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A New Method for Genomic DNA Extraction from Sclerotia of *Sclerotium rolfsii* Inciting Collar Rot of Lentil for Genomic Investigations

Purnima Singh[1], Sushma Tiwari[2], Prerana Parihar[1], Reeti Singh[1], Niraj Tripathi[3], R.K. Pandya[1], M.K. Tripathi[2]

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ABSTRACT

Background: The prevailing methods for the genomic DNA extraction of *Sclerotium rolfsii* from its mycelium mat is often time consuming and yields poor quality and quantity of genomic DNA owing to presence of the outrageous magnitude of mucilage and polysaccharides.
Methods: A new fast track method for DNA isolation from the resting structure (sclerotia) of *S. rolfsii* by modifying CTAB method deprived of supplementation of proteinase K has been standardized.
Result: The protocol produced 500ng DNA from sclerotia with purity vacillating from 1.7 to 1.9 as confirmed by A_{260}/A_{280} and A_{260}/A_{230} spectrophotometric documentation. The DNA extracted from sclerotia commissioning protocol was efficaciously used for the further downstream reactions/process like PCR-RAPD, PCR-ISSR and ITS amplification of rDNA-ITS region.

Key words: DNA extraction, ISSRITS, PCR, RAPD, Sclerotia, *Sclerotium rolfsii.*

Abbreviations: CTAB: Cetylytrimethylammonium bromide; DNA; ISSR: Inter simple sequence repeat; ITS: Internal transcribed spacer; PCR: Polymerase chain reaction; RAPD: Randomly amplified polymorphic.

INTRODUCTION

Lentil (*Lens culinaris* Medik.) originated from South Western Asia as early as 6000 B.C. It is part of human diet since Neolithic times. Archaeological corroborations have shown that lentils were consumed since 9500 to 13000 years ago (Liber *et al.,* 2021). This cool season pulse crop is cultivated throughout the world especially in Canada, India and Turkey. Canada precedes India and Turkey in area and production (Coyne and McGee, 2013). The major lentil growing regions of India are Uttar Pradesh, Madhya Pradesh, West Bengal, Bihar, Haryana and Rajasthan. In India lentil is cultivated over an area of 1.32 million hectare with production and productivity of 1.18 million tonnes and 894 kg ha^{-1} correspondingly.

Uttar Pradesh and Madhya Pradesh are two major lentil producer states in India occupying nearly 35.17% production from 28.79% area in India (Anonymous, 2019-2020). Numerous features including abiotic (Mishra *et al.,* 2021a, Sharma *et al.,* 2021, Mishra *et al.,* 2021b) and biotic (Mishra *et al.,* 2020) are answerable for yield reduction in crops including lentil. Among different biotic stresses, diverse diseases caused by pathogens like bacteria, fungi, viruses *etc*. are included and collar rot is one of the most important diseases instigated by *Sclerotium rolfsii* is a non-specialized soil borne fungal pathogen (Arya *et al.,* 2021). This fungal pathogen has a wide host range of over 500 species (Nandi *et al.,* 2017). It is a polyphagous pathogenic fungus causes substantial losses in quality and quantity of lentil. The fungus outbreaks the lentil crop at any time and any growth stage. Its ability to produce hard resting structure (sclerotia) in nature helps in its survival for many years in soil (Singh and Singh, 2021). The sclerotia formed by *S. rolfsii* are multi-hyphal structure mass comprising of three layers *viz.,* a thick and thin wall (cortex) and white medulla (Bullock *et al.,* 1980). The management of diseases in plants along with animals needs accurate identification of pathogen accountable for an exact disease. At present days, molecular (DNA or RNA) basis of pathogen detection is considered as an authentic tool for detection of a pathogen (Upadhyay *et al.,* 2020; Mandloi *et al.,* 2022; Pramanik *et al.,* 2022).

Currently, there is a demand for DNA based pathogen detection kit for accurate and authentic identification of the pathogen and genomic DNA extraction from the pathogen

[1]Department of Plant Pathology, College of Agriculture, RVS Agricultural University, Gwalior-474 002, Madhya Pradesh, India. [2]Department of Plant Molecular Biology and Biotechnology, College of Agriculture, RVS Agricultural University, Gwalior-474 002, Madhya Pradesh, India. [3]Directorate of Research Services, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur-482 004, Madhya Pradesh, India.

