

Original Research Article

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## Molecular Variability among the Isolates of *Sclerotium rolfsii* Causing Stem and Pod Rot of Groundnut Collected from Karnataka, India

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### ABSTRACT

Genetic variability among the 24 isolates of *S. rolfsii* was studied by using molecular markers like ITS-PCR and RAPD primers. Amplification of ITS region of rDNA with specific ITS1 and ITS4 universal primers produced approximately 650 to 700 bp in all the isolates of the fungus confirmed that all the isolates obtained are *Sclerotium rolfsii* and were sequenced. Identity of the isolates was confirmed with sequences of NCBI data base of *S. rolfsii*. Among the twenty four isolates, four random primers viz., UBC-467, UBC-482, UBC-485 and UBC-489 generated reproducible polymorphism. Amplified products with all the primers have showed polymorphic and distinguishable banding pattern indicating the genetic diversity among all the isolates of *S. rolfsii*. A total of 342 reproducible and scorable polymorphic bands ranging approximately as low as 150 bp to as high as 2000 bp was generated with four primers among the twenty four isolates studied. All the twenty four isolates were grouped into three main clusters indicating there is genetic diversity in the isolates of *S. rolfsii*. Cluster I contained thirteen isolates, main cluster divided into two sub clusters, sub cluster I had eight isolates (Sr21, Sr20, Sr19, Sr18, Sr22, Sr24, Sr17 and Sr23) and subcluster II had five isolates (Sr5, Sr2, Sr1, Sr6 and Sr4). Main cluster II has two sub clusters, sub cluster I consisted of six isolates (Sr8, Sr3, Sr7, Sr12, Sr15 and Sr14) and in sub cluster II three isolates (Sr16, Sr10 and Sr13) were grouped. Cluster III consisted of two isolates (Sr9 and Sr11).

#### Keywords

Groundnut, Stem and pod rot, *Sclerotium rolfsii*, Molecular variability

#### Article Info

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### Introduction

Groundnut (*Arachis hypogea* L.) is a major legume and important oil seed crop in India which is grown over an area of 52.50 lakh ha with an annual production and productivity of 94.72 lakh tons and 1804 kg ha<sup>-1</sup> respectively (Anonymous, 2014). In Karnataka, it is grown to the extent of 7.25 lakh ha with 6.58 lakh

tons production and with a productivity of 908 kg ha<sup>-1</sup> (Anonymous, 2014). The crop groundnut is affected by many diseases at different growth stages. Among the diseases, stem and pod rot caused by *Sclerotium rolfsii* Sacc. is emerging as a major problem and has become an economically important soil borne pathogen. Stem and pod rot disease is a potential threat to groundnut production and is

of considerable economic significance for groundnut grown under irrigated conditions. *Sclerotium rolfsii* Sacc. is a soil borne pathogen common in tropical and sub-tropical regions of the world where high temperature coupled with high humidity is prevalent during the rainy season causing severe damage to the crop with yield losses of over 27 per cent (Ghewande *et al.*, 2002). The symptoms of *S. rolfsii* include yellowing and wilting of branches, presence of white mycelial growth at collar region and production of mustard seed like sclerotia on infected parts (Asghari and Mayee, 1991). Studies of variability within the population in a geographical region are important because these also document the changes occurring in the population. Variability among *S. rolfsii* populations from different geographical regions was recorded by earlier workers (Harlton *et al.*, 1995; Okabe *et al.*, 1998; Sarma *et al.*, 2002).

Molecular markers play a major role in analyzing genetic basis of genotypic variation among fungal population. Welsh and McClelland (1990) described a modification of the PCR procedure referred to as the randomly amplified polymorphic DNA (RAPD) marker technique that can be used to detect genetic polymorphisms in fungi. This technique can overcome the limitations of RFLPs such as they are relatively slow, expensive and laborious, as generally only a single locus can be analyzed with each RFLP reaction. RAPD technique differs from conventional PCR in that only a single primer which is derived from an arbitrary sequence is used for amplifying DNA (Perez Moreno *et al.*, 2002). Hence the RAPD analysis being used as a powerful tool for the investigation of genetic relatedness and diversity among closely related strains and was found to be a valuable method for differentiating the genetic variability of *S. rolfsii* isolates (Saude *et al.*, 2004). To understand the present plant disease situations and for effective management, it is

essential to study as much as possible about the genetic variability in plant pathogenic fungi. The information on genetic variability among the groundnut isolates of *S. rolfsii* is limited. Thus, the present study was undertaken to assess the significant genetic variations by nucleic acid based marker techniques using ITS-PCR and RAPD.

