INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
1560–8530/2007/09–6–807–815
http://www.fspublishers.org

Characterization of *Macrophomina phaseolina* Isolates Affecting Sunflower Growth in El-Behera Governorate, Egypt

S.S. ABOSHOSHA, S.I. ATTA ALLA†, A.E. EL-KORANY[1]† AND E. EL-ARGAWY†
Plant Pathology Department, Faculty of Agriculture, El-Shatby, Alexandria University, Egypt
†Plant Pathology Department, Faculty of Agriculture, Damanhour, Alexandria University, Egypt
[1]Corresponding author's e-mail: drelkorany125@yahoo.com

ABSTRACT

Thirty three *Macrophomina phaseolina* isolates were recovered from different regions where sunflower was grown in El-Behera governorate during the 2003 and 2004 growing seasons. Housh Eisa and Abo El-Matameer were the most affected regions as 33.3% and 30.3% of the total isolates were recovered, respectively. This was followed by Etay El-Barood, where 12.1% of the isolates were recovered. Occurrence of *M. phaseolina* in rest of the surveyed regions *i.e.*, El-Dalangat, Kafr El-Dawar and El-Mahmoudia was less than 10%. The recovered *M. phaseolina* isolates were characterized for pathogenicity, colony phenotype, protein banding pattern and the random amplified polymorphic DNA (RAPD). All the recovered isolates were pathogenic to both of the tested sunflower cultivars, H11-008 and H11-009, in the pathogenicity test. However, 27.8% of the isolates (combined data) were identified as highly, 28.1% intermediately and 44.1% lowly pathogenic phenotype. Five color phenotypes and six growth patterns were recognized on PDA, Czapek-Dox and Chlorate media. These were white, grey, black, dark green and brown colony color phenotypes; grey being the most frequent (30.3%). Colony growth patterns recognized were the dense, light, sparse dense, sparse, restricted and shy growth; dense being the most frequent (40.3%). Eleven isolates *i.e.*, 33.3% of the total 33 *M. phaseolina* isolates analyzed, were Chlorate sensitive, while rest were Chlorate resistant. All the isolates showed the expression of 8 - 100 kDa polypeptides with 78.4% mean content and exhibited 6 - 20 polypeptide bands in this kDa range with 34.5% of the isolates were differentiated with unique bands. At the higher 101 - 300 kDa protein analysis, 27.4 - 51.3% of the isolates only contained polypeptides of this kDa range with little variation (2 - 6 bands) and less differentiation (6.7 - 21.7%). Moreover RAPD-PCR analysis using eight random oligonucleotide primers revealed DNA fingerprints 2 - 9 bands ranging from 350bp to 3kbp. However, no variations were revealed for number of bands between isolates with primers OPS-16 or OPA-2, while more variations were revealed with OPA-1, OPA-4, OPA-5, OPA-8, OPA-13 and OPR-14 primers. The stem type of *M. phaseolina* isolates exhibited higher pathogenicity as revealed from size of the lesions incited, plant death and plant head reduction in the pathogenicity tests as compared to the root type of isolates showing these values as 6.1 cm, 37.9% and 37.4%. However, no distinct colony phenotype or protein banding pattern was linked to any of the both types of isolates or even any of the high, intermediate and the low pathogenic phenotypes. However, the RAPD-PCR phylogenetic analysis did not reveal known genetic entities for the stem type and the root type of isolates, since it did revealed two clusters for the high pathogenic isolates and the low pathogenic isolates in the Dendrogram of the tested *M. phaseolina* isolates.

Key Words: Characterization; *Macrophomina phaseolina*; Sunflower; Egypt

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is an oilseed crop characterized with its short growing period, high yield potential, wide range of growing season, low water requirements, wide adaptability to soil condition and its high content (over 40%) of good edible oil (Weiss, 2000). Production of sunflower in Egypt was about 1627 tons in the year 2005, where El-Behera governorate was a major area for sunflower cultivation (Dept. Statistics, Ministry Agric., El-Giza, Egypt). Unfortunately, Sunflower is subjected to be attacked with a variety of fungal pathogens, which affect its yield and its oil quality (Sangawan *et al*., 2005). *Macrophomina phaseolina* (Tassi) Goid., is the most fungal pathogens affecting sunflower in Egypt and worldwide and causing the charcoal-rot disease, not only on sunflower but also on more than 500 plant species throughout the world (Purkayastha *et al*., 2006). Currently, the binomial nomenclature of *Macrophomina phaseolina* is applied to both the microsclerotial and pycnidial anamorphs, however the microsclerotial phase is the one predominantly observed worldwide (Dhingra & Sinclair, 1978). Different synonyms have been ascribed to the fungus *Macrophomina phaseolina* (Tassi). This including *Macrophomina phaseoli* (Maubl.) Ashby, *Macrophoma conchoci* Swada, *Sclerotium bataticola* Taub., and *Rhizoctonia bataticola* (Taub.) (Mihail, 1992). Although, only one species is recognized within the genus (Mihail & Taylor, 1995), great variability in morphology and pathogenicity was recognized among isolates from different host species and between isolates from different parts of the same plant (Fernandez *et al*., 2006). Efforts were made to characterize the fungus population in different parts of the world. This was based on its pathogenic variability (Karunanithi *et al*., 1999), the

morphological characteristics (Fernandez *et al*., 2006), as well as the molecular characteristics (Almeida *et al*., 2003; Jana *et al*., 2003; Purkayastha *et al*., 2006). However, little is known about characteristics of *Macrophomina phaseolina* population in Egypt. The present study, therefore was conducted to reveal characteristics of the fungus population occurring in El-Behera governorate, which is a major area for sunflower cultivation in Egypt. This was for a better understanding of the population structure of the fungus and its epidemiology in the region.

MATERIALS AND METHODS

Occurrence of *M. phaseolina* on sunflower in El-Behera governorate. In the two successive seasons of 2003 and 2004, six different regions, where sunflower was grown in El-Behera governorate were surveyed for occurrence of the charcoal-rot disease caused by *M. phaseolina* isolates. The surveyed regions were Housh Eisa, Abo El-Matameer, Etay El-Barood, El-Dalangat, El-Mahmoudia and Kafr El-Dawar. Diseased plants were collected, wherever detected throughout the two growing seasons.

Isolation and identification of *M. phaseolina* isolates. Diseased stem and root samples were washed thoroughly with running tap water, air dried and cut into small pieces. Small infected portions were surface sterilized with 2% sodium hypochlorite solution for 2 min, rinsed in sterilized distilled water, dried in sterilized filter paper and plated on PDA. Inoculated plates were then incubated at 28 ± 2ºC in darkness for 5 days and investigated for *M. phaseolina* colonies. Hyphal tips or single microsclerotium were obtained and transferred to fresh acidified PDA plates. Developed colonies were identified according to Dhingra and Sinclair (1978) and Barnett and Hunter (1987) and maintained on PDA slants.

