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Multiplex PCR assay for simultaneous detection of *Phytophthora*, *Pythium* and *Fusarium* associated with foot rot and yellowing diseases of black pepper

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Abstract

In this study, a multiplex PCR assay was developed to detect *Phytophthora*, *Pythium* and *Fusarium* infecting black pepper. Genus-specific primers were designed from the conserved region of ITS and a multiplex PCR was optimized by manipulating the annealing temperature and primer as well as $MgCl_2$ concentrations. The black pepper 18S rRNA gene-specific primers were also included in the multiplex PCR assay as internal control. The assay successfully detected the pathogens from artificially inoculated black pepper roots and did not show any cross amplification with other fungal pathogens of black pepper such as *Rhizoctonia solani*, *Sclerotium rolfsii* and *Colletotrichum* sp. Hence, the developed multiplex PCR assay will help in early diagnosis of the cause of black pepper yellowing leading to timely adoption of management strategies.

Keywords: black pepper, detection, foot rot, multiplex PCR, yellowing

Black pepper (*Piper nigrum*), a perennial flowering vine is native to the Malabar Coast of India. Foot rot incited by *Phytophthora,* the Stramenophilic hemi-biotrophic pathogen is the most devastating disease of black pepper. Two species of *Phytophthora* (*P. capsici* and *P. tropicalis*) are known to be associated with foot rot disease (Jeevalatha *et al.* 2021). Yellowing incited by *Fusarium solani* f. sp. *piperis* is considered as another important soil-borne disease which has the potential to cause substantial yield reduction. Of late, another oomycete pathogen, *Pythium* was reported to be associated with yellowing and wilt affected black pepper plants. *P. deliense* was the predominant one and *P. cucurbitacearum* and *Pythium* sp. were also found to be associated with the disease (Subila and Bhai 2020). Yellowing symptoms are often confused with nutrient deficiency, damage incited by biotic factors like foot rot disease, nematodes and also due to mechanical injury to roots. Hence, it is highly imperative to accurately identify the contributing factor in order to execute appropriate management measures to prevent crop loss.

PCR protocols are available to detect the pathogens individually from black pepper. Silvar *et al.* (2005) developed conventional and nested PCR assay for *P. capsici* detection using specific PCR primers. Duplex PCR

assay was optimized to simultaneously detect *P. capsici* and *R. similis* from infected roots of black pepper (Aravind *et al.* 2011). Pandian *et al.* (2018) reported *Phytophthora* genus-specific PCR assay to detect *Phytophthora* spp. from black pepper. Jeevalatha *et al.* (2021) also developed a PCR protocol based on *Ypt1* gene to differentiate *P. capsici* and *P. tropicalis* infecting black pepper. Similarly, a PCR protocol for *Fusarium solani* f. sp. *piperis* was developed by Costa *et al.* (2017) which could detect the pathogen from black pepper roots and soil. However, multiplex PCR assay to detect these pathogens in a single reaction tube would save time, labour and also money. Since, there is no report on simultaneous detection of *Pythium* sp., *Phytophthora* sp. and *Fusarium* sp. from black pepper, we have attempted to optimize a multiplex PCR assay and to check its specificity as well as applicability to detect these pathogens from black pepper.

Carrot agar medium (*Phytophthora*) and potato dextrose agar medium (*Pythium* and *Fusarium*) were used to grow the pathogens at 25±1°C for 3–5 days. The mycelial discs obtained from actively growing cultures were inoculated in Ribeiro's broth (*Phytophthora*) or potato dextrose broth (*Pythium* and *Fusarium*) and incubated at 25±1°C for 5–7 days. Total genomic DNA was isolated from mycelium using the method described by Sheji *et al.* (2009) with slight variations. Primers were designed manually using the ITS sequences of *P. capsici, P. tropicalis, Pythium deliense, P. cucurbitacearum* and *Fusarium solani* and the properties were checked using online software, Oligo Calc:Oligonucleotide properties calculator. The primers are listed in table 1.

Initially, all the primers designed were used in PCR assay to check the performance. Subsequently, one primer pair for each target was selected based on the size of amplicon. The specificity of the selected primer pair was analyzed by carrying out PCR assay with DNA of healthy black pepper, *Pythium* sp., *Phytophthora* sp., *Fusarium* sp. and other pathogens of black pepper such as *Rhizoctonia solani*, *Sclerotium rolfsii* and *Colletotrichum* sp. Uniplex PCR assays were optimized by performing reactions at different annealing

