Saudi Journal of Biological Sciences (2010) **17**, 347–352

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Two pathogenic species of *Pythium*: *P. aphanidermatum* and *P. diclinum* from a wheat field

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Received 22 April 2010; revised 3 May 2010; accepted 3 May 2010
Available online 21 May 2010

KEYWORDS

Egypt;
Morphological identification;
Pre- and post-emergence damping-off;
Pythium;
Rhizosphere;
Sequence of rDNA-ITS

Abstract During a survey of pathogenic and non-pathogenic *Pythium* spp. in different localities in Egypt, several isolates of *Pythia* were obtained and maintained on corn meal agar. Among these isolates, *Pythium aphanidermatum* and *Pythium diclinum* were obtained from rhizosphere of wheat plants grown in Dear Attia village, Minia, Egypt. Identification was made using morphological and molecular analyses. *P. aphanidermatum* and *P. diclinum* were able to cause reductions in emergence and adulating in wheat in laboratory scale. *P. aphanidermatum* appeared to be the most aggressive parasite under agar and pot experimental conditions.

1. Introduction

Pythium species are morphologically polymorphic, physiologically unique and ecologically versatile, which make them significant both theoretically and practically. They are ubiquitous in soil and in water, distributed worldwide, and with very diverse host ranges. They include some of the most important and destructive plant pathogens, causing losses of seeds, pre-emergence and post-emergence damping-off, rots of seedlings, roots, or basal stalks, decays of fruits and vegetables during cultivation, storage, transit or at the market, and serious damages of a wide variety of crops in Egypt (Abdelzaher et al., 1997; Yu and Ma, 1989).

Previous works revealed that the pathogenicity of *Pythium aphanidermatum* was proved. It can cause diseases of many plants from all over the world such as root rot and damping-off, stalk and rhizome rot, soft rot, fruit rot or cottony blight of *Abelmoschus esculentus*, *Basella alba*, *Carica papaya*, *Catharanthus roseus*, *Carthamus creticus*, *Chrysanthemum indicum*, *Citrus* sp., conifers, corn, a number of other crops, Crucifers, Cucurbitaceae, Leguminosae, *Fragaria* sp., *Gossypium* sp., grasses, *Ipomoea, Lactuca sativa*, *Linum usitatissimum*, peppers, poinsettia, *Solanum* sp., sugar beet, sugar cane, *Talinum fruticosum*, *Tephrosia* sp., tobacco and tomato (Plaats-Niterink, 1981; Abdelzaher et al., 2004). In case of the pathogenicity of *Pythium diclinum*, it was originally isolated from young rice plants in Japan. It has been proved a pathogen to wheat (Abdelzaher, 2004).

Pythium was recognized as a major pathogen of wheat in several countries, affecting in reduction the yields by almost 30% (Cook and Haglund, 1982). In Egypt, no comprehensive studies are available concerning losses in wheat yield due to

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doi:10.1016/j.sjbs.2010.05.001

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infection by *Pythium* despite reports of its frequent disconnection from Egyptian soils (Abdelzaher et al., 1997; Mazen et al., 1991).

This paper deals with the morphological and molecular description of *P. aphanidermatum* and *P. diclinum* isolated from an agricultural field cultivated with wheat. Pathogenicity of these fungi was also tested.

2. Materials and methods

Thirteen isolates of *P. aphanidermatum* and 17 isolates of *P. diclinum* were isolated from rhizosphere soil of wheat plants grown in Dear Attia, Minia city, Egypt (28°5′00″N, 41°30′03″E).