Pathogenicity and pathogenic variability of the recovered *M. phaseolina* isolates. Pathogenicity and pathogenic variability tests were conducted on two susceptible sunflower cultivars to charcoal-rot disease (Aboshosha, unpublished data). The tested cultivars were H118-008 (H08) and H118-009 (H09). Seeds of the tested cultivars were obtained from Seed Department, Ministry of Agriculture, El-Giza, Egypt. Seeds were surface disinfested with 2% sodium hypochlorite, rinsed in sterile distilled water and sown in 25-cm plastic pots filled with autoclaved clay and sand (1:1, v:v). Four replicate pots each was sown with two seeds of the tested sunflower cvs. were prepared for each tested isolate. Plants were watered as needed and treated according to the normal agricultural practices. The 60-days-old plants were stem inoculated with the tested isolates in the second internode using the stem-tape inoculation technique of Zazzerini and Tosi (1989). Ten days after inoculation, developed lesions were measured in cm as the longitudinal bark necrosis below and above the site of inoculation. Percentage of plant death and percentage of reduction in plant head size were also determined 21 days after inoculation according to El-Deeb *et al*. (1985). Re-isolation was conducted to insure the association of the tested isolates with the developed disease.

Colony phenotypes of the recovered *M. phaseolina* isolates. All the recovered *M. phaseolina* isolates were characterized for colony colour and growth pattern on Potato Dextrose Agar (PDA) and Czapek-Dox Agar (CD) media (Booth, 1971) as well as on the Chlorate selective medium (Pearson *et al*., 1986). Plates of the different media were inoculated with mycelia discs (0.5 mm in diameter) taken from the advancing margin of 7-days-old PDA culture of the tested isolates. Five replicate plates were prepared for each isolate on each medium and incubated at 28 ± 2ºC for seven days in darkness. The developed colonies were characterized for the colony phenotype and the growth pattern according to Pearson *et al*. (1986) and Atiq *et al*. (2001).

Molecular Characterization of the Recovered *M. phaseolina* Isolates

a. Protein analysis. All the recovered *M. phaseolina* isolates were grown on 25 mL of potato dextrose broth at 28 ± 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.

b. DNA analysis. Four *M. phaseolina* isolates representing the stem and root type of isolates and the most high and low pathogenic isolates in each group were analyzed for DNA banding pattern.

- 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 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 *M. phaseolina* isolates was amplified by the RAPD methods (Williams *et al*., 1990) using eight random oligonucleotide primers shown in (Table I). 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

Table I. Primers used in the present study and their nucleotide sequence

	Primers	Nucleotide Sequence
1-	OPA- 1	5 CAGGCCCTTC 3
2-	OPA- 2	5 TGCCGAGCTG 3
3-	OPA- 4	5 AATCGGGCTG 3
4-	OPA- 5	5 AGGGGTCTTG 3
5-	OPA- 8	5 GTGACGTAGG 3
6-	OPA-13	5 CAGCACCCAC 3
7-	OPS-16	5 AGGGGGTTCC 3
8-	OPR-14	5 CCAGCCGAAC 3

ethidium bromide and photographed under UV light (302) according to Jana *et al*. (2003). All chemicals were Bio-Rad products.

c. Phylogenetic analysis. Bands pattern of DNA developed in the RAPD-PCR analysis were scored visually for each tested isolate. 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, version 6 and means were compared by the least significant difference test (LSD).

RESULTS

1- Occurrence of *M. phaseolina* on sunflower in El-Behera governorate. A total of 33 isolates of *M. phaseolina* were recovered from sunflower plants showed charcoal-rot symptoms in six regions surveyed in El-Behera governorate. The most affected region was Housh Eisa as 11 isolate were recovered, which constituted 33.3% of the total isolates recovered. This was followed by Abo El-Matameer, where 10 isolates were made *i.e.,* 30.3% of the total isolates recovered. Four more isolates were made from Etay El-Barood and three isolates from each of El-Dalangat and Kafr El-Dawar, which constituted 12.1%, 9.0% and 9.0% of the total isolates for these regions, respectively. El-Mahmoudia region, however showed the least disease incidence as only tow isolates were recovered *i.e.,* 6.0% of the total isolates recovered (Fig. 1 & Table II).

In the 2003 first season, thirteen *M. phaseolina* isolates were recovered from the collected diseased samples six of which were occurring on stem samples, while seven were occurring on diseased roots. In the second season (2004), twenty more isolates were made. Eight of which were occurring on affected stem samples, while twelve isolates were occurring on affected root samples (Table II).

2- Pathogenicity and pathogenic variability of the recovered *M. phaseolina* isolates. All the recovered *M. phaseolina* isolates tested were pathogenic to both of the tested sunflower cvs. *i.e.,* H11-008 (H08) and H11-009 (H09) as incited stem lesions ranged between 2.9 cm and 19.7 cm in the pathogenicity test (Table III). Meantime, mean lesions size incited by stem isolates was 7.1 cm compared to 6.1 cm for the root isolates. Also, isolates were variable in their ability to cause plant death. That was in the range of 0% - 100% of the non-inoculated control on the tested cvs. with total means of 48.6% and 37.9% for the stem and the root isolates, respectively. Isolates were also variable to cause reduction in head diameter of the sunflower plants. This ranged between 4.1% and 77.6% of the non-inoculated control on the tested cvs. with means of 44.2% and 37.4% for the stem and the root isolates, respectively (Table III).

Almost 35.8% of the total (33) *M. phaseolina* recovered isolates were classified as highly pathogenic (≥ 75% aggressiveness) to cause plant death on the tested sunflower cvs. with means of 42.8% and 28.9% for the stem and the root isolates, respectively (Table IV). On the other hand, 48.4% of the isolates were low pathogenic (≤ 25% aggressiveness) with means of 46.4% for the stem isolates and 50.5% for the root isolates. The intermediate pathogenic isolates (<75% ->25% aggressiveness) were as low as 15.8% of the total isolates with means of 10.7% and 21.0% for the stem and the root isolates, respectively. However, only 19.8% of the total *M. phaseolina* isolate tested were highly pathogenic to caused ≥75% reduction in plant head size with means of 10.7% and 28.9% for the stem isolates and the root isolates, respectively. Also, 40.4% of the isolates were intermediate pathogenic with means of 57.1% for the stem isolates and 23.6% for the root isolates, while 39.7% of the isolates were low pathogenic with means of 32.1% and 47.3% for the stem and the root isolates, respectively (Table IV).

