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Short Communication
Extracellular Cystatin-like Protease Inhibitor (*EPIC1*) Gene Based PCR Primers for Specific Detection of *Phytophthora nicotianae* Infecting Citrus

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Abstract

Background: In case of genus *Phytophthora*, species identification and detection is a difficult task and requires use of taxonomic keys and knowledge of host range of the pathogen. Internal Transcribed Spacer (ITS) or intergenic mitochondrial DNA spacers (mtDNA- IGS), *Ypt1* gene, *Lpv* gene, SCAR/RAPD markers are very much in use in PCR- based molecular detection of *Phytophthora* spp. Though these genes are effective in detecting *Phytophthora* spp., but they all have their own limitations. Hence new genes are to be explored to broaden the molecular diagnostic tool box for *Phytophthora* spp., detection in infected samples. Studies on host-pathogen interaction carried out in last decade showed that the Irish potato famine pathogen, *Phytophthora infestans* secretes effector proteins viz., cystatin-like protease inhibitors (EPICs) targeting host proteases during infection. But potential of *EPIC* gene in PCR-based diagnosis of plant pathogens in infected samples was not assessed by any of the research groups earlier. Therefore, potential of *EPIC1* gene for detection of *P. nicotianae* from infected citrus samples was assessed in the study. **Materials and Methods:** The sequence of *EPIC1* region of *Phytophthora nicotianae* was retrieved from whole genomic data available at Broad institute of MIT and Harvard, Cambridge, MA website and specific primers were designed for its PCR-based identification and diagnosis. **Results:** Out of 4 primer pairs, F*EPIC1*F/F*EPIC1*R was found best suitable pair for its use in PCR-based species specific detection system. These primers were tested successfully on *P. nicotianae* infected citrus leaf, stem and root tissues. No cross reactivity of primers were observed with six other *Phytophthora* spp., viz., *P. palmivora*, *P. citrophthora*, *P. boehmeriae*, *P. lacustris*, *P. insolita*, *P. tropicalis* and three other oomycete/fungi viz., *Pythium* sp., *Colletotrichum* sp. and *Alternaria* sp. Detection by these primers was done effectively up to 100 pg μL^{-1} of DNA isolated from pure culture of *P. nicotianae*. **Conclusion:** The *EPIC1* PCR assay was found to be robust and reliable technique to detect *P. nicotianae* in infected citrus samples and thus would be useful in restricting *P. nicotianae* mediated destruction in citrus. Present investigation, also reports for the first time, isolation and annotation of *EPIC1* gene from *P. nicotianae* pathogenic to citrus.

Key words: *P. nicotianae*, oomycete, citrus, *EPIC1* region, primer design, PCR, BLAST, MEGA 5.01

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Phytophthora species, the oomycete fungal-like pathogens have been a great threat to agrarian production and natural environment[1]. Among the more than 100 *Phytophthora* species reported so far[2], *P. nicotianae* Breda de Haan (syn. *P. parasitica* Dastur) stands out since it is a threat to plant productivity on a global scale for a broad range of hosts and a wide diversity of climates[3]. The host range of *P. nicotianae* comprises 255 plant genera in 90 families including a number of economically important species, such as fruit trees, herbaceous hosts and ornamental plants[4]. The *P. nicotianae* is the most damaging soil-borne pathogen in India that attack all commercial cultivars of citrus right from damping off of seedlings in nursery beds to decay of fibrous roots, crown rot, collar rot, foot rot and gummosis in mature orchards, often leading to decline and death of citrus trees[5,6]. Symptoms of these diseases are deceptive and become apparent when underground fibrous root system has been damaged substantially. Thus it is very important to diagnose and detect the pathogen with respect to strategizing crop protection measures. Earlier used methods to detect *Phytophthora* include, plating samples onto selective agar media, baiting, immunodetection etc.[7] that are laborious, time consuming and require mycological knowledge and skill for identification. Tremendous progress has been made recently in the use of Polymerase Chain Reaction (PCR)-based methods for detection, identification and classification of *Phytophthora* species[8,9]. The PCR technique has an advantage of being specific, sensitive and most importantly rapid for *Phytophthora* detection system[10]. Commonly used loci for PCR-based molecular detection of *Phytophthora* spp., involves Internal Transcribed Spacer (ITS) or intergenic mitochondrial DNA spacers (mtDNA-IGS), *Ypt1* gene, *Lpv* gene and SCAR/RAPD marker[11]. But all of them have some or other limitations. Hence, the purpose of the study was to find a new candidate gene which can be effectively used for PCR-based molecular detection of *Phytophthora* spp., infecting citrus. Studies on host-pathogen interaction carried out in last decade showed that the Irish potato famine pathogen, *Phytophthora infestans* secretes effector proteins viz., cystatin-like protease inhibitors (EPICs) targeting host proteases during infection[12]. Data mining of genomic and cDNA sequences also revealed that *P. infestans* evolved 18 extracellular protease inhibitor genes belonging to two major structural classes: (i) Kazal-like serine protease inhibitors (*EPI1*-14) and (ii) Cystatin-like cysteine protease inhibitors (*EPIC1*-4)[13]. Work on cellular and molecular characterization of *P. nicotianae* appressorium mediated penetration showed that out of 5 sequences corresponding to putative pathogenicity factors, 3 sequences, homologous to *EPI4* and *EPI7* genes, encoded kazal-like serine protease inhibitors, whereas two homologs of the *EPIC1* and *EPIC4* genes encoded cystatin-like cysteine protease inhibitors[14]. Present study, reports mining and analysis of the whole genome data of *P. nicotianae* available at Broad Institute of MIT and Harvard, Cambridge, MA website for finding *EPIC1* gene, subsequently designing PCR primers and validated its use as a diagnostic tool for the detection of *P. nicotianae* in infected citrus samples.

