Biol Fertil Soils (2004) 40: 36–43
DOI 10.1007/s00374-004-0750-6

ORIGINAL PAPER

Steven A. Wakelin · Rosemary A. Warren · Paul R. Harvey · Maarten H. Ryder

Phosphate solubilization by *Penicillium* spp. closely associated with wheat roots

Received: 7 August 2003 / Revised: 2 February 2004 / Accepted: 2 February 2004 / Published online: 20 April 2004

Abstract In this study we found that *Penicillium* spp. exhibiting P-solubilizing activity are common both on and in the roots of wheat plants grown in southern Australian agricultural soils. From 2,500 segments of washed and surface-disinfested root pieces, 608 and 223 fungi were isolated on a selective medium, respectively. All isolates were screened for P solubilization on solid medium containing hydroxyapatite (HA); 47 isolates (5.7%) solubilized HA and were identified as isolates of *Penicillium* or its teleomorphs. These isolates were evaluated for solubilization of Idaho rock phosphate (RP) in liquid culture. *Penicillium bilaiae* strain RS7B-SD1 was the most effective, mobilizing 101.7 mg P l^{-1} after 7 days. Other effective isolates included *Penicillium simplicissimum* (58.8 mg P l^{-1}), five strains of *Penicillium griseofulvum* (56.1–47.6 mg P l^{-1}), *Talaromyces flavus* (48.6 mg P l^{-1}) and two unidentified *Penicillium* spp. (50.7 and 50 mg P l^{-1}). A newly isolated strain of *Penicillium radicum* (KC1-SD1) mobilized 43.3 mg P l^{-1}. RP solubilization, biomass production and solution pH for *P. bilaiae* RS7B-SD1, *P. radicum* FRR4718 or *Penicillium* sp. 1 KC6-W2 was determined over time. *P. bilaiae* RS7B-SD1 solubilized the greatest amount of RP (112.7 mg P l^{-1}) and had the highest RP-solubilizing activity per unit of biomass produced (up to 603.2 μg P l^{-1} mg biomass^{-1} at 7 days growth). This study has identified new isolates of *Penicillium* fungi with high mineral phosphate solubilizing activity. These fungi are being investigated for the ability to increase crop production on strong P-retaining soils in Australia.

Keywords *Penicillium bilaiae* · *Penicillium radicum* · Phosphorus · Phosphate-solubilizing microorganisms · Wheat

S. A. Wakelin (✉) · R. A. Warren · P. R. Harvey · M. H. Ryder
CSIRO Land and Water,
PMB 2,
Glen Osmond, SA, 5064, Australia
e-mail: steven.wakelin@csiro.au
Tel.: +61-8-83038708
Fax: +61-8-83038684

Introduction

P is an essential element for plant development and growth. Plants acquire P from soil solution as phosphate anions; predominantly HPO_4^{2-} and $H_2PO_4^-$. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, $Fe^{3+}$and Al^{3+}, or adsorbed to Fe-oxides and Al-oxides, Al-silicates, and Ca-carbonates, depending on the particular properties of a soil. In these forms, P is highly insoluble and unavailable to plants. Accordingly, many agricultural soils are P deficient, making P one of the most important nutrient elements limiting agricultural production.

Microorganisms play a fundamental role in the biogeochemical cycling of P in natural and agricultural ecosystems. Gerretsen (1948) initially demonstrated that microbiological activity in the rhizosphere could dissolve sparingly soluble inorganic P and increase plant growth. Subsequently, many different bacterial, actinomycete and fungal species have been shown to have P-solubilizing activity. *Penicillium* fungi (including the teleomorphic states *Talaromyces* and *Eupenicillium*) are considered to be a key group of soil microflora involved in P cycling (recently reviewed by Whitelaw 2000). This activity is generally attributable to the production of organic acids that can directly dissolve P precipitates, or chelate P-precipitating cations with the concomitant release of P into solution (Kucey et al. 1989; Gadd 1999). Furthermore, *Penicillium* spp. are often isolated as components of the root microflora of a diverse range of crop plant species. The association of P-solubilizing *Penicillium* spp. with plant roots therefore has potential to influence P nutrition to crop plant species. The use of such fungi as inoculants for increasing crop P nutrition has been demonstrated by the successful commercial release of *Penicillium bilaiae* (JumpStart; Philom Bios, Saskatoon, Canada) and *Penicillium radicum* (PR-70 Release; Bio-Care Technology, Somersby, Australia).

The aim of this work was to investigate both the occurrence and levels of phosphate solubilization activity

of *Penicillium* spp. associated with the surface of wheat roots grown in Australian soils. The work forms part of our efforts towards understanding how to manage soil microbial communities based on specific functions (P solubilization), and selection of fungi as potential microbial inoculants (biofertilizers).

Methods and materials

Soils

Single soil samples were taken from 11 conventionally managed farms under grain crops in South Australia (SA), Victoria (Vic.), New South Wales (NSW) (RS1–RS4, RS5A, RS5B, RS6A, RS6B and RS7A–7C), and at multiple sites on three organically managed properties in NSW (AD1–AD4, KC1–KC6 and PS1-PS6). A brief physical description of each soil, the state from which they were sampled, and the crop sown at the time of sampling is given in Table 1. At each location, approximately 2 kg of soil from the top 0–10 cm was collected into a plastic bag, each of which were sealed and stored at room temperature (approximately 20°C) until use (up to 6 weeks). A total of 27 soil samples were taken.

Isolation of *Penicillium* spp. from wheat roots and assay for solubilization of hydroxyapatite

For each of the soil samples, a planting container (18 cm diameter with a capacity of 1 l) was filled with soil and planted with four surface-disinfested (3% NaOCl, 2 min) wheat seeds (*Triticum aestivum* cv. Krichauff). The wheat plants were grown in a glasshouse and received water as required. After 6 weeks, the roots were recovered and washed under running water. Remaining soil was removed by shaking the roots in Schott bottles containing sterile water. Approximately half of the washed root material from each sample was surface disinfested by immersing in 70% ethanol for 1 min and 3% NaOCl for 3 min. Surface-disinfested roots were washed free of sterilant in sterile water.

