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Ypt1 gene-based recombinase polymerase amplification assay for *Phytophthora capsici* and *P. tropicalis* detection in black pepper

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Abstract Foot rot caused by *Phytophthora* is one of the major diseases of black pepper (*Piper nigrum* L.). Accurate and timely diagnosis of the disease is crucial for its successful management. Although PCR and qPCR assays are used for detection, the cost and time required to perform these assays are high. Recombinase polymerase amplification (RPA) assay has the advantage of minimal assay time and it is performed under isothermal conditions. Hence, RPA assay was developed for the detection of *P. capsici* and *P. tropicalis* and compared with newly developed end-point PCR test. Out of three sets of primers analyzed, a primer set based on the *Ypt1* gene successfully amplified a 230/231 bp product. Optimum amplification of RPA products were observed when the assay was performed at 37 °C with 14 mM magnesium acetate for 40 min. Sensitivity analysis using serial dilutions indicated that RPA is 10 times more sensitive than end-point PCR. During specificity analysis, non-specific bands were observed with other *Phytophthora* species, and hence the assay was further refined with betaine wherein addition of 1.0 M betaine avoided amplification of non-specific bands. The optimized RPA assay could detect *Phytophthora* from infected black pepper leaf, stem and root using both purified DNA and crude extracts. The end-point PCR test successfully differentiated the two species of *Phytophthora* in a validation test. These results indicate the robustness of the developed end-point PCR and RPA assays and its potential application in detection and differentiation of *P. capsici* and *P. tropicalis* infecting black pepper.

Keywords Black pepper · RPA · PCR · *P. capsici* · *P. tropicalis*

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Introduction

Black pepper (*Piper nigrum* L.) is originated in the tropical evergreen forests of the Western Ghats in India. It is an export oriented crop which is perennial in nature and vegetatively propagated through stem cuttings. Losses inflicted by diseases are the major constraints in black pepper cultivation and among the diseases, foot rot caused by *Phytophthora* is one of the major diseases of black pepper (Sarma et al. 1997). Recent studies indicated that two species, *P. capsici* and *P. tropicalis* with different symptomatology infect black pepper. *P. capcisi* infected black pepper plants show unrestricted lesions with fimbriate margins, whereas *P. tropicalis* produces restricted lesions with yellow halo. The pathogens infects all parts of black pepper and causes significant yield loss and timely diagnosis of the disease is crucial for its successful management.

Several methods for identification such as microscopic examination of phenotypic characters, leaf baiting technique, immunological assays like ELISA, PCR, real-time PCR, LAMP and real-time LAMP assays have been developed for the detection of

Phytophthora species including *P. capsici* infecting black pepper (Anandaraj and Sarma 1990; Silvar et al. 2005; Martin et al. 2012; Pandian et al. 2018; Munawar et al. 2019). Identification and differentiation of *P. capsici* and *P. tropicalis* through morphological studies is complicated as they have overlapping morphological characters and exhibit intraspecific variations. Leaf baiting technique is time consuming and it sometimes leads to isolation of non-targeted microbes. The commonly available genus-specific ELISA kits for *Phytophthora* detection are known to cross react with some *Pythium* species (Kox et al. 2007; Munawar et al. 2019). Silvar et al. (2005) and Zhang et al. (2006) developed species-specific PCR tests to detect *P. capsici* from *Capsicum annuum* using ITS region. The real-time PCR, LAMP and real-time LAMP assays developed earlier for black pepper are genus-specific and cannot differentiate *P. capsici* and *P. tropicalis* (Pandian et al. 2018). In addition, PCR and real-time PCR assays are not suitable for on-site testing since DNA extraction and thermal cycler/real-time PCR are necessary as these assays can be inhibited by substances extracted along the plant nucleic acids (Schrader et al. 2012). RPA is a rapid, sensitive and specific method, which is a promising alternative. It involves a specific combination of two core enzymes and a protein for amplification of molecular targets at a constant temperature, without the need of sophisticated equipment. This includes the recombinase, which pairs a single set of primers to the homologous sequences, resulting in 'D-loop' structure; the single stranded DNA binding protein, which stabilizes the 'D-loop' structure; and the strand-displacing DNA polymerase, which initiates DNA synthesis from the bound primers (Li et al. 2019). RPA is also more tolerant to inhibitors and the target nucleic acid can be amplified from a crude macerate of the plant tissue (Miles et al. 2015; Rojas et al. 2017).

The RPA assay has been successfully utilized to detect *Phytophthora* infecting various crop plants. *Phytophthora* genus-specific RPA assay and species-specific assays for *Phytophthora ramorum* and *P. kernoviae* were developed by Miles et al. (2015). In their study, they have designed the forward primer and the common probe from the genus-conserved region of *atp9* and the reverse primer from the intergenic spacer *atp9-nad9*. Similarly, Rojas et al. (2017) have also used *atp9- nad9* region of the mitochondrial genome to develop a highly specific RPA assay for the detection of *P. sojae* and *P. sansomeana,* root rot pathogens of soybean. A slightly different design was adopted by Munawar et al. (2019) to develop RPA assay for *P. cactorum* causing crown rot of strawberry crop. In their study, only the forward primer was from the genus conserved region of *atp9*, while the reverse primer and the overlapping probe were from the intergenic spacer *atp9-nad9*. Si Ammour et al. (2017) used ITS2 region of ribosomal RNA to develop RPA for *P. infestans* and they found cross-reaction with *P. mirabilis, P. phaseoli, P. ipomoeae* and *P. andina* due to high similarity in ITS regions among *Phytophthora* spp. (Blair et al. 2012). Of late, Dai et al. (2019) has developed RPA assay for *P. sojae* using *Ypt1* gene as target and the assay was highly specific and sensitive. Lateral flow strip-based recombinase polymerase amplification (LF-RPA) was developed for detection of *P. capsici* using *Ypt1* gene as target (Yu et al. 2019). However, there is no published report for specific detection and differentiation of *P. capsici* and *P. tropicalis* infecting black pepper. We screened different nuclear or mitochondrial genes (28srDNA, *Cox I*, *atp9-nad9*, ITS and *Ypt1*) for their possible utilization in differentiating these two species and could see a stretch of nucleotide variation only in *Ypt1* gene which was good enough to design different forward primers. Hence, in this study, these primers were utilized to develop species-specific end-point PCR and RPA assays to detect *P. capsici* and *P. tropicalis* in black pepper and the assays were compared for their sensitivity and specificity.

