

## Development of *Fusarium oxysporum* species complex (FOC) specific primers using Topoisomerase-II gene

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### Abstract

*Fusarium* is widely regarded as one of the most destructive and species-rich groups of phytopathogens. Most of the important crops affected by *Fusarium* leads to great loss to global agricultural economy. Members of the *Fusarium oxysporum* complex (FOC) are common soil-borne fungi which are known to cause wilt, crown rot and root rot in a number of crops. Therefore, accurate identification of the FOC pathogens is required for disease management in crops. In this study, 75 *Fusarium* isolates were recovered from 20 wilted plant and soil samples were taken from NBAIM fields and identified using conventional morphological techniques. Twelve representative strains of *Fusarium* spp. were identified using 28SrDNA and topoisomerase-II (topo-II) gene sequencing. Sequence analysis of both the genes were made by examining conserved sequences within species and variable sequence in different species in which no species specific sequences were found in 28S rDNA but, were present in topo-II gene sequences. FOC specific forward primer (FO-F) and reverse primer (FO-R) were designed by selecting the specific regions of topo-II gene sequence and tested with five *F. oxysporum* isolates along with some non- *F. oxysporum* isolates. The results revealed a single band size of 495 bp in all tested *F. oxysporum* isolates which was absent in other isolates. The specificity of the primer was validated by taking 23 *Fusarium* isolates from wilted plants and soil samples. The results showed positive amplification in *Fusarium oxysporum* isolates whereas no amplification observed in related species. The developed primers were able to identify and discriminate FOC from related species and can be used in ecological studies, disease diagnostics and in screening of infected plants.



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## Introduction

Genus *Fusarium* is a large group of filamentous fungi and an important phytopathogen, globally distributed in all types of soils (Aoki et al., 2014). *Fusarium* species are widely distributed in various habitats such as in soil, on living plants, on residues, air as well as in water (Almaguer et al., 2012; Donat et al., 2012; Sautour et al., 2012). Classical identification and detection of *Fusarium* has been carried-out on the basis of morphological characteristics. The diagnosis of *Fusarium* spp. based on morphological characteristics is challenging. Many *Fusarium* species are morphologically similar and identification based on morphological characteristics often restricts the number of isolates one can analyze. Moreover, these morphological methods are time-consuming and require extensive taxonomical knowledge. *Fusarium oxysporum* is one of the large, highly diverse, morphologically similar *Fusarium* species with multiple phylogenetic origins (O'Donnell et al., 1998; Baayen et al., 2000; Bogale et al., 2007). The morphology-based identification is further complicated due to existence of *Fusarium oxysporum* as, plant pathogenic, saprophytic and biocontrol strains which are morphologically indistinguishable. In addition, morphology based identification is sometime difficult to distinguish *Fusarium oxysporum* from other species belongs to section *Elegans* and *Liseola* (Fravel et al., 2003).

The molecular tools based on polymerase-chain-reaction (PCR) have been developed to support and replace morphology-based identification of phytopathogenic fungi. The PCR amplification and sequencing of DNA isolated from pure colony or directly from soil could be used for species identification (Edel-Hermann et al., 2008; Van Elsas and Boersma, 2011; Plassart et al., 2012; Suenaga, 2012). The PCR based identification and diagnosis is specific, accurate and less-time consuming in comparison to traditional culture based methods. Many studies have identified several informative loci that can be used to design PCR primers and thus resolve relationship between the *Fusarium* species (O'Donnell et al., 2009; O'Donnell et al., 2010; O'Donnell et al., 2012). Inter-species and intra-species polymorphism has been revealed in the translation elongation factor 1-alpha (EF-1 $\alpha$ ) gene that could be used to differentiate closely related *Fusarium* (O'Donnell et al., 2009; O'Donnell et al., 2012; Edel-Hermann et al., 2012). Moreover, the nucleotide sequence of EF-1 $\alpha$  could be used as BLAST query against the FUSARIUM-ID sequence database (Geiser et al., 2004 or MLST database (O'Donnell et al., 2012). However, in some closely related *Fusarium* species the nucleotide sequence variability is not suitable for designing specific primers. For accurate and specific identification and detection of *Fusarium oxysporum* many PCR and real-time PCR based assay have been developed by utilizing many gene loci. Edel et al. (2000) developed a PCR assay based on 28S rDNA for the identification of *Fusarium oxysporum*. However, Daniel Jiménez-Fernández et al. (2010), reported non-specific amplification using the Edel primers and the amplification size was too small.

