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Genetic diversity and morphological variability of *Sclerotinia sclerotiorum* isolates of oilseed Brassica in India

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Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is one of the most serious disease and causes damage to oilseed Brassica. Seventeen geographical isolates of *S. sclerotiorum* from different geographical locations of India were compared for their growth under artificial culture conditions and the result was validated through random amplified polymorphic DNA (RAPD) markers. Based on the morphological studies, the isolates were grouped into fast growing (SR-01, SR-02, SR-05, SR-16) and intermediate (SR-03, SR-04, SR-07, SR-10 to SR-13, SR-18), though the rest of the isolates showed comparatively a slow mycelial growth rate. In RAPD analysis for their genetic diversity, fifty decamer primers of arbitrary sequences produced 692 scorable amplicons ranging from 180 to 3900 bp in size. Out of these, 385 fractionated fragments were reported polymorphic. As an average, 13-14 unambiguous and reproducible amplified products were generated by single primer (minimum of 4 and maximum of 23 fragments). Hence, the present investigation confirmed the diversity among the different geographical isolates from India.

Key words: *Brassica*, *Sclerotinia sclerotiorum,* morphological variability, random amplified polymorphic DNA (RAPD).

INTRODUCTION

Sclerotinia rot, caused by *Sclerotinia sclerotiorum* (Lib.) de Bery, is one of the most devastating and cosmopolitan plant pathogen belonging to ascomycetes having a wide host range of more than 408 described plant species from 278 genera in 75 families including canola (Boland and Hall, 1994; Ziqin et al., 2008). Sclerotinia stem rot a devastating disease of rapeseed-mustard mainly since 1999 in India, caused up to 40% yield losses (Chattopadhyay et al., 2003). Sclerotinia rot is a threat to cultivation of oilseed *Brassica* in Rajasthan, Haryana, Madhya Pradesh, Uttar Pradesh, Bihar and Punjab states of India (Agarwal et al., 1997). The initial infection occurs in the late winter or /and early spring and the fungal mycelia grow within and between the plants. Host crops and their cultivars have no resistance to this broad spectrum pathogen (Zhao and Meng, 2003). Genetic diversity among the *S. sclerotiorum* population was demonstrated by few workers (Harlton et al., 1995;

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Abbreviations: PDA, Potato dextrose agar; **CTAB,** cetyl trimethyl ammonium bromide; **PDB,** potato dextrose broth; **PCR,** polymerase chain reaction; **EtBr,** ethidium bromide.

Okabe et al., 1998). Genetic variability within the isolates in different geographical regions is important for documenting the changes occurring in the population. On the basis of morphological differences in mycelial growth, sclerotia and ascospores formation, *S. sclerotiorum* isolates have been differentiated (Kohn et al., 1990). *S. sclerotiorum* is a homothallic fungus and able to produce clonal ascospores from self fertilized meiotic reproduction. Genetic analysis shows that isolates of *S. sclerotiorum* are homogenous with limited variability in 18 and 28 s rDNA regions (Kohli et al., 1995). Lack of variations in virulence among isolates from defined geographical areas has been reported by many workers (Alvarez and Molina, 2000; Atallah et al., 2004; Sexton and Howlett, 2004). For better management of the disease, it is important to understand the epidemiology and genetic diversity in the pathogen population at regional level. Differences in virulence may be detected only when isolates from widely separate geographical region are compared. There has been conclusive evidence to suggest host specialization among isolate of *S. sclerotiorum* (Kull et al., 2004).

Random amplified polymorphic DNA (RAPD) is a powerful tool for diversity analysis. On the basis of earlier studies, there is a range of markers including a multicopy repetitive element for DNA fingerprinting and nuclear gene polymorphisms (Yu et al., 2006). The aim of the present investigation is to compare different geographical isolates of *S. sclerotiorum* which originated from completely different agro-climatic conditions infecting oilseed *Brassica* in India, based on morphological variability and genetic diversity through RAPD marker.

MATERIALS AND METHODS

Seventeen isolates of *S. sclerotiorum* were originally collected from major oilseed *Brassica* growing States of India viz., Jammu & Kashmir, Rajasthan, Madhya Pradesh, West Bengal, Punjab, Uttar Pradesh, Uttarakhand and Himachal Pradesh. The isolates were designated as SR-01 to SR-18. A single sclerotium of different geographical isolate was surface sterilized separately in 1% (v/v) sodium hypochlorite and 70% ethanol for 2-3 min and thoroughly washed in sterile distilled water. Individual sclerotium was cut half and placed on potato dextrose agar (PDA) under laminar air flow (Clarkson et al., 2003). Isolates of *S. sclerotiorum* were sub-cultured and maintained at 22 ± 2°C on potato dextrose agar (PDA) under 12 h alternate fluorescent lights.