3- Colony Characteristics of the Recovered *M. phaseolina* Isolates

a. Colour phenotypes. Colour phenotype of the recovered isolates of *M. phaseolina* were variable on the different media tested (Table V & Fig. 2). Colonies were mostly grey, dark green, black, or brown for the tested fourteen stem isolates in frequencies of 4, 3, 5, 1 on PDA and 4, 5, 2, 2 on Czapek-Dox Agar (CD), for the previous colour phenotypes, respectively. The white colony phenotype, however was represented by only one isolate on each of the two tested media. The same colony colour phenotypes were revealed for the root isolates, however the grey colony phenotype constituted 7 out of the 19 isolates tested on PDA followed by 4 isolates for both the dark green and the black colony phenotype. On the CD medium, six isolates were of black colonies out of the 19 root isolates tested. This was followed by 5 isolates of the brown colony phenotype, 4 of dark green and 3 of the grey colony phenotype. The white colony phenotype was represented by only two isolates on PDA and one isolate on CD. The Chlorate selective medium however did not reveal much variation as the isolates were mostly either white or grey in almost equal frequencies with only one isolate of the black phenotype in each of the stem and the root *M. phaseolina* isolates (Table V).

b. Growth pattern phenotypes. Most of *M. phaseolina* developed colonies of the tested isolates were either dense, light dense, or sparse dense on the different media tested

Table II. Occurrence of *Macrophomina phaseolina* isolates on sunflower stem and root samples, showed charcoal rot symptoms, collected from different localities in El-Behera governorate during the 2003 and 2004 growing seasons

Season	Plant Part	Housh Eisa	Abo El-Matameer	Etay El-Barood	El-Dalangat	Kafr El-Dawar	El-Mahmoudia	Total	
2003	Stem	3	1	-	1	-	1	6	13
	Root	3	2	1	-	1	-	7	
2004	Stem	1	3	1	1	2	-	8	20
	Root	4	4	2	1	-	1	12	
Total		11	10	4	3	3	2	33	
% of occurrence*		33.3%	30.3%	12.1%	9.0%	9.0%	6.0%		

*= number of isolates recovered from this region/ Total number of isolates made x 100.
Plant Part= plant part from which the isolates were recovered.

Table III. Pathogenicity and pathogenic variability of *Macrophomina phaseolina* isolates, recovered from sunflower stem and root samples collected from different fields in El-Behera governorate during 2003 and 2004 growing seasons, and tested on sunflower cv. H11-008 (H08) and cv. H11-009 (H09) in pot experiment

Season	Plant part	No. of isolates tested	Lesion Size (cm)[1]			% Plant Death[2]			%Head Diameter Reduction[3]		
			H08	H09	M	H08	H09	M	H08	H09	M
2003	Stem	6	9.48	8.40	8.94	50.00	66.66	58.33	44.81	53.08	48.94
2004	Stem	8	6.01	4.66	5.33	37.50	40.62	39.06	39.12	39.80	39.46
Mean			7.74	6.53	7.1a	43.75	53.64	48.60a	41.96	46.44	44.20a
2003	Root	7	6.31	7.85	7.08	46.42	42.85	44.64	43.65	37.84	40.74
2004	Root	12	5.53	4.91	5.22	37.50	25.00	31.25	37.10	31.06	34.08
Mean			5.92	6.83	6.1a	41.95	33.92	37.90a	40.37	34.45	37.40a
Control (non-inoculated)			0.0	0.0	0.0	0.00	0.00	0.00	0.00	0.00	0.00

Eight replicate plants were inoculated with each tested isolate. M = mean [1] measured 10 days after inoculation. [2], [3]estimated 21 days after inoculation of the non-inoculated control, inoculation was conducted in 60-day-old plants. Means in each single column sharing the same letter are not significantly different at 0.5 of probability

Table IV. Pathogenic variability of *Macrophomina phaseolina* isolates recovered from diseased sunflower stem and root samples collected from different fields in El-Behera governorate and tested on cv. H11-008 (H08) and cv. H11-009 (H09) of sunflower in pot experiment

Isolates tested	Number of isolates tested	Sunflower cvs.	% Plant Death[1]						% Head Diameter Reduction[2]					
			H ≥75		I <75->25		L ≤25		H ≥75		I <75->25		L ≤25	
			N	%*	N	%	N	%	N	%	N	%	N	%
Stem isolates	14	H08	5	35.7	2	14.2	7	50.0	2	14.2	8	57.1	4	28.5
	14	H09	7	50.0	1	7.1	6	42.8	1	7.1	8	57.1	5	35.7
Mean %				42.8 a		10.7 b		46.4 a		10.7 b		57.1 a		32.1 b
Root isolates	19	H08	6	31.5	5	26.3	8	42.1	6	31.5	5	26.3	8	42.1
	19	H09	5	26.3	3	15.7	11	57.8	5	26.3	4	21.0	10	52.6
Mean %				28.9 b		21.0 a		50.5 a		28.9 a		23.6 b		47.3 a
				35.8		15.8		48.4		19.8		40.4		39.7

N= number of isolates. [1], [2]estimated 21 days after inoculation of the non-inoculated control, inoculation was conducted in 60-day-old plants. *calculated of the total stem type or root type of isolates tested. Means in each column followed by different letters are significantly different at 0.5 of probability.

(Fig. 2). The sparse growth phenotype was represented by only one stem isolate on each of the PDA and CD media and by two root isolates on PDA. However, on the Chlorate medium, the sparse, the restricted and the shy phenotypes were more frequent and constituted the Chlorate sensitive phenotype, which represented by 6 and 5 isolates of the stem and root isolates tested, respectively. The Dense, light dense and sparse dense phenotypes on the Chlorate medium constituted the Chlorate resistant phenotype and were represented with 8 and 14 isolates of the stem type and the root type of isolates tested, respectively (Table V).

4- Molecular Characteristics of the Recovered *M. phaseolina* Isolates

a. Protein analysis. All the 33 analyzed isolates showed polypeptides bands in 8-100 kilo Dalton (kDa) range (Fig. 3 & Table VI). This was 6 - 18 bands for stem isolates and 9 - 20 bands for root isolates with 74.0 ± 23.4% mean polypeptide content for the stem isolates and 82.8 ± 9.9% for the root isolates. Meantime, 34.5% of the total isolates showed unique bands in this kDa range *i.e.,* isolates with single different distinct bands.

