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Rapid Extraction of Genomic DNA from *Sclerotium rolfsii* Causing Collar Rot of *Amorphophallus*

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ABSTRACT

Current methods for the extraction of genomic DNA from *Sclerotium rolfsii*, a Basidiomycetous fungal pathogen, are often time-consuming 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 down stream applications. We therefore investigated a new and rapid DNA isolation method, which involves inactivation of contaminant proteins by using CTAB/Proteinase K and precipitation of polysaccharides and proteins in the presence of a high concentration of salt. This protocol yielded 0.89 ± 0.10 µg DNA mg^{-1} of mycelium from *S. rolfsii* with purity ranges from 1.83-1.95 as confirmed by $A_{260/280}$ and $A_{260/230}$ spectrophotometric readings. The new protocol can be successfully used for both small and large-scale preparation of genomic DNA which is highly suitable for further downstream processes like PCR-RAPD and ITS amplification of the rDNA-ITS region.

Keywords: genomic DNA, ITS, RAPD, *Sclerotium rolfsii*
Abbreviations: **CTAB**, cetyltrimethylammonium bromide; **EDTA**, hexadecyltri-methylammoniumbromide; **ITS**, internal transcribed spacer; **PVP**, polyvinylpolypyrrolidone; **RAPD**, randomly amplified polymorphic DNA; **RT**, Room temperature

INTRODUCTION

Elephant foot yam [*Amorphophallus paeonifolius* (Dennst.) Nicolson] is an important tuber crop of tropical and sub-tropical countries because of its yield potential and culinary properties (Ravindran and George 2008). In India, it has gained the status of a cash crop due to its high production potential, market acceptability and lucrative economic returns (Misra *et al*. 2002). Like other crops *Amorphophallus* is also prone to several field and storage diseases. Among all, the major field disease is collar rot caused by *Sclerotium rolfsii* Sacc.

S. rolfsii is a devastating soil-borne plant pathogenic fungus with a wide host range (Aycock 1966; Punja 1988). The fungus was placed in the form genus *Sclerotium* by Saccardo (1913), as it forms differentiated sclerotia and sterile mycelia. Although there are several other sclerotium-producing fungi, the fungi characterized by small tan to dark-brown or black spherical sclerotia with internally differentiated rind, cortex, and medulla were placed in the form genus *Sclerotium* (Punja and Rahe 1992). However, the teliomorphic state was discovered later (Punja 1998), confirming that the fungus was a basidiomycete. *S. rolfsii* usually causes collar rot, but spotted leaf rot with a single tiny sclerotium in the centre has also been reported (Singh and Pavgi 1965). The pathogen is soil-borne and passive type. Injury to the collar region during intercultural operations and poor drainage acts as a predisposing factor for infection by *S. rolfsii* and causing heavy yield loss (Misra and Nedunchezhiyan 2008).

The development of a PCR-based molecular marker technique 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; Okabe and Matsumoto 2000). A prerequisite for taking advantage of this method is the ability to isolate genomic DNA of superior quality and quantity for analyzing through PCR.

S. rolfsii genomic DNA isolation is difficult due to high mucilage and protein content (Cassago *et al*. 2002). In view of this, we made modifications in routinely used DNA extraction procedure and developed new DNA extraction protocol, which yields DNA of high quality and quantity from *S. rolfsii* suitable for PCR-based analysis.

The procedure involves inactivating proteins by CTAB/ proteinase K and precipitating polysaccharides and proteins in the presence of high salt potassium acetate (Kim *et al*. 1990; Cilliers *et al*. 2000). 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; Ristaina *et al*. 2007).

MATERIALS AND METHODS

Biological materials

Isolates of *S. rolfsii* used in this study were isolated from the collar region of *Amorphophallus* showing typical symptoms of collar rot disease caused by *S. rolfsii* from different regions of India (**Table 1**). *S. rolfsii* was confirmed in all isolates by comparing their morphology and further by ITS sequence information. For isolation tissue segments of 2-3 cm from collar rot infected area were ex-

Table 1 *Sclerotium rolfsii* strains used in RAPD analysis and region from which collected.

Accession number	Place of collection
S-1	CTCRI, Trivandrum, Kerala
S-2	Salepur, Bhubaneswar, Orissa
S-3	Calicut, Kerala
S-5	Rajmundary, Andra Pradesh
S-10	PDBC, Bangalore, Karnataka
S-13	Banaras, Uttar Pradesh

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cised from rotten margins. The segments were sterilized in 1% sodium hypochlorite for 2 min, rinsed twice with sterile distilled water and placed onto selective media (rye agar amended with 20 mg L^{-1} rifampicin, 200 mg L^{-1} vancomycin, 200 mg L^{-1} ampicillin, 68 mg L^{-1} pentachloronitrobenzene and 50 mg L^{-1} 50% benlate). Segments were incubated in Petri dishes for 2 to 3 days at 20°C and mycelia were then transferred and maintained on potato dextrose agar medium (PDA; 250 g L^{-1} potato, 20 g L^{-1} dextrose and 20 g L^{-1} agar).

