Direct Colony Nested-PCR for the Detection of *Fusarium oxysporum* f. sp. *Psidii* Causing Wilt Disease in *Psidium guajava* L.

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**Abstract**

Guava (*Psidium guajava* L.) is one of the important fruit crops which are grown in tropical and subtropical countries including India. Wilt is an important disease in guava which is caused by *Fusarium oxysporum* f. sp. *psidii* (Fosp) as a major obstacle for guava fruit production. It is manifested symptomatically with alterations in the development process such as premature shedding of leaves, pre-maturation of fruits, entire/whole tree defoliation and eventually death of the plant. In this study a colony PCR assay was developed for direct amplification of Internal Transcribed Spacer (ITS) region of fungal rDNA of Fop isolates. For this, two sets of primers were developed for amplification of ITS region. The sensitivity of both the primer pair was ranged up to 10^-9 and 10^-7 dilutions of the pure mycelium suspension. This study provides new insight for rapid, sensitive and specific molecular detection of Fop isolates, and also useful for earlier diagnostic for better management of guava wilt disease.

**Keywords:** *Fusarium oxysporum* f. sp. *psidii*, Diagnostic; ITS-PCR; Molecular detection; Wilt disease

**Introduction**

Guava (*Psidium guajava* L.), an arboreal shrub or a small tree, is one of the very productive and highly profitable fruit crop. It belongs to the family myrtaceae and is being grown all over the sub-tropical and tropical regions globally. It contains high dietary value and good flavor. Wilt of guava is one of the most important diseases of guava, especially in India. In earlier studies, a number of reports are available on wilt disease is caused by *Fusarium* species [1-4] but the most common fungi associated with wilt disease is *Fusarium oxysporum* f. sp. *psidii* [5, 6]. However, a need arise to identify *F. oxysporum* from guava wilt disease. The morphological and cultural characteristics are not sufficient for pathogen characterization as these are easily influenced by environmental factors and unable to emphasize genetic information of isolates [7,8]. Polymerase Chain Reaction (PCR) has been widely assessed in the field of mycology for diverse genetic analyses, specific detection, phylogenetic studies for identification and taxonomy studies [9]. These molecular studies require a number of laborious steps, including preparation of a DNA template by extraction of DNA from fungi [10]. Recently, Roberto et al. [11] reported that the omission of the DNA extraction procedure, which significantly decreases time and cost; and also avoids the risk of contamination during the DNA extraction process.

Therefore, Polymerase Chain Reaction (PCR) assay has been employed for molecular diagnostic of any pathogens [12,13]. However, the methods currently used more are often based on the analysis of ribosomal RNA (rRNA) gene (or rDNA) sequences that are universal and contain both conserved and variable regions, allowing discrimination at different taxonomic levels [7]. Thus, ribosomal RNA (rRNA) consists (18S-ITS1-5.8S-ITS2-28S) is widely used for molecular identification and phylogenetic analysis. It is the RNA component of the ribosome, the cell structure which provides the site of protein synthesis in all cells. It is present in all prokaryote and eukaryote that can be targeted for identification [7]. However, molecular approaches have been developed for fungal systematic studies, including RAPD analysis [14], specific diagnostic PCR primers [15]. Since these molecular studies require a number of laborious steps, including preparation of a DNA template by extraction of DNA from fungi [10]. Recently, culture-independent PCR assay has been developed for molecular detection of *F. oxysporum* from soil samples [16]. To avoid the time, labour, cost of PCR assay and also risk of contamination during genomic DNA preparation, a direct colony PCR for fungal cultures could be an ideal approach for early diagnosis of *F. oxysporum*. The aim of present study is to develop a colony PCR assay to amplify ITS region of *F. oxysporum* isolated from guava wilt disease.

**Materials and Methods**

**Isolation and maintenance of *Fusarium* isolates**

For the development of colony PCR assay, previously identified *Fusarium* isolates from guava wilt samples were used in this study (Table 1). The fungal isolates were deposited at National Agriculturally Important Microbial Culture Collection (NAIMCC), Mau, India and also maintained in Potato Dextrose Agar (PDA) medium at 4ºC in Molecular Diagnostics Laboratory, Central Institute for Subtropical Horticulture, Lucknow, India.
Table 1: Details of isolates of *Fusarium* spp. along with their geographic location used in present study

| Culture ID | Isolate        | Geographic origin | Colony colour  | Culture accession No. | GenBank accession No. |
|------------|----------------|-------------------|----------------|-----------------------|-----------------------|
| Fop-30     | *F. oxysporum* | Unno (U.P.)       | Light brown    | (NAIMCC-F-01998)      | KC357563              |
| Fop-44     | *F. oxysporum* | Farukhabad (U.P.) | Light pink     | (NAIMCC-F-02084)      | KC292502              |
| Fop-48     | *F. oxysporum* | Farukhabad (U.P.) | Pink           | (NAIMCC-F-00813)      | KC357565              |
| Fop-51     | *F. oxysporum* | Shamsabad (U.P.)  | Dark yellow    | (NAIMCC-F-02000)      | KC357562              |
| Fs-206     | *F. solani*    | Gopalganj (Bihar) | Pink           | -                     | -                     |
| Fop-84     | *F. oxysporum* | Kanpur (U.P.)     | Light brown    | (NAIMCC-F-00815)      | KC357561              |

Preparation of template for PCR assay

The purified and identified *F. oxysporum* isolates were grown on PDA medium at 28 ± 2°C for 5 days. Small amount of mycelia was scratched with a micropipette tip and suspended in 50 μl of 1X Tris-EDTA (pH 8.0) in 1.5 ml fresh eppendorf tube. It was vortexed thoroughly and also centrifuged at 10,000 rpm for 10 seconds and 1 μl of supernatant was used as template for PCR assay.