At the 101-200 kDa, occurrence of polypeptides of this molecular weight was 51.3% of the total 33 recovered isolates with little number of bands of 2 - 4 for stem isolates and 2 - 6 for root isolates. Isolates with unique bands of this kDa range constituted 21.7% of the total isolates analyzed. The mean polypeptide contents of the developed bands were almost equal being 12.9% for the stem and the root isolates. However, occurrence of the higher 201 - 300 kDa polypeptides was the least as only 27.4% of the total 33 isolates tested showed polypeptide bands (1 - 3) in this kDa

Table V. Frequency of colony phenotypes of *Macrophomina phaseolina* isolates recovered from sunflower stem and root samples, showed charcoal rot symptoms, collected from different fields in El-Behera governorate and tested on different media

Isolates tested	Number of isolates tested	Media	Colour phenotype					Growth pattern					
			W	G	DG	Br	Bl	D	L	SD	S	R	SH
Stem isolates	14	PDA	1*	4	3	1	5	6	5	2	1	-	-
	14	CD	1	4	5	2	2	3	7	3	1	-	-
	14	Chl	6	5	-	-	1	5	2	1	1	3	2
Mean %			19.0	30.9	19.0	7.1	19.0	33.3	33.3	14.2	7.1	7.1	4.7
Root isolates	19	PDA	2	7	4	2	4	7	9	1	2	-	-
	19	CD	1	3	4	5	6	9	6	4	-	-	-
	19	Chl	8	7	-	-	1	11	2	1	-	2	3
Mean %			19.2	29.8	14.0	12.2	19.2	47.3	29.8	10.5	3.5	3.5	5.2
			19.1	30.3	16.5	9.6	19.1	40.3	31.5	12.3	5.3	5.3	4.9

W= white G= grey DG= dark green Br= brown Bl= black D= dense LD= light dense SD= dense in a sparse pattern. S= sparse or feathery growth. R= restricted growth. SH= shy, *i.e.* no growth was occurred. CD=Czapek-Dox Agar, Chl.= Chlorate selective medium, the sparse, restricted and shy growth patterns were considered Chlorate sensitive while the dense, light, and sparse dense were considered Chlorate resistant. *= number of isolates of this phenotype. - = on isolates of this phenotype

Table VI. Protein analysis of *Macrophomina phaseolina* isolates recovered from diseased sunflower stem and root samples collected from different fields in El-Behera governorate and analyzed at different molecular weight of polypeptides

Isolates tested	No.	Molecular weight (kDa)											
		8-100				101-200				201-300			
		%I	%IUB	NB	%P	%I	%IUB	NB	%P	%I	%IUB	NB	%P
Stem isolates	14	100	42.8	6-18	74.0 ±23.4	50.0	21.9	2-4	12.9 ±4.9	28.5	7.79	1-3	9.1 ±1.8
Root isolates	19	100	26.3	9-20	82.8 ±9.9	52.6	21.5	2-6	12.9±2.7	26.3	5.74	1-2	8.1 ±6.7
Mean		100	34.5	6-20	78.4	51.3	21.7	2-6	12.9	27.4	6.76	1-3	8.6

No= total number of isolates tested. kDa= kilo Dalton. %I= percentage of isolates showed polypeptide bands at this kDa. range. %IUB= percentage of isolates showed unique bands at this kDa range of the total number of analyzed isolates, unique bands are distinct bands linked to certain isolates. NB= number of bands appeared at this kDa range. %P= mean percentage of the polypeptide content of the developed bands of the total protein content of the isolate

Table VII. DNA banding pattern of *Macrophomina phaseolina* isolates recovered from sunflower stem and root samples collected from different fields in El-Behera governorate and analyzed with the random amplified polymorphic DNA through PCR using eight random oligonucleotide primers

Isolates tested	Isolate code No.	Primers							
		OPS-16	OPA-2	OPA-4	OPA-5	OPA-13	OPA-1	OPA-8	OPR-14
Stem	9	2	5	5	6	6	4	5	4
	1	2	5	7	8	5	6	2	3
Root	18	2	5	5	6	6	9	4	2
	30	2	5	7	8	5	6	3	5
M.R.*		2	5	5-7	6-8	5-6	4-9	2-5	2-5

* mean range

Table VIII. Phenotypes of the high, intermediate, and low pathogenic isolates of *Macrophomina phaseolina* recovered from diseased sunflower stem and root samples collected from different fields in El-Behera governorate and analyzed for colony characteristics on PDA and for their molecular characteristics at different molecular weights of protein polypeptides

Isolates tested	P.	Colony Characteristics										Molecular Characteristics[1]					
		No.	Colour						Groth pattern			Number of polypeptide Bands			% Mean polypeptides content		
			W	G	Bl	DG	Br	D	SD	L	S	8-100	101-200	201-300	8-100	101- 200	201-300
Stem	H	5	1*	2	2	-	-	3	-	2	-	6-18	0-4	0-2	90.2 ± 11.7	20.4 ± 2.0	7.4**
isolates	I	4	-	1	-	-	3	3	-	-	1	6-14	0-3	0-3	86.7 ± 13.6	12.3 ± 4.5	6.7 ± 3.4
	L	5	-	1	2	2	-	-	2	3	-	10-14	0-4	0-1	83.8 ± 13.3	10.9 ± 3.4	12**
Root	H	3	-	2	1	-	-	1	1	1	-	12-20	0-2	0-1	88.1 ±9.0	14.3 ± 2.1	9.3**
isolates	I	9	2	2	3	1	1	4	-	5	-	9-20	0-5	0-2	87.4 ± 11.8	12.4 ± 2.7	9.3 ± 6.1
	L	7	-	3	1	2	1	2	-	3	2	10-19	0-6	0-1	77.9 ± 32.9	15.5 ± 1.9	6.8**
Total		33	3	11	9	5	5	13	3	14	3						

P..= pathogenicity, calculated as the mean aggressiveness in the pathogenicity test. H= high pathogenic *i.e.* ≥ 75 aggressiveness. I= intermediate pathogenic *i.e.* **<75->25** aggressiveness. L= low pathogenic *i.e.* ≤25 aggressiveness. No.= number of isolates tested W= white G= grey DG= dark green Bl= black Br= brown D= dense L= light dens SD= dense in a sparse pattern S= light growth in a sparse or feathery pattern *= number of isolates detected of this phenotype. -= on isolates of this phenotype were detected. **= single isolate. [1]analyzed at three polypeptide molecular weights, *i e.* 8-100, 101-200, and 201-300 kilo Dalton

range with 6.76% of the isolates showed unique bands. Percentage of the polypeptide content of the developed bands was 9.1 ± 1.8% and 8.1 ± 6.7% for the stem and the root isolates, respectively (Table VI).

b. DNA analysis. The RAPD analysis for the tested *M. phaseolina* isolates using eight tested primers through PCR revealed DNA fingerprints with banding pattern of 2 - 9 bands ranging in 350bp - 3kbp molecular weight (Fig. 4 & Table VII). No variations (polymorphism) were revealed for number of bands between isolates with primers OPS-16 or OPA-2 as the same banding pattern was revealed for the four isolates analyzed. However, with OPA-4, OPA-5 and OPA-13 banding pattern was similar for each of the stem and the root isolates tested, while with OPA-1, OPA-8 and OPR-14 no distinct trend was revealed between the stem and the root type of isolates (Table VII & Fig. 4).

