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Molecular characterisation, pathogenesis and fungicide sensitivity of *Pythium* spp. from table beet (*Beta vulgaris* var. *vulgaris*) grown in the Lockyer Valley, Queensland

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Abstract. Table beet production in the Lockyer Valley of south-eastern Queensland is known to be adversely affected by soilborne root disease from infection by *Pythium* spp. However, little is known regarding the species or genotypes that are the causal agents of both pre- and post-emergence damping off. Based on RFLP analysis with *Hha*I, *Hin*fI and *Mbo*I of the PCR amplified ITS region DNA from soil and diseased plant samples, the majority of 130 *Pythium* isolates could be grouped into three genotypes, designated LVP A, LVP B and LVP C. These groups comprised 43, 41 and 7% of all isolates, respectively. Deoxyribonucleic acid sequence analysis of the ITS region indicated that LVP A was a strain of *Pythium aphanidermatum*, with greater than 99% similarity to the corresponding *P. aphanidermatum* sequences from the publicly accessible databases. The DNA sequences from LVP B and LVP C were most closely related to *P. ultimum* and *P. dissotocum*, respectively. Lower frequencies of other distinct isolates with unique RFLP patterns were also obtained with high levels of similarity (>97%) to *P. heterothallicum*, *P. periplocum* and genotypes of *P. ultimum* other than LVP B. Inoculation trials of 1- and 4-week-old beet seedlings indicated that compared with isolates of the LVP B genotype, a higher frequency of LVP A isolates caused disease. Isolates with the LVP A, LVP B and LVP C genotypes were highly sensitive to the fungicide Ridomil MZ, which suppressed radial growth on V8 agar between approximately four and thirty fold at 5 µg/mL metalaxyl and 40 µg/mL mancozeb, a concentration far lower than the recommended field application rate.

Introduction

The genus *Pythium* is a member of the stramenopiles and, like the closely related oöymcota genus *Phytophthora* (Cooke *et al*. 2000), is noteworthy as the causal agent of numerous economically significant diseases of horticultural and broad acre crops (Davis and Bockus 2001; Davison and McKay 2003; Martin 1995; Moorman *et al*. 2002; Paulitz and Adams 2003; Weiland and Sundsbak 2000). Many oömycetes including the phytopathogenic *Pythium* spp. complete part of their life cycle in soil, as motile asexual zoospores that initiate infection at the root surface, and as oospores derived from sexual reproduction, which in comparison with zoospores are long-lived and more resistant to adverse conditions (Agrios 1997).

Approximately 90–95% of Australia's table beet (*Beta vulgaris* var. *vulgaris*) crop is produced in the Lockyer Valley of south-eastern Queensland, primarily for processed tinned beetroot. In 2000, this represented 35 584 t from a national production of 37 701 t (Cirillo 2001). In Queensland and other beet-growing regions of the world, *Pythium* spp. are recognised by industry, along with *Aphanomyces cochlioides* and *Rhizoctonia solani*, as the predominant soilborne pathogens implicated in the beetroot rot complex (Martin 2003; O'Brien *et al*. 1998). *Pythium* spp. cause both pre- and post-emergence damping off, typically under wet soil conditions when zoospores can migrate to the plant surface. In mature plants, infection by *Pythium* spp. can lead to poor yields and mis-shapen beets. Although *Pythium* spp. have been recognised for many years as significant soilborne pathogens of table beet in south-eastern Queensland, no work has been undertaken to date to identify and characterise the species of *Pythium* that are the causal agents.

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Traditionally, *Pythium* spp. have been identified on morphological features, particularly those of the antheridia, oogonia and associated oospores, supplemented by the structures producing zoospore-containing sporangia (Dick 1990). This method is time-consuming, cumbersome and sometimes open to subjective interpretation. More recently, DNA-based methods have been employed to identify the relevant *Pythium* spp. In contrast, DNA-based methods are often rapid and unambiguous, and in many cases do not rely on the culture and propagation of the suspected pathogen. In most of these studies, detection and identification has been based on polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) region of the genomic sequence encoding rRNA genes (rDNA), including one or more of ITS I, ITS II and 5.8S rDNA (Lévesque *et al*. 1998; Matsumoto *et al*. 1999; Moorman *et al*. 2002; Wang *et al*. 2003; Wang and White 1997; Weiland and Sundsbak 2000). Subsequent characterisation of *Pythium* isolates has been based on RFLP and/or DNA sequence analyses of the PCR amplified region or by hybridisation of the target DNA to arrays of the corresponding region from a range of oömycetes. To a lesser extent, detection and molecular characterisation of *Pythium* spp. have involved analyses using targets other than rDNA, including the genes encoding for actin and the ras-related protein and mitochondrial DNA (Weiland and Sundsbak 2000; Moorman *et al*. 2002; Wang *et al*. 2002).

