

## Molecular Variability of *Sclerotium rolfsii* Causing Foot Rot Diseases of Major Field Crops of Karnataka

Gowdra Nagamma<sup>1</sup>, A. Nagaraja<sup>2</sup>, Syeda Samina Anjum<sup>1</sup> and Sureshpatil<sup>3</sup>

<sup>1</sup>Department of Plant Pathology, UAS, GKVK, Bengaluru-560 065, India.

<sup>2</sup>Project Coordinating Unit AICSMIP (Small Millets), ICAR, GKVK, Bengaluru-560 065, India.

<sup>3</sup>Research Associate, AICSMIP (Small Millets), ICAR, GKVK, Bengaluru-560 065, India.

(Received: 11 September 2015; accepted: 03 November 2015)

*Sclerotium rolfsii* Sacc. is a well known, most destructive, soil borne, polyphagous fungus that lives as a facultative parasite. It is known to cause disease in more than 500 crops. *S. rolfsii* collected from different host and geographic locations in Karnataka, Isolates were examined for their molecular variations. Out of 37 isolates collected twelve isolates were selected and tested for their molecular variability. RFLP (Restriction Fragment Length Polymorphism) banding patterns were established for twelve isolates of *S. rolfsii* DNA amplification observed for ITS rDNA region at 550-600 bp in all the 12 isolates. The phylogenetic tree showed that four isolates Dharwad soybean, Bengaluru groundnut, Bengaluru chickpea and Davanagere sunflower belong to cluster I while one isolate Raichur groundnut belong to cluster II. PCR-RFLP of ITS region showed different patterns between the isolates of *S. rolfsii* after digestion with six enzymes *Viz.*, *AluI*, *Hinfl*, *TaqI*, *HindIII*, *EcoRI* and *HaeIII* indicating genetic variability among the isolates that varied in size of RFLP fragments and the number of fragments. Jaccards similarity co-efficient (UPGMA cluster analysis) among the twelve isolates were calculated to establish their genetic relationship. The similarity index values ranged from 31 to 93 per cent indicating the presence of high range of variability at the nucleic acid level among the isolates. The maximum genetic similarity of 93 per cent was observed between BelGn and KGn isolates, whereas least similarity of 31 per cent was observed between ChiGn and BFM isolates.

**Key words:** *Sclerotium rolfsii*, Molecular variability, foot rot, ITS-PCR-RFLP.

---

*Sclerotium rolfsii* Sacc. is a most destructive soil inhabitant, polyphagous facultative parasite. The diseases of *S. rolfsii* are a major constraint in production of groundnut, chickpea, finger millet, sunflower, soybean etc.. *S. rolfsii* has wide geographic diversity and commonly found in the tropics, subtropics and other warm temperate regions especially the Southern United States, Central and South America, West Indies, Southern European countries bordering the Mediterranean, Africa, India, Japan, Philippines and Hawaii<sup>1</sup>. The

wide host range, prolific growth and ability to produce persistent sclerotia contribute to the large economic losses associated with this pathogen. The fungus was placed in the form genus *Sclerotium*<sup>2</sup> as it formed differentiated sclerotia and sterile mycelium. The fungi included in this genus were characterized by production of small tan to dark brown or black spherical sclerotia with internally differentiated rind, cortex and medulla. *S. rolfsii* was reported as the best known member of the genus<sup>3</sup>. The cultures of *S. rolfsii* originating from various plant species and different geographical regions present wide variation in growth rate, morphological characteristics, besides genetic variability. However, the cultures of *S. rolfsii* can be identified by the size, color and

---

\* To whom all correspondence should be addressed.  
E-mail: nagamma0239@gmail.com

structure of their sclerotia. The fungus survives as sclerotia in soil and plant debris and remains viable for 2 to 3 years. High temperature 30°C and high soil moisture (77 %) encourages the disease.

Plant pathogens exhibit variation in their morphological, biological and pathogenic characters. Morphologic and pathogenic variations are known in many fungal pathogens<sup>4</sup>. The extreme variation in morphological characteristics of *S. rolfsii* has been observed in worldwide collection of this pathogen from different hosts and also from the same hosts<sup>5,6,7</sup>. Morphological and pathogenic variation among the *S. rolfsii* isolates of soybean have been studied<sup>8</sup>.

Molecular markers are increasingly being used to characterize population of plant pathogens<sup>9</sup>. Molecular markers may be used to evaluate levels of genetic diversity and phylogenetic relationships within and between species and to identify particular races and pathotypes<sup>10</sup>. Variations can be revealed in any part of the genome and data are reproducible and obtained quickly in case of RFLP. Hence, there is a need to identify variability in *S. rolfsii* so that, breeding for disease resistance is targeted against highly virulent isolate specific to the locality.

