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Distribution of *Pythium myriotylum* Drechsler causing soft rot of ginger

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Abstract

Fourteen of the 29 *Pythium* isolates pathogenic to ginger (*Zingiber officinale*) collected from various parts of India such as Assam, Karnataka, Kerala, Sikkim and Uttar Pradesh were identified as *Pythium myriotylum* based on the size of the species-specific amplicon (150 bp) using the oligo primers Pmy5 and ITS2. The suitability of the primer combination Pmy5 (5′-gTC gCT gTT ATg gCg gAg-3′) and ITS2 (5′-gCT gCg TTC TTC ATC gAT gC-3′) (Wang *et al.* 2003a) at the species level identification of *P. myriotylum* was further confirmed through this study.

Keywords: ginger, PCR, *Pythium myriotylum*, soft rot, *Zingiber officinale.*

Introduction

Ginger (*Zingiber officinale* Rosc.) is grown by small and marginal farmers in the states of Assam, Himachal Pradesh, Karnataka, Kerala, Meghalaya, Orissa, Sikkim and north eastern region of India as well as other south east Asian countries, Africa and Hawaii (USA). Diseases caused by *Pythium* spp. and *Ralstonia solanacearum* Yabuuchi(Smith) are among the major production constraints in ginger. *Pythium* sp. causing soft rot in ginger which eventually leads to rhizome loss, has been reported nearly 100 years ago from India (Butler 1907).

Pythium spp. causing soft rot in ginger have been identified and characterized, mainly based on the keys provided by Middleton (1943) and Vander Plaats-Niterink (1981). The overlapping mycelial and sporangial characters in morphological characterization hinder the identification of *Pythium* spp. Conventional methods of identification relies upon separation of species on the basis of morphology of antheridia, oogonia and sporangia, which appear to vary under different cultural conditions. Moreover, it is time consuming and expert hands are required. Hence *Pythium* species are seldom identified at the species level which limits ecological and epidemiological studies on the genus and thereby the selection and implementation of control strategies.

The use of DNA-based techniques such as polymerase chain reaction (PCR) has enabled researchers to identify and characterize *Pythium* spp. (Chen & Hoy 1993; Kageyama *et al.* 1998). The internal transcribed spacer (ITS) region of ribosomal DNA has been exploited for species-level identification of

Pythium spp. (Levesque *et al.* 1991; Chen 1992; Wang *et al.* 2002; Wang *et al* 2003a; Wang *et al.* 2003b). The present study also aims at analysing the suitability of species-specific primers available for identification of *P. myriotylum, P. aphanidermatum, P. ultimum and P. graminicolum* in a PCR-based identification assay for studying the distribution of *P. myritylum* causing soft rot of ginger in India.

Materials and methods

Isolates

Twenty nine isolates obtained from Assam, Karnataka, Kerala, Orissa, Sikkim, Uttaranchal and Uttar Pradesh were used in the study (Table 1). The pathogenicity of the isolates was confirmed on ginger under greenhouse conditions. The appearance of water soaked lesions along with yellowing and lodging of the pseudostem was adjudged as soft rot infection.

Preparation of cultures for DNA isolation

An active culture of each isolate was obtained on potato dextrose agar (PDA) plates amended with P10 VP solution upon two days of incubation at 28 ± 1° C. Two mycelial discs cut from the margins of actively growing PDA plates were transferred to flasks with pottato dextrose broth(PDB) and incubated at 28 ± 1° C till fourth day. The mycelial mats were recovered from the flasks, washed thrice in sterile distilled water to remove traces of medium, and stored at -80°C until use in DNA isolation.

DNA extraction from mycelium

DNA from mycelium was extracted by CTAB (Cetyl Tri methyl ammonium bromide) method. Fungal mycelium (200 mg) was ground with glass powder and DNA extraction buffer in 1.5 ml microfuge tubes, followed by treatments with proteinase K, 20% sodium dodecyl sulphate and extraction with chloroform: isoamyl alcohol mixture (24:1), followed by iso-propanol precipitation of nucleic acid, which after ethanol wash was dissolved in 20 ml of sterile distilled water.

Polymerase Chain Reaction (PCR)

PCR was performed with unique forward primers (Pmy5, Pa1, Pa3, Pu1, Pu2 and Pgr), in combination with universal reverse primer, ITS2, to amplify ITS region of genomic DNA of each species. The sequence of primers used is furnished in Table 2. Reagent mixture (25 ml) consisted of 20 pmoles of each primer (IDT, USA), 50 ng of template DNA, 3 units of Taq polymerase (GENEI, Bangalore), 0.08 mM dNTP's (GENEI, Bangalore), 3 mM $MgCl_2$ (GENEI, Bangalore), in a 1x polymerase buffer (GENEI, Bangalore). The reactions were carried out using a thermal cycler (Eppendorf Master Cycler, Germany), and the amplification protocol consisted of an initial denaturation of 94°C for 2 min, followed by 30 cycles of denaturation for 1 min, annealing at 57°C for 20 sec, extension at 72°C for 5 sec, and a final extension at 72°C for 1 min. PCR products were run in a 1.2% agarose gel with ethidium bromide (2 mg ml^{-1}) and bands were visualized in Alpha Imager 2200.

