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OXALIC ACID PRODUCTION BY *Sclerotinia sclerotiorum* AND ITS RELATION TO PATHOGENICITY

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ABSTRACT

It has been confirmed the association of oxalic acid with pathogenicity of *Sclerotinia sclerotiorum* isolates in the present study. The highly virulent SS 34 isolate of *Sclerotinia sclerotiorum* was found to be highly oxalic acid producer while the weakly virulent SS 2 isolate was poorly oxalic acid producer. The moderately virulent isolates, however, showed intermediate potential for oxalic acid production. These results were confirmed and explained at the molecular level analysis of *Sclerotinia sclerotiorum* isolates. The protein banding pattern based on the SDS-Page at 53-12 kDa showed ten bands for the highly virulent SS 34 isolate while only one protein band was recognized for the weakly virulent SS 2 isolate at this molecular weight. The moderately isolates however, showed intermediate number of bands of 3-6 bands at this protein range. Meantime, the highly virulent SS34 isolate showed unique protein bands at both53 kDa and 42 kDa, while, not any of the other analyzed isolates exhibited bands at this protein molecular weight. The only protein band recognized for the weakly virulent SS 2 isolate was at 24 kDa where all isolates showed protein bands at this molecular weight. Five primers were used to study the genetic variability between the tested isolates and the primer R3 showed enough potential to differentiate between the highly virulent and the weakly virulent isolates as nine bands were recognized with the highly virulent isolate while only four bands were existed for the weakly virulent one. The moderately virulent isolates, however, showed intermediate number of bands of 5-8 bands.

Meantime, on the basis of the phylogenetic analysis and the constructed dendrogram based on the SDS-Page of the isolates protein and that based on the random amplified polymorphic DNA (RAPD), It was clear that the highly virulent *Sclerotinia sclerotiorum* SS 34 isolate and the weakly virulent SS 2 isolate were located in two distinct distant clusters. The moderately virulent *S. sclerotiorum* isolates, however, were located in intermediate sub-clusters.

Keywords: *Sclerotinia sclerotiorum* – Oxaic acid – Pathogenicity- RAPD (DNA).

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a widespread fungal pathogen that causes disease on many economically important vegetables and field crops (Boland & Hall, 1994). Approximately 90% of its life cycle is spent in soil as sclerotia and their high persistence makes *S. sclerotiorum* a very successful pathogen (Adams & Ayars, 1979). This fungus produces and secretes concentrations of oxalic acid ($C_2H_2O_4$) into their surrounding media (Cessana *et al*., 2000). The production of oxalic acid was considered as a pathogenicity determinant in *S. sclerotiorum* (Goody *et al.,* 1990; Li *et al.,* 2008 and Williams *et al*., 2011). Manual injection of culture filtrate containing oxalic acid induces brown necrotic lesions similar to the symptoms caused by *Sclerotinia* disease (Dai *et al*., 2006). Oxalic acid has been reported to

disturb guard cell function during infection by *S. sclerotiorum* by inducing stomatal opening and inhibiting stomatal closure by abscisic acid (Guimaräes & Stotz, 2004). Most recently, oxalic acid secreted by *S. sclerotiorum* was shown to elicit an apoptotic-like response in the plants during the development of disease as a pathogenicity factor (Kim *et al*., 2008). Numerous methods have been demonestrated to determine oxalic acid in plant tissues and in culture filtrate*, e.g.* $KMnO_4$ titration (Bateman & Beer, 1965), enzyme-based colorimetric assay (Yriberri & Possen, 1980), spectrophotometrically (Zhou & Boland, 1999), high pressure liquid chromatography (Jarosz – Wilkolazka & Gad, 2003) and pH indicator bromophenol blue (Venu *et al*., 2009).

On the other hand, a number of molecular techniques were available for the investigation of genetic variation of the fungus (Gomes *et al*., 2010 and Qin *et al*., 2011). These included the random amplified polymorphic DNA (RAPD) analysis (Williams *et al*., 1990) which has been useful for differentiation at the species and subspecies levels of several fungal genera (Glass & Donaldson, 1995 and Gupta *et al.,* 2010). The method allowed rapid generation of reliable and reproducible DNA fingerprints and has been used to detect intraspecific polymorphisms in a wide variety of organisms and fungi (Assigbetse *et al*., 1994 and Leal *et al*., 1994). Osofee *et al*. (2005) found that RAPD-PCR analysis differed between *S. sclerotiorum* isolates. In *S. sclerotiorum*, RAPD markers were very effective for identifying genetic differences between isolates from different regions and host plants in China (Sun *et al*., 2005). Meantime, Colagar *et al*. (2010) found that RAPD profiles markedly differed between *S. sclerotiorum* isolates. The present study, therefore, was conducted to study the association of oxalic acid with pathogenicity of *S. sclerotiorum* isolates and to reveal the link between pathogenicity & oxalic acid production and characteristics of the isolates at the molecular level.

