Saudi Journal of Biological Sciences (2013) **20**, 51–56

King Saud University

Saudi Journal of Biological Sciences

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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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Received 24 July 2012; revised 29 October 2012; accepted 31 October 2012
Available online 10 November 2012

KEYWORDS

Culture independent PCR;
Fusarium species;
Guava wilt;
ITS sequences

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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Peer review under responsibility of King Saud University.

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http://dx.doi.org/10.1016/j.sjbs.2012.10.007

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

94 °C before cycling and final extension of 5 min at 72 °C after cycling. The PCR products were analyzed by 1.2% agarose gel electrophoresis.

2.5. Taxon specific primer designing

Prior to primer designing, the core parameters used in the primer design include the following: (1) the primer length is between 18 bp and 25 bp, (2) the percentage of GC is between 35% and 60%, (3) the *Tm* of the primers is over 40 °C, which was calculated using standard PCR conditions. Species-specific primers were designed using *CLUSTALW* based on the DNA sequence retrieve from the NCBI database. The specificity of the primers for *Fusarium* spp. was also validated through BLAST searching the primer sequences against the NCBI sequence database (http://www.ncbi.nlm.nih.gov/BLAST).

2.6. Specific detection of Fusarium species

PCR amplification for detection of *Fusarium* spp. in soil was performed using the DNA isolated from soil as template. Reaction volume 25 µl contains 2.5 µl of 1× PCR buffer, 2.5 mM of $MgCl_2$, 0.5 mM of each dNTPs, 5% (v/v) of DMSO (Sigma–Aldrich Inc. USA), 0.5 µM of each primers ITS1F (5′-CCAGAGGACCCCCTAACTCT-3′) and ITS1R (5′-GCCT-GAGGGTTGTAATGACG-3′), 1.25U of *Taq* polymerase (Fermentas) and 1 µl (1:10 dilution) of community DNA. Amplification was performed with an Eppendorf Thermal Cycler. PCR was performed by 34 cycles of denaturation at 94 °C for 60 s, annealing at 52 °C for 45 s, and extension at 72 °C for 60 s with an initial denaturation of 5 min at 94 °C before cycling and final extension of 5 min at 72 °C after cycling. The gel was stained with Ethidium bromide and photographed on an UV transilluminator.

3. Results and discussion

3.1. Survey of wilt disease

A total of 17 soil samples were collected from different agro-ecological regions in India. Out of 17 samples, six samples were selected due to some of samples from same regions thus, it can be considered as same soil types and agroclimatic conditions. The wilt diseases were periodically recorded and *Fusarium oxysporum* f. sp. *psidii* were identified from all the locations (Mishra et al., 2012). The percentage of wilt symptoms in guava were periodically recorded and given in Table 1 (Misra and Pandey, 2000).

3.2. DNA extraction and PCR amplification of 18S rDNA

The main objective of soil DNA extraction is that the soil DNA should be free from those PCR inhibitors or the concentration of those inhibitors must be low enough so that they do not interfere in the activity of DNA polymerase used in PCR. Soil DNA when isolated directly would accumulate impurities from soil that are potential inhibitors of restriction enzymes or polymerase enzyme (Tsai and Olson, 1992). DNA extraction from soil has three requirements: extraction of high molecular weight DNA; extraction of DNA free from inhibitors for subsequent molecular biological manipulations to be performed; and representative lysis of microorganisms within the sample (Yeates et al., 1998). The extracted DNA from six soil samples were used for PCR amplification and also obtained 500 bp bands in all samples using universal primers based on 18S rDNA sequences (Figs. 1 and 2). No variations were observed in PCR products of these samples. A series of dilutions that contain the 500 bp DNA template were made to evaluate the sensitivity of the universal primer. All the diluted templates gave the target band compared to the negative (blank) control.

3.3. Quantification and digestion of soil DNA by TaqI and MspI

The protocol standardized in the present work yielded DNA with A260/280 ratio ranging from 1.53 to 2.14 and A260/230 ratio ranging from 1.27 to 1.97 (Table 2). A high 260/230 ratio (>2) is indicative of pure DNA, while a low ratio is indicative of humic acid contamination. The yield of soil DNA was ranging from 19.20 to 40.82 µg/g of soil samples by this method which proves an efficient extraction method. None of the crude extracts could be digested by *Eco*R I or *Hind* III. Furthermore, although a reduction in the brownish color of the crude DNA was observed after gel filtration, this DNA still could not be digested by *Eco*R I or *Hind* III (Tien et al., 1999). As we assume that there is enough purity in community DNA thus, it can be completely digested by *Eco*R I or *Hind* III. It may be the high concentration of humic acid in soils that may inhibit restriction enzymes. A total of six community DNAs were digested using two restrictions such as *Eco*R I and *Hind* III. It was completely digested, and indicated that these DNA samples were highly pure (Fig. 3a and b).