## **Materials and Methods**

A survey was conducted during kharif 2013 and 2014 in groundnut growing areas of Karnataka and groundnut plants infected with stem rot pathogen, *S. rolfsii* were collected. The pathogen, *S. rolfsii* was isolated from the stems of infected groundnut plants by tissue isolation method (Rangaswami and Mahadevan, 1999) on potato dextrose agar (PDA) medium.

## **Cultural and morphological variability**

Eighty isolates collected from districts viz., Raichur, Ballari, Koppal, Gadag, Tumkur, Chitradurga, Gulbarga and Yaadgir districts of Karnataka were studied for their cultural characters such as radial growth, colony morphology, production of sclerotial bodies and pattern of production of sclerotial bodies and morphological characters such as number of days taken to produce sclerotial bodies, colour of sclerotial bodies, number of sclerotial bodies produced per plate, diameter of sclerotial bodies and sclerotial texture were studied by using potato dextrose agar medium. Further, three isolates from each district of Karnataka were selected to study molecular variability among the isolates of *S. rolfsii* by using ITS region of rDNA and Random Amplified Polymorphic DNA (RAPD).

## **Fungal cultures (*S. rolfsii*)**

Mycelial discs (5 mm diameter) of respective isolates of *S. rolfsii* from the periphery of an

actively growing 7 days old culture on PDA were inoculated in to conical flask (250 ml) containing 100 ml PDB and flasks were incubated for three days at  $27 \pm 2^\circ\text{C}$  temperature. The mycelium grown on PDB was used for isolation of genomic DNA.

### **Isolation of genomic DNA**

The DNA was extracted from 24 isolates of *S. rolfsii*. collected from eight districts of Karnataka from each district three isolates were selected. The extraction buffer used for Lysis method was composed of (400 mM TrisHcl, 60 mM EDTA 1% SDS, pH 8.0 and 150 mM Nacl). Fresh or frozen mycelial mat (100 mg) was ground to fine powder with liquid nitrogen using pre-chilled mortar and pestle. The samples were then transferred to 2 ml Eppendorf tubes and 1 ml of extraction buffer, 5  $\mu\text{l}$  of proteinase K was added to the samples and the samples are mixed by vortexing for 10 min. The samples were centrifuged at 12,000 rpm for 10 min at room temperature.

Take the supernatant add equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1). Centrifuged at 12000 rpm for 10 min. at  $4^\circ\text{C}$  then take the supernatant, equal volume of chloroform: isoamyl alcohol (24:1) was added. Centrifuged at 12000 rpm for 10 min. at room temperature, upper aqueous phase was transferred to a fresh tube. The upper aqueous layer was collected into Eppendorf and to this 3 $\mu\text{l}$  of RNase A (10 mg  $\text{ml}^{-1}$ ) was added and the mixture was incubated at  $37^\circ\text{C}$  for 30 min. Finally 2/3<sup>rd</sup> volume of ice cold isopropanol was added to the Eppendorf tubes. The contents were later mixed by tilting the tubes gently and the tubes were kept at  $-20^\circ\text{C}$  for 30 min. to allow the DNA to precipitate. Later, the samples were centrifuged at 13000 rpm for 20 min. to collect the nucleic acid precipitate. The pellet was air dried for  $10\pm 15$  min and suspend in 50 $\mu\text{l}$  TE buffer.

### **Measurement of DNA concentration**

The quality and quantity of DNA was check by using Nanodrop ND 1000 the ratio of 260/280 is 1.8 is obtained in all isolates.