Design and validation of new species-specific primers

A set of new species-specific primers were designed by using PRIMER3 tools (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) based on ITS1-5.8S-ITS2 sequence of reference isolate of *F. oxysporum* f. sp. *psidii-44* (Fop-44). The reference sequence of Fop-44 was submitted to the GenBank and assigned under accession number KC292502 (Figure 1). The specificity of the primers was also validated by BLAST for searching the primer sequences (http://www.ncbi.nlm.nih.gov/BLAST). The list of primers for *F. oxysporum* used in this study was given in Table 2.

![Figure 1: BLAST result of representative isolate Fop-44 (NAIMCC-F-2084).](image)

**Table 2: List of primers used in present study**

| Primer | Sequence (5’→3’) | Expected product size (bp) | References |
|--------|------------------|---------------------------|------------|
| ITS1   | TCCGTAGGTGAACCTGCGG TCTCCTGGCTTTATTGATATGC | 570 | [17] |
| ITS4   | CCAGAGGACCCCCTAACTCT GCCTGAGGGTTGTAAATGACG | 230 | [18] |
| ITS1F  | GATCTCTTGGCTCTGGCATC CTCTCCAGTTGCGAGGTGT | - | This study |
| ITS1R  | GATCTCTTGGCTCTGGCATC CTCTCCAGTTGCGAGGTGT | - | This study |

While, the rapid detection of *Fusarium* species was performed using specific primers ITS1F and ITS1R [16]. Thus, we have designed and synthesized a set of new primers (172F/447R) for detection of *F. oxysporum* f. sp. *psidii* isolated from guava wilt disease. A concentration of 1 × 10^5 - 1 × 10^6 spore per PCR reaction was used in our experiments as a DNA template. The PCR reaction condition was followed as initial denaturation at 94°C for 3 min, subsequently by 29 cycles of denaturation 94°C for 1 min, annealing temperature of primers at 53°C (ITS1F/ITS1R) and extension at 72°C for 1 min. A final extension at 72°C for 5 min was used. While other PCR condition perform was similar except annealing temperature at 54.5°C (172F/447R). The 10 μl of PCR product was analyzed on 1.2% agarose gel with ethidium bromide at 8V/cm and also visualized under UViPro Transilluminator.

Whereas, nested PCR included the two rounds amplification using universal primers (ITS1/ITS4) for first round and two set of ITS based internal *Fusarium* species specific primers (ITS1F/ITS1R) and *F. oxysporum* f. sp. *psidii* specific primers (172F/447R) for second round. Thus, PCR was performed in 25 μl of PCR mixture, including 1 μl of PCR product of first round of PCR using ITS1 and ITS4 primers with two different set of primers 0.5 μM ITS1F/ITS1R and 0.5 μM of 172F/447R, a 0.5 μl mixture containing 2.5 μM of each dNTP and 1.25 U Taq DNA polymerase (Fermentas). The PCR program was followed at 94°C for 3 min, 30 cycles of 94°C for 1 min, 53°C (ITS1F/ITS1R) and 54.5°C (172F/447R) for 1 min, and 72°C for 1 min while the final extension at 72°C for 5 min.
Sensitivity of PCR assay

To determine the sensitivity PCR assay, we tested a dilution series of *F. oxysporum f. sp. psidii* isolates. We tested dilution series of spores ranging from $10^{-1}$ to $10^{-7}$. These diluted samples were used as template for PCR amplification using a set of primers (ITS1F-ITS1R and 172F-447R) with above PCR conditions.

Results and Discussion

Analysis of ITS region

Guava wilt is a serious disease affected the parts of plant such as stems, twigs, leaves, roots and fruits. The causal organism has been identified as *F. oxysporum f. sp. psidii*. In the present study, we developed a colony PCR for rapid, sensitive and cost effective molecular diagnostic of guava wilt pathogen, *F. oxysporum*. This PCR assay could be useful for reducing the time, cost and laboratory practice. There is also risk of other pathogen contamination during the extraction of genomic DNA for PCR assay [10,18,19]. We focused on the ITS regions of ribosomal genes (Figure 2) for the construction of primers that can be used to identify *Fusarium* spp. In this study, we selected ITS1-5.8S-ITS2 region for amplification using previously reported primer (ITS1/ITS4) and we obtained approximately 570 bp common fragment in all isolates. This indicates that there was no size variation in ITS+5.8S rDNA region among *Fusarium* isolates. As shown in Figure 3, expected amplicon of PCR product was obtained without any PCR inhibitor.