Table 1. Primers used in the present study

Pathogen/Target	Primer	Nucleotide sequence	Amplicon size
Phytophthora capsici	PCPT-F	5′-CCTTTTAGTTGGGGGTCTTGTACC-3′	335 bp
/ *P. tropicalis*	COM-R	5′-CCACCGACTACACGGAAGGAAGAA-3	
	PHY-IT-FP	5′-CCGTATCAACCCTTTTAGTTGGG-3′	315 BP
	PHY-IT-RP	5′-AGTTTGATGTACGGACACTGATAC-3′	
Pythium sp.	PY-IT-F1	5′- TCTCTCTCGGGAGGGCTGAA-3′	373 bp
	PY-IT-R1	5′- ACTCGCGTCTTCTCCTCCGA-3′	
	PY-IT-F2	5′-CTTAATTGTGGTCTGCCGATGTA-3′	424 bp
	PY-IT-R2	5′- ATCCAAACAGATCACTGCGATTC-3′	
Fusarium sp.	FU-IT-F1	5′-AGGACCCCCTAACTCTGTTTCT-3′	222 bp
	FU-IT-R1	5′- GAGGGTTGTAATGACGCTCGA-3′	
	FU-IT-F2	5′-TGTTCGAGCGTCATTACAACC-3′	192 bp
	FU-IT-R2	5′- GTCACATTCAGAAGTTGGGTGTT-3′	
18S rRNA of black pepper	BP-18S-FP	5′ -AAGCGAACTTGTGAACCCTGGT-3′	553 bp
	BP-18S-RP	5′-AATCGGGGAGCGAACGGCA-3′	

temperatures (56, 58, 60 and 62°C) and suitable annealing temperature was selected for further study. Then multiplex PCR assay was optimized using different concentrations of primers (160-280 nM final concentrations), $MgCl_2$ concentrations (2.0 mM to 3.5 mM) and annealing temperatures (56, 58, 60 and 62°C). The multiplex PCR assay was checked to detect different isolates of *Phytophthora* sp. (*P. capsici, P. tropicalis*), *Pythium* sp. (*P. deliense, P. cucurbitacearum, P. aphanidermatum, P. myriotylum*) and *Fusarium* sp. (*F. solani, F. oxysporum*) which were available in our laboratory. Multiplex PCR assay was also performed by mixing DNA of *Phytophthora* sp., *Pythium* sp. and *Fusarium* sp. in different combinations. Then to check the suitability of the optimized multiplex PCR assay to detect the pathogens directly from plant tissues, the young roots of black pepper were infected with *Phytophthora* sp., *Pythium* sp. and *Fusarium* sp., then the root samples were mixed in combinations. The total DNA was isolated from the roots using DNeasy Plant mini kit (Qiagen, the Netherlands) and multiplex PCR assay was performed with the optimized protocol.

In PCR assay, all the primer pairs amplified the target without any non-specific amplification (Fig. 1). However, based on the size of the amplicon, primer pairs *viz.,* PCPT-F/COM-R for *Phytophthora* sp., PY-IT-F2/PY-IT-R2 for *Pythium* sp., FU-IT-F2/FU-IT-R2 for *Fusarium* sp., and BP-18S-FP/BP-18S-RP for black pepper were used for further study. In specificity analysis, these primer pairs amplified only the target pathogen and no cross amplification was noticed with the other two pathogens of the multiplex PCR assay and also with *R. solani*, *S. rolfsii* and *Colletotrichum* sp. (Fig. 2). Initially, in uniplex PCR assay all the primer pairs showed amplification at all the tested temperatures. In multiplex PCR assay, among the different primer concentrations tested, 120 nM concentration of forward and reverse primers of internal control gene, 18srRNA and *Pythium* and 280 nM concentration of

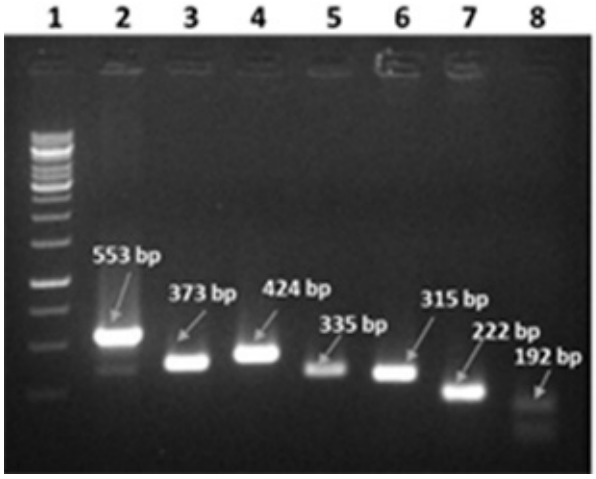

Fig. 1. Selection of primers. Lane 1. 1 Kb DNA Ladder, Lane 2. 18srRNA- black pepper, Lane 3. *Pythium* primer set I, Lane 4. *Pythium* primer set II, Lane 5. *Phytophthora* primer set I, Lane 6. *Phytophthora* primer set II, Lane 7. *Fusarium* primer set I, Lane 8. *Fusarium* primer set II

forward and reverse primers of *Phytophthora* and *Fusarium* were found to be the best and amplified all the targets in a single reaction tube (Fig. 3). Annealing temperature of 60°C and 2.0 mM $MgCl_2$ concentration was found optimum to perform multiplex PCR assay.

