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MOLECULAR ASPECTS OF SOME *Macrophomina phaseolina* ISOLATES PATHOGENIC TO SUNFLOWER IN EGYPT

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ABSTRACT

Macrophomina phaseolina is one of the most prevalent sunflower (*Helianthus annuus* L.) pathogens in Egypt. Very little is known about the genetic diversity of this pathogen. Mycelial compatibility test among 16 pathogenic isolates obtained from sunflower plants, showing charcoal rot from different government, showed that isolates of Beni-Sweif could be 2000 considered one mycelial compatibility group, but the remaining isolates gave incompatible reaction. Electrophoresis analysis of SDS protein content of mycelium of all isolates showed the presence of common bands with molecular weights of 16-17, 25-26, 28-29, 35-36, 38-39, 48-49, 52-53, 60-61, 74-75, 79-80 and 84-85 K.dal among them. These common bands could be used in quantitative determination of such pathogen in soil. RAPD-DNA analysis using two randomized primers, A (5`-CCCGTCAGCA-3`) and B (5` -CCCGTCAGCA-3`), showed very interesting figure. Four common bands were obtained by primer A, and other three common bands were found with primer B.

INTRODUCTION

Macrophomina phaseolina is one of the most important soil borne pathogens. It infects many economic host plants causing charcoal rot disease on various crops in clouding sunflower. (Kolte, 1984., Bruton, *et al.*, 1987., Mihial, *et al.*, 1988., Desai, *et al.*, 1988 and El-Fiki, *et al* 2002).Isolates of *M.phaseolina* differ from different points of view; they varied in their ability to use certain nitrogen compounds, mycelial features, production of pycinidia and their pathogenicity on different host plants. The aim of the present study is to determine mycelial compatibility groups of Egyptian isolates of *M.phaseolina* isolated from sunflower plants, as well as the patterns of soluble protein and random amplified polymorphic DNA in order to use these molecular techniques in quantitative determination of such pathogen in soil.

MATERIAL AND MATHODS

1. Isolation of *M.phaseolina* from sunflower plants:

Charcoal rot pathogen was isolated from different samples, taken from roots, crown and pith showing typical symptoms of charcoal rot infection obtained from different sunflower growing governorates in Egypt *i.e.* Kalyobia, Fayoum, and Beni–Sweif. Samples were washed thoroughly with tap water, and then surface sterilized with sodium hypochlorite 1% for 2 min and dried between sterilized absorbent papers. Small plant pieces (2-5 mm) were placed on to potato dextrose agar (PDA) supplemented with streptomycin sulfate (20 µg/ml) and incuobated for 7 days at 28C° .Hyphal tips were taken

from the developed colonies and transferred to (PDA) slants .Several isolates were established and stored as mycelial colony on (PDA) slants at 5C°. Identification of the isolates was carried out based on taxonomic criteria for *Macrophomina phaseolina* as described by (Dhingra and Sinclair, 1973).

2. Mycelial compatibility:

The Petri dishes were inoculated with four discs (5mm diameter agar) removed from actively growing cultures of individual *M.phaseolina* isolates. Cultures were incubated at 29C° and examined 7-10 days after inoculation. All discs were examined visually for hyphal fusion and other hyphal interaction, such as hyphal by passing or repelling each other. Assessments of compatibility were based on mycelial continuity between the interacting colonies without formation of either a strip of thin mycelium or aerial mycelium. Assessment of incompatibility was based on the failure of four colonies to fuse, which was indicated by the formation of a strip of thin mycelium or aerial mycelium at the interaction zone. Mycelial incompatibility could also be indicated by the formation of a dark line along the interaction zone associated with death at the point of fusion, (Earnshaw and Boland, 1997).

3. Protein pattern profile:

Protein patterns of 16 isolates of *M.phaseolina* were compared using the SDS-PAGE technique.

3.1. Protein extracts:

Protein extract of 16 pathogenic isolates was prepared according to (Rataj *et al.*, 1984). Isolates of *M.phaseolina* were grown at 29C° on liquid Czapk's medium for7 days, then mycelial mats were harvested by filtration through cheesecloth, washed with distilled water several times, and freeze-dried. This frozen mycelium was suspended in phosphate buffer pH 8.3 (1ml /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 min, to remove debris. The protein content in the supernatant extracts was estimated according to (Bradford, 1976) by using bovine serum albumin as a standard protein. Protein content was adjusted to 2 mg /ml per sample.

