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RESEARCH ARTICLE

Single tube duplex PCR for simultaneous detection of *Phytophthora capsici* and *Radopholus similis* infecting black pepper (*Piper nigrum*)

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ABSTRACT: Foot rot and slow decline diseases caused by *Phytophthora capsici* or *Radopholus similis* pose major economic threat to black pepper production. The movement of pathogens and the consequent disease outbreak is largely due to latently infected plantlets and the contaminated nursery medium. One of the strategies to prevent the spread of pathogens across the different pepper growing regions is to produce and distribute disease free vigorous plantlets. A rapid and reliable single tube duplex PCR based method to detect *P. capsici* and *R. similis* in infected roots of black pepper was standardized. This method involved use of two sets of primers designed from the conserved internally transcribed spacer (ITS) region of Oomycetes pathogen, *P. capsici* and rDNA sequences of nematode pathogen, *R. similis*. The single tube duplex PCR based technology can be adopted by developmental agencies for simultaneous indexing of both these major soil borne pathogens in plantlets of black pepper aimed for public distribution and large scale transplanting.

Key words: Black pepper, *Phytophthora capsici*, *Piper nigrum, Radopholus similis*

Foot rot disease caused by Oomycetes pathogen, *Phytophthora capsici* and slow decline caused by nematode, *Radopholus similis* are among the major constraints in the production of planting material of horticultural crops especially, black pepper plantlets (Anandaraj and Sarma, 1995). The pathogens are often carried latently to the main field through the apparently healthy rooted cuttings as well as in the rooting medium which results in reduction in the marketable plantlets and transplantation failures in the field (Anandaraj, 2000). One of the strategies to prevent the unintentional spread of pathogens across the different pepper growing regions is to produce and distribute disease free vigorous plantlets. Propagating material used in black pepper nurseries includes rooted cuttings and nodal cuttings which are known reservoirs of pathogen inoculum and often difficult to visualize and detect. In order to certify the health status of the plantlets, a reliable detection methodology for the target pathogens in the planting material as well as the potting mixture assumes significance. Conventionally, the infected plants are recognized by visual symptoms which, usually, appear during the advance stage of infection. It is, therefore, pre-requisite to select the "pathogen free" starting material followed by their protection by effective crop protection strategies (Parthasarathy *et al.*, 2007). This "clean start" can be achieved by selecting the propagating material from disease-free areas, inspecting and indexing them carefully before they enter a disease-free nursery. The symptom based indexing method is ambiguous and not reliable for large scale screening of planting materials. Rapid developments in the genomics of microbial pathogens including *Phytophthora* and *Radopholus similis* have culminated in several conserved genomic regions which can be exploited for designing molecular detection methods. In general, these methods particularly the ones based on PCR are much faster, highly specific, sensitive, and accurate, and can be performed and interpreted by personnel with no specialized taxonomical expertise. The specificity of the primers allows direct detection of a target pathogen from different plant tissues. Interpretation of the result is simple, for the presence of the target pathogen in the sample is given by the presence of the expected amplicons (Demeke *et al.*, 2003; Jurado *et al.*, 2005). Duplex PCR which detects more than one pathogens simultaneously can be one of the choices for implementation of pathogen detection in large-scale. This report describe the development of new primers that target a conserved sequences in the ITS region of *P. capsici* and ITS I region and 5.8S ribosomal RNA of *R. similis*. The objectives of this study are (i) to validate the primers sets for their specificity against *P. capsici* and *R. similis* (ii) to standardize simultaneous detection of *P. capsici* and *R. similis* in a single tube PCR, and (iii) to validate the detection system *in planta* for *P. capsici* and *R. similis* in a duplex PCR on black pepper plant roots.

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MATERIALS AND METHODS

Preparation of pathogen inoculum

Phytophthora capsici (IISR 99-166) was cultured on carrot agar for four days at room temperature (28°C) in dark. Mycelial discs (1 cm) diameter were cut from the plate and placed in another plate with mycelium on the top and sterile

distilled water was added to partially immerse the discs. The plates were incubated under continuous fluorescent light for four days. The mycelial discs were observed under an inverted microscope for the presence of mature sporangia. The plates were then given a cold shock for 10 min and observed under the microscope for release of zoospores. The zoospores were counted using a hemocytometer and the zoospore suspension of *P. capsici* (10^4 zoospores ml^{-1}) was made for plant inoculations. *P. capsici* zoospores (10^4 zoospores ml^{-1})/mycelium (0.1 mg) was used for the isolation of genomic DNA.

