

Diversity of *Macrophomina phaseolina* (Tassi) Goid Based on Chlorate Phenotypes and Pathogenicity

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Abstract

Macrophomina phaseolina (Tassi) Goid causes charcoal disease of oilseed plants. In this study 24 isolates, which were obtained from sunflower, soybean and sesame, were compared based on chlorate phenotypes and pathogenicity tests. For chlorate phenotypes, the isolates were grown on potassium chlorate and stored at 30°C in darkness. For pathogenicity test, seeds of sunflower, soybean and maize plants were placed on 6 – day – old colonies of each *Macrophomina* isolates grown on PDA and kept at 30°C in the dark. Results indicate that the sesame isolates had more colony radius rate on chlorate minimal medium in comparison to the soybean and sunflower isolates. The sesame isolates were chlorate resistant and grew normally with numerous dark microsclerotia production on the potassium chlorate. The soybean and sunflower isolates were chlorate sensitive and divided into two classes. Class 1, include the isolates that grew sparsely with a feathery like pattern, and the other one had a completely restricted radial growth meaning that *M. phaseolina* isolates differed in their ability to use certain nitrogenous compounds. Analysis of variance showed a significant difference between the colony radius rates of the isolates at 1% probability level. Based on Duncan's test, the isolates have been divided in 14 classes. Results of pathogenicity test showed that there was significant difference ($P < 0.01$) between the isolates. The results confirmed that the feathery like pattern of the isolates was more virulent on soybean and sunflower.

Keywords: *Macrophomina phaseolina*, Chlorate phenotype, Pathogenicity test, Oilseed plants

1. Introduction

Macrophomina phaseolina (Tassi) Goid, is an anamorphic and soil borne fungus with a broad host range that includes 75 plant families and more than 500 species worldwide (Salik, 2007). Many economically significant plants including legumes, vegetables, fruits and fiber crops are attacked by *M. phaseolina*, a causal agent of charcoal rot disease (Kunwar and Sin, 1986; Sinclair and Backman, 1986; Smith and Carvil, 1997). Estimates of yield reduction due to charcoal rot in the US were 1.98, 0.28, and 0.49 million metric tons in 2003, 2004, and 2005, respectively (Wrather and Koenning, 2006). *Macrophomina phaseolina* is the most fungal pathogens affecting sunflower in Egypt (Purkayastha *et al.*, 2006). 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 extremely intraspecific variations (Dhingra and Sinclair, 1972; Echavez-Badel and Perdomo, 1991). The significant differences of morphological (Mayek-Perez *et al.*, 2001), physiological (Mihali and Taylor, 1995), pathogenic (Mayek-Perez *et al.*, 2001; Su *et al.*, 2001) and genetic (Vandemark *et al.*, 2000; Mayek-Perez *et al.*, 2001; Su *et al.*, 2001; Alvaro *et al.*, 2003; Jana *et al.*, 2003; Aboshosha *et al.*, 2007) diversity have been reported. Control has not yet been achieved through resistance in spite of reports on tolerant genotypes (Smith and Carvil, 1997). Chlorate phenotypes were used as markers for identifying host-specific isolates of *M. phaseolina* (Das *et al.*, 2006). Many researchers have also found great variability in pathogenicity and morphology among isolates from the same host. It is assumed that during the hyphal fusion, heterokaryosis could occur after

mitotic segregation and recombination (Sinclair and Backman, 1986). This may explain the occurrence of cultural types or physiological races of *M. phaseolina* (Dhingra and Sinclair, 1973; Manici *et al.*, 1995). Recent efforts to classify isolates of *M. phaseolina* have centered on morphology of the colony on media amended with chlorate (Su *et al.*, 2001). Most fungi can use nitrate as a source of nitrogen. Nitrate uptake does not appear to occur without nitrate metabolism. The metabolic assimilation of nitrate is by reduction to nitrite via nitrate reductase, nitrite is then reduced to ammonia. Chlorite could restrict the growth when the nitrate reductase pathway is active. Unrestricted growth in the sectors resulted from the inactivity of one or more of the five enzymes in the nitrate reductase pathway (Cloud and Rupe, 1988; McCain and Smith, 1972; Solomonson and Vennesland, 1972). Nitrate reductase also can reduce chlorate to chlorite. The accumulation of chlorite is presumably poisonous to cells. Fungal strains that have functional nitrate reductase are chlorate sensitive, whereas those that are unable to catabolize nitrate are chlorate resistant (Pearson and Leslie, 1987). In this study we have attempted to separate and classify the isolates of *M. phaseolina* obtained from the oilseed plants on the basis of pathogenicity test, morphology and growth manner on minimal medium containing chlorate.

