MOLECULAR AND BIOLOGICAL INVESTIGATIONS OF DAMPING-OFF AND CHARCOAL-ROT DISEASES IN SUNFLOWER

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MOLECULAR AND BIOLOGICAL INVESTIGATIONS OF DAMPING-OFF AND CHARCOAL-ROT DISEASES IN SUNFLOWER

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ABSTRACT

Sunflower (*Helianthus annuus* L.), is a major oilseed in both Turkey and Egypt as well as worldwide, two of the most important diseases of sunflower are damping-off and charcoal-rot. Damping-off is caused by the following pathogenic fungi: *Fusarium oxysporum, Fusarium verticillioides, Rhizoctonia solani* and *Macrophomina phaseolina*. Charcoal-rot, on the other hand, is incited by the soil inhabiting fungus *Macrophomina phaseolina*. Damping-off as well as charcoal-rot causes important economic loss in the main production areas of sunflower around the world. In this study we selected certain sunflower fields from Egypt and Turkey to collect the fungi associated with the roots of sunflower plants so as to isolate the causal pathogens of damping-off and charcoal-rot. The isolated fungi were able to infect sunflower plants under artificial infection and cause the symptoms of pre- and post- emergence damping-off as well as charcoal-rot. To our knowledge, this is the first report of *M. phaseolina* in sunflower in Turkey. We performed the first study with sequence related amplified polymorphism (SRAP) for an overview of genetic diversity and phenetic relationships present among the isolates of *Macrophomina* and *Fusarium*. Results showed that Turkish and Egyptian isolates of *M. phaseolina* were clustered together with a genetic similarity of 60%; similarly, Turkish and Egyptian isolates of *F. oxysporum* were clustered together with a genetic similarity of 75%. Infection reactions of 41 sunflower cultivars for infection with charcoal-rot disease were also evaluated. Results indicated that certain sunflower cultivars are resistant to charcoal-rot, but the most cultivars are susceptible. A relationship between root exudates and the mechanism of sunflower plant resistance to charcoal-rot and damping-off was found. The application of biological control agents, under greenhouse conditions, significantly reduced plant mortality and increased surviving plants. These agents can readily be used to gain more resistance to charcoal-rot disease in sunflower.

Key words: Sunflower; Damping-off; Charcoal-rot; *Macrophomina phaseolina; Fusarium oxysporum*; *Fusarium verticillioides*; *Rhizoctonia solani*, Genetic diversity; SRAP.

ÖZET

Dünyada, Türkiye'de ve Mısır'da yaygın bir yağlı tohum bitkisi olarak kullanılan ayçiçeğinin (*Helianthus annuus L.*) en önemli iki hastalığı dip çürümesi/çökerten (damping-off) ve kök boğazıdır (charcoal-rot). Dip çürümesi/çökerten hastalığına *Fusarium oxysporum*, *Fusarium verticillioides, Rhizoctonia solani* ve *Macrophomina phaseolina* isimli patojenik funguslar yol açmaktadır. Kök boğazı hastalığına ise bir toprak mantarı olan *Macrophomina phaseolina* sebep olmaktadır. Her iki hastalık da dünyada ayçiçeği üretiminde önde gelen alanlarda önemli ekonomik kayıplara yol açmaktadır. Bu çalışmada, dip çürümesi/çökerten ve kök boğazı hastalıklarına yol açan patojenleri izole edebilmek amacıyla, ayçiçeği köklerinden fungusların toplanabilmesi için, Mısır ve Türkiye'de bazı ayçiçeği tarlaları seçilmiştir. Izole koşullardaki fungusların suni enfeksiyon yöntemi ile ayçiçeği bitkisini enfekte ettiği ve her iki hastalığın oluşumunda ve sonrasında görülen semptomlara yol açtığı saptanmıştır. Şu anki bilgimize gore Türkiye'de ayçiçeğinde bulunan *M. phaseolina* hastalığı ilk rapordur. Çalışmamızda, *Macrophomina* ve *Fusarium* izolatları arasındaki genetik çeşitliliği ve fenetik ilişkiyi belirlemek amacıyla, ilk kez, amplifiye edilmiş dizi bağlantılı polymorfizm (SRAP) kullanılmıştır. Sonuçlar, Türkiye ve Mısır'dan elde edilen *M. phaseolina* izolatlarının %60 genetik benzerlikle gruplandığını gösterirken Türkiye ve Mısır'dan elde edilen, *F. oxysporum* izolatlarının ise %75 genetik benzerlikle gruplandığını göstermiştir. Diger bir sonuç, SRAP markörlerinin, markore dayalı seleksiyon çalışmaları ile *Macrophomina* ve *Fusarium* izolatları arasındaki genetik çeşitliliğin ölçülmesi çalışmaları için kullanılabilirliğidir. Çalışmada, 41 ayçiçeği çeşidinin kök boğazı hastalığı enfeksiyonuna reaksiyonu da ölçülmüştür. Sonuçlar, bazı ayçiçeği türlerinin kök boğazı hastalığına dirençli olduğunu, ancak türlerin büyük çoğunluğunun hastalığa duyarlı olduğunu göstermiştir. Kök salgıları ile ayçiçeğinin dip çürümesi/çökerten ve kök boğazı hastalıklarına direnç mekanizması arasında bir bağlantı olduğu önemli bir saptamadır. Sera koşullarında, biyolojik kontrol ajanlarının uygulanmasının bitki ölümünü büyük ölçüde azalttığı ve yaşayan bitki oranını arttırdığı görülmüştür. Bu biyolojik kontrol ajanları kök boğazı hastalığına karşı dayanıklılık elde etmede kullanılabilir.

Anahtar sözcükler: ayçiçeğinin; dip çürümesi/çökerten; kök boğazı; *Macrophomina phaseolina*; *Fusarium oxysporum*; *Fusarium verticillioides*; *Rhizoctonia solani*, genetik çeşitlilik; SRAP.

To those I love with all my heart (my wife and daughter)
To my dearest family (mother, father, sisters and brother)

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TABLE OF CONTENTS

1	**INTRODUCTION**……………..…………………………………………......	1
2	**PREVIOUS STUDIES**…………..…………………………………….…..	5
2.1	Fungi associated with damping-off and charcoal-rot on sunflower……...	5
2.2	Physiological factors affecting the growth of fungi associated with sunflower roots…………………………………………………………..….	6
2.3	Determination of genetic diversity among the isolates of *Macrophomina* and *Fusarium*……………………………………………………….…….	9
2.4	Evaluation the susceptibility of sunflower cultivars for infections by *Macrophomina phaseolina*……………………………………………….……	11
2.4.1	Variation among isolates of *Macrophomina phaseolina*…………….……...	11
2.4.2	Global distribution and epidemics of charcoal-rot……………………….	12
2.4.3	Host-pathogen interaction…………….…………………………………….	13
2.5	Sunflower root exudates…………………………………….………………	14
2.6	Biological control of damping-off and charcoal-rot on sunflower….…...	15
3	**MATERIALS AND METHODS**………..….…………………………..	18
3.1	Materials……………….…………………………………………………	18
3.1.1	Sunflower seeds……………………….…………………………………	18
3.1.2	Fungal and bacterial isolates………………………………….………….	19
3.1.3	Growth media for isolation and culture of the pathogens………………..	20
3.1.3.1	Czapek's liquid medium…………………….…………………………..…..	20
3.1.3.2	Peptone-Rose Bengal Agar medium…………………………….………….	21
3.1.3.3	Nutrient Agar medium…………………………..…………………….…..	21
3.1.3.4	Potato-Dextrose Agar……………………………………….………………	21
3.1.3.5	Potato-Dextrose……………………………………………….…………….	22
3.1.3.6	Barley medium……………………………………………………………....	22
3.1.4	Reagents, buffers and solutions…………………………………………….	23
3.1.4.1	Hydrochloric acid solution……………………………………….………	23
3.1.4.2	Sodium Hydroxide solution……………………………………….……..	23
3.1.4.3	TBE Buffer………………………………………….………………………	23
3.1.4.4	Ethidium bromide solution…………………………………….…………	24
3.1.4.5	Gel preparation…………………………….……………………………..….	24
3.1.4.6	Prepare 6x DNA loading dye…………………………….…………….….	24
3.1.4.7	DNA Ladder……………………….……………………………….……….	25

3.2 Methods………………………….………………………….………… 26
3.2.1 Field survey and collection of fungal isolates associated with sunflower roots…………………………..…………………………………….... 26
3.2.1.1 Screening certain sunflower fields in Egypt for occurrence of damping-off and charcoal-rot pathogens…………………………………………... 26
3.2.1.1.1 Evaluation of Disease incidence (D.I. %)……………………………..…. 27
3.2.1.2 Screening certain sunflower fields in Turkey for occurrence of damping-off and charcoal-rot pathogens…………………….…………………..…. 27
3.2.2 Identification of fungi associated with sunflower roots………………..…. 28
3.2.3 Evaluation of Disease Severity Rating (DSR)………………...………. 28
3.2.4 Pathogenicity tests……………………….………………………………. 29
3.2.4.1 Pathogenicity test for isolates obtained from Egypt…………….………. 29
3.2.4.2 Pathogenicity test for isolates obtained from Turkey…………………..... 30
3.2.5 Physiological studies of *Macrophomina* and *Fusarium* in *vitro*……...…. 31
3.2.5.1 The influences of temperature...……………………………………...….. 31
3.2.5.2 The Influences of pH……………..……………………………………… 31
3.2.5.3 The Influences of salinity……………………..………………………. 32
3.2.6 Phenotypic variations among the isolates of *M. phaseolina, F. oxysporum* and *F. verticillioides*…….………………..…………………. 32
3.2.7 Determination of genetic diversity among the isolates of *M. phaseolina, F. oxysporum* and *F. verticillioides*……………………………………..….. 33
3.2.7.1 Growing fungi for DNA extraction……………………..…………….. 33
3.2.7.2 DNA Purification…………………..…………………………………….. 33
3.2.7.3 Determination of genetic diversity among the isolates by using restriction enzymes……………………………………………………. 35
3.2.7.4 Determination of genetic diversity among the isolates by using sequence related amplified polymorphism SRAP technique……………..……... 36
3.2.7.4.1 SRAP-PCR amplifications…………………..…………………………… 39
3.2.7.4.2 PCR reaction conditions……………………….……………………… 39
3.2.7.4.3 SRAP analysis………………...…………………………...………… 40
3.2.7.4.4 Detection of SRAP-PCR products…………………………...……….... 40
3.2.7.4.5 SRAP data analysis……………………………..……………………... 40
3.2.8 Evaluation the susceptibility of sunflower cultivars and genotypes to infection by *Macrophomina phaseolina*…………………..……..………. 41
3.2.9 Variation of sunflower root exudates…………………………….……… 41
3.2.9.1 Preparation of the exudates…………………………..………………... 41
3.2.9.2 Effect of root exudates of sunflower cultivars on the growth of

	Macrophomina and *Fusarium* in *vitro*………………………..……….. 42
3.2.9.3	Determination of amino acids quantity in the root exudates of sunflower cultivars during a 14 days growth period……………………………... 43
3.2.10	Investigate the potential biocontrol agents for the management of damping-off and charcoal-rot in sunflower……………………………… 43
3.2.10.1	Isolation and identification of the bioagents…………………………... 43
3.2.10.2	Preliminary test for efficacy of bioagents fungi against sunflower pathogens in *vitro*…………………………………………………... 44
3.2.10.3	Evaluation efficacy of *Trichoderma* and *Gliocladium* isolates against *Macrophomina phaseolina* in greenhouse………………………………. 45
3.2.10.4	Screening the effect of biological control agents on sclerotia production of *Macrophomina phaseolina*…………………………………………… 46
3.2.11	Statistical analysis…………………………………………………….. 46
4	**RESULTS**……………………………..…………………………... 47
4.1	Field survey and collection of fungal isolates associated with sunflower roots………………………………………………………………… 47
4.1.1	Screening certain sunflower fields in Egypt for occurrence of damping-off and charcoal-rot pathogens…………………………………………... 47
4.1.1.1	Percentage of disease incidence (D.I. %) during field inspection……….. 49
4.1.2	Screening certain sunflower fields in Turkey for occurrence of damping-off and charcoal-rot pathogens…………………………………………... 50
4.2	Pathogenicity tests…………………………………………………….. 51
4.2.1	Pathogenicity test for isolates obtained from Egypt……………………... 51
4.2.1.1	*Macrophomina phaseolina*……………...……………………………….. 51
4.2.1.2	*Fusarium spp*. and *Rhizoctonia solani*…..……………………………… 53
4.2.2	Pathogenicity test for isolates obtained from Turkey……………………. 55
4.3	Physiological studies of *Macrophomina* and *Fusarium* in *vitro*……….... 57
4.3.1	The influences of temperature…………………………………………… 57
4.3.2	The influences of pH………………………………………………….. 59
4.3.3	The influences of salinity……………………………………………... 61
4.4	Phenotypic variations among isolates of *M. phaseolina, F. oxysporum* and *F. verticillioides*…………………………………………………….. 63
4.5	Determination of genetic diversity among the isolates of *M. phaseolina, F. oxysporum* and *F. verticillioides*……………………………………… 66
4.5.1	DNA isolation………………………………………………………… 66
4.5.2	Determination of genetic diversity among the isolates by using restriction enzymes……………………………………………………. 66

4.5.3	Determination of genetic diversity among the isolates by using sequence related amplified polymorphism (SRAP) technique………..…………….	66
4.5.3.1	Cluster analysis……………………..……………………………………..	71
4.5.3.2	Genetic similarity as revealed by SRAP markers………………………..	74
4.5.3.3	Polymorphisms as detected by SRAP markers…………………………..	74
4.5.3.4	Principal coordinates analysis……………………………………………	75
4.6	Evaluation the susceptibility of sunflower cultivars and genotypes to infection by *Macrophomina phaseolina*…….…………………………..	77
4.7	Variation of sunflower root exudates…………………………………….	80
4.7.1	Effect of root exudates of sunflower cultivars on the growth of *Macrophomina* and *Fusarium* in *vitro*……………………………………	80
4.7.2	Determination of amino acids quantity in the root exudates of sunflower cultivars during a 14 days growth period………………………………..	81
4.8	Investigate the potential biocontrol agents for the management of damping-off and charcoal-rot in sunflower………………………………	83
4.8.1	Isolation and identification of the bioagents…………………………..	83
4.8.2	Preliminary test for efficacy of bioagents fungi against sunflower pathogens in *vitro*………………………………………………………..	85
4.8.3	Evaluation efficacy of *Trichoderma* and *Gliocladium* isolates against *Macrophomina phaseolina* in greenhouse………………………………..	89
4.8.4	Screening the effect of biological control agents on sclerotia production of *Macrophomina phaseolina*……………………………………………	91
5	**DISCUSSION**…………………..…………………………………….	93
6	**CONCLUSION**………………...……………………………………..	99
7	**REFRENCES**…………..…………………………………………….	101
8	**APPENDIX**……………………………………………………………	119
8.1	Appendix- A: (Supplies)…………………..………………………….…	119
8.2	Appendix- B: Equipment……………………………………………..….	121

LIST OF TABLES

Table (3.1) Cultivars and sources of sunflower………………………………………………… 18

Table (3.2) Isolates and sources…………………………………………………………… 19

Table (3.3) Forward and reverse SRAP primers used for this study…………………………. 38

Table (3.4) SRAP primer combinations used in this study……….……………………….. 38

Table (4.1) Screening certain sunflower fields in Egypt for occurrence of damping-off and charcoal-rot pathogens……………………………………………….…………….. 48

Table (4.2) Percentage of disease incidence (D.I. %) during field inspection……………….. 49

Table (4.3) Field survey and collection of fungal isolates associated with sunflower roots in Turkey………………………………………………………………………. 51

Table (4.4) Percentage mortality of sunflower plants, inoculated with *M. phaseolina*……..… 52

Table (4.5) Percentage mortality of sunflower plants, inoculated with *Fusarium spp*. and *Rhizoctonia solani*……………………………………………………………….. 53

Table (4.6) Percentage mortality of sunflower plants, inoculated with *M. phaseolina, F. oxysporum, R. solani* and *A. helianthi*…………………………….…………………….. 56

Table (4.7) The influences of temperature on the growth of *Macrophomina* and *Fusarium* in *vitro*………………………………………………………………………… 58

Table (4.8) The influences of pH on the growth of *Macrophomina* and *Fusarium* in *vitro*…. 60

Table (4.9) The influences of NaCL concentration on the growth of *Macrophomina* and *Fusarium* in *vitro*………………………………………………………………… 62

Table (4.10) Similarity matrix for 34 isolates of *Macrophomina* and *Fusarium* from different geographic areas, calculated using the Jaccard's coefficient…………………….. 74

Table (4.11) Levels of polymorphism among *Macrophomina* and *Fusarium* isolates as revealed by the SRAP primers………………………………………………… 75

Table (4.12) Evaluation the susceptibility of sunflower cultivars and genotypes to infection by *Macrophomina phaseolina*………………………………………………… 78

Table (4.13) Evaluation the susceptibility of sunflower cultivars and genotypes to infection by *Macrophomina phaseolina* regardless to the tested isolates………………….. 79

Table (4.14) Effect of root exudates of sunflower cultivars on the growth of *Macrophomina* and *Fusarium* isolates in *vitro*………………………………………………… 81

Table (4.15) Quantity of amino acids in the root exudates of sunflower cultivars during a 14 days growth period……………………………………………………………. 82

Table (4.16) Morphological and physiological characteristics of bacterial isolates…………... 84

Table (4.17) Preliminary test for efficacy of bioagents fungi against *Macrophomina*

	phaseolina isolates in *vitro*………………………………………………………	86
Table (4.18)	Preliminary test for efficacy of bioagents fungi against *F. verticillioides* and *F. oxysporum* in *vitro*………………………………………………………………	87
Table (4.19)	Preliminary test for efficacy of bioagents bacteria against *M. phaseolina, F. verticillioides* and *F. oxysporum* in *vitro*…………………………………………	87
Table (4.20)	Evaluation efficacy of *Trichoderma* and *Gliocladium* isolates against *M. phaseolina* in greenhouse……………………………………………………....	89
Table (4.21)	The effect of biological control agents on sclerotia production of *M. phaseolina*...	92

LIST OF FIGURES

Figure (3.1)	Diagram showing the electrophoretic profile of the 1 kb plus DNA ladder, 75-20,000 bp (fermentas: #SM1331)..	25
Figure (3.2)	Percentage of plant parts assessed based on visual rating scale (1- 6)......................	28
Figure (3.3)	Open reading frame (ORFs)……………………………………………………..	37
Figure (3.4)	SRAP primers elements……………………………………………………….	37
Figure (4.1)	Frequency of the isolated fungi associated with sunflower roots regardless to the inspected regions in Egypt..	48
Figure (4.2)	Disease symptoms of sunflower plants under artificial infections by *M. phaseolina* in both seedling and maturing stages……………………………………………….	54
Figure (4.3)	The influences of temperature on the growth of *Macrophomina* (A) and *Fusarium* (B) in vitro regardless to the tested isolates………………………………………..	59
Figure (4.4)	The influences of pH on the growth of *Macrophomina* (A) and *Fusarium* (B) in *vitro* regardless to the tested isolates……………………………………………	61
Figure (4.5)	The influences of NaCL concentration on the growth of *Macrophomina* (A) and *Fusarium* (B) in *vitro* regardless to the tested isolates……………………………..	63
Figure (4.6)	Growth patterns of *M. phaseolina* isolates (E1:E12) on PDA medium…………….	64
Figure (4.7)	Growth patterns of *M. phaseolina* isolates (M1:M10) on PDA medium…………..	64
Figure (4.8)	Growth patterns of *M. phaseolina* isolates (T8, T16, T28 and T38) on PDA medium…………………………………………………………………………	65
Figure (4.9)	Growth patterns of *F. verticillioides* isolates (E14a and E14b) and *F. oxysporum* isolates (E15a, E15b, T2, T6, T14 and T21) on PDA medium……………………..	65
Figure (4.10)	Agarose gel electrophoresis of SRAP profile generated by the primer Em6-Me4 from *Macrophomina* and *Fusarium*…………………………………………………	68
Figure (4.11)	Agarose gel electrophoresis of SRAP profile generated by the primer Em11-Me4 from *Macrophomina* and *Fusarium*…………………………………………………	68
Figure (4.12)	Agarose gel electrophoresis of SRAP profile generated by the primer Em14-Me4 from *Macrophomina* and *Fusarium*…………..…………………………………….	69
Figure (4.13)	Agarose gel electrophoresis of SRAP profile generated by the primer Em11-Me11 from *Macrophomina* and *Fusarium*…………………………………………………	69
Figure (4.14)	Agarose gel electrophoresis of SRAP profile generated by the primer Em13-Me4 from *Macrophomina* and *Fusarium*…………………..…………………………….	70
Figure (4.15)	Agarose gel electrophoresis of SRAP profile generated by the primer Em4-Me4 from *Macrophomina* and *Fusarium*……………………………………………….	70

Figure (4.16) Agarose gel electrophoresis of SRAP profile generated by the primer Em9-Me4 from *Macrophomina* and *Fusarium*………………………………………..……. 71

Figure (4.17) Dendrogram constructed with UPGMA clustering method among 34 isolates of *M. phaseolina, F. oxysporum* and *F. verticillioides* based on Jaccard's coefficient…... 73

Figure (4.18) Principal coordinates plot of 26 *Macrophomina phaseolina* isolates based on differences in the SRAP analysis………………………………………………… 76

Figure (4.19) Principal coordinates plot of 34 isolates of *M. phaseolina, F. oxysporum* and *F. verticillioides* based on differences in the SRAP analysis……………………….… 76

Figure (4.20) The above photo shows zone of inhibition produced by *T. harzianum* against *M. phaseolina* in *vitro*……………………………………………………………….. 88

Figure (4.21) The above photo shows zones of inhibition produced by *B. subtilis* against *M. phaseolina* in *vitro*……………………………………………………………….. 88

Figure (4.22) Efficacy of *Trichoderma* and *Gliocladium* isolates against *M. phaseolina* in greenhouse……………………………………………………………………….. 90

Figure (4.23) The above photo shows evaluation efficacy of *Trichoderma* against *M. phaseolina* (SANAY cultivar) under greenhouse conditions…………...………………………. 90

Figure (4.24) Sclerotia shapes of *M. phaseolina*…………………….…………………….… 92

LIST OF ABBREVIATIONS

AA:	Amino acid
A. helianthi:	*Alternaria helianthi*
AFLP:	Amplified fragment length polymorphism
Ala:	Alanine
Arg:	Arginine
Asp:	Aspartic acid
B. cereus:	*Bacillus cereus*
B. subtilis:	*Bacillus subtilis*
Bp:	Base pair
ºC:	Celsius (centigrade)
Cm:	Centimeter
Cys:	Cysteine
DI:	Disease index
dH_2O:	Distilled water
DNA:	Deoxyribonucleic acid
DSR:	Disease Severity Rating
DW:	Dry weight
ESTs:	Expressed sequence tags
F. oxysporum:	*Fusarium oxysporum*
F. verticillioides:	*Fusarium verticillioides*
FAO:	Food and Agriculture Organization
$FeSO_4.5H_2O$:	Ferrous Sulphate
G. catenulatum:	*Gliocladium catenulatum*
Glu:	Glutamic acid
Gly:	Glycine
Gm:	Gram
HCL:	Hydrochloric Acid
His:	Histidine

Ile:	Isoleucine
ISSR:	Inter-Simple Sequence Repeat
ITS:	Internal transcribed spacer
K_2HPO_4:	Potassium Hydrogen Phosphate
KCL:	Potassium Chloride
Leu:	Leucine
LSD:	least significant difference
Lys:	Lysine
M. phaseolina:	*Macrophomina phaseolina*
Met:	Methionine
mg:	Milligram
$MgSO_4.7H_2O$:	Magnesium Sulphate
ml:	Milliliter
mM:	Millimolar
mRNA:	Messenger RNA
NA:	Nutrient Agar
NaCL:	Sodium Chloride
$NaNO_3$:	Sodium Nitrate
NaOCL:	Sodium hypochlorite
NaOH:	Sodium Hydroxide
nm:	Nanometer
NPK:	Nitrogen, Phosphorus and Potassium
NTSYS-pc:	Numerical Taxonomy and Multivariate Analysis System
ORFs:	Open reading frames
P. chrysogenum:	*Penicillium chrysogenum*
P. oxalicum:	*Penicillium oxalicum*
PCR:	Polymerase chain reaction
PDA:	Potato Dextrose Agar
pH:	power of Hydrogen
Phe:	Phenylalanine
Pro:	Proline

R. solani:	*Rhizoctonia solani*
RAPD:	Random Amplified Polymorphic DNA
RCBD:	Randomized Complete Block Design
RCF:	Relative centrifugal force= (g-force)
RE:	Restriction enzymes
RNA:	Ribonucleic acid
Ser:	Serine
SRAP:	Sequence-related amplified polymorphism
SSR:	Simple Sequence Repeat
T. hamatum:	*Trichoderma hamatum*
T. harzianum:	*Trichoderma harzianum*
T. koningii:	*Trichoderma koningii*
T. pseudokoningii:	*Trichoderma pseudokoningii*
T. viride:	*Trichoderma viride*
TARI:	Trakya Agriculture Research Institute
TBE:	Tris-Borate-EDTA
Thr:	Threonine
Tyr:	Tyrosine
UV:	Ultraviolet
UPGMA:	Unweighted Pair-Group Method with Arithmetic mean
Val:	Valine
µl:	Microliter

1- INTRODUCTION

Plant pathogenic fungi are important pathogens on all kinds of crops. Without appropriate control they will cause loss of yield and quality. Chemical fungicides have been the primary management tools for over fifty years but now many products have been removed from the market or are under review. There is an urgent need for the development of alternative control options. This work is necessary to develop environmentally sound agricultural systems that minimize chemical use while maintain high production standards. Sunflower (*Helianthus annuus L.*), is a major oilseed in both Turkey and Egypt, as well as worldwide. Based on 2008 FAO statistical data, Turkey is among the top ten sunflower-producing countries in the world. The harvested areas are 577958 Ha, 8445 Ha and productions are 992000 tonnes, 21483 tonnes in both Turkey and Egypt respectively (2008, FAO Database).

Sunflower is subjected to a large number of diseases during its growing season which attack all parts of the plant (Zazzerini and Tosi, 1987, and Ahmed et al., 1994). Damping-off and charcoal-rot are two of the most important diseases of sunflower; damping-off is caused by the following pathogenic fungi: *Fusarium oxysporum, Fusarium verticillioides* and *Rhizoctonia solani* which attack plants in seedlings stage and causes pre- and post-emergence damping-off that lead to serious losses in crop productivity. Similarly, charcoal-rot disease incited by the soil inhabiting fungus *Macrophomina phaseolina* which attacks both seedlings and adult plants causing pre- and post-emergence damping-off and important economic loss in the main production areas of sunflower around the world. In general, damping-off and charcoal-rot are capable of destroying up to 10 - 15 % of plants. But under favorable conditions for the outbreak and development of the pathogen, yield losses can reached more than 75 % (Sackston, 1981 and Xiaojian et al., 1988). Yield losses claimed by charcoal-rot in Spain, United States, Uruguay and Soviet Union are up to 25 % (Orellana, 1971; Tikhonov et al., 1976 and Jimenez et al., 1983).

Sunflower plants infected with damping- off and charcoal-rot are typically invaded by the pathogen through the root system. Plants that are attacked early often die in the seedling stage, sclerotia of *Macrophomina phaseolina* observed on infected tissues can persist, as a dormant state, in the soil for long periods (more than 10 months) in the absence of the host under dry soil conditions (Khan, 2007). The asexual structures formed by *M*. *phaseolina* are pycnidia and microsclerotia. Microsclerotia are formed in infected host tissues and constitute the primary inoculum source of the pathogen. They can survive up to 15 years depending on environmental conditions and whether or not the sclerotia are associated with host residues (Cook et al., 1973; Papavizas, 1977; Short et al., 1980 and Baird et al., 2003).

Macrophomina and *Fusarium* are two of the most important fungi endemic to sunflower in Turkey and Egypt and cause damping-off and charcoal-rot diseases. Physiological factors that are influences the growth of the fungi is an important characteristic associated with its pathogenicity (Dhingra and Sinclair, 1987; Manici et al., 1995; Kok and Papert, 2002). Genetic diversity of *Macrophomina* and *Fusarium* isolates that are collected from Egypt and Turkey can be determined by using SRAP technique to elucidate the genetic relations among these isolates. SRAP analysis provides a useful tool for estimation of genetic diversity and phenetic relationships in natural and domesticated populations; SRAP is highly polymorphic and more informative when compared to AFLP, RAPD and SSR markers (Budak et al., 2004a). PCR- SRAP is an effective tool for estimating genetic diversity, identifying unique genotypes as new sources of alleles for enhancing turf characteristics (Budak et al., 2004c).

Soil-borne diseases are still a major threat to sunflower cultivation in and all over the world because of the wide host range of the pathogens and their strong survival ability in the soil (Mousa et al., 2006 and Bokor, 2007). Chemical control was massively applied; however, for the increasing public concern over the fungicide usage, alternative control methods are strongly desired for sustainable agriculture where organic amendments play an important role (Workneh and van Bruggen, 1994 and Lazarouits, 2001). Management of the diseases can be achieved with non-pathogenic microorganisms, and these can be used against the pathogenic fungi. Use of those microorganisms as biological control agents plays an important role against pathogenic isolates and increases crop yield. (Baker and

Cook, 1974; Druzhinina and Kubicek, 2005; Morsy and El-Korany, 2007 and Larralde-Corona et al., 2008).

The present investigation was designed to study fungi associated with sunflower roots, as well as some factors affecting disease incidence such as: infection reaction of sunflower cultivars and biological control. Furthermore, we also examined the genetic diversity among the isolates of *Macrophomina* and *Fusarium*, as well as the effects of physiological factors on in *vitro* growth of *Macrophomina* and *Fusarium* isolates. In this study we selected certain sunflower fields from Egypt and Turkey; to collect the fungi associated with roots of sunflower and to isolate the causal pathogens of damping-off and charcoal-rot, in order to increase our knowledge about the spread, physiological, morphological, pathogenical features and genetic conditions of those pathogens.

The specific aims of the study were:

1. Isolation and identification of fungi associated with the roots of sunflower during the growing seasons from Turkey and Egypt.
2. Pathogenicity test of the isolated fungi.
3. The influences of temperature; pH and salinity on the growth of the isolates of *Macrophomina* and *Fusarium* in *vitro.*
4. Examine the phenotypic variations among isolates of *M. phaseolina*, *F. oxysporum* and *F. verticillioides* in *vitro.*
5. Using molecular markers to determine the genetic diversity among the isolates of *Macrophomina phaseolina*, *Fusarium oxysporum* and *F. verticillioides.*
6. Evaluation of sunflower cultivars and genotypes for the infection by certain pathogenic isolates of *Macrophomina phaseolina* under greenhouse conditions.
7. Study the effects of root exudates on the growth of *Macrophomina* and *Fusarium* in *vitro*; and determine quantity of amino acids in the root exudates of sunflower cultivars.
8. Isolation and identification of biological control agents, (fungi and bacteria), from soil and the rhizosphere of sunflower.

9. In *vitro* preliminary test for antagonistic capability of biological control agents against the causal pathogens.
10. Efficiency of certain biocontrol agent in the incidence of charcoal-rot disease under greenhouse conditions.

2- PREVIOUS STUDIES

2.1- Fungi associated with damping-off and charcoal-rot on sunflower

Sunflower 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). Sunflower plants are subjected to attack by pathogenic fungi during the growing season, and inducing diseases, such as, damping-off, root-rot, charcoal-rot and wilt. Previous studies showed that *Fusarium moniliforme, F. oxysporum, Macrophomina phaseolina* and *Sclerotinia sclerotiorum* were pathogenic to germinating seeds and seedlings of sunflower (Zazzerini and Tosi, 1987, and Ahmed et al., 1994). Sunflower yield is negatively associated with responsiveness to intraspecific competition but that this relationship can be affected by fungal diseases (Sadras et al., 2000). Analysis of variance, for seed yield, of 104 sunflower genotypes under charcoal rot (*Macrophomina phaseolina*) stress, indicated highly significant differences in sunflower genotypes under study (Habib et al., 2007). Sunflower lines varied for their reaction to *S. sclerotiorum* using artificial inoculation of sunflower heads, screening for resistance in inbreds is a prerequisite for practical sunflower breeding (Hahn, 2002). Ali and Dennis, 1992 showed that, none of the tested pea varieties were immune to infection by *Macrophomina phaseolina*, but adequate sources of resistance were identified against all isolates. *Pthium sp*., *Fusarium oxysporum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Macrophomina phaseolina* were found to be associated with damping-off and charcoal-rot of sunflower in Behera governorate, Egypt. However, *Pythium* sp. was not able to incite any disease to sunflower in the pathogenicity test while the other fungi incited pre- and post- emergence damping-off in different degrees. Charcoal-rot was incited by *Macrophomina phaseolina* only (Morsy and El-Korany, 2007).

