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Full Length Research Paper

Development of specific primers for genus *Fusarium* and *F. solani* using rDNA sub-unit and transcription elongation factor (TEF-1α) gene

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Detection of *Fusarium solani* causal agent of wilt and rots in many plant species are difficult if based only on morphological characteristics. Beside this, morphological discrimination requires special skill and the expertise of taxonomists or specialists. To simplify the detection and discrimination of *F. solani* from other *Fusarium* species, end-point polymerase chain reaction (PCR) assays were developed. Consensus sequences obtained from multiple alignments of target genes, internal transcribed spacer (ITS), rDNA and transcription elongation factor (TEF-1α), were used to design the primers for rapid detection of genus *Fusarium* (amplified product 420 and 466) and *F. solani* (amplified product 658, 595 and 485). BLASTn was used for *in silico* specificity. No cross reactivity was observed when primers were checked against the near-neighbor plant pathogens. The described primer sets allowed accurate identification and discrimination of genus *Fusarium* and *F. solani.* All tests have multiple applications including screening of infected plants, breeding programs and disease diagnosis.

Key words: Inter transcribed spacer (ITS), rDNA, transcription elongation factor, beta tubulin, *Fusarium solani*.

INTRODUCTION

Fusarium species is comprised of pathogens, parasites and saprophytes, and occur on all vegetative and reproductive parts of plants. They are found on nearly all plant species in most parts of the world. *Fusarium* species are economically important as pathogens on most agricultural, horticultural and silvicultural crops grown in the world. It is a cosmopolitan soil inhabiting fungus which is known to be phylogenetically diverse. Its identification is based on morphological characteristics requiring considerable expertise in *Fusarium* taxonomy, especially to distinguish closely related species. Therefore, there is a need to develop tools which permit rapid, sensitive and specific diagnosis of *Fusarium* species in mixed contaminated samples timely disease management. Nucleic acid (NA) based techniques, especially those that rely on the polymerase chain reaction (PCR) are the most powerful for plant pathogen detection (Vincelli and Tisserat, 2008) and allow a more reliable and sensitive microbial identification when applied during surveillance programs (Kim et al., 2008). The implementation of these techniques in extension and other applied programs has been increasing yearly (Vinelli et al., 2008). The PCR based methods are also rapid as there is no need to culture the *Fusaria* prior to their identification. They are specific because identification of species is made on the basis of genotypic differences, and is highly sensitive, as target DNA molecules are detected in complex mixtures even when the mycelia are no longer viable. This research addresses the

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Table 1. Different *Fusarium* strains obtained from IMTECH, Chandigarh, India and their codes.

S/N	Strain code	Strain
1	MTCC-3871	*Fusarium solani*
2	MTCC-350	*F. solani*
3	MTCC-2088	*F. moniliforme*
4	MTCC-156	*F. moniliforme*
5	MTCC-2015	*F. moniliforme var. subglutinans*
6	MTCC-4356	*F. oxysporum*
7	MTCC-2485	*F. oxysporum*
8	MTCC-286	*F. proliferatum var. proliferatum*
9	MTCC-4649	*F. fujikuroi*
10	MTCC-349	*F. culmorum*
11	MTCC-2204	*F. udum*
12	MTCC-1893	*F. graminearum*
13	MTCC-2014	*F. compactum*
14	MTCC-2081	*F. sporotrichides*
15	MTCC-1983	*F. acuminatum*
16	-	*Trichoderma* sp.
17	-	*Pseudomonas* sp.

development of specific primer sets based on ITS-rDNA subunit and TEF-1α gene sequences for detection and discrimination of the genus *Fusarium* and *F. solani*. The strategy developed in this work consisted of newly developed group-specific PCR assays for the genus *Fusarium* and a new species-specific PCR assay for *F. solani*.

MATERIALS AND METHODS

Fusarium isolates and DNA purification

The specificity of PCR assays was tested with different *Fusarium* species strains (Table 1) obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

Potato dextrose agar (PDA) was used to revive the *Fusarium* cultures received from IMTECH. Small piece from the culture vial was transferred on PDA plates and then sealed and kept in a biochemical oxygen demand (BOD) incubator at 28 ± 2°C for four days until the fungal growth appeared. Three discs (5 mm size) of each isolate were cut from periphery of 4-day old culture grown on PDA with the help of a sterile cork borer. The discs were inoculated aseptically in Erlenmeyer flasks containing 100 ml potato dextrose broth (PDB) medium and were kept at 28 ± 2°C in an incubator shaker at 120 rpm for three to four days. After three to four days of fungal growth in PDB, the mycelia were harvested by filtering the contents of the flask through a pre-sterilized fine muslin cloth. The mass of mycelium thus obtained was used directly for DNA isolation.