2.1. Morphological studies

Portions of the rhizosphere soil were placed in Petri-dishes containing NARM [nystatin (10 mg L^{-1}), ampicillin (250 mg L^{-1}), rifampicin (10 mg L^{-1}) and miconazole (1 mg L^{-1}) in cornmeal agar (CMA)] medium for isolation of *Pythium* species selectively (Senda et al., 2009). The emerging hyphal tips were transferred to water agar (WA) medium for further purification from bacterial contamination as follows: a small block of agar medium from the distal end of a colony grown in NARM medium was cut and re-inoculating the block on 2.5% WA medium in a Petri-dish to obtain a colony of about 1 cm diameter. Then the whole agar medium in the Petri-dish was replaced upside-down with a flamed forceps in the same Petri-dish and incubated until the colony reached before the dish wall, during this procedure the mycelia penetrate the agar medium without the contaminating bacteria and reach the top of the agar medium. A thin piece of agar containing a single hyphal tip of the desired fungus was taken from the surface of the margin of the colony on water agar medium under the microscope and transferred to corn meal agar (CMA) slant for maintaining the fungus and CMA plates supplemented with 500 μg ml^{-1} wheat germ oil to check the sexual structure formation. Segments (1 × 0.5 cm) of *Zea mays* leaf blades were placed in contact with the colonial margin on the same WA dish for 24 h at 25 °C and then transferred to a sterilized distilled water and incubated at 5, 10 15, 20, 25, 30 and 35 °C to check zoospores (Waterhouse, 1967) and sexual reproductivity. The key of Plaats-Niterink (1981) and Dick (1990) were principally used for identification. Keys and descriptions by Waterhouse (1967, 1968) and Middleton (1943) and the original descriptions were also consulted for comparison or confirmation of identifications. Thirty measurements were made of each isolate wherever possible. Since structures such as antheridia and sporangia may be formed rapidly and then degenerate, cultures were observed about 8 h after inoculation and then periodically until all possible characters had been observed.

2.2. Temperature–growth relations

Minimum, optimum and maximum temperatures of each fungus were determined on CMA medium (17 gl l^{-1}, BD-BBL™) inoculated with 5 mm diam discs from stock cultures on CMA plates. All plates were incubated at 25 °C for 24 h before starting growth determinations. Cardinal temperatures were evaluated by measuring growth at different temperature (5, 10, 15, 20, 25, 30, 35, 40 and 43 °C).

2.3. Pathogenicity test

2.3.1. In vitro

Pathogenicity was evaluated in water agar as a medium for grains germination. One hundred milliliter of water agar (2%) was poured each in 250 ml Erlenmeyer flasks and then sterilized by autoclaving. Wheat grains were sterilized by surface disinfection using Clorox 2% for 3 min and then washing three times by sterilized distilled water followed by 1 min in 70% ethyl alcohol and finally by three times using sterilized distilled water. Grains were germinated to form radicles and plumules for 2 days at 25 °C and the viable ones were selected. Thereafter, in each Erlenmeyer flask, three wheat grains were planted in each flask for damping-off tests. Three mycelial discs of each fungal isolate taken from actively growing margin of *Pythium* colonies grown on CMA medium were transferred to each flask containing wheat grains under aseptic conditions. All inoculated flasks were then incubated in a growth cabinet (Precision, United States) at 25 °C with 12 h photoperiod (91 μmol m^{-2} s^{-1}). Level of damping-off was determined as the difference between seedlings emergence in the non-inoculated controls and the inoculated one.

2.3.2. In vivo

Pythium inocula were prepared with 2.5% inoculum concentration for pre-emergence damping-off test and 5% inoculum concentration for post-emergence damping-off experiments. Inoculated soil was prepared as the following: 5 g of grass blade (*Echinochloa colonum*) leaf segments (1 × 0.5 cm) and 2 g glucose were moistened by adding distilled water (10 ml) each in 250 ml Erlenmeyer flask. After autoclaving at 121 °C for 20 min, each flask was inoculated with three disks (7 mm diameter) of water agar inoculated with growing margins of *Pythium* spp. obtained from CMA culture medium. The inoculated flasks were held at 25 °C for 10 days. In pre-emergence damping-off tests, the inoculum concentration of 2.5%, was prepared by mixing throughly 1 g of colonized grass leaf segments with 50 g of oven-dried (70–80 °C for 2 days) clay loam soil using a sterilized mortar and pestle and 2.5 g of this mixture were added to 97.5 g of clay loam soil which had been sterilized by autoclaving at 121 °C for 60 min (pH 7.1) and kept in plastic bag for 2–3 weeks at room temperature with 25% water content prior to use (CL soil). Each was distributed into 10 replicate pots (500 g) and 12 wheat grains were planted in each pot for pre-emergence damping-off test, whereas in case of post-emergence damping-off experiments the inocula were added to the soil after the seedling emergence.