In this paper we report on the characterisation of isolates of *Pythium* from seedlings of table beet showing typical symptoms of damping off, and from soils where diseased beets have been grown. Isolates were characterised based on the RFLP profile and DNA sequence of the region encompassing ITS I, 5.8S rDNA and ITS II. Further, the pathogenicity of each isolate was tested via Koch's postulates using table beet seedlings as the experimental host, and the response of representative isolates to Ridomil MZ, a commercially available fungicide recommended for use against oömycetes, was determined *in vitro*.

Methods

Isolation of Pythium

Pythium spp. were isolated from soil and diseased plants obtained from the properties of seven commercial beetroot growers in the Lockyer Valley, Queensland. Initially, isolates were cultured on potato-dextrose agar (PDA) and were tentatively identified as *Pythium* spp. based on morphological characteristics. To confirm that isolates were most likely *Pythium* spp., each isolate was subcultured on 20% clarified V8 agar supplemented with the selective agents pimaricin (20 mg/L), ampicillin (125 mg/L), rifampicin (10 mg/L) and pentachloronitrobenzene (PCNB; 133 mg/L) and morphological characters re-examined.

Deoxyribonucleic acid isolation

Genomic DNA was purified from fresh mycelium of each isolate according to a method modified from McDonald *et al*. (1994). Briefly, mycelium was scraped from V8 agar plates with a scalpel blade. A 200 µL volume of extraction buffer (200 mM Tris-HCl, pH 7.5; 288 mM NaCl; 25 mM EDTA; 5 g/L SDS) was added to a 1.5 mL microfuge tube containing the mycelium. The mycelium was then coarsely ground with a micropestle. A further 800 µL of extraction buffer was added to each tube and the mycelium further disrupted by vortexing for ~30 s. The tubes were spun in a microfuge for 5 min at 16 000 *g* before the supernatant was transferred to a new microfuge tube. Genomic DNA was precipitated by the addition of 600 µL of cold (−20°C) isopropanol. After 30 min incubation on ice, DNA was collected by centrifugation for 20 min at 16 000 *g*. The DNA pellet was washed in cold 70% ethanol, dried and resuspended in 100 µL of sterile milliQ water. The DNA was further purified by the addition of 10 µL of 3 M sodium acetate (pH 5.2) and 250 µL of cold 100% ethanol. After 2 h incubation on ice, the DNA was again collected by centrifugation for 20 min at 16 000 *g*. The pellet was washed in 70% ethanol, dried and again resuspended in 100 µL of sterile milliQ water.

PCR amplification, cloning and sequence analysis of the ITS region

The ITS region encompassing the 3′ end of the 18S rDNA, ITS I, 5.8S rDNA, ITS II and the 5′ end of the 28S rDNA was amplified by PCR using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al*. 1990). In summary, using 2 µL of a 10^{-1} dilution of genomic DNA prepared as above, the ITS region was amplified in a 20 µL reaction volume containing PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), 1.5 mM $MgCl_2$, 500 µM each of dATP, dCTP, dGTP and dTTP, 500 nM each of ITS1 and ITS4, and 2.5 units of *Taq* DNA polymerase (Invitrogen, Carlsbad, USA). Reactions were carried out in a Hybaid PCR Express thermal cycler for one cycle of 94°C for 3 min, 50°C for 1 min and 72°C for 1 min, followed by 29 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. PCR products were subjected to electrophoresis in 1.0% agarose gels and DNA bands of the expected size (~500–1000 bp) were visualised on a UV transilluminator with a UVItec (Cambridge, UK) digital camera and image acquisition package after staining in ethidium bromide. The diversity of isolates was initially determined by RFLP analysis of the amplified ITS region with the restriction enzymes *Hha*I, *Hin*fI and *Mbo*I (New England Biolabs, Beverly, USA). PCR products from representative isolates of each RFLP group were cloned, sequenced and compared with the homologous sequence from other *Pythium* spp., including *P. aphanidermatum* (accession number AF452153), *P. arrhenomanes* (AJ233439), *P. catenulatum* (AF330193), *P. coloratum* (AJ233441), *P. deliense* (AF452147), *P. dissotocum* (AF452154), *P. graminicola* (AF271229), *P. heterothallicum* (AF452162), *P. hydnosporum* (AJ233445), *P. inflatum* (AJ233446), *P. myriotylum* (AF452156), *P. oligandrum* (AF364536), *P. periplocum* (AY166666), *P. torulosum* (AF452155), *P. ultimum* (AF452160), *P. ultimum* var. *sporangiiferum* (AF452157), *P. ultimum* var. *ultimum* (AJ319725), *P. undulatum* (AF271230) and *Phytophthora cinnamomi* (accession number AF266764).