Table 1 Origin and description of soils in which wheat plants were grown; number of putative *Penicillium* fungi isolated from 100 pieces each of washed and surface-disinfested root sections; and the number and frequency of hydroxyapatite (*HA*)-solubilizing activity by the isolates. *SA* South Australia, *Vic.* Victoria, *NSW* New South Wales

Sample	Soil	State	Crop[a]	Washed roots		Surface-disinfested roots	
				Total to HA[b]	HA-clearing[c]	Total to HA[b]	HA-clearing[c]
RS1	Grey calcareous earth	SA	Wheat	60	1 (1.7)	8	0 (0.0)
RS2	Brown calcareous sand	Vic.	Wheat	36	1 (2.8)	0	0
RS3	Red-brown duplex	Vic.	Wheat	16	2 (12.5)	10	3 (30.0)
RS4	Calcareous sandy earth	SA	Medic	13	1 (7.7)	2	0 (0.0)
RS5A	Red sandy earth	Vic.	Wheat	5	0 (0.0)	0	0
RS5B	Calcareous earth	SA	Wheat	23	0 (0.0)	21	0 (0.0)
RS6A	Grey cracking clay	Vic.	Wheat	5	0 (0.0)	11	0 (0.0)
RS6B	Brown siliceous sand	Vic.	Wheat	9	2 (22.2)	2	0 (0.0)
RS7A	Calcareous sandy earth	SA	Wheat	10	3 (30.0)	0	0
RS7B	Calcareous sandy earth	SA	Medic	31	5 (16.1)	6	1 (16.7)
RS7C	Sandy alkaline yellow duplex	Vic.	Wheat	1	1 (100.0)	0	0
AD1	Red-brown earth	NSW	Wheat	78	0 (0.0)	14	0 (0.0)
AD2	Red-brown earth	NSW	Wheat	12	0 (0.0)	18	0 (0.0)
AD3	Alkaline red sand	NSW	Oats	15	1 (6.7)	9	0 (0.0)
AD4	Red-brown earth	NSW	Rye	14	1 (7.1)	10	1 (10.0)
KC1	Red-brown earth	NSW	Triticale	51	2 (3.9)	27	1 (3.7)
KC2	Red-brown earth	NSW	Wheat	3	0 (0.0)	11	0 (0.0)
KC3	Neutral red sand	NSW	Oats	37	3 (8.1)	6	2 (33.3)
KC4	Red-brown earth	NSW	Pasture	11	0 (0.0)	5	0 (0.0)
KC5	Red-brown earth	NSW	Pasture	18	4 (22.2)	3	0 (0.0)
KC6	Red-brown earth	NSW	Pasture	11	3 (27.3)	0	0
PS1	Yellow duplex	NSW	Pasture	48	1 (2.1)	13	0 (0.0)
PS2	Yellow duplex	NSW	Wheat	25	0 (0.0)	8	2 (25.0)
PS3	Yellow duplex	NSW	Wheat	29	0 (0.0)	25	0 (0.0)
PS4	Yellow duplex	NSW	Wheat	14	2 (14.3)	5	0 (0.0)
PS5	Yellow duplex	NSW	Pasture	14	2 (14.3)	6	0 (0.0)
PS6	Yellow duplex	NSW	Pasture	19	2 (10.5)	3	0 (0.0)
			Totals	608	37 (6.1)	223	10 (5.4)

[a]Plant type from under which the soil was sampled

[b]Total number of putative *Penicillium* fungi isolated (from 100 root segments for each treatment) and transferred onto HA-medium

[c]Number of putative HA-solubilizing *Penicillium* isolates and percentage (*in parentheses*) of the total screened

Fungal isolations were made from both washed and surface-disinfested roots (Table 1). Roots were aseptically sliced into 3- to 7-mm segments and transferred onto Petri plates containing dichloran, rose-bengal, chloramphenicol agar (DRBC), a medium semi-selective for *Penicillium* spp. (Pitt and Hocking 1985). From each soil, 100 root segments were plated for each of the two treatments (washed and surface disinfested). Petri plates were incubated at 25°C in the dark and fungal colonies arising from the root segments were selected for assessment of P solubilization. Colonies that clearly did not resemble *Penicillium* were discarded. When multiple, culturally-identical isolates were recovered from the same sample of root material, they were treated as clones of a single isolate and only one colony was screened for P-solubilization activity.

The initial assay for P solubilization was carried out on solid medium containing hydroxyapatite ($Ca_5OH(PO_4)_3$; HA), an insoluble form of tri-calcium phosphate. Each fungal isolate was subcultured onto a Petri plate containing NBRIY medium (Nautiyal 1999) with sucrose (10 g l^{-1}) as the sole carbon source. HA was dry-autoclaved and added to the cooled molten agar medium at 5 g l^{-1} immediately prior to pouring. Petri plates were incubated at 25°C in the dark for up to 14 days and inspected every second day for the presence of a clear zone around the colony margin signifying P solubilization.

HA-solubilizing isolates were subcultured onto malt-extract agar (MEA) and incubated at 25°C for 7 days. Hyphae from each culture were mounted onto microscope slides, stained with trypan blue (0.05% in lactoglycerol), and observed under a compound microscope. Isolates producing penicilli consistent with *Penicillium* or its teleomorphs *Talaromyces* or *Eupenicillium* (Pitt 2000) were transferred to MEA slants and stored at 5°C.

