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A Simple and Efficient Method for Extracting *S. Rolfsii* DNA For PCR Based Diversity Studies

Male AS*, Kato F and Mukankusi CM

International Centre for Tropical Agriculture (CIAT), Pan Africa Bean Research Alliance (PABRA), Kampala, Uganda

Abstract

Present methods of extracting DNA from *Sclerotium rolfsii* use a lot of hazardous organic chemicals to extract high quality DNA. Extraction of the DNA is further complicated by exopolysaccharides that bind to the DNA making it mucilaginous. We developed a simple and efficient protocol for extracting DNA high quality from *S. rolfsii.* Our method uses a DNA extraction buffer that contains sodium dodecyl sulphate and proteinase K to inactivate proteins and high salt concentration to precipitate the exopolysaccharides. It uses neither phenol, chloroform nor isoamyl alcohol during the DNA extraction process. It also does not require freeze drying of the mycelia and grinding in using liquid nitrogen. Using our method, a sufficient amount of pure (mean A_{260}: A_{280}=1.91 ± 0.001) DNA (mean = 55.57 ± 0.002 ng/µl) was obtained from 100 mg of mycelia. The DNA was amenable to PCR amplification using inter-simple sequence repeat primers and primers targeting the internal transcribed spacer region of *S. rolfsii*. Our method will be very useful in laboratories that don't have access to liquid nitrogen and freeze drying facilities and will be a catalyst for PCR-based phylogenetic studies of this important pathogen of common bean.

Keywords: Exopolysaccharides; Internal transcribed spacer region; Inter-simple sequence repeat

Introduction

Common bean is the most widely consumed legume in the world [1]. It is grown and consumed by a large number of rural and urban poor in South America and Africa [2]. However, production is beset by a number of challenges especially diseases like root rots which have been implicated in yield losses of nearly 220000 tones [3]. *Sclerotium rolfsii* is a basidiomycetous fungus that causes southern blight in common bean (*Phaseolus vulgaris L)*. The asexual stage (anamorph) manifests as abundant mycelia on infected host tissue usually 4 days after infection under warm and humid conditions. Seven days after infection, the sclerotia develop and they can remain viable for several years in soil, potting media or plant debris. The sexual stage (teleomorph) is rarely seen but in this state, the pathogen produces a structure called basidium in which meiosis occurs [4]. DNA extraction is a core function of most pathology laboratories. A number of protocols for extracting DNA from plant pathogenic fungi have been developed [5-9]. However, most of them are modifications of the original protocols designed to extract DNA from plants [10-13]. Additional protocols have been developed to extract DNA from plant, fungal and bacterial specimen [14,15]. Despite the abundance of protocols for extracting DNA from plant pathogenic fungi, most of them are generic and therefore do not work for microorganisms whose DNA forms complexes with other compounds. Extraction of DNA from *S. rolfsii* is especially problematic because the fungus produces exopolysaccharides (EPS). EPS have similar precipitation behavior like other biological molecules like DNA and RNA and therefore adversely affect recovery of DNA from *S. rolfsii* [16]. Consequently, a number of protocols have been developed to deal with EPS. For instance, [16,17] used toxic organic compounds like chloroform, isoamyl alcohol and phenol to minimize EPS and improve the quality and quantity of DNA extracted. However, much as their methods yield sufficient amounts of DNA, they are unsafe for laboratories that do not have fume hoods for the safe use of hazardous organic compounds, access to liquid nitrogen and freeze drying facilities. Our experience with safer methods of DNA extraction using kits (DNeasy, Zymo) has shown that the yields are extremely low possibly because the columns are not meant to be used with microorganisms that produce high amounts of EPS like *S. rolfsii* and *X. campestris*. We therefore set out to develop a fast and safe alternative for extracting DNA from *S. rolfsii*. We also tested the suitability of the extracted DNA for PCR based analyses using inter-simple sequence repeat (ISSR) and the ribosomal DNA-internal transcribed spacer (ITS) region primers.

Materials and Methods

Plant sample collection, pathogen isolation and purification

Infected bean plants showing symptoms of *Sclerotium* root rots were collected from the fields during the cropping season of February - April 2018 at Kawanda, Uganda. Isolation of the fungus was conducted at the CIAT-Uganda laboratory at NARL-Kawanda by washing the infected roots under running tap water to remove soil and debris and blotted dry using sterile tissue (Kleen ®, Kampala SITI Industries, Uganda). Roots were then cut into small pieces ~5 mm long and placed on water agar (WA) media (13.2 g/L, Biolife Italiana, Milano, Italy) and incubated for 24 h in darkness. Emerging fungal colonies were then transferred from the WA media to potato dextrose agar (PDA), [19.5 g/L, Sigma-Aldrich Chemistry GmbH, Steinheim, Germany] supplemented with 0.03g/L Rifamycin (Sigma-Aldrich Chemistry GmbH, Steinheim, Germany)]. Actively growing agar plugs of ~3 mm of suspected *Sclerotium* spp. were again grown on WA for 24 h in darkness following single hyphal isolation. Hyphal tipping was done using a sterile needle under a stereo microscope (Leica, Wild M3B, Leica systems, Germany), and cultures grown on PDA containing 0.03 g/L of rifamycin. The resulting pure cultures were incubated in darkness at room temperature for 10 days. Nine *S. rolfsii* plugs were recovered from the PDA and inoculated in 30 ml of sterilized clarified V8 broth (200 ml of V8 juice; 3 g of calcium carbonate and 800 ml of distilled water) for 10 days at room temperature. Thereafter, 0.1 g of the mycelia were harvested using some sterile forceps and blotted on a napkin and paced in a 1.5 ml tube.