Before cloning, PCR products of selected isolates were purified using a NucleoSpin Extract Kit (Machery-Nagel, Düren, Germany) according to the manufacturer's instructions. Purified PCR products were then cloned into pGEM-T Easy vector (Promega, Madison, USA) according to the manufacturer's instructions. Ligations were performed at room temperature for 3 h followed by transformation of *Escherichia coli* strain JM109 with the respective ligation mix (Sambrook and Russell 2001). Transformants were screened on LB agar plates supplemented with 50 µg/mL ampicillin, to which 25 µL of 50 mg/mL X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside) and 40 µL of 100 mM IPTG (isopropyl-β-D-thiogalactopyranoside) had been added. Plasmid DNA was isolated from selected transformants using a Mini Plasmid Prep Kit (MoBio Laboratories Inc., Solana Beach, USA) according to the manufacturer's instructions. To identify clones with inserts of the appropriate size plasmid DNA was digested

with *Eco*RI. Plasmid DNA clones were sequenced using either the primers SP6 (5′-GCCAGGCTATTTAGGTGACACTATAG-3′) or T7 (5′-GGGTAATACGACTCACTATAGGG-3′), with the ABI Prism BigDye Terminator kit, Version 3.0 or 3.1, according to the manufacturer's instructions (Applied Biosystems, Foster City, USA). Electrophoresis of DNA sequencing products was done at the Australian Genome Research Facility (Brisbane, Australia). Initially, DNA sequence datasets were edited using BIOEDIT (Hall 1999) and characterised by BLAST analysis for the most closely related sequences (Altschul *et al*. 1990). All the DNA sequence datasets from this study together with a selection of *Pythium* sequences from the public databases were aligned using CLUSTAL-W version 1.8 (Thompson *et al*. 1994). Phylogenetic and molecular analyses were then conducted on the aligned sequences using MEGA version 2.1 (Kumar *et al*. 2001). A neighbour-joining phylogenetic tree was constructed using the Tamura-Nei model with bootstrap analysis performed by resampling the data 1000 times.

Pathogenicity testing

Pathogenicity testing of all isolates was done at Queensland Department of Primary Industries and Fisheries, Gatton Research Station, Lawes (27.55°S, 152.33°E; 94 m above sea level) from January to July 2002. To prepare inoculum, 14-day-old *Pythium* cultures grown on PDA at 25°C were flooded with de-ionised water and mycelium scraped from the plates with a glass rod. A 200 mL inoculum suspension was prepared from each culture. Beetroot seedlings (cv. Detroit Dark Red) were grown in 70-mm plastic pots filled with sterile UC mix. Four seeds were sown per pot and pots were watered twice daily and fertilised with Aquasol liquid fertiliser once a week. No fungicides were used on the seedlings during the experiment. To test the pathogenicity of each *Pythium* isolate, 1-week-old and 4-week-old seedlings were drenched with inoculum (50 mL per pot). Control plants were drenched with de-ionised water (50 mL per pot). All pots were randomly arranged on benches in a screenhouse and the plants were assessed for disease symptoms 2 and 4 weeks after drenching. At each assessment, the total number of seedlings and number of diseased and/or dead seedlings were counted in each pot. Diseased and dead seedlings were removed after each assessment.

To demonstrate that disease symptoms were attributable to the *Pythium* inoculum drenches and fulfil Koch's postulates, root/hypocotyl tissue sections from a selection of symptomatic seedlings were surface sterilised in a 1% sodium hypochlorite solution and cultured on PDA. Resulting colonies were examined microscopically to confirm they were morphologically identical to the cultures used to produce the inoculum suspensions.