Random-amplified polymorphic DNA (RAPD) markers has been used for designing primers specific for *Fusarium oxysporum* species (Alves-Santos et al., 2002; Jiménez-Gasco and Jiménez-Díaz 2003; Lievens et al., 2007). Cipriani et al. (2009) developed PCR assay for identification of biocontrol strains of *F. oxysporum*. Haegi et al. (2013) developed a species-specific real-time quantitative PCR assay using 1- $\alpha$  gene sequence for the detection of soil-borne *Fusarium oxysporum*. Real-time PCR assay has also been developed for detection of *Fusarium oxysporum* f. sp. *chrysanthemi*, *Fusarium oxysporum* f. sp. *basilica* and *Fusarium oxysporum* f. sp. *lactucae* (Pasquali et al., 2004; 2006; 2007).

Topoisomerase-II (EC 5.99.1.3) gene is part of the housekeeping genes and is essential for replication, transcription, condensation, separation of chromosomes, and for global genome stability (Nitiss et al., 1998). The Topo-II gene nucleotide sequence has been used to establish phylogenetic relationships among the pathogenic *Candida* species (Kato et al., 2001). Moreover, Hatsch et al., discriminated *Fusarium* species based on topo-II gene sequences and revealed that Topo-II gene sequences of different species of *Fusarium* could be used for development of species-specific primers for detection of *Fusarium* species (Hatsch et al., 2004). In this study we developed a PCR assay based on topo-II gene sequence for specific identification and detection of *Fusarium oxysporum* from soil and plants.

## Material and Methods

### Fungal isolation and cultural characteristics

*Fusarium* isolates were isolated from 20 wilted plant and soil samples collected from NBAIM fields. Wilted chickpea, pigeon pea, soybean, wheat, potato, tomato and maize plants were collected from nearby NBAIM fields. Symptoms such as wilting, vascular discoloration and root rotting were considered as indices for sample collection. The roots plants were cut into small pieces (2-3 cm) and washed for 20 minutes, surface sterilized in 10% sodium hypochlorite for 2 min, rinsed in sterilized distilled water and air dried on sterile filter paper. The disinfected pieces were aseptically cut into 3-5 mm pieces, placed on medium and incubated at 25°C in the dark. Five *Fusarium* specific media were used for the isolation of pure cultures Bouhot and Rouxel's medium (1971), Komada's medium (1975), Modified Czapek's Medium (Nelson et al., 1983), PCNB medium (Modified), Sharma and Singh medium (1973) and Carnation Leaf Agar (CLA). For isolation of *Fusarium* from soil, the soil samples were air-dried and passed through a 200  $\mu$ m sieve. Sub samples of ~10 mg were spread over Komada's selective medium (Komada, 1975), and incubated under fluorescent light at room temperature for 7 days.

### Morphological identification of fungal isolates

The pure isolates were initially identified based on morphological characteristics on potato dextrose agar (PDA) medium such as colony colour, growth rate, pigment formation and texture of colony.

The results were further confirmed, in accordance with other scientific reports (Gerlach and Nirenberg, 1982; Nelson et al., 1983; Samson et al., 1995). Macroscopic and microscopic characteristics of the pure isolates were studied and the species were identified using *Fusarium* illustration keys (Nelson et al., 1983; Booth, 1977). The pure isolates were deposited to the repository of National Bureau of Agriculturally Important Microorganisms, Mau India. Representative *Fusarium* isolates from each group (total 12) were selected for further study (Table-1).

