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An assessment of *Pythium* spp. associated with soft rot disease of ginger (*Zingiber officinale*) in Queensland, Australia

Duy Phu Le[1] · **Mike K. Smith**[2] · **Elizabeth A. B. Aitken**[1]

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Abstract In Australia, Pythium soft rot (PSR) outbreaks caused by *P. myriotylum* were reported in 2009 and since then this disease has remained as a major concern for the ginger industry. From 2012 to 2015, a number of *Pythium* spp. were isolated from ginger rhizomes and soil from farms affected by PSR disease and assessed for their pathogenicity on ginger. In this study, 11 distinct *Pythium* spp. were recovered from ginger farms in Queensland, Australia and species identification and confirmation were based on morphology, growth rate and ITS sequences. These *Pythium* spp. when tested showed different levels of aggressiveness on excised ginger rhizome. *P. aphanidemartum*, *P. deliense*, *P. myriotylum*, *P. splendens*, *P. spinosum* and *P. ultimum* were the most pathogenic when assessed in vitro on an array of plant species. However, *P. myriotylum* was the only pathogen, which was capable of inducing PSR symptoms on ginger at a temperature range from 20 to 35 °C. Whereas, *P. aphanidermatum* only attacked and induced PSR on ginger at 30 to 35 °C in pot trials. This is the first report of *P. aphanidermatum* inducing PSR of ginger in Australia at high temperatures. Only *P. oligandrum* and *P. perplexum*, which had been recovered only from soils and not plant tissue, appeared non-pathogenic in all assays.

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✉ Duy Phu Le
lephuduy08@gmail.com; phu.le@uqconnect.edu.au

[1] School of Agriculture and Food Sciences, The University of Queensland, Brisbane 4072, Australia

[2] Maroochy Research Station, Queensland Department of Agriculture and Fisheries, Nambour 4560, Australia

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Introduction

Ginger (*Zingiber officinale* Rosc.), belonging to the Zingiberaceae, is a cash crop for many growers in various countries including China, India, Indonesia, Fiji and Australia (Kavitha and Thomas 2008). In Australia, ginger was introduced as a crop in the early twentieth Century and has been cultivated on the Sunshine Coast, Queensland since 1916 (Hogarth 2000). Presently, production extends from Gatton to Bundaberg in southeast Queensland, but the Australian ginger industry is relatively small (less than 1 % contribution to global share) with an involvement of around 30 full time growers. Nevertheless, it was worth AUD 15.6 million at the farm gate in 2009 (Camacho and Brescia 2009) with an annual production estimated at about 8000 t. Almost half of this production is delivered to the domestic fresh market with the remainder going for processing inside the world's largest ginger factory, Buderim Ginger Ltd. (Camacho and Brescia 2009).

Pythium soft rot (PSR) of ginger was first reported around a century ago in India by Butler (1907) and has since been problematic in most ginger growing regions worldwide (Dohroo 2005). The disease caused by any one of a number of *Pythium* spp. (Dohroo 2005) is of most concern due to the destructiveness and aggressiveness of the pathogens on ginger. Losses generally vary from 5 to 30 %, but in some cases they can be up to 100 % in fields where conditions conducive for disease development, such as water logging and high temperatures, are reached (Stirling et al. 2009). Once ginger fields have been infested with *Pythium* spp., the persistence of the

pathogens leads to PSR in subsequent replanting of ginger in these fields, as reviewed in Le et al. (2014).

In Australia, *Pythium spinosum* was recorded on ginger as early as 1962 by Teakle (1962), but it was considered a secondary pathogen to *Fusarium oxysporum*, the causal agent of Fusarium yellows. During 2007, a PSR disease outbreak on ginger in the Sunshine Coast of Australia was attributed to *Pythium myriotylum* (Stirling et al. 2009) which at the time was considered to be a ubiquitous pathogen with a wide host range and consequently the relevance of the PSR outbreak may have been underestimated. However, PSR has since then continued to be a major constraint for the Australian ginger industry with up to 70 % of the growers admitting to have PSR infestation on their farms (T. Pattison pers. Comm.).

Following the first report of PSR on two of the oldest ginger farms in Australia (Stirling et al. 2009), PSR disease has since been observed on at least 11 other nearby farms. Therefore, it was questioned if there was probably more than one *Pythium* species/strains associated with PSR on ginger in Australia; and also if the pathogen has been spread from the original infested farms. In this study, a large number of PSR diseased ginger samples and soils from around infected ginger were collected from 13 infested farms for an assessment of the *Pythium* spp. diversity associated with PSR of ginger in Queensland, Australia.

Materials and methods

Pythium isolation and cultures

Diseased ginger rhizomes were sampled from 13 farms in Queensland (Fig. 1). Isolations were undertaken by excising sections (5 mm^2) of rhizome with PSR symptoms, quickly surface decontaminating (around 10 s) with 25 % bleach (1 % HOCl), then washing twice with sterilized distilled water (DW), and blotting dry with autoclaved paper towel. The sections were then transferred on to Petri plates with corn meal agar with an amendment of 50 μg/mL Penicillin, 50 μg/mL Polymyxin, and 25 μg/mL Pirmaricin (CMA + 3P). The Petri plates were incubated in the dark overnight at 27 °C, then sections taken from the growing edge of the colonies were transferred onto 1.5 % water agar (WA), incubated again under the same conditions for another night, after which hyphal tips of each of the isolates were excised out under an inverted compound microscope (Leica) and placed onto full strength potato dextrose agar (PDA, Difco).

In addition, a baiting technique based on descriptions of Stanghellini and Kronland (1985) modified by using excised ginger rhizomes was deployed in an attempt to recover *Pythium* spp. from soil collected from around ginger showing symptoms of PSR. A total 10 g of putatively infested soil was saturated with DW in a 9 cm Petri plate. Excised pieces (2 cm^2) of apparent healthy ginger rhizomes were immersed in 25 % bleach for 1 min, rinsed twice with autoclaved DW, and blotted dry with autoclaved paper towels. Ten of these excised ginger pieces were then placed onto the soil surface in each plate. On the top of each ginger piece, a plug (5 mm^2) of WA was placed. The plates were incubated at 27 °C for 2–4 days. Hyphal-colonised WA plugs were then transferred onto 1.5 % WA for single hyphal tip isolation.