Materials and methods

Phytophthora cultures and DNA extraction

The *Phytophthora* cultures maintained at National Phytophthora Repository, ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India were used for the study. The cultures were grown in carrot agar medium at 25 ± 1 °C for 3–5 days. The mycelial discs of 5 mm size from the margin of actively growing *Phytophthora* cultures were inoculated in Ribeiro's broth (Erwin and Ribeiro 1996) and incubated at 25 ± 1 °C for 5–7 days. The mycelia were harvested by filtering through sterile Whatman No.1 filter paper and washed with sterile distilled water. The DNA was isolated from the mycelium using the method described earlier with slight modifications (Sheji et al. 2009). The

mycelium was air dried and 0.1 g of mycelium was placed in a sterile motor and pestle with 50 mg glass powder and 10 mg PVPP. 750 μl of extraction buffer (1 M Tris P^H 7.5, 0.5 M EDTA, 5 M NaCl, 10% SDS) was added to the mixture and was ground into a fine paste with the pestle. The mixture was then taken in a sterile eppendorf tube and centrifuged at 13,000 rpm for 5 min. After centrifugation, the supernatant was taken in another sterile eppendorf tube. 500 μl of Tris- phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added to it and inverted gently for 2 min. The contents were centrifuged at 13,000 rpm for 5 min. The aqueous layer was transferred to a sterile eppendorf tube. Equal volume of chloroform: isoamyl alcohol (24:1) was added to it and gently inverted for 2 min. The contents were centrifuged at 13,000 rpm for 10 min. The aqueous layer was then transferred to a sterile eppendorf tube. The tube was filled with isopropanol (−20 °C) and gently inverted. The tubes were then kept at −20 °C for 30 min. The tubes were again centrifuged at 13,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 70% ethanol. The tubes were inverted gently and allowed to stand for 1 min. The tubes were centrifuged at 13,000 rpm for 2 min. The pellets were air dried and re-suspended in 100 μl sterile distilled water. To remove RNAs, 1.5 μl RNase A (Thermo Scientific™ EN0531; 10 mg/ml) was added and incubated for 30 min at 37 °C. The isolated DNA was stored at −20 °C.

Primer design and synthesis

The ITS and *Ypt1* gene sequences of different *Phytophthora* species, *P. capsici, P. tropicalis, P. palmivora, P. meadii, P. colocasiae, P. nicotianae* and *P. citrophthora,* infecting different component crops grown in black pepper-based cropping systems were obtained from NCBI (http://ncbi.nlm.nih.gov) and http://14.139.189.27/phytofura/ and aligned using BioEdit software version 7.0.9.1. RPA primers were designed manually from the region specific to *P. capsici* and *P. tropicalis* and the properties were checked using online software, Oligo Calc:Oligonucleotide properties calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html). The primers were designed according to RPA guidelines and manufacturer's instructions for Twist AmpR DNA amplification kit (Twist Dx Ltd., Cambridge, United Kingdom). Two primer combinations based on ITS (PCPT-FP/COM-RP1 & PCPT-FP/COM-RP2) and a primer combination specific to *Ypt1* gene of *P. capsici* (PC-YP-FP/COM-YR-RP) and *P. tropicalis* (PT-YR-RP/COM-YR-RP) were used to optimize the RPA assay. The RPA primers are listed in Table 1 and the position of *Ypt1* gene based primers are shown in Fig. 1. The end-point PCR primers were designed from the *Ypt1* gene region and are listed in Table 2. The primers were synthesized from integrated DNA technologies as desalted DNA oligos.

Optimization of RPA and end-point PCR

Three sets of primer combinations targeting ITS and *Ypt1*gene were used in RPA assay. The RPA mixture contained 1.2 μl of each 10 mM forward and reverse primer, 14.75 μl of rehydration buffer, 50 ng of *Phytophthora* DNA and 1.25 μl of 280 mM magnesium acetate (14 mM; final concentration) and the final volume was made up to 25 μl with sterile nuclease-free water. The assay was performed at 37 °C for 40 min followed by denaturation at 65 °C for 10 min. The *Ypt1* gene-based primer combinations which generated the expected amplicons were further used for optimization of the assay. The assay was optimized initially by setting up at different temperatures (37, 39, 41 and 43 °C), then with varying concentrations of magnesium acetate (12, 14, 16, 18, 20 mM) and at different incubation period (20, 40, 60 and 80 min).

End-point PCR test was standardized by setting up reactions at different annealing temperature (56, 58, 60 and 62 °C) using the species specific forward primers; PC-Y-FP 5′- (*P. capsici*) /PT-Y-FP (*P. tropicalis*) and common reverse primer; COM-Y-RP2 and best annealing temperature was selected. The 20 μl reaction mixture contained diluted DNA, 0.5 μl of 10 mM forward and reverse primer, 2 μl 10x Taq DNA polymerase buffer, 0.5 μl 2.5 mM dNTPs and 0.3 μl 3 U Taq DNA polymerase enzyme. The optimized PCR cycle conditions were 5 min denaturation at 94 °C, 35 cycles of initial denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. The products were run on 1% agarose gel and checked for 302 and 301 bp amplicons in case of *P. capsici* and *P. tropicalis*, respectively.

Table 1 Primers used in optimization of RPA assay

Primer	Sequence	Bases	Product size	Target	Species
PC-YR-FP	5'-CAGATTGTAAGCAACCAAAGTTCAAGACGTTT-3'	32	230 bp	Ypt1	*P.capsici*
COM-YR-RP	**5'-**TACGAACCTATCGATCTCATGCAGCCACTG-3'	30			
PT-YR-FP	5'-AAGCTCCAGATTGTAAGCACTTGTTATCCATC-3'	32	231 bp	Ypt1	*P.tropicalis*
COM-YR-RP	**5'-**TACGAACCTATCGATCTCATGCAGCCACTG-3'	30			