### DNA extraction

Fungal strains were grown in potato dextrose broth for 4 to 6 days at 28  $\pm$  2 °C in 150 ml flask, and mycelium was recovered by vacuum filtration through double layer cheese cloth. Genomic DNA was extracted by procedure previously describe by Lee and Taylor (Lee and Taylor 1992). The harvested mycelium was grounded to a fine powder using liquid nitrogen. The mycelium powder was incubated at 65°C for 10 min in 60  $\mu$ l lysis buffer (50 mM Tris-HCl, pH 7.8, 50 mM Na<sub>2</sub>-EDTA, 3% SDS) to which 1% 2-mercaptoethanol was added freshly. Following incubation, 10  $\mu$ l of proteinase K (20 mg/ml) was added and again incubated for 10 min at 65°C. Mycelial clumps were broken by vortexing the tube for 30 s. The DNA was purified by phenol-chloroform method and precipitated with ethanol. The DNA quantified and stored at 4 °C until use.

### PCR amplification of 28S rDNA and topo-II gene

PCR amplifications of 28S r-DNA gene was carried-out in 50  $\mu$ l reaction volume by mixing 20ng of template DNA, 5  $\mu$ l of 10x PCR buffer (10 mM Tris HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 0.1% triton-X100), 5 pmol of each primer, 0.2  $\mu$ l of 25 mM dNTPs, 1.2U/  $\mu$ l Taq DNA polymerase. The primer pair used was ITS-1 (White et al., 1990) and P<sub>3</sub> (Gaudet et al., 1989). Amplification were conducted in BioRad thermal cycler with an initial denaturation step of 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 50 sec, extension at 72°C for 1 min 20 sec, followed by final extension at 72°C for 10 min. PCR amplification of a part of topo-II was carried out in 50  $\mu$ l reaction mixture with 5 pmol of genus specific primers. The primer pair used were Tof 75 & 98b (Hatsch et al., 2004), with initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 1 min and extension at 72°C for 30 sec, followed by final extension at 72°C for 5 min. The PCR products were electrophoretically separated on 1.6% agarose gel in a TBE buffer. A 100 bp DNA ladder (Fermentas Inc.) was used as molecular size marker.

## Sequencing and sequence analysis

The 28S rDNA and topo-II genes of 12 *Fusarium* isolates were sequenced.

PCR amplified products were purified by using PCR purification kit and gel elution column supplied by Bangalore GeNei India.

The sequencing reactions were made with 5 µl (20-40ng) of purified amplified DNA, 4 pmol of primers, and 4 µl of Big Dye Terminator (PE Applied Biosystems) according to the manufacturer's protocol. Sequences of both strands were determined. CAP3 program (<http://www.pbil.univ-lyon1.fr/cap3.php>) was used to assemble a set of contiguous sequences using sequences of both strands (Huang et al., 1999). The sequences were cleaned of their primer sequences and aligned using Clustal X. The resulting sequences were analyzed for homology with sequences in the NCBI GenBank. Blast search was performed at NCBI (<http://www.ncbi.nih.gov/BLAST>) to establish the species identification. Sequences were submitted to NCBI GenBank and accession numbers were obtained (Table-1).

## Designing of Oligonucleotide Primers

The Topo-II gene sequences of 15 *Fusarium oxysporum* species and closely related species were aligned using Clustal W. The conserved sequences within *Fusarium oxysporum* species complex were determined manually. The PCR primers were designed using Primer3 plus software using aligned sequences. The specificity of the designed primers were checked in-silico using Primer-BLAST program against NCBI GenBank database. The secondary structure and cross dimer and self-dimer of the designed primer pair was detected using Gene Runner software (Hastings Software, USA). The primers were synthesized by Bangalore GeNei (Bangalore, India).

## PCR assay specific for *Fusarium oxysporum* complex

The designed primers were evaluated for specific amplification from genomic DNA isolated from *F. oxysporum*. The PCR reaction was carried-out in total volume of 50 µl of reaction. The reaction mixture contained 5 µl of 10X PCR buffer (10 mM Tris HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 0.1% triton-X100), 2 pmol of each forward and reverse primer, 0.2 µl of 25 mM dNTPs, 1.0 U/µl Taq DNA polymerase and 3 ng of genomic DNA. The PCR amplification reaction were as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of DNA denaturation for 1min at 95 °C, primers annealing for 1 min at 55 °C, and extension for 25 sec at 72 °C, and final extension step at 72 °C for 10 min at. The sensitivity of the PCR assay was evaluated with different concentrations of genomic DNA isolated from *Fusarium* species.