Intensive work has been done to elucidate the variability in morphology, physiology, pathogenicity and genotype of *M*. *phaseolina*. Variations in cultural characteristics and virulence to sunflower have been reported in the U.S. (Dhingra and Sinclair, 1973). *M.*

phaseolina causes seedling blight, root rot and root and stem rot of more than 500 cultivated and wild plant species including economically important crops as sunflower, common bean, sorghum, maize, cotton, peanut and cowpea (Dhingra and Sinclair, 1977; Gray et al., 1990 and Diourte et al., 1995). *Fusarium* spp., are widespread plant pathogenic fungi which commonly colonize aerial and subterranean plant parts, either as primary or secondary invaders. Species of *Fusarium* are reported as seed-borne and soil-borne plant pathogens. They cause pre- and post-emergence death of seedlings, seed abortion, seed-, root-, stem- and seedling- rots, blight, chlorosis, vascular wilt, die back, stunting and reduction in growth in a variety of host plants (Richardson, 1983). Association of *Fusarium* species with seeds results in spread of several diseases in fields such as wilting (Vijayalakshmi and Rao, 1986) foot rot, seedling blight, stunting, wilting and hypertrophy in sunflower (Shahnaz and Ghaffar, 1990). Straser, 1985 reported *Fusarium oxysporum* as seed borne pathogen of sunflower even from the endosperm of chemically treated seeds.

A new disease of sunflower caused by *Fusarium tabacinum* was reported from Italy, all sunflower cultivars tested were susceptible to the pathogen (Zazzerini and Tosi, 1987). Pathogenicity of 6 *Fusarium spp.* was tested on sunflower plants. Wilting and seedling rot were found to be the most prominent symptoms produced by all *Fusarium spp*. (Nahar and Mushtaq, 2006). Charcoal rot caused by *Macrophomina phaseolina* may kill plants prematurely and reduce yield severely in crops suffering from high temperature and drought stress, the pathogen is widely distributed in the soil in warm areas, and affects a wide range of unrelated plants (Sackston, 1983). *Rhizoctonia solani, Macrophomina phaseolina* and *Fusarium solani* were able to attack sunflower plants and cause root rot disease (Mokbel et al., 2007). *Macrophomina phaseolina* is a soil-borne pathogen that causes charcoal rot disease, of a wide range of cultivated and wild plant species; it has a wide host range and is responsible for causing losses on more than 500 species (Kunwar et al., 1986 and Day and MacDonald, 1995). *M. Phaseolina* isolated from sunflower was the most aggressive isolates. While those isolated from sesame and cotton were lowest virulent. Infection with *M. phaseolina* was varied according to the crop plants families (Suriachandraselvan et al., 2005). Among thirty three isolates of *Macrophomina phaseolina*, the causal fungus of the charcoal-rot disease of sunflower, were recovered

from the different surveyed regions, in El-Behera, Egypt (Aboshosha et al., 2007). *Macrophomina phaseolina* can survive for more than 10 months under dry soil conditions. The severity of the disease is directly related to the population of viable sclerotia in the soil. (Khan, 2007). *Macrophomina phaseolina* has been considered one of the most prevalent pathogens in sunflower and other plants (Almeida et al., 2003). Symptoms suggestive of charcoal rot were observed on oilseed sunflower plants. The first observed symptoms were confirmation of *Macrophomina phaseolina* (Tossi) Goid. as the causal agent of sunflowers charcoal rot (Gulya et al., 2002).

Root and collar rot disease (*Rhizoctonia solani*) occur destructively wherever sunflowers are grown. Severely infected plants show damping-off, seedling necrosis at the 2-4-leaf stage, rotting of the collar region and presence of dark brown spots on the stem. This is the first report of the seed-borne nature of root and collar rot disease caused by *Rhizoctonia solani* in sunflower from India (Lakshmidevi et al., 2010). *Alternaria helianthi* can cause leaf and stem lesions, seedling blight and head rot (Sackston, 1981 and Allen *et al.*, 1983). It is reported to reduce seed and oil yield, and can cause germination losses (Balasubrahmanyam and Kolte, 1980). Leaves infected with *Alternaria helianthi* showed a reduced photosynthetic performance compared with healthy leaves (Calvet et al., 2005). The leaf spot disease caused by *A. alternata* was observed in all cultivated areas in Greece. This disease and charcoal rot, (*Macrophomina phaseolina* (Tassi) were the major diseases on sunflower (Thanassoulopoulos, 1987). *A. helianthi* has been recognized as the most prevalent and damaging species worldwide (Kolte, 1985). It has been reported to reduce seed yield by 17 to 26% (Allen et al., 1981). Low quality with reduced and discolored oil contents of sunflower seeds are reported to be caused by species of *Rhizopus* (Zad, 1979), whereas seed infection during storage and reduction in germination is reported to be caused by *Alternaria alternata* (Prasad and Singh, 1983).

2.2- Physiological factors affecting the growth of fungi associated with sunflower roots

Temperature is an important factor in the growth of fungi in *vitro* (Kok and Papert, 2002). Production of antibiotics and toxic metabolites can be influenced by temperature (Lee and Magan, 1999). *Macrophomina phaseolina* causes charcoal-rot and damping-off. Its pathogenicity increases with a rise in temperature, with an optimum temperature between 28 and 35°C (Dhingra and Sinclair, 1987). Sixty-four isolates of *Macrophomina phaseolina* from sunflower, collected in four different climatic areas of Italy, were subjected to growth rate tests at 15, 25, 30, 35, and 40°C. The optimum temperature for growth was 30°C for 62 isolates and 35°C for two isolates. Isolate growth rate varied considerably at all temperatures, but the maximum variability between isolates occurred at 15 and 40°C (Manici et al., 1995.). There is a correlation between incubation temperatures and production of Fumonisin B1 in cultures by *Fusarium moniliforme*. The optimal incubation regimen for Fumonisin B1 production by *F. moniliforme* in cultures is 7 weeks at 25°C (Alberts et al., 1990). Temperature was reported to be important factor related to *Fusarium* growth and Fumonisin production on media. The optimal temperature range is 20 - 25°C (Marin et al., 1995). The optimum temperature for radial growth and spore production of some fungi in *vitro* was in the range 20-25°C. At 34°C poor mycelial growth occurred (after twelve days) with no spore production, however, mycelial growth was reduced when temperature was less than 20°C or higher than 25°C (Stephan and Shems Al-Din, 1987). It was observed that at 25°C and 30°C, the fungus attained the maximum growth. Fungi can grow at the temperature range of 10 - 35°C. However, growth of the fungus was drastically reduced below 15°C and started to decline above 35°C, no growth was observed at 5°C, as these temperatures did not favor for growth of the fungi (Farooq et al., 2005).

Fusarium grew normally at pH above 5.0, but produced little Fumonisin B1. At pH below 5.0, there was less growth but substantially more FB1. The optimal pH range for production of FB1 was between 3.0 and 4.0 (Keller et al., 1997). Increasing the concentrations of salt on the media in *vitro*, directly affected spore germination, germ tube length, and pectinolytic activity of fungi (Wisniewski et al., 1995). The tolerance of

ectomycorrhizal fungi to sodium chloride (NaCL) was studied. Four isolates were grown in liquid media with five different concentrations of NaCL and their mycelial weights were determined. The response to NaCL varied among the species. In total, the hyphal growth at 25 mM NaCL was significantly higher than those at the other NaCL concentrations (Matsuda et al., 2006). The origins of fungal isolates can affect the functional diversity or ecological plasticity of the fungi (Colpaert et al., 2004). Some arbuscular mycorrhizal fungi improve the growth of their associated plants under salt stress conditions thus may affect their range of salt tolerance (Tian et al., 2004). Salt tolerance of terrestrial fungi had been studied (Castillo and Demoulin, 1997).

2.3- Determination of genetic diversity among the isolates of *Macrophomina* and *Fusarium*

Macrophomina phaseolina (Tassi) Goid. is the most fungal pathogen affecting sunflowers in Egypt and worldwide as well as causing charcoal-rot disease, not only on the sunflower but also on the more than 500 plant species throughout the world (Purkayastha et al., 2006). The genera *Macrophomina* and *Fusarium* have some common hosts and tend to form mixed infections under conducive atmosphere conditions, resulting in charcoal root/collar rot and wilt. Both species, *M. phaseolina* and *F. oxysporum,* are soil borne, infect a range of hosts at root, stem and collar region as well as cause cortical and vascular discoloration. Therefore, there is an urgent need to devise a diagnostic tool for the identification of these pathogens at genus, species and isolate levels (Bhatti and Kraft, 1992). Despite having a wide host range, *Macrophomina* is a monotypic genus. Efforts to divide *M. phaseolina* into sub-species were unsuccessful, based on the morphology and pathogenicity; there were extreme intraspecific variations (Dhingra, and Sinclair, 1973). Recently, chlorate phenotypes were used as markers for identifying host–specific isolates of *M. phaseolina* (Das et al., 2006). Molecular markers are useful tools for detecting genetic variation within populations of *M. phaseolina*. Single primers of simple sequence repeats (SSR) or microsatellite markers have been used for the characterization of genetic variability of different populations of *M. phaseolina* obtained from soybean and cotton grown in India and the USA (Jana et al., 2005). Recently, investigations on the molecular analysis of isolates of *M. phaseolina* have contributed to the better understanding of the

relationship between the populations of the fungus and their host or geographic location (Mayek-Pérez et al., 2001). RAPD markers have been considered suitable for measuring genetic relatedness, detecting variation within and between *M. phaseolina* populations (Alvaro et al., 2003). Furthermore, Jana et al., 2003 studied the genetic variation in 43 isolates of *M. phaseolina* and 22 isolates of *Fusarium* species, collected from geographically distinct regions over a range of hosts, using random amplified polymorphic DNA (RAPD) markers. They found one primer OPA-13 which produced fingerprint profiles. The single RAPD primer OPA-13 can be used to identify and discriminate several isolates of *M. phaseolina* and *Fusarium* species obtained from 20 hosts including soybean, cotton, chickpea, pea and safflower.

Sequence related amplified polymorphism (SRAP) technology has been recognized as one of the most variable types of DNA sequences found in plants. This SRAP system has been employed for mapping and gene tagging in *Brassica* (Li and Quiros, 2001). SRAP marker is homogenously distributed in the genome and could produce higher polymorphism than those from AFLP, RAPD, and SSR markers. It has been employed to evaluate genetic diversity and phonetic relationships among turfgrass species (Budak et al., 2004a). The polymorphism produced by SRAP (95%) marker technique was higher than those produced by ISSR (81%), RAPD (79%), and SSR (87%) (Budak et al., 2004b). SRAP markers are more consistent and repeatable than RAPDs, less labor-intensive and time-consuming to produce than amplified-fragment length polymorphism techniques (Budak et al., 2004a, b, c). SRAP has proven to be more informative than AFLP (amplified fragment length polymorphism), RAPD (rapid amplified polymorphic DNA), ISSR (inter-simple sequence repeat), and SSR (simple sequence repeat) markers (Ferriol et al., 2003 and Budak et al., 2005). The SRAP marker technique was used as a new technique to assess genetic relationships and diversity among genotypes of *Saccharum*. The level of polymorphism observed proved that the SRAP system was robust at amplifying markers across species and genera and did so according to the evolutionary history interconnecting members of the Saccharum complex (Suman et al., 2008). Cloning and sequencing of a set of cDNA to visualize transcript polymorphism are reported using SRAP technology in three bentgrass species. The ESTs identified in this study could potentially be used in

turfgrass breeding and genetics programs as functional markers. Integration of these ESTs to the existing linkage map of turfgrass species provides high-density coverage in selected genomic regions. Minimum evolutionary tree clustering indicated that ESTs obtained using SRAP could be used for comparative genomics analysis of transcribed genes among the grass species (Dinler and Budak, 2008). Furthermore, Baysal et al., 2009 use SRAP primers to study the population and genetic relationships within and among *Fusarium oxysporum* f. sp. *lycopersic*i races. Mutlu et al., 2008 reported the tagging of the gene for resistance to *Fusarium* wilt (FOM) in eggplant using SRAP, RGA, SRAP-RGA and RAPD markers. Twelve single pustule isolates were generated from samples of *Puccinia striiformis* f.sp. *tritici*. They were analyzed by sequence-related amplified polymorphisms (SRAP) technique, to identify polymorphisms useful to evaluate variability among isolates. This is the first report of the application of SRAP technique to the *Uredinales* order (Pasquali et al., 2010). Molecular markers are useful tools in the analysis of genetic variation in populations of plant-pathogenic fungi. A number of molecular techniques are available for studying the genetic relationships within and among fungal populations within a species. 60 isolates of *Fusarium graminearum*, the causal pathogen of *Fusarium* head blight, were compared using vegetative compatibility analysis and polymerase chain reaction (PCR)-based sequence related amplified polymorphisms (SRAP) (Fernando et al., 2006).

2.4- Evaluation the susceptibility of sunflower cultivars for infections by *Macrophomina phaseolina*

2.4.1- Variation among isolates of *Macrophomina phaseolina*

The qualitative differences in *Macrophomina phaseolina* are related to the host specificity of the fungus. The host specificity of the fungal isolates varies from crop to crop: i.e., it may show host-specific behavior on one crop and may not on the other. The in *vitro* cultural and pathogenic differences are highly variable and are very difficult to quantify for adequate classification (Farrara et al., 1987 and Clude and Rupe, 1991). Physiological specialization in *Macrophomina phaseolina* is not well demonstrated and the fungus is known to have high degree of variation in its morphological, cultural and pathological properties even when isolated from different parts of the same plant (Dhingra and Sinclair,

1987). The genetic variation within a fungal population has been correlated with many factors, pathogen exhibits variation due to uniformity of its substrate and environmental experience. The degree of sexual reproduction within a population or species is also related to genetic variation (Newton, 1987 and Kendrick, 1992).

2.4.2- Global distribution and epidemics of charcoal-rot

Diseases are serious threat for the sunflower crop throughout world. It has been estimated that diseases can cause an average annual loss of 12 % in yield from nearly 12 million hectares of the world (Zimmer and Hoes, 1978 and Kolte, 1985). The incidence and severity of diseases are linked with the climatic factors and cultural practices. Among diseases; Rust, Sclerotium-wilt and charcoal-rot are of worldwide occurrence. Prevalence or distribution of the disease is linked with the climatic factors, cropping pattern, and cultural practices. In general, diseases cause 10-15 % net loss but under favorable conditions for the outbreak and development of the pathogen, they may claim failure of the crop (Sackston, 1981 and Xiaojian et al., 1988). *Macrophomina phaseolina* (charcoal rot) and *Rhizopus spp.* (head rot) were reported as most destructive on sunflower crop (Mirza, 1984). Yield losses on sunflower due to *M. phaseolina* up to 90 % in Pakistan (Masirvic et al., 1987). In Nigeria and Egypt; the major fungal pathogens were *Alternaria*, *Cercospora, Chlamydosporium, Curvularia, Fusarium sp.* and *Macrophomina phaseolina* (Satour, 1984 and Ado et al., 1988).

Macrophomina phaseolina causal agent of charcoal-rot is a serious threat for sunflower crop especially in the arid regions of the world (Hoes, 1985). Yield losses claimed by charcoal-rot in Spain, United States, Uruguay and Soviet Union up to 25% has been recorded under favorable conditions for the growth and development of *M. phaseolina* (Orellana, 1971; Tikhonov et al., 1976 and Jimenez et al., 1983).
Severity of the disease is characterized by drought and high temperature. However, high losses have been reported on availability of low relative humidity and high atmospheric temperature at flowering stage of the crop (Dhingra and Sinclair, 1973 and Tikhonov et al., 1976).

2.4.3- Host-pathogen interaction

Host-pathogen interaction determines the ability of host to bind a parasite and ability of parasite to injure the host. Whereas, resistance and susceptibility are heritable qualities (Yang et al*.,* 1999). Certain new aspects of pathogenic relations of *M. phaseolina* and sunflower have been identified. Therefore, understanding the genetics, behavior of host and pathogen in the process of disease development and host-pathogen relationship are crucial for reliable breeding program for disease resistance. So far many attempts have been made in order to understand various aspects of host pathogen interaction involved in infection pathway. Host-pathogen parasitic compatibility in plant pathogenic fungi is related to their antigenic similarity. However, no common antigen is found between *M. phaseolina* and resistant cultivar of soybean (Farrara et al*.,* 1987; Limpert et al*.,* 1990 and Jones et al*.,* 1996).

Extensive genetic variation and site-specific nature of *M. phaseolina* have made studies on genetics of charcoal-rot resistance difficult. Therefore, genetics of resistance against *M. phaseolina* have not been clearly demonstrated and controversies are found in the findings of various workers. Resistance in sunflower genotype is a dominant character (Olaya et al., 1996 and Michel, 2000). Severity of infection caused by a soil-borne pathogen depends upon its parasitic fitness in soil ecology. The parasitic fitness of a facultative soil-borne pathogen before invading the host is linked with its ability to compete for its survival, utilization of organic sources and colonization in the host root rhizosphere by competing with other microorganism in the vicinity. Nature of soil is also an important factor in controlling the activities of a fungus due to its adsorption capacity in utilizing the soluble nutrients. The activity of exudations from the sclerotia and utilization of soil nutrients also explain the pathogenic importance of a soil-borne fungus (Filnow and Lockwood, 1983 and Mazzola et al., 1996).

2.5- Sunflower root exudates

Root exudates have been proposed as one of the major biochemical factors that confer disease resistance to varieties (Buxton, 1962). It is one of important component of dissolved organic matter (Zhu, 1993). The general statement of plant roots secreting different sorts of compounds into exotic environment during the processes of life activities, which includes protons, organic acids, saccharide, enzymes, amino acid, polysaccharids, protein, viscose matters and so on. They are present widely in the rhizosphere environment belonging to the biologic active matters (Wei and Chen, 2001). In several studies it has been shown that root colonization by symbiotic arbuscular-mycorrhizal fungi alters the root exudation pattern and thus the effect of the exudates on soil-inhabiting microbes (Lioussanne et al., 2003 and Sood, 2003). Pinior et al., 1999 reported that root exudates from mycorrhizal plants affect the hyphal growth of arbuscular mycorrhizal fungi differently, compared to root exudates from nonmycorrhizal plants. Scheffknecht et al., 2007 reported that root colonisation by an arbuscular-mycorrhizal fungus also affects the microconidial germination of *Fusarium oxysporum.* Root exudates are known to play important roles in a number of plant-microbial associations (Inderjit and Weston, 2003). Compounds contained in root exudates interact with organic and inorganic substances to regulate not only their bioavailability in the soil environment, but also their transport. Furthermore, species composition of the rhizosphere microflora can be altered by the production of root exudates, which subsequently alter nutrient status through decomposition and mineralization of organic substances, and through the formation of soil organic matter (Hodge and Millard, 1998).

The amount of root exudates produced varies with the plant species, cultivar, age, and stress factors (Uren, 2000). Roots also exude a variety of low-molecularweight organic compounds. These include sugars and simple polysaccharides (such as arabinose, fructose, glucose, maltose, mannose, oligosaccharides), amino acids (such as arginine, asparagine, aspartic, cysteine, cystine, glutamine), organic acids (such as acetic, ascorbic, benzoic, ferulic, malic acids), and phenolic compounds. Some of these compounds, especially the phenolics, influence the growth and development of surrounding plants and soil microorganisms. In addition, higher-molecular-weight compounds such as flavonoids,

enzymes, fatty acids, growth regulators, nucleotides, tannins, carbohydrates, steroids, terpenoids, alkaloids, polyacetylenes, and vitamins are released in large quantities (Rovira, 1969; Hale et al., 1978; Fan et al., 1997 and Uren, 2000). Increase in amino acid contents has been associated with immunization in several plant-pathogen systems. Amino acids are involved in the synthesis of phytoalexins (Cui et al., 2000) and pathogenesis-related proteins (van Loon et al., 1994). Several studies indicate that Proline-rich and hydroxyproline-rich glycoproteins are implicated in plant defense against pathogens (Cassab, 1998 and Caruso et al., 1999). Root exudates are important in microbial attraction and fungal establishment on roots. Sugars and amino acids are required for the germination and growth of the fungi (Cochrane et al., 1963). Amino acid balance in fungal nutrition determines the germination, successful growth or lysis of soil fungi around roots (Schroth and Hildebrand, 1964; Snyder, 1970 and Tousson, 1970). The role of amino acids in plant diseases may be due to the correlation between these acids and plant health. Amino acids are used both for the production of new cell biomass and to produce energy, Followed by deamination into the keto acid which inter into the Tri Carboxylic acid (TCA) cycle, which plays important role in plant resistance (Bush, 1993).

2.6- Biological control of damping-off and charcoal-rot on sunflower

Biological control of plant pathogens is the use of one or more biological processes to decrease inoculums density of the pathogen or reduce the disease producing activities (Baker and Cook, 1974). Bacterial and fungal antagonists (*Pseudomonas fluorescens, Pseudomonas corrugata, Bacillus subtilis, Gliocladium virens* and *Trichoderma viride*) were used as biological control agents for damping-off caused by *Pythium, Phytophthora, Fusarium, Aphanomyces* and *Rhizoctonia solani* (Georgakopoulos et al., 2001). *Macrophomina phaseolina* (Tassi) Goid., a fungal phytopathogen, infects about 500 plant species world wide (Sinclair, 1982). Fungi belonging to the genus *Trichoderma* are important phytopathogen bioantagonists, acting especially against soil borne microorganisms (Agrios, 2001). Charcoal rot disease (*Macrophomina phaseolina* Tassi (Goid)) are controlled successfully under greenhouse conditions by treating seeds with *Trichoderma harzianum* (El-Fiki et al., 2004). The genus *Trichoderma* is a well-known

group of facultative fungal saprophytes, used for the industrial production of enzymes and as biocontrol agents (Druzhinina and Kubicek, 2005).

Trichoderma isolates are biological agents for the control of charcoal stem rot in melon caused by *Macrophomina phaseolina* and demonstrated the best result in reducing disease severity in melon plant seedlings in the glasshouse (Etebarian, 2006). *Trichoderma virens* (PDBC TVS-2) and *Pseudomonas fluorescens* (PDBC Pf1) significantly inhibited the mycelial growth of *M. phaseolina* by (78.22%) and (76.66 %) respectively in *vitro* (Lokesha and Benagi, 2007). *Trichoderma sp*. (TCBG-2) and *Trichoderma koningiopsis* (TCBG-8), respectively has a remarkable hyperparasitic behavior against *M. phaseolina* isolated from diseased bean and sorghum (Larralde-Corona et al., 2008). *Trichoderma hamatum*, *T. harzianum*, *T. polysporum* and *T. viride* were evaluated in in *vitro* condition against the Eggplant root-rot pathogen, *Macrophomina phaseolina.* Soil application of *T. harzianum*, *T. polysporum* and *T. viride* effectively controlled the root-rot of Egg-plant under field condition (Ramezani, 2008). *Trichoderma harzianum* strains are the most appropriate strains for the biocontrol of *R. solani* (Montealegre et al., 2009).

Bacteria isolated from sunflower leaves, crown and roots inhibited in vitro growth of the root rot pathogens *Rhizoctonia solani* and *Macrophomina phaseolina* (Hebbar et al., 1991). *Bacillus amyloliquefaciens* and *Brevibacterum otitidis* are produced significant antagonistic effect against in *vitro* growth of *M. phaseolina* and *Sclerotium rolfsii*. Applying those bioagents to *M. phaseolina* infested treatments reduced the severity of disease (Moussa et al., 2006). Bacterial isolates, isolated from rhizosphere, having antifungal and good plant growth-promoting attributes, *Bacillus subtilis* BN1 exhibited strong antagonistic activity against *Macrophomina phaseolina*, and other phytopathogens including *Fusarium oxysporum* and *Rhizoctonia solani* (Singh et al., 2008).

Macrophomina phaseolina produces sclerotia in root and stem tissues of its hosts which enable it to survive adverse environmental conditions (Cook et al., 1973; Meyer et al., 1974 and Short et al., 1980). After plant death, colonization by mycelia and formation of sclerotia in host tissue continue until tissues are dry. The mycelium and microsclerotia

produced in infected plant material, including plant residues are the means of propagation of the pathogen. Microsclerotia in soil, host root and stems are the main surviving propagules. After decay of root and plant debris, microsclerotia are released into the soil. They are distributed generally in clusters at the soil surface and are localized mainly at a depth of 0–20 cm (Alabouvette, 1976; Mihail, 1989 and Campbell and van der Gaag, 1993). They can survive for 2-15 years depending on environmental conditions, and whether or not the sclerotia are associated with host residue (Cook et al., 1973; Papavizas, 1977; Short et al., 1980 and Baird et al., 2003).

The resistant cultivars against the important soil-borne pathogen are lacking. In this context, biological control is increasingly capturing the attention of scientists as an alternative strategy for disease management that is also ecologically conscious and environment friendly (Colyer and Mount, 1984). Biological control is a potential non-chemical means for plant disease control by reducing the harmful effects of a pathogen through the use of other living entities (Jeyarajan et al., 1991). Use of antagonistic organisms against *Macrophomina* root rot has been well documented in several crops (Raguchander et al., 1995). It is now widely recognized that biological control of plant pathogens using antagonistic fungi and bacteria is a distinct possibility for the future and can be successfully utilized especially within the framework of integrated disease management system (Muthamilan and Jeyarajan, 1996). The effect of *Trichoderma harzianum* isolates on radial growth, sclerotia size and production of *M. phaseolina* were studied. Hyderabad isolates of *T. harzianum* was found the most effective by giving 69.48% reduction in sclerotia production and 57.36% reduction in sclerotial size.(Shekhar and Kumar, 2010).

3- MATERIALS AND METHODS

3.1- Materials

3.1.1- Sunflower seeds

Seeds of the 41 sunflower (*Helianthus annuus L.*) cultivars used in this study were obtained from Trakya Agricultural Research institute (TARI), Edirne, Turkey and the Ministry of Agriculture, Egypt.

Table (3.1): Cultivars and sources of sunflower

No.	Cultivars	Sources	No.	Cultivars	Sources
1	TARSAN1018	TARI, Edirne, Turkey	22	G3-K6-AD-CRZ-A-SN-26	TARI, Edirne, Turkey
2	PERUN		23	6545-A	
3	IYI-HA-89-7-A		24	62003-A	
4	TR-3080		25	HA-89-1-A	
5	HA-465-A		26	3009-A	
6	6626-A		27	62001-A	
7	P-4223-A		28	7675-A	
8	7751-A		29	RHA-461	
9	67372-A		30	65371-A	
10	0043-A		31	05-TR-198	
11	0704-A		32	7682-A	
12	HA-466		33	7989-A	
13	66241-A		34	BAH-4-A	
14	6163-A		35	1159-A	
15	6388-A		36	2453-A	
16	6397-A		37	6522-A	
17	7710-A		38	SANAY	
18	7990-A		39	TUNCA	
19	2517-A		40	AUROFLOR	Min. of Agri., Egypt
20	6398-A		41	VIDOIC	
21	6765-A				

3.1.2- Fungal and bacterial isolates

Isolates used in this study were isolated from Egypt and Turkey during the summer (2007 & 2009) growing seasons, respectively.

Table (3.2): Isolates and sources

No.	Numerical codes	Scientific names (Fungi/Bacteria)	Place of collection		
			Regions		Countries
1	E1	*Macrophomina phaseolina* (Tassi) Goid.	Abuteeg		Egypt
2	M1				
3	M2				
4	E2				
5	M3				
6	E3				
7	M4				
8	E4		Manfalout		
9	M5				
10	M6				
11	E5				
12	E6				
13	E7				
14	M7				
15	E8				
16	M8				
17	E9				
18	M9		Assiut		
19	M10				
20	E10				
21	E11				
22	E12				
23	E15a	*Fusarium oxysporum* Shelecht.	Assiut		
24	E15b		Manfalout		
25	E14a	*Fusarium verticillioides* Sacc.	Manfalout		
26	E14b		Abuteeg		
27	E13	*Rhizoctonia solani* Kuhn.	Assiut		
28	T8	*Macrophomina phaseolina* (Tassi) Goid.	Cavlum Village	Eskisehir	Turkey
29	T16		Gunduzler Town		
30	T28		Agapinar area		
31	T38		Sevinc area		
32	T2	*Fusarium oxysporum* Shelecht.	Cavlum Village	Eskisehir	
33	T6				
34	T14		Gunduzler Town		
35	T21		Agapinar area		

Table (3.2): Isolates and sources (Complementary)

No.	Numerical codes	Scientific names (Fungi/Bacteria)	Place of collection		
			Regions		Countries
36	T5	*Rhizoctonia solani* Kuhn.	Cavlum Village	Eskisehir	Turkey
37	T24		Agapinar area		
38	T36		Sevinc area		
39	T3	*Alternaria helianthi* (Hansford) Tubaki and Nishihara	Cavlum Village	Eskisehir	
40	T17		Gunduzler Town		
41	T34		Sevinc area		
42	E17a	*Trichoderma harzianum* Rifai.	Manfalout		Egypt
43	E17b				
44	E17c				
45	E19a	*Trichoderma hamatum* (Bonord.) Bainier			
46	E19b				
47	E20	*Trichoderma viride* Pers.			
48	E21	*Trichoderma koningii* Oudem			
49	E22	*Trichoderma pseudokoningii* Rifai			
50	E18	*Gliocladium catenulatum* Gilman. & Abbott			
51	E16	*Cunninghamella echinulata* Thaxter			
52	E25	*Penicillium oxalicum* Currie & Thom.			
53	E26	*Penicillium chrysogenum* Thom.			
54	E23	*Bacillus cereus* Frankland & Frankland			
55	E24a	*Bacillus subtilis* (Ehrenberg) Cohn.			
56	E24b				
57	E24c				

3.1.3- Growth media for isolation and culture of the pathogens

3.1.3.1- Czapek's liquid medium

Czapek's liquid medium, was prepared according to Klich and Pitt, 1988 in the following composition:

Formula	gm/liter
Sodium Nitrate ($NaNO_3$)	2.0
Potassium Chloride (KCL)	0.5
Magnesium Sulphate ($MgSO_4.7H_2O$)	0.5
Ferrous Sulphate ($FeSO_4.5H_2O$)	0.01
Potassium Hydrogen Phosphate (K_2HPO_4)	1.0
Sucrose	30.0

The final pH was adjusted to 6.8 ± 0.2.

3.1.3.2- Peptone-Rose Bengal Agar medium

Peptone-Rose Bengal Agar, a selective medium for the isolation of fungi, is prepared according to the formula of Cooke, 1954 and Martin, 1950 with some modifications: selectivity of the medium is increased by the addition of antibiotics.

Media was prepared in the following composition:

Approximate Formula	**Per Liter**
Peptone	5.0 gm
Dextrose	10.0 gm
Potassium Hydrogen Phosphate (K_2HPO_4)	1.0 gm
Magnesium Sulphate ($MgSO_4.7H_2O$)	0.50 gm
Agar	20.0 gm
Rose Bengal	30.0 mg

The final pH was adjusted to 6.8 ± 0.2; and supplemented with 40 mg streptomycin sulphate/100 ml.

3.1.3.3- Nutrient Agar medium

Nutrient Agar (N.A.) medium, for isolating bacteria, is prepared according to the formula of Clesceri et al., 1998; in the following composition:

Approximate Formula	**Per Liter**
Beef Extract	3.0 gm
Peptone	5.0 gm
Agar	20.0 gm

The final pH was adjusted to 7.2 ± 0.2.

3.1.3.4- Potato-Dextrose Agar

Potato Dextrose Agar (PDA) is a general purpose medium used for the cultivation numerous fungi and yeast; that can be supplemented with acid or antibiotics to inhibit bacterial growth. A PDA was prepared according to the methods described by Dhingra and Sinclair, 1995 in the following composition:

Approximate formula	Per liter
Potato Infusion from 200 gm	4.0 gm*
Dextrose	20.0 gm
Agar	20.0 gm

*4.0 gm of potato extract is equivalent to 200 gm of infusion from potatoes.

Final pH: 6.8 ± 0.2 at 25°C; and supplemented with 40 mg streptomycin sulphate/100 ml.

3.1.3.5- Potato-Dextrose

Potato Dextrose was prepared according to the methods described by Dhingra and Sinclair, 1995 in the following composition:

Approximate Formula	Per Liter
Potato Infusion from 200 gm	4.0 gm*
Dextrose	20.0 gm

*4.0 gm of potato extract is equivalent to 200 gm of infusion from potatoes.

Final pH: 6.8 ± 0.2 at 25°C; and supplemented with 40 mg streptomycin sulphate/100 ml.

3.1.3.6- Barley medium

Barley medium, for preparing inoculum of fungi, is prepared according to Abd-El-Moneem, 1996 and Omar, et al., 2007; in the following composition:

Barley grains	1000 gm
Glucose	20.00 gm
Yeast extract	1.00 gm
Distilled water	1000 ml

The medium was placed in conical flasks; and sterilized for 20 min. at 1.5 atmospheric pressure.

3.1.4- Reagents, buffers and solutions

3.1.4.1- Hydrochloric acid solution

1.0 N Hydrochloric acid (HCL) was prepared by diluting 101.3 ml of 36% HCL to 1000 ml using distilled water.

3.1.4.2- Sodium Hydroxide solution

1.0 N Sodium Hydroxide (NaOH) was prepared by dissolving 40 g of NaOH in 1000 ml distilled water.

3.1.4.3- TBE Buffer

Tris-Borate-EDTA (TBE) buffer is often used for agarose gel electrophoresis in the analysis of DNA products resulting from PCR amplification, DNA purification protocols, or DNA cloning experiments.

3.1.4.3.1.1- Prepare a stock solution of EDTA

- An EDTA (Ethylenediamine Tetraacetic Acid) solution is prepared ahead of time. EDTA will not go completely into solution until the pH is adjusted to about 8.0.
- For a 500 ml stock solution of 0.5M EDTA, weigh out 93.05 gm EDTA disodium salt (FW = 372.2). Dissolve in 400 ml deionized water (ddH_2O) and adjust the pH with NaOH. Top up the solution to a final volume of 500 ml.

