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MORPHOLOGICAL AND MOLECULAR DIVERSITY OF *SCLEROTINIA SCLEROTIORUM* (LIB.) DE BARY ISOLATES OF INDIA

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ABSTRACT
Sclerotinia sclerotiorum is most successful soil-inhabiting necrotropic filamentous, ascomycetes fungus with a broad host range of > 400 plant species. The morphological and molecular diversity of 9 isolates of *Sclerotinia sclerotiorum* collected from different host plants and geographical locations of India were studied by using different morphological and molecular markers. The isolates varied in morphological characters viz., colony growth, growth rate, colony appearance and sclerotia. The growth rate of isolates varied from 0.24mm-1.25mm/hr and they were grouped as fast, medium and slow growing isolates. Out of 9 isolates only four isolates produced flattened and elongated black coloured sclerotia. Extracted genomic DNA of fungus was quantified by Nano-drop and 550bp size DNA fragment was amplified with ITS primer set. The genomic DNA of fungal isolates showed no variability among the nucleotide sequences of ITS region and more homogeneity was observed with ITS-RFLP. The RAPD and URP-PCR banding pattern were analyzed by UPGMA (NTsys-PC). The dendrograms obtained by cluster analysis revealed that isolates of different geographical locations collected were distinctly diverse.

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is most successful soil inhabiting necrotropic and non-host specific fungal plant pathogen and has a broad ecological distribution with a host range of more than 400 plant species.The majority of these species are dicotyledonous, although a number of agriculturally significant monocotyledonous plants are hosts of*S. sclerotiorum* (Boland and Hall, 1994). It causes significant crop yield loss relatively more in cool and moist areas (Sharma *et al.*, 1999). Itis most common in temperate regions. It was originally believed to confine only in cool, moist areas, but is now known to occur in hot as well as dry areas because of its genetic diversity and greater adaptability.

It is important to understand the diversity of this pathogen for the development of effective screening strategies to identify and deploy host resistance as well as to know variability within the population in a geographical area is important, because these also reveal the changes occurring in the population. Several molecular methods such as amplified fragment length polymorphism (Cubeta *et al.*, 1997), random amplified fragment length polymorphism (Yli-Mattila *et al.*, 2010; Thilagavathi Rasu *et al.*, 2013), micro satellite marker (Meinhardt *et al.*, 2002) and sequence-related amplified polymorphism (SRAP) technique (Li *et al.* 2009), universal rice primer polymerase chain reaction (URP-PCR) technique (Rashmi *et al.*, 2008) were used to determine genetic diversity of fungus. The RAPD and ITS markers were suitable to determine variability among Indian population of *S. sclerotiorum* (Njambere *et al.*, 2008; Mandal *et al.*, 2012).

The objective of the present study was to understand morphological and molecular diversity among isolates of *S. sclerotiorum* collected from different host plants and geographical locations of India based on morphological and molecular characterization.

MATERIALS AND METHODS

Morphological characterization

Sclerotinia sclerotiorum isolates used for the present study were obtained from Indian Type Culture Collection (ITCC), IARI, New Delhi. These cultures were originally isolated from infected plant samples from different localities of India and stored on potato dextrose agar (PDA) slant and designated as SS-1to SS-9 (Table 1). Five mm diameter circular discs taken from the margin of an actively growing (3-4 days old) colony was punched out with the help of a gel cutter and placed onto the centre of the plate with the mycelia side facing downwards, under aseptic conditions in three replications. The plates were incubated at 24 ± 1ºC. Morphological characteristics such as colony diameter, growth type and sclerotia characters were recorded. Three days after inoculation, growth related characters were recorded whereas, sclerotia characters were recorded 15 days after inoculation (Morall *et al.*, 1972).

