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Research Article

Morphological and Molecular Description of *Phytophthora insolita* Isolated from Citrus Orchard in India

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Citrus, an important cash crop in India, is adversely affected by *Phytophthora nicotianae*, *P. palmivora,* and *P. citrophthora*. *Phytophthora insolita* is known to be associated with citrus and reported for the first time in India. It is a rare and poorly characterized *Phytophthora* species, as its natural host and pathogenic impact are unclear. Previously, it was reported only in Taiwan and China; so to confirm our suspected isolate is *P. insolita*, regions of internal transcribed spacer, elongation factor, beta-tubulin, and cytochrome oxidase genes were sequenced. This study provides description of the lone Indian *P. insolita* isolate with respect to molecular identity, morphology, mating behaviour, and pathogenicity.

1. Introduction

Phytophthora species (Greek-plant destroyer) are important plant pathogens, formerly thought to be fungus but its closest widely known relatives are brown algae and diatoms [1]. It is a representative of kingdom Chromalveolata, phylum Heterokontophyta, and class Oomycota. It affects almost every cultivated or forest vegetation. *Phytophthora* persists mainly in the soil as "chlamydospores" and spreads generally by asexual spores called "zoospores"; both of them are capable of infection by developing mycelia and parasitizing the host, but the latter is more potent. Citrus is an important tropical crop, cultivated in nearly 135 countries, and is vulnerable to more than 100 diseases and disorders [2]. *Phytophthora* induced diseases, however, cause enormous damage and economic losses in citrus production. There are 12 *Phytophthora* species known to infect citrus worldwide, namely, *P. boehmeriae, P. cactorum, P. capsici, P. cinnamomi, P. citricola, P. citrophthora, P. drechsleri, P. hibernalis, P. megasperma, P. palmivora, P. nicotianae,* and *P. syringe* [3]. There are reports of other associated *Phytophthora* species, like *P. insolita* and *P. humicola,* isolated from soil in a citrus orchard [4]. In India, *P. nicotianae, P. palmivora,* and *P. citrophthora* are major citrus pathogens [5, 6]. Recently we have reported *P. insolita* from India for the first time, which was isolated from Nagpur mandarin (*Citrus reticulata*) orchard [7]. *P. insolita* was reported for the first time from citrus soil in Changhua, Taiwan [4]. The species was noticed in southern China's Hainan Island [8] and Ohio, USA (from necrotic *Rhododendron* leaf) [9]. The species remains poorly described and characterized in the literature as compared to other *Phytophthora* species, which may be due to its infrequent isolation. Moreover, the pathological impact of this citrus associated *Phytophthora* is not clear. Here, we investigate morphological, molecular, and pathological aspect of this only Indian isolate of *P. insolita*.

2. Material and Methods

2.1. Sample Collection and Isolation. Water samples under the canopy of Nagpur mandarin trees were collected from citrus orchard of National Research Centre for Citrus (NRCC), Nagpur, India, during August 2010. Approximately, 300–500 mL water was carefully collected using sterile one-litre glass bottles without disturbing the soil below and brought to the Plant Pathology Laboratory, NRCC. Samples were processed for *Phytophthora* spp. isolation

by dispensing 1 mL of water sample on CMA-PARPH (corn meal agar supplemented with pimaricin-ampicillin-rifamycin-pentachloronitrobenzene-hymexazol) medium, with 5 replicates of each sample [10], and incubated at 25°C in dark for 5 consecutive days. Agar plugs from colonies appearing like *Phytophthora* spp. were cut and placed in petri plates containing sterile distilled water and incubated at 25°C for 48 hr, and sporangia formed were observed under microscope (200x). Agar blocks with sporangia and mycelial hyphal swelling were transferred to CMA-PARPH medium for purification of *Phytophthora* spp. The purified isolate was transferred to CMA and maintained at 25°C with accession number NRCPh-119.

2.2. Morphology and Mating Type. Investigation of sporangia with respect to nature of papilla, shape, caducity, type of branching, length and breadth and chlamydospore production, and size was done in a water culture using compound microscope (200x). Colony morphology was recorded after 4 days of growth at 25°C in the dark on PDA (potato dextrose agar), V8 juice agar, and CMA. Mating type of the isolate was investigated by the single unknown isolate method [11], for which the known mating type tester of *P. nicotianae* (A1) ATCC MYA-4036 (American Type Culture Collection) and *P. palmivora* (A1) and *P. citrophthora* (A2) isolates (Phytophthora Culture Collection, NRCC) were used. The sealed plates were directly observed after incubation of 7 days at 20°C (constant) in dark for the formation of oogonia. Average diameter of nearly 15–25 oogonia and oospores was measured.

