, 1992
Penicillium sp.				40	
P. simplicissimum				42	
Penicillium sp.				62	

[A]Effectiveness of disease control was recorded as either [1] % increase in shoot weight of diseased plants due to treatment with *Penicillium* spp. or [2] increase in % seedling emergence or, [3] reduction in % disease severity

Table 1.2 continued. Use of *Penicillium* spp. in the protection of plants against soilborne diseases.

Antagonist	Disease	Host	Description	Effectiveness[A]	Reference:
P. damascenum *P. implicatum*	Pythium root rot	*Picea glehnii*	Sterile solution culture	140[2] 15	Yamaji, 2001
P. frequentans	Pythium root rot	*B. vulgaris*	Glasshouse *Pythium* infested soil no treatment + glucose 0.22g kg^{-1} + glucose 0.74 g kg^{-1} + glucose 3.67 g kg^{-1} + sucrose 0.22 g kg^{-1} + $(NH_4)_2SO_4$ + urea + casein +peptone + NH_4^+ tartrate *Pythium* amended soil sterile non-sterile	 166[1] 36 169 -2 383 60 11 29 13 30 516 709	Liu & Vaughan, 1965
P. digitatum	Fusarium wilt	*Lycopersicon esculentum*	Field	37[1]	Khan & Khan, 2001
P. oxalicum	Fusarium wilt	*L. esculentum*	Growth chamber Glasshouse	45–49[3] 22-69	De Cal et al., 1999

[A]Effectiveness of disease control was recorded as either [1] % increase in shoot weight of diseased plants due to treatment with *Penicillium* spp. or [2] increase in % seedling emergence or, [3] reduction in % disease severity

Table 1.2 continued. Use of *Penicillium* spp. in the protection of plants against soilborne diseases.

Antagonist	Disease	Host	Description	Effectiveness[A]	Reference:
P. oxalicum	Fusarium wilt	*Lycopersicon esculentum* L.	Glasshouse sterile soil sterile soil + nutrients non-sterile soil non-sterile soil + nutrients	 40[3] 30 9.5 20	De Cal et al., 1995
P. purpurogenum			sterile soil sterile soil + nutrients non-sterile soil non-sterile soil + nutrients	25 31 12.5 0	
Talaromyces flavus	Verticillium wilt	*Solanum melongena*	Glasshouse	40[3]	Fravel & Roberts, 1991
T. flavus	Verticillium wilt	S. melongena	Glasshouse	77[3]	Fahima & Henis, 1990
P. oxalicum	root rot complex	*Pisum sativum*	Glasshouse sterile soil non-sterile soil	 0[1] 60	Windels & Kommedahl, 1978
P. funiculosum	*Phytophthora cinnamoni* *P. parasitica* *P. cinnamoni* *P. parasitica*	*Rhododendron* sp. *Citrus sinensis*	Glasshouse	238[1] 604 196 101	Fang & Tsao, 1995

[A]Effectiveness of disease control was recorded as either [1] % increase in shoot weight of diseased plants due to treatment with *Penicillium* spp. or [2] increase in % seedling emergence or, [3] reduction in % disease severity

1.7 Mechanisms of root disease suppression by plant beneficial fungi

Understanding and clarification of the mechanisms of biological control could lead to improved practical means by which biocontrol is used to improve crop production. Mechanisms involved in a fungal-fungal pathogen-antagonist interaction have been identified to include: competition, antibiosis and hyperparasitism (Lockwood, 1988; Whipps, 2001).

1.7.1 Competition

Competition as a mechanism of biocontrol may refer to the competition for nutrients, space or infection sites between fungi in the spermosphere or rhizosphere (Baker, 1968). For *Penicillium* spp., studies have suggested that competition mechanisms of disease control may be involved in the protection of germinating seedlings, i.e *Penicillium* fungi compete with the pathogen for infection sites on the root. Yamaji et al. (2001) observed that the presence of endophytic *Penicillium* spp. was a natural defence in germinating seedlings against disease damage by *Pythium* spp. *Penicillium* hyphae formed a complex network around the germinating seedlings and increased the survival of spruce seedlings in the presence of *Pythium*.

Dodd & Stewart, (1992) reported that protection of the seed was a likely mode of action by isolates of *P. raistrickii* and *P. simplicissimum* in reducing seedling disease caused by *Pythium*. *In-vitro* studies indicated that neither *Penicillium* isolates exhibited antibiotic production or mycoparasitic hyphal interactions. Hence, it was suggested that the *Penicillium* treatments applied as a seed coat acted as a seed protectant. The spores

on the seed coat grew and utilised the seed exudates as a source of carbon at the expense of the pathogen.

Windels (1981) observed the hyphal growth of *P. oxalicum* when conidia were applied as a seed coat to *Pisum sativum*. In both autoclaved and field soil *P. oxalicum* conidia germinated and a network of hyphae formed on the seed coat. While conidia were observed on the root system, they only actively germinated to form a hyphal network on the roots in autoclaved soil. This observation led the author to speculate that protection of the spermosphere was the predominant mechanism of disease control. Protection of the spermosphere was also suggested in work by Windels & Kommedahl, (1982) who observed the control of *Pythium* in pea seedlings by *P. oxalicum.* A further observation that *P. oxalicum* was more effective in controlling preemergence than postemergence damping off is consistent with a spermosphere protection mechanism (Windels & Kommedahl, 1978).

However, a pure competition (hyphal exclusion) mechanism may not entirely explain the protection of germinating seedlings against damping off following seed treatment with *Penicillium* conidia. The consumption of seed exudates for growth of the biocontrol agent would reduce the amount released into the surrounding soil for growth and chemotaxis of pathogens.

1.7.2 Antibiosis

Antibiotics are generally considered to be low molecular weight compounds produced by microbes and at low concentrations either kill or have an inhibitory effect on the growth of other microorganisms (Fravel, 1988; Haas & Keel, 2003). *Penicillium* spp.

are renowned for their attributes as antibiotic producers. For example, *Penicillium* fungi are the source of well known antibiotics such as penicillin, grisoefulvin, patulin and citrinin. The metabolic products of *Penicillium* spp. are often fungistatic and are active against fungal root pathogens (Proksa et al., 1992; De Stefano et al., 1999). De Cal et al., (1988) showed that organic solvent extracts of *P. frequentans* culture contained antibiotic type compounds that were active against a range of fungal pathogens. Similarly, De Stefano (1999) extracted a range of compounds including 3-*o*-methylfunicone from the culture supernatant and mycelia of *Penicillium pinophilum*. The compound 3-*o*-methylfunicone was found to effectively inhibit the growth of soilborne pathogens *Rhizoctonia solani, Fusarium solani, Cylindrocarpum scoparium* and *Alternaria alternata*. The organic nature of the antibiotic compounds is often typical of *Penicillium* antibiotics, however, antibiotic compounds may also be enzyme related as in the case of glucose oxidase (Ayer & Racok, 1990; Fravel & Roberts, 1991; Murray et al., 1997).

1.7.3 Mycoparasitism

Mycoparasitism occurs when one fungus interacts with another to obtain nutrients from the hyphae of the other fungus. As the parasite causes destruction or even death to the host (pathogen), mycoparasitism may act as a mechanism of disease reduction. There are a number of key components of mycoparasitism, these are briefly outlined below.

1.7.3 (i) Signalling

Mycoparasitism is characterized by recognition or signaling between the two organisms followed by a coiling and sticking of the parasite to the host. Dennis & Webster (1971) observed that *Trichoderma* spp. did not show coiling around plastic threads, but they

did coil around fungal hyphae. This suggests there is a chemical signaling between the host and parasite. The recognition between the host and parasite may be a necessary first step in the mycoparasitic process that leads to the formation of coiling structures and induction of lytic enzymes. There is some evidence that membrane bound lectins are involved in the fungal sensing process (Whipps, 2001).

1.7.3 (ii) Hyphal-hyphal interactions

Light and electron microscopic observations reveal that hyphal interactions indicative of mycoparasitism include hyphal coiling around the host, the formation of hyphal hooks, hyphal pegs and formation of haustorial type structures (McLaren et al., 1986). The formation of these structures results in contact between the host and parasite ensuring sufficient diffusion of metabolites involved in the process of cell wall degradation and hyphal death. Mycoparasitism may also involve internal structures and penetration of the host and deformation of the host cell wall may also be seen. Using light microscopy, Boosalis (1956) observed that *P. vermiculatum* penetrated the hyphae of *R. solani* by means of penetration pegs and established internal parasitic mycelium. Coagulation and granulation of the host cytoplasm has also been observed (McLaren et al., 1986).

1.7.3 (iii) Lytic enzyme complexes.

The ability of fungal hyperparasites to parasitise fungal pathogens may be related to the composition of the pathogen cell wall. Oomycetous fungal cell walls contain cellulose and glucans as the predominant polymers of their cell walls. Basidiomycete and Ascomycete fungi contain chitin and β-1,4-glucan as the main polymers of their cell

walls (Bartnicki-Garcia, 1968). The chitin is buried in glucan polymer and protein, preventing easy access of the chitin to chitinase enzymes.

As cell wall penetration and degradation frequently occur during mycoparasitism, chitinases, glucanases and other lytic enzymes (eg. proteases and lipases) have been involved in the process of fungal cell wall degradation. The loss of integrity of the cell membrane is then followed by leakage of the cytoplasm which is utilised as a nutrient source. Aside from their role in mycoparasitism, lytic enzymes were described by Jones & Hancock, (1988) as assisting in a more rapid diffusion of antibiotics produced by antagonists to the site of action in the cytoplasm.

The involvement of lytic enzymes in cell wall degradation has been demonstrated for some *Penicillium* spp. Larena & Melgarejo, (1996) detected induction of β-1,3-glucanase (up to 17 U mg protein^{-1}) by the addition of laminarin to cultures of *P. purpurogenum*. Chitinase induction was tested against synthetic chitin and non-living *Monilinia laxa* hyphae but was only induced in the presence of live *M. laxa* hyphae; only low chitinase levels were measured (25 mU g protein^{-1}). Crude preparations of enzymes from *P. purpurogenum* degraded spores and hyphae of *M. laxa* (Larena & Melgarejo, 1993; Larena & Melgarejo, 1996). When grown in the presence of cell walls of plant pathogenic *Fusarium* species, *P. frequentans* produced chitinase, glucanase and protease activity (Roberti et al., 2002).

Cell wall degrading enzymes have also been shown to be produced in cultures of the facultative mycoparasite *Talaromyces flavus* (perfect or sexual state of the fungus *Penicillium vermiculatum* or *P. dangeardii*). Inglis & Kawchuk (2002) detected

significant quantities of 1,3-, 1,4- and 1,6- glucanase activity but no significant chitinase or chitosanase activity in *T. flavus* culture. This spectrum of enzyme activity may have allowed *T. flavus,* cultured on the hyphae of *Fusarium equisetii* and *Pythium ultimum* to release significant quantities of reducing sugars into the medium indicating cell wall degradation. The absence of chitinase activity may have accounted for a lack of degradation when basidiomycete *R. solani* hyphae were used as the substrate.

Despite the widespread studies on the fungal-fungal interactions between *Penicillium* spp. and fungal root pathogens, there have been no previous studies on the *in-vitro* interaction of *P. radicum* with disease causing fungal organisms or the ability of *P. radicum* to produce antibiotics or act as a fungal hyperparasite. Such interactions may act as indicators of mechanisms that may be involved in disease suppression.

1.8 Summary

The presence of *Penicillium* spp. in the rhizosphere may be beneficial to plant growth. The beneficial effects of *Penicillium* spp. on plant growth has led to the development of seed based inoculants aimed at increasing rhizosphere populations and thereby plant growth. Various *Penicillium* spp., such as *P. bilaiae* PB-50 and *P. radicum,* possess metabolic activities such as P solubilisation, auxin production and inhibition of root pathogens that could account for increased plant growth when crops are treated with inoculants. P solubilisation could alleviate the problem of P deficiency by solubilising insoluble sources of P in the soil, thereby increasing the mineral P status of the plant. Auxin production may influence the root growth increasing root branching and root hairs so that the ability of the roots to absorb nutrients including P from the soil is increased. Various *Penicillium* spp. have been demonstrated to increase plant growth in

the presence of root pathogens such as *G. graminis* var. *tritici*, *R. solani* and *Pythium* spp. The mechanisms by which *Penicillium* spp. reduce the disease expression of root pathogens may be directly related to competition, antibiosis, mycoparasitism or increased disease tolerance due to improved plant mineral nutrition.

1.9 Background and objectives of thesis

Low soil P availability is a major constraint to plant growth. The use of P solubilising inoculants may provide a means to increase the utilisation of soluble P in crop production. The soil fungus *P. bilaiae* PB-50 has been shown to effectively increase crop growth and P fertiliser efficiency under Canadian conditions, but is ineffective under Australian conditions. In a search of a P solubilising fungus adapted to Australian conditions, *P. radicum* was isolated from a New South Wales field soil (Whitelaw, 1998). Subsequent studies on the plant growth promotion and *in-vitro* metabolic behaviour indicated that P solubilisation may have been at least partially responsible for plant growth promotion under gnotobiotic and glasshouse conditions (Whitelaw, 1998; Whitelaw et al., 1997). However, under field conditions P solubilisation did not appear to be the only mechanism in operation and it was suggested that other mechanisms could also account for plant growth promotion (Whitelaw et al., 1997).

Penicillium spp. isolated from rhizosphere soils have previously been reported to suppress disease and/or produce PGR's. It was hypothesised in this study that interactions between root pathogens and PGR production may account for the unknown plant growth promotion mechanisms following inoculation with *P. radicum*.

The plant chosen for this study was wheat as this is the main cereal crop produced in Australian agriculture and a large market therefore exists for a P solubilising inoculant. However, such an inoculant is unlikely to operate in all soil and environmental conditions of the Australian wheat belt. The area where the fungus is effective is likely to be related to the mechanisms that the fungus uses to increase plant growth. In order to increase efficacy and provide good commercial recommendations for application, it is important that the mechanisms of growth promotion are identified. This information will assist in identifying where the use of the Pr 70 Release™ is appropriate and likely to produce the most consistent response to plant growth. However, as the mechanisms by which this fungus operates are unknown, there is little predictive information on the efficacy of *P. radicum* inoculation.

The work described in this thesis aimed to investigate the P solubilising activity of *P. radicum* and identify the unknown mechanisms of plant growth promotion that were not related to P solubilisation. These aims were achieved by meeting the following experimental objectives:

- **Identify if P solubilisation acts as a mechanism of plant growth promotion by:** *In-vitro* studies of rock phosphate (RP) solubilisation (Chapter 3); glasshouse experiments that measure the P response of plants inoculated with *P. radicum* (Chapter 4) and; measurement of the effect that *P. radicum* has on the labile pool of soil P available to wheat (i.e the effect of *P. radicum* on the *L*-value, Chapter 5).
- **Identify if plant growth regulators are implicated in plant growth promotion by:** determining if *P. radicum* produces IAA and attempting to quantify the amount produced (Chapter 6).

- **Identify if *P. radicum* reduces root disease by:** examining antagonism and interactions between *P. radicum* and root disease causing fungi (Chapter 7); using a seedling bioassay to assess the effect of *P. radicum* on the severity of the cereal disease, take-all (Chapter 8).

CHAPTER 2. GENERAL MATERIALS AND METHODS

This Chapter describes methods and materials routinely used in the work described in this thesis. Any modifications to these methods are described in the relevant section.

2.1 Fungal species, culturing and inoculation techniques

2.1.1 Fungi used in the research

A list of fungi used in the research described for this thesis is given in Table 2.1. *Penicillium radicum* was routinely stored and cultured on malt extract agar (MEA) purchased from Oxoid and made according to the manufacturer's instructions. For long-term storage (greater than two months) germinated spores five days old were stored at –80 °C on solid MEA. *P. radicum* spores were readily recovered from cryogenic storage by plating onto freshly prepared MEA.

For long term storage, wheat root pathogens were maintained at –20 °C on organic substrates: *Gaeumannomyces graminis* var. *tritici, Rhizoctonia solani,* and *Bipolaris sorokiniana* were maintained on γ-irradiated ryegrass seeds, *Fusarium pseudograminearum* was maintained on wheat pollard. *Pythium irregulare* was maintained at 4 °C on corn meal agar (Difco) amended with rifampicin (40 mg L^{-1}) and ampicillin (50 mg L^{-1}). A description of the origin of these fungal isolates is given in Table 2.1.

Table 2.1 Fungal isolates used in this thesis.

Fungal species	Geographic origin	Host/substrate	Source
Penicillium radicum (Hocking et Whitelaw)	Wagga Wagga, NSW	wheat root	ASI Pty Ltd, Wodonga, NSW.
Gaeumannomyces graminis var. *tritici* (Sacc.) von Arx and Olivier isolate 8	Avon, SA, 1979.	wheat root	Rosemary Warren, CSIRO Land and Water, Glen Osmond SA
Rhizoctonia solani AG-8 (Kühn) isolate 21	Avon, SA	wheat root	CSIRO Land and Water, S. Neate, Glen Osmond, SA
Pythium irregulare (Buisman) *YDBIHR*	Young, NSW	wheat root	Paul Harvey, CSIRO Land and Water, Glen Osmond, SA.
Fusarium pseudograminearum (O'Donnell et Aoki)	northern NSW.	wheat stubble	Mark Butt, SARDI, Adelaide, SA
Bipolaris sorokiniana (Sacc. ex Sorok.)	Mt Dampier, SA.	wheat root	Mark Butt, SARDI, Adelaide, SA
Penicillium bilaiae (Chalabuda) RS7B-SD1	South East NSW	wheat root	Steven Wakelin, CSIRO Land and Water, Adelaide, SA.
Aspergillus niger (van Tieghem) FRR 4479	culture collection	MEA	CSIRO Food Sciences, North Ryde, NSW
Trichoderma koningii (Oudemans) 7a	WA.	suppressive soil	CSIRO Land and Water, Adelaide, SA.

2.1.2 Preparation of seed and ryegrass inoculum

P. radicum was introduced into pot experiments using either seed inoculation or ryegrass inoculation of soil. Seed inoculation was done by preparing a solution of spores that were lifted from a Petri dish culture of *P. radicum*. Spores were emulsified into approximately 10 mL of tween 20 solution (one drop per 100 mL of deionised water) by scratching the surface of the Petri dish culture. Wheat seeds disinfested with 4% NaOCl were then immersed into the tween 20 spore emulsion and mixed for twenty minutes. The seeds were dried overnight in a biological safety cabinet (Gelman Sciences BH – 120, class 4). Concentration of spores present on the seeds was determined by shaking the seeds for twenty minutes in 0.1% NaCl, a standard dilution

series was then plated onto Dichloran Rose Bengal Chloramphenicol agar (Pitt & Hocking, 1985).

Ryegrass inoculum was prepared as follows: after soaking γ-irradiated ryegrass seeds in deionised water overnight, the seeds were drained for approximately 2 hours through a 3 mm mesh. After draining, the seeds were autoclaved at 121 °C for twenty minutes. The process of soaking, draining and autoclaving was repeated a further two times. The sterile, moist ryegrass seeds were then inoculated with *P. radicum* in a biological safety cabinet with 1 cm squares of agar growth medium which had been colonized by the fungus for a period of approximately 10 days. The moist ryegrass seeds were then incubated in the dark at 25 °C until infestation with fungal growth appeared to be homogeneous. The infested ryegrass seeds were then dried overnight in a biological safety cabinet. Before use as a fungal inoculum, the viability and purity of the inoculum was checked by plating six separate samples onto full strength PDA or MEA. Only viable ryegrass inoculum that was free of microbial contaminants was used as an inoculum source.

2.1.3 Isolation of P. radicum *from roots using semi-selective medium*

At harvest, soil or sand adhering to the root systems was rinsed away with tapwater. After all traces of soil/sand were removed, the root systems were cut up into pieces that were 0.5 cm long. A random sample of these pieces from distances of 0-2 cm, 5-7 cm and 10-12 cm from the seed were then plated onto Dichloran Rose Bengal Chloramphenicol (DRBC, Pitt & Hocking, 1985). The medium contained per L: 10.0 g glucose, 5.0 g peptone, 1.0 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 15 g agar (Bacto), 0.5 mL of 5% w/v Rose Bengal in water, 1.0 mL of 0.2% w/v dichloran in ethanol and 100 mg

of chloramphenicol. The dry ingredients were made up to 1 L using deionised water and autoclaved for sterility.

The plates containing the root pieces were incubated at 25 °C until they were either overgrown or colonies growing from the root pieces were visible. Fungal colonies emerging from the interior of the roots that had morphologies resembling that of *P. radicum* were assumed to have originated from the colonisation of the inoculant on the roots. The identity of the *Penicillium* was then confirmed using light microscopy and was based on the branching structure of the conidiophore. A *Penicillium* isolate with identical colony morphology and conidiophore branching (*Penicillium radicum* is predominantly secondary i.e. biverticilliate) was taken as isolation of the original inoculant.

2.2 Plant growth and analysis

2.2.1 Glasshouse conditions

All pot-based experiments were performed in a temperature-controlled glasshouse. The mean ambient temperature ranged from 20-25 °C depending on the season. Light intensity ranged from 500-1100 μmol m^{-2} s^{-1}. At harvest, root material was washed to remove all traces of soil and separated from the shoots. Dry weights of roots and shoots were determined after drying fresh plant material at 60 °C for 48 hours.

2.2.2 Chemical analysis of plant material

Dry plant material was cut up into fine pieces using stainless steel scissors. Homogeneous subsamples (0.2 g) were weighed into 50 mL digestion tubes and digested in 6 mL of nitric acid at 150 °C (Tecator digestion block) for 10 hours or until

a small amount of liquid residue (approximately 0.5 mL) remained in the bottom of the digestion tube. After cooling, the digests were made up to 20 mL using 0.1 % HNO_3 in deionised water. The digest solutions were then vortexed and left overnight to settle. The digests were then decanted into polycarbonate tubes and analysed by inductively coupled plasma–atomic emission spectrophotometry (ICP-AES). A set of standard reference plant materials with known P concentrations and other elements was digested and analysed in the same manner as the experimental samples to check the accuracy of procedure (Zarcinas et al., 1987).

2.2.3 Preparation of Long Ashton nutrient solution for plant growth in sand culture

The Long-Ashton nutrient solution was used to supply mineral nutrients to wheat grown in sand culture. The Long-Ashton nutrient solution was added to the sand at a moisture of 12% w/w at the time of planting. Immediately prior to wetting the sand, the pH of the nutrient solution was adjusted to 7. The nutrient solution provided the plant with the following amounts of nutrients per kg of sand: 0.342 g $NaNO_3$, 0.528 g $(NH_4)_2SO_4$, 0.174 g K_2SO_4, 0.185 g $MgSO_4$, 0.4 mg Fe(EDTA), 2 mg $CuSO_4$, 0.6 mg $MnSO_4.4H_2O$, 0.4 mg $CoSO_4.7H_2O$, 0.5 mg H_3BO_3, 0.5 mg $H_2MoO_4.H_2O$ and 2.2 mg $ZnSO_4.7H_2O$. During the plant growth period the non-draining pots were maintained at 12% (w/w) moisture using deionised water.

2.3 Rock phosphate: preparation and chemical analysis

Idaho rock phosphate (RP) was used as a P source in fungal liquid culture and plant growth in sand culture. Prior to use the RP was treated by grinding and sieving through a 200 μm mesh. Soluble phosphate was removed by rinsing at least ten times with deionised water. The RP was air-dried and a subsample digested in $HNO_3/HClO_4$ (6:1)

and analysed for total mineral content using ICP-AES under the same instrumental and analytical conditions as described in section 2.2.2. An elemental analysis of RP was also performed using x-ray fluorescence spectroscopy. As part of this procedure, RP sample was fused in a lithium tetraborate:lithium metaborate flux mix (38:1) at 1000 °C for ten minutes. Flux discs were analysed on an XRF Phillips 1480 spectrophotometer. Calibration and matrix corrections were performed using empirically derived alpha values and line overlaps from pure reagents and certified reference materials (National Institute Standards Testing). For example, P calibration was done on natural phosphate rocks, National Bureau of Standards 56b and 120a, Washington. Two of the main components of the RP were Ca and P ions, indicating that the RP was an apatite based mineral (McClellan & Gremillon, 1980). Concentration of some of the macro constituents present in the Idaho RP were measured as 36% Ca, 13% P, 2.6% SiO_2 and 1.17% S. The following elements were measured at lower concentrations and reported as mg kg^{-1}: 7800 Na, 3800 Fe, 3200 Mg, 2000 Al, 820 K, 440 Zn, 490 B, 51 Cd, 27 Cu, 21 Mn, 18 Ni, 7.4 Mo.

2.4 Statistical Analysis

All data was analysed by ANOVA using Genstat 5th edition software. Where significant differences were found, pairwise comparisons between treatment means were calculated using the least significant differences method (l.s.d) at the 5% significance level. Where data is not significantly different, the standard error of the mean is reported. Standard errors of the mean (s.e) were calculated from the equation: SE = standard deviation/ $(n)^{0.5}$, where n is the number of replicates.

2.5 Chemical and physical analysis of field soils

Chemical and physical characteristics of soils used in this study are summarised in the following tables: particle size analysis is given in Table 2.2, soil chemical analysis is given in Table 2.3 and the total element content of these soils as determined by aqua-regia digestion and inductively coupled plasma-atomic emission spectrophotometry (ICP-AES, Zarcinas et al., 1987) analysis is given in Table 2.4.

Table 2.2 Particle size distribution of field soils used for the determination of *L*- values.

	Particle Size (%)[1]		
Field site	Clay	Silt	Sand
Mingenew (WA)	4	2	94
Minnipa (SA)	1	<1	99
Innisfail (Qld)	22	13	65
Mt Schank (SA)	22	19	59

[1] determined according to Kilmer & Alexander (1949). Particle sizes were defined as: Clay, <0.02 mm; Silt: 0.02-0.2 mm; Sand, >0.02 mm.

Table 2.3 Chemical analysis of soils used for the determination of *L*- values.

	(1:5 soil:water) EC		total [1] C	HCO_3^- [2] sol. P [2]	HCO_3^- [2] sol. K [2]	total [3] CO_3^- [3]	CEC [4] (NH_4)	Citrate-dithionite extractable [5] Al	Fe	Si	Oxalate extractable [5] Al	Fe	Si
Field site	dS m^{-1}	pH	%	mg kg^{-1}	mg kg^{-1}	(%)	cmol kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Mingenew	0.1	6.50	0.6	15	49	0	3	*	*	*	4046	2032	*
Minnipa	0.03	8.20	0.8	12	89	38	1.7	20	900	290	5210	6890	*
Innisfail	0.02	5.84	1.1	20	133	0	6.7	2940	28800	820	11104	37484	*
Mt Schank	0.3	5.51	7.0	53	455	0	15.0	7800	21400	2120	4920	88575	*

* data not recorded.
[1] determined according to Matejovic (1997).
[2] Soluble (sol.) levels of P and K determined by extraction according to Colwell (1963).
[3] determined according to Martin & Reeve (1955)
[4] Cation exchange capacity (CEC) determined according to Rhoades (1982).
[5] determined according to Sheldrick & McKeague (1975).

Table 2.4 Total concentration (mg kg^{-1}) of selected cations in experimental soils determined following aqua regia digest (3:1, $HCl:HNO_3$) and inductively coupled plasma-atomic emission spectrophotometry (ICP-AES) analysis as described in section 2.2.2

	Al	Ca	Co	Cu	Fe	K	Mg	Mn	Na	Ni	P	S	Zn
Mingenew	25375	116	2	2	7575	167	96	23	20	8	133	22	10
Minnipa	13225	151750	4	8	6850	2850	4100	179	113	7	355	355	29
Innisfail	39500	710	18	21	36000	1690	2950	500	54	50	496	41	64
Mt. Schank	29750	2525	17	14	28250	750	5025	458	29	32	1313	583	59

CHAPTER 3. SOLUBILISATION OF ROCK PHOSPHATE BY *PENICILLIUM RADICUM* AND *P. BILAIAE* RS7B-SD1 IN LIQUID CULTURE

3.1 Introduction

Rock phosphate (RP) is used as fertiliser by organic farmers. It is also used in non-developed farming areas where manufactured fertiliser is not accessible. Water soluble P fertilisers (WP) are expensive and RP is often a cheaper alternative. However, the agronomic effectiveness of RP is typically low relative to WP. While soil moisture plays an important role, the low agronomic effectiveness of RP is primarily due to the limited extent and rate of dissolution of RP in comparison with the rapid and almost complete dissolution of WP (Bolland et al., 1997).

P solubilising fungi may improve the agronomic effectiveness of RP by increasing the extent of RP dissolution by either proton attack or chelation mechanisms. The P solubilising activity of *P. radicum* was previously assessed on pure synthetic calcium phosphate (Ca-P) compounds of varying degrees of solubility (Whitelaw et al., 1999). The ability of *P. radicum* to solubilise RP either under soil or laboratory conditions is unknown.

The supply of N affects the rate of fungal RP solubilisation. For fungi that are able to produce organic acids such as oxalate and citrate, P solubilising activity was shown to be higher in the presence of NO_3^- when compared to conditions that supplied NH_4^+ (Cunningham & Kuiack, 1992; Gharieb et al., 1998; Gharieb & Gadd, 1999; Gharieb,

2000). For other fungi that did not produce significant concentrations of organic acids, P solubilisation required the presence of NH_4^+ (Asea et al., 1988; Illmer & Schinner, 1995).

Whitelaw et al. (1999) identified that *P. radicum* mainly utilised media acidification as a mechanism of P solubilisation. However, under soil conditions pH change can be buffered and this mechanism may not effectively solubilise sufficient quantities of insoluble P to affect plant growth (Gyaneshwar et al., 1998; Gyaneshwar et al., 2002). Thus, P solubilising activity of a microorganism under soil conditions may be more closely related to its P solubilising activity under buffered media conditions.

The aim of the experimental work described in part of this Chapter was to determine the RP solubilising activity of *P. radicum* under laboratory conditions. RP was used in a subsequent experiment that examined the ability of the fungus to increase plant P uptake from an insoluble P source in a sand culture assay (see Chapter 4). Knowledge of the solubilising activity that *P. radicum* has directly on RP will assist in the interpretation of the subsequent experiments. To ensure that fungus-induced RP solubilisation could be measured using this assay, the rate of P solubilisation by *P. radicum* was compared to that of a previously identified P solubilising fungus, *P. bilaiae* RS7B-SD1 (Wakelin et al., 2003).

The experimental objectives of this Chapter were to:

- study RP solubilisation by *P. radicum* and *P. bilaiae* RS7B-SD1 by measuring the amount of soluble P released in liquid culture under the supply of nitrate (NO_3^-) or ammonium (NH_4^+).
- study the effect of medium buffering on the rate of RP solubilisation by *P. radicum* and *P. bilaiae* RS7B-SD1.

3.2 Methods

Fungal isolates *P. bilaiae* RS7B-SD1 and *P. radicum* (section 2.1) and the preparation and analysis of RP (section 2.8) used in this study are as described in Chapter 2.

3.2.1 RP solubilisation assay

A 0.25 g sample of RP was weighed into 250 mL conical flasks and steam autoclaved at 121°C for 20 minutes. A total of 100 mL of sterile Pikovskaya medium (per litre of deionised water: 30 g glucose, 0.5 g $NaNO_3$ or NH_4Cl, 0.1 g $MgSO_4$, 0.2 g KCl, 0.001g $MnSO_4$, 0.001 g $FeSO_4$) was added aseptically to each flask. The total P concentration of the growth medium was approximately 325 mg L^{-1}. To examine the effect of buffering on RP solubilisation, Pikovskaya medium was buffered at pH 7.8 using 100 mM Tris-HCl (Sigma). A concentration of 100 mM Tris was chosen as initial experiments showed that 250 mM Tris was toxic to *P. radicum* but 100 mM allowed some hyphal production. The factorial experiment tested the effect of N-source (NH_4^+ or NO_3^-), buffering (+/- 100 mM Tris-HCl pH 7.8) and inoculation (nil, *P. radicum, P. bilaiae* RS7B-SD1) and included three replicate flasks per treatment. After sterilisation,

the flasks were inoculated with 5 mm agar disks taken from the edge of a 5 day old colony of each fungal species grown on malt extract agar (MEA). This method of inoculation added approximately $1x10^6$ cfu to each flask. The flasks were incubated at 25 °C on a rotary shaker at 120 rpm. The cultures were sampled daily under aseptic conditions by withdrawing 10 mL of the liquid medium. The aliquot was then replaced in a sterile laminar flow cabinet with sterile Pikovskaya medium that did not contain any RP. The sampled aliquots were filtered (0.2 μm) and analysed for pH, total soluble P by inductively coupled plasma-atomic emission spectrophotometry (see Section 2.2.2) and gluconate (Section 3.2.2). After 168 h incubation the liquid growth medium was decanted and the particulate RP was separated as much as possible from the hyphal mass. The hyphal mass was collected by suction filtering through Whatman No.1 filters. The mass of filterable solids (hereafter termed biomass) was determined after drying at 60 °C for 24 hours.

3.2.2 Identification of organic anions by capillary zone electrophoresis (CZE).

Organic anions present in the fungal exudates of the experiment described in 3.2.1 were analysed by capillary zone electrophoresis (CZE) using a Quanta 4000 instrument (Waters, Milford, USA). The system was controlled by Millennium (Waters, Milford, USA) software. Separation was carried out on fused-silica capillaries (48.5 cm x 50 μm internal diameter) with the UV detector at 214 nm. The running electrolyte contained 10 mM phthalate, 0.75 mM tetratridecylammonium bromide (TTAB) and was buffered to pH 8.0. Prior to use, a capillary was pretreated with the following cycles: 0.1 M NaOH for 20 min, 0.01 M NaOH for 20 min, deionised water for 20 min and 10 mM of the electrolyte for 30 min. The capillary was pre-conditioned with the electrolyte for 2 min

before each run. Samples were injected in the hydrostatic mode for 30 s. The capillary was held at 25 °C, and the applied constant voltage was –20 kV. The technique was used for the identification of gluconate, based on migration time and was verified by spiking samples with known standards.

3.3 Results

3.3.1 Capillary zone electrophoresis of RP solubilisation assays.

CZE was used to determine the concentration of gluconate in the RP solubilisation assays. While the CZE methodology was only calibrated for the quantitative detection of gluconate, noteworthy data on the production of other low molecular weight organic anions by *P. bilaiae* RS7B-SD1 appeared and hence a general description of the electropherograms obtained from the RP solubilisation assay is given.

Representative electropherograms of the fungal cultures, taken after 168 hours incubation is shown in Figure 3.1. Using the electrophoretic conditions as described in Section 3.2.2, gluconate could be resolved from other fungal metabolites and components of the growth medium. Gluconate responses (G) were generally detected at 3.50 to 4.00 minutes migration time.

It was found that N-source had a significant impact on the fungal production of low molecular weight anions in the growth medium. The concentration of gluconate was higher in the presence of NO_3^- than NH_4^+ (see Figures 3.2 and 3.3, Table 3.1). The effect of N-source on the organic anion profile was apparent for *P. bilaiae* RS7B-SD1 where differences between NO_3^- and NH_4^+ were distinct. When cultured with NO_3^- *P.*

bilaiae RS7B-SD1 culture contained a large peak response (marked U in Figure 3.1) at the migration time 3.1 minutes that corresponded to standard acetate; in the presence of NH_4^+ this peak was not present (see Figure 3.1). While the migration time of the U peak suggests its' identity is acetate, compound verification would require spiking the fungal culture supernatant with standard acetate. Indentification would be confirmed if both fungal and standard acetate had the same migration time and did not produce separate peaks.

Other peaks in the electrophergorams usually appeared in the migration times 1.50 to 2.50 minutes and did not appear to reveal any information on the production of organic anions in the fungal cultures.

