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Studies on the pathogenic soil-borne fungus (*Macrophomina phaseolina*)

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Macrophomina phaseolina a soil inhabiting fungus that infects many plants causing some diseases. It was isolated from the roots of infected plants cultivated in Wadi Fatma and Al-Shafa areas, Saudi Arabia.

The pathogenicity experiments of the fungus, on the test plant Squash (*Cucurbita pepo* CV Scarla) under glass house and wire green house conditions, revealed its severe pathogenicity. The pathogen has affected 23 different plant species and varieties, as its host range was tested.

The physiological studies of *M.phaseolina* indicated that the media of potato dextrose, malt and Czapek's were the best for fungal growth. Incubation temperature of 30°C, pH ranges between 5.1 and 5.9, as well as a relative humidity of 100% were responsible for maximal growth of the experimental mould. It was also able to assimilate different carbon sources of which glucose and sucrose were the best tested.

The fungus exhibited high enzymatic activity of pectin methyl estrase, polygalacturonase, cellulase and proteases. Antagonistic studies indicated that, of the microorganisms isolated from rhizophore of Squash, *Bacillus subtilis* was the most active species against *M.phaseolina* growth.

Introduction

Macrophomina phaseolina considered as one of the soil borne fungi that infect large numbers of host plants through roots, stems, and crown region, near the soil surface. It causes several plant diseases as, charcoal rot, leaf blight, damping off,...etc. (Agrawal and Gupta, 1989; Baudry and Morzieres, 1993). So, the fungus caused great economic losses. It was isolated in many countries and different types of soils, including some Arabian countries (Abdel- Hafez, 1982; Halwagy et al, 1982; Moubasher et al, 1990).

Some physiological factors that affect the growth and pathogenicity of *M. phaseolina* were indicated (Abdou et al, 1980; Das, 1988; Singh and Kaiser, 1994). Some microorganisms can inhibit or retard the growth and

hence the virulence of *M. phseolina* (Elad et al, 1986; Pineda and Gonnelas, 1988; Etheshamul and Ghaffar, 1991). So, the present work aims to isolate *M. phaseolina* from cultural area at Saudi Arabia and to characterize the isolated fungus as its pathogenicity, range of host plants, and some physiological factors that affect its growth, as well as its biological control by some soil inhabiting microorganisms.

Materials and Methods

Maintenance of *Macrophomina phaseolina*

The fungus was maintained on potato dextrose agar medium at 5 ^{0}C, with monthly transfers onto new medium.

Media used (g/l):

*Potato dextrose agar:

Potato, 100; dextrose, 20; agar, 20; and pH=5.5.

*Malt agar:

Malt extract, 30; NaCl, 10; agar, 20 and pH=5.5.

*Czapek's medium:

Sucrose, 30; $NaNO_3$, 2; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4$, 0.1; agar, 20 and pH=5.5.

*Richard's medium:

Sucrose, 50; KNO_3, 10; KH_2O_4, 5; $MgSO_4.7H_2O$, 2.5; traces of ferric chloride, agar, 20 and pH=5.5.

*Waksman's medium:

Glucose, 10; peptone, 5; KH_2O_4, 1; $MgSO_4.7H_2O$, 0.5; agar, 20 and pH=5.5.

*Peptone yeast extract:

Peptone, 5; yeast extract, 3; agar, 20 and pH=5.5.

Linear fungal growth:

The potato dextrose agar medium, 10ml/plate, was inoculated at the center by *M. phaseolina* inoculum, obtained by a sterile cork porer (5mm diameter) from homogenous 4days old cultures grown at 30 $^\circ$C, after the requested period of growth and under the tested conditions, the radial growth of the colony was determined, five replica were used and the obtained results were the arithmetic mean.