Quantification of Idaho rock phosphate-solubilizing activity by isolates of *Penicillium*

Fungal isolates capable of clearing HA were assayed in solution culture to quantify rock-phosphate (RP)-solubilizing activity. Idaho RP was ground, passed through a 200-μm mesh and washed thoroughly with deionized water. Following the method of Asea et al. (1988), 0.1 g of RP (13% P) was dry-autoclaved in 250 ml Erlenmeyer flasks and, after cooling, 100 ml of sterilized culture medium (0.1 g NaCl, 0.4 g NH_4Cl, 0.78 g KNO_3, 0.5 g $MgSO_4$, 0.1 g $CaCl_2{\cdot}2H_2O$, and 10 g sucrose l^{-1}) was added to each flask. Each isolate was inoculated into four replicate flasks and incubated at 25°C in the dark with gentle shaking. The inoculum for each flask consisted of two 3-mm-diameter agar (MEA) plugs taken from the edge of a 10-day-old fungal culture. Three control treatments were included in the experiment: (1) culture medium only, (2) culture medium+RP, and (3) culture medium+RP+two 3-mm-diameter fungal plugs. *Penicillium minioluteum* PS4-W2, an isolate that was unable to clear HA, was included in the experiment as a negative control. *P. radicum* FRR4718 was included as a P-solubilizing control.

After 7 days incubation, solution pH and soluble P levels were determined in each flask. In some instances, aliquots of the solution were first passed through a 0.2-μm filter membrane to remove thick polysaccharide-like exudates. Solution pH was measured using a Cyberscan 310 meter (Eutech Instruments, Singapore). Soluble P levels were determined colorimetrically using the Malachite-green reaction (Irving and McLaughlin 1990).

Idaho RP solubilization vs. biomass production over time

Using similar protocols as described previously, a subsequent experiment compared the P-solubilizing activities of *P. bilaiae* (RS7B-SD1), *P. radicum* (FRR4718) and *Penicillium* sp. 1 (KC6-W2) in relation to biomass production over time. A control treatment received sterile agar plugs. At each of eight sampling times (0, 1, 2, 3, 5, 7, 9, and 11 days after inoculation) four replicate flasks of each treatment were taken for analysis. Solution pH, and soluble P levels were determined as described above. Fungal biomass in each flask was determined by filtering the culture solution onto a pre-weighed Whatman no. 1 filter paper and determining the difference in mass after drying overnight at 60°C.

Identification of fungal isolates

The ten isolates most effective at solubilizing Idaho RP, two of the less effective isolates, and KC1-SD1 were identified based on nucleotide sequence data from the ITS1-5.8S-ITS2 (ITS) region of the nuclear ribosomal DNA (rDNA) gene. Mycelium from each isolate was recovered from a liquid culture (malt-extract broth, 25°C, 7 days) and ground to a fine powder in liquid nitrogen. DNA was extracted using alkaline lysis (100 mM TRIS, 100 mM NaCl, 1.0% SDS, 20 mM EDTA, 100 mM Na_2SO_3, pH 8) and phenol/chloroform extraction procedures (Harvey et al. 2000). Polymerase chain reaction (PCR) amplification was performed using the rDNA-ITS-specific primers ITS5 and ITS4 (White et al. 1990). Each reaction contained 0.2 μmol of each primer, 200 μmol of each dNTP, 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Promega) and 2.5 μl of 10×*Taq* DNA polymerase buffer and sterile deionized water to a total reaction volume of 25 μl. A 30-cycle PCR reaction was carried out on an Eppendorf mastercycler as follows: after an initial denaturation at 95°C for 4 min, each cycle consisted of 30 s denaturation at 95°C, primer annealing at 46°C for 60 s, and primer extension at 72°C for 30 s. PCR products were separated on a 1.5% (w/v) agarose gel, stained with ethidium bromide (1 μg ml^{-1}), and visualized under UV transillumination. PCR products were purified using silica-based spin columns (Concerto, LifeTechnologies), and sequenced following dye-deoxy terminator chemistry on an ABI 377 DNA Sequencer (NDNA, University of Newcastle, Australia). Nucleotide sequence data were compared with those on GenBank using the BlastN search utility. *Penicillium* isolates RS3-SD3, KC1-SD1, RS7B-W1 and KC6-W2 were sent to Food Science Australia (Sydney) for conformation of identification.

Results

Isolation of *Penicillium* spp. from wheat roots and assay for solubilization of HA

Isolates of *Penicillium* were recovered from both the surface and interior of the roots of wheat plants grown in all types of soils from different areas throughout SA, Vic. and NSW (Table 1). However, the frequency of isolation of the putative *Penicillium* isolates varied greatly across sample sites, from 60 (RS1) to 1 (RS7) per 100 root segments. Variation also occurred within individual soil types taken from under the same crops, within and between localities. For example, amongst the samples for red-brown earths cropped with wheat, the percentage of isolation ranged from 78% (AD1) to 3% (KC2). The differences in isolation frequency of *Penicillium* were therefore a result of site-specific factors.

From washed wheat roots, 608 fungi were transferred to culture medium containing HA. Of these, 37 (6.1%) formed zones of clearing around the colony margin due to solubilization of the tri-calcium phosphate (HA) (Table 1). All HA-solubilizing isolates were identified as species of *Penicillium* or its teleomorphs based on morphological features (Pitt 2000).

A total of 223 colonies from surface-disinfested wheat roots were tested for HA-solubilizing activity (Table 1);

ten were effective in solubilizing HA (4.5%). Fungal growth often initiated from the cut ends of the root segments and, in contrast to the washed root treatment, the emergence of hyphae from the surface-disinfested root segments was often slow, taking up to 2 weeks to become apparent. As such, it is likely that such isolates were occupying the internal regions of the root tissue. The selection of slowly emerging colonies was aided by the near absence of overgrowing fungi. However, actinomycetes were a common "contaminant" emerging from sections of surface-disinfested root segments and typically exhibited strong HA-solubilization activity (data not shown).

Although DRBC is semi-selective for *Penicillium* spp., fungi from a number of other genera were also isolated. *Fusarium* spp., in particular, were commonly isolated from washed root samples but did not solubilize HA.

