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Distinguished Positive Reactions of *Macrophomina phaseolina* (Tassi) Goid Host Plants, Laboratory Media, and Potting soil

Muhanna, N.A.S.

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ABSTRACT

Fourteen isolates of *Macrophomina phaseolina* (Tassi) Goid the causal of charcoal rot, were isolated from different hosts dominated at different locations in Egypt. Macromorphological features of isolates were compared by growing on different agar media at 28±2ºC. Colony appearance, growth rate, aerial mycelia, and production of sclerotia were assessed. Four isolates were scored as fast grow with cod No. M4, M9, M12, M15 and three as slow grow, *i.e.*, M1, M2, M13. The best media for growth were Lima bean Agar (LBA), Czapek's Dox Agar (CZA), Potato Dextrose Agar (PDA), Corn Agar (CA), and potato-sucrose agar (PSA) respectively. The colony characteristic of color was of black dense, light black dense, light grey and dark brown. The best media for sclorotia formation were LBA and PDA media for the isolates M4, M7, M9, M11, M14, M15. Pathogenicity experiments showed that M. *phaseolina* isolates were able to cause root rot and hypocotyl discoloration of hosts. Three fungal isolates appeared to be less virulent *i.e.*, M2, M3 and M8 whereas two isolates *i.e.*, M11and M15 were scored, highly virulent or drastically virulent to beans, cantaloupe, tomato, cotton and sunflower plants. No relationship between the morphological characteristics and pathogenicity of the isolates. In addition, protein analysis assay of *M. phaseolina* revealed different numbers of separated bands (1-10) that protein profiling for isolates of *M. phaseolina* was not related to their virulence, host variety or location. In a greenhouse study, different soil types and bean were used for pathogenicity test of the fungus. Disease severity ranged from 21.3-44.0 %.

Key words**:** *Macrophomina phaseolina,* hosts, different media, types of soil, protein analysis.

***Correspondence: Muhanna, N.A.S.**
naglaa_muhanna@yahoo.com
Naglaa A.S. Muhanna
https://orcid.org/0000-0001-9275-8406
Plant Pathology Research Institute, Agricultural Research Center, 12619, Giza, Egypt.

INTRODUCTION

Macrophomina phaseolina (Tassi) Goid (the pycnidial stage of *Sclerotium bataticola* Taub) is a soil-borne fungus having a wide host range totaling about 500 cultivated and wild plant species worldwide (Khan, 2007). Important diseases caused by *M. phaseolina* include collar rot, damping off, charcoal rot, stem rot, root rot, and seedling blight in economically important crops (Babu *et al*., 2007). Microsclerotia are usually described as spherical, black, and oblong bodies. However, they exist a great variation in their shape and size depending on substrate, isolates, and temperature (Khan, 2007). Although only one species is recognized within the genus Macrophomina, great variation in morphology and virulence has been reported among isolates from different geographical regions (Das *et al*., 2008). Variation in pathogenicity, physiology, morphology, and genotypes of *M. phaseolina* have been reported widely (Edraki and Banihashemi, 2010). The microsclerotia of the pathogen can survive in soil for extremely long period, *i.e.*, 2-15 years (Vasebi *et al*., 2013).

Polyacrylamide gel electrophoresis of soluble proteins has also been widely used for elimination of taxonomic confusion and delineation of genera, species and sub species of various fungi in general (Hall, 1973). Great variation in morphology and pathogenicity were recognized among isolates recovered from different host species and between isolates from different parts of the same plant (Fernandez *et al*., 2006) as well as the molecular characteristics (Purkayastha *et al*., 2006).

Trichoderma sp. formulated as a fungicide may be the most studied fungal bio controlling and commercially marketed as bio-pesticides (Harman, 2000). Various *Trichoderma* strains with complementary antagonistic effects can be mixed as a complex in the treatment of soil-borne plant pathogens. Aleandri *et al*., (2015) suggested that a complex of *Trichoderma* spp. activates systemic resistance in the host plants.

The present study was carried out to screen up the potentialities of the different isolate's obtained from different hosts of *M. phaseolina*, for growth on different media, protein analysis of isolates, pathogenicity on different hosts and soils.

MATERIALS AND METHODS

Isolation, purification, and identification:

Samples were collected from the infected host plant species and varieties (cantaloupe, cucumber, pepper, bean, and watermelon) grown at different locations in Egypt during the season of planting in the years 2015-2017. The fungus was isolated from tissues of different plants. The samples were routinely cut in small pieces (5-10mm), surface sterilized with 1% sodium hypochlorite for 2 minutes, washed in sterilized water and were placed on PA medium, incubated at 28±2°C for 7 days, purified and identified according to Dhingra and Sinclair, (1978). The cultures were preserved at 4°C for further studies.

Preparation of media:

The six different media i.e. Corn agar (CA), Czapek's dox agar (CZA), Lima bean agar (LBA), Potato dextrose agar (PDA), Potato sucrose agar (PSA), Water agar (WA)were prepared for propagation of 14 isolates.

Radial growth on different media:

. Potato dextrose agar (PDA), Czapek's dox agar, Potato-sucrose agar (PSA), Corn agar (CA), Lima bean agar (LBA) and Water agar (WA) plates were seeded with agar disc (5 mm) taken from 7 days old culture of *M. phaseolina* in the center of each petri plate. Three replicate plates were incubated at (28±2ºC) and the observations for growth were recorded after 72 h incubation. Radial growth of isolates grown on six different media was tried.

The fungal suspension was prepared by transferring 4 mm mycelial disc of the fungus in 10 ml sterilized distilled water. The sclerotia were counted in fungal culture suspensions under the microscope at low power (10x). The sclerotial count was grouped as: - = absent; + = 1-4; ++ = 4-8; +++ = 8-15 and ++++ = more than 15.

Screening of pathogenic potential of *M. phaseolina* on different hosts:

M. phaseolina isolates were grown in water agar (WA) medium and incubated at (28±2^{0}C) for 7 days. When dishes were completely colonized by the fungus and were covered by the microsclerotia, seeds of cantaloupe Galia cv., bean Nebraska cv., tomato Castle Rock cv., cotton G-88 cv. and sunflower G-102 cv., Table (7) were surface disinfected by immersing in 2.5% NaOCl for 1 min, rinsed in sterilized water, and air-dried. Ten seeds were planted in each petri dish containing *M. phaseolina* and incubated at 28±2°C. Each treatment was represented by three replicates. Disease severity caused by each isolate for a given cultivar was assessed after 5days of emergence using the disease rating scale (Manici *et al*., 1995) as well as hypocotyl and root lengths of the seedlings were determined.

The pathogenicity of 14 isolates was studied on some plants in laboratory.

0 = no hypocotyl discoloration & no root rot
1 = discoloration light on hypocotyl and root
2 = superficial hypocotyl rot and moderate root pruning
3 = severe hypocotyl rot and moderate root pruning
4 = severe hypocotyl rot and severe root pruning
5 = infected seed and not germinated.