MATERIALS AND METHODS

***Phytophthora* species and fungal isolates:** Nine *Phytophthora* spp., isolates (*P. nicotianae* NRCPh-133, 155 and 157, *P. palmivora* NRCPh-161, *P. citrophthora* NRCPh-147, *P. boehmeriae* NRCPh-135, *P. lacustris* NRCPh-112, *P. insolita* NRCPh-119 and *P. tropicalis* NRCPh-179) used in this study were obtained from '*Phytophthora* culture repository' at Indian Council of Agricultural Research-Central Citrus Research Institute (ICAR-CCRI), Nagpur and were routinely maintained by subculturing on Corn Meal Agar (CMA) media. These *Phytophthora* spp., isolates were recovered from rhizospheric soil and water samples collected at different citrus orchards of India. Other oomycete/fungi used in the study were *Pythium* spp., isolated from citrus nursery soil, in addition to *Colletotrichum* sp. and *Alternaria* sp. (both isolated from infected citrus fruit).

Inoculation on plant samples *in vitro*: The *P. nicotianae* NRCPh-155 agar plugs (6 mm diameter) cut from the advancing margin of colony in CMA medium were placed in sterilized petri plates containing 20 mL sterile distilled water and incubated for 2-3 days at 25°C under continuous white light. These plugs were subsequently observed under the microscope to investigate sporangial development. Once sporangia were observed the agar plugs were used for artificial inoculation on plant samples (leaf, stem and root) *in vitro*. Healthy apical leaves of 6 months old rough lemon (*Citrus jambhiri*) seedlings were infected with *P. nicotianae* culture plugs by detached leaf method[15], control leaf includes plain CMA plug without inoculum. The complete experimental setup was incubated at 25°C for 7 days. For stem infection healthy twigs (15 cm length and 8 mm diameter) of Nagpur mandarin (*C. reticulata*) were surface sterilized using 75% ethanol and air dried at room temperature. Both the ends of twigs were sealed with parafilm tape. Surface of twig was cut

(5 cm) with sterilized scalpel such a way that only vascular cambium was exposed and the green bark creates a flap. The exposed cambium was inoculated with sporulated *P. nicotianae* agar plug and covered with moist sterile cotton. The inoculated twig was then kept inside ziplock bag layered with moist tissue paper and incubated at 25°C for 15 days. Control twig includes plain CMA plug without culture. Similarly healthy roots were taken by gently removing 6 months old rough lemon seedlings from polythene bags and rinsed twice with sterile distilled water to remove all soil particles. Roots were then surface sterilized using 75% ethanol and cut into small pieces (~2-3 cm) by a sterile scalpel, placed in a sterilized petri plate with moist tissue paper and inoculated with sporulated *P. nicotianae* agar plugs. Petri plates were then sealed with parafilm tape and incubated at 25°C for 10 days. Control root includes plain CMA plug without culture. All the artificial inoculation experiments of plant samples were carried out in triplicate.

