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A Highly Sensitive Nested-PCR Method Using a Single Closed Tube for the Detection of *Colletotrichum gloeosporioides* causing Greater Yam Anthracnose

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Abstract

Greater yam is an important species of yam grown in different parts of India. Anthracnose or die back disease caused by *Colletotrichum gloeosporioides* reduces the yield up to 90 per cent. The pathogen survives in the soil debris and transmits the disease to the next season through tubers. In this study, a sensitive method for the specific diagnosis of *C. gloeosporioides* in soil and planting material was developed. The standard nested PCR previously described was re-standardized to run a single nested PCR in a closed tube. The problems of cross contamination and the increased risk of PCR product contamination, while handling the product of first PCR could be avoided. This technique could be achieved by adjusting the concentration of primers and the annealing temperature along with the standardization of PCR cycles so as to produce a single amplicon without compromising the limit of detection and specificity. The lowest amount of DNA that could be determined by this method was 200 pg ml^{-1}.

Key words: Greater yam, anthracnose, diagnosis, nested PCR

Introduction

Yams (*Dioscorea* spp.), belonging to the family Dioscoreaceae, is a multi-species, polyploid and clonally propagated crop that is cultivated for its starchy tubers. West Africa accounts for about 95% of the world production and 93% of the total area (FAO, 2002). Anthracnose disease of yam (Fig.1) adversely affects yam production worldwide (Nwakiti and Arene, 1978; Simon, 1993). Traditionally, identification and classification of *Colletotrichum* species have been based on the morphological characters such as conidial shape and size, pathogenicity tests and biochemical approaches (Mun˜oz et al., 2000). The anthracnose causing pathogen is a ubiquitous pathogen infecting several crops. The pathogen *C. gloeosporioides* overwinters in leaves, stems, seeds and infected soil (Amusa and Alabi, 1996). Accurate species identification is critical to understand the epidemiology and to develop effective control of these diseases. Morphology based identification of *Colletotrichum* species has always been problematic, because there are few reliable characters and many of these characters are not stable and depends upon methods and experimental conditions. The use of PCR has now made it possible to amplify the low copy number of DNA molecules and their molecular detection, which needs high quality DNA free from contaminating proteins and polysaccharides. The development of a PCR-based molecular marker technique has become the method of choice for plant pathologists to characterize pathogens

Fig. 1. Anthracnose infected leaf

and to understand or elucidate the principles or factors underlying molecular co-evolution, population genetics, plant-fungus interactions, or pathogen evolution at molecular level (Leung et al., 1993; Milgroom and Fry, 1997; Okabe and Matsumoto, 2000). The ability to design PCR primers to target specific regions of DNA has led to rapid, accurate and sensitive detection and management of diseases caused by *Colletotrichum* spp. (Freeman et al., 2000; Martínez-Culebras et al., 2000; Ivey et al., 2004; Azim et al., 2010). There should be more sensitive and rapid procedure to diagnose the pathogen from planting sites as well as seed tubers where the level of pathogen is too low to be detected by standard PCR (Mithun et al., 2012a) The nested-PCR assay is a frequently used strategy when the sensitivity of detection is critical. However, standard nested PCR methods are time consuming and possess an increased risk of cross-contamination due to the manipulation of previously amplified material. The objective of the present study was to develop a *C. gloeosporioides* specific nested-PCR reaction that could be performed in a single closed tube.

Materials and Methods

Fungal isolates and plant material

The isolates of pathogen used in this study were taken from the fungal culture collection of Central Tuber Crops Research Institute (CTCRI), Thiruvananthapuram, India. For artificial infection, leaves of a susceptible variety of *Dioscorea alata*, Orissa Elite, was used. The plants hardened from pathogen free tissue culture plants were used for the experiments. The plant parts were inoculated with a spore suspension of *C. gloeosporioides* (8.5×10^5spores ml^{-1}) and incubated for 24- 48 h (100% relative humidity) and observed every 8h for the development of infection. For naturally infected samples, leaves showing symptoms of anthracnose were collected from the field of CTCRI. Soil samples were collected from the mounds where diseased plants were grown and harvested.