The DNA isolated from *Fusarium oxysporum* and other related *Fusarium* species was used to evaluate the specificity of the designed PCR assay. The validation of the PCR assay was carried out with genomic DNA isolated from 23 *Fusarium* species isolated from wilted plants and soil samples.

## Results

### Isolation and morphological identification of *Fusarium* isolates

From 20 wilted plants and soil samples a total of 75 pure isolates were recovered. Among 75 pure isolates, 9 isolates recovered from wilted peginon-pea plants showed cotton smooth, yellowish-pink to deep purple colony. The macroconidia were large, crescent shaped and pointed at both sides. The microconidia were small elliptical, oval or curved with 1-2 septa (Figure 1 A). Seven *Fusarium* isolates recovered from wilted chick-pea were wooly in texture and white cream to pink coloured on PDA with microconidia were single septed and macroconidia were 2-3 septa and abundance (Figure 1 B). Eight isolates recovered from wilted tomato plants showed orange to pink colony with fast growth and floccose texture. Their macroconidia, were thin walled, three- to five-septate, fusoid-subulate and pointed at both ends. The microconidia were abundant, oval-ellipsoid, straight to curved and non-septate (Figure 1 C).

A total of 14 *Fusarium* isolates recovered from soil samples showed white and lower pink colony on PDA medium with fusiform and foot-shaped basal-cell and 3-5 septa macroconidia (Figure 1D-E). A total of 10 *Fusarium* isolates recovered from wilted wheat-plants.

The colour of colonies was variable with yellowish and aerial mycelia to red or brown base on PDA with fast growth rate of colony. The macroconidia were mostly (5-7) septate (Figure 1 F and I). Similarly, 11 isolates recovered from wilted maize-plants showed variable colony morphology from cotton coloured colony to white and orange with aerial mycelium and slow growth rate. Their macroconidia were abundant, slender shaped, thin walled, usually 3-4 septa present. The microconidia were abundance with 0-1 septa present (Figure 1 G, J and L). Eleven *Fusarium* isolates recovered from wilted soybean plants formed cream to white colour colony with round and bulgy appearance. Their apical cell was round; the basal cell was round or foot-shaped with 3-4 septa. The microconidia were abundant, mono or bi-cellular and oval or elliptical in shape (Figure 1 K).

The pure isolates were deposited in National Bureau of Agriculturally Important Microorganisms, Mau India (NBAIM) culture collection repository and accession numbers were obtained.

## PCR amplification of 28S rDNA and topo-II

PCR amplification of 28S rDNA gene from 12 representative *Fusarium* isolates using ITS-1 and P3 primers yielded a DNA band of 1123 to 1150 bp. The amplified product consists of partial sequence of ITS-1, complete sequences of 5.8S and ITS-2 region and partial 5' end of 28S rDNA gene. The PCR amplification of topo-II gene yielded a DNA fragment of 720 bp. The PCR products from two target gene were gel-eluted, purified and sequenced. The 28S rDNA gene sequences were used to confirm the identity of the *Fusarium* isolates. The BLAST search was carried out to confirm the identity of each species with homology of sequences in NCBI GenBank. The nucleotide similarity ranged between 97-99%. The sequences were submitted to NCBI Gen-Bank and accession numbers were obtained (Table-1).

On the basis of 28S r-DNA gene sequencing the 12 *Fusarium* isolates were identified as *F. oxysporum*, *F. oxysporum ciceris*, *F. oxysporum lycopersici*, *F. udum*, *F. culmorum*, *F. solani*, *F. moniliforme*, *F. pulicaris*, *F. chlamydosporum*, *F. proliferatum* and *F. cerealis*.