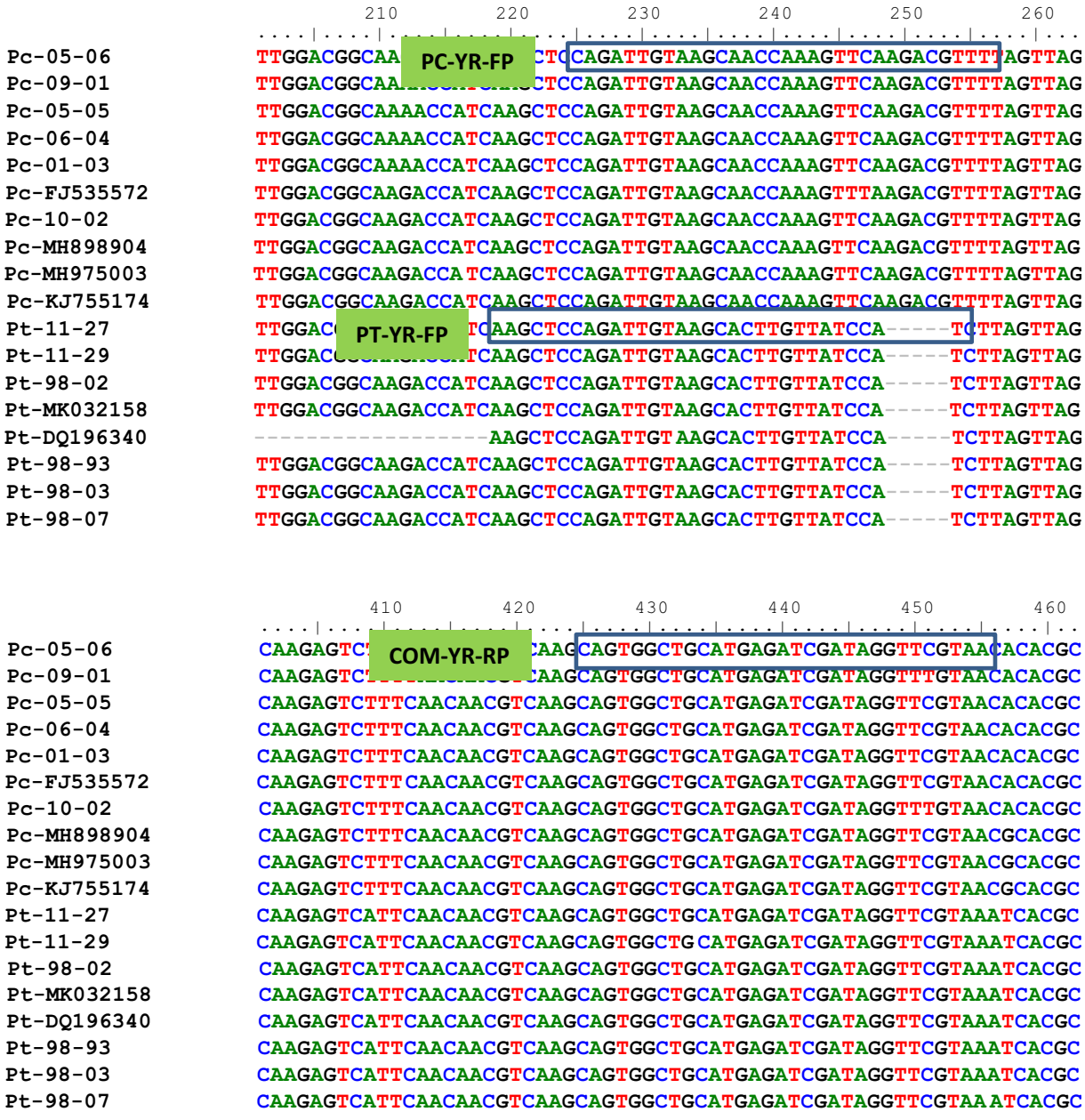


Fig. 1 Multiple sequence alignment showing position of *Ypt1* gene specific primers (Pc- *P. capsici*, Pt- *P. tropicalis*)

Table 2 Primers used in end-point PCR

Primer	Sequence	Bases	Product size	Target	Species
PC-Y-FP	5'-AAGCAACCAAAGTTCAAGACGTTT-3'	24	302 bp	Ypt1	*P.capsici*
COM-Y-RP2	5'-GCAGGCGTATCTGAAATTTGC-3'	21			
PT-Y-FP	5'-GATTGTAAGCACTTGTTATCCATC-3'	24	301 bp	Ypt1	*P.tropicalis*
COM-Y-RP2	5'-GCAGGCGTATCTGAAATTTGC-3'	21			

Specificity and sensitivity of the assays

The specificity of the assays were confirmed by performing end-point PCR and RPA assays using DNA of other *Phytophthora* species viz., *P. meadii, P. colocasiae, P. palmivora* and *P. nicotianae* and other pathogens such as *Pythium* sp., *Rhizoctonia solani*, *Fusarium oxysporum*, *Sclerotium rolfsii* and *Colletotrichum* sp. The RPA assay was performed using six isolates of *P. capsici* (03–01, 05–05, 05–06, 06–04, 03–08 & ATCC 4034) and 6 isolates of *P. tropicalis* (98–02, 11–27, 11–29, 06–08, 98–93, ATCC76651) and compared with end-point PCR test results. The sensitivity of RPA assay was compared with end-point PCR test using serially diluted *Phytophthora* DNA in healthy black pepper DNA (10^{-1} to 10^{-8} dilutions containing 10 ng to 1 fg of *Phytophthora* DNA in 50 ng of healthy black pepper DNA per µl of dilutions).Three µl of dilutions containing 30 ng to 3 fg of *Phytophthora* DNA per reaction were used in the assays. Detection limits were determined by the lowest input DNA concentration at which a positive result was obtained.

Validation of the assays

The RPA and end-point PCR tests were also validated with leaf samples collected from the fields. The total DNA was extracted from infected leaf samples using DNeasy Plant mini kit (Qiagen, Venlo, Limburg, the Netherlands) by following manufacturer's instructions and the RPA and end-point PCR tests were performed with optimized conditions. The end-point PCR test was also validated with 38 *Phytophthora* isolates from black pepper.

Phytophthora detection from infected leaf, stem and root

The leaf, stem and roots of susceptible black pepper variety Sreekara were inoculated with *P. capsici* isolate 05–06 and *P. tropicalis* isolate 98–93. The plants were observed periodically for symptom expression. Then the total DNA was extracted from infected leaf, stem and root using DNeasy Plant mini-kit (Qiagen,Venlo, Limburg, the Netherlands) following manufacturer's instructions and used in RPA. Crude DNA was extracted from infected plant tissues according to Tooley et al. (1997) with slight modifications. The crude extract was prepared by grinding 10 mg of infected leaves, stem and roots in 50 µl of 0.5 M NaOH solution (freshly prepared) in 1.5 ml centrifuge tube with a micro-pestle, followed by centrifugation at 12,000 rpm for 10 min. Two µl of supernatant was diluted in 98 µl 10 mM Tris pH 8.0. Then 3–4 µl of the crude extract was used in RPA assay.

