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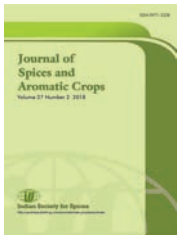

Development of diagnostic assays for rapid and sensitive detection of *Phytophthora* infecting major spices and plantation crops

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Abstract

Phytophthora, the ubiquitous s*tramenopile* phytopathogen is a major threat to several economically important horticultural crops including spices and plantation crops. Trans-seasonal survival of *Phytophthora* in plant debris and soil continuum has considerable epidemiological significance as the quiescent propagules often serve as primary foci of infection with inherent potential to trigger epiphytotics in the succeeding season favoured by conducive environmental conditions. Hence, early and rapid detection of over summering propagules is highly imperative to manage *Phytophthora*-induced diseases efficiently and economically. Twelve isolates representing different species of *Phytophthora* (*P. capsici, P. tropicalis, P. palmivora. P. citrophthora* and *P. meadii*) representing hosts such as black pepper, cardamom, nutmeg, coconut, arecanut and cocoa were used to develop nucleic acid-based diagnostic tools *viz*., polymerase chain reaction (PCR), real-time PCR, loop-mediated isothermal amplification (LAMP) and real-time LAMP. *Phytophthora* genus-specific primers were designed from the conserved region of nuclear ribosomal DNA. Each of the assays was specific and detected different species of *Phytophthora* and not other pathogens (*Rhizoctonia solani, Pythium vexans, Fusarium oxysporum* and *Colletotrichum gloeosporioides*) and plant samples. Sensitivity assays indicated that, real-time PCR detected *Phytophthora* upto 1.3 fg, followed by LAMP (13 fg) and PCR (13 pg).

Keywords: black pepper, loop-mediated isothermal amplification, *Phytophthora*, polymerase chain reaction, real-time LAMP, real-time PCR

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Introduction

Spices and plantation crop sector play vital roles in providing livelihood, national economy and contributes significantly to the foreign exchequer owing to its domestic and global importance. Spices, comprising of annual herbs and perennials are primarily valued for their medicinal properties (fungistatic, antimicrobial and antibiotic) from time immemorial and forms an essential component in several value added products and industrial by-products (Nair 2004). Plantation crops provide direct and indirect employment to the agricultural sector and supply

wide range of raw materials for various rural as well as small scale industries, besides playing a pivotal role in conserving soil and ecosystem *per se* (Chowdappa *et al*. 2014). Spices - plantation crops based cropping systems are popular and widely adopted in Southern India, especially in Karnataka and Kerala in which, spices such as cardamom, black pepper and nutmeg are intercropped with coconut, arecanut and cocoa. Though proved to be profitable and sustainable, the cropping systems often provide conducive micro-climate for the establishment, proliferation and large-scale devastation of several diseases. *Phytophthora*, the ubiquitous oomycetous hemi-biotrophic pathogen is reported to cause a broad spectrum of economically important diseases in spices as well as plantation crops (Anandaraj 2016; Erwin & Ribeiro 1996). Among the *Phytophthora* species inflicting serious damage to spices and plantation crops, *P. meadii*, *P. capsici*, *P. nicotianae* and *P. palmivora* are reported to be the major ones (Chowdappa *et al*. 2014).

Phytophthora species survives as resting spores (chlamydospores and oospores) in plant parts, debris and soil (Pratibha *et al.* 2014). It is essential to detect the perpetuating quiescent structures employing rapid and accurate tools so as to eradicate the surviving propagules thus, preventing build-up of inoculum by adopting appropriate management measures. Moreover, it is also vital to confirm identity of the pathogenic genus, especially in complex multiple cropping systems in order to employ target-specific synthetic molecules and management strategies, as symptoms induced by resident pathogens could be similar or different which might lead to wrong adoption of plant protection measures. Nucleic acid-based tools are sensitive, rapid, specific and widely used for detecting *Phytophthora* in several economically important crops (O'Brien *et al.* 2009; Silvar *et al.* 2005b; Cissin *et al.* 2016) at genus level and also at species level, *P. ramorum* (Tomlinson *et al.* 2007), *P. sojae* (Dai *et al.* 2012), *P. melonis* (Chern *et al.* 2011) and *P. capsici* (Dong *et al.* 2015). Nuclear (â-tubulin, internal transcribed spacer and *Tef*1á), mitochondrial (*cox*1, *cox*2 and NADH dehydrogenase subunit 1) and other forms (elicitin-parA1, Ras-related protein gene-Ypt1) have been widely employed for detecting *Phytophthora* species (Blair *et al.* 2008; Chern *et al.* 2011; Cooke *et al.* 2000; Dai *et al.* 2012; Kroon *et al.* 2012; Martin *et al.* 2012; Notomi *et al.* 2000). ITS gene has wide acceptance for detecting different species of *Phytophthora* including, *P. nicotianae* (Grote *et al.* 2002), *P. infestans* (Hussain *et al.* 2005; Wangsomboondee & Ristaino 2002), *P. citrophthora* (Ippolito *et al.* 2002), *P. capsici* (Silvar *et al.* 2005b), *P. sojae* (Wang *et al.* 2006). It is also used in classification and molecular phylogeny of *Phytophthora* species (Blair *et al.* 2008; Cooke *et al.* 2000; Lee & Taylor 1992; Martin & Tooley 2003).