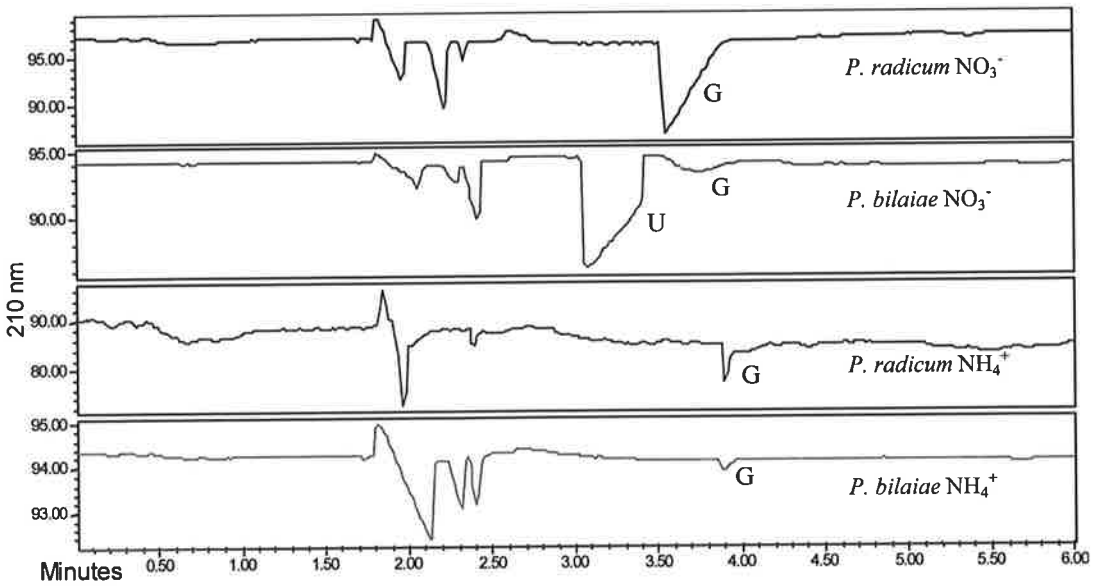


Figure 3.1 Representative electropherograms of culture filtrates of *P. radicum* and *P. bilaiae* RS7B-SD1. These samples were taken after 168 hours incubation from cultures that were supplied with either NO_3^- or NH_4^+ as the sole source of N . G = gluconate, U = main organic anion produced by *P. bilaiae* RS7B-SD1 when cultured with NO_3^-. Y axis = absorbance (210 nm), X axis = migration time (minutes).

3.3.2 RP solubilisation assay

3.3.2 (i) Non-buffered medium

The pH of uninoculated medium was stable at pH 7 ± 0.6 and contained soluble P < 1 mg L^{-1} (data not shown). For inoculated medium, changes in pH, soluble P and gluconate concentration for either *P. radicum* or *P. bilaiae* RS7B-SD1 grown on either NO_3^- or NH_4^+ is given in Figures 3.2 (a,b) & 3.3 (a,b). When comparing the two Figures, the differences in scale should be noted. Table 3.2 lists maximum values of soluble P concentration, maximum gluconate concentration, minimum pH values, biomass : soluble P ratios and correlation coefficients to enable a direct comparison across fungal and N treatments.

The ability of the two *Penicillium* spp. to solubilise RP was affected by the N source. In the presence of NO_3^-, *P. radicum* cultures had very little RP solubilising activity and the liquid medium contained up to 11.1 mg L^{-1} of soluble P by the end of the incubation period (Figure 3.2a). In NO_3^- medium, *P. bilaiae* RS7B-SD1 had a significantly better RP solubilising ability and liquid medium contained up to 37.5 mg L^{-1} (Figure 3.2b). For both fungi RP solubilisation was increased when NH_4^+ was supplied. Cultures of *P. radicum* contained up to 76.3 mg L^{-1} (Figure 3.3a) and *P. bilaiae* RS7B-SD1 up to 50.3 mg L^{-1} at the end of the experiment (Figure 3.3b & Table 3.1). The influence of N source was more pronounced for *P. radicum* than for *P. bilaiae*. While NH_4^+ supply significantly increased the P solubilising activity of *P. radicum* (in comparison to NO_3^- supply) it did not significantly raise the maximum P concentration measured in cultures of *P. bilaiae* RS7B-SD1 (Table 3.1).

The rate of acidification was significantly affected by the N-source. When N was supplied as NH_4^+, the rate of acidification was greater. In NH_4^+ medium, the pH of *P. radicum* cultures was 3.82 (Figure 3.3a) and 4.76 for *P. bilaiae* RS7B-SD1 (Figure 3.3b) after 48 hours incubation time. After 48 hours incubation in NO_3^- medium, the pH of *P. radicum* culture was 5.74 (Figure 3.2a) and for *P. bilaiae* it was 5.17 (Figure 3.2b).

The production of gluconate was significantly affected by the N source and was generally higher in NO_3^- cultures. In NO_3^- fed cultures the production of gluconate generally followed the concentration of soluble P (Figure 3.2a,b). This was reflected in the high correlation coefficient between soluble P and gluconate production (Table 3.1). In NH_4^+ cultures gluconate concentration peaked and then dropped while the concentration of soluble P was maintained (Figure 3.3a,b). This was reflected by the low correlation coefficient between gluconate production and soluble P (Table 3.1).

Table 3.1 Characteristics of RP solubilisation in cultures of *P. radicum* and *P. bilaiae* 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.

fungus	N source	pH at max. soluble P[A]	Max. sol. P ($mg\ L^{-1}$)[B]	Max. gluconate (mM)[C]	Biomass : P ratio[D]	Correlation coefficient		
						pH vs gluconate	P vs gluconate	P vs pH
non-buffered medium								
P. radicum	NO_3^-	4.75	11.24	7.72	1.18	-0.75*	0.81*	-0.68
P. bilaiae	NO_3^-	4.55	37.40	5.78	0.22	-0.65	0.87**	-0.93****
P. radicum	NH_4^+	4.04	76.33	5.64	0.05	-0.49	0.48	-0.83
P. bilaiae	NH_4^+	4.21	50.26	2.73	0.06	-0.24	-0.29	-0.92****
buffered medium								
P. radicum	NO_3^-	7.40	0.53	0.63	7.8	-0.80**	0.07	-0.46
P. bilaiae	NO_3^-	4.11	38.01	22.40	0.09	-0.56	0.50	-0.96****
P. radicum	NH_4^+	7.55	1.61	0.00	3.35	ND[E]	ND	-0.38
P. bilaiae	NH_4^+	3.97	71.19	2.81	0.07	-0.24	0.39	-0.94****
l.s.d (α 0.05)		0.39	25.61	1.94	0.89			

[A] the medium pH at sample time where the highest soluble P concentration was recorded
[B] the maximum soluble P concentration detected in the growth medium
[C] the maximum concentration of gluconate detected in the growth medium
[D] calculated as the ratio of dry mass of hyphae : concentration of soluble P present in the growth medium after 168 hours incubation.
[E] ND - gluconate not detected in these cultures.

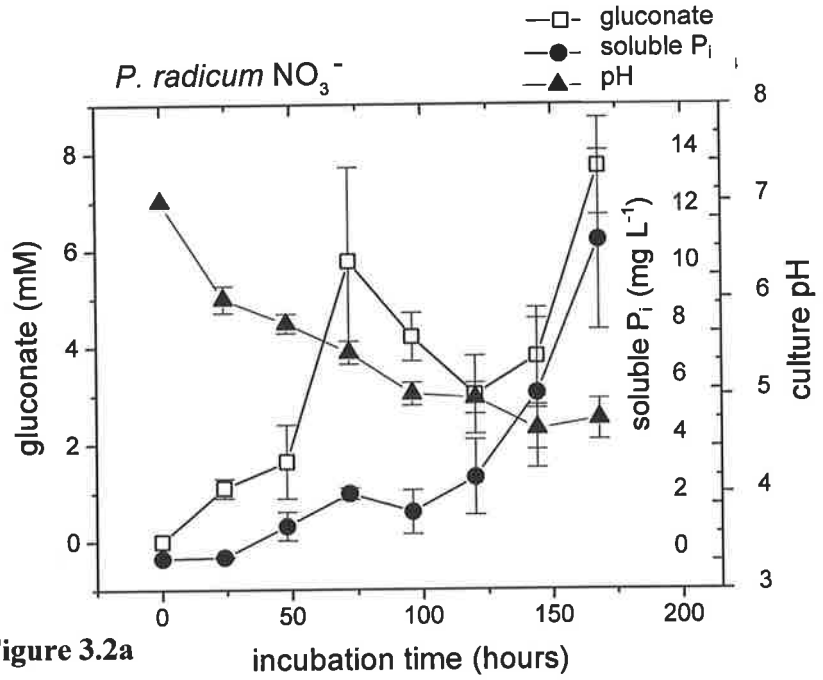


Figure 3.2a

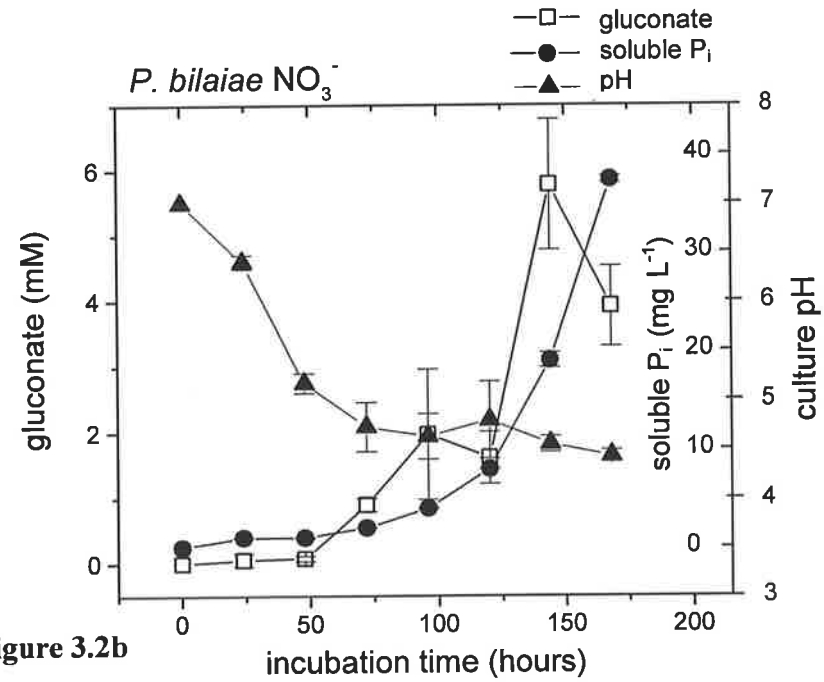


Figure 3.2b

Figure 3.2 Changes in gluconate concentration, soluble inorganic P (P_i) 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.

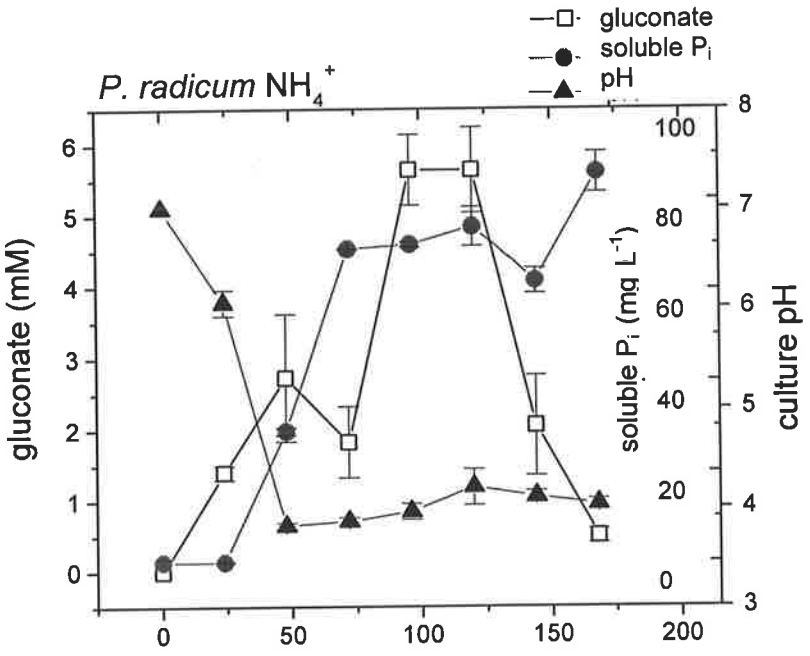


Figure 3.3a

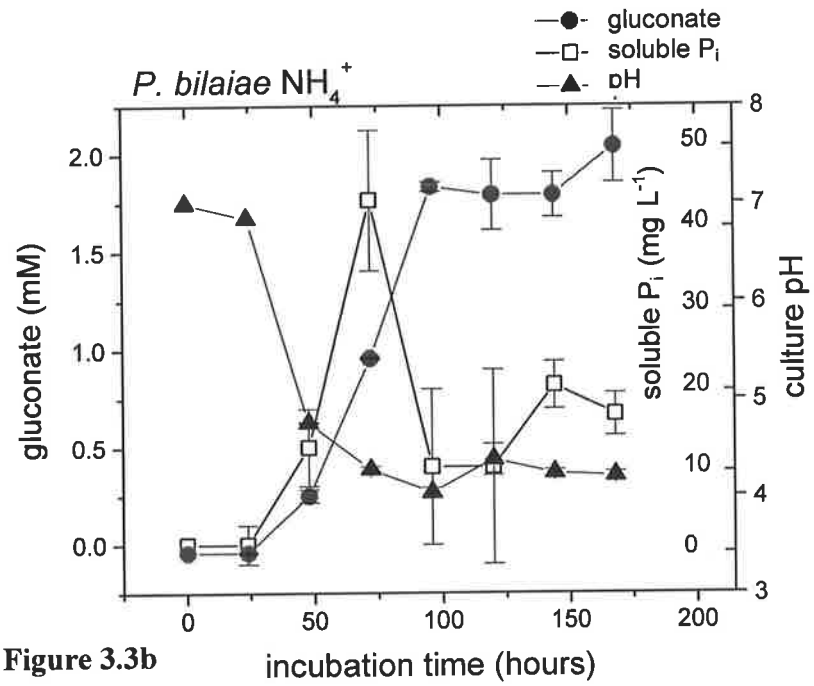


Figure 3.3b

Figure 3.3 Changes in gluconate concentration, soluble inorganic P (P_i) 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.

3.3.2 (ii) Buffered medium

In buffered medium, *P. radicum* had little effect on the medium pH or soluble P over the studied incubation time. There was generally no detectable gluconate, soluble P was unaffected and somewhat lower biomass was produced (Fig 3.5a and Table 3.1). In contrast, *P. bilaiae* RS7B-SD1 acidified the buffered medium, solubilised P and produced gluconate (Fig 3.5b). After 96 hours incubation, the pH of *P. bilaiae* RS7B-SD1 cultures had begun to drop. At this time the soluble P concentration had significantly increased and the media contained gluconate (Figures 3.5b). When the ability of *P. bilaiae* RS7B-SD1 to solubilise P and acidify the culture medium is compared between NO_3^- and NH_4^+ medium, it can be seen that the changes pH were similar in magnitude while the maximum soluble P was increased under NH_4^+ supply (Table 3.1). However, the production of gluconate was higher in NO_3^- fed cultures (up to 20.5 mM gluconate) compared to up to 2.5 mM in NH_4^+ medium (Figure 3.4b, 3.5b and Table 3.1).

As a measure of the efficiency of the fungus to solubilise RP, the ratio of total biomass to soluble P concentration was determined (Table 3.1). A higher biomass : P ratio would be indicative of a fungus with a low P solubilising efficiency as a low soluble P concentration with a high rate of hyphal P uptake would leave a low amount of solubilised P for plant uptake. A low biomass : P ratio would indicate a high concentration of soluble P relative to biomass production and would be a beneficial attribute of a fungus for effective P solubilisation i.e. a higher amount of P solubilised

and a lower rate of hyphal P assimilation would mean a higher concentration of soluble P for plant uptake.

Under all conditions tested, *P. bilaiae* RS7B-SD1 cultures had a biomass : P ratio that was 0.22 or less. Values for *P. radicum* were more variable and were significantly affected by N-source and medium buffering (Table 3.1). In non-buffered medium that supplied NH_4^+, values for *P. radicum* were not significantly different from *P. bilaiae* RS7B-SD1. Under NO_3^- supply the biomass : P ratio for *P. radicum* was significantly higher than *P. bilaiae* RS7B-SD1. Under buffered conditions, biomass : P ratios for *P. radicum* were significantly higher than *P. bilaiae* RS7B-SD1. This is indicative of the low P solubilising activity of *P. radicum* under buffered conditions.

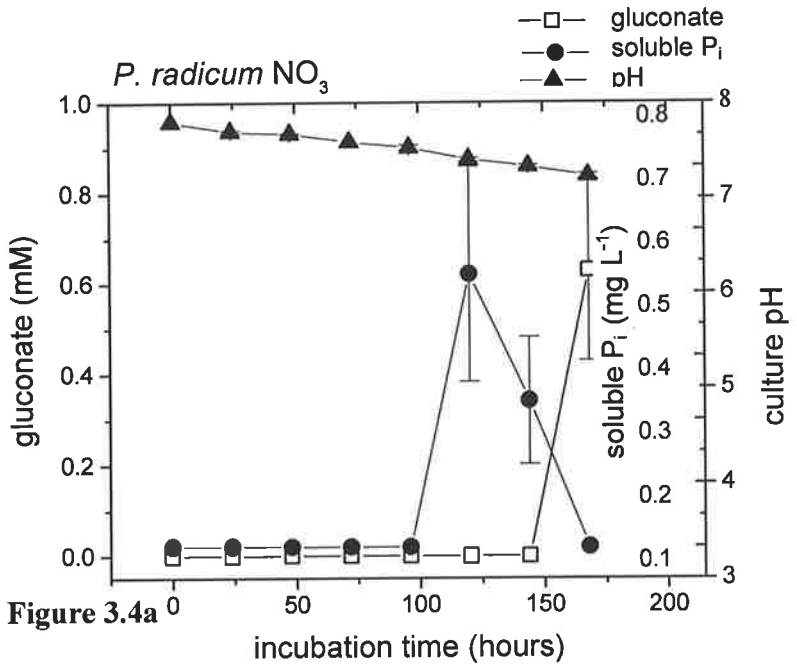


Figure 3.4a

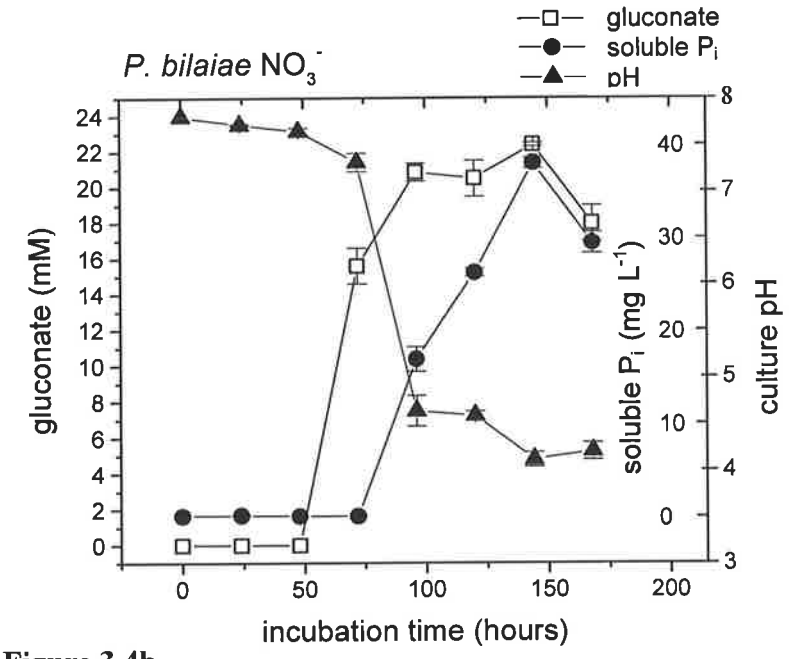


Figure 3.4b

Figure 3.4 Changes in gluconate concentration, soluble inorganic P (P_i) 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.

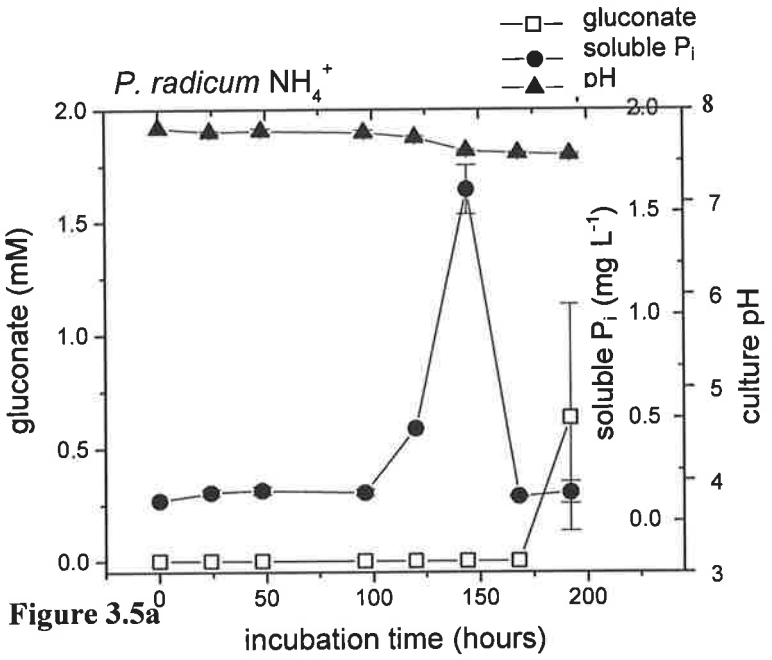


Figure 3.5a

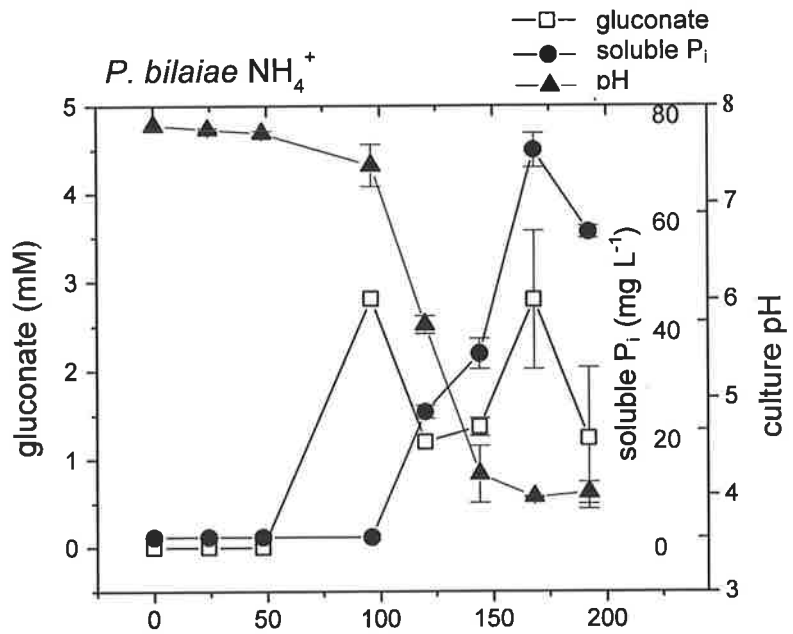


Figure 3.5b

Figure 3.5 Changes in gluconate concentration, soluble inorganic 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.

3.3.3 Fungal biomass production

Filterable solids were used as an indicator of fungal biomass production (Table 3.2). Liquid cultures fed with NO_3^- as the sole source of nitrogen produced higher biomass in comparison to cultures supplied with NH_4^+. The highest biomass production was found for NO_3^- cultures of *P. radicum* (approximately 0.619 g dry wt of total solids at the end of the incubation). In buffered culture medium, *P. radicum* produced a minimal quantity of biomass in comparison to non-buffered conditions. Biomass production by *P. bilaiae* RS7B-SD1 was also significantly affected by medium buffering.

Table 3.2 Production of biomass by *P. bilaiae* RS7B-SD1 and *P. radicum* 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.

Fungus	N source	Mean dry mass
non-buffered medium		
P. radicum	NO_3	0.619
P. bilaiae RS7B-SD1	NO_3	0.352
P. radicum	NH_4	0.418
P. bilaiae RS7B-SD1	NH_4	0.305
buffered medium		
P. radicum	NO_3	0.078
P. bilaiae RS7B-SD1	NO_3	0.272
P. radicum	NH_4	0.046
P. bilaiae RS7B-SD1	NH_4	0.361
l.s.d (α 0.05)		0.126

3.4 Discussion

For the complete dissolution of RP such as hydroxyapatite an ideal reaction would be:

$Ca_{10}(PO_4)_6(CO_3)_2$ (s) + 20HX (aq)→ $10CaX_2^-$ (aq) + $6H_3PO_4$ (aq) + $2HCO_3^-$ (aq),

where X represents an anion such as Cl^-. From the above reaction it can be seen that for every mole of hydroxyapatite consumed 20 mole of protons are consumed and 6 mole of P produced, indicating the importance of protons in the dissolution. This shows that RP dissolution is a proton-dependent process. The presence of organic anions can also increase the RP dissolution depending on the chelating ability of the anion (see Section 1.2.1).

Soluble P concentration in the flasks containing *Penicillium* would be the net result of two main processes, (1) RP solubilisation by the previously mentioned processes and (2) hyphal P assimilation. The source of N had a major impact on the soluble P concentration. In comparison to cultures fed with NO_3^-, the supply of NH_4^+ resulted in a faster rate of acidification to lower pH values and resulted in lower biomass production. Therefore soluble P depletion by hyphal assimilation would have been lower, yet RP protonation was higher leading to overall higher values of soluble P.

Under NO_3^- conditions, *P. bilaiae* RS7B-SD1 solubilised more P than *P. radicum*. This may be due to two reasons, (1) culture acidity of *P. bilaiae* RS7B-SD1 was slightly lower than *P. radicum* and (2) *P. bilaiae* RS7B-SD1 appeared to produce a greater range of organic acids. As differences in culture pH between the two fungi for NO_3^- cultures was not statistically significant, increased RP solubilisation was likely to be related to an increased production of organic acids. The greater

complexity of peaks detected in electropherograms from *P. bilaiae* RS7B-SD1 cultures suggested that a greater number of organic anions were produced. However, to ascertain the identity of these peaks would require spiking with known organic anion compounds.

Further inspection of the electropherograms of the liquid medium from the RP solubilisation assay revealed a distinctive feature of *P. bilaiae* RS7B-SD1. When this fungus was cultured in NO_3^- medium a large peak of unknown identity was produced. This reponse may be related to the production of an organic anion. The migration time of the organic anion suggested that the identity of the anion was acetate. While the stability of Ca-complexes of acetate is slightly lower than that for gluconate, the large detection response indicates that the anion was produced at a concentration that is likely to have a significant contribution to the overall dissolution of RP.

Further work should be aimed at determining the role of organic compounds in RP solubilisation by this fungus. Such information would assist in predicting where the fungus is likely to have the best effectiveness in increasing plant P uptake. Identification of organic anions and their role in RP solubilisation by *P. bilaiae* RS7B-SD1 was not performed here as the focus of the project was to determine the mechanisms of growth promotion by *P. radicum*. The production of low molecular weight anions assisting in the solubilisation of insoluble P has been reported for the isolate *P. bilaiae* PB-50 (Cunningham & Kuiack, 1992) and it is possible that such a mechanism may operate for *P. bilaiae* RS7B-SD1.

Under NH_4^+ supply, *P. radicum* was a far better RP solubiliser than *P. bilaiae* RS7B-SD1. This may be due to a higher tolerance of *P. radicum* to low pH than *P. bilaiae* RS7B-SD1. This was suggested by the higher production of biomass and slightly lower culture pH produced by *P. radicum* in NH_4^+ growth medium. If *P. bilaiae* RS7B-SD1 has a lower tolerance to acidity than *P. radicum*, it may be more reliant on mechanisms of chelation to obtain nutrients such as P from insoluble sources and this may explain the higher production of organic anions and higher concentration of soluble P in the presence of NO_3^-.

Medium buffering may be used to discriminate between the acidification and chelation mechanisms of RP solubilisation. Under buffered conditions, *P. bilaiae* RS7B-SD1 achieved similar rates of P solubilisation as in non-buffered conditions. This was evidenced by similar concentrations of soluble P and similar biomass : P ratios in buffered and non-buffered media. The rate of H^+ excretion by *P. bilaiae* RS7B-SD1 was likely greater than the buffer capacity of the medium and therefore a pH drop was seen after 48 hours incubation. In contrast, *P. radicum* could not solubilise RP and only caused a slight drop in the medium pH when cultured with Tris. While the cause of reduced RP solubilisation was likely to be due to the consumption of protons by the buffer, a toxic effect of Tris on biomass production may have contributed to the overall lower RP solubilising activity. In these experiments a concentration of 100 mM Tris was used, because in the presence of 250 mM Tris, *P. radicum* did not produce significant quantities of hyphal mass. Growth of *P. radicum* could be assessed in a range of buffers; a buffer system proven to be non-toxic to the growth of *P. radicum* would resolve the effect of buffer toxicity from the lack of soluble P as a constraint to fungal growth.

Considering that the fungal uptake of NH_4^+ is coupled with a 1:1 stoichiometric ratio of efflux of protons (Raven & Smith, 1976; Roos & Luckner, 1984), it is likely that the production of free protons was the mechanism leading to RP solubilisation in the presence of RP. Hence, this work suggests that acidification is the main mechanism of P solubilisation by *P. radicum* and that there may be a requirement of NH_4^+ for RP solubilisation. The role of acidification in the RP solubilisation was further evidenced by the calculation of correlation coefficients (Table 3.1). In the presence of NH_4^+, there was a high correlation of soluble P concentration with pH, which illustrates the importance of acidification for RP solubilisation. In the presence of NO_3^-, there was a high correlation between gluconate concentration and pH, which suggests that the production of gluconic acid was the major source of protons in the presence of NO_3^- and was the major cause of changes in pH leading to RP solubilisation. If it is assumed that all of the gluconate detected was produced as the acid form, then from the K_a of gluconate ($2.5x10^{-4}$, Merck Index 12th ed, 1996) it was calculated that concentrations of gluconate detected in these experiments could completely account for the culture pH (results not shown). In the presence of NH_4^+, which acted as the main source of acidity, changes in the concentration of gluconate were independent of pH and hence there was a low correlation of gluconate with soluble P (Table 3.1).

In summary, under NO_3^- culture *P. bilaiae* RS7B-SD1 was the superior P solubilising isolate and this activity may have been related to the production of an organic anion that assisted in its ability to solubilise extra P under buffered conditions. As soil conditions are often buffered, the ability of *P. bilaiae* RS7B-

SD1 to solubilise RP under buffered conditions suggests that further research should investigate the use of this fungus as either a general P solubilising inoculant or as a biological agent to increase the bioavailability of RP.

In the presence of NH_4^+ *P. radicum* was the superior P solubilising fungus. This suggests that NH_4^+ uptake coupled with H^+ efflux leading to acidification was the main mechanism of RP solubilisation by this fungus. This suggestion was further supported by the absence of RP solubilisation in buffered solutions and the high correlation between soluble P concentration and pH in NH_4^+ culture. Under conditions where this mechanism can effectively operate, *P. radicum* may increase the available P in the soil for plant uptake.

In-vitro studies do not take into account the relationship that the fungus has with plant roots. While *P. radicum* may possess acidification mechanisms that can effectively solubilise P, there needs to exist an ability of the fungus to either colonise the rhizosphere or rhizoplane such that the extra soluble P gained from microbial P solubilisation can be absorbed by the plant root. The ability of the fungus to solubilise P in the presence of plant roots was studied in a sand culture assay described in Chapter 4.

CHAPTER 4. THE EFFECT OF *PENICILLIUM RADICUM* ON WHEAT PHOSPHORUS UPTAKE FROM SUBSTRATES OF CONTRASTING SOLUBILITY IN SAND CULTURE

4.1 Introduction

In order to study plant growth promoting mechanisms, a reproducible assay system is required. The lack of a reliable plant growth response to *P. radicum* inoculation makes a study of the mechanisms of plant growth promotion difficult (see Section 1.4.2). Using a sand culture system, complex factors that occur in a soil environment can be largely eliminated so that plant growth response to fungal seed inoculation can be expected to be more reproducible and mechanisms of plant growth promotion may be more easily studied. In this Chapter, the P solubilising activity of *P. radicum* was interpreted from a plant growth and P response in a sand culture assay.

The principles of the sand culture assay were based on the following briefly outlined concepts. If *P. radicum* promotes plant growth via a P solubilising effect, then under conditions of low extractable P and a relatively high total P in the sand growth medium, a measurable plant response to *P. radicum* inoculation should occur. Under conditions that supply a higher concentration of soluble P, the growth response to *P. radicum* would be expected to be lower.

In Chapter 3 it was shown that N-source dramatically affected the solubilisation of RP by *P. radicum.* In this Chapter, the effect of N-source on the plant growth response to *P. radicum* was studied in a sand culture assay using P deficient and sufficient conditions for plant growth.

The experimental objective of the work described in this Chapter was to:

- study plant growth promotion and solubilisation of insoluble P sources by *P. radicum* by measuring the plant response to fungal inoculation in a sand culture assay.

This was achieved by:

- determining the types of P compounds that *P. radicum* solubilises by measuring the response to inoculation in P nutrition and dry weight of mature plants that were grown in sand culture and supplied with defined sources of P.
- determining if the type of N source as described in Chapter 3 affects the ability of *P. radicum* to solubilise P for increased plant uptake.

4.2 Methods

4.2.1 Preparation of the plant growth medium

Quartz sand, obtained commercially (Keough Sand Depot Pty Ltd, Thebarton, SA), was sieved to less than 600 μm, and soaked in 2M HCl overnight. The sand was then rinsed ten times using deionised water and autoclaved at 121 °C for twenty minutes. To test the ability of *P. radicum* to solubilise contrasting sources of insoluble P, the sand was supplemented with either Idaho rock phosphate (RP) that was prepared as outlined in Section 2.3, crystalline $FePO_4$ (Fe-P), or phytic acid (P_o). These poor sources of P for plant nutrition were compared to a relatively more soluble compound, dibasic calcium phosphate, $CaHPO_4$ (Ca-P) that would provide a better source of P for plant growth. The compounds Ca-P, Fe-P and P_o were all

purchased from Sigma-Aldrich Co., Sydney. The total P and bicarbonate extractable P contents of the amended sand growth media for the two experiments performed are given in Table 4.1. The unamended sand contained non-detectable levels of P according to the method outlined in section 2.3. In Experiment 1 the P substrates were added to produce conditions that provided the same total amount of P. In Experiment 2 P substrates were added to create P sufficient (Ca-P) and P deficient conditions (RP). Sand that was amended with the solid P sources was supplemented with other essential nutrients using Long Ashton nutrient solution as outlined in Section 2.2.3.

P. radicum ryegrass inoculum, prepared as outlined in Chapter 2, was added at a rate of 1 g kg^{-1} sand, while control growth medium contained sterile ryegrass seeds. The specific P uptake (SPU) was calculated from the ratio of total shoot P uptake (mg) to dry root mass (g). All statistical analysis was performed using Genstat 5^{th} ed. Shoot weight, root weight and shoot P data were analysed across P and inoculation treatments using two or three-way ANOVA. Specific details for each experiment are outlined below.

Table 4.1 P composition of sand growth media used in the two sand culture assay experiments.

Experiment 1

P substrate	Amount added ($g\ kg^{-1}$)	Total P ($g\ kg^{-1}$)	$NaHCO_3$ PO_4 [A] ($mg\ kg^{-1}$)
Fe-P	0.55	0.11	41.8
Ca-P	0.50	0.11	91.4
RP	1.00	0.12	10.7
P_o	0.55	0.11	ND[B]

Experiment 2

P substrate	Amount added ($g\ kg^{-1}$)	Total P ($g\ kg^{-1}$)	$NaHCO_3$ PO_4 ($mg\ kg^{-1}$)
Ca-P	0.2	0.045	38.2
RP	1.0	0.13	8.2

[A] extractable P determined by the method of Colwell (1963).
[B] ND – not determined due to the interference of P_o with the molybdenum blue method of PO_4 determination (Murphy & Riley, 1962).

4.2.2 Experiment 1. The effect of P. radicum *on plant growth and nutrient uptake from contrasting P substrates*

In Experiment 1, the effect of *P. radicum* on the growth and P nutrition of wheat was examined using a complete randomised design with 5 replications of four P sources: Ca-P, RP, Fe-P and P_o. Two wheat seeds of *T. aestivum* cv. Krichauff (seed P content 118 μg P $seed^{-1}$), were pregerminated overnight and placed in PVC cylinder pots (non-draining, 6.2 cm diameter and 26 cm long) containing 1 kg of the growth medium. After emergence, each pot was thinned to one plant. The plants were harvested at the first visible sign of heading. i.e stage 10.1 defined by Large, (1954) after 13 weeks growth. The dry weight and P nutrition was determined as outlined in Section 2.2.1 & 2.2.2.

4.2.3 Experiment 2. The effect of N source on the plant response to P. radicum *inoculation*

In the second experiment, the effect of N source on the plant growth response to *P. radicum* was investigated. Plants were supplied with either NH_4^+ (as 1.056 g kg^{-1} $(NH_4)_2SO_4$) or NO_3^- (as 0.682 g kg^{-1} $NaNO_3^-$) as the sole source of N. The total concentration of N added was the same for each N source at 8 mmol N kg^{-1} of sand. After addition of the nutrient solution to the sand, the pH between the two sources was not found to be significantly different at approximately pH 7. Seeds were coated with spores of *P. radicum* (1 x 10^4 cfu per seed) as described in section 2.1.2. Plants were grown for a period of 8 weeks (stage 5 defined by Large, 1954) and harvested as described in Section 2.2.1. The P content of these plants was then determined as described in Section 2.2.2.