MATERIALS AND METHODS

The tested *S. sclerotiorum* isolates:

Five *S. sclerotiorum* isolates*, i.e.* SS 2, SS 10, SS16, SS 22 and SS 34 representing different degrees of pathogenicity were chosen from a collection of isolates recovered from bean plants, showed basal rot, collected from different fields in EL-Behera governorate during the season 2009-2010 growing season. The collection of isolates were made by the author and previously tested for pathogenicity by the author in previous study (Shaat and EL-Argawy, 2011). These isolates SS 34, SS 2, and three , SS 10, SS 16 and SS 22 were highly, weakly, and moderately virulent isolates, respectively. These isolates were investigated for their potential to produce the oxalic acid and were analyzed at the molecular level to reveal marker linked to oxalic production and pathogenicity.

Oxalic acid production by *S. sclerotiorum* isolates:

Detection of oxalic acid production

Oxalic acid production was qualitatively investigated, using PDA+ bromophenol blue (Bb) plates inoculated with *S. sclerotiorum* isolates (SS 2,

SS 10, SS 16, SS 22 and SS 34) according to Steadman *et al.* (1994) as evident by the change of color in the medium from purple to yellow was taken as an indication for oxalic acid production by the fungus. Oxalic acid production was ranked on a scale from no production (-) to maximum production (+++) based on the visual degree of color change observed on the bromophenol blue amended PDA plates according to Steadman *et al.* (1994).

Quantification of oxalic acid production:

For oxalic acid determination, isolates were inoculated in 60 ml flasks containing 15 ml of potato dextrose broth (PDB), four flasks for each tested isolate. Flasks were incubated for 3 days at 25± 2°C. Then, cultures were vacuum filtered and oxalic acid was determinate in the supernatant of culture of each isolate according to Xu and Zhang (2000). This was conducted by preparing a mix reaction contained 0.2 ml of sample (or standard oxalic acid solution), 0.11 ml of bromophenol blue (BPB, 1 mM), 0.198 ml of sulfuric acid (1 M), 0.176 ml of potassium dichromate (100 mM) and 4.8 ml of distilled water. The reaction mixture, then, was placed in a water bath at 60 °C and quenched after 10 min by adding 0.5 ml sodium hydroxide solution (0.75 M). The absorbance was measured at 600 nm by means of a spectrophotometer (Spectronic 20DC, Spectronic Instruments, Inc. USA) and PDB was used as the blank control. Oxalic acid concentration was calculated comparing with a standard curve and was expressed as mg oxalic acid/Liter PDB medium.

Protein analysis for *S. sclerotiorum* isolates:

All the tested *S. sclerotiorum* isolates were grown on 25 ml of potato dextrose broth at 25± 2ºC in darkness for 7 days. Mycelial mats were harvested by filtering through Whatman No.1 filter paper, washed with 0.1 M phosphate buffer (pH 7), vacuum dried, frozen at-20ºC and extracted for soluble protein as described by Howard and Brown (2001). Protein extracts were then electrophoretic run with standard protein marker on polyacrylamide gel (12.5%) using Laemmli method of the sodium dodecyl sulphate (SDS) discontinuous system (Laemmli, 1970). Gels were stained according to Hames and Rickwood (1990). Banding patterns were scanned with Video Copy Processor P65E (Appligene). Quantitative determination of the resolved protein polypeptide content of
bands was carried out using the Molecular Dynamic Image Quant V3.3 Program (Appligene) according to El-Agamy(2000). All chemicals were Bio-Rad products, USA.

DNA analysis for *S. sclerotiorum* isolates:

Genomic DNA extraction:

Isolates were grown on potato dextrose broth for 10 days at 28 ± 2ºC in darkness. Mycelial mats were harvested by filtration using filter paper Whatman No. 1. Then, DNA was extracted using the hexadecyltrimethyl ammonium bromide method according to Murray and Thompson (1980). Concentration and purity of the obtained DNA were determined and adjusted using the standard methodology of Sambrook *et al*. (1989).

Random amplified polymorphic DNA (RAPD):

DNA from *S. scleotiorum* isolates was amplified by the RAPD methods (Williams *et al.,* 1990) using five random oligonucleotide primers shown in (Table 1). Amplification was conducted in a thermocycler (Eppendorf, Germany) programmed for 35 cycles. The entire reaction mixtures were loaded on 1.5% agarose gel and amplified DNA fragments were resolved by electrophoresis and stained by 809 ethidium bromide and photographed under UV light (302) according to Jana *et al*. (2003). All chemicals were Bio-Rad products.

