P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2018; 6(6): 1096-1099
© 2018 IJCS
Received: 14-09-2018
Accepted: 18-10-2018

Variability in *Sclerotinia sclerotiorum* of mustard in northern region of M.P. (India)

OP Bharti, RK Pandya, RK Singh, JC Gupta, Ashish Kumar and RC Sharma

OP Bharti
[1] JNKVV- Krishi Vigyan Kendra, Harda, Madhya Pradesh, India
[2] Department of Plant Pathology, College of Agriculture, RVSKVV, Gwalior, Madhya Pradesh, India

RK Pandya
JNKVV- Krishi Vigyan Kendra, Harda, Madhya Pradesh, India

RK Singh
Department of Plant Pathology, College of Agriculture, Indore, RVSKVV, Gwalior, Madhya Pradesh, India

JC Gupta
Zonal Agriculture Research Station, Morena, Madhya Pradesh, India

Ashish Kumar
Center for genomics, Jiwaji University, Gwalior, Madhya Pradesh, India

RC Sharma
JNKVV- Krishi Vigyan Kendra, Harda, Madhya Pradesh, India

Correspondence
OP Bharti
[1] JNKVV- Krishi Vigyan Kendra, Harda, Madhya Pradesh, India
[2] Department of Plant Pathology, College of Agriculture, RVSKVV, Gwalior, Madhya Pradesh, India

Abstract
Stem rot incited by *Sclerotinia sclerotiorum* (Lib.) de Bary is one of the drastic disease of mustard and it causes quality and quantity losses in mustard. The five isolates of *S. sclerotiorum* from different geographical locations of northern region of Madhya Pradesh were collected and compared for their cultural and morphological characters under artificial culture conditions based on radial growth. Three degenerated primers of arbitrary reverse transcription sequences produced 340 scorable amplicons through florescent labelled random amplified polymorphic DNA markers. Nucleotides range were designed for sizing DNA fragments in the 35-500 nucleotides range and provides 16 single-stranded labeled fragment. Out of these, 251 fractionated fragments were reported polymorphic. Hence, the present investigation confirmed the diversity among the different geographical isolates from five districts of northern region of Madhya Pradesh (India).

Keywords: fRAPD, Stem rot, *Sclerotinia sclerotiorum*, sclerotia, isolate

Introduction
Stem rot in mustard incited by *Sclerotinia sclerotiorum* (Lib.) de Bary is the most important devastating disease among the mustard diseases. Earlier, it was considered a minor problem in India but it has emerged a serious problem over the years in most mustard growing area of the country. It is a menace to mustard cultivation in most mustard or brassica growing area at globally. It was noticed that it causes 40-80% yield losses (Mehta *et al.,* 2010) [7] and in severe condition it can be 100% losses. Still, we don't have resistance in host crops and their cultivars to this broad pathogen (Zhao and Meng, 2003) [13]. Genetic diversity among the *S. sclerotiorum* population was demonstrated by few workers (Harlton *et al*., 1995; Okabe *et al*., 1998; Sharma *et al*., 2013) [4, 9, 10]. Genetic diversity within *Sclerotinia sclerotiorum* isolates from different geographical regions which may help as a key for over come from this devastating brassica problem. 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) [12]. The aim of the present investigation is to compare different isolates of *S. sclerotiorum* provinces in northern region of Madhya Pradesh which collected from different locations infecting oilseed *Brassica* in India based on morphological variability and genetic diversity through fluorescent RAPD marker.

Materials and Methods
The stem rot is a devastating necrotrophic threat of mustard that infects more than 400 plant species worldwide. This study was aim to find out variability in *Sclerotinia sclerotiorum* of mustard in northern region of Madhya Pradesh. The naturally infected stems of mustard were sampled from different of northern region of Madhya Pradesh provinces in 2015 and 2016. The purification of the isolated fungus was carried out by using hyphal tip technique as described by Dhingra and Sinclair (1985) [5]. 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 3, 5 and 7 days after inoculation while number of sclerotia and size of sclerotia of each isolates were recorded 10 to 15 days after inoculation. Fresh mycelial weight was taken after 10 days growth of isolates grown at 22 ± 2°C on potato dextrose broth (PDB) medium.