Identification of fungal isolates

With the exception of RS3-SD3, close matches were found between the rDNA-ITS sequences of the *Penicillium* isolates investigated in this study and existing entries in the GenBank database. With additional support from microscopic examination of sporing structures, isolate RS7B-SD1 was identified as *P. bilaiae*; KC1-W1 and KC1-W8 as *Penicillium simplicissimum*; RS7B-W1, RS7C-W1, PS5-W3, RS3-SD1, and RS3-W1 as *Penicillium griseofulvum*; PS4-W2 as *P. minioluteum*; and RS7B-W2 as *Talaromyces flavus*.

The ITS-rDNA sequence of KC1-SD1 aligned closely (99.8% similarity) to *P. radicum* FRR4718. Although similarities between *P. radicum* FRR4718 and KC1-SD1 were found upon examination of the sporing structures, the mycelium of KC1-SD1 did not exhibit the yellow to orange pigmentation considered to be distinctive of *P. radicum* (Hocking et al. 1998). However, as we have previously observed large morphological variation in hyphal pigmentation, conidiation, and exudate production in *P. radicum* FRR4718 (colony sectoring; data not shown), it is likely that KC1-SD1 is an isolate of *P. radicum* exhibiting some morphological variation from strain FRR4718.

KC6-W2 was found to be morphologically consistent with *P. simplicissimum*. However, the ITS-rDNA sequence aligned most closely with a potentially distinct but undescribed species of *Penicillium*, closely related to *P. simplicissimum* and *Penicillium ochrochloron*, isolated from decaying mushrooms (Genbank accession no. AF125941). As the classification of this isolate is uncertain, it is hereafter referred to simply as *Penicillium* sp. 1 KC6-W2.

RS3-SD3 grew slowly and sporulated poorly on all culture media. The isolate had ampulliform phialides and, on G25N medium, produced a brown pigment; characteristics indicative of *P. griseofulvum*. However, the penicilli were so aberrant from *Penicillium* as to be almost *Trichoderma*-like. Therefore, we cannot confidently place this isolate into any of the current species-concepts for *Penicillium* and it is hereafter referred to as *Penicillium* sp. 2 RS3-SD3.

Quantification of Idaho RP-solubilizing activity by isolates of *Penicillium*

Prior to addition of RP or inoculation with agar plugs, the liquid culture medium had a pH of 4.9 and undetectable levels of P (Table 2). The addition of RP at 1 g l^{-1}, lifted the pH to 7.5 and a small amount of P (0.1 mg l^{-1}) was

Table 2 Idaho rock phosphate-solubilizing activity of *Penicillium* spp. isolated from washed (*W*) and surface-disinfested (*SD*) roots of wheat plants. Values are the mean of four replicates±SEM

Fungal isolate	mg P l^{-1}[a]	Solution pH[b]
RS7B-SD1, *P. bilaiae*	101.7±4.6 (78.2)	3.4±0.0
KC1-W1, *P. simplicissimum*	58.8±0.6 (45.2)	4.3±0.1
RS7B-W1, *P. griseofulvum*	56.1±1.6 (43.1)	4.4±0.0
RS7C-W1, *P. griseofulvum*	55.5±1.8 (42.7)	4.5±0.2
RS3-SD1, *P. griseofulvum*	54.4±1.4 (41.8)	4.3±0.0
PS5-W3, *P. griseofulvum*	50.7±1.1 (39.0)	4.2±0.0
KC6-W2, *Penicillium* sp. 1	50.7±5.7 (39.0)	3.8±0.0
RS3-SD3, *Penicillium* sp. 2	50.0±1.3 (38.5)	4.5±0.1
RS7B-W2, *T. flavus*	48.6±2.4 (37.4)	6.1±0.0
RS3-W1, *P. griseofulvum*	47.6±1.6 (36.6)	4.4±0.0
PS1-W1	46.9±2.2 (36.0)	4.2±0.1
RS7B-W3	46.7±0.2 (35.9)	4.5±0.0
RS1-W1	44.7±1.1 (34.4)	4.5±0.0
KC1-SD1, *P. radicum*	43.3±8.7 (33.3)	4.1±0.0
PS4-W2	42.4±5.7 (32.7)	3.7±0.1
KC3-SD1	41.6±1.7 (32.0)	4.2±0.0
RS7A-W1	41.0±0.8 (31.5)	4.3±0.1
RS7A-W3	40.2±3.7 (30.9)	3.5±0.1
PS2-SD2	38.1±6.5 (29.3)	5.1±0.7
KC6-W1	37.8±3.7 (29.1)	3.8±0.0
PS4-W1	37.2±2.6 (28.6)	3.7±0.0
RS7B-W7	36.3±2.5 (27.9)	4.5±0.1
RS6B-W1	35.9±8.3 (27.6)	3.7±0.1
RS3-W2	33.2±1.4 (25.6)	4.4±0.0
AD4-W3	31.8±5.4 (24.5)	3.6±0.0
PS6-W3	31.7±1.9 (24.4)	4.0±0.1
KC5-W3	30.0±1.3 (23.0)	4.1±0.4
RS7A-W2	28.2±2.1 (21.7)	3.5±0.1
P. radicum FRR4718	27.1±1.3 (20.8)	4.3±0.3
KC3-W1	24.9±3.7 (19.2)	5.4±0.7
RS2-W2	22.4±2.2 (17.2)	3.6±0.0
PS4-W2, *P. minioluteum*	14.5±2.4 (11.2)	4.7±0.2
KC1-W8, *P. simplicissimum*	2.8±1.2 (2.1)	6.9±0.1
Nil-control	0.0±0.0 (0.0)	4.9±0.1

[a]Amount and % (*in parentheses*) solubilization of total available rock P after 7 days incubation. *Note* 15 isolates, solubilizing <20 mg P l^{-1}, were omitted from the table

[b]Solution pH determined at the end of the experiment

liberated into solution. The addition of two sterile MEA agar plugs to the medium released a further 0.1 mg l^{-1} into solution and did not affect solution pH.