2.3. DNA Extraction. Agar blocks containing actively growing mycelia of NRCPh-119 isolate were transferred to 25 mL of V8 broth in a conical flask and incubated in dark for 7–10 days at 25°C. Mycelia were harvested, washed with sterile distilled water, and blotted dry with sterile Whatman filter paper. Genomic DNA was extracted from approximately 100 mg of mycelium using Qiagen DNeasy Plant mini kit (Qiagen Inc, Valencia, CA).

2.4. Polymerase Chain Reaction (PCR). Multilocus PCR amplification and sequencing was carried out for precise identification of NRCPh-119 isolate. Four loci, namely, internal transcribed spacer (ITS), elongation factor-I (EF-1α), beta-tubulin (β-*tub),* and cytochrome c oxidase subunit II (*cox*-II), were selected for PCR amplification and sequencing. The ITS region of the isolate was amplified using the universal primer pair ITS-6 and ITS-4 [12]. *EF*-1α and β-*tub* genes were amplified using the primers EF1F/EF1R and BTUBF2/BTUBR2, respectively [13]. The region containing the mitochondrial cytochrome c oxidase subunit II (*cox*-II) gene fragment was amplified using Fm35 and Fmphy primer pair [14]. ITS, *EF*-1α, β-*tub,* and *cox*-II PCR products were sequenced (Chromous Biotech Pvt. Ltd, Bangalore) and were analyzed using NCBI BLAST. Sequences of all the 4 loci of the isolate were submitted to NCBI GenBank.

2.5. ITS-RFLP. ITS region of ~900 bp was amplified for *P. nicotianae, P. palmivora, P. citrophthora,* and NRCPh-119 isolates using ITS4 and ITS6 primers and further digested with *Alu* I, *Msp* I, and *Rsa* I restriction enzymes [15] according to the manufacturer's instructions and electrophoresed using 3% agarose gel.

2.6. Phylogenetic Analysis. ITS region sequence of NRCPh-119 isolate and sequences available at NCBI database (Figure 3) were compared for diversity. Sequences were aligned with ClustalW followed by construction of phylogenetic tree using maximum likelihood method with Hasegawa-Kishino-Yano model. The bootstrap consensus tree was inferred from 1000 replicates using the software MEGA 5.01 [16].

2.7. Pathogenicity. Pathogenicity was determined by artificial inoculation of NRCPh-119 on different fruits and citrus rootstocks. Healthy and mature fruits were surface sterilized with 2% sodium hypochlorite solution followed by repeated sterile distilled water washes. Fruits of Apple (*Malus domestica*), Pear (*Pyrus*), Cucumber (*Cucumis sativus*), Acid lime (*Citrus aurantifolia*), Mandarin orange (*Citrus reticulata*) and Sweet orange (*Citrus sinensis*) were inoculated in duplicate by placing 6 mm mycelial agar plug of NRCPh-119 as test and sterile CMA agar plug as control with and without injury. After infection was visible, rind portion from the site of infection of fruits (test and control) was cut into 5 mm pieces and placed into CMA-PARPH medium to reisolate NRCPh-119 in order to satisfy the Koch's postulates.

