Pl. Dis. Res. 38 (2) : 210-216
DOI No. 10.5958/2249-8788.2023.00027.7

ISSN 0970-4914 (Print)
ISSN 2249-8788 (Online)

Cultural, morphological and molecular variability of *Sclerotinia sclerotiorum* causing white rot of peas

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ABSTRACT

Thirteen isolates of *S. sclerotiorum* were obtained from pea, bean, chickpea, tomato and linseed growing areas of Himachal Pradesh and their variability *i.e.* cultural, morphological and molecular was studied . All isolates of *S. sclerotiorum* were found cross pathogenic on pea, bean, chickpea, tomato and linseed verifying the polyphagous nature of the pathogen. Cultural characteristics were used to categorize isolates into three groups, while morphological characteristics served as the basis for dividing them into two groups. On the basis of mycelial compatibility grouping (MCG), 13 isolates were placed in four groups *viz*. MCG-I, MCG-II, MCG-III and MCG-IV. Further, ISSR primers based analysis divided the isolates into three groups *viz*. Ss-I, Ss-II and Ss-III at 77 per cent similarity coefficient as cut-off point. No congruence was observed between the groups formed on the basis of cultural, morphological, mycelial compatibility and ISSR primers. However, cultural, morphological and molecular variability was observed in the present study.

Key words: *Sclerotinia sclerotiorum*, sclerotia, cross pathogenicity, pea, DNA, molecular, variability

White rot of pea caused by *Sclerotinia sclerotiorum* (Lib.) de Bary has become a limiting factor in pea cultivation due to its monoculture and extensive cultivation in some parts of Himachal Pradesh. This disease is dreaded one causing considerable losses to pea growers. The extent of ravages of this disease could be judged from the fact that under favourable conditions it could cause total failure of pea crop. The pathogen *S. sclerotiorum* is a necrotrophic and polyphagous fungus to infect over 400 plant species including important crops grown in cool and wet season (Purdy, 1979; Boland and Hall, 1994). Being a sclerotial fungus, sclerotia are formed in the pith of infected stem, on stems and roots of pea plant. These sclerotia may later get released in the soil during harvesting and serve as primary source of inoculum for next crop season (Mehta *et al.*, 2005). Sclerotia are persistent and remain viable for up to 8 years in the soil (Adams and Ayers, 1979). They germinate myceliogenically and /or carpogenically in moist – cool weather and plays a major role in disease development (Willetts and Wong, 1980). Morall *et al.* (1972) observed high degree of variability in cultural and morphological characteristics of isolates obtained from different hosts. Wide variability in virulence of different isolates of *S. sclerotiorum* has been shown across different host combination (Willetts and Wong, 1980). The pathogenic and genetic variability of this pathogen has been investigated for different crops (Auclair *et al.*, 2004; Pratt and Rowe, 1991; Hambleton *et al.*, 2002; Maltby and Mihail, 1997; Kull *et al.*, 2003). Inter Simple Sequence Repeats (ISSR) based on primers complementary to microsatellite region is believed to be useful and reliable method to determine genetic polymorphism (Meyer *et al*., 1993). Therefore, these markers were utilized to determine the genetic variability amongst the collected isolates of *S. sclerotiorum* infecting peas.

Received: 29-10-2023
Accepted: 28-12-2023

MATERIALS AND METHODS

Collection, isolation, purification and multiplication of *S. sclerotiorum* isolates

S. sclerotiorum isolates were collected in the form of hard mass called sclerotia from diseased samples of pea, bean, linseed, tomato and chickpea plants from different vegetable growing regions of Himachal Pradesh (Table 1). These hard mass sclerotia then surface sterilized for 30 seconds in 0.1 per cent of

mercuric chloride solution for 15 seconds followed by three washings in sterilized water under aseptic conditions in laminar air flow. The sterilized sclerotia were dried in two folds of sterilized filter papers to remove excess moisture and then, plated on potato dextrose agar medium (PDA) and incubated at 21±1^{0}C for three days. Single hyphal tip method was used to obtain pure culture of each isolate.

