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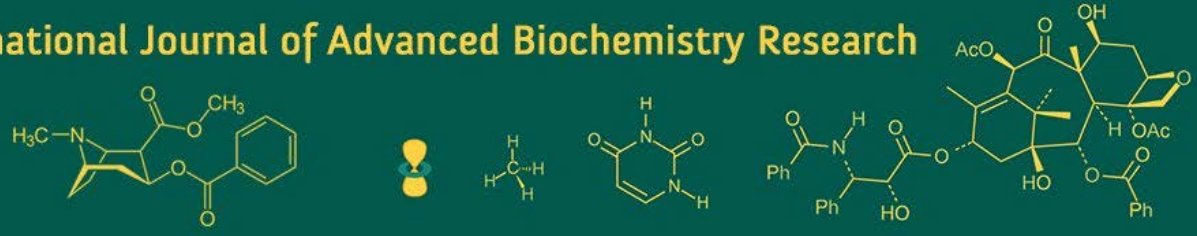

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Variability in Cultural, Morphological and Molecular Characteristics of *Sclerotium rolfsii* Associated with Stem and Pod Rot of Groundnut in Gujarat

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Abstract
Groundnut is an important oilseed crop in India, ranking first in area and second in production, with Gujarat leading in area, production and productivity. The crop is severely affected by several soil-borne diseases, among which stem and pod rot caused by *Sclerotium rolfsii* results in significant yield losses. The present study aimed to characterize the cultural, morphological and molecular variability of fifteen *S. rolfsii* isolates collected from major groundnut-growing regions of Gujarat. Considerable variation was observed among isolates in colony morphology, growth rate, sclerotial initiation, size, shape, colour, arrangement and number on PDA and CzDA media. Molecular analysis revealed high genetic diversity, with 100 per cent polymorphism detected by primers OPA-3 and OPE-1, while primers OPA-20, OPA-18 and OPA-2 showed the lowest polymorphism among the 40 primers tested.

Keywords: *Sclerotium rolfsii*, isolates, variability and RAPD

Introduction
Groundnut (*Arachis hypogaea* Linn.), a major oilseed crop, is severely affected by several soil-borne diseases, of which stem and pod rot caused by *Sclerotium rolfsii* is the most destructive. The disease, also known as sclerotium wilt or root rot (Chohan, 1974) [4], can cause yield losses ranging from 45 to 80 per cent in India and affects the crop at all growth stages, with stem infection being the most common and damaging (Maye and Datar, 1988; Rani, 2017) [11, 13]. Profuse mycelial growth and the production of long lasting brown sclerotia (Chet, 1975) [3] enable the pathogen to survive in soil and cause recurrent losses (Cilliers *et al*., 2000; Ganesan *et al*., 2007) [5, 7]. Since variability in cultural, morphological and genetic traits of *S. rolfsii* plays a crucial role in disease severity and management, the present study was undertaken to investigate the variation among *S. rolfsii* isolates collected from major groundnut growing regions of Gujarat, India, using cultural, morphological and molecular approaches.

Materials & Methods
Collection of samples/isolates
Different isolates of *Sclerotium rolfsii* were collected from major groundnut-growing areas representing various agro-ecological zones of Gujarat. Infected stem and pod samples showing typical stem and pod rot symptoms, along with mycelium and sclerotia, were collected during *kharif* 2022 and brought to the laboratory for microscopic examination.

Isolation of pathogen
The pathogen was isolated from infected groundnut stem and pod tissues showing typical stem and pod rot symptoms, including chlorosis, white mycelial growth and sclerotia formation. Infected stem pieces (5-10 mm) comprising both healthy and diseased tissues were surface sterilized with 1 per cent sodium hypochlorite, rinsed thoroughly with sterile distilled water and aseptically placed on potato dextrose agar (PDA). The inoculated plates were incubated at 28 ± 1 °C for 3-4 days and observed for fungal growth, after which developed sclerotia were sub-cultured on PDA slants for further study.