DNA extraction

Four fungal isolates, CG03, CG17, CG05 and CG20 were grown on potato dextrose broth at 27°C for 4–6 days. The mycelium was harvested and DNA was extracted as previously standardised (Mithun et al., 2012a). The DNA was extracted according to the protocol of Mithun et al. (2013a) from all plant and soil samples.

Characterization of ITS region

The genomic DNA was amplified using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primer pairs (White et al., 1990). Each 25 μl of PCR reaction consisted of 100 ng of template DNA, 100 μM each deoxynucleotide triphosphate, 20 ng of each primer, 1.5 mM $MgCl_2$, 1x Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.01% gelatin), 1U of Taq DNA polymerase (Bangalore GeNei, India). Amplifications were performed in BIORAD C1000 Thermal Cycler. The PCR profile was denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 58°C for 40 sec, and 72°C for 40 sec, then a final extension at 72°C for 5 min. Amplified products were resolved on a 1.5% agarose gel containing ethidium bromide and photograph was scanned through Gel Doc System (Alpha imager, Alpha Innotech, USA). The PCR products were eluted using QIA quick Gel extraction kit (QIAGEN, Tokyo, Japan) and cloned into the pGEM-T® vector (Promega, WI, USA). They were transformed into DH5α cells and sequenced by using T7 and SP6 promoter primers. The obtained sequences were analyzed using BLASTn program of the NCBI.

Primers for single tube nested PCR

The primers ITS1(TCCGTAGGTGAACCTGCGG) and ITS4

(TCCTCCGCTTATTG ATATGC) (White et al., 1990) and specific primers CgsF1/CgsR1 designed by the authors were used (Mithun et al., 2012b). These primers were designed from the region of high similarity within *Colletotrichum gloeosporioides* and the region of dissimilarity with all the other species of *Colletotrichum* genera available in NCBI database using Clustal W in the Bioedit package and Primer Premier software.

Standardization of single tube nested PCR

The single tube nested PCR was standardized with the following reaction parameters. Twenty five microlitres of PCR reaction consisted of 100 ng of template DNA, 100 μM each deoxynucleotide triphosphate, 0.1 f mol of universal primers, ITS1 and ITS4, 1 pmol of Specific Primers CgsF1and CgsR1, 1.5 mM $MgCl_2$, 1x Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.01% gelatin), 1.5U of Taq DNA polymerase (Bangalore GeNei, India). The PCR profile was 94°C for 2 min, followed by 15 cycles of 94°C for 30 sec, 57°C for 1 min 72°C for 1 min. Then another 30 cycles at 94°C for 30 sec, 67°C for 40 sec and 72°C for 40 sec, then a final extension at 72°C for 5 min. PCR was performed in Bio-Rad C-1000 thermal cycler in triplicates to ensure the reproducibility of the detection. To visualize the amplifications, 15 μl of the PCR products were separated by electrophoreses through 1.2% agarose gels, stained with EtBr and photographed in UV light on Alpha Imager (Alpha Innotech, USA).

Sensitivity tests

Primer Pairs were checked with the genomic DNA from many accessions of *C. gloeosporioides* from different parts of India isolated from *Dioscorea alata* and other hosts. In order to assess the primer sensitivity, a serial 10-fold dilution (100 ng μl^{-1} to 0.1 fg ml^{-1}) of the DNA was used.

Detection in artificially infected and naturally infected field samples

Plant parts of *Dioscorea alata* such as leaf, tuber and vine were inoculated with spore suspension of *C. gloeosporioides* and the total genomic DNA was isolated from them according to the protocol previously described (Mithun et al., 2012b; 2013b). In addition, yam tubers and leaves without symptoms, leaves with spots and necrosis were collected from the fields of CTCRI Farm and were used for DNA isolation and PCR assay. Soil DNA was also isolated from samples collected from the farms, where infected plants had been harvested and analyzed by the specific primers. DNA isolated from pure culture of *Colletotrichum gloeosporioides* served as positive control.

Results and Discussion

ITS characterization

PCR using the ITS 1/ITS 4 primer pair, yielded single amplicon of 580 bp in all the four isolates of *C. gloeosporioides.* This proves that designing primers from the rDNA region has greater reliability when compared to the use of random non defined probes or primers. The BLASTn search matched with *Colletotrichum gloeosporioides* isolate (Gene bank: FJ940734,) with maximum score.

