Indian Journal of Biotechnology
Vol 15, October 2016, pp 589-595

Isolation and analysis of genetic diversity amongst *Sclerotinia sclerotiorum* isolates infecting cauliflower and pea

Apoorva Kapatia, Tanvi Gupta, Mohit Sharma, Azhar Khan and Saurabh Kulshrestha*

[1]Faculty of Applied Sciences and Biotechnology
Shoolini University of Biotechnology and Management Sciences, Bajhol, Solan 173 229, India

Received 10 June 2015; revised 28 December 2015; accepted 18 January 2016

Sclerotinia sclerotiorum is an ubiquitous plant pathogen responsible for a wide range of diseases among different vegetable crops with a host range of more than 400 plant species. It continues to be the most destructive plant pathogen for many years having no significant method for its control and management. In the present study, an attempt was made to isolate and characterize *S. sclerotiorum* from infected cauliflower and pea grown in Solan and Sirmaur districts of Himachal Pradesh, India. The isolates were characterized morphologically and molecularly by the amplification of the internal transcribed spacer region (ITS) using specific primers, followed by sequencing. The genetic diversity among the sixteen isolates of *S. sclerotiorum* was also studied using random amplified polymorphic DNA (RAPD). Four random primers, *viz.*, OPA-14, OPA-16, OPA-17 and OPA-20 were used for RAPD analysis. Clustering based on RAPD fingerprint data revealed two groups and ten independent branches at 0.70 similarity value.

Keywords: Cauliflower, pea, RAPD, *Sclerotinia sclerotiorum*

Introduction

Sclerotinia sclerotiorum (Family: *Sclerotiniaceae*; Phylum: *Ascomycota*) is recognized as an important plant pathogen for its worldwide distribution, wide host range and the difficulties encountered in controlling the diseases it causes[1,2]. Plants susceptible to this pathogen encompass 75 families, 278 genera and 408 species[3]. Some common hosts of this soil borne necrotrophic fungal pathogen are broccoli, cauliflower, cabbage, carrot, common bean, citrus, mustard, celery *etc*. The *Sclerotinia* infection associated with vegetables produces a white, cottony and dense mycelial mat on the surface of host and soil. It causes a wide range of diseases, such as, stem rot, watery soft rot, cottony rot, white blight of cauliflower, white rot of pea, root rot, stem break, stalk rot, white canker *etc*.

Himachal Pradesh (HP) is a leading state for the production of different vegetables and fruits in India. Cauliflower and peas are among the main crops grown in many districts of HP including Solan and Sirmour. Stem rot, watery soft rot, cottony rot, white blight of cauliflower and white rot of pea are the major threats for the cultivation of these crops in various parts[4]. It has been observed that severe stalk rot in cauliflower resulted in remarkable reduction in seed production from 625 to 250 kg/ha in Saproon valley of Solan district as well as in other parts of HP[5]. The infection initiates in the late months of winter and in the early spring, and the fungus grows and spreads within and between the plants[6]. Various studies have also been done on field pea in case of *Sclerotinia* white rot and root rot disease. A study conducted in 1991 in HP reported *Aspergillus niger*, *Penicillium cyclopium*, *Paecilomyces lilacinus*, *Acremonium implicatum* and *Trichothecium roseum* as an antagonist against *S. sclerotiorum*[7]. Management of the disease through chemicals and other cultural practices are not very successful in case of *S. sclerotiorum* due to the formation of rigid structures called sclerotia that help fungus to survive for a long time[8,9].

