Plant Pathol. J. 21(2) : 106-110 (2005)

The Plant Pathology Journal

Relatedness Among Indiginous Members of *Sclerotinia sclerotiorum* by Mycelial Compatibility and RAPD Analysis in the Jordan Valley

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(Received on January 14, 2005; Accepted on February 14, 2005)

***Sclerotinia sclerotiorum* attacks most of the vegetable crops in the Jordan valley. Twenty-five samples/isolates were obtained in a complete coverage of that region. They were characterized for their mycelium incompatibility, and specific gene amplified using the primer SSREV/SSFWD. All isolates gave similar single band around 278 bp. Thirteen isolates were completely incompatible with the other 12 ones. The latter ones fell into four subgroups of mycelium incompatibility. RAPD analysis using three primers (OPA-2, OPA-10, and OPA-18) clustered the 25 isolates into subgroups in agreement with their morphological separation, indicating close correlation between amplified gene(s) and the gene(s) of incompatibility. All highly virulent isolates were among the group of 13, indicating a well established genomic type pathogen in this region.**

Keywords : cottony rot, *Cucurbita pipo*, Jordan valley, mycelial compatibility, RAPD assay, *Sclerotinia sclerotiorum*

Cottony rot is caused by *Sclerotinia sclerotiorum* and is responsible for losses in vegetable crops in the Jordan valley. The characterization, identification, and depicting genetic variability of *S. sclerotiorum* under natural field population were accomplished world wide on the bases of their mycelial and sclerotial morphology (Cubita et al., 1997; Morall et al., 1972; Purdy, 1955), isozyme properties (Deena and Kohn, 1994), and mycelial compatibility (Cubita et al., 1997; Freeman et al., 2002; Kohn et al., 1991; Wong and Willetts, 1975).

It is well known that ascospores are the most significant infection entities of this pathogen (Mufadi, 1981). Therefore high genetic variation is anticipated in the existing and future population of *S. sclerotiorum* (Cubita et al., 1997). Such variation may be associated with virulence, formation and germination ability of sclerotia and reaction to fungicide or the ability to resist biological control agents. Therefore the combination of mycelial compatibility and molecular techniques offers powerful tools for understanding this heterogeneity (Kohn, 1990). The present study aims to determining the extent of genetic variation among isolates of *S. sclerotiorum* by mycelial compatibility and RAPD assay.

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Materials and Methods

***Sclerotinia sclerotiorum* isolates.** Twenty-five isolates of *S. sclerotiorum* were collected from infected vegetable crops in the districts separated by a distances of more than 30 km in the Jordan valley in growing seasons of the years 2002 to 2003. For isolation, a single sclerotium or infected host tissue surface-sterilized by dipping in 5% sodium hypochlorite soln. for 5 min, followed by 70% ethanol for 2 min, and then rinsed three times with sterile distilled water, with dry out blottered for 3 min. was aseptically transferred into potato dextrose agar (PDA) plates. The plates were incubated at 24°C, and then produced sclerotia were stored in labeled 5ml screw cap glass tubes at 4°C.

Mycelial compatibility. Mycelial compatibility between all isolates was done as described by Kohn et al. (1991), in which all isolates were paired in combination including self–self pairing. Mycelial plugs (2 mm in diameter) of each isolate were cut with the aid of sterile cork borer at the growing margin of 5 days old culture plates at 24°C and transferred to the opposite ends of PDA plate (6 cm apart). The compatibility/incompatibility was scored 7 days after incubation at 24°C according to the criteria adopted by Kohn et al. (1990), and Cubita et al. (1997). Incompatibility was recognized as minor when the reaction line of the paired isolates was characterized by hyphal tufts on the colony surface, and more pronounced when there is a demarcation line on the colony reveres and the presence of discontinuity or lysis of the hyphal tips. On the other hand, complete compatibility was characterized by completely overlapping growth of both mycelium and then growing in one colony. Different degrees of incompatibility were weighed on an arbitrary scale of 0-3 where 0 means no incompatibility (or complete compatibility) and 3 means