However, pertinent information on development of similar tools and their utilization in detecting *Phytophthora* infecting spices and plantation crops-based cropping systems is scanty. Hence, the study was formulated to develop nucleic acid-based diagnostic assays such as polymerase chain reaction (PCR), real-time PCR, loop-mediated isothermal amplification (LAMP) and real-time LAMP for rapid and sensitive genus-specific detection of *Phytophthora* infecting various spices and plantation crops.

Materials and methods

Collection and maintenance of Phytophthora and cultures of other pathogens

Seven *Phytophthora* isolates belonging to different species representing different spices and plantation crops were acquired from National Phytophthora Repository (NPR), ICAR-Indian Institute of Spices Research (ICAR-IISR), Kozhikode (Table 1). The isolates were routinely cultured in carrot agar (CA) medium and maintained at 24±1°C for further studies. Other major pathogens that generally infect major spices and plantation crops *viz., Fusarium oxysporum*, *Rhizoctonia solani*, *Pythium vexans* and *Colletotrichum gloeosporioides* obtained from Biological Control Laboratory, ICAR-IISR, Kozhikode were subcultured and maintained on potato dextrose agar (PDA) medium by incubating at 28±1°C.

Plant samples

Tender leaves of spices (black pepper, nutmeg and cardamom) as well as plantation crops (coconut, arecanut and cocoa) used in the studies were collected from ICAR-IISR, Kozhikode campus.

Table 1. Particulars of *Phytophthora* and fungal isolates used in present study

Species	Host	Geographical origin	Repository ID	Year of collection
Phytophthora capsici	Black pepper	Kozhikode, Kerala	05-06	2005
Phytophthora tropicalis	Black pepper	Dakshina Kannada, Karnataka	98-93	1998
Phytophthora meadii	Cardamom	Wayanad, Kerala	13-16	2013
Phytophthora meadii	Nutmeg	Malappuram, Kerala	13-01	2013
Phytophthora meadii	Arecanut	Wayanad, Kerala	13-13	2013
Phytophthora palmivora	Coconut	Udupi, Karnataka	12-31	2012
Phytophthora meadii	Cocoa	Dakshina Kannada, Karnataka	12-19	2012
Pythium vexans	Cardamom	Kodagu, Karnataka	-	2015
Fusarium oxysporum	Cardamom	Kodagu, Karnataka	-	2015
Colletotrichum gloeosporioides	Black pepper	Kodagu, Karnataka	-	2016
Rhizoctonia solani	Cardamom	Kodagu, Karnataka	-	2016

DNA isolation

8-10 mycelial discs (5 mm size) from actively growing *Phytophthora* cultures were placed in sterile Ribeiro's media (Ribeiro 1978) and incubated at 24±1°C for 4-6 days. Mycelial mat was filtered using sterile Whatman No. 1 filter paper and DNA was extracted following the protocol described previously (Cooke & Duncan 1997). *P. vexans* and other fungal cultures were maintained in sterile potato dextrose broth (PDB) and incubated at 28±1°C for 4-6 days. Mycelial mat was filtered through sterile Whatman No. 1 filter paper and DNA was isolated using CTAB method with minor modifications as described previously (Culling 1992).

Designing primers

Nuclear ribosomal DNA (rDNA) gene sequences of different *Phytophthora* species (*P. capsici, P. palmivora, P. meadii, P. arecae, P. tropicalis, P. nicotianae, P. citrophthora, P. ramorum* and *P. heveae*), fungal pathogens (*Pythium* spp., *Fusarium* spp., *Colletotrichum* spp. and *Rhizoctonia solani*) and plant samples (coconut, arecanut, cocoa, black pepper, cardamom and nutmeg) were downloaded from various data bases *viz.*, http://Q-bank.eu, http://Phytophthora-id.org, http://Phytofura.net.in, http://Phytophthora.ucr.edu and http://ncbi.nlm.nih.gov and aligned manually in Biological sequence alignment editor tool (http://mbio.nscu.edu) to identify the conserved region among the *Phytophthora* species. Primers were designed manually to the conserved region using Bioedit tool (http://mbio.nscu.edu) for PCR. Real-time PCR primers were designed using Primer Quest tool in Integrated DNA Technologies (http://idt.org). LAMP and real-time LAMP primers were designed using Primer Explorer version 4 (http://primer explorer.jp/e/). A total of six primers including two external primers (F3 and B3), two internal primers (FIP and BIP) and two loop primers (B-loop and F-loop) were designed and submitted for desalted primer synthesis (Integrated DNA Technologies, Illinois, USA) (Table 2).

Standardization of PCR

Nuclear ribosomal DNA (rDNA) region of different *Phytophthora* isolates was PCR amplified initially using universal primers (ITS6/ITS4) while, genus-specific primers (Phyto_PCR_F/Phyto_PCR_R) (Table 2) were used to amplify *P. capsici* (05-06) target gene with initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C (ITS4/ITS6)/62°C (Phyto_PCR_F/Phyto_PCR_R) for 30 s, extension

Table 2. Particulars of primers designed from internal transcribed spacer (ITS) region of *Phytophthora* spp.