Morphological variability

Fresh cultures of isolates were subjected to detailed morphological and cultural characteristics viz., radial colony growth (mm), number of sclerotia developed in 90 mm Petri plates, size of sclerotia (mm) and weight of single sclerotia (mg). Data of radial colony growth were taken at 72 and 96 h after inoculation while number of sclerotia and size of sclerotia of each isolates were recorded 10 to 15 days after inoculation. Fresh and dry mycelial weight was taken after 10 days growth of isolates grown at 22 ± 2°C on potato dextrose broth (PDB) medium.

Genetic diversity

Mycelial mats grown in PDB and DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method of Simpson et al. (2004) with some modifications. Equal volume of tris-saturated phenol was added in the tube and mixed for 5 min then centrifuged at 11000 rpm for 20 min at 20°C. After centrifugation, aqueous layer was collected and transferred in to fresh tube. Equal volume of chloroform: iso-amyl alcohol (24:1) was added, mixed and centrifuged. The supernatant was drained out and pellet was washed with 70% alcohol. For removal of RNA and residual protein, 5 µl of RNAse (20 mg/ml) and 20 µl of protinase-K (20 mg/ml) were added in nucleic acid solution (Sambrook et al., 1989). Extracted DNA was quantified using spectrophotometer. In addition, agrose gel electrophoresis was also performed for DNA quantification.

Purified DNA was amplified with 50 primers of 10-mer arbitary sequences *viz.*, OPA01 to OPA20, OPB01 to OPB20, OPS06 to OPS10 and OPZ11 to OPZ15. Polymerase chain reaction (PCR) reaction were carried out in a final volume of 25 µl reaction mixture containing 16.0 µl MQ-water, 2 µl (50ng) template DNA, 1.5µl dNTPs, 1.5 µl $MgCl_2$, 2.5 µl 10X buffer, 0.5 µl (0.5 U) taq DNA polymerase and 1 µl primer. Amplification was performed in programmable thermo cycler (Eppendorf Mastercycler) with amplification profile. The amplified products were fractionated on 1.8% agrose gel electrophoresis with ethidium bromide (EtBr) stain (Weining and Langridge,1991; Welsh and McClelland, 1990). The fingerprints generated by different primers were compared for relatedness among the isolates.

Statistical analysis

A binary matrix was compiled using numerical system of multivariate analysis. The dendogram was constructed by the unweighted paired group method of arithmetic average (UPGMA) based on Jaccard's similarity coefficient with SHAN program of NT-sys. The morphological data were statistically analysed using analysis of variance (ANOVA).

RESULTS

Morphological variability among isolates

Results revealed that the mycelial growth rate among the isolates differed considerably. Differences in all the morphological characters of *S. sclerotiorum* were observed. Based on radial growth, the isolates were classified into three groups: Very fast, intermediate and slow growing. Data after 72 h incubations revealed that the isolates SR-01, SR-02, SR-05 and SR-16 represented significantly fast growing, isolates SR-03, SR-04, SR-07, SR-10 to SR-13 and SR-18 were intermediate, while SR-05, SR-08, SR-09 and SR-14 to SR-17 showed slow radial colony growth. However after 96 h incubation majority of isolates except SR-05, SR-12, SR-14, and SR-15 became fast growing and filled the Petri plate with mycelial growth. Maximum isolates were having whitish mycelial growth with smooth texture.

Isolates SR-09 and SR-11 were with off-white, SR-12 was gray white and SR-01 was dirty white in colour. Fresh and dry mycelial weight of different isolates

Table 1. Variability in mycelial growth and sclerotia formation (on PDA) in different geographical isolate of *Sclerotinia sclerotiorum*.

