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Phytophthora melonis Associated with Fruit and Vine Rot Disease of Pointed Gourd in India as Revealed by RFLP and Sequencing of ITS Region

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Abstract

Pointed gourd is an important tropical high value vegetable crop, which is mainly affected by fruit and vine rot disease in field conditions. Causal organism of this devastating disease is *Phytophthora melonis* as revealed through morphological criteria as well as by molecular tools based on the restriction fragment length polymorphism (RFLP) of non-coding Internal Transcriber Spacer (ITS) region and ITS sequencing. Sequencing of ITS region of our *Ph. melonis* isolate has 100% similarity with the five isolates of GenBank including a *Ph. sinensis*. The pathogen, *Ph. melonis*, is a new report from India and as regards host ranges a possible new report globally.

Introduction

Pointed gourd (*Trichosanthes dioica* Roxb, Cucurbitaceae) is a tropical perennial vegetable crop with its primary centre of origin in the Bengal-Assam area of the Indian subcontinent (Chowdhury, 1996) and cultivated widely in Bangladesh and India (West Bengal, Bihar and some parts of Uttar Pradesh). It is commonly named as 'parwal', 'palwal', 'potol' or 'parmal' in different parts of the country.

The fruit, which is the edible part of the vine-like dioecious plant, is one of the most nutritive amongst cucurbit vegetables thus earning the epithet 'King of gourds' and holds a coveted position in the Indian market due to its availability for approximately 8 months (February–September) in a year. Besides being very rich in protein, vitamin A, easily digestible and diuretic in nature, they also have certain medicinal properties (Sharma and Pant, 1998; Sheshadri and Parthasarathy, 2002).

The growing season of pointed gourd reportedly predisposes this high value crop to eight fungal, one nematode (Saha et al., 2004) and two viral diseases (Jones et al., 2000) of which fruit and vine rot of pointed gourd cause major damage (Khatua and Maiti, 1982) and is popularly known to the farmers as 'Haja'. The disease is characterized by drying of vines and fruits and with onset of rains the affected tissues become water soaked, discoloured and in all cases the fruit is covered with white mycelial growth associated with severe shrinkage at a later stage (Fig. 1d).

Earlier reports have variously assigned the causal organism of fruit rot disease of pointed gourd to *Pythium aphanidermatum* (Chattopadhay and Sengupta, 1952), *P. cucurbitacearum* (Chaudhuri, 1975), *Fusarium equiseti* (Kritagyan et al., 1980) all of which are found mostly in postharvest condition but rarely in field condition. However, reports of *Phytophthora cinnamomi* (Khatua et al., 1981) is only from field conditions where it becomes the major yield-limiting factor causing yield losses of up to 90% and might even damage the entire crop under favourable conditions of rain associated with high temperature (Fig. 1c).

Various reports on molecular identification of the *Phytophthora* pathogen based on rDNA-restriction fragment length polymorphism (RFLP) pattern or sequencing of Internal Transcriber Spacer (ITS) regions, have proved that in the past new species have been wrongly assigned to current taxa and conversely, morphological variants of existing taxa incorrectly assigned as new disease threats when the identifications were solely based on morphological criteria (Chowdappa et al., 2003a,b; Mirabolfathy et al., 2001).

Molecular identification systems based on the ITS regions of rRNA are well characterized and are based on the fact that they evolve in a neutral manner at a rate which approximates that of the speciation process. Sequences of non-coding ITS regions (ITS 1 and ITS 2) usually show interspecific variation but little or no intraspecific variation (White et al., 1990). The *Phytophthora* spp. identification process usually involves amplification of a approximately 900 bp amplicon