2. Materials and Methods

2.1 Fungal isolates

Twenty-four samples were collected from infected stems and roots of soybean, sunflower and sesame plants from Mazandaran Province in northern Iran (Table 1). Each root or stem was thoroughly washed and dried at room temperature. Four small 0.3 cm epidermal sections were excised from each sample and sterilized in 0.8% NaOCl (1min) and washed in sterile water for 1 min. Tissues were placed on potato dextrose agar (PDA) plate followed by incubation at $28\pm 1^{\circ}\text{C}$ in darkness for four days. Purification was developed by single microesclerotium culture and maintained on PDA at $28\pm 1^{\circ}\text{C}$ (Das, and Fakrudin, 2006). All 24 isolates employed in the present investigation are listed in Table 1.

2.2 Phenotypic study

A 1 mm agar plug from the 7 day-old pure culture was placed on minimal medium containing potassium chlorate with some modification (20g agar, 1.6g asparagine, 15g potassium chlorate, 30g sucrose, 2g NaNO_3 , 1g KH_2PO_4 , 0.5g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ and the final reaction volume was adjusted to 1000 ml with H_2O) and 0.2ml of trace elements solutions (95ml distilled water, 10g citric acid, 10g $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 2g $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2\cdot 6\text{H}_2\text{O}$, 0.5g $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 100mg $\text{MnSO}_4\cdot \text{H}_2\text{O}$, 100mg H_3BO_3 , 100mg $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$ and 1ml chloroform) was added and kept at 30°C in darkness. The pH of the minimal medium was adjusted to 6.5 with KOH before autoclaving (Puhalla and Spieth, 1985). The colony radius rates of the isolates were evaluated by factorial experiment based on completely randomized design (CRD) in four replications. After 48 hours, the colony radius was measured daily with ruler. The minimal medium without potassium chlorate was used as a control treatment.

2.3 Pathogenicity test

In this experiment pathogenicity test of isolates was carried out at the seedling stage of soybean (*Glycine max* L.), sunflower (*Helianthus annuus* L.) and maize (*Zea mays* L.) plants in a completely randomized block design. Each treatment (isolate) was replicated three times and included two plates with six seeds per plate. Seeds of the plants were sterilized with 2% sodium hypochlorite for 4 min and rinsed twice in sterile water. Seeds were placed on 6-day-old colonies of the *Macrophomina* isolate (on PDA plates) and incubated at 30°C in dark condition. Evaluation was done after six days, using the following severity assessment key: 0 = healthy seed; 1 = discoloration of a portion of the seedling in contact with the mycelium; 2 = seed teguments invaded by mycelium and sclerotia but healthy seedling; 3 = seed teguments free from the fungus but seedling infected; 4 = seed tegument and seedling infected; 5 = seed infected and not germinated (Manici *et al.*, 1992).

The disease index was calculated by multiplying the number of seeds by the degree of disease severity. The experimental design was a randomized complete. Analysis of variance (ANOVA) of the data was performed with the MSTAT-C package.

3. Results and Discussion

3.1 Phenotypic study

Three various growth patterns (feathery spreading growth, restricted growth and dense growth) were observed, when the isolates were grown on the minimal medium containing 120mM potassium chlorate (Table 2, Figure 1). Restricted and feathery isolates were sensitive to chlorate, whereas dense isolates were resistant to chlorate. Among soybean isolates, feathery isolates were much more abundant than restricted, whereas dense isolates predominated in sesame. Sclerotia production on chlorate medium by the sensitive isolates was too low compared

to resistant ones. Isolates of sesame grew more rapidly on the defined medium containing chlorate than did isolates from soybean or sunflower.

All isolates had dense growth when they were grown on the minimal medium without chlorate and could not be differentiated. The growth response of isolates did not alter by increasing chlorate concentrations from 120 to 240 mM, whereas dense and feathery growth appeared similar in the lower concentration (60 mM).

Su *et al.* (2001) and Pearson *et al.* (1986) reported that mycelial growth of *M. phaseolina* on chlorate medium was classified into three categories (restricted, feathery and dense). Restricted and feathery isolates were sensitive to chlorate, whereas dense isolates were resistant to chlorate. Manici *et al.* (1995) reported that four colony chlorate phenotypes were observed.

Analysis of variance of the data showed that the colony radius rate of 24 isolates of *Macrophomina phaseolina* was significantly different ($P < 0.01$) on chlorate minimal medium (Table 3).