5- Phenotypes of the High, the Intermediate and the Low Pathogenic *M. phaseolina* Isolates

a. Colony colour phenotypes on PDA. Data in (Table VIII) revealed that the white colony phenotype was linked to the high pathogenic stem isolates as well as the intermediate pathogenic root isolates. However, the grey and black colony phenotypes were common in most pathogenicity phenotypes. The dark green colony phenotype was almost confined to the low pathogenic isolates, while the brown colony phenotype almost represented the intermediate pathogenic isolates (Table VIII).

b. Growth pattern phenotypes on PDA. The dense and the light dense colony phenotypes were common among the different pathogenicity phenotypes revealed (Table VIII). However, the sparse dense colony phenotype was recorded in two low pathogenic stem isolates and in one high pathogenic root isolate. Isolates with sparse colony phenotypes were detected in the intermediate pathogenic stem isolates and the low pathogenic root isolates and represented with one and two isolates for those phenotypes, respectively (Table VIII).

c. Protein banding pattern. At the 8 - 100 kDa polypeptide molecular weight, 6 - 18 polypeptide bands were detected for the stem high pathogenic *M. phaseolina* isolates (Table VIII). This was in comparison with a smaller range of 6 - 14 and 10 - 14 bands in the intermediate and low pathogenic stem isolates, respectively. For the root isolates, a similar banding pattern was detected being 12 - 20, 9 - 20 and 10 - 19 for the low, intermediate and high pathogenic isolates, respectively (Table VIII). At the 101 - 200 kDa, the tested isolates exhibited banding pattern of maximum 4, 3 and 4 bands for the high, intermediate and low pathogenic stem isolates, respectively and maximum of 2, 5 and 6 bands in the root isolates for the same phenotypes, respectively. However, at the 201 - 300 kDa, isolates exhibited a smaller number of bands of maximum three bands in the different phenotypes (Table VIII).

d. Protein polypeptide content. Mean percentages of the polypeptide content in the detected bands at the 8 - 100 kDa range were 90.2%, 86.7% and 83.8% for the high,

Fig. 1. Occurrence of *Macrophomina phaseolina* on sunflower in different regions in El-Behera governorate during the 2003 and 2004 growing seasons

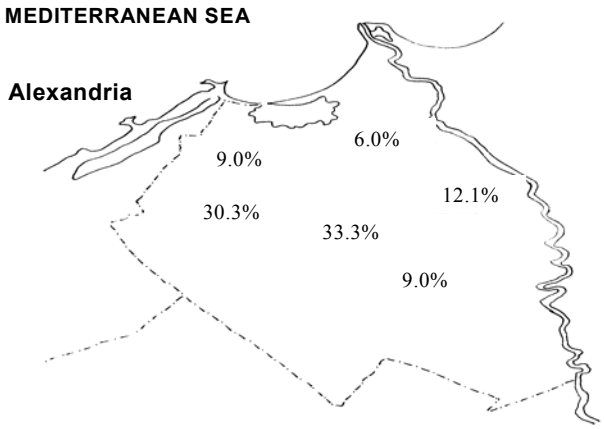


Fig. 2. Colour phenotypes (a) and growth patterns (b) of *Macrophomina phaseolina* isolates recovered from sunflower plants collected from different fields in El-Behera governorate during the 2003 and 2004 growing season and tested on different media. W.= white, G.= grey, Bl.= black, Br.= brown, Dg.= dark green, D= dense, L= light dense, SD.= dense in a sparse pattern, S.= sparse or feathery growth, R.= restricted growth, Sh.= shy growth

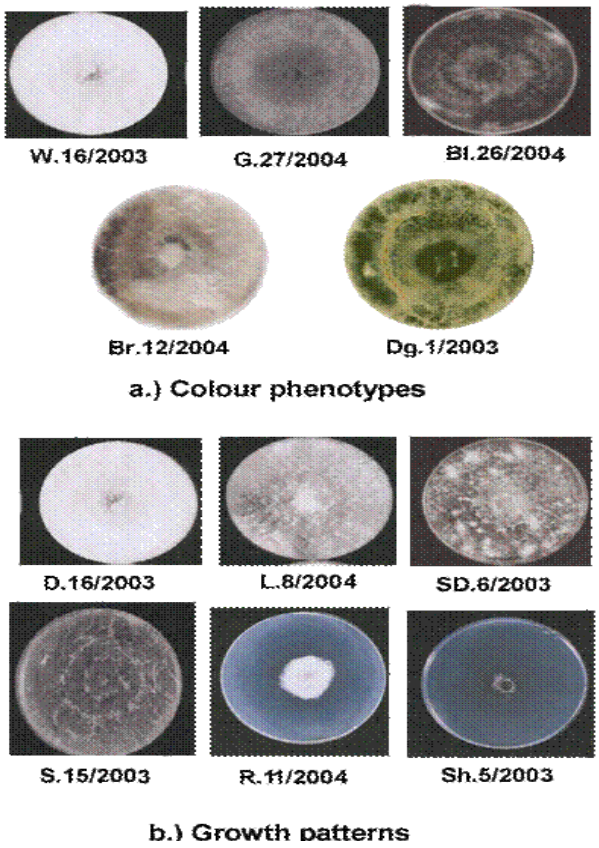
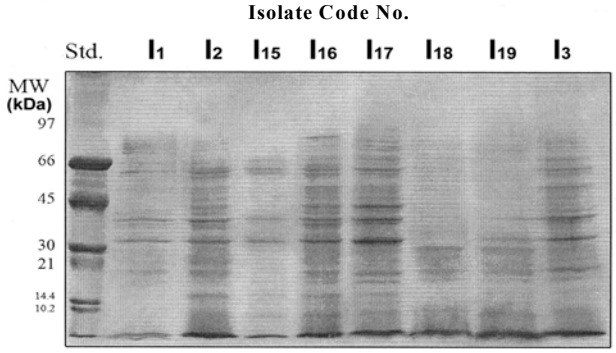