In addition to representative isolates of each of *Pythium* spp. recovered in this study, reference specimens of *P. myriotylum* CBS254.70 and *P. zingiberis* NBRC30817, as well as *P. myriotylum* UQ5993 recovered from sudden wilt capsicum in Australia were also included in pot trials on ginger plants. Where the reference isolates were used for pot trials, the assays were conducted in a contained controlled environment facility, as required by quarantine for the overseas isolates. All cultures were placed on half strength corn meal agar (CMA) prepared from maize meal (polenta), based on the protocol of Dhingra and Sinclair (1995). The culture on the CMA was then submerged in autoclaved DW and stored at room temperature for future use.

Hyphal growth rate

For each *Pythium* spp. tested, daily mycelial growth rate under a temperature range from 5 to 45 °C was assessed on 9 cm Petri plates. The growth rate was assessed at 5 °C intervals and carried out as descriptions previously in Le et al. (2015). Generally, 0.5 cm^2 plugs of tested cultures (one-week-old) were subcultured onto PDA contained in Petri plates. Two plates were used for isolate, representing two replicates. After subculturing, initial growth of the cultures were manipulated by leaving the plates at room temperature for at least 8 h. Growing edges were marked before the plates were incubated at each designated temperature for 24 h. Growing edges again were marked after 24 h of incubation; radial growth of the colonies was measured at four points in mm using two transecting lines from the first mark to the next. Daily growth rate was presented as a mean of the four measurements. The growth rates were assessed at each of the designated temperature twice.

Morphology

Sexual and asexual structures of *Pythium* spp. were produced in soil extract cultures amended with two pieces (1 cm^2) of grass leaves (*Pennisetum setaceum*). To make the soil extract, 20 g of air-dried sandy soil was soaked in 1 L DW overnight. The filtrate sieved through 150 mm Whatman filter paper (No. 1) was made up to a volume of 1 L with DW and autoclaved at 121 °C for 20 min (McLeod et al. 2009). Active growing cultures of *Pythium* spp. on PDA were excised and the plugs (0.5 cm^2) were submerged into the soil extract cultures contained in Petri plates. The plates were kept at 27 °C and

Fig. 1 A sketch map indicating relative locations (black squares) of 13 ginger farms in which ginger with symptoms of PSR and soils around the ginger were sampled for this study

checked daily for development of taxonomic characteristics. The resultant mycelial mats were mounted on microscopic slides, stained with cotton blue, and observed under a BH2 (Olympus) microscope where measurements were recorded of structures at 400× magnification. For each isolate, 20 randomly selected structures were measured.

DNA extraction and amplification

The CTAB protocol of Doyle and Doyle (1990) was employed to extract DNA from mycelia mats growing in potato dextrose broth. The ITS regions including the 5.8S rRNA subunit were amplified in a Mastercycler by using two universal primers: ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). In every 20 μL PCR reaction, there was 1 μL of DNA template (25 ng/μL) and 19 μL of master mix, the latter which included 0.18 μL of *Taq* DNA polymerase (5 u/μL), 1 μL of primer ITS1 and primer ITS4 each (10 μM), 2.83 μL of 5X Green GoTaq flexi buffer, 0.72 μL of 25 mM $MgCl_2$, 1.05 μL of 10 mM dNTPs and 13.2 μL of dH_2O. The reaction cycle was first denatured for 5 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s. The reaction was finally extended for 10 min at 68 °C.

The *Cox*I gene was amplified by using a primer set of OomCoxILevup (5′-TCAWCWMGATGGCTTTTTTCAAC-3′) and Fm85mod (5′-RRHWACKTGACTDATRATACCAAA-3′) (Robideau et al. 2011). The reaction cycle for *Cox*I included a first step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min. The reaction was finally extended for 10 min at 72 °C.

The *Cox*II gene was amplified by using two primers: FM66 (5′ TAGGATTTCAAGATCCTGC 3′) and FM58 (5′ CCACAAATTTCACTACATTGA 3′) (Martin 2000). The reaction cycle was first denatured for 5 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 90 s. The reaction was finally extended for 10 min at 68 °C.

To amplify the *β-tubulin* gene, two primers TUBUF2 (5′ CGGTAACAACTGGGCCAAGG 3′) and TUBUR1 (5′ CCTGGTACTGCTGGTACTCAG 3′) were used (Kroon et al. 2004). The cycling program was based on descriptions of Mu et al. (1999) with some modifications. The reaction cycle was first denatured for 5 min at 94 °C, followed by eight cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min. The reaction was then continued with another 22 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 2 min. The program was completed with a 7 min step at 72 °C. All PCR products were run at 110 V in a 1.5 % agarose gel for 40 min to confirm the presence of an amplified product.

Sequencing and analysing sequences

Both forward and reverse directions were sequenced directly from PCR products by Macrogen (Korea) and the consensus sequences were created after manual alignment and comparison using Clustal 2.0.2. The Geneious 7.1.4 was used to align the sequences of the different *Pythium* isolates collected from ginger fields. Comparisons were made among

the sequences in this study with those of deposited sequences in Genbank to confirm species identification and determine whether there were any genetic variations. A maximum likelihood tree of the ITS region was drawn to assess the phylogenetic relationship among the isolates and species.