### **PCR amplification of ITS region**

PCR amplification of Internal Transcribed Spacers (ITS) region of rDNA was performed using universal primers ITS-1 (5' - TCC GTA GGT GGA CCT GCG G - 3') as forward primer and ITS-4 (5' - TCC TCC GCT TAT TGA TAT GC - 3') as reverse primer (White *et al.*, 1990) in Eppendorf PCR master cycler. Amplification was carried out in 0.2 mL Eppendorf tubes with 25  $\mu\text{L}$  reaction mixture containing 2.5  $\mu\text{L}$  of 10x Taq buffer, 4  $\mu\text{L}$  of 25 mM  $\text{MgCl}_2$ , 1.0  $\mu\text{L}$  of ITS1 primer (5 picomolar/ $\mu\text{L}$ ), 1.0  $\mu\text{L}$  of ITS-4 primer (5 picomolar/ $\mu\text{L}$ ), 1  $\mu\text{L}$  of 5 mM dNTP mix, 0.5  $\mu\text{L}$  of Taq polymerase (conc. 5 U  $\mu\text{L}^{-1}$ ) and 12.00  $\mu\text{L}$  of Nuclease free water (Genei, Bangalore) and 3  $\mu\text{L}$  (100-200 ng) of DNA sample. The PCR amplification was carried out by 5 min of initial denaturation at  $94^\circ\text{C}$  followed by 35 cycles of denaturation of  $94^\circ\text{C}$  for 1 min; annealing at  $55^\circ\text{C}$  for 2 min; extension at  $72^\circ\text{C}$  for 1 min with final elongation at  $72^\circ\text{C}$  for 5 min. Amplified PCR products were subjected to 1.2 (w/v) agarose gel using TBE (electrophoresis buffer, 40mM Tris 2mM EDTA, pH 8) containing ethidiumbromide (0.5 $\mu\text{g}/\text{ml}$ ). The size of the PCR product was estimated by comparison with known DNA marker of 1 kb DNA ladder. The banding profiles of ITS-PCR products were documented in gel documentation system.

### **Sequencing of ITS region and *in silico* analysis**

The ITS region was sequenced from eighteen isolates belonging to different locations from Karnataka to confirm organism and to know

the variability present in them. The PCR product was sequenced using forward and reverse primers at Chromos Biotech Ltd., Bengaluru. Homology search was done using BLAST algorithm available at the <http://www.ncbi.nlm.nih.gov>. Multiple alignments for homology search were performed using the Cluster W algorithm software and the phylogenetic tree was constructed (Patil, 2009).

### **RAPD profiles through Polymerized Chain Reaction (PCR)**

Four random primers UBC-467, UBC-482, UBC-485 and UBC-489 were screened for generating polymorphism among the isolates under the study. The experiment was repeated thrice and results were reproducible. The Oligonucleotide primer sequences used in RAPD technique are given below:

PCR amplifications were carried out in 0.2 mL Eppendorf tubes with 25  $\mu$ L reaction mixture which consists of 2.5  $\mu$ L of 10x Taq buffer, 2.0  $\mu$ L of 25 mM MgCl<sub>2</sub>, 1.0  $\mu$ L of primer (5 picomolar/ $\mu$ l), 1.0  $\mu$ l of 5 mM dNTP mix, 0.5  $\mu$ L of Taq polymerase enzyme (conc. 5 U  $\mu$ L<sup>-1</sup>) and 15.5  $\mu$ L of Nuclease free water (Genei, Bangalore) and 2.5  $\mu$ L (40-50 ng) of DNA sample. PCR amplification was carried out by 5 min of initial denaturation at 94°C followed by 40 cycles of denaturation of 94°C for 1 min; annealing at 37°C for 1 min; extension at 72°C for 2 min with final elongation at 72°C for 5 min.

Amplified PCR products were subjected to 1.5 per cent agarose gel electrophoresis with 1.0 x TBE as running buffer. The banding patterns were visualized under UV trans-illuminator with ethidium bromide (10 mg mL<sup>-1</sup>) staining. The DNA banding profiles were documented in the gel documentation system (Alpha Innotech) and compared with 1 kb DNA ladder (Genei, Bangalore).