Cross pathogenicity test of *S. sclerotiorum*

Cross pathogenicity tests were carried out by inoculating twenty days old seedling of pea, bean, linseed, tomato and chickpea seedlings raised in pot culture under controlled conditions by inoculating with the bit of *S. sclerotiorum* (5.0 mm) placed near the collar region of the seedlings. The inoculated seedlings were kept at 21±1ºC temperature in net house and covered with polythene bags to maintain required relative humidity of 85 per cent or above for disease development.

Table 1. *S. sclerotiorum* isolates collected from different crops grown in Himachal Pradesh

Isolate	Crop	Place of collection
SsP-1	Pea	Nagrota-Kangra
SsP-2	Pea	Jamanabad-Kangra
SsP-3	Pea	Kukumseri-Lahul and Spiti
SsP-4	Pea	Trilokinath-Lahul and Spiti
SsP-5	Pea	Jhalma-Lahul and Spiti
SsP-6	Pea	Tharot-Lahul and Spiti
SsB-7	Bean	Department of Plant Pathology-Kangra
SsB-8	Bean	Sangla- Kinnaur
SsB-9	Bean	Kinnaur
SsB-10	Bean	Palampur-Kangra
SsL-11	Linseed	Jogindernagar-Mandi
SsCh-12	Chickpea	Hamirpur
SsT-13	Tomato	Bada-Hamirpur

Cultural variability

Pure cultures of isolate were used to obtain mycelial disc of 5.0 mm diameter from actively growing colony of 4 days old culture and further, transferred to PDA in Petri plates (90.0 mm diameter). All cultures were replicated thrice, incubated at 21±1 ^{0}C in B.O.D. incubator and observations on the cultural characters *viz*., colony colour, type and growth in diameter (mm) observed at 24, 48, 72 and 96 hours.

Morphological variability

The morphological characteristics of thirteen isolates in above experiment were examined for sclerotial formation i.e. initiation of sclerotia, size of sclerotia; length and width (mm) measured with help of Vernier caliper and sclerotia formation pattern in Petri plates.

Mycelial compatibility groups

Mycelial compatibility and/or incompatibility was assessed by pairing thirteen isolates in all possible combinations on potato dextrose agar (PDA) in Petri plates. Mycelial discs measuring 5 mm in diameter were sampled from the outer edge of actively growing colonies of each paired isolate, replicated three times, and positioned 40.0 mm apart in Petri plates. The plates were then incubated at 21±10°C in a B.O.D. incubator. After 7 days of incubation, mycelial reactions were documented to determine compatibility and/or incompatibility. Incompatibility was identified if a visible line of demarcation, a barrage zone, or a mycelia-free zone appeared between the paired isolates in confrontation. Conversely, compatibility was recognized when no line of demarcation was observed.

Genetic variability

Isolates were inoculated on sterilized 50 ml potato dextrose broth (200 g peeled potato, 20 g dextrose in one liter distilled water) and incubated at 21±1^0 C in B.O.D. incubator for 6 days. Mycelia was harvested by filtration through double layers of filter paper and then, dried and stored at -20^o C till DNA extraction. CTAB (Cetyl Trimethyl Ammonium Bromide) method of Saghai-Maroof *et al.* (1984) and SDS (Sodium Dodecyl Sulphate) method were used for the extraction of total genomic DNA of different isolates of *S. sclerotiorum*. Agarose gel electrophoresis (1.5 per cent) was used to check quality and quantity of genomic DNA. The gel was visualized in a UV trans illuminator. PCR reaction was carried out for obtaining the best amplification in DNA of thirteen isolates of *S. sclerotiorum* by ISSR (Inter Simple Sequence Repeats) primers. The PCR reaction was performed in 12.5 µl of reaction mixture containing 1.0 µl of DNA, 1.25 µl of Taq buffer, 0.4 µl of dNTPs (dATP, dCTP, dGTP, dTTP), 0.5 µl of each ISSR primer, 0.1 µl Taq DNA polymerase and

8.25 µl double distilled autoclaved water. The DNA amplification was performed by using thermal cycler as follows for 40 cycles. Initial denaturation 94° C for 2mins, denaturation 94° C for 1min, annealing 47° C for 1 min, extension 72° C for 1.5 min, final extension 72° C for 6 min and amplified products were stored at -20 ° C till further use. Forty-eight ISSR primers were screened among two isolates for polymorphism (Table 2). Out of which five primers had shown polymorphism. Then PCR products were subjected to agarose gel i.e. 2% was used for ISSR primers to check polymorphic reaction of DNA with the seven primers. Samples and ladder i.e. of 1 kb+ were loaded in the wells and electrophoresis was carried out at constant voltage (150V) for 30-45 minutes, and after this data were obtained by scoring i.e. presence of band given number 1, absence given 0 and total absence or no amplification given number 9. The data of genetic variability i.e. similarity coefficient of thirteen isolates were calculated by using NTSYS and dendrogram of thirteen isolates was constructed by UPGMA analysis.

