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Rapid and Efficient Method for the Extraction of Fungal and Oomycetes Genomic DNA

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ABSTRACT

An improved protocol for the isolation of high-quality DNA from fungi is described. The method involves inactivating proteins by SDS/proteinase K and precipitating polysaccharides in the presence of high salt. Further purification is based on differential solubility of DNA and high molecular weight polysaccharides in aqueous media. The procedure does not use the toxic and potentially hazardous chemical such as phenol and as many as 50 samples can be processed per day. The purity of isolated genomic DNA was confirmed by means of spectrophotometer analysis ($A_{260/280}$ ratio of 1.80-1.96), indicated a minimal presence of contaminating metabolites. The method yielded 0.55-0.92 µg DNA mg^{-1} freeze dried mycelia, when tested on three fungal species *Fusarium solani*, *Colletotrichum capsici*, and *Rhizoctonia solani* and two oomycetes *Phytophthora colocasiae* and *Pythium aphanidermatum*. The DNA was completely digested with *Eco*RI and *Hin*dIII. PCR-based technique such as random amplification of polymorphic DNA (RAPD), showed the DNA's compatibility with downstream applications.

Keywords: DNA extraction, fungi, RAPD, restriction enzyme digestion

INTRODUCTION

The development of PCR-based molecular marker techniques have become the method of choice for plant pathologists to characterize pathogens to understand or elucidate the principles or factors underlying molecular evolution, population genetics, plant fungus interactions, or pathogen evolution at molecular level (Leung *et al*. 1993; Milgroom and Fry 1997). A prerequisite for taking advantage of this method 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 lack of an easy DNA extraction method suited to conditions commonly encountered in many laboratories, which are not equipped to handle toxic organic substances such as phenol, commonly used during DNA extraction (Graham *et al*. 1994; 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, etc., which limit the sensitivity of different reactions in which isolated DNA is applied (Sambrook *et al*. 1989). The major challenge for isolation of DNA of good quality from fungi lies in breaking the rigid cell walls, as they are often resistant to traditional DNA extraction procedures (Fredricks *et al*. 2005). Fungal nucleases and high polysaccharide contents add to the difficulties in isolating DNA from filamentous fungi (Zhang *et al*. 1996; Muller *et al*. 1998). The methods include use of SDS, CTAB or proteinase K (Wilson 1990), SDS lysis (Syn and Swarup 2000), lysozyme or SDS (Flamm *et al*. 1984), high-speed cell disruption (Muller *et al*. 1998) and bead-vortexing or SDS lysis (Sambrook and Russel 2001). Additionally, some give poor yields of DNA, as cell walls or capsule are difficult to lyse (Muller *et al*. 1998).

The method that we developed for extracting DNA from filamentous fungi 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 pulverizing the mycelia by vortexing with glass beads as this reduces the shearing of DNA (Hollick *et al*. 2004), 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).

MATERIALS AND METHODS

Biological materials

This study included three fungal species (*Fusarium solani*, *Colletotrichum capsici* and *Rhizactonia solani*) and two oomycetes (*Phytophthora colocasiae* and *Pythium aphanidermatum*). Mycelium was 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 removed from actively growing cultures. The cultures were placed on a rotary shaker (100 rpm) and incubated at 27°C for 4-5 d. Mycelia were harvested by filtration through cheesecloth, blotted dry with sterile paper towels and used immediately for DNA extraction.

Extraction of genomic DNA

50 mg of mycelia was ground to a fine powder in 2 ml of extraction buffer (Tris-HCl, 100 mM; EDTA, 10 mM; NaCl, 1M; SDS, 1%; proteinase K, 0.05 mg ml^{-1}; pH 8.0) and 10% (v/v) glass beads. Samples were vortexed and incubated at 65°C for 30 min. After incubation, samples were centrifuged at 10,000 × *g* for 15 min and supernatant was transferred to a fresh tube. To the supernatant, 150 µl of 3 M guanidine hydrochloride was added and incubated at -20°C for 10 min. Samples were centrifuged at 10,000 × *g* for 10 min. After centrifugation supernatant was transferred to a fresh tube, and an equal volume of isopropanol was added. Samples were incubated at -20°C for 1 h. The samples were then centrifuged for 10 min at 10,000 × *g* and 70% ethanol was added and

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centrifuged once more for 10 min at 10,000 × *g*. The pellet obtained were air dried and dissolved in 50 µl of TE buffer (Tris-HCl, 10 mM, pH 8; EDTA, 1 mM). The nucleic acid dissolved in TE buffer was treated with 3 µl of RNase A (10 mg ml^{-1}), incubated at 37°C, and stored at -20°C until use.