Technique	Primer name	Sequence (5′-3′)	Annealing tempera -ture (°C)	Expected product size	Remarks
PCR	ITS_4	TCCTCCGCTTATTGATATGC	60	~900 bp	White *et al.* (1990)
	ITS_6	GAAGGTGAAGTCGTAACAAGG	60		
PCR	Phyto_PCR_F	TGAACTGTACTTCTCTTTGCTCG	62	~309 bp	This study
	Phyto_PCR_R	GAGATGCGCACCGAAGTGCA	62		
*q*PCR	Phyto_qPCR_F	CTGAACAGGCGCTTATTGAATG	62	~106 bp	This study
	Phyto_qPCR_R	CTACTTCGCAACAGCAAAGC	62		
LAMP	LF	CCTGTTCAGCCGAAGCCA	66	Ladder-like pattern	This study
	LB	TGCTGTTGCGAAGTAGGGTGG	66		
	F3	GCTGCGGCGTTTAAAGGA	62		
	B3	AGTGCACACAAAGTTCCCAA	62		
	FIP	ACGCCACAGCAGGAAAAGCA TTGAGTGTTCGATTCGCGGTA	-		
	BIP	GGCTTGGCTTTTGAATCGGCTTT GGATCGACCCTCGACAG			

*q*PCR=quantitative PCR; F=Forward; R=Reverse; LF=Forward Loop; LB=Backward Loop; FIP=Forward Inner Primer; BIP=Backward Inner Primer; bp=Base pair

at 72°C for 1 min and a final extension period at 72°C for 7 min (Eppendorf Vapo Protect™ MasterCycler® Pro S Thermocycler, Germany). The 25 µL PCR reaction mixture was prepared with 12.5 µL master mix (TaKaRa Taq™ DNA Polymerase, USA), 1.0 µL each of forward and reverse primers (10 pM), 1.0 µL of genomic DNA and 9.5 µL of sterile water. PCR amplified products were electrophoretically separated on 1.2 per cent (w/v) agarose gel containing ethidium bromide (0.5 µg μL^{-1}) for 60 min at constant 100 V (Sambrook & Russel 2001). Gel documentation of the amplified products was carried out (Bio-Rad Laboratories, CA, USA). Further, to standardize other assays like, conventional PCR with newly designed primers (*Phytophthora*-specific), real-time PCR, LAMP and real-time LAMP as well as sensitivity determination, the representative isolate of black pepper *viz*., *P. capsici* (05-06) was used.

Standardization of real-time PCR

SYBR green dye (QuantiTect® SYBR® Green RT-PCR kit Qiagen, Germany) based real-time PCR was performed (Rotor-Gene® Q, Qiagen, Germany) using diluted *P. capsici* DNA. The 25 µL of real-time PCR mix contained 12.5 µL of master mix, 1.0 µL each of forward and reverse primers (10 pM), 1.0 µL of diluted genomic DNA and 9.5 µL of water. Reaction was performed with the following conditions, 95°C for 10 min followed by 95°C for 10 s, 60°C for 20 s, 60°C for 20 s and 65-95°C for 1-5 s. Additionally, non-template control was also maintained.

Standardization of LAMP assay

The LAMP reaction was standardized with *P. capsici* (05-06) isolate. Different concentrations of $MgSO_4$ (4-10 mM) and betaine (0.4-1.0 M) (Sigma Chemicals, Bengaluru, India) were used to optimize the reaction. To standardize $MgSO_4$ concentration, betaine was maintained at 0.6 M while, $MgSO_4$ was maintained at 6 mM to standardize betaine concentration. The 25 µL of LAMP reaction included, 1 µL template (150 ng), 2x thermopol buffer (New England Bio Labs,

USA), 1.4 mM dNTPs, 6 mM $MgSO_4$, 0.8 M betaine, 0.2 µM external primers, 2 µM internal primers, 1 µM loop primers and 8 U of *Bst* polymerase (New England Bio Labs, USA). The reaction was performed at 65°C for 60 min followed by incubation at 80°C for 5 min to inactivate the *Bst* polymerase (Eppendorf Vapo Protect™ MasterCycler® ProS Thermocycler, Germany). LAMP products were analysed through agarose gel electrophoresis.

Standardization of real-time LAMP assay

Optimization of the real-time LAMP assay was carried out in 0.2 ml strips (Optigene, UK) using *P. capsici* (05-06) isolate. The real-time LAMP reaction mixture contained 1 µl template, 15 µl isothermal master mix (Optigene, UK) and primers as mentioned in the LAMP assay. The reaction was performed at 65°C for 60 min in real-time LAMP instrument (Genie II, Optigene, UK). The results were analyzed in terms of Tp values which denote the time taken to generate positive result based on the fluorescence. Annealing/melting temperature analysis from 98 to 80°C was performed to validate authenticity of the LAMP products. The optimized LAMP assay was further performed with representative species of *Phytophthora* from black pepper, arecanut, nutmeg, cocoa, coconut, cardamom and *P. vexans* to analyze the specificity. Specificity of the real-time LAMP product was further confirmed through gel electrophoresis.

