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Rapid and Efficient Method for the Extraction of Genomic DNA from *Colletotrichum* spp. Suitable for PCR Analysis

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ABSTRACT

Contemporary approaches for the extraction of genomic DNA from *Colletotrichum* spp., the filamentous fungal pathogen genera infecting many important crop plants, are often expensive and yield poor quantity and quality of DNA. The high mucilage and polysaccharide content in this fungus add difficulties in genomic DNA isolation, and further downstream applications. We therefore investigated a new and rapid DNA isolation method, which involves inactivation of contaminant proteins by using guanidine hydrochloride/Proteinase K and precipitation of DNA using ice cold isopropanol. This protocol yielded 0.89 ± 0.10 μg DNA mg^{-1} of mycelium with purity ranges from 1.75-2.05 as confirmed by $A_{260/280}$ spectrophotometric readings. An advantage of this protocol is its compliance even without a refrigerated centrifuge. The new protocol can be successfully used for both mini and maxi preparation of genomic DNA which meet the quality parameters for further downstream processes like PCR-RAPD, AFLP, SSR and ITS amplification of the rDNA-ITS region.

Keywords: anthracnose, RAPD, ITS, rDNA, molecular detection, filamentous fungi, guanidine hydrochloride, restriction enzyme digestion

INTRODUCTION

The genus *Colletotrichum* is a member of the subdivision Deuteromycotina of the form order Melanconiales (Sutton 1992). Members of this genus are parasitic on several plants causing a group of diseases called anthracnose. *Colletotrichum* spp. is the causal agent of anthracnose and other diseases on leaves, stems and fruits of numerous plant species, including several important crops. Species of this genus have been used as models for studying infection strategies and host-parasite interactions (Perfect *et al*. 1999), defining the genetic basis of fungal symbiotic life styles (Rodríguez *et al*. 2000), and for developing infection (Fitzell *et al*. 1984) and disease forecasting systems (Dannenberger *et al*. 1984; Timmer and Zitko 1993; Monroe *et al*. 1997; Adaskaveg *et al*. 2002; Uddin *et al*. 2002). Accurate species identification is critical to understand the epidemiology and to develop effective control of these diseases. Morphologically based identification of *Colletotrichum* species has always been problematic, because there are few reliable characters and many of these characters are plastic, dependent 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 need high quality DNA free from contaminating proteins and polysaccharides. The development of a PCR-based molecular marker technique become the method of choice for plant pathologists to characterize pathogens and to understand or elucidate the principles or factors underlying molecular coevolution, 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 and Katan 1997; Martínez-Culebras *et al*. 2000; Ivey *et al*. 2004; Azim *et al*. 2010). A prerequisite for taking advantage of these methods is the ability to isolate genomic DNA of superior quality and quantity for analyzing through PCR, restriction enzyme digestion and subsequent Southern blot hybridization. The isolation of genomic DNA from filamentous fungi is often difficult due to high mucilage and protein content (Cassago *et al*. 2002). The lack of an easy DNA extraction method is commonly encountered in many laboratories, which are not equipped to handle toxic organic substances such as phenol and chloroform commonly used during DNA extraction (Zhang *et al*. 1996). Thus the method must limit the use of toxic chemicals as much as possible and should potentially remove inhibitory materials, i.e. polysaccharides, proteins, salts etc., which limit the sensitivity of different reactions in which isolated DNA is applied (Sambrook *et al*. 1989). In view of this, we made amendments in routinely used DNA extraction technique and developed a reliable DNA extraction protocol, which yields DNA of high quality and quantity from different species of *Colletotrichum* with relatively simple, rapid and has easy methodology which can be used for isolation of total DNA from mycelia scraped from agar plates, grown in liquid media or from infected plant parts.

The method that we developed for extracting DNA from the filamentous fungi *Colletotrichum* does not use organic reagents such as phenol, chloroform or isoamyl alcohol but yields DNA of high quality and purity suitable for restriction digestion and PCR-based analysis. The procedure involves homogenization of fungal mycelia using liquid nitrogen or micro pestle, inactivating proteins by SDS or proteinase K and precipitating polysaccharides and proteins in the presence of high salt (Kim *et al*. 1990). The removal of polysaccharides and other contaminating hydrates is based on the differential solubility of DNA versus the high-molecular weight polysaccharides in aqueous media (Rozman and Komel 1994).

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MATERIALS AND METHODS

Biological materials

Five species of *Colletotrichum* were used in this study (*Colletotrichum gloeosporioides*, *C. lindemuthianum*, *C. dematium*, *C. truncatum and C. capsisi*). The isolates were confirmed as *Colletotrichum* by comparing their morphology, spore shape and further by ITS sequence information. Mycelium of each species were grown on potato dextrose agar medium (PDA; 250 g L^{-1} potato, 20 g L^{-1} dextrose and 20 g L^{-1} agar) at 25°C for 1 week. Erlenmeyer flasks (250 mL) containing 100 mL of potato dextrose broth were inoculated with two 1-cm discs of actively growing cultures. The cultures were placed on a rotary shaker (100 rpm) and incubated at 27°C for 4-7 days or till approximately 100 mg wet weight of mycelia was obtained. Mycelia were harvested by filtration through cheesecloth, blotted dry with sterile paper towels and used immediately for DNA extraction. Likewise fungal mycelia scraped from agar plates, or from infected plant parts were also used for genomic DNA extraction.