Preparation, inoculation and cultivation the soil:

The homogenous agricultural soil of Wadi Fatma, Saudi Arabia was dispensed in pots (20cm diameter) in mixture 1:1 to peat moss. The soil was infected by 4days old cultures of *M. phaseolina* (Rodrigues-Viviane et al, 1997). Thereafter, the seeds (4-5 seeds/pot) of the tested plant, Squash CV. Scarla (*Cucurbita pepo*) were cultivated 7days after fungal inoculation. The pots receive suitable equal amounts of water every 3days and the growing plants sprayed by a suitable insecticide to prevent insects attack. The Squash

plants either grown under green- house conditions (temperature 25-27°C, light intensity 1500 candle/feet^2, and relative humidity 70-80%) or wire green house, natural conditions of Jeddah, Saudi Arabia, (atmospheric temperature 35-37°C, and relative humidity 70-80%) were let to grow for 3 weeks, thereafter, the necessary analyses were carried out.

Estimation of enzymatic activity:

The enzymatic activity of pectinolytic enzymes (pectin methyl estrase – PME and polygalacturonase -PG) were estimated by growing *M. phaseolina* on Dhingra and Sinclair (1985) medium for 14 days at 30°C under stagnant conditions. The filtrate was separated by centrifugation (4000 rpm for 20 min) to obtain the crude enzyme. The PME activity was determined by titration method (Kartesz, 1951; Matta and Diamond, 1963), and PG activity was also estimated according to Talboys and Busch (1970). Cellulase activity of *M. phaseolina* was estimated (Talboys and Busch, 1970). While, protease activity was assayed colourimetrically using casein as a substrate.

Antagonistic studies:

The antagonism between the tested microorganisms (fungi and bacteria) and *M. phaseolina*, grown on potato dextrose agar medium (in vitro) was carried out as described by Royse and Rise (1978); Chand and Logan (1984). While, the antagonistic relations between them in vivo was done as described by Sellam et al (1978).

Results and Discussion

Isolation and identification of *Macrophomina phaseolina*:

The fungus was isolated from plants suffering from the symptoms of damping off, decay and root rot, and brown or black colour of the crown region. The fungus was isolated, according to the method described by Louw and Webly (1959), on potato dextrose agar medium (PDA). It was purified by hyphal tip and single spore isolation (Brown, 1924). It was identified (Barnett, 1972; Alexopoulus and Mimis, 1979; Webster, 1980) as *Macrophomina phaseolina*, and verified by the International Mycological Institute Ferrylane; Kew, Surry, England.

Pathogenicity test:

It was carried out by growing the test plant, Squash CV scarla (*Cucurbita pepo*), in sterilized and non-sterilized soil under glass-house conditions and in wire green house (natural conditions). Soil samples (250g/pot) was infested by the fungal inoculum. The results (Table 1) revealed that *M. phaseolina* severely infected the test plant when applied to the soil a week before planting the seeds in both temperatures and also in sterilized and non-sterilized soils. The highest pathogenicity percentage was recorded in the non-sterilized soil under green house conditions (67%). The

pathogenicity was decreased when the fungus was applied to the soil at the same time of Squash planting and reached its maximum decrease as the fungus was added to the soil 20 days after planting, in all treatments. The glass house treatments were concomitant with higher pathogenicity as compared to the other treatments. It may due to the suitability of temperature (27°C) for fungal growth than that of the wire green house (37°C), and to higher humidity under the first conditions. The effect of temperature and humidity on the growth of *M. phaseolina* was reported (Kaiser and Das, 1988; Viana-Francisco and Souza-Nilton, 1997).

Host range:

It was carried out on 23 different plant species belonging to 6 families (Table 2). The soil was infested by *M.phaseolina* 7 days before planting. The symptoms were variable in the tested plants and Squash plants were the most severely infected ones. The results indicated that the susceptibility of infection was independent on the plant genus and also its family, but depend mainly on the plant species. The wide range of host plants of *M.phaseolina* was indicated by many workers (Tosi et al, 1993; Kamlesh, 1993; Baird et al, 1994; Pratt et al, 1998).

Physiological studies:

Each microbial system has its own physiological features that control its different activities. Impressed by this fact some physiological factors as suitability of the growth medium, incubation temperature, initial pH value, relative humidity and different carbon sources, were studied.