3.1.4.3.1.2- Prepare a stock solution of TBE

- Make a concentrated (5x) stock solution of TBE by weighing 54 gm Tris base (FW = 121.14) and 27.5 gm boric acid (FW = 61.83) and dissolving both in approximately 900 ml deionized water.
- Add 20 ml of 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1000 ml. This solution can be stored at room temperature but a precipitate will form in older solutions. Store the buffer in glass bottles and discard if a precipitate has formed.

3.1.4.3.1.3- Prepare a working solution of TBE

For agarose gel electrophoresis, TBE can be used at a concentration of 0.5x (1:10 dilution of the concentrated stock). Dilute the stock solution by 10x in deionized water. Final solute concentrations are 45 mM Tris-borate and 1 mM EDTA.

3.1.4.4- Ethidium bromide solution

This solution was prepared by dissolving 1 gm ethidium bromide in 100 ml dist. H_2O.

3.1.4.5- Gel preparation

Electrophoresis was made on 1% agarose gel (100 ml TBE buffer (0.5x) + 1 gm agarose + 2 µl Ethidium bromide.

3.1.4.6- Prepare 6x DNA loading dye

3.1.4.6.1- Prepare stock solutions:

10 ml of a 2% bromophenol blue stock solution.

10 ml of a 2% xylene cyanol stock solution.

50% glycerol solution.

3.1.4.6.2- Dilute the stock solutions with H_2O to prepare 10ml of the final 6x loading dye with the following component concentrations:

0.3% bromophenol blue.

0.3% xylene cyanol.

30% glycerol.

The 6x DNA loading dye is added to DNA samples to achieve a final dye concentration of 1x.

3.1.4.7- DNA Ladder

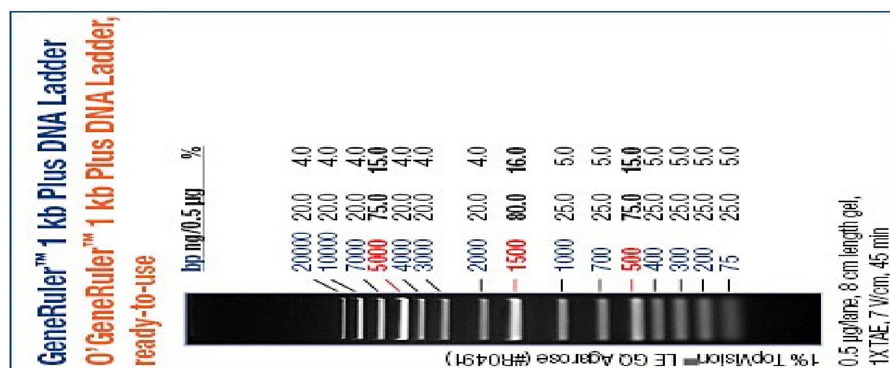


Figure (3.1): Diagram showing the electrophoretic profile of the 1 kb plus DNA ladder, 75-20,000 bp (fermentas: #SM1331).

3.2- Methods

3.2.1- Field survey and collection of fungal isolates associated with sunflower roots

3.2.1.1- Screening certain sunflower fields in Egypt for occurrence of damping-off and charcoal-rot pathogens

Naturally diseased plants of sunflower showing symptoms of seedling damping –off and charcoal rot were collected from three locations Assiut, Manfalout and Abuteeg in Assiut Governorate, Egypt. Samples of the diseased sunflower plants were collected during the summer growing season, 2007. Fields were inspected at four different times covering the different stages of plant growth (from seedling to mature plants), as follows:

(1) 30 days after planting
(2) 45 days after planting
(3) 60 days after planting
(4) 90 days after planting

Ten diseased sunflower samples were collected from each locality in different stages. Plant samples, (roots or stems), were washed carefully in cold running water to remove adhering soil particles. Small pieces of the diseased tissues (2-3 mm. Long) from roots or stems were taken. The samples were washed thoroughly with tap water, and then the surface was sterilized with 2 % solution of sodium hypochlorite (NaOCL) for 3 minutes, and then washed several times with sterilized distilled water. After surface sterilization, outer tissues were removed and discarded. The remaining pieces were plated in Potato Dextrose Agar medium, (2 segments / Petri dish), and incubated at 28±2°C for 5-10 days. In order to prevent bacterial contamination, the antibiotic streptomycin sulphate was added to the medium after autoclaving as the rate of 1200 antibiotic units / 20 ml medium in all isolation procedures.

Ten Petri dishes were used for each sample to determine the frequency of associated fungi with sunflower. Pure cultures of the developing fungi were obtained using single spore or hyphal-tip isolation technique (Dhingra and Sinclair, 1995) and kept at 5°C in the refrigerator for further studies.

3.2.1.1.1- Evaluation of Disease incidence (D.I. %)

Disease Incidence (%) is an indicator of percentage of plants in a field showing disease symptoms. Two fields were selected randomly from each zone (Assiut, Manfalout and Abuteeg) in Assiut Governorate, Egypt. Fields were inspected at different times during isolation procedures from June to September, 2007. Three plots from each field were used as replicates. Each plot contains approximately 150 – 250 plants.

Percentage of disease incidence was calculated during samples collection procedures, according to the following formula:

$$D.I.\ (\%) = \frac{\text{Infected plants}}{\text{Total plants}} \times 100$$

3.2.1.2- Screening certain sunflower fields in Turkey for occurrence of damping-off and charcoal-rot pathogens

During 2009, summer growing season, naturally diseased plants of sunflower showing symptoms of seedling damping-off and charcoal-rot diseases were collected from the fields of four sunflower production areas (Cavlum Village, Gunduzler Town, Agapinar area and Sevinc area) of Eskisehir regions in Turkey. Stems and roots of symptomatic plants were sampled from farmer's fields. Depending on the number of diseased foci in inspect fields, 5 - 10 plants were sampled per field.

Plant samples, (roots or stems), were washed carefully in cold running water to remove adhering soil particles. Small pieces of the diseased tissues (2-3 mm. long) from roots or stems were taken. The samples were washed thoroughly with tap water, and then the surface was sterilized with 2 % of sodium hypochlorite solution (NaOCL) for 3 minutes, and then washed several times with sterilized distilled water. After surface sterilization, outer samples tissues were removed and discarded. The remaining pieces were plated in Potato Dextrose Agar medium, (2 segments / Petri dish), and incubated at 28±2°C for 5-10 days. In order to prevent bacterial contamination, the antibiotic streptomycin sulphate was added to the medium after autoclaving as the rate of 1200 antibiotic units / 20 ml medium in all isolation procedures.

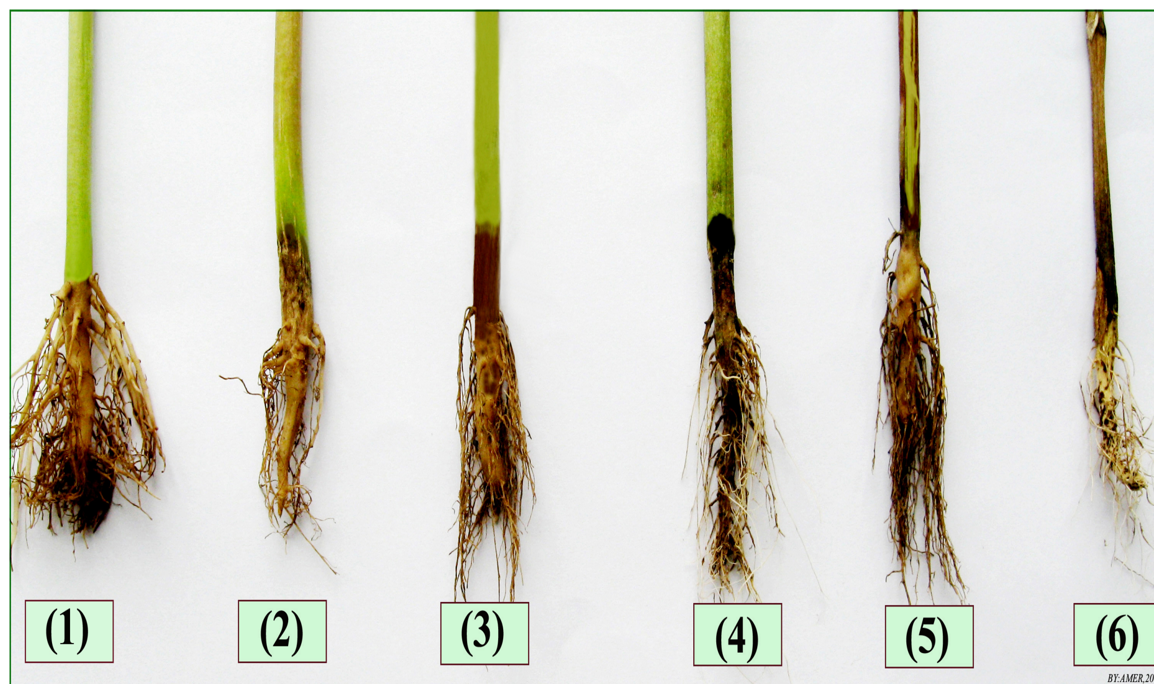
Ten Petri dishes were used for each sample to determine the frequency of associated fungi with sunflower plants. Pure cultures of the developing fungi were obtained using single spore or hyphal-tip isolation technique (Dhingra and Sinclair, 1995) and kept at 5°C in the refrigerator for further studies.

3.2.2- Identification of fungi associated with sunflower roots

Identification of the isolated fungi was carried out on 5-12 days old culture using the morphological and microscopic characteristics of mycelium and spores according to Booth, 1977; Pitt, 1979; Domsch et al., 1980; Sivansen, 1984; Agrios, 1997 and Lucas, 1998.

3.2.3- Evaluation of Disease Severity Rating (DSR)

Severity of disease was assessed visually for plants which showing symptoms of charcoal-rot or root-rot diseases according to the following rating scale in Figure (3.2). Disease severity rating scale was designed according to the description of James, 1971.



(1)= No symptoms on plant.
(2)= 1 - < 10 % area infected
(3)= 10 - < 25 % area infected
(4)= 25 - < 50 % area infected
(5)= 50 - < 75 % area infected
(6)= 75 - < 100 % area infected or deceased plants.

Figure (3.2): Percentage of plant parts assessed based on visual rating scale (1- 6).

A Mean of Disease Severity Rating (MDSR) for each replicate was calculated according to the following formula:

$$MDSR = \frac{(n \times 1) + (n \times 2) + \ldots\ldots\ldots (n \times 6)}{tn}$$

Where, tn = The total number of plants,

n = Number of plants in each group of diseased plants,

(1, 2, ... 6) = Rating scale of diseased plants.

3.2.4- Pathogenicity tests

3.2.4.1- Pathogenicity test for isolates obtained from Egypt

The total numbers of the obtained isolates were 180 isolates. The isolates were divided into four categories, of *Macrophomina phaseolina, Fusarium oxysporum, Fusarium verticillioides* and *Rhizoctonia solani*. Twenty seven isolates were selected randomly, to be tested for their virulence on SANAY, TUNCA and TR-3080 sunflower cultivars, in order to verify their virulence capabilities. The isolates were selected according to their cultural morphology and growing characteristics. The selected isolates were: twenty two isolates of *Macrophomina phaseolina*, two isolates from each of *Fusarium oxysporum* and *F. verticillioides* and one isolates from *Rhizoctonia solani*.

Sterilized pots (No.7) containing sterilized soil were seeded with sterilized seeds. Soil was prepared by mixing the soil carefully with nitrogen, phosphorus and potassium (NPK) at the ratio of 2 % of soil weight. Sterilization of pots and soil was carried out by using 5 % formaldehyde solution. Treated soil was covered with polyethylene for 7 days to retain the gas. The soil was not planted until all traces of formaldehyde disappeared (after 2 weeks). Seed disinfestation was carried out by dipping the seed in 1% sodium hypochlorite solution for 3 minutes, and then rinsed for several times with sterilized water.

Inoculum for each tested fungi was prepared by inoculating sterilized conical flasks 500 ml containing barley medium (100 g barley supplemented with 2 g glucose + 0.1 g yeast extract + 100 ml distilled water) with the tested fungal isolates and incubated at 28±2°C for 12 days (Abd-El-Moneem, 1996 and Omar, et al., 2007).

Soil infestation was carried out one day before sowing by adding the fungal inoculum separately to each pot at the rate of 2 % soil weight, mixed well and thoroughly irrigated. Pots containing non infested soil that mixed with 2 % sterile barley medium were used as control. Each pot was sown with 5 seeds. Three pots were used for each treatment as replicates. Pathogenicity tests were carried out under greenhouse conditions, the prevailing temperatures during Pathogenicity tests were 24°C (minimum) and 28°C (maximum). The plants were irrigated when necessary and daily observed for infection.

Percentages of plant mortalities, (pre - and post - emergence damping-off), were measured after 2 and 4 weeks from planting respectively. Disease severity of charcoal-rot or root-rot was measured after 10 weeks as mentioned before. Reisolation was made from the infected plants in order to verify the involved pathogens (Koch's postulates).

3.2.4.2- Pathogenicity test for isolates obtained from Turkey

The total numbers of the obtained isolates were 40 isolates. Four isolates of each of *Macrophomina phaseolina* and *Fusarium oxysporum* as well as three isolates of each of *Rhizoctonia solani* and *Alternaria helianthi* were selected randomly, to be tested for their virulence on SANAY, TUNCA and TR-3080 sunflower cultivars, in order to verify their virulence capabilities. The isolates were selected according to their cultural morphology and growing characteristics. Randomized Complete Block Design (RCBD) with three replications was used. Sterilized pots (No.7) containing sterilized soil were used. Five sterilized seeds of sunflower cultivars were sown per pot containing infested and non-infested soils (control). Pots were placed in a greenhouse and watered as needed. Pre- and post emergence damping-off was recorded for two and four weeks after sowing respectively. Disease severity of charcoal-rot or root-rot was measured after 10 weeks as mentioned before. Inoculum of the isolates for the bioassays; seed disinfestations as well as soil sterilization were prepared as we mentioned before. Reisolation was made from the infected plants in order to verify the involved pathogens (Koch's postulates).

3.2.5- Physiological studies of *Macrophomina* and *Fusarium* in *vitro*

3.2.5.1- The influences of temperature

In the laboratory test, the effect of temperature on the radial growth of 26 isolates of *Macrophomina phaseolina*, 6 isolates of *Fusarium oxysporum* and 2 isolates of *Fusarium verticillioides* was studied. Petri dishes (9 cm in diameter) containing PDA medium were inoculated in the center with disks (5 mm diameter mycelium piece) of each isolate growing 5 days old culture. Plates were incubated at several temperatures (5, 10, 15, 20, 25, 30 and 40 °C). Three plates were placed in each temperature as replicates. The linear growth of fungi was measured in cm. after 7 days of incubation. The longest and shortest diameters of hyphal colonies were measured and the hyphal growth was evaluated as Follows:

$$\text{Hyphal growth (cm)} = \frac{LD - SD}{2}$$

Where, LD = Longest diameter,
SD = Shortest diameter.

3.2.5.2- The Influences of pH

Laboratory works were directed to study the effect of different pH levels on the linear growth of *M. phaseolina*, *F. verticillioides* and *F.* oxysporum. The tested isolates were inoculated on Petri dishes (9 cm in diameter) containing a PDA medium whose pH was adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0, respectively using 1.0 N HCL or 1.0 N NaOH. Three replicates were conducted for each treatment. Plates were inoculated by placing 5 mm diameter agar medium containing active mycelium of the fungi and were placed in the center of the Petri dishes. Plates were incubated at 27±1°C. Observations on linear growth were recorded after 7 days of inoculation, as we mentioned before.

3.2.5.3- The Influences of salinity

Twenty-six isolates of *M. phaseolina*, 6 isolates of *F. oxysporum* and 2 isolates of *F. verticillioides* were grown singly on conical flasks 250 ml. each containing 100 ml Czapek's liquid medium amended with different concentrations of NaCL (0, 25, 50, 100, 200, 500 and 1000 mM). Molar mass of NaCL = 58.44 g/mol.

The flasks were sterilized for 20 min. at 1.5 atmospheric pressure and inoculated after cooling with 5 mm. in diameter agar discs obtained from 7 days old cultures of the tested isolates. The cultures were incubated at 26±2°C for 7 days. There were three replicate flasks for each isolate, in seven treatments. Following incubation, grown mycelium was filtered by sterile filter paper (Whatman No. 1) and weighed after drying for 48 hours at 60 °C to determine the dry weight of fungal growth, in order to investigate the effect of salinity on the growth of fungi.

3.2.6- Phenotypic variations among the isolates of *M. phaseolina*, *F. oxysporum* and *F. verticillioides*

All the recovered isolates of *Macrophomina* and *Fusarium* were characterized for colony colour and growth pattern on Potato Dextrose Agar medium (Booth, 1971), to determine phenotypic variations among the different isolates of *Macrophomina* and *Fusarium*. Petri dishes containing PDA 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. The plates were incubated at 26 ± 2ºC for 7 days in darkness.

3.2.7- Determination of genetic diversity among the isolates of *M. phaseolina*, *F. oxysporum* and *F. Verticillioides*

3.2.7.1- Growing fungi for DNA extraction

The *Macrophomina phaseolina*, *Fusarium oxysporum* and *Fusarium verticillioides* isolates were grown in liquid medium. Falcon tubes (50 ml), each containing 40 ml of Potato Dextrose medium, were used. The tubes were sterilized for 20 min. at 1.5 atmospheric pressure and inoculated after cooling with 2 mm. in diameter agar discs obtained from 7 days old cultures of the tested isolates. The cultures were incubated at 25±2°C in Shaker Incubator for 5 days. There were two tubes for each isolate.

Potato Dextrose liquid medium was used instead of Potato Dextrose Agar for growth fungal cultures; because DNA extraction is complicated with Agar.

3.2.7.2- DNA Purification

Total genomic DNA was isolated from 60 mg of each isolate of fungi using the Promega purification kit for DNA extraction (http://www.promega.com). We modified the existing protocol described in Promega kit for isolation of high quality DNA from fungi. The new extraction method was performed as follows:

1- Centrifuge the Falcon tubes, (contains the grown mycelium), at 4000× g for 15 minutes to pellet the mycelium and then remove the supernatant.
2- Add 25 ml of sterile ddH_2O to Falcon tubes to wash the mycelium.
3- Centrifuge the samples at 4000× g for 15 minutes. Remove the supernatant.
4- Dry the mycelium under vacuum (15-30 min.).
5- Place 60 mg of mycelium in a 2 ml centrifuge tube.
6- Fill tubes with liquid nitrogen and let it evaporate.
7- Place a sterile metal rod and 10 mg glass powder. Grind the lyophilized pad by the brief shaking (Tissue Lyser: 30× frequency for 30 second; twice).
8- Immediately add 600µl of Nuclei Lysis Solution to the powdered mycelium and gently pipette to mix.
9- Incubate at 65°C for 15 minutes on a water bath or heating thermostat.

10- Add 200µl of protein precipitation solution and vortex vigorously at high speed for 20 seconds.

11- Let the sample sit on ice for 5 minutes.

12- Centrifuge at 12000 (rcf) for 15 minutes at room temperature.

13- Transfer the supernatant, (containing the DNA), carefully to a clean 1.5 ml microcentrifuge tube.

Note-1: Some supernatant may remain in the original tube containing the protein pellet. Leave this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.

Note-2: If the supernatant is not clean enough; repeat steps from 10-13.

14- Add the same volume of cold isopropanol (approximately 600µl).

15- Gently mix by inversion until the thread-like strands of DNA form a visible mass

16- Put the microcentrifuge tubes on ice for 15-30 min (optional).

17- Centrifuge at 16000 (rcf) for 5 minutes at 4°C.

18- Carefully decant the supernatant and drain the tube on clean absorbent paper.

19- Add the same volume of room temperature 70% ethanol, (approximately 600µl), and gently invert the tube several times to wash the DNA pellet.

20- Centrifuge at 14000 for 2 minutes at room temperature. Carefully aspirate all of the ethanol.

Note-3: If the DNA pellet still looks dirty; repeat steps from 19-20 to wash again with ethanol.

21- Drain the tube on clean absorbent paper and allow the pellet to air-dry for 15-20 minutes.

22- Add 100µl of DNA Rehydration Solution. And mix the solution by gently tapping the tube.

23- Add 3µl of RNase Solution to the purified DNA sample.

24- Centrifuge briefly in a microcentrifuge at 10000 (rcf) for 5 seconds to collect the liquid.

25- Incubate at 37°C for 2 hours to rehydrate the DNA.

26- Incubating the solution overnight at room temperature or at 4°C.

27- Store the DNA at 2-8°C.

In order to check the quality of DNA in terms of the presence of degradation, electrophoresis was made on 1% agarose gel (100 ml TBE buffer (0.5x) + 1 gm agarose + 2 µl ethidium bromide) at 100 Volt for 30 min and DNA bands were visualized by UV Bio-RAD unit. Quality of DNA samples were checked by loading (2 µl DNA + 8 µl ddH_2O + 2 µl loading dye) on agarose gel. Good DNA extractions are characterized by the presence of a distinct high molecular weight DNA band with little or no evidence of lower molecular weight degradation products.

The concentration and purity measurements of DNA samples at 260 nm wavelength were performed via using the Nano-Drop spectrophotometer (ND-1000). The concentration was adjusted at 100 ng/µl for all samples using DNA Rehydration Solution. For the subsequent molecular studies, the samples were kept either at -20 ºC for short-term or at -80 ºC for long-term storage.

3.2.7.3- Determination of genetic diversity among the isolates by using restriction enzymes

Restriction enzymes (RE) are a class of enzyme that cut DNA molecules. Each enzyme recognizes a unique sequence of nucleotides in the DNA strand, usually about 4-6 base-pairs long. Restriction enzymes are used to cut DNA into smaller strands and use of agarose gel electrophoresis for separation of the DNA fragments in order to study fragment length differences among individuals. The minimum amount of enzyme necessary should be used. The one unit of enzyme activity is defined as the amount needed to digest 1 µg of a specific DNA in a 50 µl digest at the appropriate temperature. For large genomic digests, use 0.5 unit per µg DNA and digest overnight. Standard digestion temperature is 37 ºC.

RE reaction was performed in a 20 µl digest volume containing the following Components:

1-	7.6 µl ddH_2O
2-	2.0 µl restriction buffer (10X)
3-	10 µl DNA sample (100 ng/µl)
4-	0.4 µl restriction enzyme

EcoRI, EcoRV, HinIII and BamHI restriction enzymes were used for single and double digestion. Reaction was incubated at 37 ºC in water bath for 2- 3 hours.

The samples were prepared as follows:

1- Add loading dye (6x) with amount of 1.0 µl per 5.0 µl of sample. Mix the mixtures by pipette.

2- Load 10 µl of the sample in the wells of the gel. 1.0 µl of the DNA ladder was loaded in the side lanes of the gel.

3- Start the run at 100 volt for 30 minutes.

4- Transfer the gel to Bio-RAD unit to take the photo and check the bands under UV.

3.2.7.4- Determination of genetic diversity among the isolates by using sequence related amplified polymorphism SRAP technique

Sequence-related amplified polymorphism (SRAP) is a molecular marker technique that has been employed in genetic diversity and phylogenetic studies for many crop species (Riaz et al., 2001 and Budak et al., 2004a, b, c). In this study SRAP analysis was used to determine the genetic variability among *Macrophomina phaseolina* and *Fusarium spp.* isolates.

SRAP is based on two-primer amplification to amplify the ORFs. SRAP is a portion of an organism's genome which contains a sequence of bases that could potentially encode a protein. The start and stop ends of the ORF are not equivalent to the ends of the mRNA, but they are usually contained within the mRNA. In a gene, ORFs are located between the start-code sequence (initiation codon) and the stop-code sequence (termination codon) (Li and Quiros, 2001).

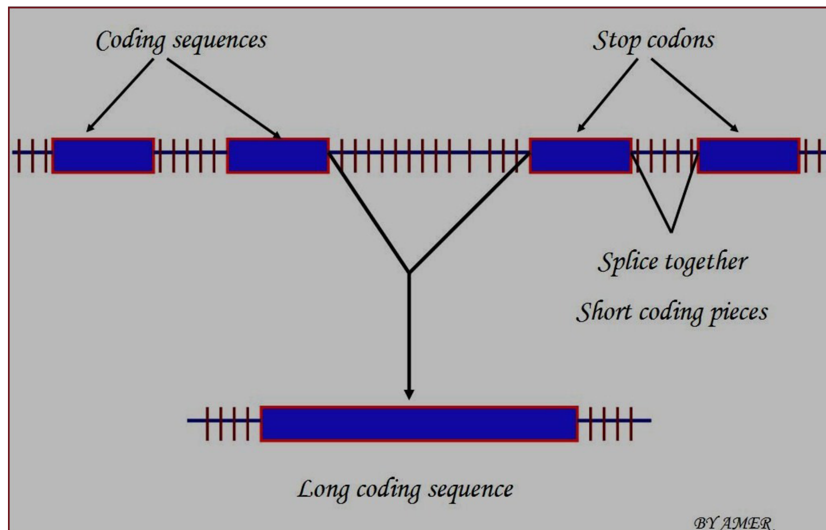


Figure (3.3): Open reading frame (ORFs)

The primers are 17 or 18 nucleotides long and consist of the following elements. Core sequences, which are 13 to 14 bases long, where the first 10 or 11 bases starting at the 5′ end, are sequences of no specific constitution (filler sequences), followed by the sequence CCGG in the forward primer and AATT in the reverse primer. The core is followed by three selective nucleotides at the 3′ end. The filler sequences of the forward and reverse primers must be different from each other and can be 10 or 11 bases long (Li and Quiros, 2001).

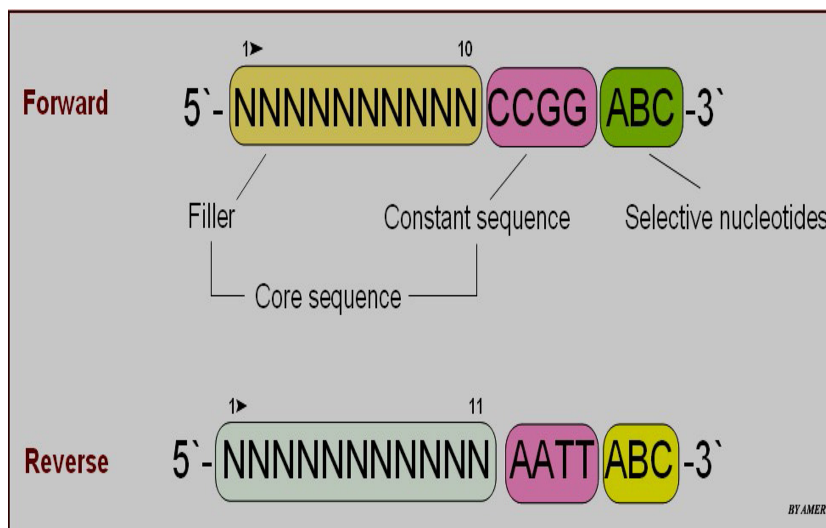


Figure (3.4): SRAP primers elements

Table (3.3): Forward and reverse SRAP primers used for this study

Forward		Reverse	
Primer code	Primer sequence 5′–3′	Primer code	Primer sequence 5′–3′
Me1	TGA GTC CAA ACC GGA TA	Em1	GAC TGC GTA CGA ATT AAT
Me2	TGA GTC CAA ACC GGA GC	Em2	GAC TGC GTA CGA ATT TGC
Me3	TGA GTC CAA ACC GGA AT	Em3	GAC TGC GTA CGA ATT GAC
Me4	TGA GTC CAA ACC GGA CC	Em4	GAC TGC GTA CGA ATT TGA
Me5	TGA GTC CAA ACC GGA AG	Em5	GAC TGC GTA CGA ATT AAC
Me6	TGA GTC CAA ACC GGA CA	Em6	GAC TGC GTA CGA ATT GCA
Me7	TGA GTC CAA ACC GGA CG	Em7	GAC TGC GTA CGA ATT CAA
Me8	TGA GTC CAA ACC GGA CT	Em8	GAC TGC GTA CGA ATT CAC
Me9	TGA GTC CAA ACC GGA GG	Em9	GAC TGC GTA CGA ATT CAG
Me10	TGA GTC CAA ACC GGA AA	Em10	GAC TGC GTA CGA ATT CAT
Me11	TGA GTC CAA ACC GGA AC	Em11	GAC TGC GTA CGA ATT CTA
Me12	TGA GTC CAA ACC GGA GA	Em12	GAC TGC GTA CGA ATT CTC
		Em13	GAC TGC GTA CGA ATT CTG
		Em14	GAC TGC GTA CGA ATT.CTT
		Em15	GAC TGC GTA CGA ATT.GAT
		Em16	GAC TGC GTA CGA ATT.GTC

Table (3.4): SRAP primer combinations used in this study

No.	Primer combinations	No.	Primer combinations
1-	Em1- Me1	18-	Em15-Me10
2-	Em1- Me3	19-	Em16- Me11
3-	Em10- Me4	20-	Em2- Me1
4-	Em10- Me5	21-	Em2- Me8
5-	Em11- Me11	22-	Em3- Me10
6-	Em11- Me2	23-	Em3-Me3
7-	Em11- Me4	24-	Em4- Me1
8-	Em11- Me6	25-	Em4- Me4
9-	Em11- Me7	26-	Em5- Me10
10-	Em11- Me8	27-	Em6- Me2
11-	Em12- Me4	28-	Em6- Me4
12-	Em13- Me12	29-	Em7- Me6
13-	Em13- Me4	30-	Em7-Me8
14-	Em14- Me12	31-	Em8- Me6
15-	Em14- Me3	32-	Em8- Me7
16-	Em14- Me4	33-	Em9- Me4
17-	Em15- Me9	34-	Em9-Me8

3.2.7.4.1- SRAP-PCR amplifications

The SRAP-PCR amplifications were performed in a 25 µl reaction volume. The PCR mixture reaction consisted of the following:

Components		For 1 sample
1-	Sterile ddH_2O	16.7 µl
2-	Reaction buffer $(NH)_4SO_4$. (10x)	2.5 µl
3-	dNTPs mix. (10 mM)	0.6 µl
4-	Primer (Forward). (10 µM)	0.5 µl
5-	Primer (Reveres). (10 µM)	0.5 µl
6-	$MgCL_2$. (25 mM)	2.0 µl
7-	Taq polymerase	0.2 µl
8-	DNA sample. (100 ng/µl)	2.0 µl

3.2.7.4.2- PCR reaction conditions

The amplification protocol was carried out as follows using PCR-unit, PTC-100 Peltier Thermal Cycler:

a) Initial denaturation at 95 °C for 5 min.

b) 35 cycles each consists of the following steps:

1- Denaturation at 94 °C for 1 min.

2- Annealing at 47 °C for 1 min.

3- Extension at 72 °C for 1 min.

C) Final extension at 72 °C for 5 min.

D) Hold at 4 C for ever.

3.2.7.4.3- SRAP analysis

The SRAP analysis was carried out using 28 primers including 12 forward (Me 1-12) and 16 reverse (Em 1-16) for a total of 34 primer combinations according to Li and Quiros, 2001; Budak et al., 2004a, b, c; Budak et al., 2005 and Dinler and Budak, 2008.

3.2.7.4.4- Detection of SRAP-PCR products

The amplification products of the SRAP reaction were prepared as the following:

1- Add loading dye (6x) with amount of 1.0 µl per 5.0 µl of sample. Mix the mixtures by pipette.

2- Load 10 µl of the sample in the wells of the gel. 1.0 µl of the DNA ladder was loaded in the side lanes of the gel.

3- Start the run at 100 volt for 30 minutes.

4- Transfer the gel to Bio-RAD unit to take the photo and check the bands under UV.

3.2.7.4.5- SRAP data analysis

Digital images were scored as ‘1’ for presence and ‘0’ for absence of clear and unambiguous DNA fragments. Similarity matrices were constructed from the binary data with Jaccard’s coefficients (Jaccard, 1908). The genetic distance matrix was subjected to cluster analysis using the Unweighted Pair-Group Method with Arithmetic mean (UPGMA) in NTSYS-pc version 2.1 program (Rohlf, 2000).

A graphical display of the genetic relationships was also constructed by principal coordinate analysis to provide another means of testing relationships among isolates (Gower, 1966). These statistical analyses were performed by NTSYS-pc version 2.2 program.

3.2.8- Evaluation the susceptibility of sunflower cultivars and genotypes to infection by *Macrophomina phaseolina*

In this part, one of the most important factors that may affect the incidence of sunflower damping-off and charcoal-rot caused by *M. phaseolina* was studied. Three isolates of *M. phaseolina,* each of them are from different location were selected according to their pathogenicity test. Infection reactions of 41 sunflower cultivars were studied. Two cultivars, (AUROFLOR and VIDOIC), were obtained from the Ministry of Agriculture, Egypt; and 39 cultivars were obtained from Trakya Agricultural Research Institute (TARI), Edirne, Turkey.

This experiment was carried out in the greenhouse of Anadolu Institute Research, Eskişehir, Turkey during spring, 2009. Sterilized pots (No.6) were filled with sterilized soil which mixed thoroughly with equal amounts of inoculum of each fungus at the ratio of 2 % of soil weight, and then pots were irrigated. Soil was prepared by mixing the soil carefully with nitrogen, phosphorus and potassium (NPK) at the ratio of 3 % of soil weight.