Extraction of genomic DNA

50 to 100 mg of mycelia was ground to a fine powder using liquid nitrogen or into a fine paste using glass beads using a pre sterilized mortar and pestle. 1.5 mL of extraction buffer (100 mM Tris-HCl, 10 mM EDTA, 1M NaCl, 1% SDS, adjusted to pH 8.0 and autoclaved), prewarmed at 37°C and 5 µl of β-mercaptoethanol and 0.25 g of polyvinylpyrrolidone (Mr. 40,000) was added to the samples and ground once more. All the samples were transferred to 2.0-mL Eppendorf tubes and, 50 µL Proteinase K (10 mg/mL) was added to each of the sample, vortexed gently for 1min and was incubated in 37°C for 15 min and then at 65°C for another 20 min with gentle vortexing in between. After incubation, samples were centrifuged at 10,000 × g for 15 min, the pellet was discarded and supernatant was transferred to a fresh tube. 200 µl of 3M guanidine hydrochloride was added to the supernatant and the contents were mixed by gentle inversion 20-30 times and incubated at -20°C for 15 min or at 4°C for 30 min. Samples were centrifuged at 10,000 × g for 10 min and the supernatant was collected in fresh tube and the pellet was discarded. To the supernatant 100 µl of 3M sodium acetate was added, mixed well by inversion and incubated at -20 for 10 min or 4°C for 15-20 min. 150 µl of pre chilled isopropanol was added and samples were incubated at -20°C for 30 min or 4°C for 1 h. The samples were then centrifuged for 10 min at 10,000 × g and the supernatant was discarded. The pellet obtained was washed in 70% ethanol by centrifugation for 5 min at 7500 × g. The nucleic acid pellet obtained was air-dried and dissolved in about 25-50 µl of TE buffer (according to the size of the pellet). The nucleic acid dissolved in TE buffer were treated with 3 µl ribonuclease (RNase, 10 mg/ml), incubated at 37°C for 20 min and stored at –20°C or 4°C until use. The experiment was repeated thrice and result described as the mean of three independent experiments.

Measurement of amount and purity of DNA

The quality of the isolated DNA was analyzed by using 0.8% agarose gel containing 0.1 µg mL^{-1} ethidium bromide. DNA quantity was determined by calculating the absorbance ratio $A_{260/280}$ (Quant-iT™ assay, Invitrogen, USA). DNA purity was further confirmed by digestion with Eco RI, incubating reaction mixture at 37°C overnight and followed by 0.8% agarose gel electrophoresis.

RAPD analysis

Suitability of the isolated DNA for downstream analysis was assessed by a RAPD decamer primer OPG-07 (5′-GAACCTGCGG-3′) (Operon Technologies) based on its ability to reveal the variability in *Colletotrichum* genera (Ford *et al*. 2004). The PCR reaction mixtures were heated at an initial step of 94ºC for 2 min and then subjected to 35 cycles of {94ºC for 30 s, 48ºC for 2 min, 72ºC for 1 min 45 s}. After the last cycle, temperature was maintained at 72ºC for 8 min and final hold at 4°C. Amplified products were resolved on a 2% agarose gel containing 0.5 mg mL^{-1} ethidium bromide and image was visualized by ethidium bromide staining through Gel Doc System (Alpha imager).

ITS amplification

The isolated DNA was also used for amplification of the rDNA-ITS region using ITS1 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS2, (5′-GAAGGTGAAGTCGTAACAAGG-3′) primers (White *et al.* 1990). PCR reaction for the ITS amplification was performed in a 25 µL volume containing 20 ng of genomic DNA, 2.5 µL of 10X reaction buffer, 4 µL of 25 mM $MgCl_2$, 2.5 mM dNTPs, 200 ng of each primer. The reaction mixture was heated in an initial step of 94°C for 2 min and subjected to 35 cycles of {94°C for 30 s, 55°C for 1 min, 72°C for 1.5 min}. After the last cycle, the temperature was maintained at 72°C for 8 min and then final hold at 4°C. Amplified DNA was electrophoresed in a 1.5% agarose gel containing 0.5 mg mL^{-1} ethidium bromide and photographed on Gel Doc System (Alpha imager).

PCR amplification with genus-specific primers

Colletotrichum specific primer was used to amplify the genomic DNA of all the five isolates. The specific primer Col F: 5′-AAC CCTTTGTGAACATACCT-3′, Col R: 5′-CCACTCAGAAGA AACGTCGTT-3′ (Cano *et al*. 2004). Each 25 µL of PCR reaction consisted of 10 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, Bangalore). Amplifications were performed in Biorad C1000 Thermal Cycler. Mixtures were subjected to 35 cycles of {94ºC at 30 sec, 2 min at 52ºC and 1.30 sec at 72ºC}, a final extension of 72ºC for 1 min was given at the end of the cycles. Amplified products were resolved on a 1.5% agarose gel and the image was visualized by ethidium bromide staining through a Gel Doc System (Alpha imager).