The yield of DNA ranging from 19.20 to 40.82 µg/g of soil samples by this method therefore proved an efficient extraction method like described by Roh et al. (2006). The humic materials in soil have similar size and charge characteristics to DNA resulting in their co-purification, evident by the extractions

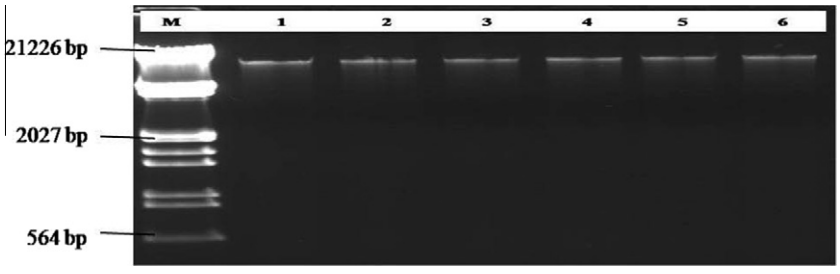

Figure 1 DNA extracted from six soil samples of wilted guava plant, Lane M: DNA size marker (DNA double digested with *Eco*RI and *Hind* III) (Fermentas). Lanes 1–6: Soil samples.

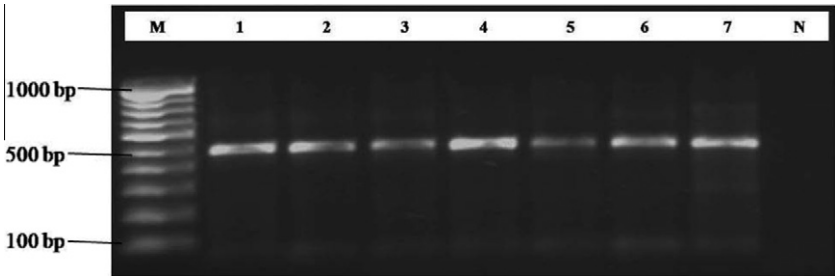

Figure 2 18S rDNA PCR amplification of uncultured fungi, Lane M: 100 bp DNA Ladder (Fermentas), Lanes 1–6 soil samples, Lane 7: Positive control, Lane N: Negative control.

Table 2 Amount and purity of DNA extracted from different soil samples.

Sample ID	Purity		Yield of DNA (μg/g)
	A_{260}/A_{280}	A_{260}/A_{230}	
W-1	1.84 ± 0.07	1.27 ± 0.00	26.56
W-2	1.86 ± 0.05	1.97 ± 0.02	40.82
W-3	1.87 ± 0.05	1.91 ± 0.07	35.62
W-4	1.85 ± 0.04	1.95 ± 0.00	38.64
W-5	1.53 ± 0.01	1.78 ± 0.01	33.03
W-6	2.13 ± 0.12	1.37 ± 0.02	19.20

being brown in color (Holben, 1994). Humic contaminants also interfere in DNA quantification since this exhibit absorbance at both 230 nm and at 260 nm, the latter used to quantitate DNA (Liesack et al., 1997; Olson, 1992). Zhou et al. (1996) have reported 0.91 and 1.35 for A260/230 and A260/280, respectively. While More et al. (1994) reported soil DNA yield as high as 11.8 and 5.2 μg g^{-1} in the bead beating and freeze thawing methods, respectively. The bead beating direct lysis method described by Yeates et al. (1998) yielded DNA between 15 and 23.5 μg/g of soil. Extraction methods using small soil samples ranging from 5 to 100 mg of soil have extracted 9–25 μg/g (Porteous and Armstrong, 1991), 12 μg/g (Tsai and Olson, 1992), 1–100 μg/g (Porteous et al., 1994) and 2.5–26.9 μg/g of soil (Zhou et al., 1996).

3.4. Specific detection of Fusarium spp. in soil samples

PCR-based assays have already been applied to microbial ecology and environmental sciences for detection and monitoring of microorganisms in rhizosphere, soils and diagnose plant diseases. In the present study, culture independent PCR techniques were used for the specific detection of *Fusarium* species. Out of 17 soil samples, six were selected from different locations (Table 1) and used to evaluate the specificity and sensitivity of the newly developed primer.

Among these samples one of them (W-1) was collected from the rhizosphere of partially wilted host plant that shows wilt symptoms and five of them (W-3, W-5, W-7, W-10 and W-13) were collected from the rhizosphere of completely wilted host plant. All six samples produced the target amplicon size of 230 bp (Fig. 4) which showed the presence of *Fusarium* spp. in the soil. Specificity and sensitivity of the primer are evaluated with several *Fusarium* isolates. The predicted amplicon size of 230 bp amplifies in all *Fusarium* isolates. No amplification was observed in control sample (Fig. 4). To determine the sensitivity of the newly designed primers based on ITS region they were tested with serial dilutions of genomic DNA from cultured *Fusarium* species. This PCR amplification was obtained up to 10^{-6} dilutions of DNA sample. ITS region was sequenced and found the homology with *F. oxysporum* (Mishra et al., 2012; Pandey et al., 2010). According to the results we can make a conclusion that this primer is sensitive enough to produce the target fragment even from one copy of the template DNA.