S. sclerotiorum has been affecting many crops like cauliflower and pea for many years in HP. The extent of infection and losses due to *S. sclerotiorum* in different areas of HP are very high and devastative. The very early signs and symptoms of the disease caused by *S. sclerotiorum* are almost impossible to identify in the fields. Variations occurring at the genetic level between the isolates from different geographical regions are essential for documenting the changes evolving in the population. Wide variability in the

*Author for correspondence:
Tel: +91-1792-308000 Ext. 2022; Fax: +91-1792-308000
Mobile: +91-9459241393
saurabh_kul2000@yahoo.co.in
sourabhkulshreshtha@shooliniuniversity.com

population of *S. sclerotiorum* has been found using mycelium compatibility groups and DNA (RAPD markers) in a study carried out by Litholdo *et al*[10] based on analysis of morphological, physiological and molecular traits of *S. sclerotiorum* population collected from different areas and different hosts. Similar variations in case of virulence among isolates of *S. sclerotiorum* from a defined geographical area have been observed[11,12]. Osofee *et al*[13] also found variations among isolates based on their ability to produce oxalic acid, which is responsible for the extent of virulence in *S. sclerotiorum*.

RAPD technique has been widely used and helps to identify a large number of markers used for estimating genetic variations[13]. A firm understanding of the genetic diversity and population structure of *S. sclerotiorum* in different hosts may provide important information about the spread of this deadly fungal disease as well as help understanding the different modes of reproduction and severity of *S. sclerotiorum*[14].

The studies regarding the genetic diversity of *S. sclerotiorum* in HP has not been reported in the past. Keeping this in view, the present investigation was carried out to study the genetic diversity in *S. sclerotiorum* isolates taken from two different hosts [*Brassica oleracea var*. (cauliflower) and *Pisum sativum* (pea)] grown in different locations of Solan and Sirmour districts of HP.

Material and Methods

Sample Collection

Cauliflower and pea fields in the district of Solan and Sirmour were visited in the month of March/April 2015. Infected cauliflower and pea samples were collected in zip bags and transported to Molecular Plant Microbe Interaction Laboratory, Shoolini University, Solan for further isolation of *S. sclerotiorum*.

Isolation of *S. sclerotiorum* and Morphological Characterization

Sclerotia of *S. sclerotiorum* were collected from the infected samples, air dried and stored at room temperature for further use. The sclerotia were surface sterilized using 10% commercial bleach (0.5% NaHCl) for 3 min, followed by washing with ethanol and 3 washes in sterile distilled water. Then surface sterilized sclerotia were placed on the PDA plates and incubated at 25°C for 3-5 d. All the isolates were analyzed morphologically under the microscope for their characterization.

Isolation of Total DNA from Selected Isolates of *S. sclerotiorum*

After successful isolation of *S. sclerotiorum* from all the samples, total DNA was extracted using protocol described by Sambrook *et al*[15]. Bands corresponding to genomic DNA were observed by performing electrophoresis in 1% agarose gel.

Molecular Characterization of Isolates by PCR Amplification of ITS Region

The ITS region of the isolates was amplified with *S. sclerotiorum* specific forward/reverse primers[16] ITS4SS (5'-TCCTCCGCTTATTGATATGC-3')- F, ITS5SS 5'-GGAAGTAAAAGTCGTAACAAGG-3')-R. PCR was carried out in automated thermal cycler (Applied Biosystems) with 20 µL of total reaction mixture containing: 1 µL of genomic DNA (~50 ng), 10 pM of upstream (forward) primer, 10 pM of downstream (reverse) primer, 2 µL of 10× PCR buffer, 2 µL of 1.5 mM each dNTP mix and 1 unit of Taq DNA polymerase. Cycling conditions for PCR were: initial denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 30 sec, 50°C for 30 sec and 72°C for 1 min. One cycle of final elongation was performed at 72°C for 10 min. The PCR product was analyzed using 1% agarose gel electrophoresis.

Purification of PCR Amplified DNA Product and DNA Sequencing

Of sixteen characterized samples, two samples, one from cauliflower (SSC4) and one from pea (SSP67), were randomly selected for further identification by sequencing of the amplified ITS region. PCR amplified DNA products of both the samples (SSC4 & SSP67) were purified from gel using gel purification kit (DNA gel/PCR purification miniprep kit, XcelGen). Purified PCR product was then sent for sequencing to Xcelris Genomics. BLAST was performed for sequence analysis using BLASTn suite with nucleotide collection (nr/nt) as database and for search Megablast program was used.