To assess the pathogenicity of NRCPh-119 on citrus roots, six-month old rough lemon (*Citrus jambhiri*), Rangpur lime (*Citrus limonia*), sweet orange (*Citrus sinensis*), and acid lime (*Citrus aurantifolia*) plants were used, each in triplicate for both test and control setup. Fifteen 6 mm agar plugs of NRCPh-119 (5 days old) grown on CMA were dispensed in 100 mL sterile distilled water and kept under fluorescent illumination at 25–28°C for 7 days and thereafter observed under compound microscope for sporangial development. Following ample sporangia formation, zoospores were released by keeping the water containing agar plugs at 4°C for 45 min and confirmed microscopically. Roots were thoroughly washed with sterile distilled water, and test plants were dipped in the beaker containing 100 mL zoospore suspension, while control plants were dipped in the beaker containing 100 mL sterile distilled water with the same number of sterile CMA agar plugs. Furthermore, 400 mL of sterile distilled water was added to respective beakers to completely immerse the roots. The setup was kept for 48 hours at 25°C, and then the plants were transferred in polyethylene bags (30 cm × 15 cm) containing sterile soilrite mix (Chowgule Industries Ltd., Bangalore, India) and sand (1 : 1) along with their respective water in which the roots were dipped. Plants were kept in glasshouse at 30 ± 2°C and observed for root rotting in test plants after 20 days. Roots from both test and control groups were carefully washed with sterile distilled water to clean the soil adhered to them. To confirm whether the rotting in test roots was caused due to NRCPh-119 infection, 6 root segments of about 1-2 cm each were placed per plate of CMA-PARPH medium and incubated at 25°C for 3-4 days.