The burrowing nematode, *R. similis* was extracted from infected black pepper roots. The collected nematodes were surface sterilized in 1 % sodium hypochlorite, washed in sterile water and subsequently treated with 0.01 % streptomycin sulphate and washed again with sterile water. Then the nematodes were inoculated on carrot discs placed on 2% water agar (O'Bannon and Taylor, 1968) and were maintained at 28±1°C and were used for template preparation for in PCR or for plant inoculation experiments.

DNA isolation from *P. capsici* and *R. simlis*

Genomic DNA was isolated from *P. capsici* and *R. similis* using the procedure described by Pastrick et al. (1995) with some modifications. Briefly mycelium of *P. capsici* or nematode suspension of *R. similis* was incubated in 100 ml of extraction buffer (100mM Tris-Cl pH 8, 100mM EDTA, 100mM NaCl, 0.5% SDS and 200 mg of proteinase K) at 55°C for 3 h. Mycelium or nematode suspension was ground using a micro homogenizer with pinch of glass powder to facilitate lysis before incubation. The samples were then diluted with buffer saturated phenol and DNA was extracted with chloroform- isoamyl alcohol (24:1). The DNA was then precipitated with isopropanol and was dissolved in TE buffer (10mM and was dissolved in TE buffer (10mM Tris-Cl, 0.1mM EDTA pH 8). The DNA was quantified in Biophotometer (Eppendorf Ltd).

Primer designing

The *P. capsici* specific primer was designed based on conserved ITS region (accession Nos. AB359950, AY726623, AY726623, EU015123, EU162757, EU162758, EU162758) and other closely related species and genus (*Pythium* spp.) available in the NCBI database using BLASTn and the nucleotide sequences were compared by multiple alignments using CLUSTAL X (Thompson *et al.*, 1997). The aligned sequence with the variable region was found among the isolates of *Phytophthora capsici*. Those variable regions were exploited in the designing of forward and reverse primers specific for *P. capsici* and these primers were designated as PcapF and PcapR. The designed forward and reverse primers were subjected to *in silico* validation and analysis such as the Tm values and percent GC determined, and stem loop formation and self-complementary (primer dimer) within the sequence were checked using Oligonucleotide Properties Calculator (www.basic.northwestern.edu/biotools/oligocalc.html). Similarly *R. similis* specific primers were designed from the conserved ribosomal RNA and these were designated as RsimF and RsimR. Primer blast analysis confirmed the specificity of the primers for *Phytophthora capsici* or *Radopholus similis* that are documented in the database. The annealing temperature was optimized for each primer set by performing PCR over an annealing temperature gradient from 50 to 60°C as derived from Tm values.

Primer validation

The designed specific primers for each of the pathogens were custom synthesized (IDT Incorp, USA) and validated in PCR. Direct cell PCR was performed using mycelium and zoospores as template. Reaction mixture (10µl) consisting of Taq buffer (10mM Tris pH 9, 50mM KCl, 0.01% gelatin), 3mM $MgCl_2$, 200µM dNTP's mix, 5pM each primer (PcapF and PcapR), 0.5 units of Taq DNA polymerase and 100ng DNA. The thermocycling condition consisted of an initial denaturation at 92°C for 2min 10s, 92°C for 1 min 10s, 54°C for 30s, 72°C for 2 min 10s, 35 amplification cycles and a final polymerization step of 72°C for 6 min 10s with Eppendorf master thermal cycler.

Similarly PCR was setup for validation of RsimF and RsimR for specific amplification of *R. similis*. The thermocycling conditions consisted of an initial denaturation at 94°C for 2 min, 35 amplification cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min and a final polymerization step of 72°C for 10 min with Eppendorf master thermal cycler. The final PCR product was resolved in 1.5% agarose in Tris Acetate EDTA buffer at 4V/cm.

Validation of primers in duplex PCR

The designed primers were validated for simultaneous amplification of *P. capsici* and *R. similis* in a single reaction tube. Two sets of specific primers were used for the detection of the targets. Reaction mixture (10µl) 1X buffer (10mM Tris pH 9, 50mM KCl, 0.01% gelatin), 3mM $MgCl_2$, 200µm dNTP's mix, 5pM each primer (*P. capsici and R. similis*), 0.5 units of Taq DNA polymerase and 100ng template DNA of *P. capsici and R. similis*. The duplex thermocycling conditions are 94°C for 2 min, 35 amplification cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min and a final polymerization step of 72°C for 10 min. The common annealing temperature was derived from gradient PCR ranged from temperatures 50-60°C (data not shown).