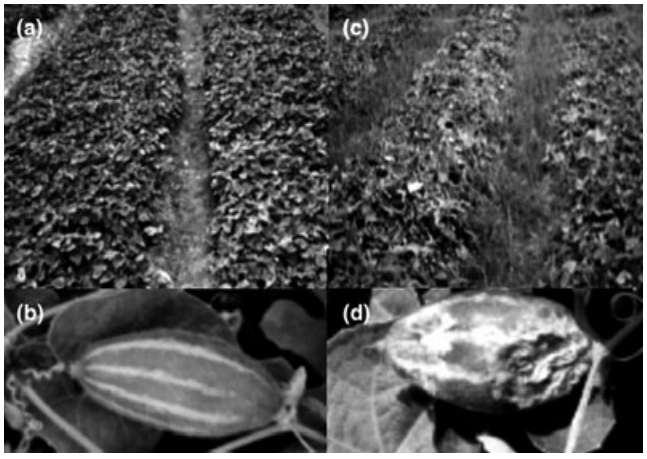

Fig. 1 (a) Healthy pointed gourd field at harvest, (b) healthy pointed gourd fruit, (c) fruit and vine rot in the field showing total loss of the crop, (d) symptom of *Phytophthora melonis* fruit rot of pointed gourd

which includes ITS 1, the 5.8s region and ITS 2 and its digestion with restriction enzymes to produce a species-specific characteristic profile which can be compared against a reference collection (Cooke et al., 2000b).

Additionally, DNA sequence data of the ITS region of rDNA have also been used to differentiate *Phytophthora* spp. (Crawford et al., 1996; Cooke and Duncan, 1997; Cooke et al., 2000a; Zhang et al., 2004) by homology search against the sequences present in GenBank database.

The objective of this study was to identify the *Phytophthora* spp. associated with fruit and vine rot of pointed gourd disease in India through sporangial morphology and validate the identification based on ITS regions of rDNA (Cooke et al., 2000a,b) which is one of the most comphrensive systems to date (Martin and Tooley, 2003).

Materials and Methods

Collection and isolation of fungal isolates

Ten pathogen isolates were collected from farmers' fields in the six agro-climatic zones of this region where pointed gourd is extensively cultivated from diseased fruits showing typical symptoms as mentioned earlier. Collection was spread over a period of two cropping seasons. Diseased fruits and vines were washed in running water, dried, subsequently washed with 90% ethanol, and kept in a moist chamber for a day or 2 till the surface was covered with mycelium. Pathogen isolation was carried out after surface sterilization of the infected part with 0.1% Mercuric Chloride solution and then by plating on a modified P_{10}ARP having V_8 Juice Agar (V_8JA) ammended with Pimaricin 10 p.p.m., Ampicillin 250 p.p.m., Rifampicin 10 p.p.m. and Carbendazim 100 p.p.m.. The Petri plates were incubated in dark with cellophane overlays for 7 days at 28 ± 1°C. Sporangium morphology was checked by 'agar-disk-in-water' technique (Erwin and Riberio, 1996) and then the cultures routinely maintained on V_8JA medium. Pathogenity of the isolated pathogen were established through Koch's postulates. One representative isolate was deposited in the World Phytophthora Collection (WPC; USA#P10994) and the same isolate was also deposited in Virginia Polytechnic and State University (USA#33C9).

DNA extraction, ITS region amplification and its sequence determination

DNA was extracted from fungal mycelium which was harvested directly from the V_8JA medium amended with antibiotic under aseptic conditions, washed in sterilized deionized water, freeze-dried and stored in −20°C freezer for future extraction. DNA extraction was performed according to Cooke et al. (2000b) with minor modifications.

For amplification of ITS region of rDNA, the genomic DNA was amplified by DNA thermal cycler 2400 (Perkin-Elmer Applied Biosystem, Foster City, CA, USA) using primers ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS 6 (5′-GAAGGTGAAGTCGTAACAAGG-3′; Cooke et al., 2000b). The PCR amplification consisted of an initial denaturation step at 94°C for 5 min followed by 35 cycles with conditions of 94°C for 30 s (denaturation), 55°C for 45 s (annealing), 72°C for 1 min (extension) and a final extension at 72°C for 7 min. The PCR mixture (25 μl) contained 20 ng template DNA, 15 mM dNTPs, 2.5 μl 10X PCR buffer, 100 ng each ITS 4 and ITS 6 primers, 1.5 units *Taq* DNA polymerase (Genei, Bangalore, India).