Pathogenicity tests

In vitro pathogenicity test on excised ginger sticks

The experiment was adapted from methods described in Le et al. (2015) to test for colonization and aggressiveness of *Pythium* spp. on ginger pieces at 27 ± 2 °C. Briefly, disease free ginger rhizomes were cut into sticks (5.5 cm long × 1 cm wide × 1 cm high), which were surface disinfected in 25 % bleach for 1 min, washed twice again with autoclaved DW for another min, and blotted dry on autoclaved paper towels. Three replicate sticks were prepared for each isolate by plating together in 9 cm Petri plates containing wet autoclaved filter paper. The sticks were inoculated by placing small squares (0.5 cm^2) of active growing *Pythium* cultures on PDA at one end of each ginger stick. Three days after incubation, each stick was chopped up into five smaller pieces (1 × 1 × 1 cm) under sterile conditions which were then plated (except for the one end containing the culture) onto PDA in a Petri plate in order of distance from the inoculated end of the ginger sticks. These plates were then incubated at the same temperature in the dark for 24 h and observed for recovery of *Pythium* spp. from each excised piece. The negative control treatment included the same procedure but with uninoculated PDA squares (non-cultured). The data were recorded determining the colonization of *Pythium* spp.to 1, 2, 3, 4 or 5 cm sections on the ginger sticks. The assay was independently repeated.

In vitro pre-emerging damping off test

The pathogenicity of the *Pythium* spp. was assessed by screening seedlings of 13 different plant species in Petri plates against cultures of each isolate. For each isolate-plant species interaction assessed, a one-week-old culture that had been grown on PDA and was then sub-cultured onto three replicate 9 cm Petri plates of 1.5 % WA, and kept in an incubator set at 27 °C for a week. On each WA plate, ten surface disinfected seeds were placed one of the following 13 plant species: rye (*Secalecereale*), wheat (*Triticum* sp.), millet (*Panicum miliaceum*), barley (*Hordeum vulgare*), buck wheat (*Fagopyrum esculentum*), beet-root (*Beta vulgaris*), spring onion (*Allium fistulosum*), carrot (*Daucus carota*), cauliflower (*Brassica oleracea botrytis*), cucumber (*Cucumis sativus*), eggplant (*Solanum melongena*), lettuce (*Lactuca sativa*), and gypsophila (*Gypsophila elegans*). The plates were then incubated at 27 °C for 7 days before checking for disease indices. The disease index was calculated using the equation of Zhang and Yang (2000).

$$DI = \sum_{i=1}^{10} Xi/40$$

DI is disease index rating from 0 (all seedlings healthy after germination) to 1 (all seeds dead before germination).

X_i is disease rating of the ith replicate (from 1 to 10).

40 is equal to the number of replicates multiplying with the highest rating scale (from 0 to 4).

The rating scale used was as follows: 0 = seed germinated and healthy seedlings with no obvious symptoms; 1 = seed germinated and seedlings with light brown lesion on roots; 2 = seed germinated and seedlings with short and enlarging brown lesion on roots; 3 = seedlings died after germination; 4 = seed died with no apparent germination.

Glasshouse pathogenicity test on ginger plants

Ginger (cv 'Queensland') plants derived from tissue culture were used in pot trials to assess their reaction to a subset of *Pythium* spp. isolates. Inoculum for pot trials was prepared using sorghum seeds that had been soaked in water for 24 h in the dark and autoclaved twice at 121 °C for 20 min. The sorghum seeds were then plated onto 1 to 2-day-old *Pythium* cultures grown on PDA in Petri plates and kept at 27 °C for a week or until the seeds were fully covered with mycelia of *Pythium*. For each isolate of *Pythium* spp. tested, ginger plants (about 4-month-old after deflasking) that had been grown in 140 mL pots were inoculated by inserting the two sorghum seeds under the soil to a depth of 20–30 mm, which were fully colonized with *Pythium* spp. The pots of inoculated ginger were kept saturated with water from time of inoculation onwards by placing on saucers where the water was maintained. Plants were monitored daily for disease development based on aboveground symptoms applying the following scale: 0 = plants remain green and healthy; 1 = leaf sheath collar discoloured and lower leaves turned yellow; 2 = plants alive, but shoots either totally yellow or dead; and 3 = all shoots dead (Stirling et al. 2009). Attempts were made to re-isolate the *Pythium* spp. either from diseased rhizomes or from soil, in the latter case by baiting with carrot pieces placed on the soil. The pathogenicity assays were conducted in growth cabinets, which were assigned two temperature ranges 20/25 °C and 30/35 °C (night/day) both with 10 h photoperiod under fluorescent light. The assays were undertaken second time on cv 'Canton' due to the shortage of cv 'Queensland'.

All data from pathogenicity assays and the growth rate experiments were subjected to analysis of variance (ANOVA) and the means were compared using Tukey's least significant difference (LSD) test ($P \leq 0.05$). Where stated, data from repeated assays were pooled and analyzed together if

there were no significant difference between the two repetitions. The ANOVA and comparisons of means were performed with Minitab 16.

Results

Isolation and identification

A total of 173 isolates of *Pythium* spp. and 15 of *Pythiogeton ramosum* isolates were obtained either directly from PSR ginger or from baiting of soil from around infected ginger. Eleven different species were initially identified based on morphological characteristics by using the keys of van der Plaats-Niterink (1981) and Dick (1990). Of these 11 different *Pythium* spp. and one *Pythiogeton ramosum*, only three of the *Pythium* spp. as well as the *Pythiogeton ramosum* were recovered directly from PSR ginger tissue (Table 1). PSR ginger sampled from a single location only ever yielded one single species of *Pythium* sp. When using ginger as bait from the soil, *P. spinosum* and *P. splendens* were recovered most frequently followed by *P. aphanidermatum*, *P. deliense*, *P. heterothallicum*, *P. oligandrum*, *P. perplexum*, *P. torulusum*, and *P. ultimum*. However, using ginger baits from the soil failed to retrieve either *P. graminicola* or *P. myriotylum*.

All of the species recovered grew well at temperature ranges above 5 °C and below 40 °C, except for *P. graminicola* which grew poorly below 10 °C, and *P. aphanidermatum* which grew normally even at 40 °C and above (Table 2). *P. aphanidermatum* was exceptional in that it still grew at 45 °C.