Glasshouse assay

Rooted cuttings: Rooted cuttings (IISR Sreekara) of black pepper were planted in non sterile potting mixture containing soil, sand and farmyard manure at 2:1:1 ratio in poly bags (2 kg soil per bag) in a glasshouse. The rooted cuttings were maintained in a temperature controlled growth chamber (25°C, 70% relative humidity, 16 h of day light). Rooted cuttings were challenge inoculated with pathogens while set of control plants were treated with distilled water.

Challenge inoculation: The bacterized plants were challenge inoculated with the pathogens, *P. capsici* (10^4 zoospores g^{-1}) bag^{-1}, *R. similis* @ 100 nematodes bag^{-1} and both the pathogens together (*P. capsici* and *R. similis*). After three months, root rotting and root lesion indices were

Table 1. PCR primers for selective amplification of *P. capsici* and *R. similis*

Pathogen	Primer Sequences	Expected product size	Length	GC (%)	Tm
Phytophthora capsici (PC)	PcapF 5'- ACCCTATCATGGCGAATGTTTGG-3'	573bp	23	48	63
	PcapR 5'- GTTCACCAGCCCATCACGC -3'		19	63	62
Radopholus similis (RS)	RsimF 5'- GATTCCGTCCTTTGGTGGGCA-3'	398bp	21	57	63
	RsimR 5'- GAACCAGGCGTGCCAGAGG-3'		19	68	64

calculated the infected roots of black pepper plants by destructive sampling.

Duplex PCR for simultaneous detection *P. capsici* and *R. similis* in roots

Isolation of total genomic DNA from infected roots: Total genomic DNA from the infected roots was extracted by modified method of Doyle and Doyle (1990). The infected roots were washed in running tap water and surface sterilized aseptically, ground in mortar and pestle and DNA isolated using extraction buffer (Tris-Cl 100mM; EDTA 20mM; NaCl 1.4M; cetyl trimethyl ammonium bromide (CTAB) 2%; polyvinyl pyrrolidone (PVP) 1%; Mercaptoethanol 0.1%; Sodium sulphite 0.5% pH 8.0). DNA pellet was dissolved in 50µl sterile distilled water, and concentration of DNA determined using spectrophotometer (Biophotometer, Eppendorf). The DNA was reconstituted as 100ng template for PCR.

The duplex PCR was performed as mentioned above with purified DNA as template. The PCR product was resolved in 2% agarose stained with ethidium bromide in 1x TAE at 4°C for 2 h. The size of the amplicon was determined using the DNA size marker and the results were documented in alpha imager documentation system.

RESULTS

Primer designing and validation

The primers, PcapF and PcapR designed for *P. capsici* was predicted to yield an amplicon size of 573bp and the specific amplicon for *R. similis* with primers, RsimF and RsimR was predicted to be 398bp (Table 1). Upon validation *in silico*, the primers could hit the predicted sequences of *Phytophthora capsici* and *Radopholus similis* which was confirmed in wet lab trials with purified DNA of both the pathogens. The primers specific for *P. capsici* could successfully amplify 573bp of genomic region in *P. capsici* and *R. similis* specific primers could yield expected amplicon size of 398bp (Fig. 1 and 2). Thus, an annealing temperature of 54°C was found to yield two discrete amplicons for expected size of 573bp and 398bp in a duplex PCR (Fig. 3).

Duplex PCR for detection *P. capsici* and *R. similis* in black pepper roots

Having confirmed the specificity of the primers both *in silico* and wet lab conditions, PCR based detection of *P. capsici* and *R. similis* in black pepper roots was validated *in planta*. The duplex PCR was performed with template DNA extracted from infected roots. Total DNA extracted from the asymptomatic black pepper cuttings collected from nursery infected either by *P. capsici* or *R. similis* alone or in their combination. Single amplicon size of 573bp and 398bp could be observed when the plantlets were infected by *P. capsici* alone and *R. similis* alone, respectively. Two amplicons of size, 573bp and 398bp, could be observed when the plantlets were infected by both the pathogens (Fig. 4). Interestingly the PCR did not yield any non specific or back ground amplification indicating their high specificity towards the target pathogens.