Sensitivity assay

The degree of sensitivity of diagnostic assays developed (conventional PCR, real-time PCR, LAMP and real-time LAMP) was evaluated with serially diluted DNA of *P. capsici* (05-06). Dilutions were made from 10^{o} (130 ng) to 10^{-10} (0.013 fg). The PCR and LAMP assays were performed using 1 µL each of the above dilutions with specific primers as indicated above. The specificity of the products was confirmed through gel electrophoresis.

Results and discussion

DNA isolation and PCR amplification

The DNA was isolated from all *Phytophthora* isolates, fungal pathogens and plant samples. PCR amplification of rDNA region with universal ITS primers (ITS4/ITS6) resulted in amplicons of ~900 bp, ~900 bp, ~600 bp and ~800 bp in all the *Phytophthora* species (Fig. 1) representing black pepper, arecanut, coconut, nutmeg, cocoa and cardamom. PCR amplification with newly designed primers *viz*., Phyto_PCR F/Phyto_PCR R resulted in the amplification of product ~300 bp in the representative *Phytophthora* species *viz*., *P. capsici* (05-06). While, no amplification was noticed in plant samples (Fig. 2A) and fungal pathogens (Fig. 2B). Subsequently, the representative isolate of black pepper *viz*., *P. capsici* (05-06) was employed to

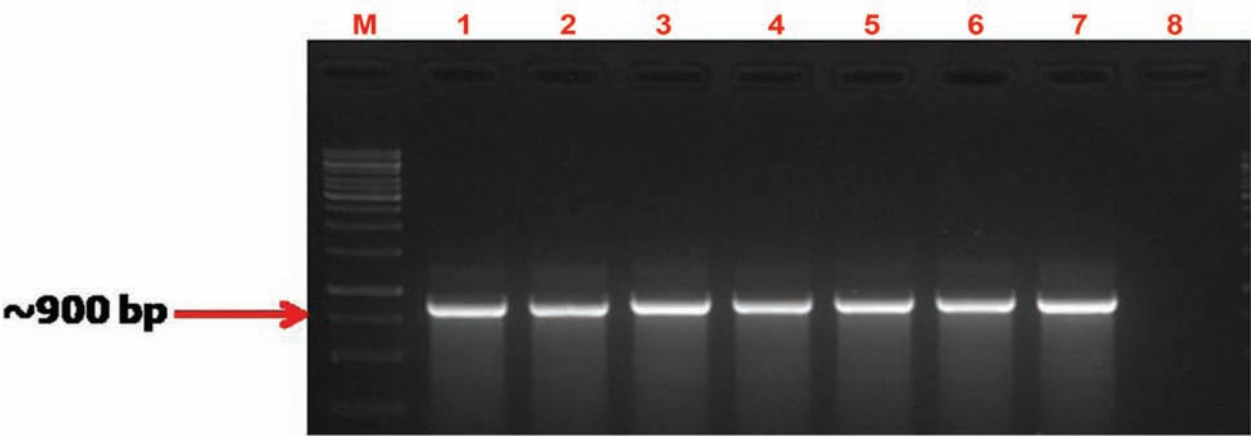


Fig. 1. Amplification profile of ribosomal RNA (rRNA) region with universal internal transcribed spacer (ITS) primers ITS4/ITS6 of representative *Phytophthora* species Lane M: 1Kb DNA ladder Lane 1: black pepper (05-06) Lane 2: black pepper (98-93) Lane 3: arecanut (13-13) Lane 4: nutmeg (13-01) Lane 5: cocoa (12-19) Lane 6: coconut (12-31) Lane 7: cardamom (13-16) Lane 8: non-template control.

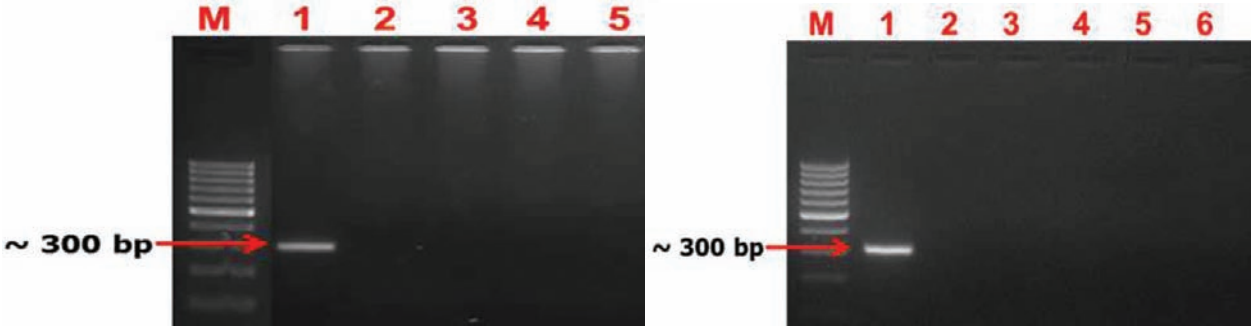


Fig. 2. (A) PCR amplification with *Phytophthora* specific primers (Phyto_PCR F/R) in plant genomic DNA Lane M: 1Kb DNA ladder Lane 1: *Phytophthora capsici* (05-06) Lane 2: Black pepper var. Sreekara Lane 3: Black pepper var. Panniyur 1 Lane 4: Cardamom Lane 5: Non-template control.
(B) PCR amplification with *Phytophthora* specific primers (Phyto_PCR F/R) in fungal genomic DNA Lane M: 1Kb DNA ladder Lane 1: *Phytophthora capsici* (05-06) Lane 2: *Fusarium oxysporum* Lane 3: *Pythium vexans* Lane 4: *Colletotrichum gloeosporioides* Lane 5: *Rhizoctonia solani* Lane 6: Non-template control.

standardize conventional PCR with newly designed *Phytophthora*-specific primers, real-time PCR, LAMP, real-time LAMP assays and determining the sensitivity levels.