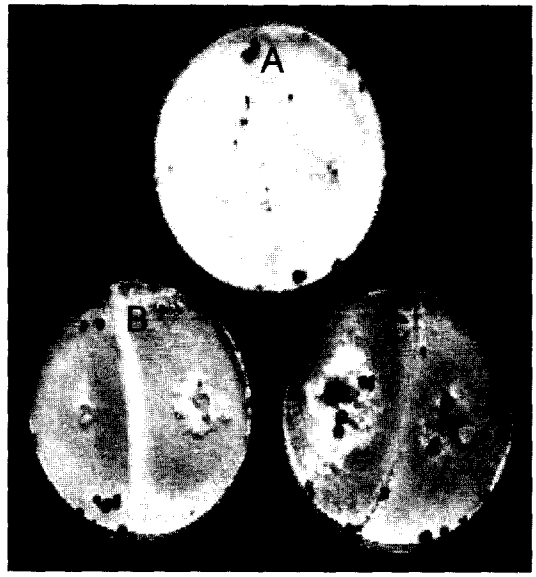

Fig. 1. Representative 3 types of mycelial compatibility of *Sclerotinia sclerotiorum*. Panels A-C represent complete compatible (level 0), intermediate incompatibility (levels 1-2), and incompatibility (level 3), respectively.

complete incompatibility (no compatibility) and documented by photography (Fig. 1).

DNA Extraction. For DNA extraction, fungal mycelia were obtained from 5-days old stationary cultures of *S. sclerotiorum* grown on PDB medium at 24°C. Mycelium mats were collected by filtration and approximately 30 mg (wet weight) was processed for genomic DNA extraction following the protocol of yeast genomic DNA extraction with one exception of using liquid nitrogen through freezing and thawing process instead of lyticase enzyme to disrupt the cell wall of fungal hyphae (Wizard genomic DNA purification kit, Promega, U.S.A). The extracted DNA was adjusted to 10 ng/μl before amplification with either specific primers or RAPD primers.

Specific PCR based assay. The isolates were identified at the molecular level by using a specific primer set previously described by Freeman et al. (2002). A set of primers (SSFWD 5'-GCTGCTCTTCGGGGCCTTGTATGC-3' and SSREV 5'-TGACATGGACTCTCAATACCAAGCTC-3') which amplifies a segment of 278bp in the region between 18S and 28S rDNA was used to identify *S. sclerotiorum*.

The reaction mixture was prepared in 25 μl volume as described by Freeman et al. (2002) containing the following compounds, 1 μl of each primer (5 pmols), 0.5 μl of Taq DNA polymerase, 2.5 μl of 10X PCR Mg free buffer, 0.5 μl of 10 mM dNTPs mix, 1 μl of 50 mM $MgCl_2$, 2.5 μl of DNA template (25 ng), and nuclease free water was used to bring the reaction volume to 25 μl. A negative control in which nuclease free water was added instead of DNA was used for the specific PCR. A touch-down PCR program was used in the amplification with an annealing temperature range from 72 to 65°C decreasing by 1°C after each two cycles with 16 cycles at the minimum annealing temperature (65°C). Therefore, the cycling condition were as follows: 95°C for 1min initial denaturation; then a total of 30 cycles of denaturation at 94°C for 30 sec, annealing (as described above) for 1min, extension at 72°C for 1 min; and followed by final extension at 72°C for 10 min. Then, the tubes were held at 4°C for direct use, or stored at –20°C until needed.