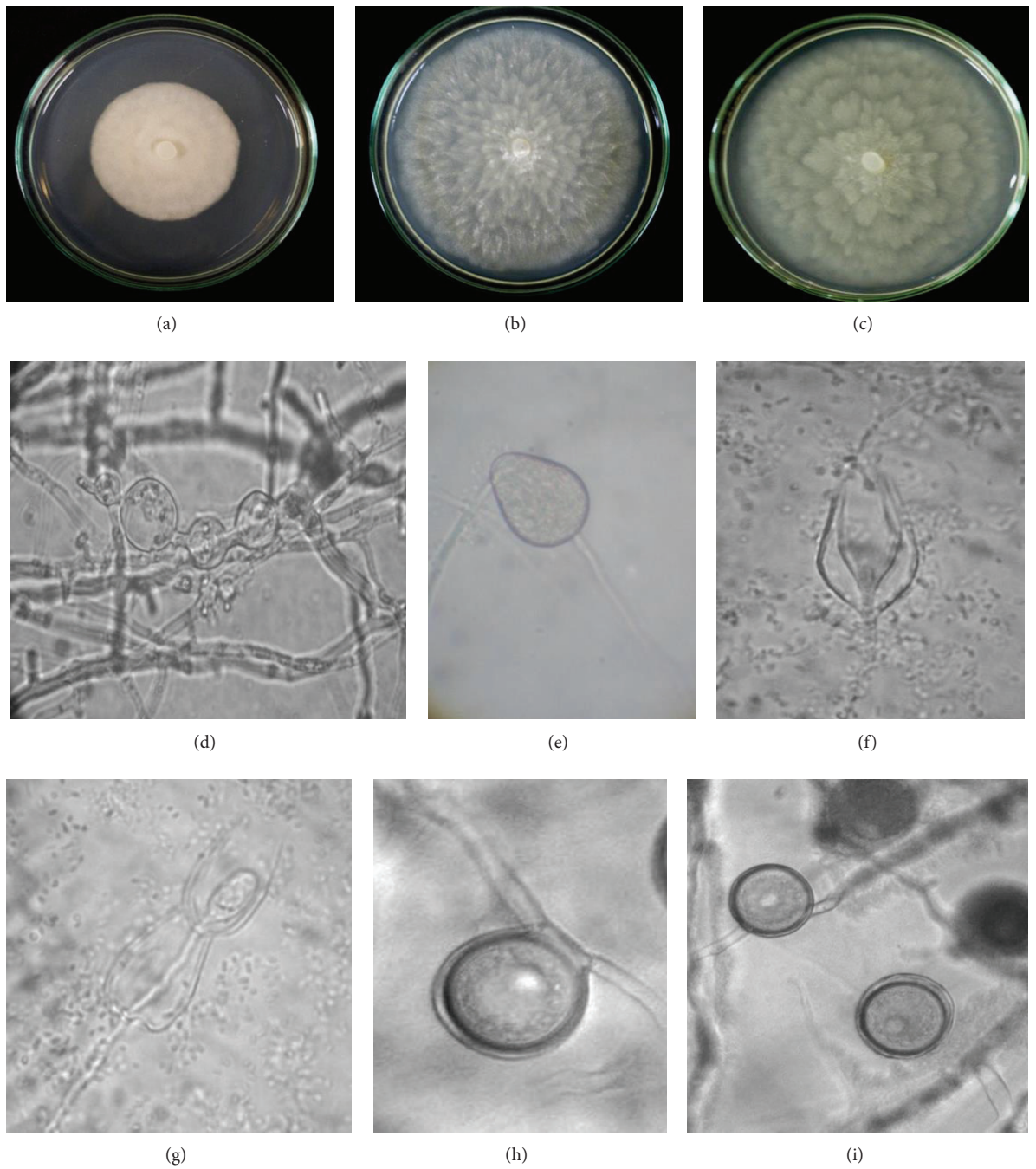

Figure 1: Colony morphology of *P. insolita* on (a) PDA, (b) V8, and (c) CMA. Asexual structures of *P. insolita* in water: (d) hyphal swelling, (e) sporangium, (f) nested proliferation of sporangium, and (g) internal proliferation of sporangium. ((h) and (i)) Gametangia formed on carrot agar by *P. insolita*.

2.8. Effect of Fungicide. To control *Phytophthora* in citrus, 4% metalaxyl + 64% mancozeb (Ridomil gold-Syngenta Corp, Mumbai, India) is recommended. The effect of this fungicide at 25°C on growth rate of NRCPh-119 was studied by amending CMA with fungicide concentration of 1 mg/l, 5 mg/l, 10 mg/l, and 50 mg/l and compared to control plates (CMA without fungicide).

3. Results and Discussion

3.1. Morphological and Sexual Characterization. Asexual and mycelial features were studied by following the "agar-disk-in-water" method [3]. Chlamydospores were infrequently observed, which were spherical in shape having an average diameter of 38.8 μm, which was in agreement with Ann and

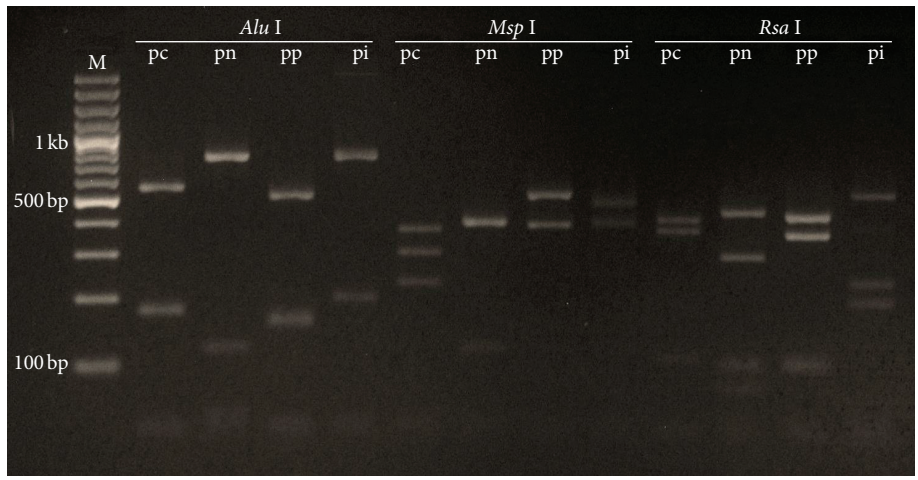

Figure 2: Restriction digestion pattern of ITS region for *P. citrophthora* (pc), *P. nicotianae* (pn), *P. palmivora* (pp), and *P. insolita* (pi) with *Alu* I, *Msp* I, and *Rsa* I restriction enzymes. M-100 bp ladder.