DNA extraction: For DNA isolation from pure mycelial culture, 6 mm agar discs of actively growing mycelium from single hyphal tip of *Phytophthora* spp., isolates were transferred to 25 mL of V8 broth in a conical flask and incubated in dark for 3-4 days at 25+1°C. Mycelial mats were washed with sterile distilled water and dried briefly under vacuum before being frozen at -20°C. Frozen mats were ground with a sterile mortar and pestle. Similarly after the appearance of visible symptoms (Fig. 1) of infection on leaf, twig and root, in the *in vitro* inoculation experiments, the tissues were blot dried on sterilized tissue paper and crushed with the help of sterile mortar and pestle by adding liquid nitrogen to form a powder. Total genomic DNA from 100 mg of ground mycelium/homogenized tissue was extracted using Qiagen DNeasy Plant mini kit (Qiagen Inc., Valencia, CA) according to manufacturer's directions. DNA concentrations were estimated using Nanovue plus spectrophotometer (GE Life Sciences, USA).

Mining of *EPIC1* region and primer design: Two nucleotide sequences of *EPIC1* cystatine like cysteine protease inhibitor gene, unique to *P. infestans*[12], AY935250 and XM_002903434 were retrieved from NCBI GenBank database by searching the term "*EPIC1 Phytophthora infestans*" in nucleotide resources (http://www.ncbi.nlm.nih.gov/nuccore/). Subsequently, to find out whether *EPIC1* gene was present in *P. nicotianae* whole genome sequence, homologous sequence for both the above sequences was searched in *P. nicotianae* INRA-310 transcripts database using nucleotide BLAST tool (http://www.broadinstitute.org/annotation/genome/Phytophthora_parasitica/Blast.html). The *P. nicotianae* (isolate INRA-310) genome/transcripts sequence data was obtained from Broad Institute Genome database (http://www.broadinstitute.org/annotation/genome/Phytophthora_parasitica /MultiHome.html). The three sequences i.e., *EPIC1* reference sequences from *P. infestans* (XM_002903434 and AY935250) and the sequence mined from *P. nicotianae* genome (PPTG_03489T0) were aligned using ClustalW module in MEGA 5.01 software[16] (Fig. 2) to identify conserved and differing regions. On the

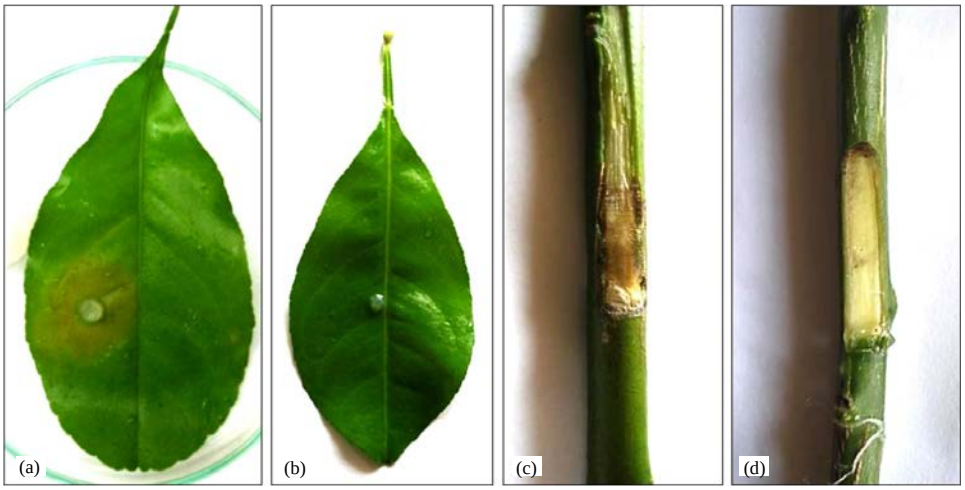

Fig. 1(a-d): Development of disease symptoms on citrus leaf and stem after *in vitro* inoculation with *P. nicotianae* (isolate NRCPh-155), (a) Inoculated rough lemon leaf showing brown lesion, (b) Control rough lemon leaf, (c) Inoculated Nagpur mandarin stem showing progressive stem lesion and (d) Control Nagpur mandarin stem