Table 1: Nucleotide sequences of 5 primers used to screen the polymorphism of *S. scleotiorum* isolates tested.

Primer code	Nucleotide sequence (5′ to 3′)
R 1	GGTGCGGGAA
R 2	GTTTCGCTCC
R 3	GTAGACCCGT
R 4	AACGCGCAAC
R 5	CCCGTCAGCA

Phylogenetic analysis:

The RAPD banding patterns as well as the protein banding pattern were analyzed using UPGMA method to construct a similarity matrix and to generate a dendrogram indicating the relationship between the five experimental isolates. The presence or absence of any particular bands was the only factor considered in the computer analysis. Dendrogram of the phylogenetic relationship was produced using the software program "Statistica version 5.0" according to Rholf (2000).

Statitical analysis:

The obtained data were statistically analyzed using the American SAS/STAT Software (SAS Institute. 2000), version 6 and means were compared by the least significant difference test (LSD).

RESULTS

Oxalic acid production by *S. sclerotiorum* isolates:

Detection of oxalic acid production:

Data illustrated in Fig. (1) showed that all *Sclerotinia sclerotiorum* isolates were able to produce oxalic acid after one day of inoculation as evident by the change of color of bromophenol blue of PDB medium from purple to yellow. Meantime, oxalic acid production increased by the following days up to five days as indicated by the visual degree of color change. The highly virulent SS 34 isolate of *Sclerotinia sclerotiorum* was found to be highly oxalic acid producer showed deep bright yellow (+++) while the weakly virulent SS 2 isolate was poorly oxalic acid producer pale, faint yellow (+). The moderately virulent isolates SS 22, SS 10 and SS16 showed intermediate yellow color for oxalic acid production (++). However, the uninoculated PDA plates amended with bromophenol blue (control) remained purple in color.

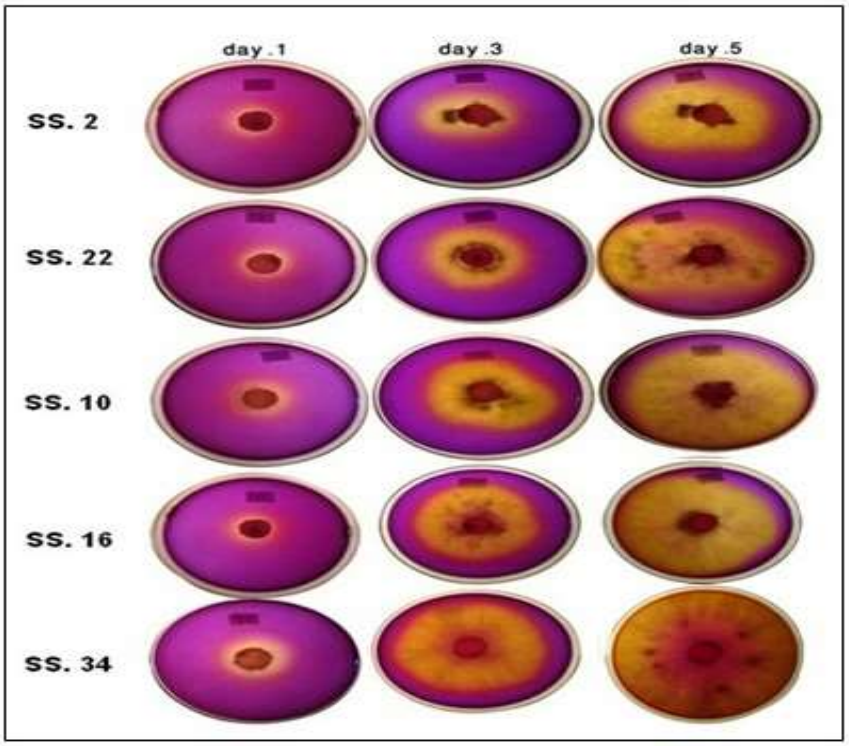

Fig.1: Detection of oxalic acid production by using PDA amended with bromophenol blue inoculated with *S. sclerotiorum* isolates 1, 3 and 5 days after inoculation.

Quantification of oxalic acid production:

Data tabulated in Table (2), illustrated in Fig. (2) showed that oxalic acid production by the tested *S. sclerotiorum* isolates was obviously correlated with their pathogenicity degree. The highly virulent *S. sclerotiorum* isolate SS 34 was the most highly oxalic acid producer (5.46 mg/L). While, the most weakly virulent isolate, SS 2 was poorly oxalic acid producer (2.46 mg/L). The moderately virulent isolates, SS16, SS 10 and SS 22 were also moderately oxalic acid producer (3.68 – 3.33 mg/L).