Results

Optimization of RPA and end-point PCR

Three primer pairs targeting ITS and *Ypt1* gene of *P. capsici* and *P. tropicalis* were tested to select the best primer pair. However, only the primer pair (PC-YR-FP/PT-YR-FP & COM-YR-RP) based on *Ypt1* gene showed expected product of 230/231 bp size in RPA assay (Fig. 2) and it was used further for RPA assay optimization. No amplification was found in other two primer pairs. The assay temperature was selected by setting up RPA assay at 37, 39, 41 & 43 °C. RPA products were observed in all temperatures. However, the product at 37 °C was brighter and hence 37 °C was selected as optimum temperature. Then the effect of different MgOAc concentrations (12 mM to 20 mM) was checked and there was no much variation in amplification. Then the assay was carried out for 20, 40, 60 and 80 min and 40 min incubation period was found optimum (Fig. 3). End-point PCR test was performed at different annealing temperatures (56, 58, 60 and 62 °C) and 60 °C was found optimum.

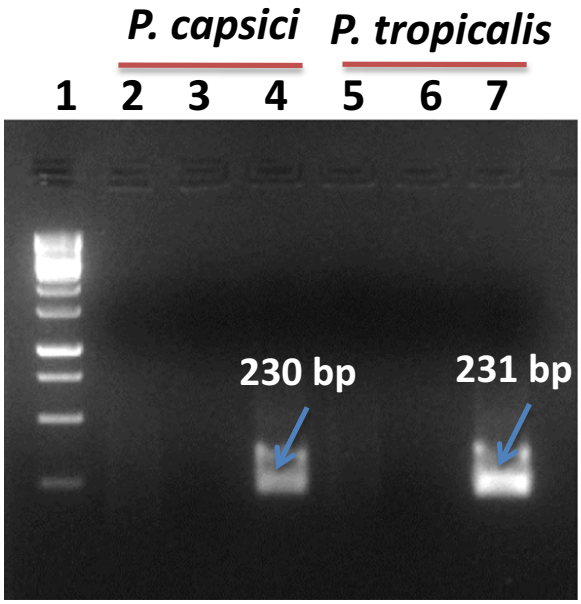


Fig. 2 Screening of primers for RPA assay. Lane 1. 1 Kb DNA Ladder, Lane 2 &5. Primer PCPT-FP & COM-RP1 (ITS), Lane 3 &6. Primer PCPT-FP & COM-RP2 (ITS), Lane 4. Primer PC-YR-FP & COM-YR-RP (Ypt1 gene), Lane 7. PT-YR-FP & COM-YR-RP (Ypt1 gene)

Specificity and sensitivity of the assays

In specificity analysis with other *Phytophthora* species such as *P. palmivora, P. meadii, P. colocasiae* and *P. nicotianae* and other pathogens of black pepper, *Pythium* sp., *Rhizoctonia solani, Fusarium oxysporum, Sclerotium rolfsii* and *Colletotrichum* sp., non-specific amplification was observed in some cases and therefore, betaine (0.8 and 1.0 M) was included in RPA assay to increase specificity. Our results indicated that, addition of 1.0 M betaine could avoid such non-specific amplification (Fig. 4a). End-point PCR test did not show any cross amplification with other fungal pathogens; however when the test was performed with different *Phytophthora* species, non-specific amplification was observed with *P. meadii* in both primers (Fig. 4b). The RPA assay was performed with *P. capsici* and *P. tropicalis* isolates maintained at the National Repository along with ATCC cultures. In case of PC-YR-FP/ COM-YR-RP, RPA product amplification was observed only in *P. capsici* isolates (01–03, 05–05, 06–04, 03–08, 05–06 and ATCC4034). Primer pair, PT-YR-FP/ COM-YR-RP amplified expected 231 bp amplicon only in *P. tropicalis* isolates (98–02, 11–27, 11–29, 06–08, 98–93 and ATCC76651). Hence the

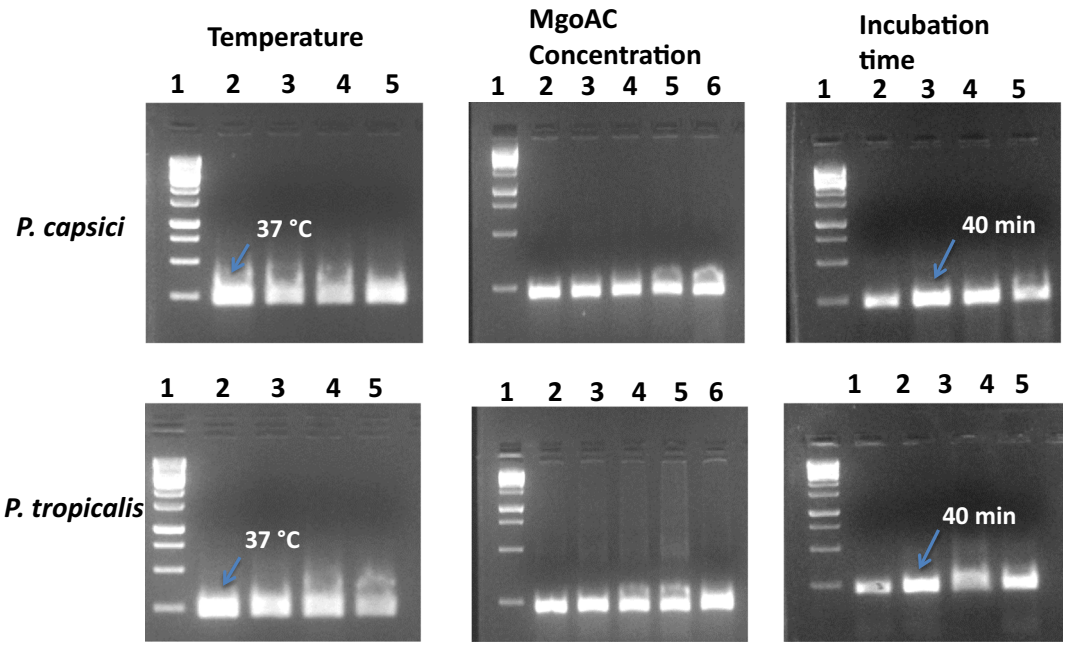
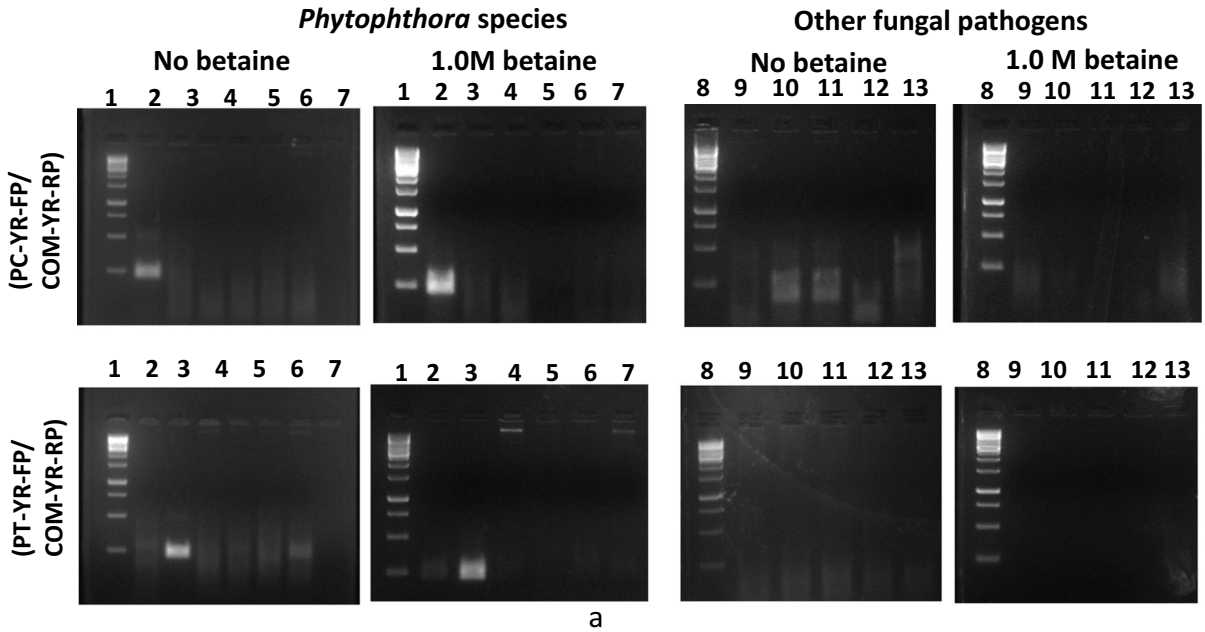