The disease rotting was calculated by multiplying the number of seeds by each degree of disease severity, divided by total number multiplied by the highest grade.

$$\textbf{Disease severity \%} = \Sigma(fv)/nx \times 100$$

F = number of seeds tested in each grade.
V= numerical rating of the scale (1-5), grade.
nX = total number of seeds tested multiplied by (5) *i.e.*, the highest grade

Characterization of *M. phaseolina* isolates by protein analysis:

All the recovered *M. phaseolina* isolates were grown on 50 mL of Czapek's dox broth incubated at 28±2ºC for 7 days. Mycelial mat was harvested by filtering through Whatman No.1 filter paper, washed with 0.1 M phosphate buffer (pH 7), vacuum dried, frozen at (-20ºC) and extracted for soluble protein as described by Howard and Brown (2001). Protein extracts were then electrophoretic run with standard protein marker on polyacrylamide gel (12.5%) using Laemmli method of the sodium dodecyl sulphate (SDS) discontinuous system (Laemmli, 1970).

Computing numerical data:

Analysis was carried out at the Biotechnology Lab Faculty of Agriculture, (CURP). Gel documentation (G:Box), Cairo University Research Park, (SYNGENF model 680 XHR).

The Lab Image 1D gel analysis was captured using a flexible software solution with strong image analysis, the molecular weight of each protein band was determined by molecular weight analysis software of this system. Cluster analysis was performed with a computerized program. Molecular weight markers used in SDS- PAGE were (175, 130, 95, 70, 62, 51, 42, 29, 22 and 14) KD.

Pathogenicity test using different soil types:

The highly pathogenic isolate (No. M15) was used for pathogenicity test under greenhouse conditions. Seeds of bean cultivar (Nebraska cv.) obtained from Veg. Res. Dept., Hort. Res. Inst., ARC., Giza, were used. Soil was homogenized autoclaved, and infestation was made by the fungal inoculum at the upper layer of different formulations of soil treatments (Table 1) at the rate of 3% (w/w) active inoculum, in pots (12.5 cm diam.) containing approximately 1 kg soil possible. Variation in soil content may be attributed to specific weight or density of contents used. Ten seeds were sown per pot, and three replicates were used for each treatment, the seeds were treated separately with a fungicide named Bio-control (T34 formulation) label 12% was used as 0.3g /100g seed, added as soil application (100ml /pots) application once time after sowing. Percentage of pre- and post-emergence damping-off 15, 30 days after sowing, and survivals after 45 days were recorded as mentioned by El-Helaly *et al*., (1970). The disease severity was determined as charcoal rot in common beans according to Pastor-Corrales and Abawi (1988). Using the rating scale of 1-5 grads as follows:

0 = absence of symptoms
1 = lesions limited to the cotyledon tissues
2 = lesions on roots, cotyledons at approximately 2.0 cm
3 = lesions above 2.0 cm in length in the region of the plant stem base
4 = stem with entire diameter colonized by the fungus and/or with the presence of pycnidia
5 = ungerminated seeds and collapse of seedlings.

Disease severity % = Σ(fv)/nx × 100

F = number of plants tested in each grade.
V = numerical rating of the scale (1-5), grade.
nX = total number of plants tasted multiplied by (5) *i.e.*, the highest grade

Preparation of inoculum:

The isolate No. M15 was grown on PDA medium for 7 days at 28ºC. Two discs (5 mm) of agar with mycelium were taken from 7-days old culture and were transferred onto the surface of autoclaved cornmeal sand medium (75 g grinded corn meal, 25 g fine washed sand and 50 ml tap water) in glass bottles (500 ml) and incubated at 28°C for 15 days.

Table (1): Treatments used in the present study.

No	Treatments	Weight of soil/pot (g)
1	Peat moss only (Control)	250 g
2	Peat moss infested with *M. phaseolina*	“
3	Peat moss infested with *M. phaseolina* + *(Bio control)	“
4	Sandy soil only (Control)	1.300 g
5	Sandy soil infested with *M. phaseolina*	“
6	Sandy soil infested with *M. phaseolina* + * (Bio control)	“
7	Clay soil only (Control)	800 g
8	Clay soil infested with *M. phaseolina*	“
9	Clay soil infested with *M. phaseolina* +* (Bio control)	“
10	Peat moss + Sandy soil (Control) 1:1	915 g
11	(Peat moss + Sandy soil) infested with *M. phaseolina*	“
12	(Peat moss + Sandy soil) infested with *M. phaseolina* +*(Bio control)	“
13	(Peat moss + Clay soil) (Control) 1:1	525 g
14	(Peat moss + Clay soil) infested with *M. phaseolina*	“
15	(Peat moss + Clay soil) infested with *M. phaseolina*) +*(Bio control)	“
16	(Sandy soil + Clay soil) (Control) 1:1	1050 g
17	(Sandy soil + Clay soil) infested with *M. phaseolina*	“
18	(Sandy soil + Clay soil) infested with *M. phaseolina* +*(Bio control)	“
19	(Sandy soil + Clay soil + Peat moss) (Control) 1:1:1	784 g
20	(Sandy soil + Clay soil + Peat moss) infested with *M. phaseolina*	“
21	(Sandy soil + Clay soil + Peat moss) infested with *M. phaseolina* + *(Bio-control)	“

* Fungicide named (Bio- control T 34)

Statistical analysis:

Data were compared by the analysis of variance according to the procedures of Snedecor and Cochran (1980). Means of treatments were compared by the least significant difference LSD at 5% level.

RESULTS

Isolation, purification, and identification:

During 2015 to 2017, root samples representing different host species of bean, cantaloupe, pepper, watermelon, tomato were collected from different locations Table (2). Isolation trials were carried out, the isolated fungi were purified and identified. Data presented in Table, (2) indicate that *Macrophomina phaseolina* (Tassi) Goid obtained from the surveyed localities was found elsewhere. Isolates Nos.M1 and M7 *M. phaseolina* were isolated from Ismailia, isolates Nos. M2, M9 and M13 were isolated from Noubariya, isolates Nos. M3 and M15 were isolated from Qaluobiya, isolates Nos. M4 and M11 were isolated from Damietta, isolate No. M12 was isolated from Giza, isolates Nos. M6, M8 and M10 were isolated from Monofia and isolate No. M14 was isolated from Fayoum.

Table (2): Isolate number, host and location of isolates of *M. phaseolina.*

isolates No.	Host	location
M1	Cantaloupe (*Cucumis melo* var. *cantalupensis*)	Ismailia
M2	Cantaloupe (*Cucumis melo* var. *cantalupensis*)	Noubariya
M3	Cantaloupe (*Cucumis melo* var. *cantalupensis*)	Qaluobiya
M4	Cantaloupe (*Cucumis melo* var. *cantalupensis*)	Damietta
M6	Cantaloupe (*Cucumis melo* var. *cantalupensis*)	Monofia
M7	Bean (*Phaseolus vulgaris*)	Ismailia
M8	Pepper (*Capsicum annuum*)	Monofia
M9	Bean (*Phaseolus vulgaris*)	Noubariya
M10	Bean (*Phaseolus vulgaris*)	Monofia
M11	Watermelon (*Citrullus lanatus*)	Damietta
M12	Watermelon (*Citrullus lanatus*)	Giza
M13	Pepper (*Capsicum annuum*)	Noubariya
M14	Tomato (*Solanum lycopersicum*)	Fayoum
M15	Bean (*Phaseolus vulgaris*)	Qaluobiya

Radial growth on different media

Data in Table (3) and Fig. (1) show clear differences among the 14 isolates of *M. phaseolina* due to their ability to grow on media with different compositions. Isolates Nos. M1, M2, M6, M7, M8, M10 and M11 and M13 grown on WA medium showed poor growth compared to isolates Nos. M4, M9, M12 and M15 that grew faster after 72 h incubation on different media. The most favorable medium for growth was LBA that supported average radial growth ranging between (5.7 to 9.0cm.), followed by growth on Czapek's dox agar that showed maximum radial growth ranging between (5.2 to 9.0cm.), corn agar (CA) which supported maximum radial growth ranging from (4.8 to 9.0 cm.), potato- dextrose agar (PDA) ranged from (3.0 to 9.0 cm.), potato-sucrose agar (PSA) that gave radial growth ranged from (3.0 to 9.0cm.) and water agar (WA) that ranged from (2.8 to 6.8cm.) respectively. Accordingly, data in Table (3) show the ascending sequence of growth as WA, PSA, CA, PDA, CZA and LBA, respectively.