3.2. Electrophoresis of protein:

The thawed protein extract supernatant was mixed with an equal volume of a solution containing 20 % glycerol (v/v) and.1 % bromphenol blue (v/v) in 0.15 M Tris-Hcl, pH 6.8. Twenty micro liters of the resulting suspension (40-60 µg of protein) was subjected to electrophoresis in 25 mM Tris–buffer containing 192 mM glycin at pH 8.3. Electrophoresis was conducted at room temperature (approximately 20-22C°), for 9 hrs on an 15 % polyacrylamide gel with a 6 % stacking gel at 20 and 10 mM, respectively, until the dye reached the bottom of the separating gel. Electrophoresis was performed in a vertical slab model (16.5 x 14.5 x 0.1 cm) and the gel was stained using the silver nitrate methods (Sammons *et al.*, 1981).

3.3. Cluster analysis:

Electrophoresis protein patterns of the16 isolates (obtained by the SDS-PAGE technique) were clustered (Jobseph *et al.*, 1992), by the average

linked technique (un-weighed pair-group , method). The results were expressed as phonograms; cluster analysis was performed with a computerized program.

3.4. Gel analysis;

All the obtained gels were scanned for band RF using gel documentation system (AAB Advanced American Biotechnology 1166E. Valencia Dr. Unit6 C, Fullerton CA 92631). The different M.W. of bands were determined against PCR marker Pro mega G 317A.

4. Deoxyribonucleic acid (DNA) analysis:

4.1. DNA isolation:

DNA was isolated from 50 mg of mycelial growth 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 quanta" system-Pharmacia Bio-tech. The purity of the DNA for all samples were between 90-97 % and the ratio between 1.7-1.8. Concentration was adjusted at 6 ng /µl for all samples using TE buffer pH 8.0.

4.2. Random amplified polymorphic DNA (RAPD) technique:

Thirty ng from the DNA were used for the amplification reaction. The polymerase chain reaction (PCR) mixture contained PCR-beads as tablets (manufactured by Amessham Pharmacia Biotech), which containing all of the required reagents except the primer and the DNA which were added to the tablet. The primer was added at 5µL.Their sequences were Primer A (5$^{\backslash}$ -GTTTCGCTCC-3$^{\backslash}$) and Primer B (5$^{\backslash}$-CCCGTCAGCA-3$^{\backslash}$). 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.

4.3. Amplification product analysis:

All the amplified DNA samples were electrophoresed (15 µl) using electrophoresis unit (WIDE mini-sub-cell GT Bio-RAD) on 1 % agarose containing ethedium bromide (0.5 µg /ml), at 75 constant volt, and determined with UV transilluminator.

4.4. Gel analysis;

All the obtained gels were scanned for band RF using gel documentation system (IAS Imaging and Analysis Software Bio-Rad. Diversity data base V.2 Release 4. CA 92631). The different M.W. of bands were determined against PCR marker Pro mega G 317A.

RESULTS

Sixteen pathogenic isolates of *M.phaseolina* were isolated from sunflower plants showed charcoal rot symptoms. These isolates were announced by the letter M. Isolates M1, M2, M3 and M4 were isolated from different locations belong to Beni-Sweif governorate during 2000 agricultural season. Isolates M5, M6, M7 and M8 were isolated from plants collected from different regions of Fayoum governorate. Isolates M9, M10, M11, M12, M13 and M14 were isolated from Kalyobia governorate during 2001 agricultural season. Isolates M15 and M16 were isolated from Beni-Sweif governorate during 2001 agricultural season.