Phylogenetic Analysis

The phylogenetic tree was constructed using maximum likelihood method based on the Tamura-Nei model[17]. The analysis was performed with ITS region sequences of the selected two isolates (SSC4 & SSP67) and with twenty-three other ITS sequences of the related taxa retrieved from GenBank database. The degree of similarity between the sequences was found out using EMBOSS Needle method.

Diversity Analysis of Isolated Strains Using RAPD

Isolated DNA from *S. sclerotiorum* was used to study the genetic variation using RAPD along with

DNA from *Fusarium* spp. as outgroup. The standard RAPD reaction was set up by using standard RAPD primers. Four RAPD primers [OPA-14 **(**5'-TCTGTGCTGG-3'), OPA-16 **(**5'-AGCCAGCGAA-3'), OPA-17 **(**5'-GACCGCTTGT-3') and OPA-20 (5'-GTTGCGATCC-3')] were used in this study. PCR was conducted in 25 µL mixture containing: 1 µL DNA (~50 ng), 2.5 µL of 2 mM-dNTPs, 2 µL of (10 pM) RAPD primer and 1 unit of Taq polymerase with 10× PCR buffer. Cycling conditions for PCR were: initial denaturation at 94°C for 5 min, 40 cycles at 94°C for 30 sec, 36°C for 1 min, 72°C for 1 min and a final elongation at 72°C for 7 min. The PCR product was analyzed in 1% agarose gel electrophoresis.

The RAPD fragments obtained after the amplification of genomic DNA were scored for their presence (1) and absence (0) of amplification for each sample. The matrix was designed for all the seventeen samples. The data of similarity coefficients generated were used to construct the dendrogram indicating the genetic relatedness or differentiation among the *Sclerotinia* isolates. Data was compiled as binary 0-1 matrix, (1) represents the presence of a band and (0) represents the absence of band at a particular position. All RAPD bands were considered in statistical analysis. Dendogram was produced from the distance matrix by unweighted pair group method with arithmetic average (UPGMA), contained in the software package NTSYS 2.2 version.

Results and Discussion

Isolation of *S. sclerotiorum*

S. sclerotiorum was successfully isolated from infected cauliflower and pea using surface sterilized slerotium. A total of eleven isolates were collected from cauliflower and five isolates from pea representing different places of district Solan and Sirmour. The identity of all the isolates was confirmed by morphological characters of the fungus. The sclerotia were formed in all the isolates which were rigid and appeared drop like at the initial stage, which turned black at the maturity (Fig. 1). Microscopic characterization of all the isolates revealed the presence of granules in the hyphae (Fig. 2). These two characters are typical to *S. sclerotiorum*[12]. This confirms the identification of the fungus as *S. sclerotiorum.*

Molecular Characterization of Isolates

Good quality, total DNA was extracted from all the sixteen samples of *S. sclerotiorum* isolated from infected cauliflower and pea samples of district Solan and Sirmour. Isolated genomic DNA was used in PCR reactions with *S. sclerotiorum* specific primers for the amplification of respective ITS region. Primers used in the reaction specifically amplified the ITS region of all the putative isolates giving an amplification product of ~600 bp, which further confirmed the identity of all the sixteen isolates as *S. sclerotiorum.*

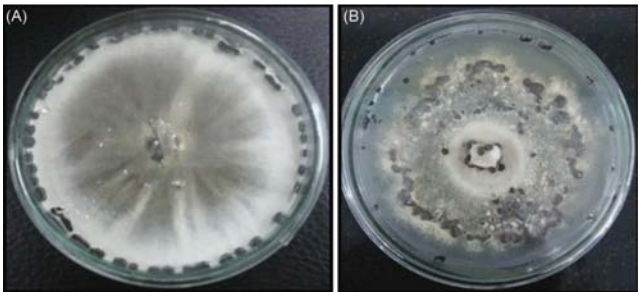

Fig 1—PDA plates showing sclerotial growth of fungus after 3-5 d of incubation at 25°C.