Colony characteristics of *M. phaseolina*:

Data in Table (4) show different colony morphology characteristics of *M. phaseolina* in terms of color ranging between black dense, light black dense, light grey and dark brown. Several isolates were structurally variable that formed dirty white aerial hyphae, grey or dense grey color on different media and not on others.

Some isolates have had slight surface mycelium of the colony that was observed on PSA, CA, PDA, media, i.e. M2, M12, and M14, while for isolate No. M9 slight surface mycelium was observed on CA, PDA, CZA, LBA medium, and for isolates No. M4, M15 observed on PDA, LBA media. While Some isolates produced dirty white aerial hyphae *i.e.*, isolate Nos. M1, M10 on PSA media, isolates Nos. M6, M8 and M13 on CA media, isolates Nos. M7, M8, M13 on PDA media, isolates Nos. M10, M12 on CZA media, and on LBA media isolates Nos. M7, M8, M11. While all isolates were not able to produce aerial hyphae on WA. and isolate Nos. M3, M4 and M15 (colony black dense) was not able to produce aerial hyphae on PSA, CA and CZA media.

Table (3): Radial growth of *M. phaseolina* isolates grown on different media after 72h at 28±2ºC.

isolates No.	Host	Radial growth on medium (cm.)					
		CA	CZA	LBA	PDA	PSA	WA
M1	Cantaloupe	4.8	5.2	9.0	5.3	5.8	4.3
M2	Cantaloupe	5.1	5.8	5.7	3.0	3.0	2.8
M3	Cantaloupe	5.8	9.0	7.3	5.8	5.8	6.0
M4	Cantaloupe	9.0	9.0	9.0	9.0	9.0	5.8
M6	Cantaloupe	8.8	7.1	7.0	8.3	6.0	4.3
M7	Bean	6.3	7.0	9.0	5.8	4.5	3.7
M8	Pepper	5.8	7.1	9.0	4.6	3.3	3.0
M9	Bean	9.0	9.0	9.0	9.0	7.3	5.0
M10	Bean	4.8	9.0	9.0	9.0	8.8	4.4
M11	Watermelon	7.8	9.0	9.0	6.8	8.3	4.5
M12	Watermelon	8.8	9.0	9.0	9.0	8.3	6.0
M13	Pepper	6.3	9.0	9.0	4.3	3.3	2.8
M14	Tomato	9.0	8.8	8.8	6.3	6.7	6.8
M15	Bean	9.0	9.0	9.0	9.0	9.0	6.5
LSD at 5%		0.49	0.61	0.69	0.67	0.51	0.68

CA = Corn Agar; CZA = Czapek's Dox Agar; LBA = Lima bean Agar; PDA = Potato Dextrose Agar; PSA = Potato sucrose Agar; WA= Water Agar.

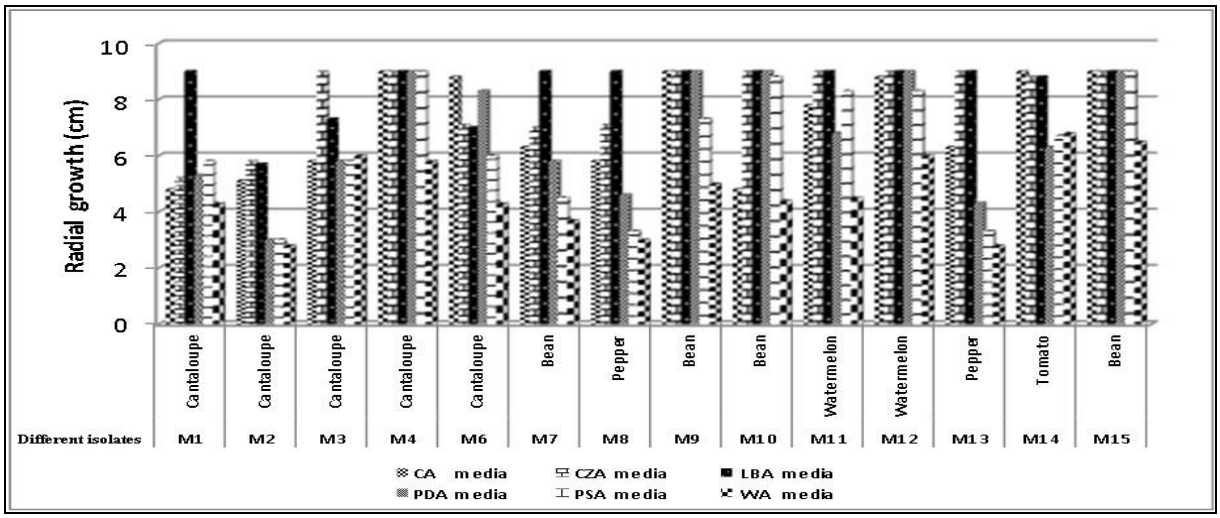

Fig (1): Radial growth of fourteen *M. Phaseolina* isolates on different media after 72h at 28±2°C.

Table (4): Colony characteristics of *M. phaseolina* isolates grown on different media after 15 days at 28±2ºC.

isolates No.	Host	Colony characteristics of *M. phaseolina*:					
		CA	CZA	LBA	PDA	PSA	WA
M1	Cantaloupe	B	B+	B	G+	B+++	LB
M2	Cantaloupe	DB+	LB+	DB+	DB+	DB+	LB
M3	Cantaloupe	B	B	B+	B+	B	LB
M4	Cantaloupe	B	B	B+	B+	B	LB
M6	Cantaloupe	B+++	B+	B++	B++	B+	LG
M7	Bean	B	B+	B+++	B+++	B	LB
M8	Pepper	B+++	B+	B+++	B+++	B+	LG
M9	Bean	DB+	DB+	DB+	DB+	DB++	LB
M10	Bean	B++	B+++	B++	B++	B+++	LB
M11	Watermelon	B++	B+	B+++	DB+	B++	LG
M12	Watermelon	B+	B+++	B++	DB+	B+	LG
M13	Pepper	B+++	B+	B	B+++	B+	LB
M14	Tomato	G+	LG	G+	G+	G+	LG
M15	Bean	B	B	B+	B+	B	LB

LB = Light Black dense; B = Black dense; LG = Light Grey; B + = Black with grey hyphae on the surface of colonies; B ++ = Black with dense grey aerial hyphae; B +++ = Black with dirty white aerial hyphae; G + = Gray with grey hyphae on the surface of colonies; G++ = Gray with dense grey aerial hyphae; DB+ = Dark brown with grey hyphae on the surface of colonies.