PCR optimization, specificity and sensitivity

The objective of this study was to re-standardise the two step nested PCR protocol to a single tube nested PCR. Since then a couple of reports have described the usefulness of the method in the detection of plant pathogenic bacteria (Llop et al., 2000; Bertolini et al., 2003). Primers were selected so that the first pair in the nested PCR reaction should have an annealing temperature close to 70°C and should be at least 10°C higher than the second primer pair. In the standardised conditions the designed *C. gloeosporioides* specific CgsF1/CgsR1 gave an approximately 310-bp product in all isolates of *C. gloeosporioides* (Fig. 2). No species other than *C. gloeosporioides* was amplified with the designed primer, which indicated that no corresponding sites of the designed primer existed in the genomic DNA of any other organism. The lowest amount of DNA that could be determined by the method was 200 pg ml^{-1}.The sensitivity of this test was comparable to previously published PCR assays (Peter et al., 1992; Mithun et al., 2012a; 2013a). The principle on which this PCR assay is based, i.e. primers used in different concentrations and with sequences adjusted to different annealing temperatures, was first described by Orou et al. (1995) in the detection of mutations in human genetic studies.

Detection of pathogen from infected soil and tuber samples

Detection of pathogen was observed in artificially

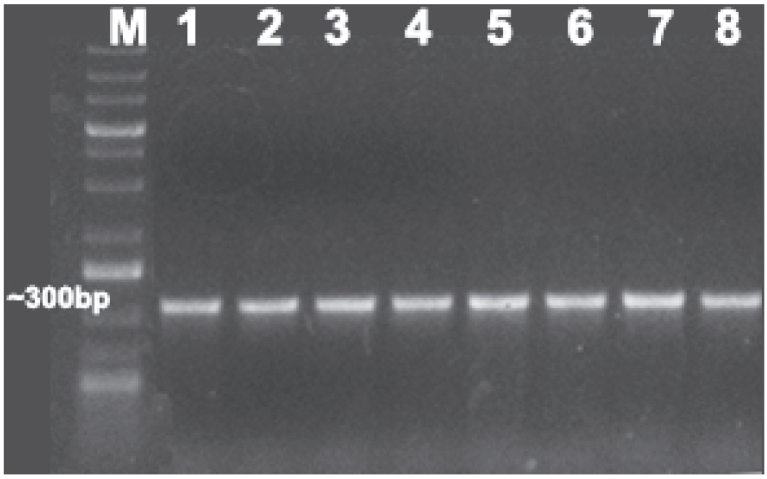

Fig. 2. Specific detection of pathogen using single tube nested PCR from isolates from different places of India

Lane No.	Isolate name and place of collection	State
M	100bp ladder	Fermentas
1	CG33 Kollam	Kerala
2	CG40 Pathanamthitta	Kerala
3	CG32 CTCRI Trivandrum	Kerala
4	CG01 Karnataka	Karnataka
5	CG19 Berhampur	Odhisa
6	CG17 Navasari	Gujarat
7	CG48 Umiam	Meghalaya
8	CG51 West Godavari	Andhra Pradesh

infected and naturally infected tubers and leaves of greater yam by the nested and conventional PCR. The DNA samples isolated from soil samples collected in the same farms with infection showed the specific detection of *C. gloeosporioides* in them. The nested PCR has shown to be more sensitive and could be used for the detection of pathogen even before the onset of visible symptoms. The detection time for the nested PCR was 16 h post inoculation. The control samples did not record any amplification.

Conclusion

The single tube nested PCR is more sensitive compared to previous reports and reduces the costs, the risk of contamination and the hands-on time required compared to standard nested-PCR protocols. Combined with an efficient DNA extraction protocol previously developed in our laboratory, this procedure could be used as a rapid and sensitive technique for the routine diagnosis of *Colletotrichum gloeosporioides* in planting material. The single tube nested PCR method was found to be useful in the identification and characterisation of *C. gloeosporioides* from yam.

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