Penicillium isolates varied in their capacity to mobilize P from the Idaho RP source, ranging from 2.8 mg P l^{-1} for *P. simplicissimum* KC1-W8 to 101.7 mg P l^{-1} for *P. bilaiae* RS7B-SD1 (Table 2). Whilst *P. simplicissimum* KC1-W8 was the least effective at P mobilization, another isolate of the same species was very effective (KC1-W1; 58.8 mg P l^{-1}) indicating that the activity is not necessarily a species-wide trait. *P. minioluteum* PS4-W2, unable to clear HA on solid agar plates, was relatively ineffective at solubilizing RP in liquid culture (14.5 mg P l^{-1}).

There was a large difference between the level of RP solubilization between the strongest isolate, *P. bilaiae* RS7B-SD1 (101.7 mg P l^{-1}) and the second strongest, *P. simplicissimum* (58.8 mg P l^{-1}). *P. radicum* FRR4718 mobilized 20.8% (27.1 mg l^{-1}) of the available P into the culture solution.

All *Penicillium* isolates reduced the pH of the culture medium relative to the control treatments (Table 2). Overall, however, there was little relationship between the lowering of solution pH and increasing solution P (R^2=0.25; P<0.001). Despite this, the isolate that was least effective at P solubilization had the highest pH and vice versa (Table 2). In some instances, for example RS2-W2, a substantial reduction of solution pH was not associated with high levels of soluble P (pH 3.6, 22.4 mg P l^{-1}). Moreover, *T. flavus* RS7B-W2 released 48.6 mg P l^{-1} yet the final solution pH was 6.1.

P. minioluteum PS4-W2, unable to solubilize HA on solid medium, liberated a small quantity (14.5 mg P l^{-1}) of P from Idaho RP. Fifteen isolates ranked less effective at P solubilization than *P. minioluteum* PS4-W2 (data not shown in Table 2). These isolates were most likely to be those that demonstrated marginal or transient clearing on culture medium containing HA.

Idaho RP solubilization vs. biomass production over time

The Idaho RP-solubilizing activity of *P. bilaiae* RS7B-SD1, *P. radicum* FRR4718 and *Penicillium* sp. 1 KC6-W2 was measured in relation to biomass during the course of a solution culture experiment. After inoculation, each of the three *Penicillium* spp. significantly (P<0.05) decreased the pH of the growth solution relative to the control, reaching

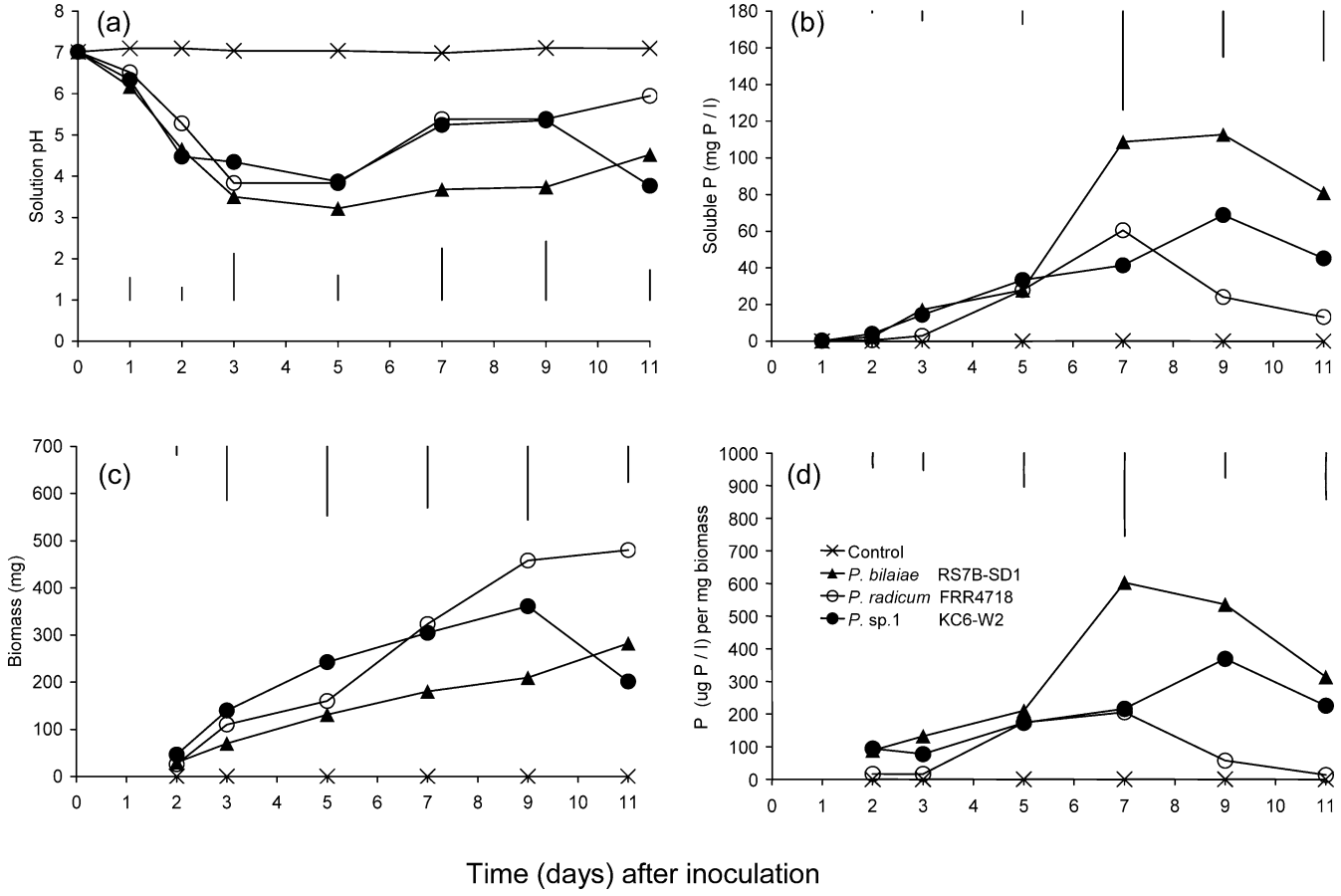

Fig. 1 Effect of *Penicillium bilaiae* RS7B-SD1, *Penicillium radicum* FRR4718 and *Penicillium* sp. KC6-W2 on **a** solution pH, **b** total P solubilization, **c** biomass production, and **d** P solubilization mg^{-1} fungal biomass production over time. Experiments were carried out at 25°C using Idaho rock phosphate as the sole P source. Each *data point* represents a mean of four replicates; *error bars* represent the LSD at the 5% level. The control treatment was inoculated with sterile agar plugs only