1- Effect of different growth media:

The effect of different 6 fermentation media on the linear growth, density of growth, mycelium and colony colour as well as the pigmentation of the fungus (Table 3) indicated that potato dextrose agar (PDA) medium favoured *M.phaseolina* growth as compared to the other tested media, where Richard's medium was conducive to the lowest growth values. Therefore, PDA medium will be used in the subsequent work.

The linear growth of *M.phaseolina* on PDA was also estimated for sex days at 30°C. It indicated that the maximal growth was attained after 4 days of incubation.

2- Effect of incubation temperature, initial pH value, relative humidity and carbon sources on the growth of *M.phaseolina*:

The results (Table 4) revealed that incubation temperature of 30°C was responsible for maximal growth values. It was reported that a temperature of 30°C was optimal for *M.phaseolina* growth and activities (Kaiser and Das, 1988; Viana-Francisco and Souza-Nilton, 1997).

The data (Table 4) indicated that the optimal pH value for the growth of the tested fungus lies in the slightly acidic range (pH 5.1- 5.9). The effect of pH on the activities and growth of *M.phaseolina* was reported

(Mukheerjee et al, 1983; Kaiser and Das, 1988; Viana-Francisco and Souza-Nilton, 1997).

As the relative humidity was considered, its increase to 100% was concomitant with the increase of linear fungal growth. It is well known that humidity plays an important role in the microbial growth (Garcia et al, 1986).

M.phaseolina linear growth showed different responses to the tested carbon sources (equivalent weights of carbon). The monosaccharide glucose followed by fructose appeared to be the most favourable carbon sources, even in their combination in the disaccharide sucrose. While glucose polymer (starch) was relatively inhibitory to the fungal growth. The effect of different carbon sources in the activities of *M.phaseolina* was reported (Griffin, 1981; Reddy et al, 1987; Das, 1988).

Some enzyme activities of *M.phaseolina*:

As *M.phaseolina* a plant pathogen, it secretes enzymes catalyzing the hydrolysis of cell wall materials. Therefore, the ability of the fungus to secrete pectinolytic enzymes (pectin methyl esterase -PME, polygalacturonase - PG), cellulases and proteases was studied.

The PME activity of the fungus was estimated in the filtrate after growth in pectin containing medium. The fungus proved to have high PME activity (relative activity = 5.7% = 51.3 unit/h/mM). The results (Table 5) indicated high ability of *M.phaseolina* to secrete PG. Thus, after 20m of crude enzyme incubation with 1% polygalacturonic acid at pH=5, the viscosity showed about 37.4% decrease (relative activity = 37.4%). Using carboxy methyl cellulose as enzyme substrate, the fungus have high cellulase activity (Table 5). *M.phaseolina* showed high protease activity (150 microgram tyrosine =25 enzyme unit). The high activity of *M.phaseolina*, as a plant pathogen, to produce pectinolytic enzymes, cellulases and proteases indicated its high efficiency to induce plant diseases (Lumsden, 1976; Abdela et al, 1986).

Antagonistic studies:

The antagonistic effect of the rhizosphere microflora of the test plant Squash on the growth of *M.phaseolina* was studied. This goal of work started by isolation, purification and identification of the isolated bacteria and fungi from the rhizosphere of Squash plants, grown in infected and non-infected soils (diseased and healthy plants) for 30days. The isolated bacteria were 6 isolates of *Bacillus subtilis*, 3 species of *Streptomyces*, and one species of *Bacillus megaterium*, *Corynebacterium*, and *Sarcina*. While, the isolated fungi were *Alternaria* sp., *Aspergillus niger*, *A. fumigatus*, *Fusarium oxysporum*, *Penicillium* sp., *Trichoderma harizianum*, *T. viride*, and *Rhizoctonia solani*.