For DNA isolation, Erlenmeyer flasks (250 ml) containing 100 ml of potato dextrose broth were inoculated with two 1-cm discs of actively growing cultures of *S. rolfsii*. The cultures were placed on a rotary shaker (100 revs min^{-1}) and incubated at 27°C for 2-3 d. Mycelia were harvested by filtration through cheesecloth, blotted dry with sterile paper towels and used immediately for DNA extraction.

Reagents and chemicals

All chemicals were analytical grade and purchased from Sigma and Merck.

The following buffers and solutions were prepared: Extraction buffer (100 mM Tris-HCl (pH 8); 20 mM EDTA (pH 8); 2 M NaCl; 3% CTAB (w/v); 1% PVP (Mr. 40,000); 2% β-mercaptoethanol (v/v)); phenol: chloroform (24:1); potassium acetate 7.5 M; proteinase K, 0.05 mg ml^{-1}; wash solution (15 mM ammonium acetate in 75% (v/v) ethanol]; TE buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8)).

Genomic DNA extraction

100 mg of mycelia was ground to a fine powder using liquid nitrogen. Prewarmed extraction buffer (1 ml) was added to the samples and it was ground once more in the buffer. All the samples were transferred to 2.0 ml Eppendorf tubes, 5 µL Proteinase K (10 mg/ml) was added. The tube was incubated in 37°C for 30 min and then at 65°C for another 30 min with frequent swirling. Samples were centrifuged at 10,000 × *g* for 10 min at RT and supernatant was transferred to a fresh Eppendorf tube. To the supernatant, 100 µl of 7.5 M potassium acetate was added and incubated at 4°C for 30 min. It was observed that addition of this solution and incubation of the samples for at least 15 min at 4°C increased the recovery of DNA yield with high quality. The samples were centrifuged at 13,000 × *g* for 10 min at RT; the supernatant was transferred to a fresh tube, an equal volume of chloroform: isoamyl-alcohol was added and mixed by gentle inversion 30-40 times. The samples were centrifuged at 10,000 × *g* for 10 min at RT. The supernatant was transferred to a fresh tube and precipitated with 2/3 volume of isopropanol. The precipitated nucleic acids were collected and washed twice with wash solution. The obtained nucleic acid pellet was air-dried until the ethanol was removed and dissolved in an appropriate amount of TE buffer (50-70 µL). The nucleic acid dissolved in TE buffer were treated with ribonuclease (RNase, 10 mg/ml), incubated at 37°C for 30 min and stored at –20°C until use. The experiment was repeated thrice and result described as the mean of three independent experiments.

DNA analysis

The quality of extracted DNA was analyzed by means of agarose gel electrophoresis (0.8%), followed by ethidium bromide staining (0.5 mg/ml). The purity of the DNA was estimated by spectrophotometry by calculating the $A_{260/280}$ and $A_{260/230}$ ratios, and the yield was estimated by measuring absorbance at 260 nm.

To check the suitability of extracted DNA for downstream analysis, RAPD analysis was done with screened OPA-8 primer, 5′-GTGACGTAGG-3′ (Imperial Bio Medics, Coralville, USA). Each PCR reaction mixture of 25 µL consisted of 200 ng genomic DNA, 2.5 µL of 10X reaction buffer, 4 µL of 25 mM $MgCl_2$, 2 µL of 2.5 mM dNTPs, 200 ng primer and 1U *Taq* DNA polymerase (Promega Corporation, USA). PCR amplification was performed in an Eppendorf thermal cycler (Eppendorf Ltd, Germany). The PCR reaction mixtures were heated at an initial step of 94°C for 2 min and then subjected to 35 cycles of {94°C for 30 s, 37°C for 1 min, 72°C for 1 min 45 s}. After the last cycle, the temperature was maintained at 72°C for 8 min. Amplified products were resolved on a 1.5% agarose gel containing 0.5 mg/ml ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (Alpha imager, Alpha Innotech, USA).