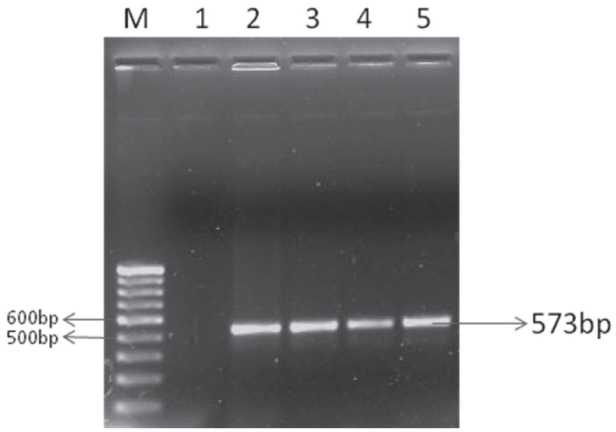

Fig. 1. Validation of detection of *P. capsici* using species specific primers, PcapF and PcapR. M - 100 bp ladder; Lane 1 – Negative control; Lanes 2-3 DNA from *P. capsici* mycelium; Lanes 4-5 DNA from *P. capsici* zoospores

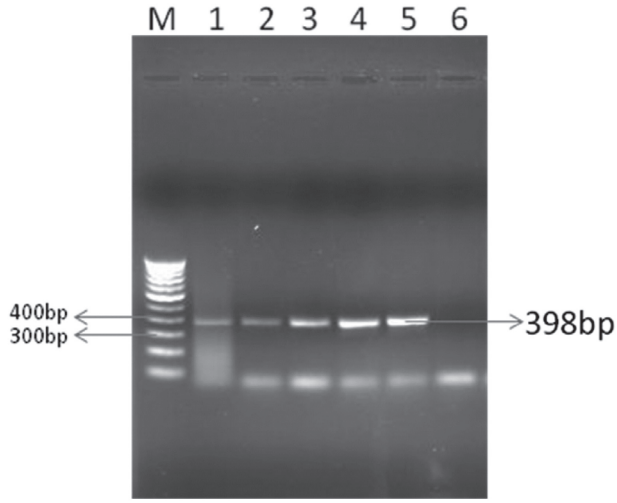

Fig. 2. Validation of *R. similis* using species specific primers, RsimF and RsimR. M - 100 bp ladder; Lanes 1-5 *R. similis* genomic DNA in different concentrations (10ng, 25ng, 50ng, 75ng and 100ng respectively); Lane 6 – negative control

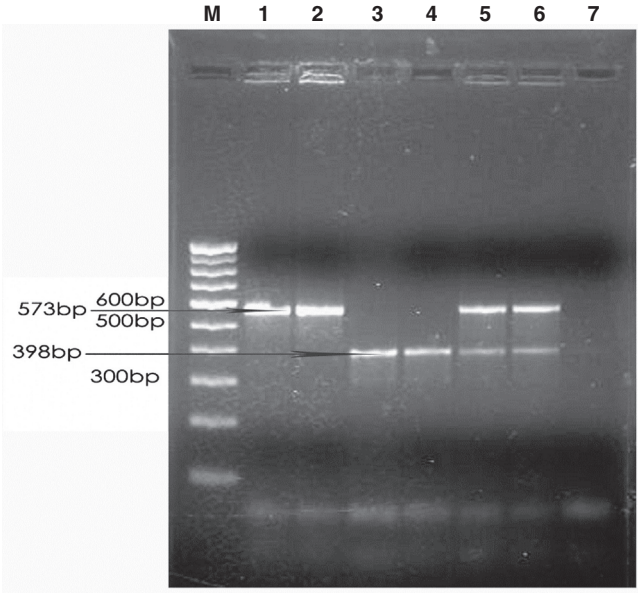

Fig. 3. Validation of duplex PCR for simultaneous detection *P. capsici* and *R. similis*. M: Marker (100 bp ladder), Lanes 1: *Phytophthora capsici*, 2: *Phytophthora capsici*, 3: *Radopholus similis*, 4: *Radopholus similis*, 5: *Phytophthora capsici* + *Radopholus similis*, 6: *Phytophthora capsici* + *Radopholus similis* and 7: Negative control

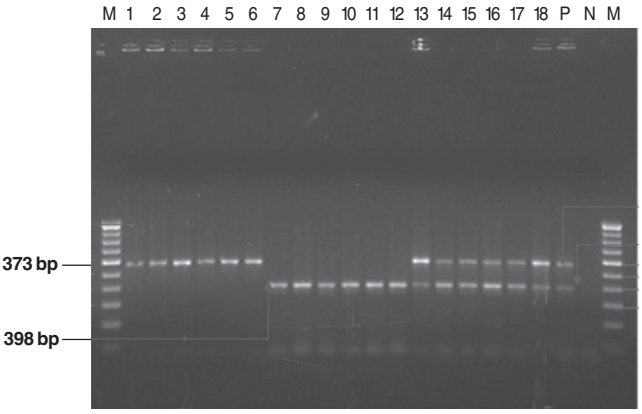

Fig. 4. Simultaneous detection of *P. capsici* and *R. similis* in infected black pepper rooted cuttings by duplex PCR. M - 100 bp ladder; Lanes 1-6 DNA extracted from roots of *P. capsici* inoculated plants; Lanes 7-12 DNA extracted from roots of *R. similis* inoculated plants; Lanes 13-18 DNA extracted from roots of *P. capsici* and *R. similis* inoculated plants; Lane P – positive control; Lane N – negative control.