Isolate	Mycelium						Sclerotia			
	Growth on PDA (mm)		Colour	Texture	Growth on PDB		No. of days formation	Sclerotia /plate	Weight/ sclerotia (mg)	Diameter (mm)
	72 h	96 h			Fresh wt (g)	Dry Wt (g)				
SR-01	41.4	90.0	Dirty white	Scattered	3.03	0.12	9	7	1.2	1.83
SR-02	44.2	90.0	Whitish	Smooth	3.42	0.21	9	12	0.3	1.47
SR-03	31.8	90.0	Whitish	Smooth	3.55	0.27	8	32	0.6	1.39
SR-04	33.4	90.0	Whitish	Smooth	5.50	0.35	14	27	0.3	1.08
SR-05	26.9	35.0	Whitish	Smooth	4.57	0.40	9	16	0.6	1.62
SR-07	30.9	90.0	Whitish	Fluffy	4.40	0.39	11	29	1.7	1.87
SR-08	27.8	90.0	Whitish	Smooth	3.93	0.40	6	32	0.6	1.89
SR-09	27.1	90.0	Off white	Fluffy	3.47	0.32	8	67	0.6	1.77
SR-10	30.7	90.0	Whitish	Smooth	4.55	0.33	9	29	0.9	1.63
SR-11	30.8	90.0	Off white	Smooth	3.54	0.22	6	39	1.1	1.84
SR-12	32.8	34.9	Gray white	Scattered	4.54	0.21	7	17	0.9	2.08
SR-13	32.6	90.0	Whitish	Smooth	4.65	0.34	6	20	0.6	2.03
SR-14	14.7	38.8	Whitish	Smooth	7.87	0.43	9	27	0.6	1.89
SR-15	26.5	30.3	Whitish	Fluffy	4.87	0.37	10	28	0.5	2.08
SR-16	42.9	90.0	Whitish	Fluffy	5.15	0.35	7	41	1.1	1.16
SR-17	27.6	90.0	Whitish	Smooth	7.50	0.38	6	17	1.1	1.63
SR-18	30.8	90.0	Whitish	Smooth	4.70	0.47	8	70	0.5	1.42
SE (m)±	0.90	1.48			0.29	0.04	0.16	2.88	0.01	
LSD P<0.05)	2.56	0.51			0.83	0.11	0.46	8.22	0.02	

significantly varied. Maximum fresh mycelial weight was observed in isolate SR-14 (7.87 g) followed by SR-17 (7.50 g), while the minimum fresh and maximum dry weight was in isolate SR-01 (3.03, 0.12g). On the basis of number of sclerotia produced by the isolates of *S. sclerotiorum* SR-09, SR-11, SR-16 and SR-18 ranked as higher producer of sclerotia, SR-03, SR-04, SR-07, SR-08, SR-10, SR-14 and SR-15 were intermediate and SR-01, SR-02, SR-05, SR-12, SR-13 and SR-17 showed least number of sclerotial formation. Majority of isolates produced sclerotia in 6-9 d, however in isolate SR-04 sclerotia appeared on 14 d. As for size of sclerotia is concerned, isolates SR-12, SR-13 and SR-15 were bigger with diameter 2.03-2.08 mm while SR-04 and SR-16 showed least size of sclerotia. Maximum weight of sclerotia was observed in isolate SR-07 (1.7 mg), however other isolates varied in their sclerotial weight ranging 0.3 - 1.2 mg (Table 1 and Figure 1).

Molecular diversity

DNA of *S. sclerotiorum* was amplified with 50 decamer primers of arbitrary sequences. Primers were selected that produced a reasonably good number of countable and reproducible PCR products. The RAPD analysis is prone to