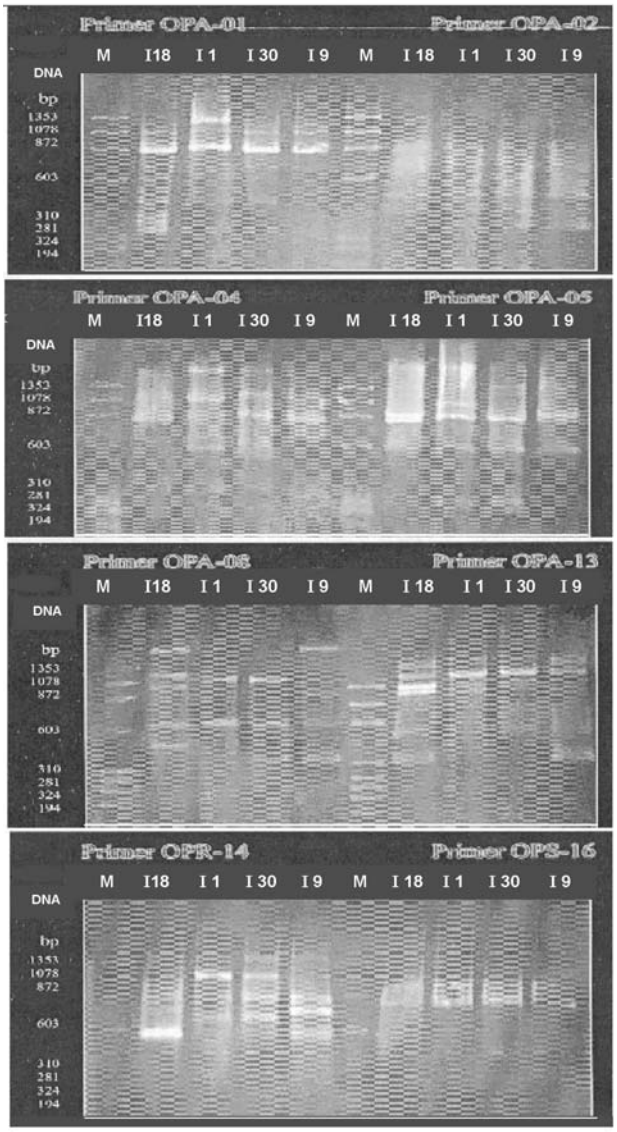
Fig. 3. SDS-Page of protein banding pattern of certain *Macrophomina phaseolina* isolates recovered from sunflower plants collected from different fields in El-Behera governorate during the 2003 and 2004 growing season. I1, I2, I3, I15, I16, I17, I18 and I19 are code numbers of the analyzed isolates= stem isolate1/2003, stem isolate 2/2003, stem isolate 3/2003, root isolate15/2003, root isolate16/2003, root isolate17/2003, root isolate18/2004 and root isolate19/2003, respectively



intermediate and low pathogenic stem isolates, respectively. This was in comparison with 88.1%, 87.4% and 77.9% for the root isolates for the same phenotypes, respectively. At the 101 - 200 kDa, smaller values were revealed being 20.4%, 12.3%, 10.9% for the stem isolates and 14.3%, 12.4%, 15.5% for the root isolates for the same phenotypes, respectively. However, at the 201 - 300 kDa, only one isolate of each of the high pathogenic and low pathogenic phenotypes showed bands of polypeptide content of 7.4% and 12.0% for the stem isolates and also 9.3% and 6.8% for the root isolates. For the intermediate pathogenic isolates, the mean polypeptide contents were 6.7% and 9.3% for the stem and root isolates, respectively (Table VIII).

e. Phylogenetic analysis. The phylogenetic relationship based on the banding patter obtained by the RAPD-PCR showed that the four isolates analyzed were grouped into two clusters. One cluster contained the two high pathogenic isolates (stem isolate 9/2003 & root isolate 18/2004) with 0.350 genetic similarity coefficient, while the other cluster contained the two low pathogenic isolates (stem isolate 1/2003 & root isolate 30/2004) with 0.300 genetic similarity coefficient (Fig. 5).

Fig. 4. DNA fingerprint of certain *Macrophomina phaseolina* isolates recovered from sunflower plants collected from different fields in El-Behera governorate and analyzed with the RAPD-PCR using certain random oligonucleotide primers. M= marker, I18= root isolate 18/2004, I1= stem isolate 1/2003, I30= root isolate 30/ 2004, I9= stem isolate 9/2003

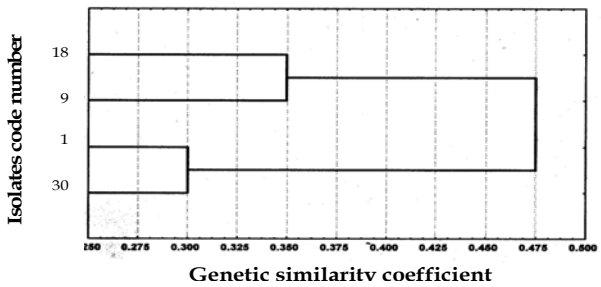


DISCUSSION

The *Macrophomina phaseolina*, the causal fungus of the charcoal-rot disease of sunflower, is a soil-borne and a wide host range pathogen, which distributed worldwide. In the present study, 33 *Macrophomina phaseolina* isolates were recovered from the different regions surveyed, where sunflower was grown in El-Behera governorate during the 2003 and 2004 growing seasons. Housh Eisa and Abo El-Matameer were the most affected regions as 33.3% and 30.3% of the isolates were recovered, respectively. This was followed by Etay El-Baroud region, where 12.1% of the isolates were made. Occurrence of *Macrophomina phaseolina* in the rest of the surveyed regions *i.e.*, El-Dalangat, Kafr El-Dawar and El-Mahmoudia was as less than 10%. These findings corroborate reports from various parts of the world including some parts in Egypt (Eisa *et al*., 1981; El-Deeb *et al*. 1985; Manici *et al*., 1995; Desai, 1998; Atiq *et al*., 2001; Almeida *et al*., 2003; Ibrahim, 2006; Fernandez *et al*., 2006).

Although only one species was recognized within the genus *Macrophomina* (Mihail, 1992), great variability in cultural morphology, the pathogenicity and on the molecular level were recorded on which efforts were made worldwide