Purification maintenance of isolates

The cultures obtained were purified using the single-sclerotia isolation method and maintained on PDA slants for further studies. Based on cultural and morphological characteristics, the pathogen was identified as *Sclerotium rolfsii* (Barnett and Hunter, 1972) [1]. The fifteen purified isolates were designated as SR1 to SR15, corresponding to Jamnagar, Junagadh, Kalavad, Arni, Beraja, Tarvada, Jamvadi, Satudad, Majevadi, Dawli, Pipartoda, Dal-devdiya, Dhoraji, Visavadar and Kesod, respectively.

Morphological variability

The morphological characteristics of different *S. rolfsii* isolates, including sclerotial size, number, shape and colour, were recorded. Sclerotial size was measured using an ocular micrometer, and identification was carried out with the help of standard laboratory manuals, including the *Pictorial Atlas of Soil and Seed Fungi* (Watanabe, 1993) [16].

Molecular variability

Isolation of genomic DNA

Total genomic DNA was extracted from seven-day-old fungal mycelia using the CTAB method. About 300 mg of mycelium was ground in liquid nitrogen and mixed with pre-warmed CTAB buffer, followed by incubation at 65 °C. The extract was purified using chloroform: isoamyl alcohol, and DNA was precipitated with absolute ethanol, washed with 70 per cent ethanol, air-dried and dissolved in TE buffer. The DNA was treated with RNase, heat inactivated and stored at −20 °C for further molecular analysis.

Qualitative and quantitative analysis of DNA

The quality of extracted DNA was assessed by 0.8 per cent agarose gel electrophoresis followed by ethidium bromide staining. Quantitative analysis was performed using a NanoDrop spectrophotometer by measuring absorbance at 230, 260 and 280 nm, and DNA purity and concentration were evaluated using A260/A280 and A260/A230 ratios.

RAPD analysis of *S. rolfsii* isolates

The PCR master mix was prepared by adding nuclease-free water, PCR master mix (12.50 µl), primer (5 µl), and template DNA (5 µl), followed by gentle vortexing and brief centrifugation. Amplification was carried out in a thermal cycler using two programs: primers OPA-2, OPA-3, and OPE-1 were amplified with an initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 37 °C for 50 s, extension at 72 °C for 50 s, and a final extension at 72 °C for 10 min; primers OPA-18 and OPA-20 were amplified with an initial denaturation at 96 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 37 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The RAPD products were resolved on 3% agarose gel in 1× TBE buffer, stained with ethidium bromide (0.5 µg/ml), run at 80 V for 45 min, and visualized using a gel documentation system. Band sizes were estimated using a 100-bp ladder, and polymorphism was assessed by comparing RAPD profiles of *S. rolfsii* isolates.

Data scoring and analysis

The RAPD data were scored as presence (1) or absence (0) of bands for each genotype and recorded in an Excel sheet. Polymorphism percentage was calculated using the formula (Blair *et al*., 1999) [2]:

$$\text{Polymorphism (\%)} = \frac{\text{Total bands} - \text{Monomorphic bands}}{\text{Total bands}} \times 100$$

The binary data were used to generate a matrix for analysis using the Numerical Taxonomy and Multivariate Analysis System. A similarity matrix among isolates was calculated using Jaccard's coefficient, and clustering was performed with the UPGMA method. The resulting dendrogram was drawn using NTSYS-pc 1.8 (Applied Biostatistics Inc., Setauket, NY, USA) (Rohlf, 1993) [14].

Results and Discussion

The pathogen was isolated from all the infected plant samples collected from major groundnut growing regions of Gujarat and variability was determined on two different culture media i.e. Potato Dextrose Agar (PDA) and Czapek's Dox Agar (CzDA).

Cultural Variability on PDA Media

On PDA media, the 15 isolates showed variation in colony morphology, with 8 isolates (SR2, SR4, SR6, SR7, SR8, SR9, SR11, SR14) exhibiting fluffy colonies and 7 isolates (SR1, SR3, SR5, SR10, SR12, SR13, SR15) showing flat colonies. Mycelial growth rates ranged from 15.00 mm/day (SR14) to 19.50 mm/day (SR4), with all isolates demonstrating fast growth. Colony color was white for all isolates, and sclerotia initiation varied between 5 and 7 days, with most isolates initiating on the 6th day (Table 1).