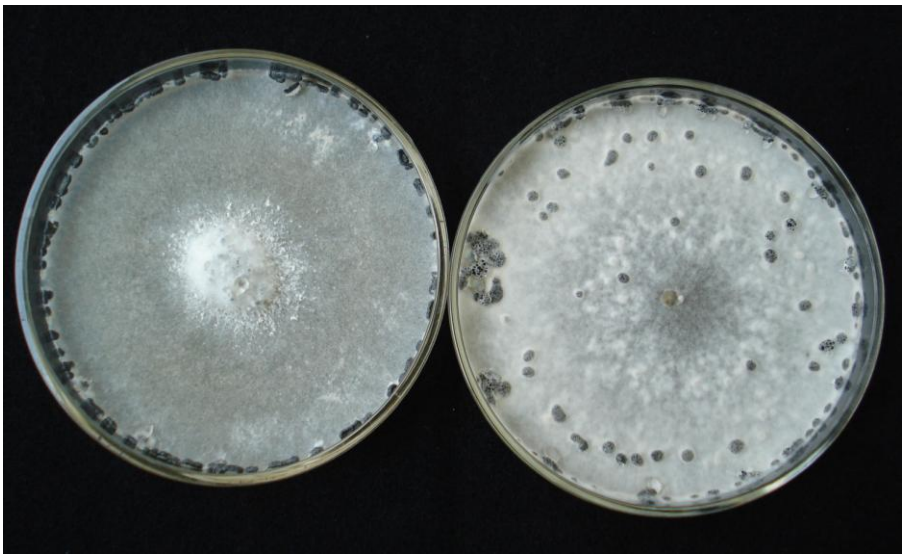

Figure 1. Mycelial growth of *S. sclerotiorum*.

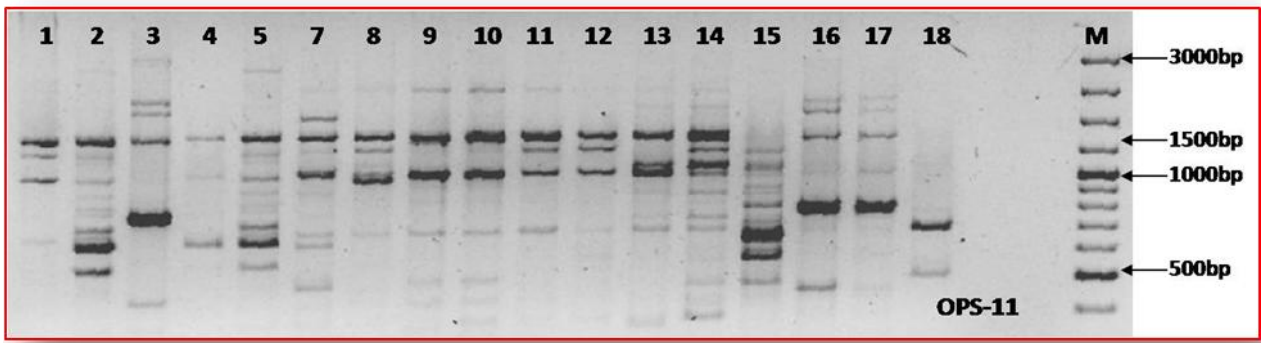

Figure 2. RAPD profile of different geographical isolates of *S. sclerotiorum*.

reproducibility problems across the laboratories but the results obtained in the investigation were consistent and repeatable. 50 primers produced 692 scorable amplicons ranging in size from 190 to 3600 bp. Out of these, 385 fractionated fragments were reported polymorphic. The Polymorphism was detected for 1.3 to 2.7 kb amplified product as in primer OPS-11 (5'-AGTCGGGTGG-3') (Figure 2). On an average, 13-14 unambiguous and reproducible amplified products were generated by single primer (minimum of 4 and maximum of 23 fragments). The present investigation confirmed the diversity in *S. sclerotiorum* isolates determined on the basis of morphological criteria. The segregation of RAPD marker among the isolates is provided (Table 2). The RAPD bands segregation was estimated by theoretical ratio of segregation using χ^2 (qui square) analysis.

Based on Jaccard's similarity coefficient, dendogram for different isolates was prepared using 50 primers (Figure 3). The genetic relationships among the isolates based on the results of RAPD analysis, were also examined. All the 17 isolates of *S. sclerotiorum* were placed into four groups based on the presence and absence of the amplified products. These major clusters were further divided into sub-cluster. RAPD-PCR analysis proved to be useful in determining the extent of genetic diversity among the *S. sclerotiorum* isolates under investigation. Values of similarity coefficient of RAPD data ranged from 0.18 to 0.87 (Table 3). Isolates SR-02, SR-05, SR-13, SR-14 and SR-15 formed first clear group with 70% diversity. Second cluster framed the isolates SR-01, SR-07, SR-08, SR-09, SR-10 and SR-11. SR-04 and SR-12 isolates formed the third cluster. Fourth cluster was framed by SR-03, SR-16, SR-17 and SR-18 isolates. This is the first report indicating the existence of three genetically different population of *S. sclerotiorum* in India collected from oilseed *Brassica*.

Table 2. Qui squire analysis for allele frequency.