Not much work is done so far on *S. rolfsii* with respect to molecular variability studies. Hence, an attempt was made for systematic understanding molecular variations which attribute to the genetic variability of *S. rolfsii* in different hosts were also initiated.

## MATERIALS AND METHODS

### Molecular variability studies among the isolates of *S. rolfsii*

#### Reagents and chemicals

All the chemicals were of analytical grade and were 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 per cent CTAB (w/v); 1 per cent PVP (Mr. 40,000); 2 per cent  $\beta$ -mercaptoethanol (v/v)); phenol: chloroform (24:1); potassium acetate 7.5 M; proteinase K, 0.05 mg ml<sup>-1</sup>; wash solution (15 mM ammonium acetate in 75 per cent (v/v) ethanol]; TE buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8))<sup>11</sup>).

### Genomic DNA extraction

100 mg of mycelia was ground to fine powder using liquid nitrogen. Pre warmed 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  $\mu$ L Proteinase K (10 mgml<sup>-1</sup>) 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  $\times$ g for 10 min at RT and the supernatant was transferred to fresh Eppendorf tube. To the supernatant, 100  $\mu$ L 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  $\times$ 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  $\times$  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 the wash solution. The nucleic acid pellet so obtained was air-dried until the traces of ethanol was removed and dissolved in an appropriate amount of TE buffer (50-70  $\mu$ L). The nucleic acid dissolved in TE buffer were treated with ribonuclease (RNase, 10 mg ml<sup>-1</sup>), incubated at 37 °C for 30 min and stored at -20 °C until further use. The experiment was repeated thrice and the results described as the mean of three independent experiments.

### Qualitative and quantitative verification of DNA

The quality and quantity of DNA was analyzed by running 2  $\mu$ L of each sample mixed with 2  $\mu$ L of 10 x loading dye in 1 per cent agarose gel. The DNA from all the isolates produced clear sharp bands in one per cent agarose gel indicating good quality of the DNA. The DNA has been quantified by comparing with the 1 kb size marker (Genei, Bangalore) and by spectrophotometer (Nanodrop ND1000).

### PCR amplification of ITS region

The ribosomal DNA (rDNA) unit contains genetic and non-genetic or spacer region. Each repeat unit consists of a copy of 18S, 5.8S and 28S like rDNA and its spacer like Internal Transcribed

Spacers (ITS) and Inter-Genic Spacers (IGS). The rDNA have been employed to analyze evolutionary events because it is highly conserved, whereas ITS rDNA is more variable. Hence, it has been used for investigating the species level relationships.

The primers for amplification were custom synthesized at Bangalore Genie Pvt. Ltd. Bengaluru and supplied as lyophilized products of desalted oligos. Primer sequences used are as follows.

Organism	Sequence
Universal	ITS-1 - F 5'-AACGTTACCAAACACTGTTG-3'
fungal ITS	ITS-4 - R 5'-AAGTTCAGCGGGTATTCCT-3'

PCR reaction mixture		PCR condition for ITS region amplification		
Reaction mixture	Quantity	Step	Universal ITS Temp (°C)	Duration (min)
Template DNA (40 ng)	1 µl	Initial denaturation	94	5
Primers (5pmol)-F and R	1 µl	Denaturation	94	1
dNTP's (2 mM)	2 µl	Annealing	54	1
Taq buffer A (10X)	2 µl	Extension	72	2
TaqDNA polymerase (3U/µl)	0.3 µl	Final extension	72	10
Sterile water	13.7 µl	Hold	4	20
Total	20 µl	No. of cycles	30	
		Denaturation		
		Annealing Extension		

### Separation of amplified products by agarose gel electrophoresis

Agarose gel electrophoresis was performed to resolve the amplified product using 1.4 per cent agarose in 1X TBE (Tris Borate EDTA) buffer, 0.5 µgml<sup>-1</sup> of ethidium bromide and loading buffer (0.25 % Bromophenol Blue in 40 % sucrose). Four µL of the loading dye was added to 20 µL of PCR product and loaded to the agarose gel. Electrophoresis was carried at 65 V for 1.5 h. The gel was observed under UV light and documented using gel documentation unit.

### Sequencing of ITS region

The ITS region was sequenced from five isolates belonging to five different crops and regions to confirm organism and to know the variability present in them.

### Sequencing and *in silico* analysis

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 Clustal

W algorithm software and the phylogenetic tree was constructed (Patel, 2009).