Different colony radius rates were observed in *M. phaseolina* isolates on chlorate minimal media. Based on Duncan's test, comparison of the means of colony radius rates grouped the 24 isolates in 14 classes on chlorate minimal media (Table 4). Isolate 4 and 7 on chlorate minimal medium had the highest colony radius rate. The chlorate resistant isolates had more radius rates than those in chlorate minimal medium. The results were similar to the reported by Manici (Manici *et al.*, 1992) (Table 4).

3.2 Pathogenicity test

The experiment of the pathogenicity test demonstrated that none of the isolates were pathogenic on maize while all isolates showed pathogenic ability on soybean and sunflower (Figure 2).

Analysis of variance showed that the pathogenicity of the 24 isolates of *M. phaseolina* was significantly different ($P < 0.01$) on the plant species (Table 5).

Disease indices on sunflower and soybean range between 19 - 24 and 27- 30 respectively. The means comparison of the different isolates indicate that there was significant difference among isolates on the rate of the disease index. Isolate 2, 5, 11, 23 and 24 indicate maximum disease index (27.00) and were placed in class A, while isolates 10 showed minimum disease index (23.17) and were grouped in class K (Table 6). Fernandez *et al.*, (2006) reported that great variability in pathogenicity was recognized among isolates from different host species and between isolates (Fernandez *et al.*, 2006). Das *et al.*, (2006) was investigated pathogenicity of some isolates of *M. phaseolina* that belong to different country. The results showed the pathogenicity of isolates was different and the most aggressive isolates were from Mexico, Brezil and Colombia (Das *et al.*, 2006).

(Table 6)

Means comparison of different plants species reaction showed that there was significant difference ($P < 0.01$) on the rate of disease index, hence, the plant species were placed in different groups (Table 7). Soybean with 28.56 had more level of sensitivity (class A) than sunflower (class B) with 22.48.

(Table 7)

Mean comparison of interaction between host plants and isolates showed significant difference ($P < 0.01$). All of isolates were pathogenic on soybean and sunflower. Isolates 24, 5, 11 and 23 showed most intensity on both soybean and sunflower, and the intensity of all isolates was more on soybean than on sunflower (Table 8).

The pathogenicity test showed that soybean and sunflower plants are susceptible while maize plant is resistant to *Macrophomina*. All the 24 isolates that were tested for charcoal rot reaction could infect soybean and sunflower. A broad pathogenic and phenotypic diversity was noticed among Iranian *M. phaseolina*.

Mayek-Prez *et al.* (2001) studied 84 isolates of *M. phaseolina* from different geographical regions of Mexico, and identified 43 distinct pathotypes. Su *et al.* (2001) found high levels of variation in pathogenicity of *M. phaseolina*. Manici (1995) investigated pathogenicity of *M. phaseolina* on eight plant species, and all were pathogenic on other plant species except on maize. Isolates were highly virulent on soybean and virulent on sunflower, safflower, sorghum and melon. Studies on *M. phaseolina* have investigated variations in morphology and pathogenicity among isolates from soybean, common bean and cluster bean (Purkayastha *et al.*, 2006).

In our study the most aggressive isolates originated from North of Mazadaran province were mainly isolated from soybean plants. The study also demonstrated that some chlorate sensitivity in *M. phaseolina* had some relation with charcoal rot severity in soybean and sunflower. Similar results were reported by Mihali and Taylor (1995) and Mayek-Perez *et al.* (2001).

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Table 1. *Macrophomina phaseolina* isolates characteristics, used in this study

No. of Isolates	Hosts	Geographic origin
1	Soybean	Ghamemshar
2	Soybean	Behshar
3	Sunflower	Neka
4	Sesame	Behshar
5	Soybean	Behshar
6	Soybean	Ghamemshar
7	Sesame	Neka
8	Soybean	Galoga
9	Soybean	Goybar
10	Sesame	Galoga
11	Soybean	Galoga
12	Soybean	Goybar
13	Soybean	Sari
14	Soybean	Sari
15	Soybean	Galoga
16	Soybean	Goybar
17	Soybean	Ghamemshar
18	Soybean	Sari
19	Soybean	Sari
20	Soybean	Sari
21	Soybean	Neka
22	Soybean	Neka
23	Soybean	Galoga
24	Soybean	Behshar

Table 2. Chlorate phenotypes of *Macrophomina phaseolina* isolates obtained from different hosts

