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MOLECULAR CHARACTERIZATION OF *Sclerotium rolfsii* Sacc. INCITING STEM ROT OF GROUNDNUT PLANT (*Arachis hypogea* L.)

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ABSTRACT

In the present study, stem rot disease of groundnut was observed in many crop fields of Telangana, India during survey. The most distinguishable symptom is a white fan shaped abundant fungal mycelium mat that appears on the soil surface, leaf fragments and at stem bases of infected plants. The mycelial mat may be seen on the stem a few millimeters above the soil line. Abundant mustard seed-sized, brown to dark brown, spherical sclerotia, initially white later turned to dark brown on maturation was recorded. The fungal pathogen was isolated on potato dextrose agar (PDA) medium. Genomic DNA was isolated and internal transcribed region of ribosomal DNA was amplified using ITS1 universal primers. Based on cultural, morphological and molecular characteristics, the fungal pathogen was identified as *Sclerotium rolfsii*. The rDNA sequence results also showed 98% similarity with reference sequence OR911599 and JF966208.1 confirming pathogen identity. The pathogenicity test was conducted on healthy plants for symptom expression after 6-8 days of inoculation. *S. rolfsii* is known to infect many economically important crop plants at various stages of its growth and development.

KEYWORDS: Groundnut, stem rot, sclerotia, pathogenicity

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop cultivated worldwide in tropical, subtropical and warm areas. Groundnut kernels contain about 26 per cent protein, 48 per cent edible oil, 20 per cent carbohydrates and 3 per cent fiber and also rich in calcium, thiamine and niacin (Haveri, 2017). Globally, groundnut covers an area of 327 lakh ha with the production of 539 lakh tonnes and productivity of 1648 kg per hectare (FAOSTAT, 2021). Groundnut crop suffers from several diseases incited by many fungal, bacterial and viral pathogens, but stem rot caused by *Sclerotium rolfsii* is one of the most important seed and soilborne diseases causing huge economic loss in India and abroad.

According to Grichar and Boswell (1987) yield losses in severely infected areas can range between 10 and 25% and in certain areas, up to 80% losses due to groundnut stem rot occured in India (Mayee and Datar, 1988). In India, stem rot of groundnut was first recorded by Butler and Bisby (1931).

Recently, taxonomic position of *Sclerotium rolfsii* has been assigned by Kirk *et al*. (2008) to the Kingdom-Fungi, Phylum-Basidiomycota, Class-Agaricomycetes, Order-Polyporales, Family-Atheliaceae, Genus- *Sclerotium* and Species- *rolfsii*. The most distinguishable symptom is a white fan-shaped fungal mycelial mat that appears on the soil surface, leaf fragments and near stem bases of infected plants. The mycelial mat may be seen on the stem a few millimeters above the soil line. Abundant mustard seed-sized, tan to brown, spherical sclerotia grow on infected plant material and on the surface of the soil.

This paper describes the isolation and molecular characterization of stem rot disease based on molecular characterization and pathogenicity test.

MATERIAL AND METHODS

Groundnut plants showing typical stem rot symptoms were collected from field during survey in Telangana state. Isolation of fungus was made on PDA medium following standard tissue isolation technique (Wilson, 1953). Infected portion of the root was cut into small pieces (3-4 mm size) and surface sterilized by dipping in freshly prepared 1% sodium hypochlorite solution for one minute followed by rinsing

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three times with sterilized distilled water to remove the traces of disinfectant and dried on sterilized blotter paper. These bits were transferred aseptically into Petri plates containing PDA and incubated in BOD incubator at 25 ± 1°C. After 72 hours, the pure culture of the fungus was obtained by following the hyphal tip culture method (Rangaswami, 1971).

A disc of fungal colony was cut with the help of cork borer and placed in the middle of Petri plate containing plain agar and incubated for 2 days. Petri plate was placed under dissecting microscope and mycelial threads of pathogen were located at higher magnification. The pure culture was obtained by hyphal tip method (about 1 mm) and was transferred to PDA plate. After two weeks period, pure culture was prepared by transferring a single sclerotial body on PDA in Petri plates and the pure cultures were maintained in BOD incubator at 25 ± 1°C for further studies (Dhingra and Sinclair, 1985).