Real-time PCR assay

Real-time PCR assay was standardized with the target region in diluted *P. capsici* (05-06) DNA and the sensitivity limit was found to be 10^{-8} (1.3 fg) (Table 3).

Table 3. Real-time PCR data with respect to different dilutions of *Phytophthora capsici* (05-06) genomic DNA

Dilutions of genomic DNA	DNA concentration	Ct value
1	130 ng	5.51
10^{-1}	13 ng	7.87
10^{-2}	1.3 ng	10.47
10^{-3}	0.13 ng	14.38
10^{-4}	0.013 ng	17.51
10^{-5}	1.3 pg	20.47
10^{-6}	0.13 pg	23.43
10^{-7}	0.013 pg	26.71
10^{-8}	1.3 fg	29.28
10^{-9}	-	-
10^{-10}	-	-
Water control	-	-

LAMP assay

LAMP primers were standardized with *P. capsici* (05-06) DNA which exhibited a characteristic ladder-like pattern in agarose gel electrophoresis while no amplification was observed in the negative control (Fig. 3A). The optimum concentration of $MgSO_4$ was found to be 6 mM and that of betaine was found to be 0.8 M (Fig. 3B & 3C). Amplification was greater at 60 min with respect to number and intensity of bands. LAMP assay confirmed specificity of the LAMP primers in detecting *Phytophthora* species representing different crops including black pepper, arecanut, nutmeg, cocoa, coconut and cardamom (Fig. 3D).

Real-time LAMP assay

Real-time LAMP detected the target region in *P. capsici* (05-06) DNA within 6 min and a single peak of annealing curve was observed at T_m 89°C. While, amplification was not observed in non-template control (Fig. 4A). The real-time LAMP reaction detected target region in all the *Phytophthora* spp. used but not in *P. vexans* and water control (Fig. 4B). This assay was found specific for target regions and the detection time varied from 6 to 12 min in different samples. Specificity of the real-time LAMP product was subsequently confirmed through annealing curve which exhibited a single peak at 89°C for different *Phytophthora* species.