Fungicide testing

Fungicide sensitivity was tested by inoculating selected *Pythium* isolates onto V8 agar supplemented with Ridomil MZ (80 g/kg a.i. metalaxyl; 640 g/kg a.i. mancozeb). Agar blocks ~25 mm^2, colonised by mycelium of the respective *Pythium* isolates were cut from V8 agar plates and transferred mycelium side down to V8 agar plates supplemented with Ridomil MZ at concentrations corresponding to 0.1, 0.5, 1.0 or 5.0 µg/mL metalaxyl, and 0.8, 4.0, 8.0 or 40 µg/mL mancozeb, respectively. The radial growth of each isolate was measured after 24 h incubation in the dark at 25°C. Inhibition of growth was determined against control cultures in which no Ridomil MZ had been added.

Results

PCR-RFLP analysis

The vast majority (>95%) of isolates from soil and diseased plants initially grown on PDA successfully grew on V8 agar supplemented with pimaricin, ampicillin, rifampicin and PCNB. All 130 presumptive Lockyer Valley *Pythium* (LVP) isolates exhibited colony morphologies on V8 agar typical of this genus. For each isolate, a single DNA fragment was obtained by PCR using the primers ITS1 and ITS4 and a 1 : 10 dilution of the genomic DNA. These DNA fragments ranged in size from ~500 bp to almost 1 kb. Each PCR product was digested separately with *Hha*I, *Hin*fI and *Mbo*I to determine the overall genetic diversity of the isolates (Fig. 1). Although a range of RFLP profiles was obtained, 91% of the isolates could be allocated to three genotypic groups, LVP A (43%), LVP B (41%) and LVP C (7%).

Deoxyribonucleic acid sequence analysis of ITS region

The ITS sequence of representative isolates of each RFLP group was compared with the public database using BLAST searches. For the LVP A isolates, the region encompassed by the primers ITS1 and ITS4 was 849 bp in length. With the numbering of the DNA sequence beginning at the first nucleotide of the ITS1 primer sequence, the LVP A sequence was 99% identical to the corresponding sequence from *P. aphanidermatum* (AF452153) for nucleotides 8 to 244 and 100% identical to the *P. aphanidermatum* sequence over a stretch of 565 bp beginning at nucleotide 254 of the LVP A sequence. The corresponding sequence for the LVP B isolates was 909 bp and 96% identical to the closest

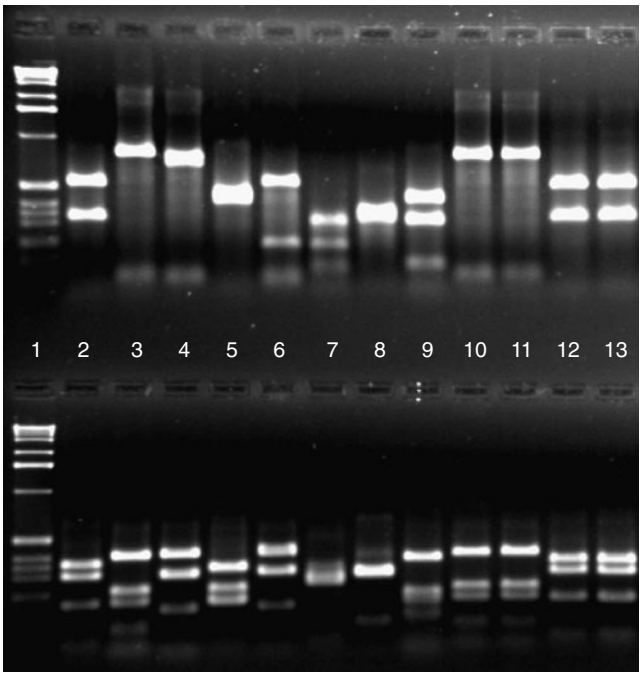

Fig. 1. RFLP analysis of the PCR-amplified ITS region DNA from representative *Pythium* isolates, digested with *Hha*I (top) or *Hin*fI (bottom). Lane 1 = 1 kb DNA ladder; lanes 2, 12, and 13 = LVP A isolates; lanes 3, 10 and 11 = LVP B; lane 4 = LVP C; lanes 5–9 = unique LVP isolates.

database sequence of *P. ultimum* (AF452160) over an 820 bp region from nucleotides 1 to 818, including two single nucleotide gaps in the LVP B sequence generated in the pairwise BLAST alignment. The terminal 3′ 68 bp was 100% identical to the respective *P. ultimum* sequence. The LVP C sequence was 866 bp in length and was 98% identical to the corresponding sequence from *P. dissotocum* (AF452154) over 763 nucleotides starting at nucleotide 45 of the LVP C sequence.