### **Scoring and data analysis**

Each amplified band was considered as RAPD marker and recorded for all samples. Data was entered using a matrix in which all observed bands or characters were listed. The RAPD pattern of each isolate was evaluated, assigning character state '1' to all the bands that could be reproducible and detected in the gel and '0' for the absence of band. The binary data were thus obtained was used as an input for the construction of dendrogram using the Unweighted Neighbourhood-joining method using the program DARwin 6.0 software (Perrier *et al.*, 2003).

### **Results and Discussion**

#### **Cultural variability of *S. rolfsii* isolates**

Based on radial mycelial growth among eighty isolates of *S. rolfsii*, seventy three isolates were found fast growing (75-90 mm), five isolates were moderate growing (60-75 mm) and two isolates were slow growing (45-60 mm). Further, fifteen isolates showed compact growth and sixty five showed fluffy growth. With respect to pattern of production of sclerotial bodies, sixty four isolates produced sclerotial bodies scattered all over the plate, whereas thirteen at the periphery region and two at the centre region of Petriplate.

#### **Morphological variability of *S. rolfsii* isolates**

Based on colour of sclerotial bodies isolates were categorized into four groups, thirteen isolates produced dark brown, thirty three isolates light brown, four reddish brown and three isolates produced all the three coloured sclerotial bodies. Sixty two isolates were produced (0- 100) sclerotial bodies per plate, fourteen isolates produced 101-200 sclerotial bodies per plate, two isolates 201-300 sclerotial bodies per plate and isolate Sr15

produced more than 300 sclerotial bodies per plate. Further, twenty five isolates produced 1-2 mm sized sclerotial bodies, forty five 0.5-1 mm and nine isolates produced more than 0.5 mm sized sclerotial bodies.

### **Identification of *S. rolfsii* isolates by ITS – PCR**

ITS-1 and ITS-4 primers were used for PCR amplification of ITS region of rDNA clusters (ITS-1, 5.8S and ITS-4 regions) of all twenty four isolates. Both the primers produced amplified product size of 650-700 bp in all the twenty four isolates indicating that all the isolates belong to genus *Sclerotium* (Fig. 1).

Amplification of the ITS region of rDNA produced an approximately 650–700 bp fragment which is specific to *S. rolfsii*. Our results are in agreement with those of Adandonon *et al.*, (2005) who studied genetic variation among *S. rolfsii* isolates of cowpea by using mycelial compatibility and ITS rDNA sequence data and obtained an amplification fragment of about 700 bp which is specific for *S. rolfsii*. In the present study, all isolates gave the same size of the fragment that is 650–700 bp, which suggests that these isolates are the same species.

Harlton *et al.*, (1995) screened a world-wide collection of *S. rolfsii*, using universal primer pairs ITS1-ITS4, ITS1-ITS2 and ITS3-ITS4, and revealed variation in ITS regions with 12 sub-groups. *Sclerotium rolfsii* and *S. delphinii* yielded a common unique band of about 720 bp.

### **Nucleotide sequence accession number**

The BLAST data results revealed that the *S. rolfsii* isolates matched with the reference strains of NCBI results and identified as *Sclerotium rolfsii* and isolates were deposited in NCBI GenBank, Maryland, USA along

with location of the isolates. Accession numbers obtained are: KT337415, KT337409, KT337424, KT337411, KT337413, KT337425, KT337414, KT337423, KT337420, KT337419, KT337421, KT337412, KT337426, KT337418, KT337422, KT337416, KT337410, and KT337417 (Table 1).

The BLAST data results revealed that the *Sclerotium* species matched with the reference strains of NCBI results and identified as *Sclerotium rolfsii* and represented in the phylogenetic tree (Fig. 3). Out of 18 isolates, all the isolates were distinct from one another in the cluster but identical to each other in homology and divergence with different nodes.

### **Molecular diversity of *S. rolfsii* isolates by RAPD**

Among the twenty four isolates, four random primers *viz.*, UBC-467, UBC-482, UBC-485 and UBC-489 generated reproducible polymorphism. Amplified products with all the primers have showed polymorphic and distinguishable banding pattern indicating the genetic diversity among all the isolates of *S. rolfsii* (Fig. 2).