Table 2. Nucleotide sequence of ISSR primers used for DNA fingerprinting

S. No.	Name of the primer	Sequence 5'-3'	No. of base pairs
1	ISSR 807	AGA GAG AGA GAG AGA GT	17
2	ISSR 808	AGA GAG AGA GAG AGA GC	17
3	ISSR 809	AGA GAG AGA GAG AGA GG	17
4	ISSR 810	GAG AGA GAG AGA GAG AT	17
5	ISSR 811	GAG AGA GAG AGA GAG AC	17
6	ISSR 812	GAG AGA GAG AGA GAG AA	17
7	ISSR 813	CTC TCT CTC TCT CTC TTA	17
8	ISSR 814	CTC TCT CTC TCT CTC TA	17
9	ISSR 815	CTC TCT CTC TCT CTC TG	17
10	ISSR 816	CAC ACA CAC ACA CAC AT	17
11	ISSR 817	CAC ACA CAC ACA CAC AA	17
12	ISSR 818	CAC ACA CAC ACA CAC AG	17
13	ISSR 819	GTG TGT GTG TGT GTG TA	17
14	ISSR 820	GTG TGT GTG TGT GTG TC	17
15	ISSR 821	GTG TGT GTG TGT GTG TT	17
16	ISSR 822	TCT CTC TCT CTC TCT CA	17
17	ISSR 823	TCT CTC TCT CTC TCT CC	17
18	ISSR 824	TCT CTC TCT CTC TCT CG	17
19	ISSR 825	ACA CAC ACA CAC ACA CT	17
20	ISSR 826	ACA CAC ACA CAC ACA CC	17
21	ISSR 827	ACA CAC ACA CAC ACA CG	17
22	ISSR 828	ATA TAT ATA TAT ATA TYA	17
23	ISSR 829	ATA TAT ATA TAT ATA TYC	17
24	ISSR 830	ATA TAT ATA TAT ATA TYG	17
25	ISSR 831	AGA GAG AGA GAG AGA GYT	17
26	ISSR 832	AGA GAG AGA GAG AGA GYC	17
27	ISSR 833	AGA GAG AGA GAG AGA GYA	17
28	ISSR 834	TAT ATA TAT ATA TAT ART	17
29	ISSR 835	TAT ATA TAT ATA TAT ARC	17
30	ISSR 836	TAT ATA TAT ATA TAT ARG	17
31	ISSR 837	GAG AGA GAG AGA GAG AYT	17
32	ISSR 838	GAG AGA GAG AGA GAG AYC	17
33	ISSR 839	GAG AGA GAG AGA GAG AYG	17
34	ISSR 840	CTC TCT CTC TCT CTC TRA	17
35	ISSR 841	CTC TCT CTC TCT CTC TRC	17
36	ISSR 842	CTC TCT CTC TCT CTC.TRG	17
37	ISSR 843	CAC ACA CAC ACA CAC ART	17
38	ISSR 844	CAC ACA CAC ACA CAC ARC	17
39	ISSR 845	CAC ACA CAC ACA CAC ARG	17
40	ISSR 846	GTG TGT GTG TGT GTG TYA	17
41	ISSR 847	GTG TGT GTG TGT GTG TYC	17
42	ISSR 848	GTG TGT GTG TGT GTG TYG	17
43	ISSR 849	TCT CTC TCT CTC TCT CRA	17
44	ISSR 850	TCT CTC TCT CTC TCT CRT	17
45	ISSR 851	TCT CTC TCT CTC TCT CRG	17
46	ISSR 852	ACA CAC ACA CAC ACA CYT	17
47	ISSR 853	ACA CAC ACA CAC ACA CYA	17
48	ISSR 854	ACA CAC ACA CAC ACA CYG	17

RESULTS AND DISCUSSION

Cross pathogenicity

Cross pathogenicity of each test isolate was carried out *in vivo* on different host plants and were found cross pathogenic on each other. SsP-6 isolate of pea was found to be aggressive among all isolates and formed apothecial cups at 0.5-1 cm depth in soil to release ascospores from ascus.