Measurement of amount and purity of DNA

DNA was quantified by spectrophotometric measurement of UV absorption at 260 nm (Shimadzu UV-260). DNA was also quantified by means of 0.8% agarose gel electrophoresis followed by ethidium bromide visualization using 1-kbp DNA ladder (Fermentas) as DNA size marker. DNA purity was determined by calculating the absorbance ratio $A_{260/280}$. DNA purity was further confirmed by digestion with *Eco*RI and *Hin*dIII, incubating reaction mixture at 37°C for 3 h and followed by 0.8% agarose gel electrophoresis.

RAPD analysis of DNA

Suitability of the isolated DNA for downstream analysis was assessed by RAPD. PCR reactions for RAPD analysis were performed in a 25 µl volume containing 200 ng of genomic DNA, 2.5 µl of 10 x reaction buffers, 4 µl of 25 mM $MgCl_2$, 2 µl of 2.5 mM dNTPs, 200 ng screened random decamer oligonucleotide primer OPA-1 (5′-CAGGCCCTTC-3′). Amplification was performed in a thermal cycler (Techne progene). The reactions were heated in an initial step of 94°C for 2 min and subjected to 4 cycles of the following program 94°C for 30 s, 37°C for 1 min, 72°C for 2 min and further to 40 cycles of same program. After the last cycle, the temperature was maintained at 72°C for 8 min. Amplified DNA was electrophoresed in a 1.4% agarose gel containing 0.5 mg ml^{-1} ethidium bromide and photographed on UV transilluminator.

RESULTS AND DISCUSSION

The present extraction method yielded good quantity and quality of pure, high molecular weight DNA (**Table 1**, **Fig. 1**). The present extraction method yielded 0.55-0.92 µg DNA mg^{-1} freeze dried mycelium (**Table 1**). The $A_{260/280}$ ratio ranged from 1.80-1.96, showing that DNA was of high purity (**Table 1**). The purity of DNA was further confirmed by means of digestion by two restriction enzymes *Eco*RI and *Hin*dIII and monitoring the banding profile of digested DNA (**Fig. 2**). This indicated that isolated DNA was amenable for further downstream applications. Successful and reproducible amplification was obtained by using screened random decamer primer OPA-1 (5′-CAGGCCCTTC-3′) in RAPD analysis for all the samples (**Fig. 3**).

The purpose of this study was to improve and simplify the currently available DNA extraction method for filamentous fungi (van Burik *et al.* 1998; Haugland *et al.* 1999; Al-samarrari and Schmidt 2000) for use by researchers in developing countries. 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 key step of modified protocol is the use of guanidine hydrochloride and proteinase K to remove protein completely. This step precludes 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 for handling such chemical do not exist. These simple modifications make the method faster and provide another effective and rapid nuc-

Table 1 DNA yield by following present DNA extraction protocol.

Biological species	Mean yield of DNA (µg per mg mycelium) ± SD by presented DNA extraction protocol	$A_{260/280}$
C. capsici	0.55 ± 0.05	1.82 ± 0.03
P. colocasiae	0.76 ± 0.05	1.87 ± 0.06
F. solani	0.90 ± 0.2	1.89 ± 0.05
P. aphanidermatum	0.72 ± 0.1	1.90 ± 0.02
R. solani	0.79 ± 0.03	1.88 ± 0.03