To generate deferential RAPD profiles of five fungal isolates (Fig.- 1), genomic DNA was isolated by using chromus genomic DNA isolation kit (RKN11/12). Each of the DNA fragments in a single peak when run under denaturing conditions. With the 5th dye LIZ marker fragments can be labeled with the dyes FAM, VIC, NED or PET. Each kit contains enough standard for 800 analyses. The quality of DNA validated on 1% agarose gel. PCR amplification was performed by using florescent labeled RAPD primers; (Primer-1: 5'-CCCHGCAMCTGMTCGCACHC-3': Primers 2- 5'- AGGHCTCGATAHCM GBY-3': Primer-3: 5'-MTGTAMGCTCCTGGGGATTCHC-3'). Genetic analysis of flourescent labeled PCR products were conducted on ABIC3130 genetic analyzer. Isolated genomic DNA was amplified by PCR using following conditions (Table 1), PCR cycle conditions (Table 2) and Kit (Table 3) was used for RAPD perform once. A binary matrix was compiled using numerical system of multivariate analysis. The dendogram was constructed by the unweighted paired group method of arithmatic average (UPGMA) based on Jaccard's similarity coefficient with SHAN program of NT-sys, (Jaccard, 1912) [6].

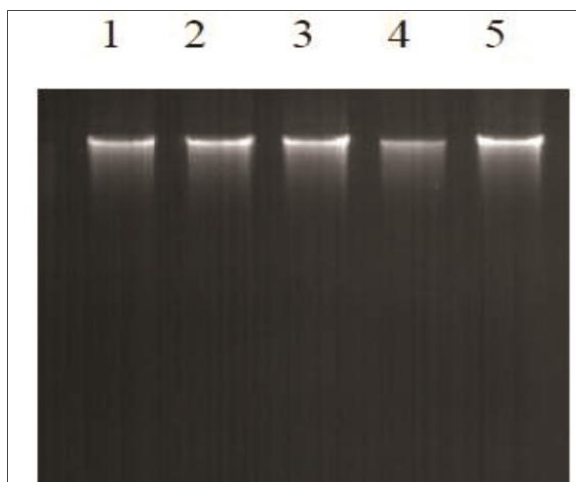


Fig.-1 : Genomic DNA extracted from fungal Isolates loaded on 1% agarose gel
Isolate1- Bhind
Isolate 2- Morena
Isolate 3- Gwalior
Isolate 4- Datia
Isolate 5- Sheopur

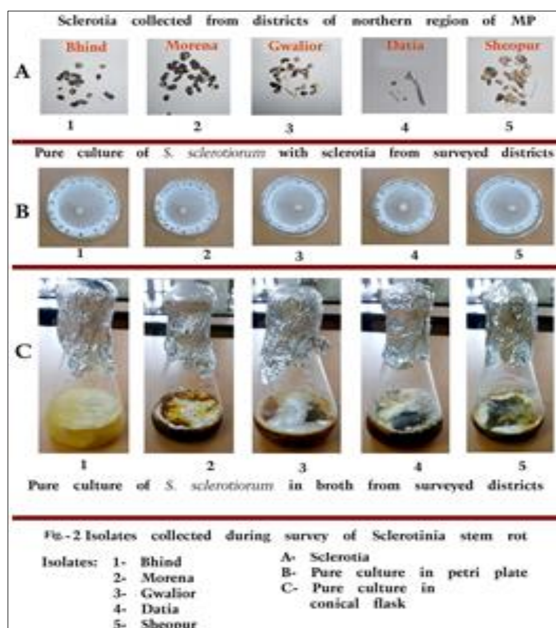
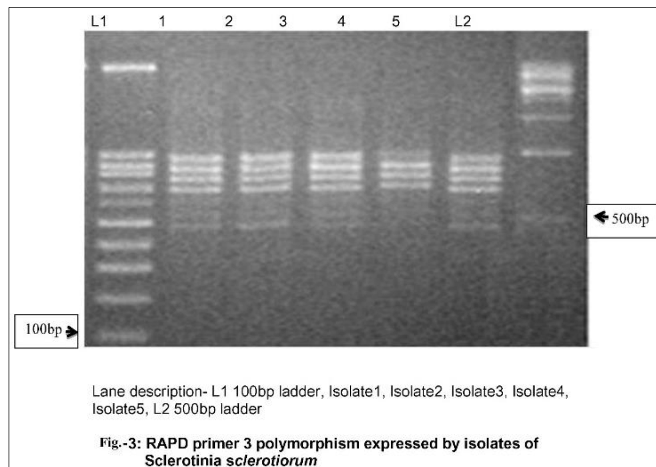