Table 2: Oxalic acid production on PDB medium by *S. sclerotiorum* isolates of different degrees of pathogenicity.

Isolates	Pathogenicity	Oxalic acid (mg/L) of medium
SS 34	**Highly**	5.46 [a]
SS 16	**Moderately**	3.68 [b]
SS 10	**Moderately**	3.46 [b]
SS 22	**Moderately**	3.33 [b]
SS 2	**Weakly**	2.46 [c]

Data are means of four replicates.
Data with the same letters are not significant at probability = 0.05

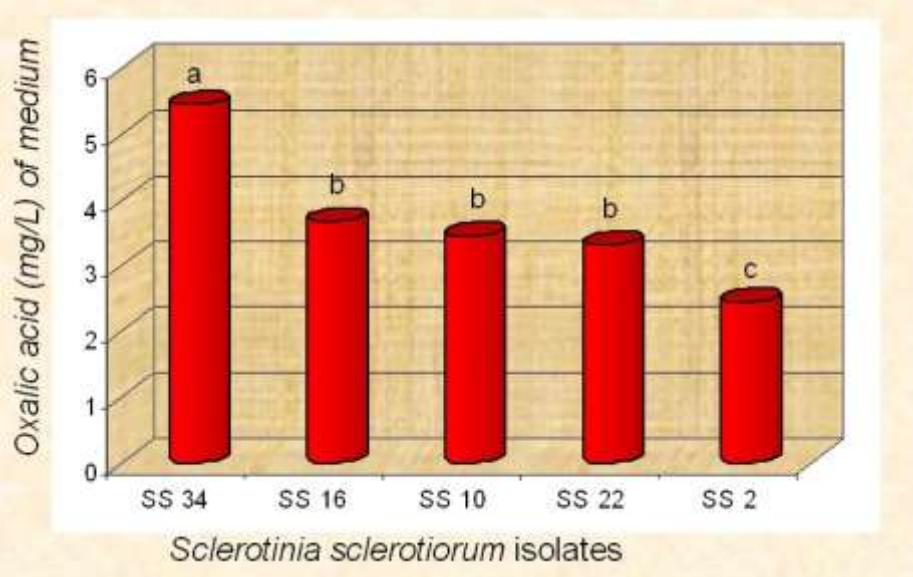

Fig. 2: Oxalic acid production by the tested *S. sclerotiorum* tested isolates, detected three days after PDB medium inoculation.

Protein analysis for *S. sclerotiorum* isolates:

The SDS- Page of the protein banding pattern of the analyzed *S. sclerortiorum* isolates (Fig. 3), and the data tabulated in Table (3) showed that at the protein molecular weight of 53 -12 kDa, ten bands were recognized for the highly virulent SS34 isolate. On the other hand, the weakly virulent SS 2 isolate exhibited only one band at this range of protein molecular weight. The moderately virulent isolates, however, showed intermediate number of bands of 3 – 6 at this protein range. Meantime, the highly virulent SS 34 isolate showed unique two protein bands at both 53 kDa and 48 kDa, while not any of the other isolates exhibited bands at this molecular weight. The only protein band recognized for the weakly virulent SS 2 was at 24 kDa where all isolates exhibited a protein bands at this molecular weight.

The phylogenetic analysis based on SDS- Page:

Its is quite clear from dendrogram (Figure 4) constructed according to the SDS- Page showing the phylogenetic relationship of the analyzed *S. sclerotiorum* isolates that the highly virulent SS 34 and the weakly virulent isolate SS 2 were located in two distant clusters. The moderately virulent isolates, SS 22, SS 10 and SS 10 were located in intermediate sub-clusters.

