Using of RAPD-PCR for Separation of *Pythium spinosum* Sawada into Two Varieties: var. *spinosum* and var. *sporangiferum*

Youssuf A. M. H. Gherbawy and Hani M. A. Abdelzaher

1 Botany Department, Faculty of Science, South Valley University, Qena, Egypt
2 Botany Department, Faculty of Science, El-Minia University, El-Minia City 61519, Egypt

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**Summary**  *Pythium spinosum* var. *spinosum* and *Pythium spinosum* var. *sporangiferum*, 2 varieties of *Pythium spinosum* were isolated from the rhizosphere soil of wheat grown in Bear-Elnansura zone (The New Valley Governorate, Egypt) and from the mud soil at the bank of Sheatan Lake (Tunisia), respectively. Cardinal temperatures, mycelial growth, zoospore production, and morphological features using light and electron microscopy under different conditions revealed that the 2 isolates studied were distinct different varieties. The randomly amplified polymorphic DNA (RAPD) banding patterns of 8 isolates of *Pythium spinosum* generated by 3 random oligonucleotides showed a very high level of variation, allowing these isolates to be separated into 2 groups. These results support the separation of *Pythium spinosum* into 2 varieties. The 2 varieties were proved to be highly pre-emergence damping-off pathogenic *pythia* against cucumber germinating seeds.

**Key words**  Morphological features, *Pythium spinosum* var. *spinosum*, *Pythium spinosum* var. *sporangiferum*, RAPD-PCR.

*Pythium spinosum* Sawada is a cosmopolitan species and has a wide host range (Van der Plaats-Niterink 1981). It infects the seeds and young roots of a broad range of plant species and is found in nearly every country of the world (Van der Plaats-Niterink 1981, Botha 1993). It was originally isolated from seedlings of *Antirrhinum majus* in Formosa, Taiwan (Sawada and Chen 1926). It was recorded not only from numerous different plants but also soil and water (Van der Plaats-Niterink 1981).

*Pythium spinosum* has been isolated from many countries as Taiwan, Japan, Queensland, New Zealand, India, South Africa, USA, Fidji Island, Argentina, Hawaii, England, The Netherlands and the former Czechoslovakia (Van der Plaats-Niterink 1981, Abdelzaher 1994, 1995). Keys and monographs of the genus *Pythium* (Middleton 1943, Waterhouse 1967, Van der Plaats-Niterink 1981, Dick 1990) treated *Pythium spinosum* as a non-zoospore forming *Pythium* species, however, terminal and intercalary hyphal swellings have been described by Sawada and Chen (1926). Since that time no reports were given concerning zoospore production by this species.

Botha (1993) reported that when *Pythium spinosum* exposed to a non-sterile soil extract and light, 6 isolates from South Africa and 2 isolates from culture collections produced zoospores. He added that autoclaved and filter sterilized soil extract, as well as a defined salt solution failed to induce sporangial formation. In addition, he reported that the temperature growth response of the isolates studied different, but the dimensions of sexual and asexual reproductive structures were closely similar. Before and after Botha’s (1993) claim no reports have been given concerning zoospore formation by *P. spinosum*.

Identification of the closely similar species of *Pythium* using traditional mycological methods based on morphological differences or pathogenicity tests can be difficult and time consuming. Sev-
eral molecular techniques has been developed to detect and identify different *Pythium* species, protein analysis (Martin 1995), immunological methods (Yuen et al. 1993), DNA probe (Matthew et al. 1995), analysis of PCR (polymerase chain reaction) amplified rDNA (Rafin et al. 1995), RAPD-PCR (Herrero and Klemsdal 1997) and restriction fragment length polymorphism (RFLP) analysis of the internal transcribed spacer region of ribosomal DNA (Wang and White 1997). The advent of molecular methods has supplemented traditional taxonomic methods with DNA-based tools with which to examine phylogenetics and systematics of fungi. During the last years, numerous papers have described PCR using short arbitrary primers (RAPD) as a useful method in genetic mapping and diagnosis, and in molecular taxonomy and evolutionary studies.