Cultural Variability on CzDA Media

On CzDA media, colony morphology differed slightly, with 10 isolates (SR1, SR2, SR3, SR5, SR7, SR9, SR10, SR12, SR13, SR15) producing flat colonies and 5 isolates (SR4, SR6, SR8, SR11, SR14) showing fluffy colonies. Growth rates ranged from 11.20 mm/day (SR2) to 19.00 mm/day (SR4), all colonies were white, and sclerotia initiation occurred between 5 and 9 days, with most isolates initiating between the 6th and 8th day (Table 1).

Morphological Variability on PDA Media

The size of sclerotia varied from 0.98 mm (SR11) to 2.15 mm (SR9). Most isolates (10 isolates: SR3, SR4, SR5, SR7, SR8, SR9, SR10, SR13, SR14, SR15) produced round to irregular sclerotia, while the remaining five (SR1, SR2, SR6, SR11, SR12) were round. Sclerotia color ranged from light brown to dark brown, with six isolates (SR2, SR11, SR12, SR13, SR14, SR15) showing brown, four isolates (SR6, SR7, SR8, SR9) dark brown, three isolates (SR3, SR5, SR10) brown to dark brown, and two isolates (SR1, SR4) light brown to brown. Sclerotia arrangement was mostly scattered (11 isolates), while three isolates (SR6, SR8, SR14) had central arrangement and one (SR2) peripheral. Number of sclerotia per plate varied widely; five isolates (SR1, SR2, SR4, SR10, SR11) showed excellent formation (++++) with 153-250 sclerotia, four isolates (SR3, SR5, SR7, SR15) good (+++) with 106-119 sclerotia, and the remaining six showed moderate formation (++) with 52-95 sclerotia (Table 2).

Morphological Variability on CzDA Media

Sclerotial size ranged from 0.80 mm (SR11) to 1.80 mm (SR9). The majority of isolates (10 isolates: SR3, SR4, SR5,

SR7, SR8, SR9, SR10, SR13, SR14, SR15) had round to irregular sclerotia, while five isolates (SR1, SR2, SR6, SR11, SR12) were round. Sclerotia color varied from light brown to dark brown, with nine isolates (SR1, SR2, SR8, SR9, SR11, SR12, SR13, SR14, SR15) brown, three isolates (SR5, SR6, SR10) brown to dark brown, two isolates (SR3, SR4) light brown to brown, and one isolate (SR7) dark brown. Sclerotia arrangement was mostly scattered (10 isolates), three isolates (SR6, SR8, SR14) had central arrangement, and two (SR1, SR2) peripheral. Sclerotial formation grade varied; only two isolates (SR2, SR11) were excellent (++++) with 154-243 sclerotia, one isolate (SR10) good (+++) with 128 sclerotia, nine isolates moderate (++) with 51-95 sclerotia, and three isolates (SR3, SR7, SR14) poor (+) with 26-45 sclerotia (Table 2). These results are in conformity with the findings of those reported earlier by several workers against *S. rolfsii*. Cultural and morphological variability studied on PDA and CzDA and observed variation in mycelial sclerotial characters (Kumar *et al*., 2012). Variation observed in colony morphology, colony diameter, sclerotial arrangement, scleroctial shape, size of sclerotia and number of sclerotia/plate in S. rolfsii causing stem and pod rot of groundnut (Sivakumar *et al*., 2016; Manu *et al*., 2018; Praveen and Kannan, 2021).

Molecular variability

Random Amplified Polymorphic DNA study

Molecular variability among 15 *S. rolfsii* isolates was assessed using 40 RAPD primers. Initial screening of SR1 (Jamnagar) and SR2 (Junagadh) showed that five primers produced clear, scorable bands, which were then used for all isolates. Fragment sizes were compared with a 100 bp DNA ladder. The study revealed significant genetic diversity, with primers OPA 3 and OPE 1 showing 100% polymorphism, OPA 2 and OPA 18 showing 83.33% and 70%, and OPA 20 showing the lowest at 62.50%. A total of 36 loci were amplified, 29 of which were polymorphic. The number of alleles per primer ranged from 6 to 10, demonstrating the discriminatory power of RAPD for distinguishing isolates and potential for species identification (Table 3).