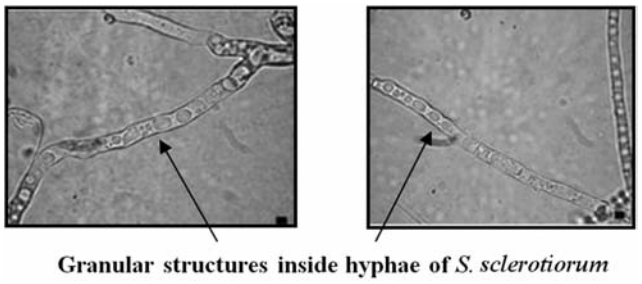

Fig 2—Hyphal structure of *S. sclerotiorum* under light compound microscope.

The ITS region of fungal ribosomal DNA (rDNA) has conserved sequences of a species and has great importance in distinguishing fungal species by PCR analysis. Jeon and coworkers[18] reported the use of ITS region for amplification and subsequent identification of *S. sclerotiorum* isolates taken from paprika. Similarly, amplification of ITS region has been extensively used by a number of researchers as a tool to confirm the identity of the isolates upto genus and species level[18,19,16]. PCR product from isolate SSC4 (isolate of cauliflower) and SSP67 (isolate of pea) were eluted and sequenced using PCR primers.

DNA Sequencing and Data Analysis

Purified PCR product was sequenced and a sequence of 509 bp corresponding to cauliflower isolate SSC4 was obtained. The sequence was then analyzed using BLASTn tool, which revealed that ITS region of isolate SSC4 was 100% identical to the ITS region of *S. sclerotiorum* (acc. no. KF859932 & AY187066) reported from Canada and USA.

Similarly, 512 bp sequence corresponding to pea isolate SSP67 was obtained after sequencing. The sequence was then analyzed using BLASTn tool, which revealed that ITS region of isolate SSP67 was 99% identical to the ITS region of *S. sclerotiorum* isolates (acc. no. KF859932 & KJ614564) reported from Canada and South Korea. The sequence corresponding to ITS region of isolate SSC67 was submitted to EMBL database under acc. no. LN898438.

Phylogenetic Analysis

The evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model[17]. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 397 positions in the final dataset. Evolutionary analyses were conducted in MEGA6[20].

Four clads were formed in the phylogenetic analysis (Fig. 3), which were showing similarity with each other. To find out the degree of similarity between the sequences forming a clad, pairwise sequence alignment was performed using EMBOSS Needle method and results were obtained, which showed 97.7% similarity between *Brassica carinata* isolate JX307092 reported from Florida and isolate AY187066 reported from USA. The 96.4% similarity was found between *Trifolium ambiguum* isolate KT224660 reported from USA and *B. napus* isolate JQ480621 reported from China. Similarly 98.9% similarity was found between *T. alexandrinum* isolate KT595415 reported from Pakistan and *Sesamum indicum* isolate KT595414 reported from Pakistan. Cauliflower isolate SSC4 and Pea isolate SSP67 clustered together with an isolate (KF859932) infecting *B. oleracea* var. *botrytis* with 93.3% similarity and thus concluded that all these isolates are similar to each other.

RAPD Analysis

Isolated DNA from all *S. sclerotiorum* isolates were used to study the genetic variations using RAPD. The standard RAPD reaction was set up using four RAPD primers, *viz.*, OPA-14, OPA-16, OPA-17 and OPA-20, and reproducible RAPD patterns were generated. *Fusarium* sp. was used as an out-group. All the sixteen isolates showed excellent polymorphism (Fig. 4). *Fusarium* sp. showed totally different amplifications with all the primers in comparison to amplification obtained with *S. sclerotiorum* isolates.