Table 1 Species of oomycetes, including *Pythium* and *Pythiogeton* isolated from ginger fields in Queensland, Australia

Species	Number of isolates obtained	Material used to source isolates
P. aphanidermatum	2	Soil
P. deliense	1	Soil
P. graminicola	3	Ginger
P. heterothallicum	2	Soil
P. myriotylum	79	Ginger
P. oligandrum	3	Soil
P. perplexum	1	Soil
P. spinosum	51	Soil
P. spinosum	12	Ginger
P. splendens	27	Soil
P. torulosum	1	Soil
P. ultimum	1	Soil
Pythiogeton ramosum	15	Ginger

Species as determined by morphology and ITS sequence-based similarity

All recovered isolates had the ability to be cultured on different media including PDA, CMA, WA, potato broth and all isolates were stored and kept in a collection at Plant Pathology lab, School of Agriculture and Food Sciences, The University of Queensland (UQ).

For species confirmation, the ITS sequences, which were deposited to GenBank (Table S1) were between 761 to 910 bp and once compared with sequences on the Genbank database, all sequences were above 99 to 100 % identical with reference species (CBS isolates) on the Genbank, so confirming the status of *Pythium* spp. in this study. A phylogenetic tree built on the ITS region showed very high bootstrap values (98–100 %) between the morphologically identified and the reference species; subsequently, the species identification was verified (Fig. 2). Of those UQ isolates considered to be *P. myriotylum*, sequences of nuclear and mitochondrial loci, including of ITS (57 sequences), *Cox*I (47 sequences), *Cox*II (67 sequences) and *β-tubulin* (19 sequences) were 99.8 to 100 % homologous to each other (Fig. S1-S4).

Pathogenicity tests

In vitro tests on excised ginger

The different *Pythium* spp. showed varying levels of aggressiveness when inoculated onto ginger sticks. Once colonized, the ginger became soft then discoloured and then white mycelia of pathogenic *Pythium* spp. covered the tissue. Most of the isolates identified as *P. myriotylum* showed a high level of aggressiveness as assessed by rate of development on the excised ginger rhizome the exception being *P. myriotylum* UQ5892 isolate (Fig. 3). Isolates of *P. deliense* and *P. aphanidermatum* also showed a high level of aggressiveness followed by *P. spinosum*, *P. splendens*, *P. ultimum*, *P. graminicola*. *P. heterothallicum* and *P. torulosum* were the least aggressive. Those isolates identified as *P. perplexum* and *P. oligandrum* were not able to grow on the ginger sticks and colonize the tissue in the conditions tested at 27 ± 2 °C; subsequently, neither of these species were recovered from the inoculated ginger sticks. None of the control sticks yielded *Pythium* spp. when samples were plated out onto PDA plates (Fig. 3).

In vitro pre-emerging damping off

The seeds of all tested plant species, except for beet-root germinated well in the control treatments (disease index, DI ≤ 0.2) and the subsequent seedlings remained healthy afterwards. The DIs in control treatments were significantly less ($P = 0.05$) than those recorded for seeds treated with *Pythium* spp., excluding *P. oligandrum* in which DIs on tested plant species were not different from those of the control. Similarly, DI recorded on millet infected with *P. torulosum* was as low as

Table 2 Growth rate and analysis of morphological characters of selected isolates of *Pythium* spp. obtained from PSR ginger and from baiting from soil around PSR ginger in Queensland, Australia

Pythium species[a]	Hyphal width (μm)	Sporangia (μm)	Hyphal swelling (μm)	Colony appearance on PDA	Temp. response (°C)			Oogonia (μm)	Antheridia	Oospores (μm)
					Max	Opt.	Growth at 25 (mm)			
P. aphanidermatum UQ6082	4–6 (−10)	I, 10–16 (−20)	Nil	Dense-cottony	≤ 45	35–40	38.67	S, 16–27 μm (av. 22.05)	M, 1(−2)/oog.	A, 14–22 (av. 18.65)
P. deliense UQ6021	4–6 (−8)	I, 6–10 (−12)	Nil	Slight radiate	≤ 40	35	25.71	S, 18–22 (av. 20.45)	M, 1(−2)/oog.	A, 12–19 (av. 15.70)
P. graminicola UQ6049	2–4 (−6)	I, 6–10 (−12)	Nil	Dense-cottony	≤ 40	35	16.71	S, 19–24 (av. 21.05)	M, D (occ.), 1–4 (−6)/oog.	A, P, 14–23 (av. 18.65)
P. heterothallicum UQ6290	2–5 (−7)	Nil	G, 8–15 (−24)	Radiate	≤ 35	25	10.41	Nil	Nil	Nil
P. oligandrum UQ6085	2–5 (−7)	Ir, 8–30 (−36)	Nil	Radiate	≤ 40	30	26.13	O, 14–23 (av. 18.55)	Nil	A, 12–20 (av. 14.85)
P. perplexum UQ6250	2–4 (−6)	Ir, up to 30	Nil	Radiate	≤ 30	20	9.27	S, 14–26 (av. 21.10)	M, 1/oog.	A, P (oc.), 14–22 (av. 18.20)
P. spinosum UQ6244	2–5 (−7)	Nil	G, up to 30	Dense-cottony	≤ 35	25	34.06	O, 12–24 (av. 18.70)	M, D (occ.), 1 (−2)/oog.	P, A (oc.), 10–21 (av. 16.65)
P. splendens UQ6036	2–6 (−7)	Nil	G, 14–30 (−42)	Dense-cottony	≤ 35	30	38.67	Nil	Nil	Nil
P. torulosum UQ6291	2–4 (−6)	I, 4–10 (−12)	Nil	Radiate	≤ 35	30	12.59	S, 12–20 (av. 15.80)	M, D (occ.), 1–2(−3)/oog.	P, 12–18 av. 15.00)
P. ultimum UQ6023	4–6 (−10)	Nil	G, 12–20 (−25)	Dense-cottony	≤ 35	30	30.96	S, 19–26 μm (av. 22.35)	M, 1/oog.	A, P (oc.), 16–20 (av. 18.85)