Antagonism between rhizospheric microflora of Squash and *M.phaseolina* (in vitro):

The effect of the isolated bacteria and fungi on the growth of *M.phaseolina,* on potato dextrose agar medium (in vitro), Table 6 indicated that *B.subtilis* isolates were the most active species that inhibiting the growth of *M.phaseolina* (especially *B. subtilis* VI). While species of *Corynebacterium* and *Sarcina* failed to be so. On the other hand, *Trichoderma harizianum* was the most active fungus that can inhibit the growth of *M.phaseolina* (96.6% inhibition). However, *Alternaria* sp. and *Fusarium oxysporum* failed to inhibit *M.phaseolina* growth. The previous results indicated that *B. subtilis* VI, *T. harizianum* and *T. viride* were the most active isolates that inhibit the growth of *M. phaseolina* (100, 96.6 and 77.3%, respectively) on agar medium (in vitro).

The influence of these isolates to inhibit the growth of *M.phaseolina* in soil planted with the test plant Squash (in vivo) was studied. The results (Table 7) revealed that *B.subtilis* can inhibit the growth of *M.phaseolina* by about 74%, followed by *T.harizianum* 63% and *T.viride* showed 47% reduction in *M.phaseolina* growth. It was reported that the non-pathogenic microbes compete or antagonize with the pathogenic group and reduce their distribution and pathogenicity (Kalznelson, 1965). The biological control of *M.phaseolina* by bacteria and fungi was reported by some workers (Perveen et al, 1994; Mathur, 1995; Perdomo et al, 1995; Khan and Gupta, 1998). It was also indicated by some workers that antagonism due to antibiosis of the metabolites and toxins of the organism beside parasitism and prey (Elad et al, 1982; Gangopadhyay and Grover, 1985; Adetuyi and Olowoyo, 1993).

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Table 1. Number of infected plants/200 plant in both green house and wire green house, in sterilized and non-sterilized soils, as infected by *M.phaseolina*, during and after planting of the seeds of Squash.

Time of fungal infection into the soil	Glass house				Wire green house			
	Sterilized soil		Non-sterilized soil		Sterilized soil		Non-sterilized soil	
	Infected plants	% of infection	Infected plants	% of infection	Infected plants	% of infection	Infected plants	% of infection
7 days before planting	117	58.5	134	67.0	106	53.0	118	59.0
At the same time of planting	95	45.5	119	59.5	85	42.5	103	51.5
20 days after planting	33	16.5	44	22.0	25	12.5	29	14.5

Table 2. Susceptibility of some plants for infection with *M.phaseolina*.

Plant	Symptoms		
	Damping off		Wilting and root rot
	Pre-emergence	Post-emergence	
Family: *Chenopodiaceae*			
Beta vulgaris Early wonder	+	+	+
Family: *Crucifereae*			
Brassica oeracoae Capitata	+	-	-
Family: *Cucurbitaceae*			
Citrulius lanatus Crimson sweet	+	+	+
Cucumis melon Ananas	+	-	+
C. sativus Mycogreen MT	+	-	-
Cucurbita maxima Butternut	+	-	-
C. pepo Bottle guard	+	+	+
C. pepo Scarla	+	+	+
Cucumis melo Nabolsi	+	+	+
Family: *Gramineae*			
Zea mays Golden beauty	+	-	-
Triticum vulgare Gonion	+	+	+
Family: *Malvaceae*			
Hibiscus esculentus Clemson	+	+	+
Sub-family: *Papilionoideae*			
Cicer arietinum	+	+	+
Arachis hypogaea	+	-	-
Lupinus termis	+	-	+
Phaseolus vulgaris	+	-	+
Pisum sativum Calfornia	+	+	+
Vicia faba Giza-2	+	+	+
Vigna sinensis Azmerly	+	+	+
Family: *Solanaceae*			
Capsicum fruitescens Long stem	+	+	+
Lycopersicum esculentum Person improved	+	-	-
Solanum melogena Black beauty	+	+	+
Seasanum indicum Giza-25	+	+	+

+ Symptoms clear. **- No symptoms**

Table 3. Effect of different growth media on the linear growth and some parameters of *M.phaseolina*.