Morphological characterization

The fungal culture was observed for 4-5 days and the slides of fungal mycelia in the reproductive stage was prepared using lactophenol and cotton blue and observed under the microscope and the colony characteristics were noted (Raper and Fennell, 1965).

Mass multiplication

Sorghum seeds were thoroughly washed with running water and soaked overnight in 1% sucrose solution. Next day, the soaked seeds were transferred to 250 ml conical flasks and autoclaved twice at 15 *psi* pressure (121.6°C) for 20 minutes. After autoclaving, flasks were allowed to cool down. The bits of pure culture mycelium were placed to the conical flask under aseptic condition and were incubated at 25±1°C for 15 days. These seeds were shaken periodically to get uniform growth of fungus.

Pathogenicity test

Susceptible groundnut variety K6 was used for testing the pathogenicity of *S. rolfsii* following soil inoculation technique. The pots were filled with sterilized soil and then each pot was covered with a polyethene cover after being artificially inoculated with 40 g of inoculum that had been multiplied on sorghum grains and carefully mixed. Sterilized soil without inoculation served as control. After 3 days, five groundnut seeds were sown randomly in each pot and three replications were maintained. The pots were maintained under controlled conditions in glass house with adequate soil moisture and watered regularly as per the requirement.

Molecular characterization

DNA extraction, PCR amplification and sequencing

Mycelial disc (5 mm diameter) of an actively growing 5 days old culture was cut off and transferred onto the fresh PDB (Potato Dextrose Broth) and the culture was incubated in shaker at 125 rpm for 3 days. Subsequently, the growing mycelium was scraped with the help of sterilized forceps from the surface and used for genomic DNA extraction by following Cetyltrimethyl Ammonium bromide (CTAB) method.

Protocol for fungal genomic DNA isolation

Mycelial mat was harvested by filtration and stored at -70°C for further use. Mycelial mat was lyophilized and ground in a sterile pre-chilled mortar and pestle to a fine powder. The fine powder was then transferred into a 2 ml Eppendorf tube and 1 ml of CTAB extraction buffer preheated at 65°C was added, mixed thoroughly and incubated in a water bath at 45°C for 45 minutes. After incubation, contents of the tube were cooled to room temperature and an equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed by inversion and centrifuged at 12000 rpm for 15 minutes at room temperature. The upper aqueous phase was transferred to a new tube and an equal volume of chloroform:isoamyl alcohol (24:1) was again added and mixed by inversion and centrifuged at 12000 rpm for 15 minutes at room temperature. The upper aqueous phase was recovered in a fresh tube and DNA was precipitated by adding approximately an equal volume of isopropanol and incubating at room temperature for 30 minutes. After incubation, content in eppendorf tube was centrifuged at 12000 rpm for 20 minutes to pellet DNA. The supernatant was discarded and the DNA pellet was washed with 70 per cent chilled ethanol and centrifuged at 12000 rpm for 5 minutes at 4°C. DNA pellet was dried under vacuum, dissolved in 20 μl of TE buffer and stored at -20°C for further use.

Quality check and quantification of genomic DNA

A small quantity of extracted genomic DNA was loaded on 0.8 per cent agarose gel stained with ethidium bromide (0.5 μg/ml) and it was run on gel electrophoresis unit and visualized in gel documentation

system. PCR product was quantified in nanodrop, concentration and purity of DNA were estimated by measuring absorbances at A_{260} and A_{280} nm. Concentration was expressed in ng/μl . The remaining DNA was stored at -20°C for further use.