Marker	Frequency*	Level of significance					
		1%			5%		
		Expected χ2		P	Expected χ2		P
OPA -06	53	43	0.153	0.808	56.7	59.31	0
OPA -12	34	43	6.432	0.004	56.7	127.2	0
OPB- 08	62	45.3	5.183	0.041	57.5	21.93	0
OPZ-11	37	45.3	0.014	0.904	57.5	52.83	0
OPZ-15	63	54.1	26.8	0	57.5	0.235	0.628

*Number of bands.

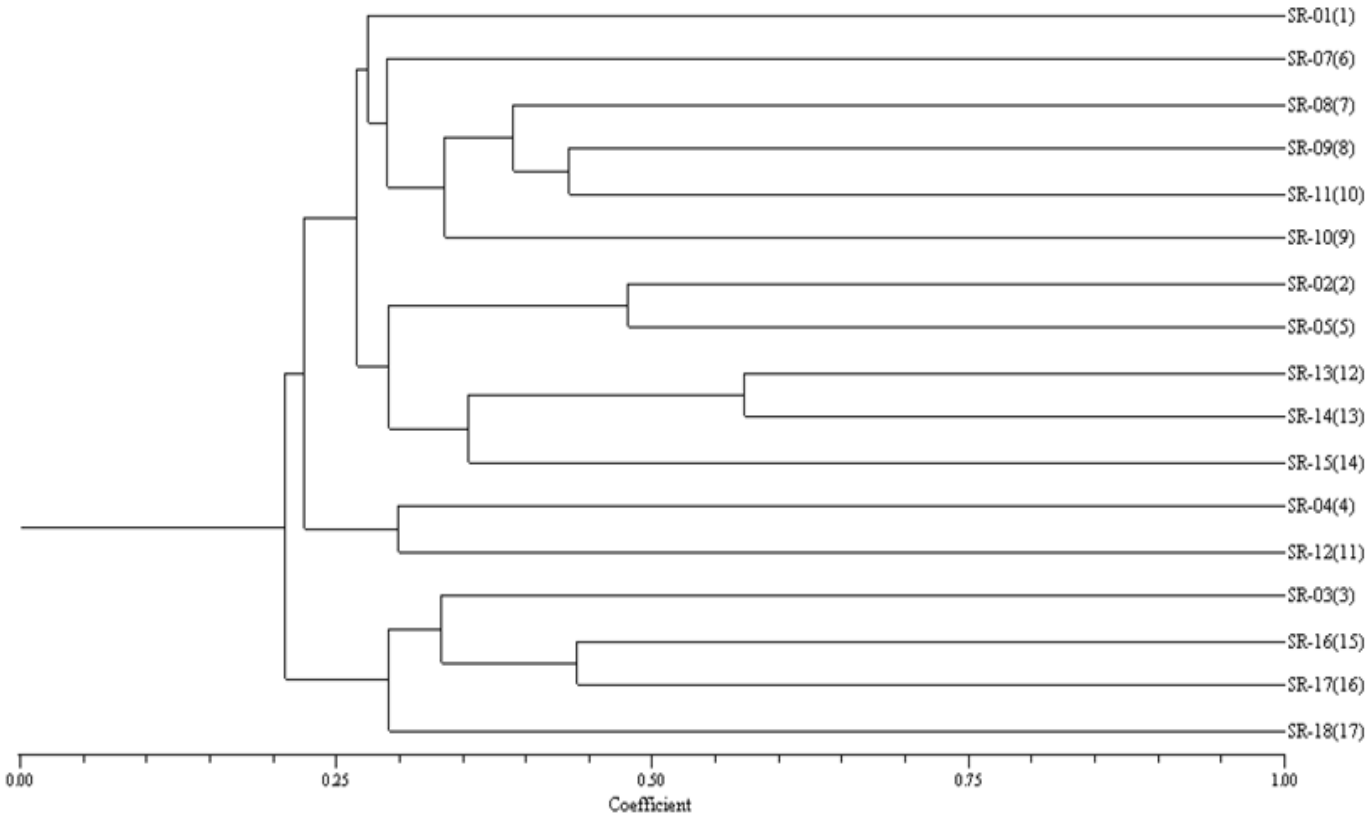

Figure 3. Cluster diagram showing grouping of *Sclerotinia sclerotiorum* based on RAPD**.**

DISCUSSION

Diversity among the Indian geographical isolates of *S. sclerotiorum* based on their growth behavior with validation using RAPD markers was confirmed. Variability among the isolates has already been reported by Carpenter et al. (1999). All isolates were darkly pigmented. However, intensity of melanisation varied among the pigmented isolates. Primarily, the dark colour of the sclerotia results from the production of melanin. The main role of melanin in pathogen is to protect the sclerotia from adverse biological and environmental conditions (Butler and Day, 1998). Punja and Damiani (1996) and Zarani and Christensin (1997) recorded differences in growth rates among different isolates obtained from various host species. Based on sclerotial diameter, several workers recorded variation in size of sclerotia among different isolates of the fungus (Dhingra and Sinclair, 1973; Mirza et al., 1985).