## Sequence analysis of 28S rDNA and topo-II for primer designing

In order to design species specific PCR primers, the DNA sequence of 28S rDNA and topo-II genes were aligned using Clustal W. The conserved sequences within species and variable sequence in different species were examined in which no species specific sequences were found in 28S rDNA, whereas, conserved sequences within species and variable sequences in between species were found in topo-II gene sequences. The 15 topo-II gene sequences (11 used in this study and 4 retrieved from NCBI GenBank) were aligned. The sequence analysis showed variable regions that were conserved within *Fusarium oxysporum* species and variable with other species. The sequence specific regions were selected for designing forward and reverse primer. The forward primer (FO-F; 5'- AGGGTCATGCTCTGAAGCAGAG-3') was picked from left specific region which was conserved within (intra-species conserved) *Fusarium oxysporum* isolates and variable with other species (inter-species variable). The variability of sequence was kept at 3' end of the primer to increase sequence discrimination during primer annealing. The reverse primer (FO-R; GAACTGTTAATGTTCTGAATTTCA) was picked from right specific region which showed conserved sequence within *Fusarium oxysporum* isolates and variable at 3'end with other closely related species (Figure 2 A and B). The in-silico analysis of primers using primer-BLAST revealed that primers were specific for *Fusarium oxysporum* and no homology of the primer with sequences other than *Fusarium oxysporum* was detected. In addition, no primer dimer and self-complementary structure were detected in the designed primers.

## Detection of *Fusarium oxysporum* using specific primers

The specificity of the PCR assay with *F. oxysporum* specific primers (FO-F & FO-R) was tested with genomic DNA isolated from a panel of *Fusarium oxysporum* isolates (*F. oxysporum*, *F. oxysporum ciceris*, *F. oxysporum lycopersici* and *F. oxysporum melonis*) and non-*Fusarium oxysporum* isolates. The PCR reaction was carried out in total volume of 50 µl reaction. The reaction mixture consists of 5 ng DNA template, 5 µl of 10x PCR buffer (10 mM Tris HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 0.1% triton-X100), 4 pmol of each primer, 0.2 µl of 25 mM dNTPs, 1.2U/µl Taq DNA polymerase.

Amplification were carried out with an initial denaturation step of 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 30 sec, extension at 72°C for 1 min 20 sec, followed by final extension at 72°C for 10 min. The PCR amplification results revealed a single band of 495 bp only in *Fusarium oxysporum* isolates whereas, no amplification was observed in closely related non-*Fusarium oxysporum* isolates (Figure 3).

## Validation of PCR assay using *Fusarium* specific primers

The validation of PCR assay was performed with DNA extracted from 23 *Fusarium* isolates recovered in this study from wilted plants and soil sample. The DNA was isolated and subjected for PCR amplification. The results showed positive amplification of 10.

*Fusarium oxysporum* isolates and no PCR amplification was detected in closed related species (Figure 4). However, the PCR assay was negative for 3 *Fusarium* isolates that were detected *Fusarium oxysporum* on the basis of morphological characters (Figure 4).

## Discussion

The application of PCR and real-time PCR has been increasing recently for identification and detection of *Fusarium* species. Although, many real-time PCR methods has been developed for identification and detection of *Fusarium* (De Sousa et al., 2015; Li et al., 2014; Luchi et al., 2016), due to higher cost it is still not commonly used for plant-pathogen detection in developing countries. The conventional PCR is still in-use for detection and identification of fungi in plants and soil. Most of the developed PCR assays rely on rDNA gene sequences, which sometime shows less polymorphism among species. In such circumstances, the other target genes such as  $\beta$ -tubulin gene (Ma et al. 2005), translation elongation factor 1- $\alpha$  (TEF) gene (Mogens et al., 2009), and cellobiohydrolase-C gene (Hatsch et al., 2004) have been used for the design of specific PCR primers. However, in some species less variation in DNA sequence limits the possibility to design specific primers which necessitates looking for new target gene. In this study we have isolated and identified 75 *Fusarium* isolates from wilted plants and soil sample using morphological methods. The isolates were further identified using conserved 28S rDNA gene sequencing and a PCR assay was designed using Topo-II gene sequences for specific identification and detection of *Fusarium oxysporum*. Identification of *Fusarium* spp. using 28SrDNA from different environmental samples were previously reported by many researchers (Balajee et al., 2009; Sheikha et al., 2011; Kwiatkowski et al., 2012).