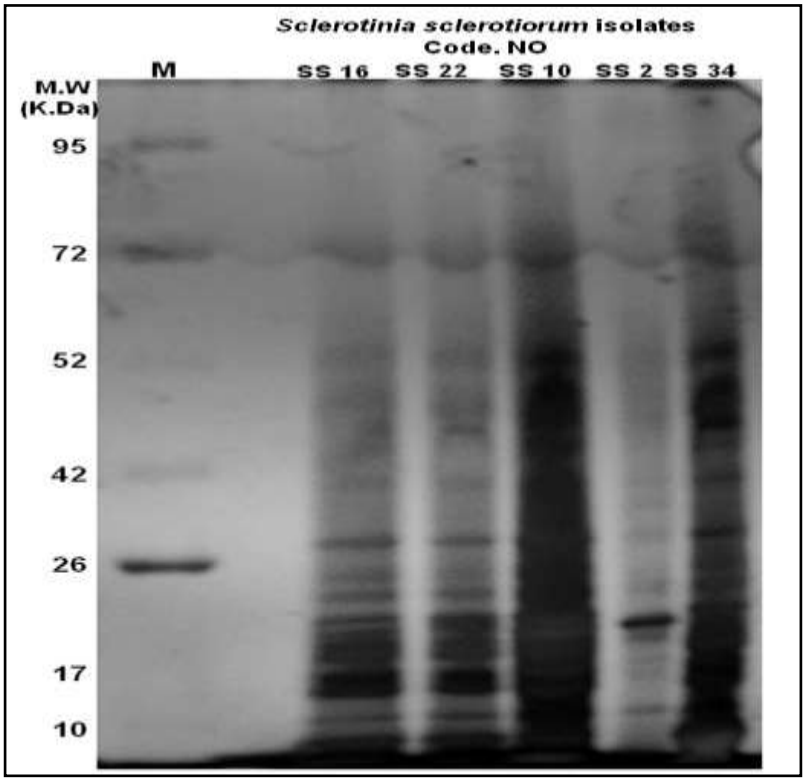

Fig. 3: SDS-Page of protein banding pattern of five *Sclerotinia sclerotiorum* isolates recovered from bean plants collected from different fields in El-Behera governorate.

Table 3: Characterization of protein bands of the SDS-Page analysis in five *Sclerotinia sclerotiorum* isolates of different degrees of pathogenicity showing unique positive and/or negative markers.

Molecular weight of protein bands (kDa)	Number of protein bands of *Sclerotinia sclerotiorum* isolates				
	SS 34 (H.v.)	SS 16 (M.v.)	SS 22 (M.v.)	SS 10 (M.v.)	SS 2 (W.v.)
53	1	0	0	1	0
48	1	0	0	0	0
45	1	0	1	0	0
44	1	0	0	1	0
40	1	1	0	1	0
25	1	1	0	0	0
24	1	1	1	1	1
20	1	1	1	0	0
17	1	1	1	1	0
12	1	1	0	1	0
Number of bands	10	5	3	6	1

H.v. =highly virulent isolate, M.v. =moderately virulent isolate,
W.v. =weakly virulent isolate.

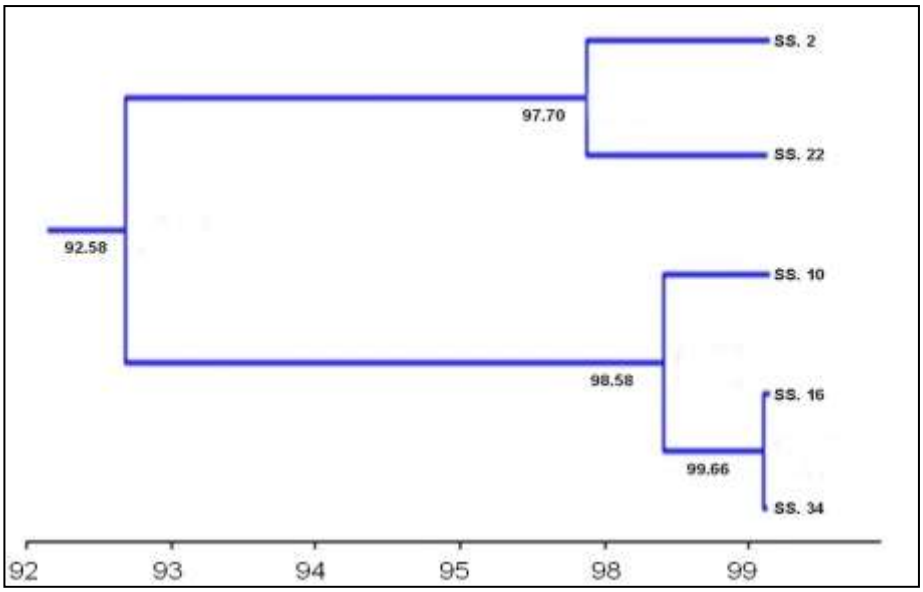

Fig. 4: Dendrogram based on the protein analysis showing the phylogenetic relationship of the analyzed *S. sclerotiorum* isolates recovered from bean plants in El-Behera governorate.