The aim of this study was to compare RAPD patterns of some *Pythium spinosum* isolates to determine the relation between morphological (the ability of zoospore formation) and molecular characteristics.

**Materials and methods**

**Morphological studies**

Isolation and maintenance of cultures: For selective isolation of the studies pythia from the soil, portions of the rhizosphere soil were placed in Petri-dishes containing VP3 medium of the following composition (g/l): Sucrose 20, CaCl₂ 0.01, MgSO₄·7H₂O 0.01, ZnCl₂ 0.001, CuSO₄·7H₂O 0.00002, MoO₃ 0.00002, MnCl₂ 0.00002, FeSO₄·4H₂O 0.00002, Corn meal agar (CMA) 14, Agar 23, antibiotics (mg/l): Pimaricin 5, Vancomycin 45, Penicillin 50, PCNB 100, Thiamin HCl was add at the rate of 100 mg/l (Ali-Shtayeh et al. 1986). 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 growing in the VP3 medium was cut and re-inoculated on 2.5–3% WA medium in a Petri-dish to obtain a colony of about 1 cm diameter. The whole agar medium in the Petri-dish was then turned 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 non-contaminated mycelia penetrated the agar medium and reached the top of the agar medium. Thin pieces of the agar containing a single hyphal tip of the fungus were taken from the margin of the colony and transferred to CMA slants for maintaining the fungus and to CMA plates supplemented with 500 µg/ml wheat germ oil to check the formation of sexual structure. *Zea mays* leaf blades were placed in contact with the colony margin on the same WA dish for 24 h at 25°C and then transferred to sterile distilled water and incubated at 5, 10, 15, 20, 25, 30 and 35°C to check the formation of zoospores (Waterhouse 1967) and sexual reproductively.

Identification of *Pythium* species: The keys of Van der Plaat-Niterink (1981) and Dick (1990) were principally used for identification. Keys and descriptions by Middleton (1943) and Waterhouse (1967, 1968) were also consulted for comparison or confirmation of identifications. Fungal maintenance was performed as described previously (Ichitani and Kang 1988, Abdelzaher et al. 1997a).

Thirty measurements were made of 1 of each variety 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.

Cardinal temperature studies: Minimum, optimum and maximum temperatures of mycelial growth of 4 isolates of each variety were concluded on corn meal agar (CMA 17 g l⁻¹, Difco) inoculated with 5 mm diam discs from stock cultures on water agar (WA) plates. All plates were incubated at 25°C for 24 h before starting growth determinations. Cardinal temperature were evaluated at 2–40°C as (2, 4, 7, 10, 15, 20, 25, 30–40°C) with 5 replicates for each temperature.

Production of zoospores: Four isolates of each varieties of *P. spinosum* were inoculated in Petri-dishes containing 3% WA until the developing colonies reached about 4 cm diam. The auto-
claved pieces (3×14 mm) of Zea mays leaf blades were laid over each colony and in contact with the actively growing margin and incubated at 20°C. After 24 h incubation, colonized Zea mays leaf blades were transferred to Petri-dishes containing 10 ml of heat sterilized distilled water and incubate at different temperatures (5, 10, 10, 20, 25, 30, 35°C).

Induced solutions recommended by Botha (1993) were tested for inducing zoospore formation by 4 isolates of P. spinosum var. spinosum which were not able to produce zoospores by normal conditions. Colonized Zea mays leaf blades by isolates of P. spinosum var. spinosum were places in 7 cm diam Petri-dishes filled with 20 ml soil extract from 3 different types of soil (sandy, sandy loam, clay loam) and incubated for 48 h. Soil extract was prepared from 5 g air-dried soil suspended in 11 distilled water. The suspension was left overnight and the clear supernatant was filtered through Whatman No. 1 filter paper or sterilized by filtration through a 0.45 μm Millipore filter. In addition, colonized leaf blades of Zea mays were incubated for 12 h in 1/4 strength dilute salt solution (Roberson and Howard 1987) and replaced with full-strength dilute salt solution every 12 h. All water cultures were incubated at (10, 15, 50, 25, 25, 30, 35°C) under dark and white fluorescent light (1400 lux) for 48 h, cooled to 4°C for 30 min and rewarmed to the desired temperature to re-lease zoospores.