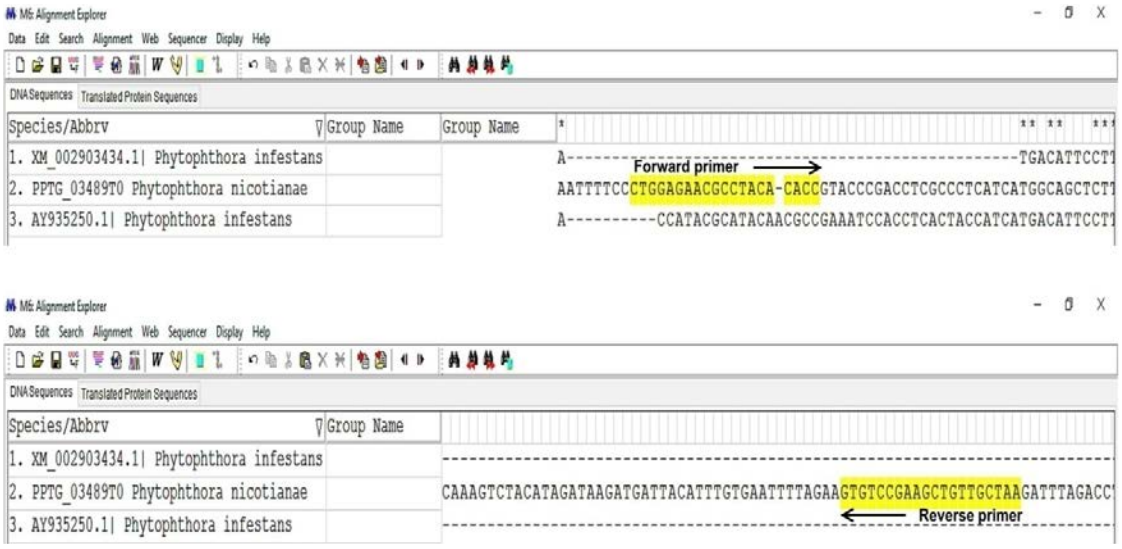

Fig. 2: Snapshot of MEGA 5.01 software window showing multiple sequence alignment and location of F*EPIC1*F/F*EPIC1*R primer sets targeting the PPTG_03489T0 *P. nicotianae EPIC1* region, arrows indicate the direction of extension

basis of regions of divergence in *P. nicotianae*, 4 primer pairs for *EPIC1* regions were designed using Fast PCR software[17], a stand alone primer designing tool. For validation of these *in silico* designed primers, PCR was performed using template DNA from pure mycelial cultures of *Phytophthora* spp., *Pythium* spp., *Colletotrichum* spp. and *Alternaria* spp., isolates and artificially infected citrus tissue samples.

PCR amplification: The PCR reactions were performed in a total volume of 25 µL containing 2.5 µL of 10X PCR buffer (Fermentas Inc., Maryland, USA), 0.5 µL of 10 mM dNTPs (Fermentas Inc., Maryland, USA), 2.0 µL of 25 mM $MgCl_2$ (Fermentas Inc., Maryland, USA), 1.25 µL each of 10 µM primers (Integrated DNA Technologies, Coralville, USA), 0.2 µL of 20 mg mL^{-1} BSA (Fermentas Inc., Maryland, USA), 0.2 µL of Taq DNA polymerase (5 U μL^{-1}) (Invitrogen, Life Technologies Corporation, USA) and 1 µL template DNA (20-40 ng μL^{-1}). Sterile distilled water was added to make up the final volume. The PCR conditions were optimized for number of cycles (20-40), temperature (gradient 52-60°C) and annealing time (30-60 sec). Ten microliters of PCR products were resolved by electrophoresis in 1.5% agarose gel (Amresco, Cleveland, USA) for 90 min at 5-6 V cm^{-1}, stained with ethidium bromide and visualized under gel documentation system (Biovis Gel, Expert Vision Labs Pvt Ltd., Mumbai, India). Out of 4 primer pairs designed, only F*EPIC1*F/F*EPIC1*R primers were found to produce a clear PCR amplification and hence subjected to further analysis.

Primer specificity and sensitivity: To determine specificity of the primers, PCR was conducted on the extracted genomic DNA of *P. nicotianae*, 6 different *Phytophthora* species and 3 other oomycete/fungi as described earlier using the specific primer sets F*EPIC1*F/F*EPIC1*R. Sensitivity of the primer pair F*EPIC1*F/F*EPIC1*R was determined by using series of DNA dilutions from pure culture of *P. nicotianae* (NRCPh-155) as templates ranging from 100 ng-100 pg μL^{-1}. Negative (sterile water) controls lacking template DNA were also included in each experiment to eliminate the possibility of carryover contamination.