Effect of culture media on sclerotia formation of *M. phaseolina* isolates:

In general, Table (5) and Figs (2,3 and 4) show that all isolates produced sclerotia on LBA, PDA, Czapek's dox agar medium, CA, and PSA respectively. But on WA medium isolates Nos. M2, M8 and M13 failed to produce sclerotia. isolate No. M15 produced the highest number of sclerotia followed by isolates Nos. M4, M14, M13, M7 and M11 while, isolates M8 and M1 gave the lowest number of sclerotia.

Table (5): Sclerotial formation of 14 *M. phaseolina* isolates grown on different media after 15 days of incubation at 28±2ºC.

isolates No.	Host	The Formed sclerotia on (medium)/1 mm					
		CA	CZA	LBA	PDA	PSA	WA
M1	Cantaloupe	6.2	2.0	7.0	6.0	3.4	3.0
M2	Cantaloupe	10.2	5.2	11.4	9.0	10.2	0.0
M3	Cantaloupe	7.0	2.6	16.4	15.6	6.0	3.8
M4	Cantaloupe	11.8	11.0	15.2	12.6	8.0	2.4
M6	Cantaloupe	9.6	3.0	14.2	13.0	7.2	3.2
M7	Bean	8.8	5.6	16.2	15.8	6.0	3.8
M8	Pepper	2.6	6.4	6.0	6.2	2.2	0.0
M9	Bean	7.2	6.0	10.4	6.8	6.2	5.0
M10	Bean	8.2	7.0	15.6	10.2	5.2	3.2
M11	Watermelon	10.4	6.6	14.6	10.0	6.2	5.4
M12	Watermelon	6.8	5.8	13.8	11.4	5.6	2.8
M13	Pepper	11.2	6.8	16.0	15.4	6.6	0.0
M14	Tomato	11.4	6.6	15.4	13.4	9.6	3.2
M15	Bean	12.4	11.6	18.0	17.2	14.4	6.6
LSD at 5%		1.76	1.66	1.95	2.99	1.28	0.86

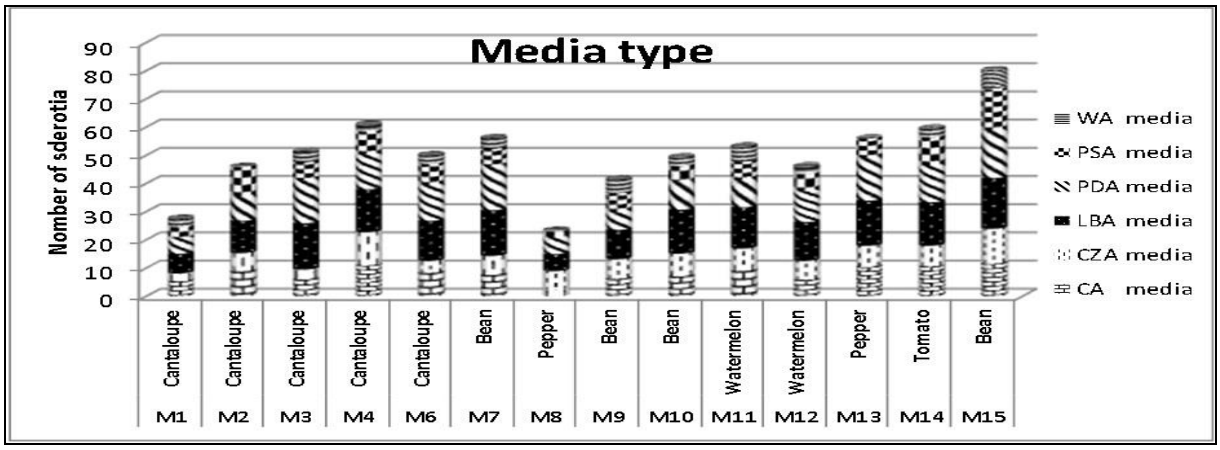

Fig (2): Relationship between the types of media and the number of formed sclerotia

It seems possible that they are exactly a correlation between media composition and sclerotia formation. Statically the correlation was found positive between different media and sclerotia formation as shown in Fig (3).

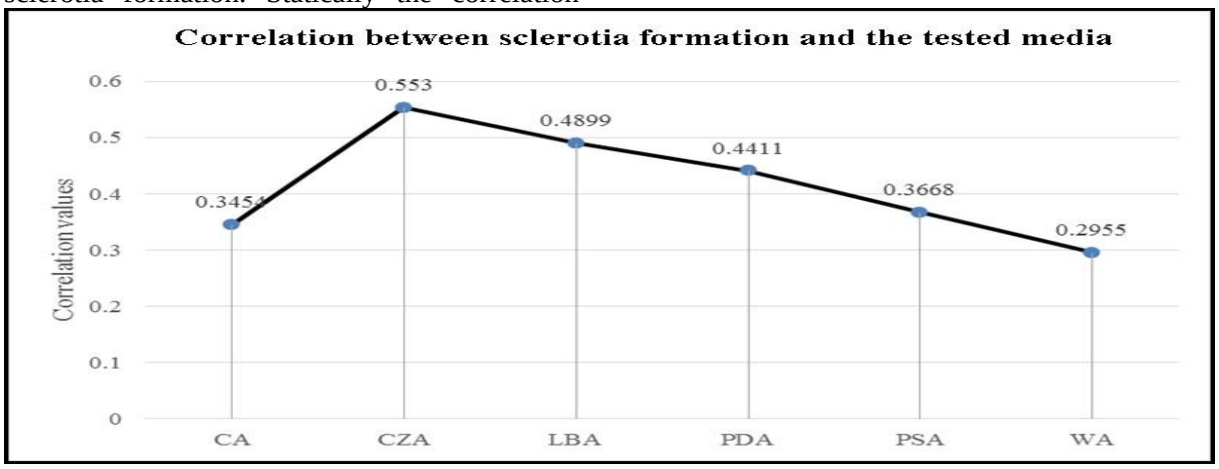

Fig (3): Correlation between the different media and sclerotia formation by various isolates.