Fig. 3 Optimization of RPA assay **a** Temperature: Lane 1. 1 Kb DNA ladder, Lane 2–37 °C, Lane 3–39 °C, Lane 4–41 °C, Lane 5–43 °C, **b** Magnesium acetate concentration: Lane 1. 1 Kb DNA ladder, Lane 2. 12 mM, Lane 3. 14 mM, Lane 4. 16 mM, Lane 5. 18 mM, Lane 6. 20 mM, **c** Incubation time: Lane 1. 1 Kb DNA ladder, Lane 2. 20 min, Lane 3. 40 min, Lane 4. 60 min, Lane 5. 80 min



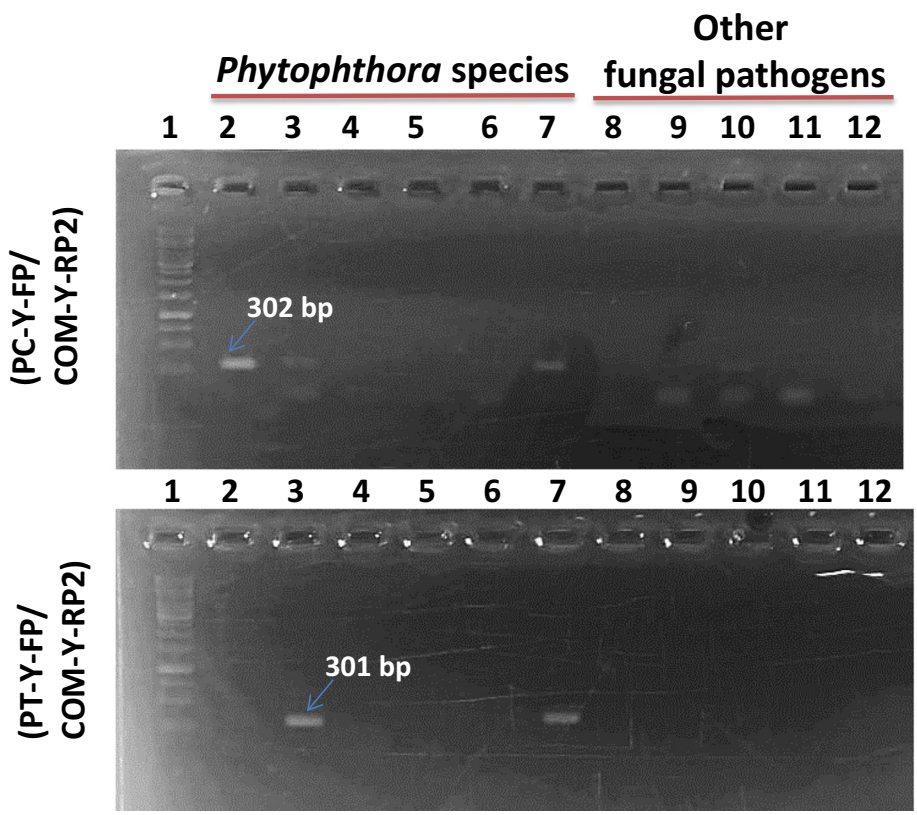


Fig. 4 a Specificity analysis of the optimized RPA assay. RPA assay with other *Phytophthora* species: Lane 1. 1 Kb DNA Ladder, Lane 2. *P. capsici,* Lane 3. *P. tropicalis,* Lane 4. *P. meadii,* Lane 5. *P. colocasiae,* Lane 6. *P. palmivora,* Lane 7. *P. nicotianae*, RPA assay with other fungal pathogens of black pepper: Lane 8. 1 Kb DNA Ladder Lane 9. *Pythium* sp., Lane 10. *R. solani,* Lane 11. *F. oxysporum,* Lane 12. *S. rolfsii,* Lane 13. *Colletotrichum* sp. **b** Specificity analysis of the optimized end-point PCR test. End-point PCR test with other *Phytophthora* species: Lane 1. 1 Kb DNA Ladder, Lane 2. *P. capsici,* Lane 3. *P. tropicalis,* Lane 4. *P. colocasiae,* Lane 5. *P. palmivora,* Lane 6. *P. nicotianae*, Lane 7. *P. meadii;* End-point PCR test with other fungal pathogens of black pepper: Lane 8. *Pythium* sp., Lane 9. *R. solani,* Lane 10. *F. oxysporum,* Lane 11. *S. rolfsii,* Lane 12. *Colletotrichum* sp.

developed RPA primers did not cross react with other species and were highly specific to the species to which they were developed (Fig. 5).