A total of 88, 53, 54 and 74 bands were scored by using primers OPA-14, OPA-16, OPA-17 and OPA-20, respectively. The number and size of the amplified products resulted from random primers varied between 53-88 and 300-1750 base pairs. Clustering based on RAPD fingerprinting data revealed that isolate SSC2 and SSC12; SSC4 and SSC17, SSC22 and SSP67; and SSP72 and SSP77 showed maximum homology. Two independent groups were formed at similarity value 0.75 (Fig. 5), where one group comprises of cauliflower isolates

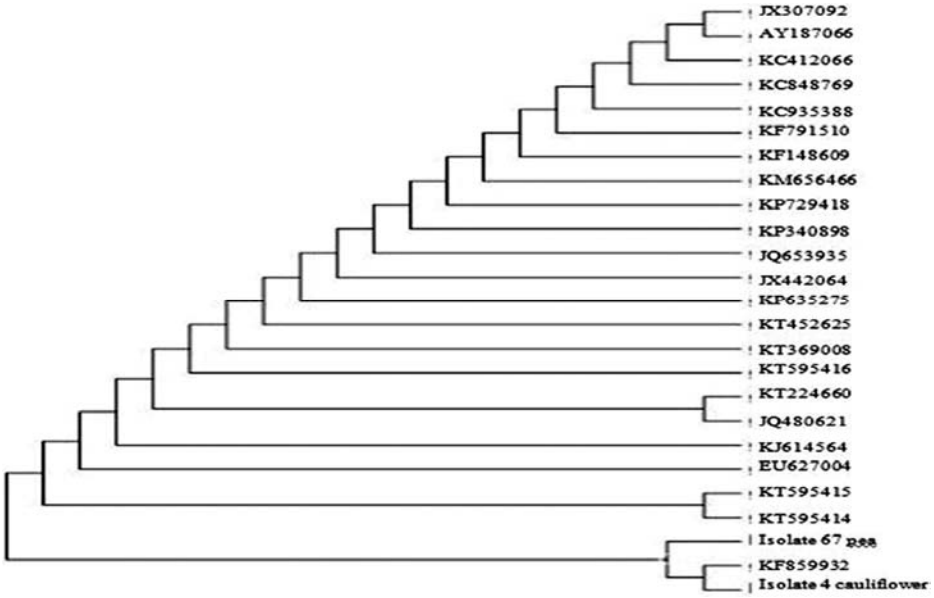

Fig 3—Phylogenetic tree showing evolutionary relationship of *S. sclerotiorum* isolates based on Tamura-Nei model.

SSC2, SSC12, SSC4 and SSC17; and the other group comprises of pea isolates SSP72, SSP77, SSP75 and SSP73. However, at similarity value nearly 0.55, two groups were formed, which were distantly related to one another. The first group comprises of only cauliflower isolates (SSC2, SSC12, SSC4, SSC17, SSC20, SSC10, SSC7 & SSC11) and the second group comprises of some cauliflower and all pea isolates (SSC22, SSP67, SSC27, SSC47, SSP72, SSP77, SSP75 & SSP73). Isolate SSC2 (Fig. 6A) and SSC4 (Fig. 6B), which showed similar morphology also clustered together in RAPD analysis. Relatively slow growing isolates from pea (SSP72, SSP73, SSP75 & SSP77) also clustered together in RAPD analysis. However, isolate SSC12 (Fig. 6C) and SSC17 (Fig. 6D), which showed different morphology, clustered together in RAPD. Similarly, isolate SSC22 (Fig. 6E) also clustered together with slow growing isolate of pea SSP67 (Fig. 6F). Four cauliflower isolates, ***viz.***, SSC2, SSC12, SSC4 and SSC17, formed a group, similarly four pea isolates, ***viz.***, SSP72, SSP77, SSP75 and SSP73 (Fig. 6G) formed a separate group, which confirmed that all these isolates were closely related to one another. The clustering of all the pea isolates together was possibly because all the pea isolates were collected from the nearby locations and had similar morphology and growth patterns; whereas in case of cauliflower, the isolates were taken from distant fields. The results obtained clearly show the differences between the *Sclerotinia* isolates obtained from cauliflower and pea on the basis of variability obtained through RAPD fingerprinting data. In comparison to all the isolates, *Fusarium* sp. formed a different cluster, which was joined to the rest of the isolates at very initial point on the scale value. This showed *Fusarium* sp. was an outlier and that it was least related to the rest of the clusters formed.