1. Compatibility groups of *M.phaseolina* isolates:

Compatibility test between all 16 isolates were carried out. Fig (1) and Data in Table (1) showed that isolates M1, M2, M3 and M4 which isolated from Beni-Sweif governorate during 2000 agriculture season belong to the same mycelial compatibility group, therefore, margins of their colony was interfered between each other. Other 12 isolates were found to belong to different mycelial compatibility groups, whereas, their mycelial margins did not interfere between each, this led to appearance of inhibition zoon between their margins. Although isolates M15 and M16 were isolated from the same governorate (Beni-Sweif), but from other regions during 2001 agriculture season, they appeared as distinct mycelial compatibility groups.

Fig. 1. Example of mycelial compatibility test between isolates of *Macrophomina phaseolina* (A= Compatible reaction & B= Incompatible reaction).

Table (1): Mycelial compatibility among 16 isolates of *Macrophomina phaseolina*, from sunflower plants in Egypt.

Isolate code	Isolate code															
	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16
M 1	C	C	C	C	I	I	I	I	I	I	I	I	I	I	I	I
M 2		C	C	C	I	I	I	I	I	I	I	I	I	I	I	I
M 3			C	C	I	I	I	I	I	I	I	I	I	I	I	I
M 4				C	I	I	I	I	I	I	I	I	I	I	I	I
M 5					C	I	I	I	I	I	I	I	I	I	I	I
M 6						C	I	I	I	I	I	I	I	I	I	I
M 7							C	I	I	I	I	I	I	I	I	I
M 8								C	I	I	I	I	I	I	I	I
M 9									C	I	I	I	I	I	I	I
M 10										C	I	I	I	I	I	I
M 11											C	I	I	I	I	I
M 12												C	I	I	I	I
M 13													C	I	I	I
M 14														C	I	I
M 15															C	I
M 16																C

I = Incompatible reaction. C = Compatible reaction.

2. SDS profile of protein pattern of *M.phaseolina* isolates:

SDS electropheretic pattern protein of all tested isolates showed that all isolates shared in bands with molecular weights (16-17, 25-26, 28-29, 35-36, 38-39, 48-49, 52-53, 60-61, 74-75, 79-80, 84-85 K.dal) Fig (2).

The resulting protein profiles after SDS-PAGE (Fig 2) were normalized and analyzed with the AAB software. This programe calculated the genetic similarities (GS) and differences among each protein profile, with the Pearson's product moment correlation coefficient (r) among samples to construct a matrix. The samples were then clustered using the UV weighted pair group method of arithmetic average (UPGMA) which resulted in a dendrogram. Based on protein profile's markers, genetic similarities (GS) were calculated among the sixteen isolates of *M. phaseolina*. Cluster analysis of the protein markers data placed the *M. phaseolina* isolates into two main groups. The genetic similarity among *M. phaseolina* isolates ranged from (7.5 to 96.8 %) the first main group cluster included isolate M8 (from Fayoum) with genetic similarity (7.5%) with other isolates. The second main group cluster included all the remaining isolates at the genetic similarity (57.4%). The second main group cluster was divided into two subgroups, the first included M1 (Beni-Sweif 2000) M7 (Fayoum 2000) M9, M10, M11 (Kalyobia2001) and M16 (Beni-Sweif 2001) with 96.7% genetic similarity. The second subgroup included M2, M3, M4 (Bine-Sweif 2000) M5, M6 (Fayoum 2000) M12, M13, M14 (Kalyobia 2001) and M15 (Beni-Sweif 2001) with 82.7% genetic similarity.

3. RAPD analysis of nine isolates of *M.phaseolina*

RAPD analysis of genomic DNA was performed on nine isolates of *M. phaseolina* (M1, M4 from Beni-Sweif 2000 and M16 from Beni-Sweif 2001), (M5, M6 and M8 from Fayoum 2000) and (M9, M12 and M13 from Kalyobia 2001) using two primers A (5`-GTTTCGCTCC-3`) and B (5`-CCCGTCAGCA-3`).Analysis of DNA fragments obtained from RAPD using both primers are shown in (Fig 3).