a minimum of 3.2 for *P. bilaiae*, 3.8 for *P. radicum* and 3.8 for (Fig. 1a). The pH then gradually rose towards neutrality for each of the fungal isolates (Fig. 1a). Solution pH in flasks inoculated with *P. bilaiae* was significantly ($P<0.05$) lower that the other treatments at days 5 and 7.

The soluble P at the beginning of the experiment was approximately 0.01 mg l^{-1} (Fig. 1b). *P. bilaiae* released significantly ($P<0.05$) more P from Idaho RP than the other isolates, reaching a maximum of 112.7 mg P l^{-1} after 9 days incubation. The maximum solution P in flasks inoculated with *Penicillium* sp. 1 KC6-W2 was 68.8 mg l^{-1}, significantly ($P<0.05$) greater than that in flasks inoculated with *P. radicum* (24.1 mg l^{-1}) at the same sampling time (day 9, Fig. 1b). The increase in solution P did not appear to be related to solution pH, as the soluble P peaks occurred well after minimum pH levels were measured. Furthermore, the largest increase in solution P, for example between days 3 and 7 for *P. radicum*, was associated with an increase in pH from 3.8 to 5.4 (Fig. 1a, b).

At the end of the experiment, *P. radicum* had produced significantly ($P<0.05$) more biomass than the other fungal treatments. Maximum biomass production for *P. radicum* was 480 mg and *P. bilaiae* 283 mg, both occurring at the end of the experiment. For *Penicillium* sp. 1 KC6-W2, maximum biomass production of 361 mg occurred 9 days after inoculation.

When P solubilization was considered with respect to fungal biomass production (Fig. 1d), it was clear that *P. bilaiae* RS7B-SD1 had a higher intrinsic ability to solubilize Idaho RP than the other two isolates. At each sampling time, the level of P solubilization by *P. bilaiae* RS7B-SD1 was significantly ($P<0.05$) greater than those of *P. radicum* (except at day 5), and greater than *Penicillium* sp. 1 KC6-W2 at days 2, 5 and 11. The maximum level of solubilization by *P. bilaiae* was 603.2 μg P l^{-1} mg biomass^{-1} at day 7. For each of the fungi, the capacity of each unit-biomass to solubilize Idaho RP varied over time. Therefore, P-solubilizing activity is related not only to biomass production, but the physiological state of the biomass (possibly the secretion of organic acids, etc.).

Discussion

Penicillium spp. (including *Talaromyces* and *Eupenicillium*), were frequently isolated from both washed and surface-disinfested wheat roots grown in a range of cropping soils. A sub-population of these root-associated *Penicillium* spp. had P-solubilizing activity (5.7%) and, as such, may be able to affect P availability to crop plants. We intentionally isolated fungi closely associated with the root surface (epiphytes or rhizoplane colonists) or within the root (endophytes or root invaders/inhabitors; sensu Garrett 1970) for the following reasons: (1) mineral P cycling in these regions is most likely to influence plant nutrition; (2) accessibility to root exudates that are required to drive the production of metabolites, such as organic acids, involved in P solubilization; and (3) potential to influence plant health through other means, such as increasing the root's absorptive capacity for minerals or nutrients (viz. mycorrhiza), altering the root architecture (Gulden and Vessey 2000), by controlling plant pathogens directly or stimulating plant defence mechanisms (Whipps 1997).

Fungi were isolated from organically and conventionally managed soils. By isolating from a diverse range of soils (soil types, localities, etc.) and from soils managed using different techniques (organic vs. conventional) it was anticipated that a more diverse range of *Penicillium* would be encountered. It was also thought that organically managed soils might have a higher frequency of P-solubilizing microorganisms as the soils can only be fertilized with low-grade inorganic (e.g. RP) or organic P. As low levels of plant-available P are often the result of such practices, plants grown on organically-managed farms may be dependent on enhanced soil biological activity as a mechanism to supply P (Ryan et al. 1994). In this study, we found large variability in the recovery of *Penicillium* between soils of the same type irrespective of the management system employed. Therefore, it was inappropriate to compare either the recovery of *Penicillium* from roots grown in conventional or organic soils taken from different areas, or the frequency of phosphate solubilization among those isolates. However, the isolation of *Penicillium* spp. from across a wide range of agricultural soils reflects the widespread distribution of this genus. Although *Penicillium* spp. are generally not reported as being root inhabitants, we made repeated isolations from surface-disinfested root segments. In most instances, hyphal growth initially appeared at the cut ends indicating that the fungi occupied the internal tissue of the host roots. Although it was not possible to determine the exact nature of the relationships fungi had with plant roots (i.e. epiphytic commensals, mutualistic symbionts, endophytes, pathogens, or merely casual invaders), it is clear that the roots of wheat plants are often closely associated with *Penicillium*.