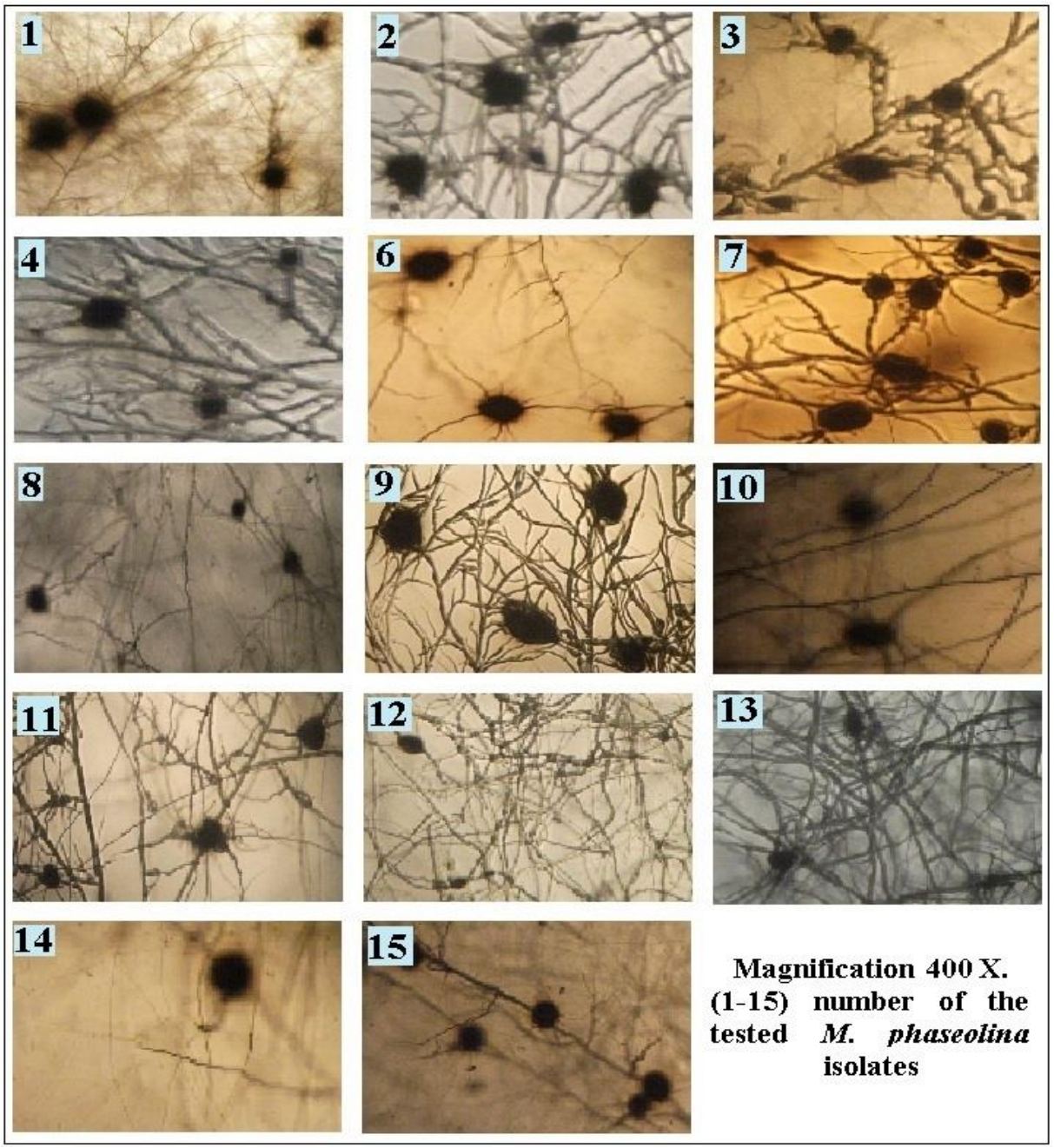

Fig (4): Sclerotia formation by 14 *M. phaseolina* isolates on PDA medium 15 days after incubation at 28±2ºC.

Differential response of selected hosts against various isolates of *M. phaseolina in vitro:*

Significant response (Table 6) was observed between 14 *M. phaseolina* isolates and five different hosts, which resulted in different disease severity levels. Isolates M11 and M15 were highly pathogenic on bean, cotton, cantaloupe, tomato and sunflower, respectively. While the low ones were isolates M2, M3, M8, M13 and the moderate isolates were M1, M4, M9 on different hosts.

Bean:

Bean seedlings responded positively to seven isolates, M6, M7, M10, M11, M12, M14 and M15 and were considered virulent as they showed disease severity 30.0%. While three isolates, M2, M8 and M13 were weak pathogens where they recorded 0.6% on root and hypocotyl of bean, and the remaining isolates, M1, M3, M4 and M9 showed disease percentages ranged between (11.1 to 18.9 %) on root, (0.6 to 18.9%) on hypocotyl and are considered to be intermediate in this regard.

Cantaloupe:

Seedlings responded positively to infection by five isolates, M9, M11, M12, M14 and M15 with an average disease percentage ranging between 14.2 to 21.1 % on hypocotyl and 15.0

to 21.1% on roots, in addition seven isolates, M1, M3, M4, M7, M8, M10 and M13 were weak pathogens that recorded 0.6% on hypocotyl but disease severity on roots ranged from 11.1 to18.9%. On the other hands M2 recorded (0.6%) on both hypocotyl and root.

Tomato:

Data (Table 6) show that tomato responded to infection by six isolates, M4, M6, M11, M12, M14 and M15 that were scored highly virulent where disease severity ranged between 15.0 to 18.9% on hypocotyl and 10.6 to 18.9% on roots, and five isolates, M1, M2, M3, M8 and M13 which are considered as weak pathogens (0.6%) on hypocotyl, but disease severity on root ranged from (8.9 to 13.1%).

Cotton:

Cotton seedlings responded to infection by almost all isolates which are considered highly pathogenic as expressed by disease severity values which ranged between 13.1 to 21.1% on hypocotyl and 8.9 to 21.1% on roots.

Sunflower:

Seedlings responded to infection by four isolates, M4, M9, M11 and M15 that were considered extremely virulent where disease severity ranged between 15.0 to 18.9% on the hypocotyl and 14.2 to 18.9% on roots, and nine isolates, M1, M2, M3, M6, M7, M8, M10, M12 and M13 were weak pathogens and recorded 0.6% on hypocotyl, but disease severity percentage on root ranged from 11.1 to 15.8%

Table (6): Screening of pathogenic potential(s) of *M. phaseolina* on different host plants.

isolates No.	Plant organ	% Disease Severity				
		Bean	Cantaloupe	Tomato	Cotton	Sunflower
M1	Hypocotyl	15.0	0.6	0.6	15.0	0.6
	Root	15.0	11.1	8.9	16.5	15.8
M2	Hypocotyl	0.6	0.6	0.6	16.9	0.6
	Root	0.6	0.6	8.9	11.1	15.0
M3	Hypocotyl	0.6	0.6	0.6	16.9	0.6
	Root	11.1	11.1	8.9	11.1	11.1
M4	Hypocotyl	0.6	0.6	18.9	21.1	15.0
	Root	18.9	18.9	18.9	21.1	18.9
M6	Hypocotyl	30.0	15.0	15.0	18.9	0.6
	Root	30.0	0.6	11.1	15.0	11.1
M7	Hypocotyl	30.0	0.6	11.1	15.0	0.6
	Root	30.0	11.1	0.6	15.0	11.1
M8	Hypocotyl	0.6	0.6	0.6	13.1	0.6
	Root	0.6	11.1	13.1	8.9	15.0
M9	Hypocotyl	18.9	19.2	15.0	19.7	16.5
	Root	18.9	18.9	8.9	18.9	15.0
M10	Hypocotyl	30.0	0.6	14.6	16.9	0.6
	Root	30.0	18.9	0.6	13.1	14.6
M11	Hypocotyl	30.0	18.9	18.5	21.1	16.9
	Root	30.0	18.9	10.6	21.1	14.2
M12	Hypocotyl	30.0	14.2	18.9	21.1	0.6
	Root	30.0	18.9	11.1	21.1	16.5
M13	Hypocotyl	0.6	0.6	0.6	16.9	0.6
	Root	0.6	11.1	11.1	16.9	15.0
M14	Hypocotyl	30.0	15.0	16.9	18.9	11.1
	Root	30.0	15.0	16.9	13.1	0.6
M15	Hypocotyl	30.0	21.1	18.9	21.1	18.9
	Root	30.0	21.1	18.9	21.1	18.9
Cont.	Hypocotyl	0.6	0.6	0.6	0.6	0.6
	Root	0.6	0.6	0.6	0.6	0.6
LSD at 5%						
Isolates (I)		1.49	0.79	0.96	1.34	0.84
Plant organs		0.53	0.28	0.34	0.47	0.29
I × P		2.11	1.12	1.36	1.89	1.19

Hosts response expressed as hypocotyl and root length due to infection by some *M. phaseolina* isolates:

Response of five hosts (Table 7) expressed as degree of elongation of hypocotyl and root length in the presence of 14 isolates representing *M. phaseolina* isolates.