Three web-accessible resources available for DNA sequence-based identification of *Fusarium* are: FUSARIUM-ID (<http://isolate.fusariumdb.org/>); Geiser et al., 2004; Park et al., 2010), *Fusarium* MLST (<http://www.cbs.knaw.nl/fusarium/>); O'Donnell et al., 2010), and NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>). The BLAST analysis using FUSARIUM-ID or, *Fusarium* MLST is considered advantageous over NCBI GenBank searches as they contain well characterized, phylogenetically informative sequences from broadly sampled *Fusarium* isolates which can be obtained from the ARS Culture Collection (NRRL, <http://nrri.ncaur.usda.gov/cgi-bin/usda>) or, FRC (<http://plantpath.psu.edu/facilities/fusarium-research-center/>) (O'Donnell et al., 2015).

*Fusarium oxysporum* is a well recognized pathogenic plant fungus that affects a wide range of crops. It is cosmopolitan in nature and is commonly found to be associated with plant diseases (Demers et al., 2015). However, the rhizosphere consist of pathogenic and non-pathogenic *Fusarium oxysporum* (NPS) together and it was suggested that the population of NPF isolates give rise to pathogenic species by gaining pathogenicity (Inami et al., 2014). Therefore, it has become important to identify the pathogenic and pathogenic *Fusarium oxysporum*.

In this study the specific primers for the detection of FOC were designed by targeting nucleotide variability of Topo-II gene, which encodes the carboxy-terminal of half of the topoisomerase-II ATP binding domain. Previously, Hatsch et al. (2004) used Topo-II sequences for phylogenetic analysis of *Fusarium* spp. and suggested that despite the relatively short size; the topo-II gene parts contained a great deal of variability in between different *Fusarium* spp. that can be used for species identification (Hatsch et al., 2004). Similarly, Kato et al., (2001) also used Topo-II gene for phylogeny study of *Candida* spp. Our study also clearly showed the existence of higher variability in different species on the basis of topo-II sequences (56.78%). Oligonucleotide primers were designed by keeping the nucleotide variability of *Fusarium oxysporum* with other closely species at 3' end of the primers. The mismatch at 3' end of primers is vital for successful amplification and imparts specificity while annealing with non-target template. Previously, allele specific PCR assays have been designed for identification of phytopathogenic fungi using the mismatch between in the closely non-target species at the 3' end in one or both PCR primers .

(Ma et al., 2007). Moreover, the single nucleotide polymorphism in *Fusarium* spp. has been widely used for designing PCR primers (Bogale et al., 2007; Mehl and Epstein, 2007) and for phylogenetic studies (O'Donnell et al., 1998; Fourie et al., 2009). Similar approach has been previously used for identification and detection of *Fusarium* spp. and *Fusarium oxysporum* (Kulik et al., 2008; Dita et al., 2010).

The PCR assay was specific to *Fusarium oxysporum* species complex and, positive amplification were detected in *Fusarium oxysporum* (two isolates of *F. oxysporum lycopersici*, *F. oxysporum ciceris*, *F. oxysporum melonis*) and no amplification was detected in non-target DNA sample. The results show that PCR assay developed in this study is specific for *Fusarium oxysporum* and formae specialis of *Fusarium oxysporum*. Furthermore, the validation of assay with sample DNA isolated from infected plant roots and soil sample indicates on field application of the PCR assay.