### Analysis of the genetic variability among *S. rolfii* isolates using ITS-PCR-RFLP

PCR products of ITS region of the isolates under study were digested with six different restriction enzymes *viz.*, *AluI*, *HinfI*, *EcoRI*, *Hind III*, *HaeIII* and *TaqI*. Digestion was carried out with 20 µL reaction mixture containing 7 µL of ITSPCR product, 1 µL of enzyme (10 U/µL), 2 µL of 10X enzyme buffer and 10 µL of sterile PCR water. Aliquots of four µL of the amplicons were subjected to enzymatic digestion with the restriction enzymes *AluI*, *HinfI*, *EcoRI*, *Hind III*, *HaeIII* and *TaqI* (Invitrogen), as per the instructions. Agarose gel electrophoresis was performed to resolve the restricted product using 3.0 per cent agarose in 1X TBE (Tris Borate EDTA buffer, 0.5 µg ml<sup>-1</sup> of ethidium bromide and loading buffer (0.25 % Bromophenol Blue in 40 % sucrose). Electrophoresis was carried at 65 V for 1.5 h. The gel was observed under UV light and documented using gel documentation unit. ITS-RFLP fragments were visualized under UV light. The fragment sizes

Table 1. Homology of *S. rolfsii* isolates with gene bank in NCBI BLAST programme

Sl. No.	Sequence isolates	Gen bank accession number	Gen bank ref accession number	NCBI BLAST Hit results	Max. Ident. (%)
1	Bengaluru finger millet	KT319123	JN241560.1	Athelia rolfsii isolate 1125 internal transcribed spacer 1	90
2	Bengaluru finger millet	KT319123	HQ895869.1	Athelia rolfsii strain HUGS18T1 18S ribosomal RNA gene	90
3	Bengaluru chickpea	KT319124	KJ546416.1	Athelia rolfsii isolate BOSC-R-1 18S ribosomal RNA gene	94
4	Bengaluru chickpea	KT319124	HQ420816.1	Athelia rolfsii isolate SR001 18S ribosomal RNA gene	94
5	Dharwad soybean	KT319125	JN017199.1	Athelia rolfsii strain 09-044 18S ribosomal RNA gene	94
6	Dharwad soybean	KT319125	EU338381.1	Athelia rolfsii isolate SR-SC1 18S ribosomal RNA gene	94
7	Raichur groundnut	KT319126	HQ420816.1	Athelia rolfsii isolate SR001 18S ribosomal RNA gene	95
8	Raichur groundnut	KT319126	KJ546416.1	Athelia rolfsii isolate BOSC-R-1 18S ribosomal RNA gene	95
9	Davanagere sunflower	KT319127	HQ420816.1	Athelia rolfsii isolate SR001 18S ribosomal RNA gene	96
10	Davanagere sunflower	KT319124	AY684917.1	Athelia rolfsii strain FSR-052 18S ribosomal RNA gene	95

were estimated by comparing with a 100 bp marker.

#### Scoring and statistical analysis

Clearly visible RFLP products were scored from the image of ethidium bromide stained gels. The presence or absence of bands was recorded. For cluster analysis, the NTSYS.PC (Numerical Taxonomy System Applied Bio-statistics, Setauket, New York) computer programme was used. The generated pair wise similarity matrix was used to group strains by the unweighted pair-group method with arithmetic average (UPGMA). A dendrogram was derived from the similarity matrix.

## RESULTS AND DISCUSSION

### Isolation of Genomic DNA

Genomic DNA of the fungus was isolated by CTAB method. The DNA so obtained was observed by running through 1.5 per cent agarose gel electrophoresis. The DNA was about 7000-8000 bp (whole genomic DNA) of *S. rolfsii* with approximate concentration of 91 µg/µg.

### Amplification of ITS1 and ITS4 region

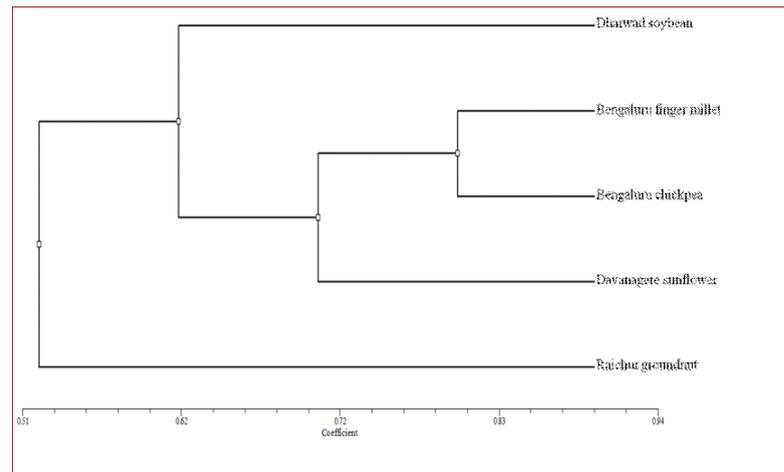
The full length ITS rDNA region was amplified with ITS1 (5'-TCCGTAGGTG AACCTGCGG-3') and ITS4 (5'-TCCTCCGC TTATTGATATGC-3') primers for all the 12 isolates of *S. rolfsii*. DNA amplicon was observed at the region of 600 to 650 bp with a concentration of around 430 µg/µl. The amplified products were checked on 1.4 per cent agarose gel electrophoresis.