A total of 342 reproducible and scorable polymorphic bands ranging approximately as low as 150 bp to as high as 2000 bp were generated with four primers among the twenty four isolates studied. The primer 467 amplified two unique fragments of approximately 350 bp in Sr1, Sr2, Sr5 and 400bp in Sr2 isolate. Primer 482 amplified specific bands of 200 bp in Sr14, Sr15 and 550 bp in Sr4 and Sr9, 1000bp in Sr7, Sr12, Sr14 and Sr15. Primer 485 amplified specific band of 500 bp in Sr13 and 550 bp in Sr18 primer, 489 amplified specific band of 450 bp in Sr10. However, 500 bp fragment was absent in case of Sr22 compared to other isolates (Fig. 4).

**RAPD profiles through Polymerized Chain Reaction (PCR)**

Sl. No.	Primer	Primer Sequence (5' - 3')
1	UBC-467	GAG GAA GCTT
2	UBC-482	AGA CGG CCGG
3	UBC-485	AGC ACG GGCA
4	UBC-489	CTA TAG GCCG

**Table.1** Eighteen isolates of *Sclerotium rolfsii* infecting groundnut along with their accession numbers

Sl. No.	Isolates designation	Identified as	Location	Accession No.
1	<i>S. rolfsii</i> - Sr1	<i>Sclerotium rolfsii</i>	Raichur	KT337415
2	<i>S. rolfsii</i> - Sr2	<i>Sclerotium rolfsii</i>	Raichur	KT337409
3	<i>S. rolfsii</i> - Sr3	<i>Sclerotium rolfsii</i>	Koppal	KT337424
4	<i>S. rolfsii</i> - Sr4	<i>Sclerotium rolfsii</i>	Koppal	KT337411
5	<i>S. rolfsii</i> - Sr5	<i>Sclerotium rolfsii</i>	Bellary	KT337413
6	<i>S. rolfsii</i> - Sr6	<i>Sclerotium rolfsii</i>	Bellary	KT337425
7	<i>S. rolfsii</i> - Sr7	<i>Sclerotium rolfsii</i>	Bellary	KT337414
8	<i>S. rolfsii</i> - Sr8	<i>Sclerotium rolfsii</i>	Gadag	KT337423
9	<i>S. rolfsii</i> -Sr9	<i>Sclerotium rolfsii</i>	Gadag	KT337420
10	<i>S. rolfsii</i> - Sr10	<i>Sclerotium rolfsii</i>	Gadag	KT337419
11	<i>S. rolfsii</i> - Sr11	<i>Sclerotium rolfsii</i>	Kalburgi	KT337421
12	<i>S. rolfsii</i> - Sr12	<i>Sclerotium rolfsii</i>	Kalburgi	KT337412
13	<i>S. rolfsii</i> - Sr13	<i>Sclerotium rolfsii</i>	Kalburgi	KT337426
14	<i>S. rolfsii</i> - Sr14	<i>Sclerotium rolfsii</i>	Yadgir	KT337418
15	<i>S. rolfsii</i> - Sr15	<i>Sclerotium rolfsii</i>	Chitradurga	KT337422
16	<i>S. rolfsii</i> - Sr16	<i>Sclerotium rolfsii</i>	Chitradurga	KT337416
17	<i>S. rolfsii</i> - Sr17	<i>Sclerotium rolfsii</i>	Tumkur	KT337410
18	<i>S. rolfsii</i> - Sr18	<i>Sclerotium rolfsii</i>	Tumkur	KT337417

Fig.1 Amplification product of Internal Transcribed Spacer (ITS) with ITS 1 and ITS 4 primers