Bean:

After infection by any of the tested isolated isolates, M1, M3 and M13 hypocotyl length ranged between 1.3 to 1.5 cm and the root length that ranged between 0.5 to 1.5 cm on the average. In this regard, isolates, *i.e.*, M2, M8 and M9 were considered to be of moderate effect, hypocotyl length ranged between 1.5 to 3.5 cm and the root length recorded 2.5 to4.5cm on the average. Meanwhile, bean seeds failed to germinate in the presence of any of the tested isolates, i.e. M6, M7, M10, M11, M12, M14 and M15**.**

Cantaloupe:

The effect of M2, M4, M11 on hypocotyl and root length recorded 0.0 cm and 0.5cm on the average, respectively, followed by isolates M8, M9, M13, M15 on hypocotyl elongation that ranged from 1.0 to 2.5cm and on the root length that ranged between 1.5 to 2.0 cm. Isolates M3, M6, M10 were less effective on hypocotyl elongation and recorded 2.5 to 9.6 cm and root length recorded 6.5 to 9.0 cm. Isolates M1, M7, M12, M14 showed moderate elongation of hypocotyl, being 1.5 to 4.0 cm while root length recorded 2.0 to 4.0 cm compared to control.

Tomato:

Five isolates, M4, M8, M12, M13, M14 induced elongation of tomato hypocotyl that ranged from 1.5 to 3.2 cm and root that ranged from 0.5 to 2.5 cm, followed by isolates M1, M2, M3, M9, M11, M15 which induced elongation of the hypocotyl that recorded 4.0 to 9.8 cm. and (2.8 to 6.5 cm.) for the root, and isolates M6, M7, M10 showed moderate effect on the hypocotyl (7.0 to 7.8 cm.) and the root (5.5 to 7.4 cm.) compared to control.

Cotton:

Four isolates, M4, M12, M13, M15 were more effective on the elongation of hypocotyl that ranged from 0.5 to 1.0 cm. while root length ranged from 1.0 to 1.5cm, followed by M7, M8, M9, M11, M14 where hypocotyl elongation recorded 3.0 to 6.0 cm. and root length recorded 3.5 to 8.0 cm. Isolates M1, M2, M3, M6, M10 showed moderate effect on the hypocotyl and recorded 3.5 to 10.0 cm and for the root (8.0 to 10.5cm) compared to control.

Sunflower:

Four isolates M4, M8, M9, M13 were more effective on the elongation of hypocotyl which ranged from 2.0 to 3.8 cm and on root that ranged from 1.5 to 2.0 cm., followed isolates M12, M14, M15 where the of elongation of hypocotyl recorded 3.8 to 5.5 cm and 3.0 to 4.5 cm on root. Isolates M1, M2, M3, M6, M7, M10, M11showed moderate effect on hypocotyl and recorded 5.5 to 8.5cm and 2.2 to 11.5 cm. on root.

Differentiation of *M. phaseolina* isolates by protein banding obtained by SDS-PAGE:

Protein analyses of *M. phaseolina* isolates showed different numbers of separated bands (Fig 5, and Table 8). Molecular masses of polypeptides were shown to range in size from 22 to167 KD on SDS- PAGE. Moreover, the polypeptides accumulation and pattern were changed for isolates (1-10 bands). Protein analysis (Table 8) shows the detected common proteins with molecular weights and protein bands (47, 32, 30, 29, 24 K.D). showed a greater number of protein bands (10) for isolate no. M1 compared to isolate M2 and showed unique bands with molecular weight of 154 K.D.

It was concluded that isolates which were separated into a greater number of bands 1-10 could be arranged in five groups. The first included 7 isolates M1, M3, M4, M6, M7, M9, and M10 with band (24 K D), the second embraces 5 isolates (M11, M12, M13, M14 and M15 with band (30 KD), the third included 4 isolates M11, M12, M13 and M14 with band 32 KD, the fourth included 4 isolates M11, M13, M14, and M15 with band 47 KD and 4 isolates M8, M9, M13 and M14 with band 29 KD, and the fifth included 3 isolates M1, M11, M15 with band 31KD.

Pathogenicity test of *M. phaseolina* on different soil types under greenhouse:

Results in Table (9) clearly indicate that sowing bean seeds in any soil infested by *M. phaseolina* caused pre- and post- emergence damping off. Data in Table (9) show that soil infestation with *M. phaseolina* isolate M15 gave the highest percentages of pre-emergence damping off in bean sown in peat moss + clay soil (73.3%), followed by sandy soil + clay soil treatment (66.7%), clay soil treatment (60.0%), peat moss treatment (53.3%) while sand soil with either peat moss + sand soil + clay soil treatment gave 40.0% compared to control treatment. Meanwhile, the highest percentages of pre-emergence damping off were obtained from soil infested by *M. phaseolina* and treated

with fungicide (Bio-control) ranged from 13.3 to 40.0%.

The post emergence percentages ranged from 13.3 to 33.3%, with *M. phaseolina* (M15 isolate) alone on different soil types. On the other hand, with fungicide (Bio-control T34), the percentage of post-emergence damping off ranged between 6.7 to 13.3%. The percentage of survived seedlings ranged from 6.7 to 60.0% in the presence of *M. phaseolina* alone in different soil type treatments. The recorded percentage of survived seedlings with bio-control ranged from 46.7 to 86.7% compared to control. Pathogenicity by virulent isolate of *M. phaseolina* no. M15 was successfully studied by evaluating the seven types of soil treatments. Results clearly indicate that all types of soil treatments caused high disease severity with *M. phaseolina* treatment alone ranged from 21.3 to 44.0 % and decreased the disease due to fungicide (Bio-control) application which recorded 12.0 to 26.7 %.