Fig-2 Isolates collected during survey of Sclerotinia stem rot



Lane description- L1 100bp ladder, Isolate1, Isolate2, Isolate3, Isolate4, Isolate5, L2 500bp ladder

Fig.-3: RAPD primer 3 polymorphism expressed by isolates of Sclerotinia *sclerotiorum*

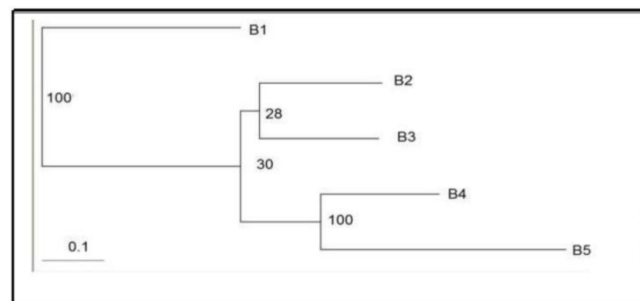


Fig.- 4: Dendogram showing grouping of *Sclerotinia sclerotiorum* based on RAPD polymorphism

Table 1: PCR Reaction conditions.

gDNA (50ng)	1.0 µl
RAPD primer (100ng/ µl)	2.0 µl
dNTPs (10mM)	1.0 µl
Taq Pol Assay buffer (10X)	2.5 µl
Taq DNA Polymerase (3U/µl)	0.5 µl
Water	18.0 µl
Total:	25 µl

Table 2: PCR Cycle conditions.

94 °C	94 °C	40 °C	72 °C	72 °C
5 min	1 min	1 min	2 min	5 min
	40 Cycles			

Results and Discussion

The data results revealed that the mycelial growth rate among the isolates differed considerably. The pathogen is widely spread and well established in the different geographical regions. The isolates of *S. sclerotiorum* based on their growth behaviour and morphological characters may be differed due to environmental factors. Based on radial growth, the isolates were classified into three groups on the basis of days taken to fill plate (90mm) *viz.,* (7 Days) very fast, (8 Days) intermediate and (9 and above) slow growing (Table-4 and Fig. -2). The data were taken after 7 days after incubations revealed that the isolates B2 (Isolate-2) represented significantly fast growing which were taken 7 days, while, isolates B1 (Isolate-1) and B3 (Isolate-3) were intermediate which were taken 8.5 days, but B4 (Isolate-4) showed slow radial mycelial growth which was taken 9 days taken to fill plate respectively. The mycelial and sclerotia colour was whitish and light Black, respectively in all isolates, while the sclerotia shape was round. Among five isolates, maximum isolates showed whitish mycelial growth with smooth texture. While, the sclerotia colour did not differ in all the isolates and

the shape of the sclerotia was mostly round. Fresh mycelial mat weight of different isolates significantly varied.

