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ORIGINAL ARTICLE

Detection of *Fusarium* wilt pathogens of *Psidium guajava* L. in soil using culture independent PCR (ciPCR)

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Abstract Traditional culturing methods take a long time for identification of pathogenic isolates. A protocol has been developed for the detection of *Fusarium* from soil samples in the early stage of infection. Seventeen soil samples from different locations were collected before the onset of rains to find out the presence of *Fusarium* spp. population present in the soil of guava orchards and to correlate its presence with incidence of wilt. A PCR based method was developed for the molecular characterization of *Fusarium* using *Fusarium* spp. specific primer. DNA extracted by this method was free from protein and other contaminations and the yield was sufficient for PCR amplification. The primer developed in this study was amplifying ~230 bp in all infected samples while not in healthy soil. The specificity and sensitivity of primer were tested on several *Fusarium* spp. and found that this primer was amplifying 10^{-6} dilution of the fungal DNA. The present study facilitates the rapid detection of *Fusarium* spp. from infected soil samples of guava collected from different agro-climatic regions in India. A rapid detection method for pathogens and a diagnostic assay for disease would facilitate an early detection of pathogen and lead to more effective control strategies.

1. Introduction

Guava (*Psidium guajava* Linn.), is considered as nutrient rich sources for humans globally as it contains vitamin C, pectin, calcium, phosphorous and trace elements. It has been grown in all regions of India while good quality of guava is produced in Allahabad, Uttar Pradesh.

Due to the wide occurrence of microbial pathogens the production is now decreasing drastically as about 177 different pathogens including fungi, bacteria, algae, nematodes and epiphyte, causing various pre and post-harvest diseases, are reported on various parts of guava plant (Misra and Prakash, 1990). *Fusarium* spp., one of the most important pathogens which causes wilt disease of guava (*P. guajava* L.) is a major threat to guava cultivation (Misra and Pandey, 1996; Misra, 2006). Varied chemical and non-chemical control measures have been applied to control the *Fusarium* spp., which has resulted in heterogeneity among the isolates (Misra, 2006; Misra

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and Gupta, 2007). *Fusarium* is a cosmopolitan soil borne fungus that colonizes into the vascular system of the host plant and thereby blocks the movement of water to the upper part of the host plant, which in turn causes yellowing, wilting and finally death of the host plant. It is very difficult to manage this disease in the early infection of the pathogen because symptoms are not visualized during the early stage of infection (Pandey et al., 2010). But recently molecular tools have become valuable for specific detection of the pathogen in the early stage of infection and analyzing of microbial populations or communities (Kumar and Anandaraj, 2006; Louws et al., 1999). Specific taxonomic groups can be identified and detected by using nucleic acid probes without culturing microbes. Previously, studies on the development of microbial communities required their isolation from soil samples, followed by a series of morphological and biochemical tests to identify them. Furthermore, culture dependent community structure analysis produces spatial and heavily biased results (Fatima et al., 2011). Molecular techniques allow to access the metabolic potential of microorganisms via the isolation of DNA from environmental samples, i.e., without the application of microbial culture techniques such as PCR using pathogen specific probes or oligo primers to detect the pathogens (Louws et al., 1999).

Various procedures for extracting microbial DNA from soil have been reported and these techniques employ extensive purification steps to ensure that the DNA is suitable for PCR (Tsai and Olson, 1991; Holben, 1994; Zhou et al., 1996; Miller et al., 1999; Roose-Amsaleg et al., 2001). Thus, the selection of an appropriate DNA extraction and purification procedure remains a major problem in the application of molecular techniques for studying of soil and sediment microbial communities.

In the present investigation an attempt was made to isolate fungal DNA from soil and to detect guava wilt pathogen '*Fusarium* spp.' in soil using specific primer. We described the development of PCR primer derived from ITS sequences for the specific detection of *Fusarium* spp. from soil. The specificity and sensitivity of the reaction were tested on a range of wild *Fusarium* species. The sensitivity of the PCR assay was determined, and the PCR protocols were tested for their ability to detect *Fusarium* in diseased soil samples and *Fusarium* isolates collected in the field.