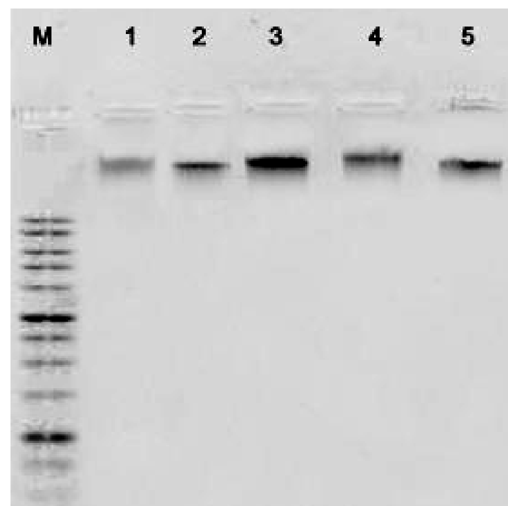

Fig. 1 Agarose gel electrophoresis of extracted genomic DNA. Lanes 1, 2, 3, 4, 5: DNA from *C. capsici*, *P. colocasiae*, *F. solani*, *P. aphanidermatum* and *R. solani*, respectively. Lane M: molecular weight marker (1 kb).

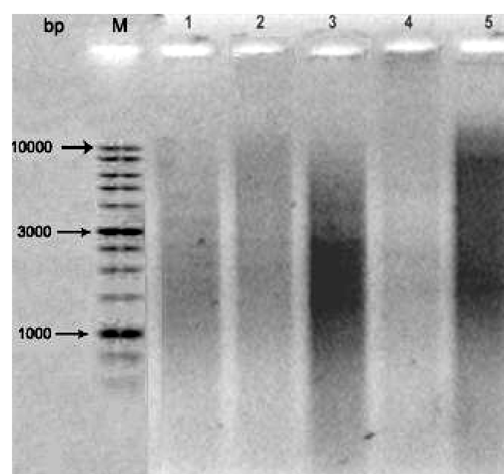

Fig. 2 Restriction digestion analysis of DNA prepared by using present method. Lanes 1, 2, 3, 4, 5: double digest with *Eco*RI and *Hin*dIII of DNA products from *C. capsici*, *P. colocasiae*, *F. solani*, *P. aphanidermatum* and *R. solani*, respectively. Lane M: molecular weight marker (1 kb).

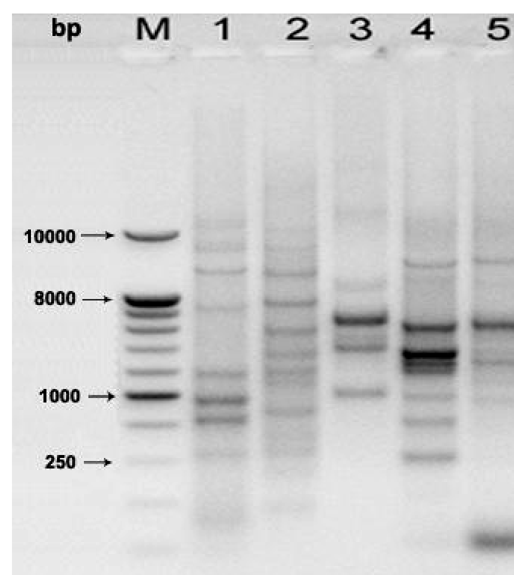

Fig. 3 RAPD assay conducted with the DNA prepared by means of the present method. Lanes 1, 2, 3, 4, 5: amplification product from *C. capsici*, *P. colocasiae*, *F. solani*, *P. aphanidermatum* and *R. solani* respectively. Lane M: molecular weight marker (1 kb).

leic acid extraction procedure. The protocol was successfully extended to recover DNA from *Sclerotium rolfsii* and *Trichoderma*, where the genomic DNA isolation is difficult due to high mucilage and protein content (Cassago *et al.* 2002) and thus we believe that it can easily be adapted to other filamentous fungi.

The amount and quality of the DNA obtained by this method were suitable for PCR amplification, restriction digestion and further downstream analysis. This DNA extraction method has several advantages: a) the number of DNA extraction steps is minimal and thus large number of samples can be processed in parallel without any contamination risk and loss of DNA, b) it is efficient and cost effective because as little as 50 mg of mycelium gives good DNA yields with small amount of chemicals and little equipment.

The method described here is rapid, reliable and use of this procedure resulting in extraction of DNA sufficiently pure for PCR and other molecular assay.

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