Primer A (5` - GTTTCGCTCC-3`): data illustrated by Fig (4) and represented in Table (2 A) clearly shown that primer A gave 9 bands for all isolates, common band with 292, 347, 508, 671 and 960 bp, were found among all tested isolates. Dendrogram derived from RAPD profile analysis indicated that isolates were represented into two clads, the first contained M9 (Kalyobia, 2001), M16 (Beni-Sweif, 2001), M12 (Kalyobia, 2001), M5 (Fayoum, 2000), M8 (Fayoum, 2000), M6 (Fayoum, 2000), M4 (Beni-Sweif, 2000) and M1 (Beni-Sweif, 2000) the second clad contained only one isolate M13 (Kalyobia, 2001). Great similarities were shown among isolates {(M9-M16) Kalyobia 2001-Beni-Sweif 2001}, {(M8-M6) Fayoum2000} and {(M4-M1) Beni-Sweif 2000}.

Primer B (5` -CCCGTCAGCA-3`): gave 10 bands for all isolates represented in Table (2 B). Common bands with 508, 792 and 1189 bp among all tested isolates were shown. Dendrogram of the tested isolates was divided into two clads, the first contained isolates M8 (Fayoum 2000), M6 (Fayoum 2000), M12 (Kalyobia 2001) and M4 (Beni-Sweif 2000), the second contained isolates M13 (Kalyobia 2001), M16 (Beni-Sweif 2001), M5 (Fayoum 2000), M9 (Kalyobia 2001) and M1 (Bine-Sweif 2000). Similarities

were found among isolates M8 (Fayoum2000), M6 (Fayoum2000) and M12 (Kalyobia 2001) and between isolates M16 (Beni-Sweif 2001), M5 (Fayoum 2000), M9 (Kalyobia2001) and M1 (Beni-Sweif 2000) (Fig 5).

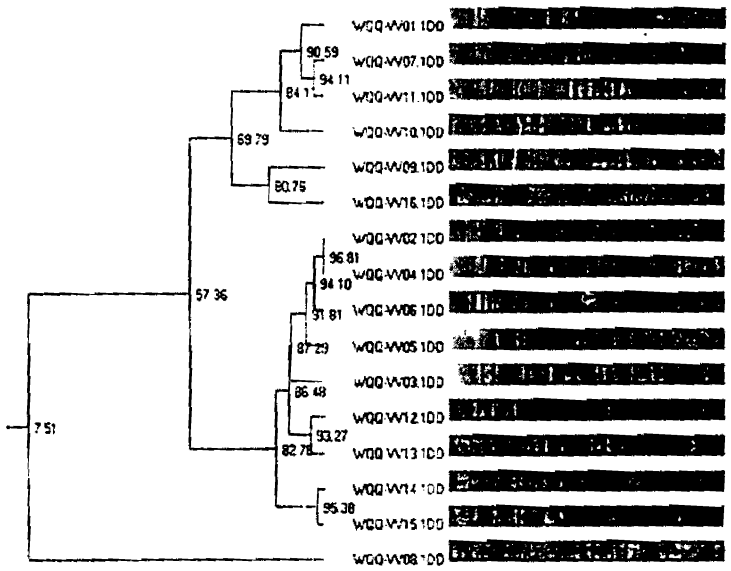

Fig. 2.
Dendrogram for protein profile of 16 Egyptian isolates of *Macrophomina phaseolina* from sunflower plants.

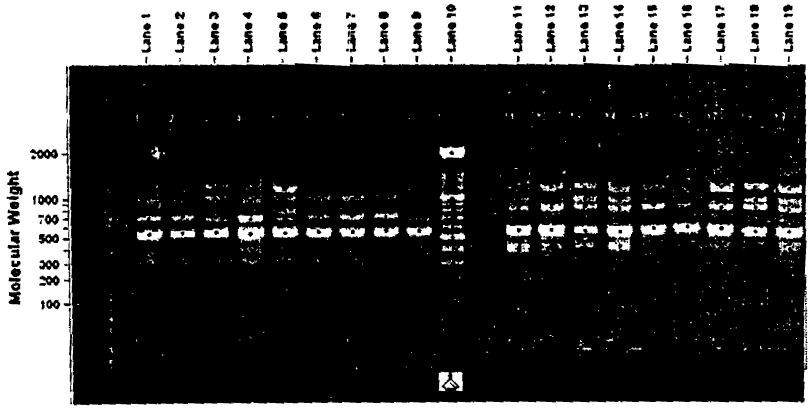

Fig. 3. RAPD profile analysis of nine *Macrophomina phaseolina* isolates using two primers A (5' - GTTTCGCTCC- 3') & B (5' - CCCGTCAGCA- 3').