To detect *Fusarium* species and also *F. oxysporum*, a set of primer pairs (ITS1F/ITS1R and newly developed primers: 172F/447R) were used for amplification of conserved region of ITS. We successfully obtained expected size of amplicon in both 230 bp for *Fusarium* species and 280 bp for *Fop* isolates. Therefore, isolate Fs-206 was isolated from Gopalganj (Bihar) which was amplified using primers (ITS1F/ITS1R), while no amplification was obtained using primers (172F/447R). This isolate may be other species of *Fusarium*. The expected size of amplicon was shown in Figure 4A and Figure 4B. In earlier report, PCR assays have been developed for disease diagnosis, monitoring and forecasting programs [20]. A PCR assay has been developed based on amplification of ITS-5.8S rDNA using a set of primers such as ITS1F-ITS1R [16].

Nested PCR amplification and analysis

In the present study, a *nested PCR* was developed to amplify the variable region of ITS of all *F. oxysporum* which infect wilt disease in guava. Nested PCR uses two sets of primers, one set is external region
Sensitivity and sensitive and new

Also in nested PCR no simplify the process of detection was designed based on the of the detection method has to be at high levels in order to detect sequence with primers 172F/447R.

Specificity

agarose gel electrophoresis of representative PCR products of the serial dilution of the fungal strains using a set of primers ITS1F/ITS1R and 172F/447R, and the sensitivity of each set of the tests ranged up to 10^-6 and 10^-7, respectively (B). Lane M, 100-bp DNA ladder marker.

sensitivity of the primer sets ITS1F/ITSR and 172F/447R was shown by amplification of 230 and 280 bp products from second round of PCR amplification in all the isolates of Fusarium spp. (Figure 5A and 5B). Also in nested PCR no amplification was obtained in isolate Fs-206 with primers 172F/447R.

Figure 5: Agarose gel electrophoresis of Nested PCR-amplified products using the primers ITS1F/ITS1R (A) and 172F/447R (B). Lane M, 100-bp DNA ladder, Lane 1-4 and 6: F. oxysporum f. sp. psidii isolates, Lane 5: Fusarium solani isolates and Lane N: Negative control (dH2O).

In the present study, the sensitivity of detection of fungal pathogens can be appreciably enhanced by nested PCR. The conventional nested PCR reaction was shown by amplification of 230 and 280 bp products from Fusarium suspension of all isolates. It is a powerful tool for sensitive and specific molecular diagnostic of F. oxysporum. In earlier studies, nested PCR showed that microsatellite regions could be used for developing highly sensitive PCR detection systems for other fungal pathogens infecting various crops [21]. As the isolation based methods are time consuming, a nested PCR was developed to accelerate and simplify the process of detection was designed based on the genome-specific sequence of Fusarium.

Sensitivity and specificity of nested PCR assay

To determine the sensitivity and specificity of the direct colony PCR, a serial dilution of reference fungal isolate from corresponding species were tested. Figure 6A and Figure 6B showed the results of agarose gel electrophoresis of representative PCR products of the serial dilution of the fungal strains using a set of primers ITS1F/ITS1R and 172F/447R, and the sensitivity of each set of the tests ranged up to 10^-6 and 10^-7 dilutions, respectively. It could be used for amplification of F. oxysporum isolates directly from mycelium suspension. The sensitivity of the detection method has to be at high levels in order to detect fungal pathogens, especially those causing wilts. We can be further used developed nested PCR for specific detection of not only F. oxysporum but also other guava pathogens or similar approach can be applied for other pathogens.

In the previous report suggested that the possibility of direct PCR from hyphae of a limited number of mold species [19, 21]. Although, several simplification of DNA extraction protocols have been reported, most of them involve the mechanical disruption of mycelia, use of organic solvents, microwave irradiation and various centrifugation steps. The most of process is time-consuming usually DNA extraction and can hinder its effective deployment when either a large number samples need to be analyzed [22], disrupting the cell and need excessive amounts of material [23]. This approach was adopted for detection of Fusarium species that are difficult to identify based on the morphological characteristics. The developed nested PCR assay facilitates the detection and confirmation of Fusarium species and applied during pathogen control activities. The nested PCR assay is a rapid and low-cost, and efficient for the identification and discrimination of genus F. oxysporum f. sp. psidii.

Conclusion

In conclusion, a wide range of diagnostic techniques have been applied for detection, identification and quantification of fungal pathogens which are present in the infected plants, propagative plant materials and postharvest. This assay will be helpful in detection of pathogens directly from infected plant leaf or tissue to avoid DNA extraction protocol. However, time, specificity, sensitivity and cost-effectiveness are major challenge that may determine the suitability and choice of the diagnostic assay. Our newly designed nested PCR based assay could be useful for earlier, rapid, specific and sensitive molecular diagnostic of F. oxysporum f. sp. psidii that may be helpful in management of guava wilt.

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