Inocula were prepared as mentioned before in the pathogenicity test. After one day, each pot was sown with 4 sterilized seeds for each cultivar. Pots containing non infested soil which mixed with 2 % sterile barley medium were used as a control. Three pots were used for each treatment as replicates. The plants were irrigated when necessary and daily observed for infection. Pots were placed in the greenhouse at 24°C (minimum) and 28°C (maximum). Percentages of survival plants were recorded after 2 and 4 weeks from sowing. Disease severity of charcoal-rot was determined at the end of the experiment, (after 10 weeks from planting), as mentioned before.

3.2.9- Variation of sunflower root exudates

3.2.9.1- Preparation of the exudates

Thirteen sunflower cultivars were selected according to their reaction on incidence by damping-off and charcoal-rot diseases during the experiment of cultivars evaluation which mentioned before. The selected cultivars were: four cultivars that proved to be resistant cultivars (BAH-4-A, P-4223-A, RHA-461 and 62003-A), four cultivars which showed to

have moderate resistance (1159-A, 0043-A, 7675-A and 7989-A) as well as fives Susceptible cultivars (HA-465-A, 66241-A, 7710-A, 7990-A and TUNCA).

Sunflower root exudates were prepared by placing 100 seeds of surface sterilized seeds of the tested cultivars in sterilized Petri dishes (10 cm in diameter) each containing 20 ml of distilled sterile water and were incubated in seed germinator at 25±2°C (I.S.T.A.,1959) for 48 hours.

Preparation of root exudates was done according to the method described by Mohamed et al., 1981 and Li et al., 2009 with some modifications. Conical flasks (1000 ml in capacity) each containing 120 gm of glass beads (0.2 cm average diameter), were moistened with 150 ml distilled water then autoclaved. After autoclaving, incubated seeds which previously disinfested and soaked in water for 48 hrs were seeded in the conical flasks. Seeded flasks were incubated in a seed germinator at 25±2°C. After 14 days from seeding, contents of the flasks were examined. Contaminated flasks, which containing contaminated seeds that had internal fungal infection were discarded. After two days from the test, the exudates were collected and filtered through sterile filter paper (Whatman No. 1). The collected exudates were concentrated to 100 ml using a vacuum rotary evaporator and 50 ml were used to determine the amino acid contents, while the other 50 ml were used to determine the effect on the growth of *M. phaseolina, F. oxysporum* and *F. verticillioides.*

3.2.9.2- Effect of root exudates of sunflower cultivars on the growth of *Macrophomina* and *Fusarium* in *vitro*

The collected exudates were filtered through 0.22 µm sterile filters (PES Syringe filter). Sterilized flasks of 100 ml in capacity containing 10 ml of autoclaved Czapek's medium supplemented by 10 ml of the tested root exudates samples were inoculated with 5 mm in diameter agar discs bearing 96 hours old cultures of tested fungus. In control treatments, root exudates were replaced by sterile distilled water. Three replicates were used for each treatment. Dry weight of fungal growth was determined after incubation at 26±2°C for 7 days. The tested fungi were: three isolates of *Macrophomina phaseolina* and one isolate of each of *Fusarium oxysporum* and *Fusarium verticillioides.*

3.2.9.3- Determination of amino acids quantity in the root exudates of sunflower cultivars during a 14 days growth period

Sunflower root exudates were filtered through a 0.22 µm sterile filter (PES Syringe filter) prior to the determination of the contents of amino acids. The components and contents of the amino acids were determined using BIOCHROM 30 (amino acid analyzer).

The analytical conditions were as follows: pH was adjusted to 2.2 using sodium citrate loading buffer, for all exudates.

3.2.10- Investigate the potential biocontrol agents for the management of damping-off and charcoal-rot in sunflower

3.2.10.1- Isolation and identification of the bioagents

Isolation of fungal and bacterial microorganisms from rhizosphere of sunflower plants was carried out according to the method described by Dhingra and Sinclair, 1995. For collecting the soil samples, sunflower plants were collected from different locations from Egypt and Turkey. Plants were carefully uprooted, then the excess soil gently was shaked off, discarded and only that soil which was adhering closely to the root system was leaved. Roots were removed, cut to pieces and placed in flasks (500 ml) containing 200 ml. sterile water. Flasks were gently shook until most of the closely adhering rhizosphere soil was removed, then roots were removed and placed into another flasks containing 200 ml sterile water and then flasks were shook again. Suspensions from both flasks were mixed and serial dilutions were prepared. One milliliter from each dilution was used for isolating the fungi and bacteria associated with sunflower roots. Peptone-Rose Bengal Agar medium was used for isolating fungi (Martin, 1950) whereas; Nutrient Agar (N.A.) medium was used for isolating bacteria (Clesceri et al., 1998).

Pure cultures of the isolated fungi, which belong to the genus *Trichoderma spp., Gliocladium spp. Cunninghamella spp*., and *Penicillium spp*., were obtained by using single spore isolation technique described by Brown, 1924 and kept at 5°C on PDA slants for further studies. The antagonistic fungi were identified according to their morphological characteristics of mycelia and conidiophores as described by Domsch et al., 1980 and Dhingra and Sinclair, 1995.

Pure cultures of the isolated bacteria were obtained by using single colony technique and were kept at 5°C on nutrient agar slants for further studies. The antagonistic bacterial isolates were identified according to their morphological, culture and biochemical activities according to Skinner and Lovelock, 1979 and Sneath et al., 1986.

3.2.10.2- Preliminary test for efficacy of bioagents fungi against sunflower pathogens in *vitro*

Antagonistic capability of twelve fungal isolates and four isolates of endospore-forming bacteria were tested against the pathogenic isolates of sunflower damping-off and charcoal rot diseases in *vitro.* For studying the effect of bioagents fungi on the growth of the tested pathogenic fungi, plates were inoculated with equal disks (5 mm. in diameter) of each pathogenic fungus obtained from 5 days old cultures on a side of each plate. One equal disk of each antagonistic fungus was also placed at opposite sides, at the periphery, of the same plate. Each sterilized Petri dishes (9 cm. in diameter) containing 10 ml. of PDA medium (pH7) was used in this study. For studying the antagonistic effect of bacterial isolates on growth of the tested highly pathogenic isolates, plates were streaked with the bacterial growth obtained from two days old culture on a side of each plate by using needle. At the same time, one disk of each pathogen was placed on the opposite side of the same plate.

Plates inoculated with pathogenic fungi only were used as control. The inoculated plates were incubated at 26±2°C. Three replicates were used for each treatment. Observation of inhibition and/or antagonism of the tested fungi were recorded after incubation period, when the control plates reached its maximum growth, and covered the plate surface (9.0 cm in diameter).

In case of antagonistic fungi, the diameter of inhibition zones was measured using a ruler, to measure the diameter of the clear inhibition zone area. After measuring all the replicates, the averages of the treatments were calculated according to following formula:

Percentage of inhibition zones = 100 - [A+B]

Where: (A) = The percentage of bioagents growth,

(B) = The percentage of pathogen growth.

In case of antagonistic bacteria, percentages of mycelial growth inhibition were calculated according to the following formula:

$$\text{Percentage of mycelial growth inhibition} = \frac{A - B}{A} \times 100$$

Where: (A) = Length of the control hyphal growth,

(B) = Length of the treated hyphal growth.

3.2.10.3- Evaluation efficacy of *Trichoderma* and *Gliocladium* isolates against *Macrophomina phaseolina* in greenhouse

The efficiency of the highly antagonistic isolates of *Trichoderma harzianum* (E17b & E17c), *T. hamatum* (E19a) *T. koningii* (E21) and *Gliocladium catenulatum* (E18) as biological control agents, on incidence of sunflower damping –off and charcoal rot diseases caused by *Macrophomina phaseolina* was carried out under greenhouse conditions.

Pathogenic and antagonistic fungus inocula were prepared by inoculating sterilized conical flasks 500 ml containing Barley medium (75 gm barley + 25 gm clean sand + 2 gm glucose + 0.1 gm yeast extract + 100 ml dH_2O) with the tested fungal isolates and incubated at 26±2°C for two weeks.

Sterilized pots (No.5) were filled with sterilized sandy-loam soil which mixed thoroughly with equal amounts of inoculums of *Macrophomina phaseolina* isolates at the ratio of 1% of soil weight, and then pots were irrigated. After 7 days, inoculation of infested pots with antagonistic fungus was done. Soil surface in each pot was dug, and then 5gm. of inoculums of each of biocontrol agents were added separately to each pot and were seeded with sterilized sunflower seeds. Four surface disinfected seeds of SANAY and TUNCA (commercial sunflower cultivar) were sown in each pot. Uninoculated Pots (containing uninfested sterile barley medium), pots containing only *M. phaseolina* isolates and pots containing only biocontrol agents were used as control. Three replicates were

used for each treatment. Plants were irrigated when it necessary and daily observed for infection. Percentages of survival plants were recorded after 2 and 4 weeks from sowing.

3.2.10.4- Screening the effect of biological control agents on sclerotia production of *Macrophomina phaseolina*

The effect of *Trichoderma harzianum* (E17b), *T. harzianum* (E17c), *T. hamatum* (E19a), *T. koningii* (E21) and *Gliocladium catenulatum* (E18) on sclerotia production of *Macrophomina phaseolina* were studied by counting sclerotia in the soil during the period of biological control experiment which mentioned before. The numbers of sclerotia were estimated in one gram of the soil under (4x) microscopic field. Three replicates were used for each treatment. The soil samples were taken at three times 10 days, 20 days and 30 days after soil infestation. Percentages of reduction in number of sclerotia were calculated according to the following formula:

$$\text{Percentage of reduction in number of sclerotia} = \frac{A - B}{A} \times 100$$

Where: (A) = Sclerotia number on the control,

(B) = Sclerotia number on treatment.

3.2.11- Statistical analysis

Data analysis presented in this study, were statistically analyzed with MSTAT and GENSTAT software. RCBD design was used for greenhouse experiments according to Gomez and Gomez, 1984.

4- RESULTS

4.1- Field survey and collection of fungal isolates associated with sunflower roots

4.1.1- Screening certain sunflower fields in Egypt for occurrence of damping-off and charcoal-rot pathogens

The causal pathogens of seedling damping-off and charcoal-rot diseases of sunflower are varied according to the plant ages and area of collection. Therefore, this study was carried out to determine the fungi associated with the roots of sunflower plants in three different zones in Assiut Governorate, Egypt. The survey was done during the summer growing season, 2007.

The total numbers of the obtained isolates were 180 isolates. Results of this study, Table (4.1) and Figure (4.1), showed that the most frequently isolated fungi from naturally diseased sunflower plants were *Macrophomina phaseolina* (Tassi) Goid., *Fusarium oxysporum* Shelecht., *Fusarium verticillioides* Sacc. (*F. moniliforme* Sheldon.) and *Rhizoctonia solani* Kuhn. Other fungal species which recorded as weakly pathogenic or saprophytic fungi were also commonly recorded; they included *Rhizopus spp., Aspergillus spp.* and *Penicillium spp*.

The frequency of the causal fungi of sunflower damping-off and charcoal-rot diseases varies from cultivated area to another according to the environmental factors, soil type and plant age. The major causal pathogens of sunflower damping-off and charcoal-rot were *Macrophomina phaseolina*. The highest frequency of *M. phaseolina* was occurred in the adult stage. The occurrence and frequency of *F. oxysporum, F. verticillioides* and *R. solani* reached their maximum in the seedling stage and decreased by increasing in plant age. They varied also according to the investigated area. Other common isolated fungi, which regarded as weakly pathogenic fungi may infect sunflower plants as secondary infection after invasion of sunflower roots by *M. phaseolina* and *Fusarium* or *Rhizoctonia spp.*

Table (4.1): Screening certain sunflower fields in Egypt for occurrence of damping-off and charcoal-rot pathogens

Regions	*Age of plants	Frequency of isolated fungi (%)				
		M. phaseolina	*F. oxysporum*	*F. verticillioides*	*R. solani*	**Other fungi
Assiut	(1)	9.09	18.18	9.09	18.18	45.46
	(2)	6.67	26.66	20.00	6.67	40.00
	(3)	33.34	16.66	16.66	8.34	25.00
	(4)	20.00	20.00	10.00	10.00	40.00
Manfalout	(1)	13.33	20.00	13.33	20.00	33.34
	(2)	23.53	23.53	17.65	5.88	29.41
	(3)	50.00	12.50	6.25	6.25	25.00
	(4)	47.62	4.76	4.76	9.52	33.34
Abuteeg	(1)	23.07	7.70	15.38	15.38	38.47
	(2)	37.50	18.75	12.5	6.25	25.00
	(3)	42.10	15.79	15.79	5.26	21.06
	(4)	53.33	6.67	13.33	6.67	20.00
Means		29.97	15.93	12.89	9.87	31.34

*: Different times of fields inspecting to covering the different stages of plant growth, as follows:

(1) = 30 days after planting (3) = 60 days after planting

(2) = 45 days after planting (4) = 90 days after planting

**: weakly pathogenic or saprophytic fungi: (*Rhizopus spp., Penicillium spp.* and *Aspergillus spp.*).

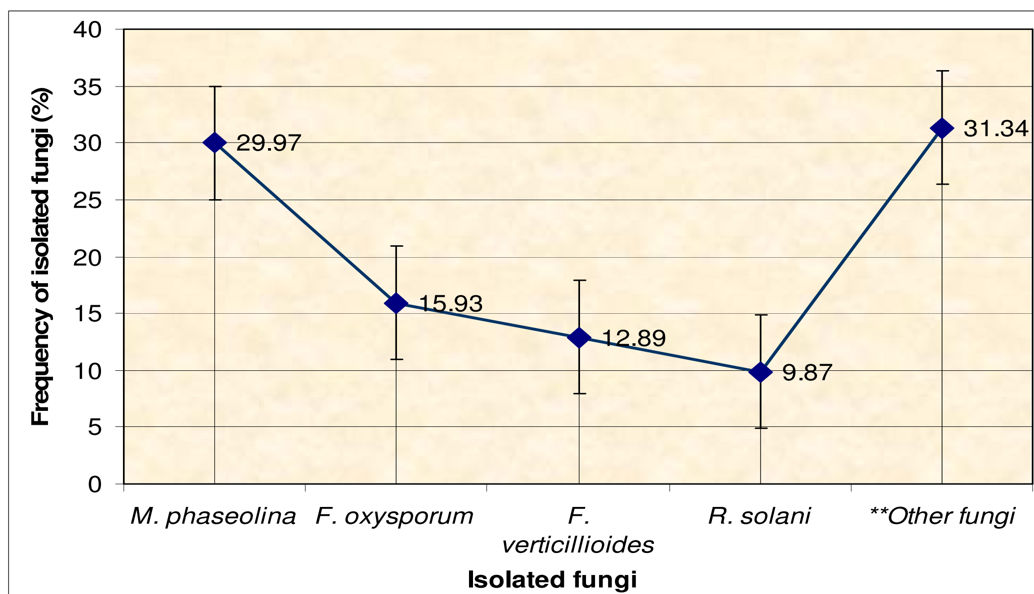


Figure (4.1): Frequency of the isolated fungi associated with sunflower roots regardless to the inspected regions in Egypt.

4.1.1.1- Percentage of disease incidence (D.I. %) during field inspection

Field inspection data in Table (4.2) indicated that, in general, inspection of the plants during the growing season is an essential step to verify severity of the disease and to advice/guide the grower to remove the infected plants and to design standard program for control the disease.

The results obtained showed differences in the disease incidence according to the age of the plants and the growing area. The percentages of disease incidence were increased during progressing age of sunflower. The highest disease incidence (%) was occurred in the adult stage (at the age of 90 days). On the other hand, disease incidence was also varied according to the investigated area. Manfalout was the highest DI region followed by Assiut, while Abuteeg showed the lowest DI. The occurrence and frequency of fungi associated with sunflower roots reached their maximum at the harvest time.

Table (4.2): Percentage of disease incidence (D.I. %) during field inspection

Zones	*Age of plants	**Percentage of disease incidence		
		Field No. 1	Field No. 2	Means
Assiut	(1)	5.04	3.14	4.09
	(2)	7.39	5.89	6.64
	(3)	10.90	9.01	9.95
	(4)	21.04	14.73	17.88
Manfalout	(1)	2.64	5.14	3.89
	(2)	4.93	8.25	6.59
	(3)	8.00	12.85	10.42
	(4)	16.86	19.21	18.03
Abuteeg	(1)	2.45	4.75	3.60
	(2)	5.15	6.09	5.62
	(3)	6.69	10.48	8.58
	(4)	17.73	16.77	17.25

*: Different times of field inspecting to covering the different stages of plant growth, as follows:
(1) = 30 days after planting (3) = 60 days after planting
(2) = 45 days after planting (4) = 90 days after planting
**: Disease incidence (%) = percentage of plants in a field showing disease symptoms.

4.1.2- Screening certain sunflower fields in Turkey for occurrence of damping-off and charcoal-rot pathogens

In June 2009, symptoms suggestive of charcoal-rot were observed on oilseed sunflower plants in Eskişehir Province. Symptoms first observed on plants approaching physiological maturity consisted of silver-gray lesions girdling the stem at the soil line, premature plant death, and reduced head diameter. The pith in the lower stem was completely absent or compressed into horizontal layers. Black, spherical microsclerotia were observed in the pith of the lower stem, underneath the epidermis, and on the exterior of the taproot. The internal stem a shredded appearance. Later, the vascular bundles become covered with small, black flecks or sclerotia of the fungus. The first observed symptoms were confirmation of *Macrophomina phaseolina* (Tossi) Goid. as the causal agent of sunflower charcoal-rot based on the size of the microsclerotia, which ranged from 80 to 100 µm in diameter, from both infected sunflowers and pure cultures (Holliday and Punithalingam, 1970). This is the first report of *M. phaseolina* on sunflower in Turkey.

Results of this study are presented in Table (4.3) and showed that the total numbers of the obtained isolates were 40 isolates; the most frequently isolated fungi from naturally diseased sunflower were *Macrophomina phaseolina*, *Fusarium oxysporum, Rhizoctonia solani* and *Alternaria helianthi* (Hansford) Tubaki and Nishihara. Other fungal species which recorded as weakly pathogenic or saprophytic fungi were also commonly recorded; they included *Rhizopus spp., Aspergillus spp.* and *Penicillium spp*.

The frequency of the causal fungi of sunflower root-rot and charcoal-rot diseases varies from cultivated area to another. The major causal pathogen of sunflower damping-off and charcoal-rot was *Macrophomina phaseolina*. The highest frequency of *M. phaseolina* was occurred in Cavlum Village followed by Sevinc area. The occurrence and frequency of *F. oxysporum* and *Rhizoctonia solani* also varied according to the investigated area. The highest frequency of *F. oxysporum* was occurred in Cavlum Village followed by Gunduzler Town, furthermore, *R. solani* reached its maximum in Sevinc area. Data also revealed that, *F. oxysporum* and *R. solani* not occurred in Sevinc area and Gunduzler Town respectively. Other common isolated fungi, which regarded as weakly

pathogenic fungi may infect sunflower plants as secondary infection after invasion of sunflower roots by *M. phaseolina* and *Fusarium* or *Rhizoctonia spp.*

Table (4.3): Field survey and collection of fungal isolates associated with sunflower roots in Turkey

Regions	Area of the field	Age of plants	Frequency of isolated fungi (%)				
			M. phaseolina	*F. oxysporum*	*R. solani*	*A. helianthi*	*Other fungi
Cavlum Village	5.0 Km^2	10 weeks	14.00	20.00	10.00	10.00	46.00
Gunduzler Town	2.5 km^2	6 weeks	10.00	12.00	0.0	10.00	68.00
Agapinar area	3.0 km^2	6 weeks	10.00	10.00	10.00	0.0	70.00
Sevinc area	2.0 Km^2	8 weeks	12.00	0.0	12.00	14.00	62.00
Means			11.50	10.50	8.00	8.50	61.50

*: weakly pathogenic or saprophytic fungi: (*Rhizopus spp., Penicillium spp.* and *Aspergillus spp.*).

4.2- Pathogenicity tests

4.2.1- Pathogenicity test for isolates obtained from Egypt

4.2.1.1- *Macrophomina phaseolina*

Pathogenicity of 22 isolates of *Macrophomina phaseolina* was tested on three sunflower cultivars, (SANAY, TUNCA and TR-3080), under greenhouse conditions. Isolates x cultivars interaction revealed that all tested isolates of *M. phaseolina* were pathogenic to all tested cultivars of sunflower. All of the tested isolates were pathogenic at the pre- and post- emergence damping-off stages. Such results indicated that *M. phaseolina* was considered the major causal pathogen of sunflower charcoal-rot disease.

Data presented in Table (4.4) and Figure (4.2), indicated that isolates No. E3, E6 and M9 were the highest virulent, that caused 100% mortalities. Other tested isolates differed in their virulence from highly pathogenic (95.55 %) in case of isolates No. E2, E4 and E5 to moderately (93.33 %) in case of isolates No. E1, M3, M10 and E12.

Table (4.4): Percentage mortality of sunflower plants, inoculated with *M. phaseolina*

Isolates		Mortality of sunflower plants (%)								Severity of Disease			
Source	N0.	2 weeks				4 weeks				SANAY	TUNCA	TR-3080	Means
		SANAY	TUNCA	TR-3080	Means	SANAY	TUNCA	TR-3080	Means				
Assiut	M9	66.66	80.00	60.00	68.88	100	100	100	100	6.00	6.00	6.00	6.00
	M10	73.33	80.00	60.00	71.11	93.33	100	86.66	93.33	5.80	6.00	5.60	5.80
	E10	53.33	60.00	66.66	59.99	86.66	93.33	93.33	91.10	5.60	5.73	5.80	5.71
	E11	40.00	66.66	66.66	57.77	86.66	93.33	93.33	91.10	5.66	5.80	5.86	5.77
	E12	73.33	53.33	66.66	64.44	93.33	93.33	93.33	93.33	5.73	5.86	5.93	5.84
Manfalout	E4	86.66	80.00	53.33	73.33	100	100	86.66	95.55	6.00	6.00	5.60	5.86
	M5	60.00	53.33	40.00	51.11	93.33	80.00	86.66	86.66	5.86	5.26	5.80	5.64
	M6	53.33	53.33	53.33	53.33	93.33	86.66	93.33	91.10	5.73	5.80	5.86	5.79
	E5	46.66	60.00	73.33	59.99	86.66	100	100	95.55	5.60	6.00	6.00	5.86
	E6	73.33	86.66	93.33	84.44	100	100	100	100	6.00	6.00	6.00	6.00
	E7	53.33	60.00	73.33	62.22	86.66	93.33	93.33	91.10	5.60	5.80	5.73	5.71
	M7	46.66	66.66	46.66	53.32	86.66	93.33	86.66	88.88	5.66	5.80	5.73	5.73
	E8	53.33	46.66	66.66	55.55	86.66	86.66	93.33	88.88	5.73	5.80	5.86	5.79
	M8	46.66	46.66	66.66	53.32	93.33	86.66	93.33	91.10	5.86	5.53	5.93	5.77
	E9	66.66	40.00	53.33	53.33	86.66	80.00	93.33	86.66	5.66	5.46	5.86	5.66
Abuteeg	E1	53.33	80.00	80.00	71.11	86.66	93.33	100	93.33	5.73	5.73	6.00	5.82
	M1	46.66	66.66	53.33	55.55	86.66	93.33	86.66	88.88	5.73	5.73	5.66	5.70
	M2	66.66	33.33	60.00	53.33	86.66	73.33	93.33	84.44	5.60	5.26	5.73	5.53
	E2	73.33	53.33	73.33	66.66	100	86.66	100	95.55	6.00	5.46	6.00	5.82
	M3	73.33	66.66	60.00	66.66	93.33	93.33	93.33	93.33	5.86	5.86	5.73	5.81
	E3	73.33	73.33	86.66	77.77	100	100	100	100	6.00	6.00	6.00	6.00
	M4	53.33	53.33	80.00	62.22	86.66	86.66	100	91.10	5.66	5.66	6.00	5.77
Control (C)		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.00	1.00	1.00	1.00
Means		57.96	59.12	62.31	59.80	87.53	87.53	89.85	88.30	5.56	5.54	5.63	5.58
LSD		0.05		0.01		0.05		0.01		0.05		0.01	
Cultivars (C)		10.18		16.84		3.12		5.16		0.11		0.19	
Isolates (I)		13.81		18.10		8.67		11.37		0.26		0.34	
C x I		23.92		31.36		15.02		19.70		0.46		0.60	

4.2.1.2- *Fusarium spp.* and *Rhizoctonia solani*

Results in Table (4.5) revealed that, percent of infection was significantly affected according to the differences among the isolates. All of the tested isolated fungi were able to infect sunflower plants causing pre-and post-emergence damping-off and root-rot. The fungal isolates varied in their virulence. *Fusarium verticillioides* proved to be the most aggressive one followed by *Fusarium oxysporum* whereas; *Rhizoctonia solani* was the lowest one.

Table (4.5): Percentage mortality of sunflower plants, inoculated with *Fusarium spp.* and *Rhizoctonia solani*

Isolates		Mortality of sunflower plants (%)								Severity of Disease			
Source	N0.	2 weeks				4 weeks				SANAY	TUNCA	TR-3080	Means
		SANAY	TUNCA	TR-3080	Means	SANAY	TUNCA	TR-3080	Means				
F. oxysporum													
Assiut	E15a	53.33	33.33	46.66	44.44	66.66	60.00	73.33	66.66	5.00	5.20	5.33	5.17
Manfalout	E15b	20.00	33.33	33.33	28.88	53.33	73.33	73.33	66.66	4.53	5.26	5.20	4.99
F. verticillioides													
Manfalout	E14a	20.00	46.66	40.00	35.55	53.33	66.66	66.66	62.21	5.00	4.80	5.26	5.02
Abuteeg	E14b	40.00	53.33	33.33	42.22	66.66	93.33	86.66	82.21	5.20	5.73	5.80	5.57
R. solani													
Assiut	E13	46.66	26.66	46.66	39.99	73.33	80.00	93.33	82.22	5.33	5.53	5.73	5.53
Control (C)		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.00	1.00	1.00	1.00
Means		29.99	32.21	33.33	31.84	52.21	62.22	65.55	59.99	4.34	4.58	4.72	4.54
LSD		0.05		0.01		0.05		0.01		0.05		0.01	
Cultivars (C)		22.41		37.09		15.13		25.03		0.38		0.63	
Isolates (I)		10.66		14.37		11.99		16.16		0.43		0.57	
C x I		18.46		24.89		20.77		27.99		0.74		1.00	

Figure (4.2): Disease symptoms of sunflower plants under artificial infections by *M. phaseolina* in both seedling and maturing stages.

4.2.2- Pathogenicity test for isolates obtained from Turkey

Pathogenicity of four isolates of *Macrophomina phaseolina* and *Fusarium oxysporum* as well as three isolates of *Rhizoctonia solani* and *Alternaria helianthi* were tested on three sunflower cultivars, (SANAY, TUNCA and TR-3080), under greenhouse conditions. Isolates x cultivars interaction revealed that all tested isolates except *A. helianthi* were able to infect sunflower plants causing pre-and post-emergence damping-off as well as charcoal/root-rot. Fungal isolates varied in their virulence. The percent of infection was significantly affected according to the differences among the isolates.

Data presented in Table (4.6) indicated that, *M. phaseolina* is the major causal pathogens of sunflower charcoal-rot disease. *M. phaseolina* isolates were differentiated into pathogenicity test. Isolate No. T38 followed by isolate No. T28 caused the highest infection rates. Other tested isolates showed to have less infection rates. *F. oxysporum* was significantly reduced the percentage of survival plants after 2 and 4 weeks and increased disease severity rate. Also we found that, there is a quite limited variation among *F. oxysporum* isolates in their virulence. *R. solani* isolates proved to be lower aggressive than *M. phaseolina* and *F. oxysporum*. *Alternaria helianthi* isolates proved to be weakly or non pathogenic fungus. Data also revealed that, none of the tested sunflower varieties were immune to infect by the tested isolates.

Table (4.6): Percentage mortality of sunflower plants, inoculated with *M. phaseolina, F. oxysporum*, *R. solani* and *A. helianthi*

Isolates		Mortality of sunflower plants (%)								Severity of Disease			
		2 weeks				4 weeks							
Sources	No.	SANAY	TUNCA	TR-3080	Means	SANAY	TUNCA	TR-3080	Means	SANAY	TUNCA	TR-3080	Means
M. phaseolina													
Cavlum Village	T8	40.00	46.66	33.33	39.99	46.66	53.33	60.00	53.33	3.33	3.86	4.00	3.73
Gunduzler Town	T16	26.66	53.33	33.33	37.77	40.00	60.00	46.66	48.88	3.53	4.13	3.93	3.86
Agapinar area	T28	40.00	33.33	46.66	39.99	53.33	53.33	60.00	55.55	3.66	4.13	4.40	4.06
Sevinc area	T38	46.66	40.00	46.66	44.44	66.66	46.66	53.33	55.55	4.46	4.13	3.80	4.13
F. oxysporum													
Cavlum Village	T2	40.00	46.66	40.00	42.22	53.33	66.66	60.00	59.99	4.00	4.40	4.13	4.17
	T6	40.00	33.33	33.33	35.55	73.33	66.66	66.66	68.88	4.73	4.53	4.46	4.57
Gunduzler Town	T14	33.33	60.00	46.66	46.66	66.66	73.33	60.00	66.66	4.33	4.33	4.33	4.33
Agapinar area	T21	66.66	53.33	46.66	55.55	80.00	66.66	66.66	71.10	4.66	4.46	4.33	4.48
R. solani													
Cavlum Village	T5	46.66	40.00	46.66	44.44	73.33	40.00	60.00	57.77	4.40	4.46	3.93	4.26
Agapinar area	T24	40.00	53.33	40.00	44.44	60.00	66.66	53.33	59.99	4.33	4.33	3.73	4.13
Sevinc area	T36	66.66	46.66	40.00	51.10	73.33	66.66	53.33	64.44	4.66	4.33	4.13	4.37
A. helianthi													
Cavlum Village	T3	6.66	26.66	13.33	15.55	13.33	33.33	20.00	22.22	2.06	3.26	2.73	2.68
Gunduzler Town	T17	26.66	40.00	13.33	26.66	33.33	46.66	26.66	35.55	3.00	3.53	2.86	3.13
Sevinc area	T34	26.66	33.33	6.66	22.21	26.66	40.00	13.33	26.66	2.73	3.20	2.20	2.71
Control		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.00	1.00	1.00	1.00
LSD		0.05		0.01		0.05		0.01		0.05		0.01	
Cultivars (C)		7.09		11.74		5.52		9.14		0.29		0.49	
Isolates (I)		13.28		17.62		12.44		16.50		0.56		0.74	
C x I		23.01		30.53		21.54		28.58		0.97		1.29	

4.3- Physiological studies of *Macrophomina* and *Fusarium* in *vitro*

4.3.1- The influences of temperature

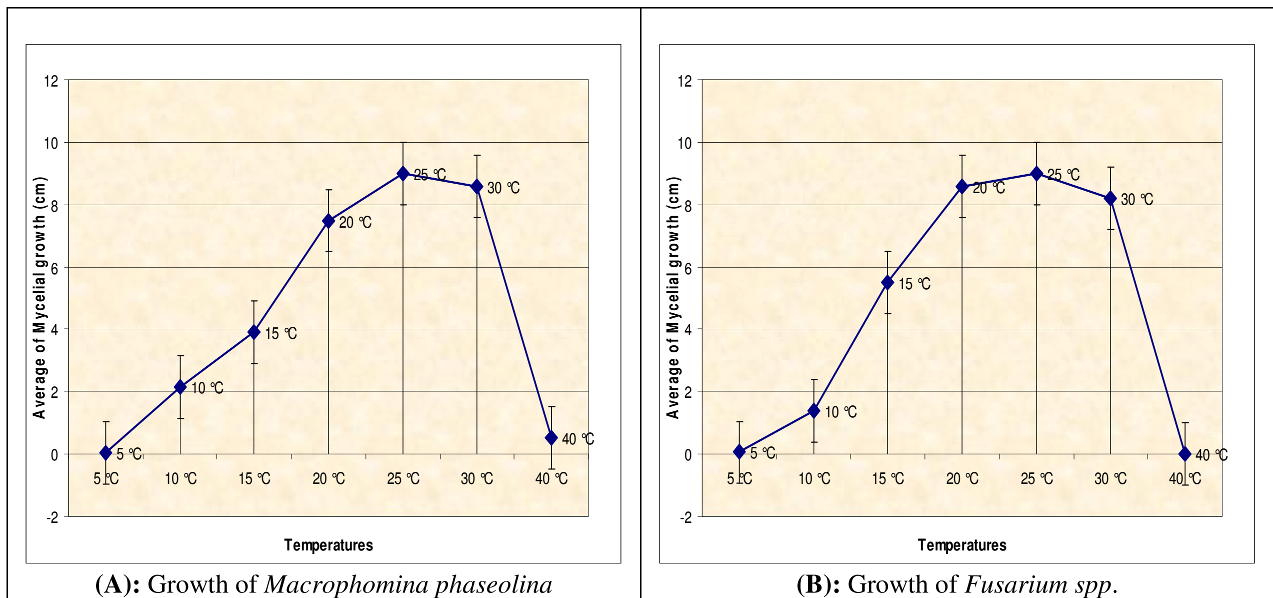
The aim of this study was to investigate a range of temperatures on radial growth of *M. phaseolina* and *Fusarium spp*. in order to determine the optimum temperature for those fungi, and take these information into consideration on the control of the diseases which caused by such microorganisms.