This appears to be the first report of amplification of ITS rDNA region of *S. rolfsii* from different crops and regions in Karnataka. Since, there is less number of gene sequences of *S. rolfsii* in genbank; the sequences of ITS rDNA region isolate of finger millet from Bengaluru were deposited (Table 1).

### DNA sequencing

The DNA sequences were obtained for ITS rDNA. The sequences of these isolates are as given below.

Accession no: KT319123, KT319124, KT319125, KT319126 and KT319127 DNA sequences of selected isolates were compared using bioinformatics tools like NCBI (National Centre for Bioinformatics) BLAST programme. Based on the sequence comparison, the



**Fig. 1.** Dendrogram of selected *S. rolfsii* isolates based on sequencedata

identification of *Sclerotium* isolates was confirmed and all the ITS rDNA sequences of the isolates were confirmed as of *S. rolfsii*. The list of isolates, accession number, per cent homology and name identified are given in Table 1.

#### Phylogenetic Analysis

The ITS rDNA region sequence was used in these analysis because it has been shown to be more informative and closest phylogenetic relative in the genus *Sclerotium*. In order to evaluate whether the grouping pattern obtained on the basis of the ITS -sequences of the ex-type strains would be useful frame to identify and align, a total of five *Sclerotium* isolates were used and most of which had been identified as *Sclerotium rolfsii*. These isolates were identified at the species level by morphological characters using the existing taxonomic criteria analysis and analysis of their ITS rDNA region gene sequences.

In the present case, five isolates are in two main clusters I and II. One consists of only one isolate, while two consists of four isolates which was again divided in to two sub clusters. Subcluster I with Dharwad soybean and remains three in sub cluster II. The subcluster II is further divided in to two subclusters. Only Raichur groundnut formed a separate cluster and distinct from other four. Bengaluru chickpea and Bengaluru finger millet had more similarity (Fig. 1). A glance of phylogenetic tree reveals that, there is not much diversity among the isolates of *S. rolfsii*. However, Dharwad soybean and Raichur groundnut isolate formed a separate cluster showing divergence to

other *S. rolfsii* isolates. As expected the clustering of *Sclerotium* was altogether different having wide divergence with separate branch in the phylogenetic tree.

Abundance and uniform distribution of genetic markers in any pathogen is necessary for applications like diversity analysis at various levels. Presently, DNA based markers are a class by themselves. Almost unlimited in number, they are widely and evenly distributed in the genome. Unaffected by other genes and environment, the genotype of any individual of a population with respect to DNA based markers can be determined unequivocally, at any stage of the development non-destructively. In addition, it is possible to generate markers to suit specific applications without altering the genotype of the individuals. It is difficult to distinguish these species using traditional morphological and physiological differences. To understand existence of variation among the isolates of pathogens, PCR based technique *i.e.*, ITS (Internal Transcribed Sequence) was used in the present investigation.

The genus *Sclerotium* poses a major challenge for systematics because the phylogenetic relationships of many of its members still are unclear. In these the isolates identified as *S. rolfsii* are markedly similar to the reference strain of NCBI BLAST Genbank. The information provided by these studies gives more useful tips for assessing the taxonomy. In the present case, isolates of *S. rolfsii*, *S. delphinii* and *Athelia rolfsii*, was used as an out group vice versa to interpret