RESULTS AND DISCUSSION

The method was standardized for five species of *Colletotrichum*. The standardized extraction method yielded good quantity of pure, high molecular weight DNA from mycelia harvested from liquid media, mycelia from agar plates and from infected plant parts. The method employed for grinding of mycelia, whether liquid nitrogen or glass bead showed no significant change in the quality of DNA isolated. The $A_{260/280}$ ratio ranged from 1.75-2.05, showing that DNA was of high purity. Successful amplification and variation among species was obtained by using primer OPG-7 in RAPD analysis, when tested with genomic DNA isolated using this new protocol from different species of *Colletotrichum* (**Fig. 1**). The purity of DNA was further confirmed by means of digestion by restriction enzyme Eco RI which completely digested the genomic DNA (**Fig. 2**) ITS amplification with extracted genomic DNA of isolates yielded a single product of ~ 700-bp (**Fig. 3**) in all of the isolate, demonstrating that DNA extracted with this new method is suitable for PCR-based analysis and further downstream processing. The genus specific primer yielded a single amplicon of ~150 bp in all the five isolates (**Fig. 4**). This proves the adeptness of this protocol for routine DNA isolation useful in identification and characterization of *Colletotrichum* spp. The aim of this work was to develop a highly reliable, simple and efficient method for isolation of genomic DNA from the phytopathogenic fungi *Colletotrichum* spp. The DNA extraction protocol described here is rapid and technically easy for preparing high molecular weight DNA without any ultra centrifugation or column purification steps. The most important steps in this protocol are the use of guanidine hydrochloride and proteinase K to remove protein completely and to use pre chilled isopropanol and ethanol which will retain the quality of DNA. These steps avoid the use of organic solvent (e.g., phenol, chloroform) that is normally used during DNA isolation in other methods, making it suitable for use in areas where facilities

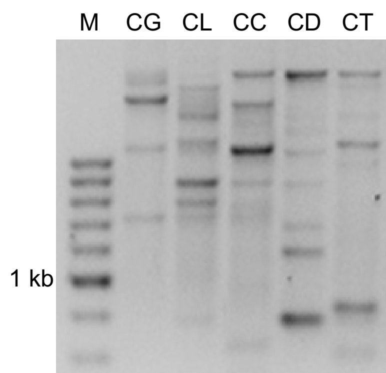

Fig. 1 RAPD analysis, with different *Colletotrichum* species. M: 1Kb DNA ladder, CG: *Colletotrichum gloeosporioides*, CL: *C. lindemuthianum*, CC: *C. capsisi*, CD: *C. dematium*, CT: *C. truncatum*.

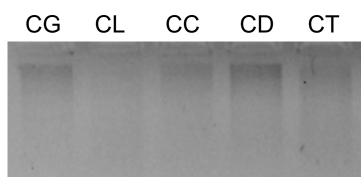

Fig. 2 Restriction digestion using *Eco*RI. CG: *Colletotrichum gloeosporioides*, CL: *C. lindemuthianum*, CC: *C. capsisi*, CD: *C. dematium*, CT: *C. truncatum*.

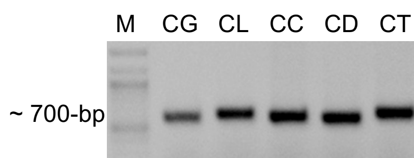

Fig. 3 ITS amplification with extracted genomic DNA. M: 100-bp DNA ladder, CG: *Colletotrichum gloeosporioides*, CL: *C. lindemuthianum*, CC: *C. capsisi*, CD: *C. dematium*, CT: *C. truncatum*.

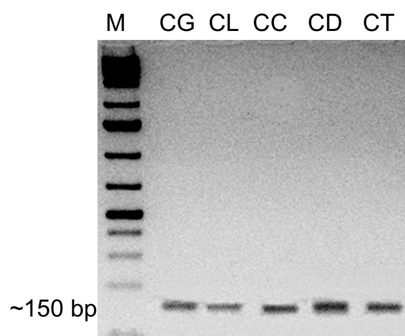

Fig. 4 PCR amplification using genus specific primers Col F/Col R. M: 1-kb DNA ladder, CG: *Colletotrichum gloeosporioides*, CL: *C. lindemuthianum*, CC: *C. capsisi*, CD: *C. dematium*, CT: *C. truncatum*.

for handling such chemical do not exist. Another major achievement is that this protocol can be used even in small labs were the facilities of -20 deep freezer or ultracentrifuge is unavailable. It can serve as a low cost and effective alternative to kit based DNA extraction methods or high end protocols. Since this protocol was successfully used to isolate DNA from five species of *Colletotrichum* it can easily be adapted to other species in this genus and even to other filamentous fungi or basidiomycetes which could be the next step of this research.

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