Ko [4]. Irregular, globose, inflated, and catenulate shaped hyphal swelling of varying sizes was observed at the terminal and intercalary positions of mycelia. Sporangia were 29.1 μm and 24.25 μm in length (L) and breadth (B), respectively, with L : B ratio of 1.20. These average measures were not in accordance with the prior reports of *P. insolita* for the length and breadth of sporangia range from 31 to 68 μm and 24 to 44 μm, respectively, and the L : B ratio between 1.3 and 1.7 [8] with nested (Figure 1(f)) as well as internal proliferation (Figure 1(g)). NRCPh-119 isolate showed stellate striated pattern with uniform margin and a growth rate of 12.06 mm/day on V8 agar (Figure 1(b)); on PDA, the growth rate was 7.00 mm/day. It did not exhibit any mycelial pattern but had uniform margin (Figure 1(a)), whereas on CMA it showed less petalloid pattern (Figure 1(c)). Effect of fungicide (4% metalaxyl and 64% mancozeb) at concentrations of 1 mg/l, 5 mg/l, 10 mg/l, and 50 mg/l showed deviation in growth pattern and reduction in growth rate (Figure 4) by up to 44.04%, 69.04%, 82.14%, and 100%, respectively when compared with control. The isolate was homothallic as abundant gametangia were developed in the self-crossed mating plate after 88 hrs of setup; the oogonia and oospore were 35.36 μm and 29.67 μm in diameter, respectively. On mating with *P. nicotianae* A1, it formed oogonia measuring 38.41 μm and oospore 35.43 μm in diameter. Mating with *P. nicotianae* A2, oogonia (35.78 μm) and oospore (33.15 μm) were found smaller in diameter compared to *P. nicotianae* A1 mating type. It also formed oogonia with *P. palmivora* A1 and *P. citrophthora* A2. The measures of oogonia and oospore were in the range as previously described [4, 8]. Types of proliferation, mating behavior, and absence of antheridium (Figures 1(h) and 1(i)) observed in NRCPh-119 confirmed its identity as *P. insolita*. Previously oospore formation was induced when *P. insolita* was paired with *P. nicotianae* A2 mating type on V8 juice agar but not when paired with an A1 mating type [17]. But our isolate of *P. insolita* formed oospores when crossed with both A1 and A2 mating types of *P. nicotianae* along with other *Phytophthora* species—*P. palmivora* (A1), *P. citrophthora* (A2). Ann and Ko [18] suggested that in nature both sexual and asexual isolates of *P. insolita* exist; moreover asexual isolates of *P. insolita* probably originated from sexual isolates by losing their ability to produce oospores. This hypothesis was supported by Ho et al. [8] as there was production of oospores in aged culture of some asexual isolates of *P. insolita* from Hainan Island, China. Our *P. insolita* isolate showed some sexual behaviour even after one year.

Table 1: Approximate fragment size (base pairs) of internal transcribed spacer region digested with *Alu* I, *Msp* I, and *Rsa* I restriction enzymes for 4 *Phytophthora* species.

Species	*Alu* I	*Msp* I	*Rsa* I
P. citrophthora	575, 175	370, 300, 225	400, 360, 110
P. nicotianae	750, 120	410, 120	430, 290, 90, 70
P. palmivora	525, 150	525, 390	410, 340, 90
P. insolita	750, 210	480, 400	510, 210, 180

3.2. Molecular Characterization and Phylogeny. The amplicon of ~900 bp was amplified from ITS regions of *P. nicotianae, P. palmivora, P. citrophthora,* and *P. insolita* isolates. Restriction digestion of *P. insolita* ITS region (Figure 2) with enzyme *Alu I* showed two bands of 750 bp and 210 bp and with *Msp I* two bands of 480 bp and 400 bp, whereas *Rsa I* digestion showed 3 bands of 510 bp, 210 bp, and 180 bp (Table 1). Amplicons from ITS, *EF*-1α, β-*tub,* and *cox*-II regions of NCPh-119 isolate were sequenced and compared using BLAST with existing GenBank accessions which showed maximum identity of 97% with GU993897 for ITS region, 99% with EU080177 for *EF*-1α, 99% with EU080210 for β-*tub,* and 98% with GU222041 for *cox*-II. The submitted nucleotide sequences of ITS, *EF*-1α, β-*tub,* and *cox*-II regions were assigned with the following GenBank accession numbers: JN655559, JN807441, JN807437,