Fig. 5. Dendrogram based on the RAPD-PCR showing the phylogenetic relationship of the analyzed high and low pathogenic *Macrophomina phaseolina* isolates recovered from sunflower plants in El-Behera governorate during the 2003 and 2004 growing seasons. Isolates code number 18, 9, 1 and 30 are root isolate 18/2004, stem isolate 9/2003, stem isolate 1/2003, root isolate 30/2004, respectively



to characterize and classify *Macrophomina phaseolina* isolates (Atiq *et al*., 2001; Almeida *et al*., 2003; Fernandez *et al*., 2006). In the present study, all the recovered *M. phaseolina* isolates tested (33 isolates) were pathogenic to both of the tested sunflower cvs. *i.e.,* H11-008 (H08) and H11-009 (H09) and incited stem lesions ranged between 2.9 cm and 19.7 cm in the pathogenicity test. However, 35.8% of the isolates were classified as high pathogenic as caused ≥75% mean plant death in comparison with 19.8% caused ≥75% reduction in plant head size. However, 15.8% and 40.4% of the isolates were designated intermediate pathogenic (<75%-<25%) to cause plant death and reduction in plant head size, respectively. This was compared with 48.4% and 39.7% low pathogenic (≤25%) isolates for the same previous parameters, respectively. More variations were identified for the isolates colony phenotypes as five colour phenotypes and six growth patterns were recognized on the media tested (PDA; CD; Chlorate). These were white, grey, black, dark green and brown colony colour phenotypes with the most frequent was the grey phenotype (30.3%), while the colony growth patterns were the dense, light, sparse dense, sparse, restricted and shy growth with the most frequent phenotype was the dense phenotype (40.3%). The use of the Chlorate selective medium to differentiate strains of *M. phaseolina* was suggested (Pearson *et al*., 1986). The reduction of chlorate, an analog of nitrate, to chlorite in fungi, where the nitrate reductase pathway was functional can result in toxicity, which differentiate fungal isolates to sensitive and resistant strains. Eleven isolates *i.e*., 33.3% of the total 33 *M. phaseolina* isolates analyzed were Chlorate sensitive, while rest of the isolates were Chlorate sensitive. Meantime, since soluble protein analysis was used to differentiate fungal species (Clare, 1963) quantitative and qualitative differences in protein pattern among fungal isolates were reported (Milton *et al*., 1971; Avivo & Govannetti, 1998), which provided a conservative estimation of the amount of variation within a genome. The *M. phaseolina* isolates analyzed in the present study showed that 100% of the isolates contained the light polypeptides of 8 - 100 kDa in mean percentage of 78.4% of the total polypeptides and exhibited 6 - 20 bands in the different isolates with 34.5% of the isolates were differentiated with unique bands, while at the higher 101 - 300 kDa protein analysis, little variation (0 - 6 bands) and less differentiation (6.7 - 21.7%) were revealed and 27.4 - 51.3% of the isolates only contained polypeptides of this kDa range. Moreover, the RAPD-PCR analysis of the tested *M. phaseolina* isolates using eight random oligonucleotide primers revealed DNA fingerprints with banding pattern of 2 - 9 bands ranging in 350bp - 3kbp molecular weight. However, no variations were revealed for number of bands between isolates with primers OPS-16 or OPA-2, while with OPA-1, OPA-4, OPA-5, OPA-8, OPA-13 and OPR-14 more variations were revealed. These findings were in agreement with reports concerning *M. phaseolina* population in several parts of the world including very recent studies in Egypt (Dhingra & Sinclair, 1972; Pearson *et al*., 1986; Chase *et al*., 1994; Mihail & Taylor, 1995; Weising *et al*., 1995; Selvan & Seetharaman, 2000; Atiq *et al*., 2001; Almeida *et al*., 2003; Khalifa, 2003; Ramadan, 2005; Fernandez *et al*., 2006; Franco *et al*., 2006; Ibrahim, 2006; Purkayastha *et al*., 2006).

The *M. phaseolina* isolates occurred on the stem part of the sunflower plant constituted 42.4% of the total 33 isolates recovered compared to 57.6% of the isolates occurred on roots. The stem type of isolates exhibited higher pathogenicity as size of the lesion incited, percentage of plant death and percentage of plant head reduction, in the pathogenicity tests, were 7.1 cm, 48.6% and 44.2%, respectively for stem isolates compared to 6.1 cm, 37.9% and 37.4%, respectively for the root type of isolates. Meantime, the stem type of isolates were more variable for the protein banding pattern as 42.8% of stem isolates exhibited unique bands in comparison with 26.3% for the root isolates at the 8 - 100 kDa. However, no distinct colony phenotype or protein banding pattern were linked to any of the both types of isolates. Besides, the RAPD-PCR analysis revealed that the same cluster in the developed Dendogram contained both the stem and the root isolates. These results did not show enough support to the hypothesis of the stem type and the root type of *M. phaeolina* isolates, which were in harmony with Khare *et al*. (1970), Dhingra and Sinclair (1973) and Ibrahim (2006).

The high, intermediate and the low pathogenic phenotypes were recognized among the recovered *M. phaeolina* isolates. These were in frequencies of 27.8%, 28.1.4% and 44.1% (combined data), respectively. These phenotypes shared the same colony phenotypes and the protein banding pattern. However, Dendrogram of the RAPD-PCR Phylogenetic analysis showed that the high pathogenic isolates constituted one cluster with 0.350 genetic similarity coefficient, while the low pathogenic isolates constituted another cluster with 0.300 genetic similarity coefficient. These results were in agreement with reports of several investigators (Mihail & Taylor, 1995; Atiq *et al*., 2001; Fernandez *et al*., 2006; Purkayastha, 2006).

The amount of variation identified in the present study was in harmony with such deuteromycetous fungus and was suggested (Manici *et al*., 1995; Mihail & Taylor, 1995) to be due to its heterokaryotic nature. Probably, study with bigger number of isolates and the establishment of techniques such the restriction fragment length polymorphism (RFLP) of the nuclear, ribosomal and the mitochondrial DNA for *M. phaseolina* isolates could certainly help to reveal more variations for better understanding of the population structure of *M. phaseolina* isolates. Such characterization and understanding of the population genetic structure of a pathogen is a vital consideration in elucidating disease epidemiology and could be a strategy for devising a stable disease management in a specific region (Purkayastha *et al*., 2006).

REFERENCES

Almeida, A., R. Abdelnoor, C. Arias, V. Carvalho, D. Filho, S. Marin, S. Benato, M. Pinto and C. Carvalho, 2003. Genotypic diversity among Brazilian isolates of *Macrophomina phaseolina* revealed by RAPD. *Fitopatol. Bras*., 28: 279-85

Atiq, M., A. Shabeer and I. Ahmed, 2001. Pathogenic and cultural variation in *Macrophomina phaeolina*, the cause of charcoal rot in sunflower. *Sarhad J. Agric*., 2: 253–5

Avivo, L. and M. Govannetti, 1998. The protein pattern of spores of arbuscular mycorrhizal fungi: Comparison of species, isolates, and physiological stages. *Mycol. Res.*, 102: 985–90

Barnett, H.I. and B.B. Hunter, 1987. *Illustrated Genera of Imperfect Fungi,* 4th edition. Millan Publication Co. Inc. USA

Booth, C., 1971. Fungal culture media, *In*: Booth, C. (ed.), *Methods in Microbiology*. Academic Press, London, UK

Chase, T.E., Y. Jiang and J. Mihail, 1994. Molecular variability in *Macrophomina phaseolina*. *Phytopathol.,* 84: 1149