On the basis of number of sclerotia produced and weight of sclerotia per plate by the isolates of *S. Sclerotiorum.* The Isolate-2 produced maximum (31g and 1024.50mg) number of sclerotia and weight of sclerotia per plate followed by Isolate-3 (14g and 622.25mg), Isolate-5 (13g and 570.75mg) and Isolate-4 (12.50g and 568.5mg) recorded, respectively. The Isolate-1 (12g and 350.25 mg) showed least number of sclerotial formations and weight of sclerotia per plate. Majority of isolates produced sclerotia in 15 days after inoculation, however in isolate Isolate-2 sclerotia appeared on 11 days after inoculation. During the study, bigger sizes of sclerotia were observed in the same morphological group and genetic cluster. Earlier, Carpenter *et al.* (1999) [2] the variability among the isolates has already been reported. This findings supported by Dhingra and Sinclair, (1973) [5] and Mirza *et al*., (1985) [8] based on sclerotial diameter, several workers recorded variation in size of sclerotia among different isolates of the fungus. This finding closely related with Sharma *et al.* (2013) [10] the 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.

The genomic DNA for different fungal isolates extract and custom processed the sample for RAPD analysis. The DNA of *S. sclerotiorum* was amplified with 3 fluorescent primer sets and used for PCR condition for RAPD analysis and observed significant discrimination among different fungal isolates (Fig.- 3). The distance matrix analysis (Table-5) was drawn phylogenetic tree by PCR data showed genetic distance among the fungal isolates. The phylogenetic tree was clearly showed genetically variation in different isolates. The reference fungal isolate was marked as Isolate-1 among all the other isolate. The value of Isolate-1 differs was indicated as 100 per cent. The genetic distance showed for Isolate-2 was 70% and Isolate-3 was 72 per cent. Further Isolate-4 and Isolate -5 were 100 per cent similar to Isolate -2 and Isolate -4 and 70 and 72per cent genetic distance for Isolate-1(Fig. – 4).

The Gene Scan 500 LIZ size standard is a fifth dye-labeled size standard for the reproducible sizing of fragment analysis data. This standard size was used for fragments between 35 and 500 bp. The standard contains 16 LIZ dye-labeled, single-stranded DNA fragments. By using of the three florescent decamer primers to amplify genomic DNA from 5 isolates of the *S. sclerotiorum* species a total of 251 fractionated fragments were reported polymorphic that contained ranging in size from 51.43 to 499.56 nucleotides which were provided 16 single-stranded labeled fragments of 160.253, 249.523, 244.118, 234.958, 229.155, 241.897, 227.875, 183.539, 237.35, 135.339, 253.014, 240.998, 140.384, 159.045, 153.171 and 243.994 bp nucleotides. As an average, reproducible amplified products were generated by single primer (minimum of 5 and maximum of 41 fragments) 19 haploid groups detected among 5 isolates with 340 alleles polymorphism like polymorphic loci containing 2–10 alleles at each locus. The sizing curve was generated from these fragments for a variety of fragment analysis of fragment length, polymorphisms and relative fluorescent quantitation. The RAPD bands segregation was estimated by theoretical ratio of segregation using $\chi 2$ (qui square) analysis. The morphological variations were strongly correlated to genotypic variations. The pathogen is widely spread and well established in the different districts geographical regions of northern Madhya Pradesh. Therefore it dealt with the relatedness among five *S. sclerotiorum* isolates as representative samples in different districts of northern Madhya Pradesh. This finding closely related with Sharma *et al.* (2013) [10] the 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. Sun *et al*. (2005) [11] compared *S. sclerotiorum* 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) [12], who attempted to unlock the polymorphism of *S. sclerotiorum* isolated from sunflower in China. Ziqin *et al*. (2009) [14] 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) [1].

Table 3: Kit components.

Component	Concentration
Fungal gDNA Suspension Buffer	5 X
Fungal gDNA Lysis Buffer	1 X
Wash Buffer	5 X
Elution Buffer	1 X
Spin Column	-
Collection Tube	-