The sensitivity of the assay was checked by performing RPA assay with serially diluted total DNA of infected black pepper leaves in healthy black pepper leaf DNA. RPA assay could detect *P. capsici* up to 10^{-3} dilution (300 pg of *Phytophthora* DNA in 50 ng of healthy black pepper leaf total DNA) and *P. tropicalis* up to 10^{-4} dilution (30 pg of *Phytophthora* DNA in 50 ng of healthy black pepper leaf total DNA). Whereas the detection limit of end-point PCR test was 3 ng and 0.3 ng of *Phytophthora* DNA in case of *P. capsici* and *P. tropicalis*, respectively (Table 3). Thus the RPA assay was 10 times more sensitive than end-point PCR test.

Validation of the assays

RPA was carried out with both total DNA and crude extracts of black pepper plants inoculated with *P. capsici* and *P. tropicalis* isolates. The assay could detect the pathogens from infected leaf, stem and root using crude extracts also (Fig. 6). RPA assay could not detect *P. tropicalis* from root using both total DNA and also crude extract and the reason may be due to low inoculum density. The assay was also validated with field samples collected from experimental farms of ICAR-IISR located at Chelavoor and Peruvannamuzhi, Kozhikode, Kerala, India (Table 4). Out of 7 field samples, all the three *Phytophthora* infected leaf samples were found to be infected with *P. capsici* both in RPA and end-point PCR test. No cross amplification was observed with other species and also with *Pythium* and *Colletotrichum* sp. infected leaf samples. End-point PCR test was also performed with 38 isolates of *Phytophthora* isolated from black pepper. Among them, 21 isolates were found to be *P. capsici* as they showed amplification of an expected 302 bp band using *P. capsici* primers, PC-Y-FP/COM-Y-RP2. Remaining 17 isolates did not show amplification with *P. capsici* primers; however showed amplification of 301 bp band with *P. tropicalis* primers PT-Y-FP/COM-Y-RP2 (Table 5).

Discussion

Black pepper (*Piper nigrum* L), one of the major spice crops, is known to be affected by several diseases of which foot rot caused by *Phytophthora* is a major threat to its cultivation worldwide, including India. Both morphological characters and molecular methods are used to identify and detect *Phytophthora* infecting black pepper.

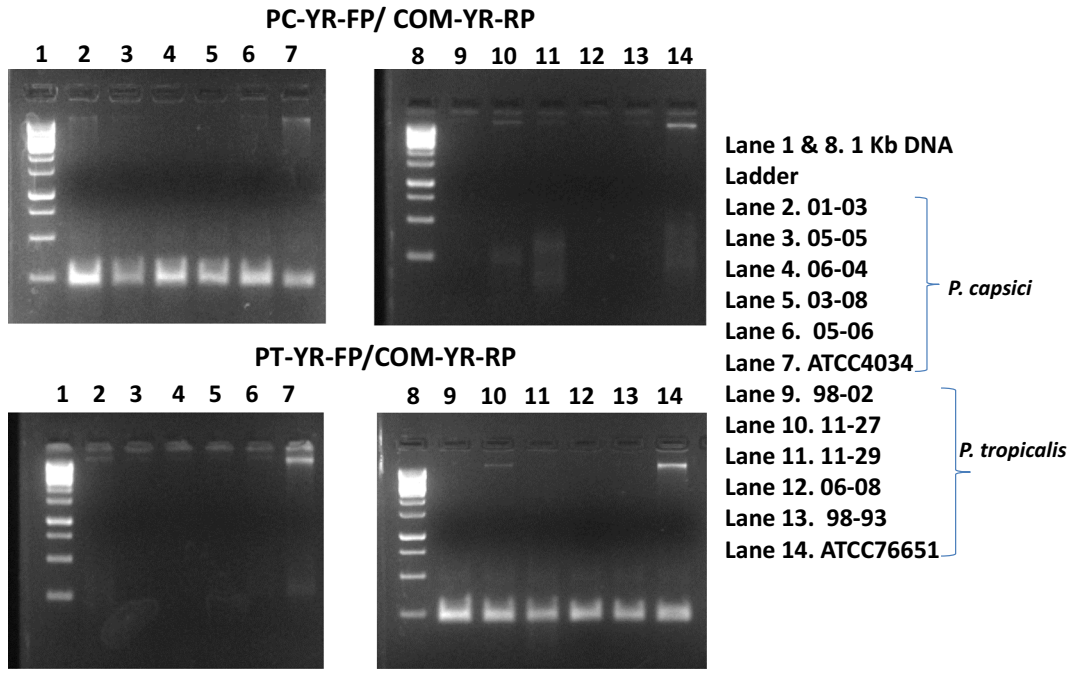


Fig. 5 Validation of the RPA assay for specificity using *Phytophthora* repository isolates

Table 3 Sensitivity analysis of RPA assay and comparison with end-point PCR

Dilutions		*P. capsici*		*P. tropicalis*	
		RPA	PCR	RPA	PCR
10^{-1}	(30ng)*	+	+	+	+
10^{-2}	(3ng)	+	+	+	+
10^{-3}	(0.3ng)	+	-	+	+
10^{-4}	(0.03ng)	-	-	+	-
10^{-5}	(3pg)	-	-	-	-
10^{-6}	(0.3pg)	-	-	-	-
10^{-7}	(0.03pg)	-	-	-	-
10^{-8}	(3fg)	-	-	-	-

* final concentration of *Phytophthora* DNA (+) – Positive; (-)-Negative

Recently, two species, *P. capsici* and *P. tropicalis,* have been found to be associated with foot rot of black pepper. In this study, RPA assays were developed to detect and differentiate these two species of *Phytophthora* and compared with newly developed end-point PCR test.