PCR amplification of genomic DNA

PCR amplification of the isolated fungal DNA was amplified at internal transcribed spacer region by ITS1 and ITS4 primers (Table 1). Initially, 10 μl of small

Table 1. Base sequences of ITS primers used

Pri-mers	Sequence	Base pairs
ITS-1	5'TCCGTAGGTGAACCTGCGG-3'	19
ITS-4	5'TCCTCCGCTTATTGATATGC-3'	20

volume PCR product was used followed by 50 μl volume PCR on gel electrophoresis unit and visualized in gel documentation system. DNA ladders (100 bp) were loaded on first well as a size standard. The gel was checked to know whether the PCR amplicon is of the correct size.

Sequencing of PCR products

PCR products of ITS regions of fungal isolate were obtained through amplification and were outsourced for purification and sequencing to Eurofins Genomics, Bengaluru, India.

Table 2. PCR mixtures for 10 μl and 50 μl reaction volumes

Components		Quality for one reaction	
		Total volume (10 μl)	Total volume (50 μl)
EmeraldAmp GT PCR Master Mix (2X premix)		5 μl	25 μl
Primers (2.5 pmol/ μl)	Forward	1 μl	5 μl
	Reverse	1 μl	5 μl
Template DNA(100 ng/ μl)		1 μl	5 μl
dH_2O		2 μl	10 μl
Total volume		10 μl	50 μl

Table 3. Cycling conditions and amplicon size for ITS amplification

Steps	ITS
Initial denaturation	94°C for 5 minutes
35 cycles of	
Final denaturation	94°C for 45 seconds
Primer annealing	55°C for 45 seconds
Extension	72°C for 1 minute
End of cycle	
Final Extension	72°C for 5 minutes
Amplicon size	~ 600 bp

Sequence data analysis

Sequence results obtained from Eurofins Genomics, Bengaluru, India was analysed using BioEdit, MEGA11 and NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi#). A consensus sequence was generated from forward and reverse sequence data using BioEdit software. The consensus sequence was used to perform BLAST against the NCBI GenBank database. The first ten sequences were chosen based on their maximum identity score and aligned using the multiple alignment software program ClustalW. MEGA 11 was used to create the distance matrix and the phylogenetic tree. Using distance matrix and the phylogenetic tree thus formed closest sequence and isolate was identified.

RESULTS AND DISCUSSION

Isolation and Identification of pathogen

The groundnut plants showing typical symptoms were collected during *kharif* 2021-2022 survey and brought to the laboratory. Pure culture of the pathogen was obtained by hyphal tip technique. The mycelium of the isolated organism showed fluffly white growth on PDA medium (Fig.1a and Fig.1b). Based on cultural and morphological characteristics the isolated test pathogen was identified as *S. rolfsii* (Punja, 1985).

In pure culture, the fungal mycelium is initially silky white, but it gradually lost its lustre and later assumes dull appearance. The mycelium is seen

radiating with abundant aerial hyphae that exhibits rapid growth habit. Often, the aerial hyphae appeared as dense tufts and seen dispersed all over the culture medium. The mycelium completely disappeared over a period of three months leaving only sclerotia. Sclerotia are at first white, became light brown to dark brown at maturity. The sclerotia are sub spherical with the surface finely wrinkled and sometimes flattened.

The primary symptoms of stem rot in groundnut are observed as browning and wilting of leaves and branches while still being attached to the plant. The fungus predominantely infected the stem by forming a whitish mycelial mat. However, the infection was also observed in all plant parts including leaf, root and stem (Fig.1a and Fig.1b). The results are in accordance with the findings of Pande and Rao (2000), Rakholia and Jadeja, 2011 and Divya Rani *et al. (*2017) who have noticed similar symptoms on infected groundnut plants in their research experiments.

In pathogenicity test, the inoculated groundnut plants exhibited typical symptoms of stem rot and the pathogen was reisolated and Koch postulates were proved. The pathogen was reisolated and Koch postulate was proved. The results are in similarity with the findings of Le *et al.* (2012) and Eslami *et al.* (2015) on groundnut.