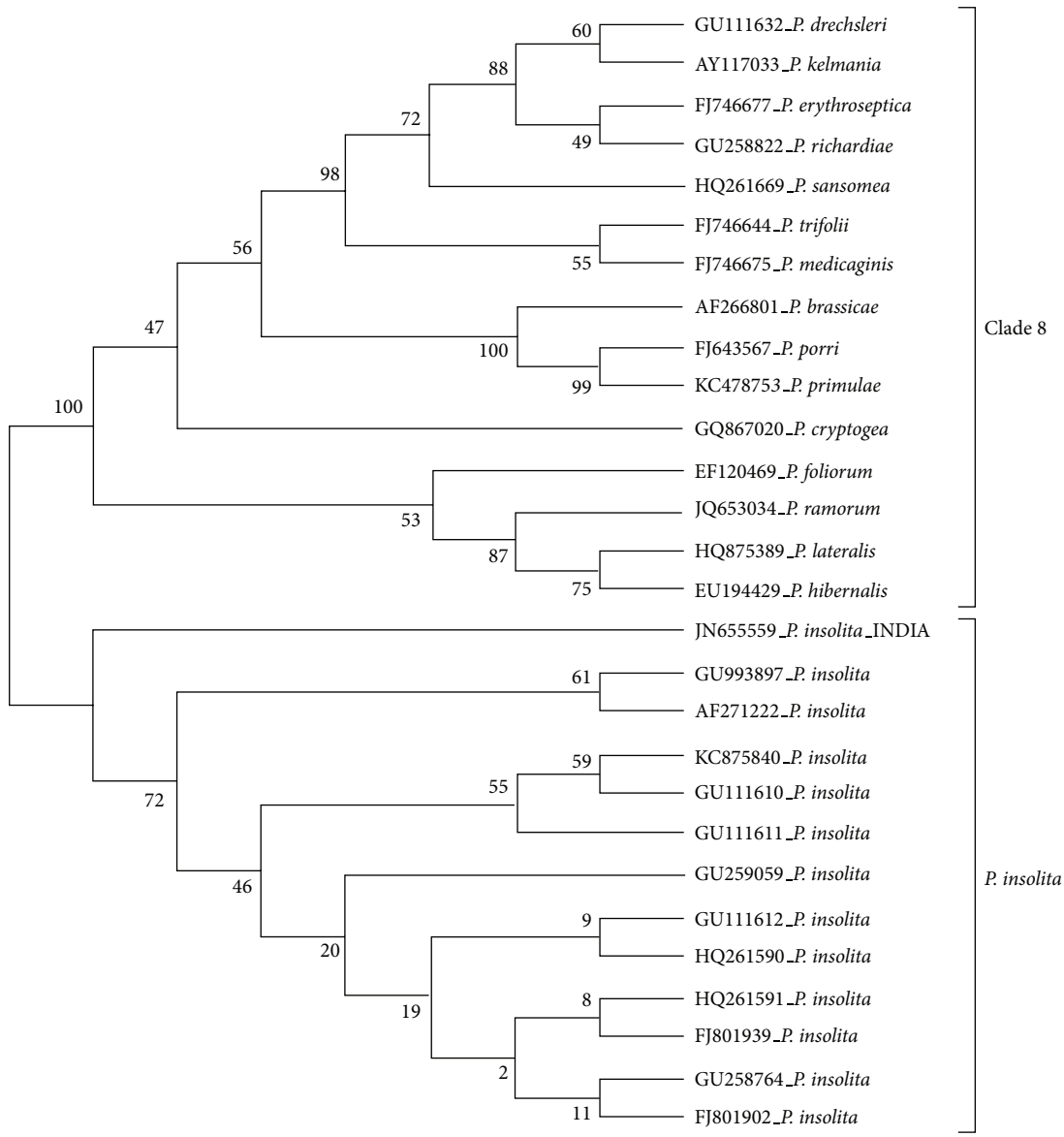

Figure 3: Phylogenetic tree derived from the internal transcribed spacer region of *P. insolita* isolates using Maximum Likelihood method based on the Hasegawa-Kishino-Yano model at 1000 bootstraps.

and JN865172, respectively. ITS sequence based phylogeny revealed that our *P. insolita* isolate is distinct from other *P. insolita* isolates, but all the *P. insolita* isolates segregated in one cluster when compared with other *Phytophthora* species of clade 8 (Figure 3).