Detection in host tissues: In order to detect *P. nicotianae* in infected tissues, extracted DNA from diseased and healthy citrus leaf stem and roots was tested. The PCR reactions were carried out in 25 µL volume containing 2.5 µL of 10×PCR buffer (Fermentas Inc., Maryland, USA), 0.5 µL of 10 mM dNTPs (Fermentas Inc., Maryland, USA), 2.0 µL of 25 mM MgCl2 (Fermentas Inc., Maryland, USA), 1.25 µL each of 10 µM primers (F*EPIC1*F/ F*EPIC1*R)-(Integrated DNA Technologies, Coralville, USA), 0.2 µL of 20 mg mL^{-1} BSA (Fermentas Inc., Maryland, USA), 0.2 µL of Taq DNA polymerase (5 U μL^{-1}) (Invitrogen, Life Technologies Corporation, USA) and 1 µL of template DNA. Sterile distilled water was added to make up the final volume. One microliter of DNA template extracted from control samples of leaf, twig and roots were used as non-target control to see the cross reactivity with uninfected (healthy) citrus samples.

Sequencing, annotation and NCBI submission: The *EPIC1* PCR products of three *P. nicotianae* (NRCPh-133, NRCPh-155, NRCPh-157) isolates were purified through QiaQuick PCR purification kit (Qiagen) to remove excess primers and nucleotides and were sequenced in forward and reverse orientation using the primers used for amplification at the commercially available automated DNA sequencing facility (Chromous Biotech Pvt Ltd., Bengaluru, India). The sequenced products were assembled using the software[16] MEGA 5.01. Open Reading Frame (ORF) was investigated by an online tool "ORF finder" provided by National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The program BLASTp on UniProtKB (http://www.uniprot.org/blast/) was used to know the percentage identity of the amino acid sequence with the existing sequences in UniProt database. All these sequences were submitted to NCBI GenBank database.

RESULTS AND DISCUSSION

The BLAST search of *P. infestans* sequences (both AY935250 and XM_002903434) showed 80% similarity with the *P. nicotianae EPIC1* Supercontig 4: 1741299-1742007 after mining of cystatin-like protease inhibitor (*EPIC1*) gene from *P. nicotianae* genome data available in public domain (Broad Institute genome database). The PCR assay with 4 primer pairs showed that only one primer set F*EPIC1*F: CTGGAGAAC GCCTACACACC/F*EPIC1*R: TTAGCAACAG CTTCGGACAC could amplify a single clear PCR product having size of 700 bp. Results of PCR optimization showed that ideal temperature and time for annealing of primers F*EPIC1*F/F *EPIC1*R was 58°C for 30 sec and 25 cycles showed enough amplification which was clearly seen on 1.5% agarose gel (Fig. 3a). Therefore, the PCR assay was optimized under following conditions: an initial denaturation at 94°C for 3 min, 25 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 10 min. The specificity of primers was determined against *P. nicotianae*, *P. palmivora*, *P. citrophthora*, *P. boehmeriae*, *P. lacustris, P. insolita P. tropicalis*, *Pythium* spp., *Colletotrichum* spp. and *Alternaria* spp., isolates. Primers F*EPIC1*F and F*EPIC1*R amplified a single DNA fragment of 700 bp from *P. nicotianae* isolates and no amplicon was observed in case of other oomycete and