Cluster analysis of RAPD data

Cluster analysis using Jaccard's similarity coefficient and UPGMA revealed two main clusters (A and B) among the isolates. A total of 339 reproducible fragments were amplified by the five primers. The highest similarity (0.92) was observed between SR14 (Visavadar) and SR15 (Keshod), while the lowest (0.41) was between SR3 (Kalavad) and SR15 (Keshod), with an average similarity of 0.67. Cluster A contained sub-clusters A1 (SR1) and A2, which was further divided into a1 (SR3, SR11) and a2 (SR10). Cluster B included B1 and B2, with further sub-division showing genetic relationships among isolates, including SR14 and SR15 forming a highly similar pair. The dendrogram illustrated clear genetic differentiation, confirming RAPD's effectiveness in discriminating among *S. rolfsii* isolates (Table 4 & figure 1).

Table 1: Cultural variability among the isolates of *S. rolfsii* on different solid media

Sr. No.	Isolates	Culture media	Colony morphology	Colony diameter (mm)*	Growth rate	Colony colour	Days to sclerotia initiation
1.	SR1	PDA	Flat	18.00	Fast	White	6
		CzDA	Flat	15.40	Fast	White	7
2.	SR2	PDA	Fluffy	15.90	Fast	White	7
		CzDA	Flat	11.20	Fast	White	9
3.	SR3	PDA	Fluffy	18.20	Fast	White	6
		CzDA	Flat	12.80	Fast	White	8
4.	SR4	PDA	Fluffy	19.50	Fast	White	5
		CzDA	Fluffy	19.00	Fast	White	5
5.	SR5	PDA	Flat	17.60	Fast	White	6
		CzDA	Flat	15.40	Fast	White	7
6.	SR6	PDA	Fluffy	17.80	Fast	White	6
		CzDA	Fluffy	17.65	Fast	White	6
7.	SR7	PDA	Fluffy	17.00	Fast	White	6
		CzDA	Flat	12.85	Fast	White	8
8.	SR8	PDA	Fluffy	18.00	Fast	White	6
		CzDA	Fluffy	15.30	Fast	White	7
Sr. No.	**Isolates**	**Culture media**	**Colony morphology**	**Colony diameter (mm)***	**Growth rate**	**Colony colour**	**Days to sclerotia initiation**
9.	SR9	PDA	Fluffy	17.50	Fast	White	6
		CzDA	Flat	16.45	Fast	White	8
10.	SR10	PDA	Flat	17.00	Fast	White	7
		CzDA	Flat	16.70	Fast	White	7
11.	SR11	PDA	Fluffy	18.15	Fast	White	6
		CzDA	Fluffy	17.40	Fast	White	6
12.	SR12	3PDA	Flat	18.10	Fast	White	6
		CzDA	Flat	15.40	Fast	White	7
13.	SR13	PDA	Flat	15.50	Fast	White	7
		CzDA	Flat	13.00	Fast	White	8
14.	SR14	PDA	Fluffy	15.35	Fast	White	7
		CzDA	Fluffy	15.00	Fast	White	7
15.	SR15	PDA	Flat	17.00	Fast	White	5
		CzDA	Flat	15.70	Fast	White	6

Note: * Average mycelial growth of 5 days

Table 2: Morphological variability among the isolates of *S. rolfsii* on different solid media