The PCR products were first checked on a 1% agarose gel for a single band of approximately 900 bp PCR product (2 μl) was digested for 4 h (reaction mixture volume 20 μl) with *Alu*I and *Msp*I restriction enzymes at 37°C. The digested products were directly run slowly on 2.5% agarose gel at constant low voltage (50 V) in 1X TBE buffer. The characteristic-banding pattern produced was compared with the PhytID database (Cooke et al., 2000b).

For rDNA sequence determination of the two isolates P10994 or 33C9 and PG2, the PCR products were sequenced with primers in reverse ITS 7 (AGCGTTCTTCATCGATGTGC) and in forward ITS 8 (GCACATCGATGAAGAACGCT) primers which were located in the 5.8s gene (Cooke et al., 2000a) and compared with published GenBank sequences, analysed, using BLAST algorithm. The sequences from P10994 or 33C9 were deposited in GenBank, accession numbers DQ075216, DQ075217 and AH015039. The sequence from PG2 isolate was not submitted, as it was identical.

Results

Sporangium morphology

Analysis of sporangial morphology showed that there was very little variation in sporangial dimensions, averaging 40.8×24.85 μm; and pedicel l/b ratio (length : breadth) ≤1.64; but much variation was

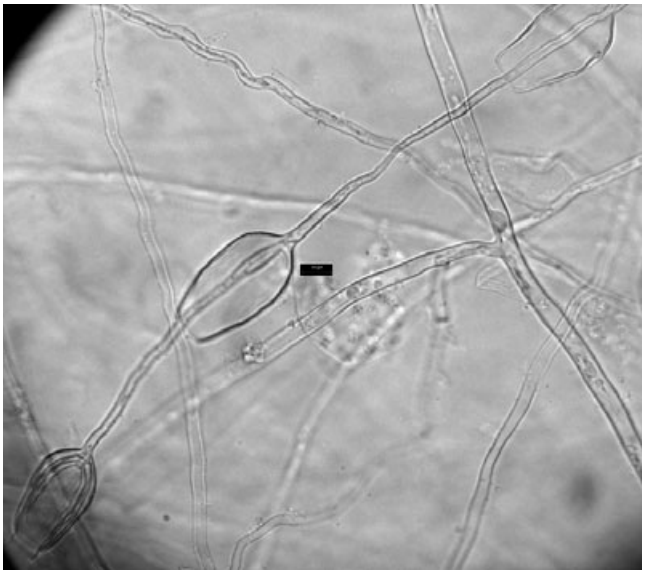

Fig. 2 Sporangiophore showing nested internal elongated proliferation (bar = 10 μm)

observed in shape. The sporangia are globose, obpyriform, ovoid to ellipsoid with rounded bases, non-caducous; non-papillate with pore and producing sporangia by nested elongated internal proliferation (Fig. 2). Sporangiophores are indeterminate in simple sympodia. No oospores were detected in natural conditions or in cultures. Colony morphology in some isolates is of slightly rosaceous type and others had no discernible pattern. Most of these characteristics are similar to that of *Ph. melonis* (Erwin and Riberio, 1996). When these data were fed into a computerized database, for identification of *Phytophthora* species based on morphological features maintained by Indian Institute of Spices Research (Calicut, India), it returned similarity values of 80% for both *Ph. melonis* and *Ph. drechsleri*.

ITS-RFLP and sequencing of ITS 1 and ITS 2 of rDNA region

ITS-RFLP pattern of two of the isolates are illustrated in Fig. 3. Digestion with *Alu*I yielded two bands of approximately 122 and 814 bp and *Msp*I digestion

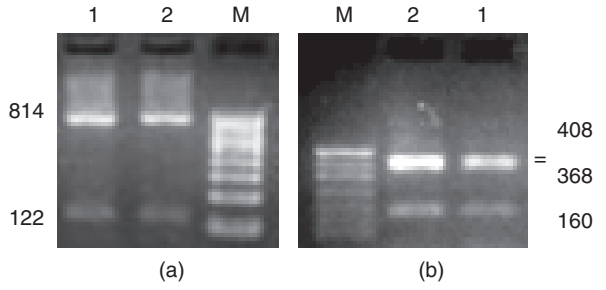

Fig. 3 Digestion of amplicon generated by primers (ITS 4 and ITS 6) with restriction enzymes (a) *Alu*I, (b) *Msp*I, and then separated on 2.5% agarose gels. Lanes 1 and 2, two different isolates; lane M of (a) 100 bp ladder; lane M of (b) pUC19/*Msp*I

