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Molecular Diversity of *Sclerotinia sclerotiorum*, the Incitant of Sclerotinia Rot of Common Bean in Upper Egypt

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Key words: Common bean, Sclerotinia rot, *S. sclerotiorum*, VCGs, proteins, Esterase, DNA.

Abstract:

The tested isolates of *S. sclerotiorum* obtained from different localities of Upper Egypt were able to infect common bean Balady cv. causing Sclerotinia rot disease with different degrees of disease severities ranging from moderate to sever. Isolates consisted of eight vegetative compatible groups (VCGs); three of them consisted of two different isolates (3EB, 5EB), (3EB, 6ED) and (5EB, 6ED) and the remaining 5 VCGs each consist one isolate, compatible only with itself. Cluster analysis of electrophoretic patterns of proteins obtained by PAGE and SDS-PAGE and isozyme obtained by PAGE were able to differentiate the tested isolates of the pathogen, according to similarity levels, to different groups, however, they unable to detect differences among isolates in DNA when RAPD-PCR was adopted using two primers.

Introduction:

Sclerotioria sclerotiorum (Lib.) de Bary is a cosmopolitan necrotrophic plant pathogen, that infect 64 families, 225 genera and 361 species (Purdy, 1979). It causes Sclerotinia rot (white mold) of common bean (*Phaseolus vulgaris* L.) and in extreme cases, yield losses approach 100% (Hall, 1994). The disease was originally believed to occur only in cool, moist areas (Boland and Hall, 1994), but is now known to be occur in hot dry areas including Egypt (Abd El-Moity *et al*., 1993).

Various levels of genetic diversity in *S. sclerotiorum* field population was recorded in different countries (Kohn *et al*. 1991; Laurence, 1996; Li *et al*., 2005; El-Blasy, 2006; Li *et al*., 2008 and Barari *et al*., 2010). However, little is known about the genetic diversity in the pathogenic isolates in Egypt (El-Blasy, 2006). It is possible that the genetic variability of this pathogen may influence the success of control measures. Immunological techniques, which are being developed for the prediction of disease risk, may also be affected by pathogen variability. Therefore, the aim of the present work was to identify genetic variability in field populations of *S. sclerotiorum* isolates, affecting common bean in Upper Egypt by proteins, esterase and DNA analysis.

Materials and Methods:

Source of *Sclerotinia sclerotiorum* isolates:

Eight isolates of the fungus were used in the present work. Seven of them were isolated from diseased common bean plants, showing typical symptoms of Sclerotinia rot, collected from different fields of El-Minia Governorate and an isolate was isolated from infected lettuce plants showing lettuce drop, symptoms obtained from a lettuce field located in Assiut Governorate. Isolation was done on PDA at 20°C.

Identification of the fungal isolates was carried out by using the morphological characteristics of mycelia and sclerotia as described by Saharan and Naresh Mehta (2008) and confirmed by Assiut University Mycological Center (AUMC), Assiut, Egypt.

Pathogenicity tests:

Pathogenicity tests were carried out under greenhouse conditions in 2007-2008 season. Fungal inocula were prepared by growing discs (5 mm in diameter) of 7-day-old culture of the fungus isolates in 250 ml glass bottles containing autoclaved ground sorghum seeds-sand medium (50 g. ground sorghum seeds + 200 g. sand + 100 ml water) and incubated at 20°±2°C for 15 days. Pots (25 cm in diameter) were sterilized by dipping in 5% formalin solution for 10 minutes then washed several times with tap water and left several days to get rid of formalin. Soil was autoclaved at 121°C for 2 hr for 3 days and left for two weeks before cultivation. Sterilized pots were filled with sterilized soil and infested with the tested fungal isolates at the rate of 5% (w/w) (Omar *et al.*, 1992 and Mazen, 1995). Non-infested soil was used as a control. Surface sterilized seeds of Balady common bean cv. were sown in each pot (5 seeds/pot) and four replicates were used for each treatment. Surface sterilization of seeds was carried out by dipping seeds in 2% sodium hypochlorite for 2 min before sowing. Percentage of infection was recorded after 60 days from sowing date.