Pre-emergence damping-off was determined as the difference in seedling emergence between healthy control soil and infested soil. Post-emergence damping-off was determined from the number of diseased plants as a percentage of the emerged ones. The experiments were carried out in a growth cabinet (Precision, United States) at 25 °C with 12 h photoperiod (91 μmol m^{-2} s^{-1}). Healthy and diseased seedlings were counted at regular intervals until the development of the first two leaves. Pre-emergence damping-off level was determined as difference in seedling emergence between healthy control soil and infested soil. The experiments were repeated twice.

2.4. Molecular studies

2.4.1. DNA extraction

Mycelia were grown in V8 agar medium at 25 °C for 7 days or until adequate growth was observed. To extract the total genomic DNA, mycelia from the edge of *Pythium* colony from a culture plate were suspended in 200 µl of PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, CN, USA) in a 2.0 ml microcentrifuge tube. Samples were vortexed for 10–30 s and then heated for 10 min at 100 °C in dry thermo unit or water bath. Samples were spin for 30 min at 15,000*g*. Supernatants were transferred into a new microcentrifuge tubes and were ready for (PCR) amplification by the polymerase chain reaction (Senda et al., 2009).

2.4.2. DNA amplification and sequencing

The nuclear rDNA region of the internal transcribed spacer (ITS), including the 5.8S rDNA, was amplified with the universal primers ITS 4-(5′TCCTCCGCTTATTGATATGC3′) and ITS 5-(5′GGAAGTAAAAGTCGTAACAAGG3′). Depending on the experiment, sometimes, primers of ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and ITS2 (5′GCTGCGTTCTTCATCGATGC3′) were used as described by White et al. (1990) and Matsumoto et al. (1999). The amplicons were 700–900 bp long. On the other hand, 563 bp of the *cox II* gene was amplified in certain *Pythia* with the primer pair FM66 (5′TAGGATTTCAAGATCCTGC3′) and FM58 (5′CCACAAATTTCACTACATTGA3′) (Martin, 2000). Amplification of the sequencing template was carried out with DNA Thermal Cycler 2700 (Applied Biosystems) with a cycling profile of pre-PCR at 94 °C for 5 min, followed by denaturation at 94 °C for 1 min, 1 min primer annealing at 55 °C for ITS, 52 °C for *cox II* and elongation at 72 °C, 2 min for 40 cycles, with a 7 min extension at 72 °C after the final cycle. To check the presence of PCR products, 5 µl of the PCR reaction mixture was loaded in 2% L03 (Takara Bio) agarose gel, electrophoresed at 100 V, 20–30 min, and stained with ethidium bromide. The sequencing templates were purified with GenElute PCR clean-up kit (Sigma Chemical Co., St. Louis, Missouri, USA) following the manufacturer's instructions. Sequencing was performed with BigDye Terminator v3.1 Cycle Sequencing Reaction kit (Applied Biosystems) using the same primers in the initial PCR step. After purifying the sequencing reaction mixture through ethanol precipitation it was run on ABI 3100 DNA Sequencer (Applied Biosystems).

3. Results

3.1. Morphological description of isolated Pythia

Description of each fungus was based on a single isolate in water culture.

3.1.1. P. aphanidermatum (Edson) Fitzp. (Plate 1)

Colonies on cornmeal agar have a cottony aerial mycelium, on potato–carrot agar they have some loose aerial mycelia without a special pattern and with heavy aerial mycelia on potato dextrose agar (PDA). Main hyphae are up to 10 µm wide. Zoosporangia consist of terminal complexes of swollen hyphal branches of varying length and up to 22 µm wide. Zoospores are formed at 15–30 °C. Encysted zoospores have a diameter of 12 µm. Oogonia are terminal, globose, smooth and of (20–) 22–25 (–26) µm (average 24 µm) diameter Antheridia are mostly intercalary, sometimes terminal, broadly sac-shaped, 11–15 µm long and 9–15 µm wide, 1(–2) per oogonium and monoclinous or diclinous. Oospores are aplerotic, (19–) 20–23 µm (average 21 µm) in diameter and their walls are 1–2 µm thick.