Molecular characterization

Results showed that the test fungal isolate had similar morphological characteristics and its pathogenicity was proved. DNA from the test pathogen isolated from stem rot affected groundnut plants was successfully amplified using universal primer pairs ITS1 targeting the ITS region. The total size of ITS1 region studied was 681 bp. The ITS sequences for *Sclerotium* isolate were compared against the sequences in the NCBI nucleotide database using BLAST tool to confirm the identity of test pathogen. The BLAST results indicated that the pathogen isolated from stem rot infected plants as *Sclerotium rolfsii* Sacc. The query cover of isolate identity was 98% homology to that of *S. rolfsii*. This indicated that the test pathogen belongs to *Sclerotium rolfsii*. The nucleotide sequences of ITS1-4 regions had been deposited in the NCBI database as accession number of OR911599 for the test pathogen.

The present findings were at par with rDNA sequence which showed 99-100% similarity with reference sequence AB075298.1 and confirmed the pathogen identity (Tejaswini *et al*., 2022).

Morphological approaches can be used for species identification, however this method is not appropriate because high degree of accuracy is required at the species level where most isolates of the same genus are nearly identical. *Sclerotium rolfsii* morphologically very similar and in many cases are phenotypically indistinguishable and can only be reliably identified by molecular characterization. Therefore, consistent and reliable pathogen identification is an essential indicator in plant disease epidemiology and development of management strategies. Currently, molecular methods focusing on genotypic characteristics are used to confirm identification method and accelerate detection of species.