During the study, bigger sizes of sclerotia were observed in the same morphological group and genetic cluster. Sun et al. (2005) compared *S. sclerotiorum*

Table 3. Similarity matrix generated through Jacquard coefficient.

	SR-01	SR-02	SR-03	SR-04	SR-05	SR-07	SR-08	SR-09	SR-10	SR-11	SR-12	SR-13	SR-14	SR-15	SR-16	SR-17	SR-18
SR-01																	
SR-02	0.537																
SR-03	0.773	0.4639															
SR-04	0.619	0.5679	0.53														
SR-05	0.529	0.434	0.57	0.536													
SR-07	0.574	0.5357	0.64	0.54	0.609												
SR-08	0.64	0.5402	0.62	0.611	0.518	0.815											
SR-09	0.529	0.434	0.57	0.536	0.55	0.609	0.486										
SR-10	0.574	0.5357	0.64	0.54	0.616	0.611	0.518	0.815									
SR-11	0.619	0.6186	0.43	0.619	0.619	0.619	0.574	0.536	0.64								
SR-12	0.619	0.5294	0.61	0.486	0.619	0.529	0.619	0.568	354	0.577							
SR-13	0.815	0.5743	0.61	0.518	0.815	0.574	0.483	0.434	0.43	0.496	0.619						
SR-14	0.43	0.6186	0.62	0.619	0.568	0.529	0.619	0.568	0.529	0.55	0.574	0.574					
SR-15	0.609	0.4865	0.62	0.529	0.434	0.574	0.529	0.434	0.574	0.616	0.619	0.64	0.529				
SR-16	0.611	0.5179	0.81	0.574	0.536	0.64	0.574	0.536	0.64	0.619	0.619	0.619	0.568	0.529			
SR-17	0.55	0.6092	0.57	0.616	0.54	0.616	0.619	0.574	0.574	0.486	0.619	0.529	0.434	0.574	0.529		
SR-18	0.616	0.6111	0.64	0.616	0.529	0.434	0.574	0.536	0.55	0.518	0.815	0.574	0.536	0.64	0.574	0.536	

population in oilseed rape from China, Canada and Poland with RAPD and reported highly significant genetic diversity within the population. Molecular diagnosis and classification of plant pathogen have greatly influenced epidemiology and disease management studies. The results confirmed our presumption about link of different morphological characters and genetic variations within the isolates. The very fast growing group of isolates was also within the same genetic cluster except SR-16. The molecular biology techniques have added to the analysis of variability in that organism where stable morphological markers are absent. Our finding is also supported by Yu et al. (2006), who attempted to unlock the polymorphism of *S. sclerotiorum* isolated from sunflower in China. Ziqin et al. (2009) also reported genetic diversity and differentiation of *S. sclerotiorum* population. The population differentiation may results from the reproductive isolation due to geographical separation studied by Bowden and Leslie (1999). They reported that sexual reproduction is considered as an important assumption for molecular divergence among the population from different geographical regions. Isolates of *S. sclerotiorum* (Lib) de Bary from various agro-climatic conditions has been compared by morphological and genetic analysis. Morphological variations observed in the study in general were strongly correlated to genotypic variations. The pathogen is widely spread and well established in the different geographical regions. Therefore it dealt with the relatedness among 17 *S.* sclerotiorum isolates as representative samples of the pathogen.

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REFERENCES

Agarwal RA, Kumar K, Thakur LC (1997). Effect of Sclerotinia rot on oil quality in low erucic acid cultivar of rapeseed.