Although the vast majority of *Pythium* isolates were from the above three groups, there were other distinct and unique isolates obtained from both diseased beets and soil samples. The sequence for the single LVP D isolate was 503 bp and 98% identical to the corresponding sequence from *P. heterothallicum* (AF452162) over 444 nucleotides from nucleotide 1 of the LVP D sequence. This region corresponded to nucleotides 354 to 796 of the *P. heterothallicum* sequence. Further analysis of the LVP D sequence indicated that mis-priming by the forward primer ITS1 was the likely explanation for this result, as this sequence contained only the 3′ 11 bp of the 5.8S rDNA, the entire ITS II spacer region and the 5′ 58 bp of the 28S rDNA. The sequence for the single LVP E isolate was 914 bp and 100% identical to the corresponding sequence from *P. ultimum* (AF452160) over the entire sequence. The sequence for the single LVP F isolate was 853 bp and 97% identical to the corresponding sequence from *P. periplocum* (AY166666) over 860 nucleotides, with gaps at seven nucleotide positions in the LVP F sequence in the BLAST alignment of the two sequences.

Despite the fact that all isolates characterised were cultured on V8 agar supplemented with agents selective for oömycetes, two isolates were obtained that gave DNA sequences unrelated to any *Pythium* spp. For the first of these isolates the PCR product was 576 bp and was 100% identical over a region of 509 nucleotides with the corresponding sequence from *Nectria haematococca* (AF165874). More interesting was the second isolate for which a PCR product of 670 bp was obtained. This sequence was 99% identical to the corresponding sequence from the oil producing fungus *Mortierella alpina* (AJ271630; Mackenzie *et al*. 2000). Although pimaricin is active against most fungi, it is not active against some *Mortierella* spp. (Erwin and Ribeiro 1996).

The DNA sequences for all six *Pythium* genotypes (LVP A-F) were aligned with the corresponding sequences for 18 other *Pythium* spp. of interest and *Phytophthora cinnamomi*, these sequences being obtained from the public database (http://www.ncbi.nlm.nih.gov). A phylogenetic tree based on the aligned sequences was constructed (Fig. 2). Overall, the pool of *Pythium* isolates from diseased plant and soil samples described in this study was widely dispersed among this group of phylogenetically divergent, previously characterised *Pythium* spp.

Accession numbers

The DNA sequence data that were generated during the course of this study have been given the accession numbers AY310437–AY310442 and AY445122.

In vitro *pathogenicity testing*

The pathogenicity was determined for 94 *Pythium* isolates, corresponding to 32, 54 and 8 isolates each from the groups LVP A, LVP B and LVP C, respectively. Isolates were scored as either those failing to cause any mortality, those for which up to 50% of seedlings died, or those for which greater than 50% of seedlings died. As a group, the LVP A isolates were more pathogenic to beetroot seedlings than both the LVP B and LVP C isolates (Table 1). The LVP A group contained the greatest proportion of isolates that killed more than 50% of plants. In addition, LVP A isolates were more pathogenic on younger seedlings. Hence, at 4 weeks after inoculation, only three of 32 LVP A isolates killed greater than 50% of plants inoculated at 4 weeks of age, compared with 12 of 32 that killed greater than 50% of plants when inoculated at 1 week of age. The less pathogenic LVP B isolates appeared to kill a similar proportion of plants regardless of the age at which the plants were inoculated.