Medium	Linear growth (cm)	Growth density	Mycelium colour	Form of colony	Pigmentatn.
Potato dextrose agar (PDA)	8.5	Heavy	White	Circular	Dark
Malt agar	8.2	Heavy	White	Circular	Dark
Waksman's agar	7.2	Surface	White	Circular	Dark
Peptone yeast extract	8.0	Heavy	White	Circular	Dark
Czapek's	8.1	Heavy	White	Circular	Dark
Richard's	4.4	Poor	White	Circular	Dark

Table 4. Linear growth of *M. phaseolina* as influenced by incubation temperature, pH value, relative humidity and different carbon sources after 4 days of incubation.

Tested parameter	Linear growth (cm)	Tested parameter	Linear growth (cm)
Incubation temp. (°C):		**Relative humidity (%):**	
5	1.18	15	1.80
10	4.48	50	4.90
15	6.38	65	6.66
20	7.32	73	6.68
25	8.06	80	7.30
30	8.48	85	7.86
35	7.46	90	8.20
40	0.00	100	8.50
pH value:		**Carbon source**:	
3.1	1.80	Glucose	8.50
4.1	5.12	Fructose	8.20
4.6	6.94	Galactose	4.20
5.1	8.50	Maltose	4.10
5.9	8.50	Sucrose	8.50
6.3	6.20	Cellulose	7.10
6.8	5.18	Starch	3.50
7.1	3.12		
8.1	0.00		

Table 5. Relative activity of polygalcturonase and cellulase (carboxy methyl cellulase) of *M. phaseolina*.

Polygalacturonase		Cellulase	
Reaction time (min)	**Relative activity* (%)**	**Reaction time (min)**	**Relative activity** (%)**
0	8.7	5	49.8
5	19.2	10	58.1
10	33.6	20	60.0
20	37.4	30	61.2

*** Percentage of decrease of viscosity of 1% polygalcturonic acid at pH=5 under the action of the crude polygalacturonase.**

**** Percentage of decrease of viscosity of 1.2% carboxy methyl cellulose at pH=5 as influenced by the action of crude carboxy methyl cellulose.**

Table 6. The antagonistic effect of the rhizospheric microflora of Squask on the growth *M. phaseolina* (in vitro).

Serial number	Microorganism	% of inhibition of *M. phaseolina* *
	BACTERIA:	
BS I	*Bacillus subtilis*	54.2
BS II	*Bacillus subtilis*	58.2
BS III	*Bacillus subtilis*	71.6
BS IV	*Bacillus subtilis*	61.8
BS V	*Bacillus subtilis*	68.8
BS VI	*Bacillus subtilis*	100.0
I	*Bacillus megaterium*	30.2
I	*Corynebacterium* sp.	0.0
I	*Sarcina* sp.	0.0
S I	*Streptomyces* sp.	48.3
S II	*Streptomyces* sp.	28.6
S II	*Streptomyces* sp.	69.2
	FUNGI:	
1	*Alternaria* sp.	0.0
2	*Aspergillus fungiatus*	58.2
3	*A. niger*	21.4
4	*Fusarium oxysporium*	0.0
5	*Penicillium* sp.	25.6
6	*Trichoderma harizianum*	96.6
7	*T. viride*	77.3
8	*Rhizoctonia solani*	0.0

% of growth inhibition = $(R_1 - R_2)/R_1 \times 100$

R_1 = Linear growth (mm) of *M. phaseolina* in the farthest of the tested microorganism, at the edge of the petri-dish.

R_2 = Linear growth (mm) of *M. phaseolina* to the center of the petri-dish.

Table 7. Antagonistic effect of the isolated Squash rhizospheric microorganisms on the pathogencity of *M. phasolina* in the soil (in vivo).

Soil infection	Pathogenecity			
	Damping off (%)			% to *M. phaseolina* pathogenecity
	Pre-emergence	Post-emergence	Total	
None	0.0	0.0	0.0	0.0
M.phaseolina	37.0	21.0	58.0	100
M.phaseolina + *B. subtilis*	9.5	5.5	15.0	74.1
M. phaseolina + *T. harizianum*	12.6	8.9	21.5	63.0
M. phaseolina + *T.viride*	18.3	12.2	30.5	47.4