The PCR assay with genomic DNA isolated from *Fusarium oxysporum* isolates of infected plants roots and soil reproducibly produced a single DNA band of 495 bp, which shows the specificity of the primers. The PCR assay developed in this study has a number of advantages over other assay developed for identification and detection of *Fusarium oxysporum*. PCR assay developed in this study is simple, cost effective and could be applicable to large number of samples in compare to real-time PCR assay (De Sousa et al., 2015; Li et al., 2014; Luchi et al., 2016). By using 28S rDNA sequences Edel et al., (2000) developed specific primers for

*F. oxysporum* which amplified a DNA band of 70 bp. However, the product size was very small for detection and RFLP analysis and required 4% agarose gel for visualization. Moreover, while using this protocol for the characterization of a large collection of *F. oxysporum* isolates, Daniel Jiménez-Fernández et al. (2010) found non- target amplification. Similarly, another PCR assay was developed for specific detection of FOSC using ITS region by Mishra et al. (2003) that showed non-specific amplification. The validation of PCR assay for detection of DNA from infected plant roots and soil sample showed 90% specificity for detection FOC.

## Conclusion

The results of this study demonstrate that molecular techniques are important tools for specific, accurate and quick identification and detection of *Fusarium* spp. The PCR assay developed in this study could be used for evaluation of pathogenic *Fusarium oxysporum* in infected plants and soil and thus helpful in making strategies for the management of disease.

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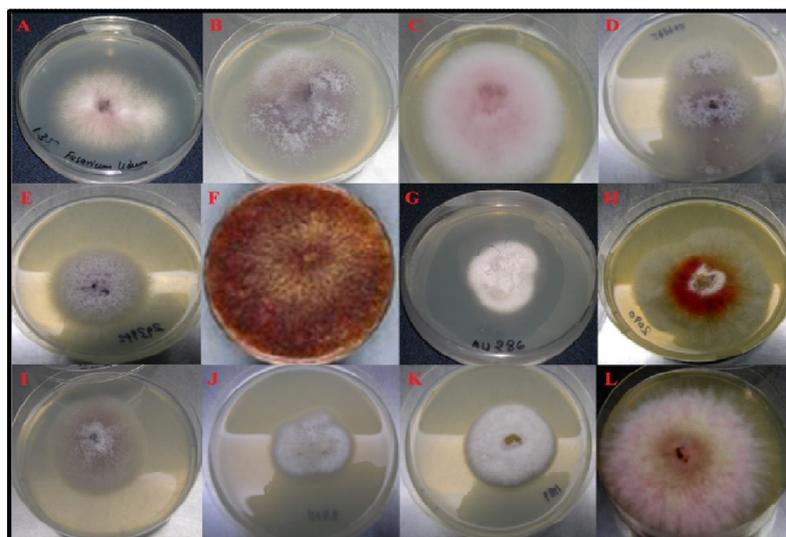
## Tables

Species and NBAIM Accession number	NCBI Accession Number (Ribosomal DNA)	NCBI Accession Number (Topoisomerase-II)	Isolated from
<i>Fusarium oxysporum</i> (NBAIM, F-1281)	EU214567	EU214576	Soil
<i>Fusarium oxysporum</i> (NBAIM, F- 4998)	EU214568	EU214570	Soil
<i>Fusarium oxysporum</i> f. sp.ciceris (NBAIM, F-2791)	EU214563	EU214577	Chickpea
<i>Fusarium udum</i> (NBAIM, F-138)	EU214566	EU214580	Pigeon pea
<i>Fusarium culmorum</i> (NBAIM, F- 349)	EU214562	EU214575	Cereals
<i>Fusarium solani</i> (NBAIM, F- 350)	EU214559	EU214571	Potato
<i>Fusarium moniliforme</i> (NBAIM, F-110)	EU214560	EU214572	Maize
<i>Fusarium oxysporum</i> f. sp. lycopersici (NABIM, F- 690)	EU214564	EU214578	Tomato
<i>Fusarium pulicaris</i> (NBAIM, F- 455)	EU214565	EU214579	Wheat
<i>Fusarium chlamydosporium</i> (NBAIM F-236)	EU214561	EU214574	Rice field
<i>Fusarium proliferatum</i> (NBAIM, F-344)	X94171 *	EU214581	Maize
<i>Fusarium cerealis</i> (NBAIM, F- 784)	EU214569		Maize
<i>Fusarium oxysporum</i> f. sp. melonis		AF546133 *	
<i>Gibberella pulicaris</i>		AF546125 *	
<i>Gibberella avenacea</i>		AF546129 *	
<i>Gibberella fujikuroi</i>		AF546131 *	