Determination of vegetative compatibility groups (VCG) of tested isolates:

The tested isolates of *S. sclerotiorum* were grown in 9 cm Petri dishes containing PDA medium for five days at 20°C. Isolates were paired in all possible combinations on potato dextrose agar (PDA) medium according to the procedure of Kohn *et al*. (1991). Discs (5 mm in diameter) from the tested isolates were inoculated 1-1.5 cm from the edge of the plate and incubated at 20°C. for 15 days. Mycelial reactions were recorded as incompatible when an apparent line of demarcation was observed between the confronting paired isolates. The compatible and incompatible isolates were determined.

Identification of major proteins in tested isolates and electrophoretic patterns of esterase enzymes:

Extraction of fungal protein:

Protein extracts from tested isolates were prepared according

to Guseva and Gromova (1982), Rataj-Guranowska *et al*. (1984) and Hussein (1992). Fungal isolates were grown for 22 days at 20°C on liquid Czapek's medium. The mycelium was harvested by filtration through cheesecloth, washed with distilled water several times and freeze-dried. The frozen mycelium was suspended in phosphate buffer pH 8.3 (1-3 mL/g mycelium), mixed thoroughly with glass beads, and ground in liquid nitrogen to a fine powder. The ground mycelium was centrifuged at 19.000 rpm for 30 minutes at 0°C. The protein content in supernatant was estimated according to Bradfrod (1976) by using bovine serum albumin as a standard protein..

Electrophorests of native protein (PAGE):

The protein-extract supernatant was mixed with equal volume of a solution containing 20% glycerol (v/v) and 0.1% bromophenol blue (v/v) in 0.15 M Tris-HCl, pH 6.8. Twenty microliters of the resulting suspension (40 to 60 mg of protein) was subjected to electrophoresis in 2.5 mM Tris buffer containing 192 mM glycin at pH 8.3 Electrophoresis was conducted at room temperature (approximately 20+2°C), for 9 hr on an 15% polyacrylamide gel with a 6% stacking gel, at 20 and 10 mA, respectively, until the dye reached the bottom of the separating gel. Electrophoresis was performed in a vertical slab mold (16.5 x 14.5 x 0.1 cm). Gel was stained with silver nitrate for the detection of protein bands Sammons *et al*. (1981).

Electrophoresis of disociated protein (SDS-PAGE):

For electrophoresis of disociated protein, each supernatant was mixed with an equal volume of a solution consisting of (by volume) 64% buffer (0.15 MT, pH 6.8); 20% glycerol; 6% sodium dodecyl sulfate (SDS); 10% 2-6-mercaptoethanol; and 0.1% bromphenol blue, before boiling in a water bath for 3 min. Twenty-microliter samples (40 µg of protein) were subjected to electrophoresis in 15% polyacrylamide prepared in 0.1% SDS Laemmli (1970) and Latorre *et al*. (1995). The electrophoresis, staining were conducted as described for undisociated proteins.

Electrophoretic patterns of esterase:

For detection of esterase, the following staining solutions were used:

0.05 M phosphate buffer pH 7.2 100 ml
a Naphathyl acetate (dissolved in 1 ml of acetone) 10 mg.
Fast Blue RR salt 50 mg

Determination of nucleic acids in tested isolates:

This experiment was carried out in Agriculture Research Center, Giza, Egypt.

DNA isolation and random amplified polymorphism DNA (RAPD) technique.

Czapek's broth was distributed in 250 ml. Erlenmeyer flasks, 50 ml each and autoclaved for 15 min. Flasks were inoculated with a 5 mm fungal discs from 7 days

old cultures of tested isolates. Flasks were inoculated at 20-22°C for 10 day, then the mycelial growth was filtered using filter paper, washed three times with deionzed distilled water and dried in a freeze drier for 30 min. Two grams of the dried mycelia were homogenized with a pestle in liquid nitrogen using aprechilled mortar. Two ml of Tris-HCl buffer solution (pH 6.8 and 0.0625 M, containing 10% glycerol and 1 ml M.