Cultural variability

The cultural characteristics of mycelium i.e. colony colour and type of growth (pattern and diameter) of *S. sclerotiorum* isolates were examined. All isolates had shown white cottony growth and covered the complete surface of Petri plates (90mm) at 96 hours of incubation (Table 3). Isolates SsP-2, SsP-4, SsP-5, SsB-7, SsB-8, SsB-10, SsCh-12 showed sparse and regular growth, while SsP-1, SsP-3, SsP-6, SsB-9, SsL-11, and SsT-13 showed sparse and irregular growth and whereas, SsP-6 showed fluffy and irregular type of growth. Isolate SsP-6 was found fast growing i.e. 74.3 mm whereas, all others showed slow mycelial growth ranging from 64.3mm to 70.6 mm at 72 hours of incubation and almost statistically at par with each other except SsP-2 (57.0 mm).

Morphological variability

The data (Table 4) revealed the non-significant differences among the isolates for initiation of sclerotia. However, significant variations were observed for number of sclerotia formed among the isolates. The maximum number of sclerotia (25) were formed in SsP-3 isolate followed by SsB-7(23), SsB-9(21), SsB-8(20), SsL-11(20), SsT-13(17) and SsCh-12(16), while minimum number (10) were found in SsB-10 isolate followed by SsP-2(11), SsP-5(12), SsP-6(12), SsP-1(13) and SsP-4(15). Size of sclerotia also varied significantly among the isolates. SsP-6 isolate produced large sized sclerotia of 5.8 x 12.7 mm while SsT-13 isolate produced a small size of sclerotia of 4.0 x 5.3mm. SsP-2, SsP-5 and SsB-9 isolates formed scattered sclerotia pattern while SsP-1, SsP-3, SsP-4, SsP-6, SsB-7, SsB-8, SsB-10, SsL-11, SsCh-12 and SsT-13 formed circular pattern in Petri plate. Thus ten isolates produced sclerotia in circular and three in scattered pattern.

Basha and Chatterjee (2007) documented differences in colony morphology, mycelial growth rate, and sclerotial formation among isolates of *S. sclerotiorum* gathered from diverse host/soil samples across various locations in India.

Mycelial compatibility groups

The variability within *S. sclerotiorum* isolates was also determined based on mycelial compatibility and incompatibility reactions under *in vitro* conditions. In compatible reaction no line of demarcation was formed while in incompatible reaction line of demarcation was formed. The isolates SsP-1, SsP-2, SsP-3, SsP-4 and SsP-5 from pea were compatible with each other and incompatible to others forming group MCG-I except SsP-6 which was exclusively incompatible to all the isolates forming another group MCG-IV. The isolate SsB-7 was found compatible with the isolates of tomato (SsT-13), linseed (SsL-11) and chickpea (SsCh-12) and incompatible to others forming group MCG-II. Though the isolates SsB-8, SsB-9 and SsB-10 from beans were compatible with each other and incompatible with others form group MCG-III. Thus based on mycelial compatibility reactions four distinct groups namely MCG-I, MCG-II, MCG-III and MCG-IV were established among thirteen isolates of *S. sclerotiorum*.