In vitro *fungicide sensitivity testing*

The amended-agar assay for determining the response to metalaxyl and mancozeb was preferred to a leaf disk assay primarily because of the ease and simplicity with which *Pythium* isolates could be assessed. Isolates that were pathogenic on beet seedlings were selected for assessment of fungicide sensitivity. Four LVP A isolates, three LVP B isolates, and a single LVP C isolate were tested on at least four independent occasions. Clearly, the fungicide Ridomil MZ had a strong negative effect on the vegetative growth of all three genotypes (Fig. 3). The highest concentration of Ridomil MZ used in this assay equated to a metalaxyl concentration of 5 µg/mL and a mancozeb concentration of 40 µg/mL, the recommended dose for field applications being 20–40 times higher (Novartis, Basel, Switzerland). At this concentration, all three genotypes were sensitive to the combination of metalaxyl and mancozeb, with radial growth over 24 h being inhibited by between 72% (LVP C) and 97% (LVP B). Although all three genotypes exhibited a sensitive phenotype, there was some variation in the response to Ridomil MZ between genotypes. For example, the slowest growing genotype, LVP C, was inhibited the least by the fungicide. Thus, growth of LVP C was little affected by Ridomil MZ at metalaxyl concentrations of 0.1 and 0.5 µg/mL and was inhibited only appreciably at metalaxyl concentrations of 1.0 and 5.0 µg/mL resulting in 29.2 and 72.4% inhibition, respectively. In comparison, the growth of the LVP B genotype was most affected by Ridomil MZ and was inhibited by 39.7, 59.1, 67.9

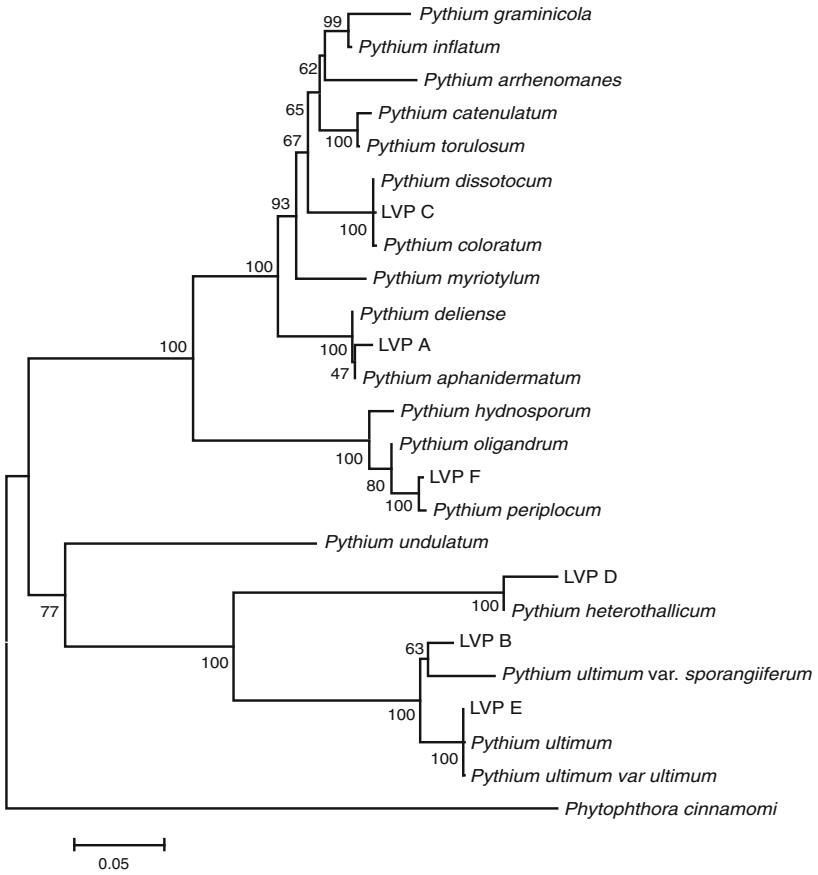

Fig. 2. Phylogenetic tree of *Pythium* isolates from this study and previously characterised *Pythium* spp. based on DNA sequence analysis of the region encompassing ITS I, 5.8S rDNA and ITS II. The numbers at each branch point are bootstrap values following resampling of the data 1000 times.

Table 1. Pathogenicity of *Pythium* isolates towards table beet seedlings

Pythium isolate	Pathogenicity trial[A]	% Mortality (no. of isolates)[B] Zero	≤50	>50
LVP A	1 week seedlings 2 wpi	12	11	9
	1 week seedlings 4 wpi	6	14	12
	4 week seedlings 2 wpi	19	10	3
	4 week seedlings 4 wpi	15	14	3
LVP B	1 week seedlings 2 wpi	49	5	0
	1 week seedlings 4 wpi	40	11	3
	4 week seedlings 2 wpi	47	4	3
	4 week seedlings 4 wpi	41	9	4
LVP C	1 week seedlings 2 wpi	6	0	2
	1 week seedlings 4 wpi	5	1	2
	4 week seedlings 2 wpi	7	1	0
	4 week seedlings 4 wpi	6	2	0

[A] 1-week-old and 4-week-old seedlings were challenged with *Pythium* at 2 and 4 weeks post infection (wpi).
[B] The number of isolates causing mortality of zero, ≤50 and >50% of seedlings, respectively.