Data presented in Table (4.7) and Figure (4.3) showed that, all tested isolates of *Macrophomina* and *Fusarium* could grow at a wide temperature range (10 - 30°C), but growth was drastically inhibited at 5 and 40°C. The optimum temperature for growth of all isolates was determined to be between 25 and 30°C. In general, the mycelial growth was found to be significantly influenced by, temperature. The growth increased with increasing temperature, reaching optima at 25 and 30°C respectively. The growth rate of all isolates dropped at 5 and 40°C.

The maximum variability between isolates occurred at 10 and 40°C. Isolates from Turkey grew better at lower temperatures than other isolates and also showed inability to grow at 40°C. Isolates from Egypt showed a good adaptability to grow at the highest temperatures. Results also, showed that, among *M. phaseolina* isolates, E3, E6, M9, and T38 isolates significantly grown faster than the other isolates at 10, 15, 20, 25 and 30°C. And they cause the high percentage of pre- and post- emergence damping-off on sunflower. The *Fusarium spp*. isolates did not show any significant differences between their growth rates.

Table (4.7): The influences of temperature on the growth of *Macrophomina* and *Fusarium* in *vitro*

Isolates		Average of mycelial growth (cm)						
Sources	No.	5 °C	10 °C	15 °C	20 °C	25 °C	30 °C	40 °C
Macrophomina phaseolina								
Egypt	E1	0.0	2.00	3.26	6.60	9.00	8.30	0.43
	E2	0.0	2.10	3.73	6.90	9.00	8.76	0.46
	E3	0.0	2.60	4.20	7.76	9.00	9.00	2.53
	E4	0.0	2.80	4.03	7.43	9.00	9.00	1.60
	E5	0.0	2.56	3.60	7.73	9.00	9.00	1.86
	E6	0.0	3.30	4.46	7.80	9.00	9.00	2.20
	E7	0.0	1.70	3.30	6.50	9.00	8.60	0.0
	E8	0.0	2.13	3.30	7.40	9.00	8.46	0.0
	E9	0.0	1.66	3.66	7.03	9.00	8.20	0.0
	E10	0.0	2.23	4.03	7.20	9.00	8.80	0.46
	E11	0.0	1.53	3.60	7.23	9.00	7.86	0.0
	E12	0.0	2.26	3.96	7.26	9.00	7.73	0.60
	M1	0.0	1.63	3.80	6.86	9.00	8.33	0.0
	M2	0.0	1.90	3.53	6.90	9.00	8.30	0.0
	M3	0.0	2.30	3.60	7.53	9.00	8.90	0.70
	M4	0.0	1.56	3.50	7.33	9.00	8.40	0.0
	M5	0.0	1.73	3.23	7.40	9.00	8.66	0.0
	M6	0.0	2.30	3.86	7.73	9.00	9.00	0.90
	M7	0.0	1.70	3.30	7.30	9.00	8.70	0.0
	M8	0.0	1.46	3.50	7.26	9.00	8.46	0.0
	M9	0.0	3.00	4.46	8.23	9.00	9.00	2.10
	M10	0.0	1.66	3.60	7.36	9.00	8.63	0.0
Turkey	T8	0.0	2.06	4.66	8.30	9.00	8.60	0.0
	T16	0.0	1.83	4.53	8.23	9.00	8.50	0.0
	T28	0.53	2.36	5.10	8.60	9.00	8.63	0.0
	T38	0.60	3.13	5.53	8.83	9.00	8.53	0.0
Fusarium oxysporum								
Egypt	E15a	0.0	1.06	4.60	8.43	9.00	8.46	0.0
	E15b	0.0	0.83	4.73	8.26	9.00	8.76	0.0
Turkey	T2	0.0	1.30	5.56	8.20	9.00	7.40	0.0
	T6	0.46	1.76	6.20	9.00	9.00	8.16	0.0
	T14	0.0	1.26	5.60	8.60	9.00	7.26	0.0
	T21	0.0	1.83	6.63	9.00	9.00	8.13	0.0
Fusarium verticillioides								
Egypt	E14a	0.0	1.40	5.20	8.53	9.00	8.53	0.0
	E14b	0.0	1.70	5.46	8.63	9.00	9.00	0.0
LSD			0.05			0.01		
Temperatures(T)			0.041			0.055		
Isolates (I)			0.092			0.121		



(A): Growth of *Macrophomina phaseolina* | **(B):** Growth of *Fusarium spp*.

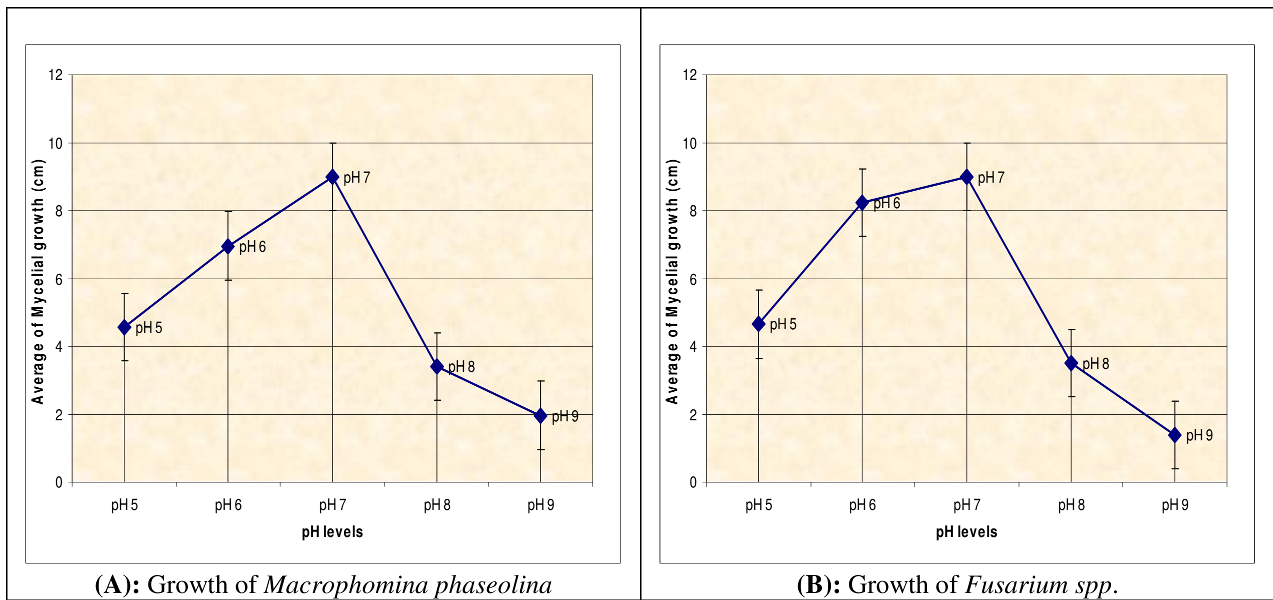
Figure (4.3): The influences of temperature on the growth of *Macrophomina* (A) and *Fusarium* (B) in *vitro* regardless to the tested isolates.

4.3.2- The influences of pH

The study was conducted to determine the effect of pH on the growth of *Macrophomina* and *Fusarium* isolates. Data presented in Table (4.8) and Figure (4.4) demonstrates that, the tested isolates were grown at all pH levels. Their maximum growth was obtained at pH 7 after seven days of inoculation. The pH 6 was also favorable. Growth of the isolates was decreased by increasing or decreasing the pH level from the neutral level. There were no differences in the radial growth among the tested isolates at pH 7.

Table (4.8): The influences of pH on the growth of *Macrophomina* and *Fusarium* in *vitro*

Isolates		Average of Mycelial growth (cm)				
Sources	No.	pH 5	pH 6	pH 7	pH 8	pH 9
Macrophomina phaseolina						
Egypt	E1	4.30	6.06	9.00	2.96	1.80
	E2	4.36	6.36	9.00	3.43	2.10
	E3	5.16	7.23	9.00	3.90	2.46
	E4	4.86	6.90	9.00	3.73	2.33
	E5	4.30	7.20	9.00	3.30	2.56
	E6	5.30	7.26	9.00	4.16	2.36
	E7	4.16	5.96	9.00	3.00	1.60
	E8	4.33	6.86	9.00	3.00	2.13
	E9	4.40	6.50	9.00	3.36	1.66
	E10	4.86	6.66	9.00	3.73	2.23
	E11	4.26	6.70	9.00	3.30	1.53
	E12	4.66	6.73	9.00	3.66	2.26
	M1	4.63	6.33	9.00	3.50	1.63
	M2	4.50	6.36	9.00	3.23	1.73
	M3	4.30	7.00	9.00	3.30	2.26
	M4	4.23	6.80	9.00	3.20	1.56
	M5	4.23	6.86	9.00	2.93	1.60
	M6	4.43	7.20	9.00	3.56	2.30
	M7	4.26	6.76	9.00	3.00	1.60
	M8	4.36	6.73	9.00	3.20	1.46
	M9	5.20	7.70	9.00	3.40	2.33
	M10	4.23	6.83	9.00	3.13	1.56
Turkey	T8	5.40	7.76	9.00	3.86	1.96
	T16	5.23	7.70	9.00	3.73	1.53
	T28	4.23	8.06	9.00	3.53	2.30
	T38	4.36	8.30	9.00	3.40	2.13
Fusarium oxysporum						
Egypt	E15a	4.36	8.06	9.00	3.43	1.06
	E15b	5.16	8.03	9.00	2.76	0.83
Turkey	T2	4.60	8.23	9.00	3.56	1.30
	T6	4.60	8.16	9.00	3.53	1.76
	T14	4.70	8.43	9.00	3.40	1.26
	T21	4.70	8.23	9.00	3.96	1.83
Fusarium verticillioides						
Egypt	E14a	4.50	8.36	9.00	3.53	1.40
	E14b	4.60	8.40	9.00	3.80	1.70
LSD			0.05		0.01	
pH			0.063		0.084	
Isolates (I)			0.166		0.219	



(A): Growth of *Macrophomina phaseolina* | **(B):** Growth of *Fusarium spp*.

Figure (4.4): The influences of pH on the growth of *Macrophomina* (A) and *Fusarium* (B) in *vitro* regardless to the tested isolates.

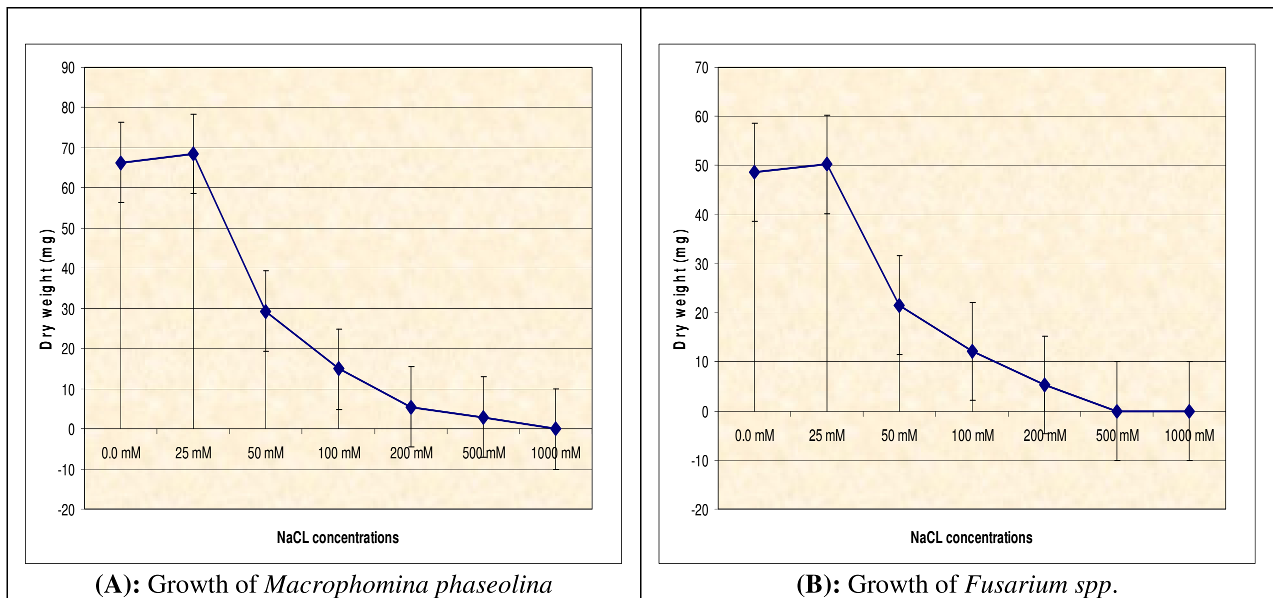
4.3.3- The influences of salinity

The present study was aimed to investigate the influence of NaCL on the fungal growth to clearly understand the modes of tolerance towards salt (NaCL) stress. For this purpose, we measured the growth of 26 isolates of *M. phaseolina* and 8 *Fusarium* isolates on Czapek's liquid media with different concentrations of NaCL. Moreover, data obtained from this study helps to understand the favorable conditions for fungal growth; hence we can make use of this information to design a good program to prevent the infection.

The results of this study were presented in Table (4.9) and Figure (4.5) and data demonstrate that, in general, the fungal responses towards NaCL stress seemed to vary with the concentration of NaCL. Similar sensitivities were observed at high concentrations of NaCL. *Fusarium* isolates displayed a greater sensitivity than *Macrophomina* isolates to high concentration of NaCL. The results showed that there are variations among the isolates in the response to NaCL.

Table (4.9): The influences of NaCL concentration on the growth of *Macrophomina* and *Fusarium* in *vitro*

Isolates		Dry weight (mg)						
Sources	No.	0.0 mM	25 mM	50 mM	100 mM	200 mM	500 mM	1000 mM
Macrophomina phaseolina								
Egypt	E1	62.23	68.25	27.39	12.03	2.87	1.77	0.0
	E2	64.96	71.29	29.29	14.11	4.50	2.82	0.0
	E3	72.60	79.91	33.75	18.01	5.85	4.02	0.0
	E4	61.23	64.11	22.32	8.44	3.80	2.91	0.0
	E5	66.96	67.72	30.34	14.46	5.53	2.87	0.0
	E6	70.63	73.03	31.02	16.73	7.79	3.61	0.0
	E7	63.46	69.25	30.49	14.92	5.87	2.31	0.0
	E8	63.43	64.90	24.06	9.51	3.96	2.27	0.0
	E9	63.90	66.48	31.30	17.11	6.97	2.51	0.0
	E10	72.23	72.50	34.79	22.53	6.49	3.50	0.0
	E11	65.56	66.76	32.19	18.34	6.67	2.54	0.0
	E12	62.13	63.29	26.46	12.51	4.81	2.82	0.0
	M1	69.90	70.71	34.23	19.00	6.57	2.79	0.0
	M2	59.96	65.29	24.49	7.56	3.40	3.26	0.0
	M3	66.56	68.20	27.53	13.04	5.07	2.82	0.0
	M4	70.40	71.78	34.14	16.04	6.21	3.25	0.0
	M5	62.26	64.31	27.39	13.77	4.64	3.61	0.0
	M6	63.03	64.31	25.58	11.27	4.04	2.57	0.0
	M7	65.60	67.40	28.69	14.58	5.67	2.60	0.0
	M8	66.03	66.60	26.91	13.20	5.91	2.57	0.0
	M9	74.53	75.50	35.58	25.04	7.54	4.64	0.0
	M10	66.33	69.80	27.97	15.00	5.81	2.71	0.0
Turkey	T8	65.96	67.52	27.00	13.50	5.34	2.88	0.0
	T16	68.70	66.33	29.49	14.84	5.54	2.01	0.0
	T28	69.70	67.45	30.78	17.37	5.57	3.62	0.0
	T38	64.83	67.08	27.18	14.42	5.21	2.64	0.0
Fusarium oxysporum								
Egypt	E15a	43.76	46.82	18.17	8.15	4.10	0.0	0.0
	E15b	49.70	50.99	21.36	11.45	5.17	0.0	0.0
Turkey	T2	51.53	54.69	22.39	13.46	5.49	0.0	0.0
	T6	52.26	56.02	25.50	18.04	5.90	0.0	0.0
	T14	47.06	46.69	19.41	7.36	3.84	0.0	0.0
	T21	51.06	51.64	21.60	12.39	6.30	0.0	0.0
Fusarium verticillioides								
Egypt	E14a	46.56	47.54	21.21	12.66	6.30	0.0	0.0
	E14b	47.43	47.29	22.81	13.54	5.04	0.0	0.0
LSD			0.05			0.01		
NaCL conc. (C)			0.517			0.681		
Isolates (I)			1.140			1.500		



(A): Growth of *Macrophomina phaseolina* | **(B):** Growth of *Fusarium spp*.

Figure (4.5): The influences of NaCL concentration on the growth of *Macrophomina* (A) and *Fusarium* (B) in *vitro* regardless to the tested isolates.

4.4- Phenotypic variations among isolates of *M. phaseolina*, *F. oxysporum* and *F. verticillioides*

Efforts were made to characterize the fungi associated with sunflower in different regions in Egypt and Turkey. This characterization was based on its pathogenic variability, morphological characteristics as well as the molecular characteristics. For this purpose we study the phenotypic variations among the isolates of *Macrophomina* and *Fusarium.*

The following photos showed the phenotypic variations among 26 *M. phaseolina* isolates, 6 *oxysporum* isolates and 2 isolates of *F. verticillioides*.

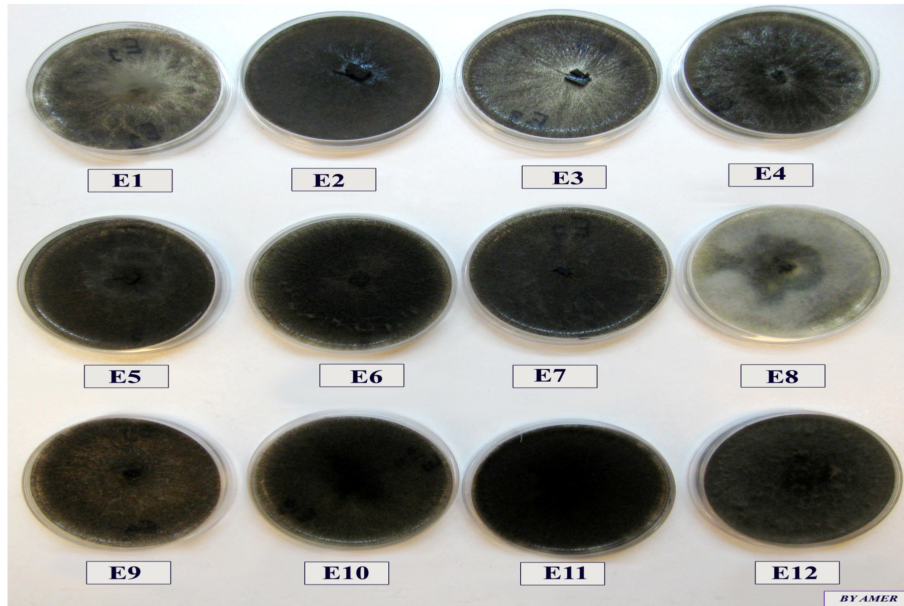


Figure (4.6): Growth patterns of *M. phaseolina* isolates (E1-E12) on PDA medium.

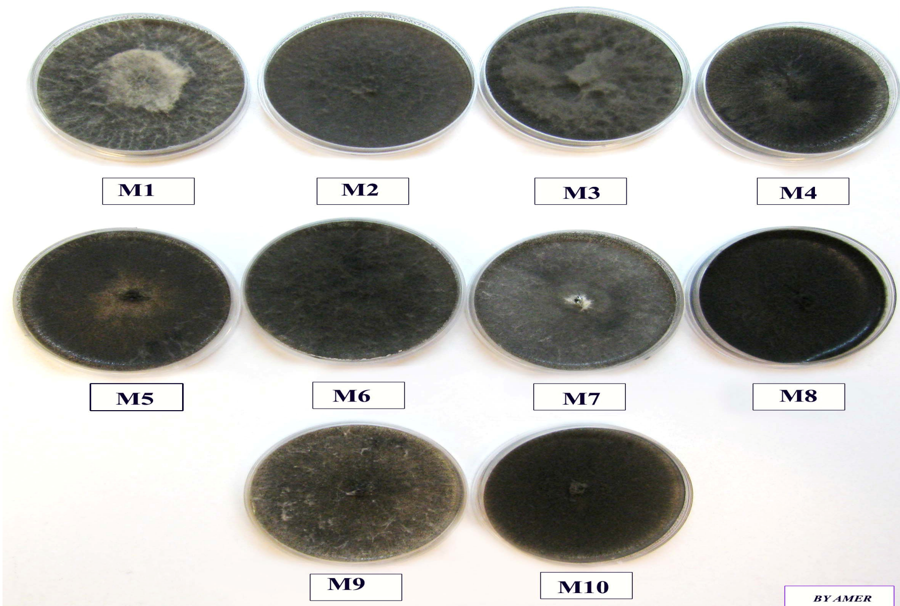


Figure (4.7): Growth patterns of *M. phaseolina* isolates (M1-M10) on PDA medium.

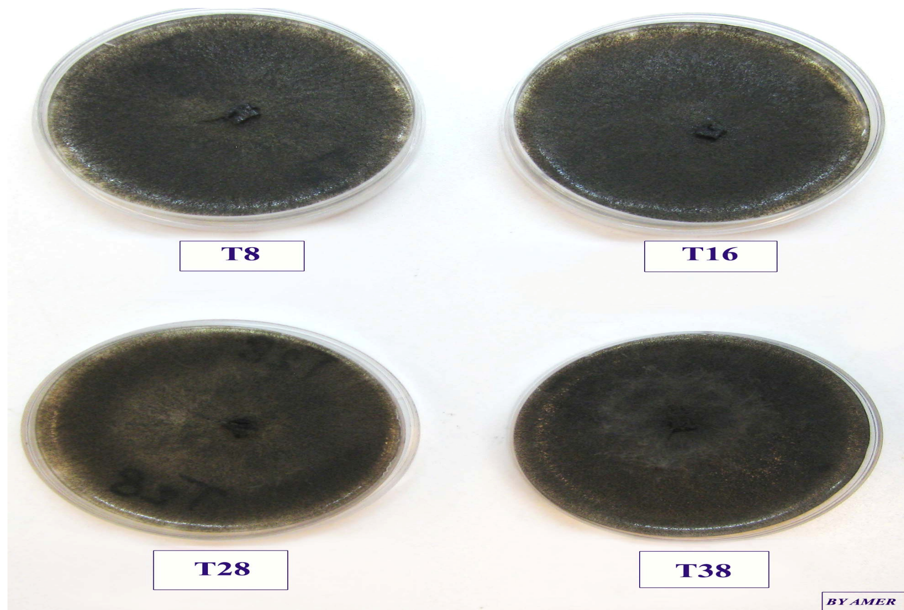


Figure (4.8): Growth patterns of *M. phaseolina* isolates (T8, T16, T28 and T38) on PDA medium.

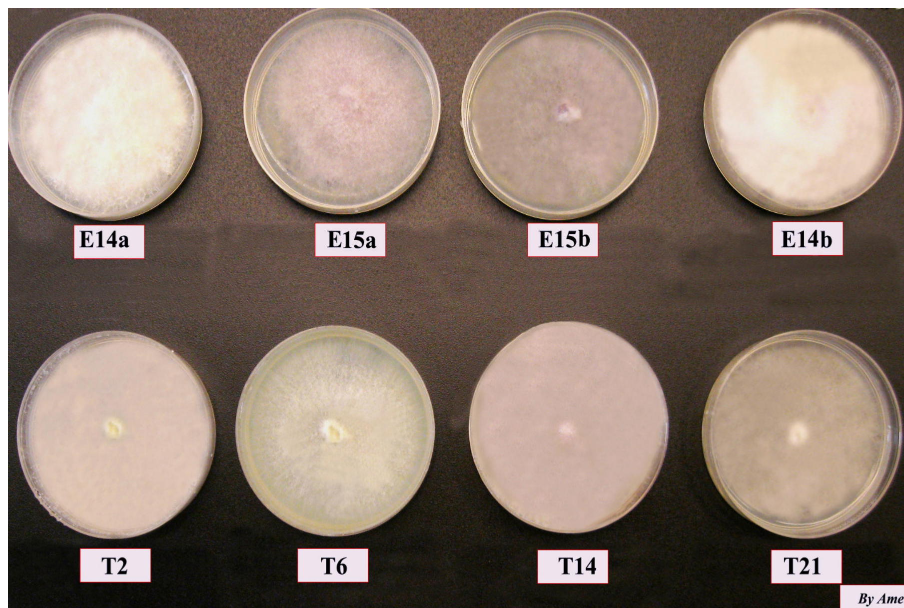


Figure (4.9): Growth patterns of *F. verticillioides* isolates (E14a and E14b) and *F. oxysporum* isolates (E15a, E15b, T2, T6, T14 and T21) on PDA medium.

4.5- Determination of genetic diversity among the isolates of *M. phaseolina*, *F. oxysporum* and *F. verticillioides*

4.5.1- DNA isolation

DNA successfully isolated from the fungal species of *Macrophomina phaseolina*, *Fusarium oxysporum* and *Fusarium verticillioides* by the use of young lyophilized mycelial (5 days growth). A rapid procedure for isolating fungal DNA from multiple isolates of *M. phaseolina*, *F. oxysporum* and *F. verticillioides* has been modified in our study. This technique is useful for diagnosis and screening of natural populations of multiple fungal isolates. The average yield of extracted DNA concentration ranged from 290 to 1400 ng/ul. The purity ratio of the DNA for all samples was between 1.8 – 2.0. The procedure described here works well for extracting high-quality DNA from *Macrophomina* and *Fusarium* as well as other fungi.

4.5.2- Determination of genetic diversity among the isolates by using restriction enzymes

Extracted DNA is digested with specific restriction enzymes. Restriction fragments were separated by electrophoresis in an agarose gel, to be used as markers for studying genetic variation within and among multiple isolates of *M. phaseolina*, *F. oxysporum* and *F. verticillioides*. We found that using only restriction enzymes, (EcoRI, EcoRV, HinIII and BamHI), did not give a polymorphism in this study. The fragments were seen as a continuous 'smear'.

4.5.3- Determination of genetic diversity among the isolates by using sequence related amplified polymorphism (SRAP) technique

The objective of this study was to characterize genetic variability within isolates of *M. phaseolina* and different isolates of *Fusarium species* collected from different geographical regions in Egypt and Turkey. This is the first study on the utility of the SRAP marker

technique to assess genetic relationships and diversity among isolates of *Macrophomina* and *Fusarium*.

Thirty four SRAP primer combinations were used in SRAP-PCR to determine the genetic variability among 26 isolates of *M. phaseolina*, 6 isolates of *F. oxysporum* and 2 isolates of *F. verticillioides*. The obtained results showed that SRAP analysis appeared to be useful tool for identification and characterization the genetic differences among *Macrophomina* and *Fusarium* isolates as well as study the differences between species and strains.

Among the 34 SRAP primer combinations, 12 were amplified the genomic DNA of *Macrophomina* and *Fusarium*, and produced bands ranging from 75-5000 bp. The primers Em11-Me11, Em11-Me4, Em13-Me4, Em14-Me4, Em4-Me4 and Em6-Me4 amplified the genomic DNA of all the isolates of *Macrophomina* and *Fusarium* producing 2- 9 bands of size between 75-5000 bp. While, primers Em11-Me6, Em12-Me4, Em14-Me3, Em2-Me8, Em6-Me2 and Em9-Me4 amplified the genomic DNA of most of the isolates, but not all, producing 1- 4 bands of size between 75-5000 bp. Nearly all isolates of *M. phaseolina*, irrespective of their geographical origin, exhibited a common band of 700 bp.

The DNA fragments generated by the primers Em6-Me4, Em11-Me4, Em14-Me4, Em11-Me11, Em13-Me4, Em4-Me4 and Em9-Me4 from the 34 isolates of *Macrophomina* and *Fusarium* were illustrated in Figures 4.10, 4.11, 4.12, 4.13, 4.14, 4.15 & 4.16, respectively, show potential for discriminating among the different isolates of *M. phaseolina, F. oxysporum* and *F. verticillioides.* The primers Em6-Me4, Em11-Me4, Em14-Me4 and Em11-Me11 produced fingerprint profiles, which were clearly distinguished among the different isolates of *M. phaseolina*, *F. oxysporum* and *F. verticillioides*, the causal pathogens of damping-off and charcoal-rot diseases in sunflower. The primers Em13-Me4 and E14-Me4 gave the highest number of fragments (10, 9) amplicons respectively, while the lowest number of fragments (one amplicon) was amplified with the primer Em11-Me6. Those molecular markers used in this study are useful for distinguishing between *Fusarium* and *Macrophomina*.

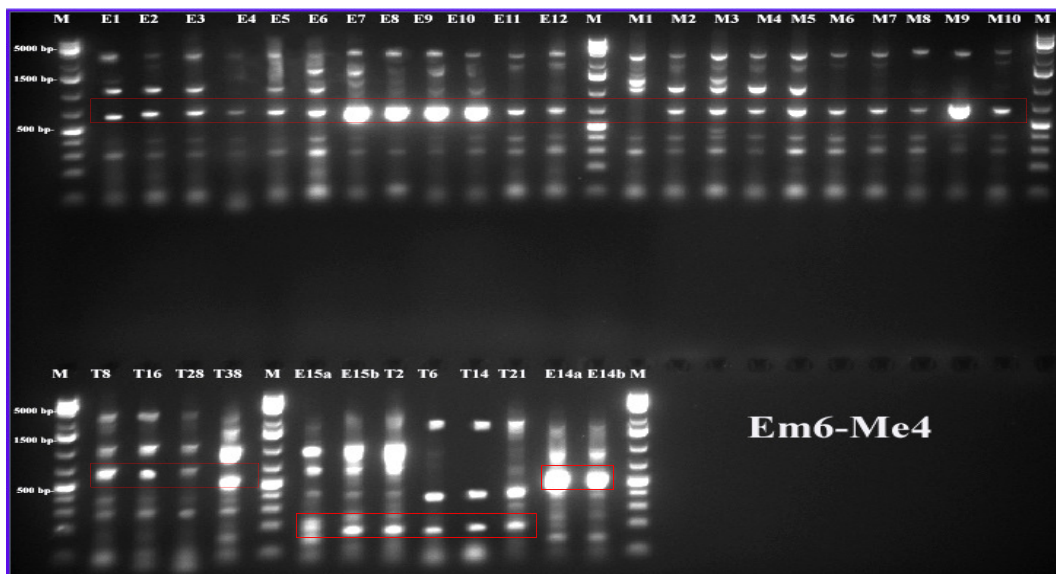


Figure (4.10): Agarose gel electrophoresis of SRAP profile generated by the primer Em6-Me4 from *Macrophomina* and *Fusarium*, where:
(M): Molecular weight marker,
(E1-E12), (M1:M10) and (T8, T16, T28 & T38): *M. phaseolina* isolates,
(E15a, E15b, T2, T6, T14 & T21): *F. oxysporum* isolates,
(E14a & E14b): *F. verticillioides* isolates.

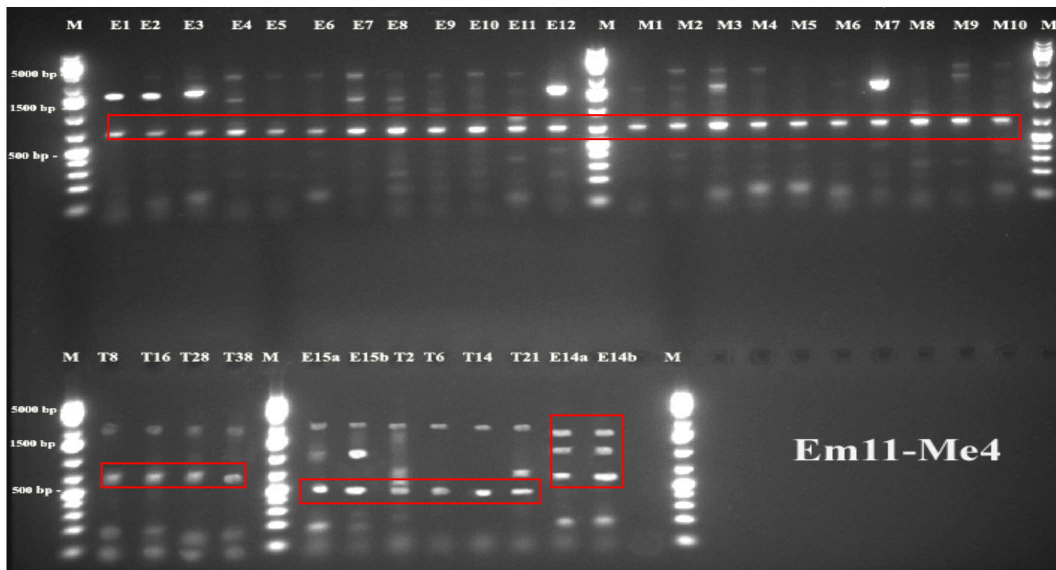


Figure (4.11): Agarose gel electrophoresis of SRAP profile generated by the primer Em11-Me4 from *Macrophomina* and *Fusarium*, where:
(M): Molecular weight marker,
(E1-E12), (M1:M10) and (T8, T16, T28 & T38): *M. phaseolina* isolates,
(E15a, E15b, T2, T6, T14 & T21): *F. oxysporum* isolates,
(E14a & E14b): *F. verticillioides* isolates.

