

Identification of Molecular Marker for Detection of *Sclerotinia sclerotiorum* Causing Stem Rot of Chickpea and Other Host

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Abstract The *Sclerotinia sclerotiorum* is a soil-inhabiting plant pathogen. It is considered as one of the most devastating plant pathogens infecting various crop plants including chickpea. PCR assays of *S. sclerotiorum* and other fungi species was carried out using 21 random primers. In our present study we found that OPA-8, OPA-20 and OPA-16 primers produced almost identical genetic fingerprints in all 24 isolates of *S. sclerotiorum*. Two bands of same sizes~1.65 to 1.70 kb were present in the fingerprint generated by primer OPA-8 amplified only in DNA from *S. sclerotiorum* and not from any of the other species tested like *Rhizoctonia bataticola*, *R. solani* and *Fusarium oxysporum* f. sp. *ciceris*. The fingerprint of primer OPC-3 was able to differentiate in between non-sclerotia forming and sclerotia forming isolates through presence of ~ 1.9 kb band in sclerotia forming isolates. Therefore, this marker may be tagged with factor responsible for sclerotia formation in *S. sclerotiorum*. Similarly, the RAPD primer OPA-8 could be used for identification of *S.*

sclerotiorum as it produced~1.5 kb band in *S. sclerotiorum* isolates only.

Keywords Chickpea, *Sclerotinia sclerotiorum*, RAPD, Molecular marker, Stem rot.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is the most cosmopolitan and versatile pathogen and it infects more than 400 species of plants, including stem rot of chickpea (*Cicer arietinum* L.). During 1986, its occurrence was reported in Himachal Pradesh with considerable yield losses of chickpea. In India, chickpea (*Cicer arietinum* L.) is prone to many diseases, amongst them stem rot caused by *S. sclerotiorum* is one of the most devastating diseases and causes significant yield loss in cool and moist areas. The disease causes total crop failure where chickpea is grown in the same paddock in successive years. Dense crops are likely to be the most severely affected by the disease, particularly under moist condition. It is also frequently found to be associated with the root rot complex in chickpea. The extent of damage ranged from 21.3% to 46.7% at pod bearing stage of the crop. Besides chickpea, it also causes damage to vegetables and oilseed crops. *S. sclerotiorum* is a cosmopolitan, homothallic and necrotrophic, ascomycetous fungus dispersed by air borne ascospores or soil borne sclerotia. Concerns *S. sclerotiorum* are also justified because it is a necrotrophic pathogen with a very wide host range and could prevent the growing of crops in areas for periods of until 10 years.

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Table 1. List of isolates of *Sclerotinia sclerotiorum* collected from different states of India.

Isolates number	Location	State	Host
SS1	IARI, New Delhi	Delhi	Chickpea
SS2	IARI, New Delhi	Delhi	Chickpea
SS3	IARI, New Delhi	Delhi	Pea
SS4	ITCC (6094), New Delhi	Delhi	Pea
SS5	ITCC (4042), New Delhi	Delhi	Mustard
SS6	Delhi University, New Delhi	Delhi	Mustard
SS7	Ureeka, Jhunjhun	Rajasthan	Chickpea
SS8	ARS, Sriganaganagar	Rajasthan	Chickpea
SS9	Jhunjhun	Rajasthan	Mustard
SS10	Muktsar	Punjab	Chickpea
SS11	Gidderanwali, Firozpur	Punjab	Chickpea
SS12	BHU, Varanasi	Uttar Pradesh	Chickpea
SS13	Bhagwanpur, Varanasi	Uttar Pradesh	Rajma
SS14	Aligarh	Uttar Pradesh	Menthool
SS15	Dholaquan	Himachal Pradesh	Chickpea
SS16	Ranichauri	Uttarakhand	Chickpea
SS17	Ranichauri	Uttarakhand	Pea
SS18	Ranichauri	Uttarakhand	Pea
SS19	Pantnagar	Uttarakhand	Mustard
SS20	Hisar	Haryana	Coriander
SS21	Hisar	Haryana	Chickpea
SS22	Srinagar	Jammu & Kashmir	Chickpea
SS23	Samastipur	Bihar	Chickpea
SS24	Coochbehar	West Bengal	Tobacco

S. sclerotiorum survives in the soil as sclerotia, which are produced in infected tissue. Ascospores produced by carpogenic germination of sclerotia are the major source of inoculum for infection by *S. sclerotiorum*. Infection occurs when ascospores germinate on nonliving or senescent plant parts and then infect healthy plant tissue. Traditional methods of detection of airborne inoculum are time-consuming, labor intensive and subjective since they rely on identification of spores by microscopy or culture-based techniques. Ascospores of *S. sclerotiorum* are small, non-descript and similar to spores of a number of other fungal species and therefore are extremely difficult to identify morphologically with certainty.

