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Morphological, pathogenic and molecular characterisation of *Pythium aphanidermatum*: A causal pathogen of coriander damping-off in India

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Abstract
Damping-off caused by several soilborne fungi and oomycetes is dangerous threatening disease affecting coriander plants in India and presumably in many other countries. The objective of the present investigation was to characterize the pathogen morphologically *in vitro* and to confirm the pathogenicity of *Pythium aphanidermatum* isolated from several coriander growing regions of Tamil Nadu, India. Pathogenic *P. aphanidermatum* isolates were characterized using molecular methods based on ITS-PCR. Based on the morphological and molecular characters, all tested isolates were confirmed as *Pythium aphanidermatum*. According to the pathogenic test done on young coriander plants under glasshouse conditions, all the isolates of *P. aphanidermatum* were found to be pathogenic in nature. To the best of our knowledge, this is the first work that shows that *P. aphanidermatum* might be a major agent causing damping-off disease of coriander plants in India.

Keywords: coriander, damping-off, morphology, molecular, *Pythium aphanidermatum*

Introduction

Coriander (*Coriandrum sativum* L.), one of the important seed spices generally known as "Dhania" belonging to the family Apiaceae is a native of the Mediterranean region. It is an annual herbaceous plant, used as a common food flavouring agent. Coriander seeds have medicinal properties used as a carminative and diuretic. Being a tropical crop, these plants prefer a frost-free tropical climate at the time of flowering and seed formation. Pests and diseases are the major constraints in the production of coriander. Coriander cultivation is affected by several diseases like wilt caused by *Fusarium oxysporum* [24], stem gall caused by *Protomyces macrospores* [6], grain mould diseases caused by *Helminthosporium* spp., *Fusarium* spp., *Curvularia* spp. and *Alternaria* spp. [21], powdery mildew (*Erisyphe polygoni*), rust and leaf spots. Among the diseases, damping-off caused by *Pythium* spp. are the most important diseases which cause a yield loss of 10-60% [20]. The genus *Pythium* consists of diverse and ubiquitous species, many of which are important soil-borne plant pathogens that cause disease of plants in agriculture, forestry, and greenhouse environments [9].There are about 140 recognized *Pythium* species [3,11]. *Pythium* is a member of the Pythiaceae in the class Oomycota and order Peronosporales, which was recently placed in the kingdom Chromista of the phylum Stramenopiles [10]. Plant pathogenic *Pythium* species can affect a wide variety of economically important agricultural crops [1, 9]. These species are most commonly identified as causal agents of pre- and post-emergence damping-off, leading to poor stands and low crop vigour [1]. Soil moisture and temperature are two factors that have great influence on the growth of many *Pythium* species and on damping-off development [4]. Some *Pythium* species can infect mature plants, causing significant damage to yields [26]. *Pythium* species can survive in the soil *via* oospores or sporangia [9, 28]. Identification of *Pythium* species traditionally has been done based on morphological characteristics *viz*., sporangia, oogonia, and antheridia, the type and size of oospores, homothallism *vs*. heterothallism, growth habit, and rate of growth in culture media are some of the common criteria used to differentiate among species [7, 28]. These characteristics also can vary under different culture conditions, and many species are very similar in morphological characters [5]. Some of these characteristics can also change or be acquired or lost readily. Also, the criteria traditionally used for species differentiation has not always correlated with the major clades in *Pythium* determined by molecular methods [12, 13]. The use of molecular methods for the identification of *Pythium* species began more than a decade ago [14, 12]. The ITS region of the nuclear rDNA has been established to be variable at

the family, genus, and species level for *Pythium* [5]. However, characterization of *Pythium* spp. associated with certain crops has largely depended on sequences of the internal transcribed region (ITS) of the ribosomal DNA (rDNA) [27, 17, 18]. Use of the ITS region seems to be the most popular choice of many researchers working with detection of this pathogen [11, 22]. In India *Pythium* spp. is cosmopolitan and one of the most common plant pathogen of a number of various crop plants. All these observations have attracted the attention of researchers about an epidemic pathogenic form that may have evolved from other species infecting horticultural crops. This paper will furnish useful information towards a clear understanding of the current crop management status, thus lending to the design of better management practices in these infected areas. In this regard, the present study aims to characterize morphologically and molecularly by polymerase chain reaction of *Pythium* spp. isolates recovered from infected coriander plants.