No. of isolates	Source	Collection site	Chlorate reaction	Phenotype
5	Soybean	Behshar	Sensitive	Feathery
2	Soybean	Behshar	Sensitive	Feathery
24	Soybean	Behshar	Sensitive	Feathery
11	Soybean	Galoga	Sensitive	Feathery
23	Soybean	Galoga	Sensitive	Feathery
15	Soybean	Galoga	Sensitive	Feathery
8	Soybean	Galoga	Sensitive	Restricted
1	Soybean	Ghamemshar	Sensitive	Feathery
6	Soybean	Ghamemshar	Sensitive	Feathery
17	Soybean	Ghamemshar	Sensitive	Feathery
12	Soybean	Goybar	Sensitive	Feathery
9	Soybean	Goybar	Sensitive	Feathery
16	Soybean	Goybar	Sensitive	Feathery
22	Soybean	Neka	Sensitive	Feathery
21	Soybean	Neka	Sensitive	Feathery
18	Soybean	Sari	Sensitive	Feathery
19	Soybean	Sari	Sensitive	Feathery
13	Soybean	Sari	Sensitive	Feathery
20	Soybean	Sari	Sensitive	Feathery
14	Soybean	Sari	Sensitive	Feathery
3	Sunflower	Neka	Sensitive	Feathery
4	Sesame	Behshar	Resistant	Dense
10	Sesame	Galoga	Sensitive	Feathery
7	Sesame	Neka	Resistant	Dense

Table 3. Analysis of variance colony radius rate of 24 isolates of *M. phaseolina*

K Value	Source	Degrees of Freedom	Sum of Square	Mean of Square	F Value	Prob
2	Replication	2	66.778	33.389	5418.9271	0.0000
4	Treatment	23	199.075	8.655	1404.7420	0.0000
6	Replication* Treatment	46	15.417	0.335	54.3956	0.0040
7	Error	216	1.331	0.006		
	Total	287	282.601			

Coefficient of Variation: 4.52%.

Table 4. The mean comparison of 24 isolates based on Duncan's test for colony radius rate at minimal medium

Isolates code	Source	Means	class
4	Sesame	3.150	A
7	Sesame	3.141	A
1	Soybean	3.096	A
10	Sesame	2.950	B
5	Soybean	2.633	C
17	Soybean	2.534	D
22	Soybean	2.400	E
6	Soybean	2.162	F
16	Soybean	2.033	G
3	Sunflower	1.967	G
18	Soybean	1.702	H
19	Soybean	1.533	I
23	Soybean	1.500	I
24	Soybean	1.299	J
15	Soybean	1.267	J
20	Soybean	1.182	K
13	Soybean	1.048	L
2	Soybean	1.034	L
12	Soybean	1.033	L
14	Soybean	1.033	L
21	Soybean	1.023	L
11	Soybean	0.998	L
9	Soybean	0.733	M
8	Soybean	0.233	N

Table 5. Analysis of variance pathogenicity test of *M. phaseolina* on the two plant species

K Value	Source	Degrees of Freedom	Sum of Square	Mean Square of	F Value	Prob
1	Replication	2	0.181	0.090	0.1826	
2	Factor A	1	1290.007	1290.007	2608.5351	0.0000
4	Factor B	23	168.326	7.319	14.7989	0.0000
6	AB	23	25.160	1.094	2.2120	0.0040
7	Error	94	46.486	0.495		
Total	Total	143	1530.160			

Coefficient of Variation: 2. 74%.

Table 6. Mean comparison of *M. phaseolina* pathogenicity on the different plants species

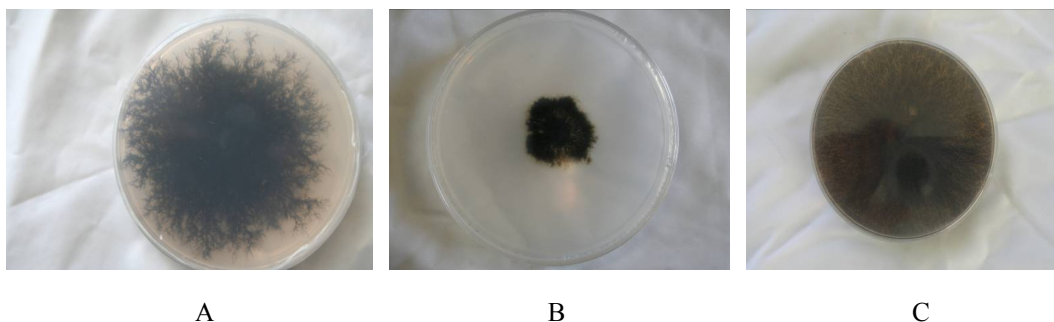
No. of isolates	Disease index	$\alpha = 0.01$
5	27.00	A
2	27.00	A
24	27.00	A
11	27.00	A
23	27.00	A
15	26.67	AB
8	26.50	ABC
1	26.50	ABC
6	26.17	ABCD
17	25.00	BCDE
12	26.00	BCDE
9	26.00	BCDE
16	25.83	BCDEF
22	25.67	CDEFG
21	25.50	DEFG
18	25.50	DEFG
19	25.33	DEFG
13	25.17	EFG
20	25.00	FGH
14	24.83	GHI
3	24.17	HIJ
4	24.00	IJK
10	23.83	JK
7	23.17	K