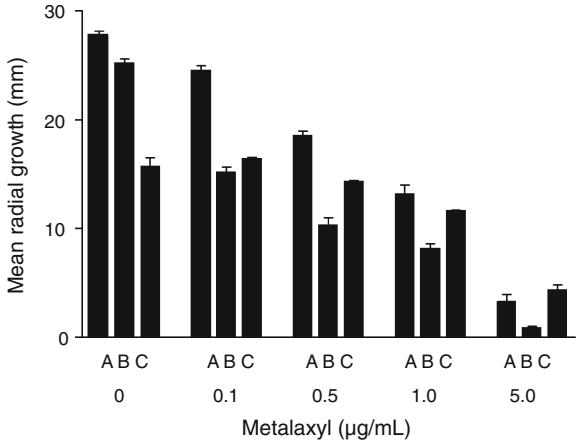

Fig. 3. *In vitro* response of LVP A, LVP B and LVP C isolates (A, B and C) to the fungicide Ridomil MZ. The radial growth of each isolate was measured after 24 h incubation on V8 agar with and without Ridomil MZ. Results are shown as the final concentration of metalaxyl in the medium; the corresponding concentrations of mancozeb are given in the methods section. Four LVP A, three LVP B and a single LVP C isolate that were demonstrated to be pathogenic were tested on at least four separate occasions with standard error bars shown.

and 96.8% at metalaxyl concentrations of 0.1, 0.5, 1.0 and 5.0 µg/mL, respectively. For the dominant and fastest growing beetroot pathogen, LVP A, radial growth was inhibited by 11.9, 33.5, 52.5 and 88% at metalaxyl concentrations of 0.1, 0.5, 1.0 and 5.0 µg/mL, respectively, and was intermediate between LVP C and LVP B in its response to Ridomil MZ. The focus of this study was the predominant *Pythium* spp. and so we did not test the fungicide sensitivity of the LVP D, LVP E or LVP F isolates.

Discussion

Root rot of beetroot is a disease complex that is currently having a significant effect on the long-term viability of the Australian beetroot industry (Martin 2003). Implicated as a major contributor to this disease complex are previously uncharacterised *Pythium* spp. Therefore, the main objective of this study was to characterise *Pythium* isolated from diseased plants and soil from farms in the Lockyer Valley with a history of beetroot rot complex. *Pythium* isolates were characterised using a molecular approach based on RFLP pattern and DNA sequence in the ITS region. The ITS region, encompassing the ITS I and ITS II and 5.8S rDNA, is a useful target for characterisation as it is highly conserved within species but has sufficient sequence divergence to enable taxonomic designations to be made between species (Lévesque and de Cock 2004). Although other genes have been the subject of analysis of *Pythium* spp., most studies have focussed on the ITS region of rDNA, whether it be for the purposes of characterising populations or for the development of diagnostic assays (Matsumoto *et al*. 1999; Paulitz and Adams 2003; Wang and Chang 2003; Wang *et al*. 2003; Wang and White 1997; Weiland and Sundsbak 2000).

The most important outcome from this study is the identification of LVP A (*P. aphanidermatum*) and LVP B (*P. ultimum*) as the dominant phytopathogenic *Pythium* spp. of beetroot in the Lockyer Valley. Although these species can be distinguished from one another morphologically, molecular characterisation allows for rapid, accurate identification of species by workers with only limited training in mycology or by those who have only limited experience in working with the genus *Pythium*. The predominance of these two species in the beetroot rot complex is perhaps not all that surprising. Both *P. aphanidermatum* and *P. ultimum* are known to have a broad host range (Martin and Loper 1999), and have been previously reported as the causal agents of seedling beet disease in other parts of the world (Babai-Ahary *et al*. 2004; Brantner and Windels 1998; Kuznia and Windels 1993; Leach 1986). Although diseases caused by *P. ultimum* have been most often reported in soils of cool to moderate temperature (<25°C), *P. aphanidermatum* has been shown to cause disease in soils above 25°C, temperatures typically occurring in the Lockyer Valley for much of the year. The pathogenicity testing in this study was done from January to July 2002. We acknowledge that this was not ideal, since temperatures varied substantially throughout this period, with maximum average monthly temperatures falling from 33.5°C in January to 21.7°C in July. Consequently, it is possible that the data presented in Table 1 underestimate the pathogenicity of each group, since some isolates from each group were tested for pathogenicity at temperatures that were not optimal for disease development.