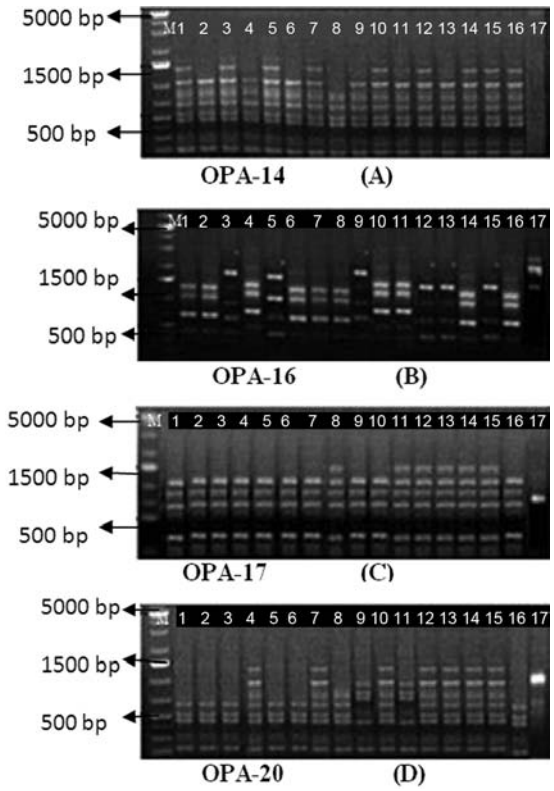

Fig 4—RAPD banding pattern of *S. sclerotiorum* isolates using random primers.

In the present study, two different hosts of *S. sclerotiorum* were taken [*Brassica oleracea var.* (cauliflower) and *Pisum sativum* (pea)]. Both types of isolates were having different morphological characteristics and growth patterns, and possibly the same were reflected through the RAPD fingerprint data.

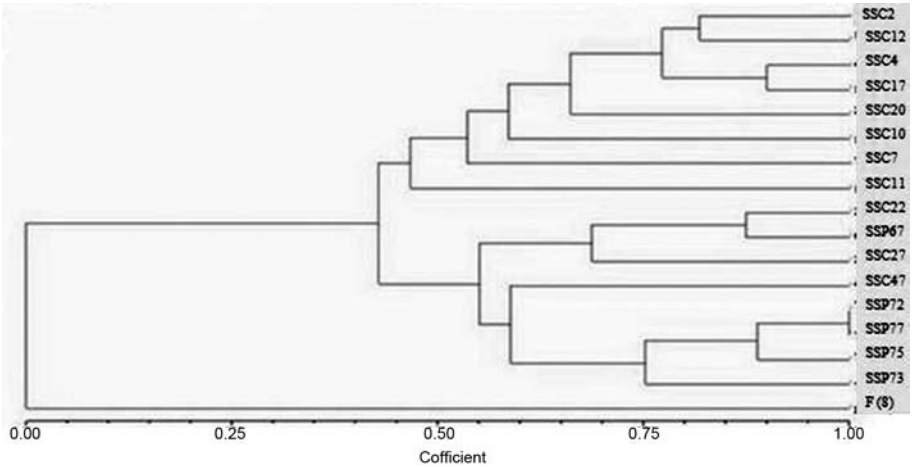

Fig 5—Dendogram of *S. sclerotiorum* isolats derived from RAPD fingerprint data using UPGMA.