Results and discussion

In India, at least six pathogenic species of *Pythium* have been reported to cause soft rot in ginger, and these include *P. myriotylum, P. aphanidermatum, P. deliense, P. perilium, P. vexans, P. ultimum* and *P. butleri* (Shahare & Asthana 1962; Haware & Joshi 1974; Dohroo 1987). The present study attempted at identifying *Pythium* spp. by PCR-based method isolated from soft rot affected ginger collected from various ginger growing tracts of India. Typical water soaked lesions and yellowing and lodging of sprouts was observed within 2 weeks of inoculation with the *Pythium* isolates. A specific amplicon of 150 bp was obtained in 14 isolates by primer combination Pmy 5/ITS2 (Fig. 1) which was identified as *P. myriotylum* as reported by Wang *et al.* (2003a). Four isolates such as IISR-610, 611, 612 and 612a from a heavily infected ginger field in Parappanangadi (Wayanad District, Kerala) were identified as *P. myriotylum.* No isolate from eastern districts (Idukki) of Kerala was identified as

Table 1. Isolates of *Pythium* spp. used in the study

Isolate	Source	Remarks
IISR-520	Thodupuzha, Kerala	Reisolated from culture collection
IISR-527	Thodupuzha, Kerala	Reisolated from culture collection
IISR-54	Wayanad, Kerala	Reisolated from culture collection
IISR-580	Ambalavayal, Wayanad, Kerala	Isolated in this study
IISR-584	Kalpetta, Wayanad, Kerala	Isolated in this study
IISR-609	Wayanad, Kerala	Isolated in this study
IISR-610	Parappanangadi, Wayanad, Kerala	Isolated in this study
IISR-611	Parappanangadi, Wayanad, Kerala	Isolated in this study
IISR-612	Parappanangadi, Wayanad, Kerala	Isolated in this study
IISR-612a	Parappanangadi, Wayanad, Kerala	Isolated in this study
IISR-607	Mananthavady, Wayanad, Kerala	Isolated in this study
IISR-575	Upper Aho, East Sikkim	Isolated in this study
IISR-577	Lower Aho, East Sikkim	Isolated in this study
IISR-578	Lower Aho, East Sikkim	Isolated in this study
IISR-579	Lower Aho, East Sikkim	Isolated in this study
IISR-591	Upper Aho, East Sikkim	Isolated in this study
IISR-587	Gom, South Sikkim	Isolated in this study
IISR-588	Gom, South Sikkim	Isolated in this study
IISR-585	Kabi, North Sikkim	Isolated in this study
IISR-589	Kabi, North Sikkim	Isolated in this study
IISR-590	Suldung, West Sikkim	Isolated in this study
IISR-593	Gey Singh, West Sikkim	Isolated in this study
IISR-600	Pottangi, Orissa	Isolated in this study
IISR-606	Assam	Dr. Y Rathaiah, Jorhat
IISR-606a	Assam	Dr. Y Rathaiah, Jorhat
IISR-608	Uttaranchal	Dr. J Kumar, Uttranchal
IISR-613	Kodagu, Karnataka	Isolated in this study
IISR-613a	Kodagu, Karnataka	Isolated in this study
IISR-614	Kumarganj, Uttar Pradesh	Isolated in this study

Table 2. Primer sequence reported for species-specific identification of *Pythium*

Primer	Sequence	Reference
ITS2 (Universal reverse primer)	5′-gCT gCg TTC ATC gAT gC-3′	Wang *et al.* 2003b
Pmy 5-*P. myriotylum*	5′-gTC gCT gTT ATg gCg gAg-3′	Wang *et al.* 2003a
Pa1-*P. aphanidermatum*	5′-TCC ACg TgA ACC gTT gAA ATC-3′	Wang *et al.* 2002
Pa3-*P. aphanidermatum*	5′-ATT TTT CAA ACC CAT TTA CC-3′	Wang *et al.* 2002
Pu1-*P. ultimum*	5′-ACg AAg gTT g gT CTg TTg-3′	Wang *et al.* 2003b
Pu2-*P. ultimum*	5′-TCT CTA CgC AAC TAA ATg C-3′	Wang *et al.* 2003b
Pgr-*P. graminicolum*	5′-ACg AAg gTg ggC TgC ATg TA-3′	Wang *et al.* 2003b