Primer design for RPA assays is very similar to the conventional PCR except that the length of the primer is longer. However, to optimize specificity, it may be necessary to evaluate several primers (Miles et al. 2015). In our study, since our aim is to develop species-specific RPA assays which can differentiate the two *Phytophthora* species, *P. capsici* and *P. tropicalis* infecting black pepper, only one specific primer pair each for *P. capsici* and *P. tropicalis* from *Ypt1* gene was designed and used along with two common ITS primer pairs. Out of three primer pairs used, only *Ypt1* gene-based primers could amplify the expected amplicon. This might be due to specificity of recombinase enzyme to form a complex with the primers and subsequent scanning of the template for annealing. In recent studies, the *Ypt1* gene was successfully used to develop RPA assay for *P. sojae* (Dai et al. 2019) and RPA-LFA for *P. capsici* detection (Yu et al. 2019). The simplicity of primer design in RPA has an advantage over LAMP, where the primer design can be complex and requires specific software for designing (Miles et al. 2015). LAMP also requires several primers which increase cost of the assay compared to use of a single primer pair in RPA. The primer development for RPA assay is also critical in assay optimization. Rojas et al. (2017) found faster amplification of RPA product with 29 mer primer than 35 mer primer. Most of the developed RPA assays have used amplicons of length between 100 and 250 bps which actually facilitates faster amplification and increased sensitivity (Li et al. 2019). In this study, the size of the amplicons using *Ypt1* gene-based primers were 230/231 bp which falls within the above mentioned range.

Among the different incubation periods tested, the RPA product could be detected even with 20 min

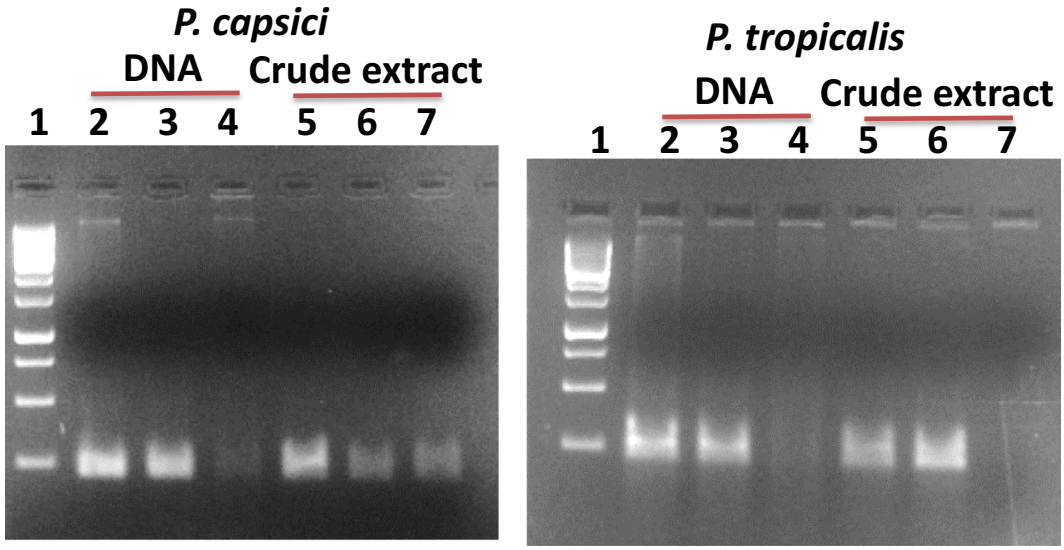


Fig. 6 Detection of *P. capsici* and *P. tropicalis* from infected leaf, stem and roots using total DNA and crude extracts using RPA assay. Lane 1. 1 Kb DNA ladder, Lane 2 & 5 leaf, Lane 3 & 6 stem, Lane 4 & 7 root

Table 4 Details of the isolates from Phytophthora National Repository and field samples used for validation and results of RPA and end-point PCR

Isolate	Place of collection	Plant part	Species	RPA & end-point PCR	
				(*P. capsici*)	(*P. tropicalis*)
01-03	Chelavoor, Kozhikode, Kerala	Leaf	*P. capsici*	+	-
05-05	Peruvannamuzhi, Kozhikode, Kerala	Leaf	*P. capsici*	+	-
06-04	Puthuppadi, Kozhikode, Kerala	Leaf	*P. capsici*	+	-
03-08	Yythiri, Wayanad, Kerala	Stem	*P. capsici*	+	-
05-06	Peruvannamuzhi, Kozhikode, Kerala	Spike	*P. capsici*	+	-
ATCC4034	American Type Culture Collection		*P. capsici*	+	-
98-02	Peruvannamuzhi, Kozhikode, Kerala	Soil	*P. tropicalis*	-	+
11-27	Wayanad, Kerala	Leaf	*P. tropicalis*	-	+
11-29	Wayanad, Kerala	Soil	*P. tropicalis*	-	+
06-08	Thiruvampadi, Kozhikode, Kerala	Leaf	*P. tropicalis*	-	+
98-93	Adyanadka, Dakishna Kannada, Karnataka	Leaf	*P. tropicalis*	-	+
ATCC76651	American Type Culture Collection		*P. tropicalis*	+	+
Phytophthora infected sample 1	Chelavoor, Kozhikode, Kerala	Leaf	*P. capsici*	+	-
Phytophthora infected sample 2	Chelavoor, Kozhikode, Kerala	Leaf	*P. capsici*	+	-
Phytophthora infected sample 3	Peruvannamuzhi, Kozhikode, Kerala	Leaf	*P. capsici*	+	-
Colletotrichum infected sample 1	Chelavoor, Kozhikode, Kerala	Leaf	-	-	-
Colletotrichum infected sample 2	Peruvannamuzhi, Kozhikode, Kerala	Leaf	-	-	-
Pythium infected sample 1	Chelavoor, Kozhikode, Kerala	Leaf	-	-	-
Pythium infected sample 2	Chelavoor, Kozhikode, Kerala	Leaf	-	-	-

(+) – Positive; (-)- Negative

incubation time; however 40 min incubation period was selected for better amplification. Similarly, Zhao et al. (2019) detected rice black-streaked dwarf virus using RT-RPA assay within 20 min incubation time, however, further incubation upto 40 min resulted in better signal. Depending on the initial copy number of the target templates and size of the amplicon, the RPA assay time can be completed in 5–20 min (Babu et al. 2018). In most of the studies, the assay time was as low as 20 min for RPA (Rojas et al. 2017; Dai et al. 2019). Although it is recommended to remove DNA binding proteins before visualization in agarose gels, in our study heat denaturation of RPA products at 65 °C for 10 min was sufficient. This saves assay time and cost involved in RPA assays. Similarly, several researchers have used heat denaturation and addition of SDS instead of purification of RPA product (Londoño et al. 2016; Kapoor et al. 2017).