RAPD analysis. RAPD-PCR was performed using three random primers of 10 nucleotides each obtained from Operon Technologies: OPA2, OPA10, and OPA18 using the manufacturer recommended protocol. The reaction was performed in 25 μl volume containing 2.5 μl of DNA template (25 ng), 2 μl of 10 pmol primer, 2.5 μl of 10 X PCR Mg free buffers, 1 μl of 50 mM $MgCl_2$, 0.5 μl Taq DNA polymerase, and 0.5 μl of 10 mM dNTPs mix. Nuclease free water was used to bring the reaction volume to 25 μl. The PCR program was set as follow: A hot start at 95°C for 3 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 33°C for 30 sec, extension at 72°C for 2 min, and final extension step at 72°C for 7 min. After amplification the tubes were held at 4°C for future use or stored at –20°C until needed. PCR products were separated on 2% w/v agarose gel in 0.5X TBE buffer and visualized using ethedium promide staining. The molecular weight of DNA bands was estimated using standard 100bp DNA ladder (Promega, USA), and documented by photography under Fotodyne U. V illuminator using 667 black and white film.

Results

Mycelial compatibility. The mycelial types from pairing of 25 isolates of *S. sclerotiorum* in all possible combinations are summarized in Table 1. These phenotypes of interaction are weighed in arbitrary scale 0-3 as 0 for completely compatible to 3 for completely incompatible.

Level scales are as follows: Level 0, incompatibility (100% compatible): when the two isolates intermingled freely and merged to form one colony, no distinct zone of incompatibility was visible where the mycelia came into contact (Fig. 1A). Level 1, incompatibility (not completely compatible): when sharp distinct thin band of mycelia was observed in the interaction zone (Fig. 1B); Level 2, incompatibility (not completely incompatible): reaction line was visible as abundant, tufts, white patches of aerial

Table 1. Pairing of the 25 isolates of *Sclerotinia sclerotiorum* in all combination testing their incompatibility

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Sst-1	0[a]	1	2	2	2	3	1	1	2	2	2	3	3	2	2	2	2	3	1	1	2	2	2	2	1
Sst-2		0	2	2	2	2	2	1	2	1	2	2	2	3	2	2	2	2	2	1	2	1	2	2	2
Sst-3			0	0	2	1	1	2	0	2	3	1	1	2	2	2	2	1	2	2	1	2	2	2	2
Sst-4				0	2	1	1	2	0	2	3	1	1	2	2	2	2	1	2	2	2	2	2	2	2
Ssb-5					0	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Ssc-6						0	2	2	1	2	2	0	0	3	2	1	2	0	2	2	1	2	2	2	2
Ssc-7							0	2	1	2	1	2	2	2	2	2	2	2	2	2	2	3	1	2	2
Ssc-8								0	2	2	1	2	2	2	2	2	2	2	2	0	2	2	2	2	2
Ssc-9									0	2	3	1	1	2	2	2	2	1	2	2	2	2	2	2	2
Ssc-10										0	2	2	2	2	2	2	1	2	1	2	2	2	1	1	2
Ssc-11											0	2	2	2	2	1	2	2	2	1	2	2	2	2	2
Sse-12												0	0	3	2	1	1	0	2	2	2	2	1	1	3
Sse-13													0	3	2	1	2	0	2	2	2	2	2	2	2
Sse-14														0	2	2	2	3	2	2	2	2	2	2	2
Sse-15															0	2	2	2	2	2	2	2	2	2	2
Sse-16																0	2	1	2	2	2	2	2	2	2
Sse-17																	0	2	2	2	2	2	0	0	2
Sse-18																		0	2	2	1	2	2	2	2
Sss-19																			0	2	2	2	2	2	2
Sss-20																				0	2	2	2	2	2
Sss-21																					0	2	2	2	2
Sss-22																						0	2	2	2
Sss-23																							0	0	2
Sss-24																								0	2
Sss-25																									0

[a] Their interaction was rated on a 0-3 scale where, 0 = completely compatible and 3 = completely incompatible, while 1 and 2 = degrees of incompatibility between those two extremes.

mycelium in the reaction zone on the colony surface; and Level 3, incompatibility (100% incompatible): when relatively clear zone, devoid of significant mycelia growth, separated one mycelium from the other, distinct band of hyphal lyses in the reaction zone (Fig. 1C).