ITS-RFLP analysis

The *Sclerotinia* isolates were cultured in potato dextrose broth (PDB) to obtain mycelia mat for DNA extraction. Agar discs were cut out from an actively growing fungal colony with a 5 mm diameter cork borer and placed into 250 ml conical flask containing 100 ml of PDB. These cultures were incubated at 24 ± 1ºC in an incubator. The mycelial mats were harvested by filtration through Whatman filter paper No.1 and washed repeatedly with distilled water. The obtained mycelial mats were stored at -20°C for genomic DNA extraction. Genomic DNA of the fungus was extracted using CTAB method (Murray and Thompson, 1980) with a slight modification and stored at -20 ºC in small aliquots for further use. The extracted DNA was also quantified with the help of Nano-drop and readings were taken at two wavelengths i.e. at 260nm and 280nm.

The ITS region of *S. sclerotiorum* isolates was amplified with a set of primers ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as described by White *et. al.*(1990).The PCR reaction was carried out in a 100µl volume containing 10µl of 1X PCR buffer, 1µl of 2.5 mM Mg Cl_2, 1.5µl of 0.6 mM dNTP, 1µl 0f 5 pmol of each primer(ITS1 and ITS4), 2µl of template DNA 1µl of (3U/µl) Taq polymerase (Bangalore Genei, India) and 83.5µl nuclease free water. PCR was performed using AB, (BIOER GenePro) Thermo cycler. The cycle parameter included an initial denaturation at 94 ºC for 5 min and followed by 35 cycles containing denaturation at 94 ºC for 1min; annealing at 58 ºC for 1min; extension at 72 ºC for 1min and the final extension was set up at 72°C for 10 min. Amplified products were electrophoresed on a 1.2% gel in TAE buffer and photographed using a Genius Gel Documentation System (SyngeneInc, Cambridge,UK). The size of fragments were analyzed by comparison with 100bp DNA ladder

Amplified products of *S. sclerotiorum* isolates were digested individually with 6 different restriction enzymes viz., *HaeIII, Eco RI, Bsp143I, RsaI, PstI* and *PvuII* according to the manufacturer's instructions. Digested products were separated on a 1.2% gel in TAE buffer using ethidium bromide visualized stain.

RAPD and URP -PCR analysis

Genomic DNA of *S. sclerotiorum* isolates was amplified by using 10-mer arbitrary oligonucleotide primers (Williams *et al.,* 1990) and URP (universal rice primer) primers with 20 oligonucleotides (Kang *et al.,* 2002). These primers were obtained by Operon Technology, USA and Genuine Chemical Corporation (GCC), India, respectively (Table 2).

The reaction mixture was prepared by using standardized protocol of Rashmi *et al.*, 2008. The reaction was carried out in a 100µl volume containing 10µl of 1X PCR buffer, 1µl of 2.5 mM MgCl2, 1.5µL of 0.6 mMdNTP, 1µl of primer (oligonucleotide primers), 2il of template DNA, 1µl of (3U/µl)Taq polymerase (Bangalore Genei, India) and 83.5µl nuclease free water.

RAPD and URP-PCR were performed separately using AB, (BIOER Gene Pro) Thermo cycler. Thermal cycling conditions were: initial denaturation at 94ºC for 4 min, followed by 35 denaturation cycles at 94ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 2 min. A final extension step at 72ºC for 7 min was also performed. The amplified products were separated on a 1.2% gel in TAE buffer stained with ethidium bromide. 100bp DNA ladder used as a marker.

Relatedness among the isolates of *S. sclerotiorum* was determined by means of scorable DNA bands which were amplified from different 10 mer oligonucleotide primers and URP's with 20 oligonucleotides. Each band was considered as character and was scored on basis of presence (coded as1) or absence (coded as 0). The data obtained from banding pattern was analysed by using the computer programme NTSYS-PC (version 2.02e). Cluster analysis was performed on the similarity matrix with the SAHN program using UPGMA (Rohlf, 1998) and dendrograms were constructed.

RESULTS AND DISCUSSION

The variability among different isolates of *S. sclerotiorum* based on radial growth rate has already been reported by various researchers (Carpenter *et al.*, 1999; Akram *et al.*, 2008). Morphological characteristics of *S. sclerotiorum* isolates grown on PDA varied considerably (Table3). Isolates showed different colony appearance on PDA such as, white fluffy (SS-3, SS-4, SS-6, SS-7), white suppressed (SS-2, SS-8, SS-10)) and dull white suppressed (SS-1, SS-5, SS-9). The entire plate was covered with mycelium within 72 hours by fast growing isolates.