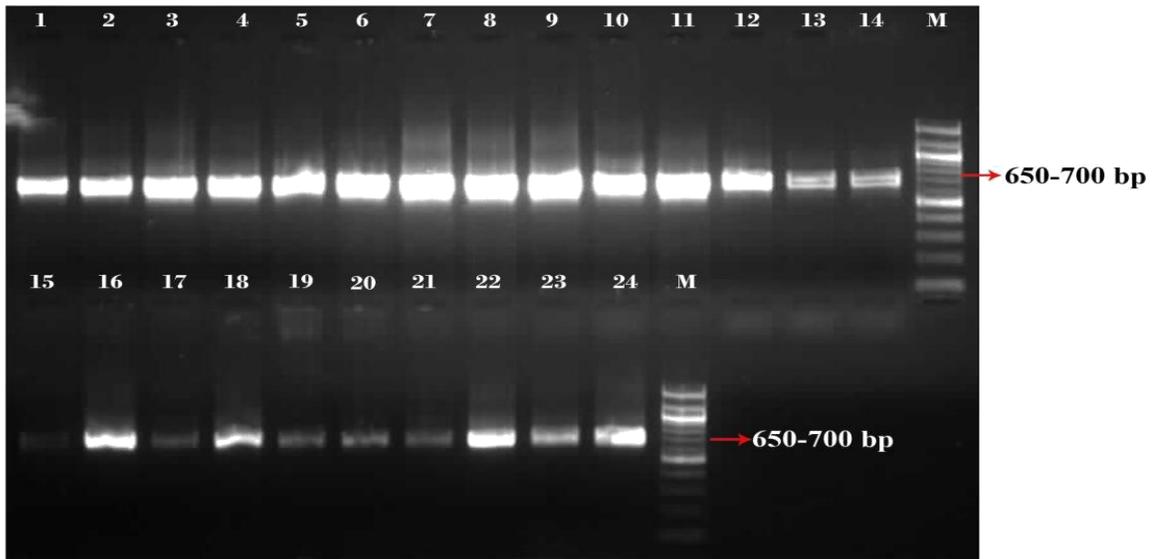
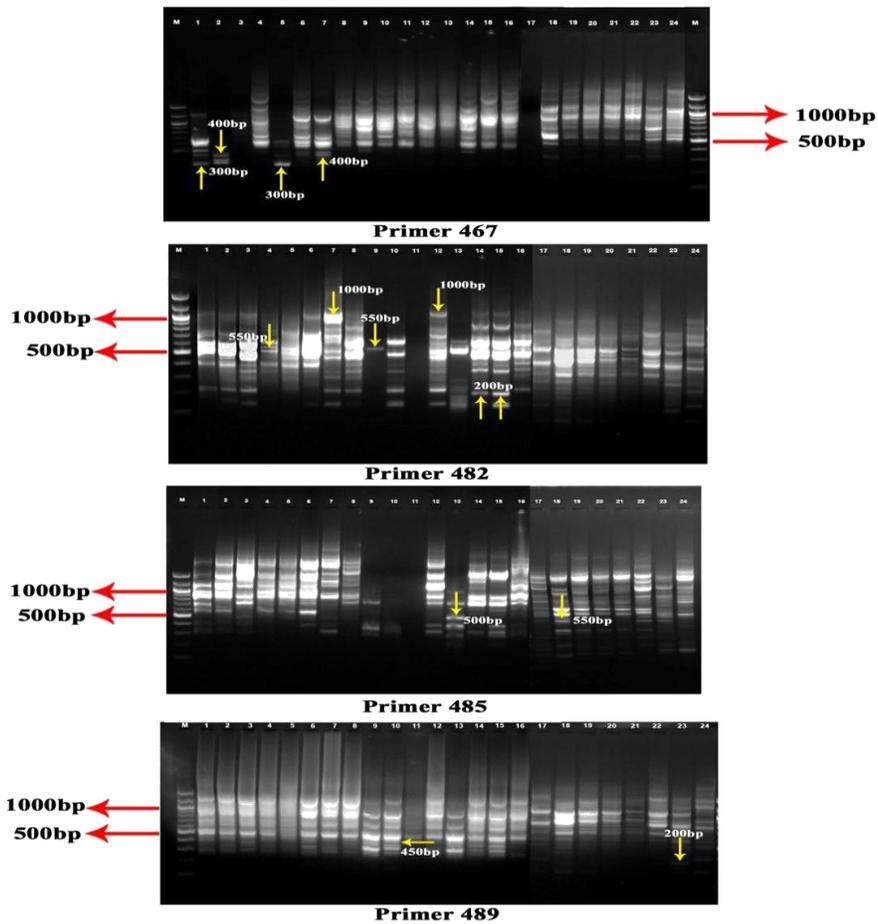
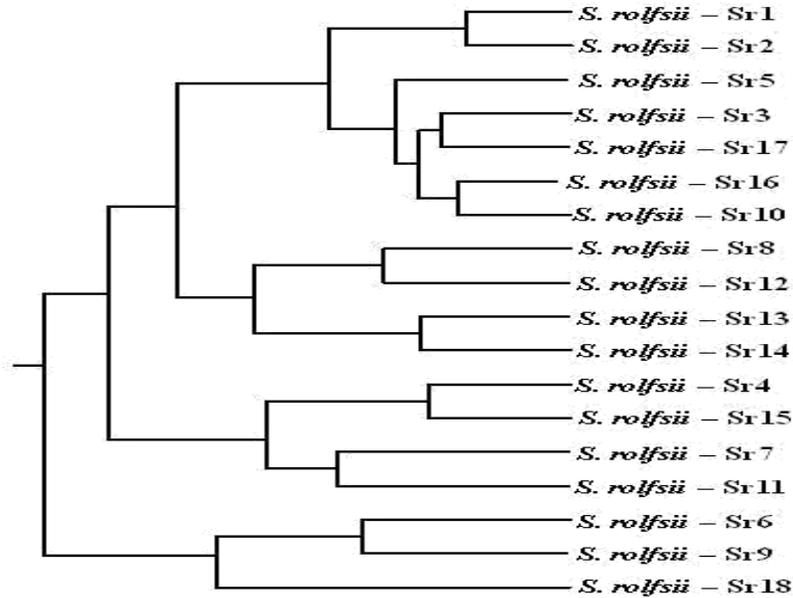