Corresponding Author: M.K. Tripathi, Department of Plant Molecular Biology and Biotechnology, College of Agriculture, RVS Agricultural University, Gwalior-474 002, Madhya Pradesh, India. Email: drmanojtripathi64@gmail.com

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is the pre-requisite for development of desired kit. Genomic DNA isolation from *S. rolfsii* is tedious because presence of high mucilage and protein content (Cassago *et al.,* 2002). In view of these facts, it is essential to develop a DNA extraction protocol from this fungal pathogen. Existing DNA extraction protocols comprehending use of proteinase K as an essential component. Applications of proteinase K based DNA extraction is the competent process for DNA extraction from different microorganisms. Proteinase K possess the properties to catalyze fungal cell lysis owing to function of β-1,3-glucanase and a specific alkaline protease activity. But the DNA extracted employing proteinase K based method needs purification before applying it further for molecular analysis (Gautam, 2022). Furthermore, one of more important things is that Proteinase K is a costly ingredient being used in the DNA extraction process. Removal of this component from the procedure may reduce the cost of DNA extraction from sclerotia of *S. rolfsii*. Therefore, in the present investigation, an effort has been made to modify in routinely employed DNA extraction procedure and develop a new DNA extraction protocol without using proteinase K.

MATERIALS AND METHODS

Biological material

Isolates of *S. rolfsii* used in this investigation were isolated from the collar region of *Lens culinaris* showing typical symptoms of collar rot disease caused by *S. rolfsii* from different regions of Madhya Pradesh (Table 1). *S. rolfsii* was confirmed in all isolates by comparing their morphology and further by ITS sequence information. The molecular work was carried out at Plant Molecular Biology Laboratory, Department of Plant Molecular Biology and Biotechnology, College of Agriculture, Rajmata Vijayaraje Scindia Agricultural University, Gwalior, India during the year 2021-22. For isolation of genomic DNA, tissue segments of 2-3 cm from collar rot disease infected plants were excised from rotten margins. The segments were sterilized with 1% sodium hypochlorite solution for 2 min followed by rinsing twice with sterilized double distilled water and placed onto Potato Dextrose Agar (PDA) medium. Then segments were transferred in Petri dishes and incubated at 25±2°C for 2 to 3 days and mycelia were then transferred and maintained on PDA for 15 days to allow sclerotia development. Twenty-five to thirty days old sclerotia (mature) were employed for DNA extraction.

Reagents and chemicals

The following buffers and solutions were prepared for DNA extraction:[Extraction buffer (1M Tris-HCl (pH 8); 0.5 M EDTA (pH 8); % M NaCl; 2% CTAB (w/v); 1% PVP (Mr. 40,000); 2% β-mercaptoethanol (v/v)]; phenol: chloroform (24:1); wash solution (3M ammonium acetate in 70% (v/v) ethanol. The pH of DNA extraction buffer (pH-5.2) was adjusted and volume was made up to 100 ml with distilled water.

Genomic DNA extraction

About 150 mg of sclerotia was ground to a fine powder by using liquid nitrogen. The powder was directly added to 2 ml Eppendorf tube containing 500 µl of pre-warmed DNA extraction buffer (DEB). The tube was incubated for about 15 min at 55°C in water bath with frequent swirling. The samples were centrifuged @12000 rpm for 5 min and supernatant obtained was transferred into clean micro-centrifuge tube. To each tube 250 µl of chloroform: isoamyl alcohol (24:1) was added and solution was mixed by the gentle inversion. After mixing, the tube was spinned at 13000 rpm for 10 minutes. By using cut tips transfer the upper aqueous phase transferred to new 1.5 ml Eppendorf tube. To each tube 50 µl 3M sodium acetate was added tracked by 500 µl of ice-cold absolute ethanol. Tube was inverted several times to precipitate the DNA. These tubes were placed at -20°C for 1 hr after addition of ethanol to precipitate out the DNA. DNA was pelleted by centrifugation at 13000 rpm for 1 min and the supernatant was discarded. The DNA was washed twice by 70% ethanol and again centrifuged at 13000 rpm for a min. The supernatant was discarded and allowed the DNA to dry till the smell of ethanol gone away. The DNA was dissolved in nuclease free water (50 µl - 100 µl). The dissolved DNA was stored at -20°C for further downstream reactions (PCR). The experiment was repeated thrice and result described as the mean of three independent experiments.

DNA analysis

The quality of extracted DNA was checked by means of 0.8% gel electrophoresis tracked by ethidium bromide staining

Table 1: List *Sclerotium rolfsii* isolates collected from locations of Madhya Pradesh, India.