DNA analysis of *S. sclerotiorum* isolates:

Fingerprinting of isolates using random amplified polymorphic DNA (RAPD):

Considerable variations were revealed among the *S. sclerotiorum* isolates analysed for RAPD by using the tested five primers, R1, R2, R3, R4 and R5 (Fig. 5& Table 4). All the tested primers succeed to reveal polymorphic patterns among the isolates. However, primers R1 and R4 were the most efficient to reveal polymorphism in the *S. sclerotiorum* isolates as 37 DNA fragments were identified in the analyzed five isolates. On the other hand, primer R3 was the most efficient to reveal differences among isolates of different degrees of pathogenicity as the nine bands were appeared for the highly virulent (SS 34) while only four bands were revealed for the weakly virulent (SS 2). The moderately virulent isolates, SS 16, SS 10, and SS 22, exhibited intermediate number, 5-8 bands.

Phylogenetic analysis of RAPD:

According to the RAPD banding patterns of the isolates analyzed using UPAGM program analysis a dendrogram (Fig. 6) was constructed. This was quiet clear from the phylogenetic analysis, and the dendrogram that the highly virulent SS 34 isolate and the weakly virulent SS 2 isolate were located in two distant clusters. The moderately virulent isolates, SS 10, SS 16 and SS 22, on the other hand, were located in intermediate sub clusters (Fig. 6).

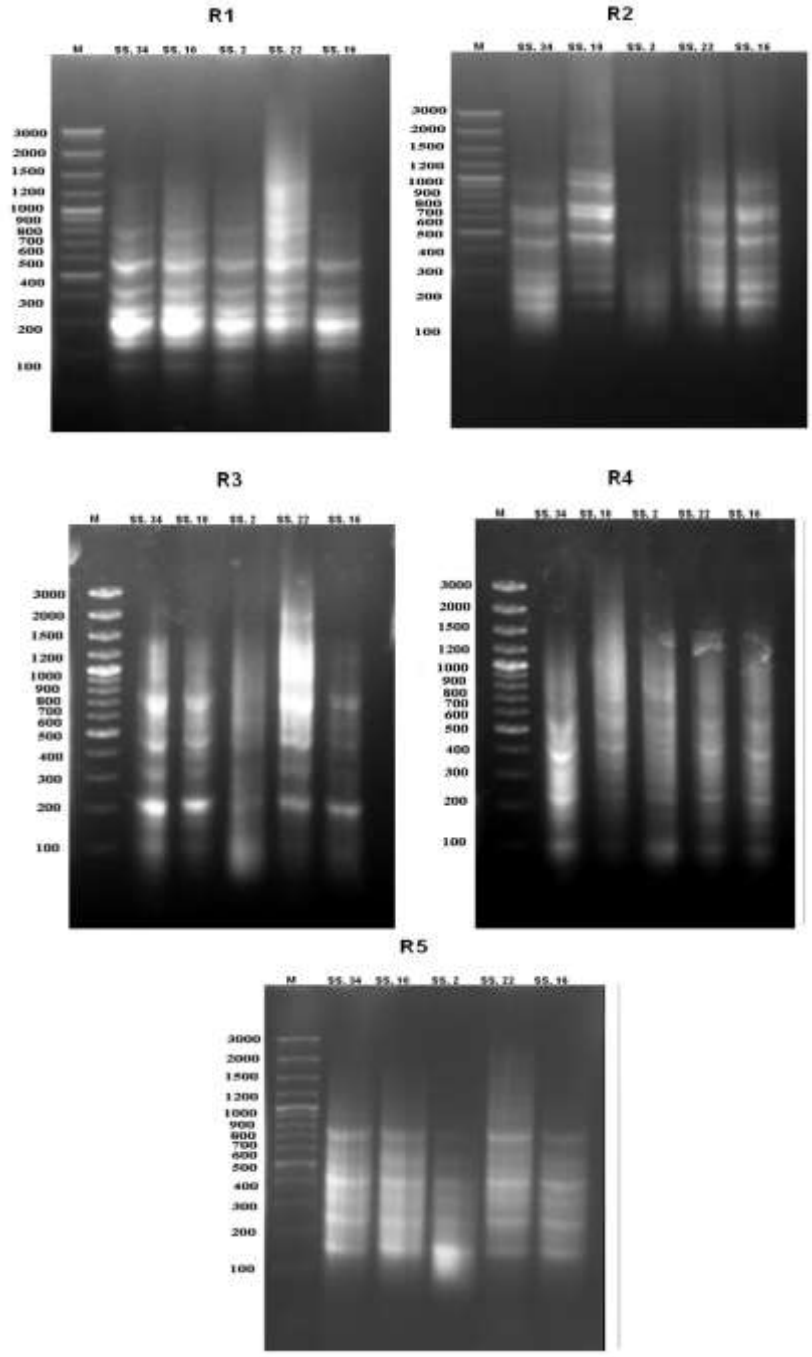

Fig. 5: DNA banding pattern of *Sclerotinia sclerotiorum* isolates recovered from bean plants collected from different fields in El-Behera governorate and analyzed with the random amplified polymorphic DNA through PCR using five random oligonucleotide primers, R1, R2, R3, R4 and R5.