Table (7): Host response expressed as hypocotyl and root length in the presence of 14 isolates of *M. phaseolina,* each alone.

isolates No.	Plant organ	Average of length hypocotyl and root (cm.)				
		Bean	Cantaloupe	Tomato	Cotton	Sunflower
M1	Hypocotyl	1.5	1.5	6.5	9.5	8.0
	Root	1.5	3.0	4.7	8.5	4.5
M2	Hypocotyl	1.5	0.0	4.5	3.5	7.5
	Root	4.5	0.5	3.6	8.0	3.9
M3	Hypocotyl	1.3	9.5	9.8	7.5	8.5
	Root	0.5	6.5	2.8	10.5	3.5
M4	Hypocotyl	0.0	0.0	3.2	1.0	2.8
	Root	0.5	0.5	1.7	1.5	1.5
M6	Hypocotyl	0.0	2.5	7.0	10.0	7.0
	Root	0.0	7.5	7.4	9.5	2.2
M7	Hypocotyl	0.0	4.0	7.8	5.0	6.0
	Root	0.0	2.0	6.0	6.0	2.5
M8	Hypocotyl	1.5	2.5	2.8	6.0	2.0
	Root	4.5	1.5	1.3	6.5	1.5
M9	Hypocotyl	3.5	1.0	5.0	3.0	3.0
	Root	2.5	2.0	6.5	4.5	2.0
M10	Hypocotyl	0.0	3.0	7.5	9.5	7.8
	Root	0.0	9.0	5.5	8.5	5.5
M11	Hypocotyl	0.0	0.0	5.5	6.0	5.5
	Root	0.0	0.5	3.3	8.0	11.5
M12	Hypocotyl	0.0	1.5	1.5	1.0	3.8
	Root	0.0	3.0	2.5	1.5	3.0
M13	Hypocotyl	1.5	1.0	3.5	1.0	3.8
	Root	1.0	2.0	0.5	1.0	1.5
M14	Hypocotyl	0.0	3.0	1.5	4.5	5.0
	Root	0.0	4.0	1.5	3.5	4.5
M15	Hypocotyl	0.0	1.0	4.5	0.5	5.5
	Root	0.0	1.5	3.0	1.0	3.5
control	Hypocotyl	8.0	12.0	12.0	15.0	14.0
	Root	7.0	10.0	9.0	14.0	13.0
LSD at 5%						
Isolates (I)		0.33	0.63	6.34	0.68	0.69
Plant organs (P)		0.12	0.22	2.24	0.24	0.25
I × P		0.46	0.89	8.96	0.96	0.98

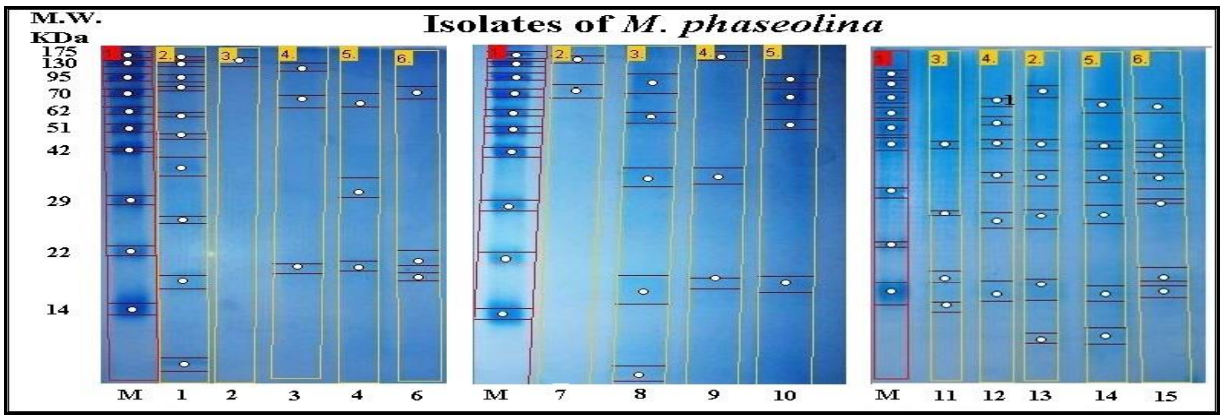

Fig (5): Protein profiles of 14 *M. phaseolina* isolates as separated by polyacrylamide gel electrophoresis of SDS-dissociated proteins.

M = Marker; M.W = molecular weight; Isolates of *M. phaseolina* no. (M1, M2, M3, M4, M6, M7, M8, M9, M10, M11, M12, M13, M14, M15).

Table (8): Electrophoretic analysis of soluble proteins of 14 isolates *M. phaseolina.*

Bands No.	MW(KDa)	Raw volume													
		Number of *M. phaseolina* isolates													
		1	2	3	4	6	7	8	9	10	11	12	13	14	15
1	175														
2	167	391													
3	159								1851						
4	158						2318								
5	154		2113												
6	140	974													
7	130														
8	126			1781											
9	114												4236		
10	106	1691													
11	98									1365					
12	95														
13	94							4632							
14	93											2536			
15	87	1134												4522	
16	84														4680
17	83					2853									
18	82						4271								
19	71									2474					
20	70														
21	68			3527											
22	64				3626										
23	62											2658			
24	54	1334													
25	53							2978							
26	51														
27	48											2893			
28	47										2643		3510	2571	2331
29	46									2025					
30	42	1580													2905
31	37											2578			
32	36												4122	3582	4325
33	33														1475
34	32										1574	3936	5339	5472	
35	31	5185									2799				4453
36	30											6304	6198	4514	3721
37	29							5540	4226				2702	4631	
38	27				5926										
39	25	2349													
40	24	4394		3123	2899	4984		10520	3553	4366					
41	23										2389				
42	22					2626									
43	14	3547						5302							
Number of bands		10	1	3	3	3	2	5	3	4	4	6	6	6	7

Table (9): Effect of different soil types and treatments on the incidence of damping-off of bean seedlings caused by *M. phaseolina* (M15 isolate) under greenhouse conditions.

Type of soil	Treatments	Bean			
		% Damping-off			
		Pre-	Post-	% Plant survival	Disease Severity %
Peat moss	*M. phaseolina*	53.3	13.3	33.4	44.0
	M. phaseolina + Bio-control	13.3	0.0	86.7	18.7
	Control	6.7	0.0	93.3	0.0
Sandy soil	*M. phaseolina*	40.0	20.0	40.0	34.7
	M. phaseolina + Bio-control	20.0	6.7	73.3	26.7
	Control	0.0	0.0	100.0	0.0
Clay soil	*M. phaseolina*	60.0	26.7	13.3	36.0
	M. phaseolina + Bio- control	40.0	13.3	46.7	18.7
	Control	33.3	0.0	66.7	0.0
Peat moss + sand soil	*M. phaseolina*	46.7	33.3	20.0	38.7
	M. phaseolina + Bio- control	33.3	13.3	53.4	16.0
	Control	6.7	0.0	93.3	0.0
Peat moss + clay soil	*M. phaseolina*	73.3	20.0	6.7	21.3
	M. phaseolina +Bio-control	40.0	0.0	60.0	16.0
	Control	26.7	0.0	73.3	0.0
Sandy soil + clay soil	*M. phaseolina*	66.7	20.0	13.3	36.0
	M. phaseolina + Bio-control	26.7	0.0	73.3	18.7
	Control	13.3	0.0	86.7	0.0
Peat moss + sand soil + clay soil	*M. phaseolina*	40.0	0.0	60.0	41.3
	M. phaseolina + Bio-control	33.3	0.0	66.7	12.0
	control	20.0	0.0	80.0.	0.0
LSD at 5%					
Types of soil		1.55	0.25	0.93	0.65
Treatments		1.02	0.16	0.61	0.42
Soil types x Treatments		2.69	0.43	1.61	1.12

DISCUSSION:

This study was carried out to investigate the morphological variations and pathological differences as related to protein differences of *M. phaseolina* isolates recovered from different host plants and different locations in Egypt. The study showed variations in the morphological characteristics, including intensity of aerial mycelia, dense of sclerotia formation spreading progress of colony and influence of different solid media on their pathogenicity.