Isolates	Source	Location	Coordinates
Sr-G	Lentil field	Gwalior	26.2231°N, 78.1909°E
Sr-A	Lentil field	Ashok Nagar	24.5775°N, 77.7318°E
Sr-Gj	Lentil field	Ganjbasoda	23.8494°N, 77.9371°E
Sr-M	Lentil field	Morena	26.4947°N, 77.9940°E
Sr-B	Lentil field	Bhind	26.5638°N, 78.7861°E
Sr-S	Lentil field	Shivpuri	25.4320°N, 77.6644°E
Sr-J	Lentil field	Jabalpur	23.1815°N, 79.9864°E
Sr-R	Lentil field	Ratlam	23.3315°N, 75.0367°E
Sr-Sh	Lentil field	Sehore	23.2032°N, 77.0844°E

(0.5 mg ml^{-1}). The purity of DNA was estimated by 1.7 to 1.9 by calculating the A_{260}/A_{280} ratios and the yield was estimated by measuring absorbance at 260 nm.

To check the suitability of extracted DNA for downstream analysis, RAPD, ISSR and rDNA-ITS primers were employed for amplifications of extracted DNA samples. For this purpose, RAPD (OPA-8) primer, 5′-GTGACGTAGG-3′ (Imperial Bio Medics, Coralville, USA), ISSR (ISSR-12) 5′-$(GA)_8$T-3′) and universal primers ITS-1 (5′-TCCGTAGGTGG ACCTGCGG-3′) as forward primer and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) were used. Each PCR reaction mixture of 10 µl consisted of 200 ng genomic DNA, 1 µl of 6X green *Taq* reaction buffer with $MgCl_2$ (Thermofisher), 0.2 µl of 2.5 mM dNTPs, 100 ng primer. PCR amplification was performed in an Eppendorf thermal cycler (Bio-Rad).

The temperature profiles used for RAPD amplification were: an initial denaturation at 94°C for 5 min and then subjected to 30 cycles of (94°C for 1 min, 36°C for 2 min, 72°C for 2 min). After the last cycle, the final extension was carried out 72°C for 5 min. The temperature profiles employed for ISSR amplification were: an initial denaturation at 94°C for 5 min and then subjected to 30 cycles of (95°C for 30 sec, 48°C for 30 sec, 72°C for 1.5 min). After the last cycle, the final extension was carried out 72°C for 10 min. The amplified product was resolved on 1.5% gel comprehending 0.5 mg ml^{-1} ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (SYNGENE, USA). The temperature profiles used for rDNA-ITS region amplification were: an initial denaturation at 94°C for 4 min and then subjected to 35 cycles of (94°C for 1 min, 56°C for 1 min, 72°C for 1.5 min). Afterward the last cycle, the final extension was carried out at 72°C for 6 min. The amplified product was resolved on 1.8% gel containing 0.5 mg ml^{-1} ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (SYNGENE, USA).

RESULTS AND DISCUSSION

The method was standardized for isolation of genomic DNA from sclerotia of *S. rolfsii* collected from diverse regions of Madhya Pradesh, India (Table 1). The standardized method yielded better quality of pure, high molecular weight DNA (Fig 1). The current method produced 480-510 ng DNA mg^{-1}. The A_{260}/A_{280} ranged from 1.7 to 1.9, displaying that DNA was of high purity and suitability for PCR-based analysis (Table 2). The procedure involves inactivating proteins by CTAB and precipitating polysaccharides and proteins in the presence of high salt potassium acetate (Kim *et al.,* 1990; Cilliers *et al.,* 2000). The exclusion of polysaccharides and other contaminating hydrates is based on the differential solubility of DNA versus the higher-molecular weight polysaccharides in aqueous media (Rozman and Komel 1994).

Successful amplification and variation were obtained by employing OPA-8 and ISSR12 markers (Fig 2) when verified with extracted genomic DNA without performing any purification process. ITS amplification with extracted DNA produced amplicons between 640-700 bp of sizes from all of the nine isolates (Fig 1), signifying that DNA extracted

Table 2: Qualitative and quantitative analysis of extracted genomic DNA.