**Table 2.** Restriction digestion of ITS-PCR-RFLP pattern (approximate band sizes)

Isolates	No of Restriction sites	AluI Size	No of Restriction sites	HinfI Size	Restriction site	TaqI Size	Restriction site	HindIII Size	Restriction site	EcoRI Size	Restriction site	HaeIII Size
1	2	350bp, 150bp, 480bp, 100bp	2	310bp, 180bp, 150bp	1	350bp, 280bp	1	410bp, 190bp	0	-	0	-
2	2	350bp, 150bp, 480bp, 100bp	2	310bp, 180bp, 150bp	1	350bp, 280bp	1	410bp, 190bp	0	-	0	-
3	2	350bp, 150bp, 480bp, 100bp	2	310bp, 180bp, 150bp	1	350bp, 280bp	1	410bp, 190bp	0	-	0	-
4	2	480bp, 350	2	310bp, 180bp, 150bp	1	350bp, 280bp	1	410bp, 190bp	0	-	0	-
5	2	480bp, 350bp	2	310bp, 180bp, 150bp	0	600	1	410bp	0	-	0	-
6	2	480bp, 350bp	2	310bp, 180bp, 150bp	1	350bp, 280bp	1	410bp	0	-	0	-
7	2	480bp, 350bp	2	310bp, 180bp, 150bp	1	350bp, 280bp	1	410bp	0	-	0	-
8	2	480bp, 350bp	2	310bp, 180bp, 150bp	0	600	1	410bp	0	-	0	-
9	2	480bp, 350bp	1	310bp, 180bp, 150bp	1	350bp, 280bp	1	410bp, 200bp	0	-	0	-
10	2	480bp	1	310bp, 180bp, 150bp	1	350bp, 280bp	1	410bp	0	-	0	-
11	2	480bp	1	310bp, 180bp, 150bp	1	350bp, 280bp	1	410bp	0	-	0	-
12	2	480bp, 350bp	1	310bp, 180bp, 150bp	1	350bp, 280bp	1	410bp	0	-	0	-

the clustering of isolates as distinct or related out group of genus. Phylogenetic analysis of ITS sequences showed three clusters (Fig. 1).

Since, there is less number of gene sequences of *S. rolfsii* infecting finger millet and groundnut causing foot rot and collar rot diseases respectively, our sequences are deposited in the Genbank and the accession numbers are awaited. This is the first such information in sequencing ITS rDNA region and on molecular variability of pathogen causing the foot rot disease in Karnataka.

### Specific amplification of *S. rolfsii*

DNA amplification from *S. rolfsii*, was observed at good specificity for the genus *Sclerotium* and approximately 550-600 bp product was exclusively amplified in all the twelve isolates (Plate 1). These results confirmed that all the isolates belonged to the genus *Sclerotium*. Inter and intra-specific variation within the ITS regions have been reported in several fungi, and are mostly due to deletion or insertion events in the ITS 1 and ITS 4 regions<sup>12,13,14</sup> screened a worldwide collection of *S. rolfsii* which revealed variation in

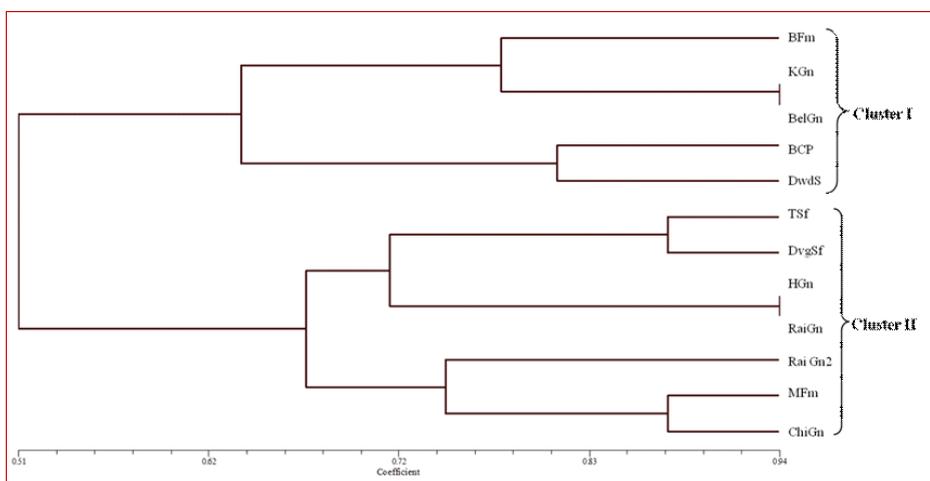
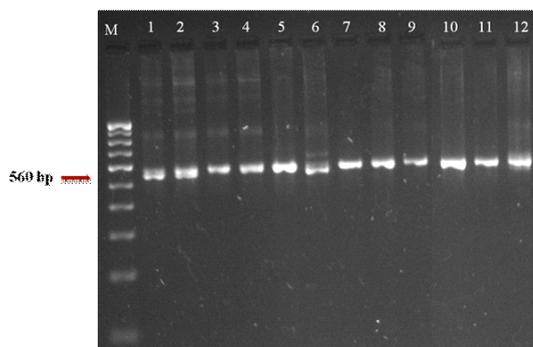


Fig. 2. Relationship between *S. rolfsii* isolates using different molecular RFLP marker through UPGMA analysis



M 100 bp marker 1. Bengaluru finger millet (BFm) 2 Tumkur sunflower (TSf) 3 Koppal groundnut (KGn) 4 Haveri groundnut (HGn) 5 Raichur groundnut 2 (RaiGn2) 6 Mandya finger millet (MFm) 7 Belagavi groundnut (BelGn) 8 Davanagere sunflower (DvgSf) 9 Chitradurga groundnut (ChiGn) 10 Bengaluru chickpea (BCP) 11 Dharwad soybean (DwdS) 12 Raichur groundnut (RaiGn)

**Plate 1.** Amplification of ITS1 and ITS4 region of representative *S. rolfsii* isolates

ITS regions of 12 sub-groups of *S. rolfsii*<sup>15</sup>. Studied the variability among 30 isolates of *S. rolfsii* by RAPD and differentiated into distinct groups by ITS-PCR.