DNA isolation and RAPD technique:

DNA was isolated from 50 mg of organism using Qiagen Kit for DNA extraction. The extracted DNA was dissolved in 100 µl of elution buffer. The concentration and purity of the obtained DNA was determined by using "Gen qunta" system-pharmacia Bio-tech. The purity of the DNA for all samples was between 90-97% and the ratio between 1.7-1.8. Concentration was adjusted at 6 ng/ul for samples using TE buffer pH 8.0.

Random amplified polymorphism DNA technique (RAPD):

Thirty ng from the extracted DNA were used for amplification reaction. The polymerase chain reaction (PCR) mixture contained PCR beads tablet (manufactured by Amessham Pharmacia Biotech), which containing all of the necessary reagents except the primer and the DNA which add to the tablet.

The kits of Amessham Pharmacia Biotech were also included the following primers. Five microliters of the primer (10 mer) were added. The sequences of used primers were:

RAPD analysis Primer 2: 6-d (GTTTCGCTCC)-3

RAPD analysis Primer 4: 6-d (AAGACCCGT)-3

The total volume was completed to 25 µl using sterile distilled water. The amplification protocol was carried out as follows using PCR unit II biometra.

a)Denaturation at 95°C for5 min.

b) 45 cycles each consists of the following steps:

1- Denaturation at 95°C for 1 min.

2- Annealing at 36°C for 1 min.

3- Extension at 72°C for 2 min.

c)Final extension at 72°C for 5 min.

d) Hold at 4°C

7 µl of 6X tracking buffer (manufactured by Qiagen Kit) were added to 25 µl of the amplification product.

Amplification product analysis:

The amplified DNA for all samples were electrophorased (15 µl) using electrophoresis unit (WIDE mini-sub-cell GT Bio-RAD) on 2% agarose containing ethedium bromide (0.5 µg/ml). At 75 constant volt, and determined with UV transilluminator.

Gel analysis:

All kinds of gels (protein, isozyme, and DNA) were scanned for band R_f using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia Dr. Unit 6 C, Fullerton CA 92631). The different Mol.W. of bands were determined against PCR marker

promega G 317A by unweighted pair-group method based on arithmetic mean (UPGMA).

Results and Discussion:

Variation existed among *S. sclerotiorum* isolates:

1- Pathogenicity of isolates:

Data presented in Table (1) indicate that, the tested isolates of *S. sclerotiorum* were able to infect Balady common bean plants with different degrees of infection causing Sclerotinia rot. Isolates No. 1EB, 2ED, 4EB, 5EB, 7ED and 8AA caused the highest % of infection (90-100%) followed by isolates No. 6ED (80%) then isolate No. 3EB (60%).

Variation in pathogenicity among isolates of *S. sclerotiorum* are in agreement with those reported by Adams and Tate (1975) and El-Blasy (2006) and it may be associated with the production of pectolytic enzymes, cellulase, hemicellulase, phosphatidase and oxalic acid by different isolates of the pathogen (Lumsden, 1969, 1970 and 1979).

Table (1): Pathogenicity of *S. sclerotiorum* isolates on Balady common bean cultivar.

Isolates	Source	% of infection
1EB	El-Minia, Elbergaia	100
2ED	El-Minia, Damshmeer	100
3EB	El-Minia, Elbergaia	60
4EB	El-Minia, Elbergaia	100
5EB	El-Minia, Elbergaia	100
6ED	El-Minia, Damarees	80
7ED	El-Minia, Damarees	90
8AA	Assiut, Assiut	100
Control	--	**0**
L.S.D. at 0.05		**19.86**

2- Vegetative compatibility groups (VCGs) of different isolates of *S. sclerotiorum in vitro*:

Mycelial compatibility, *in vitro*, of the eight different isolates of the pathogen obtained from different hosts and localities in Upper Egypt presented in Table (2) show that tested isolates consisted of 8 vegetative compatible groups (VCGs), three of them consisted of two different isolates (3EB and 5EB), (3EB and 6ED) and (5EB and 6ED) and the remaining 5 VCGs were each made up of one isolate, compatible only with itself. There was no correlation between mycelial compatibility grouping and virulence of isolates. Such results partially agree with those reported by Kohn *et al*. (1991), Kull *et al*. (2000), Meinhardt *et al*. (2002), Kull *et al*. (2004) and El-Blasy (2006).