Table 4: Number of amplified and polymorphic DNA-fragments generated by five RAPD primers from five *Sclerotinia sclerotiorum* isolates.

Primers	Number of DNA fragments of different isolates					Total
	SS 34	SS 10	SS 2	SS 22	SS 16	
R_1	7	7	7	9	7	**37**
R_2	7	8	3	7	7	**32**
R_3	9	7	4	8	5	**33**
R_4	7	6	9	8	7	**37**
R_5	7	6	5	7	7	**32**
Total	**37**	**34**	**28**	**39**	**33**	

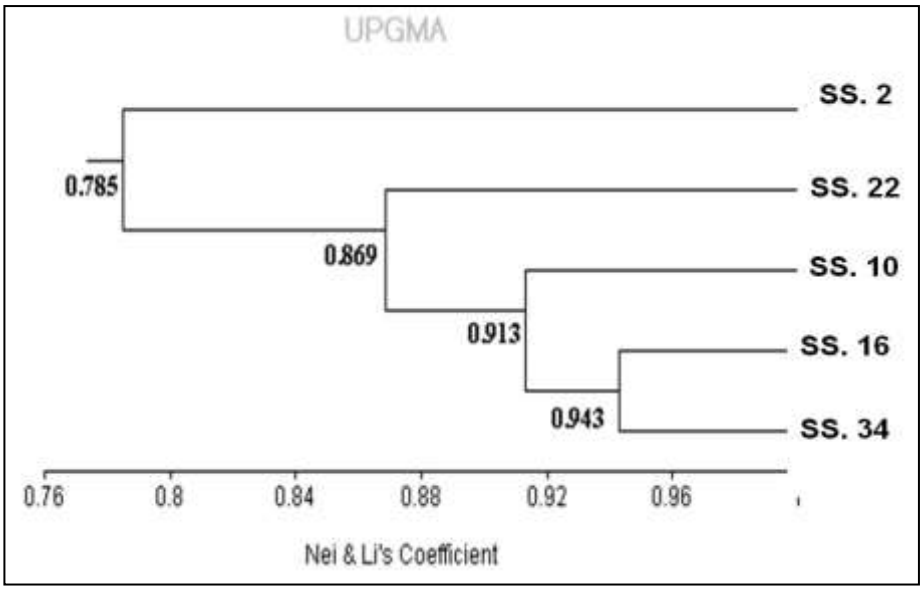

Fig. 6: Dendrogram of five *Sclerotinia sclerotiorum* isolates developed by RAPD data using UPGMA analysis.

DISCUSSION

The hypothesis that oxalic acid is a pathogenicity determinant in *S. sclerotiorum* isolates (Durman *et al.,* 2005; Xiao-Ting *et al.,* 2009 and Williams *et al.,* 2011) has been confirmed in the present study.

The highly virulent SS 34 isolate of *Sclerotinia sclerotiorum* was a highly oxalic acid producer while the weakly virulent SS 2 isolate was poorly oxalic acid producer. The moderately virulent isolates, however, showed intermediate potential for oxalic acid production. These findings were in agreement with several reports worldwide (Callahan and Row, 1991; Harel *et al*., 2006 Li *et al.,* 2008). Considerable research interest has been focused on oxalic acid production by this pathogen and the following mechanisms of action have been proposed to explain its involvement in pathogenesis: (1) lowering infected tissues pH, which enhances the activity of extracellular

enzymes produced by the pathogen (Bateman & Beer, 1965), (2) chelating cell wall Ca^{+2} by the oxalate anion, which softens plant cell wall and compromises the function of Ca^{+2} -dependent defense responses (Bateman& Beer, 1965), (3) directing toxicity to host plants, which weakens the plant and facilitates invasion (Noyes & Hancock, 1981), (4) suppressing the host plant oxidative brust (Cessna *et al*., 2000). Also, oxalic acid inhibits the activities of polyphenol oxidase of host plants (Xiong *et al*., 1998).

These results were confirmed and explained at the molecular level analysis of *Sclerotinia sclerotiorum* isolates. The protein banding pattern based on the SDS-Page at 53-12 kDa showed ten bands for the highly virulent SS 34 isolate while, only one protein band was recognized for the weakly virulent SS 2 isolate at this molecular weight. The moderately virulent isolates, however, showed intermediate number of bands of 3-6 bands at this protein range. Meantime, the highly virulent SS34 isolate showed unique protein bands at both53 kDa and 42 kDa, while, not any of the other analyzed isolates exhibited bands at this protein molecular weight. The existence of bands recognized in the highly virulent SS 34 isolate may indicate a special enzymes or signals could be involved in virulence potentially. The only protein band recognized for the weakly virulent SS 2 isolate was at 24 kDa where all isolates showed protein bands at this molecular weight. These results were in agreement with those of Novak & Kohn (1991) and Aboshosha *et al.* (2007).