Isolate	A_{260}/A_{280}	Yield of DNA (ngml^{-1})
Sr-G	1.83	498
Sr-A	1.84	502
Sr-Gj	1.79	490
Sr-M	1.77	480
Sr-B	1.71	510
Sr-S	1.75	488
Sr-J	1.79	500
Sr-R	1.72	495
Sr-Sh	1.89	487

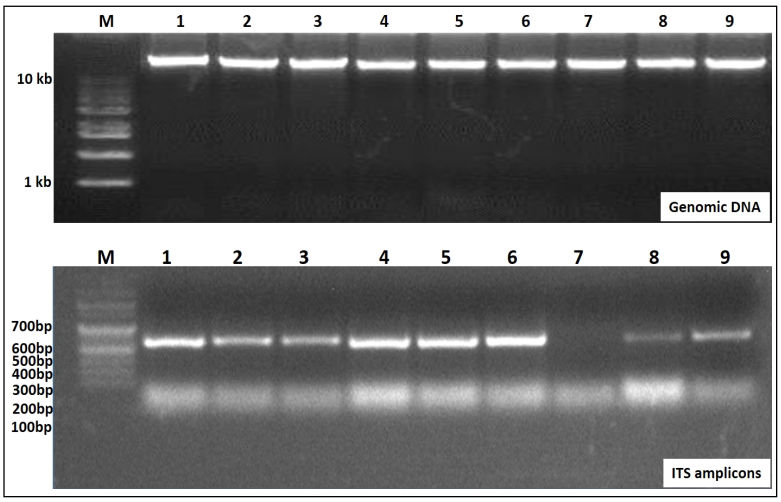

Fig 1: Illustration of A.) genomic DNA extracted from *Sclerotium rolfsii* using standardized protocol without proteinase K, B.) Amplification of extracted DNA samples with ITS primer.

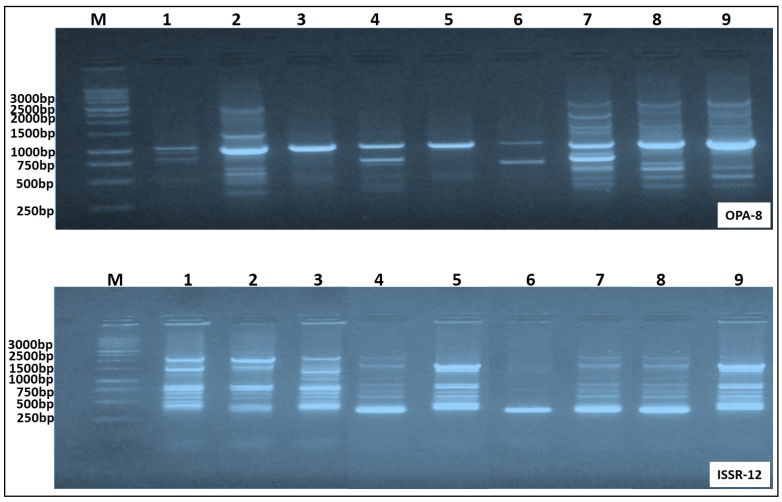

Fig 2: Successful amplification of extracted DNA from *Sclerotium rolfsii* using standardized protocol. A). Amplification with RAPD primer OPA-8 and B). Amplification with ISSR primer ISSR-12.

from the resting structure (sclerotia) of *S. rolfsii* is appropriate for PCR-based analysis.

The persistence of this investigation was to develop improved, simple and to get over the problem of high mucilage polysaccharides (Jeeva *et al.,* 2008) and protein content which adds difficulties in the genomic DNA isolation (Tiwari *et al.,* 2017). The chief reason behind choosing the sclerotia for the extraction of genomic DNA was to solve the prime complications caused by polysaccharides secreted by fungal mat in broth culture which interfere in genomic DNA isolation. Secondly, we did not use Proteinase K during the extraction of genomic DNA owing to the attribute that sclerotia have a smaller number of ribosomes (Henis and Kislev, 1969). Moreover, mature sclerotia have meager amount protein-containing structures (Henis and Kislev, 1969) in comparison to hyphal cells. While the researchers who developed DNA extraction protocols employing fungal mats of *S. rolfsii* used proteinase K (Jeeva *et al.,* 2008). Our method yielded 500 ng μl^{-1} of DNA from 150 mg of sclerotia while as 100 mg of mycelia yielded only 55.57± 0.002 ng μl^{-1} DNA in the experiment of Male *et al.* (2018).

CONCLUSION

The DNA extraction protocol described here is technically easy, economic and rapid for preparing high molecular weight DNA without any column precipitation step. Nevertheless, DNA extracted from the sclerotia of *S. rolfsii* ensuing this procedure has been promptly amplified by PCR. To the best of our knowledge the genomic DNA extraction from resting structure (sclerotic) of *S. rolfsii* has not yet been reported by any other researcher and it is plausible that this protocol may be employed for the extraction of genomic DNA from many other fungal cultures producing sclerotia as an alternative to the existing genomic DNA extraction protocols from the mycelium mat.

Conflict of interest: None.

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