Molecular methods based on the technique of Polymerase Chain Reaction (PCR) have been investigated for the detection of different pathogens whose morphological characteristics are very similar [1, 2]. Recently, several molecular based detection assay of *S. sclerotiorum* were developed for soybean seeds [3] ; canola [4]. A marker for differentiation of *S. sclerotiorum* and *S. trifoliorum* using comparative

transcriptome analysis and RNA sequencing [5]. Highly benzimidazole-resistant isolates of *S. sclerotiorum* was identified by molecular marker developed using loop-mediated isothermal amplification [6]. These techniques show promise for this purpose, since they have high sensitivity, specificity, rapid, reliable methods and they have the advantage of evaluating a large number of samples in a short period time. It provides an accurate assessment in pathogens that cannot be cultured and most importantly in earlier stages of the disease development [7]. Ascospores of *S. sclerotiorum* are small, non-descript and similar to spores of a number of other fungal species and therefore are extremely difficult to identify morphologically with certainty. Therefore, the present investigation was undertaken to develop a PCR based easy sophisticated detection protocol for *S. sclerotiorum*.

Materials and Methods

Fungal cultures

Diseased specimens of chickpea as well as other

Table 2. Analysis of polymorphism obtained with random primers in isolates of *Sclerotinia sclerotiorum*. Figures in parentheses are percentage of polymorphic bands.

Sl. No.	Name	Sequence (5'-3')	Total No. of band	Polymorphic band
1	OPA-02	TGCCGAGCTG	5	1 (20.00)
2	OPA-03	AGTCAGCCAC	5	5 (100.00)
3	OPA-08	GTGACGTAGG	2	0 (0.00)
4	OPA-10	GTGATCGCAG	5	2 (40.00)
5	OPA-18	AGGTGACCGT	7	1 (14.29)
6	OPA-20	GTTGCGATCC	4	2 (50.00)
7	OPC-03	GGGGGTCTTT	6	4 (66.67)
8	OPC-04	CCGCATCTAC	7	7 (100.00)
9	OPC-15	GACGGATCAG	6	4 (66.67)
10	OPC-16	CACACTCCAG	4	2 (50.00)
11	OPF-10	GGAAGCTTGG	3	1 (33.33)
12	OPF-16	GGAGTACTGG	5	2 (40.00)
13	OPI-9	TGGAGAGCAG	4	0 (0.00)
14	OPL-07	AGGCGGGAAC	6	0 (0.00)
15	OPL-08	AGCAGGTGGA	4	0 (0.00)
16	OPL-14	GTGACAGGCT	6	2 (33.33)
17	OPL-15	AAGAGAGGGG	9	5 (55.56)
18	OPM-6	CTGGGCAACT	7	5 (71.43)
19	OPM-20	AGGTCTTGGG	5	3 (60.00)
20	OPM-4	GACCGACCCA	4	2 (50.00)
21	OPM-20	GGTGCTCCGT	5	5 (100.00)
Total			109	53 (48.62)

crops were collected from different states of India for isolation *S. sclerotiorum* isolates. Twenty four isolates including 12 isolates were from chickpea and 12 were purified by single sclerotium or hyphal tip method (Table 1). The isolates were identified as on the basis of mycelial and sclerotial characters. All the isolates were proved to be pathogenic on chickpea cultivar BDG 1005 under artificial inoculated condition. The isolates were maintained in potato dextrose agar (PDA) slant at 4°C for further studies.

DNA extraction

Single sclerotium culture of *S. sclerotiorum* isolates were inoculated in 100 ml potato dextrose broth in the 250 ml flasks and incubated at 24 ± 1°C in shaking incubator for 7 days. Mycelial mats were harvested by filtering through Whatman filter Paper No. 1, washed repeatedly with distilled sterilized water, blot dried and stored in Al-foils at -80°C for genomic DNA extraction. DNA extraction was based on the cetrinide tetradecyl trimethyl ammonium bromide (CTAB) method. After quantification of DNA, working solutions with 25 ng 11-1 were prepared for each isolate.