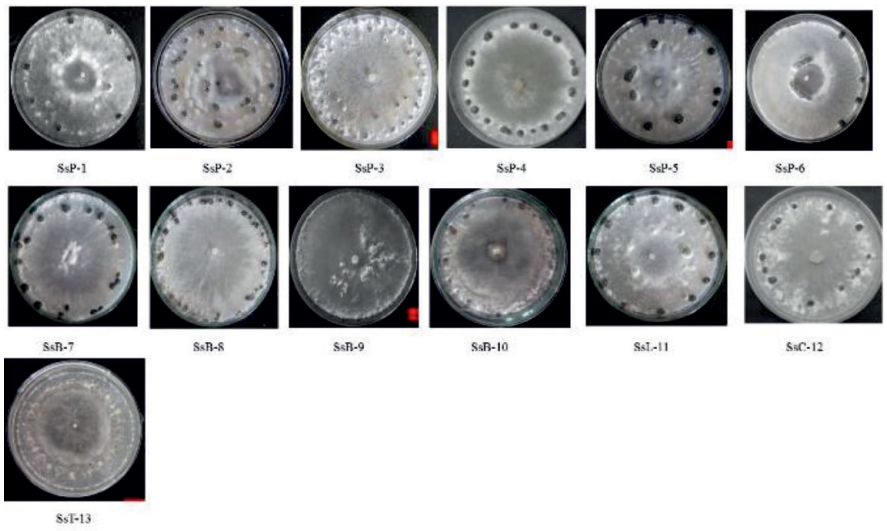

Fig. 1. Morphological variability of thirteen different isolates of *S. sclerotiorum*

Table 3. Cultural characteristics of thirteen isolates of *S. sclerotiorum*

Isolates	Colony colour	Types of growth	Mycelial growth at different intervals (mm)			
			24 hours	48 hours	72 hours	96 hours
SsP-1	White	Sparse and irregular	11.0	47.3	66.6	90
SsP-2	White	Sparse and regular	7.0	24.6	57.0	90
SsP-3	White	Sparse and irregular	17.6	45.6	68.6	90
SsP-4	White	Sparse and regular	19.0	47.6	70.6	90
SsP-5	White	Sparse and regular	21.0	44.0	72.0	90
SsP-6	White	Fluffy and irregular	25.0	53.3	74.3	90
SsB-7	White	Sparse and regular	11.3	43.3	66.3	90
SsB-8	White	Sparse and regular	14.6	51.6	67.0	90
SsB-9	White	Sparse and irregular	11.6	41.3	67.0	90
SsB-10	White	Sparse and regular	16.3	41.6	66.6	90
SsL-11	White	Sparse and irregular	17.0	47.6	70.0	90
SsCh-12	White	Sparse and regular	11.0	37.0	64.3	90
SsT-13	White	Sparse and irregular	11.6	42.6	68.09	90
CD (p=0.05)			2.372	2.490	5.608	NS

Table 4. Morphological characteristics of thirteen isolates of *S. sclerotiorum*

Isolate	Sclerotial formation				
	Initiation (Days)	No. of sclerotia / plate	Dimension(mm)		Pattern
			Width	Length	
SsP-1	6	13.0	4.5	5.7	Circular
SsP-2	8	11.0	5.5	5.6	Scattered
SsP-3	6	25.0	4.6	6.7	Circular
SsP-4	6	15.0	6.2	7.7	Circular
SsP-5	6	12.0	5.2	11.0	Scattered
SsP-6	7	12.0	5.8	12.7	Circular
SsB-7	6	23.0	6.5	8.5	Circular
SsB-8	7	20.0	4.7	7.2	Circular
SsB-9	7	21.0	4.8	7.8	Scattered
SsB-10	6	10.0	4.4	7.4	Circular
SsL-11	6	20.0	6.0	7.0	Circular
SsCh-12	7	16.0	4.7	5.5	Circular
SsT-13	7	17.0	4.0	5.3	Circular
CD (p=0.05)	NS	1.04			

Genetic variability

All the ISSR primers mentioned in the Table 2 were used for amplifying two DNA samples randomly picked and primers showing polymorphism were further used for amplifying all the 13 DNA samples of the *S. sclerotiorum* isolates. Primers *viz*. ISSR 838, ISSR 837, ISSR 809, ISSR 854 and ISSR 820 showed polymorphism (Fig.3) in case of two DNA samples, thus these primers were further used for variability analysis of the test isolates. Two clusters (A and B) were formed, cluster (A) possessing SsP-1, SsP-2, SsP-4, SsB-7, SsL-11, SsB-9, SsCh-12, SsP-5, SsB-8, SsT-13, SsP-3 and SsB-10 isolates and cluster (B) possessing SsP-6 isolate respectively. Cluster (A) was further sub grouped into A1 and A2 sub cluster. Isolates placed in sub cluster A1 and A2 showed further divergence. All 13 isolates were