The growth rate of the isolates varied from 0.24 to 1.25 mm/hr. Based on differences in growth rate, the isolates were classified into three groups; fast growing (>1mm/hr), medium growing (0.5-1mm/hr) and slow growing (<0.5mm/hr). The data after 72hr indicated that the isolates SS-2, SS-4, SS-5, SS-6 and SS-7 were representing fast growing, SS-1and SS-8 were representing medium growing and isolates SS-3 and SS-9 were significantly representing slow growing isolate.

The formation of sclerotia initiated 8 days after inoculation, out of 9 isolates only 4 isolates were produced sclerotia. The

Table 1: List of isolates collected from ITCC, IARI and New Delhi

ITCC No.	Isolates No.	Host	Location
5575	SS1	-	Srinagar
5425	SS2	-	Varanasi
4042	SS3	Mustard	New Delhi
4043	SS4	Carrot	New Delhi
5492	SS5	Potato	Ludhiana
4727	SS5	*Tagetus minuta*	Nainital
4929	SS7	Coriander	Hissar
6583	SS8	Berseem	Jhansi
6094	SS9	Pea	New Delhi

Table 2: RAPD and URP primers and their sequences

Sl.No.	RAPD primers	Sequence(5'-3')
1	OP04	CCGCATCTAC
2	OP14	GTGACAGGCT
3	OP15	AAGAGAGGGG
4	OP18	AGGTGACCGT
Sl.No.	URP primers	Sequence(5'-3')
1	URP-1F	ATCCAAGGTCCGAGACAACC
2	URP2R	CCCAGCAACTGATCGCACAC
3	URP-4R	AGGACTCGATAACAGGCTCC
4	URP17R	AATGTGGGCAAGCTGGTGGT

Table 3: Different morphological characters of *Sclerotinia sclerotiorum* isolates on PDA at 24 ± 1°C

Isolate No.	*Colony diameter(mm) 48 hr	72hr	Growth rate(mm/hr)	**Growth type	Colony appearance	*Sclerotia arrangement	*No. of sclerotia	*Sclerotia diameter (mm)	*Sclerotia Weight (mg)
SS1	18.0	65.0	0.90	Medium	Dull suppressed	-	-	-	-
SS2	70.0	87.5	1.21	Fast	White suppressed	Scattered	15.5	2.5	0.01
SS3	12.5	17.5	0.24	Slow	white fluffy	-	-	-	-
SS4	27.5	82.5	1.14	Fast	White fluffy	Peripheral	3.0	3.0	0.18
SS5	37.5	86.5	0.93	Fast	Dull white suppressed	Peripheral	14.5	2.0	0.02
SS6	40.0	90.0	1.25	Fast	White fluffy	-	-	-	-
SS7	27.5	87.5	1.21	Fast	White fluffy	-	-	-	-
SS8	15.0	37.5	0.52	Medium	White fluffy	Scattered	26.5	1.8	0.01
SS9	25.0	30.0	0.41	Slow	White suppressed	-	-	-	-

** (Fast growth - > 1.0mm/hr, Medium growth- 0.5 -1.0mm/hr, Slow growth- < 0.5mm/hr); *(Mean of three replications)

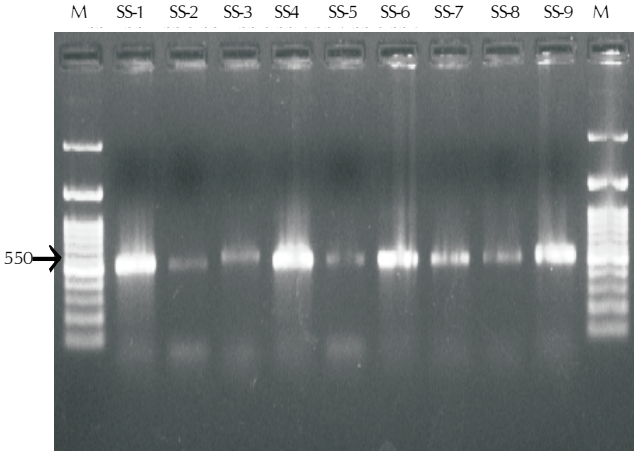

Figure 1: Amplification of ITS region using primer set ITS1 and ITS4. Lane M, 100bp DNA and Lanes SS-1 to SS-9, isolates of *S. sclerotiorum*.