Random amplified polymorphic DNA (RAPD) analysis

DNA of different isolates was amplified by RAPD method using 10-mer arbitrary oli gonucleotide primers (Table 2) obtained from Operon Technology, USA. The reaction was performed in 25µl volume containing DNA template 75 ng, *Taq* DNA polymerase 1 U, primer 10 pmol (10 mer primer), MgCl₂ 2.5 mM, dNTPs mix 0.6 mM (10 mM–2.5 mM each of dATP, dCTP, dGTP, dTTP) and 1 × PCR Mg free buffers. Nuclease free water was used to bring the reaction volume to 25 µl. The DNA amplification was performed by using Mastercycler™ epgradient thermal cycler (Eppendorf AG, Germany) and PCR conditions were consisted of 5 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 35°C, and 2 min at 72°C with a final extension for 5 min at 72°C. Amplified fragment were resolved in 1.2% agarose gels in 1×TAE electrophoresis buffer, stained with ethidium bromide along with 1 kb DNA ladder (Fermentas Life Sciences, Canada) as standard molecular weight marker. The electrophoresis was carried out at a constant voltage of 70 V for 1.5 h and

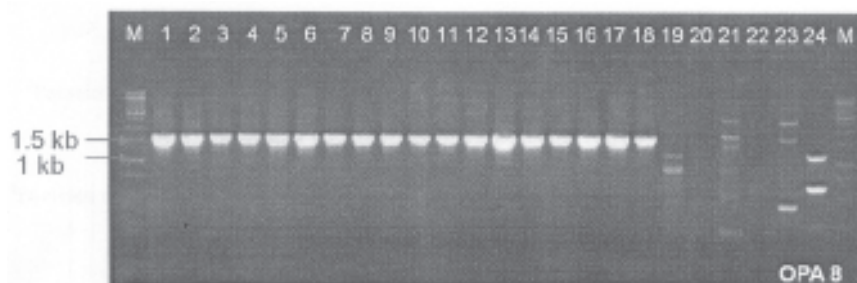


Fig. 1. RAPD profile generated by OPA 8 primer for *Sclerotinia sclerotiorum* and other fungi. Delhi (SS1–SS6) ; lane 7–9 : Rajasthan (SS7–SS9) ; lane 10–11 : Punjab (SS10–SS11) ; lane 12–14 : Uttar Pradesh (SS12–SS14) ; lane 15–18 : Uttarakhand (SS15–SS18) isolates of *Sclerotinia sclerotiorum* ; lane 19 and lane 20 : isolates of *Rhizoctonia bataticola* ; lane 21 and lane 22 : isolate of *Fusarium oxysporum* f. sp. *ciceri* ; lane 23 and lane 24 : isolate of *R. solani* and M = 1 kb Marker.

visualized using a digital gel documentation system (Bio Rad Gel Doc system). Phylogenetic tree was constructed based on the maximum nucleotide sequence similarity by using CLUSTALW 1.8 sequence alignment selecting Bootstrap neighbor-joining by MEGA 4.1 program.

Results and Discussion

In our present study we also found that OPA-8 primers produced the identical genetic fingerprints in all 24 isolates of *S. sclerotiorum*. But OPA-8 produced different genetic fingerprints in case of different isolates of *Rhizoctonia bataticola*, *R. solani* and *Fusarium oxysporum* f. sp. *ciceris*. It indicates that Indian isolates of *S. sclerotiorum* is more genetic relatedness as compare to other fungi like *Rhizoctonia bataticola*, *R. solani* and *Fusarium oxysporum* f. sp. *ciceris*. Several molecular methods such as amplified fragment length polymorphism random amplified fragment length polymorphism [8] ; microsatellite marker [9] ; sequence-related amplified polymorphism (SRAP) tech nique [10] were used to reveal genetic polymorphism of *S. sclerotiorum* isolated from different hosts. The isolates of *S. sclerotiorum* isolated from canola were found to be homogenous with limited variability in respect of 18S and 28S r DNA regions as well as internal transcribed spacers (ITS) sequence [11].

Table 3. Specificity tests on the *S. sclerotiorum*-specific PCR assay. DNA purified from a number of *S. sclerotiorum* isolates and other fungi was tested in PCR assay with the consensus fungal primers ITS 4/5 and the *S. sclerotiorum*-specific primers OPA-8.