Cruciferae Newsl. 19:103-104.
Alvarez E, Molina ML (2000). Characterizing the Sphaceloma fungus, causal agent of super elongation disease in cassava. Plant Dis. 84:423-428.
Atallah ZK, Larget B, Chen X, Johnson DA (2004). High genetic diversity, phenotypic uniformity and evidence of outcrossing in *Sclerotinia sclerotiorum* in the Columbia basin of Washington state. Phytopathology 94:737-742.
Boland GJ, Hall R (1994). Index of plant host of *Sclerotinia sclerotiorum.* Can. J. Plant Pathol. 16:94-108.
Bowden RL, Leslie JF (1999). Sexual recombination in *Gibberella zeae*. Phytopathology 89:182-188.
Butler MJ, Day AW (1998). Fungal melanins: a review. Can. J. Microbiol. 44:1115-1136.
Carpenter MA, Frampton C, Stewart A (1999). Genetic variation in New Zealand population of pathogen *Sclerotinia sclerotiorum*. New Zealand J. Crop Hort. 27:13-21.
Chattopadhyay C, Meena PD, Sastry RK, Meena RL (2003). Relationship among pathological and agronomic attributes for soilborne diseases of three oilseed crops. Indian J. Pl Prot. 31(2):127-128.
Clarkson JP, Staveley J, Phelps K, Young CS, Whipps JM (2003). Ascospore release and survival in *Sclerotinia sclerotiorum.* Mycol. Res. 107:213-222.
Dhingra OD, Sinclair JB (1973). Variation among isolates of *Macrophomina phaseolina* (*Rhizoctonia bataticola*) from different regions. Phytopathology 76:200-204.
Harlton CE, Levesque CA, Punja ZK (1995). Genetic diversity in *Sclerotium (Athelia) rolfsii* and related species. Phytopathology 85:1269-1281.
Kohli Y, Brunner JL, Yoell H, Milgroom MG, Anderson JB, Morall RA, Kohn A (1995). Clonal dispersal and spatial mixing in population of plant pathogenic fungi *Sclerotinia sclerotiorum.* Mol. Ecol. 15:255-267.
Kohn L, Carbone I, Anderson J (1990). Mycelia interaction in *Sclerotinia sclerotiorum*. Exp. Mycol. 14:292-298.
Kull LS, Pederson WL, Palmquist D, Hartman GL (2004). Mycelial compatibility grouping and aggressiveness of *Sclerotinia sclerotiorum.* Plant Dis. 88:325-332.
Mirza MS, Ahmad Y, Beg A (1985). Sclerotinia stalk rot of sunflower. Pak. J. Agric. Res. 6:286-288.
Okabe I, Morikawa C, Matsumoto N, Yokoyama K (1998). Variation *Sclerotinia rolfsii* isolate in Japan. Mycoscience 39:399-407.
Punja ZK, Damiani A (1996). Comparative growth, morphology and physiology of three *Sclerotinia* species. Mycologia 88: 694-704.
Sambrook J, Fritsch EF, Miniatis T (1989). Molecular cloning a laboratory manual 2nd edition, Cold Spring Harbour Laboratory Press, pp. 856- 965.
Sexton AC, Howlett BJ (2004). Microsatellite marker reveals genetic differentiation among population of *Sclerotinia sclerotiorum* from Australian canola fields. Curr. Genet. 46:357-365
Sun J, Irzykowaski W, Jedryczka M, Han F (2005). Analysis of genetic structure of *Sclerotinia sclerotiorum* population from different region and host plant by RAPD markers. J. Int. Plant Biol. 47:385-395.
Weining S, Langridge L (1991). Identification and mapping of polymorphism in cereals based on polymerase chain reaction. Theor. Appl. Genet. 82:209-216.
Welsh J, McClelland M (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucl. Acid Res. 18:7213-7218.
Yu X, Wang G, Zhang N (2006). The polymorphism of nuclear DNA of *Sclerotinia sclerotiorum* from different area of our country. J. Hebei. N. Univ. 122:24-26.
Zarani F, Christensin C (1997). Sclerotial biogenesis in basidiomycetes *Sclerotium rolfsii*. Scanning electron microscopic study. Mycologia 89:598-602.
Zhao J, Meng J (2003). Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed mustard (*Brassica napus* L.). Theor. Appl. Genet. 106:759-764.
Ziqin L, Zhang M, Wang Y, Li R (2008). Mycelial compatibility group and pathogenicity variation of *Sclerotinia sclerotiorum* population in Sunflower from China, Canada and England. J. Plant. Pathol. 7:131-139.
Ziqin L, Wang Y, Chen Y, Zhang JW, Fernando D (2009). Genetic diversity and differentiation of *Sclerotinia sclerotiorum* population of sunflower. Phytoparasitica 37:77-85.