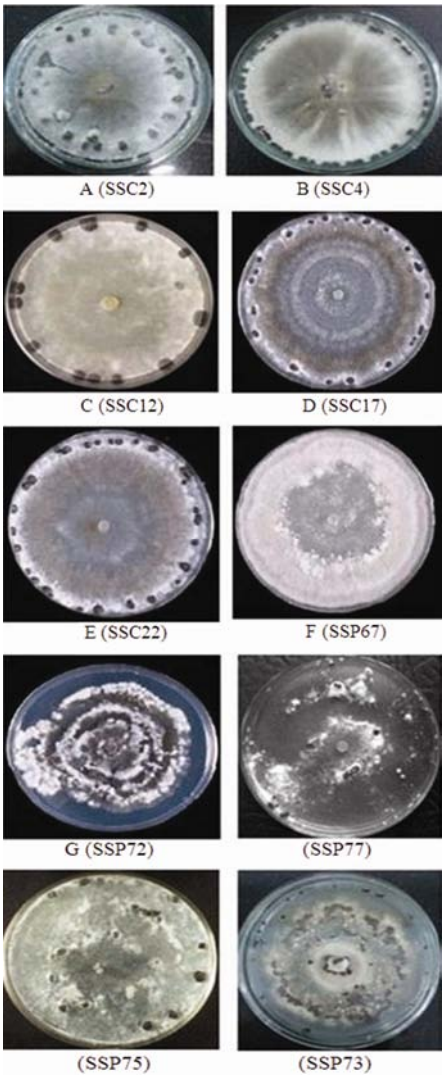

Fig 6—PDA plates showing different isolates of *S. sclerotiorum.*

Similar studies to analyze genetic diversity using the arbitrary sequence decamer primers for RAPD and minisatellite core sequence primers[21] have also been reported in the past. Similar to our findings Sun and coworkers[22] also reported the effectiveness of RAPD markers for identifying genetic differences between isolates of *S. sclerotiorum* from different regions and host plants in China. Futher, Litholdo *et al*[10] reported a high level of variability among isolates of *S. sclerotiorum* obtained by using MCG and RAPD markers, suggesting the occurrence of sexual recombination in the population in addition to clonal reproduction. Saxena and coworkers[23] also reported the use of RAPD markers in molecular tagging of gene for resistance to stalk rot in cauliflower. RAPD analysis along with morphological characterization can be used as a reliable and specific tool for identifying variations occurring within a species and gives a firm understanding about genetic diversity and population structure of the species, which may provide important aspects about the epidemiology and modes of reproduction of the concerned species.

The dendogram generated on the basis of similarity matrix, in the present study, was used to evaluate the degree of polymorphism and genetic relationship between *Sclerotinia* isolates and revealed significant amount of genetic variations among the isoltes. Our results also suggests the existence of variations among the isolates of *S. sclerotiorum* isolated from cauliflower and pea and revealed that all the isolates were divided into two main clusters where some of the isolates were found to be homologous, while some are found to be in a distant relationship with each other. The results of the present study also demonstrates the effectiveness of RAPD in identifying the genetic diversity among *S. sclerotiorum* isolates.

Acknowledgement

The authors would like to thank Professor P K Khosla, the Hon'ble Vice-Chancellor, Shoolini University of Biotechnology and Management Sciences, Solan, and Foundation for Life Sciences and Business Management (FLSBM), Solan for providing financial support and necessary facilities.

References

1. Saharan G S & Mehta N, *Sclerotinia diseases of crop plants: Biology, ecology and disease management* (Springer + Business Media B.V., The Netherlands) 2008, p 485.
2. Sharma P, Worldwide new host record of *Sclerotinia sclerotiorum*, *Sarson News*, 18 (2014) 9.
3. Li Z, Zhang M, Wang Y, Li R & Fernando W G D, Mycelial compatibility group and pathogenicity variation of *Sclerotinia sclerotiorum* population in Sunflower from China, Canada and England, *Plant Pathol J*, 7 (2008) 131-139.
4. Kumar A & Thakur K S, *Effect of nitrogen levels on Sclerotinia stem rot in canola and non-canola types cultivars of rapeseed-mustard*, paper presented in *Natl Seminar on Oilseeds and Oil-Research and Development Needs in the Millennium*, Directorate of Oil Research, Hyderabad, India, 2000, 224-225.
5. Asia News International (ANI), Off-seasonal vegetable production takes-off in Himachal Pradesh, ANI Shimla, November 2, 2013. [http://www.aninews.in]
6. Zhao J & Meng J, Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus* L.), *Theor Appl Genet*, 106 (2003) 759-764.
7. Singh D, Biocontrol of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Trichoderma harzianum*, *Trop Pest Manage*, 37 (1991) 374-378.
8. Bolton M D, Thomma B P & Nelson B D, *Sclerotinia sclerotiorum* (Lib.) de Bary: Biology and molecular traits of a cosmopolitan pathogen, *Mol Plant Pathol*, 7 (2006) 1-16.