Table (2): Vegetative compatibility reaction between *S.sclerotiorum* isolates, *in vitro*.

Isolates	1EB	2ED	3EB	4EB	5EB	6ED	7ED	8AA
1EB	+	-	-	-	-	-	-	-
2ED		+	-	-	-	-	-	-
3EB			+	-	+	+	-	-
4EB				+	-	-	-	-
5EB					+	+	-	-
6ED						+	-	-
7ED							+	-
8AA								+

+ compatible reaction. - incompatible reaction.

3- Identification of major protein of *S. sclerotiorum* isolates:

(a) Electrophoretic protein banding patterns obtained by PAGE:

Fig. (2) showed the phenogram constructed based on similarity levels (SLs) generated from cluster analysis of electrophoretic banding patterns of native (undisociated) proteins shown in Fig. (1). The greater the LSs the more closely the isolates were related in their protein composition. The isolates in the phenogram formed two remotely related lager groups at SLs 30%. The first group included isolates No. 7ED, 6ED, 5EB and 3EB, while the second group included isolates No. 4EB, 2ED and 1EB. Within the second group, the highest SLs (45%) was found between isolates No. 1EB and 2ED. Isolate No. 8AA from Assiut was remotely related to the other isolates forming a separate sub-cluster.

(b) Electrophoretic protein banding patterns obtained by SDS-PAGE:

The phenogram shown in Fig. (4) was constructed based on LSs generated from cluster analysis of electrophoretic banding patterns of dissociated proteins shown in Fig. (3). Cluster analysis placed the isolates in three distinct groups. The first one included isolates No. 1EB, 5EB, 3EB and 2ED, the second one included isolates No. 6ED and 7ED, and the third one included isolates No. 4EB and 8AA. Isolates No. 6ED and 7ED showed the highest SLs (90%).

The results of electrophoresis indicated that PAGE and SDS-PAGE showed variation in protein levels in the tested isolates of the pathogen and able to separate the isolates into groups. Grouping the isolates by PAGE was not related to their virulence or geographic origin, however, grouping them by SDS-PAGE was partially successful in geographical origin. Thus, 75% of Elbergaia isolates were placed in the same group (SLs 58%) and the two isolates from Damshmeer were placed in the same group showing the highest SLs (90%)

Such results are in line with results of Novak & Kohn (1988), Saeed and Abo-Elseoud (1990), Lima and Menezes (2002) and Sallam, Nashwa (2004) who

worked on another different plant pathogenic fungi.

(c) Electrophoretic pattern of esterase enzymes produced by *S. sclerotiorum* isolates:

According to electrophoretic patterns of esterase isozyme obtained by PAGE (Fig. 5) from the 8 isolates of the pathogen and cluster analysis (Fig. 4), isolate No. 4EB formed a separate sub-cluster while the remaining isolates formed 3 sub-clusters at SLs 30, 40 and 38% including isolates No. 1EB and 5EB; isolates No. 2ED, 6ED and 8AA and isolates No. 3EB and 7ED, respectively.

Such results are in agreement with those of Cao & Ye (2001), Henning & Orlicz-Luthordt (2002), Lima & Menzes (2002), Sallam, Nashwa (2004) and Cao and Ye (2001) who worked on other plant pathogenic fungi and showed that neither virulence nor geographic origins of isolates were correlated to esterase patterns of isolates.

DNA diversity among isolates of *S. sclerotiorum* using RAPD-PCR:

The phenograms shown in Fig. (10) and (8) were constructed based on LSs generated from cluster analysis of RAPD patterns shown in Fig. (9) and (7).

Primers 2 and 4 were not able to detect appreciable differences in DNA banding patterns of the tested isolates. Thus, the overall SLs among the isolates were 98.92% by primer No. 2 and 93.92 by primer No. 4 (Fig. 10 and 8).

Such results are in agreement with results of Barari *et al.* (2010) who reported that phynotypic and molecular analysis of the pathogen isolates were similar.