Data revealed that the 25 isolates tested for incompatibility interaction involves 13 isolates completely incompatible with all other isolates. The remaining 12 isolates are grouped into four distinct groups with appreciable degrees of incompatibility among groups. But members within each sub-group are compatible among themselves only. All the isolates tested were self-compatible.

Identification of *S. sclerotiorum* by using specipic-PCR based assay. All the isolates were previously identified by conventional methods; also they were subjected to PCR assay as a selective method for identification and detection of *S. sclerotiorum* species. PCR was carried out with the specific primer pair SSFWD/SSREV, which amplifies a segment of 278bp in the region between 18S and 28S rDNA. This amplified segment of DNA appeared as one band in 2% agarose gel in the amplified DNA of all isolates Fig. 2 lane 2-19. No band was observed in the negative control in the PCR assay (Fig. 2, lane 1). Therefore the results of the specific PCR assay product revealed that all the isolates belong to *S. sclerotiorum* species.

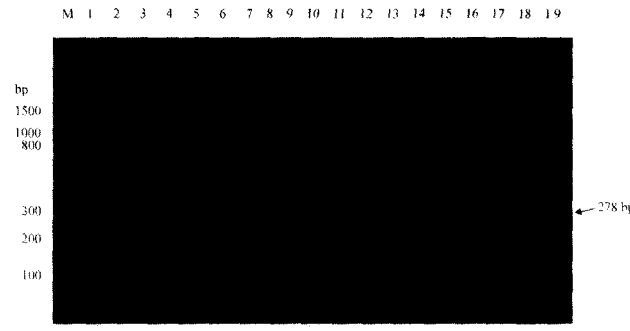

Fig. 2. 2% agarose gel of PCR products amplified using the SSFWD/SSREV specific primer set. Lane M: 100bp DNA ladder, Lane 1: Negative control, Lane 2-19: *Sclerotinia sclerotiorum* isolates.

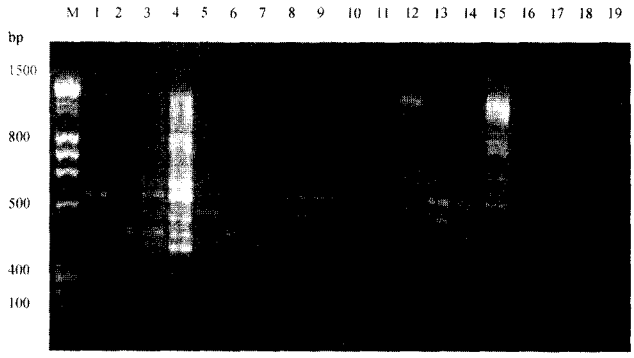

Fig. 3. Electrophoretic patterens showing amplification products generated from the *Sclerotinia sclerotiorum* isolates with primer OPA-2 on 2.0% agarose gel. M is 100bp molecular weight marker, Lane 1-19 *Sclerotinia sclerotiorum* of isolates.

RAPD analysis. The genomic DNA of the selected *S. sclerotiorum* isolates was amplified using three 10-mer random primers. The three primers produced a total of 148 polymorphic bands. Nineteen representative isolates were subjected to RAPD-PCR analysis (Fig. 3). The number of amplified products generated by each primer varied between one and 11 bands and the size of the product were within the range 110 to 1600bp. Both faint and distinct bands were considered in the scoring.

Based on Jaccard measurement, genetic similarity matrix was constructed using the RAPD data to assess the genetic relatedness among the isolates. Values of the similarity coefficient (Table 1) of RAPD data ranged from 0.18 between Ss-t4 and Ss-c11 to 0.87 between Ss-t2 and Ss-c10; Ss-c6 and Ss-c10. All evaluated isolates showed an overall mean of similarity of 0.45.