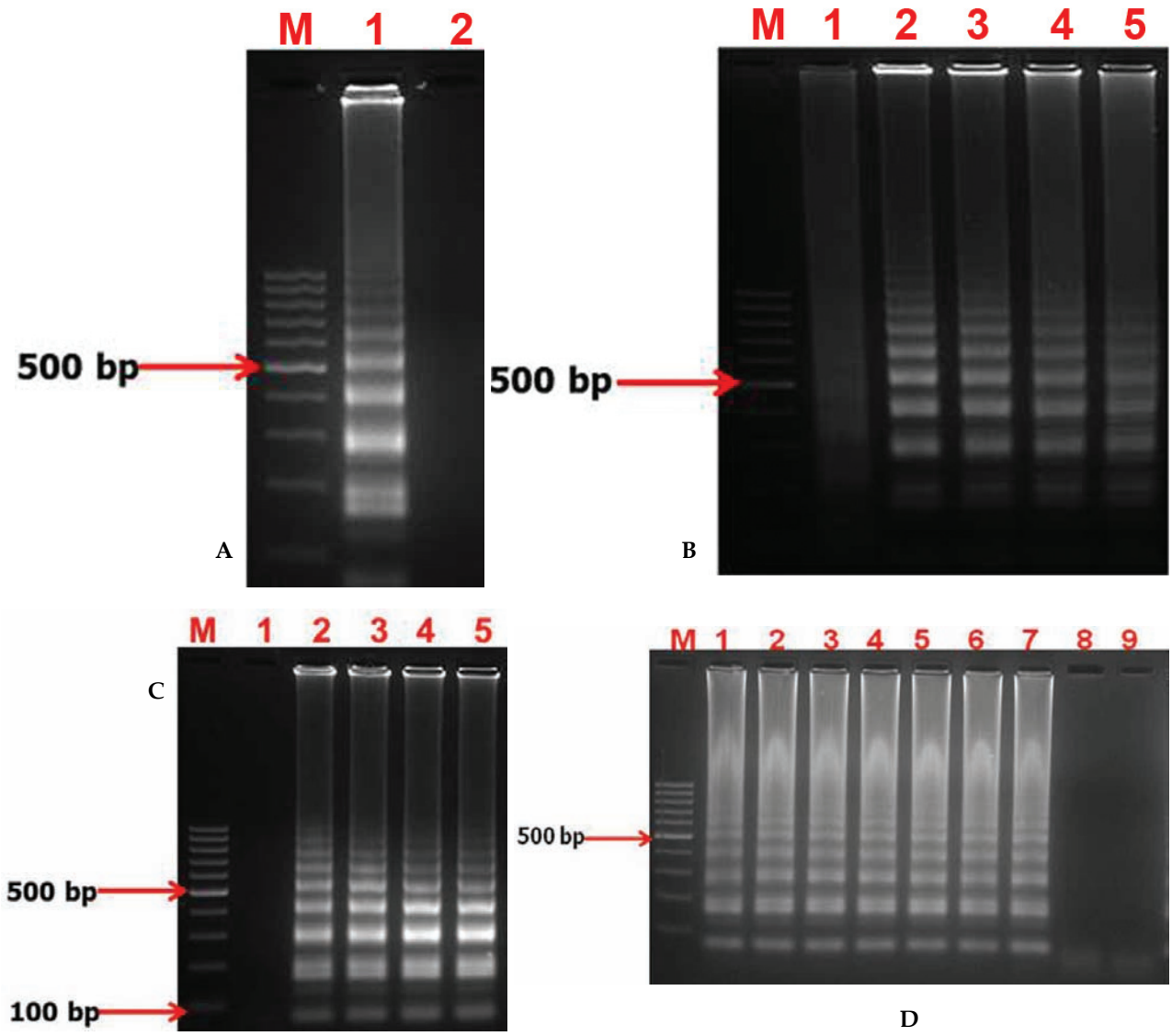
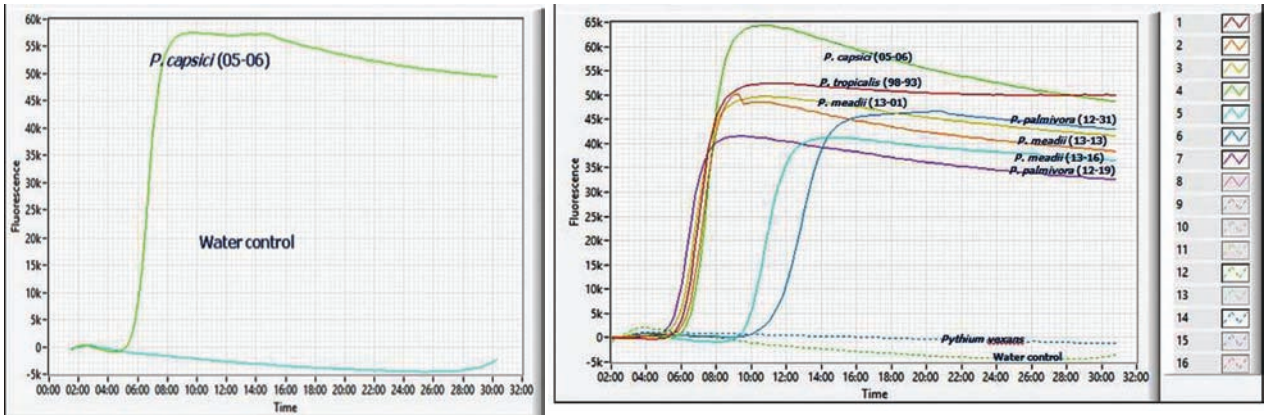


Fig. 3. Standardization of loop-mediated isothermal amplification (LAMP) for the detection of *Phytophthora* from spices-plantation based cropping system (A) LAMP assay with *Phytophthora capsici* (05-06) genomic DNA Lane M: 100 bp DNA ladder Lane 1: *P. capsic*i genomic DNA Lane 2: non-template control (B) optimization of $MgSO_4$ concentration Lane M: 100 bp DNA ladder, Lane 1: non-template control Lane 2: 4 mM Lane 3: 6 mM $MgSO_4$ Lane 4: 8 mM $MgSO_4$ and Lane 5: 10 mM $MgSO_4$ (C) optimization of betaine concentration, Lane 1: non-template control Lane 2: 0.4 M betaine Lane 3: 0.6 M betaine Lane 4: 0.8 M betaine, and Lane 5: 1.0 M (d) Gel electrophoresis pattern of LAMP assay products of *Phytophthora* isolates representing different crops: black pepper (05-06) (Lane 1), black pepper (98-93) (Lane 2), arecanut (13-13) (Lane 3), nutmeg (13-01) (Lane 4), cocoa (12-19) (Lane 5), coconut (12-31) (Lane 6), cardamom (13-16) (Lane 7), Negative control (*Pythium vexans*) (Lane 8), Non-template control (water control) (Lane 9), Lane M: 1Kb DNA ladder.

Sensitivity assay

The detection limits for the target region in diluted *P. capsici* (05-06) DNA was found to be 10^{-4} (13 pg) in PCR, 10^{-7} (13 fg) using LAMP assay and 10^{-8} (1.3 fg) in real-time PCR (Fig. 5). Specificity of the product was further confirmed by gel electrophoresis.

Phytophthora survives in plants, crop debris and soil across seasons. Such infected materials could possibly serve as randomly distributed primary sources of inoculum which have the potential to endure adverse weather conditions and initiate disease in the succeeding season. Diagnosis of *Phytophthora* based on their



A **B**

Fig. 4. (A) Standardization of real-time LAMP assay for the detection of *Phytophthora* using specific primers (PhytoPCR F/R) using *P. capsici* (05-06) genomic DNA. (B) Real-time LAMP assay with *Phytophthora* specific primers (PhytoPCR F/R) using genomic DNA of different representative *Phytophthora* species.

morphological characteristics and isozyme pattern based detection methods are time consuming and requires expertise. In the present study, protocols for genus-specific detection of *Phytophthora* species infecting spices and plantation crops based on nucleic acid-based techniques *i.e.* PCR, real-time PCR, LAMP and Real-time LAMP were developed and sensitivity levels were determined.