The optimized 25 µl reaction mix contained 0.3 µl of 10 mM forward and reverse primers of internal control gene and *Pythium* (120 nM) and 0.7 µl of 10 mM forward and reverse primers of *Phytophthora* and *Fusarium* (280 nM), 2.5 µl 10x Taq DNA polymerase buffer, 2 µl 2.0 mM dNTPs and 0.5 µl 3 U Taq DNA polymerase enzyme, approximately 100 ng of DNA and sterile distilled water. The standardized PCR cycle conditions were as follows: initial denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. The PCR products were run on 1% agarose gel.

The optimized multiplex PCR assay could detect different isolates of *Phytophthora* sp. (*P. capsici, P. tropicalis*), *Pythium* sp. (*P. deliense,*

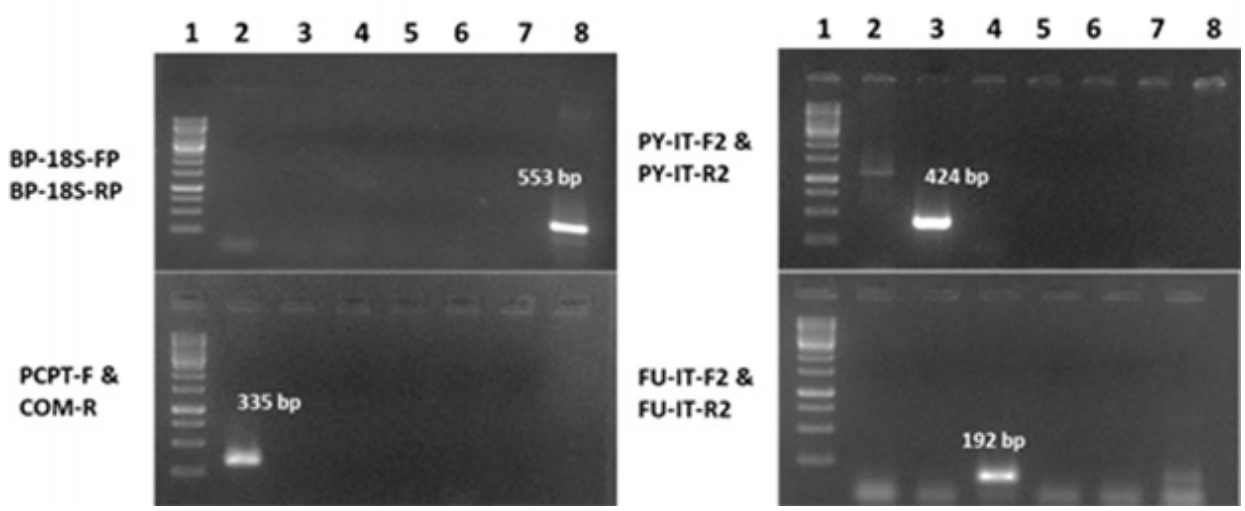

Fig. 2. Specificity analysis of selected primer pairs. Lane 1. 1 Kb DNA Ladder, Lane 2. *Phytophthora* sp., Lane 3. *Pythium deliense*, Lane 4. *Fusarium* sp., Lane 5. *Rhizoctonia solani*, Lane 6. *Sclerotium rolfsii*, Lane 7. *Colletotrichum* sp, Lane 8. Black pepper