***Corresponding author:** Male Allan Ssekamattec, International Centre for Tropical Agriculture (CIAT), Pan Africa Bean Research Alliance (PABRA), P. O. Box 6247, Kampala, Uganda, Tel: +57 2 4450000; Email: a.m.ssekamate@cgiar.org

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DNA extraction and visualization

A DNA extraction buffer (2M NaCl, 0.2M EDTA, 0.2M Tris-HCl [pH 8] and 1% SDS) was made and autoclaved. After autoclaving, 500 µl of the buffer was added to the mycelia (0.1 g) in a mortar and ground with a pestle in the presence of sterile acid washed sand. Thereafter, 1.5 µl of 20 mg/ml proteinase K was added, the solution vortexed and incubated in a water bath at 65°C for 30 min. Half volume (250 µl) of a mixture of 1.2 M NaCl and 5 M ammonium acetate (precipitation solution) was added and the solution incubated on ice for 5 min. The solution was then centrifuged at 13000 rpm for 5 min, the supernatant (400 µl) transferred to a new 1.5 ml tube and an equal volume of ice cold isopropanol added. The mixture was incubated at -20°C for 2 h and centrifuged at 13000 rpm for 10min to recover the DNA pellet. The pellet was then washed twice with 70% ethanol and left to dry at room temperature for 20 min. It was finally dissolved in 100 µl 1X TE buffer (10 mM Tris-HC1 [pH 8], 1 mM EDTA) and treated with 5 µl RNase A (20 mg/ml) for 30 min. Quality of the DNA extracted was checked by electrophoresis on 1% agarose gel in 1X TAE (0.04 M Tris – Acetate, 0.001 M EDTA) at 100 V for 40 min. The gel was then stained in 0.5 µg/ml ethidium bromide for 40 min and the image captured using the G: BOX gel documentation system (Syngene, Fredrick, MD).

DNA analysis

DNA was quantified using a DyNa Quant DQ300 fluorometer (Hoefer, Holliston, MA) with Hoechst 33342 as fluorescent dye following the manufacturers recommendations. Prior to quantification of the DNA, a standard curve from duplicate readings of various concentrations of 0.5 µg/µl calf thymus DNA was determined. The standard curve was estimated using the GraphPad prism software v.5.00 (GraphPad Software, La Jolla California USA). Afterwards, duplicate readings of the fluorescence of the extracted DNA were taken the average of which was used to estimate the DNA concentration by extrapolating from the standard curve. Purity of the DNA was ascertained by the ratio of the absorbance at 260nm to the absorbance at 280 nm (A_{260}: A_{280}) using the Nano Drop 2000 c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The suitability of the extracted for PCR of the rDNA-ITS region of *S. rolfsii* was checked using the primers ITS4 (5′-TCCTCCGCTTATT GATATGC-3′) and ITS1 (5'-TCCCTAGGTGAACCTGCGG-3'). A 20 µl reaction volume consisting of 2 mM $MgCl_2$, 0.2mM dNTPs, 0.5U Top DNA polymerase (Bioneer, Daejeon, Korea), 0.3 µM of each of the primer and 1X PCR buffer was set up and amplified in a Techne Prime thermal cycler (Cole-Parmer, Staffordshire, UK) using a thermal profile that consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s with a final extension at 72°C for 5 min. The primer ISSR 2 (5'-GAC$(CAC)_4$CA-3') was used to amplify random segments of the *S. rolfsii* genome. Similarly, a 20 µl PCR reaction volume consisting of 3 mM $MgCl_2$, 0.4 mM dNTPs, 1U *Top* DNA polymerase (Bioneer, Daejeon, Korea), 0.5 µM of each the primer and 1X PCR buffer was also amplified in the Techne Prime thermal cycler using a thermal profile that consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 53°C for 60s and extension at 72°C for 60 s with a final extension at 72°C for 5 min. All the amplicons were resolved on 1.5% agarose gels in 1X TAE buffer (0.04 M Tris - Acetate 0.001 M EDTA) in 1X TAE at 70 V for 80 min. The gels were then stained in 0.5 µg/ml ethidium bromide for 40 min and the images captured using the Syngene G: BOX gel documentation system.