Species (Isolate)	Assay	
	ITS1/ ITS 4 PCR	OPA-8 PCR
<i>S. sclerotiorum</i> (SS1)	+	+
<i>S. sclerotiorum</i> (SS2)	+	+
<i>S. sclerotiorum</i> (SS3)	+	+
<i>S. sclerotiorum</i> (SS4)	+	+
<i>S. sclerotiorum</i> (SS5)	+	+
<i>S. sclerotiorum</i> (SS6)	+	+
<i>S. sclerotiorum</i> (SS7)	+	+
<i>S. sclerotiorum</i> (SS8)	+	+
<i>S. sclerotiorum</i> (SS9)	+	+
<i>S. sclerotiorum</i> (SS10)	+	+
<i>S. sclerotiorum</i> (SS11)	+	+
<i>S. sclerotiorum</i> (SS12)	+	+
<i>S. sclerotiorum</i> (SS13)	+	+
<i>S. sclerotiorum</i> (SS14)	+	+
<i>S. sclerotiorum</i> (SS15)	+	+
<i>S. sclerotiorum</i> (SS16)	+	+
<i>S. sclerotiorum</i> (SS17)	+	+
<i>S. sclerotiorum</i> (SS18)	+	+
<i>Rhizoctonia bataticola</i>	+	–
<i>Rhizoctonia bataticola</i>	+	–
<i>Fusarium oxysporum</i> f. sp. <i>ciceri</i>	+	–
<i>Fusarium oxysporum</i> f. sp. <i>ciceri</i>	+	–
<i>R. solani</i>	+	–
<i>R. solani</i>	+	–

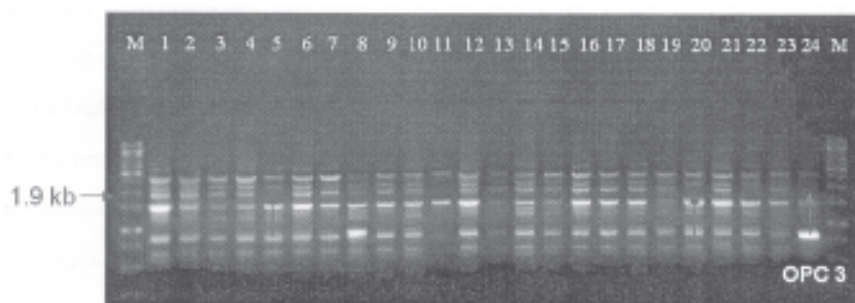


Fig. 2. RAPD profile generated by OPC 3 primer. Lane 1—6 : Delhi (SS1–SS6) ; lane 7–9 : Rajasthan (SS7–SS9) ; lane 10–11: Punjab (SS10–SS11) ; lane 12–14 : Uttar Pradesh (SS12–SS14) ; lane 15–19 : Uttarakhand (SS15–SS19) ; lane 20 —21 : Haryana (SS20–SS21) ; lane 22 : Jammu & Kashmir (SS22) ; lane 23 : Bihar (SS23) and lane 24 : West Bengal (SS24) isolate of *Sclerotinia sclerotiorum* and M = 1 kb Marker.

PCR assays using consensus fungal primers (ITS1/ITS4) and *S. sclerotiorum*-specific primers (OPA-8) were carried out using DNA of a range of different fungal species. DNA from all of the tested species was amplified using the consensus fungal primers (ITS1 / ITS4) and primers OPA-8 (Table 3). But two bands of same sizes ~1.65 to 1.70 kb were present in the fingerprint generated by primer OPA-8 amplified only in DNA from *S. sclerotiorum*. Further, these bands were absent in the other soil borne plant pathogenic fungi like *Rhizoctonia bataticola*, *R. solani* and *Fusarium oxysporum* f. sp. *ciceris* (Fig. 1). The fingerprint of primer OPC-3 was able to differentiate between non-sclerotia forming and sclerotia forming isolates through presence of ~1.9 kb band in sclerotia forming isolates (Fig. 2). Therefore, this marker may be tagged with factor responsible for sclerotia formation in *S. sclerotiorum*. Similarly, the RAPD primer OPA-8 could be used for identification of *S. sclerotiorum* as it produced ~1.5 kb band in *S. sclerotiorum* isolates only. *S. sclerotiorum* specific molecular marker SSFWD5'GCTGCTCTTTCGGGG-CCTTGTA3' and SSREV5'TGACATGCACTCAATACCAAGCTG3' was developed by J. Freeman which amplify a fragment with 278 base pairs but since primer has larger base pair, it is more difficult for standardization and amplification. In present study we identified the smaller primer OPA-8 which is very easy for standardization and amplification.

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