Development of nucleic acid based diagnostic assays was attempted by various researchers employing PCR and its variants. Mitochondrial (*cox*), nuclear (ITS) and various other genes (*Ypt*1 and *ParA*1) were targeted to detect *Phytophthora* at genus and species level. Earlier investigations clearly indicated that, ITS region could be of immense use for *Phytophthora* genus and/or species-specific detection. Ribosomal DNA of *Phytophthora* species contains highly conserved ITS region (ITS1 and ITS2) and 5.8S RNA in between 18S RNA and 28S RNA region (Cooke & Duncan 1997). ITS region contains high interspecific and low intraspecific variations which are highly suitable for species-specific detection (Bruns *et al.* 1991). Moreover, multiple copies of ribosomal RNA genes make it an appropriate candidate for sensitive detection of the species. PCR-based assays developed for the detection of various species of *Phytophthora* resulted in variation in terms of the sensitivity ranging from 0.5 pg in *P. infestans* (Hussain *et al.* 2005) to 150 pg in generic *Phytophthora* (Schena *et al.* 2008) which indicates that, designing of primers plays a vital role in development of highly sensitive assay (O'Brian *et al.* 2009).

In this study, real-time PCR and LAMP assays developed were found more sensitive in detecting the *Phytophthora* species as compared with conventional PCR. Real-time PCR could detect upto 1.3 fg of genomic DNA as compared to LAMP (13 fg) and PCR (13 pg). This clearly indicated that, LAMP assay is 1000 times more sensitive and real-time PCR is 10000 times more sensitive than PCR. This increased sensitivity in detecting low concentration of the targets in *Phytophthora* spp. makes real-time PCR and LAMP assays as most preferable alternative compared to PCR.

The LAMP assay sensitivity in detecting different *Phytophthora* species varies as reported in several *Phytophthora* species. The detection limit was observed as 2 pg in *P. infestans* (Hansen *et al.* 2016), 10 pg each in *P. sojae* (Dai *et al.* 2012) and *P. ramorum* (Tomlinson *et al.* 2007), 10 fg in *P. melonis* (Chen *et al.* 2013) and 100 fg in *P. capsici* (Dong *et al.* 2015) by targeting various genes. In this study, a total of six LAMP primers including a pair of loop primers (forward and backward) for better specificity of the assay targeting the ITS region were developed. Our study suggests that, LAMP assays developed were more specific and