4.2.4 Isolation of P. radicum from wheat roots

The isolation of *P. radicum* from wheat roots was performed as described in Section 2.1.3 using semi-selective DRBC agar to show that plant growth responses to inoculation occurred with the colonisation of plant roots by *P. radicum*.

4.3 Results

4.3.1 Experiment 1

4.3.1 (i) Plant growth response to P source

For non-inoculated plants, it was identified that P source significantly affected plant growth. Plants grown in Ca-P medium had the highest total mean mass of 1.69 g pot^{-1} and were significantly different from the P_o, RP and Fe-P treatments; these did not significantly differ from each other in plant dry weight (see Figure 4.1).

4.3.1 (ii) Plant growth response to fungal inoculation

Plant dry weight response to *P. radicum* inoculation for each of the individual P sources is summarised in Figure 4.1. *P. radicum* significantly decreased the root weight of plants grown in Ca-P amended growth medium and increased the shoot weight of these plants. Although the effect of *P. radicum* on shoot weight of plants grown in Ca-P medium was not significant at the 5% level, it was significant at the 10% level. For plants grown in Fe-P medium, *P. radicum* increased the mean shoot weight and root weight, but the effects were not statistically significant. When the increase in plant dry weight for Fe-P grown plants was determined as the total dry mass pot^{-1}, inoculation significantly increased the total plant dry mass from 1.19 to 1.45 g pot^{-1}. In the RP and P_o growth media there were marginal increases in the mean shoot and root weight in response to inoculation, however, these effects were not statistically significant (Figure 4.1).

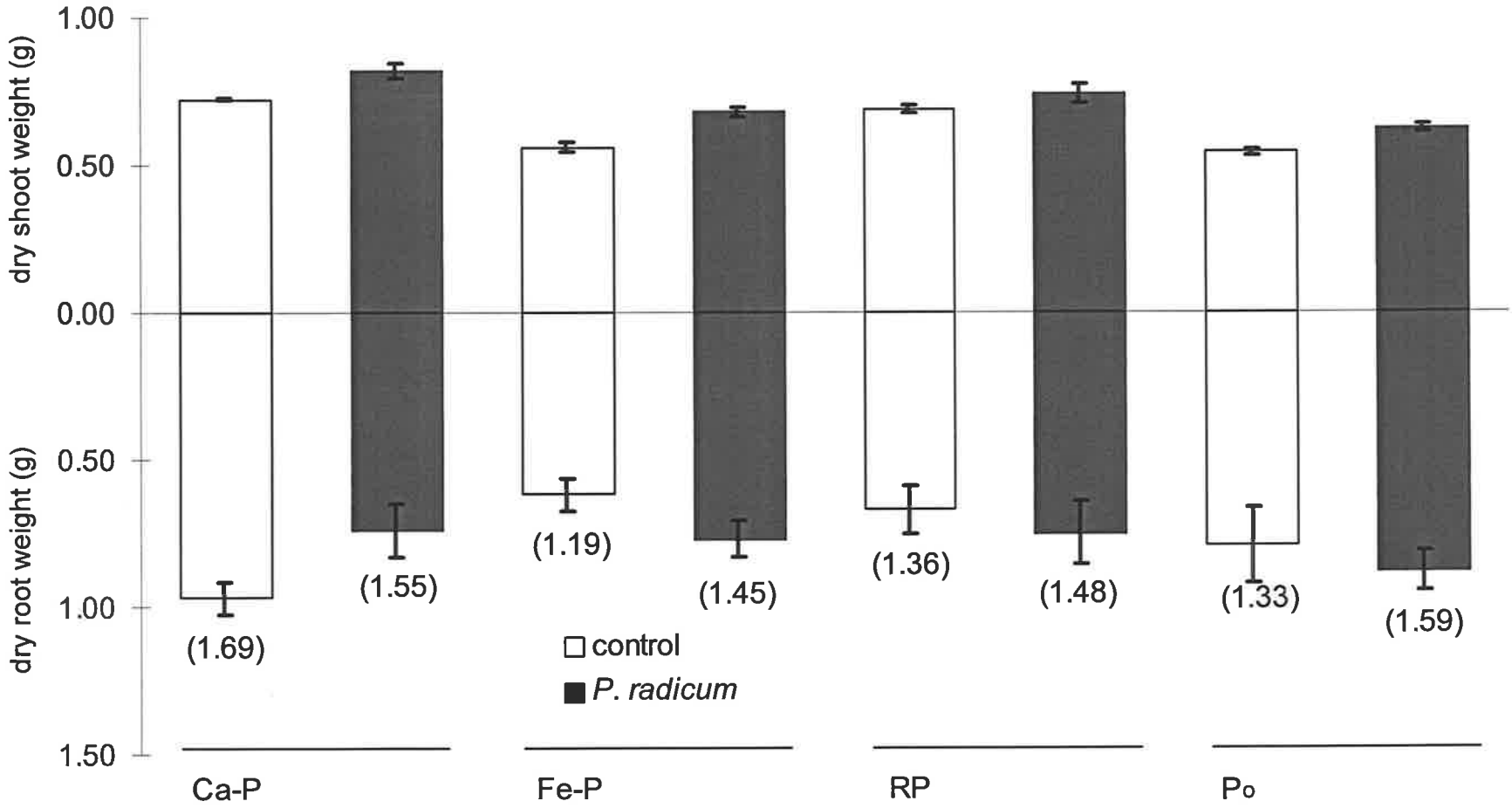


Figure 4.1 The effect of *P. radicum* on plant dry weight in sand culture supplying P substrates of varying degrees of solubility (Experiment 1). P sources were: Ca-P = $CaHPO_4$; Fe-P = $FePO_4$, RP = rock phosphate and; P_o = phytic acid. Values in parentheses indicate total dry weight pot^{-1} (g), 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).

4.3.1 (iii) Phosphorus nutrition

For non-inoculated plants, the type of P substrate present in the plant growth medium significantly affected shoot P concentration (Figure 4.2). The highest shoot P concentrations were recorded for plants in the P_o amended media, while the Ca-P, Fe-P and RP media produced plants with significantly lower shoot P concentrations. Inoculation significantly increased the shoot P concentration of plants grown in the Ca-P medium. There were marginal non-significant increases in the shoot P concentration due to inoculation of plants grown in the RP and P_o media. *P. radicum* had no effect on the shoot P concentration of plants grown in Fe-P medium.

The greatest shoot P uptake was seen for plants that were supplied with Ca-P as the P source. Plants grown in the P_o medium did not have a significantly different amount of P uptake in comparison to Ca-P plants. The lowest shoot P uptake was measured for plants that were grown with Fe-P and RP substrates and values were significantly less for plants grown in the Ca-P medium. *P. radicum* inoculation significantly increased the total shoot P uptake of plants grown in the Ca-P medium. Shoot P uptake responses to inoculation in the other growth media were not statistically significant (see Figure 4.2).

As shown in Figure 4.2, *P. radicum* significantly increased the SPU for plants grown in the Ca-P medium. For the other substrates (Fe-P, RP & P_o) there was no significant effect of inoculation on SPU.

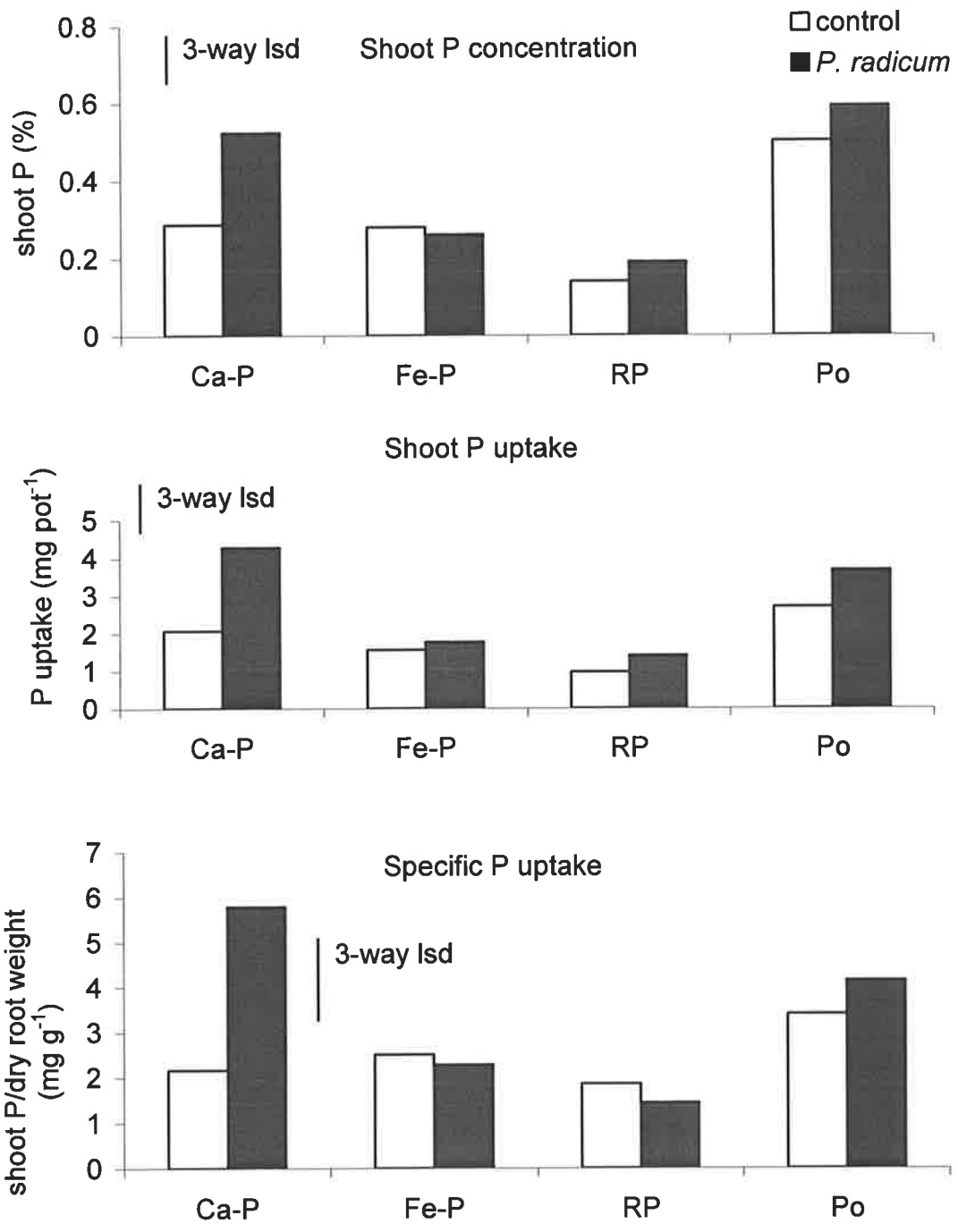


Figure 4.2 The effect of *P. radicum* on the P nutrition of plants grown in sand culture that supplied P substrates of varying solubility. P sources were: Ca-P = $CaHPO_4$; Fe-P = $FePO_4$, RP = rock phosphate and; P_o = phytic acid. Bars within the figure indicate 3-way l.s.d (α 0.05).

4.3.2 Experiment 2

Plants grown in Experiment 2 were harvested at 8 weeks growth rather than 13 weeks (Experiment 1). In Experiment 1 N was applied as a combination of NH_4^+ and NO_3^-. In Experiment 2, the effect of N source on the plant response to *P. radicum* was tested by supplying either NH_4^+ or NO_3^- separately as the sole source of N.

4.3.2 (i) Plant growth

After 4 weeks, a growth response to *P. radicum* inoculation was apparent as increased plant height. However, as shown in Figure 4.3, the effect of *P. radicum* inoculation on plant height was only seen for plants supplied with NO_3^-. Plants supplied with NH_4^+ were taller overall than NO_3^- fed plants but did not show any height difference due to treatment with *P. radicum.*

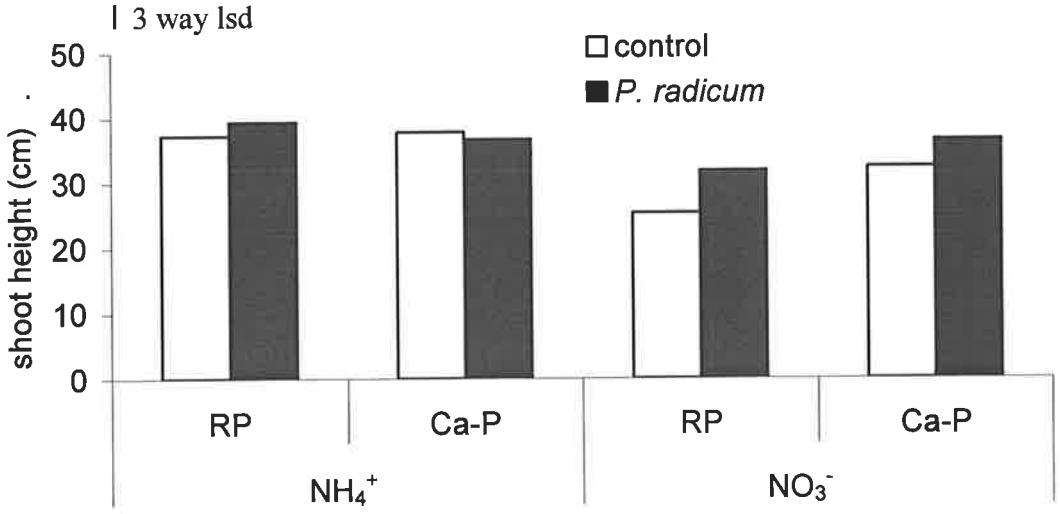


Figure 4.3 Shoot height (cm) after 4 weeks growth of wheat plants supplied with either RP (rock phosphate) or Ca-P ($CaHPO_4$) as the P source and supplied with either NH_4^+ or NO_3^- as the sole source of N. Bar within the figure indicates 3-way l.s.d (α 0.05).

Figure 4.4a summarises the shoot dry weight production at harvest. *P. radicum* increased the dry shoot weight of wheat supplied with RP regardless of the source of N. For plants supplied with Ca-P, a positive response to inoculation was only seen in plants fed with nitrate.

There was a significant effect of P source on root weight; plants grown with RP had root systems of lower mass than plants with Ca-P (Figure 4.4b). While *P. radicum* inoculation did not have a statistically significant effect on root growth, there was a clear trend towards lower root weight for most inoculated groups following inoculation. However, there was an exception to this trend as inoculated plants grown in RP medium and fed with NH_4^+ had root systems of a higher mass than non-inoculated controls (Figure 4.4b).

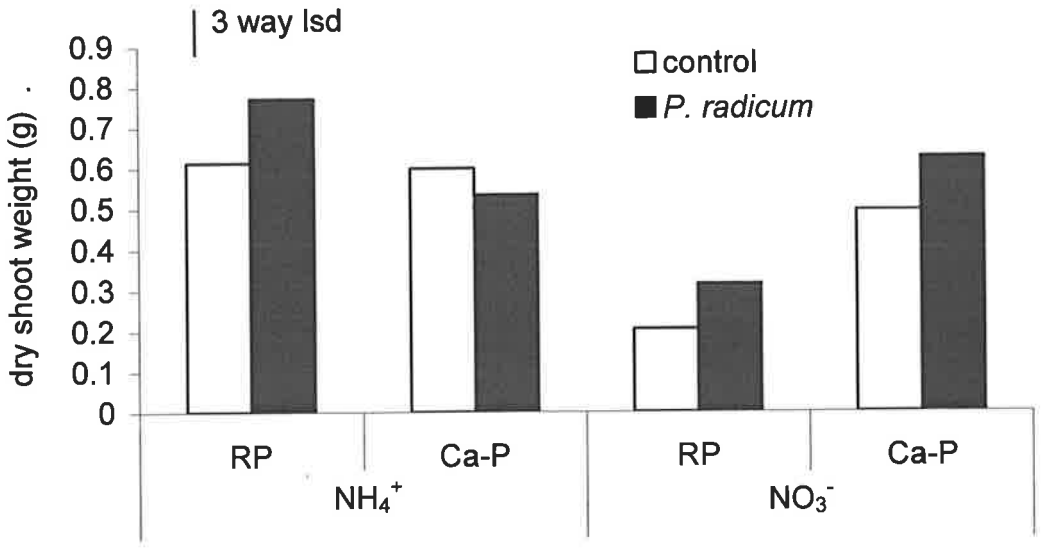


Figure 4.4(a)

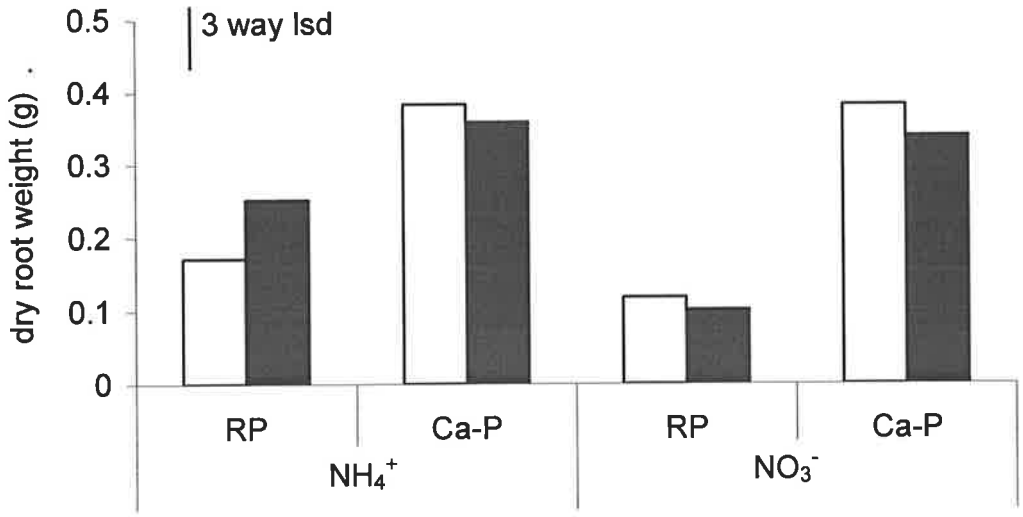


Figure 4.4(b)

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. Bars within the figure indicate 3-way l.s.d (α 0.05).

4.3.2 (ii) Phosphorus nutrition.

The P source present in the sand growth medium significantly affected plant P nutrition. The shoot concentration, plant uptake and specific root uptake of P from either RP or Ca-P was related to the relative solubilities of these two substrates (Figure 4.5). Plants grown with RP had lower concentrations of shoot P than plants grown with Ca-P.

The N-source also had a significant effect on P nutrition. Plants supplied with NH_4^+ were more efficient at accessing P than plants grown with NO_3^-. Plants supplied with the P source of lower solubility (RP) and NO_3^-, contained lower concentrations of P than plants supplied with NH_4^+ and Ca-P.

The effect of *P. radicum* on plant P nutrition was dependent on the N source. In the presence of NH_4^+, *P. radicum* increased the shoot P concentration and shoot P uptake. Similar trends were also seen with the specific P uptake. However, differences in the SPU were not statistically significant due to large errors. The error associated with the calculation of SPU is an accumulation of errors in P concentration, total shoot P uptake and root weight determination. With NO_3^- supply, *P. radicum* had no effect on P nutrition measured as either shoot P concentration, shoot P uptake or specific P uptake (Figure 4.5).

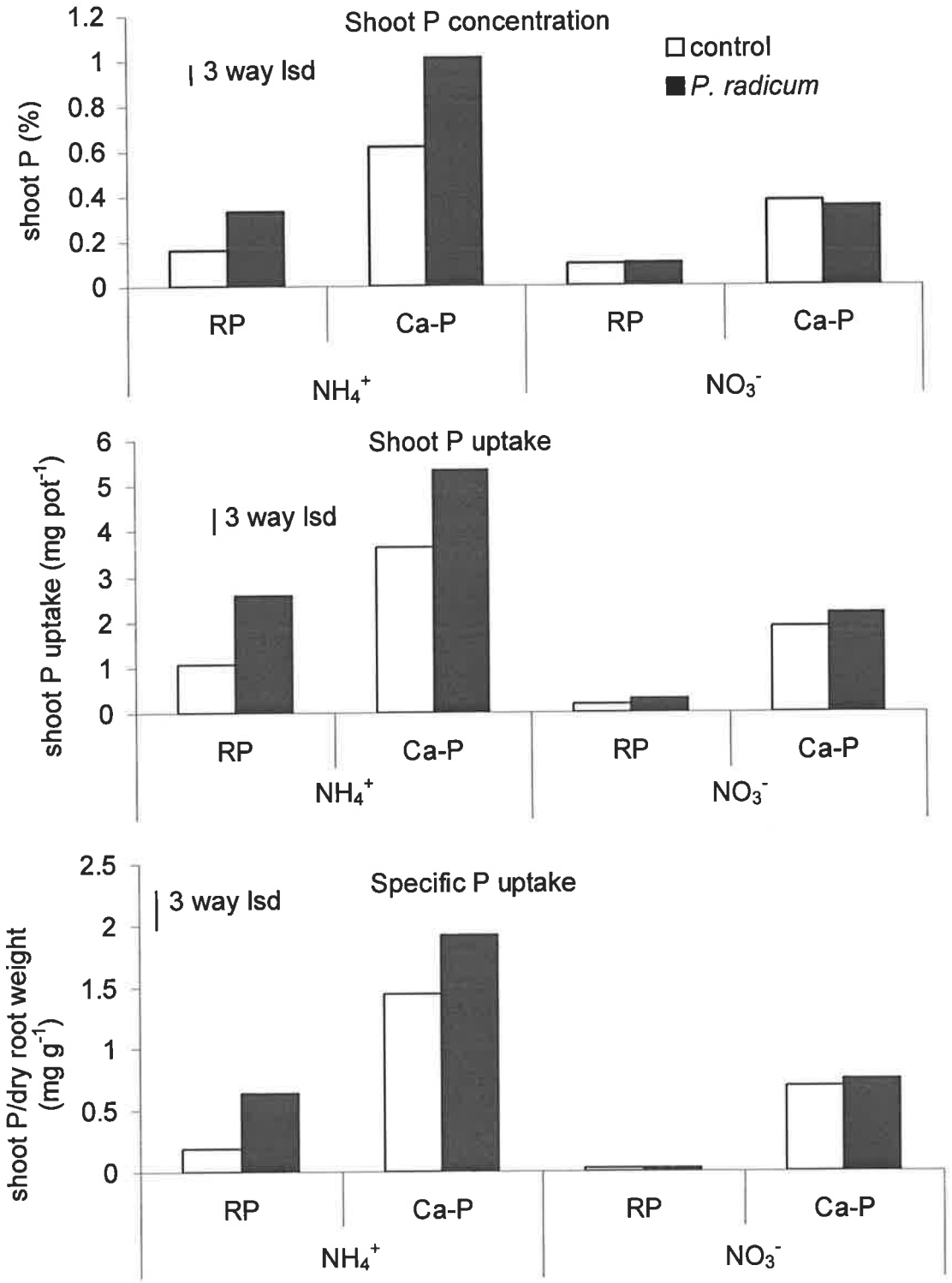


Figure 4.5 The effect of *P. radicum* on the P nutrition of plants grown with either RP (rock phosphate) or Ca-P ($CaHPO_4$) as the P source and supplied with either NH_4^+ or NO_3^- as the source of N. Bars within the figure indicate 3-way l.s.d (α 0.05).

4.3.3(iii) *Isolation of P. radicum from root sections*

Data on isolation of *P. radicum* from roots are given in Table 4.2. There was a high variability in the mean $\log_{10}$ cfu of *P. radicum* g fresh wt root^{-1} within each treatment group. The variability of *P. radicum* root isolations meant effects of either N-source or P-source on the levels of *P. radicum* root colonisation were not significant. However, there was an overall trend where the mean *P. radicum* populations increased as the root was sampled closer to the seed.

Table 4.2 Estimation of root colonisation by *P. radicum* as the mean $\log_{10}$ colony forming unit (cfu) g fresh root wt^{-1} at distances of either a (0-2 cm), b (5-7 cm) or c (10-12 cm) from the seed in the seminal roots 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 sources of N.

	$\log_{10}$ *P. radicum* cfu				
Distance from seed (cm)	NH_4^+		NO_3^-		
	RP	Ca-P	RP	Ca-P	s.e.
a (0-2)	3.12	4.28	3.31	3.32	0.62
b (5-7)	2.11	2.05	0.82	2.10	0.76
c (10-12)	2.01	1.66	0.50	1.84	0.79

4.4 Discussion

In these experiments, a sand culture assay was used to investigate the types of compounds that *P. radicum* could solubilise for improved plant P nutrition. These compounds were chosen to be representative of contrasting types of soil P retention. In addition, the sand culture assay was used to determine the effect of N-source on the plant P response and to inoculation. Data from the sand culture assay was used to distinguish plant growth promotion mechanisms from P solubilisation.

To eliminate any potential variability with introducing a rhizosphere inoculant as a seed coat and to ensure that *P. radicum* had come into contact with the roots (to encourage colonisation), the fungus was introduced to the experimental system as ryegrass propagules and mixed throughout the growth medium (Experiment 1). Whilst this is not the conventional procedure of inoculation in wheat production, the experimental system was designed to examine mechanisms of *P. radicum*-plant interaction. To accurately interpret experimental results, there was a requirement that the fungus was repeatably established onto the roots. Introducing *P. radicum* as ryegrass propagules distributed throughout the growth medium would have delivered an overall higher population of *P. radicum* to the experimental system and increased the chance that the fungus would colonise the roots and influence plant growth. In comparison, introducing the fungus as a seed coat may have required that the fungus grow and proliferate on the roots to ensure a plant growth response. Variability in the colonisation of the roots from the seed coat would have created anomalies in the data rendering interpretation more difficult. Having established that a plant growth response to *P. radicum* inoculation could be observed in the sand culture assay, the fungus was then introduced as a seed coat (in Experiment 2) to closer model the typical method of delivering the fungus in broadacre cereal production.

In Experiment 1, the P solubilising effect of *P. radicum* was investigated by growing plants on defined P sources. The plant uptake of these P sources gave information on the direct availability of these substrates to wheat plants. The availability of the substrates was similar to other studies where it was found that Fe-P, Ca-P, RP and P_o

had different mobilities that could be related to their solubility in water (Armstrong & Helyar, 1993; Yan et al., 1996).

The plants in experiment 1, were harvested at an early heading stage of growth to determine if the P solubilising effects were translated to near mature plants. In mature plants it was found that *P. radicum* significantly increased the P uptake from Ca-P and had little P solubilising effect on the P sources RP, Fe-P and P_o. However, for plants grown with Fe-P, growth promotion effects were independent of increases in P nutrition suggesting that growth promotion mechanisms other than P solubilisation were in operation.

Despite information that was obtained on the availability of P substrates and the ability of *P. radicum* to influence plant growth in the presence of these substrates, the experimental design may be criticised in that it lacked a 'no P' control and therefore the residual P present in the growth medium and seed could not be accounted for. Hence, any subtle effects that *P. radicum* may have had on the availability of the more insoluble substrates Fe-P and RP could have been masked. Inclusion of a 'no P' treatment in the experimental design would have allowed the degree to which *P. radicum* increased the availability of Ca-P to have been accurately determined.

Results from Chapter 3 showed that NH_4^+ was required by *P. radicum* for solubilisation of RP. However, it was not known if this mechanism would operate when *P. radicum* was growing in association with the roots and if it did, whether the

effect would improve plant P nutrition. Hence, in the second experiment the effect of N-source on plant P response to inoculation was investigated. In Experiment 2, plants were harvested relatively early (8 weeks) so that effects of treatments could be assessed at a stage when P uptake and accumulation were high relative to plant growth. In agreement with P solubilising activity in liquid culture observed in Chapter 3, it was found that *P. radicum* required NH_4^+ to significantly improve plant P nutrition. In the absence of NH_4^+, growth promotion was independent of P solubilisation. These results suggested that at least two mechanisms of plant growth promotion by *P. radicum* were in operation. One mechanism required NH_4^+ and increased the P nutrition of plants; the other mechanism was independent of changes in plant P nutrition.

The P solubilisation effect of *P. radicum* only occured with Ca-P type compounds, Ca-P and RP, and was absent with Fe-P and P_o. This pattern of P solubilisation is consistent with the findings of Whitelaw et al. (1999) who identitied that *in-vitro P. radicum* solubilised Ca-P compounds but did not significantly solubilise Fe-P and in the presence of NH_4^+, P solubilisation was increased.

To check that a plant growth response to inoculation was due to fungal effects, the roots were examined for *P. radicum* colonisation. Spatial variation in the populations of *P. radicum* associated with the roots suggested that the fungus had germinated from the inoculum source and proliferated on the wheat roots. The levels of *P. radicum* colonisation associated with the root were variable. Consequently, there was no observable effect of N or P source on the levels of root colonisation. The variable

nature of the root colonisation data is a likely reflection of the formation of clusters or discrete patches of sporulation by *P. radicum* along the root. Despite an overall variability, it was clear that *P. radicum* colonisation was highest near the seed. This pattern of root colonisation is typical for microorganisms introduced as a seed coating as in Experiment 2. For example, Ahmad & Baker (1987) found that when seeds were treated with conidia from strains of *Trichoderma harzianum,* the inoculated *T. harzianum* was only recovered in rhizosphere soil up to 3 cm from the seed. Certain benomyl-resistant mutants of *T. harzianum* strains were termed 'rhizosphere competent' if they had an increased capacity to colonise the rhizosphere and were found to colonise rhizosphere soil along the entire section of the root system. If seed coated fungi are able to colonise rhizosphere soil along the entire length of the root, they may be more effective than seed inoculants that just colonise the spermosphere as they are more likely to protect the entire seedling root system and solubilise P from a greater area of soil in comparison to just the seed.

In Experiment 2 growth promotion effects were well defined, while in Experiment 1 dry weight responses to inoculation were only marginal. As the plants in Experiment 1 were harvested at a growth stage that was close to maturity it was hypothesised that dry weight responses to *P. radicum* inoculation become lower as plants approached maturity. There is anecdotal evidence for a diminishing effect of *P. radicum* inoculation with plant maturity under field conditions (Peter Grieve, Bio-Care Technology, Pty Ltd., personal communication).

Another plant response to *P. radicum* inoculation was an increase in the SPU value, particularly for the Ca-P substrates (Experiment 1) and in the presence of NH_4^+ (Experiment 2). This may be the result of the P solubilising (acidifying) effect of *P. radicum*. Following acidification, the Ca-P would have been solubilised and the concentration of *ortho*-P available for root absorption was likely to be increased enabling the concentration of P absorbed per unit of root mass to increase. Increases in the SPU may have also been related to altered root morphology. However, further research is needed to identify the effects of *P. radicum* on root morphology.

Phytate was added to the growth medium in Experiment 1 to determine the ability of *P. radicum* to mineralise this major component of unavailable soil organic P (see Section 1.1.2). However, the use of sand culture to study the ability of *P. radicum* to mineralise P_o was not found to be appropriate. In sand culture phytate amendment supplied sufficient amounts of plant available P (evidenced by the high shoot P concentration and shoot P uptake) and plants would not have responded to any phytate mineralisation effects by *P. radicum*.

In this experiment it was suggested that the high availability of P from phytate is an artefact of sand culture as the addition of phytate to sand does not reflect the behaviour of phytate under soil conditions. As phytate is highly competitive with *ortho*- P for sorption sites in soils, it is the precipitation and sorption of phytate that determines its plant availability (McKercher & Anderson, 1989). In sand culture there are likely to be a much lower set of sorption sites in comparison to soil. Hence, in the sand culture

assay, phytate would have existed in a soluble from. In solution, phytate would have been available for dephosphorylation (to form *ortho*-P available for plant uptake) by phytase producing microorganisms in the sand growth medium.

Plants have a limited ability to utilise phytate as a source of P due to the low rate of excretion of phytase enzymes from the root into the rhizosphere (McLachlan, 1980; Asmar, 1997; Hayes et al., 2000). Hence, plants may have a reliance on microorganisms to mineralise phytate in the soil so that they are available for absorption by roots. Hence, further research should investigate the potential of microorganisms to increase the plant P nutrition from phytate. Under soil conditions the main processes increasing organic P for plant uptake is desorption or release from insoluble precipitates rather than susceptibility to phosphatases (Adams & Pate, 1992; George et al., 2002). This suggests that normal sorption-desorption and solubilisation reactions that are important parameters regulating the availability of *ortho*-P are also likely to be important for P_o. Hence, organic acids, in addition to phytase enzymes, may be important for the solubilisation/mineralisation of phytate. Investigations to determine the ability of *P. radicum* to mineralise phytate for plant uptake should be performed in soil. Using this approach, the precipitation and sorption of phytate will be more accurately modelled and provide a closer representation of the performance of a *P. radicum* inoculant under field conditions.

The P solubilisation mechanism of *P. radicum* required the presence of NH_4^+ to be effective. This activity is consistent with the proton extrusion mechanism of microbial

P solubilisation proposed by Illmer & Schinner (1995). The effectiveness of this mechanism has been criticised as inoperable under buffered soil conditions (Tinker, 1984; Gyaneshwar et al., 1998) and it is unlikely that P solubilising microorganisms will affect the pH of the bulk soil. However, it has been suggested that P solubilising microorganisms may create temporary microsites of P solubilisation that may exist long enough to increase plant P uptake (Kucey et al., 1989). The persistence and extent of these microsites is likely to be related to soil buffering capacity. Hence, soil buffering is likely to be a key component of the effectiveness of P solubilising microorganisms to successfully solubilise P for plant uptake. This was demonstrated in Chapter 3 and in previous studies where it was shown that P solubilisation effects were lost when activity of P solubilising microorganisms were examined in buffered medium (Gyaneshwar et al., 1998; Gyaneshwar et al., 2002). While the impact of *P. radicum* on the rhizosphere pH is unknown, research performed to date suggests that a number of constraints on the ability of *P. radicum* to acidify the rhizosphere are likely to exist. For example. *P. radicum* lost its ability to solubilise RP *in-vitro* under buffered conditions (see Chapter 3). In addition, available NH_4^+ would need to be present in sufficient concentrations in the rhizosphere for effective P solubilisation.

If the uptake of NH_4^+ and the subsequent export of H^+ leading to acidification is the principal mechanism of P solubilisation it is expected that the range of soil types in which *P. radicum* solubilises P is likely to be limited. Root zone acidification would be an effective mechanism of P solubilisation under alkaline soil conditions and limited by the buffering capacity of the soil. Under acid soil conditions, further acidification

may have little or no effect on P availability. Surprisingly, inoculation with *P. radicum* in the field has been met with most success under acid soil conditions (see Section 1.4.2). As it is unlikely that precipitated forms of Ca-P exist in acid soil conditions, these compounds are unlikely to contribute to any P solubilisation effect by *P. radicum* when inoculated into acid soils. This suggests that mechanisms other than P solubilisation are in operation.

For most trials, *P. radicum* performance under field conditions has not been subject to scientific review. Hence, there is still some doubt as to whether *P. radicum* can solubilise P under alkaline conditions. For example, when *P. radicum* was evaluated under alkaline calacareous soil conditions in the Eyre peninsula, SA, the mean yield was increased 25% for plants that received *P. radicum*. Despite the apparently large mean yield increase, the yield response to inoculation was reported as not statistically significant (Alison Frischke, Eyre Peninsula Farming Systems 2002 Summary, Minnipa Agricultural Centre, SARDI).

This study has demonstrated that *P. radicum* could contribute to acidification effects of wheat roots in the presence of NH_4^+. Further work should investigate the operation of this mechanism under soil conditions and the use of ammonium fertilizer strategies to increase P solubilising effectiveness of *P. radicum* inoculation. The NH_4^+ mediated P solubilising effect may assist in increasing the efficiency of P fertiliser application, particularly where *P. radicum* is applied with an ammoniated phosphatic fertiliser as the acidification effects of the fungus could assist in keeping the P fertiliser in the soil

solution. However, further research is required to identify the compatibility of *P. radicum* with NH_4^+ fertiliser application under field conditions.