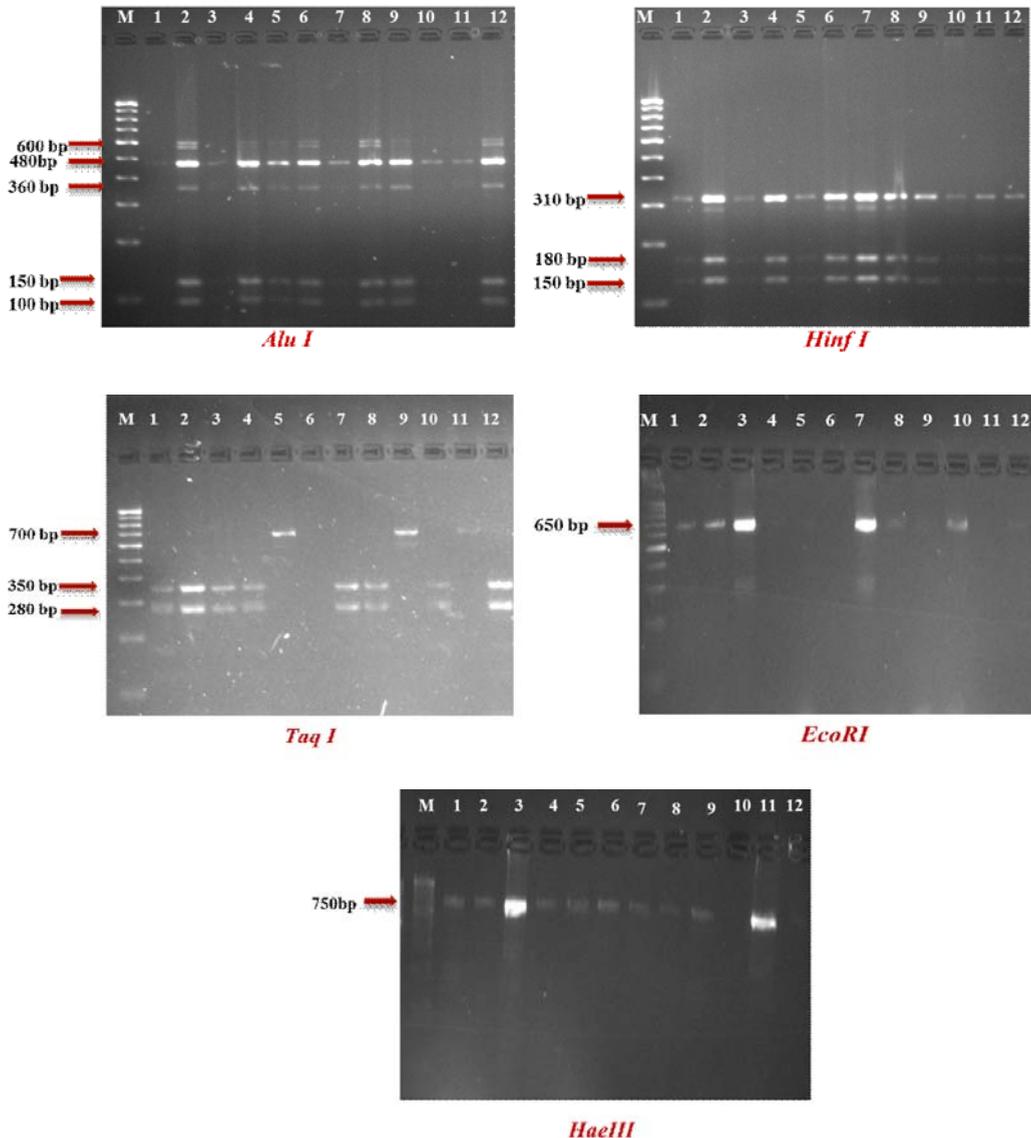
### Analysis of the genetic variability among the isolates of *S. rolfsii* by using PCR-RFLP

Twelve selected isolates of *S. rolfsii* from different crops and regions were analyzed by internal transcribed spacer-polymerase chain reaction-restriction fragment length polymorphism (ITS-PCR-RFLP) to assess the genetic variability among the isolates.