Materials and Methods

Isolation and identification of pathogen

Damping-off infected samples were collected from different coriander growing districts of Tamil Nadu *viz*., Coimbatore, Tirupur and Dharmapuri. These infected samples were used for the isolation of pathogens. Infected collar portion of the seedlings were cut into small pieces and surface sterilized with 0.1% mercuric chloride for 30 seconds and subsequently three washings were given with sterile distilled water. Then, they were placed in sterilized Petri dishes containing Potato dextrose agar (PDA) medium by half plate technique and incubated at 25 ± 2ºC. Pure cultures were aseptically maintained in PDA slants in a refrigerator at 4ºC for further studies (Plate 1). The isolates of *Pythium* spp. were grown on PDA medium to study their growth and variability in colony characters using microscopic image analyser (Mediline Scientifics). From the three day-old culture plates, a five mm disc of the fungus was cut using a sterilized cork borer and placed at the centre of each sterile Petridish (90-mm-dia) containing 15 ml of sterilized and solidified PDA. The plates were incubated at 28 ± 2ºC for 3 days. The mycelial growth, colony characters, and spore characters were recorded three days after inoculation (DAI).

Pathogenicity Study

The *Pythium* isolates (P1 – P5) was multiplied in sand maize medium [23]. Sand and ground maize seeds were mixed at the ratio of 19:1 respectively. The mixture was moistured with water and sterilized at 121ºC at 15 psi for 2 h. The *Pythium* isolates were inoculated into sand maize medium and incubated for 15 days at 28º ± 2ºC for multiplication. Potting soil (Red soil: sand: cow dung manure @ 1:1:1 w/w/w) was sterilized in an autoclave at 121ºC at 15 psi for 2 h for consecutive days. The isolates of *P. aphanidermatum* was multiplied on sand maize medium were incorporated separately into the sterilized soil at the rate of 5% (w/w). Coriander (Co.3) seeds were sown at the rate of 10 seeds/ pot and maintained under glass house conditions. Three replications were maintained for each isolate and monitored regularly.

DNA Extraction

DNA was extracted from five pathogenic *P. aphanidermatum* isolates. The mycelium of each isolate was collected by harvesting the mat grown on the surface of inoculated PD broth. After grinding 100 mg of fungal mycelia from each isolate in liquid nitrogen, the powder was incubated in 5 ml, 2 % CTAB extraction buffer [10 mM Tris base (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, CTAB (2 %), mercaptoethanol (0.1%) and PVP (0.2%)] at 65ºC for 1 h. An equal volume of phenol-chloroform-isoamylalcohol (25:24:1) mixture was added. The mix was vortexed to mix two phases, followed by centrifugation at 12,000 rpm for 5 min. The supernatant was transferred to clean tube and mixed with an equal volume of ice cold isopropanol and incubated at 25ºC for DNA precipitation. The precipitate was collected by centrifugation and the pellet was washed with 0.1 M ammonium acetate in 70% ethanol. Again incubation was for 15 min. The pellet was resuspended in TE buffer (10mM Tris, 1mM EDTA, pH 8.0) [30] and the DNA concentration and purity was determined using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc). Aliquots of samples were also analyzed on a 1% agarose gel to check DNA quality.

PCR amplification

The universal primers Pa 1 (Forward) and ITS 2 (Reverse) were used to amplify the ITS regions of *P. aphanidermatum* [29, 30].

Pa1 - 5’-TCCACGTGAACCGTTGAAATC-3’

ITS 2 -5’-GCTGCGTTCTTCATCGATGC-3’

PCR reaction mixture consisted of 10µl *viz*., 5 µl of PCR master mix, 1µl of forward primer and 1 µl reverse primer, template DNA 1 µl and 2 µl of sterile water. PCR was performed with an initial denaturation step at 95 ºC for 5 min, 40 cycles of amplification with denaturation at 94 ºC for 1 min, annealing at 67 ºC for 1 min and 72 ºC for 1 min for extension with final extension 72 ºC for 10 min, in PCR Palm Cycler (Corbett Research, Australia). The amplified PCR products were run on 1.5% agarose gel in Tris-acetate buffer. The gel was stained with ethidium bromide, visualized on an UV-transilluminator and photographed in a gel documentation unit (Alpha Innotech Corp, USA).