To some extent the growth response of *P. radicum* inoculation with NH_4^+ fertilisation has been assessed. In a trial performed on the Eyre Peninsula, South Australia, the potential of *P. radicum* in combination with diammonium phosphate (DAP) fertilisation to increase the yield of wheat grown in alkaline calcareous soil was evaluated (Peter Hitchcock, Nathan Hitchcock & Neil Cordon, Eyre Peninsula Farming Systems 2002 Summary, Minnipa Agricultural Centre, SARDI). In comparison to untreated wheat, *P. radicum* inoculation did not increase yield and when *P. radicum* was applied in combination with DAP there was no significant increase in yield in comparison to plants that received DAP alone. However, the bias of the results is unknown as the trial was performed in unreplicated strips. In addition, the yield of the tested wheat was well below the potential suggesting that other factors (most likely limitation of soil moisture) were constraining plant growth. Given that a higher response was observed in younger plants in sand culture experiments reported here, it is possible that a seedling growth response diminished as the plants matured and became limited by water. Had conditions been more conducive to support the increased demand of plants stimulated by an inoculant, an increased yield may have been observed in *P. radicum* treated strips.

In summary, growing plants in a clean sand culture is a simplification of field conditions, but this study has provided data on the mechanisms of plant growth

promotion by *P. radicum*. Results suggest that proton cxtrusion under NH_4^+ nutrition is an important component of P solubilisation by *P. radicum* and that P solubilising mechanisms of *P. radicum* were reduced in the absence of NH_4^+. In addition, it was identified that there was a general plant growth promotion mechanism that was independent of P solubilisation. This suggests that under field conditions where the plant is P deficient and NH_4^+ availability is low, the P solubilising activity of *P. radicum* is also likely to be low and the general plant growth promotion mechanism may become a more important means for *P. radicum* to influence plant growth.

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 *P. RADICUM*?

5.1 Introduction

In most cases the P solubilising activity of a microorganism is determined in synthetic medium and measured as the release of *ortho*-phosphate from insoluble sources of P such as rock phosphate or calcium, aluminium or iron phosphate sources. As synthetic media and laboratory conditions do not effectively mimic rhizosphere/soil conditions, it is not surprising that in many cases inoculation with P solubilising microorganisms has not affected plant growth or P uptake. Essential proof of microbial P solubilisation would be the finding of clear and consistent differences in the *L*-value (defined as: the ratio of the amount of ^{32}P added to the soil to the specific activity of ^{32}P in the plant tissue, see Section 1.2.3) of soil grown plants.

In Chapter 4 it was determined using sand culture that *P. radicum* did not necessarily influence plant growth by P solubilisation. In this regard, sand culture was found to be a useful tool to identify mechanisms of plant growth promotion. However, sand culture cannot fully reflect the complexity of the rhizosphere of field grown wheat. To more closely ascertain the P solubilising activity of *P. radicum* under field conditions, the effect of *P. radicum* on the *L*-value of soil grown wheat was determined.

Where the effect of microbial P solubilisation is determined using isotopic dilution techniques, it is possible to determine if increased plant growth and P uptake is due to a

solubilisation mechanism (i.e changes in the *L*-valuc) or an indirect effect of general growth promotion (*L*-value unaffected).

The experimental objective of this Chapter was to:

- determine if *P. radicum* affected the *L*-value of wheat seedlings grown in soil and hence to determine if P solubilisation acts as a mechanism of plant growth promotion in soil.

5.2 Methods

5.2.1 Seed preparation

Wheat seeds (*Triticum aestivum* L. cv. Krichauff) were prepared and inoculated as outlined in section 2.1.2. The final inoculum density of *P. radicum* on the seeds was 1.35×10^6 cfu $seed^{-1}$. Control seeds were treated with tween 20.

5.2.2 Soils.

The soils used in this experiment were from Mingenew, Innisfail, Mt. Schank and Minnipa. Soil properties are outlined in section 2.5.

5.2.3 L-*value determination*

5.2.3 (i) Soil preparation, plant growth and analysis

252 g of each soil was weighed into plastic bags. Each bag of air dry soil was then spiked with carrier-free $KH_2{}^{32}PO_4$. The $KH_2{}^{32}PO_4$ was added to the soil by spraying 20 mL of a stock solution of $KH_2{}^{32}PO_4$ diluted with deionised water. During spraying, the soil in each bag was mixed to evenly distribute the $KH_2{}^{32}PO_4$. A volume of 7 mL of nutrient solution containing (per litre of deionised water) 17.43 g NH_4NO_3, 1.92 g

$MgSO_4.7H_2O$, 0.663 g $MnCl_2.4H_2O$, 0.123 g $ZnSO_4.7H_2O$, 0.0624 g H_3BO_4, and 0.1014 g $CuSO_4.5H_2O$ was added to each bag. Deionised water was also added to bring each soil to 80% water holding capacity. The soil from each plastic bag was used to fill one non-draining plastic pot (300 mL, 6.5 cm diameter x 10 cm high). To determine the level and homogeneity of ^{32}P spiking, 0.5 g of soil was sampled from each pot, digested (conc. nitric acid) and analysed using the procedure outlined in Section 2.2.2. The mean ^{32}P activity in the pots at planting was 1110 ± 427 kBq kg^{-1} soil. The plastic containers were sown with four wheat seeds and thinned to two seedlings per pot after emergence. The seedlings were maintained at 80% water holding capacity with deionised water for a period of four to five weeks in a controlled environment chamber (16/21 °C, 8/16 h dark/light cycle). At harvesting the seedling roots were rinsed from the soil and separated from the shoots. Shoot and root dry weight and P tissue analysis was performed as outlined in Section 2.2.2.

The dried plant material and seeds of the same weight as those sown were analysed for P content using the procedure described in Section 2.2.2. The radioactivity in plant and soil digests was determined by scintillation counting using a Wallac Winspectral α\β Liquid scintillation counter controlled by Winspectral software.

The effect of *P. radicum* on the P taken up by the plants was determined according to Larsen (1952). The specific activity of the plant (SpA) was calculated as:

$$\text{SpA (kBq mg}^{-1}) = \frac{\text{Plant Activity (kBq)}}{[P_{\text{shoot uptake}}\text{ (mg)} - P_{\text{seed}}\text{ (mg)}]}$$

The *L*-value was calculated as:

$$L\ (\text{mg kg}^{-1}) = \frac{\text{R (kBq kg}^{-1})}{\text{SpA (kBq mg}^{-1})}$$

Where R is the radioactivity introduced into the soil.

The *L*-value was analysed using a three-way ANOVA across soil type, irradiation (see 5.2.3 iii) and fungal inoculant using Genstat 5th edition. While there was not always a significant 3-way interaction, mean data from treatment groups is displayed (with standard errors) to illustrate data trends.

5.2.3 (ii) Experiment 1

Two *L*-value experiments were performed as part of this Chapter. In Experiment 1, the soils Innisfail, Mt Schank and Minnipa were labelled and prepared as described in Section 5.2.3(i). Pots filled with ^{32}P labelled soil were sown with either control or treated wheat seeds at a replication of five pots each. Plants were harvested 5 weeks after sowing and analysed for shoot dry weight, P content and specific ^{32}P activity as described in Section 5.2.3(i).

5.2.3 (iii) Experiment 2

In Experiment 2, Mingenew, Innisfail, Mt Schank and Minnipa soils were sterilised by γ-irradiation (2.4 MBq, Steritech Pty Ltd, Australia). To determine if indigenous microflora influenced the plant growth response to *P. radicum* the irradiated soil was spiked with a non-sterile sample of the original soil (10 g of non-sterile to 1 kg of γ-irradiated soil). Both irradiated and re-inoculated soils were moistened to 20% water

holding capacity and incubated for one week at 25 °C. Following sowing with either treated or control wheat seeds, plants were left to grow for a period of four weeks and analysed for shoot and root dry weight, P content and specific ^{32}P activity as described in Section 5.2.3(i).

5.2.4 Isolation of P. radicum *seed inoculant*

To determine if seed treatment had introduced detectable populations to the root system, *P. radicum* was isolated from the root system using semiselective medium DRBC agar as described in Section 2.1.3.

5.3. Results

5.3.1 Experiment 1

In Experiment 1 it was found that there was no statistically significant effect of *P. radicum* inoculation on plant growth (Table 5.1). Although there was a marginal dry shoot weight increase in the Innisfail and Mt. Schank soil due to inoculation, this was not significant (α 0.05). Tissue analysis indicated that inoculation had no effect on the shoot P concentration. Similarly, there was no effect of *P. radicum* inoculation on the *L*-value.

Table 5.1 The effect of *P. radicum* 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).

Soil	Dry shoot weight ($g\ pot^{-1}$)		Dry shoot P concentration (%)		*L*-value ($mg\ kg^{-1}$)	
	control	*P. radicum*	control	*P. radicum*	control	*P. radicum*
Innisfail	0.23 ± 0.01	0.26 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	36.9 ± 1.51	27.7 ± 0.92
Mt Schank	0.31 ± 0.01	0.33 ± 0.01	0.26 ± 0.01	0.26 ± 0.01	49.4 ± 2.32	50.2 ± 3.01
Minnipa	0.27 ± 0.01	0.23 ± 0.02	0.26 ± 0.01	0.25 ± 0.01	23.9 ± 0.82	24.6 ± 0.72

5.3.2 Experiment 2

5.3.2 (i) Plant growth

When averaged across all soil types, *P. radicum* significantly increased shoot dry weight (0.15 g, mean shoot weight of control plants; 0.17 g, mean shoot weight of *P. radicum* inoculated plants). However, there was no interaction between *P. radicum* and soil type or re-inoculation of soils i.e. *P. radicum* had no significant effect on each individual soil type and there was no effect on the plant response to *P. radicum* by re-inoculating γ-irradiated soils with non-sterile soil. Despite only marginal differences,

means for each of the treatment groups is given in Figure 5.1 to illustrate trends. The greatest increase in shoot dry weight was seen in Minnipa and Mt Schank soils.

For root weight (Figure 5.2), when data across all soil types and irradiation treatments was pooled, *P. radicum* showed a significant increase in dry root weight. The greatest effect of *P. radicum* inoculation on root weight was seen in Minnipa soils. There was no significant effect on *P. radicum* inoculation by re-inoculating sterile soils with non sterile samples.

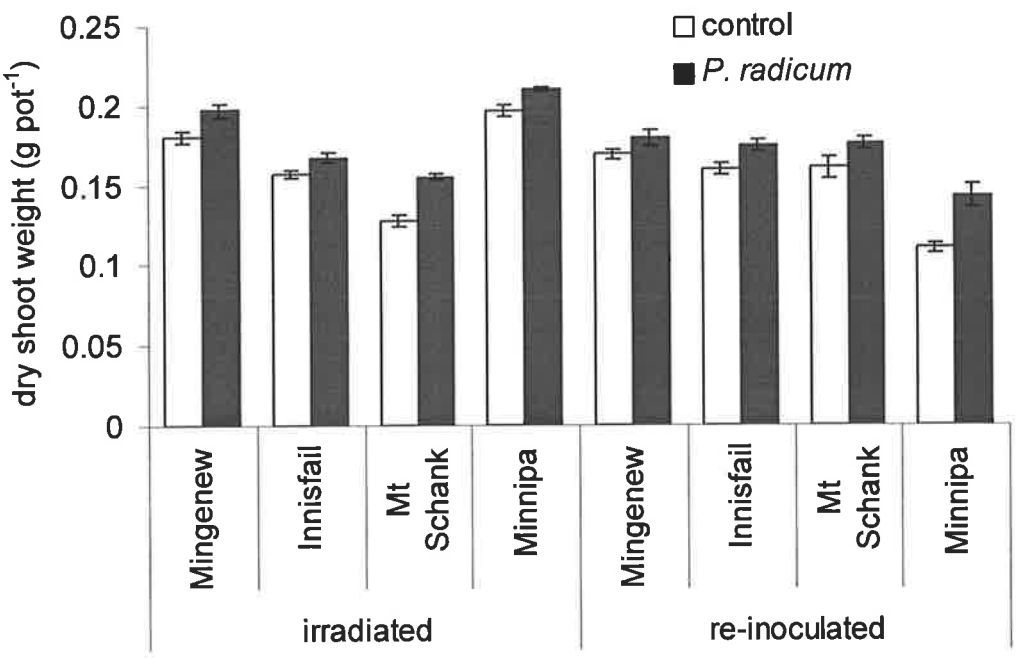


Figure 5.1 The effect of *P. radicum* inoculation on dry shoot weight of wheat seedlings grown in four γ-irradiated soils with or without re-inoculation with non-sterile soil. Error bars indicate s.e. (n=5).

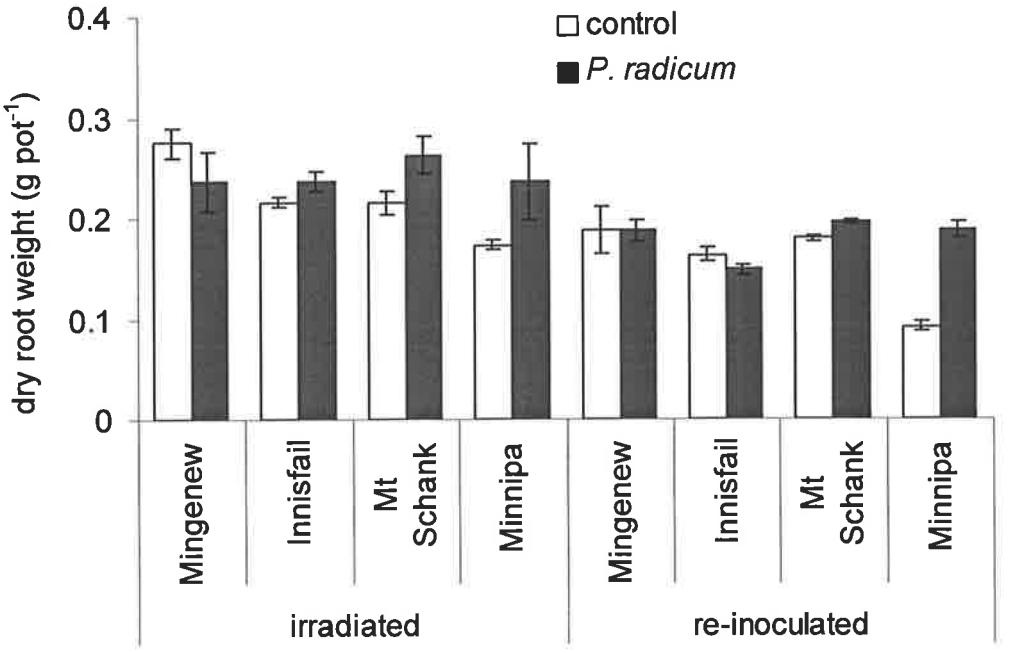


Figure 5.2 The effect of *P. radicum* inoculation on dry root weight of wheat seedlings grown in four γ-irradiated soils with or without re-inoculation with non-sterile soil. Error bars indicate s.e. (n=5).

5.3.2 (ii) P uptake

Shoot P concentration (% P) was significantly affected by soil type but was not significantly affected by *P. radicum* inoculation or re-inoculation with non-sterile soil. The effect of soil type on shoot P concentration is illustrated in Figure 5.3. Wheat seedlings grown in Innisfail soil had the highest P concentration (0.33%), seedlings grown in Mt. Schank and Minnipa soils were similar (0.24%), while plants grown in Mingenew soils had the lowest P concentration of 0.19%.

Shoot P uptake (mg P pot^{-1}) varied significantly between the four soils (Figure 5.4). The mean P uptake of control seedlings grown in Innisfail soil was 0.53 mg, while for Minnipa it was 0.40 mg, Mt Schank 0.37 mg and Mingenew 0.34 mg. At the 10% level there was a significant one-way interaction for *P. radicum* inoculation (P=0.055). However, two way (*P. radicum* x soil type, *P. radicum* x soil re-inoculation) and three-way interactions were not statistically significant. The largest differences in mean P uptake values between *P. radicum* and control plants were found for plants grown in Mt Schank irradiated soil and Minnipa re-inoculated soil, for other soil groups the differences in the mean values were much less. All differences between *P. radicum* and control plants for P nutrition data failed on significance tests (P = 0.05).

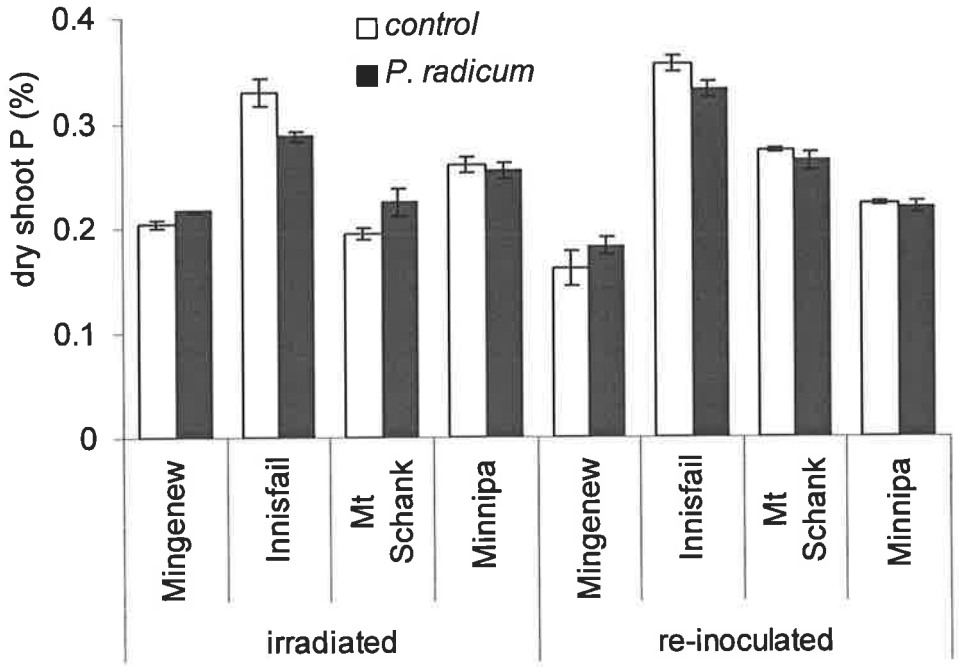


Figure 5.3 The effect of *P. radicum* on shoot P concentration of wheat seedlings grown in γ-irradiated soil with or without re-inoculation with 10% non-sterile soil to re-introduce indigenous microflora. Error bars indicate s.e. (n=5).

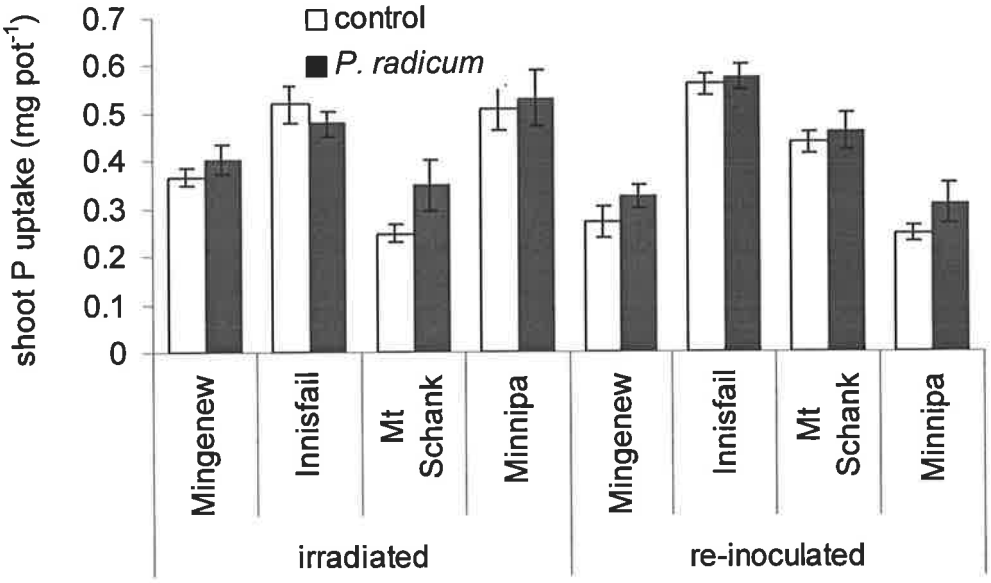


Figure 5.4 The effect of *P. radicum* on shoot P uptake of wheat seedlings grown in γ-irradiated soil with or without re-inoculation with 10% non-sterile soil to re-introduce indigenous microflora. Error bars indicate s.e. (n=5).

5.3.3 (iii) L-values

As shown in Figure 5.5, the *L*-values were significantly different for the three soil types, but were not affected by *P. radicum* or re-inoculation with non-sterile soil. Mt Schank and Innisfail soils had similar *L*-values at 47 mg kg^{-1}, while Minnipa and Mingenew had lower *L*-values at 21 and 18 mg kg^{-1}, respectively. In this experiment *P. radicum* increased the *L*-value of Mt Schank soils by marginal amounts. This effect was not consistent between Experiment 1 and Experiment 2.

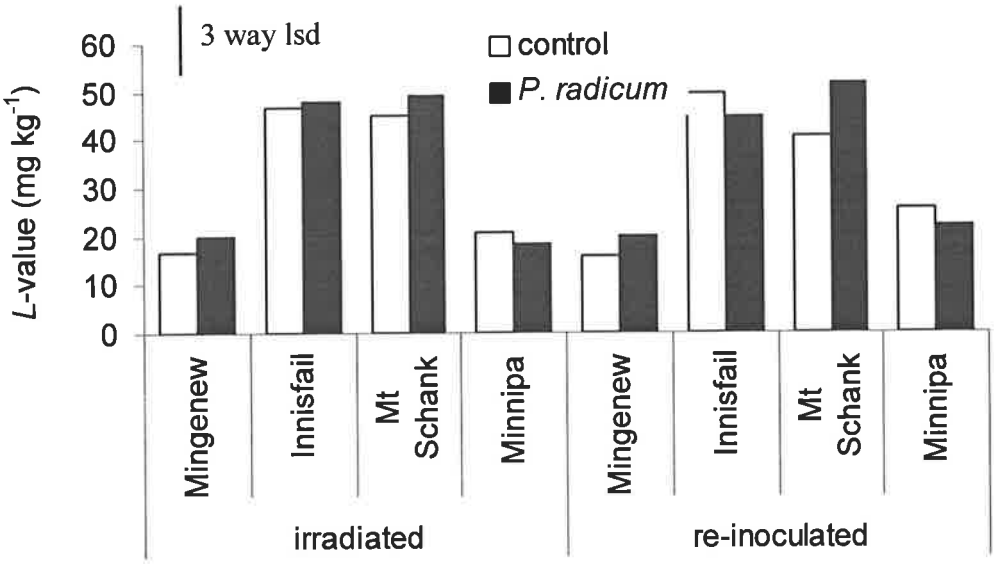


Figure 5.5 Mean *L*-values of wheat seed treated with *P. radicum* and grown in γ-irradiated soils with or without re-inoculation with 10% non-sterile soil. Bar within the figure indicates 3-way lsd (α 0.05)

5.3.4 (iv) Isolation of P. radicum *from roots*

To check for growth of the inoculant onto the root system, *P. radicum* was isolated from the root crown at a distance of 0 – 2 cm from the seed. There was no effect of either soil type or re-inoculation with non sterile soil on the level of *P. radicum* associated with this section of the root system (Table 5.2).

Table 5.2 Isolation of *P. radicum* 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.

Soil	γ-irradiated soil		re-inoculated soil	
	$\log_{10}$ cfu g fresh root^{-1}	s.e	$\log_{10}$ cfu g fresh root^{-1}	s.e
Mingenew	4.74	0.06	4.41	0.67
Innisfail	5.03	0.44	4.51	0.57
Mt Schank	3.85	0.81	4.46	0.33
Minnipa	4.51	0.35	3.53	0.49

5.4 Discussion

In Experiment 1, there was no significant effect of *P. radicum* on plant growth. For Experiment 2, *P. radicum* had a significant one-way effect on shoot and root dry weight (data pooled across soil type and soil re-inoculation treatments). However, there was no significant effect of *P. radicum* on the shoot P concentration and effects on shoot P uptake were only significant at $P = 0.1$. This result suggests that *P. radicum* did not significantly increase P nutrition and that the small increases in plant dry weight (growth promotion) were independent of increases in P nutrition. If *P. radicum* was able to solubilise soil P and hence increase plant P uptake, then an increase in the *L*-value should have occurred. This was not seen and such a result suggests that P solubilisation does not account for the growth response observed following the introduction of *P. radicum* under previous greenhouse and field trials (Whitelaw et al. 1997).

To determine if the lack of a substantial plant growth response to *P. radicum* inoculation was due to a failure to establish the inoculant on the roots, the inoculant (*P. radicum*) was re-isolated from the root crown at harvest. In all cases, *P. radicum* was successfully re-isolated from the roots in reasonable densities (approximately 4 $\log_{10}$ cfu g frt wt^{-1}). This indicated that inoculation had successfully introduced *P. radicum* to the rhizosphere where it had persisted over the plant growth period. This finding suggests that a lack of effect in inoculated treatments was related to the inability of the fungus to influence plant growth and not a failure of the inoculation procedure to introduce *P. radicum* to the root system.

The four soils chosen in this experiment are all of low P availability and had contrasting mechanisms of P retention. This is reflected in the chemical characteristics of these soils (see Table 2.4). The ability of *P. radicum* to affect the *L*-value for plants grown in the different soils would be a reflection of the mechanisms that *P. radicum* uses to increase bioavailable P in the soil for plant uptake. In the Minnipa soils, the predominant mechanism of P retention is the precipitation of Ca-P type compounds. The solubilisation of P in this soil would occur through a localised acidification in addition to Ca chelation by organic anions. While *P. radicum* could solubilise calcium phosphate compounds in solution culture (Chapter 3) and sand culture (Chapter 4), this effect was not transferred to alkaline soil conditions, i.e there was no change in the *L*-value of wheat seedling grown in Minnipa soils. The inability of *P. radicum* to solubilise P in Minnipa soil was probably related to the high buffering capacity of the Minnipa soil, which contains approximately 38% CO_3^- (see Chapter 3). Secondly, the high Ca content of the soil would immediately precipitate any soluble PO_4^{3-} that *P. radicum* may have been able to solubilise.

The P fixation in Mt Schank is governed by the adsorption and precipitation of P with Al oxyhydroxide compounds. Despite previous indications (see Whitelaw et al., 1999) that Al-chelation from amorphous Al-P compounds may have a minor contribution to P solubilisation, there was no significant increase in the *L*-value in these soils.

In Innisfail soils, P fixation is dominated by reaction with Fe based compounds. Hence, if *P. radicum* could solubilise Fe-P compounds this would be reflected in the *L*-values.

There was no effect on the *L*-values of wheat seedlings and it appears that *P. radicum* did not solubilise Fe-P compounds in this soil.

The Mingenew soil was included as, under field conditions, *P. radicum* has been reported to increase the growth and yield of wheat in this soil (see Section 1.6). Hence, this soil was chosen to determine if P solubilisation was responsible for the reported increase in yield. The results here suggest that *P. radicum* did not increase the fraction of soil P available for plant uptake in Mingenew soils and therefore other mechanisms may have been involved in plant growth promotion.

P. radicum could increase the P nutrition of plants without an effect on the *L*-value. Such an effect would occur if P absorption was enhanced by either (1) an alteration in root morphology such as increased root hair production that would increase the specific P uptake of the root ie. the amount of P that is able to be absorbed per unit of root weight, or (2) if the rate of P diffusion to the root was increased to allow a larger uptake rate or possibly uptake of P from a larger volume of soil (Tinker, 1980).

The influence of root hairs on P uptake has been previously discussed in this thesis (see Section 1.5.3). The rate of P diffusion to the root could be increased by the secretion of organic anions that form complexes that keep P in solution (Gardner et al., 1983). For *P. radicum*, the only organic anion that is present in sufficient concentration to perform such a function is gluconate. To effectively increase the rate of P diffusion to the plant, gluconate would need to diffuse away from the point of secretion (presumably from hyphae near the surface of the root), and allow P to remain in solution such that it can

diffuse to the root. While the fate of gluconate in these soils was not experimentally investigated, it appears that gluconate would be unlikely to perform such a function, due to two reasons. Firstly, gluconate is readily biodegradable. As mentioned by Whitelaw et al., (1999) gluconate is likely to be rapidly degraded when released into the rhizosphere. Secondly, gluconate has a low chelating ability (Moghimi & Tate, 1978) and is therefore unlikely to form complexes that are of sufficient stability to remain intact for any period to influence P availability.

In Chapters 3 & 4, it was identified that *P. radicum* increased plant P uptake from Ca-P and RP when also supplied with NH_4^+ under sand culture conditions. Further experiments could use ^{32}P labelled Ca-P and RP substrates added to soil to determine if *P. radicum* increases the plant P uptake from these substrates under soil conditions. When plants are grown in irradiated soil the addition of these substrates could be used to identify if *P. radicum* increases the plant uptake from these sources within the complex physical soil environment. The addition of non-sterile soil to re-introduce microflora could then be used to determine if the presence of microflora removes the influence of *P. radicum* on P nutrition of either Ca-P or RP under non-sterile soil conditions. Information produced from these proposed experiments would be of greater value to *P. radicum* inoculant use under field soil conditions in comparison to sand culture data.

In summary, the results that *P. radicum* had no effect on the P concentration, shoot P uptake, or *L*-values indicates that *P. radicum* had no effect on either the P absorption or

the availability of P in the soil. In Experiment 2, for all of the soils tcsted there were slight increases in the seedling shoot dry weight that were not associated with increased plant P nutrition. It is therefore suggested that *P. radicum* increased seedling mass due to mechanisms that were independent of P solubilisation.

These results justify an approach to define other mechanisms of growth promotion by *P. radicum* that are not related to P solubilisation. Hence, the ability of *P. radicum* to produce the plant growth regulator indole-3-acetic (IAA) acid was investigated (Chapter 6). While IAA may increase root hairs and influence root growth, it may also be related to a direct plant growth promotion effect. The ability of *P. radicum* to suppress root pathogens was also investigated as an additional mechanism of plant growth that was not related to P solubilisation (see Chapters 7 and 8).

CHAPTER 6. EVIDENCE FOR PRODUCTION OF AUXIN BY *PENICILLIUM RADICUM*

6.1 Introduction

Although *P. radicum* did solubilise P in pure culture, mechanisms of growth promotion utilised by this fungus are not necessarily related to P solubilisation. Wheat seedlings grown in field soils had marginal increases in the shoot weight, without a concomitant change in the *L*-value or P status in response to *P. radicum* inoculation (Chapter 5). In a sand culture assay where the only source of P was insoluble (RP and Fe-P), *P. radicum* increased the shoot weight but had no effect on the P status of the plants (Chapter 4). Taken together these results suggest that a plant growth promotion mechanism other than P solubilisation was in operation.

Auxin production is a common feature of root-associated microorganisms and is frequently related to the ability of rhizosphere microorganisms to influence plant growth (see Section 1.5). For most cases, rhizosphere microorganisms require the presence of a precursor, tryptophan (TRP), to synthesise detectable quantities of IAA (see Section 1.5.4). The purpose of the experiments reported in this Chapter was to determine if *P. radicum* produces auxin with supply of the precursor TRP.

The experimental aim of the work reported in this Chapter was:

- to determine if *P. radicum* produces auxin-like activity (IAA) *in-vitro* and to validate production and the chemical identity of IAA *in-vitro* using analytical techniques.

6.2 Methods

6.2.1 P. radicum *culture, extraction and thin layer chromatography*

P. radicum was inoculated in 500 mL of malt extract broth (MEB) that contained (per litre of deionised water): 20 g malt extract (Oxoid), 5 g peptone (Bacto). The effect of TRP addition on indole production was investigated by adding 30 mg L^{-1} TRP to the MEB. Control treatments contained no TRP. The MEB (+,- TRP addition) was inoculated by adding a 5 mm agar plug taken from a *P. radicum* colony actively growing on MEA. The inoculated MEB was incubated at 25 °C on a rotating shaker (180 rpm) for a period of five days. On selected days of *P. radicum* incubation in MEB, 50 mL aliquots were withdrawn, centrifuged and the supernatant acidified to pH 4 with 1 M HCl to protonate any IAA present (pK_a IAA, 4.27). The acidified aliquot was extracted using two 20 mL volumes of diethyl ether (Merck) stabilised with 0.2% (w/v) of the antioxidant, *tert*-butylated hydroxytoluene (BHT), to minimise the oxidative degradation of IAA. The diethyl ether extracts were dried under vacuum at 40 °C and the residue was made up to 2 mL using pure methanol (BDH). The methanol suspensions were analysed using thin-layer chromatography (TLC). Approximately 25 µL of the methanol suspension was spotted onto 0.2 cm silica gel G plates (Merck), dried at 40 °C for twenty minutes and developed in chloroform : ethanol (80 : 20) to a height of 12 cm above the spotting line (approximately 1 hour). After drying the silica gel plates at 60 °C for one minute, they were visualised for indole compounds with Salkowski reagent (Kaldewey 1968, 163 mL of reagent was prepared by adding 3 mL of 1.5 M aqueous $FeCl_3$ to 100 mL of water and 60 mL of 10 M H_2SO_4). The zones of Salkowski reaction detected in *P. radicum* culture were compared to 10 µL spots of

standard indole compounds (100 mM) tryptophan (TRP), tryptamine (TAM), indole-3-acetic acid (IAA), indole-3-carboxylic acid (ICA) and tryptophan-3-ethanol (TOL).

6.2.2 Avena *coleoptile straight growth assay*

Acidified diethyl ether extracts of 5 day old cultures of *P. radicum* grown in MEB were spotted onto silica gel plates (10 cm wide x 12 cm height) and eluted in 80:20 $CHCl_3$: EtOH solvent (as described in Section 6.2.1). The silica gel plates were divided into ten equal sections representing units of retention factor (R_f). Each R_f section was then assayed for auxin-like activity using the *Avena* coleoptile straight growth assay (Bentley, 1962).

Coleoptiles used in the straight growth assay were generated by placing *Avena* caryopses on moistened paper towel and covering with several strips of paper towel that were moistened. The paper towel was moistened daily. After incubating for one week in the dark at 25 °C, the coleoptiles were detached from the seed using a sterile scalpel. A total of five coleoptiles, each 40 mm long, were placed into sterile 4.5 cm diameter Petri dishes with the R_f sections of silica gel plate that had been developed with *P. radicum* culture supernatant. To each Petri dish was added 5 mL of sterile 4% sucrose citrate-phosphate buffer (SCP) that was adjusted to pH 5. The coleoptiles were incubated for 36 hours in the dark at 25 °C. The growth of the coleoptiles immersed in *P. radicum* culture supernatant was compared to the growth of control coleoptiles grown in sterile SCP. The coleoptiles were then individually measured and elongation expressed as percentage extension in buffer according to the following equation:

% coleoptile extension =

$$\frac{(\text{coleoptile length after incubation}) - (\text{original coleoptile length, 40mm})}{(\text{original coleoptile length, 40 mm})} \times 100$$

6.2.3 Generation of diazomethane

Analysis of samples by ELISA (Section 6.2.4) required methylation as pretreatment. Indole standards and extracts of *P. radicum* culture were methylated using diazomethane. Diazomethane (CH_2N_2) was generated as an ethereal solution by collecting distillate under a constant stream of N_2 from the reaction of alkali with the precursor Diazald^{TM} (Sigma). The reaction has been described previously by Vogel (1956). The alkali solution was prepared by mixing 10 mL of ethanol, 2 g of KOH and 3.2 mL of Milli-Q water and reacting with 2.14 g of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald^{TM}) in 13.0 mL of diethyl ether. Derivatisation of samples was performed within four days of generation of the ethereal diazomethane according to methods described by Vogel (1956).

6.2.4 Enzyme linked immunosorbent assay (ELISA).

Two agar plugs (10 mm diameter) of 5 day old cultures of *P. radicum* growing on MEA were used to inoculate 500 mL of both MEB and a glucose minimal salts (GMS) fungal growth medium that consisted of (per litre of deionised water): 7 g glucose, 1 g NH_4NO_3, 1 g K_2HPO_4, 0.5 $MgSO_4$ 0.2 g KCl, 0.1 g NaCl, 0.01 g $MnSO_4$ and 0.01 g $FeSO_4$. The inoculated liquid medium was incubated in darkness with stirring (280 rpm) for 5 days at 25 °C. At this time, 50 mL acidified aliquots were withdrawn, extracted with 2 x 20 mL volumes of acidified diethyl ether (pH 4) and methylated according to procedures described in section 6.2.3. Quantification of IAA present in

derivatised samples was determined using competitive ELISA that was purchased in the kit form (Agdia, Elkhardt Industries, USA). The kit was used according to the manufacturers instructions. The monoclonal antibody in this kit is documented to show high specificity to the methylated form of IAA (Mertens et al., 1985). The ELISA procedure will be briefly described here, for specific technical and experimental details, the reader is referred to Weiler et al. (1981).