fungal species (Fig. 3a). Detection by these primers was done effectively up to 100 pg μL^{-1} of DNA isolated from pure culture of *P. nicotianae* (NRCPh-155) (Fig. 3b). The PCR results from artificially *P. nicotianae* inoculated citrus samples showed that a single amplicon of 700 bp was amplified only from infected leaf, bark and root tissues (Fig. 3c). Plants contain a great number of cystatins and a diagnostic technique would only be useful when it is directly applied on plant material and not cross-reacting with plant DNA. The *EPIC1* primers study showed that there was no amplification with uninfected (healthy) citrus DNA (Fig. 3c) indicating no cross-reaction with plant cystatins. A clean forward and reverse read was acquired from the sequence data on assembling of raw sequences and a final output of 681-691 bp *EPIC1* gene sequence was obtained. The *P. nicotianae EPIC1* gene sequences generated in this study were deposited in GenBank under the accession numbers KP412980 (NRCPh-133), KP412981 (NRCPh-155) and KP412982 (NRCPh-157). Annotation of *EPIC1* gene showed that there was 126 amino acid intronless coding region (GenBank accessions KP412980-KP412982). UniProt BLASTp search showed 100% identity with *P. nicotianae* extracellular cystatin-like protease inhibitor gene *EPIC1* (Uniprot ID: A0A0E3TKP5). When NCBI nucleotide database was searched for *EPIC1* sequences of other *Phytophthora* species, only 3 other *Phytophthora* spp., viz., *P. infestans*, *P. mirabilis* and *P. iopomoeae* sequences were found. The study therefore reports for the first time, isolation, annotation and PCR based diagnostic potential of *EPIC1* gene from *P. nicotianae* pathogenic to citrus. Using existing methods for rapid extraction and purification of DNA, single-round amplification was found appropriate for detection of target *Phytophthora* species in leaf, stem and roots. This technique gives an extension to a range of DNA-based markers to aid *Phytophthora* identification in plant materials. More significantly it presents an ideal one-step PCR alternative and time-saving advantage over widely followed classical morphological identification, ITS sequencing and PCR-RFLP techniques[18,19]. The importance of *EPIC* gene family in host-pathogen interaction studies is explored only in recent years[12-14,20]. But potential of *EPIC* gene in PCR-based diagnosis of plant pathogens in infected samples was not assessed by any of the research groups earlier. The *EPIC1* gene based PCR primers developed in this study can be used for specific detection of *P. nicotianae* and has potential to replace or supplement previously reported ITS primers[21] and *Ypt1* gene based primers[22] used in molecular diagnostic of *Phytophthora* spp. Further in addition to citrus, this primer pair F*EPIC1*F/F*EPIC1*R can be tested on other susceptible economically important crop plants (viz., tobacco, ornamentals etc.) to help detecting *P. nicotianae* in infected samples. In future, similar to ITS based real-time PCR[23] and *Ypt1* based lab-on-a-chip DNA hybridization array[24], *EPIC1* based rapid detection tools can be developed for *P. nicotianae*.