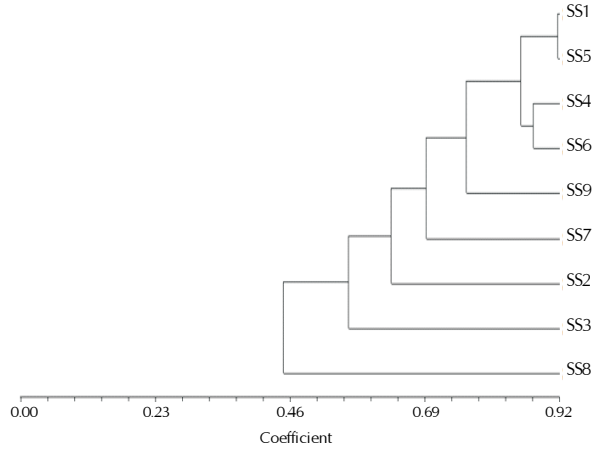

Figure 2: Dendrogram derived from RAPD analysis of isolates of *Sclerotinias clerotiorum*(SS1-SS9) with 4 primers by UPGMA (Unweighted Paired-Group method with Arithmetic average)

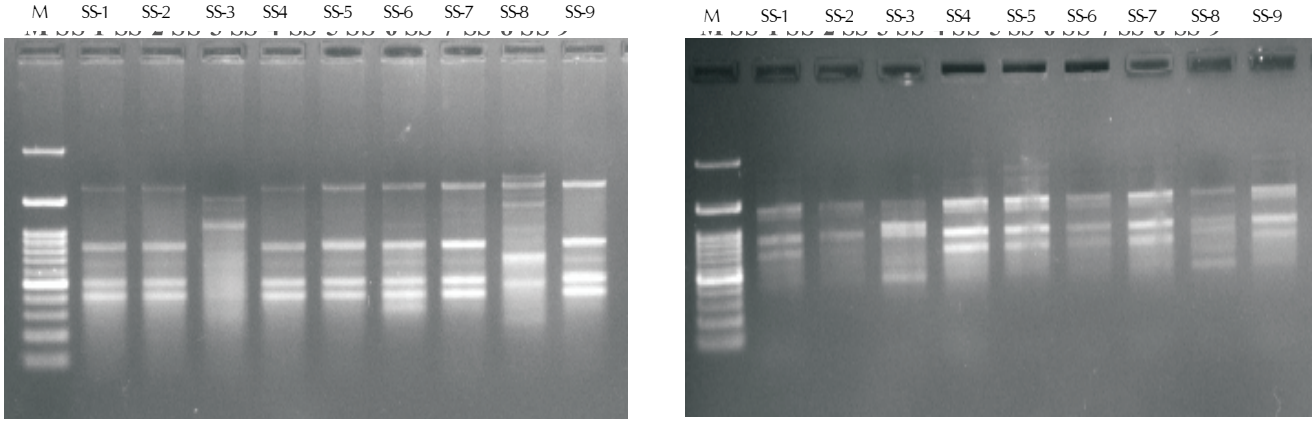

Figure 3: (a) DNA profile of *S. sclerotiorum* isolates obtained with primer URP-17R.(b) DNA profile of *S. sclerotiorum* isolates obtained with primer URP-2R. Lane M, 100 bp DNA ladder and Lanes SS-1 to SS-9 isolates of *S. sclerotiorum*

failure of sclerotia may be due to unfavourable conditions or sterile mycelium. The colour of sclerotia was initially light brown in colour and later turned to black after maturity. The arrangement of sclerotia on PDA differed with isolates such as scattered and peripheral ring form. As for the sclerotia number, size and diameter concerned were also varied considerably among sclerotia formed isolates. Based on sclerotial characters several workers recorded variation among different isolates of *S. sclerotiorum*(Dhingra and Sinclair, 1973; Mirza*et al.*, 1985, Akram*et al.*, 2008).