The immunoassay uses anti-IAA immunoglobulins that are adsorbed to the surface of polystyrene microtitre 96 well plates. In non-specific terms, the procedure requires that the sample containing an unknown quantity of IAA to be added to the microwell. The IAA present in the sample then binds to the surface adsorbed immunoglobulins. After rinsing the, IAA coupled (covalently linked) to alkaline phosphatase is added to the microwell and binds to the remaining unreacted surface adsorbed immunoglobulins. After rinsing, alkaline phosphatase substrate (*para*-nitrophenol phosphate) is added. After incubation, the enzyme catalysed development of colour is measured using a spectrophotometer.

The quantitative determination of IAA using the ELISA is based on the rclationship between the enzyme activity for incubations without added IAA and that of similar incubations with increasing concentrations of a standard IAA solution. The relationship is represented by the formula:

$$\frac{A_B - A_{UB}}{A_{B_0} - A_{UB}} \times 100 = \%\left(B/B_0\right)$$

Where A_B is the absorbance of a known standard; A_{UB} is the absorbance in the presence of a large excess of standard IAA; B is binding of the sample or standard and; B_0 is the maximum enzyme activity determined by the addition of excess conjugated IAA alkaline phosphatase in the absence of standard IAA. A linear standard curve can then be obtained by a log transformation of the equation to obtain:

$$\text{logit}\,\%\left(B/B_0\right) = \ln \frac{B/B_0}{100 - B/B_0}$$

6.3 Results

6.3.1 Thin-layer chromatography

Salkowski reactive zones of *P. radicum* culture filtrate were compared to those of standard indole compounds. Using this approach it was found that *P. radicum* culture extract did not contain detectable quantities of compounds that had a similar identity to either tryptophol (TOL) or indole-3-carboxylic acid (ICA, see Figure 6.1). While the polar compounds tryptamine (TAM) and tryptophan (TRP), have a distinctive reaction with Salkowski reagent (golden yellow), they could not be resolved from other components of the malt extract growth medium that produced a dark brown reaction with Salkowski reagent.

When *P. radicum* was cultured in MEB amended with TRP, a faint pink band with a similar R_f to IAA was detected after 5 days of incubation (see Figure 6.1a). When *P. radicum* was cultured in MEB with no TRP addition, there was no detectable compound that had a similar R_f or colour reaction to Salkowski reaction as IAA (Figure 6.1b). The *Avena* straight growth assay was used to determine if compounds produced by *P. radicum* that had a chromatographically close identity to IAA were also biologically active with auxin-like activity.

Figures 6.1 a and b (opposite page) Thin layer chromatograms 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 with 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).

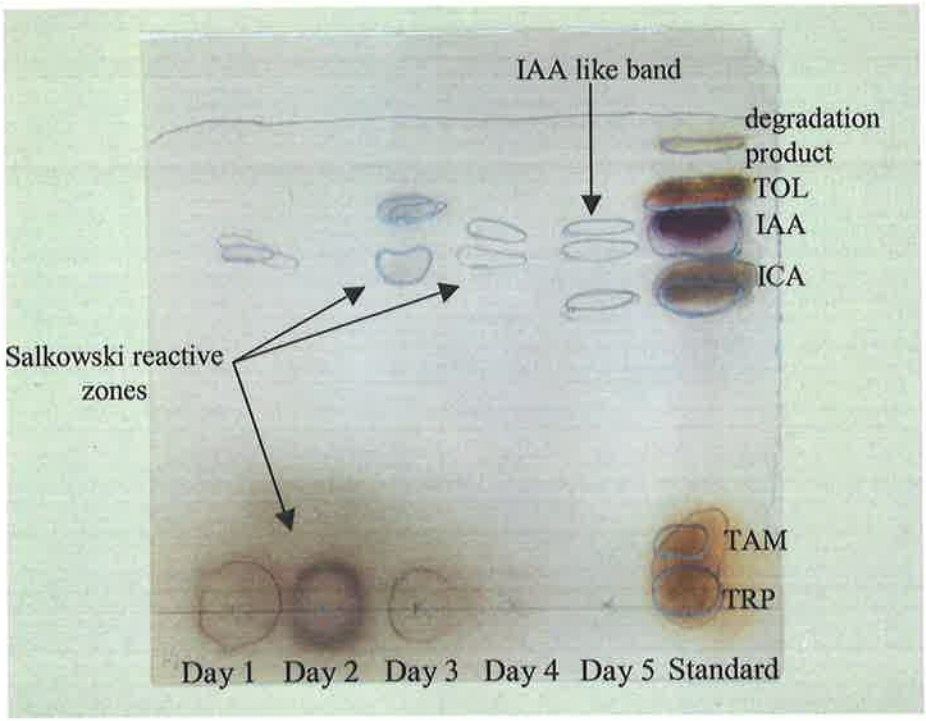


Figure 6.1a Production of Salkowski reactive compounds by *P. radicum* cultured in MEB with 30 mg L^{-1} TRP amendment.

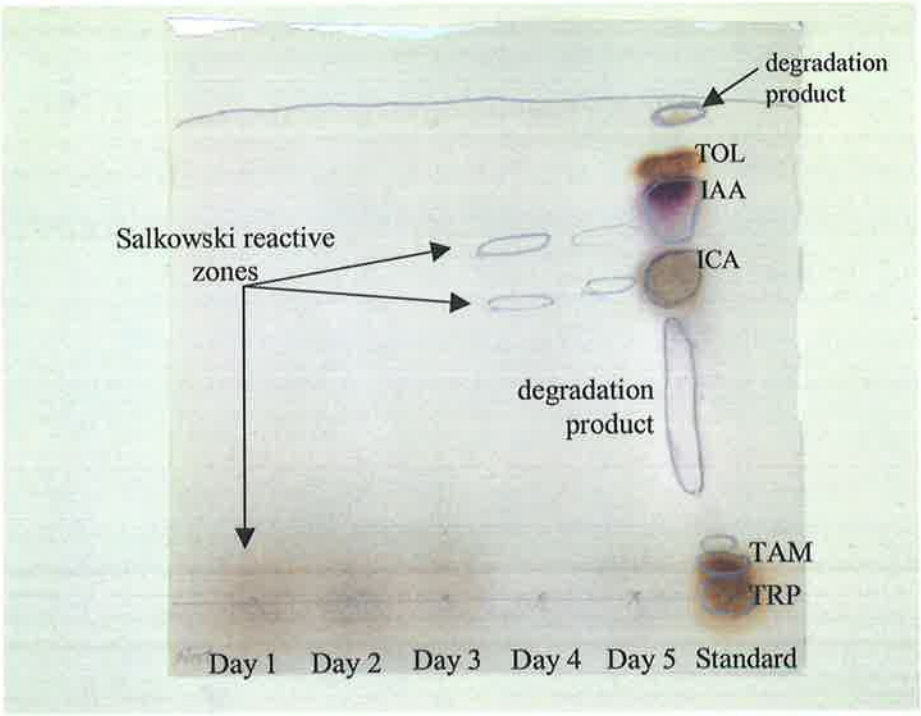


Figure 6.1b Production of Salkowski reactive compounds by *P. radicum* cultured in MEB with no TRP amendment.

6.3.2 Avena *straight growth assay*

6.3.2 (i) Response to synthetic indole acetic acid

A standard activity curve of the *Avena* straight growth assay constructed using dilutions of pure synthetic IAA in SCP is shown in Figure 6.2. The coleoptile growth response to auxin-like activity was dependent on the concentration of IAA present. At concentrations of IAA from $1x10^{-2}$ M to $4x10^{-4}$ M, reduction of coleoptile growth was measured. In the concentration range $8x10^{-5}$ M to $6.4x10^{-7}$ M, an increase in coleoptile growth from approximately 30% to 40% was seen. The extension of coleoptiles in neat SCP was 25 ± 2%.

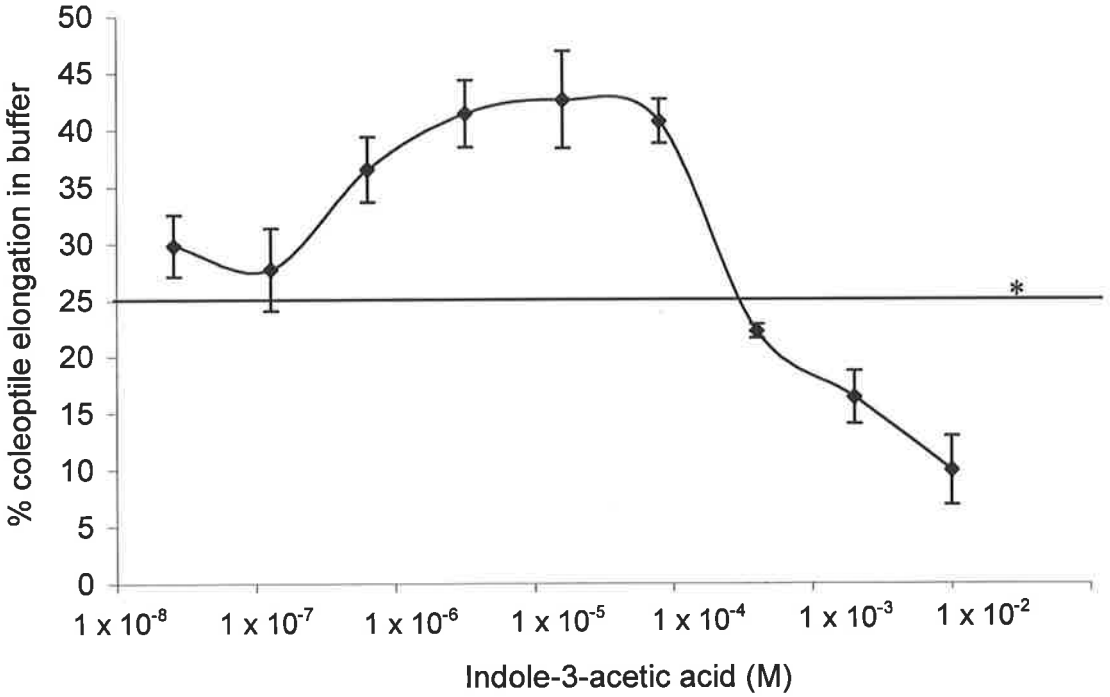


Figure 6.2 Activity curve of coleoptile extension for the *Avena* straight growth assay using standard indole-3-acetic acid (IAA) over a concentration range of 6.4 x 10^{-7} M to 0.01 M. The horizontal line marked with an asterisk (*) indicates extension of coleoptiles in 4% sucrose citrate phosphate buffer. Error bars show s.e (n=5).

6.3.2 (ii) Application to P. radicum *culture extracts*

Avena straight growth assay indicated that auxin-like activity was present in cultures of *P. radicum* grown in MEB that was not amended with TRP (Figure 6.3). Zones of auxin activity corresponding to the Rf of IAA were detected on sample times day 1 and day 2. However, this was not consistent with the presence of Salkowski reactive zones detected for incubations reported in Figure 6.1. Various reasons accounting for these differences are introduced in Section 6.4.

In MEB spiked with 30 mg L^{-1} TRP coleoptile growth was generally less variable than samples with no TRP addition. On days 1 to 4 there was a mean coleoptile extension that ranged from 20 – 30% and no significant auxin activity was detected. On day 5 at

R_f 0.6, which corresponded to the R_f of IAA, coleoptile elongation was approximatcly 12% greater than the mean coleoptile growth. This result suggested that auxin activity was present at this sampling time (see Figure 6.4). There was no Salkowski reactive zone that corresponded with this section of auxin-like activity.

The Salkowski reaction did not appear to be a reliable indicator of biological auxin-like activity. Zones of reaction to Salkowski reagent in samples taken from cultures both amended and unamended with TRP did not always correspond to sections that contained auxin-like activity. This suggests that a wide range of compounds can potentially form Salkowski reactive zones. Inconsistency in the presence of Salkowski reactive zones between different experimental incubations and the lack of correlation between the position of Salkowski reactive zones with the detection of auxin like activity in the same R_f section is considered further in Sction 6.4.

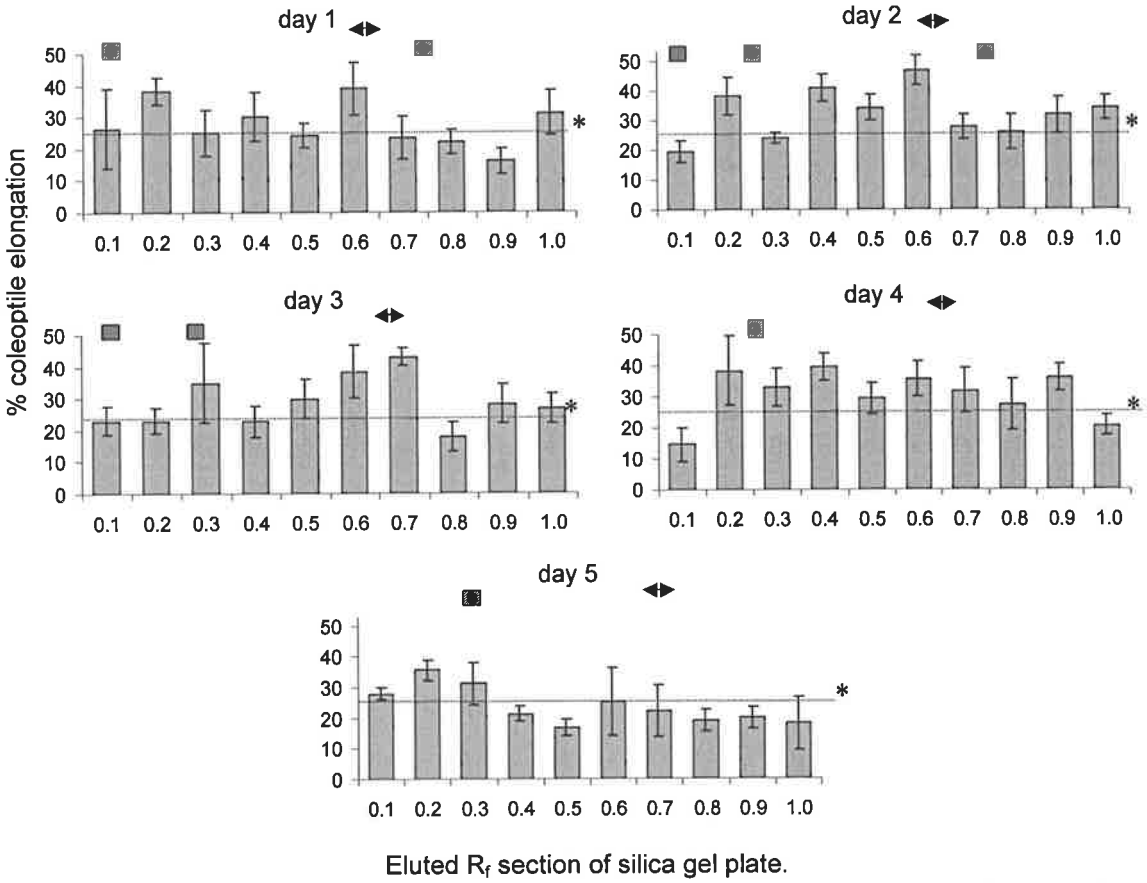


Figure 6.3 Auxin-like activity in cultures of *P. radicum* (MEB) as determined by the *Avena* coleoptile straight growth assay. Fungal culture medium was sampled following 1, 2, 3, 4 and 5 days of incubation. The samples were acidified (pH 4), extracted with diethyl ether and developed on silica gel 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 asterix (*) represents the mean percent coleoptile extension of coleoptiles grown in sterile 4% SCP. Error bars indicate s.e. (n=5).

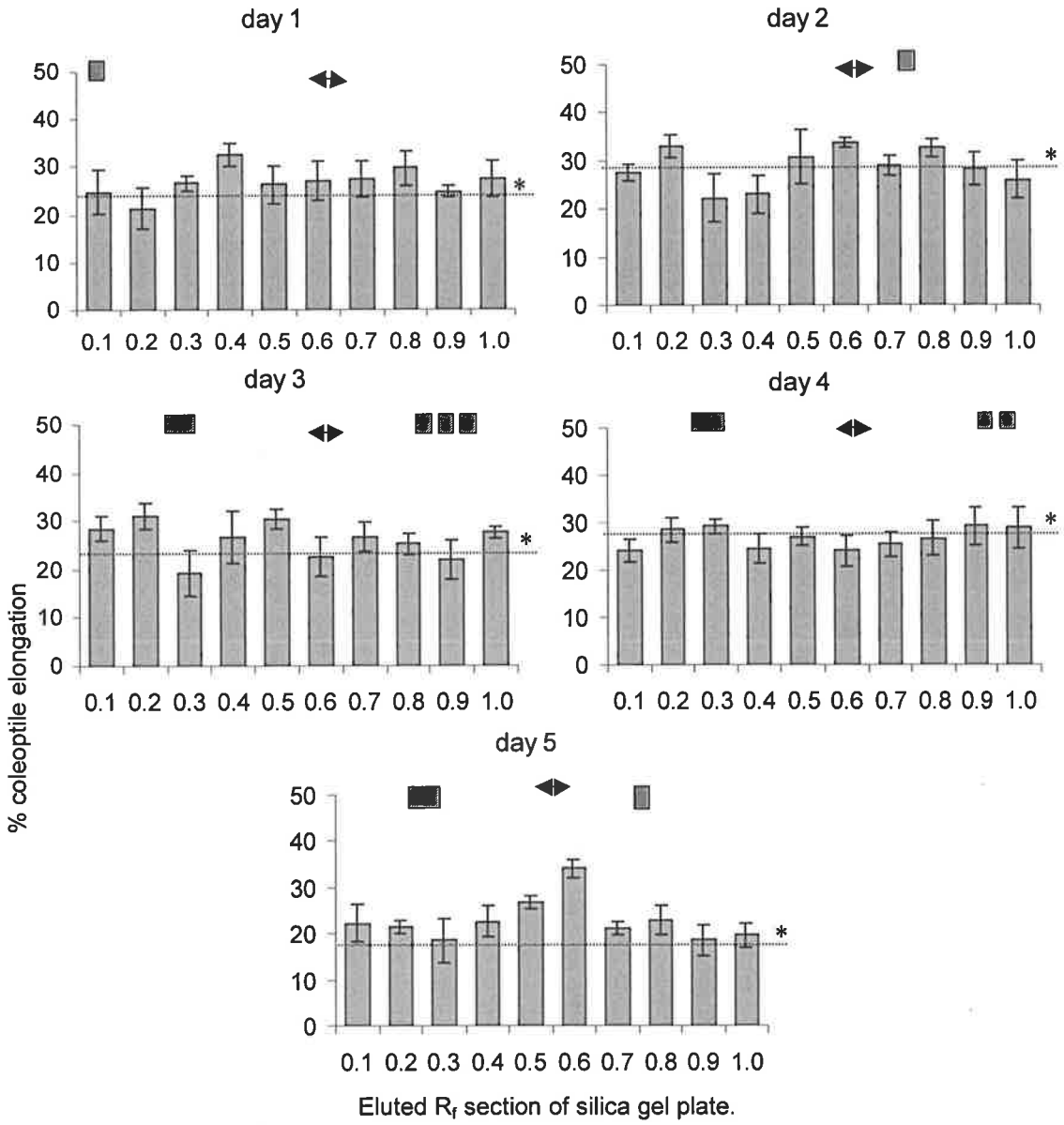


Figure 6.4 Auxin-like activity in cultures of *P. radicum* (MEB + 30 mg L^{-1} TRP) as determined by the *Avena* coleoptile straight growth assay. Fungal culture was sampled following 1, 2, 3, 4 and 5 days of *P. radicum* incubation and extracted using acidified (pH 4) diethyl ether and developed on silica gel plates. Plates were visualised by spraying with Salkowski reagent. Zones that developed are marked as diagonally striped 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).

6.3.3 ELISA

To obtain a more accurate determination of IAA production in *P. radicum* culture, an immunological approach was used. Samples from *P. radicum* cultured in two growth media were selected for examination using a specific mono-clonal anti-IAA antibody (Weiler et al. 1981). According to the manufacturer, the limit of detection is set at 78 nM. Hence, where no activity was detected using the ELISA, the result is reported as <78 nM.

The type of growth medium influenced the production of IAA in liquid cultures of *P. radicum* (Table 6.1). While *P. radicum* produced abundant hyphae in both of the tested media, IAA production was only found in MEB medium. When MEB was supplemented with TRP, the concentration of IAA produced was increased.

Table 6.1. Use of ELISA to determine the production of IAA by *P. radicum* in Malt Extract Broth (MEB) and Glucose minimal salts (GMS) either with or without TRP addition. Errors (±) indicate s.e (n=3).

	Growth medium	
Treatment	MEB	GMS
sterile medium	<78 nM	<78 nM
P. radicum	139 ± 39 nM	<78 nM
P. radicum + 30 mg L^{-1} TRP	406 ± 11 nM	<78 nM

6.4 Discussion

A number of techniques were used to detect IAA production by *P. radicum*. Based on R_f and reaction to Salkowski reagent it was identified that a compound with similar characteristics to IAA was found in *P. radicum* cultures.

By application of the *Avena* straight growth assay, it was found that R_f fractions of *P. radicum* culture corresponding to that of standard IAA also contained auxin-like activity. This gave further evidence that IAA was produced by *P. radicum.* However, the *Avena* coleoptile straight growth assay is not analytically specific and a variety of compounds could have auxin-like activity or act as a nutrient for coleoptile elongation. Despite the lack of specificity, TLC and *Avena* straight growth assay were employed as they are inexpensive, rapid and require non-specialised equipment. These characteristics make them appropriate techniques to perform an initial screen for the presence of IAA. When the results of TLC and *Avena* straight growth assay were taken together there was sufficient positive evidence to warrant using highly specific methods of detection for IAA.

The application of ELISA gave further evidence that *P. radicum* produced IAA. Using ELISA it was found that IAA was produced in concentrations up to 406 nM, which is sufficient to cause a physiological effect on plant growth (see Section 1.2.5). For example,in Figure 6.1 it is illustrated that coleoptiles exposed to this concentration ($4x10^{-6}M$) are increased in elongation rate by approximately 37% while Figure 1.1 shows that the growth of stem and bud tissue is promoted. However, it is uncertain how the concentration of IAA produced in culture relates to the concentration of IAA found in the rhizosphere. In non-sterile soil the IAA produced would be quickly degraded by other organisms and/or adsorbed onto soil surfaces and unavailable for plant uptake.

While the presence of IAA in *P. radicum* culture was generally indicated, further confirmation may be established using other analytical techniques such as high performance liquid chromatography and gas chromatography-mass spectrometry (Lebuhn et al., 1997; Pilet & Saugy, 1987; Frankenberger & Poth, 1987). In combination with the data reported here the use of these techniques would provide irrefutable evidence of IAA production in *P. radicum* culture.

While the presence of auxin like activity and auxin like compounds was detected in these experiments there were inconsistencies within the results. A discrepancy was observed for the presence of Salkowski reactive zones between experiments 6.2.1 and 6.2.2. For these two experiments, Salkowski reactive zones were observed at different times for the two separate incubations. These differences may be related to a number of different reasons including extraction efficiency and the growth rate of *P. radicum*.

As the extraction efficiency was not measured it is possible that samples taken on different incubation times had varying extraction efficiencies. Consequently, compounds forming reaction zones may have been present at too low a concentration to be detected in some instances. Differences in extraction efficiencies may have been related to the chemical instability of IAA. The nonezymatic degradation of IAA occurs in the presence of light, oxygen or peroxide and it may also be degraded by acids (see references cited within Sandberg et al. 1987). While *tert*-butylated hydroxytoluene was added to minimise losses to nonenzymatic degradation, IAA decomposition during the extraction still may have occurred.

A further explanation accounting for the variation in the detection of Salkowski reactive zones may be related to variable growth rates of the fungus between the separate experiments. As the reactive zones are likely to represent organic metabolites it is possible their existence in liquid medium is temporal and dependant on the growth stage of the fungus. If the reactive zones are biosynthetic intermediates of metabolites such as IAA they may rapidly undergo conversion by the fungus or alternatively they may be catabolised. For instance, Arshad & Frankenberger (1998) report that a number of studies have shown that TRP derived IAA formation was only a transitory intermediate in microbial culture as IAA was rapidly conjugated to IAA-lysine and other products. Hence, it is unlikely that IAA would be maintained at a constant concentration throughout the incubation.

There were also inconsistencies between the presence of Salkowski reactive zones and zones of auxin activity detected by the *Avena* coleoptile assay. This lack of agreement in detection by the two methods would be primarily explained by two reasons: lack of specificity and different limits of detection. While it has been previously reported that the Salkowski reagent will react with a number of indole compounds (Bric et al., 1995; Glickmann & Dessaux, 1995), the reagent was chosen as it is known to be the most sensitive colorimetric reaction with IAA (Bric et al., 1991). The reaction of Salkowski with indole derivatives produces bright distinctive colours that are typically shaded purple to pink. Colour shades of the reactive zones observed in the experiments conducted as part of this Chapter were often brown-black and hence they are not likely to be indoles. However, the position of the reactive zones was recorded as it was

hypothesised that they may have a relationship with the presence of auxin activity in *P. radicum* cultures. For example, these zones may have been the degradation products of IAA biosynthetic intermediates. However, there was no observable relationship for these zones with auxin-like activity and the variability in the appearance of these zones was such that they did not provide any meaningful information on the production of auxin activity in *P. radicum* culture. It is suggested that only those zones forming purple-pink zones be reported as Salkowski reactive zones for the presence of auxins.

ELISA revealed that the addition of TRP stimulated IAA production. This indicates that TRP is used as a precursor for IAA synthesis. As MEB contains peptone (10 g L^{-1}) this would have supplied a small amount of TRP. Consequently, IAA was detected in MEB without specific TRP amendment. To determine if TRP was an essential requirement for IAA synthesis, *P. radicum* was cultured in a defined growth medium (GMS) that did not contain TRP. However, IAA was not detected in GMS medium either with or without TRP added. This suggests that although TRP stimulates IAA production there are other factors required for IAA production. Presumably, these IAA biosynthetic growth factors would have been supplied by the peptone and malt extract present in the MEB growth medium. These biosynthetic factors may be amino acids other than TRP. For example, Martens & Frankenberger (1993) found that addition of the amino acids L-lysine, L-asparagine and L-alanine increased the TRP derived IAA formation in soil and that these amino acids also aided in the production of IAA by bacterial endophytes of Graminae. If other growth factors such as a mixture of amino

acids are required by *P. radicum* to produce IAA, then biosynthesis of IAA would not occur in GMS growth medium.

If TRP and other factors are required for detectable IAA synthesis it is likely that these compounds will be a key component that influences the effectiveness of IAA production as a mechanism of growth promotion. Hence, one of the parameters that is likely to be influencing the production of IAA by *P. radicum* is the availability of TRP in the rhizosphere. While amino acids have been previously identified as components of root exudates (Jaeger, 1999), the main components of root exudates are saccharide based compounds and organic acids. Typical concentrations of TRP found in the exudates of wheat roots are much lower than those supplied in the fungal growth media used in the experiments reported in this Chapter. For example, Martens & Frankenberger (1994) found that in 17-day-old wheat seedlings, 0.6 nM of TRP was produced per plant. The addition of 30 mg L^{-1} provides a solution that is approximately 170,000 nM. Hence, the conditions used in these experiments are not comparable to the rhizosphere. The concentration of TRP addition was chosen at such high values to ensure that any IAA production by *P. radicum* would be above the limits of detection for the *Avena* coleoptile assay and Salkowski reagent.

The low concentration of tryptophan available in the rhizosphere suggests that TRP may only be available to microorganisms that are closely associated with the root. As concentrations of TRP supplied in the growth medium was much higher than TRP concentrations found in root exudates this suggests that the actual concentration of IAA

produced by *P. radicum* in the rhizosphere may be much lower than that measured in the growth medium. However, due to physical, biological and chemical differences between laboratory growth medium and the rhizosphere it is difficult to relate concentrations of IAA produced *in-vitro* to activity in the rhizosphere.

The concentration of IAA produced is an important aspect that will determine the effect that IAA production has on plant growth. At high concentration there is an inhibitory effect of IAA on plant growth (see Section 1.5.2, Fig 6.2). The deleterious effect of high IAA production by pathogenic microorganisms is well documented. For example the mycoherbicide, *Colletotrichum gloeosporoides* f. sp. *aeschynomene* (*Cga*) produces high concentrations of IAA. Robinson et al. (1998) screened a range of *Cga* isolates in liquid culture and found IAA production to be within in the range 11 – 186 μM; if *Cga* could produce these concentrations *in planta*, IAA would at least be partly responsible for its pathogenicity. It is also known that IAA production is a feature of bacterial plant pathogens. The formation of olive and oleander knot by *Pseudomonas syringae* is due to the production of IAA. Glickmann et al. (1998) investigated IAA production by 57 pathovars of *P. syringae* and found that IAA concentration in bacterial culture supernatant ranged from 4 – 405 μM, with a mean concentration for all strains that were tested at 55 μM. Direct comparison of the concentrations of IAA produced in cultures of plant pathogens with that found in *P. radicum* cultures shows that IAA production is in most cases dramatically lower for *P. radicum*. This is consistent with a plant growth promoting effect due to IAA production by *P. radicum*.

To understand the significance of IAA production as a mechanism of plant growth promotion, further research needs to identify the effect that IAA produced by *P. radicum* has on the growth and development of the plant. The effect of *P. radicum* on root morphology was studied in sterile liquid culture by the method of Ridge & Rovira (1971). In the presence of TRP within a concentration range of 1 to 100 μM TRP, *P. radicum* visibly reduced root elongation and increased the length of root hairs and the area where root hairs were distributed over the roots (results not shown) an effect that is associated with the effects of auxin on root growth. This preliminary experiment suggests that *P. radicum* may be involved in the alteration of root morphology. These experiments were performed under sterile conditions and further research is required to identify if such an effect can occur in field soils.

At concentrations below 1 μM TRP, *P. radicum* had no observable effect on the morphology or root hair distribution in the liquid culture assay system. Direct comparison to the concentration of TRP in the exudate of wheat seedlings reported by Martens & Frankenberger (1993) at 0.6 nM, suggests that availability of TRP is likely to limit the ability of *P. radicum* to increase root hair production.

Although IAA production may be related to the changes in root morphology (see Section 1.5.5), auxins produced in the rhizosphere by *P. radicum* may be absorbed by the roots and translocated to the shoots increasing shoot weight (Scott, 1972; Salisbury & Ross, 1978; Martens & Frankenberger, 1994). This may explain why *P. radicum* inoculated onto wheat seeds increased the shoot weight of seedlings grown in

Experiment 5.3.2 and increased the mass of plants in the absence of any increase in P nutrition under sand culture conditions (see section 4.3.2).

Studies by Wakelin et al., (2003) suggest that there is a loose non-specific association between *Penicillium* spp. and plant roots. As *P. radicum* is isolated from the interior of recently inoculated wheat roots (see Table 5.2, Table 4.2 and Whitelaw et al., 1997), this is indicative of the ability of *P. radicum* to colonise the interior of the root. The ability to colonise the interior of the root may be of importance where IAA production is a mechanism of plant growth promotion. The colonisation of the roots by *P. radicum* would ensure that IAA produced by root associated *P. radicum* is taken up by the root cells and less subject to chemical degradation and/or degradation by other soil microorganisms.

6.5 Conclusion

This study has provided evidence and validation with a sensitive and specific technique that *P. radicum* produces IAA. The production of IAA by *P. radicum* needs to be linked with effects on plant growth and development under non-sterile soil conditions to determine the significance of IAA as a mechanism of plant growth promotion.

CHAPTER 7. *IN-VITRO* INDICATORS OF THE DISEASE SUPPRESSING ACTIVITIES OF *PENICILLIUM RADICUM*

7.1 Introduction

In previous Chapters 4 and 5, it was identified that the mechanisms of plant growth promotion by *P. radicum* were not necessarily related to P solubilisation and other mechanisms are needed to account for growth promotion. In Section 1.6.4 it was identified that various *Penicillium* spp. may alleviate the deleterious effects of soilborne root pathogens. Hence, it was hypothesised that a plant growth response to *P. radicum* inoculation under field conditions may be related to the suppression of plant disease or the direct inhibition of the fungal agents causing disease.

Antagonistic potential and mechanisms of suppression between fungi are frequently examined using solid agar growth medium as it is a convenient method of obtaining fungal growth data and observing interactions (Upadhay & Rai, 1987; Antal et al., 2000; Perello et al., 2001; Innocenti et al., 2003). Typical mechanisms of inhibition observed *in-vitro,* are antibiosis and the production of glucanase, chitinase and protease enzymes. These enzymes are associated with the degradation of cell walls, a process implicated in the hyperparasitism of fungal pathogens (see Section 1.7). This Chapter describes a variety of *in-vitro* experiments conducted to assess the interaction of *P. radicum* with soil- borne root pathogens and to identify mechanisms that may be involved in the suppression of root disease caused by these organisms.

While *in-vitro* conditions do not simulate the ecological and environmental factors present in agricultural fields, this approach can provide important information on the

potential mechanisms of pathogen suppression (see section 1.7). There are no published data on the mechanisms of disease suppression by *P. radicum* or the interaction of *P. radicum* with other soilborne fungi.

The experimental objectives of this Chapter were to:

- Ascertain the potential of *P. radicum* to inhibit the growth of cereal root pathogens *in-vitro*.
- Identify mechanisms that *P. radicum* may use to suppress the growth of cereal root pathogens, as these mechanisms may be related to the reduction of root disease. Mycoparasitism was studied by evaluating hyphal interactions in dual inoculated agar medium and activity of various enzymes which are commonly involved in this process. Antibiosis mechanisms were studied by evaluating the growth of cereal root pathogens in the presence of diffusible *P. radicum* metabolites.

7.2 Methods

7.2.1 Experiment 1. In-vitro *inhibition of cereal root pathogens* Gaeumannomyces graminis *var.* tritici, Pythium irregulare *and* Rhizoctonia solani *by* P. radicum.

The first experiment was performed to determine if *P. radicum* had an effect on the growth of three cereal root pathogens *Gaeumannomyces graminis* var. *tritici* (*Ggt*), *Pythium irregulare* and *Rhizoctonia solani*. Observations were performed on two media of contrasting nutrient composition; malt extract agar (MEA, that contained per litre of deionised water: 30 g malt extract, 10 g mycological peptone and 15 g agar) represented adequate nutrient conditions and; 0.2% glucose agar (0.2% G) represented low nutrient availability (2 g glucose and 15 g agar diluted to 1 L with deionised water).

The colony interactions of *P. radicum* with either *Ggt, Py. irregulare* or *R. solani* were studied by placing a 5 mm agar plug of test pathogen and a 5 mm agar plug of *P. radicum* on a Petri dish at a distance of 20 mm (three replicate Petri dishes for each pathogen). The inhibition of cereal root pathogens against established *P. radicum* colonies was studied by inoculating Petri dishes of either MEA or 0.2% G agar with 5 mm agar plugs of *P. radicum* and left to incubate for a period of 5 days at 25 °C in the dark. After 5 days incubation a second inoculation of either *Ggt, Py. irregulare* or *R. solani* (three Petri dishes for each pathogen-*P. radicum* interaction) was performed so that a plug of agar (5 mm) for the pathogen was 20 mm from the edge of the *P. radicum* agar plug. The inoculated dishes were incubated at 25 °C in the dark. Colony interactions were observed following the second inoculation. The timing of colony

interaction varied according to the growth rate of the pathogen. The effect of *P. radicum* on the pathogen colony growth was determined as the % inhibition rating (% I.R.) calculated as: 100 x $(r_1-r_2)/r_1$; where r_1 is the distance of radial growth of *P. radicum* fungus away from the test pathogen and r_2 is the distance of radial growth of *P. radicum* toward the colony of the test pathogen. Similarly, tr_1 and tr_2, the radial colony extension of the test pathogen away and toward *P. radicum*, respectively, were also measured. The %IR for the test pathogen in the presence of *P. radicum* was also calculated. A schematic diagram illustrating the inoculation and direction of measurement is shown in Figure 7.1.

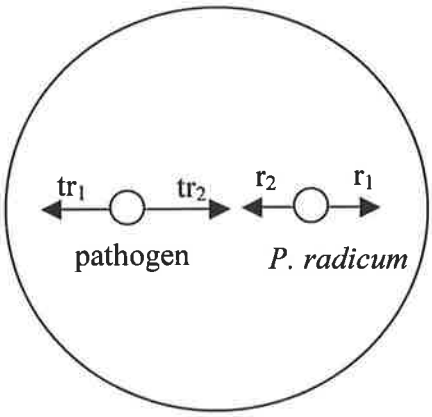


Figure 7.1 Schematic diagram of dual inoculated Petri dish indicating the direction of measurements r_1 and r_2 for equation given in Section 7.2.1.