DNA was extracted from the fungal isolates using the DNA isolation protocol developed by Lee and Tayler (1990). RNAse treatment was performed by adding 2 µl of RNAse (10 mg/ml) to the Eppendorf tube containing 100 µl of extracted DNA and then incubated for 3 h at 37°C in a water bath. The concentration of DNA was determined by UV visible spectrophotometer (Biomate, Thermo Spectronic, Madison, WI).

Primer design

The nucleotide sequence search program located in the 'Entrez' browser provided by the National Center for Biotechnology Information (NCBI) (http://www3.ncbi.nlm.nih.gov/Entrez) (Bethesda, MD) was used to retrieve the sequences of different *Fusarium* species. Nucleotide sequences of all the GenBank isolates were aligned using the program CLUSTALX2 (Larkin et al., 2007) and were examined for the conserved regions. Specific nucleotide regions were selected to design the primers for the rapid detection of *Fusarium genus and F. solani*. Compatible PCR primers for genus *Fusarium and F. solani* were designed from consensus sequences using Primer3 program (Rozenand and Skaletsky, 2000) and are listed in Table 2. The specificity of each primer was confirmed *in silico* by screening the primer sequences with BLASTn (Altschul et al., 1990).

PCR amplification

PCR assays were carried out in 50 µl reaction mixtures containing 20 µl 2.5X master mix (Eppendorf) and 1 µl of each forward and reverse primer (30 ng.µL^{-1}), 1 µl genomic DNA template (40 ng.µL^{-1}) and 27 µl sterilized water. PCR program was standardized for each primer set (Table 2) and amplification was carried out in Eppendorf thermal cycler (Eppendorf, Hauppauge, NY).

A volume of 20 µl of amplified PCR product was electrophoresed in a 1.4% agarose gel containing ethidium bromide (0.25 mg.mL^{-1}) in 1X TAE buffer, and amplicon sizes were estimated using 100 bp ladders (Fermentas, Glen Burnie, MD). The PCR amplicons were visualized using UV gel documentation system (Bio-Rad, Hercules, CA).

RESULTS AND DISCUSSION

We reported the development of PCR assays for the identification and discrimination of genus *Fusarium* and *F. solani*. Initially, 16 primer sets were used (data not shown), but only five primer sets were specific to the genus *Fusarium* (TEF-Fu3 and ITS-Fu1) and, *F. solani* (TEF-Fs4, ITS-Fu2 and ITS-Fs5). PCR conditions of each primer set were standardized (Table 2). Primer set TEF-Fs4 showed a specific 658 bp amplicon on agarose gel only with *F. solani* (Figure 1), while primer set ITS-Fu2 and ITS-Fs5 showed 595 and 485 bp amplicon, respectively. Primer set TEF-Fu3 showed ~420 bp product size (Figure 2) with 15 *Fusarium* isolates belonging to 11 *Fusarium* species including *F. solani, Fusarium moniliforme, F. moniliforme* var. *subglutinans, Fusarium oxysporum, Fusarium proliferatum* var. *proliferatum, Fusarium fujikuroi, Fusarium culmorum, Fusarium udum, Fusarium graminearum, Fusarium compactum, Fusarium sporotrichides* and *Fusarium acuminatum* (Table 1). About 466 bp amplicon was obtained with each genomic DNA of 15 *Fusarium* isolates (Table 1) when used against the primer set ITS-Fu1 (Figure not shown). No PCR products were amplified from other species, although all five primer sets specifically amplified genus *Fusarium* and *F. solani* genomic DNA. The developed primers sets successfully identified the causal organism of die-back disease of *Dalbergia sissoo* and will be also informative to identify the *Fusarium* from other diseased

Table 2. List of primers developed for rapid detection of *Fusarium* sp. and *F. solani*.