Table: (2A,B). Digitized patterns and dendrogram derived from RAPD profile analysis of nine *Macrophomina phaseolina* isolates using two primers A & B.

		Molecular	Isolate No.								
		weight	1	9	5	16	4	12	13	6	8
	1	1189	1	1	1	1	1	0	0	0	0
	2	960	1	1	1	1	1	1	1	1	1
	3	792	0	0	0	0	1	0	0	0	0
A	4	671	1	1	1	1	1	1	1	1	1
	5	508	1	1	1	1	1	1	1	1	1
	6	397	0	0	0	0	0	0	0	0	0
	7	369	0	0	0	0	1	1	0	1	1
	8	347	1	1	1	1	0	0	1	0	0
	9	292	1	1	1	1	1	1	1	1	1
		Molecular	Isolate No.								
		weight	1	6	4	8	12	13	16	5	9
	1	2222	0	0	0	0	0	0	1	0	1
	2	1670	0	0	0	0	1	0	1	0	1
	3	1189	1	1	1	1	1	1	1	1	1
B	4	960	1	0	1	1	1	0	1	1	1
	5	792	1	1	1	1	1	1	1	1	1
	6	671	0	0	0	0	0	0	0	1	1
	7	508	1	1	1	1	1	1	1	1	1
	8	397	1	0	1	0	0	0	0	1	1
	9	369	1	1	1	1	0	0	1	0	1
	10	347	1	1	0	1	0	0	0	0	0

Analysis of RAPD-DNA of the two primers together gave another dendrogram (Fig 6). It is clearly shown from this dendrogram that the tested isolates were isolated into two clades. The first contained isolates M9 (Kalyobia 2001), M16 (Beni-Sweif 2001), the similarity 87% M12 (Kalyobia 2001), M8, M6 (Fayoum 2000), the similarity 91% and M4 M1 (Beni-Sweif 2000) the similarity 92.5% and the second clade contained isolate M13 only.

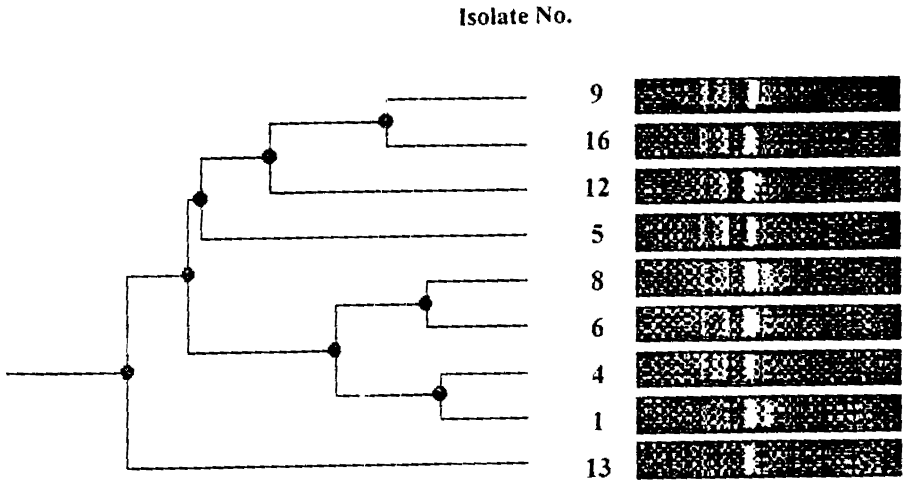

Fig. 4. Digitized patterns and dendrogram derived from RAPD profile analysis of nine *Macrophomina phaseolina* isolates using primer A(5'-GTTTCGCTCC-3').