PCR-RFLP of ITS region showed different patterns between the isolates of *S. rolfsii* after digestion with six enzymes viz., *AluI*, *HinfI*, *TaqI*, *HindIII*, *EcoRI* and *HaeIII* indicating genetic variability among the isolates that varied in size of RFLP fragments and the number of fragments (Plate 2).

sizes ranges from 310bp, 180bp and 150 bp. *HindIII* had one restriction site in all the isolates producing two bands of sizes 480 and 200bp. *TaqI* has produced polymorphic bands in the restriction site. It produces bands of 350-280 bp. However both groundnut from Raichur and Chitradurga isolates did not showed any restriction site therefore produced parental band approximately 600 bp.

Out of six restrictions enzyme tried on PCR ITS rDNA digestion two amplified product did not have restriction site in any of the isolates and enzymes viz., *EcoRI* and *HaeIII*. Remaining four *AluI* and *HinfI* had two restriction sites in all the isolates at the same site producing four bands in all the isolates. *AluI* has produced 480-100 and 350-150 bp. Similarly *HinfI* it produced three bands



M 100 bpmarker 1. Bengaluru finger millet (BFm) 2 Tumkur sunflower (TSf) 3 Koppal groundnut (KGn) 4Haveri groundnut (HGn) 5 Raichur groundnut 2 (RaiGn2) 6 Mandya finger millet (MFm) 7 Belagavi groundnut (BelGn)8 Davanagere sunflower (DvgSf) 9 Chitradurga groundnut (ChiGn) 10 Bengaluru chickpea (BCP) 11 Dharawad soybean (DwdS) 12 Raichur groundnut ( RaiGn)

**Plate 2.** PCR-RFLP banding pattern of ITS region in different isolates of *S.rolfsii*

Difference in the banding pattern indicates that variability exist between the isolates. In the present investigation, all the enzymes above have shown different restriction pattern but the each enzyme has the same restriction site among all the isolates under the study. It is worthwhile to use more number of enzymes to study the polymorphism (Table 2).

These results are conformity <sup>16</sup> who conducted an experiment to know the variation in the internal transcribed spacer by RFLP using the restriction enzymes *RsaI*, *HpaII*, *Sau3AI*, and *MspI*. Combined banding patterns for the four enzymes were used to characterize isolate variation and to compare these results with the combing banding patterns of the eleven isolates from the world wide collection. It was found that twelve isolates from Mexico belonged to some of the RFLP-ITS groups of the worldwide collection. According to <sup>17</sup> ITS-RFLP analysis using four restriction enzymes (*AluI*, *Hpa2*, *RsaI* and *MboI*) displayed a low degree of variability among the isolates of *S. rolfsii*.

Genetic variability among the virulent isolates of *S. rolfsii* using molecular techniques like RAPD, ITS-PCR and RFLP. 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 are of *S. rolfsii* <sup>18</sup>. Studies by ITS-RFLP indicated that there was no polymorphism in restriction banding pattern among the isolates with the restriction endo nucleases (*AluI*, *HinfI* and *MseI*) used. Ramesal *et al.* (2013) ITS-RFLP analysis using four restriction enzymes (*AluI*, *HpaII*, *RsaI*, and *MboI*) displayed a low degree of variability among MCGs. Only three different restriction profiles were identified among *S. rolfsii* isolates, with no correlation to MCG or to geographic origin.

Sixty seven isolates of the southern blight fungus from Japan into five groups based on ITS-RFLP analysis of nuclear rDNA. Three groups were re-identified as *S. rolfsii* and two resembled *S. delphinii* in RFLP patterns. In the present investigation, all the enzymes used have shown different restriction pattern indicating the existence of polymorphism between the isolates collected from different crops and regions <sup>19</sup>.

Relationships among the isolates were evaluated by cluster analysis of the data based on the similarity matrix. The dendrogram was

generated using UPGMA method, based on the results all the twelve isolates were grouped into two main and six sub clusters. The isolates in Cluster-I included BFM, KGn, BelGn, BCP, DwdS; cluster – II included RCRGn, DvgSf, HGn, TGn, RCRGn2, MFM and ChiGn. Whereas isolate BFM fell into Sub cluster IA, KGn and BelGn into sub cluster IB, RCRGn and DvgSf into sub cluster IC, TSf and DvgSf into sub cluster IIA, HGn and RCRGn into sub cluster IIB; RCRGn2, MFM and ChiGn in sub cluster IIC (Fig.2).

Jaccards similarity co-efficient among the twelve isolates were calculated to establish their genetic relationship. The similarity index values ranged from 31 to 93 per cent indicating the presence of high range of variability at the nucleic acid level among the isolates (Table 2). The maximum genetic similarity of 93 per cent was observed between BelGn and KGn isolates, whereas least similarity of 31 per cent was observed between ChiGn and BFM isolates.