7.2.2 Experiment 2. Hyphal and colony interactions of P. radicum *with cereal root pathogens.*

Colony and hyphal interactions of *P. radicum* with the cereal root pathogens *Ggt, R. solani, Py. irregulare, Fusarium pseudograminearum* and *Bipolaris sorokiniana* were investigated by dual inoculation with 5 mm plugs of agar (one for *P. radicum* and one for the test pathogen) placed 45 mm apart. To determine if the interaction of *P. radicum* with plant pathogens was similar for a non-plant pathogen, the hyphal

interaction of *P. radicum* with a non-plant pathogen *Aspergillus niger* was also observed. The dual inoculation test was conducted on two formulations of solid growth media: ¼ PDA that consisted of potato dextrose agar (Difco) made to quarter strength of the manufacturers recommendation and supplemented with 7g L^{-1} Difco agar and 0.2% G agar (defined previously). The effect of dual inoculation on colony growth was measured as the % I.R. (as above, see Figure 7.1). Antibiosis was also recorded as the zone of clearance that separated the two confronting colonies and recorded as either less than or greater than 1 mm. Observations were performed at varying times of incubation according to the growth rate of the pathogenic fungi.

Hyphal interactions were observed using bright field light microscopy performed directly on the agar dishes with a Leitz light microscope at magnification 320x. Scanning electron microscopy (SEM) was done using a Philips XL30 Field Emission Scanning Electron Microscope, equipped with an Oxford Instruments CT1500 HF Cryo Preparation System. A sample was attached to the holder using Tissue-Tek OCT compound mixed with powdered carbon. It was then frozen in liquid nitrogen, and transferred under vacuum to the preparation chamber. The temperature of the sample was raised to – 92 °C and held for approximately three minutes to allow any ice on the surface to sublime. The temperature was then lowered to – 110 °C (at which sublimation ceases) and the sample coated with platinum to make it electrically conductive. It was then loaded onto the microscope stage (held at a temperature lower than –150 °C) and examined.

7.2.3 Experiment 3. Growth inhibition of cereal root pathogens by diffusible P. radicum *exudates.*

Cellophane obtrained commercially from a general stationary supplier was cut to the size of 90 mm Petri dishes and sterilised. These cellophane discs were aseptically laid onto full strength MEA. *P. radicum* was inoculated onto the cellophane covered medium and incubated in the dark at 25 °C for a period of four weeks. After the incubation period, the cellophane was lifted from the MEA plates. A sterile cork borer (12 mm diameter) was used to take samples from the sterile underlying agar. To test for the presence of inhibiting metabolites, two of these cores were placed next to a 5 mm agar plug of the root pathogen to be tested. The percent inhibition was measured by taking two radial colony growth measurements, r_1 and r_2. These measurements are defined as: r_1, distance from the colony center to the edge in a direction away from the agar core and r_2, distance from the colony center to the edge in a direction toward the agar core. This antibiosis assay was performed on two media, ¼ PDA and 0.2% G agar.

7.2.4 Experiment 4. In-vitro *examination of the lytic enzyme activity of* P. radicum *culture.*

7.2.4 (i) Chitinase activity.

Chromogenically labelled chitin (chitin azure – Sigma) was used to determine the chitinase activity of *P. radicum* culture. Agar medium was prepared by adding (per L of deionised water) 0.5 g NH_4NO_3, 1 g K_2HPO_4, 0.5 g $MgSO_4$ and 15 g agar. Under sterile conditions, 10 mL of the agar medium was dispensed into sterile McCartney tubes followed by a 1 mL overlay that consisted of 20 g L^{-1} of chitin azure and 15 g L^{-1}

of agar. The chitin tubes were stab-inoculated with either *Trichoderma koningii* 7a (chitinase positive reference fungus), *P. radicum* or *P. bilaiae* RS7B-SD1. All tubes were incubated in the dark at 25 °C and observed for enzyme activity. Under assay by chromogenically labelled substrate, enzyme activity is demonstrated as leaking of the azure dye to the underlying agar following the hydrolysis of the polymer into smaller molecular weight fragments that have a higher mobility through the agar medium. If the polymer remains intact there is no leakage of azure dye into the underlying agar. In some instances if the organism acidifies the agar medium, the azure dye may become pink. However, without enzymatic hyrdolysis, there will be no diffusion to the lower layer. Hence, evidence for enzyme activity can only be taken as leakage to the underlying agar. The tubes were observed for enzyme activity for a period of up to two months.

Chitinase activity was also determined on a solid agar medium (Hankin & Anagnostakis, 1975). By this method, clearing of the colloidal chitin around the colony is taken as demonstrating chitinase activity. Chitin (Sigma) was prepared by the method of Rossner (1995). Briefly, 15 g of chitin was wet with acetone for 5 minutes, to remove organic contaminants, dried and placed in concentrated HCl with stirring for 3 hours. The dissolved chitin was then filtered through a filter funnel (Whatman 4 filter paper). The colloidal chitin was filled into dialysis tubing and rinsed for 8 hours with running deionised water. The suspended chitin was centrifuged, the supernatant discarded and the pellet of chitin redissolved in deionised water. The process of centrifugation and rinsing was repeated until the pH of the suspension was 5.5 to 6.

The suspension of chitin was added to a growth medium of (per L): 5 g glucose, 1 g K_2HPO_4, 0.5 g $MgSO_4$ and 15 g agar. The colloidal chitin medium was prepared as a 2 mm thick overlay on 0.2% G agar.

7.2.4 (ii) β-1,3-glucanase activity.

Medium containing (per L of deionised water): 6.8 g KH_2PO_4, 17.9 g K_2HPO_4, 5 g yeast extract, 4 g laminarin (Sigma) and 14 g agar, was autoclaved and poured into Petri dishes. *P. radicum* was inoculated with a pin point of spores and incubated at 25 °C for 10 to 12 days. At this time, the plate was flooded with 0.25% congo red in 50% ethanol. β-1,3-glucanase activity was demonstrated by the orange zones where the reducing sugars that were liberated from the carbohydrate polymer reacted with the congo red.

7.2.4 (iii) β-1,4-glucanase activity

Cellulose azure (Sigma) was used as a surrogate for the fungal cell wall polymer, β-1,4-glucan. The fungal production of β-1,4-glucanase was assayed using azure labelled cellulose (Sigma) by a similar procedure to that described in the use of azure labelled chitin in Section 7.2.4 (i).

7.2.4 (iv) Protease production.

Proteolytic enzymes were detected using two substrates; skim milk and gelatin agar plates. Skim milk plates were prepared according to Hopwood (1970) and consisted of potato dextrose agar 20 g L^{-1} plus 10% w/v skim milk powder solution (100 mL per litre of PDA). Each solution was autoclaved separately and then mixed aseptically.

Gelatin plates were prepared according to Hankin & Anagnostakis (1975). Nutrient broth (Difco) 8 g was added to 4 g of commercial grade gelatin per 100 mL of deionised water. Plates were stab inoculated with a pin point of *P. radicum* conidia. After 10 days of incubation, a saturated solution of $(NH_4)_2SO_4$ was added to enhance the zones of clearing surrounding the colonies. The production of protease was demonstrated by the formation of clearing zones around the colony of *P. radicum*.

7.2.5 Experiment 5. Glucose oxidase activity of P. radicum *culture supernatant.*

P. radicum was cultured in 250 mL conical flasks at 25 °C on a stirring shaker (120 rpm) in 100 mL of a liquid medium that contained 40 g glucose, 5 g $NaNO_3$, 0.5 g KCl, 1 g KH_2PO_4, 0.05 g $FeSO_4$ and 1 g mycological peptone. The medium was inoculated with *P. radicum* using 5 mL of conidiospore suspensions that contained 2 x 10^7 cfu mL^{-1}. After 120 hours incubation, glucose oxidase activity was assayed by the method of Ciucu & Patroescu (1984). The assay was performed by adding 1.0 mL of 1 M glucose, 0.5 mL of 0.1% benzoquinone and 0.45 mL 0.1 M Na-citrate buffer (Na-citrate buffer was prepared by adding 52 mL of 0.1 M citric acid and 48 mL of 0.2 M Na_2HPO_4). The enzyme reaction was initiated by the addition of 50 µL of culture supernatant. The reagents were mixed and immediately added to a 1 cm quartz cuvette inside a spectrophotometer (Beckmann). Enzyme activity was measured as the formation of hydroquinone and measured as the increase in absorbance at 290 nm for 120 s. Glucose oxidase activity was expressed as µmol hydroquinone min^{-1}.

7.3 Results

7.3.1 Experiment 1. In-vitro *inhibition of cereal root pathogens* Gaeumannomyces graminis *var.* tritici, Rhizoctonia solani *and* Pythium irregulare *by* P. radicum *in solid media.*

7.3.1 (i) Inhibition ratings of cereal root pathogens in dual inoculation with P. radicum.

The ability of *P. radicum* to inhibit root pathogenic fungi was examined on MEA and 0.2% G agar. When inoculation of the fungal root pathogen onto the agar was delayed, and the pathogen was inoculated against an established colony of *P. radicum*, the inhibition potential of *P. radicum* increased (Table 7.1).

Table 7.1 The effect of *P. radicum* 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 (±) indicate s.e (n=3).

		% I.R. of pathogen colony growth.	
Medium	Pathogen	Simultaneous inoculation	5 day pre-established *P. radicum* [C]
MEA	*Ggt* [A]	8 ± 3	56 ± 4
	Py. irregulare [B]	0 ± 0	67 ± 4
	R. solani [A]	7 ± 2	20 ± 4
0.2% G agar	*Py. irregulare* [B]	0 ± 0	31 ± 5
	R. solani [A]	35 ± 5	58 ± 5

Interactions were observed according to growth rates of pathogens, i.e. when the two colonies met.
[A] observed 5 days from the time of dual inoculation
[B] observed 2 days from the time of dual inoculation
[C] pathogen was inoculated against a 5 day old colony of *P. radicum*

7.3.1 (ii) Description of colony interactions between P. radicum *and cereal root pathogens.*

When studied on MEA, colony edges of *Ggt* and *P. radicum* grew to meet each other. A zone of clearance was never apparent between these two fungi on MEA. Typical colony morphology of these two fungi in dual culture on MEA is given in Figure 7.2a. As *Ggt* did not produce appreciable colony growth on water agar, no interaction was recorded between *P. radicum* and *Ggt*.

Under simultaneous inoculation on MEA, *P. radicum* had little effect on the growth of *Py. irregulare* (Table 7.1). *Py. irregulare* grew so rapidly on MEA that before *P. radicum* had established a reasonable colony, *Py. irregulare* had overgrown the Petri dish. The colony diameter of *P. radicum* was generally reduced when in dual inoculation with *Py. irregulare* on MEA (mean reduction 4 mm after 5 days). A

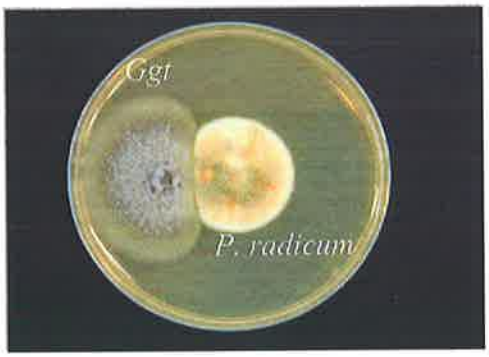
comparison of plates that consist of *Py. irregulare* inoculated alone and a *Py. irregulare* and *P. radicum* dual inoculation on MEA is shown in Figure 7.2b.

When an MEA plate was preincubated with *P. radicum* for a period of five days and then inoculated with *Py. irregulare*, there was significant inhibition of growth of *Py. irregulare* (Figure 7.2c & Table 7.1).

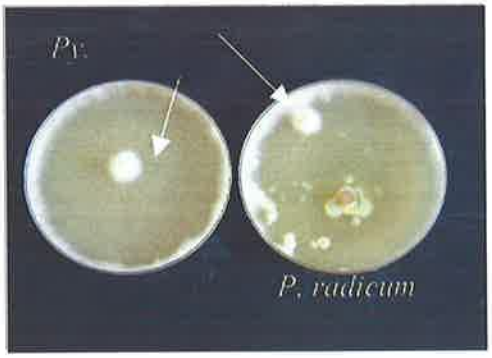
When *R. solani* was dual inoculated with *P. radicum* on MEA, *R. solani* did not show inhibition and hyphae extended to the edge of the *P. radicum* colony. However, the hyphae of *R. solani* never grew over the top of the *P. radicum* surface despite the higher growth rate of *R. solani* hyphae. A typical colony interaction of *P. radicum* with *R. solani* is given in Figure 7.2d.

On 0.2% G agar medium, *Py. irregulare* was not inhibited when simultaneously inoculated with *P. radicum* (Table 7.1, Petri dish cultures not shown). However, when the interaction of these pathogens was studied on 0.2% G agar pre-incubated with *P. radicum*, *Py. irregulare* was significantly inhibited (Table 7.1).

On 0.2% G agar, *R. solani* appeared to be more sensitive to the presence of *P. radicum*. The hyphae of *R. solani* tended to grow away from the *P. radicum* colony and this led to a measured % I.R. of 35% after 5 days incubation. Growth inhibition of *R. solani* was increased for plates preincubated with *P. radicum* for both MEA and 0.2% G agar (Table 7.1).

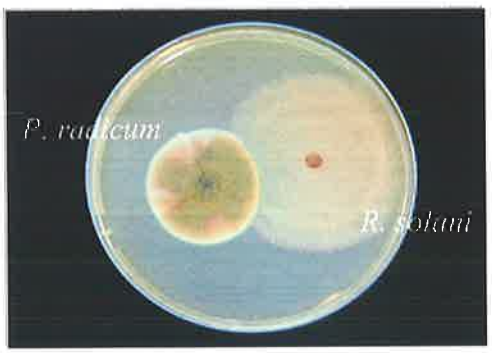


7.2a *P. radicum* and *Ggt* after 5 days incubation; simultaneous inoculation.



7.2b *P. radicum* and *Py. irregulare*, typical colony interaction. The Petri dish on the left shows *Py. irregulare* alone and the dish on the right shows a dual inoculation of *P. radicum* with *Py. irregulare*. Arrows indicate *Py. irregulare* hyphal

7.2c *Py. irregulare* inoculated against a 5 day old colony of *P. radicum* on MEA and incubated for 48 hours.



7.2d *R. solani* inoculated against a 5 day old colony of *P. radicum* and incubated for 48 hours.

Figure 7.2 Growth and interaction of *P. radicum* and cereal root pathogens on MEA at 25 °C.

7.3.2 Experiment 2. Hyphal and colony interactions of P. radicum *with cereal root pathogens.*

In-vitro interactions of *P. radicum* with soilborne root pathogens were investigated further by studying the hyphal and colony interactions of *P. radicum* with the pathogens *Ggt, R. solani, Py. irregulare, Fusarium pseudograminearum* and *Bipolaris sorokiniana* on two media, ¼ PDA and 0.2% G agar. The % I.R. and the presence of clearance zones between the two colonies from all of these *P. radicum*-pathogen interactions is summarised in Table 7.2. Descriptions of these interactions are given in the text below.

7.3.2 (i) Colony interactions on ¼ PDA.

Typical *P. radicum* colony growth on ¼ PDA is shown in Figure 7.3a. When *P. radicum* was grown in dual culture with cereal root pathogens on this medium, inhibition of colony growth was often observed for both *P. radicum* and the pathogen. *P. radicum* colony growth was inhibited in dual culture with *Py. irregulare, Ggt, B. sorokiniana* and *F. pseudograminearum.* While there was no inhibition of *Py. irregulare* growth, *P. radicum* inhibited the colony growth of the other test pathogens by 44 to 58% (Table 7.2).

On ¼ PDA, the formation of clearance zones was most apparent in *Ggt*- and *B. sorokiniana-P. radicum* interactions. When *P. radicum* was grown in dual culture with either of these organisms, the zone of clearance formed was typically greater than 1 mm. Colonies of *B. sorokiniana* produced a flat, velutinous black colony morphology that spread evenly from the point of inoculation in the centre of the Petri dish (Figure

7.3b). When grown in a dual inoculated plate with *P. radicum*, the black colony formed around the *P. radicum* colony forming a zone of clearance (Figure 7.3c). Similarly, typical morphology of a *Ggt* colony is given in Figure 7.3d, while interaction with *P. radicum* is illustrated in Figure 7.3e.

Table 7.2 Inhibition rating (% I.R.) and antibiosis colony interactions between *P. radicum* and cereal root pathogens in dual inoculation on 0.2% G agar and ¼ PDA. Errors (±) refer to s.e. of three experimental replicates.

¼ PDA	% I.R.	zone of clearance (mm)
Ggt [1]	58 ± 10	>1
P. radicum [1]	57 ± 5	
R. solani [2]	44 ± 4	0
P. radicum [2]	0 ± 0	
Py. irregulare [3]	0 ± 0	0
P. radicum [3]	56 ± 5	
F. pseudograminearum[4]	48 ± 3	variable
P. radicum [4]	11 ± 3	
B. sorokiniana [1]	44 ± 16	>1
P. radicum [1]	52 ± 6	
0.2% G agar		
Ggt	ng[A]	ng
P. radicum		
R. solani [2]	15 ± 17	>1
P. radicum [2]	0 ± 0	
Py. irregulare [3]	41 ± 2	>1
P. radicum [3]	22 ± 38	
F. pseudograminearum[4]	21 ± 13	>1
P. radicum	0 ± 0	
B. sorokiniana [1]	50 ± 9	>1
P. radicum [1]	27 ± 25	

ng[A] – *Ggt* did not grow on 0.2 % G agar.
Interactions were observed at varying times depending on the growth rate of the pathogen to *P. radicum*.
[1] determined after 6 days of growth.
[2] determined after 4 days growth.
[3] determined after 2 days growth.
[4] determined after 5 days growth.

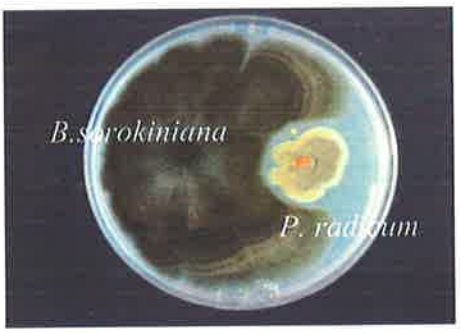
In pure culture on ¼ PDA, *R. solani* covered a 90 mm Petri dish by four days of inoculation. However, in a dual inoculation with *P. radicum*, the growth of *R. solani* was interrupted at the edge of the *P. radicum* colony and *R. solani* generally took 7 days to completely fill a 90 mm Petri dish (Figure 7.3f). There was generally no zone of clearance between these two fungi and hyphae of *R. solani* that were adjacent to the *P. radicum* colony often took on a yellow discolouration (Figure 7.3f).

In some cases a zone of clearance formed between colonies of *F. pseudograminearum* and *P. radicum* (see Figure 7.3g). However, the size of the zone was not consistently greater than 1 mm in width (Table 7.2). The *F. pseudograminearum* hyphae closest to *P. radicum* took on a yellow colouration which may be indicative of hyphal stress following exposure to colonies of *P. radicum*.

P. radicum did not inhibit the colony growth of *Py. irregulare* in dual cultures on ¼ PDA. By day 3 of incubation, *Py. irregulare* had completely filled the plate and inhibited the radial growth of *P. radicum* (Figure 7.3h & Table 7.2).

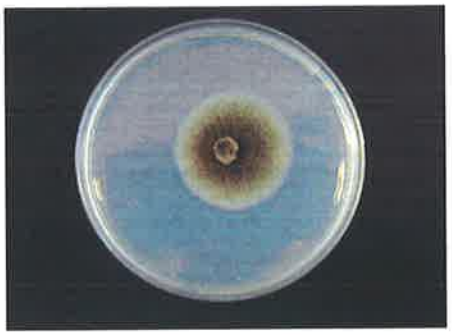
7.3a Colony morphology of *P. radicum* in a single inoculation

7.3b Colony morphology of *B. sorokiniana* in a single inoculation.

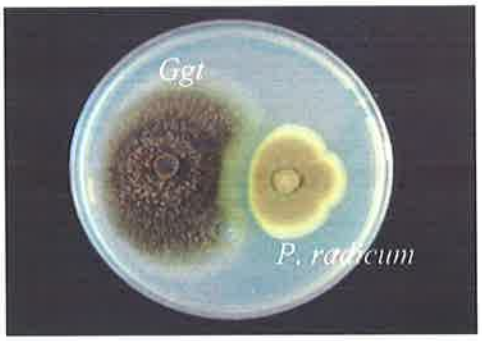


7.3c Interactions of *P. radicum* with *B. sorokiniana* in a dual inoculation.

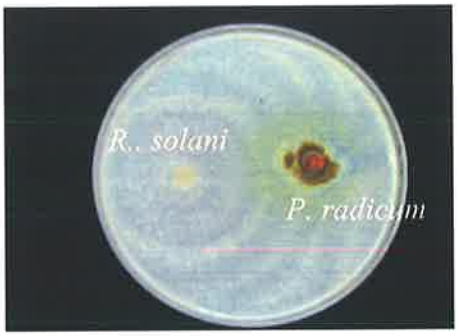
Figure 7.3 Typical growth and colony interaction of *P. radicum* and cereal root pathogens on ¼ PDA at 25 °C. Incubation time was 5 days.



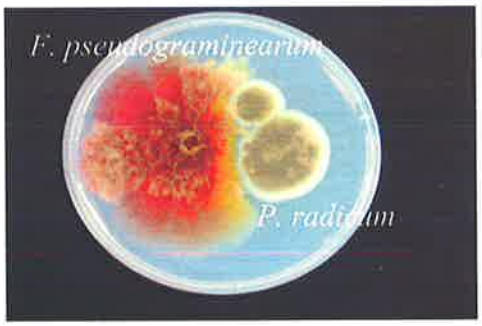
7.3d Typical colony morphology of *Ggt* in a single inoculation.



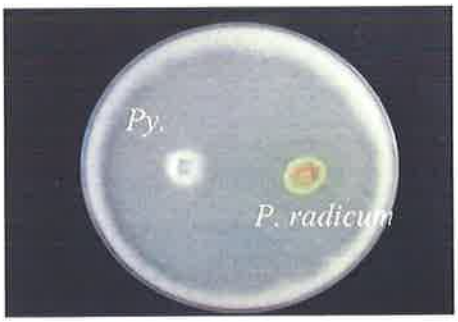
7.3e Interaction of *P. radicum* with *Ggt* in a dual inoculation .



7.3f Interaction of *P. radicum* in a dual inoculation with *R. solani* after 7 days incubation.



7.3g Interaction of *P. radicum* with *F. pseudograminearum* after 4 days incubation.



7.3h Interaction of *P. radicum* with *Py. irregulare* in a dual inoculation after 3 days incubation.

Figure 7.3 continued. Typical growth and colony interaction of *P. radicum* and cereal root pathogens on ¼ PDA at 25 °C. Incubation time was 5 days unless otherwise stated

7.3.2 (ii) Colony interactions on 0.2% G agar

When cultured on 0.2% G agar, *P. radicum* was slow growing; after 3 days colonies were approximately 4 mm in diameter. On this medium, vegetative growth of *P. radicum* was minimal, but sporulation was prolific and colonies were khaki, with a granular appearance while the surface was flat and rough. A general picture of colony growth of *P. radicum* on this medium is shown in Figure 7.4a, but this figure does not illustrate fine morphological details.

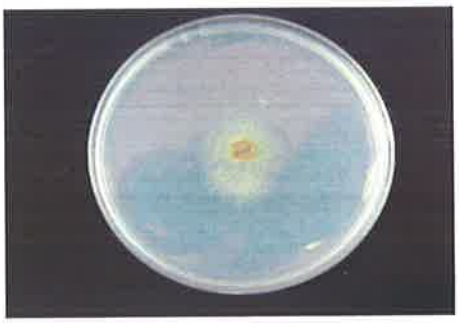
F. pseudograminearum colony growth on 0.2%G agar generally covered the entire Petri dish within 7 days. Colonies were smooth and had a distinctive pink pigmentation (Figure 7.4b). The inhibitory effect of *P. radicum* in a dual inoculation with *F. pseudograminearum* was clearly visible. The growth of pink hyphae toward the colony of *P. radicum* was halted and at the edge of the colony, they became yellow or developed an aerial growth habit (Figure 7.4c).

In contrast to more nutrient rich media (such as MEA or ¼ PDA), *Py. irregulare* cultures showed clearly visible inhibition by *P. radicum* when the interaction of these two fungi was observed on 0.2% G agar. After 10 days incubation the zone of inhibition was clearly visible (Figure 7.4d). Hyphae next to the zone of inhibition appear more fluffy than the hyphae on the rest of the plate due to increased aerial growth. Aerial growth of the *Py. irregulare* hyphae was probably a response to inhibitory compounds secreted by *P. radicum*. The inhibition was on the side closest to the *Penicillium* colony which indicates that the compound causing the inhibition is

likely diffusing into the agar. This contrasts with a volatile compound that would restrict growth of the colony evenly on all sides.

There were some inhibitory effects on the growth of *R. solani* in the presence of *P. radicum*. *R. solani* hyphae did not grow near the colony of *P. radicum* and generally grew around or away from *P. radicum*. A typical colony interaction between these two fungi is given in Figure 7.4e.

B. sorokiniana growth over 0.2 % G agar was sparse but the hyphae spread evenly over the Petri dish (Figure 7.4f). In dual inoculation with *P. radicum*, the growth of *B. sorokiniana* was prevented from covering the entire Petri dish (Figure 7.4g). As shown in Figure 7.4g, the hyphae that were growing away from the point of *P. radicum* inoculation grew to the edge of the Petri dish, while hyphae growing toward *P. radicum* were halted. The inhibition was maintained even after sustained incubation of up to 14 days.

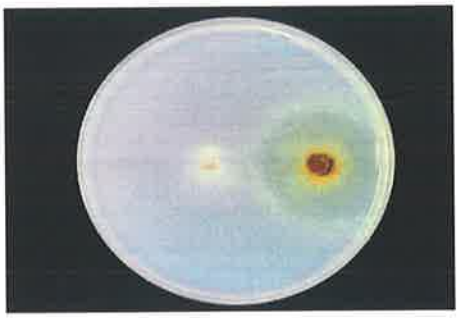


7.4a *P. radicum* after 7 days.

Figure 7.4b *F. pseudograminearum* after 5 days

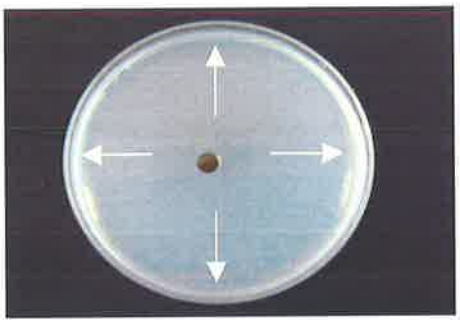
Figure 7.4c Dual inoculation of *F. pseudograminearum* and *P. radicum* after 5 days incubation.

Figure 7.4 Growth and interactions of *P. radicum* with cereal root pathogens on 0.2% G agar at 25 °C.

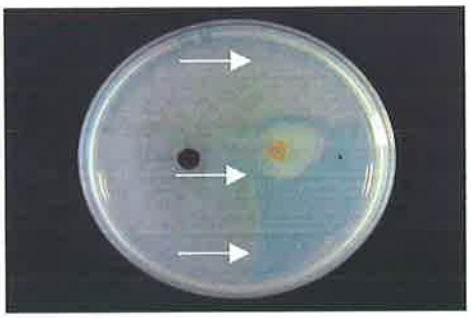


7.4d The interaction of *Py. irregulare* in a dual inoculation with *P. radicum* after 10 days incubation.

7.4e Dual inoculation of *R. solani* and *P. radicum* after 3 days incubation. As the colony edge is not visible in the photograph, black arrows are used to indicate where the edge of the *R. solani* colony would appear.



7.4f *B. sorokiniana* after 5 days growth. The white arrows indicate where the edge of the colony has reached on the plate.



7.4g The interaction of *B. sorokiniana* and *P. radicum* in a dual inoculation after 5 days incubation. White arrows indicate the edge of the *B. sorokiniana* colony.

Figure 7.4 continued. Growth and interactions of *P. radicum* with cereal root pathogens on 0.2% G agar at 25 °C

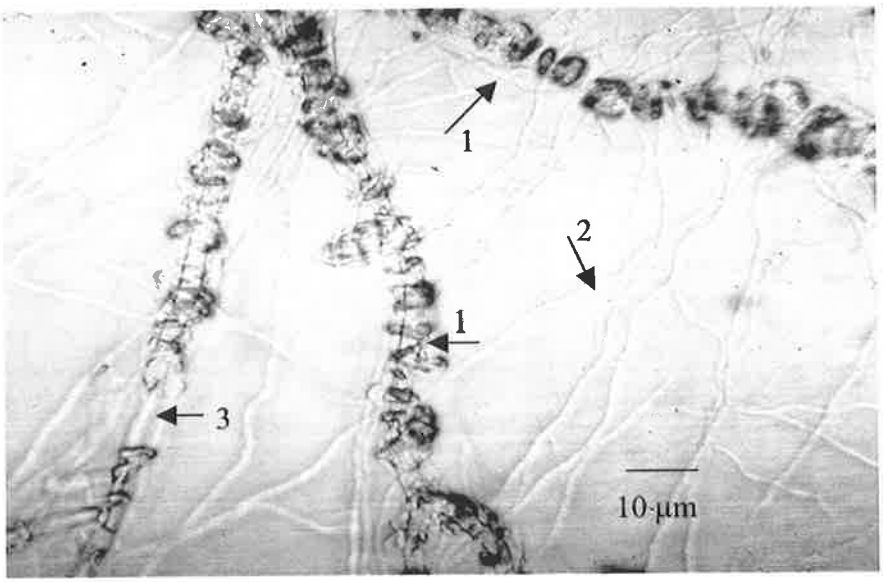
7.3.2 (iii) Hyphal interactions

Areas of the dual cultures where the hyphae of *P. radicum* and other fungi intermingled were examined using light microscopy. This revealed that *P. radicum* produced hyphae that either coiled or grew along the hyphae of the fungal pathogen that was in dual inoculation with *P. radicum*. Coiling effects, typical of hyperparasitism, were readily observed in hyphal interactions of *P. radicum* with *R. solani, F. pseudograminearum, Py. irregulare* and the non plant pathogen, *A. niger*. A typical light microscopic image of *P. radicum* hyphae in dual culture is shown in Figure 7.5a where *P. radicum* hyphae are coiled on *A. niger* hyphae in a dual inoculation of the growth medium, 0.2 % G agar.

To obtain further detail on the association between the coiling of the host and hyperparasite, hyphal interactions between *R. solani* and *P. radicum* were studied using electron microscopy. These two fungi were chosen as the difference in hyphal diameter (≈10 μm for *R. solani* and ≈ 2 μm for *P. radicum*) allowed the two fungi to be clearly distinguished.

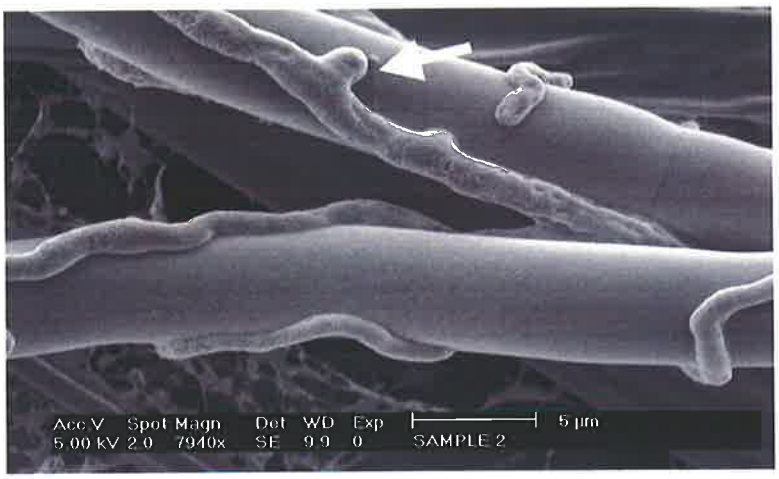
Observations with SEM revealed that *P. radicum* hyphae appeared to be closely associated with the *R. solani* hyphae (Figure 7.5b). Rather than growing randomly when coming in contact with a hypha of *R. solani*, *P. radicum* hyphae appeared to extend along the surface of *R. solani*. Branches formed along the surface of the *R. solani* hyphae (Figure 7.5b and 7.5c). In some areas the *R. solani* hyphae became infested with extensive coiling of *P. radicum* (Figures 7.5c and 7.5d) and may have

been associated with the collapse and deterioration of the *R. solani* (host) hyphae. In some instances, residues were observed along the perimeter of the interface of the coiling and host hyphae (Figure 7.5d). The residues may have been exudate from *P. radicum* that contained substances that would weaken the host hyphae and assist in the parasitism process. These components may include antibiotics, and/or gelling substances that would aid the *P. radicum* hyphae to stick to the host. In addition, the exudate may contain hydrolytic enzymes (i.e. chitinase, glucanase, protease) that would degrade the cell wall. All of these components would presumably act together to assist in the apparent hyperparasitism and nutrient transfer from the host hyphae to *P. radicum*.



7.5a Light microscopic photograph of *P. radicum* coiling on hyphae of *Aspergillus niger* in dual culture on 0.2% agar. This image is representative of typical hyphal interaction of *P. radicum* with other fungi including *Py. irregulare*, *R. solani*, *F. pseudograminearum*. Hyphal coiling of *P. radicum* against *A. niger* is shown by arrow (1). The hyphal coiling is often blurred by shadowing. Normal straight growth of *P. radicum* hyphae can be seen in the background as indicated by arrow (2). Arrow (3) indicates the host *A. niger* hyphae.

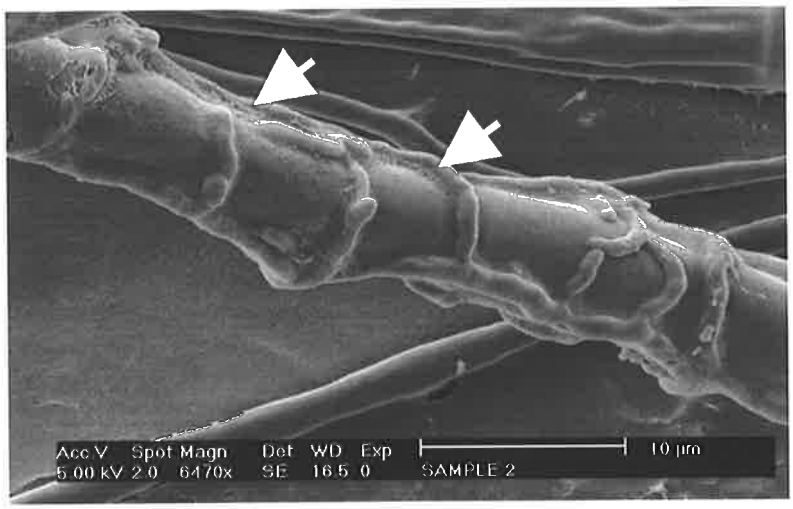
Figure 7.5 Hyphal interactions between *P. radicum* and other fungi in dual inoculation on 0.2% G agar at 25 °C.



7.5b Scanning electron micrograph of *P. radicum* and *R. solani* in dual inoculation. *P. radicum* hyphae appeared to be closely associated with the *R. solani* hyphae. Rather than growing randomly *P. radicum* hyphae have extended along the surface of the *R. solani*. Branches (an example marked by an arrow) can be seen forming on the surface of the *R. solani* hyphae.

7.5c Host hyphae (*R. solani*) are infested with coiled hyphae of *P. radicum*. At the top of the image (marked 1) *R. solani* hyphae appear rough which contrasts with other areas where the hyphae are smooth. This coarse appearance may be due to loss of cell wall integrity as a result of *P. radicum* parasitic activity.

Figure 7.5 continued. Hyphal interactions between *P. radicum* and other fungi in dual inoculation on 0.2% G agar at 25 °C after 5 days.



7.5d Scanning electron micrograph of *P. radicum* and *R. solani* in dual inoculation showing coiling and branching along a portion of *R. solani* hypha. Residues of organic compounds (indicated by arrows) can be observed near the interface of the two fungi.

Figure 7.5 continued. Hyphal interactions between *P. radicum* and other fungi in dual inoculation on 0.2% G agar at 25 °C after 5 days incubation

7.3.3 Experiment 3. Fungal growth inhibition by diffusible P. radicum *metabolites.*

The inhibitory effect of *P. radicum* exudates on the colony growth of cereal pathogens is summarised in Table 7.3. This assay was effective in detecting an inhibitory effect of diffusible *P. radicum* exudates on the growth of pathogens *Py. irregulare, F. pseudograminearum, Ggt, R. solani* and *B. sorokiniana.*

Table 7.3. % Inhibition rating of cereal root pathogen colony growth on either ¼ PDA or 0.2% G agar by diffusible *P. radicum* exudates after 3 days incubation at 25 °C. Errors (±) indicate s.e of four experimental replicates.