I inflated, *Ir* irregular, *G* globose, *S* smooth, *O* ornamental, *M* monoclinous, *D* diclinous, *A* aplerotic, *P* plerotic, *Occ* occasionally, *Opt.* optimum, *Nil* not observed

[a] *P. myriotylum* were excluded for separate descriptions (unpublished data)

that of the control. *P. aphanidermatum*, *P. myriotylum*, *P. graminicola*, *P. spinosum*, *P. splendens* and *P. ultimum* appeared the most aggressive on the tested plant species, except on barley which was less vulnerable to attack by *P. splendens* and *P. ultimum* (DI < 0.5). All these pathogenic *Pythium* spp. attacked and killed the seeds before germination and also young seedlings of plant species tested in the assays (DI > 0.5–1) (Table 3). All eight tested *P. myriotylum* isolates were highly aggressive on the 12 different tested plant species (DI > 0.5), except for *P. myriotylum* UQ6152 and UQ6349 which were less aggressive on lettuce (DI = 0.42–0.43). The isolate *P. myriotylum* UQ5892 was least aggressive, compared to other *P. myriotylum* isolates on ginger sticks, but it was comparable to the others in attacking and killing seeds and young seedlings of tested plant species in this assay (Table 3). Where tested, the isolate of *P. torulosum* was only highly aggressive as classified by Zhang and Yang (2000) on wheat (DI > 0.5). *P. heterothallicum* and *P. perplexum* appeared to be least aggressive to those plant species on which they were tested.

Pathogenicity on ginger plants

Reference isolates of *P. myriotylum* CBS254.70 and *P. zingiberis* NBRC30817 were included in this pot trial after an additional permit was granted. Only *P. myriotylum* isolates, excluding *P. myriotylum* CBS254.70, all of which were obtained from PSR ginger, were able to infect and kill ginger plants at the two different temperature ranges, regardless of ginger cultivars tested; the only exception was *P. myriotylum* UQ5892 which showed a low disease severity level at both temperatures and more so at the lower of the two temperature regimes. None of the other *Pythium* spp. caused disease when assessed at the lower temperature. Where disease developed, initially above ground symptoms such as water soaking at collar regions and yellowing of lower leaves were observed as early as 5 and 10 DAI on ginger plants grown at 30/35 °C and 20/25 °C, respectively. However, by 21 DAI, disease severity eventually was not significantly different between the two temperature ranges, except for the one recorded on *P. myriotylum* UQ5892. *P. myriotylum* UQ5993 from capsicum, *P. myriotylum* CBS254.70, *P. zingiberis* NBRC30817, and *P. aphanidermatum* UQ6082 showed different levels of disease severity on ginger plants from the control only when the temperatures were higher than 30 °C (Fig. 4). All other *Pythium* spp. were non-pathogenic on ginger plants in the bioassays regardless of temperature. In all assays, *Pythium* spp. were re-isolated and identified based on morphology from infested potting mix by baiting confirming successful colonization of tested *Pythium* spp. in soil. Where applicable, the pathogenic *Pythium* spp. were also recovered from artificially induced PSR ginger in the bioassays.