ITS-RFLP analysis

Extracted DNA was quantified by Nano-drop. PCR amplification of *S. sclerotiorum* isolates yielded single DNA

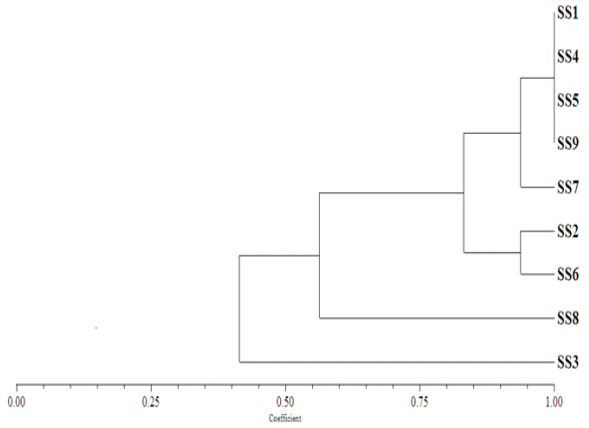

Figure 4: Dendrogram obtained from 9 isolates of *Sclerotinia sclerotiorum* with UPGMA-based similarity coefficient, using primer URP-2R and 17R

fragment of approximately 550bp size with primer set ITS1 and ITS4 (Fig. 1). The ITS –RFLP profiles showed high level similarity in ITS region of different isolates. Out of 6 restriction enzymes used *PvuII* and PstI didn't show any restriction site in ITS region of the fungus. Remaining 4 enzymes namely *Bsp143I, EcoRI, HaeIII,* and *RsaII* had 2, 1, 2 and 2 restriction sites respectively. The present findings are agreed with the observation of several researcher (Njambere*et al*. 2008; Mandal, *et al*., 2011). They also observed limited variation in ITS region of the pathogen. The similarity among *Sclerotinia sclerotiorum* isolates based on ITS-RFLP profiling by has already been studied by several research workers (Carpenter, 1999; Li *et al*., 2009; Litholdo Junior, 2011).

RAPD and URP -PCR analysis

S. sclerotiorum isolates evaluated in the present study were characterized using 4 random primers and they produced reproducible and scorable bands properly. The UPGMA cluster analyses of isolates did not show higher similarity to each other (Fig.2). The clustering of the isolates indicated clear pattern on geographical origin and host association even though isolates of Delhi partially related which were grouped in one cluster. Dendrogarm obtained gave clear correlation towards geographical origin and host association. Therefore similarity or diversity depends on the geographical location, host association and reproduction of fungus as well. Genetic diversity of *S. sclerotiorum* isolates with respect to geographical origin and host association based on RAPD markers has already been reported by several workers (Hambelton *et al.*, 2002; Yli-Mathla, 2010; Litholdo junior, 2011). The same work in other pathogens has already been clearly discussed by different researchers (Saude *et al.*, 2004; Prasad *et al.*, 2010; Adhipathi *et al.*, 2013)

Four URP primers were used for molecular characterization of *S. sclerotiorum* isolates. 2 primers out of 4 URP primers gave good banding pattern (Fig. 3). The data on banding pattern was analysed through UPGMA cluster analyses. The resulted dendrogram revealed that, isolates were genetically diverse and different clusters were obtained. The genetic variation to different geographical locations and host association of isolates which were studied. The genetic differentiation of isolates using URP-PCR has already been reported (Rashm *et al.*, 2008).The majority of isolates (SS1, SS4, SS5, SS9) showed more than 95% similarity formed in one cluster, isolates (SS7, SS2, SS6, SS3) showed less similarity separated from the cluster one and isolate SS3(Mustard, New Delhi) was totally separated as a outlier (Fig. 4).

The present study showed that morphological and molecular characters were keys to determine diversity among population of *S. sclerotiorum*. The RAPD and URP-PCR were suitable markers for rapid differentiation of fungal isolates. The results generated by molecular characterization revealed that their is less correlation with geographical location of isolates and more with their host association.

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