3.3. Pathogenicity. P. insolita is known to have no natural hosts [3]. Earlier studies on the pathogenicity suggest that it was isolated from soil associated with citrus, and it was not pathogenic naturally or when inoculated to sweet orange. *P. insolita* could not infect unwounded fruits but was pathogenic to wounded apple, avocado, cucumber, eggplant, green pepper, and tomato [8]. We observed similar results where *P. insolita* could not infect any of the nonwounded fruits, but artificial wound inoculation resulted in water-soaked dark brown lesions on pear and acid lime fruit (Figures 4(e) and 4(f), resp.), apple and mandarin orange [7], but not on cucumber and sweet orange fruits. *P. insolita* was reisolated (identification confirmed by agar-disk-in-water method) from the rotted portion of fruit as well as roots on PARPH-CMA medium fulfilling the Koch's postulates. It is not very clear how significantly *P. insolita* contributes as a plant pathogen in nature [8]; however, it is reported to infect alfalfa root and poinsettia and is associated with strawberry fruit rot [17, 19]. It was also reported to be isolated from necrotic tissue of *Rhododendron* [9]. However, our *in vivo* glasshouse experiment showed *P. insolita* infection only in rough lemon roots, whereas roots of Rangpur lime, sweet orange, and acid lime remained uninfected. Stunted

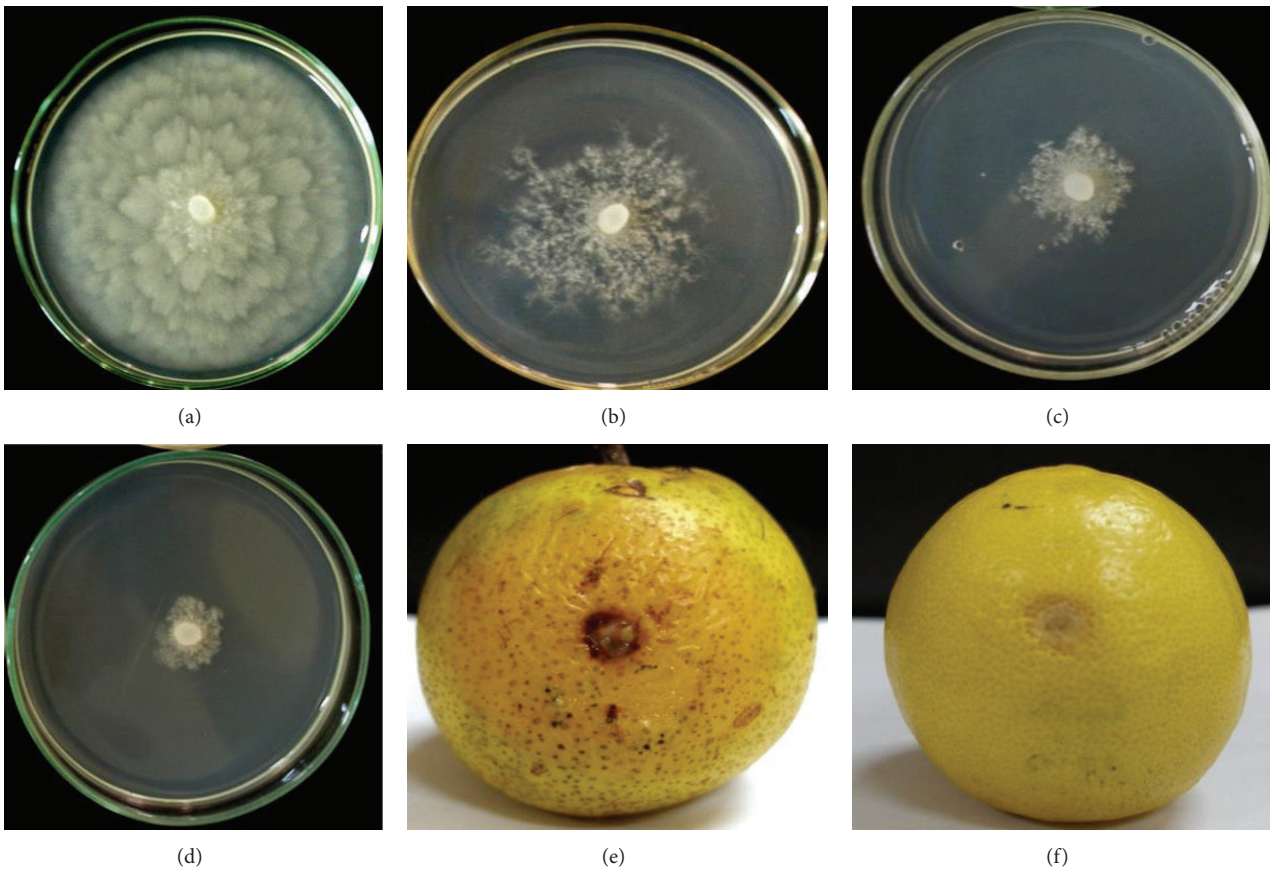

Figure 4: Growth pattern and inhibition (a) on CMA, (b) on CMA + 1 mg/l fungicide, (c) on CMA + 5 mg/l fungicide, and (d) on CMA + 10 mg/l fungicide. ((e) and (f)) Pathogenicity of *P. insolita* on artificially inoculated fruits of pear and acid lime, respectively.