DISCUSSION

The broad range of conventional methods for detection of pathogenic microbial species are largely based on isolation, cultivation and subsequent observation of morphological traits/or cultural characteristics which is ambiguous and often less discriminatory (Wu *et al.*, 2002). Genomic data and the consequent genomic tools have yielded several molecular techniques with the inherent potential to address these issues. Among them, PCR based one has emerged as powerful tool for the identification and the consequent detection of microbial pathogens in environmental samples owing to its sensitivity, selectivity, robustness, rapidity, and ease of methodology (Kaplan *et al.*, 1996, 2000; Mercado-Blanco *et al.*, 2003; Zhang *et al.*, 2005). PCR techniques have advantages over the traditional methods of detection and diagnosis, because the pathogen in question need not be cultured and puriûed (Zhang *et al.*, 2005). The capacity of the method to amplify DNA from infected but symptomless plants suggests that it could be a useful method to index planting material meant for public distribution and the consequent large scale transplantation. Transplantation failures in black pepper garden are largely attributed to soil borne pathogens such as *P. capsici* and *R. similis* which normally are associated with the potting mix and often latently carried in the plantlets (Anandaraj, 2000; Ramana and Eapen, 2000).

The traditional methods of pathogen detection is often not possible due to its inherent ambiguity and poor resolving power which often lead to “false positive” and “false negative” test results. One of the criteria for successful pathogen detection by PCR is the specificity of the primers used and the amenability of the template DNA or other representatives for a primer binding. Species specific signature sequences in the pathogen genome can be targeted for development of PCR based detection methods. Among the DNA sequences deployed for specific primer development, the highly conserved rDNA and the intergenic regions, also called as internally transcribed spaces (ITS) region are the easy targets (Wu *et al.*, 2002).

We have designed specific primers from ITS region of documented sequences of *P. capsici* and *R. similis*. The present study has clearly proved that there is sufficient sequence stability and divergence in the ITS region of *P. capsici* and *R. similis* for PCR amplification with species specific primers. Selectivity of the primers was validated both *in silico* as well as in wet-lab trials. Briefly, the *in silico* validation was carried out by blasting the designed primer sequence with the Genbank database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The primer blasting had resulted perfect hit (100% identity) with the target sequences of *P.capsici* or *R. similis* indicating their specificity. The same was confirmed in wet lab trial where the pure DNA of the respective pathogen could yield ‘noise less specific amplicon’ indicating their specificity towards *P. capsici* or *R. similis*.

One of the major issues in pathogen detection in environmental samples is the ability of the primers to detect the pathogen in the complex DNA representing the whole microbial community. The primers PcapF and PcapR could amplify the target, the *P. capsici*, in the complex total DNA extracted from roots of black pepper plantlets obtained from nursery soil. Similarly the primers, RsimF and RsimR could selectively amplify the target, *R. similis* thus confirming the sensitivity and the consequent utility of these primers for selective detection of the pathogens in the plant samples. Specific detection method of *P. capsici* has been reported by several workers (Zhang *et al.*, 2006; Silvar *et al.*, 2005; Judelson and Tooley, 2000). Plant parasitic nematode specific primers have been reported in several crops (Syarifah *et al.*, 2010; Qiu, 2008). Perusal of records showed that this is the first report of simultaneous detection of two different kinds of pathogens in a single tube PCR. Such a dual pathogen detection method is not only economical for

adoption but also saves considerable time in indexing planting material of black pepper. The duplex PCR method can be a step towards a development of crop specific disease diagnosis wherein the entire range of pathogens can be detected in a single PCR reaction.

In conclusion, this study demonstrated that Pcap F/R and Rsim F/R primer sets were found suitable for *P. capsici* and *R. similis*. Use of these new primers sets and single tube duplex PCR will improve detection of *P. capsici* and *R. similis* in field and laboratory assays and thus contribute to improved foot rot and slow decline disease management in black pepper.

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