Sr. No.	Isolates	Culture media	Sclerotia size (mm)	Shape	Colour	Sclerotial arrangement	Sclerotial Formation grade	No. of sclerotia per plate	Category
1	SR1	PDA	1.10	Round	Light brown to brown	Scattered	Excellent	240	++++
		CzDA	0.90	Round	Brown	Peripheral	Moderate	89	++
2	SR2	PDA	1.14	Round	Brown	Peripheral	Excellent	250	++++
		CzDA	0.98	Round	Light brown	Peripheral	Excellent	243	++++
3	SR3	PDA	1.64	Round to irregular	Brown to dark brown	Scattered	Good	118	+++
		CzDA	1.27	Round to irregular	Light brown to brown	Scattered	Poor	45	+
4	SR4	PDA	1.34	Round to irregular	Light brown to brown	Scattered	Excellent	153	++++
		CzDA	1.18	Round to irregular	Light brown to brown	Scattered	Moderate	95	++
5	SR5	PDA	1.41	Round to irregular	Brown to dark brown	Scattered	Good	119	+++
		CzDA	1.12	Round to irregular	Brown to dark brown	Scattered	Moderate	60	++
6	SR6	PDA	1.85	Round	Dark brown	Central	Moderate	85	++
		CzDA	1.77	Round	Brown to dark brown	Central	Moderate	51	++
7	SR7	PDA	1.95	Round to irregular	Dark brown	Scattered	Good	108	+++
		CzDA	1.51	Round to irregular	Dark brown	Scattered	Poor	26	+
8	SR8	PDA	1.40	Round to irregular	Dark brown	Central	Moderate	78	++
		CzDA	1.21	Round to irregular	Brown	Central	Moderate	55	++
9	SR9	PDA	2.15	Round to irregular	Dark brown	Scattered	Moderate	95	++
		CzDA	1.80	Round to irregular	Brown	Scattered	Moderate	80	++
10	SR10	PDA	1.87	Round to irregular	Brown to dark brown	Scattered	Excellent	169	++++
		CzDA	1.63	Round to irregular	Brown to dark brown	Scattered	Good	128	+++
11	SR11	PDA	0.98	Round	Brown	Scattered	Excellent	165	++++
		CzDA	0.80	Round	Brown	Scattered	Excellent	154	++++
12	SR12	PDA	1.22	Round	Brown	Scattered	Moderate	79	++
		CzDA	1.00	Round	Brown	Scattered	Moderate	72	++
13	SR13	PDA	1.34	Round to irregular	Brown	Scattered	Moderate	73	++
		CzDA	1.00	Round to irregular	Brown	Scattered	Moderate	65	++
14	SR14	PDA	1.40	Round to irregular	Brown	Central	Moderate	52	++
		CzDA	1.20	Round to irregular	Brown	Central	Poor	45	+
15	SR15	PDA	1.67	Round to irregular	Brown	Scattered	Good	106	+++
		CzDA	1.09	Round to irregular	Brown	Scattered	Moderate	89	++

Note: +: poor sclerotial formation grade, ++: moderate sclerotial formation grade, +++: good sclerotial formation grade, ++++: excellent sclerotial formation grade

Table 3: Amplification obtained with different RAPD primers

Sr. No.	Primers	Primer sequence (5'-3')	Total number of bands	Total no of loci	Number of polymorphic loci	Number of monomorphic loci	Polymorphism (%)	PIC value
1.	OPA 2	TGCCGAGCTG	52	6	5	1	83.33	0.81
2.	OPA 3	AGTCAGCCAC	39	6	6	0	100.00	0.79
3.	OPA 18	AGGTGACCGT	115	10	7	3	70.00	0.90
4.	OPA 20	GTTGCGATCC	88	8	5	3	62.50	0.86
5.	OPE 1	CCCAAGGTCC	45	6	6	0	100.00	0.78
Total			339	36	29	7	-	4.14
Average			67.8	7.2	5.8	1.4	98.41	0.82

PIC- Polymorphism Information Content

Table 4: Jaccard's similarity coefficient between fifteen isolates of *S. rolfsii* based on RAPD data