The fingerprinting of the isolates using the random ampilified polymorphic DNA (RAPD) using five primers, R1, R2, R3, R4 and R5 showed that the tested primers were all capable to reveal variations among isolates. The primer R3, however, showed enough potential to differentiate between the highly virulent and the weakly virulent isolates as nine bands were recognized in case of the highly virulent isolate while only four bands were existed in the weakly virulent one. The moderately virulent isolates, however, showed intermediate number of bands of 5-8 bands.

Meantime, on the basis of the phylogenetic analysis and the constructed dendrogram based on the SDS-Page of the isolates protein and that based on the random amplified polymorphic DNA (RAPD), It was clear that the highly virulent SS 34 isolate and the weakly virulent SS 2 isolate were located in two distinct distant clusters. The moderately virulent *S. sclerotiorum* isolates, however, were located in intermediate sub-clusters. These findings where in harmony with those of several investigators (Osofee *et al*., 2005; Aboshosha *et al*., 2007 and Arbaoui *et al*., 2008).

The understanding of the pathogenicity – related substance could be an approach for elucidating strategies for the basal rot control of bean.

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إنتاج حمض الأوكزاليك بواسطه سكليروتينيا سكليروتيورم وعلاقة بالمقدرة المرضية

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أثبتت الدراسة العلاقة بين أنتاج حمض الأوكزاليك والمقدره المرضيه لعزلات سكليروتينيا سكليروتيورم.

فقد كانت العزلة SS 34 الشديدة الشراسة المرضية أيضا عالية المقدرة علي أنتاج حمض الأوكزاليك بينما كانت العزلة SS 2 المنخفضة الشراسة المرضية منخفضة في أنتاج حمض الأوكزاليك وقد أظهرت العزلات المتوسطة في شراستها المرضية مقدرة متوسطة علي أنتاج حمض الأوكزاليك.

تم تفسير النتائج المتحصل عليها من الدراسة علي المستوي الجزيئي لعزلات سكليروتينيا سكليروتيورم فقد أظهر نمط الحزم البروتينيه بواسطة تقنية الصوديوم دودي سايل سالفات بيج SDS-Page . في المدي من 12 – 53 كيلو دالتون عشرة حزم بروتينية للعزلة عالية الشراسة المرضية SS 34 بينما ظهرت حزمه بروتينيه واحدة للعزلة SS 2 المنخفضة في شراستها المرضية و أظهرت العزلات المتوسطة المقدرة في أفراز حمض الأوكزاليك عدد من الحزم البروتينيه يتراوح من 3 – 6 حزم بروتينيه في هذا المدي من الحزم البروتينية وقد أظهرت العزلة عالية الشراسه المرضية حزمتان مميزتان عند الأوزان الجزيئيه 42 و 53 كيلو دالتون بينما لم تظهر أي حزم بروتينيه أخري لباقي العزلات عند نفس الأوزان الجزيئيه . وفي هذه الأثناء أظهرت العزلة SS 2 المنخفضة الشراسة المرضية حزمة بروتينية عند 24 كيلو دالتون حيث اظهرت باقي العزلات نفس الحزمة البروتينيه عند نفس المستوي.

تم أختيار خمسة بادئات جزيئية لدراسة الأختلافات الوراثية بين العزلات المختبرة وقد اظهر البادىء R3 المقدرة الكافية للتفرقة مابين العزله عالية الشراسة المرضية والعزلة منخفضة الشراسة المرضية حيث ظهرت تسعة حزم للعزلة عالية الشراسة المرضية وأربعة حزم فقط للعزلة منخفضة الشراسة المرضية وأظهرت العزلات المتوسطة عدد من الحزم تراوح مابين خمس الي ثماني حزم.

وعلي أساس تحليل القرابة الوراثية المبني علي تقنية الصوديوم دودي سايل سالفات بيج SDS-Page لبروتين العزلات وكذلك تقنية التضاعف العشوائي للحمض النووي الدنا RAPD (DNA) كان من الواضح أن عزلة سكليروتينيا سكليروتيورم SS 34 عالية الشراسة المرضية والعزلة SS 2 منخفضة الشراسة المرضيه قد تم وضعهما في مجموعتين وراثيتين متباعدتين بينما تم وضع العزلات المتوسطة المقدرة المرضية في تحت مجموعات وراثيه متوسطه بينهما.

قام بتحكيم البحث

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