During specificity analysis, non-specific amplification was observed in some samples and addition of 1.0 M betaine could avoid such non-specific amplification and increased the assay specificity. Betaine is known to increase specificity in several detection assays like PCR and isothermal amplification methods such as LAMP and RPA (Henke et al. 1997; Notomi et al. 2000; Mok et al. 2016; Luo et al. 2019). Luo et al. (2019) used betaine to improve the specificity of RPA and their results demonstrated that the addition of 0.8 M betaine can significantly enhance the specificity of RPA.

Miles et al. (2015) developed RPA assays, which are specific to *Phytophthora* genus and specific to the species, *P. ramorum* and *P. kernoviae* and the detection limit of RPA assays using purified DNA were 200 to 300 fg. In another study, the sensitivity of the RPA assays was around 1 to 10 pg of total DNA from both, *P. sojae* and *P. sansomeana* (Rojas et al. 2017). RPA-LFD was also found to be sensitive, detecting as low as 10 pg of *P. sojae* genomic DNA in a 50-mL reaction which was 100 and 10 time more sensitive than conventional PCR test and LAMP (Dai et al. 2019). Munawar et al. (2019) could develop a highly specific and sensitive RPA assay with lower detection limit of 10 fg for

Table 5 Details of the isolates from Phytophthora National Repository used for validation of end-point PCR

Isolate	Place of collection	Isolated from	end-point PCR	
			(*P. capsici*)	(*P. tropicalis*)
98-48	Asoka Plantations, Madikeri, Karnataka	Leaf	+	-
98-49	Asoka Plantations, Madikeri, Karnataka	Berry	+	-
98-81	Malappuram, Kerala	Leaf	+	-
98-87	Kunnamangalam, Kozhikode, Kerala	Leaf	+	-
98-142	Nukkal, Sirsi, Karnataka	Root	+	-
98-155	Isloor, Karnataka	Soil	+	-
98-164	Kangode, Sirsi, Karnataka	Leaf	+	-
98-174	Isloor, Sirsi, Karnataka	Leaf	+	-
98-185	Puttanamana, Sirsi, Karnataka	Stem	+	-
03-10	Thikkodi, Kerala	Root	+	-
05-05	Peruvannamoozhi, Kozhikode, Kerala	Leaf	+	-
05-09	Chelavoor, Kozhikode, kerala	Collar region	+	-
05-13	Appangala, Karnataka	Leaf	+	-
05-14	Appangala, Karnataka	Leaf	+	-
05-19	Chettalli, Karnataka	Leaf	+	-
06-01	Koothali farm, Kozhikode, Kerala	Stem	+	-
06-03	IISR- Nursery, Kozhikode, Kerala	Seedlings	+	-
07-02	Peruvannamoozhi, Kozhikode, Kerala	Stem	+	-
07-06	Wyanad, Kerala	Leaf	+	-
08-03	Peruvannamoozhi, Kozhikode, Kerala	Stem	+	-
08-05	Koppa, Karnataka	Collar region	+	-
97-11	Pulpally, Wayand, Kerala	Leaf	-	+
97-53	Pulpally, Wayand, Kerala	Leaf	-	+
97-54	Aigoor, Karnataka	Stem	-	+
98-59	Chettalli, Karnataka	Leaf	-	+
98-66	Valnoor, Kodagu, Kartanaka	Leaf	-	+
98-71	Kodagu, Karnataka	Leaf	-	+
98-128	Kunnamangalam, Kozhikode, Kerala	Stem	-	+
98-135	Theli, Sirsi, Karnataka	Root	-	+
98-143	Yadally, Sirsi, Karnataka	Root	-	+
98-145	Puttanamana, Karnataka	Root	-	+
98-172	Experimental plot II.2, Kozhikode	Leaf	-	+
98-177	Rayerpet, Karnataka	Root	-	+
98-182	Isloor, Sirsi, Karnataka	Leaf	-	+
99-136	Silver Cloud, Wayanad, Kerala	Leaf	-	+
06-17	Rasi Estate, Yercaud, Tamil Nadu	Leaf	-	+
09-11	Peruvannamoozhi, Kozhikode, Kerala	Leaf	-	+
09-36	Mudigere, Karnataka	Soil	-	+

(+) – Positive; (-)- Negative

P. cactorum. The detection limit of RPA-LFA developed for the detection *P. capsici* was found to be 10 pg of genomic DNA (Yu et al. 2019). In our study, the detection limit of RPA assays were 300 pg for *P. capsici*

and 30 pg for *P. tropicalis,* which was 10 times more than the end-point PCR test detection limit. The sensitivities of the developed RPA assays were comparatively lower than the earlier reported assays for other *Phytophthora* species. However, the RPA assays developed in this study were highly specific and could differentiate *P. capsici* and *P. tropicalis* and no cross-reaction was observed with other tested *Phytophthora* species. Also the detection limit of the developed assays could be improved with the addition of fluorescent molecule. End-Point PCR tests showed cross amplification with *P. meadii*. PCR primer developed for *P.capsici* had only four nucleotide variation with *P. meadii* and this might be the reason for cross amplification. Surprisingly, cross amplification was also found with *P. tropicalis* primer, which needs to be addressed with further optimization studies. Even though, end-point PCR test showed non-specific amplification with *P. meadii*, it can also be used to detect these two species in black pepper (Tables 4 and 5). *P. meadii* is a pathogen of cardamom and rubber. In some places, black pepper and cardamom are grown together and there is a chance of cross infection. However, the likelihood of finding *P. meadii* in black pepper is very less.

Some of the recently developed isothermal amplification assays are tolerant to PCR inhibitors present in plant extracts (Craw and Balachandran 2012; Ahmed et al. 2018). Hence, these assays could be used to detect pathogens in crude extracts reducing the time required to prepare pure genomic DNA (Miles et al. 2015). RPA can be applied in field because the method requires minimal sample preparation and can be performed under constant temperature (Babu et al. 2018). In the present study also, *P. capsici* and *P. tropicalis* were detected using both purified DNA and crude extracts indicating that the inhibitors present in the crude extract did not affect RPA reaction.

In conclusion, the RPA assay developed was more specific and sensitive than end-point PCR. The developed RPA assay was used to detect these two species of *Phytophthora* from infected black pepper leaf, stem and root using both purified DNA and crude extracts. The results of the study indicate the robustness of the developed RPA assay and its potential application in detection and differentiation of *P. capsici* and *P. tropicalis* infecting black pepper.

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Declarations

Research involving human participants and/or animals Not applicable.

Informed consent All authors have reviewed the manuscript and approved its submission to the European Journal of Plant Pathology.

Conflict of interest The authors declare that they have no conflict of interest.

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