The initial assay for P solubilization was carried out on solid medium containing precipitated calcium phosphate (HA). HA has previously been used in such studies (Illmer and Schinner 1992; Kucey 1983), however the use of calcium phosphates with higher solubility is far more common (reviewed in Whitelaw 2000). This may lead to overestimation of the P-solubilizing characteristics of microorganisms. For example, many microorganisms can mobilize P from inorganic compounds simply by lowering pH occurring as a result of general metabolic activity. Cellular H^+exudation to balance NH_4^+ uptake may also lower solution pH and propitiously increase the solubility of some forms of calcium phosphate (Asea et al. 1988). It is probable that such mechanisms will be neither expressed strongly nor constitutively in soil, so that their role in P mobilization would be of trivial value to plants. The high degree of insolubility of HA should provide a more robust test for P solubilization activity of microorganisms when used in such assays. A dramatic shift in pH would be

required to increase its solubility, and, therefore mechanisms such as secretion of strongly chelating organic acids (or the conjugate anions) must be expressed to effect P solubilization.

Of the *Penicillium* spp. associated with wheat roots, *P. bilaiae* RS7B-SD1 mobilized the most P from Idaho RP. A strain of *P. bilaiae*, sometimes referred to as *P. bilaii* or *P. bilaji*, has been found to increase P nutrition of wheat and other crop species (Kucey 1987; Kucey and Leggett 1989; Downey and van Kessel 1990), and is available commercially in Canada as an inoculant (JumpStart; Philom Bios). Under the assay conditions described in this work, *P. bilaiae* RS7B-SD1 released 101.7 mg P l^{-1} (7 days incubation) in the first assay, and up to 112 mg P l^{-1} in the second assay (9 days incubation). Using a similar type of RP, Asea et al. (1988) found the commercially developed strain solubilized 46–298 mg P l^{-1}; the variation being due to the constitution of the solution media, particularly NH_4^+availability. Given that culture composition and conditions qualitatively and quantitatively affect organic acid production (Cunningham and Kuiack 1992) and thereby P solubilization, comparing P solubilization rates between studies can be inappropriate.

Penicillium isolates were ranked for Idaho RP-solubilizing activity in a solution culture experiment. However, the experiment was potentially biased towards fast-growing isolates, as no allowances were made for biomass production between the isolates. Conceivably, *Penicillium* isolates with high growth rates would have a greater opportunity to solubilize P than slow-growing isolates. A second assay was therefore conducted to investigate the importance of such factors, by testing P solubilization in relation to biomass for three selected isolates (*P. bilaiae* RS7B-SD1, *Penicillium* sp. 1 KC6-W2, and *P. radicum* FRR4718) over time. The results showed that *P. bilaiae* RS7B-SD1 solubilized significantly more P from Idaho RP than the other two isolates whilst producing less biomass. As such, under the experimental conditions described, *P. bilaiae* RS7B-SD1 can be regarded as having a greater efficacy to solubilize P per unit biomass than either *P. radicum* FRR4718 or *Penicillium* sp. 1 KC6-W2. It is possible that some of the P solubilized from RP by the fungal isolates was incorporated into the biomass thereby underestimating their P-solubilizing activities. However, Illmer and Schinner (1992) found that only a small fraction (about 1%) of the P solubilized by isolates of *Penicillium aurantiogriseum* and a *Pseudomonas* sp. was incorporated into the biomass of the microorganisms despite virtually all of the P being solubilized.

The differences in P-solubilizing activity between the *Penicillium* isolates may be attributable to differences in the types and/or levels of organic acids produced in culture. *P. radicum* has been found to produce gluconic acid (Whitelaw et al. 1999), and *P. bilaiae* citric and oxalic acids (Cunningham and Kuiack 1992). The power of organic acids to complex with cations in RPs (thereby liberating P) varies considerably, with citric and oxalic acids among the strongest (Kpomblekou and Tabatabai 1994). Gluconic acid appears to have a very limited ability to release P complexed with Ca^{2+} (Illmer and Schinner 1992); however, it may have a role in P solubilization where Al^{3+} is the major cation binding P (Whitelaw et al. 1999).

The P-solubilizing ability of *P. radicum* varied between experiments and between isolates. In the first solution culture experiment *P. radicum* FRR4718 mobilized 27.1 mg P l^{-1} after 7 days incubation, yet in the second experiment mobilized 60.5 mg P l^{-1} after the same incubation time. *P. radicum* isolate KC1-SD1 mobilized 43.3 mg P l^{-1}. Much of this variation, however, could be accounted for by variability of the growth of *P. radicum* in culture. In some flasks, the fungus produced large amounts of a thick polysaccharide-like compound. In these instances, the solution P level was typically low, suggesting that the fungi were at different physiological states or that the P potentially had been incorporated into the organic matrix. In other flasks, the production of the polysaccharide was minimal and the solution P was much greater. The level of soluble P in the solution cultures fluctuated over time. It is eminently preferable, therefore, to measure P levels at time intervals over the duration of experiments when possible. The importance of this has been shown previously for *P. radicum* and other phosphate-solubilizing microorganisms (Illmer and Schinner 1992; Whitelaw et al. 1999) and is reiterated by the findings described in this study. On solid culture medium, we have previously observed culture sectoring by *P. radicum*, suggesting that the strain may have some inherent variability. This presents opportunities for enhancing the P-solubilizing characteristics of this isolate through selection of naturally arising variants. *P. radicum* KC1-SD1, for example, mobilized approximately 12% more P from RP than *P. radicum* FRR4718. However, mechanisms other than P solubilization account for a significant portion of plant growth promotion by *P. radicum* FRR4718 (Whitelaw et al. 1997).

P-solubilizing isolates of *P. griseofulvum* were commonly isolated from washed wheat roots. This species is common on the roots of wheat plants (Dewan and Sivasithamparam 1988) and may be an important component of the root microflora involved in P cycling. As *P. griseofulvum* is known to produce mycotoxins (Pitt 2000), and isolates have been found to cause disease on plant roots (Dewan and Sivasithamparam 1988), it is unlikely that this species will be of value as a microbial inoculant.

P-solubilizing isolates of *T. flavus* and *P. simplicissimum* were also associated with wheat roots. *T. flavus* has been shown to exhibit strong biological control activity towards soil-borne plant pathogens, particularly *Verticillium dahliae* (Marois et al. 1984) and other sclerotia-forming fungi. Isolates of *T. flavus* that can increase the supply of nutrients to plants in addition to having biological control characteristics would be desirable. Isolates of *P. simplicissimum* have previously been found to solubilize Al-phosphates and other metal-phosphates (Illmer et al. 1995; Sayer et al. 1995).