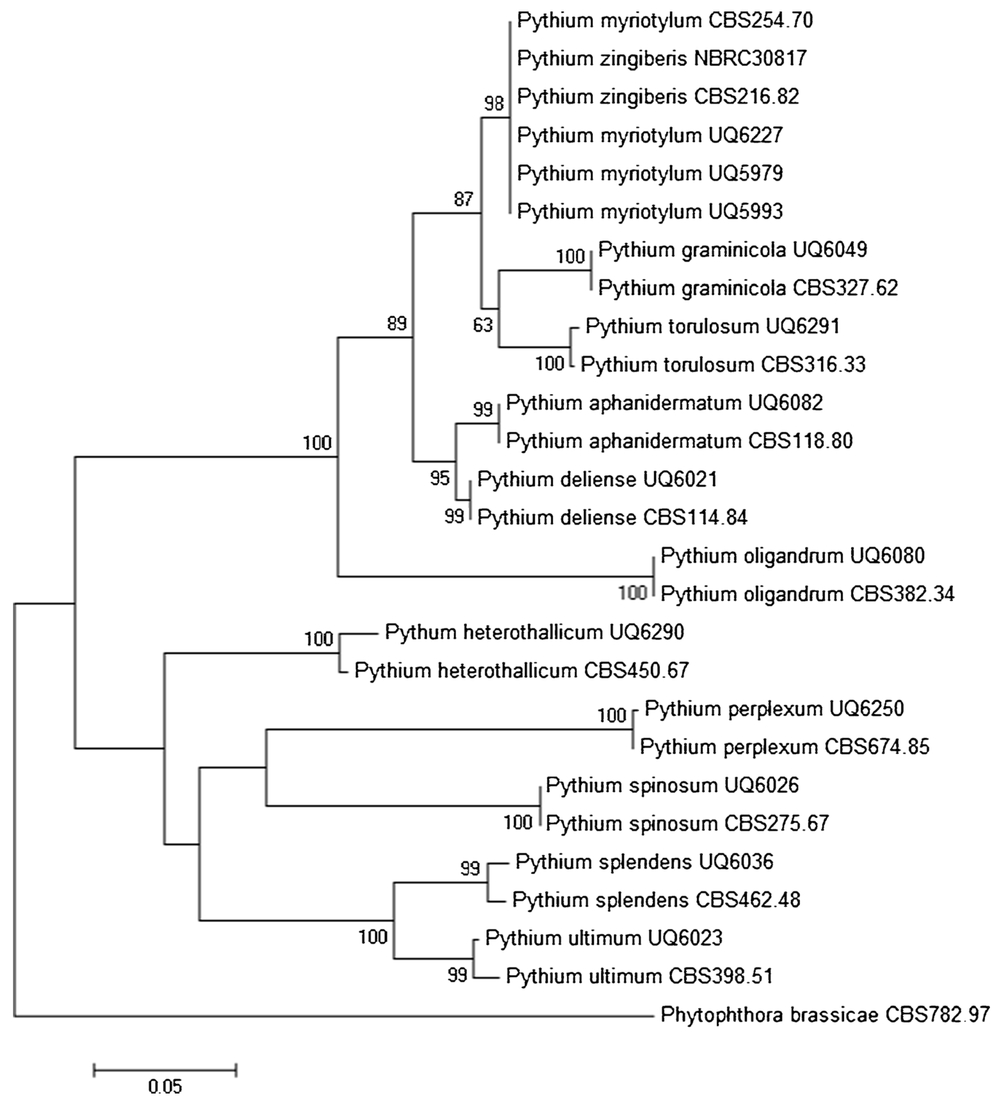

Fig. 2 A maximum likelihood tree showing phylogenetic relationships among *Pythium* spp. (only representatives presented) using DNA sequences encoded from ITS region. The numbers at the nodes are the percentage of the trees from bootstrap analysis (1000 replications)

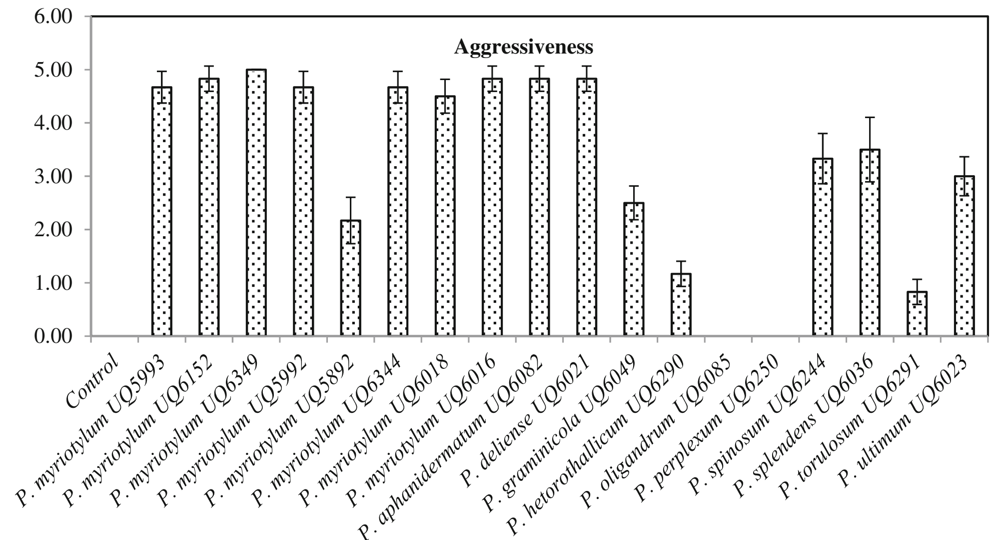

Fig. 3 Aggressiveness of selected *Pythium* spp. assessed based on mean colonization and recovery of *Pythium* spp. on 1 cm segments excised from 5.5 cm long ginger sticks at 27 ± 2 °C at 3 DAI (bars represent standard deviations, $n = 6$). Means were combined from two independent assays due to no significant difference between the two repetitions ($P = 0.05$)

Table 3 Comparisons of disease indices (0–1) recorded on seeds and seedlings of flower, vegetable and plant species in common rotation with ginger inoculated with *Pythium* spp.

Isolates	Beet root	Carrot	Cucumber	Eggplant	Lettuce	Spring onion	Gypsophllia	Barley	Buck wheat	Millet	Rye	Wheat
P. myriotylum UQ5993	0.95 a	0.99 a	0.83 bc	0.88 a	0.78 a	0.83 a	0.83 a	0.88 a	0.74 ab	0.98 a	0.91 ab	1.00 a
P. myriotylum UQ6152	0.88 a	0.58 a	0.78 c	0.84 a	0.43 bc	0.82 a	0.77 ab	0.73 ab	0.67 abc	0.95 a	0.89 ab	0.94 a
P. myriotylum UQ6349	0.89 a	0.58 a	0.88 abc	0.81 a	0.42 c	0.80 a	0.78 ab	0.75 ab	0.64 bc	0.88 ab	0.86 abcd	0.87 a
P. myriotylum UQ5992	0.97 a	0.64 a	0.91 ab	0.78 a	0.65 abc	0.88 a	0.83 a	0.71 abc	0.74 ab	0.97 a	0.86 abcd	0.87 a
P. myriotylum UQ5892	0.89 a	0.87 a	0.77 c	0.83 a	0.61 abc	0.76 a	0.70 b	0.57 bcd	0.53 c	0.98 a	0.72 bcde	0.87 a
P. myriotylum UQ6344	0.98 a	0.69 a	0.91 ab	0.88 a	0.68 ab	0.79 a	0.78 ab	0.73 ab	0.72 abc	0.94 a	0.91 ab	0.92 a
P. myriotylum UQ6018	0.95 a	0.67 a	0.88 abc	0.79 a	0.84 a	0.85 a	0.79 ab	0.76 ab	0.78 ab	0.98 a	0.87 abc	0.92 a
P. myriotylum UQ6016	0.91 a	0.76 a	0.87 bc	0.86 a	0.82 a	0.72 a	0.82 a	0.74 ab	0.70 abc	1.00 a	0.86 abcd	0.93 a
P. spinosum UQ6244	0.96 a	0.98 a	0.94 ab	0.80 a	0.81 a	0.80 a	0.83 a	0.58 bcd	0.77 ab	0.88 ab	0.63 cde	0.92 a
P. splendens UQ6036	0.94 a	0.91 a	0.94 ab	0.79 a	0.83 a	0.74 a	0.77 ab	0.37 d	0.74 ab	0.93 a	0.62 de	0.84 ab
P. ultimum UQ6023	0.95 a	0.91 a	0.99 a	0.84 a	0.83 a	0.79 a	0.79 ab	0.49 d	0.86 a	0.98 a	0.88 ab	0.95 a
P. aphanidermatum UQ6082	-	-	-	-	-	-	-	-	-	0.92 a	0.87 abc	0.85 a
P. graminicola UQ6049	-	-	-	-	-	-	-	-	-	0.94 a	1.00 a	0.86 a
P. heterothallicum UQ6290	-	-	-	-	-	-	-	0.39 d	-	0.32 c	-	0.38 d
P. oligandrum UQ6085	-	-	-	-	-	-	-	-	-	0.00 d	0.10 f	0.00 e
P. perplexum UQ6250	-	-	-	-	-	-	-	0.42 d	-	0.33 c	-	0.47 cd
P. torulosum UQ6291	-	-	-	-	-	-	-	0.50 cd	-	0.08 d	-	0.62 bc
Control	0.43 b	0.15 b	0.03 d	0.13 b	0.10 d	0.17 b	0.10 c	0.07 e	0.20 d	0.00 d	0.07 f	0.13 e