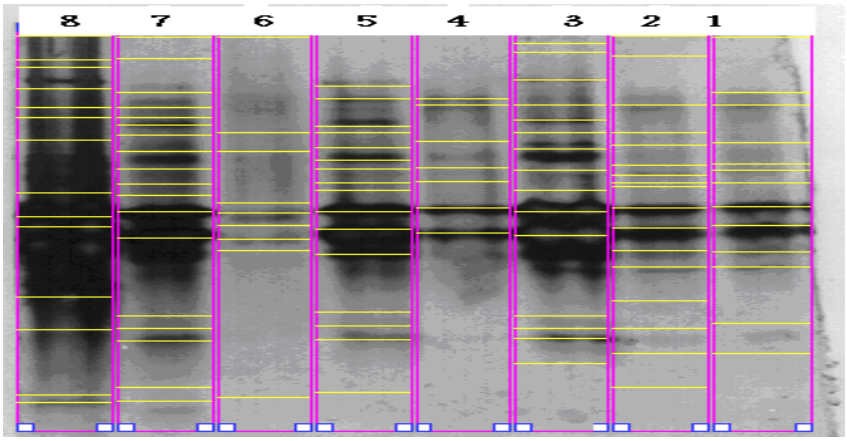

Fig. (1):Protein banding patterns obtained by PAGE from 8 isolates of *S.sclerotiorum*.

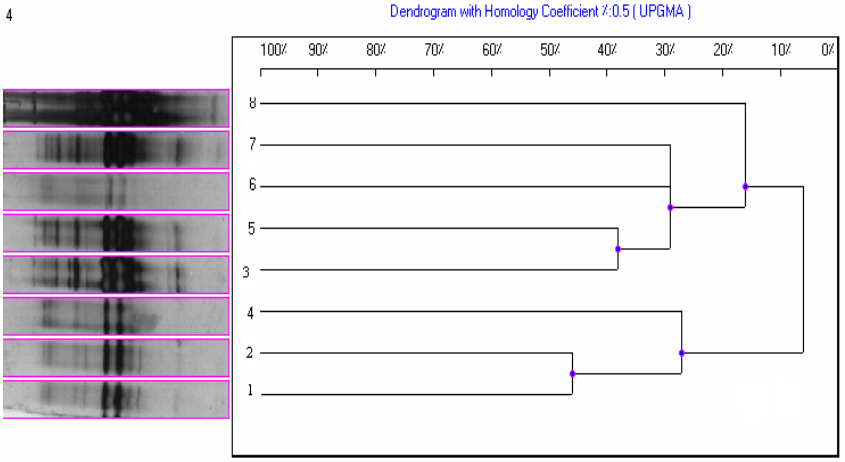

Fig. (2):Phenogram based on average linkage cluster analysis of electrophoretic protein banding patterns obtained by PAGE from 8 isolates of *S.sclerotiorum*.

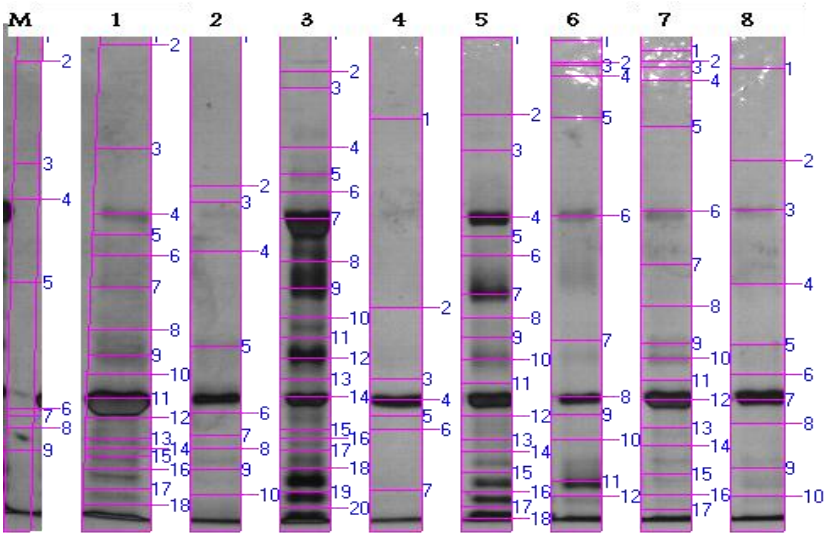

Fig. (3): Protein banding patterns obtained by SDS-PAGE from 8 isolates of *S.sclerotiorum*