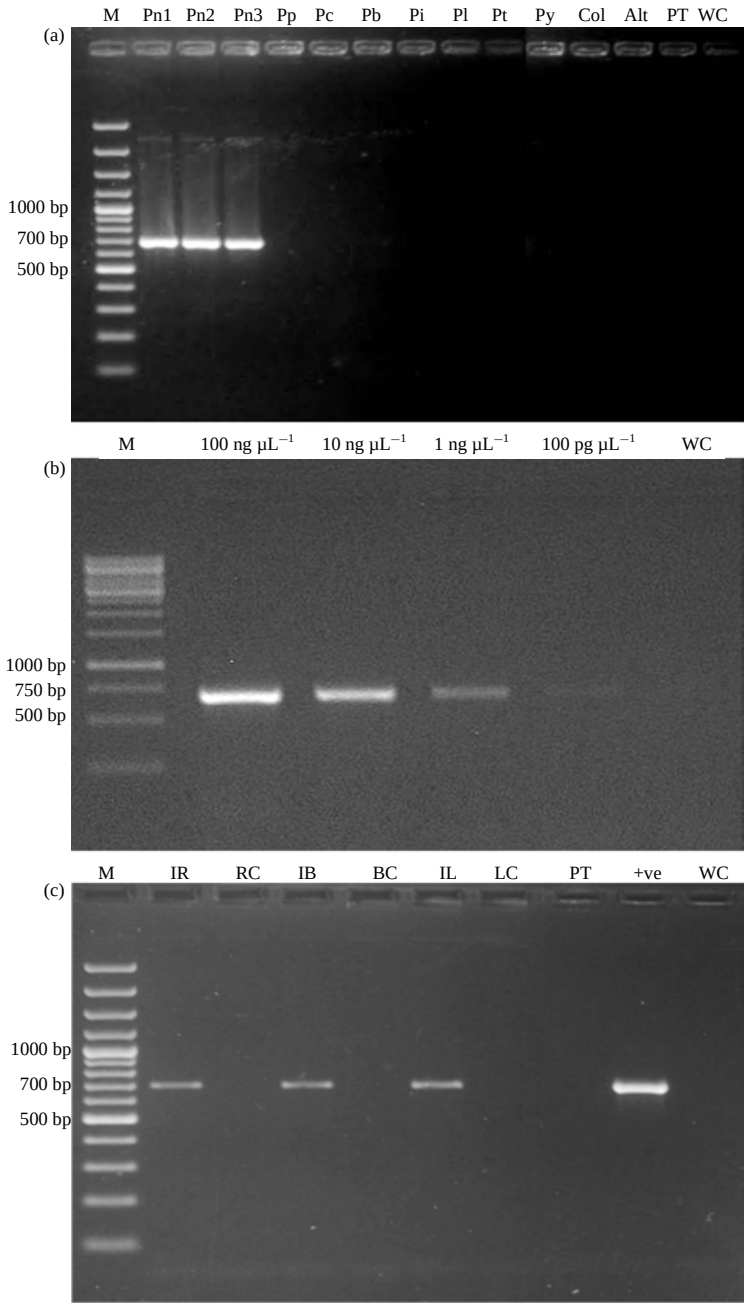

Fig. 3(a-c): Specificity, sensitivity and host tissue infection detection capacity of primer pair F*EPIC1*F/F*EPIC1*R, (a) Specificity of primer pair F*EPIC1*F/F*EPIC1*R for detection of *P. nicotianae.* Only *P. nicotianae* isolates (Pn1, Pn2, Pn3) show expected band of 700 bp, M: 100 bp ladder, Pn1: *P. nicotianae* (NRCPh-133), Pn2: *P. nicotianae* (NRCPh-155), Pn3: *P. nicotianae* (NRCPh-157), Pp: *P. palmivora*, Pc: *P. citrophthora*, Pb: *P. boehmeriae*, Pi: *P. insolita* , Pl: *P. lacustris*, Pt: *P. tropicalis*, Py: *Pythium* spp., Col: *Colletotrichum* spp., Alt: *Alternaria* spp., PT: Citrus plant DNA control and WC: No template (water) control, (b) Detection sensitivity of F*EPIC1*F/F*EPIC1*R primers for *P. nicotianae* (NRCPh-155), WC: No template (water) control, M: 1 kb ladder and (c) Detection of *P. nicotianae* in leaf, bark and root tissues using F*EPIC1*F/F*EPIC1*R primers, M:100 bp ladder, IR: Infected root, RC: Root control, IB: Infected bark, BC: Bark control, IL: Infected leaf, LC: Leaf control, PT: Citrus plant DNA control, +ve: *P. nicotianae* (NRCPh-155) and WC: No template (water) control

CONCLUSION

The *EPIC1* gene based PCR detection technique reported here will be useful for rapid and sensitive detection of *P. nicotianae* in citrus nursery planting material as well as in orchard trees. This will provide huge advantage in the management strategies against citrus root rot, gummosis and dieback diseases as appropriate preventive and quarantine measures can be, launched more quickly before the spread of the pathogen.

SIGNIFICANT STATEMENTS

Phytophthora nicotianae is a very common and destructive pathogen of numerous field and horticultural crops such as citrus. This pathogen mainly causes damping off of seedlings in nursery beds to decay of fibrous roots, crown rot, collar rot, foot rot and gummosis in mature orchards, often leading to decline and death of citrus trees. This manuscript reports one-step PCR diagnosis of *P. nicotianae* in infected citrus samples. The technique involved mining cystatin-like protease inhibitor gene specific (*EPIC1*) gene from genome database available in public domain, designing its specific primers and finally showing their potential to replace or supplement previously reported *ITS* and *Ypt1* gene based primers. This technique gives an extension to a range of DNA-based markers to simplify *P. nicotianae* identification in plant material and presents an ideal single step PCR alternative and time advantage over widely followed classical morphological identification and DNA-based ITS sequencing and PCR-RFLP techniques.