Values followed by different letters within a column show a statistically significant difference at the 5 % - level as determined by Tukey's test

P. deliense was excluded from this table as most data were presented in Le et al. (2015)

- not tested

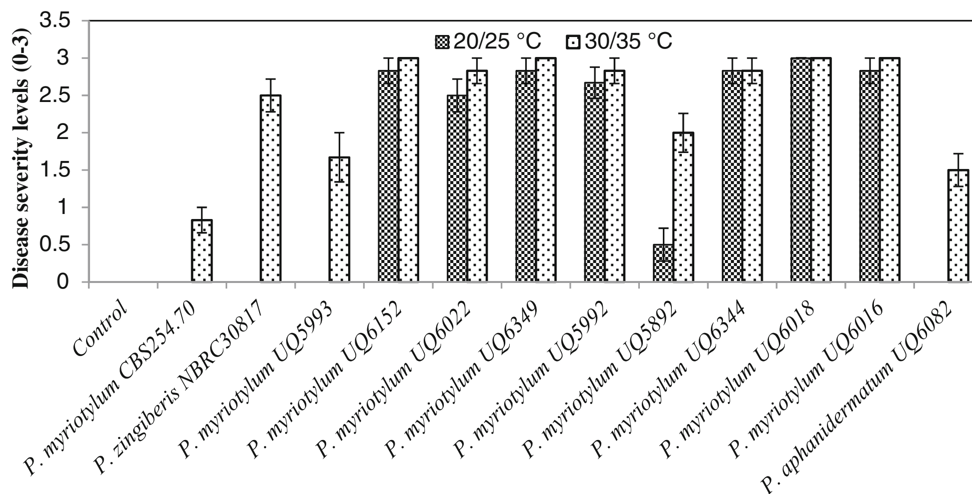

Fig. 4 Disease severity (0–3) which was recorded on ginger plants inoculated with *Pythium* spp. (only pathogenic *Pythium* spp. to ginger plants were presented) at 21DAI at two temperature ranges 20/25 °C and 30/35 °C with 10 h of photoperiods (bars represent standard errors, $n = 6$). Data presented the combination of two pot trials due to no significant difference in disease severity recorded on the two ginger cultivars tested ($P = 0.05$)

Discussion

Several oomycetes, namely *Pythium* spp. and *Pythiogeton ramosum* were isolated directly from PSR ginger in Queensland, Australia. *P. myriotylum* was isolated from most of the farms assessed. *P. spinosum* and *P. graminicola* were also occasionally recovered from PSR ginger. Unlike studies on other crops, including rooibos, bell pepper, common bean and soybean, of which more than one *Pythium* sp. was often recovered from diseased plants (Bahramisharif et al. 2013; Chellemi et al. 2000; Li et al. 2014b; Zitnick-Anderson and Nelson 2014), with PSR on ginger in this study only one particular *Pythium* species was isolated per site. In some cases, mixed infections of *Fusarium* sp. and *Pythium* sp. and *Pythiogeton* sp., were recorded although our findings indicated that PSR in ginger in Australia was not caused by such a disease complex.

Other *Pythium* spp., including *P. aphanideramtum*, *P. deliense*, *P. splendens* and *P. ultimum* have been reported as causal agents of PSR on ginger around the world (Dohroo 2005); in this study however, their recovery was only obtained from ginger baits. Ginger baits were quite effective in isolating *Pythium* spp. from soils around ginger, but the species of interest, *P. myriotylum* was not trapped from the field soils. This is possibly due to either the use of unsuitable baiting conditions or use of substrates which may have favoured the overgrowth of other more saprophytic species, namely *P. splendens* and *P. spinosum*. If this was the case, soil plating dilution with a *Pythium* selective medium will possibly be effective since *P. myriotylum* populations were assumed to be dominant in the soil habitat, and it will be important from a ginger farming system perspective to evaluate *P. myriotylum* population levels before planting ginger back into infested fields.

When potting mix was inoculated with a single *Pythium* species in an artificially inoculated pathogenicity assays in the pot trials, the same baiting technique successfully allowed recovery of *P. myriotylum*. Therefore in the absence of other competitors, recovery of *P. myriotylum* was obtained without much effort from the simple baiting technique. The success of recovering *Pythium* spp. from soil has been shown in the literature to be dependent on the target species and for *P. myriotylum* in particular, others have also reported difficulty (Lumsden et al. 1975; Wang and Chang 2003) and our experience with field baits support the assumption that *P. myriotylum* is often overgrown by other saprophytes and will prove difficult to isolate from soils.

In most cases, morphology and growth rate allowed us to identify to species status the *Pythium* spp. isolated in this study. All 11 *Pythium* spp. were identified using the keys of van der Plaats-Niterink (1981) and Dick (1990), although identification of some species, including *P. perplexum*, *P. splendens* and *P. heterothallicum* were at first not assured due to morphological similarities to others as well as a lack of sexual structures. The identified and putative species were further confirmed by sequences of the ITS region. The ITS sequence analysis is now used worldwide for *Pythium* identification, but for many cases, species boundaries could not be revealed by the ITS locus solely (Levesque and De Cock 2004; Robideau et al. 2011). In this study, a BLAST search from the Genbank database resulted in very high levels of identity, namely over 99 to 100 % homology to well-known reference isolates, so multiple gene sequencing was not required. The only exception was that species boundary of *P. myriotylum* and *P. zingiberis* was not clearly distinguished using only ITS sequence data. However, this was addressed by analyzing morphology, whole genome data and pathogenicity exclusively (unpublished data).