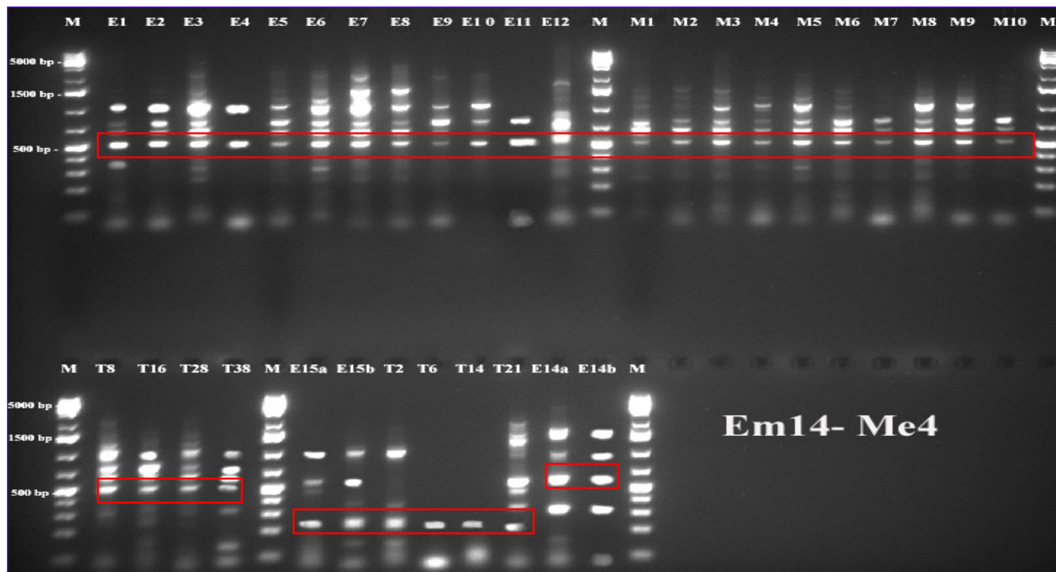


Figure (4.12): Agarose gel electrophoresis of SRAP profile generated by the primer Em14-Me4 from *Macrophomina* and *Fusarium*, where:
(M): Molecular weight marker,
(E1-E12), (M1:M10) and (T8, T16, T28 & T38): *M. phaseolina* isolates,
(E15a, E15b, T2, T6, T14 & T21): *F. oxysporum* isolates,
(E14a & E14b): *F. verticillioides* isolates.

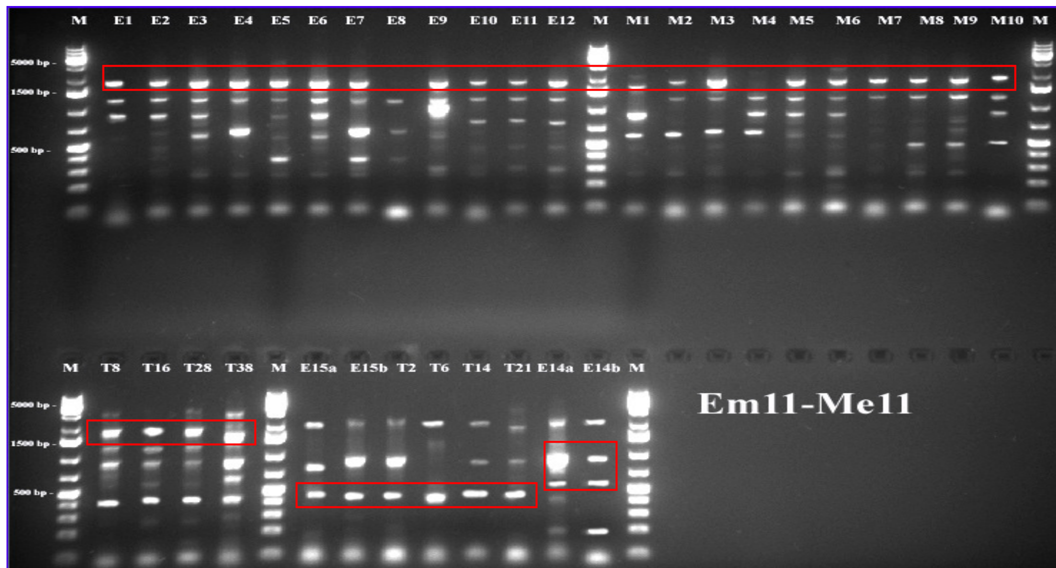


Figure (4.13): Agarose gel electrophoresis of SRAP profile generated by the primer Em11-Me11 from *Macrophomina* and *Fusarium*, where:
(M): Molecular weight marker,
(E1-E12), (M1:M10) and (T8, T16, T28 & T38): *M. phaseolina* isolates,
(E15a, E15b, T2, T6, T14 & T21): *F. oxysporum* isolates,
(E14a & E14b): *F. verticillioides* isolates.

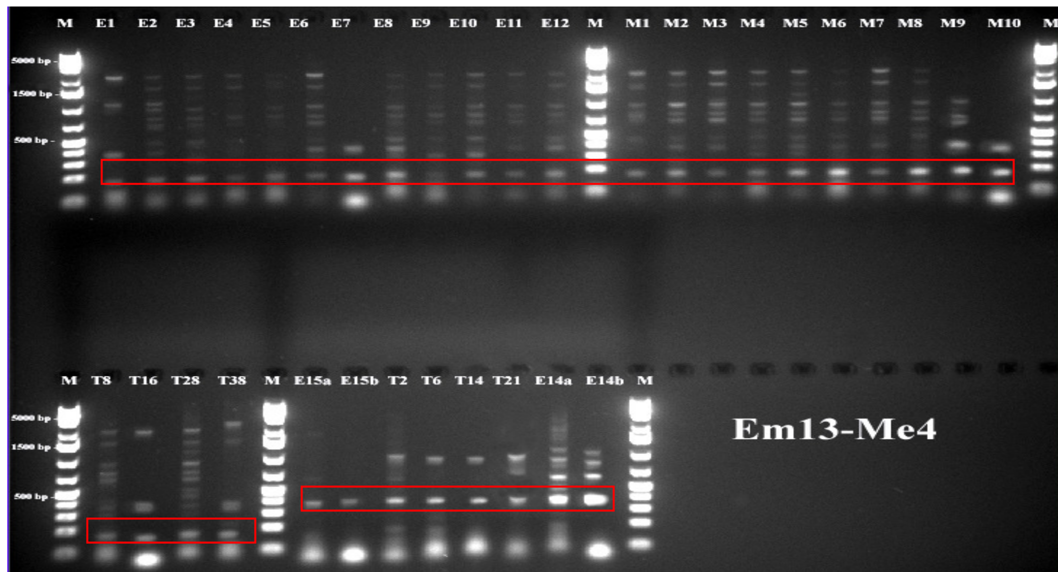


Figure (4.14): Agarose gel electrophoresis of SRAP profile generated by the primer Em13-Me4 from *Macrophomina* and *Fusarium*, where:
(M): Molecular weight marker,
(E1-E12), (M1:M10) and (T8, T16, T28 & T38): *M. phaseolina* isolates,
(E15a, E15b, T2, T6, T14 & T21): *F. oxysporum* isolates,
(E14a & E14b): *F. verticillioides* isolates.

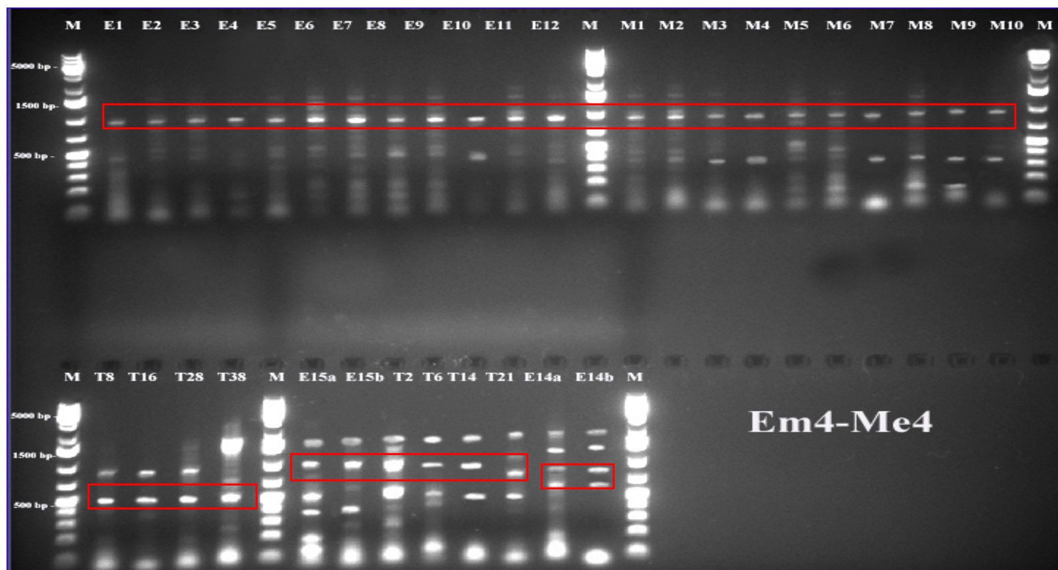


Figure (4.15): Agarose gel electrophoresis of SRAP profile generated by the primer Em4-Me4 from *Macrophomina* and *Fusarium*, where:
(M): Molecular weight marker,
(E1-E12), (M1:M10) and (T8, T16, T28 & T38): *M. phaseolina* isolates,
(E15a, E15b, T2, T6, T14 & T21): *F. oxysporum* isolates,
(E14a & E14b): *F. verticillioides* isolates.

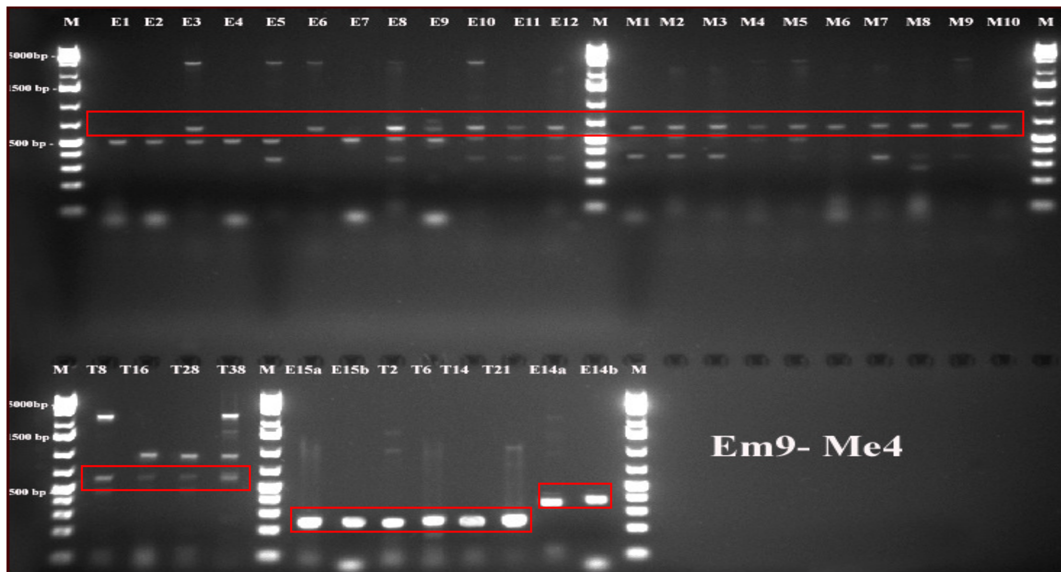


Figure (4.16): Agarose gel electrophoresis of SRAP profile generated by the primer Em9-Me4 from *Macrophomina* and *Fusarium*, where:
(M): Molecular weight marker,
(E1-E12), (M1:M10) and (T8, T16, T28 & T38): *M. phaseolina* isolates,
(E15a, E15b, T2, T6, T14 & T21): *F. oxysporum* isolates,
(E14a & E14b): *F. verticillioides* isolates.

4.5.3.1- Cluster analysis

Macrophomina phaseolina, *Fusarium oxysporum* and *Fusarium verticillioides* a filamentous fungi, that are widely distributed in the soil and causes damping-off and charcoal-rot in sunflower. Those fungi are associated with the rhizosphere of many plant species, for this purpose we compared the genetic similarity among and within the different isolates of *Macrophomina* and *Fusarium.*

Unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis showed that all tested isolates of *Macrophomina* and *Fusarium* were clustered together with a branched-off at genetic similarity of 40%. The isolates of *Macrophomina phaseolina* were clustered together with a branched-off at genetic similarity of 60%. The isolates of *F. oxysporum* and *F. verticillioides* were clustered together with a branched-off at genetic similarity of 55%. UPGMA analysis classified these isolates into five major groups.

The isolates of *F. oxysporum* were clustered together with a branched-off at genetic similarity of 75%, including the isolates obtained from Egypt (E15a & E15b) and the isolates obtained from Turkey (T2, T6, T14 & T21). The isolates E15a & E15b were clustered together and showed very high similarity of 100%, while, Turkish isolates of *F. oxysporum* (T2, T6, T14 & T21) were clustered together with a branched-off at genetic similarity of 85%. The two isolates of *F. verticillioides* (E14a & E14b) were clustered together and showed very high similarity of 100%, Figure (4.17).

Egyptian and Turkish isolates of *M. phaseolina* were clustered together with a branched-off at genetic similarity of 60%. Isolates obtained from Turkey (T8, T16, T28 & T38) were clustered together with a branched-off at genetic similarity of 80%. While, isolates of *M. phaseolina* which obtained from Egypt were clustered together with a branched-off at genetic similarity of 65%. Isolates E2, M7, M1 & M2 were clustered together in sub-clusters and displayed high genetic similarity of 80%. However, isolates M5 & M4 showed high genetic similarity of 81%. Isolates E11 & E12 were clustered together in sub-clusters and displayed genetic similarity of 76%, while the genetic similarity was 75% between E3 and E6. In general, the UPGMA cluster analysis showed that all tested isolates were clustered together according to the genus and geographic areas. The results of cluster analysis were presented in Figure (4.17) and Table (4.10). Levels of polymorphism among and within *Macrophomina* and *Fusarium* isolates were presented in Table (4.11).

The UPGMA cluster analysis also showed that a high similarity, (100%) between Egyptian isolates of *F. oxysporum* (E15a and E15b), was observed. The same similarity, (100%) was observed between two of Turkish isolates of *F. oxysporum* (T6 and T14) From the results obtained, we can declared that the isolates of *Fusarium oxysporum* from Egypt were similar in molecular genetic features to *F. oxysporum* isolates from Turkey, all the tested isolates of *F. oxysporum* were clustered together with a similarity of (75%).

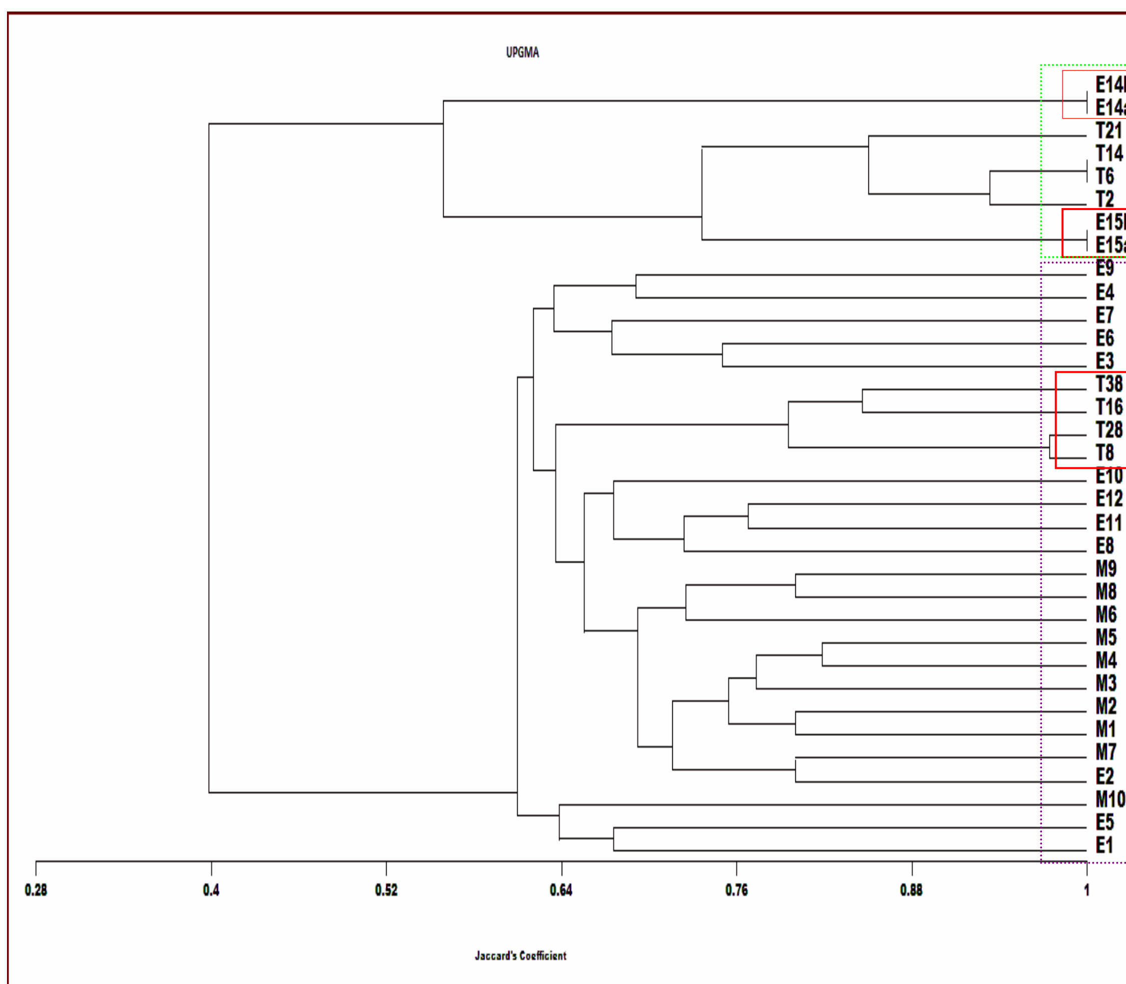


Figure (4.17): Dendrogram constructed with UPGMA clustering method among 34 isolates of *M. phaseolina, F. oxysporum* and *F. verticillioides* based on Jaccard's coefficient. Branches are labeled by isolate numbers. The line below the dendrogram represents the similarity index. Dendrogram constructed based on polymorphism of 12 SRAP primer combinations (Em11-Me11, Em11-Me4, Em11-Me6, Em12-Me4, Em13-Me4, Em14-Me3, Em14-Me4, Em2-Me8, Em4-Me4, Em6-Me2, Em6-Me4 and Em9-Me4).
Where,
(E1-E12) and (M1-M10): *M. phaseolina* isolates, collected from Egypt,
(E15a & E15b): *F. oxysporum* isolates, collected from Egypt,
(E14a & E14b): *F. verticillioides* isolates, collected from Egypt,
(T8, T16, T28 & T38): *M. phaseolina* isolates, collected from Turkey,
(T2, T6, T14 & T21): *F. oxysporum* isolates, collected from Turkey.

4.5.3.2- Genetic similarity as revealed by SRAP markers

In the present study, the genetic relationships among 34 isolates of *Macrophomina* and *Fusarium* were determined by Jaccard’s coefficient, Table (4.10). The matrix of similarity index ranged from 30 % to 100%. The highest genetic similarity (100%) was recorded between E14a and E14b; E15a and E15a; T6 and T14 followed by (97.4 %) was recorded between T8 and T28, while the lowest genetic similarity (30 %) was recorded between E8 and T6; E8 and T14.

Table (4.10): Similarity matrix for 34 isolates of *Macrophomina* and *Fusarium* from different geographic areas, calculated using the Jaccard's coefficient

	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	T8	T16	T28	T38	E15a	E15b	T2	T6	T14	T21	E14a	E14b
E1	1																																	
E2	0.737	1																																
E3	0.591	0.733	1																															
E4	0.605	0.643	0.587	1																														
E5	0.676	0.667	0.682	0.585	1																													
E6	0.532	0.667	0.75	0.596	0.617	1																												
E7	0.605	0.571	0.62	0.636	0.587	0.729	1																											
E8	0.571	0.644	0.66	0.643	0.628	0.569	0.571	1																										
E9	0.581	0.583	0.6	0.69	0.636	0.708	0.681	0.652	1																									
E10	0.444	0.622	0.638	0.619	0.568	0.646	0.52	0.659	0.63	1																								
E11	0.477	0.587	0.571	0.545	0.643	0.549	0.583	0.698	0.63	0.714	1																							
E12	0.533	0.711	0.723	0.565	0.659	0.627	0.538	0.75	0.646	0.652	0.767	1																						
M1	0.595	0.705	0.681	0.591	0.651	0.653	0.56	0.563	0.711	0.644	0.644	0.66	1																					
M2	0.636	0.739	0.647	0.705	0.617	0.654	0.627	0.702	0.745	0.717	0.681	0.66	0.8	1																				
M3	0.558	0.705	0.717	0.628	0.69	0.688	0.592	0.667	0.674	0.644	0.644	0.733	0.689	0.761	1																			
M4	0.571	0.721	0.66	0.605	0.75	0.633	0.51	0.682	0.652	0.622	0.622	0.674	0.744	0.739	0.786	1																		
M5	0.565	0.667	0.75	0.596	0.652	0.72	0.596	0.667	0.708	0.646	0.612	0.729	0.8	0.792	0.761	0.818	1																	
M6	0.659	0.727	0.6	0.578	0.636	0.608	0.549	0.583	0.625	0.563	0.596	0.681	0.75	0.745	0.571	0.689	0.745	1																
M7	0.6	0.8	0.689	0.595	0.619	0.56	0.471	0.674	0.574	0.614	0.614	0.744	0.698	0.733	0.738	0.756	0.696	0.721	1															
M8	0.595	0.705	0.646	0.667	0.614	0.62	0.592	0.705	0.674	0.574	0.644	0.733	0.727	0.761	0.689	0.667	0.723	0.711	0.698	1														
M9	0.615	0.651	0.63	0.61	0.718	0.54	0.609	0.69	0.622	0.591	0.707	0.721	0.674	0.674	0.636	0.651	0.638	0.738	0.683	0.8	1													
M10	0.629	0.625	0.605	0.538	0.649	0.511	0.511	0.585	0.523	0.524	0.561	0.581	0.61	0.578	0.571	0.667	0.614	0.675	0.703	0.65	0.722	1												
T8	0.558	0.705	0.681	0.628	0.69	0.62	0.56	0.705	0.604	0.609	0.609	0.696	0.652	0.688	0.652	0.705	0.688	0.638	0.659	0.689	0.636	0.61	1											
T16	0.615	0.614	0.596	0.571	0.634	0.54	0.609	0.651	0.587	0.522	0.591	0.609	0.6	0.604	0.532	0.614	0.571	0.622	0.568	0.565	0.659	0.632	0.8	1										
T28	0.545	0.727	0.667	0.614	0.674	0.64	0.549	0.689	0.592	0.63	0.63	0.717	0.674	0.708	0.674	0.727	0.708	0.66	0.682	0.674	0.622	0.595	0.974	0.78	1									
T38	0.558	0.596	0.681	0.556	0.69	0.653	0.625	0.63	0.604	0.574	0.609	0.66	0.617	0.588	0.583	0.63	0.653	0.571	0.587	0.551	0.6	0.61	0.81	0.846	0.791	1								
E15a	0.395	0.36	0.358	0.313	0.362	0.396	0.449	0.333	0.458	0.288	0.314	0.34	0.438	0.396	0.327	0.417	0.451	0.458	0.347	0.353	0.354	0.372	0.353	0.444	0.346	0.438	1							
E15b	0.395	0.36	0.358	0.313	0.362	0.396	0.449	0.333	0.458	0.288	0.314	0.34	0.438	0.396	0.327	0.417	0.451	0.458	0.347	0.353	0.354	0.372	0.353	0.444	0.346	0.438	1	1						
T2	0.341	0.396	0.365	0.319	0.37	0.352	0.4	0.34	0.408	0.347	0.347	0.346	0.447	0.404	0.333	0.396	0.404	0.468	0.354	0.388	0.391	0.381	0.36	0.391	0.353	0.36	0.743	0.743	1					
T6	0.326	0.354	0.327	0.333	0.326	0.34	0.388	0.3	0.396	0.306	0.306	0.308	0.404	0.365	0.294	0.354	0.365	0.426	0.313	0.347	0.348	0.366	0.32	0.378	0.314	0.347	0.735	0.735	0.933	1				
T14	0.326	0.354	0.327	0.333	0.326	0.34	0.388	0.3	0.396	0.306	0.306	0.308	0.404	0.365	0.294	0.354	0.365	0.426	0.313	0.347	0.348	0.366	0.32	0.378	0.314	0.347	0.735	0.735	0.933	1	1			
T21	0.378	0.4	0.423	0.327	0.375	0.434	0.49	0.373	0.469	0.302	0.327	0.377	0.449	0.407	0.365	0.4	0.434	0.469	0.36	0.392	0.396	0.386	0.365	0.426	0.358	0.392	0.73	0.73	0.853	0.848	0.848	1		
E14a	0.391	0.5	0.49	0.457	0.478	0.5	0.471	0.412	0.542	0.392	0.42	0.531	0.587	0.56	0.553	0.565	0.592	0.574	0.458	0.521	0.468	0.37	0.46	0.408	0.48	0.404	0.5	0.5	0.585	0.575	0.575	0.619	1	
E14b	0.391	0.5	0.49	0.457	0.478	0.5	0.471	0.412	0.542	0.392	0.42	0.531	0.587	0.56	0.553	0.565	0.592	0.574	0.458	0.521	0.468	0.37	0.46	0.408	0.48	0.404	0.5	0.5	0.585	0.575	0.575	0.619	1	1
	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	T8	T16	T28	T38	E15a	E15b	T2	T6	T14	T21	E14a	E14b

4.5.3.3- Polymorphisms as detected by SRAP markers

One of the most important applications of the SRAP technique is detecting DNA sequence polymorphism so as this application has been used in this study. In the present study, 34 primer combinations were applied to investigate the level of polymorphism among 34 isolates of *Macrophomina* and *Fusarium* collected from different regions in Egypt and Turkey. Twelve primer combinations were given polymorphic bands with different levels of polymorphism.

Polymorphism detected by SRAP analysis (91.67%) is given in Table (4.11). The highest percentage of polymorphism (75.86%) was revealed between Egyptian and Turkish isolates of *Macrophomina phaseolina* followed by (75.44%) revealed within Egyptian isolates of *M. Phaseolina,* while the lowest percentage of polymorphism (14.71%) was revealed within Turkish isolates of *Fusarium oxysporum*.

Table (4.11): Levels of polymorphism among *Macrophomina* and *Fusarium* isolates as revealed by the SRAP primers

Category		*TNB	**% Poly
Fusarium	Within *Fusarium spp*. (Egypt and Turkey)	45	55.56
	Within *F. oxysporum* (Turkey)	34	14.71
	F. oxysporum (Egypt) vs *F. Verticillioides* (Egypt)	44	50.00
	F. oxysporum (Egypt) vs *F. oxysporum* (Turkey)	38	31.58
	F. Verticillioides (Egypt) vs *F. oxysporum* (Turkey)	42	45.24
Macrophomina	Within *M. phaseolina* (Egypt and Turkey)	58	75.86
	Within *M. phaseolina* (Egypt)	57	75.44
	Within *M. phaseolina* (Turkey)	44	27.27
Total	*Fusarium* vs *Macrophomina* (Egypt and Turkey)	60	91.67

Legend: *=TNB: Total number of bands. **=% Poly: percentage of polymorphism.

4.5.3.4- Principal coordinates analysis

This analysis was constructed to provide another means of testing relationships among the isolates. Analysis through principal coordinate analysis confirmed the cluster profile of genetic diversity among and within the isolates of *Macrophomina* and *Fusarium*. *Macrophomina phaseolina* isolates from Egypt were clustered together, while the isolates from Turkey were clustered together, Figure (4.18). Results presented in Figure (4.19) showed that the isolates were clustered into three groups of *Macrophomina phaseolina, Fusarium oxysporum* and *Fusarium verticillioides* based on differences in SRAP analysis.

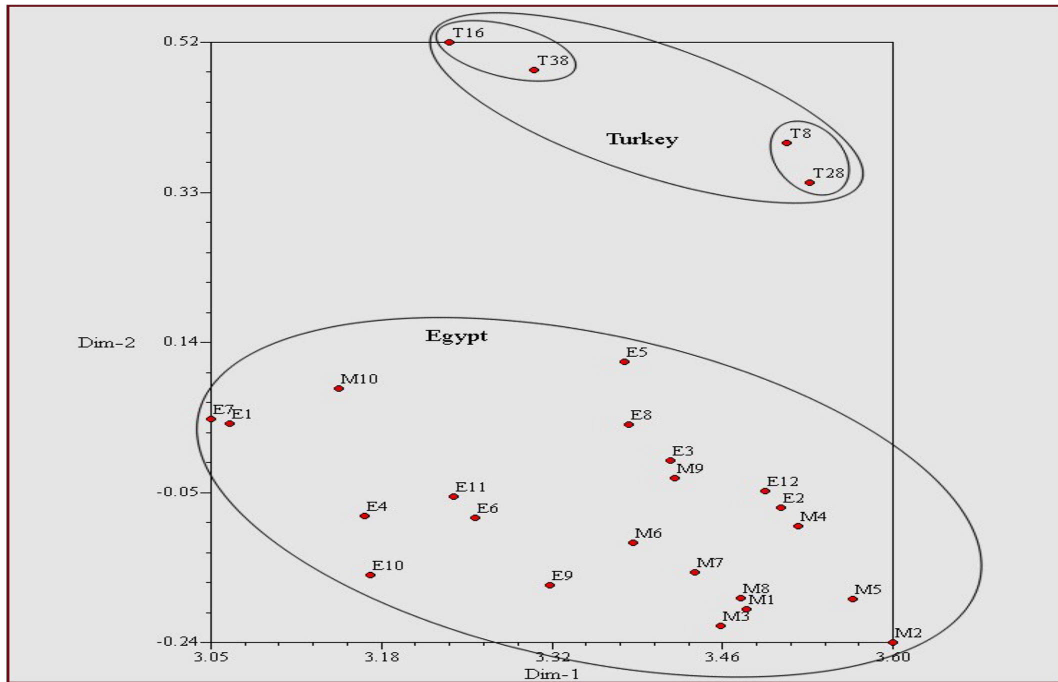


Figure (4.18): Principal coordinates plot of 26 *Macrophomina phaseolina* isolates based on differences in the SRAP analysis.

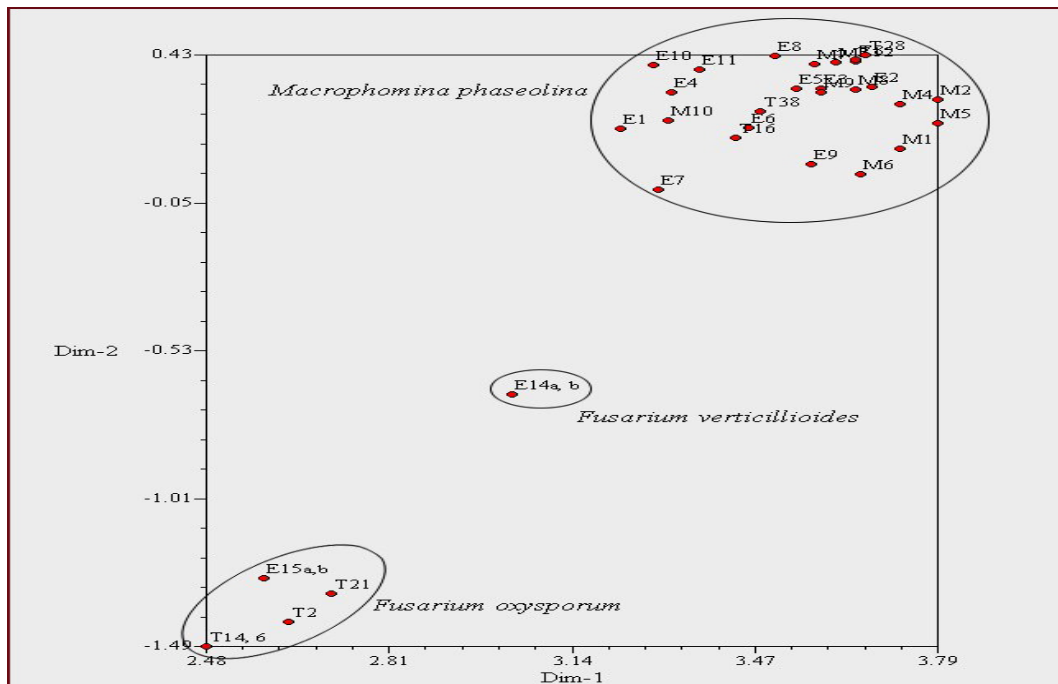


Figure (4.19): Principal coordinates plot of 34 isolates of *M. phaseolina, F. oxysporum* and *F. verticillioides* based on differences in the SRAP analysis.