Penicillium spp. are often reported to exhibit traits such as mineral solubilization, biological control, and are

known to produce a wide range of secondary metabolites. As such, their potential to impact plant growth when in close association with the root system is clear. The challenge is how to make use of such biological resources to increase crop productivity. This study has identified several isolates of *Penicillium* fungi with high mineral phosphate-solubilizing activity. These isolates, particularly *P. bilaiae* RS7B-SD1, *Penicillium* sp. 1 KC6-W2 and *P. radicum*, are undergoing further testing for the ability to increase crop production on P-retaining soils in Australia.

Acknowledgements Dr Ailsa D Hocking, Food Science Australia, for *Penicillium* taxonomy. Bio-Care Technology, Somersby, NSW, Australia, for providing cultures of *Penicillium radicum* FRR4718. Dr Gupta Vadakattu (CSIRO) and Dr Stephen Barnett (SARDI) for helpful comments on this manuscript. This project is funded by the Grains Research & Development Corporation of Australia (project CS0223), and Bio-Care Technology, Australia.

References

Asea PEA, Kucey RMN, Stewart JWB (1988) Inorganic phosphate solubilization by two *Penicillium* species in solution culture and soil. Soil Biol Biochem 20:459–464

Cunningham JE, Kuiack C (1992) Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaii*. Appl Environ Microbiol 58:1451–1458

Dewan MM, Sivasithamparam K (1988) Occurrence of species of *Aspergillus* and *Penicillium* in roots of wheat and ryegrass and their effect on root rot caused by *Gaeumannomyces graminis* var. *tritici*. Aust J Bot 36:701–710

Downey J, van Kessel C (1990) Dual inoculation of *Pisum sativum* with *Rhizobium leguminosarum*and *Penicillium bilaji*. Biol Fertil Soils 10:194–196

Gadd GM (1999) Fungal production of citric and oxalic acid: importance in metal speciation, physiology and biogeochemical processes. Adv Microb Physiol 41:47–92

Garrett SD (1970) Pathogenic root-infecting fungi. Cambridge University Press, London, p 294

Gerretsen FC (1948) The influence of microorganisms on the phosphate intake by the plant. Plant Soil 1:51–81

Gulden RH, Vessey JK (2000) *Penicillium bilaii* inoculation increases root-hair production in field pea. Can J Plant Sci 80:801–804

Harvey PR, Butterworth PJ, Hawke BG, Pankhurst CE (2000) Genetic variation among populations of *Pythium irregulare*in southern Australia. Plant Pathol 49:619–627

Hocking AD, Whitelaw M, Harden TJ (1998) *Penicillium radicum* sp. nov. from the rhizosphere of Australian wheat. Mycol Res 102:801–806

Illmer P, Schinner F (1992) Solubilization of inorganic phosphates by microorganisms isolated from forest soils. Soil Biol Biochem 24:389–395

Illmer P, Barbato A, Schinner F (1995) Solubilization of hardly-soluble $AlPO_4$ with P-solubilizing microorganisms. Soil Biol Biochem 27:265–270

Irving GCJ, McLaughlin MJ (1990) A rapid and simple field test for phosphorus in Olsen and Bray No. 1 extracts of soil. Commun Soil Sci Plant Anal 21:2245–2255

Kpomblekou AK, Tabatabai MA (1994) Effect of organic acids on release of phosphorus from phosphate rocks. Soil Sci 158:442–453

Kucey RMN (1983) Phosphate-solubilizing bacteria and fungi in various cultivated and virgin Alberta soils. Can J Soil Sci 63:671–678

Kucey RMN (1987) Increased phosphorus uptake by wheat and field beans inoculated with a phosphorus-solubilizing *Penicillium bilaji* strain and with vesicular-arbuscular mycorrhizal fungi. Appl Environ Microbiol 53:2699–2703

Kucey RMN, Leggett ME (1989) Increased yields and phosphorus uptake by Westar canola (*Brassica napus* L.) inoculated with a phosphate-solubilizing isolate of *Penicillium bilaji*. Can J Soil Sci 69:425–432

Kucey RMN, Janzen HH, Leggett ME (1989) Microbially mediated increases in plant-available phosphorus. Adv Agron 42:199–228

Marois JJ, Fravel DR, Papavizas GC (1984) Ability of *Talaromyces flavus* to occupy the rhizosphere and its interaction with *Verticillium dahliae*. Soil Biol Biochem 16:387–390

Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265–270

Pitt JI (2000) A laboratory guide to common *Penicillium* species. Food Science Australia, North Ryde, NSW, p 197

Pitt JI, Hocking AD (1985) Fungi and food spoilage. CSIRO division of food processing, North Ryde, NSW, p 413

Ryan MH, Chilvers GA, Dumaresq DC (1994) Colonisation of wheat by VA-mycorrhizal fungi was found to be higher on a farm managed in an organic manner than on a conventional neighbour. Plant Soil 160:33–40

Sayer JA, Raggett SL, Gadd GM (1995) Solubilization of insoluble metal compounds by soil fungi: development of a screening method for solubilizing ability and metal tolerance. Mycol Res 99:987–993

Whipps J (1997) Developments in the biological control of soil-borne plant pathogens. Adv Bot Res 26:1–134

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, Calif., pp 315–322

Whitelaw MA (2000) Growth promotion of plants inoculated with phosphate-solubilizing fungi. Adv Agron 69:99–151

Whitelaw MA, Harden TJ, Bender GL (1997) Plant growth promotion of wheat inoculated with *Penicillium radicum* sp. nov. Aust J Soil Res 35:291–300

Whitelaw MA, Harden TJ, Helyar KR (1999) Phosphate solubilisation in solution culture by the soil fungus *Penicillium radicum*. Soil Biol Biochem 31:655–665