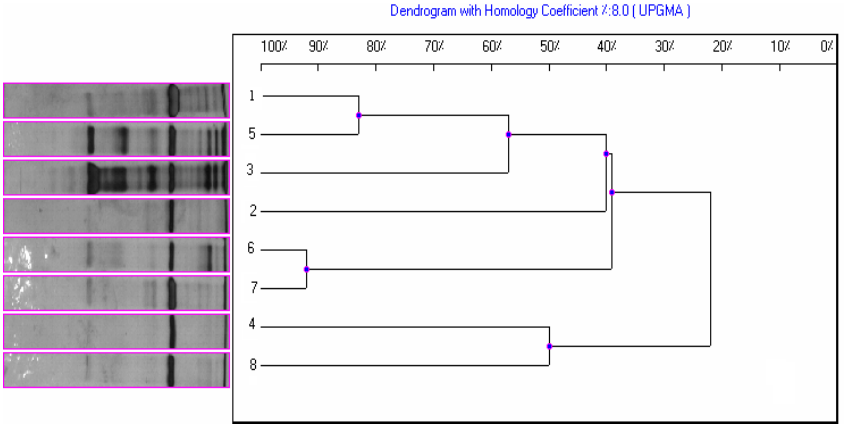

Fig. (4): Phenogram based on average linkage cluster analysis of electrophoretic protein banding patterns obtained by SDS-PAGE from 8 isolates of *S.sclerotiorum*.

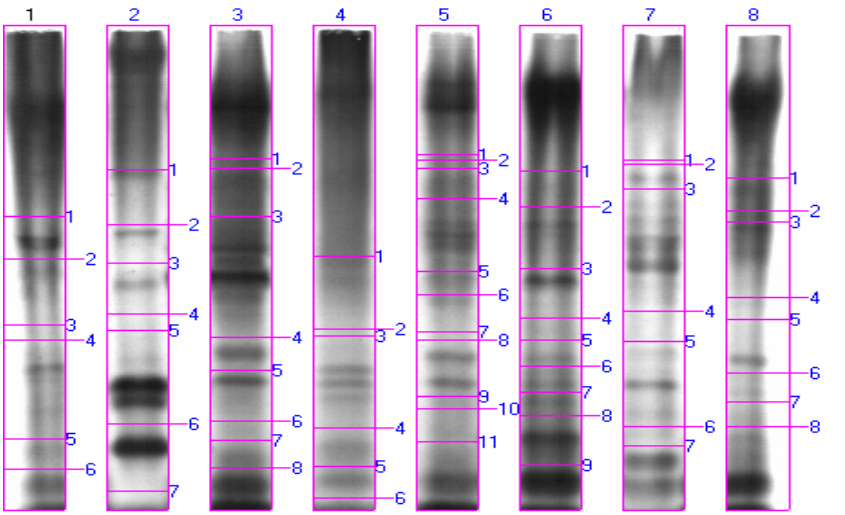

Fig. (5):Esterase isozyme patterns obtained by PAGE from 8 isolates of *S.sclerotiorum*

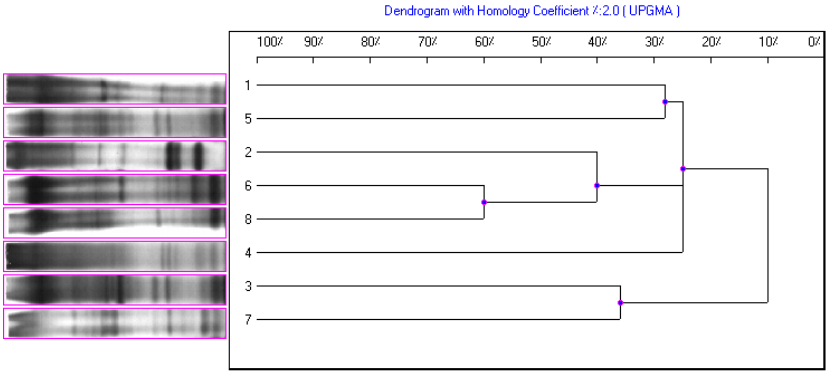

Fig. (6): Phenogram based on average linkage cluster analysis of electrophoretic esterase isozyme patterns obtained by PAGE from 8 isolates of *S.sclerotiorum*.