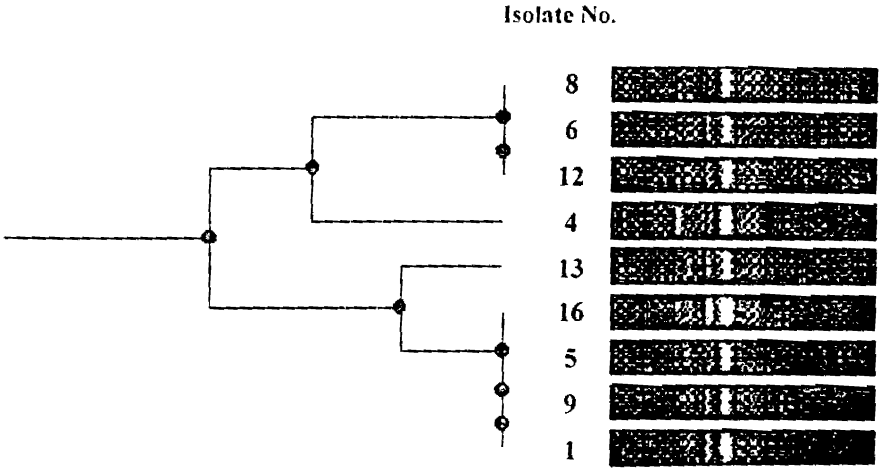

Fig. 5. Digitized patterns and dendrogram derived from RAPD profile analysis of nine *Macrophomina phaseolina* isolates using primer B (5' - CCCGTCAGCA- 3').

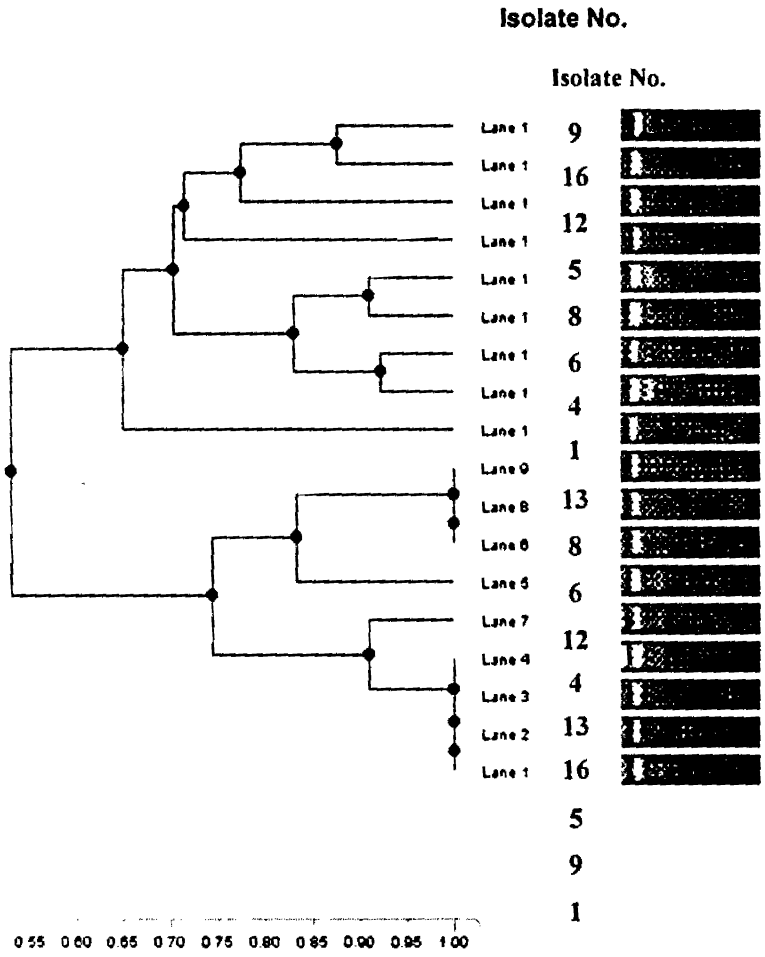

Fig. 6. Digitized patterns and dendrogram derived from RAPD profile analysis of nine *Macrophomina phaseolina* isolates using tow primers A (5' - GTTTCGCTCC- 3') & B (5' - CCCGTCAGCA- 3').