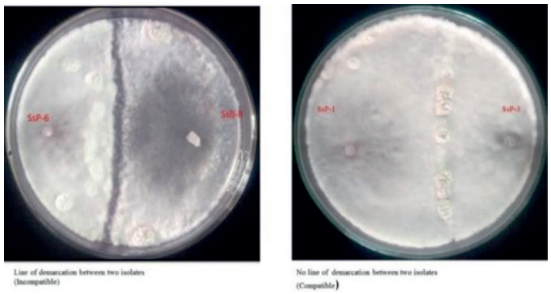

Fig. 2. Mycelial compatibility and incompatibility reaction among different isolate of *S. sclerotiorum*

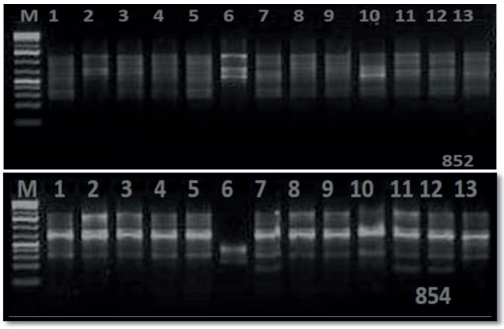

Fig. 3. ISSR primers showing polymorphic reaction

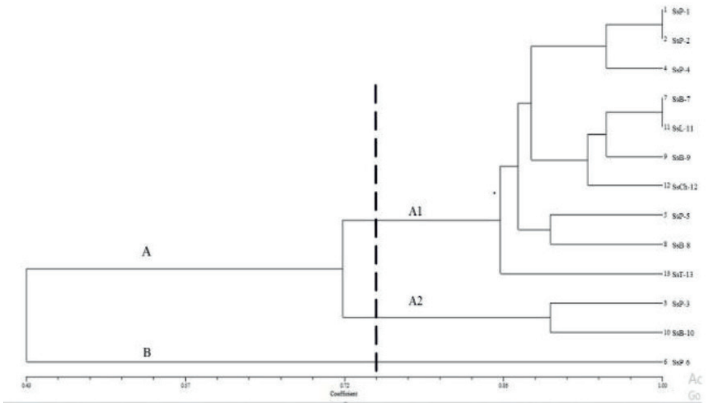

Fig. 4. Dendrogram showing similarity between thirteen different isolates of *S. sclerotiorum*

placed in three groups *viz*., Ss-I, Ss-II and Ss-III keeping 77 per cent as cut off point for grouping. Mandal and Dubey (2012) used RAPD, ITS-RFLP and ITS sequence to determine the genetic diversity of *S. sclerotiorum* causing stem rot in chickpea and found that the isolates showed more than 90 per cent genetic similarity.

Grouping of *Sclerotinia sclerotiorum* isolates

The test isolates underwent grouping according to cultural, morphological, and molecular features (ISSR markers) as well as mycelial compatibility groups. The clustering patterns based on cultural and morphological variations, such as the type of mycelial growth and the pattern of sclerotia formation, along with mycelial compatibility groups, did not align with the clusters observed in the dendrogram (Fig. 4). This discrepancy became apparent when analyzing the amplification patterns of all isolates using ISSR markers. Notably, Mycelial Compatibility Groups II and III did not correlate with the groups formed by molecular markers. This can be inferred from the present studies that there is no congruence between cultural and morphological groups, MCGs and molecular markers group except isolate SsP-6. On the basis of molecular marker analysis, SsP-6 was out grouped in the dendrogram. Hambleton *et al.* (2002) also observed similar differences in DNA fingerprints and MCGs of *Sclerotinia sclerotiorum*. Mandel and Dubey (2012) also reported that RAPD markers were suitable to determine genetic variability among *S. sclerotiorum* but groups formed by them did not correspond to the MCGs.

The study of variability helps to know the evolutionary trends of new strains and races in a pathogen in different geographical regions. Hence, the molecular aspects are needed to characterize the genetic variability of *Sclerotinia sclerotiorum* isolates within host range of field crops to develop resistant varieties and for effective and ecofriendly management modules.

ACKNOWLEDGMENT

The first author gratefully acknowledges the financial help granted by the Department of Plant Pathology, College of Agriculture, CSK HPAU,

Palampur, H.P. 176062, India, for carrying out the research work.

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