Fig.1a: Stem rot symptoms observed on groundnut

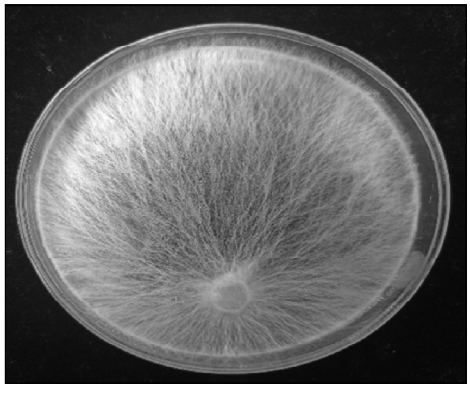

Fig.1b: Pure culture of *Sclerotium rolfsii*

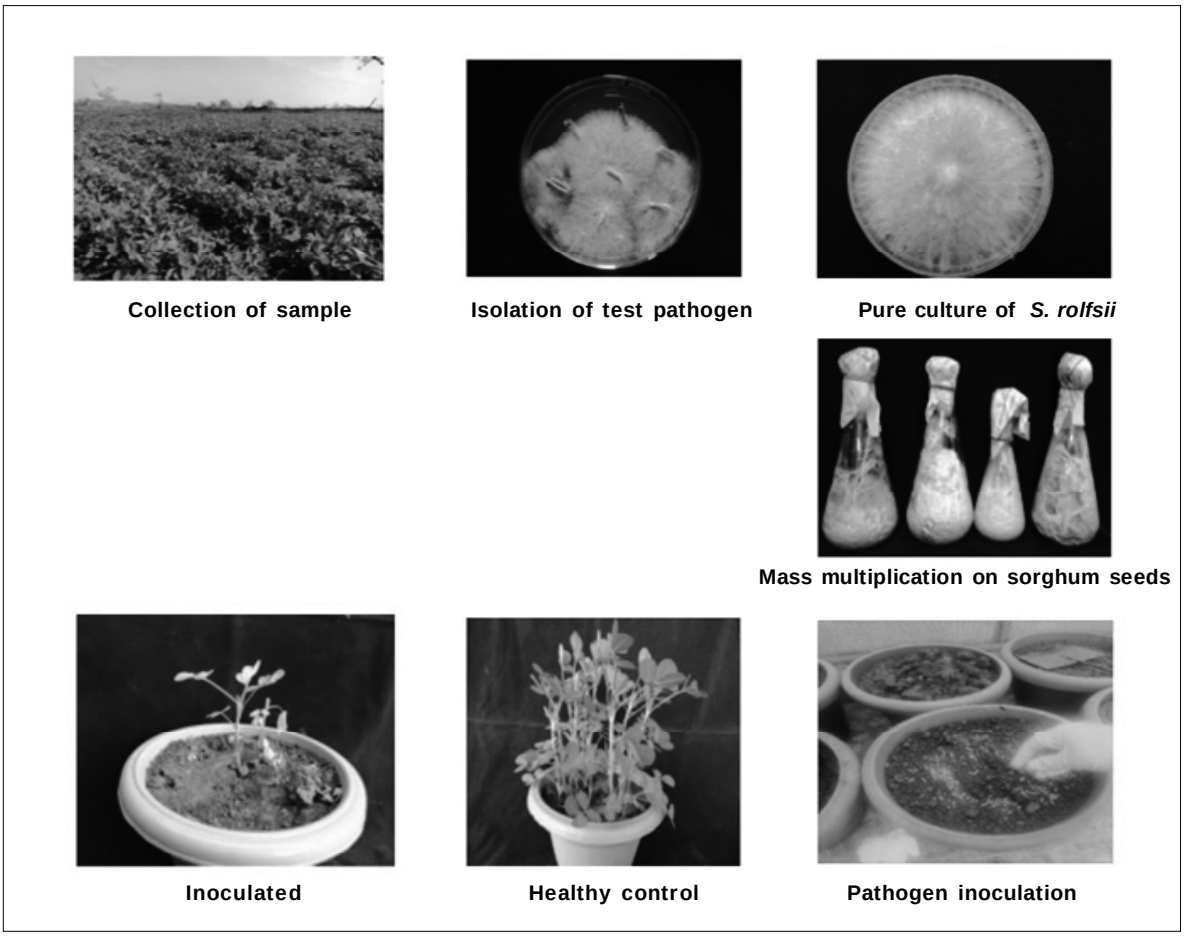

Fig.2: Pathogenicity test of *S. rolfsii*

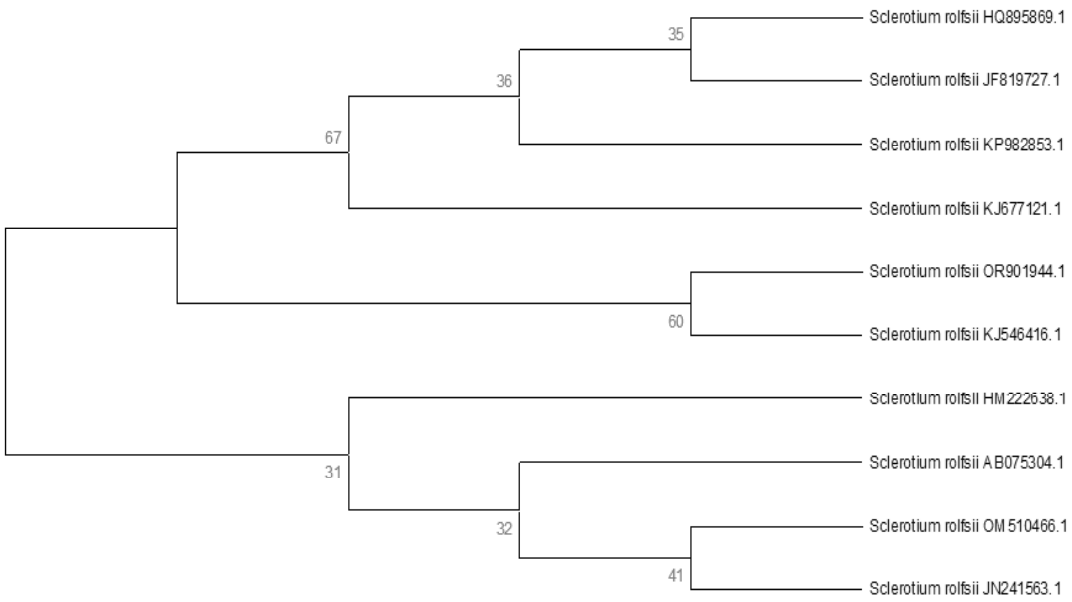

Fig.3: Phylogenetic tree based on ITS sequence of *Sclerotium* isolate

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