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REFERENCES

1. Hansen, E.M., P.W. Reeser and W. Sutton, 2012. *Phytophthora* beyond agriculture. Annu. Rev. Phytopathol., 50: 359-378.
2. Martin, F.N., J.E. Blair and M.D. Coffey, 2014. A combined mitochondrial and nuclear multilocus phylogeny of the genus *Phytophthora*. Fungal Genet. Biol., 66: 19-32.
3. Panabieres, F., G.S. Ali, M.B. Allagui, R.J.D. Dalio and N.C. Gudmestad *et al*., 2016. *Phytophthora nicotianae* diseases worldwide: New knowledge of a long-recognised pathogen. Phytopathol. Mediterr., 55: 20-40.
4. Cline, E.T., D.F. Farr and A.Y. Rossman, 2008. A synopsis of *Phytophthora* with accurate scientific names, host range and geographic distribution. Plant Health Prog. 10.1094/PHP-2008-0318-01-RS
5. Das, A.K., A. Kumar, A.A. Ingle and S.G. Nerkar, 2011. Molecular identification of *Phytophthora* spp. causing citrus decline in Vidarbha region of Maharashtra. Indian Phytopathol., 64: 342-345.
6. Naqvi, S., 2006. Distribution of citrus *Phytophthora* spp. and mating types pathogenic to citrus in central India. J. Mycol. Plant Pathol., 36: 44-48.
7. O'Brien, P.A., N. Williams and G.E. Hardy, 2009. Detecting *Phytophthora*. Crit. Rev. Microbiol., 5: 169-181.
8. Grunwald, N.J., F.N. Martin, M.M. Larsen, C.M. Sullivan and M.D. Press *et al*., 2011. Phytophthora-ID.org: A sequence-based *Phytophthora* identification tool. Plant Dis., 95: 337-342.
9. Kroon, L.P.N.M., H. Brouwer, A.W.A.M. de Cock and F. Govers, 2012. The genus *Phytophthora* anno 2012. Phytopathology, 102: 348-364.
10. Nath, V.S., V.M. Hegde, M.L. Jeeva, R.S. Misra and S.S. Veena *et al*., 2014. Rapid and sensitive detection of *Phytophthora colocasiae* responsible for the taro leaf blight using conventional and real-time PCR assay. FEMS Microbiol. Lett., 352: 174-183.
11. Schena, L., J.M. Duncan and D.E.L. Cooke, 2008. Development and application of a PCR based 'molecular tool box' for the identification of *Phytophthora* species damaging forests and natural ecosystems. Plant Pathol., 57: 64-75.
12. Tian, M., J. Win, J. Song, R. van der Hoorn, E. van der Knaap and S. Kamoun, 2007. A *Phytophthora infestans* cystatin-like protein targets a novel tomato papain-like apoplastic protease. Plant Physiol., 143: 364-377.
13. Tian, M., E. Huitema, L. da Cunha, T. Torto-Alalibo and S. Kamoun, 2004. A kazal-like extracellular serine protease inhibitor from *Phytophthora infestans* targets the tomato pathogenesis-related protease P69B. J. Biol. Chem., 279: 26370-26377.
14. Kebdani, N., L. Pieuchot, E. Deleury, F. Panabieres, J.Y. Le Berre and M. Gourgues, 2010. Cellular and molecular characterization of *Phytophthora parasitica* appressorium mediated penetration. New Phytol., 185: 248-257.
15. Tedford, E.C., T.L. Miller and M.T. Nielsen, 1990. A detached-leaf technique for detecting resistance to *Phytophthora parasitica* var. *nicotianae* in tobacco. Plant Dis., 74: 313-316.
16. Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Mol. Biol. Evol., 28: 2731-2739.

17. Kalendar, R., D. Lee and A.H. Schulman, 2014. Fast PCR Software for PCR, *In silico* PCR and Oligonucleotide Assembly and Analysis. In: DNA Cloning and Assembly Methods, Valla, S. and R. Lale (Eds.). Chapter 18, Springer, New York, USA., ISBN-13: 9781627037631, pp: 271-302.
18. Bowman, K.D., U. Albrecht, J.H. Graham and D.B. Bright, 2007. Detection of *Phytophthora nicotianae* and *P. palmivora* in citrus roots using PCR-RFLP in comparison with other methods. Eur. J. Plant Pathol., 119: 143-158.
19. Cooke, D.E.L., J.M. Duncan, N.A. de Williams, M.H. Weerdt and P.J.M. Bonants, 2000. Identification of *Phytophthora* species on the basis of restriction enzyme fragment analysis of the internal transcribed spacer regions of ribosomal RNA. EPPO Bull., 30: 519-523.
20. Dong, S., R. Stam, L.M. Cano, J. Song and J. Sklenar *et al*., 2014. Effector specialization in a lineage of the Irish potato famine pathogen. Science, 343: 552-555.
21. Grote, D., A. Olmos, A. Kofoet, J.J. Tuset, E. Bertolini and M. Cambra, 2002. Specific and sensitive detection of *Phytophthora nicotianae* By simple and nested-PCR. Eur. J. Plant Pathol., 108: 197-207.
22. Das, A.K., S.S. Bawage, S.G. Nerkar and A. Kumar, 2013. Detection of *Phytophthora nicotianae* in water used for irrigating citrus trees by *Ypt*1 gene based nested PCR. Indian Phytopathol., 66: 132-134.
23. Nowakowska, J.A., T. Malewski, A. Tereba and T. Oszako, 2016. Rapid diagnosis of pathogenic *Phytophthora* species in soil by real time PCR. For. Pathol. 10.1111/efp.12303
24. Konig, S., L. Schwenkbier, S. Pollok, M. Riedel and S. Wagner *et al*., 2015. Potential of *Ypt1* and ITS gene regions for the detection of *Phytophthora* species in a lab on a chip DNA hybridization array. Plant Pathol., 64: 1176-1189.