Babu *et al*. (2010) reported such differences among the isolates from hosts as sorghum, soybean, chickpea, and corn. *M. phaseolina* is one of the most pathogens that infect more than 500 plant species throughout the world (Khan, 2007).

The obtained data revealed that all the tested isolates of *M. phaseolina* (14), have different growth rates on solid media based on the results of the molecular identification of the 14 isolates collected from five different hosts grown at different governorates.

In the present study, the maximum mycelial growth for the 14 *M. phaseolina* isolates was obtained on Lima bean Agar (LBA), Czapek's medium (CZA), Potato dextrose agar (PDA), followed by Corn agar (CA), Potato-sucrose agar (PSA), and Water agar (WA) medium that supported good growth of *M phaseolina.* Colony characteristics of *M. phaseolina* and color among black dense, light black dense, light grey and dark brown on different media. Some isolates have aerial hyphae dense grey hyphae or dirty white hyphae. While all isolates were unable to produce aerial hyphae on WA medium. On the other hand, isolates No. M3, M4 and M15 were unable to produce aerial hyphae on PSA, CA, CZA media. Excellent sclerotial formation was observed on potato dextrose agar and Lima bean agar. Moreover, exist a positive statistical correlation between the media used and number of sclerotia formed.

The variations in morphology might be due to differences in temperature, moisture and soil types. Riaz *et al*. (2007) reported that morphological variability has also included

growth, color, pycnidium production, and chlorate sensitivity among different isolates of *M. phaseolina* on different hosts. Jha and Dubey (2000) obtained the highest growth and sclerotial formation of *M. phaseolina* on potato dextrose agar followed by Richard's medium, also reported flat mycelial growth with white to dirty white mycelium color in all media except Water agar (WA) media. Sundravadana *et al*., (2011) reported that the color of microsclerotia produced by the isolates was usually black, the variability exists in microsclerotia formation of *M. phaseolina*, confirming that the size and number of microsclerotia depend on the nutrients available in the substrate. Differences were also found in the production of microsclerotia. It was observed that the fungus formed the largest number of microsclerotia / microscopic field on different media. According to the production of microsclerotia the highest number was found due to sucrose component of Czapek-Dox agar (CDA), and moderate in glucose (Das, 1988).

The hosts *i.e.,* bean, cantaloupe, tomato, cotton and sunflower expressed a range of resistance or susceptibility responses to infection with the 14 isolates of *M. phaseolina*. Pathogenicity of fourteen isolates of *M. phaseolina* was successfully studied by evaluating the hypocotyls and root discoloration of the hosts. Two isolates, *i.e.,* M11, M15, were highly virulent or pathogenic on the five tested host plants.

The hypocotyl and root damage caused by isolates no. M1, M3, M9 was greater due to disease severity than the control which suggests the reason for the greater disease in bean. Five isolates *i.e.,* M9, M11, M12, M14, M15 caused damage in hypocotyl and root of cantaloupe compared to control, in addition six isolates *i.e.,* M4, M6, M11, M12, M14, M15 were able to infect tomato, while all isolates caused damage of hypocotyl and root on cotton, and four isolates *i.e.,* M4, M9, M11, and M15 caused great damage to hypocotyl and root of sunflower.

Moreover, in this study infection with *M. phaseolina* resulted in significant decrease in the growth readings such as hypocotyl and root lengths. The inhibition of root growth especially on bean with isolates M6, M7, M10, M11, M12, M14, M15, the failure of seed germination, and reduction of root and hypocotyl growth with isolates *i.e.,* M1, T2, T3, T8, T9, T13. The decrease of root and hypocotyl growth of cantaloupe was caused by ten isolates *i.e.,* M1, M6, M7, M8, M9, M10, M12, M13, M14, M15. The decrease of root and hypocotyl growth of tomato with isolates M4, M8, M12, M13, M14 was recognized. Decrease of growth, and the decrease of root and hypocotyl on cotton with all isolates were documented. The reduction of root and hypocotyl growth on sunflower with all isolates compared to control was clearly recorded.

The symptoms of the charcoal rot disease include seedling blight, root and stem rots, damping-off, wilting, death, and failure of seed germination (Papavizas, 1977). Su *et al*. (2001) found host specialization in maize on the basis of pathogenic, genetic, and physiological differences in *M. phaseolina*. Hypocotyls and root injuries or damage are a good indicator for pathogenicity of *M. phaseolina*. Because *M. phaseolina* affects the host plant at all stages of development the pathogen causes major damage to susceptible crops throughout the growing seasons (Baird *et al*., 2003). Charcoal rot severity is directly related to viable sclerotia produced in soil (Salik, 2007). It was reported that isolates producing fewer sclerotia were less pathogenic on bean (Purkayastha *et al.,* 2004).

Jones *et al*. (1996) found that host-pathogen parasitic compatibility in plant pathogenic fungi is related to their antigenic similarity. Host-pathogen interaction determines the ability of host to bind a parasitic and ability of parasite to injure the host. Whereas resistance and susceptibility are heritable qualities (Yang *et al*., 1999).

Protein analysis of *M. phaseolina* isolates assayed different numbers of separated bands. The obtained results showed that protein profiling for isolates of *M. phaseolina* is not related to their virulence, respective host, or location.

The genetic variations and site-specific nature of resistance against *M. phaseolina* have not been clear (Michel, 2000). Pathogenesis along with genetic diversity plays a specific role in host-plant resistance. Isolates having morphological similarity are not necessarily identical genetically, they might have some differences. The variable genetic pattern contributes to variation in morphology and pathogenesis, which has been confirmed by using different molecular tools (Reyes-Franco *et al*., 2006). These variations may be explained by the geographic origin (Sexton *et al*., 2016), as soil temperatures may have led to the selection of individuals which were adapted to local conditions.

Based on the present study, the results suggest that isolate No.15 *M. phaseolina* showed showed faster radial growth on LBA, Czapek's dox agar and PDA medium, respectively, morphological characteristics colony black dense non able to produce aerial hyphae, dense of sclerotia formation with different solid media, more pathogenic on five hosts, the effect of different types of soil treatment was found to cause high severity with infection (*M. phaseolina*) alone, while the maximum disease severity decrease was observed in pots treated with the fungicide (bio-control T 34).

Dubey *et al*. (2007) reported that biocontrol with potential of *Trichoderma* sp. have been successfully agent for seed germination and seedling vigour in addition to manage and suppression of soil-borne plant pathogenic fungi causing diseases such as, Phytophthora, Pythium, Sclerotinia, Botrytis, Rhizoctonia and Fusarium of crops i.e. lettuce, tomato, onion, cotton,grapes, peas, apples, sweet corn, carrots. Vinale *et al*., (2008) found the suppresses the activity of pathogenic microorganisms by enzymatic activities and protect plants through different modes of action, *Trichoderma* spp. may release siderophore compounds for induce cell elongation in plants.

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