2. Materials and methods

2.1. Sample collection and maintenance

Seventeen soil samples from different locations (Table 1) were collected before the onset of rains to find out the presence of *Fusarium* spp. population present in the soil of guava orchards and to correlate its presence with incidence of wilt. *Fusarium* spp. could be isolated from all the locations. All isolates were stored on potato dextrose agar (PDA) at 4 °C and maintained in collection at the Department of Molecular Plant Pathology, Central Institute for Subtropical Horticulture, Rehmankhera, Lucknow, UP, India.

2.2. DNA extraction method

DNA was prepared by the modified method of Fatima et al. (2011). Approximately 500 mg of soil samples were suspended in 0.5 ml DNA extraction buffer containing 200 mM Tris–HCl (pH 8.0), 0.02 M Na_2EDTA (pH 8.0), 5 M NaCl, 10% SDS, 10% CTAB, 10 µl of Proteinase K (10 mg/ml) and 1 M mannitol in centrifuge tubes and incubated at 65 °C in a water bath for 1 h with occasional stirring and homogenizing the slurry horizontally at 37 °C on a vortex mixture for 10 min. This was followed by centrifugation at 12,000 rpm for 15 min at 4 °C. The supernatant was extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) followed by centrifugation at 12,000 rpm at 4 °C. Aqueous layer of PCI was precipitated with 1/10th volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol and the pellet was recovered by centrifugation at 12,000 rpm and dried, dissolved in 50 µl of sterile water or 1× TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0), and used as a template for PCR amplification or stored at −20 °C until use.

Table 1 Soil samples collected from different guava orchards.

Sample ID	Location	Wilt (%)
W-1	1st Block, CISH, Lucknow (UP)	70–80
W-2	3rd Block, CISH, Lucknow (UP)	50–70
W-3	3rd Block, CISH, Lucknow (UP)	100
W-4	RB road campus, CISH, Lucknow (UP)	70–75
W-5	RB road campus, CISH, Lucknow (UP)	100
W-6	RB road campus, CISH, Lucknow (UP)	100
W-7	Puskar (Rajasthan)	100
W-8	Puskar (Rajasthan)	80–90
W-9	Puskar (Rajasthan)	100
W-10	Muzaffarnagar (UP)	100
W-11	Muzaffarnagar (UP)	100
W-12	Bihar sample (Bihar)	90
W-13	Bihar sample (Bihar)	100
W-14	Allahabad sample (UP)	60–70
W-15	Allahabad sample (UP)	100
W-16	Meadow orchard CISH, Lucknow	100
W-17	Meadow orchard CISH, Lucknow	100

2.3. Quantification of DNA

The concentration of the DNA was determined by following the absorbance at 260 nm (Sambrook et al., 1989). Sample dilution was adjusted to get the absorbance between 0.1 and 1.0. The ratio of the readings at 260 nm and 280 nm provides an estimate of the purity of DNA with respect to contaminants that absorb UV.

2.4. PCR amplification of 18S rDNA regions

The 18S rDNA regions were amplified using 18SF (5′-ATTGGAGGGCAAGTCTGGTG-3′) and 18SR (5′-CCGATC CCTAGTCGGCATAG-3′) primer pair (Einsele et al., 1997). Amplification was carried out in 25 µl reaction mixture containing 2.5 µl of 1× PCR buffer, 2.5 mM of $MgCl_2$, 0.5 mM of each dNTPs, 0.5 µM of each primers (10 pmol), 1.25U of *Taq* polymerase (Fermentas), 5% (v/v) of DMSO (Sigma–Aldrich Inc. USA) and 1 µl (1:10 dilution) of community DNA. Amplification was performed with an Eppendorf Thermal Cycler in a program comprising of 34 cycles of denaturation at 94 °C for 60 s, annealing at 53 °C for 60 s, and extension at 72 °C for 1.5 min with an initial denaturation of 5 min at