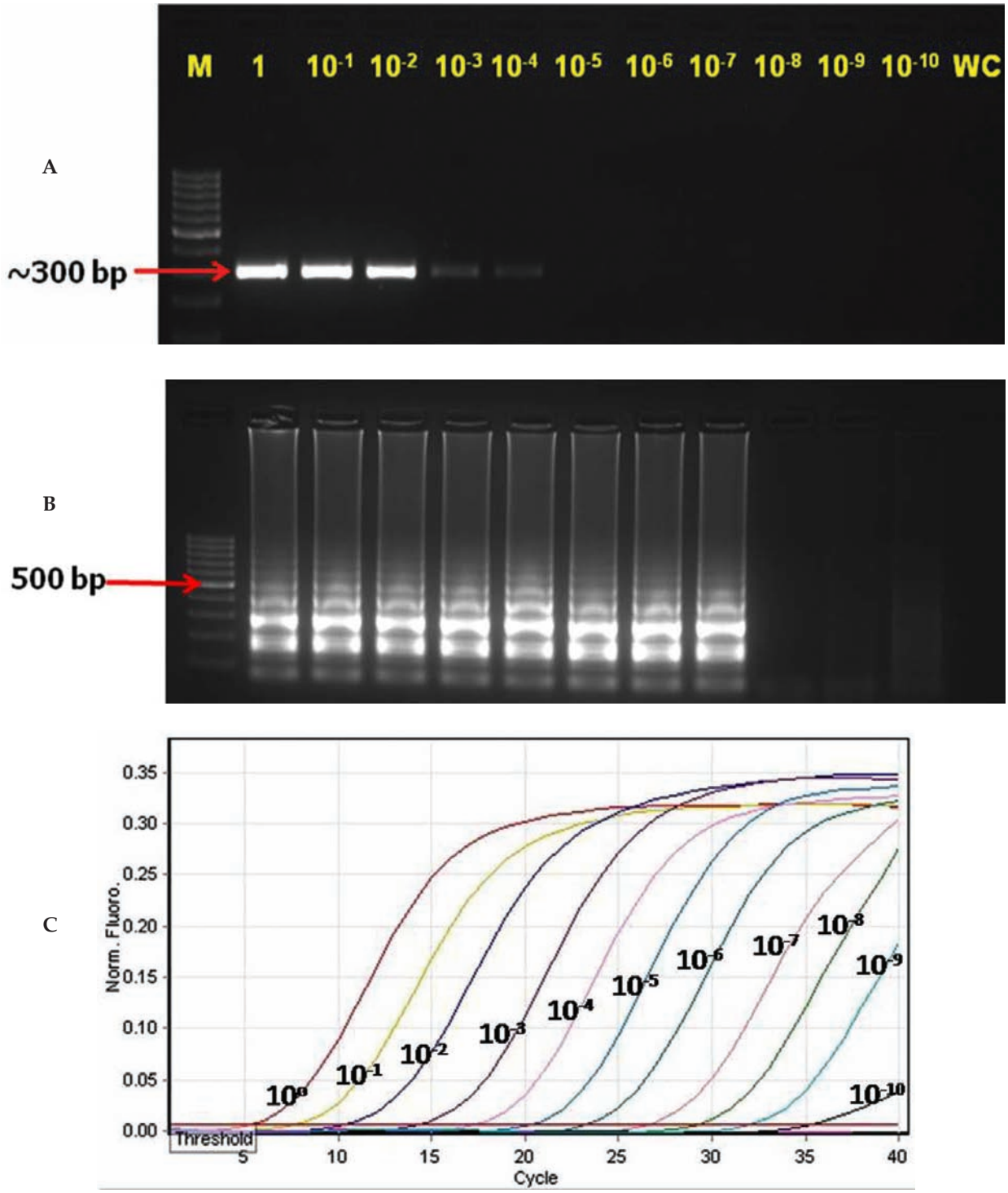


Fig. 5. Comparison of sensitivity assay of different diagnostic tools developed on diluted concentrations of representative *Phytophthora capsici* (05-06) genomic DNA
(A) conventional PCR
(B) LAMP assay; Lane M: 1Kb DNA ladder Lane 1: 10^{o} Lane 2: 10^{-1} Lane 3: 10^{-2} Lane 4: 10^{-3} Lane 5: 10^{-4} Lane 6: 10^{-5} Lane 7: 10^{-6} Lane 8: 10^{-7} Lane 9: 10^{-8} Lane 10: 10^{-9} Lane 11: 10^{-10} dilutions Lane 12: non-template control
(C) Real-time PCR graph showing amplification curve of different dilutions in *Phytophthora capsici* (05-06) genomic DNA.

sensitive to detect all *Phytophthora* species associated with spices-plantation crops based cropping systems. Moreover, it required lesser time i.e. <1 hour to detect the target gene in the sample. Hence, it will be helpful to detect the cryptic propagules and other oversummering structures of *Phytophthora* species within a short span of time.

Diagnostic assays developed should be more precise in terms of sensitivity and time. Real-time PCR based assays could detect relatively low concentration of the targets in lesser duration as compared to conventional PCR and LAMP assays. SYBR green dye-based real-time PCR assay was developed for the detection of ITS gene in *P. ramorum* (Hayden *et al.* 2006). This assay detected upto 15 fg genomic DNA. Whereas, Taq-Man probe-based assay detected the ITS gene ranged from 10 fg in generic *Phytophthora* (Kox *et al.* 2007) to 1000 fg in *P. fragariae* (Bonants *et al.* 2004). In case of molecular beacons, *P. ramorum* was detected upto 500 fg (Tomlinson *et al.* 2007) whereas, Scorpion probe detected upto 25000 fg in *P. citrophthora* and *P. nicotianae* (Ippolito *et al.* 2004) and 500 fg in *P. ramorum* (Tomlinson *et al.* 2007). Mitochondrial (*cox*I and *cox*II) and other unique genes like, â-tubulin, *GPA*1, *ParA*1, *TRP* and *Ypt*1 also have been assessed (Blair *et al.* 2008) and the sensitivity ranged from 1 fg to 2006 fg genomic DNA (O'Brian *et al.* 2009). This sensitivity range clearly indicated that, selecting the appropriate gene target, designing more specific primers and sensitive method plays critical roles in the development of highly sensitive diagnostic assays. In this study, a highly sensitive SYBR green dye based real-time PCR assay for the detection of *Phytophthora* species was developed and the sensitivity was observed upto 1.3 fg DNA.

The present study constitutes the first report on development of nucleic acid-based diagnostic tools for genus-specific detection of *Phytophthora* species infecting spices-plantation crops targeting ribosomal DNA region. In terms of duration required for different assays, real-time PCR assay proved to be superior compared with conventional PCR in which the former required <1 hour to detect the target region. Whereas, the latter required minimum reaction time of 3 hours. LAMP was also found to be simple and more sensitive technique with a short period of reaction time *i.e.* ~1 hour. In case of sensitivity of the assays developed, LAMP was 10^3 times more sensitive than conventional PCR while, real-time PCR was 10^4 times more sensitive than conventional PCR and 10 times that of LAMP, suggesting that real-time PCR and LAMP assays are more sensitive than conventional PCR. Real-time LAMP assay was tried with *P. capsici* genomic DNA which could detect the gene targets in relatively lesser duration *i.e.* 10 min (data not presented). The results clearly indicate that, real-time LAMP was highly sensitive and required short span of time in detecting the target. In the present study, quick, sensitive and reliable protocols for detecting different *Phytophthora* species infecting major spices and plantation crops were developed and standardized which subsequently warrants validation using field samples originating from various spices and plantation crops growing regions to further confirm specificity as well as sensitivity.

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