Primer code	Primer sequence (5'→3')	Gene	Amplified product size	Specificity	PCR programme
TEF-Fs4f	**ATCGGCCACGTCGACTCT**	TEF-1α	658 bp	*F. solani* (Fig.1)	40 cycles; 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min
TEF-Fs4r	GGCGTCTGTTGATTGTTAGC				
ITS-Fu2f	CCAGAGGACCCCCTAACTCT	ITS1, ITS2	595 bp	*F. solani* (Fig. not shown)	42 cycles; 94°C for 1 min, 63.5°C for 1 min, and 72°C for 2 min
ITS-Fu2r	CTCTCCAGTTGCGAGGTGTT				
ITS-Fs5f	CGTCCCCCAAATACAGTGG	ITS2-rDNA subunit	485 bp	*F. solani* (Fig. not shown)	42 cycles; 94°C for 1 min, 61°C for 50 s, and 72°C for 2 min
ITS-Fs5r	TCCTCCGCTTATTGATATGCTT				
TEF-Fu3f	GGTATCGACAAGCGAACCAT	TEF-1α	~420 bp	*Fusarium* species (Fig. 2)	40 cycles; 94°C for 1 min, 58°C for 1 min, and72°C for 2 min.
TEF-Fu3r	TAGTAGCGGGGAGTCTCGAA				
ITS-Fu1f	ACAACTCATAACCCTGTGAACAT	ITS2-rDNA subunit	~466 bp	*Fusarium* species (Fig. not shown)	40 cycles; 94°C for 1 min, 58°C for 1 min, and72°C for 2 min.
ITS-Fu1r	CAGAAGTTGGGTGTTTTACGG				

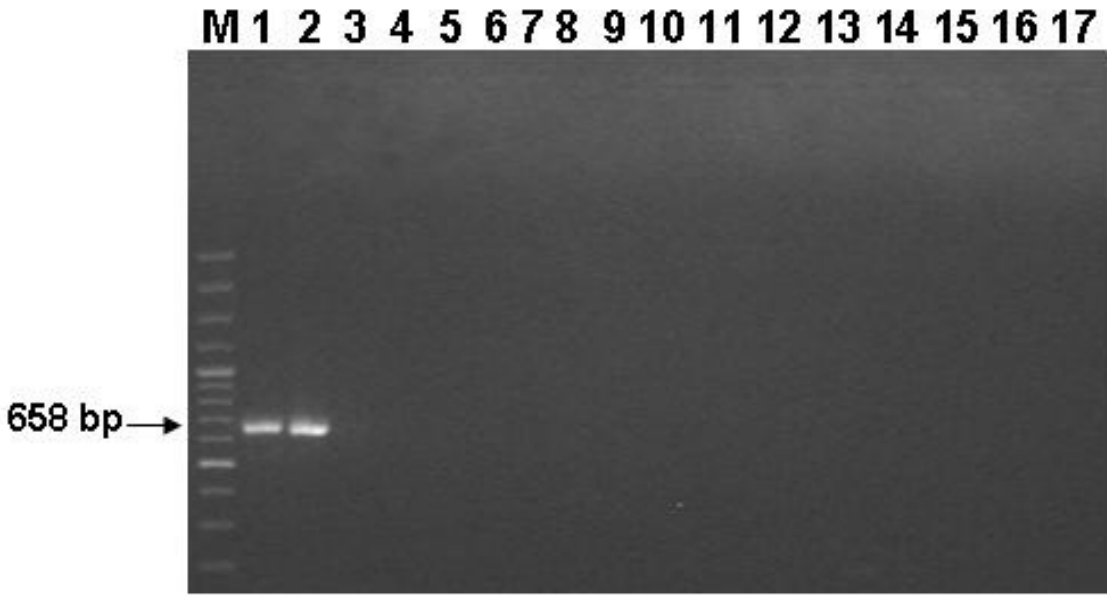

Figure 1. Profile of *F. solani* specific marker obtained using primer TEF-Fs4. Lane M is 100 bp ladder and lanes 1 to 17 represent different strains as listed in Table 1.

plants. These primer sets are particularly useful because Fusaria are widely distributed in the soil environment and *F. solani* causes infection on a variety of plants (Arif et al., 2008a, b; Romberg and Davids, 2007) and have also been implicated in mycotic ocular keratitis (Godoy et al., 2004). The developed assays will also be useful for determining the population's biology of *Fusarium* or *F. solani* and can be unambiquously discri-minated and identified using sequencing of amplified PCR products.