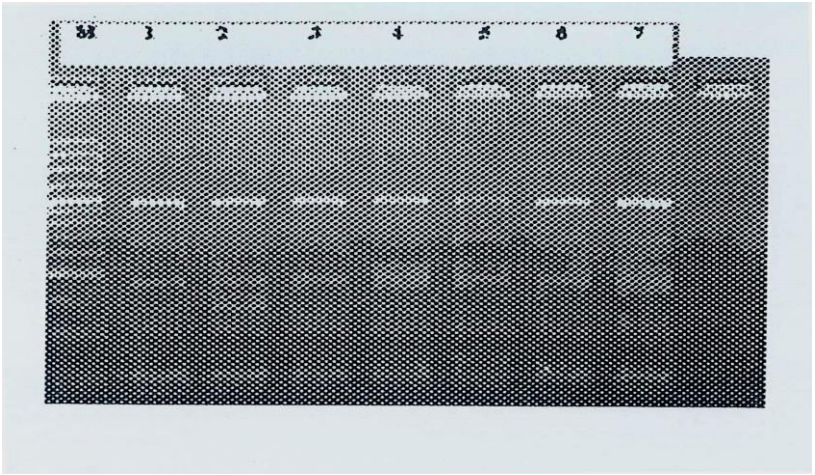

Fig. (7): RAPD banding patterns of *S.sclerotiorum* isolates obtained by the primer No. 2 and electrophoresed on polyacrylamide gel.

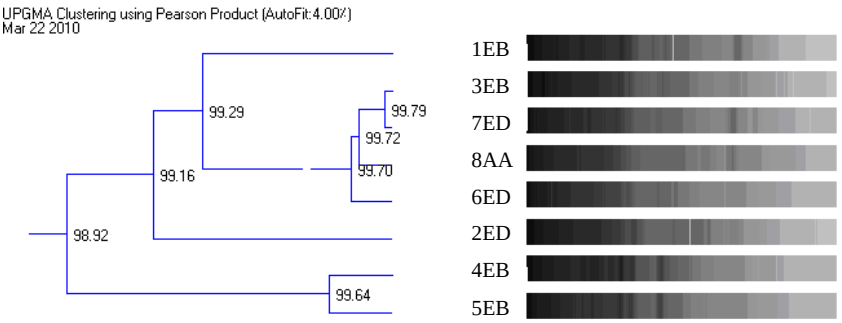

Fig. (8): Phenogram based on cluster analysis of RAPD banding patterns of *S.sclerotiorum* isolates obtained by the primer No. 2 and electrophoresed on polyacrylamide gel.

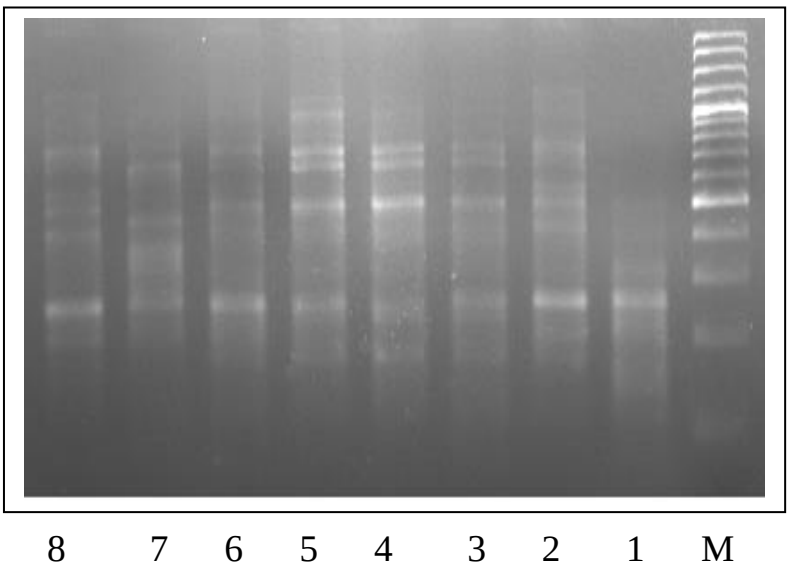

Fig. (9): RAPD banding patterns of *S.sclerotiorum* isolates obtained by the primer No. 4 and electrophoresed on polyacrylamide gel.