Table 4: Morphological characteristics of isolates of *S. sclerotinia*

District	Days to fill plate radial growth in mm	Sclerotia			Colour	Shape of sclerotia
		No. per plate	Size (Avg. dia.) (mm)	Mycelial	sclerotia	
Bhind (Isolate-1)	8.50	12.00	4.75	whitish	Light Black	Round
Morena (Isolate -2)	7.00	31.00	3.75	whitish	Light Black	Round
Gwalior (Isolate -3)	8.50	14.00	3.00	whitish	Light Black	Round
Datia (Isolate -4)	9.00	12.50	3.00	whitish	Light Black	Round
Sheopur (Isolate -5)	7.00	13.00	3.50	whitish	Light Black	Round
CV	9.12	6.45	23.24			
CD 5%	1.10	1.60	NS			

Table 5: Similarity Distance Matrix

	B1	B2	B3	B4	B5
BI		0.57517	0.51876	0.66361	0.82469
B2	0.57517		0.39017	0.49040	0.76151
B3	0.51876	0.39017		0.58132	0.76426
B4	0.66361	0.49040	0.58132		0.58722
B5	0.82469	0.76151	0.76426	0.58722	

Conclusion

The present investigation was concluded that the morphological, cultural and pathological was reported distinguish and the diversity in *S. sclerotiorum* was present in northern region of Madhya Pradesh. The intermediate growing behavior isolates were reported late infection rather than fast growing behavior isolates in mustard crop.

Acknowledgements

Author gratefully acknowledges the financial support of the Rajiv Gandhi National Fellowship under UGC. I also acknowledge the Chromous biotech for performing RAPD for generation information.

References

1. Bowden RL, Leslie JF. Sexual recombination in *Gibberella zeae*. Phytopathology. 1999; 89:182-188.
2. Carpenter MA, Frampton C, Stewart A. Genetic variation in New Zealand population of pathogen *Sclerotinia sclerotiorum*. New Zealand J Crop Hort. 1999; 27:13-21.
3. Dhingra OD, Sinclair JB. Variation among isolates of *Macrophomina phaseolina* (*Rhizoctonia bataticola*) from different regions. Phytopathology. 1973; 76:200-204.
4. Harlton CE, Levesque CA, Punja ZK. Genetic diversity in *Sclerotium (Athelia) rolfsii* and related species. Phytopathology. 1995; 85:1269-1281.
5. Dhingra, OD, Sinclair JB. Basic Plant Pathology Methods. CRC Press, Inc. Boca Raton, Florida. 1985, 132-163.
6. Jaccard P. The distribution of the flora in the alpine zone, New Phytologist. 1912; 11:37-50.
7. Mehta N, Hieu NT, Sangwan MS. Efficacy of botanicals against *Sclerotinia sclerotiorum* inciting white stem rot of rapeseed-mustard. Pl. Dis. Res. 2010; 26(1):82-86.
8. Mirza MS, Ahmad Yand Beg A. Sclerotinia stalk rot of sunflower. Pak. J Agric. Res. 1985; 6:286-288.
9. Okabe I, Morikawa C, Matsumoto N, Yokoyama K. Variation *Sclerotinia rolfsii* isolate in Japan. Mycoscience. 1998; 39:399-407.
10. Sharma Pankaj, Meena PD, Kumar Sandeep, Chauhan JS. Genetic diversity and morphological variability of *Sclerotinia sclerotiorum* isolates of oilseed Brassica in India. Afr. J. Microbiol. Res. 2013; 7(18):1827-1833.
11. Sun J, Irzykowaski W, Jedryczka M, Han F. Analysis of genetic structure of *Sclerotinia sclerotiorum* population from different region and host plant by RAPD markers. J Int. Plant Biol. 2005; 47:385-395.
12. Yu X, Wang G, Zhang N. The polymorphism of nuclear DNA of *Sclerotinia sclerotiorum* from different area of our country. J Hebei. N. Univ. 2006; 122:24-26.
13. Zhao J, Meng J. Genetic analysis of loci associated with partial resistance to *Sclerotinia sclerotiorum* in rapeseed (*Brassica napus* L.). Theor. Appl. Genet. 2003; 106:759-764.
14. Ziqin L, Wang Y, Chen Y, Zhang JW, Fernando D. Genetic diversity and differentiation of *Sclerotinia sclerotiorum* population of sunflower. Phytoparasitica. 2009; 37:77-85.