4.6- Evaluation the susceptibility of sunflower cultivars and genotypes to infection by *Macrophomina phaseolina*

This study was conducted to screen sunflower cultivars for susceptibility/resistance to damping-off and charcoal-rot diseases caused by *Macrophomina phaseolina*. Forty-one sunflower cultivars were evaluated for *M. phaseolina* infection. Two cultivars, (AUROFLOR and VIDOIC), were obtained from the Ministry of Agriculture, Egypt; and 39 cultivars were obtained from Trakya Agricultural Research Institute (TARI), Edirne, Turkey.

Results of this study are presented in Table (4.12) and revealed that in general, with few exceptions, it seems there are a few completely resistant cultivars to damping-off and charcoal-rot diseases caused by *M. phaseolina*. Certain cultivars proved to be resistant to damping-off and charcoal-rot diseases, but the most tested cultivars proved to be susceptible. The susceptibility of the tested cultivars to the disease was variable and varied according to the tested cultivars and the tested isolates.

Sunflower cultivars: BAH-4-A, P-4223-A, RHA-461 and 62003-A proved to be the highest resistant cultivars against *M. phaseolina* infection while, cultivars: HA-465-A, 66241-A, 7710-A, 7990-A, 6545-A, 65371-A, 2453-A and AUROFLOR proved to be very susceptible cultivars to *M. phaseolina* infestation, followed by the other tested cultivars. On the other hand, sunflower cultivars: 1159-A, 0043-A, 7675-A, 7989-A, 6397-A and 6522-A showed to have moderate resistant.

Data in Table (4.13) indicated that regardless to the tested isolates, cultivars: BAH-4-A and P-4223-A gave the lowest percentage of damping-off as well as charcoal-rot severity whereas, cultivars: HA-465-A, 66241-A, 7710-A, 7990-A, 6545-A, 65371-A, 2453-A and AUROFLOR gave the highest reduction in survival plants after 2 and 4 weeks. There were significant differences in the rate of susceptibility among the tested sunflower cultivars to damping-off and charcoal-rot diseases, these differences may be due to the variations in their root system, hypocotyl diameter and/or variation in their root exudates components. Results of the presented work indicate that there are certain resistant cultivars against damping-off and charcoal-rot diseases of sunflower.

Table (4.12): Evaluation the susceptibility of sunflower cultivars and genotypes to infection by *Macrophomina phaseolina*

	Cultivars	*Macrophomina phaseolina* isolates No.									Control		
		E3			E6			M9					
		% of Survival plants after		Severity of Disease	% of Survival plants after		Severity of Disease	% of Survival plants after		Severity of Disease	% of Survival plants after		Severity of Disease
		2 Ws	4 Ws		2 Ws	4 Ws		2 Ws	4 Ws		2 Ws	4 Ws	
1	TARSAN1018	58.33	41.66	4.41	91.66	75.00	3.41	66.66	50.00	4.58	100	100	1.33
2	PERUN	58.33	41.66	4.58	66.66	50.00	5.00	50.00	33.33	5.08	100	100	1.50
3	IYI-HA-89-7-A	33.33	0.0	6.00	41.66	0.0	6.00	16.66	0.0	6.00	83.33	83.33	2.58
4	TR-3080	25.00	0.0	6.00	16.66	0.0	6.00	50.00	25.00	5.25	91.66	91.66	2.16
5	HA-465-A	0.0	0.0	6.00	0.0	0.0	6.00	0.0	0.0	6.00	91.66	91.66	1.66
6	6626-A	50.00	25.00	5.08	58.33	50.00	4.75	50.00	25.00	5.25	100	100	1.50
7	P-4223-A	75.00	66.66	3.50	91.66	75.00	3.75	100	100	2.91	100	100	1.50
8	7751-A	8.33	0.0	6.00	33.33	0.0	6.00	25.00	0.0	6.00	91.66	91.66	1.75
9	67372-A	58.33	41.66	5.00	58.33	41.66	4.66	58.33	25.00	5.25	100	100	1.83
10	0043-A	66.66	58.33	4.75	83.33	66.66	4.33	100	75.00	4.00	100	100	1.33
11	0704-A	16.66	0.0	6.00	25.00	0.0	6.00	41.66	0.0	6.00	91.66	91.66	1.83
12	HA-466	41.66	25.00	5.50	50.00	25.00	5.08	41.66	25.00	5.16	100	100	1.16
13	66241-A	0.0	0.0	6.00	0.0	0.0	6.00	0.0	0.0	6.00	83.33	83.33	2.41
14	6163-A	41.66	25.00	5.41	33.33	0.0	6.00	41.66	25.00	5.25	100	100	1.16
15	6388-A	0.0	0.0	6.00	0.0	0.0	6.00	25.00	25.00	5.33	91.66	91.66	1.83
16	6397-A	58.33	50.00	4.58	91.66	75.00	3.83	66.66	50.00	4.41	100	100	1.66
17	7710-A	0.0	0.0	6.00	0.0	0.0	6.00	0.0	0.0	6.00	83.33	83.33	2.25
18	7990-A	0.0	0.0	6.00	0.0	0.0	6.00	0.0	0.0	6.00	91.66	91.66	1.41
19	2517-A	58.33	41.66	5.16	83.33	75.00	4.33	66.66	50.00	4.41	100	100	1.33
20	6398-A	8.33	0.0	6.00	16.66	0.0	6.00	25.00	0.0	6.00	100	100	2.00
21	6765-A	41.66	33.33	5.33	41.66	25.00	5.50	0.0	0.0	6.00	100	100	1.91
22	G3-K6-AD-CRZ-A-SN-26	33.33	33.33	5.00	58.33	50.00	4.50	41.66	25.00	5.33	100	100	1.33
23	6545-A	0.0	0.0	6.00	0.0	0.0	6.00	0.0	0.0	6.00	83.33	83.33	2.25
24	62003-A	66.66	50.00	4.66	100	100	3.25	83.33	75.00	4.08	100	100	1.16
25	HA-89-1-A	41.66	25.00	5.25	8.33	0.0	6.00	8.33	0.0	6.00	91.66	91.66	1.41
26	3009-A	25.00	0.0	6.00	41.66	25.00	5.16	16.66	0.0	6.00	100	100	1.66
27	62001-A	41.66	25.00	5.33	58.33	50.00	4.66	33.33	25.00	5.16	100	100	1.50
28	7675-A	66.66	66.66	4.50	83.33	75.00	4.33	58.33	50.00	4.58	100	100	1.75
29	RHA-461	66.66	50.00	4.66	91.66	75.00	4.00	100	100	2.75	100	100	1.66
30	65371-A	0.0	0.0	6.00	0.0	0.0	6.00	0.0	0.0	6.00	83.33	83.33	2.41
31	05-TR-198	0.0	0.0	6.00	33.33	25.00	5.33	0.0	0.0	6.00	100	100	1.33
32	7682-A	33.33	25.00	5.41	66.66	58.33	4.41	58.33	50.00	4.66	100	100	1.33
33	7989-A	58.33	50.00	5.00	66.66	50.00	4.83	91.66	91.66	3.00	100	100	1.16
34	BAH-4-A	83.33	75.00	3.83	100	100	2.50	91.66	75.00	4.00	100	100	1.33
35	1159-A	66.66	58.33	5.08	83.33	75.00	4.50	91.66	75.00	4.50	100	100	1.16
36	2453-A	0.0	0.0	6.00	0.0	0.0	6.00	0.0	0.0	6.00	83.33	83.33	2.25
37	6522-A	83.33	75.00	4.41	66.66	50.00	4.25	58.33	50.00	4.33	100	100	1.16
38	SANAY	33.33	25.00	5.66	41.66	25.00	5.16	41.66	0.0	6.00	91.66	91.66	1.41
39	TUNCA	41.66	16.66	5.50	58.33	16.66	5.58	33.33	8.33	5.83	100	91.66	1.91
40	AUROFLOR	0.0	0.0	6.00	0.0	0.0	6.00	0.0	0.0	6.00	91.66	91.66	1.66
41	VIDOIC	25.00	0.0	6.00	41.66	0.0	6.00	41.66	25.00	5.16	100	100	2.00

LSD	0.05	0.01	0.05	0.01	0.05	0.01
Isolates (I)	5.4	8.18	4.70	7.12	0.14	0.22
Cultivars (C)	10.88	15.26	10.57	13.86	0.43	0.57
I x C	21.76	28.53	21.14	27.72	0.87	1.14

Table (4.13): Evaluation the susceptibility of sunflower cultivars and genotypes to infection by *Macrophomina phaseolina* regardless to the tested isolates

Cultivars		% of survival sunflower plants relative to control after		Severity of Disease
		2 Weeks	4 Weeks	
1	TARSAN1018	72.21	55.55	4.13
2	PERUN	58.33	41.66	4.88
3	IYI-HA-89-7-A	36.66	0.0	6.00
4	TR-3080	33.33	9.09	5.75
5	HA-465-A	0.0	0.0	6.00
6	6626-A	52.77	33.33	5.02
7	P-4223-A	88.88	80.55	3.38
8	7751-A	24.24	0.0	6.00
9	67372-A	58.33	36.10	4.97
10	0043-A	83.33	66.66	4.36
11	0704-A	30.30	0.0	6.00
12	HA-466	44.44	25.00	5.24
13	66241-A	0.0	0.0	6.00
14	6163-A	38.88	16.66	5.55
15	6388-A	9.09	9.09	5.77
16	6397-A	72.21	58.33	4.27
17	7710-A	0.0	0.0	6.00
18	7990-A	0.0	0.0	6.00
19	2517-A	69.44	55.55	4.63
20	6398-A	16.66	0.0	6.00
21	6765-A	27.77	19.44	5.61
22	G3-K6-AD-CRZ-A-SN-26	44.44	36.11	4.94
23	6545-A	0.0	0.0	6.00
24	62003-A	83.33	75.00	3.99
25	HA-89-1-A	21.20	9.09	5.75
26	3009-A	27.77	8.33	5.72
27	62001-A	44.44	33.33	5.05
28	7675-A	69.44	63.88	4.47
29	RHA-461	86.10	75.00	3.80
30	65371-A	0.0	0.0	6.00
31	05-TR-198	11.11	8.33	5.77
32	7682-A	52.77	44.44	4.82
33	7989-A	72.21	63.88	4.27
34	BAH-4-A	91.66	83.33	3.44
35	1159-A	80.55	69.44	4.69
36	2453-A	0.0	0.0	6.00
37	6522-A	69.44	58.33	4.33
38	SANAY	42.42	18.18	5.60
39	TUNCA	44.44	15.14	5.63
40	AUROFLOR	0.0	0.0	6.00
41	VIDOIC	36.10	8.33	5.72
LSD 0.05		14.77	14.63	0.49
LSD 0.01		19.37	19.19	0.65

4.7- Variation of sunflower root exudates

4.7.1- Effect of root exudates of sunflower cultivars on the growth of *Macrophomina* and *Fusarium* in *vitro*

This experiment was conducted to study the role of root exudates on the physiology of sunflower resistance to damping-off and charcoal-rot diseases. The effect of root exudates of thirteen sunflower cultivars on the growth of *Macrophomina* and *Fusarium* isolates was investigated in *vitro*. The tested cultivars were: four cultivars that proved to be resistant cultivars (BAH-4-A, P-4223-A, RHA-461 and 62003-A), four cultivars which showed to have moderate resistant (1159-A, 0043-A, 7675-A and 7989-A) as well as five susceptible cultivars (HA-465-A, 66241-A, 7710-A, 7990-A and TUNCA). The tested fungi were: three isolates of *Macrophomina phaseolina* and one isolate of each of *Fusarium verticillioides* and *Fusarium oxysporum*.

Data of this study is presented in Table (4.14) and showed that, with few exceptions, root exudates of almost all tested cultivars had significant effect on mycelial growth of all tested isolates compared with the control. The root exudates of sunflower cultivars: HA-465-A, 66241-A, 7710-A, 7990-A and TUNCA increased significantly the mycelial growth of all tested isolates and reached its maximum stimulation with *M. phaseolina* (E3) followed by *M. phaseolina* (M9). While, root exudates of sunflower cultivars: BAH-4-A, P-4223-A, RHA-461 and 62003-A decreased significantly the mycelial growth of all tested isolates.

Data also reveals that, regardless tested fungi, root exudates of sunflower cultivars: HA-465-A, 66241-A, 7710-A, 7990-A and TUNCA (susceptible cultivars) stimulated mycelial growth of the tested fungi. Sunflower cultivars: BAH-4-A, P-4223-A, RHA-461 and 62003-A (resistant cultivars) decreased the mycelial growth of all tested fungi while, sunflower cultivars: 1159-A, 0043-A, 7675-A and 7989-A (moderate resistant) showed to have intermediate effect. These results are in compatible with the results which found in the experiment of cultivars evaluation

Table (4.14): Effect of root exudates of sunflower cultivars on the growth of *Macrophomina* and *Fusarium* isolates in *vitro*

Cultivars	Dry weight (mg)					
	M. phaseolina (E3)	*M. phaseolina* (E6)	*M. phaseolina* (M9)	*F. verticillioides* (E14b)	*F. oxysporum* (E15a)	Mean
BAH-4-A*	87.40	89.96	84.16	56.56	72.10	78.03
P-4223-A*	90.00	93.23	91.40	57.43	81.03	82.61
RHA-461*	76.56	90.40	83.53	53.63	70.20	74.86
62003-A*	84.06	90.93	86.23	57.96	74.26	78.68
1159-A**	92.06	93.03	91.63	57.36	79.40	82.69
0043-A**	91.63	92.26	93.13	61.36	82.73	84.22
7675-A**	91.80	90.70	93.90	66.63	78.26	84.25
7989-A**	90.10	94.53	88.76	65.20	84.93	84.70
HA-465-A***	113.90	98.00	103.30	66.76	96.63	95.71
66241-A***	128.90	104.63	115.00	65.63	82.46	99.32
7710-A***	112.23	96.03	105.06	68.60	84.33	93.25
7990-A***	122.13	98.70	110.73	71.73	100.40	100.73
TUNCA***	118.56	111.50	115.56	68.53	97.20	102.27
Control	91.43	94.13	96.43	59.36	85.60	85.39
Mean	99.34	95.57	97.05	62.62	83.53	
LSD	0.05			0.01		
Cultivars(C)	1.802			2.380		
Isolates (I)	1.077			1.422		
(C x I)	4.029			5.322		

Legend: * Resistant cultivars;** Moderate resistant; *** Susceptible cultivars.

4.7.2- Determination of amino acids quantity in the root exudates of sunflower cultivars during a 14 days growth period

Seventeen amino acids were detected in the root exudates of thirteen cultivars of sunflower, Table (4.15), including: Aspartic acid (Asp), Threonine (Thr), Serine (Ser), Glutamic acid (Glu), Proline (Pro), Glycine (Gly), Alanine (Ala), Cysteine (Cys), Valine (Val), Methionine (Met), Isoleucine (Ile), Leucine (Leu), Tyrosine (Tyr), Phenylalanine (Phe), Histidine (His), Lysine (Lys), Arginine (Arg).

In general, with few exceptions, the most abundant amino acids were Proline, Valine, Phenylalanine and Alanine. The concentration of total amino acid on sunflower cultivars which proved to be resistant were higher than the cultivars which showed to have moderate resistant as well as susceptible cultivars. Significant differences were found between

resistant and susceptible cultivars in some kinds and contents of amino acids in their root exudates.

The root exudates of sunflower cultivars: BAH-4-A, P-4223-A, RHA-461 and 62003-A contained high concentration of Proline, Valine and Phenylalanine than the root exudates of other cultivars, therefore, we can declare that, the resistance on those cultivars may be due to their high concentrations of Proline, Valine and Phenylalanine. The total amount of amino acid in the root exudates of sunflower cultivars: BAH-4-A and P-4223-A was higher than sunflower cultivars: RHA-461 and 62003-A. Root exudates of sunflower cultivars: BAH-4-A, P-4223-A, RHA-461 and 0043-A not contained Cysteine. In the susceptible cultivars we found that the total amount of amino acids was equal in both sunflower cultivars: HA-465-A and 66241-A; in addition, Threonine was absent in root exudates of sunflower cultivars: 7710-A and 7990-A.

Table (4.15): Quantity of amino acids in the root exudates of sunflower cultivars during a 14 days growth period

Cultivars	Amino acids concentration (µmol/150 ml)																	
	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Total
BAH-4-A*	2.45	12.50	6.22	16.20	32.07	18.95	23.87	0.0	25.85	18.10	5.84	1.40	15.37	24.72	2.00	8.10	4.00	217.64
P-4223-A*	4.84	13.37	4.86	14.66	27.29	15.69	27.41	0.0	25.51	15.36	1.89	2.65	17.30	28.83	4.59	6.46	5.81	216.52
RHA-461*	2.73	8.48	4.88	17.61	36.00	14.00	37.12	0.0	21.93	10.96	2.27	2.75	13.06	26.51	2.85	8.40	1.80	211.35
62003-A*	5.47	11.87	5.67	23.47	32.40	15.11	27.76	1.18	18.48	12.78	1.20	7.99	14.50	28.17	4.19	4.14	3.28	217.66
1159-A**	5.85	5.36	5.65	16.48	27.35	17.01	19.20	1.29	19.26	15.04	2.83	8.96	20.62	22.46	7.09	2.55	7.98	204.98
0043-A**	7.14	4.46	6.22	17.22	26.76	11.72	11.06	0.0	21.00	3.60	1.57	4.99	16.68	24.92	4.57	14.19	4.20	180.30
7675-A**	4.34	6.08	9.22	12.91	25.82	14.30	18.12	2.50	11.40	3.45	6.11	10.16	17.65	19.59	6.35	12.15	8.44	188.59
7989-A**	5.03	7.50	8.81	18.91	22.60	12.93	13.77	2.51	22.71	7.88	11.32	17.67	17.54	18.97	7.26	12.99	2.08	210.48
HA-465-A***	5.65	2.16	6.77	10.28	12.33	10.80	8.02	3.85	10.45	1.91	15.37	17.32	14.91	19.41	3.40	13.51	3.35	159.49
66241-A***	7.62	1.34	3.87	5.76	11.87	2.45	4.41	4.05	15.20	7.11	18.39	23.78	10.76	11.04	6.02	22.43	3.59	159.69
7710-A***	8.34	0.0	4.65	4.23	7.05	5.17	3.47	6.26	13.75	5.14	12.05	16.23	17.21	6.00	6.98	14.17	5.36	136.06
7990-A***	12.20	0.0	7.86	2.52	10.59	6.22	6.23	7.32	12.32	5.30	19.90	20.05	19.16	3.88	10.28	19.76	4.71	168.30
TUNCA***	11.12	1.04	5.34	7.39	8.90	4.89	2.47	4.10	13.92	11.00	18.34	18.83	12.98	7.43	6.27	13.86	3.48	151.36

Legend: * Resistant cultivars; ** Moderate resistant; *** Susceptible cultivars

4.8- Investigate the potential biocontrol agents for the management of damping-off and charcoal-rot in sunflower

4.8.1-Isolation and identification of the bioagents

Twelve fungal isolates and four isolates of endospore-forming bacteria were isolated from rhizosphere of healthy sunflower plants. Isolated fungi were identified according to their morphological and characteristics of mycelia and conidiophores as described by Domsch et al., 1980. The obtained isolates were: three isolates of *Trichoderma harzianum* Rifai, two isolates of *Trichoderma hamatum* (Bonord.) Bainier and one isolate from each of *Trichoderma viride* Pers, *Trichoderma koningii* Oudem, *Trichoderma pseudokoningii* Rifai, *Gliocladium catenulatum* Gilman. & Abbott, *Cunninghamella echinulata* Thaxter, *Penicillium oxalicum* Currie & Thom, and *Penicillium chrysogenum* Thom.

Concerning the identification of isolated bacteria, morphological and physiological properties of four isolates of bacteria are presented in Table (4.16). Bacterial isolates were identified by a series of morphological, biochemical as well as physiological characteristics using the standard characterization procedure of Skinner and Lovelock, 1979, Sneath et al., 1986 and Bergey's Manual of systematic bacteriology, 2001. The isolated endospore-forming bacteria could be identified as *Bacillus cereus* Frankland & Frankland (isolate No. E23) and *Bacillus subtilis* (Ehrenberg) Cohn (isolates No. E24a, E24b and E24c).

Table (4.16): Morphological and physiological characteristics of bacterial isolates

	Characteristics	Isolates No.			
		E23	E24a	E24b	E24c
1	Shape of cell	Rods	Rods	Rods	Rods
2	Motility	+Ve	+Ve	+Ve	+Ve
3	Gram staining	+Ve	+Ve	+Ve	+Ve
4	Endospore production	+Ve	+Ve	+Ve	+Ve
5	Hydrolysis of casein	+Ve	+Ve	+Ve	+Ve
6	Gelatin liquefaction	+Ve	+Ve	+Ve	+Ve
7	Urea test	-Ve	-Ve	-Ve	-Ve
8	Nitrate reduction	+Ve	+Ve	+Ve	+Ve
9	Starch hydrolysis	+Ve	+Ve	+Ve	+Ve
10	Levan production	+Ve	-Ve	-Ve	-Ve
11	Catalase test	+Ve	+Ve	+Ve	+Ve
12	Indole formation	-Ve	-Ve	-Ve	-Ve
13	Esculin hydrolysis	+Ve	+Ve	+Ve	+Ve
14	Anaerobic growth	+Ve	-Ve	-Ve	-Ve
15	Methyl red test	+Ve	+Ve	+Ve	+Ve
16	Oxidase	-Ve	-Ve	-Ve	-Ve
17	Acid from:				
-	D- Glucose	+Ve	+Ve	+Ve	+Ve
-	L- Arabinose	-Ve	+Ve	+Ve	+Ve
-	D- Xylose	-Ve	+Ve	+Ve	+Ve
-	D- Mannitol	-Ve	+Ve	+Ve	+Ve
18	Growth at pH:				
-	6.8	+Ve	+Ve	+Ve	+Ve
-	5.7	+Ve	+Ve	+Ve	+Ve
19	Growth in NaCl:				
-	2.0 %	-Ve	+Ve	+Ve	+Ve
-	5.0 %	-Ve	+Ve	+Ve	+Ve
-	7.0 %	+Ve	+Ve	+Ve	+Ve
-	10.0 %	-Ve	-Ve	-Ve	-Ve
20	Growth at:				
-	5.0°C	-Ve	-Ve	-Ve	-Ve
-	10 °C	+Ve	+Ve	+Ve	+Ve
-	30 °C	+Ve	+Ve	+Ve	+Ve
-	40 °C	+Ve	+Ve	+Ve	+Ve
-	50 °C	-Ve	-Ve	-Ve	-Ve
Probable		*Bacillus*	*Bacillus*	*Bacillus*	*Bacillus*
Identity		*cereus*	*subtilis*	*subtilis*	*subtilis*

Legend: -Ve = Negative reaction
+Ve = positive reaction.

4.8.2- Preliminary test for efficacy of bioagents fungi against sunflower pathogens in *vitro*

The antagonistic capability of three isolates of *Trichoderma harzianum* and *Bacillus subtilis*, two isolates of *T. hamatum* and one isolate of *T. viride*, *T. koningii*, *T. pseudokoningii*, *Gliocladium catenulatum, Cunninghamella echinulata*, *Penicillium oxalicum*, *P. chrysogenum* and *B. cereus* obtained from the roots and rhizosphere of healthy sunflower plants were tested against *Macrophomina phaseolina, Fusarium verticillioides*, and *Fusarium oxysporum,* the causal pathogens of sunflower damping-off and charcoal-rot diseases, in *vitro*.

With respect to antagonistic fungal isolates, data presented in Tables (4.17) & (4.18) and Figure (4.20) indicated that, the tested antagonists showed different inhibitory effect against *M. phaseolina*, *F. verticillioides* and *F. oxysporum* isolates. All tested antagonistic fungi were able to inhibit the growth of all tested pathogenic isolates. *T. harzianum* (E17c) produced the maximum inhibition zone of average of 22 % followed by *T. harzianum* (E17b & E17a) and *G. catenulatum* (E18). Data also indicate that *T. hamatum* (E19a) and *T. koningii* (E21) displayed great inhibitory action to the tested pathogens whereas, *T. hamatum* (E19b) and *T. pseudokoningii* (E22) showed to have moderate inhibitory effect. Other bioagents showed slightly inhibitory effect.

Concerning antagonistic bacteria, results are presented in Table (4.19) and figure (4.21) indicated that, the tested *Bacillus subtilis* isolates inhibited the mycelial growth of all tested pathogenic fungi, and their antagonistic effect differed according to the tested antagonistic bacterial isolates and the pathogenic fungi. *B. cereus* gave moderate effect with all tested pathogenic fungi. In general, with few exceptions, *Bacillus subtilis* (E24b) gave the highest antagonistic effect with all tested pathogenic isolates. The antagonistic effect may be due to direct influence of antagonists fungi against the pathogens through coiling their hyphae around the hyphae of the pathogens to prevent their continued growth and/or producing antagonistic substance which can play an important role in lysis of the cell wall components of the pathogenic fungi to help the antagonists to penetrate the host

hyphae and grow on it as hyper parasite (Sivan and Chet, 1989 and Ikotun and Adekunle, 1990).

Table (4.17): Preliminary test for efficacy of bioagents fungi against *Macrophomina phaseolina* isolates in *vitro*

Treatments		Growth %		Inhibition zones %
Bioagents	Pathogens	Bioagents	pathogens	
T. harzianum (E17a)	*M. phaseolina* (E3)	67.40	13.96	18.64
T. harzianum (E17b)		68.57	13.22	18.21
T. harzianum (E17c)		66.21	11.40	22.39
T. hamatum (E19a)		64.68	18.15	17.17
T. hamatum (E19b)		65.59	20.55	13.86
T. viride (E20)		68.86	21.46	9.68
T. koningii (E21)		68.15	20.15	11.70
T. pseudokoningii (E22)		58.24	29.87	11.89
G. catenulatum (E18)		64.54	18.43	17.03
C. echinulata (E16)		73.94	23.22	2.84
P. oxalicum (E25)		41.13	48.38	10.49
P. chrysogenum (E26)		39.09	47.79	13.12
T. harzianum (E17a)	*M. phaseolina* (E6)	68.09	13.32	18.59
T. harzianum (E17b)		66.57	14.31	19.12
T. harzianum (E17c)		65.86	12.41	21.73
T. hamatum (E19a)		65.52	17.42	17.06
T. hamatum (E19b)		65.81	20.21	13.98
T. viride (E20)		69.43	21.46	9.11
T. koningii (E21)		65.58	18.82	15.60
T. pseudokoningii (E22)		58.17	30.14	11.69
G. catenulatum (E18)		62.87	17.86	19.27
C. echinulata (E16)		74.02	23.36	2.62
P. oxalicum (E25)		41.11	48.21	10.68
P. chrysogenum (E26)		38.22	45.96	15.82
LSD		0.05 0.01 4.12 5.51	0.05 0.01 2.39 3.19	0.05 0.01 4.74 6.34

Table (4.18): Preliminary test for efficacy of bioagents fungi against *F. verticillioides* and *F. oxysporum* in *vitro*

Treatments		Growth %		Inhibition zones %
Bioagents	Pathogens	Bioagents	Pathogens	
T. harzianum (E17a)	*F. verticillioides* (E14b)	64.38	16.39	19.23
T. harzianum (E17b)		67.56	13.73	18.71
T. harzianum (E17c)		66.77	10.63	22.60
T. hamatum (E19a)		58.35	25.43	16.22
T. hamatum (E19b)		60.14	28.01	11.85
T. viride (E20)		59.15	28.25	12.60
T. koningii (E21)		55.00	30.11	14.89
T. pseudokoningii (E22)		57.88	38.12	4.00
G. catenulatum (E18)		56.50	28.44	15.06
C. echinulata (E16)		67.89	27.15	4.96
P. oxalicum (E25)		42.13	48.07	9.80
P. chrysogenum (E26)		40.24	53.12	6.64
T. harzianum (E17a)	*F. oxysporum* (E15a)	65.31	18.52	16.17
T. harzianum (E17b)		66.90	14.73	18.37
T. harzianum (E17c)		66.13	12.03	21.84
T. hamatum (E19a)		59.10	27.46	13.44
T. hamatum (E19b)		60.81	28.01	11.18
T. viride (E20)		56.83	30.25	12.92
T. koningii (E21)		56.05	34.11	9.84
T. pseudokoningii (E22)		57.90	38.44	3.66
G. catenulatum (E18)		57.21	31.92	10.87
C. echinulata (E16)		69.55	28.27	2.18
P. oxalicum (E25)		43.13	50.41	6.46
P. chrysogenum (E26)		42.09	52.70	5.21
LSD		0.05 4.42 / 0.01 5.91	0.05 5.21 / 0.01 6.97	0.05 4.81 / 0.01 6.44

Table (4.19): Preliminary test for efficacy of bioagents bacteria against *M. phaseolina*, *F. verticillioides* and *F. oxysporum* in *vitro*

Bioagents	Mycelial growth inhibition (%)				Mean
	M. phaseolina (E3)	*M. phaseolina* (E6)	*F. verticillioides* (E14b)	*F. oxysporum* (E15a)	
B. cereus (E23)	50.83	53.33	48.10	42.50	48.69
B. subtilis (E24a)	46.10	51.93	59.43	60.93	54.59
B. subtilis (E24b)	46.66	61.23	65.46	63.50	59.21
B. subtilis (E24c)	41.83	43.40	52.30	45.76	45.82
Mean	46.35	52.47	56.32	53.17	
LSD	0.05		0.01		
Bioagents (B)	2.47		3.36		
Pathogens (P)	1.61		2.45		
Interactions (BxP)	4.94		6.72		

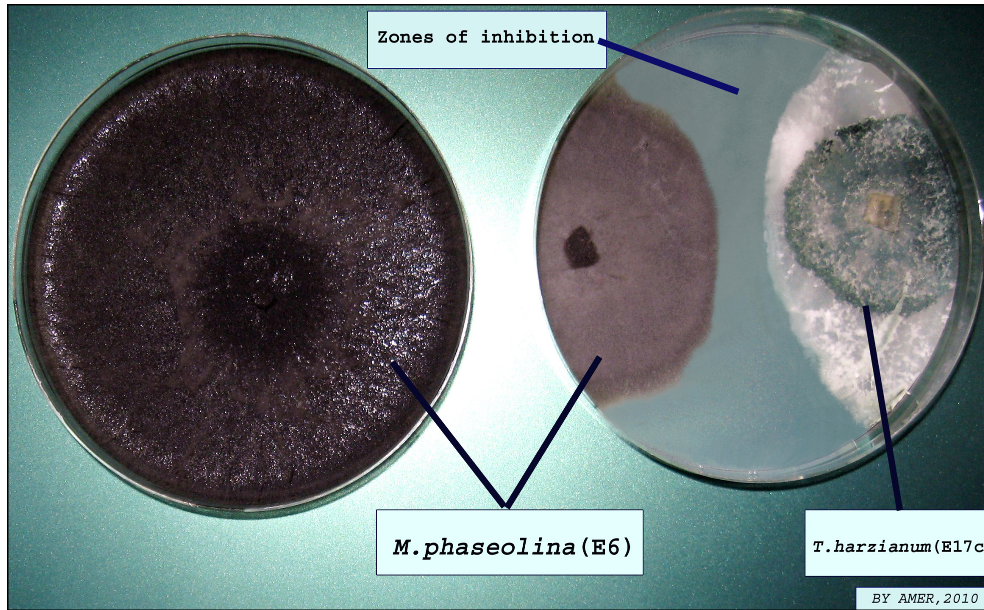


Figure (4.20): The above photo shows zone of inhibition produced by *T. harzianum* against *M. phaseolina in vitro.*

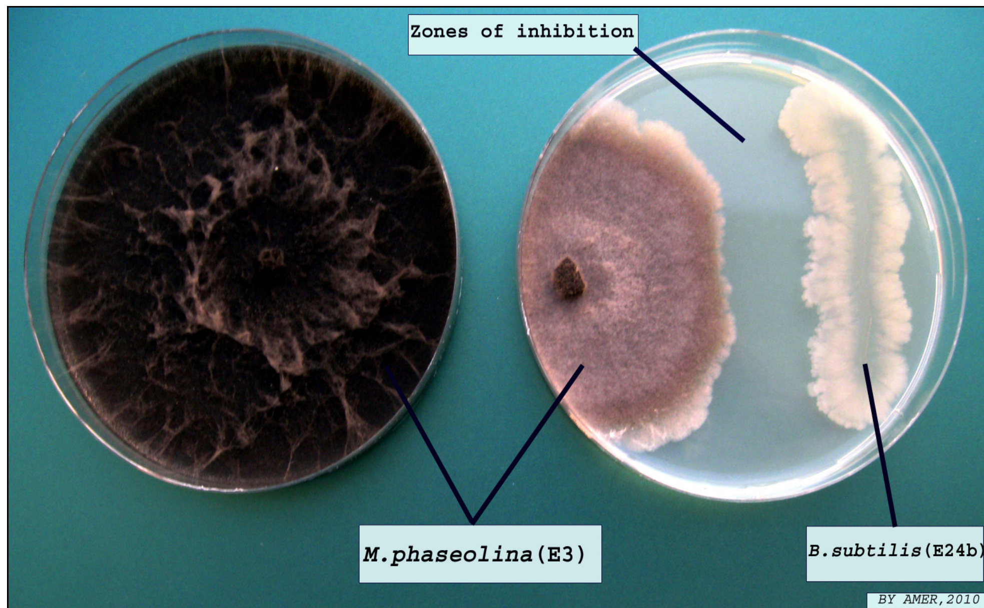


Figure (4.21): The above photo shows zones of inhibition produced by *B. subtilis* against *M. phaseolina in vitro.*

4.8.3- Evaluation efficacy of *Trichoderma* and *Gliocladium* isolates against *Macrophomina phaseolina* in greenhouse

The efficiency of *Trichoderma* and *Gliocladium* as biological control agents, on the incidence of sunflower damping-off and charcoal-rot diseases caused by *Macrophomina phaseolina* was carried out under greenhouse conditions.