	Test medium	
Pathogen	**¼ PDA**	**0.2% G agar**
Py. irregulare	92 ± 4	97 ± 1
R. solani	44 ± 4	35 ± 8
F. pseudograminearum	0 ± 0	76 ± 5
B. sorokiniana	57 ± 5	75 ± 7
Ggt	60 ± 10	ng[A]

[A] ng indicates no detectable growth.

7.3.3 (i) Growth inhibition on ¼ PDA

When *Py. irregulare* was grown on ¼ PDA in the presence of sterile agar plugs, there was no inhibitory effect on the normal colony growth (Figures 7.6a). When plugs of agar that contained *P. radicum* exudates were placed next to the *Py. irregulare* colony, significant inhibition could be seen after 24 hours (Figure 7.6b). The effect of diffusible exudates on colony growth was less marked for the other pathogens studied. In the case of *R. solani*, sterile agar plugs had no effect on radial hyphal extension (Figure 7.6c) while *P. radicum* exudates caused disruption in the radial growth of the *R. solani* colony (Figure 7.6d). For *B. sorokiniana*, the majority of the hyphae grew

away from the plugs of agar that contained that fungal exudates (compare Figures 7.6e and f). *Ggt* showed a similar response where the hyphae grew away from the agar containing exudates and did not grow over the plugs of agar containing the *P. radicum* exudates (Table 7.3, plates not shown). There was no effect of *P. radicum* diffusible exudates on the radial growth of *F. pseudograminearum* (see Figures 7.6g and h).

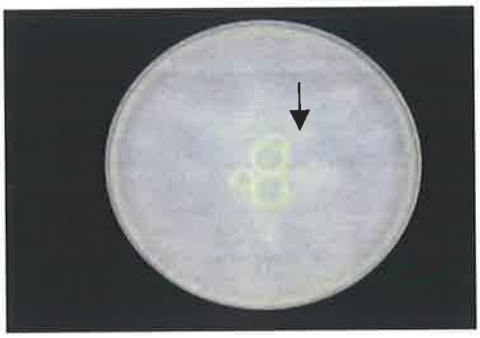


Fig 7.6a Growth of *Py. irregulare* against sterile agar plugs after 3 days.

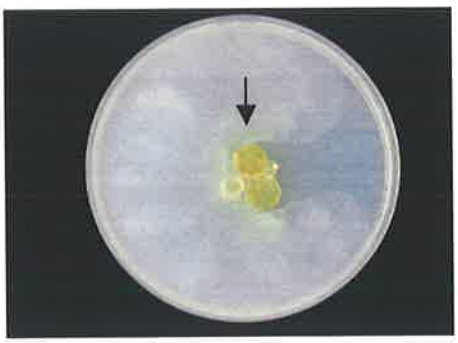


Fig 7.6b Growth of *Py. irregulare* against agar plugs that contain diffusible *P. radicum* exudates after 3 days (c.f Fig 7.6a).

Fig 7.6c Growth of *R. solani* against sterile agar plugs

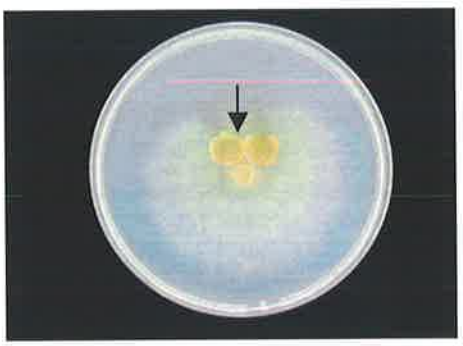
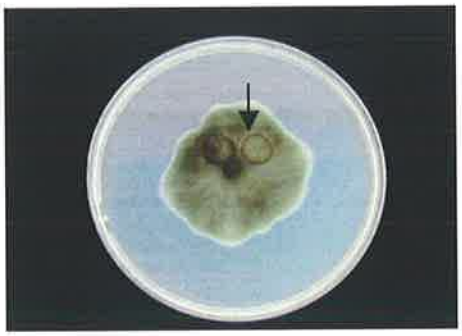
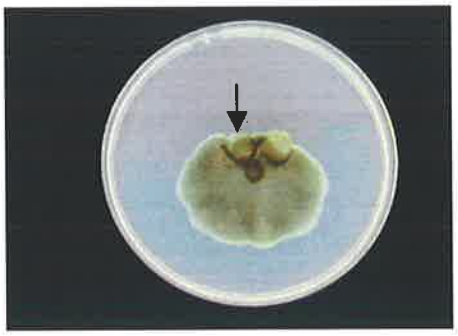


Fig 7.6d Growth of *R. solani* against agar plugs that contain diffusible *P. radicum* exudates (c.f Fig 7.6c). *R. solani* hyphae are not seen to grow either side of the black arrow.

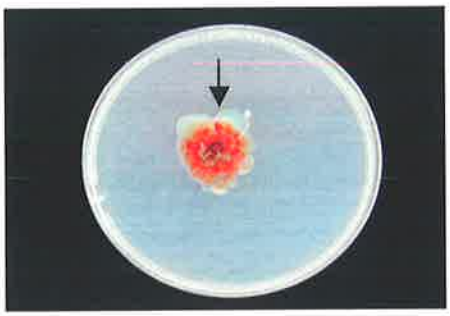
Figure 7.6 Growth inhibition of cereal root pathogens by diffusible *P. radicum* exudates when tested on ¼ PDA agar at 25 °C. Black arrow indicates the position of test agar plugs



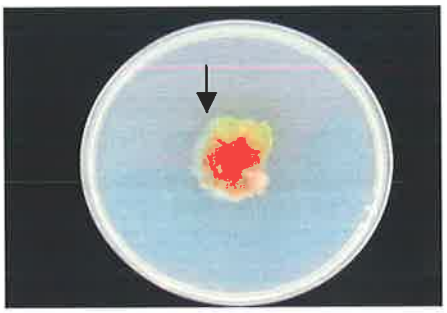
7.6e Growth of *B. sorokiniana* against sterile agar plugs.



7.6f Growth of B. sorokiniana against plugs of agar that contain diffusible *P. radicum* exudates (c.f Fig 7.6e).



7.6g Growth of *F. pseudograminearum* against sterile agar plugs.



7.6h Growth of *F. pseudograminearum* against agar plugs that contain diffusible *P. radicum* exudates (c.f Fig 7.6g).

Figure 7.6 continued. Growth inhibition of cereal root pathogens by diffusible *P. radicum* exudates when tested on ¼ PDA agar at 25 °C. Black arrow indicates the position of test agar plugs.

7.3.3 (ii) Growth inhibition on 0.2% G agar

On 0.2% G agar, all of the tested pathogens were inhibited due to the presence of diffusible *P. radicum* exudates (Table 7.3). Figures illustrating the growth inhibition for *Py. irregulare* are given in Figures 7.7a,b and for *R. solani* in Figures 7.7c,d. Growth inhibition of *B. sorokiniana* and *F. pseudograminearum* from diffusible exudates of *P. radicum* culture were also apparent on this medium (Table 7.3).

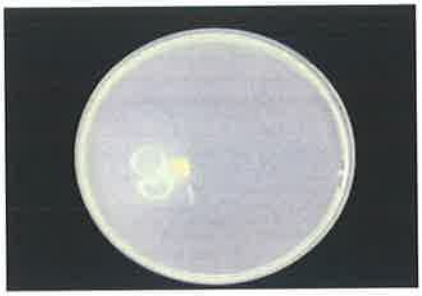


Fig 7.7a Growth of *Py. irregulare* against sterile agar plugs after 3 days.

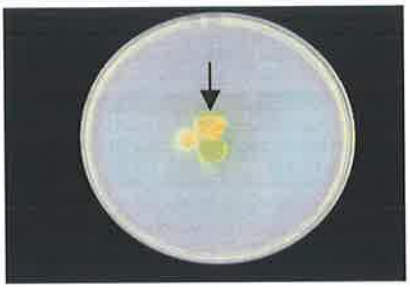


Fig 7.7b Growth *of Py. irregulare* against agar plugs that contain diffusible *P. radicum* exudates (c.f Fig 7.7a).

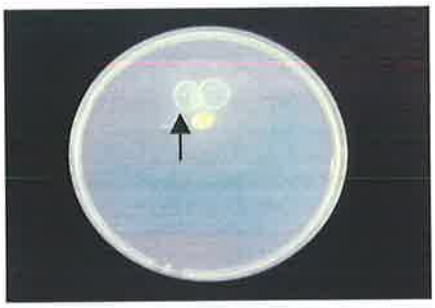


Fig 7.7c Growth of *R. solani* against sterile agar plugs after 2 days.

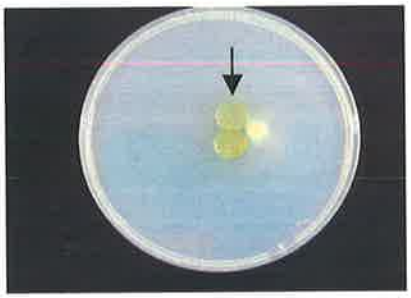


Fig 7.7d Growth of *R. solani* against agar plugs containing diffusible *P. radicum* exudates after 2 days incubation (c.f Fig 7.7c).

Figure 7.7 Growth inhibition of cereal root pathogens by diffusible exudates of *P. radicum* when tested on 0.2% G agar at 25 °C. Black arrows indicate position of agar plugs.

7.3.4 Experiment 4. Production of cell wall lysing and glucose oxidase enzymes by P. radicum.

It was found that *P. radicum* produced a range of enzymes that can be implicated in the inhibition of pathogenic fungi. A summary of enzymes produced in cultures of *P. radicum* is given in Table 7.4. Details on assay results for chitinase, β-1,3-, β-1,4-glucanase and protease activities are presented in Sections 7.3.4 (i) to 7.3.4 (iv), respectively.

Table 7.4 The production of cell wall degrading enzymes by *P. radicum.*

Enzyme	Substrate	Activity
chitinase	chromogenically labelled chitin	negative[A]
	colloidal crab shell chitin	negative[A]
β-1,3 glucanase	laminarin	9 ± 4 mm[C]
β-1,4 glucanase	chromogenically labelled cellulose	positive
protease	milk protein	3 ± 1 mm [B]
	gelatin	3 ± 1 mm [B]

[A] chitinase was observed after prolonged incubation (after 4 weeks) of *P. radicum* on the test medium.
[B] width of reactive zone detected from *P. radicum* colony edge on solid growth medium after 7 days incubation at 25 °C
[C] after 10 days incubation.

7.3.4 (i) Chitinase activity

For the chitinase azure substrate, significant chitinase activity by *Trichoderma koningii* 7a was observed after 5 days, as the entire McCartney tube had turned blue. For *P. bilaiae* RS7B-SD1, the substrate had turned pink (due to fungal acidification) after 7 days and some of the enzymatically cleaved substrate had leaked into the agar medium below indicating enzyme activity. For *P. radicum*, there was no apparent difference between inoculated tubes and the control tubes which appeared stable and there was no

appearance of the dye in the agar medium at 7 days. After four weeks of incubation, *P. radicum* had caused the chitin-azure to become pink (indicating acidification) and the dye had diffused into the growth medium below. As control substrates had retained their blue colour and did not leak to the agar below, this indicated that the degradation of the azure labelled substrate was due to a low level of chitinase activity released by *P. radicum*.

Native chitin prepared from crab shells was also used to assess chitinase activity. On this medium, *P. radicum* produced very low, but detectable chitinase activities (observable as a zone of clearance when the plate was placed against a dark background) after 4 weeks incubation. It can be concluded from both assays that chitinase production by *P. radicum* is very low.

7.3.4 (ii) β-1,3-glucanase activity

The activity of *P. radicum* cultures indicated that enzymes had degraded the β-1,3-glucan substrate. After 10 days of incubation, *P. radicum* colonies grew to a mean diameter of 11 ± 6 mm. An orange zone around the colonies after flooding with congo red had a mean diameter of 9 ± 4 mm.

7.3.4 (iii) β-1,4-glucanase production

After 5 days, *P. radicum* had degraded the blue colour of the cellulose azure substrate to a similar extent seen for the positive control organisms *P. bilaiae* RS7B SD1 and *Trichoderma koningii* 7a. The dye had turned pink and had sunk through the agar

medium (McCartney bottle). The control remained blue indicating that degradation of the substrate was due to fungal activity.

7.3.4 (iv) Protease activity

On both the skim milk and gelatin media, *P. radicum* produced a distinct zone of clearing, indicating protease production. After 10 days incubation colony diameters and zones of clearance were measured. For the skim milk medium, *P. radicum* grew to a colony diameter of 7 ± 3 mm from a pinpoint inoculation. The size of the halo of clearing was 3 ± 1 mm. On the gelatine medium, the mean colony size was 5 ± 2 mm and the zone of clearing 3 ± 1 mm in width from the edge of the colony.

7.3.5 Experiment 5. Production of glucose oxidase activity

Assay of the sterile growth medium gave no increase in absorbance at 290 nm. Assay of the medium supernatant after *P. radicum* culture revealed that the fungus produced a mean glucose oxidase activity of 0.93 ± 0.26 μmol mL^{-1} min^{-1} (data of three experimental replicates). The method used to determine glucose oxidase activity in *P. radicum* cultures was also employed by Petruccioli et al., (1993) to determine the glucose oxidase activity in a number of *Apergillus* and *Penicillium* spp cultures (Table 7.5). The glucose oxidase activity found within *P. radicum* culture were found to be similar to the values reported by Petruccioli et al., (1993).

Table 7.5. Glucose oxidase activity in *P. radicum* culture supernatant compared with reference *Penicillium* and *Aspergillus* strains reported by Petruccioli et al. (1993).

Species	GO activity μmol mL^{-1} min^{-1}
P. chrysogenum NRRL 811	1.37
P. italicum NRRL 983	0.89
P. variabile NRRL 1048	2.04
P. radicum	0.93
A. niger	1.21

7.4 Discussion

As a first examination of the ability of *P. radicum* to reduce root disease, the interaction of *P. radicum* with pathogens causing root disease was investigated using simple *in-vitro* tests.

Under *in-vitro* dual inoculation *P. radicum* inhibited a variety of cereal root pathogens. However, the effect was dependent on the nutrient content of the medium in which the interaction was studied. Under adequate nutrition, fungi are likely to have a greater resistance to stress related to fungal antagonism. Hence, the ability of *P. radicum* to inhibit pathogens was lower when the nutritional supply of the medium was increased. This effect was most pronounced for *Py. irregulare* where in complete media such as ¼ PDA and MEA, colonies of *P. radicum* were swamped by the rapid growth of *Py. irregulare*. In contrast, when the interaction was studied on 0.2% G, *P. radicum* produced a persistent zone of clearance in the growth of *Py. irregulare*. The nutrition conditions of 0.2% G are likely to be closer to the nutritional supply of the rhizosphere in comparison to either ¼ PDA or MEA. Hence, the results of 0.2% G may hold more relevance to rhizosphere interactions. Data from interactions on 0.2% G agar suggest

that *P. radicum* may inhibit the growth and possibly pathogenicity of *Py. irregulare, B. sorokiniana, F. pseudograminearum* and *R. solani*. While *Ggt* did not grow on 0.2% G agar, inhibition effects seen on ¼ PDA suggest that *P. radicum* may also inhibit the growth of *Ggt* and may affect the development of the take-all disease.

Where hyphae on dual inoculated plates were found to intermingle it was observed that *P. radicum* produced hyphal coiling around the pathogen. This response was found against *Py. irregulare, F. pseudograminearum*, *R. solani* and a non plant-pathogenic fungus, *A. niger*. This suggests that hyphal coiling is a general response of *P. radicum* to competing fungi. Hyphal coiling is usually interpreted as mycoparasitism (Fahima et al., 1992; Alagesaboopathi,1994; Pandey & Upadhyay, 2000). As *P. radicum* produced cell wall degrading enzymes, β-1,3 and β-1,4-glucanase with protease, this gave further evidence that *P. radicum* may act as a mycoparasite. The pattern of lytic enzyme activity suggests that *P. radicum* may degrade the cell walls of fungi belonging to the phylum Oomycetes (*Pythium*) that have cell walls predominantly composed of glucan polymers.

The % I.R. was used as an index of the ability of *P. radicum* to antagonise pathogenic fungi. The inhibition in the growth of the plant pathogenic fungi in dual culture with *P. radicum* may have been due to a combination of the following effects: (1) production of inhibitory metabolites, (2) slower growth rate of the pathogen across agar that has been depleted of nutrients, (3) formation of hyphae that act as a physical barrier preventing the spread of the pathogen in a symmetrical fashion across the Petri dish.

To distinguish between the production of inhibitory metabolites from the other two mechanisms an antibiosis assay was used. Using this assay it was identified that *Py. irregulare* was the most sensitive of the fungi studied to the presence of diffusible *P. radicum* metabolites. It was also identified that the pathogens *Ggt*, *B. sorokiniana* and *R. solani* were inhibited by *P. radicum* exudates.

F. pseudograminearum was not inhibited on ¼ PDA by diffusible *P. radicum* exudates but was significantly inhibited on 0.2% G agar, suggesting that the susceptibility of this pathogen to inhibition by *P. radicum* was significantly influenced by the physiological state of the pathogen. However, in dual inoculated plates the colony inhibition of *F. pseudograminearum* was markedly different from inhibition effects due to *P. radicum* exudates alone. In dual inoculation with *P. radicum* the % I.R. of *F. pseudograminearum* colony growth was greater on ¼ PDA in comparison to 0.2% G agar. This suggests that in the presence of *P. radicum* other factors inhibited the colony growth of *F. pseudograminearum.* For example, *P. radicum* may have required the presence of *F. pseudograminearum* to elicit the production of metabolites toxic to *F. pseudograminearum.* Another explanation would be that the rate of colony extension by *F. pseudograminearum* away from *P. radicum* was greater on ¼ PDA than for 0.2% G agar. This growth response would then account for the increased % I.R.

It is interesting to note that the inhibition of *Py. irregulare* was greater by *P. radicum* metabolites than inhibition by the intact organism on both of the test media. This result

could be explained as the application of a plug taken from a mature colony or *P. radicum* would have a concentration of inhibitory metabolites while the simultaneous inoculation of the pathogen against *P. radicum* would occur on a medium that contained no inhibitory metabolites. This would allow the *Py. irregulare* colony to quickly establish on a dual inoculated plate while in comparison the colony against the plug of *P. radicum* exudates would be inhibited. Another more interesting explanation for this observation would suggest that an interaction between the two organisms occurs where *Py. irregulare* recognises *P. radicum* and attenuates the antagonism or the toxicity of the metabolites produced by *P. radicum*. Evidence for this hypothesis comes from other studies between pathogen and biocontrol agents where pathogen signals or pathogen self-defense compounds modulate gene expression in biocontrol agents to counteract their antagonism. For example, in strains of *Ps. fluorescens* the antibiotic 2,4-diacetylphloroglucinol (DAPG) was shown to play a key role in the biological control of a broad range of plant pathogens (Keel et al., 1992; Duffy et al., 2004). However, for some *Ps. fluorescens* strains the presence of the metabolite, fusaric acid, produced by *Fusarium oxysporum* f. sp. *radicis-lycopersici*, blocked the expression of DAPG biosynthesis genes halting the synthesis of DAPG (Duffy et al., 2004). Similarly, the presence of intact *P. radicum* cells may elicit the production of factors by *Py. irregulare* that down regulate synthesis or detoxify metabolites that are inhibitory to growth of the pathogen.

When averaged across the two test media, *R. solani* had the lowest overall % I.R. in the presence of diffusible *P. radicum* metabolites. This was consistent with observations

on dual inoculated Petri dishes where there were no clearance zones in the interface of the two fungal colonies. Hence, biological control effects of *R. solani* by *P. radicum*, if present, may be more associated with mycoparasitism as seen under SEM. However, as *P. radicum* produces only low chitinase activity, *P. radicum* may not be effective as a biological control agent of *R. solani* or as a mycoparasite of Basidiomycetes or Ascomycetes (*Ggt*; *F. pseudograminearum*; *B. sorokiniana*) as chitin is an important component of the cell wall of fungi belonging to these two phyla.

Glucose oxidase activity was assayed as it may act as a mechanism that *P. radicum* uses to inhibit the growth of competing fungi. The glucose oxidase enzyme catalyses the oxidation of β-D-glucose to gluconic acid and hydrogen peroxide. The toxic effect of glucose oxidase is associated with the by-product hydrogen peroxide (Ayer & Racok, 1990). In *P. radicum* culture it was found that the levels of glucose oxidase were within the ranges of glucose oxidase activity that were detected for other *Penicillium* spp. by Petruccioli et al. (1993) who found that *P. italicum* NRRL 983 produced 0.89 and *P. variabile* NRRL 1048, 2.04 μmol mL^{-1} min^{-1}, respectively.

Glucose oxidase activity has been found to be an important mechanism of biological control by *Talaromyces flavus* (Klocker) Samson and Stolk (anamorph *P. dangeardii* Pitt, synonym *P. vermiculatum* Dang.) which has potential for control of several soilborne diseases such as verticillium wilt (Fahima & Henis, 1990; Fahima et al., 1992; Nagtzaam & Bollen, 1997). As *P. radicum* is a closely related fungus (for the asexual or anamorphic growth phase, both fungi produce biverticilliate conidiophores

and produce characteristic yellow pigmentation) similarities may also exist between the two fungi in mechanisms that inhibit the growth of soilborne fungal pathogens. Hence, it is worth considering the mechanisms that *T. flavus* uses to inhibit soilborne pathogens as there may be similarities for *P. radicum*.

While glucose oxidase may act as an important biological control mechanism by *T. flavus*, the antagonism of fungal pathogens by products of glucose oxidase activity in the rhizosphere is likely to be limited by the production and availability of the enzyme substrate, glucose (Kim et al., 1988; Fravel & Roberts, 1991; Murray et al., 1997). Therefore, glucose oxidase activity may not always function as the predominant mechanism of biological control. As a result it is considered that a number of factors are associated with the biocontrol activity of *T. flavus*. For example, a range of isolates of *T. flavus* have been shown to be rhizosphere competent (Marois et al., 1984; Tjamos & Fravel, 1997) and exhibit hyperparasitic behaviour towards pathogens such as *R. solani* (Boosalis, 1956), *Verticillium* (Fahima & Henis, 1990; Fahima et al., 1992) and *Sclerotinia* (McLaren et al., 1986). The role of mycoparastism as a mechanism of biocontrol activity may be reflected in the production of lytic enzymes (Madi et al., 1997; Inglis & Kawchuk 2002). The presence of other antifungal metabolites such as 2-methylsorbic acid in culture filtrates suggests that a range of other potentially inhibitory metabolites are produced by *T. flavus* cultures (Proksa et al., 1992). Thus, when the mechanisms of biocontrol by *T. flavus* are taken with experimental data presented on *P. radicum* in part of this Chapter, it is likely that the mechanism of pathogen inhibition by *P. radicum* is likely to involve a number of components.

In addition to morphological similarities between *P. radicum* and *T. flavus*, a similarity also appears to exist in the production of enzymes by the two fungi. In this study it was found that *P. radicum* produced β 1,3- and β 1,4-glucanase activity but only low levels of chitinase activity. This profile of enzyme activity (for these three enzymes) was also reported for *T. flavus* by Inglis & Kawchuk (2002). This pattern of enzyme activity suggests that both of these fungi may degrade the cell walls of fungi that are composed of glucans (Oomycetes) but may not be effective against fungi with chitin based cell walls (Ascomycetes & Basidiomycetes). Fungal isolates of either *P. radicum* or *T. flavus* with higher chitinase activity may be more effective biocontrol agents against Ascomycete and Basidiomycete fungi. For example, Madi et al. (1997) correlated lytic enzyme activity (glucanase, cellulase and chitinase) of a number of different *T. flavus* wild type isolates and benomyl mutants against reduction of stem rot caused by *Sclerotium rolfsii* and found that biocontrol was related to chitinase activity.

While biocontrol studies most frequently focus on pathogen inhibition by the biocontrol agent, the converse (i.e inhibition of the biocontrol agent by the pathogen) should also be considered. Pathogens possess self defense mechanisms to counteract antagonism and encourage niché colonisation in the presence of antagonists. Duffy et al., (2003) considered this and suggested that the inhibition of biocontrol agents by pathogens could be a contributing factor to the low efficacy of biocontrol agents. Work conducted in this Chapter has shown that depending on a range of factors, including nutrient supply and the relative timing of colony establishment, the presence of

pathogens may inhibit the growth of *P. radicum*. If *in-vitro* inhibition is reflected under soil/rhizosphere conditions, the presence of soilborne pathogens could prevent the establishment of *P. radicum* inoculant in the rhizosphere and may account for some of the variability pertaining to performance of *P. radicum* under field conditions.

The experimental data presented here suggests that *P. radicum* may act as an effective control agent of *Py. irregulare*. The production of cell wall degrading enzymes and direct inhibition of colony growth indicated that *P. radicum* may suppress the pathogenicity of *Py. irregulare* via at least two mechanisms (mycoparasitism & production of inhibitory metabolites). As shown in this Chapter, the outcome of a *P. radicum* - *Py. irregulare* interaction is likely to depend on the nutrient status of the two fungi and other factors such as timing of colony establishment and the presence of the intact organism versus the metabolites. Therefore, to determine the effect of *P. radicum* on the plant pathogenicity of *Py. irregulare* the interaction of the two fungi needs to be studied in the presence of plant roots. Further research should seek to identify the effect of *P. radicum* on the pathogenicity of *Pythium* spp so that use of *P. radicum* in this capacity can be optimised. Further work to identify the effect of *P. radicum* on root disease caused by *Py. irregulare* in a bioassay is required.

While *Py. irregulare* is a potential candidate as a target pathogen for disease control by *P. radicum, in-vitro* data suggests that *P. radicum* may also inhibit the growth and reduce the pathogenicity of a range of soilborne pathogens such as *Ggt*, *R. solani*, *B. sorokiniana* and *F. pseudograminearum*. As *Ggt* is a widespread root pathogen and

causes loss to cereal production for Australian agriculture (see Table 1.1) a number of bioassays are already in existence that can be utilised as a tool to screen *P. radicum* for biocontrol activity. The effect of *P. radicum* on the development of disease caused by this pathogen was further studied in Chapter 8.

In summary, if *in-vitro* mechanisms of fungal antagonism operate under soil conditions, there is evidence to suggest that *P. radicum* may inhibit the growth of soilborne root pathogens *Ggt, R. solani, Py. irregulare, F. pseudograminearum* and *B. sorokiniana* and therefore assist in the suppression of root diseases. The suppression of root diseases may then account for growth promotion effects seen by *P. radicum* under field conditions.

Despite data on the mechanisms of fungal interaction that can be obtained from *in-vitro* studies, they are readily criticised as not simulating environmental or field conditions and they do not consider the interaction of fungi with the plant host within the complexity of the rhizosphere of field soils. As result there may not be a high correlation between *in-vitro* antagonism and suppression of disease observed in the field (Papavizas & Lumsden, 1986). Glasshouse trials need to be performed to validate the ability of *P. radicum* to suppress the severity of cereal root disease caused by the pathogens *Ggt, R. solani, Py. irregulare, B. sorokiniana* and *F. pseudograminearum* before *in-vitro* antibiosis data can be reported as a mechanism of cereal root disease suppression. The ability of *P. radicum* to control the cereal root disease, take-all, in a wheat root environment is investigated in the next Chapter.

CHAPTER 8. THE EFFECT OF *PENICILLIUM RADICUM* ON ROOT DISEASE CAUSED BY *GAEUMANNOMYCES GRAMINIS* VAR. *TRITICI* IN A WHEAT SEEDLING BIOASSAY

8.1 Introduction

Results presented in Chapter 7 indicated that *P. radicum* grown *in-vitro* possessed several mechanisms (i.e. production of compounds that inhibited the growth of root pathogens and mycoparasitic behaviour) that may confer on the fungus an ability to suppress cereal root disease. However, *in-vitro* tests are frequently criticised as providing an inappropriate assessment of disease control mechanisms as they do not consider the relationship of the antagonist and fungal pathogen to the plant host nor do they include environmental variables of field conditions. Hence, the effect of *P. radicum* on plant growth in the presence of root disease needs to be evaluated in a root environment before there is evidence of disease suppressing activity.

The fungus *Gaeumannomyces graminis* var. *tritici (Ggt)*, is the causal agent of take-all disease. Take-all is a widespread and economically important cereal root disease in Australian wheat production (see section 1.6). Under *in-vitro* conditions, *P. radicum* inhibited the colony growth of *Ggt* and diffusible *P. radicum* metabolites also inhibited the colony growth of *Ggt*. Hence, *P. radicum* may reduce the severity of take-all by directly inhibiting the growth of the causative pathogen. If *P. radicum* inhibits the growth of *Ggt* under field conditions, then a reduction in the severity of root disease caused by this fungus may account for plant growth promotion in response to *P. radicum* inoculation.

In this Chapter the ability of *P. radicum* to suppress root disease was investigated using two experiments that varied in the patterns of *P. radicum* and *Ggt* inoculation. In the first experiment *P. radicum* was distributed evenly throughout a sand/ field soil (70:30, sand:field soil) growth medium and *Ggt* was placed in a band that was located 1 cm below the germinating seed. It was estimated that seedling roots had approximately 1 day to allow *P. radicum* to become established on the roots before they came into contact with *Ggt*. In the second experiment, the effect of *P. radicum* on the severity of take-all was determined on a field soil that was not mixed with sand in order to more closely simulate interactions under field conditions. In this experiment, both *P. radicum* and *Ggt* were mixed evenly throughout the soil.

The experimental objective of this Chapter was to:

- to determine if the reduction of root disease could account for the plant growth response to inoculation by studying the effect of *P. radicum* on the severity of root disease caused by *Ggt* in a sand based wheat seedling bioassay and in an artificially infested field soil.

8.2 Methods

8.2.1 Experiment 1. Effect of P. radicum *on take-all severity in a wheat seedling bioassay using a sand/soil medium.*

The methods used in this bioassay, described below, was based on that described in Ryder & Rovira (1993). The soil used in this experiment was obtained from the topsoil (10 cm) of an experimental agriculture research site in Kapunda, South Australia. The plant growth medium consisted of non-sterile quartz sand mixed with non-sterile

Kapunda soil that had been sieved through 3 mm. The sand was mixed with the field soil at a ratio of 70:30 (w/w), sand: soil.

Inocula of *Ggt* and *P. radicum* were both prepared as infested ryegrass propagules as described in Section 2.1.2. The ryegrass inocula were then added directly to the growth medium and distributed evenly throughout the growth medium. The effect of *P. radicum* inoculation on the severity of take-all disease was assessed by a factorial experiment of *Ggt* addition (+,-) at a rate of 0.26 g kg^{-1} with different rates of *P. radicum* inoculum addition (0, 0.07, 0.13, 0.26, 0.52 & 1.0 g kg^{-1}). Each experimental treatment had four replicates. Inoculated soil or non-inoculated soils were used to fill pots (300 mL capacity, 6.5 cm height x 10 cm depth) according to the following banding procedure:

1. FIRST BAND (7 cm): 280 g of sand/soil inoculated with *Ggt* plus the different amounts of *P. radicum* added
2. SECOND BAND (1 cm): 50 g of sand/soil inoculated with *P. radicum*.
3. Seven wheat seeds (*T. aestivum* L. cv. Frame) that had been pregerminated overnight on filter paper moistened with sterile deionised water and placed at the top of the second band.
4. THIRD BAND (1 cm): top layer of sand/soil mix (50 g) contained no inoculum.
5. Plastic beads added to the surface to standardise the total weight of all the pots and reduce evaporation.

A schematic diagram of the banding pattern of inoculated and non-inoculated soil used to fill the pots is given in Figure 8.1.

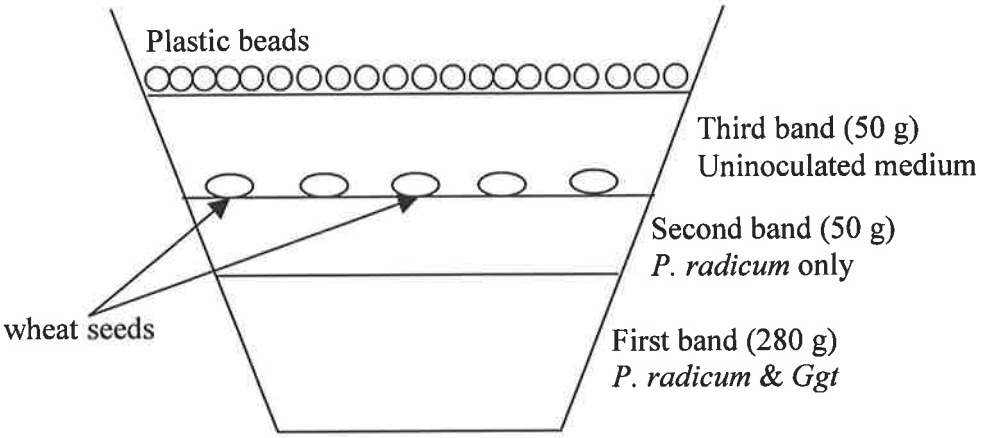


Figure 8.1. Banding procedure used to fill the pots in Experiment 1. In the first band both *P. radicum* and *Ggt* were added, for the second medium only *P. radicum* was added. Seven pregerminated seeds, thinned to five, were added on top of the second band. In the third band, used to cover the seeds, the soil mix was not inoculated. Plastic beads were added as the final layer to reduce moisture loss by evaporation.

After the growth medium was filled into the pots, Hoaglands nutrient solution (Hoagland & Arnon, 1938) was added to each pot to bring the final moisture content of the growth medium to 6.7% (w/w). Deionised water was then used to maintain soil moisture throughout the growing period. Seedlings were grown for 4 weeks at 15 °C in a controlled environment chamber at humidity of approximately 41% and set on a 16/8 hour day/night light regime with a light intensity of approximately 700 µmol m^{-2} s^{-1}. Plants were harvested after 4 weeks, dry weight and P contents were determined as outlined in Section 2.2.2 and root disease was determined as outlined in Section 8.2.3.

8.2.2 Experiment 2. Effect of P. radicum *on take-all severity in a field soil.*

In the second experiment the effect of *P. radicum* on a *Ggt* population artificially introduced into a non-sterile field soil was determined. Field soil taken from the top 10 cm from Kapunda, SA, was sieved to less than 3 mm. Characteristics of the field soil

(red-brown earth, pH $_{(water\ 1:5)}$ 6.2, extractable P (Colwell, 1963) 46 mg kg^{-1}) were also as reported previously by Ryder & Rovira (1993). A factorial design of *Ggt* (+,-) added at a rate of 0.26 g kg^{-1} and *P. radicum* (+,-) added at a rate of 1 g kg^{-1}, mixed evenly throughout the soil was used. Each treatment was replicated four times. A total of 1 kg of the inoculated sieved soil was added to pots (12 cm depth x 12 cm width). A total of 5 wheat seeds (cv Frame) were added to each pot (depth 2 cm) and the field soil was then moistened to 8% w/w using deionised water. The plants were left to grow in a controlled temperature water bath (15 °C) that was located in a temperature controlled glasshouse (Section 2.2.1). Following seedling emergence the number of plants were thinned to three per pot. Plants were harvested at four and eight weeks. Plant dry weight and tissue P were analysed as described in Section 2.2.2 and root disease determined as described in Section 8.2.3.

8.2.3 Root disease analysis

After separation from the shoots, the roots were analysed for root disease. The seminal root length was measured in a shallow water bath with a Perspex ruler. The lesioned section of the root was determined as the length of blackened section of the seminal root. The severity of take-all was assessed as a ratio of the length of lesioned seminal roots to the total seminal root length and expressed as the % seminal root lesioning. All data was subjected to an analysis of variance using Genstat 5.

8. 3 Results

8.3.1 Experiment 1. The effect of P. radicum *on take-all severity in a wheat seedling bioassay*

8.3.1 (i) Plant growth and water consumption

The addition of *P. radicum* had no significant effect on the dry shoot weight of either non-inoculated control plants or those inoculated with *Ggt*. Plants inoculated with *Ggt* had lower shoot weights.

Trends in the dry shoot weight were generally reflected in the seedling water consumption; the addition of *P. radicum* alone did not significantly influence water consumption and plants inoculated with *Ggt* had significantly lower water consumption than control plants (Table 8.1). However, in some cases *P. radicum* inoculation significantly increased water consumption of plants that were also inoculated with *Ggt* (Table 8.1) suggesting that *P. radicum* increased the growth of diseased plants. However, this trend was not reflected in shoot dry weights.