According to the specific detection results we can say that the primer we used was sensitive enough to detect the infection of *Fusarium* spp. in soil. Specificity and sensitivity of the primer pair were investigated using *Fusarium* isolates from these soil samples and we found 230 bp amplicon in all the *Fusarium* isolates tested. DNA detection tests from soil have been developed for several pathogens like *Phytophthora* species (Stammler and Seemuller, 1994; Grote et al., 2002; Hussain et al., 2005; Bilodeau et al., 2007), *Ralstonia solanacearum* (Kumar and Anandaraj, 2006), *Phytophthora cinnamomi* (O'Brien, 2008), *Histoplasma capsulatum* (Reid and Schafer, 1999) and *Agrobacterium tumefaciens* (Yang et al., 2011).

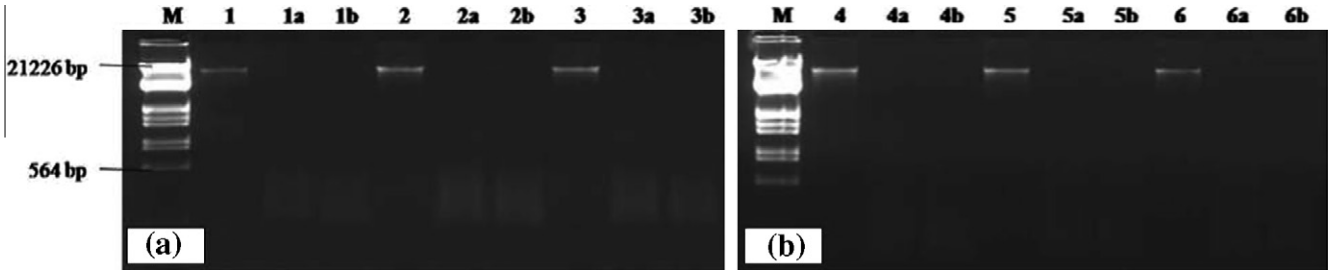

Figure 3 (a and b) Lane M: DNA size marker (DNA double digested with *EcoR*I and *Hind* III) (Fermentas), Lanes 1, 2, 3, 4, 5, 6 undigested soil DNA, Lanes la, 2a, 3a, 4a, 5a, 6a, *Taq*I digested soil DNA and Lanes 1b, 2b, 3b, 4b, 5b, 6b, soil DNA digested with *Msp*I.

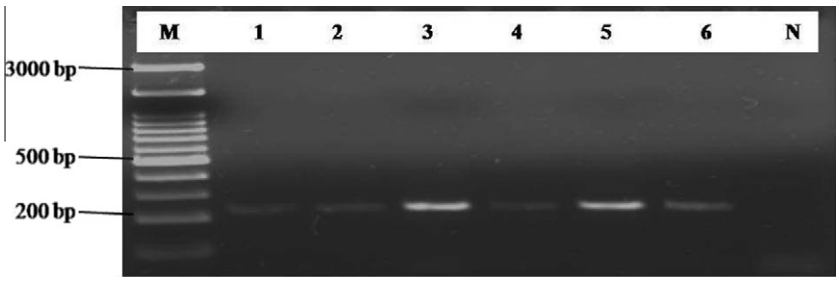

Figure 4 Specificity of PCR based assay for the detection of *Fusarium* spp. in soil samples. Lane M: 100 bp DNA Ladder (Biochem), Lane 1: W-1, Lane 2: W-3, Lane 3: W-5, Lane 4: W-7, Lane 5: W-10, Lane 6: W-13 soil samples, Lane N: control (DNA from healthy plant).

In present study, all these community DNAs were completely digested. Similarly, a number of reports are available on humic acids that pose a considerable problem and interfere in enzymatic manipulations of DNA and various protocols have been published in the past for the successful isolation of PCR amplifiable DNA from soil (Yeates and Gillings, 1998; Amorim et al., 2008). Due to the low level of contamination, DNA extracted by this method was easily digested by restriction enzymes *Taq*I and *Msp*I and this DNA can be successfully employed for metagenomic studies of soil from wilted guava plant.

4. Conclusion

The PCR assay reported here is a sensitive, specific, efficient, very convenient and easy to be developed in actual diagnosis technique for detecting *Fusarium* spp. from the soil. Because this PCR assay also has significant practical applications in the detection of *Fusarium* infected soils as well as isolates, it constitutes a powerful tool for controlling the dispersion of *Fusarium* and developing disease control strategies. Detection of *Fusarium* spp. from soil is a time consuming process by traditional isolation methods, which can delay disease management decisions. The PCR detection method reported here can provide a definitive diagnosis of this pathogen in soils within hours, and can be used to more accurately survey the occurrence and distribution of the pathogen in soil. Moreover, the molecular markers developed in this study have potential to overcome the problems associated with existing methods and can even be used by non-expert biologists.

Acknowledgements

We are thankful to Director and Head, Division of Crop Protection, Central Institute for Subtropical Horticulture, Rehmankhera, Lucknow for their encouragement and support. We gratefully acknowledged the Indian Council of Agricultural Research for Financial support in the form of Senior Research Fellowship and Vice Chancellor, Integral University for necessary facilities.

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