Pre-emergence damping-off pathogenicity of the 2 varieties of P. spinosum to cucumber seeds: Four isolates of each variety were tested for their pathogenicity against cucumber germinating seeds. For preparation of inocula, the method of Tojo et al. (1993) was followed in which inoculum concentration of 2.5% was employed. Corn powder (1 g) was moistened by adding distilled water in 250 ml Erlenmeyer flask. After autoclaving at 121°C for 20 min, each flask was inoculated with 3 discs (7 mm dia.) of WA with growing margins of Pythium spp. The inoculated corn powder was held at 25°C for 10 d. The inoculum of 2.5% was obtained by mixing thoroughly 1 g of colonized corn powder in the Erlenmeyer flask with 50 g of over-dried (70–80°C for 2 d) clay loamy soil using a sterilized mortar and pestle. Two and half grams of this mixture was added to 97.5 g of clay loam soil which has been sterilized by autoclaving at 121°C for 60 min (pH 7 after autoclaving) and kept in a plastic bag for 2–3 weeks at room temperature with 25% water content prior to use.

Soil (300 g) with inocula of each fungal treatment was put into 5 replicate plastic pots (30 ml capacity and 12 cm dia.) For each fungal treatment, 6 cucumber (Telegraph) seeds which are highly susceptible to Pythium species (Takahashi et al. 1970) were planted in each pot. Pre-emergence damping-off was determined as the difference in emergence between non-inoculated control soil and inoculated soil.

**Molecular studies**

DNA extraction: Fungal cultures were grown in 125 ml of potato dextrose broth (PDB) containing 100 ppm streptomycin sulphate and incubated at 20°C with shaking on an orbital shaker. After 5 d mycelium was collected by vacuum filtration and ground to fine powder in a liquid N₂ 50 mg of the powder transferred to 1.5 ml Eppendorf tube and mixed with 700 μl/2×CTAB buffer. The tubes incubated at 65°C for 30 min, then 700 μl of chloroform were added and the mixture vortexed briefly. The resulting mixture centrifuged at a maximum speed of 15000 rpm for 30 min and the cleared supernatant was mixed with 600 μl of isopropanol chilled to −20°C. The mixture was centrifuged at the maximum speed for 5 min and the resulting pellet washed twice with 1 ml of 70% ethanol the pellet was dried under vacuum and dissolved in 100 μl TE (10 mM Tris, 1 mM EDTA, pH 7.5) buffer. The DNA was visualized by electrophoresis in 1% agarose in Tris Borate EDTA (TBE) buffer: 89 mM Tris-HCl (pH 8.3), 89 mM boric acid, 20 mM EDTA (Maniatis et al. 1989). Agarose gels were stained with ethidium bromide and examined under ultraviolet light.

RAPD-amplification: PCR conditioned and separation of RAPD-PCR fragments were carried out according to Messner et al. (1994). Using the primers of V5 (5'dTGCCGAGCTG; Caetano-Anolles et al. 1992). V6 (5'dTGCGACCGTGG; Lopandic et al. 1996) and M13
(5’dGAGGGTGGCGGTCT; O’Donnell et al. 1999). Synthesis of primers performed by (Codon Genetical Systems, Vienna, Austria), using a model 392 DNA synthesizer (Applied Biosystems, Foster City, CA, USA). The temperature profile of primers was subjected for denaturation at 98°C for 15 sec, annealing at 40°C for 90 sec and extension at 72°C for 100 sec to a total of 40 cycles.

Analysis of RAPD profiles: RAPD profiles were scored by visually comparing RAPD amplification profiles and scoring the presence or absence of each band in each profile according to Halmschlager et al. (1994). Basically the formation obtained from agarose gel electrophoresis was digitalized by hand to a two-discrete-character-matrix (0 and 1 for absence and presence of RAPD-markers). For running cluster analysis, the 2 discrete characters of 0 and 1 had to be Guanine and Thymine in the RAPD data matrix. Complete alignment of data was performed with CLUSTALX software, then cluster analysis with be ready by using Treecon programme (Van der Peer 1994).