Statistical Analysis

The data obtained were statistically analysed [8] and the treatment means were compared by Duncan’ package used for analysis (IRRISTAT version 92) developed by the International Rice Research Institute, Biometrics Unit, The Philippines.

Results and Discussion

Occurrence of coriander wilt has been reported from several parts of India *viz*., Gwalior and Guna districts of Madhya Pradesh, Kota division of Rajasthan and Coimbatore district of Tamil Nadu [20]. The present study, five isolates of *Pythium* spp. were isolated from infected coriander samples using PDA medium by half plate technique. Pathogenicity tests were conducted for *Pythium* spp. as per Koch’s postulates. Isolated *Pythium* spp. from infected plants were multiplied in sand maize medium and used as inoculum for pathogenicity assay. The pathogen inoculated plants showed reddish brown lesions at collar portion, girdling and toppling down of the seedlings on 15th day (Plate 2; Table 1). The pathogens were re-isolated from artificially infected coriander plants. The re-isolated pathogens were similar to the original culture. Species of *Pythium* spp. can live saprophytically or parasitically. Their parasitic role often depends on external factors. When conditions are favorable for the fungus but less

for the host, *Pythium* species can become very pathogenic and cause pre- or post-emergence damping-off of seeds and seedlings, rotting of roots, stems and fruits. Young or watery tissue is preferentially attacked. Infection takes place when zoospores produce germ tubes [23] or hyphal elements form appressoria and penetrate the plant by means of infection pegs [16].Pathogenic *Pythium* spp. may survive from season to season on bermudagrass and under favorable conditions, it may cause damping-off or blight on the overseeded cool-season turfgrass [25]. All five isolates of *Pythium* on PDA produced a dense, white cottony mycelial growth with fluffy topography. Each produced aseptate, hyaline mycelium (3.4 µm to and double 6.2 µm), oogonia terminal, globose and smooth 20-25 µ diameter. Antheridia mostly intercalary, sometimes, broadly sac shaped, 10-14 µ long and 10-14 µ wide, 2 per oogonium, monoclinous or diclinous, thick walled aplerotic oospores (17 to 19µm) and lobed sporangia (Plate 3). Hence, this pathogen was identified as *P. aphanidermatum* [7]. *Pythium* species have been traditionally identified and classified based on the morphology of asexual and sexual structures [28]. Morphological characters of *Pythium viz.,* structures of sporangia and oospore vary between species [22]. *Pythium* consistently isolated from diseased ripe tomato fruits collected from the field and fruits exposed to infested soil in the laboratory, have been identified as *P. ultimum* and *P. aphanidermatum* [15, 19]. *P. aphanidermatum* can be distinguished from *P. deliense* on the basis of oospore wall. It is thin in *P. aphanidermatum* and thick in *P. deliense*. Also, *P. aphanidermatum*, *P. deliense* and *P. indigoferae* are characterized by the presence of turuloid sporangia, apleurotic oospores and intercalary antheridia [7]. PCR amplification of ITS region in the five isolates of *P. aphanidermatum* was performed using the specific forward and reverse were used to amplify the ITS regions of *P. aphanidermatum.* It was amplified as a fragment of 210 bp which confirmed as *P. aphanidermatum* (Plate 4). Similarly, *P. aphanidermatum* was amplified by using specific primers ITS 2 and Pa1 with an amplicon size of 210 bp [29]. *P. aphanidermatum* has also been identified based on morphological, molecular characteristics using specific conventional PCR primers for the DNA-mediated detection from infected cucumber tissue [2].