Data in Table (4.20) and Figures (4.22) & (4.23) indicated that, *M. phaseolina* (E6) was able to infect sunflower plants causing significant increasing in damping-off disease and reduce the percentage of surviving plants after 2 and 4 weeks compared with the control whereas, application of both *Trichoderma* and *Gliocladium* isolates gave the reverse effect. They significantly increased the surviving rate of sunflower plants and depressed the percent of mortality in plants compared with the tested pathogen only. Data also reveal that, all the tested antagonistic isolates of *Trichoderma* and *Gliocladium* were not able to infect sunflower plants and gave similar results as the control.

In general, success of the biocontrol agents in controlling the soil borne pathogenic fungi depends on many factors i.e., the ability of the antagonists fungi to penetrate the root tissues faster than the pathogen and the activity of the microorganisms to absorb the nutrients from the soil faster than the pathogen (Fisher, 1977; and Huang, 1992).

Table (4.20): Evaluation efficacy of *Trichoderma* and *Gliocladium* isolates against *M. phaseolina* in greenhouse

Treatments	% Seedling surviving			
	After 2 weeks		After 4 weeks	
	SANAY	TUNCA	SANAY	TUNCA
Uninoculated control	83.33	91.66	83.33	91.66
M. phaseolina (E6)	25.00	41.66	16.66	25.00
T. harzianum (E17b)	100	100	100	100
T. harzianum (E17c)	100	91.66	100	91.66
T. hamatum (E19a)	100	100	100	100
T. koningii (E21)	91.66	100	91.66	91.66
G. catenulatum (E18)	91.66	100	91.66	100
T. harzianum (E17b) + *M. phaseolina* (E6)	75.00	83.33	75.00	75.00
T. harzianum (E17c) + *M. phaseolina* (E6)	75.00	75.00	58.33	66.66
T. hamatum (E19a) + *M. phaseolina* (E6)	83.33	83.33	75.00	75.00
T. koningii (E21) + *M. phaseolina* (E6)	66.66	75.00	58.33	75.00
G. catenulatum (E18) + *M. phaseolina* (E6)	83.33	83.33	75.00	83.33
LSD	0.05 24.05	0.01 32.15	0.05 19.30	0.01 25.80

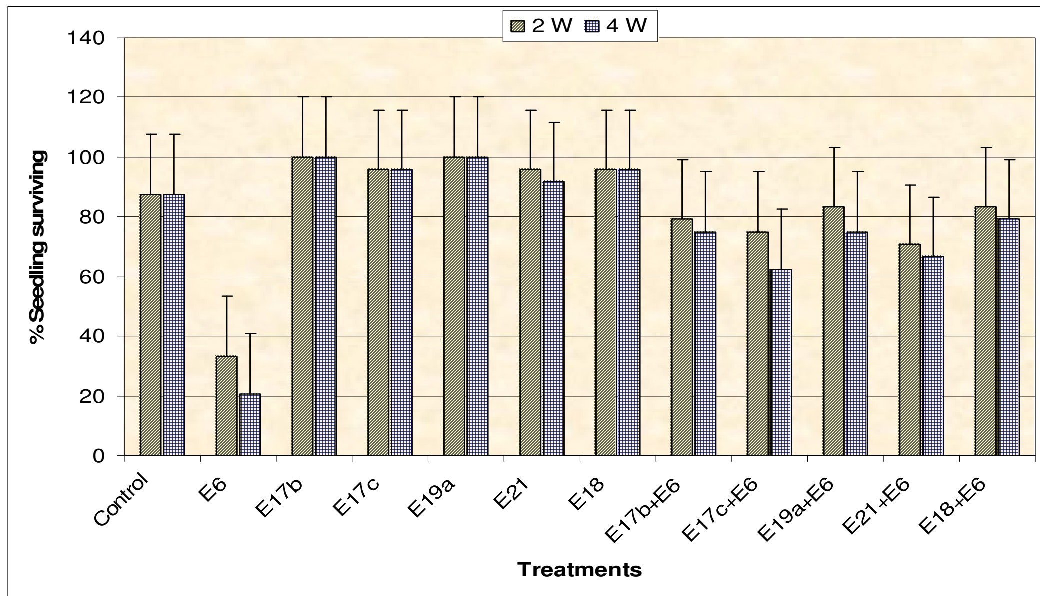


Figure (4.22): Efficacy of *Trichoderma* and *Gliocladium* isolates against *M. phaseolina* in greenhouse

Figure (4.23): The above photo shows evaluation efficacy of *Trichoderma* against *M. phaseolina* (SANAY cultivar) under greenhouse conditions (4 Weeks).
(1)= Pathogen only: *M. phaseolina* (E6),
(2)= *T. harzianum* (E17c) + *M. phaseolina* (E6),
(3)= *T. harzianum* (E17c) only

4.8.4- Screening the effect of biological control agents on sclerotia production of *Macrophomina phaseolina*

Results presented in Table (4.21) showed that, all tested bioagents were found to be effective in reducing the sclerotia production of *Macrophomina phaseolina* in the soil. Among the five tested antagonists, *Gliocladium catenulatum* was found significantly superior in reducing the sclerotia production (69.30%) of *M. phaseolina* followed by *Trichoderma hamatum* (67.98%) and *Trichoderma harzianum* (67.32% & 65.78%) and 62.28 percent reduction by *Trichoderma koningii*.

T. harzianum (E17b &E17c), *T. hamatum* (E19a), *T. koningii* (E21) and *G. catenulatum* (E18) isolates achieved highly control of the pathogen by growing fast and stopping further growth of the pathogen. Therefore, *Trichoderma* and *Gliocladium* isolates can be used as a potential biocontrol agent for minimizing pathogen growth and also in the reduction of primary inoculum in the field by restricting the production of sclerotia. Results of this experiment are in agreement with those reported by Majumdar et al., 1996; Patil et al., 2003 and Shekhar et al., 2006, reported that *T. harzianum* isolates were capable to inhibit microsclerotia production of *M. phaseolina*. *Trichoderma* is the most effective and antagonistic against *M. phaseolina* in *vitro* and in *vivo*.

Further conclusions from the results obtained from this in *vitro* and in *vivo* study showed that, the isolates of *Trichoderma* and *Gliocladium* could be used effectively to control the damping-off and charcoal-rot disease on sunflower caused by *M. phaseolina*. Applications of those bioagents were effective and safe in controlling sunflower damping-off and charcoal-rot diseases.

Table (4.21): The effect of biological control agents on sclerotia production of *M. phaseolina*

Treatments	No. of sclerotia			% Reduction in No. of sclerotia		
	10 days	20 days	30 days	10 days	20 days	30 days
T. harzianum (E17b) + *M. phaseolina* (E6)	71.00	63.00	49.66	13.76	42.54	67.32
T. harzianum (E17c) + *M. phaseolina* (E6)	72.66	63.66	52.00	11.74	41.94	65.78
T. hamatum (E19a) + *M. phaseolina* (E6)	70.33	59.33	48.66	14.57	45.89	67.98
T. koningii (E21) + *M. phaseolina* (E6)	79.66	68.33	57.33	3.24	37.68	62.28
G. catenulatum (E18) + *M. phaseolina* (E6)	68.66	63.00	46.66	16.60	42.54	69.30
M. phaseolina (E6) (Control)	82.33	109.66	152.00	--	--	--

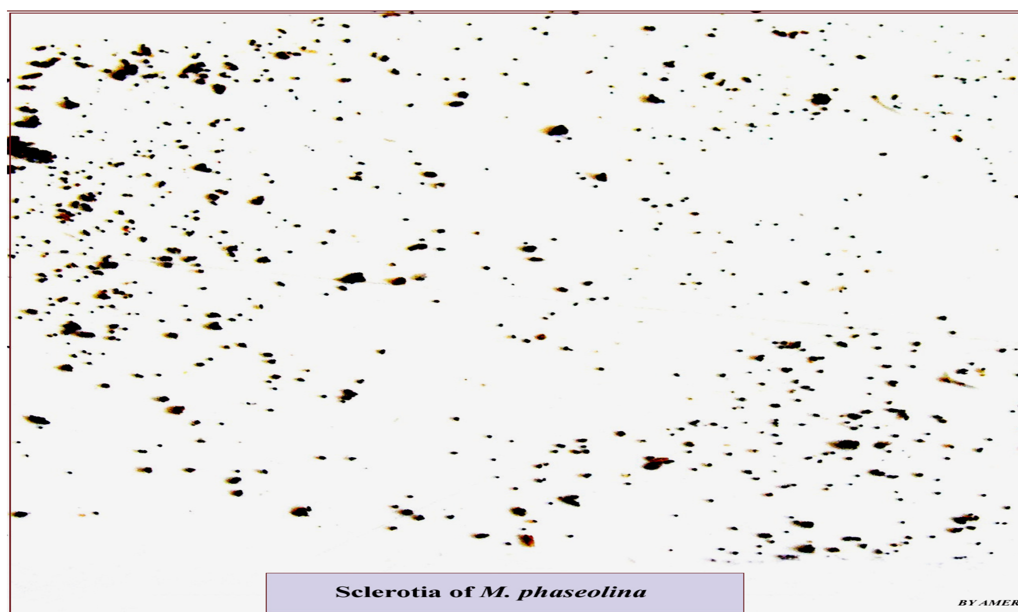


Figure (4.24): Sclerotia shapes of *M. phaseolina*

5- DISCUSSION

In both Turkey and Egypt, a little attention has been directed to study sunflower damping-off and charcoal-rot diseases, in spite of their widespread and importance therefore, this study was achieved to study those important diseases. Damping-off and charcoal-rot diseases cause important economic loss in the main production areas of sunflower around the world. Sunflower plants infected with damping-off and charcoal-rot is typically invaded by the pathogen through root system. Plants that are attacked early often die in seedling stage (Sackston, 1981; Xiaojian et al., 1988; Orellana, 1971; Tikhonov et al., 1976 and Jimenez et al., 1983). Our objective was to screen selected sunflower fields from Egypt and Turkey, to determine the fungi associated with the roots of sunflower so as to isolate the causal pathogens of damping-off and charcoal-rot, in order to increase our knowledge about the spread, physiological, morphological, pathogenical features and genetic diversity of those pathogens.

Several fungi were found to be associated with damping-off and charcoal-rot of sunflower in Egypt and Turkey. The most dominating and pathogenic fungi were *Macrophomina phaseolina, Fusarium oxysporum*, *Fusarium verticillioides* and *Rhizoctonia solani*. However, although *Alternaria helianthi* was also isolated in Turkey, the fungus was not able to incite remarkable disease to sunflower in the pathogenicity test. *Macrophomina phaseolina, Fusarium oxysporum*, *Fusarium verticillioides* and *Rhizoctonia solani* incited pre- and post- emergence damping-off at different degrees. Meantime, charcoal-rot disease, as evident by the presence of black sclerotia in plant stem base, was only developed with *Macrophomina phaseolina.* These findings are in agreement with Dhingra and Sinclair, 1974; El-Zarka, 1976 and Bokor, 2007. Our study reveals that 220 isolates belonging to *Macrophomina phaseolina*, *Fusarium oxysporum*, *Fusarium verticillioides*, *Rhizoctonia solani, Alternaria helianthi, Rhizopus spp., Aspergillus spp.* and *Penicillium spp.* were isolated from Egypt, (180 isolates), and Turkey, (40 isolates). Fungi of the genera *Macrophomina phaseolina* was the most prevalent fungus, followed by *Fusarium oxysporum, Fusarium verticillioides*, *Rhizoctonia solani* and *Alternaria helianthi*.

There was appreciable variation in pathogenicity among the tested isolates of *Macrophomina phaseolina, Fusarium oxysporum, Fusarium verticillioides* and *Rhizoctonia solani*, such results are in agreement with Mihail and Taylor, 1995 and Mayek-Pérez et al., 2001 they have also found high variability in the pathogenicity and population of *M. phaseolina* with respect to host reaction. In general, with few exceptions, our study showed that isolates collected from the cultivation area in Egypt were more virulent than isolates collected from Turkish cultivation area.

The influences of temperature, pH and salinity on the growth of *Macrophomina* and *Fusarium* were achieved on Agar. Physical and chemical factors cause either dimorphic switching or monomorphic growth of these organisms. We examined the response of 26 isolates of *Macrophomina phaseolina*, 6 isolates of *Fusarium oxysporum* and 2 isolates of *Fusarium verticillioides* to temperatures, pH and NaCL in *vitro*.

The mycelial growth was found to be significantly influenced by temperature. The growth increased as did temperature, reaching optima at 25 and 30°C respectively. The growth rate of all isolates dropped at 5 and 40°C. *Macrophomina* isolates grew faster than did *Fusarium* isolates at high temperatures (30 and 40°C) while *Fusarium* isolates are grew faster at low temperature (15 and 20°C). These findings are supported by the data presented by Hong et al., 2000; Kiewnick, 2001 and Meikle et al., 2003. They found similar results when studying the effect of growth conditions (temperature and moisture) on the formulation process of fungal spore and the growth of fungi. Results are in agreement with Manici et al., 1995; Gupta et al., 1986 and Farooq et al., 2005 reported similar findings regarding temperature requirements to *Macrophomina phaseolina* and other fungi.

The obtained results indicated that the most suitable pH levels for growth of all tested isolates were 6.0 and 7.0.These results are in agreement with those achieved by Hayes, 1978. The fungal responses towards NaCL stress seemed to vary with the concentration of NaCL. Similar sensitivities were observed at high concentrations of NaCL. *Fusarium* isolates displayed a greater sensitivity than did *Macrophomina* isolates to a high concentration of NaCL. Results showed that there are variations among the isolates in the

response to NaCL. Our results were consistent with those of Chen et al., 2001 who found growth variations among 18 strains at various salinities.

Isolates originating from Egyptian sunflower fields had a faster growth rate than isolates collected from Turkish fields as well as they were more virulent in their pathogenicity on sunflower. The most suitable temperature for *M. phaseolina* growth was 27°C. Growth was slower at 15 and 20°C. At 40°C, growth was slight or absent. Studying in *vitro* growth of *M. phaseolina* isolates from sunflower grown in different climatic regions of Italy, Manici et al., 1995 noticed that some isolates from the south (Mediterranean climate) grew better at 35°C. These results are in agreement with those found by Armstrong and Armstrong, 1981.

Determination of genetic diversity among the isolates of *Macrophomina phaseolina*, *Fusarium oxysporum* and *Fusarium verticillioides* aimed to study the genetic variations for 34 fungal isolates collected from different origins grown under the Egyptian and Turkish environments using sequence related amplified polymorphism (SRAP) technique, to elucidate the genetic relations among these isolates. *Macrophomina* and *Fusarium* are a filamentous fungi, that are widely distributed in the soil and causes damping-off and charcoal-rot in sunflower (Dhingra and Sinclair, 1977; Sinclair and Backman, 1989), those fungi are associated with the rhizosphere of many plant species, for this purpose we compared the genetic similarity among and within the different isolates of *M. phaseolina*, *F. oxysporum* and *F.* verticillioides.

As we expected, due to the geographic distinction, there was a high level of genetic diversity among the tested genotypes. In this study, genetic diversity was observed among isolates from the same as well as different areas, showing that in the same geographic areas, exhibited the same haplotypes. Genetic diversity among isolates from different regions was classified as different haplotypes and established in different clusters. Our results demonstrated that SRAP markers are suitable for measuring genetic relatedness, detecting variation within and between *M. phaseolina* and *Fusarium* populations. SRAP analysis provides a useful tool for estimation of genetic diversity and phenetic relationships in natural and domesticated populations; SRAP is highly polymorphic and more informative when compared to AFLP, RAPD and SSR markers (Budak et al., 2004a). PCR- SRAP is an

effective tool for estimating genetic diversity, identifying unique genotypes as new sources of alleles for enhancing turf characteristics (Budak et al., 2004c). Variability in the pathogen population may reflect the lack of resistance among current commercial sunflower cultivars. A principal component analysis strongly supported these clusters. Mayek-Peréz et al., 2001 studied the variability among 84 isolates and found that 43 pathotypes of *M*. *phaseolina* had a unique AFLP-analyzed genotype indicating an absence of correlation between pathotypes and genotypes of isolates of *M*. *phaseolina*. The results are in disagreement with Su et al., 2001 who were unable to find variation in restriction and length polymorphisms of the ITS region among 45 isolates of *M*. *phaseolina* obtained from maize, cotton, sorghum and soybean.

Infection reactions of 41 sunflower cultivars for susceptibility/resistance to *Macrophomina phaseolina*, the causal pathogen of damping-off and charcoal-rot, were evaluated under greenhouse conditions. The aim of this study was to identify resistant cultivars of sunflower to *M. phaseolina* infection. Certain sunflower cultivars proved to be resistant to damping-off and charcoal-rot diseases, but the most cultivars were susceptible. This study showed significant variation in susceptibility/resistance among sunflower cultivars to *M. phaseolina* infection. The differences of susceptibility/resistance to *M. phaseolina* infection in sunflower cultivars were observed by the variations on surviving plants in stages of pre- and post- emergence damping-off, as well as disease severity. Sunflower cultivars that proved to be resistant cultivars yield a high number of survival plants after 2 and 4 weeks, as well as a low value of disease severity. The previous studies showed that extensive genetic variation and site-specific nature of *M. phaseolina* have made studies on genetics of charcoal-rot resistance difficult. Therefore, genetics of resistance against *M. phaseolina* have not been clearly demonstrated and controversies are found in the findings by other researchers. Resistance in sunflower genotype is a dominant character (Olaya et al., 1996; Jones et al., 1996; Yang et al., 1999 and Michel, 2000). The resistant cultivars that were identified in this study could be used for breeding programs; therefore, plant breeders should exert more efforts to improve and produce these cultivars to be used widely to overcome damping-off and charcoal-rot diseases. These results are in

agreement with results that reported by Edmunds, 1964; Orellana, 1971; Tikhonov et al., 1976; Jimenez et al., 1983; Olaya et al., 1996 and Michel, 2000.

Investigation of the root exudates of sunflower cultivars were conducted to provide quantitative information about the amino acids (AA) on root exudates of sunflower cultivars, as well as, their influences on the growth of *Macrophomina* and *Fusarium* in *vitro*. A small change of root exudates will lead to great changes in the system of microorganisms. Therefore, by studying the root exudates components of sunflower cultivars with different levels of disease resistance, the pathogenesis of *Macrophomina* and *Fusarium* may be revealed. Furthermore, the physiological and biochemical index of sunflower resistance to charcoal-rot and damping-off may be discovered, which is useful for sunflower breeding and disease control. Developing and utilizing the disease-resistant cultivars is the most economic method to control this disease. Amino acids are fundamental ingredients in the process of protein synthesis. About 20 important amino acids are involved in the process of each function. Studies have proven that amino acids can directly or indirectly affect the physiological activates of the plant because the amino acid pool is only a small portion of the total dissolved organic nitrogen pool, which generally contains less than 10% free amino acids in temperate ecosystems (Qualls and Haines, 1991 and Yu et al., 2002). Amino acids help to increase the chlorophyll concentration in the plant leading to higher degree of photosynthesis. Also amino acids are associated with phenolic compounds which play a major role in plant defense (Hahlbrock and Scheel, 1989). The resistance which is found in certain sunflower cultivars to damping-off and charcoal-rot diseases may be due to their high concentrations of some kinds of amino acids such as Proline and Valine. Several studies indicate that Proline-rich and hydroxyproline-rich glycoproteins are implicated in plant defense against pathogens (Cassab, 1998 and Caruso et al., 1999). Root exudates are important in microbial attraction and fungal establishment on roots (Cochrane et al., 1963). The role of amino acids in plant diseases may be due to the correlation between these acids and plant health (Bush, 1993). Amino acids are involved in the synthesis of phytoalexins (Cui et al., 2000) and pathogenesis-related proteins (van Loon et al., 1994).

The results indicate that the pathogenic strains of *Macrophomina phaseolina, Fusarium verticillioides* and *Fusarium oxysporum* trigger different growth with Root exudates of sunflower. These results in agreement with Scheffknecht et al., 2007; they showed that root exudates of many plants, colonized by the arbuscular-mycorrhizal fungus, exhibited a different effect on the microconidial germination *Fusarium oxysporum.*

Biological control of sunflower pathogens aims to lower the inoculum density of the pathogen to reduce the disease incidence. *Trichoderma spp*, *Gliocladium spp* as well as *Bacillus spp*. were isolated from soil. Some of these isolates showed antagonistic effect against sunflower pathogens in *vitro* and in *vivo*. Its antagonism may be due to stimulation of plant defense mechanism, production of some antibiotics which may play remarkable role in the biological control and mycoparasitism processes against the pathogen, which begins to initiate a response to biological control.

Bioagents fungi which populate the area around the plants are like having a second set of roots for the plants. Bioagents fungi increase a plant's immune system, making it resistant to soil-borne pathogens. Plants with a well established bioagents fungus can survive better in their non-native environments; they also absorb more nutrients from the soil. Bioagents fungi were able to inhibit the growth of *Macrophomina phaseolina*, *Fusarium oxysporum, Fusarium verticillioides* and *Rhizoctonia solani* the causal pathogens of sunflower damping-off and charcoal-rot in *vitro*. *Trichoderma spp*. isolates gave highest antagonistic effect than other bioagents isolates. Under greenhouse conditions, application of *T. harzianum*, *T. hamatum*, *T. koningii* and *G. catenulatum* separately to soil infested with pathogens, caused significant increase in plant survival. The results showed that the percentage of infection was significantly affected by bioagents. The application of bioagents reduced the percentages of infection as compared to the control. In general the application of *G. catenulatum* produced the highest value (83.33%). The observation of the effect of *Trichoderma* and *Gliocladium*, as biological control agents, on sclerotia production of *Macrophomina phaseolina* revealed that the tested bioagents were found to be effective in reducing the sclerotia production of *M. phaseolina* in the soil. These results are in agreement with Baker and Cook, 1974; Druzhinina and Kubicek, 2005; Morsy and El-Korany, 2007 and Larralde-Corona et al., 2008.

6- CONCLUSION

The pathogenicity, physiological, as well as the genetic diversity of isolates of *M. phaseolina, F. oxysporum* and *F. verticillioides* responsible for damping-off and charcoal-rot of sunflower was described. The isolates were collected from naturally infected roots of sunflower both in Egypt and Turkey. The isolated fungi were able to infect sunflower plants under artificial infection and cause the symptoms of pre- and post- emergence damping-off, as well as charcoal-rot. The isolates were characterized by their growth potential at different temperatures, their morphology on PDA medium, their virulence on three sunflower cultivars, and a SRAP analysis. We estimated the growing of mycelia of 26 isolates of *M. phaseolina*, 6 isolates of *F. oxysporum* and 2 isolates of *F. verticillioides* to temperatures, pH and NaCL to determine the adaptability of those fungi to different Turkish climatic conditions under which sunflower grows. The mycelial growth was found to be significantly influenced by temperature; the growth increased as did temperature, reaching optima at 25 and 30°C respectively. The most suitable pH levels for growth of all tested isolates were 6.0 and 7.0. The fungal responses towards NaCL stress seemed to vary with the concentration of NaCL. Sensitivities were observed at high concentrations of NaCL. Furthermore, we determined the phenotypic variations among the isolates of *Macrophomina* and *Fusarium* that are collected from different regions in Turkey and Egypt.

We performed the first study with sequence related amplified polymorphism (SRAP) for an overview of genetic diversity and phenetic relationships present among *Macrophomina* and *Fusarium* isolates that are collected from Egypt and Turkey. Results showed that Turkish and Egyptian isolates of *M. phaseolina* were clustered together with a genetic similarity of 60%; on the other hand, Turkish and Egyptian isolates of *F. oxysporum* were clustered together with a genetic similarity of 75%. The study also indicated that *M. phaseolina* isolates from different cultivation areas in Egypt and Turkey vary with respect to virulence and ability to cause infection on sunflower cultivars. Furthermore, we found certain sunflower cultivars are resistant to *M. phaseolina*. A relationship between root exudates and the mechanism of sunflower plant resistance to

charcoal-rot and damping-off was found. In addition, certain isolates of *Trichoderma spp*. and *Gliocladium catenulatum* were found to be efficient in controlling *M. phaseolina*. These agents can readily be used to gain more resistance to charcoal-rot diseases in sunflower.

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8- APPENDIX

8.1- Appendix- A: (Supplies):

Disposable Labware:

Centrifuge tubes:	ISOLAB, Germany, (Lot: TPPD9004).
ClickFit Cap microtubes:	TreffLab, Switzerland, (Cat: 96.7246.9.01, 96.4625.9.01, 96.9329.9.01, 96.7514.9.01, 96.7811.9.03, 96.8185.9.03).
Diamond® Tips:	Gilson, USA, (D10, D200, D1000).
Filter paper circles:	125 mm,whatman® Schleicher & Schuell, Germany, (Lot: 300011).
Microscope slides:	ISOLAB Laborgeräte GmbH, Germany.
PCR-tubes:	TreffLab, Switzerland, (Cat: 96.9852.9.01).
Petri dishes:	ISOLAB Laborgeräte GmbH, Germany, (081.02.091).
Polystyrene round-bottom test tubes:	Becton Dickinson Falcon™, USA (352096).
Syringe filter:	Spritzen, 0.22µl, membrane:PES, Germany, (lot: 20080292).
Syringe filter:	Single use, 0.45µl, Germany, (Lot: 1655580390103).
Tips for pipettes:	TreffLab, Switzerland, (Cat: 96.9515.9.02, 96.9085.4.01, 96.10308.9.02).

Chemical Supplies:

2- Propanol (Isopropyl alcohol):	PESTANAL®, Sigma-Aldrich, Riedel-de Haën®, Germany, (Cat: 34486).
Agar-Agar:	Merck, Germany, (Cat: 1.01613; Lot:

	VM909613).
Agarose low EO :	Applichem, Germany, (Cat: A2114).
Agarose:	Basica LE agarose, Prona, EU, (Lot: 084543PR).
Ampicillin:	Sigma, Germany, (Cat: A9518).
BamHI:	Fermentas, EU, (Cat: ER0051).
Boric acid for molecular biology, ≥98.5%:	Sigma-Aldrich, Germany, (B6768).
Buffer BamHI:	Fermentas, EU, (Cat: B57).
Buffer EcoRI:	Fermentas, EU, (Cat: B12).
Buffer R:	Fermentas, EU, (Cat: BR5).
Buffer Tango™ :	Fermentas, EU, (Cat: BY5).
Crystal Violet:	Sigma, Germany, (Cat: C3886).
D-Glucose (Dextrose):	GIBCO™, USA, (Lot: 512011).
di-Potassium hydrogen phosphate:	Merck, Germany, (Code: 137010).
DNA Rehydration solution:	Promega, USA, (Cat: A796A).
dNTP mix:	Fermentas, EU, (Cat: R0192; Lot: 00030191).
EcoRI:	Fermentas, EU, (Cat: ER0271).
EcoRV:	Fermentas, EU, (Cat: ER0301).
EDTA:	Riedel-de Haén, Germany, (Cat: 27248).
Ethanol absolute:	Sigma-Aldrich, Riedel-de Haën®, Germany, (Cat: 40274).
Ethidium Bromide:	Merck, Germany, (Code: OCO28942).
Ferrous sulfate:	Merck, Germany, (Lot: A421365246).
Gene Ruler 1 Kb DNA ladder plus:	Fermentas, EU, (Cat: SM1333; SM1331).
Glycerol:	Riedel-de Haén, Germany, (Cat: 15523).
HCL:	Merck, Germany, (Cat:100314).
HinIII:	Fermentas, EU, (Cat: ER0501).
Liquid nitrogen:	Karbogaz, Turkey.
Loading Dye Solution:	Qiagen, (Lot: 130184025).
Magnesium sulfate extra pure:	Merck, Germany, (Code: 172572).
Meat extract:	Biolab®, Germany, (Code: MEE10; Lot:

	07/A/41).
Nuclei lysis solution:	Promega, USA, (Cat: A7941).
Peptone from casein:	Applichem, Darmstadt, Germany, (Lot: 5G004687).
Potassium chloride:	Merck, Germany, (Code: 104936).
Potassium dihydrogen phosphate:	Merck, Germany, (Code: 105108).
Protein precipitation solution:	Promega, USA, (Cat: A795A).
RNase solution:	Promega, USA, (Cat: A797A).
Sodium chloride:	Merck, Germany, (Code: 1.06400).
Sodium hydroxide pellets pure:	Merck, Germany, (Code: 1.06462).
Sodium nitrate:	Merck, Germany, (Code: 106546).
Streptophenicol antibiotic:	L-chloramphenicol and streptomycin, Misr Co. for Pharm-Ind.SAE.
Sucrose:	Sigma, Germany, (Cat: S0389).
Taq DNA polymerase (recombinant):	Fermentas, EU, (EP0402; Lot: 00059899).
Tris(hydroxymethyl)aminomethane:	Merck, Germany, (Cat: 1.08387).
Yeast extract:	AppliChem, GmbH, Germany, (Code: A1552).

Commercial Kits:

Promega, DNA purification Kit, USA, (Cat: A1125; Lot: 258656).

8.2- Appendix- B: Equipment

Autoclave:	Certoclav, Table Top Autoclave CV-EL-12L, AUSTRIA.
	Hirayama, Hiclave HV-110, JAPAN.
	Nüve, OT 032, TURKEY.
Balance:	Sartorius, BP610, BP221S, BP221D, GERMANY.
	Schimadzu, Libror EB-3200 HU, JAPAN.
Biochrom 30:	Amino acid analyzer, UK.

Cassette:	Kodak Biomax MS casette, USA.
Centrifuge:	Beckman Coultier ™ Microfuge® 18 Centrifuge, USA.
	Hitachi, Sorvall RC5C Plus, USA.
	Eppendorf, 5415D, GERMANY.
	Eppendorf, 5415R, GERMANY.
	Eppendorf, 5415C, GERMANY.
	Kendro Lab. Prod., Heraeus Multifuge 3L, GERMANY.
Cold room:	Alarko Carrier, TURKEY.
Deep-freeze:	-80ºC, Thermo Electron Corporation, USA.
	-20ºC, Bosch, TURKEY.
Deionized water:	Millipore, MilliQ Academic, FRANCE.
Digital Camera:	Canon, Power-Shot SD 400, USA.
Electrophoresis:	Biogen Inc., USA.
	Biorad Inc., USA.
	SCIE-PLAS, TURKEY.
Gel documentatiton:	UVITEC, UVIdoc Gel Documentation System, UK.
	BioRad, Universal Hood II, USA.
	BioRad, Quantity One, USA.
	BIO-RAD, UV-Transilluminator 2000, USA.
Heating block:	Bioblock Scientific, FRANCE.
	Bio TDB-100 Dry Block Heating Thermostat, HVD Life.
	Sciences, AUSTRIA.
Ice machine:	Scotsman Inc., AF20, USA.
Incubator:	Memmert, Modell 300, GERMANY.
	Memmert, Modell 600, GERMANY.
	Nüve EN 120, TURKEY.
	Shaker, New Brunswick Scientific, Innova 4330, USA.
Laminar flow:	Kendro Lab. Prod., Heraeus, Herasafe HS12, GERMANY.
Liquid nitrogen tank:	DEWAR, Flask and container, ENGLAND.
Magnetic stirrer:	VELP Scientifica, ARE Heating Magnetic Stirrer, ITALY.
	VELP Scientifica, Microstirrer, ITALY.

Micropipette:	Gilson, Pipetman, FRANCE.
	Eppendorf, GERMANY.
	Olympus, SZ61, USA.
Microwave Oven:	Bosch, TURKEY.
	Vestel, TURKEY.
pH meter:	Hanna, pH213 microprocessor pH meter, GERMANY.
	WTW, pH540 GLP Multical®, GERMANY.
Power Supply:	Wealtec, Elite 300, USA.
	Biogen, AELEX, USA.
Refrigerator:	+4ºC, Bosch, TURKEY.
Shaker:	Excella E24 Shaker Series, New Brunswick Sci., USA.
	GFL, Shaker 3011, USA.
	Innova™ 4330, New Brunswick Sci., USA.
Spectrophotometer:	Bio-RAD, SmartSpec™ 3000, USA.
	Schimadzu, UV-3150, JAPAN.
	Nanodrop, ND-1000, USA.
	Varian, Cary 300 Bio Uvi-visible spec., AUSTRALIA.
Speed vacuum:	Savant, Refrigerated Vapor Trap RVT 400, USA.
Thermocycler:	PE Applied biosystems, GeneAmp PCR System 9700, USA.
	MJ Research, PTC-100, USA.
	TECHNE, TC 512, UK.
Tissuelyser:	QIAGEN, Retsch®.
Vortex:	VELP Scientifica, 2x3, ITALY.
Water bath:	TECHNE, Refrigerated Bath RB-5A, UK.
	JULABO, TW 20, USA.