RFLP is suitable to detect the variations among the *S. rolfsii* isolates. In the present investigation, six restriction enzymes were used to determine genetic diversity between the isolates and construct a dendrogram. The dendrogram of RFLP data differentiated the twelve isolates into two major clusters. In Cluster I the similarity index between the clusters varied from 62 to 100 per cent whereas in Cluster II it ranged from 31 to 50 per cent. The results also revealed that the isolates are related irrespective of the geographical locations. The diversity observed in this study could mainly be attributed to genetic dissimilarity revealed by the different banding patterns in RFLP profile exhibited by each isolate.

## REFERENCES

1. Aycock, R., Stem rot and other diseases caused by *Sclerotium rolfsii*. N. C. Agric. Exp. Tech. Bull., 1966; **174**: 202.
2. Saccardo, P. A., *Sclerotium rolfsii*. *Sylogae Fungorum* 22. Pavia, Italy, 1913; p. 1500.
3. Punja, Z. K. and Damiani, A., Comparative growth, morphology and physiology of three *Sclerotium* species. *Mycologia*, 1996; **88**: 694-706.
4. Kumar, S., Thind, T. S. And Mohan Chander, Morphogenic and pathogenic variation in *Gloeosporium ampelophagum*. *Indian*

- Phytopathol.*, 1955; **48**: 331-334.
5. Cooper, W. E., Strains of *Sclerotium rolfsii* resistance to antagonists. *Phytopathology*, 1961; **51**: 113-116.
  6. Kim, K., Studies on *Sclerotium rolfsii* Sacc. isolated from *Mangolia kubus* DC in Korea. *Korean J. Pl. Protec.*, 1974; **13**: 105-113.
  7. Punja, Z. K., The biology, ecology and control of *Sclerotium rolfsii*. *Annu. Rev. Phytopathol.*, 1985; **23**: 97-127.
  8. Ansari, M. M. And Agnihotri, S. K., Morphological, physiological and pathogenic variations among *Sclerotium rolfsii* isolates of soybean. *Indian Phytopathol.*, 2000; **53**: 65-67.
  9. Mcdonald, B. A., The population genetics of fungi: Tools and technique. *Phytopathology*, 1987; **87**: 448-453.
  10. Majer, D., Mithen, R., Lewis, B. G., Vor, P. And Oliver, P. R., The use of AFLP fingerprinting for the detection of genetic variation in fungi. *Micol. Res.*, 1996; **100**: 1107-1111.
  11. Liu, D., Coloe, S., Baird, R. And Pederson, J., 2000, Rapid mini preparation of fungal DNA for PCR. *J. Clin. Microbiol.* **38**: 471.
  12. Carbone, I. And Kohn, L. M., Ribosomal DNA sequence divergence within internal transcribed spacer1 of *Sclerotiniaceae*. *Mycologia*, 1993; **85**: 415-427.
  13. Chen, W., Hoy, J. W. And Schneider, R. W., Species-specific polymorphisms in transcribed ribosomal DNA of five *Pythium* species. *Exp. Mycol.*, 1992; **16**:22-34.
  14. Harlton, C. E., Levesque, C. A. And Punja, Z. K., Genetic diversity in *Sclerotium (Athelia) rolfsii* and related species. *Phytopathology*, 1995; **85**(10):1269-1281.
  15. Almeida, A. M. R., Abdelnoor, R. V., Calvo, E. S., Tessnman, D. And Yorinori, J. T., Genotypic diversity among Brazilian isolates of *Sclerotium rolfsii*. *J. Phytopathol.* 2001; **149**(9): 493-502.
  16. Flores-moctezuma, H. E., Montes-belmont, R., Rogel- Hernandez, M. A. And Martinez-romero, M. E., Diversidad genetica de *Sclerotium rolfsii* Sacc. en Mexico. *Revista Mexicana de Fitopatologia*, 2008; **26**:7-14.
  17. Ramesal, E., Landa, B. B., Jiménez-gasco, M. M. And Navas-cortés, J. A., Sequence variation in two protein-coding genes correlates with mycelial compatibility groupings in *Sclerotium rolfsii*. *Phytopathology*, 2013; **103**: 479-487.
  18. Durgaprasad, S., Basha, S. T., Peddanarappa, N. And Eswara Reddy, G., Molecular variability among the isolates of *Sclerotium rolfsii* causing stem rot of groundnut by RAPD, ITS-PCR and RFLP. *EurAsia J. BioSci.*, 2010; **4**: 80-87.
  19. Okabe, I., Morikawa, C., Motsumoto, N. And Yokoyama, K., *Sclerotium rolfsii* isolates in Japan. *Mycosci.*, 1998; **39**: 399-407.