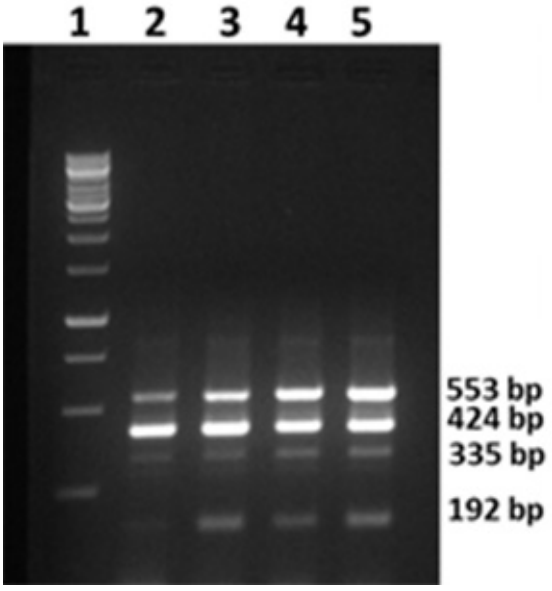

Fig. 3. Optimization of multiplex PCR. Lane 1. 1 Kb DNA Ladder, Final concentration of each primers 18s rRNA+ *Pythium* sp. +*Phytophthora* sp + *Fusarium* sp, Lane 2. 200 nm FP & RP + 200 nm FP & RP + 200 nm FP & RP 200 nm FP & RP, Lane 3. 160 nm FP & RP + 160 nm FP & RP + 200 nm FP & RP 200 nm FP & RP, Lane 4. 160 nm FP & RP + 160 nm FP & RP + 240 nm FP & RP 240 nm FP & RP, Lane 5. 120 nm FP & RP + 120 nm FP & RP + 280 nm FP & RP 280 nm FP & RP.

P. cucurbitacearum, P. aphanidermatum, P. myriotylum) and *Fusarium* sp. (*F. solani, F. oxysporum*). The assay amplified only the specific targets when the pathogen DNAs were used either alone or in different combinations (Fig. 4a). Also, the assay performed with DNA from pathogen inoculated root samples also showed only the expected size of amplicons (Fig. 4b) indicating its specificity and robustness in detecting the pathogens from field samples. PCR assay was developed to simultaneously detect *P. capsici* and *R. similis* from black pepper roots by Aravind *et al.* (2011) in which, ITS region was used to design primers for *P. capsici.* Pandian *et al.* (2018) also used ITS region-based primers to develop PCR assay for *Phytophthora* sp. detection in black pepper. In the present study also, we have successfully used ITS region to design genus-specific primers to detect *Phytophthora*, *Pythium* and *Fusarium* from black pepper. However, Costa *et al.* (2017) used *Ef1-α* region to design primers specific to *Fusarium solani* f. sp. *piperis* and developed PCR protocol to detect the pathogen from black pepper roots and soil. The multiplex PCR assay developed in this study will be useful to accurately diagnose the cause of the symptoms and to adopt appropriate

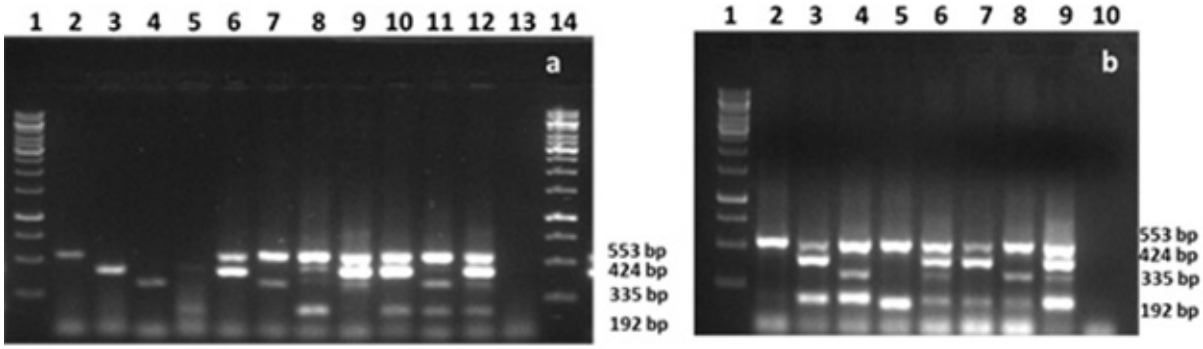

Fig. 4a. Multiplex PCR detection using mixture of pathogen's DNA. Lane 1& 14. 1 Kb DNA Ladder, Lane 2. Black pepper, Lane 3. *Pythium* sp, Lane 4. *Phytophthora* sp, Lane 5. *Fusarium* sp, Lane 6. Black pepper+ *Pythium* sp, Lane 7. Black pepper + *Phytophthora* sp, Lane 8. Black pepper + *Fusarium* sp, *Lane 9.* Black pepper +*Pythium* sp + *Phytophthora* sp, Lane 10. Black pepper +*Pythium* sp + *Fusarium* sp, Lane 11. Black pepper + *Phytophthora* sp + *Fusarium* sp, Lane 12. Black pepper+ *Pythium* sp + *Phytophthora* sp + *Fusarium* sp, Lane 13. Negative control.
Fig. 4b. Multiplex PCR detection of pathogens from artificially inoculated roots. Lane 1. 1 Kb DNA Ladder, Lane 2. Healthy root, Lane 3-9. Mixed infection of pathogens in different combinations, Lane 10. Water control

management strategies. This is also the first report of simultaneous detection of three major pathogens in black pepper.

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