9. Li C X, Liu S Y, Sivasithamparam K & Barbetti M J, New sources of resistance to *Sclerotinia* stem rot caused by *Sclerotinia sclerotiorum* in Chinese and Australian *Brassica napus* and *Brassica juncea* germplasm screened under Western Australian conditions, *Australas Plant Pathol*, 38 (2008) 149-152.
10. Litholdo C G Jn, Gomes E V, Lobo M Jn, Nasser L C & Petrofeza S, Genetic diversity and mycelia compatibility groups of the plant-pathogenic fungus *Sclerotinia sclerotiorum* in Brazil, *Genet Mol Res*, 10 (2011) 868-877.
11. Alvarez E & Molina M L, Characterizing the *Sphaceloma* fungus, causal agent of superelongation disease in cassava, *Plant Dis*, 84 (2000) 423-428.
12. Pellegrini S, Patrignani G & Gerola F M, Structures of *Sclerotinia sclerotiorum* ascocarp and its septal pore ultrastructure, *Caryologia*, 42 (1989) 275-283.
13. Osofee H, Hameed K M & Mahasneh A, Relatedness among indigenous members of *Sclerotinia sclerotiorum* by mycelial compatibility and RAPD analysis in the Jordan valley, *Plant Pathol J*, 21 (2005) 106-110.
14. Aldrich-Wolfe L, Travers S & Nelson B D Jr, Genetic variation of *Sclerotinia sclerotiorum* from multiple crops in the North Central United States, *PLoS One*, 10 (2015) e0139188.
15. Sambrook J, Fritsch E F & Maniatis T, *Molecular cloning: A laboratory manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA) 1989, p 1626.
16. Grabicoski E M G, Filho D S J, Pileggi M, Henneberg L, Pierre M L C *et al*, Rapid PCR-based assay for *Sclerotinia sclerotiorum* detection on soybean seeds, *Sci Agric (Piracicaba, Braz)*, 72 (2015) 69-74.
17. Tamura K & Nei M, Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees, *Mol Biol Evol*, 10 (1993) 512-526.
18. Jeon Y-J, Kwon H-W, Nam J-S & Kim S H, Characterization of *Sclerotinia sclerotiorum* isolated from Paprika, *Mycobiology*, 34 (2006) 154-157.
19. Schoch C L, Seifert K A, Huhndorf S, Robert V, Spouge J L *et al*, Fungal barcoding consortium. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi, *Proc Natl Acad Sci USA*, 109 (2012) 6241-6246.
20. Tamura K, Stecher G, Peterson D, Filipski A & Kumar S, MEGA6: Molecular evolutionary genetics analysis, version 6.0, *Mol Biol Evol*, 30 (2013) 2725-2729.
21. Bhattacharya E & Ranade S A, RAPD and DAMD profile differences amongst mulberry varieties, *BMC Plant Biol*, 1 (2001) 3.
22. Sun J-M, Irzykowski W, Jedryczka M & Han F-X, Analysis of the genetic structure of *Sclerotinia sclerotiorum* (Lib.) de Bary populations from different regions and host plants by random amplified polymorphic DNA markers, *J Integr Plant Biol*, 47 (2005) 385-395.
23. Saxena B, Kaur R, Shivani, Kanwar H S, Dohroo N P *et al*, Molecular tagging of gene for resistance to stalk rot (*Sclerotinia sclerotiorum* de Bary) in cauliflower (*Brassica oleracea var. botrytis*) using RAPD markers, *Adv Hortic Sci*, 23 (2009) 108-112.