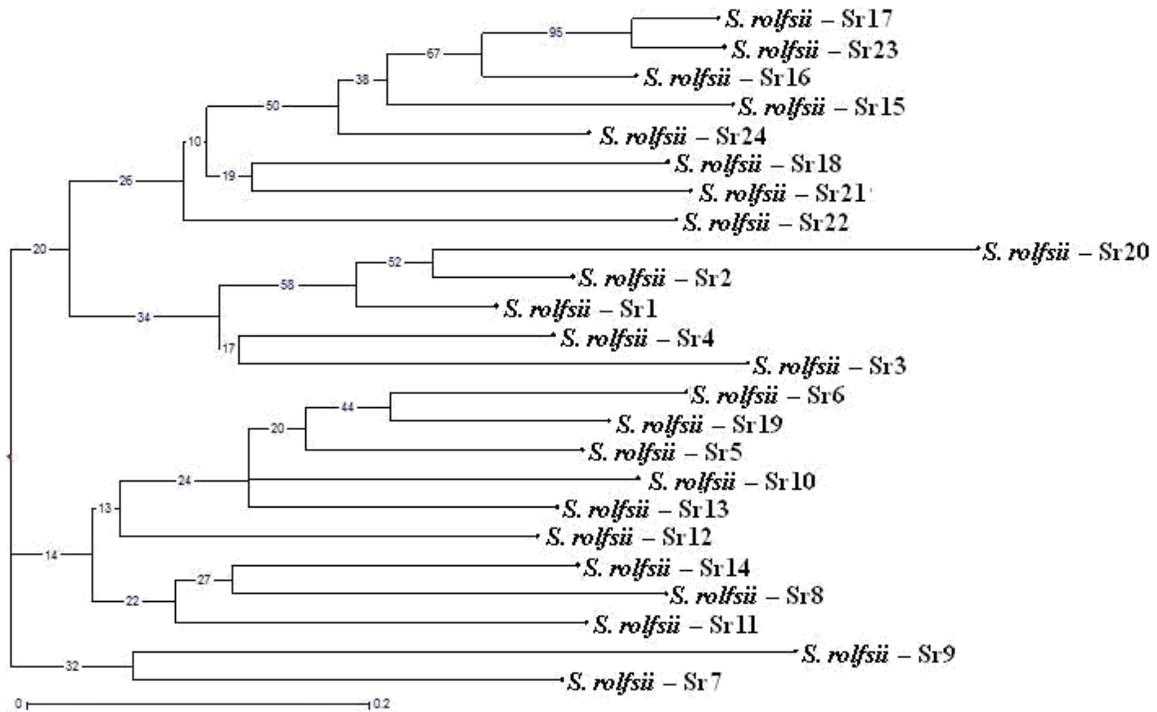
Fig.2 DNA profiling of *S. rolf sii* isolates banding on RAPD pattern using UBC-467, UBC-482, UBC-485 and UBC-489 primers