Results and discussion

Morphology and description of Pythia studied

Eight isolates of both P. spinosum var. spinosum and var. sporangiiferum (4 from each one) were obtained in pure cultures. The following description is based on studies of isolates of each variety on water cultures and soil agar media such as CMA, potato carrot agar (PCR) and V8 agar,

Fig. 1. Scanning electron micrographs of oogonia of P. spinosum var. sporangiiferum (A, B) and P. spinosum var. spinosum (C, D).
Fig. 2. Random amplified polymorphic DNA patterns obtained by using the primer V6 (5'dT-GCAGCGTG; Lopandic et al. 1996) from Pythium spinosum var. spinosum (lanes 1–4), P. spinosum var. sporangiferum (lanes 5–8), P. deliense (lanes 9–12) and P. vitale (lanes 13–16).

Fig. 3. Random amplified polymorphic DNA patterns obtained by using the primer M13 (GAGGTTGCGTTCT; K. O'Donnell et al. 1999) from Pythium spinosum var. spinosum (lanes 1–4), P. spinosum var. sporangiferum (lanes 5–8), P. deliense (lanes 9–12) and P. vitale (lanes 13–16).

each supplemented with 500 μg/ml wheat germ oil.

Pythium spinosum var. spinosum

This fungus was originally described by Sawada and Chen (1926) in Taiwan and has not been isolated from Egypt before. A detailed description is as follows (Fig. 4A–J, Fig. 1C, D). Colonies showing a radiate pattern with some aerial mycelium (somewhat arachnoid-cottony), thin on Difco-CMA, thick on Biolife-PDA. Main hyphae up to 9 μm wide, septate when old (Fig. 4A). Hyphal swellings usually limoniform, rarely globose, up to 26 μm dia., intercalary, rarely terminal, mostly smooth, sometimes with projections, germination by germ tubes (Fig. 4B, C). Oogonia rarely fusiform, mostly intercalary, 18–26 μm, average 22 μm dia. (Fig. 4E–I). Oogonia provided with a varying number of blunt, digitate projections, 2.0–10.0 μm long and 2.0–4.0 μm dia. at the base (Fig. 1C, D). Antheridia usually 1, sometimes 2, rarely 3 per oogonium, originating at various dis-

stances from the oogonium, clavate, crook-necked, rarely hypogenous, making apical contact with the oogonium, predominantly monoclinous, sometimes diclinous, soon disappearing after fertilization (Fig. 4D–I). Oospore plerotic, 16–24 μm, average 20 μm dia., wall thin up to 1.5 μm thick (Fig. 4J).

Daily mycelial growth on Difco-CMA at 25°C = 29 mm.

Description: based on E1-UOO20 (deposited in the Botany Dept., Faculty of Science, El-Minia University, Egypt).

Highly-parasitic on pre-emergent cucumber "Telegraph" potted in growth chamber at 22°C (see pathogenicity test).

*Pythium spinosum var. sporangiiferum*

A detailed description is as follows (Fig. 5A–N, Fig. 1A, B)

Colonies showing a radiate pattern with some aerial mycelium (somewhat arachnoid-cottony), thin on Difco-CMA, thick on Biolife-PDA. Main hyphae up to 10 μm wide, septate when old (Fig. 5A). Hyphal swellings usually irregular in shape, rarely globose, up to 40 μm dia., intercalary, rarely terminal, mostly smooth, sometimes with projections, germinating by germ tubes (Fig. 5B–D). Zoosporangia and zoospores readily formed; zoosporangia globose, mostly terminal, 15–20 μm, average 18 μm dia., with one, rarely 2 short discharge tubes, (3–6)×(2–3) μm (Fig. 5E–H). Zoospores kidney shaped, longitudinally grooved, laterally flagellate, after encysting, mostly 9–11 μm dia. Encysted zoospores germinated mostly by one germ-tube (Fig. 21). Zoospores easi-
ly released at 15°C after 12 h. Oogonia globose, rarely fusiform, mostly intercalary, 16–15 μm, average 20 μm (Fig. 5J–M). Oogonia provided with a varying number of blunt, digitate projections, 2.0–8.0 μm long and 2.0–3.0 μm dia. at the base (Fig. 1A, B). Antheridia usually 1, rarely 2 per oogonium, originating and various distances from the oogonium, clavate, crook-necked, rarely hypogenous, making apical contact with the oogonium, predominantly monoclinous, sometimes dicipitous, soon disappearing after fertilization (Fig. 5J–M). Oospores plerotic, 14–23 μm, average 18 μm dia.; wall thin, up to 1.5 μm thick (Fig. 5N).