3.2. Molecular description of P. aphanidermatum

Sequence of rDNA-ITS including the 5.8S rDNA for *P. aphanidermatum* studied by the method of Kageyama et al. (2003) to confirm the species identification. Results showed the presence of 777 bases and a BLAST search showed complete resemblance to the reference isolate with 100% similarity (Genbank accession no. AB160845.1).

3.3. Morphological description of P. diclinum Tokunaga (Plate 2)

Colonies on cornmeal agar and potato–carrot agar submerged and with a more or less radiate pattern. Main hyphae up to 6 µm wide. Appressoria club-shaped. Sporangia filamentous non-inflated, branched or unbranched. At 5 °C forming vesicles containing zoospores. Encysted zoospores 6–7 µm diameter. Oogonia spherical or ovoid, smooth, mostly terminal or subterminal, occasionally intercalary, (18–) 19–23 (average 20.5) µm diameter. Antheridia typically diclinous, 1–2 per oogonium, about 12×5 µm. Antheridial stalks not branched. Oospores single, aplerotic, 17–19 (average 17.5) µm diameter, wall up to 3 µm thick.

3.4. Molecular description of P. diclinum

Sequence of rDNA-ITS including the 5.8S rDNA for *P. diclinum* studied by the method of Kageyama et al. (2003) to confirm the species identification. Results showed the presence of 777 bases and a BLAST search showed complete resemblance to the reference isolate with 99% similarity (Genbank accession no. AY598690.1).

3.5. Temperature–growth relations

As shown in Table 1, *P. aphanidermatum* started to grow at 10 °C with optimum between (25–30 °C). The maximum temperature for mycelia growth of *P. aphanidermatum* was above 43 °C. *P. diclinum* started to grow at 5 °C with optimum at 25 °C and maximum at 35 °C.

3.6. Pathogenicity tests

3.6.1. In vitro (agar flasks)

Pathogenicity of *Pythia* was tested on wheat grains. *P. aphanidermatum* was highly pathogenic to wheat grains causing (100%). *P. diclinum* was less pathogenic to wheat grains causing (67%) damping-off at the two studied inoculum concentrations.

3.6.2. In vivo (pot experiment)

Pathogenicity tests (pre-emergence damping-off) were performed also in pots using 2.5% inoculums concentration of the tested *Pythia* added to soil and incubated for 2–3 weeks be-