The isolated DNA was also used for amplification of the rDNA-ITS region of *S. rolfsii* using ITS4 (5′-TCCTCCGCTTATT GATATGC-3′) and ITS 6, (5′-GAAGGTGAAGTCGTAACAAGG -3′) primers (Jeeva *et al*. 2008). PCR reaction for the ITS amplification was performed in a 25 µL volume containing 200 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 30s, 52.5°C for 1 min, 72°C for 1.5 min}. After the last cycle, the temperature was maintained at 72°C for 8 min. Amplified DNA was electrophoresed in a 1.0% agarose gel containing 0.5 mg ml^{-1} EtBr and photographed on Gel Doc System (Alpha imager).

RESULTS AND DISCUSSION

The method was standardized for *S. rolfsii* collected from different regions of India (**Table 1**). The standardized extraction method yielded good quantity of pure, high molecular weight DNA (**Fig. 1**). The present extraction method yielded 0.89 ± 0.10 µg DNA mg^{-1} of *S. rolfsii* mycelium. The $A_{260/280}$ ratio ranged from 1.83-1.95, showing that DNA was of high purity. Successful amplification and variation was obtained by using primer OPA-8 in RAPD analysis,

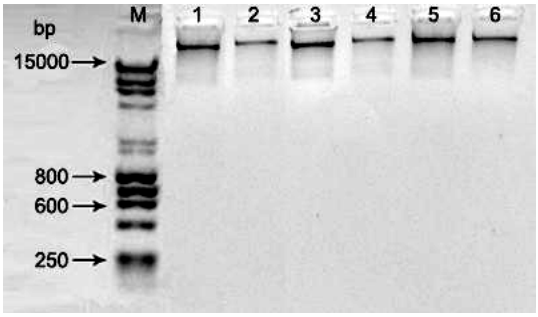

Fig. 1 *Sclerotium rolfsii* DNA prepared by using this method. Lanes 1-6 show DNA from different *S. rolfsii* strains collected from different regions of India (**Table 1**). Lane M: molecular weight marker (1.5 kb, Fermentas).

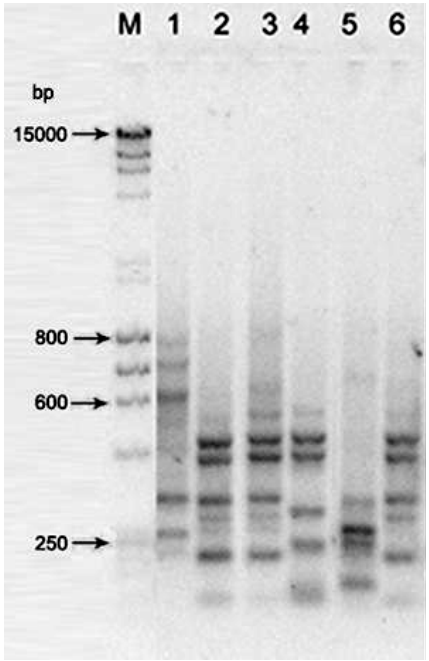

Fig. 2 RAPD assay conducted with the DNA prepared by this method. Lanes 1-6 show DNA from different *S. rolfsii* strains collected from different regions of India (**Table 1**). Lane M: molecular weight marker (1.5 kb, Fermentas).

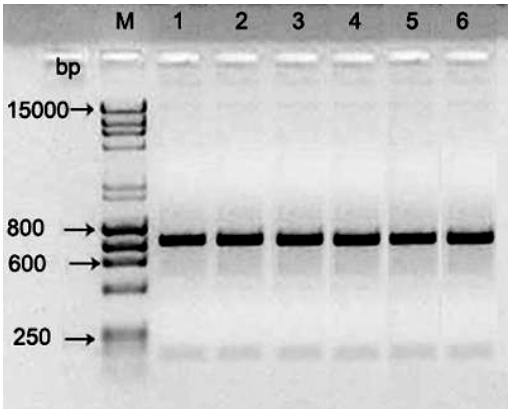

Fig. 3 ITS amplification conducted with the DNA prepared by this method. Lane M: molecular weight marker (1.5 kb, Fermentas).

when tested with extracted genomic DNA of different isolates of *S. rolfsii* (**Fig. 2**). ITS amplification with extracted genomic DNA of isolates yielded a single 700-bp product in both of the isolate, demonstrating that DNA extracted with this new method is suitable for PCR-based analysis (**Fig. 3**).

The purpose of this study was to improve and simplify the currently available DNA extraction method for *S. rolfsii* 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. Nevertheless, genomic DNA extracted from *S. rolfsii* by the procedure has been readily amplified by PCR. It is likely that this procedure could be applied to the examination of many other fungal cultures containing high mucilage and polysaccharides as it provides a rapid, reliable, and low-cost alternative to the existing DNA purification protocols used in research.

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