Daily mycelial growth on Difco-CMA at 25°C = 25 mm.

Description: based on El-UOO21 (deposited in the Botany Dept., Faculty of Science, El-Minia University, Egypt).

Isolation: on VP3 medium selectively from mud soil at the bank of Shean lake, Tunisia.

Highly-parasitic on pre-emergent cucumber “Telegraph” potted in growth chamber at 22°C (see pathogenicity test).

**Cardinal temperature studies**

The minumum, optimum and maximum temperatures of 8 isolates of the 2 varieties studied are listed in Table 1. Minimum and optimum temperatures were similar for the 2 varieties while maximum temperatures were 34 and 37°C for var. spinosum and var. sporangiiferum, respectively.
Table 1. Cardinal temperature response of mycelial growth of *Pythium spinosum* varieties

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<tr>
<th>Variety</th>
<th>Temperature (°C)</th>
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<td>2</td>
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<tr>
<td><em>v. spinosum</em></td>
<td>NG</td>
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<tr>
<td><em>v. sporangiferum</em></td>
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a) There is no differences in measurements within the 4 isolates in the same variety. b) Colony diameter after 24 h mycelial growth is the mean of 5 replicates. c) Standard error. NG, no growth.
Production of zoospores

All of the isolates tested of *P. s*. var. *spinosum* failed to produce zoospores when exposed to the following solutions:

- Non-sterile soil extract (3 types).
- Non-sterile soil extract (3 types) under white fluorescent light.
- Filter-sterilized soil extract.
- Filter-sterilized soil extract + distilled water (1:1).
- Roberson and Howard’s solution.
- Distilled water.

All of these solutions were used at different incubation temperatures (5, 10, 15, 20, 25, 30, 35°C) or even after the cultures were cooled to 4°C and rewarmed at 25°C.

On the other hand, isolates of *Pythium spinosum* var. *sporangiferum* produced zoospores after 12 h of incubation at 15°C in the dark using only distilled water. Production of zoospores was failed when temperature exceed 15°C.

Pathogenicity test

Pre-emergency damping-off pathogenicity test indicates that the 8 isolates of the 2 varieties employed in this study were highly virulent against cucumber germination seeds with 100% damping-off. The results of the pathogenicity test indicate that this criteria was not significant factor for separation of the 2 varieties.

*P. spinosum* and *P. ultimum* have been described as non-zoospore forming pythia in their original descriptions (Trow 1901, Sawada and Chen 1926). They have similar charters 1 of which is that they are pathogenic to germinating seeds and seedlings (Van der Plaats-Niterink 1981). *P. ultimum* was identified as non-zoospore forming *Pythium* and placed in this section in the key and monograph of the genus *Pythium*. In 1960, Drechsler divided *P. ultimum* into 2 varieties: *P. ultimum* var. *ultimum* and *P. ultimum* var. *sporangiferum*. He pointed out that *P. ultimum* var. *sporangiferum* mainly differs from *P. ultimum* var. *ultimum* by its capacity to produce zoospores at room temperature.

*P. spinosum* was firstly isolated by Sawada in 1926. He noticed that there was no zoospore formation by this fungus. No materials and results were available concerning zoospore formation by this taxon, till 1993 when Botha reported zoospore production by 6 South African isolates of *P. spinosum* under special conditions. He mentioned that, when these isolates of *P. spinosum* exposed to a non-sterile soil extract and light they produced zoospores while autoclaved and filter sterilized soil extract, as well as, a defined salt solution, failed to induce sporangial formation.