Clare, B.G., 1963. Starch-gel electrophoresis of protein as an aid in identifying fungi. *Nature*, 200: 803–4

Desai, S.A., 1998. Virulent isolates of *Macrophomina phaeolina* (Tassi) Goid. from sunflower infecting sorghum in India. *Karnataka J. Agric. Sci.*, 11: 1094–5

Dhingra, O.D. and J.B. Sinclair, 1972. Variation among isolates of *Macrophomina phaseolina* (*Rhzoctonia bataticola*) from the same soybean plant. *Phytopathol.,* 62: 1108

Dhingra, O.D. and J.B. Sinclair, 1973. Location of *Macrophomina phaseolina* on soybean plants related to cultural characteristics and virulence. *Phytopathol*., 63: 934–6

Dhingra, O.D. and J.B. Sinclair, 1978. *Biology and Pathology of Macrophomina phaseolina*. Viscosa, Minosa, Brasil

Eisa, N., K.G. Ahmed and M.A. Shaarawy, 1981. *Pathological Effects of Macrophomina phaseolina (Tassi) Goid. on Sunflower (Helianthus annus L.),* pp: 1–12. Reserch Bulletin, Faculty of Agriculture, Ain Shams University, Egypt

El-Agamy, S., 2000. Phsiocochemical, molecular and immunological characterization of camel rennet: a comparison with buffalo rennet. *J. Dair. Res*., 67: 73–81

El-Deeb, A., H. Mohamed and A. Hilal, 1985. *Studies on Charcoal Rot Disease of Sunflower in Egypt,* pp: 999–1012. The 1stNational Conference Pests and Dis. of Veg. and Field Crops in Egypt, Ismailia

Fernandez, R.B., A. De Santiago, S.H. Delgado and N.M. Perez, 2006. Characterization of Mexican and non-Mexican isolates of *Macrophomina phaseolina* based on morphological characteristics, pathogenicity on bean seeds and endoglucanase gene. *J. Pl. Path.,* 88: 1

Franco, M.R., S.H. Delgado, R.B. Fernandez, J. Simpson and N.M. Perez, 2006. Pathogenic and genetic variability within *Macrophomina phaseolina* from Mexico and other countries. *J. Phytopathol*., 154: 447–53

Hames, B. and D. Rickwood, 1990. *Gel Electrophoresis of Protein: a Practical Approach.* TRL Publishing Co., London

Howard, G.C. and W.E. Brown, 2001. *Modern Protein Chemistry: Practical Aspects*. ISBN, CRC Press, USA

Ibrahim, M.M., 2006. Studies of charcoal rot disease caused by *Macrophomina phaseolina* on sunflower and its control. *Ph. D. Thesis*, Faculty of Agriculture, Ain Shams University, Egypt

Jana, T.K., T.R. Sharma, R.D. Prasad and D.K. Arora, 2003. Molecular characterization of *Macrophomina phaseolina* and *Fusarium* species by using a single primer RAPD technique. *Microbiol. Res*., 158: 249–57

Karunanithi, K., M. Muthusamy and K. Seetharaman, 1999. Cultural and pathogenic variability among the isolates of *Macrophomina phaseolina* causing root rot of sesame. *Pl. Dis.,* 14: 113–7

Khalifa, M.A., 2003. Pathological studies on charcoal rot disease of sesame. *Ph. D. Thesis*, Faculty of Agriculture, Moshtohor, Zagazig University, Egypt

Khare, M.N., N.K. Jain and H.C. Sharma, 1970. Variation among *Rhizoctonia bataticola* isolates from Urid bean plant parts soil. *Phytopathol.*, 60: 1298

Laemmli, K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680–5

Manici, L.M., F. Caputo and C. Castro, 1995. Temperature responses of isolates of *Macrophomina phaseolina* from different climatic regions of sunflower production in Italy. *Pl. Dis*., 79: 838–9

Mihail, J.D., 1992. *Macrophomina*. *In*: Singleton, L., J. Mihail and C. Rush, (eds.), *Methods for Research on Soil-borne Phytopathogenic Fungi,* pp: 134–6. American Phytopathology Society, St. Paul, USA

Mihail, J.D. and S.T. Taylor, 1995. Interpreting variability among isolates of *Macrophomina phaseolina* in pathogenicity, pycnidium production and chlorate utilization. *Canadian J. Bot.,* 73: 1596–603

Milton, J.M., W.G. Rogers and I. Isaac, 1971. Application of acrylamide gel electrophoresis of soluble fungal proteins to taxonomy of *Verticillium* species. *Trans. Br. Mycol. Soc*., 56: 61–5

Murray, M.G. and W.F. Thompson, 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Res*., 8: 4321–5

Pearson, C.A., J.F. Leslie and F.W. Schwenk, 1986. Variable chlorate-resistance in *Macrophomina phaseolina* from corn, soybean and soil. *Phytopathol.,* 76: 646–9

Purkayastha, S., B. Kaur, N. Dilbaghi and A. Chaudthury, 2006. Characterization of *Macrophomina phaseolina*, the charcoal rot pathogen of cluster bean, using conventional techniques and PCR-based molecular markers. *Pl. Pathol.,* 55: 106–16

Ramadan, M.R., 2005. Pathological and biochemical studies on *Macrophomina phaseolina* on cotton. *Ph. D. Thesis*, Faculty of Agriculture, Suez Canal University, Egypt

Rholf, F.J., 2000. *NTSYS-PC Numerical Taxonomy and Multivariable Analysis System*. Version 2.1, Exeter Publishing, USA

Sambrook, J., E. Fritsch and T. Maniatis, 1989. *Molecular Cloning: A Laboratory Manual*, 2nd edition. Harber Press, New York

Sangawan, M., N. Metha and G. Saharan, 2005. *Diseases of Oil Seed Crops*. Indus Publication Co. India

Selvan, S.M. and K. Seetharaman, 2000. Relationship among pigment synthesis, culture media growth and virulence of the geographical isolates of *Macrophomina phaseolina* causing charcoal rot of sunflower. *Canadian J. Pl. Pathol*., 13: 370–4

Weising, K., H. Nybom, K. Wolff and W. Meyer, 1995. *DNA Fingerprinting in Plants and Fungi*. CRC Press, Boca Raton, USA

Weiss, E.A., 2000. *Oil Seed Crops,* 2nd edition. Blackwell Science Publication, UK

Williams, J., A. Kubelik, R. Livak, J. Rafalski and S. Tingey, 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.,* 18: 6531–5

Zazzerini, A. and L. Tosi, 1989. Chlorate sensitivity of *Sclerotium bataticola* isolates from different hosts. *J. Phytopathol.,* 126: 219–24

(Received 31 May 2007; Accepted 17 August 2007)