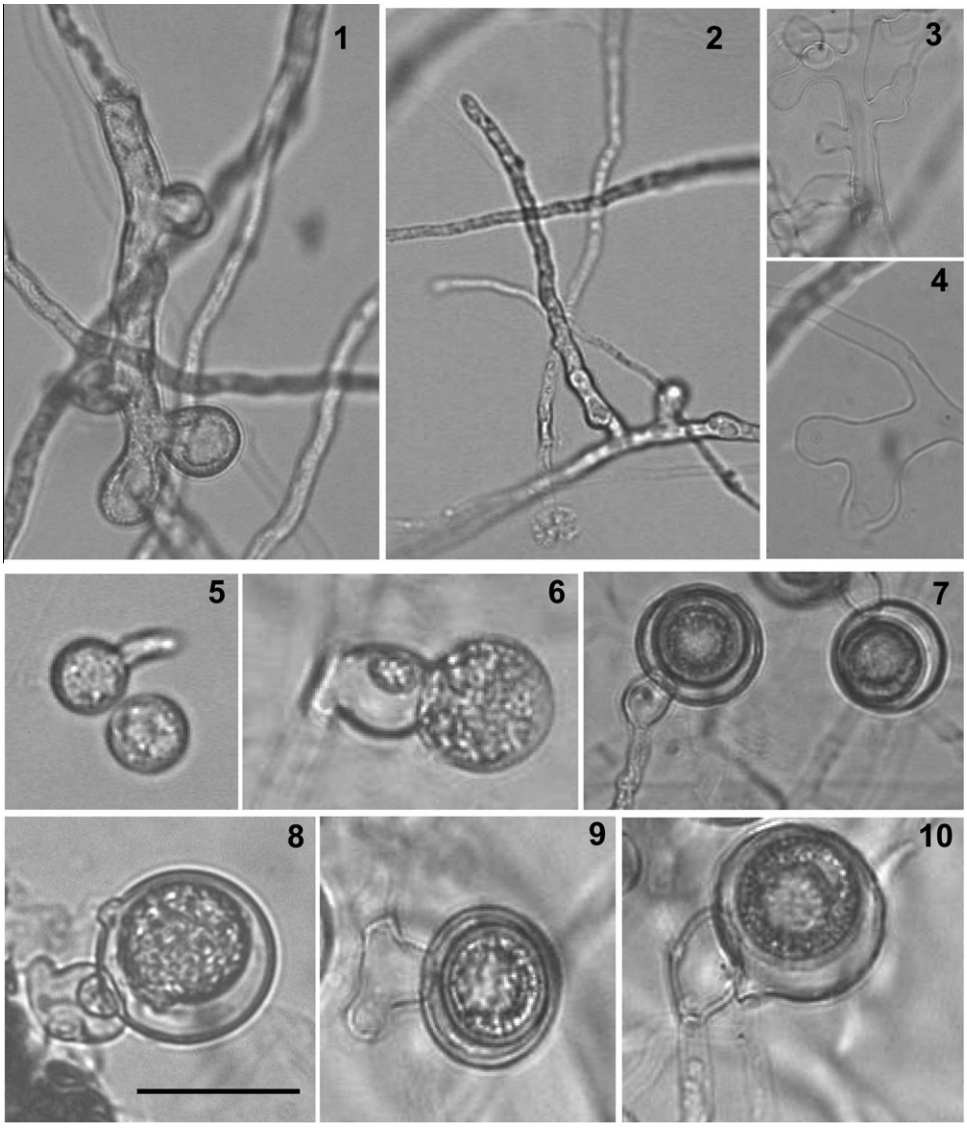

Plate 1 Morphology of *Pythium aphanidermatum*. (1 and 2) A toruloid zoosporangium. (3 and 4) Empty toruloid zoosporangia. (5) Encysted and germinating zoospores. (6) Young oogonium and intercalary antheridium. (8 and 9) Intercalary antheridia. (7, 9 and 10) Aplerotic oospores. Bar (20 μm) on photo 8 is applicable to the rest photos.

fore cultivated wheat grains. *P. aphanidermatum* was highly pathogenic to wheat grains causing 100% damping-off. *P. diclinum* was moderately pathogenic producing 60% damping-off. Post-emergence damping-off experiments indicated that *P. aphanidermatum* was highly pathogenic to wheat seedlings causing 100% damping-off while *P. diclinum* caused 60% damping-off.

4. Discussion

The first announcement of diseased wheat in Minia, Egypt was in 1997 when Abdelzaher and his co-workers reported damping-off of wheat caused by *P. ultimum* var. *ultimum* (Abdelzaher et al., 1997). They isolated *P. ultimum* var. *ultimum* from the infected wheat plants while *P. diclinum* was only obtained from rhizosphere soil of the infected wheat seedlings. They also tested the pathogenicity of *P. diclinum* against wheat germinating seeds and seedlings and reported that *P. diclinum* was a non-pathogenic fungus (Abdelzaher et al., 1997). Our findings here revealed the occurrence of *P. diclinum* in a wheat field in Dear Attia village, Minia city, Egypt. Plaats-Niterink (1981) pointed out that nothing is known about the pathogenicity of *P. diclinum*. Later, Abdelzaher et al. (2004) reported the incident of *P. diclinum* as a pathogen to wheat. Avirulent *Pythium* species can become a very pathogenic agent especially when the environmental factors favor the disease prevalence. For this reason, occurrence of *P. diclinum* in rhizosphere soil of wheat plant represents a risk factor to wheat production.