There have been many different *Pythium* spp. reported worldwide as major pathogens of PSR of ginger (Dohroo 2005). In Australia, *P. myriotylum* was reported in 2009 (Stirling et al. 2009) and in this study, the prevalence and

importance of the pathogen were revealed from all infested farms from the Queensland, Australia. Moreover, *P. myriotylum* also caused major losses to ginger crops in many other countries that being China, India, Fiji, Korea and Taiwan (Kim et al. 1997; Kumar et al. 2008; Stirling et al. 2009; Tsai 1991; Yuan et al. 2013). *P. myriotylum* is well-known for its high temperature preferences and it is most pathogenic at about its temperature optimum (37 °C) (van der Plaats-Niterink 1981). This was supported by the pot trial results, from which PSR symptoms were only observed on ginger plants inoculated with *P. myriotylum* CBS254.70, *P. zingiberis* NBRC30817 and *P. myriotylum* UQ5993 (capsicum isolate) at 30–35 °C. However, isolates of *P. myriotylum* initially obtained from PSR ginger in Australia appeared more aggressive at a wide temperature range, 20–35 °C. Likewise, Perneel et al. (2006) found that *P. myriotylum* recovered from cocoyam was most pathogenic to cocoyam at 28 °C. Interestingly, cocoyam was also more vulnerable to *P. myriotylum* isolates, which were initially isolated from diseased cocoyam, than those recovered from other hosts (Perneel et al. 2006). In addition, from our unpublished pot trial data, *P. myriotylum* from capsicum and *P. myriotylum* from ginger were strongly pathogenic to capsicum and ginger, respectively regardless of testing temperatures, but they were much less pathogenic when cross inoculations were undertaken. This could be implied that the *P. myriotylum* isolated from PSR infected ginger in this study possibly exhibited some level of host preference, so more pot trail screenings for their pathogenicity on other crops is warranted. If the theory is supported by future results, it may have a significant contribution to PSR control programs through crop rotations.

In addition to the ITS region, sequences of *Cox*I, *Cox*II and *β-tubulin* regions of representatives of *P. myriotylum* obtained from PSR ginger revealed genetic uniformity of the population. Unlike the *P. myriotylum* population recovered also from PSR ginger in China, certain genetic differences were noticed within sequences of the ITS region (Yuan et al. 2013). Because of the genetically uniform population of *P. myriotylum* on ginger farms in Australia, it could be implied that *P. myriotylum* may have been spread from one original source. Stirling et al. (2009) argued that the incidence of PSR on ginger in 2007 was a sporadic event in a very wet year on two of the oldest farms under continuous ginger cultivation, and so specific control methods were not recommended at the time apart from paying better attention to drainage and soil health. However, PSR is now observed on many farms (up to 70 % of the ginger farmers in the Sunshine Coast region have admitted to having PSR on their farms according T. Pattison (pers. Comm.)) and is of major concern for the ginger industry. Although quarantine approaches have been deployed in the regions, stricter on farm biosecurity to prevent further spread is necessary.

In this study, *P. aphanidermatum*, *P. deliense*, *P. graminicola*, *P. spinosum*, *P. splendens* and *P. ultimum*, which were isolated from either soil or diseased ginger or both, have also been found associated with PSR on ginger elsewhere around the world (Dohroo 2005). Isolates of these species were able to colonize and cause soft rot on ginger sticks at a single incubation temperature (27 °C) and were also highly pathogenic on a wide host range in vitro assays. However, none of these listed species were able to induce symptoms on ginger plants, except for *P. aphaniderамtum* which only attacked and induced PSR on ginger at 30/35 °C. This is also the first report of *P. aphanidermatum* attacking ginger in Australia. The results here are therefore in agreement with the literature that *P. aphanidermatum* is a high temperature preference species (van der Plaats-Niterink 1981). Nonetheless, Li et al. (2014a) found that *P. aphanidermatum* recovered initially from PSR ginger in China was capable of inducing symptoms of PSR on ginger at 24–26 °C. Therefore, it could be of interest to undertake population studies of *P. aphanidermatum* and ginger around major ginger growing regions since findings might lead to a significant impact for PSR control. The current study could not induce PSR symptoms on ginger plants inoculated with *P. deliense*, *P. graminicola*, *P. spinosum*, *P. splendens* and *P. ultimum*, but presence of these pathogenic species should not be underestimated. Except for *P. spinosum* and *P. ultimum* which were reported as secondary and postharvest pathogens on ginger, respectively (Dohroo 2001; Le et al., 2010; Teakle 1962), presence of other *Pythium* spp. on Australian ginger farms might pose additional threats to the ginger industry in Australia once favorable environmental conditions for these species to thrive are met.

The recovery of non-pathogenic *P. oligandrum*, which is in fact a well-known wide host range mycoparasite and capable of parasitizing species within the same genus, that being *Pythium* spp. (Gerbore et al. 2014), might be of interest for further interaction studies between the main PSR pathogen, *P. myriotylum* and the mycoparasite.

In conclusion, PSR disease has remained as a main constraint for ginger industry in Australia since the first outbreaks recorded in 2007. Although a number of *Pythium* spp. have been found to be associated with PSR in certain conditions, the PSR on ginger in Australia was mainly attributed to *P. myriotylum*. Pathogenicity of *P. myriotylum* across a wide temperature range might indicate for an occurrence of a new more aggressive strain of *P. myriotylum*. Therefore, it is worth practicing a stricter quarantine approach to contain the spread, as well as looking for alternative control strategies, including biocontrol.

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