**Table 1. NCBI accession number of Fusarium isolates used in this study**

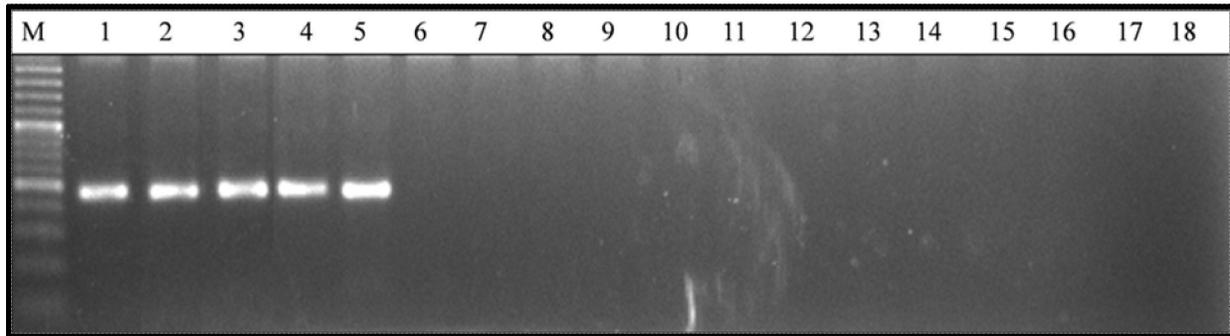
\* Accession no. used from NCBI

## Figures

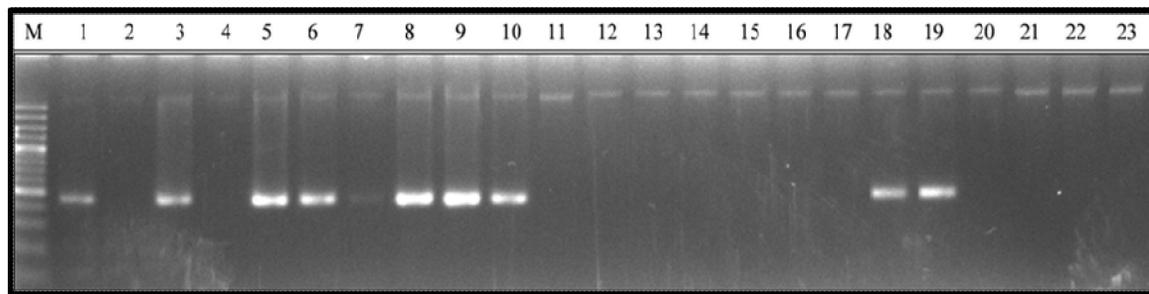


**Figure1.** Colony morphology of the Fusarium isolates A: *F. udum*, B: *F. oxysporum* f. sp. ciceri, C: *F. oxysporum* f. sp. lycopersici, D & E: *F. oxysporum*, F: *F. culmorum*, G: *F. proliferatum*, H: *F. chlamydosporium*, I: *F. sambucinum*, J: *F. moniliforme*, K: *F. solani*, L: *F. cerealis*





**Figure 3.** Detection of *Fusarium* species with *Fusarium oxysporum* specific PCR primers. M is 100 bp marker. Lane 1 and 2. *Fusarium oxysporum*, lane 3 *F. oxysporum ciceris*, lane 3 *F. oxysporum lycopersicum* and lane 5 *F. oxysporum meloni*. The five *Fusarium* isolates showed positive amplification and produced DNA band of 495 bp.



**Figure 4.** Validation of the PCR assay with sample DNA isolated from soil samples and infected plants roots. M is 100bp ladder. Lane 1-3 DNA isolated from wilted chickpea roots. Lane 4-6 DNA isolated from tomato plants roots. Lane 7-10 DNA isolated from soil. Lane 11-13 DNA isolated from wilted pigeon-pea roots, Lane 13-17 DNA isolated from wilted potato roots. Lane 17 to 19 DNA isolated from wilted melon. Lane 20-23 wilted maize plants root.