8.3.1 (ii) P nutrition

Inoculation with *P. radicum* significantly increased the total shoot P uptake (there was no effect of inoculation on shoot P concentration) of control seedlings at all rates of *P. radicum* inoculation (Table 8.1). The tissue P concentrations of plants inoculated with *Ggt* was not analysed.

8.3.1 (iii) Root disease

Inspection of a random sample of roots that were not treated with *Ggt* inoculum indicated that there were no disease symptoms and hence no background *Ggt* in the plant growth medium. When *Ggt* was added to the plant growth medium in the absence of *P. radicum*, there was a mean 35% seminal root lesioning.

In the presence of *Ggt*, *P. radicum* inoculation increased the mean total seminal root length from 37.8 cm to 44.2 cm. Increasing the inoculation rates of *P. radicum* significantly reduced the % lesioning of seminal roots. *P. radicum* inoculated at a rate of 1 mg kg^{-1} reduced % seminal root lesioning from 35% (no *P. radicum*) to 23.5%. This corresponded to a 32% reduction in disease severity.

Table 8.1 The effect of *P. radicum* on wheat seedling growth and take-all disease severity in a sand : soil (70 : 30) based seedling bioassay with artificially added populations of *Ggt* (summary of results from Experiment 1). *P. radicum* was added as infested ryegrass propagules to a soil-sand mix at five rates. Data is the mean of four replicates.

P. radicum inoculation	Water consumption (mL)		Dry shoot weight (mg pot^{-1})		Shoot P (mg kg^{-1})		Total shoot P (µg pot^{-1})		Seminal root length (cm)		% seminal root lesioning	
(g kg^{-1})	control	*Ggt*	control	*Ggt*	control	*Ggt*	control	*Ggt*	control	*Ggt*	control	*Ggt*
0	67	48.3	0.38	0.29	0.15	x	585	x	x	37.8	0	35.3
0.07	64	54.6	0.37	0.33	0.20	x	740	x	x	49.1	0	29.9
0.13	67	50.8	0.39	0.30	0.17	x	663	x	x	43.1	0	26.2
0.26	68	51.5	0.37	0.31	0.19	x	703	x	x	42.7	0	26.8
0.52	67	56.2	0.42	0.33	0.17	x	714	x	x	42.8	0	25.1
1	70	51.0	0.38	0.31	0.19	x	703	x	x	42.9	0	23.5
l.s.d (α 0.05)	4	3.0	0.05	0.05	0.04		57			4.9		6.1

x – not determined.

8.3.2 Experiment 2. The effect of P. radicum *on take-all severity in a field soil.*

8.3.2 (i) Plant growth

After four weeks, the mean dry shoot weight of control plants was 0.16 g and inoculation with *P. radicum* increased the mean shoot weight to 0.19 g (Table 8.2). Seedlings inoculated with *Ggt* had significantly lower dry shoot weight. *P. radicum* did not significantly affect the dry shoot weight of *Ggt* inoculated plants. These trends in dry shoot weight were also seen in shoot heights, where *P. radicum* increased the mean shoot height by 2.3 cm but had no effect on the shoot height of plants inoculated with *Ggt*. *P. radicum* had no effect on the root weight compared to non-diseased uninoculated controls, but root weight was significantly reduced in the presence of *Ggt*.

8.3.2 (ii) P nutrition

Shoot P nutrition (Table 8.2) of the seedlings followed trends seen in the shoot dry weight. Inoculation with *P. radicum* did not significantly influence shoot P concentration there was however, a significant increase in shoot P uptake. *Ggt* reduced the shoot P concentration and uptake. In the presence of *Ggt*, *P. radicum* had no significant effect on shoot P content.

P nutrition of the roots was significantly affected by all fungal treatments. *P. radicum* reduced concentration of P in root tissue, while *Ggt* increased the P concentration.

8.3.2 (iii) Root disease

The total seminal root length was not altered by any of the treatments at the four-week harvest. The grand mean of the total seminal root length across all treatments was 31 cm. The % seminal root lesioning caused by the addition of *Ggt* was 19%, the addition of *P. radicum* increased the root lesioning to 26%.

8.3.2 (iv) Seedling and disease development after 8 weeks growth

A harvest was conducted at 8 weeks to determine if *P. radicum* had any effects on plant growth and disease severity with longer incubation time. *P. radicum* significantly increased the shoot weight of control plants. While the dry shoot weight of plants that received *Ggt* treatments were lower in comparison to controls, the dry shoot weight of *Ggt* inoculated plants was not significantly affected by *P. radicum* inoculation.

For control plants, *P. radicum* had an effect on P nutrition. The mean shoot P concentration of plants that were inoculated with *P. radicum* was higher than those plants that were not inoculated, but the difference was not statistically significant. The total shoot P uptake was significantly increased by *P. radicum* inoculation. Plants inoculated with *Ggt* had an overall lower P nutrition that was unaffected by *P. radicum* inoculation.

The mean total seminal root length of *Ggt* inoculated plants was 44 ± 1.4 cm (± s.e) while dual inoculation of *P. radicum* and *Ggt* resulted in a mean root length of 40 ± 1.2 cm. The % seminal root lesioning of *Ggt* treated roots was 43.5% while the seminal

root lesioning in the combined treatment of *Ggt* and *P. radicum* was not altered at 45.7% infection.

Table 8.2. The effect of *P. radicum* 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.

Table 8.2a 4 week harvest

treatment	Shoot height (cm)	Dry shoot weight (g pot^{-1})	Dry root weight (g pot^{-1})	Shoot P		Root P		Seminal root length (cm)	% seminal root lesioning
				(mg kg^{-1})	(µg P pot^{-1})	(mg kg^{-1})	(µg P pot^{-1})		
Control	25.9	0.16	0.06	3800	610	1098	75.7	31.8	nil
P. radicum	28.4	0.19	0.06	4050	785	888	46.4	29.8	nil
Ggt	25.0	0.14	0.07	2950	400	1815	129.6	29.6	19
P. radicum + *Ggt*	24.4	0.15	0.03	2673	388	2425	77.7	31.5	26
l.s.d. (α 0.05)	1.7	0.02	0.02	295	71	55	38.8	4.1	4

Table 8.2b 8 week harvest

treatment	Dry shoot wt (g pot^{-1})	Shoot P (mg kg^{-1})	Shoot P uptake (µg P pot^{-1})	Seminal root length (cm)	% seminal root lesioning
Control	0.41	413	1721	> 50[A]	0
P. radicum	0.47	495	2347	> 50	0
Ggt	0.18	358	666	45	19.5
P. radicum + *Ggt*	0.19	338	629	40	18.3
l.s.d. (α 0.05)	0.04	101	348	nd	7.7

[A]roots were measured to 50 cm in total length of all seminal roots (10 cm maximum depth of pots used to grow seedlings).

8.4 Discussion

In field soil that was not inoculated with *Ggt*, *P. radicum* increased the shoot dry weight of plants, providing further evidence of the growth promoting activity of this fungus. However, the plant growth promotion of *P. radicum* was inconsistent between the two Experiments. In Experiment 1, *P. radicum* did not increase the shoot dry weight but did increase shoot P uptake. For Experiment 2, *P. radicum* increased both shoot dry weight and P uptake. An increase in the P uptake of the plants suggested that increases in the plant growth were related to improved plant P nutrition.

In addition to differences in plant growth, the two experiments gave different results with respect to the effect of *P. radicum* on the disease development by *Ggt,* expressed as % seminal root length with take-all lesions. This result was likely to be related to the banding procedure (Expt 1) and the different conditions associated with use of a field soil and a soil:sand mix that were used to grow the wheat seedlings. For Experiment 1, *P. radicum* was allowed to colonise the germinating roots before *Ggt*. Colonisation of the germinating seed and juvenile roots in the absence of *Ggt* would have allowed *P. radicum* access to nutrients such as C and N provided in seed and root exudates and may have facilitated establishment in the rhizosphere. In Experiment 1, increasing doses of *P. radicum* decreased the size of lesions caused by *Ggt*. Reduction in lesion size may have been due to either A) direct antagonism by either antibiosis or mycoparasitism (as seen in the previous Chapter) or B) improved nutritional status of the plant. As no plant growth trend occurred with reduction in lesion size it is possible that direct interaction between the two fungi rather than factors affecting plant growth were responsible for

reduced root disease. However, there are no data on the interaction of these two fungi in the rhizosphere to confirm this theory. Further work, such as direct observation of *P. radicum-Ggt* interactions on the surface of wheat roots, could identify whether direct antagonism is associated with reduction of take-all lesions.

If *P. radicum* increased the nutritional status of the plants, this could have increased the tolerance of the plant to disease. A number of studies have shown that P deficient soils tend to favour take-all and that the application of P fertiliser to such soils is to an extent beneficial in reducing the damage caused by take-all (Stumbo et al., 1942; Brennan, 1988; Brennan, 1989; Brennan, 1995). Increasing the P content may allow the roots to outgrow the disease, or reduce the leakiness of the cells so that less substrate is released from the root for growth of *Ggt* (Brennan, 1989; Neate, 1994). However, the P nutrition of diseased plants was not measured in the first experiment where *P. radicum* reduced the lesion size of diseased roots. Consequently, there is no evidence to show that *P. radicum* increases the tolerance of plants to *Ggt* by improved nutrition.

In the second experiment, the growth promoting effect of *P. radicum* was withdrawn in the presence of *Ggt* and dual treatment (i.e. *Ggt* and *P. radicum*) reduced root weight and enhanced lesion size beyond the effect of *Ggt* alone. In this experiment both *Ggt* and *P. radicum* had the opportunity to colonise the germinated seedling simultaneously. The early establishment of *Ggt* in and around the germinating seed and developing roots, may have suppressed the establishment of *P. radicum* in the rhizosphere. *Ggt* has a far greater root penetration capacity than *P. radicum* and colonises the root cortex,

penetrates the suberised endodermis and enters the stele, whereas *P. radicum* is not likely to colonise the root beyond the epidermal layer. At the stage where *Ggt* hyphae are colonising internal tissues of the root, it is probable that they are protected from antagonism by *P. radicum* and have access to nutrients from decaying root cells. At this stage any competitive interaction between the two fungi would favour *Ggt*. The competitive interaction between *P. radicum* and *Ggt* would be dependent on their relative nutritional status. In Chapter 7, it was shown that on a complete medium, such as MEA, *P. radicum* had a low antagonistic potential while on ¼ strength PDA, growth inhibition by *P. radicum* was more pronounced.

Although *in-vitro* inhibition of *Ggt* by *P. radicum* was more prominent on ¼ PDA than in comparison to MEA, *Ggt* also inhibited the growth of *P. radicum*. Hence, it is possible that under soil conditions *Ggt* inhibited the growth of *P. radicum* from the ryegrass propagules. If this is so the germination of *P. radicum* from the ryegrass propagules may have been minimised in the presence of *Ggt*, therefore reducing the probability of contact between *P. radicum* and the roots. The inhibition of *P. radicum* by *Ggt* may be related to the production of antibiotic like compounds by *Ggt*. The production of inhibitory compounds by *Ggt* was found to reduce the growth of a range of fungi (McGinty et al., 1984) and may also be active against *P. radicum* under soil conditions. Hence, under soil conditions with a high take-all potential, the application of *P. radicum* may achieve a more consistent response when used in conjunction with other management strategies that reduce the damage of take-all.

If early rhizosphere establishment and root colonisation are important for seedling protection against *Ggt* by *P. radicum*, it is possible that a mechanism of disease suppression occurs on the rhizoplane (root surface) of young root tissue and not in the bulk soil. In this respect *P. radicum* may have acted as a biological protectant of the root surface (Cook & Baker, 1984) by colonising in or around infection courts reducing the extent of *Ggt* hyphae entering the root. This mechanism is thought to occur for rhizosphere competent fungi such as *Trichoderma* spp. that proliferate through the rhizosphere to colonise outer surfaces of the root (Ahmad & Baker, 1987). However, *P. radicum* is not a fast growing fungus like *Trichoderma* spp. and as such the growing roots are likely to exceed the growth rate of *P. radicum* so that only a small proportion of the root would be protected by the hyphae of *P. radicum*. This would leave large areas of the root where infection courts would still be open to infection by the pathogen.

In the first experiment, there was approximately 1 cm between *P. radicum* inoculum located next to the seed and the band of *Ggt* inoculum. This spatial separation may have provided sufficient space and time for *P. radicum* to become established on the roots without competition from *Ggt*. In addition, the *Ggt* may have needed to grow further from the propagule to the root before infection occurred. When *Ggt* hyphae are growing through the soil they have poor saprophytic competitiveness (Simon, 1989; Cook, 1994) and hence the fungus is vulnerable to inhibition by antagonists such as *P. radicum*. As the *P. radicum* inoculum increased in density its inhibition activity increased and the ability of the *Ggt* hyphae to grow through the soil decreased resulting in reduced root infection.

In summary, these experiments have demonstrated that *P. radicum* has the potential to suppress *Ggt*. However, early root colonisation and rhizosphere establishment appears to be very important for *P. radicum* to reduce the severity of take-all. This may be because *P. radicum* has a low competitiveness against *Ggt* in the rhizosphere and a low ability to inhibit growth of the pathogen if *Ggt* has already parasitised the root. Consequently, in the presence of *Ggt*, *P. radicum* is prevented from achieving populations in the rhizosphere that are sufficient to achieve disease suppression and hence growth promotion.

Results from these experiments suggest that to encourage growth enhancement and increase the effectiveness of *P. radicum* for disease suppression, the method of inoculation employed by farmers should ensure early rhizosphere establishment and root colonisation. This may be best achieved by the current practice where *P. radicum* is applied as a seed coating. However, seed coat inoculation was not used in the experiments reported here as seed coating was not a part of the established methodology for the bioassay system used.

When the efficacy of *P. radicum* seed coat treatment was evaluated in a field soil that was naturally infested with the *Ggt* pathogen, it was found that *P. radicum* did not significantly influence plant weight, but increased the extent of root lesioning caused by *Ggt* (Anstis et al., 2003). This response was in agreement with Experiment 2 of this

Chapter. This suggests that there are factors other than early root colonisation that are in important in reducing *Ggt* root disease by *P. radicum*.

The ability of *P. radicum* to reduce root lesioning may also be related to the greater level of soil colonisation achieved by adding *P. radicum* as ryegrass propagules that were distributed through the soil. The level of *P. radicum* populations achieved by adding the fungus as organic propagules mixed throughout the soil would be greater than the soil populations where the fungus was inoculated as a seed coat. The higher level of *P. radicum* populations in the soil may explain why increasing rates of ryegrass addition further reduced root lesioning.

If the level of *P. radicum* populations present in the soil were important for reduction of root lesioning, it is possible that the antagonism of *Ggt* in the bulk soil (outside of the rhizosphere) may have played a part in reducing *Ggt* hyphal growth from the propagule so that initial root infection was also reduced. This hypothesis is supported from *in-vitro* data where the two fungi competitively inhibited each other and in some instances the inhibition of *Ggt* was much greater than for *P. radicum*. On growth medium that supplied 0.2% glucose as the only added nutrient, *P. radicum* produced hyphal growth and abundant sporulation while *Ggt* did not produce visible colonies. This suggests that where carbon is at a low availability (such as in the bulk soil), *P. radicum* will outgrow *Ggt* and therefore have an advantage in a competitive interaction.

If the level of *P. radicum* colonisation in the soil/rhizosphere plays an important role in the competitive interaction with *Ggt*, the addition of a food base with inoculation to specifically enhance the growth of *P. radicum* may increase the efficacy of *P. radicum* inoculation by encouraging a quicker and greater establishment in the rhizosphere. However, in light of these experiments, the C source provided in the food base must encourage the growth of *P. radicum* while at the same time not act as a C source for competing root pathogens such as *Ggt* that may eliminate the beneficial effect of *P. radicum* inoculation. Further research into the growth of *P. radicum* relative to *Ggt* in the presence of formulation additions should be conducted so that a selective enhancement can be obtained.

CHAPTER 9. GENERAL DISCUSSION

9.1 Introduction: Project aims & background

The aim of this project was to identify the mechanisms of plant growth promotion by *P. radicum*. In this thesis, three plant growth promotion mechanisms deemed relevant to the action of beneficial rhizosphere *Penicillium* sp. were investigated: P solubilisation, production of auxin, and interaction of *P. radicum* with root pathogens.

9.2 P solubilisation

Characteristics of P solubilisation by *P. radicum* were measured in liquid medium and compared to that of a known P solubilising fungus, *P. bilaiae* RS7B-SD1. In liquid culture, rock phosphate (RP) solubilisation by *P. radicum* was increased by NH_4^+ supply (in comparison to NO_3^- supply) and was removed in the presence of buffering. These results suggested that *P. radicum* used acidification as the main mechanism of P solubilisation. Acidification is a widely recognised mechanism of P solubilisation by soil microorganisms (Halvorson et al., 1990; Illmer & Schinner, 1992; Illmer & Schinner, 1995; Whitelaw et al., 1999). The source of acidification often originates from the export of protons following NH_4^+ assimilation (Roos & Luckner, 1984).

If acidification is the primary source of P solubilisation, *P. radicum* is likely to be an effective P solubiliser under alkaline conditions. However, P solubilisation mediated by *P. radicum* is only likely to occur where fungal acidification can overcome the buffering capacity of the soil and where there supply of NH_4^+ ions is adequate to drive this acidification. Under alkaline soil conditions a low pH buffering and high NH_4^+

concentration is unlikely to exist and hence, P solubilisation is unlikely to operate. In agreement with this statement, other studies have shown that (Eyre Peninsula Farming Systems 2002 Summary, Minnipa Agricultural Centre, SARDI; Bio-Care Technology, field trials, unpublished results) *P. radicum* did not solubilise P in alkaline soils. While other studies have suggested that fungal P solubilisation can operate under alkaline conditions (Salih et al., 1989; Kucey & Leggett, 1989; Kucey, 1987), these P solubilising microorganisms are likely to possess different mechanisms of P solubilisation such as the release of organic acids (e.g citric acid) that can effectively mobilise P by chelation in addition to acidification mechanisms. Further research could investigate improving inoculant formulation to increase the growth of *P. radicum* under alkaline soil pH or amending the formulation with nutrients such as NH_4^+ ions that could increase the P solubilising activity of *P. radicum*. These strategies may improve the P solubilising and/or plant growth response of *P. radicum* under alkaline soil conditions.

Studies on RP solubilisation in liquid culture showed that for the isolate *P. bilaiae* RS7B-SD1, the N source or medium buffering did not significantly affect the rate of P solubilisation. As this isolate, *P. bilaiae* RS7B-SD1, had a consistent RP solubilising activity over a range of cultural conditions and given that there is a market opportunity and an environmental need for a P solubilising inoculant, further research should look to assess the potential of this fungus to solubilise P under glasshouse and field conditions. The results of such trials could then be used to evaluate the development of a commercially available P solubilising inoculant based on this fungus.

Characteristics of P solubilisation by *P. radicum* in liquid culture (Chapter 3) were also seen under sand culture assay (Chapter 4). In sand culture assay as for liquid culture, RP solubilisation was most effective in the presence of NH_4^+ as the sole N supply. Under sand culture conditions *P. radicum* increased the solubility and plant P uptake of $CaHPO_4$ but had no effect on the solubility and plant uptake of Fe-P. This P solubilising activity was also seen in liquid culture of *P. radicum* (Whitelaw et al., 1999). This suggests that *in-vitro* studies accurately reflect the metabolic activity of the fungus in the presence of plant roots.

While the complexity of soil P chemistry and the presence of other microorganisms is not completely modelled under sand culture conditions, the sand culture method provided a useful tool to show that *P. radicum* used at least two discrete plant growth promoting mechanisms; P solubilisation and a general mechanism of plant growth promotion. This finding is similar to other studies where microorganisms that solubilised P *in-vitro* did not always have an effect on the plant P uptake and hence P solubilisation did not always explain increases in plant growth after inoculation with P solubilising microorganisms. For example, de Freitas et al. (1997) found that plant growth promoting microorganisms from the rhizosphere of canola solubilised P *in-vitro*. However, none of these P solubilising microorganisms increased plant P uptake from a P deficient soil. Cattelan et al. (1999) screened bacterial isolates from bulk soil and rhizosphere of soybean. Of 116 isolates, they found that five solubilised insoluble forms of P *in-vitro*. However, when the ability of these P solubilising isolates were tested for

their effects on soybean P uptake, only two isolates increased shoot P content. While *in-vitro* P solubilisation may be a common attribute of beneficial rhizosphere microorganisms, P solubilisation may not necessarily form the main mechanism of plant growth promotion.

Section 1.2.3 gave examples where isotopic labelling with ^{32}P had been used to determine if solubilisation mechanisms increased the amount of P available for plant uptake. If *P. radicum* had solubilised insoluble P for increased plant uptake there should have been clear and consistent differences in the plant *L*-values (Chapter 5). As there was no effect on the *L*-value in response to inoculation it is concluded that *P. radicum* does not have P solubilising ability as a major mode of action. When this data was taken together with the results of the sand culture assay (Chapter 4) that separated a P response from a general plant growth increase, there was strong evidence to investigate mechanisms of growth promotion not related to P solubilisation. As the production of auxins has previously been implicated as a mechanism of microbial induced plant growth promotion (Frankenberger & Poth, 1987; Arshad & Frankenberger, 1998; Asghar et al., 2002) the ability of *P. radicum* to produce the auxin IAA was investigated.

9.3 Production of auxin

In liquid medium supplemented with the precursor tryptophan (TRP), *P. radicum* produced IAA at physiologically active concentrations. However, further research is needed to correlate the effects of IAA production by *P. radicum* with effects on plant growth.

The effect of IAA production of *P. radicum* as a mechanism of plant growth promotion may be obtained by generating *P. radicum* mutants that have lost the ability to produce IAA. By studying the interaction of these (IAA^-) mutants with plant growth and development it may be possible to identify the effects that IAA production has on the plant. Dobbelaere et al. (1999) used different genetically modified strains of *Azospirillum brasilense* to determine the effects of IAA production in the plant growth effects of this root associated diazotrophic bacterium. Mutants that did not express an IAA synthesising enzyme did not alter root morphology. On the other hand, transconjugants that contained extra copies of this gene had enhanced effects on the root morphology. Dobbelaere et al. (1999) demonstrated that the use of IAA mutants can be used to determine the effects of IAA production on plant growth and development.

While alterations in root growth and morphology may be the result of microbial IAA production, IAA that is absorbed by the roots may be transported to active sites elsewhere in the plant up in the shoots (Scott, 1972; Martens & Frankenberger, 1994). Alternatively, IAA may be absorbed and conjugated to a biologically inactive compound to be hydrolysed at a later time upon requirement by the cell (Frankenberger & Arshad, 1995). Future research in this area should address these potential fates of IAA. This may be achieved by the use of isotopically labelled ^{14}C-TRP. Isotopically labelled precursor ^{14}C-TRP could be added to the sand culture system defined in Chapter 4. If the presence of isotopically labelled ^{14}C-IAA is found in the tissue of inoculated plants that had higher rates of growth, this would provide some evidence that

P. radicum had converted TRP to IAA that had been absorbed by the plants. However, such an approach would need to take into account that IAA may be degraded by the plant or simple oxidative degradation. To minimise losses of ^{14}C-IAA plants should be harvested after a short growth period of three to four weeks.

If *P. radicum* produced IAA (or IAA induced the production of ethylene, see Section 1.5.6) that stimulated root growth and initiated the formation of extra lateral roots or root hairs, this would create extra sites for infection by root pathogens. Hence, in the presence of root pathogens *P. radicum* inoculation would lead to a higher rate of infection. This hypothesis may explain why the inoculation of *P. radicum* increased the root infection by *Ggt* in comparison with *Ggt* inoculation alone (Chapter 8). Similarly, other studies have observed that the exposure roots to ethylene that were infected with *F. oxysporum* increased the development of root disease incited by this pathogen (Collins & Scheffer, 1958; Graham & Linderman 1981).

9.4 Interaction of *P. radicum* with soilborne pathogens

As mentioned in Section 1.6.4, several *Penicillium* spp. have been shown to suppress root diseases in a number of plant species caused by a variety of soilborne pathogens (see Table 1.2). Given that *Penicillium* spp. have previously been associated with the reduction of root diseases, the interaction of *P. radicum* with root pathogens was studied as a potential mechanism of plant growth promotion.

Under laboratory culture *P. radicum* possessed a variety of metabolic and growth activities that are sought in an effective biological control agent. These were: (a)

characteristics of a facultative mycoparasite that was evidenced by hyphal coiling and (b) the production of cell wall degrading enzymes glucanase, cellulase, protease and; (c) thc production of antibiotic exudates including glucose oxidase.

There is a large body of literature that supports the role of mycoparasitism and the production of lytic enzymes as a mechanism of biocontrol (Sundheim & Tronsmo, 1988; Fahima et al., 1992; Whipps, 2001). However, for *P. radicum* further research is required to identify if mycoparasitism effectively operates as a mode of biocontrol under field soil conditions.

The role of antibiosis in biocontrol has been demonstrated in a number of cases (for a review on the topic see Whipps, 2001; Haas & Keel, 2003). For example production of antibiotics such as phenazine carboxylic acid (Thomashow & Weller, 1988) and 2,4-diacetlyphloroglucinol (Keel et al., 1992) has been shown to be an important component in the suppression of take-all by *Pseudomonas* spp. For fungi such as *Trichoderma* spp. production of antibiotics (eg pyrone-type compounds) is likely to have a major role in biocontrol (Ghisalberti et al., 1990; Screenivasaprasad & Manibhushanrao, 1990; Perello et al., 2003). Given that antibiosis has been established for a number of other biocontrol microorganisms and that *Penicillium* spp. are well known antibiotic producers, it would not be surprising if *P. radicum* uses antibiosis to antagonise root pathogens.

While *in-vitro* studies generated data on the interaction of *P. radicum* with soilborne pathogens, *in-vitro* conditions do not mimic the complexity of the wheat rhizosphere nor do they include the host plant. As a result there may not be a high degree of correlation between *in-vitro* pathogen interaction and suppression of root disease in non-sterile conditions. Renwick et al. (1991) found that less than 50% of rhizosphere isolates capable of reducing take-all *in-vivo* inhibited the growth of *Ggt in-vitro*. This may be because *in-vitro* interactions typically focus on a single or simplified mechanism, whereas the actual mode of biocontrol may be more complex or involve other mechanisms.

Further reasons for a lack of correlation between *in-vitro* activity and disease suppression under soil conditions may be related to the availability of C substrates to the organism and the fate of microbial metabolites. For example, the production of antibiotics would require adequate nutrition for which *P. radicum* would be in competition with resident organisms. If *P. radicum* does produce antibiotic compounds, their effectiveness to inhibit pathogens would also depend on inactivation of the antibiotics by processes such as adsorption to clay and humus surfaces, microbial degradation and instability or inactivation due to pH (Baker, 1968).

To gain a greater understanding of the interaction of *P. radicum* with pathogens in the presence of a plant host, the ability of *P. radicum* to reduce take-all disease was investigated in a glasshouse bioassay. A reduction in the root lesioning caused by take-all was seen when *P. radicum* was given access to the wheat seedling roots prior to

colonisation by *Ggt*. This may have allowed *P. radicum* to establish itself in the rhizosphere and on the juvenile roots. However, when *Ggt* was allowed access to the roots at the same time and in competition with *P. radicum* at the seed germination stage, there was no reduction in the severity of the take-all disease.

This result suggested that *P. radicum* reduced root infection by inhibiting the saprophytic growth of *Ggt* hyphae through the soil. By increasing the inoculum density of *P. radicum* in a band between the *Ggt* propagules and the seed, the inhibition potential toward *Ggt* was increased and the amount of seminal root infection that *Ggt* caused was reduced. However, if *Ggt* inoculum was mixed uniformly throughout the soil *P. radicum* had little effect on disease. Mixing *Ggt* inoculum throughout the soil would have placed *Ggt* propagules in close proximity to the roots where they readily cause primary infection of the seedling (Kabbage & Bockus, 2002). Once *Ggt* had infected the root it would have access to adequate nutrients and protection from fungal antagonism.

While *P. radicum* inhibited a range of pathogens *in-vitro*, further *in-vivo* trials are needed to clarify the effect of *P. radicum* on disease caused by fungi such as *Fusarium* spp., *Pythium* spp and *Bipolaris sorokiniana*. This clarification could be achieved via glasshouse and field based plant disease trials.

9.5 Further mechanisms to account for plant growth promotion by *P. radicum*.

Despite data indicating that P solubilisation was not the major mode of action, there are numerous examples that may be found within literature (Whitelaw et al., 1997) and

throughout this thesis that demonstrate *P. radicum* can increase the P nutrition of plants. While a P solubilisation mechanism may not satisfactorily explain this response it is possible that *P. radicum* stimulates root growth either via hormone production such as auxin (as suggested in Chapter 6) or protection from root pathogens (as suggested in Chapters 7 & 8). The stimulated root growth due to either auxin production or reduction in root pathogen damage would increase the volume of soil that could be foraged for P and hence increase the amount of P that was taken up by the plant. This suggestion is supported by the *L*-value experiments where in some instances, *P. radicum* increased the root mass. In addition, the increased utilisation of Ca-P in the sand culture assay (Chapter 4) may have also been the result of stimulated root growth.

Another explanation that may account for the variable plant P response to *P. radicum* inoculation, is a *P. radicum*-VAM interaction. As noted in Section 1.3.4, Kucey (1983) observed that a rhizosphere biological P solubilising system exists and the presence of mycorrhizae were required the introduction of a P solubilising microorganism to achieve the maximum benefit to plants. Hence, it is hypothesised that VAM could significantly increase the effectiveness of *P. radicum* inoculation. This hypothesis is supported by a number of studies that have identified that the co-inoculation of VAM and P solubilising microorganisms resulted in greater plant benefits than inoculation with either organism alone (Azcon et al., 1976; Kucey. 1983; Toro et al., 1996; Toro et al., 1997; Barea et al., 2000). Unfortunately, the interaction of VAM with *P. radicum* was not considered in this thesis and to date there are no published studies on the interaction of *P. radicum* with VAM. Hence, it is unknown if *P. radicum* requires VAM

to achieve a significant influence on plant growth or P nutrition. If such a *P. radicum*-VAM interaction exists, the absence of adequate mycorrhizal colonisation may account for the lack of a significant P response measured in Chapter 5 (*L*-value experiments). For Chapter 8 where *P. radicum* significantly increased shoot P uptake, the soil:sand growth medium may have contained sufficient VAM propagules to allow the extra P solubilised by *P. radicum* to be transported to the roots. If *P. radicum* requires the presence of VAM, the lack of active mycorrhizal populations may also account for the variability to *P. radicum* inoculation as observed under field conditions (see Section 1.4.2).

For an inoculant to be effective, it should establish stable populations in the rhizosphere. However, the establishment of an introduced microorganism in the rhizosphere is likely to produce an altered rhizosphere community (in comparison to a non-inoculated rhizosphere). A number of studies have identified that the introduction of a microbial inoculant has altered the rhizosphere community and function (Mar-Vazquez et al., 2000; Cicillo et al., 2002; Reyes et al., 2002). If the rhizosphere community is altered in such a way that the proportion of pathogen antagonists or plant growth stimulators is increased (plant growth promoting rhizobacteria), an interaction of rhizosphere microflora with the inoculant may account for increased plant growth. As there is very little published data on the interaction of *P. radicum* with other groups of rhizosphere microorganisms, it is possible that the modification of the rhizosphere community following *P. radicum* inoculation may account for plant growth promotion. For example, Whitelaw et al., (1997) found that in comparison to uninoculated controls,

the incidence of P solubilising fungi was higher in the rhizosphere of field grown wheat plants that had been sown as seed inoculated with *P. radicum*. This increased P solubilising population may have been responsible for an increased P nutrition in *P. radicum* inoculated plots. In an analogous manner to P solubilising fungi reported by Whitelaw et al. (1997), *P. radicum* may interact with deleterious rhizosphere bacteria to reduce their activity or interact with plant beneficial rhizosphere bacteria to increase their activity. Unfortunately, the interaction of *P. radicum* with other groups of rhizosphere microorganisms was not considered in this thesis and to date there is no published research on this topic.

9.6 Future Research

As mentioned previously future research on mechanisms of plant growth promotion could focus on the use of *P. radicum* IAA^- mutants, studies on P solubilisation in other soils and the disease reduction caused by other fungi such as *Fusarium* spp., *Pythium* spp. and *Bipolaris sorokiniana*.

The greatest hurdle to the widespread adoption of biological inoculants as a standard farming practice is the lack of a consistent plant growth response to inoculation. Further research could improve the consistency of inoculant response by (a) creating better inoculant strains or (b) defining conditions under which *P. radicum* can be expected to produce a plant growth response. Currently, only a single strain of *P. radicum* has been rigorously evaluated for plant growth promotion, the isolation or identification of other strains with a greater P solubilising or biocontrol potential could improve the efficacy to inoculation. While different studies have focused on the genetic improvement of

inoculants and the use of techniques such as mutagenesis or protoplast fusion to develop improved strains (Pe'er & Chet, 1990; Taylor & Harman, 1990; Sivan & Harman, 1991; Lalithakumari et al., 1996), the low likelihood of generating protoplast hybrids or mutants that are more active than the parent strains may prohibit this avenue of inoculant development (Migheli et al., 1995).

Further research should continue on the development of the Pr70 Release™ inoculant formulation. Previous studies have shown that appropriate inoculant formulation is a critical factor leading to improved inoculant efficacy. A good example where appropriate formulation of a biological inoculant has improved inoculation response can be found with the use of *Pseudomonas* spp. for biological control. For instance, Vidhyasekaran & Muthamilan (1995) showed that identification of the appropriate formulation of *Ps. fluorescens* increased the ability of inoculation to reduce the yield loss to chickpea wilt. Biocontrol with the appropriate formulations was as effective or more effective than control with chemical fungicides. In another study, inoculum preparation and addition of nutrients were shown to be key factors in optimising the biocontrol response of a *Ps. fluorescens* inoculant formulation (Moenne-Loccoz et al., 1999).

The aim of the inoculant formulation should be to allow persistence of the inoculant in the rhizosphere for as long as possible to obtain the maximum benefit for the plant. A critical factor in influencing the growth and persistence of *P. radicum* in the rhizosphere may be related to the addition of a food base to the inoculant formulation. This has been

previously shown for the biocontrol agent *T. flavus* where the addition of a bran food source to an alginate formulation increased the population densities in comparison to formulations that did not contain an organic food source (Papavizas, et al., 1987). After 16 weeks incubation in field soil, the population was approximately 800% of the original numbers added. In comparison there was no significant difference in the inoculant population when the condia were added as an alginate formulation that did not contain an organic food source.

The inoculant formulation should as much as possible selectively enhance the growth of *P. radicum* in the rhizosphere. No additive or component of the inoculant formulation should enhance the growth or act as a food source for other root pathogens or soil microbes that may competitively displace or inhibit the colonisation of *P. radicum* in the rhizosphere. For example, Harman et al., (1981) attempted to find the conditions required for maximum biocontrol activity of *Trichoderma hamatum.* The addition of chitin, cellulose and peat to the inoculant formulation all resulted in the increased growth of the biocontrol agent, but only chitin was effective in enhancing biocontrol activity. In contrast, the addition of cellulose increased disease as it served as a food source for the pathogen.

9.7 Conclusion

These studies have shown that *P. radicum* possesses a number of characteristics that may contribute to its ability to promote plant growth and yield. These were P solubilisation, production of auxin and antagonism of root pathogens. However, none of these attributes were shown to dramatically increase plant growth alone over the condition tested. Hence, the interaction between *P. radicum* and wheat roots is likely to involve multiple mechanisms.

Previous to the work described in this thesis it was regarded that P solubilisation was a major component of the plant growth promotion mechanism utilised by *P. radicum*. However, these studies showed that P solubilisation did not operate in four out of five soils and also had a requirement for NH_4^+. To improve the P solubilising response to inoculation it is suggested that inoculation be applied with ammoniacal fertiliser.

In this thesis it was shown that *P. radicum* increases plant growth via a general plant growth mechanism that was independent of P solubilisation. The general plant growth promotion mechanism may be related to the production of IAA. If the use of IAA mutants reveals that IAA production is responsible for growth promotion, an increased field consistency to inoculation may be obtained with the inclusion of the IAA precursor tryptophan in the inoculant formulation. Under laboratory and glasshouse conditions, *P. radicum* inhibited the growth of several important soilborne pathogens of cereal crops. Further research is needed to identify if this translates to an effective reduction of disease under field conditions.

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