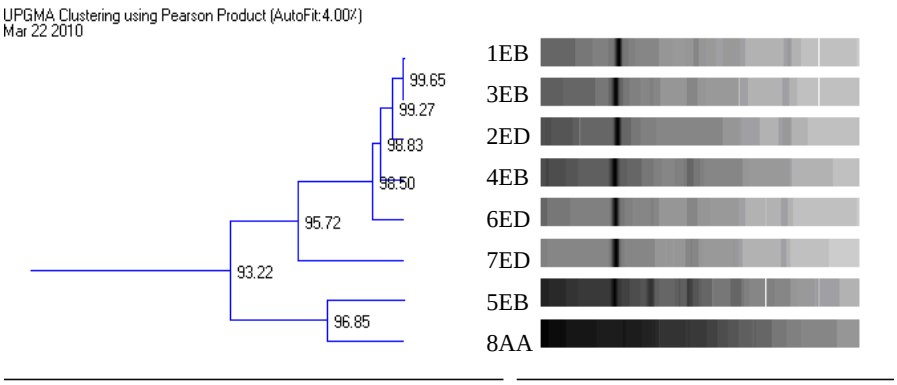

Fig. (10):Phenogram based on cluster analysis of RAPD banding patterns of *S.sclerotiorum* isolates obtained by the primer No. 4 and electrophoresed on polyacrylamide gel.

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الاختلافات الجزيئية فى فطر *Sclerotinia sclerotiorum* المسبب لعفن الأسكليروتينيا فى الفاصوليا العادية فى صعيد مصر

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يعتبر مرض عفن سكليروتينيا فى الفاصوليا العادية من الأمراض الهامة التى تصيب هذا المحصول فى مصر والتى تسبب خسائر كبيرة فى المحصول . ولقد أجرى هذا البحث بهدف دراسة الاختلافات الوراثية والقدرة المرضية بين ثمانية عزلات للفطر *Sclerotinia sclerotiorum* المسبب لعفن الأسكليروتينيا فى الفاصوليا العادية والتى عزلت من مناطق مختلفة من محافظة المنيا وأسيوط وذلك بإجراء تحليل للبروتين وأنزيم الاستيريز والحمض النووى DNA للعزلات المختبرة .

وتتلخص النتائج فيما يلى :

1- كانت جميع العزلات المختبرة من فطر *S. sclerotiorum* لها القدرة على إصابة الصنف بلدى من الفاصوليا العادية وأعطت أعراض عفن الأسكليروتينيا وتراوحت قدرة الإصابة من متوسطة إلى شديدة (60-100%) .

2- أظهرت نتائج التوافق الميسليومى بين العزلات المختبرة أن هذه العزلات يمكن وضعها فى ثمانية مجموعات توافقية كما يلى :
ثلاثة من هذه المجموعات تحتوى كل مجموعة على عزلتين مختلفتين (3EB, 5EB) و (3EB , 6ED) و (5EB , 6ED) أما باقى المجموعات فتحتوى كل منهم عزلة واحدة متوافقة مع نفسها .

3- أظهر التحليل العنقودى الكهربائى لأنماط البروتين الخام والمفكك وكذلك التحليل الكهربائى لأنماط طرز أنزيم استيريز لعزلات فطر *S. sclerotiorum* أن هذه العزلات يمكن وضعها فى مجموعات مختلفة فيما بينها من حيث درجة التماثل .

4- أظهر الفحص باستخدام تكنيك الإكثار العشوائى للحامض النووى متعدد الأشكال (RAPD) باستخدام نوعين من البادئ أن البادئين ليس لهما القدرة على إظهار الفروق بين طرز الحامض النووى فى العزلات المختبرة وذلك لأن درجة التماثل عند استخدام البادئ رقم 2 هى 98.92% بينما كانت 93.92% عند استخدام البادئ رقم 4 .