Abdelzaher et al. (1997b), found that distilled water was very efficient to produce zoospores by 3 pythia namely: *P. aphanidermatum*, *P. oligandrum* and *Pythium* “group F”. They used different NaCl concentrations and different osmotic pressures and concluded that distilled water is an excellent aquatic phase for producing zoospores. Their results was to the similar to the same experiment which had been done before using 3 aquatic pythia namely: *P. fluminum* var. *fluminum*, *P. marsipium* and *Pythium* “group F” (Abdelzaher et al. 1994).

Our results indicate clear evidence that *P. s*. var. *spinosum* failed to produce zoospores under all of the conditions used. On the other hand, var. *sporangiferum* produced zoospores easily using distilled water at 15°C under dark condition. It is worth to mention that temperature was very critical for zoospore production by var. *sporangiferum*. No zoospore production has been released at 20°C or more but at 15°C plenty of zoosporangial vesicles contain vital zoospores were produced. For this reason, investigators must use different incubation temperature to test zoospore producing ability of pythia studied and this may be the reason of delay of discovering var. *sporangiferum* and furthermore misidentification of many pythia investigated.

It was found that the maximum temperatures of the 2 varieties studied were different. The
maximum temperature of *P. s. var. spinosum* was under 35°C whereas it was above 35°C in case of var. *sporangiferum*. Middleton (1943) in his key of the genus *Pythium* concluded that *Pythium* spp. can be segregated into groups based on maximum temperature tolerance.

The 2 varieties of *P. spinosum* had different dimensions of mycelia, a sexual and sexual structures as well as var. *sporangiferum* was slower than var. *spinosum* in mycelial growth rate.

PCR amplification of DNA from 16 isolates of each of *Pythium spinosum* (8 isolates), *P. deliense* (4 isolates) and *P. violae* (4 isolates) from diverse geographic locations and hosts was carried out using 3 primers and the randomly amplified DNA fragments were separated by gel electrophoresis. The RAPD patterns showed a high degree of variation and enabled to the 16 isolates to be separated into 4 groups (Figs. 2, 3, 6). A number of primers gave a rise to RAPD bands which clearly enabled isolates in *Pythium spinosum* to be distinguished into 2 distinctive groups (Figs. 2, 3, 6). RAPD analysis of 16 isolates of each of *Pythium spinosum, P. deliense* and *P. violae* demonstrated considerable genetic heterogeneity within *Pythium spinosum* species group.

Although Van der Plaats-Niterink (1981) reported that *Pythium ultimum* var. *sporangiferum* differs *P. ultimum* var. *ultimum* by its capacity to produce globose sporangia and to easily release zoospore, Rafin et al. (1995) by using restriction patterns generated by enzymes *AluI,* *Hinfl* and *HaeIII* could differentiate easily *P. ultimum* and *Pythium* strains producing filamentous sporangia. These enzymes could not differentiate between *P. ultimum* var. *ultimum* and *P. ultimum* var. *sporangiferum* because they had not only the same ITS length but also exhibited the same restriction patterns. Although previous reports also indicated that variation in length of amplified rDNA coincides with morphological differences, especially the type of sporangia (Chen et al. 1992). In addition, Wang and White (1997) reported that both of *P. ultimum* var. *ultimum* and *P. ultimum* var. *sporangiferum* clustered together by using *CfoI, EcoR1, HaeIII, HindIII, Hinfl, Mbol* and *TaqI*.

In summary, the molecular analysis reported here, not only support the separation of *Pythium spinosum* Sawada into 2 varieties: *P. spinosum* var. *spinosum* and *P. spinosum* var. *sporangiferum* but reveal considerable genetically differences between them. A more definitive study of the taxonomic and phylogenetic relationships in *Pythium* species will require sequencing aligning, and comparison of the appropriate regions of rDNA and ITS.

References