DNA-based detection and identification methods desc-ribed here can be used to supplement or confirm the morphological identification of genus *Fusarium* and *F. solani*. The developed methods can facilitate detection, confirm genus *Fusarium* and *F. solani* identity, and be applied during pathogen control activities. The assays are practical, rapid and low-cost, and efficient for the identi-fication and discrimination of genus *Fusarium* and *F. solani.*

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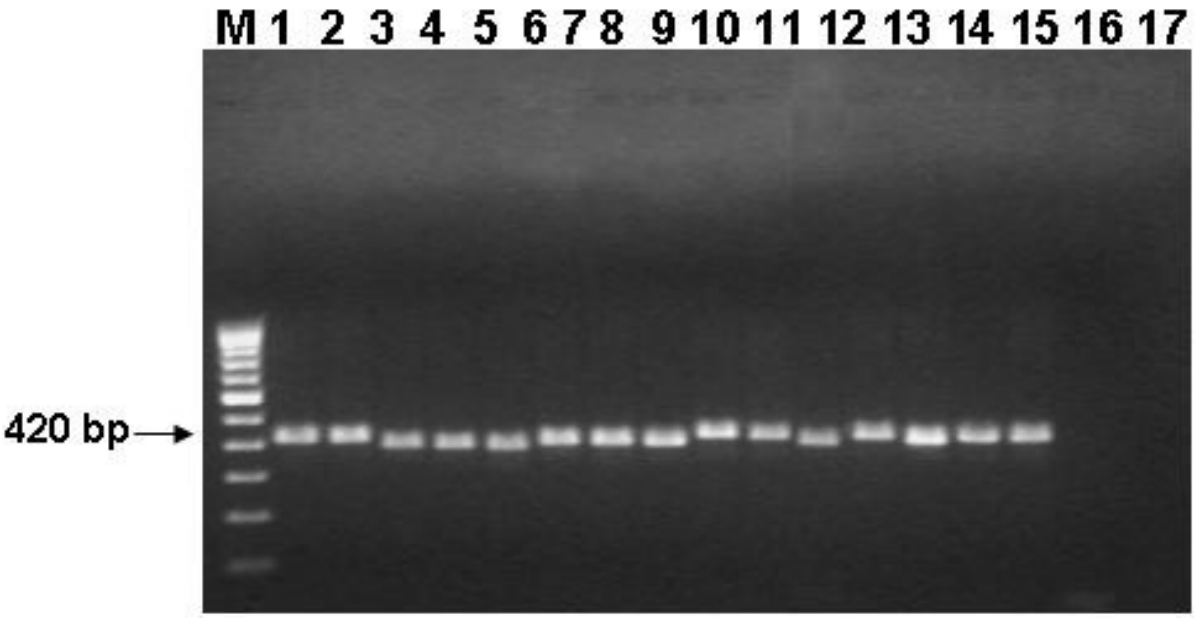

Figure 2. Profile of *Fusarium* species specific marker obtained using primer TEF-Fu3. Lane M is 100 bp ladder and lanes 1 to 17 represent different strains as listed in Table 1.

Department of Biotechnology, Government of India.

REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. J. Mol. Biol. 215(3): 403-410.

Arif M, Zaidi NW, Haq QMR, Singh US (2008a). Genetic variability within *Fusarium solani* as revealed by PCR- fingerprinting based on ISSR markers. Indian Phytopath. 61(3): 305-310.

Arif M, Zaidi NW, Haq QMR, Singh US (2008b). Molecular diversity in *Fusarium solani* associated with sissoo (*Dalbergia sissoo*) wilt assessed by RAPD DNA markers. J. Mycol. Pl. Pathol. 38(2): 258-260.

Godoy P, Cano J, Gené J, Guarro J, Höfling-Lima A, Colombo A (2004). Genotyping of 44 Isolates of *Fusarium solani*, the Main Agent of Fungal Keratitis in Brazil. J. Clin. Microbiol. 42(10): 4494-4497.

Kim JS, Lee GG, Kim J, Kwon JY, Kwon ST (2008). The development of rapid real-time PCR detection system for *Vibrio parahaemolyticus* in raw oyster. Lett. Appl. Microbiol. 46(6): 649-654.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007). Clustal W and Clustal X version 2.0. Bioinformatics, 23: 2947-2948.

Lee SB, Taylor JW (1990). In: The PCR protocols: A guide to methods and applications. (eds. nnis MA, Gelfand DH, Sninsky JJ, White TJ), Academic Press, New York, pp. 282-314.

Romberg MK, Davis RM (2007). Host range and phylogeny of *Fusarium solani* f. sp. *eumartii* from potato and tomato in California. Plant Dis. 91: 585-592.

Rozenand S, Skaletsky HJ (2000). Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 365-386.

Vincelli P, Tisserat N (2008). Nucleic acid-based pathogen detection in applied plant pathology. Plant Dis. 92(5): 660-669.