P. diclinum was originally isolated from flooded young rice plants in Japan (Tokunaga, 1932). In the species with non-inflated filamentous zoosporangia, *P. diclinum* is characterized by its typically diclinous antheridia and aplerotic thick-walled oospores. Schenk described from algae a fungus with only filamentous zoosporangia and named it *Pythium gracile* (Schenk, 1859). A year later, De Bary (1860) described a fungus from dead insects which he called *Pythium reptans*, but he considered that this isolate was synonymous with Schenk's *P. gracile* in a footnote. In 1881, De Bary described another isolate from

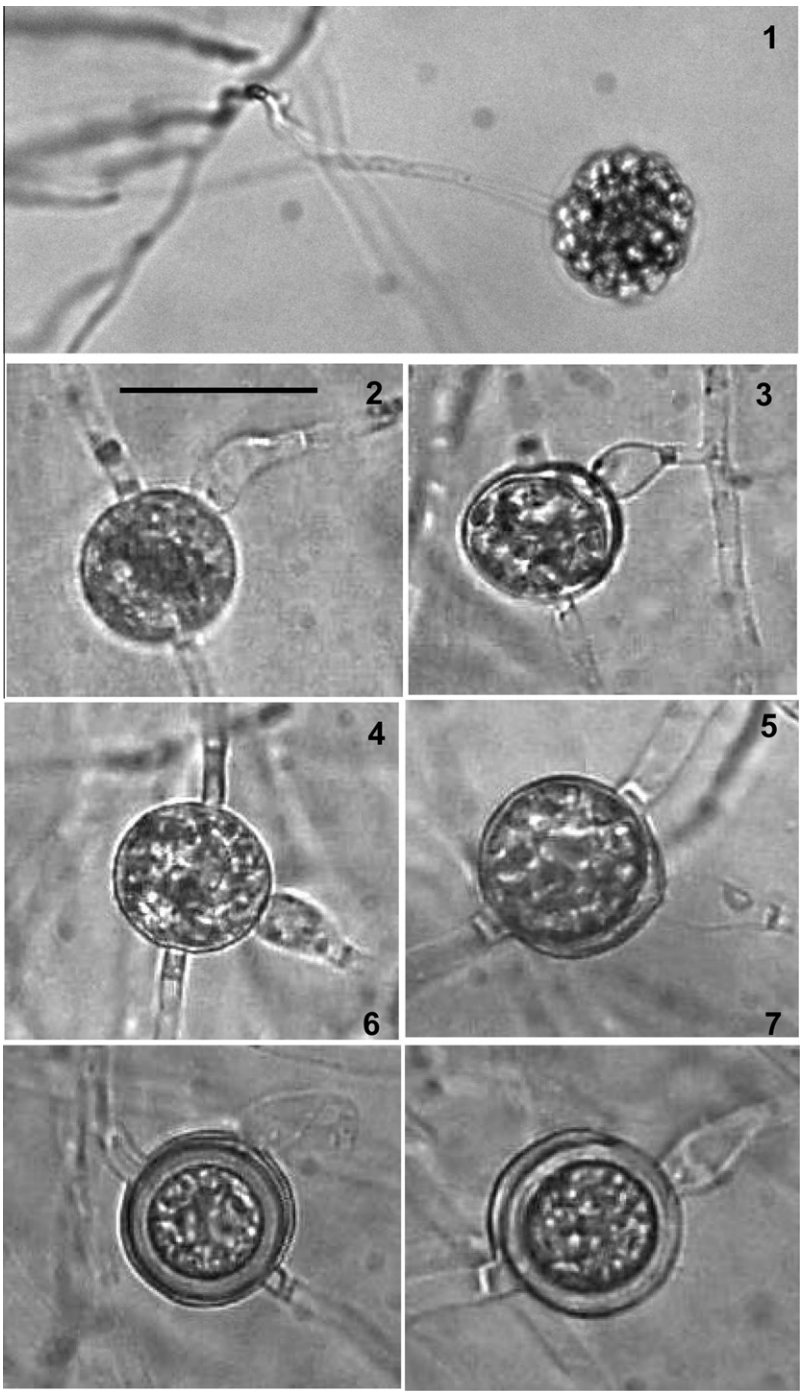

Plate 2 Morphology of *Pythium diclinum*. (1) Filamentous zoosporangium with evacuation tube carrying a vesicle containing zoospores. (2–5) Diclinous antheridia attached to intercalary oogonia. (6 and 7) Thick-walled aplerotic oospores. Bar (20 μm) on photo 2 is applicable to the rest photos.