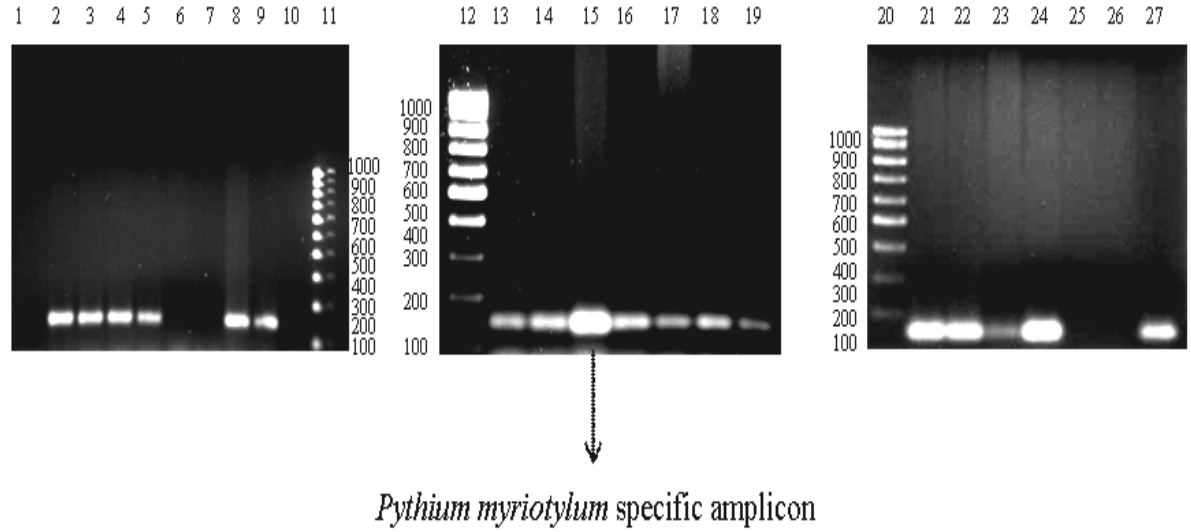

Fig. 1. PCR based identification of *P. myriotylum* causing soft rot of ginger
Lane 1: IISR-54 (Kerala), Lane 2: IISR-613 (Karnataka), Lane 3: IISR-614 (Uttar Pradesh), Lane 4: IISR-609 (Kerala), Lane 5: IISR-593 (Sikkim), Lane 6: IISR-600 (Orissa), Lane 7: IISR-578 (Sikkim), Lane 8: IISR-612 (Kerala), Lane 9: IISR-607 (Kerala), Lane 11: 100 bp ladder
Lane 12: 100 bp ladder, Lane 13: IISR-613 (Karnataka), Lane 14: IISR-614 (Uttar Pradesh), Lane 15: IISR-612(Kerala), Lane 16: IISR-607 (Kerala), Lane 17: IISR-587 (Sikkim), Lane 18: IISR-588 (Sikkim), Lane 19: IISR-590 (Sikkim)
Lane 20: 100 bp ladder, Lane 21: IISR-611 (Kerala), Lane 22: IISR-610 (Kerala), Lane 23: IISR-606a (Assam), Lane 24: IISR-613a (Karnataka), Lane 25: IISR-609 (Kerala), Lane 26: IISR-593 (Sikkim), Lane 27: IISR-612a (Kerala)

P. myriotylum. Two of the isolates, IISR-587 and 588 were from Gom, a southern district of Sikkim. The Sikkim isolates identified as *P. myriotylum* exclusively represented the southern and western districts (Gom and Suldung) and no isolate from eastern districts (Upper Aho and Lower Aho) were identified as *P. myriotylum*. Among the two isolates from Assam, one designated as IISR-606a was found to be *P. myriotylum* (Table 3). From these observations it was inferred that the isolates from different geographical locations were identified as *P. myriotylum*. *P. myriotylum* caused typical soft rot symptoms on 30 days old sprouts within 2 weeks of soil inoculation of mycelial macerate (data not shown). The study also supported the prevalence of the pathogen in warm and humid areas. *P. splendons* is reported from soft rot affected plants of Sikkim (Dr. Graham Jackson, personal communication).

The remaining 15 samples did not give a positive result with any of the five other species-specific primers used in the study. They might belong to other species of *Pythium*. In another study, 20 isolates out of

Table 3. Distribution of *Pythium myriotylum* in various ginger growing states of India

Isolate	Source
IISR-587	Gom, Sikkim
IISR-588	Gom, Sikkim
IISR-590	Suldung, Sikkim
IISR-593	Geysingh, Sikkim
IISR-606a	Assam
IISR-607	Mananthavady, Kerala
IISR-609	Wayanad, Kerala
IISR-610	Parappanangadi, Kerala
IISR-611	Parappanangadi, Kerala
IISR-612	Parappanangadi, Kerala
IISR-612a	Parappanangadi, Kerala
IISR-613	Kodagu, Karnataka
IISR-613a	Kodagu, Karnataka
IISR-614	Kumarganj, Uttar Pradesh

29 were found to be *P. myriotylum*, which indicates the predominance of *P. myriotylum* in Kerala (Jooju 2005), who found that *P. myriotylum* and *P. deliense* were among the most virulent strains of *Pythium* causing extensive damage to ginger.

P. myriotylum is reported to cause soft rot in ginger in many other ginger growing countries such as Fiji, Taiwan and other south eastern Asian countries (Parham 1935; Park 1937; Takahashi *et al.* 1954; Lin *et al.* 1971; Wang & Chang 2003). It was inferred from the present study that soft rot disease causing widespread crop loss to ginger in Kerala, Karnataka, Uttar Pradesh, Assam and Sikkim in India was caused by *P. myriotylum*.

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