From a disease management and crop production standpoint the characterisation of *Pythium* species pathogenic to table beet has important implications, particularly in regards to the development of rapid species-specific molecular diagnostic assays. Identification of *Pythium* species in a field soil before a beet crop is planted will provide information useful to the management of disease epidemics. To illustrate this principle, preliminary experiments we have conducted indicate that *P. aphanidermatum* causes more disease at relatively high soil temperatures (>27°C), whereas *P. ultimum* causes more disease at lower soil temperatures (Hancock 1977; Kuznia and Windels 1993). It seems likely that *Pythium* epidemics may be prevented or minimised in fields that are known to be infested with *P. aphanidermatum* if planting of beets can be delayed to the cooler months of the year when temperature conditions do not favour infections by this species. In contrast, planting beets in soils infested with *P. ultimum* when soil temperatures are low is likely to increase the likelihood of disease epidemics.

In addition to the effects of temperature on infection, pathogenicity testing has shown that 1-week-old beet seedlings are more susceptible to *P. aphanidermatum* than 4-week-old seedlings. Therefore, it is particularly important that seeds are not sown until soil temperatures fall to a temperature less favourable to the infection of young plants (i.e. <27°C). The higher susceptibility of young plants to *P. aphanidermatum* may explain why, in the Lockyer Valley of south-east Queensland, beetroot losses tend to be highest in young stands planted during the summer months of February and March. At this time of year, the plants are not only susceptible to infection because of their age, but the soil temperatures are also highly favourable for *P. aphanidermatum* epidemics. At the end of the season (October to December), losses are less, presumably because the plants are mature at this time and are, therefore, less susceptible to infection, despite high soil temperatures.

A similar relationship between disease susceptibility and plant age did not appear to hold for *P. ultimum* in this study, and this is reflected in field observations. In the cooler months, when *P. ultimum* is more likely to cause disease than *P. aphanidermatum*, *P. ultimum* is commonly isolated from both young and mature symptomatic plants. In addition, in the Lockyer Valley, the greatest losses due to disease generally occur in the summer months when

P. aphanidermatum appears to be more prevalent. The above correlations on the seasonal isolation of *Pythium* species, together with the pathogenicity testing in this study, indicate that *P. aphanidermatum* is a more virulent pathogen than *P. ultimum*.

A major concern in crop management is the development of resistance to fungicides following field and/or seed applications. Ridomil MZ contains metalaxyl, a fungicide active against species in the Peronopsorales (including *Pythium* spp.), and the broad-spectrum fungicide, mancozeb. In this study, it was clear that the *Pythium* isolates tested *in vitro* were not resistant to this commonly used commercial fungicide at concentrations far less than those recommended by the manufacturer (Novartis). Regardless of whether fungicide sensitivity was due to metalaxyl and/or mancozeb, current farm practices in the Lockyer Valley have not yet lead to the selection of strains of *Pythium* that are resistant to this fungicide. Previous studies have demonstrated variability in the response of *Pythium* to metalaxyl (Brantner and Windels 1998; Cook and Zhang 1985; Sanders 1984; White *et al*. 1988). These workers showed that metalaxyl inhibited the growth of *P. aphanidermatum* and *P. ultimum* isolated from sugar beet on corn-meal agar by 50% (EC_{50}) at concentrations that ranged from 0.05 to 3.12 µg/mL (Brantner and Windels 1998). However, there was no correlation between the EC_{50} of an isolate and its phytopathogenicity.

The characterisation of *Pythium* spp. associated with the beetroot rot complex has substantiated the importance of this group of oömycetes, and should aid in the management of this disease. In particular, DNA sequence determination of the respective ITS regions for the phytopathogenic *Pythium* spp. will enable molecular diagnostic systems to be developed that will provide rapid and reliable data on the pathogen load of soils and the potential for establishment of disease.

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