height and reduced root density were observed in rough lemon infected with *P. insolita* when compared to control. Sweet orange, acid lime, Rangpur lime, and rough lemon are *Phytophthora* susceptible rootstocks, but Rangpur lime is somewhat more resistant than others [2]. Also it was very interesting to know that *P. insolita* isolate infected only rough lemon plant among all the above mentioned *Phytophthora* susceptible rootstocks. Though the direct proof of citrus as a natural host for this pathogen is not yet clear; this is the first evidence of *P. insolita* pathogenesis in citrus plant (rough lemon). Infrequent isolation of *P. insolita* from citrus associated soil [4, 8] and pathogenicity towards rough lemon seems to hint its mode of survival and propagation. Kong et al. reported the role of zoospore interspecific signalling in promoting plant infection by *Phytophthora* [20]. They found that signal molecules present in zoospore-free fluids (ZFF) from *Phytophthora capsici*, *P. hydropathica*, *P. nicotianae*, *P. sojae,* and *Pythium aphanidermatum* could act in an interspecific manner. Due to limited resources this self-interested cooperation among related species gives individual pathogens of the same group a competitive advantage over pathogens and microbes from other groups. These findings would help in understanding why these pathogens often are individually undetectable until severe disease symptoms have developed [20]. In India, *P. nicotianae, P. palmivora,* and *P. citrophthora* are common citrus infecting *Phytophthora* spp. [5, 6], and it is likely that *P. insolita* may persist with them as masked and insignificant pathogen escaping detection. In a series of sampling (water, soil, root, bark, leaf, and fruit) from different citrus cultivating regions of India, we were able to isolate only the predominant three *Phytophthora* species—*P. nicotianae*, *P. palmivora,* and *P. citrophthora.* We could not detect any other *Phytophthora* species in the *P. insolita* positive sample, but two *P. nicotianae* isolates were recovered from the remaining samples. Also, many of our previous samplings have recovered *P. nicotianae* (but no *P. insolita*) from these and nearby orchards. It is astonishing to know that we were able to isolate only one *P. insolita* isolate during four years of sampling from nearly more than 200 locations across different states of India (data not shown). Similar was the case with *P. lacustris,* which we have reported for the first time in India from citrus orchard [21]. The study on *P. captiosa* and *P. fallax* suggests that they were not found in Australia, but there is possibility that they exist in an "obscure equilibrium with hosts and environment" without causing any notable disease [22]. We did not explore the validity of hypothesis discussed above truly for *P. insolita*, but we take this opportunity to suspect.

4. Conclusion

Our findings contribute to the understanding of *P. insolita* species associated with citrus, which is one of the most important horticultural crops in India. Although a single isolate was obtained, there are very few reports of this species worldwide, which explains its singular occurrence. Our study provides very comprehensive method for multi-gene sequence based identification, which is the most preferred method for identification of *Phytophthora* species nowadays. Also ITS-RFLP with *Alu* I, *Msp* I, and *Rsa* I restriction enzymes and ITS sequence based phylogeny provides a precise molecular status of this Indian *P. insolita* isolate amongst other *Phytophthora* species in India and different parts of the world. Our study also showed interesting fact about pathogenesis of this rare species. Pathogenesis study on commonly used citrus rootstocks, that is, rough lemon (*Citrus jambhiri*) and Rangpur lime (*Citrus limonia*), and cultivars, that is, sweet orange (*Citrus sinensis*) and acid lime (*Citrus aurantifolia*), showed that out of all four citrus varieties mentioned, *P. insolita* selectively infected only rough lemon (*Citrus jambhiri*), but still this fact needs further investigations to evaluate the significance of this *Phytophthora* species with respect to citrus pathogenesis.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper, and there is absolutely no conflict of interests for any of the authors nor any direct financial relation with the commercial firm mentioned in the paper.

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