The dendrogram revealed that these isolates could be clustered into five major clusters with sub-clusters within each (data not shown). These major clusters are also divided further into sub clusters. For example the third main cluster is divided into 2 sub-clusters; first one included isolates Ss-e16 and Ss-s21 with 0.85 degree of similarity and the other sub-cluster included one isolate Ss-e17. These results also revealed that some isolates belonging to the same mycelium incompatibility group (ICG) fell into the same main cluster, such as the isolates Ss-c6, Ss-e13, Ss-e18 belonged to the ICG1 and fell in the first main cluster with similarity coefficient ranging from 0.6 to 0.82.

Discussion

The present investigation addressed the issue of the nature of *S. sclerotiorum* population in the northern Jordan valley. This pathogen is widely spread and well established in that area. Therefore it dealt with the relatedness among 25 *S. sclerotiorum* isolates as representative sample of the population of that pathogen. We expected the variation exist in population of *S. sclerotiorum* because the primary and most significant inoculum of this pathogen is the ascospores (Mufadi, 1981). This is substantiated here by the results of compatibility tests and the RAPD-PCR analysis. Using this combination allowed an efficient segregation among those isolates in a similar manner previously established by Tariqu et al. (1985). Phenotypic variations observed in this study in general were strongly correlated to genotypic variation. This could be due to genetic recombination during reproduction processes of *S. sclerotiorum* in field and these changes might have affected the phenotypic products in the progeny.

The fully mycelial compatibility reactions are probably due to a component of vegetative compatibility. However, that 13 isolates of the 25 total isolates were completely incompatible with the rest of the isolate strongly indicate a predominant factor(s) of incompatibility at the level of whole population of *S. sclerotiorum* in the Jordan valley. Furthermore those 13 isolates were not compatibile among them selves indicating some other secondary factors of incompatibility are existing. Mycelial compatibility groups are thought to be determined by the alleles at several loci in the genome. Therefore that the high level of incompatibility exists among the 25 isolates of *S. sclerotiorum* indicates that genetic heterogeneity exist within this species. However the genetic regulation of mycelial compatibility in *S. sclerotiorum* is still not known (Cubita et al., 1997). The genetic variation among the 25 isolates of *S. sclerotiorum* by the RAPD-PCR analysis was some consistent with patterns of mycelial compatibility. For instance, isolates Ss-c6, Ss-e13, Ss-e18 remained clustered close together in all means of segregation, indicating that those isolates are indeed closely related. However the isolates Ss-e17, Ss-s23, and Ss-s24 were compatible among themselves but they belonged to different genetic groups. Even though the RAPD-PCR analysis, proved to be useful to determine the extent of genetic diversity among *S. sclerotiorum*, but it is still a non-specific random detection of such phenomenon. Therefore the weak correlation between groups of incompatibility, and phenotypes with those presented from RAPD analysis may be due to the mechanisms of RAPD-PCR technique. RAPD markers are distributed throughout the genome. The 10bp primer can bind to any region in the genome that contains matching sequence. However the combination of molecular criteria, RAPD analysis and mycelium compatibility/incompatibility provided powerful understanding of the genetic heterogeneity in the *S. sclerotiorum* population under the Jordan valley condition. Furthermore, grouping of the 19 isolates of *S. sclerotiorum*, 13 isolates incompatible with all other isolates, yet they are

not compatible among themselves, plus 4 other compatibility groups is closely correlated to their genome. However this grouping is not correlated to their distribution and original locations of collection or even, to host plant associated with. This comes in full agreement with the description of this pathogen as omnivorous and not specific pathogen with a wide host rang and ecological distribution.

Successful application of specific PCR-based assay in identification those isolates also, provided usefully, quick technique, to detect natural existing inoculum of *S. sclerotiorum* in the feild, even in the presence of large amount of other biological material. Therefore this technique could be recommended to be used in detecting ascospores of *S. sclerotiorum*, the inoculum prior to infection. Further characterization of the nature of the incompatibility factor(s) which seem to be existing in the 50% of the *S. sclerotiorum* in the Jordan valley, presents legitimate target for future breeding and/ or transgenic manipulation in the control measures against this pathogen.

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