Table 7. Pathogenicity of 24 isolates of *M. phaseolina* on two plant species

Species	Cultivars	Average of disease index
<i>Glycine max</i>	willyams	28.56a
<i>Helianthus annuus</i>	Master	22.48b

Table 8. Mean comparison of interaction among different plants species and *M. phaseolina* isolates

Name of host and NO. of isolates	Mean diseases index	$\alpha=0.01$	Name of host and NO. of isolates	Mean diseases index	$\alpha=0.01$
5*soybean	30	A	2*sunflower	24.67	F
23*soybean	30	A	5 *sunflower	24.00	FG
24*soybean	30	A	24*sunflower	24.00	FG
11*soybean	30	A	11*sunflower	24.00	FG
15*soybean	29.67	A	23*sunflower	24.00	FG
1*soybean	29.67	A	8*sunflower	24.00	FG
2 *soybean	29.33	AB	15*sunflower	23.67	FGH
6*soybean	29.33	AB	17*sunflower	23.33	GH
12*soybean	29.00	ABC	1*sunflower	23.33	GH
8*soybean	29.00	ABC	16*sunflower	23.00	GHI
22*soybean	29.00	ABC	12*sunflower	23.00	GHI
9*soybean	29.00	ABC	19*sunflower	23.00	GHI
17*soybean	28.67	ABCD	21*sunflower	23.00	GHI
16*soybean	28.67	ABCD	9*sunflower	23.00	GHI
18*soybean	28.67	ABCD	6*sunflower	23.00	GHI
21*soybean	28.00	BCDE	13*sunflower	22.67	GHI
14*soybean	28.00	BCDE	22*sunflower	22.33	HI
13*soybean	27.67	CDE	18*sunflower	22.33	HI
20*soybean	27.67	CDE	20*sunflower	22.33	HI
4*soybean	27.67	CDE	14*sunflower	21.67	IJ
19*soybean	27.67	CDE	10*sunflower	20.67	JK
3*soybean	27.67	CDE	3*sunflower	20.67	JK
7*soybean	27.33	DE	4*sunflower	20.33	K
10*soybean	27.00	E	7*sunflower	19.00	L

Figure 1. Owth patterns of *Macrophomina phaseolina* on a minimal medium containing 120 mM potassium chlorate. A, Feathery, B, restricted and C, dense

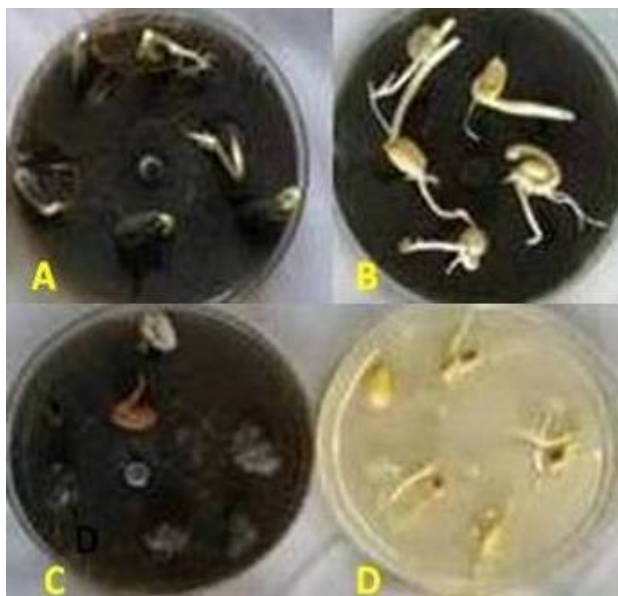


Figure 2. The pathogenicity test of *M. phaseolina* on sunflower (A), maize (B), soybean (C) and check (D)