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	1.00														
2	0.76	1.00													
3	0.78	0.65	1.00												
4	0.54	0.78	0.49	1.00											
5	0.57	0.76	0.46	0.81	1.00										
6	0.59	0.84	0.54	0.78	0.86	1.00									
7	0.54	0.73	0.54	0.73	0.76	0.84	1.00								
8	0.57	0.76	0.62	0.70	0.73	0.76	0.70	1.00							
9	0.59	0.73	0.59	0.62	0.65	0.73	0.73	0.59	1.00						
10	0.73	0.76	0.78	0.54	0.51	0.59	0.65	0.60	0.70	1.00					
11	0.73	0.59	0.89	0.49	0.46	0.59	0.59	0.57	0.65	0.78	1.00				
12	0.54	0.68	0.54	0.62	0.70	0.73	0.73	0.70	0.78	0.70	0.54	1.00			
13	0.54	0.68	0.49	0.68	0.76	0.73	0.62	0.65	0.57	0.59	0.43	0.78	1.00		
14	0.54	0.57	0.43	0.51	0.59	0.62	0.73	0.59	0.68	0.65	0.49	0.78	0.68	1.00	
15	0.46	0.54	0.41	0.49	0.62	0.65	0.76	0.57	0.70	0.57	0.46	0.76	0.70	0.92	1.00

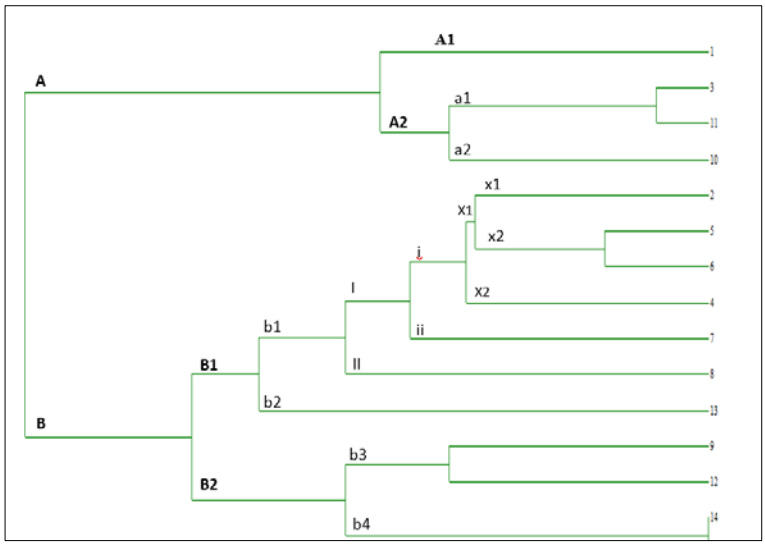

Fig 1: RAPD UPGMA dendrogram of fifteen isolates of *S. rolfsii* based on Jaccard's similarity coefficient

Several studies have shown similar results indicating genetic variability of the *S. rolfsii* cause stem and pod rot of groundnut by RAPD and showed that dendrogram analysis of data had a tendency of isolates to group according to the geographic precedence (Durgaprasad *et al*., 2008) [6]. RAPD profiles of *Sclerotium* spp. among which 13 were *S. rolfsii* and 4 were *S. delphinii* by using RAPD markers to determine the genetic diversity based on the amplification pattern and reproducibility of eleven RAPD premiers viz., OPB-11, OPB-12, OPB-20, OPD-7, OPD-13, OPD-16, OPD-20, OPE-12, OPE-16, OPB-16 and OPE-20. The primer OPB-16 was found to be the best primer for determination of variability among *Sclerotium* spp (Gawande *et al*., 2013) [8].

Conclusion

The study of 15 *Sclerotium rolfsii* isolates revealed a high degree of variability across cultural, morphological, and molecular traits. Cultural and morphological analyses indicated that mycelial growth, sclerotial size, and the number of sclerotia per plate were higher on PDA media, with sclerotia initiation occurring between 5 to 7 days. Molecular analysis showed the highest genetic similarity (0.92) between isolates SR14 and SR15, while the lowest similarity (0.41) was observed between SR3 and SR15, highlighting substantial genetic diversity among the isolates.

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Conflict of Interest

The authors declare no conflict of interest.

Statement on Human and Animal Rights

This article does not involve any studies with human participants or animals performed by the authors.

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