**Fig.3** Dendrogram based on UPGMA cluster analysis obtained from multiple sequences of ITS region of *Sclerotium rolfsii* isolated from groundnut



**Fig.4** Hierarchical horizontal dendrogram of RAPD region showing clustering of 24 *S. rolfsii* isolates by DARwin V.6 software



The genetic dissimilarity estimates for twenty four isolates were employed to generate dendrogram by using tree construction using the Unweighted Neighbourhood-joining method using the program DARwin 6.0 software (Fig 4). Based on the results obtained, all the twenty four isolates were grouped into three main clusters indicating there is genetic diversity in the isolates of *S. rolfssii*. Cluster I contained thirteen isolates, main cluster divided into two sub clusters, sub cluster I had eight isolates (Sr21, Sr20, Sr19, Sr18, Sr22, Sr24, Sr17 and Sr23) and subcluster II had five isolates (Sr5, Sr2, Sr1, Sr6 and Sr4). Main cluster II has two sub clusters, sub cluster I consisted of six isolates (Sr8, Sr3, Sr7, Sr12, Sr15 and Sr14) and in sub cluster II three isolates (Sr16, Sr10 and Sr13) were grouped. Cluster III consisted of two isolates (Sr9 and Sr11).

Among the twenty four isolates, four random primers viz., UBC-467, UBC-482, UBC-485 and UBC-489 generated reproducible polymorphism. Amplified products with all the primers showed polymorphic and distinguishable banding pattern indicating the genetic diversity among all the isolates of *S. rolfssii*. A total of 342 reproducible and scorable polymorphic bands ranging approximately as low as 150 bp to as high as 2000 bp were generated with four primers among 24 isolates characterized.

Based on the results obtained, all twenty four isolates were grouped into three main clusters (Fig. 4). Cluster I contains 13 isolates main cluster divided into two sub cluster, sub cluster I had eight isolates viz., Sr21, Sr20, Sr19, Sr18, Sr22, Sr24, Sr17, Sr23 and subcluster II had five isolates viz., Sr5, Sr2, Sr1, Sr6 and Sr4. Main cluster II has two sub cluster. The sub cluster I consists of six isolates (Sr8, Sr3, Sr7, Sr12, Sr15, and Sr14) and in sub cluster II had three isolates (Sr16, Sr10 and Sr13) and cluster III consists of two isolates (Sr9 and Sr11). Similarly, Prasad *et al.*, (2010) studied the genetic variability among the virulent isolates of *Sclerotium rolfssii* by using molecular techniques like RAPD, ITS-PCR and RFLP.

The RAPD banding pattern reflected the genetic diversity among the isolates by formation of two clusters. A total of about 221 reproducible and scorable polymorphic bands ranging approximately as low as 100 bp to as high as 2500 bp was generated with five RAPD primers.

ITS region of rDNA amplification with specific ITS1 and ITS4 universal primers produced approximately 650 to 700 bp in all the isolates confirmed that all the isolates obtained were *Sclerotium rolfssii*. Le *et al.*, (2012) studied the genetic and phenotypic diversity among the isolates of *Sclerotium rolfssii* isolated from groundnut crop. Based on ITS-rDNA sequence analyses, three distinct groups were identified among a total of 103 randomly selected *S. rolfssii* field isolates, with the majority of the isolates (n=90) in one ITS group. Rasu *et al.*, (2013) studied genetic diversity of *Sclerotium rolfssii* from different hosts by using RAPD primers. RAPD banding patterns were established for 10 isolates of *S. rolfssii* using five random primers. Size of DNA fragments amplified by all five primers ranged from 100 bp to > 1 kb indicating polymorphism among *S. rolfssii* isolates.

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