Table 1 Linear growth of *Pythium aphanidermatum* and *Pythium diclinum* at various temperatures.

Pythium spp.	Average growth (mm) after 24 h on cornmeal agar									
	2 °C	5 °C	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C	40 °C	43 °C
P. aphanidermatum	0	0	3*	15	25	32	33	30	29	4
P. diclinum	0	2	7	9	16	20	16	2	0	0

* Growth is the mean of five replicates and no significant difference in diameter within replicates of each measurement was observed.

decaying insects, which he identified as *P. gracile* (De Bary, 1881). He pointed out that this isolate seems to be *Pythium monospermum* based upon its sexual structures. Butler (1907) gave a further description of *P. gracile*, including three varieties. The description of *P. gracile* by keys of Matthews (1931) and Middleton (1943) agree well with cultures of *P. diclinum* in the CBS collection examined by Plaats-Niterink (1981) who proposed CBS 66479 as the neotype of this species. Many researchers thereafter agreed with Plaats-Niterink's treatment (Yu and Ma, 1989; Abdelzaher, 1999). The ITS region of the nuclear ribosomal DNA of our isolate of *P. diclinum* is comprised of 777 bases and a BLAST search showed complete resemblance of 99% similarity (Genbank accession no. AY598689.1). Subsequently, we report here that this fungus is an independent species of *Pythium* and confirm the identification of *P. diclinum*.

P. aphanidermatum are typical plant parasites of plants in warm regions (Plaats-Niterink, 1981). It characterized by inflated zoosporangia and intercalary antheridia. *P. aphanidermatum* is similar to *Pythium deliense* but oogonia in the latter pend toward antheridia wears the oogonial stalk is upright in the former. This character is not a clear criterion during identification. Subsequently, researchers always face problem in distinguish between identification between *P. aphanidermatum* and *P. deliense*. For this reason, identification of *P. aphanidermatum* using molecular criteria is important. The results show that 13 isolates of *P. aphanidermatum* were isolated from the rhizosphere soil of wheat. The ITS region of the nuclear ribosomal DNA of our isolate of *P. aphanidermatum* is comprised of 777 bases and a BLAST search showed complete resemblance of 100% similarity (Genbank accession no. AB274404.1). Subsequently, we confirm the identification of *P. aphanidermatum* using morphological and molecular identification.

Our results here confirm the pathogenicity of *P. aphanidermatum* and *P. diclinum* to wheat germinating seeds and seedlings. For this reason, occurrence of these two pathogenic *Pythium* spp. in rhizosphere soil of wheat plants represents a risk factor especially when environmental conditions favour disease prevalence.

The experimental work described here demonstrates that *P. aphanidermatum* and *P. diclinum* are able to cause reductions in emergence and adulting in wheat. *P. aphanidermatum* appeared to be the most aggressive parasite under the given experimental conditions.

The occurrence of such species in the rhizosphere of wheat plants represents a very dangerous element in the soil. When the conditions favor a disease proceeding, *Pythium* species became a main source of pre- and post-emergence damping-off of germinating seeds and seedlings. Therefore, studying the occurrence of *Pythia* in cultivated fields is of important to draw a map of its distribution in fields for cultivation. Distribution of pathogenic *Pythium* species in agricultural soil should studied further.

Acknowledgment

Thanks are due to Dr. Hani M.A. Abdelzaher, Professor at Department of Botany and Microbiology, Faculty of Science, Minia University, Egypt for kind assistance during soil sampling.

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