Isolates in the first clad were divided into two sub-clads, the first contained isolates M8, M6 (Fayoum 2000), M12 (Kalyobia 2001), and M13 (Kalyobia 2001) and the second contained isolates M16 (Beni-Sweif 2001), M5 (Fayoum 2000), M9 (Kalyobia 2001) and M1 (Beni-Sweif 2000). It is clearly shown the presence of strong similarity 100% among isolates M8, M6 and M12. The similarity 100% among isolates M16, M5, M9 and M1.

DISCUSSION

Sixteen pathogenic isolates of *Macrophomina phaseolina* were isolated from sunflower plants showed charcoal rot symptoms during 2000

and 2001 agricultural seasons. Since the isolates of fungi are differ in many morphological and physiological features, the concept of mycelial compatibility and incompatibility was put in order to grouping the isolates belong to the same species in groups (Earnshaw and Boland, 1997).The results of this investigation demonstrated that the isolates of *M.phaseolina* (M1, M2, M3 and M4) from Bine-Sweif were showed compatibility between them this may be due to the heterogeneicty is large in this location. The results also indicated that the heterogeneicty within other locations is not exists and that may be due to some other factors changing their compatible reaction through mutation, hybridization etc. All this causes led to incompatibility.

Electrophoresis analysis of SDS protein content of mycelial mates of all isolates showed the presence of common band with molecular weights 16-17, 25-26, 28-29, 35-36, 38-39, 48-49, 52-53, 60-61, 74-75, 79-80 and 84-85 K.dal among them. These common bands could be used in quantitative determination of such pathogen in soil using ELISA test. Phenograms of SDS protein indicated the separation of all isolates into two groups; the first contained all isolates except isolate M8 which separated in district group. The first subgroup was separated into two philials, the first contained isolates M1, M7, M11, M10, M9 and M16 and the second philial contained isolates M2, M4, M6, M5, M3, M12, M13, M14, and M15.

Fortunately, RAPD-DNA analyses using two randomize primers, the first consisted of (5\-CCCGTCAGCA-3\) and the second consisted of (5\-CCCGTCAGCA-3\) showed very interesting figure. Four common bands were obtained by primer A, and other three common bands were found with primer B. The pattern of DNA separation has significant importance. Comparing this pattern of *M.phaseolina* using these two primers with other pattern obtained by these two primers but in other fungi,(EL-harrany M. Omnia personal communication), clearly indicated the ability of such primers two identify *M.phaseolina* and may also be used in detection of such dangerous fungus in the soil.

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السمات الجزيئيه لبعض عـزلات فطـر *Macrophomina phaseolina* الممرضة لعباد الشمس في مصر

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يعتبر فطر ماكروفومينا فاصولينا من أهم الممرضات التي تصيب عباد الشمس في مصر، وهنـاك من المعلومات القليل عن التركيب الجينى لهذا الممرض. تم اختبار التوافق الخضري الميسليوم٣ى لعـزلات الفطر المعزولة من نباتات عباد الشمس المصابة بالعفن الفحمي من محافظات مختلفة حيث أعطت العزلات المتحصل عليها من محافظة بنى سويف عام ٢٠٠٠ م توافق خضري ميسليومى بينمـا لـم تعطـى بـاقي العزلات أي توافق خضري ميسليومى ، أظهر التفريد الكهربي للمحتوى الكلى للبروتين بطريقـة SDS أن هناك حزما مشتركة بين العزلات و كانت اوزانها الجزيئية١٦-١٧، ٢٥-٢٦،٢٨-٢٩، ٣٥-٣٦، ٣٨-٣٩، ٤٨-٤٩، ٥٢-٥٣، ٦٠-٦١، ٧٤-٧٥، ٧٩-٨٠، ٨٤-٨٥ كيلو دالتون. وقد تـستخدم هذه الحزم في تقدير الفطر بالتربة. كما أظهر التفريد الكهربي العشوائي لل DNA باستخدام ٢ بادئ عشوائي: الأول تركيبة -5') GTTTCGCTCC-3') والثاني تركيبة (5')-CCCGTCAGCA-3') وجود تباين بين عزلات الفطر، وأعطى كل من البادئ الأول أربعة حزم رئيسية بينما البادئ الثاني أعطى ثلاث حزم رئيسية وكانـت هـذه الحزم مشتركة بين عزلات الفطرالمختلفة.