Table 1: Pathogenicity of *P. aphanidermatum* isolates causing damping-off in coriander

Isolates	Source	Incidence of post-emergence damping-off (%)
P1	Mettupalayam, Coimbatore District	83.24a (66.29)
P2	Idikarai, Coimbatore District	68.90ab (56.19)
P3	TNAU orchard, Coimbatore District	74.89ab (60.09)
P4	Palladam, Tirupur District	81.00a (64.50)
P5	Kongalnagar, Dharmapuri District	60.94b (51.35)

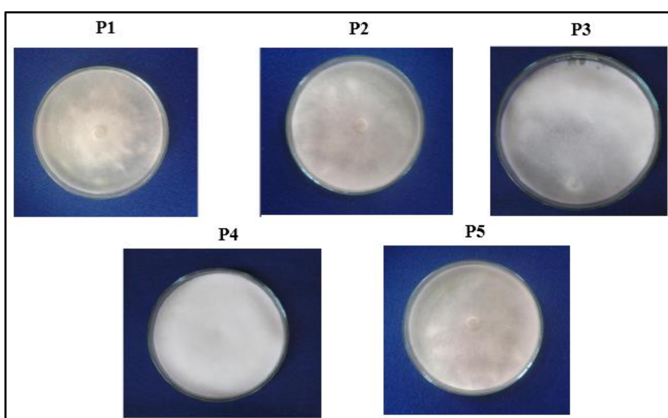
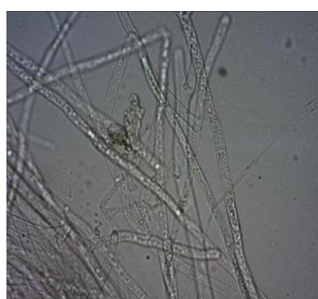
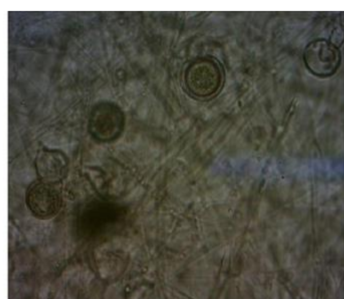


Plate 1: Isolates of *Pythium aphanidermatum*

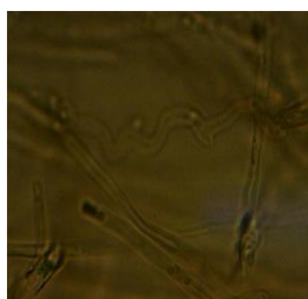
Plate 2: Pathogenicity test for *Pythium aphanidermatum*



A) Aseptate hyaline mycelia

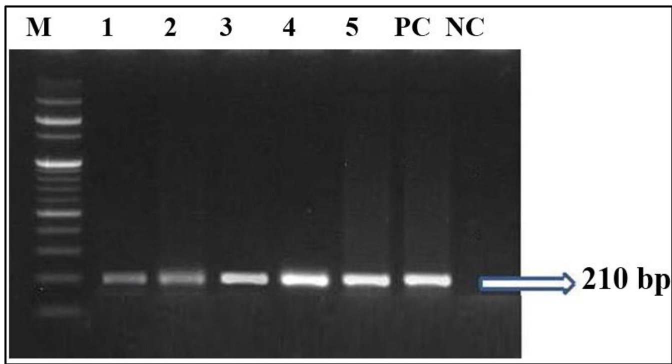


B) Oospores



C) Lobed sporangia

Plate 3: Morphological characters of *Pythium aphanidermatum*



Lane
M - 100 bp ladder, L1 - P1 (Mettupalayam), L2 - P2 (Idikarai), L3 - P3 (TNAU Orchard), L4 - P4 (Palladam), L5 - P5 (Kongal Nagar), L6 – Positive Control, L7 – Negative Control

Plate 4: Molecular characterization of *Pythium aphanidermatum*

Conclusion
The present study demonstrates the association of pathogenic *Pythium aphanidermatum* with damping-off of coriander in India. It reveals evidence for the presence of severity in epidemic of *P. aphanidermatum* infecting coriander. However, because the number of isolates involved in the pathogenicity study was limited, a detailed study on the morphological and molecular characterization of *P. aphanidermatum* will be necessary to establish data on the infestation of *P. aphanidermatum* infecting coriander in India. Investigating the characterization and distribution of *P. aphanidermatum* inoculum in field condition may help to avoidance the primary inoculum of the pathogen.

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