Table 1
Comparision of sequence position of ITS 1 and ITS 2 among four *Phytophthora melonis* species (nine sequences were compared), showing position of different nucleotides in comparision with our isolates P10994 or 33C9 and PG2 (data not shown)

	ITS 1			ITS 2			
GenBank accession number	16	46	97	43	87	209	423
DQ075216 and DQ075217 (our isolates)	G	C	C	G	C	G	T
AF266767.1	G	C	G	G	x	G	T
AF403504	G	x	C	G	C	A	T
AY739021.1	x	C	C	G	C	G	T
AY745753.1	G	C	C	G	C	G	A

Deletion or substitution mutations are underlined.

revealed three bands of 160, 368 and 408 bp. All isolates showed similar RFLP pattern. When compared with PhytID database (Cooke et al., 2000b) these results conclusively showed the identity of these isolates to be that of *Ph. melonis* as *Ph. drechsleri* would have given three bands on digestion with *Alu*I and seven bands on digestion with *Msp*I. Sequencing resolved the exact length of the ITS 1 (228 bp) and ITS 2 (433 bp) region of our isolates of *Phy. melonis*. The two isolates sequenced in this study are found to be 100% identical to each other. These isolates had almost 100% sequence similarity, both at ITS 1 and ITS 2 regions with the five isolates of GenBank (AF228094, AF403507, AF403508, AF403509) including one *Phy. sinensis* (AF266768.1). Sequence variation due to single base pair deletion or substitution was also observed with four isolates (Table 1). Sequence variation was evident both in ITS 1 and ITS 2, but as base pair change does not alter or create any new *Alu*I and *Msp*I site inside the rDNA region, therefore, analysis of RFLP pattern of ITS region for the identification of *Phy. melonis* might be sufficient.

Discussion

Analysis of morphological data shows that in some cases the results of *Ph. melonis* are very similar to that of *Ph. drechsleri* and *Ph. sinensis*. This is as because *Ph. melonis*, *Ph. sinensis* and *Ph. drechsleri* are a closely related group of non-papillate species (Erwin and Riberio, 1996; Mirabolfathy et al., 2001), forming a sort of 'morphospecies group' (Brasier, 1997) designated as the *P. drechsleri* which attacks crops of Old World origin. In fact, there has been much debate whether to retain *Ph. melonis*, *Ph. sinensis* and *Ph. drechsleri* as separate taxa.

This stramenopile oomycetous pathogen *Ph. melonis* was first reported in Japan (Katsura, 1968) from diseased cucumber plants. While some workers have (Mills et al., 1991) recommended not merging *Ph. melonis* into *Ph. drechsleri*, there is no clear consensus whether the cucumber isolates should be retained and classified as *Ph. melonis* (Katsura, 1968, 1976). 100% sequence similarity between our isolates and *Ph. sinensis* confirm that *Ph. melonis* and *Ph. sinensis* are the same with *Ph. melonis* to have priority over

Ph. sinensis. Increasing reports (Mirabolfathy et al., 2001; Alvarez et al., 2002) including this present study, which show an extended host and geographical range than that originally described attest to the fact that if *Ph. melonis* is to be maintained as a separate species; it should be re-described (Erwin and Riberio, 1996).

Thus, *Ph. melonis* is the causal organism of fruit and vine rot of pointed gourd in India, and presently there is no evidence in support of *Ph. cinnamomi* or *Ph. drechsleri*. A possible cause for misidentification of *Ph. melonis* as *Ph. cinnamomi* by earlier workers (Khatua et al., 1981; Saha et al., 2004) is the fact that although not within this clade it is one of the most closely related species (Mirabolfathy et al., 2001). This is the first report of *Ph. melonis* causing fruit and vine rot of pointed gourd in India and also as regards the host range a possible new report globally. Further work for estimation of variation, if present, at molecular level among all the isolates is in progress.

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