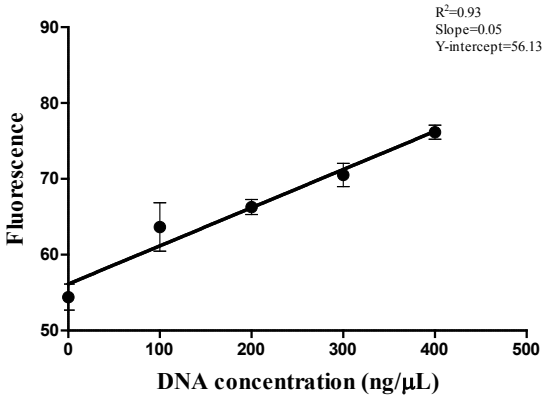

Figure 1: Standard curve showing fluorescence values at different concentrations of DNA.

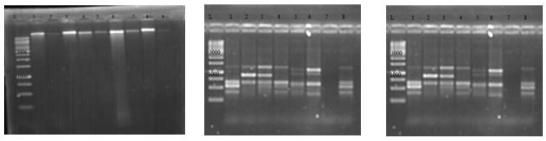

Figure 2: Ethidium bromide stained agarose gels showing quality of DNA extracted (Left), amplification profiles obtained with ISSR marker RAMS 3 (middle) and amplicons obtained using the ITS primers (right). In all images, L is the 1kb DNA ladder (Thermo Fisher Scientific, Wilmington, DE).

Sample ID	Fluorescence reading 1	Fluorescence reading 2	Mean of Fluorescent readings	DNA Concentration (ng/µl)	A_{260}: A_{280}
1	51.72	52.48	52.1	55.68	1.95
2	51.58	58.12	54.85	55.84	1.94
3	47.54	46.72	47.13	55.38	1.95
4	49.24	51.12	50.18	55.56	1.96
5	42.5	41.37	41.94	55.07	1.82
6	45.04	45.93	45.49	55.28	1.91
7	57.68	56.78	57.23	55.98	1.9
8	56.18	51.56	53.87	55.78	1.9

Table 1: Values of *S. rolfsii* DNA extracted using our method.

Result

Our method produced sufficient amounts of highly pure (mean A_{260}: A_{280}=1.91 ± 0.001), easily soluble and non-viscous DNA (Table 1). The method of quantifying DNA using the Hoechst dye (Figure 1) was also very accurate (R^2=0.93). In addition, the DNA showed very minimum degradation and it was suitable for PCR analysis using the ISSR and ITS primers (Figure 2).

Discussion

The main objective of this study was to develop a protocol for extracting high quality DNA from *S. rolfsii* and to assess its suitability for analysis using PCR. The DNA obtained was free of EPS. This is supported by values of the A_{260}: A_{280} ratio which were within the limits (1.8-2) of pure DNA. Purity was achieved by using a very high salt (NaCl) concentration in the DNA extraction buffer which precipitated most of the EPS but kept the DNA in solution. To ensure that any remnant EPS did not co-precipitate with the DNA, a precipitation solution containing a high concentration of NaCl was used and the EPS free DNA was precipitated from the solution using ammonium

acetate. Since polysaccharides also make DNA viscous, the appearance of a clear, non-viscous solution also indicated absence of EPS. It has been reported [18] that SDS and high salt concentrations remove proteins, hydrates and polysaccharides. High concentrations of NaCl and SDS effectively remove scleroglucan obviating the need of either growing *S. rolfsii* on special media that minimizes its production or washing the mycelia with hot water during the DNA extraction process [16] The DNA was suitable for PCR assays as shown by the amplicons obtained using the ITS and ISSR markers. Although it has been reported that concentrations of NaCl >25 mM inhibit PCR [19], our PCR analyses were successful despite the very high concentrations of NaCl used. Since washing the DNA pellet with ethanol removes NaCl [20], multiple washes with ethanol greatly reduce the concentration of NaCl. High concentration of EDTA prevented DNA degradation. Available methods [16,17] for the extraction of DNA from *S. rolfsii* use much lower concentrations of EDTA than our method but also require freeze drying and grinding mycelia using liquid nitrogen. EDTA is a chelating agent that deprives DNases of divalent metal ions. Since the divalent ions are cofactors for the action of DNases, the DNA degrading enzymes are rendered inactive. In the absence of liquid nitrogen, very high concentrations of EDTA enable the recovery of good quality DNA.

Conclusion

A protocol that does not require freeze drying of mycelia and toxic organic compounds like phenol, chloroform or isoamyl alcohol to extract high quality DNA from *S. rolfsii* for routine analysis by PCR was developed. Our methodology will be useful for research groups which conduct genomic DNA extraction from *S. rolfsii* and other microorganisms that produce large amounts of exopolysaccharides for PCR based phylogenetic studies.

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