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Pathogenic Variability among *Macrophomina Phaseolina* Isolates Associated With Soybeans

[1]Lord Famiyeh, [2]Todd Mlsna

[1,2]Department of Chemistry
[1,2]Mississippi State University, Starkville, United State of America

Abstract: **In this greenhouse study eight (8) isolates of *Macrophomina phaseolina,* MP120, MP30, MP218, MP210, MP29, MP32, MP24, and MP23 isolated from soybeans grown in Mississippi were compared based on their pathogenicity potential. Soybeans (Viking 2265(OG)) were planted in pots infested with one of the eight isolates of *M. phaseolina* and uninfested control pots in six replicates randomized black design at Mississippi State University North Farm. One important parameter that was monitored in this study is temperature. Temperatures measured in pots across all replicates were not significantly different (P≥0.05) according to analysis of variance (ANOVA). The pathogenicity was determined using seedling survival and growth at 9, 14, 22, and 28 days after planting, plant height, and root-hypocotyls discoloration at 28 days. The results showed that there were no significant difference (P≥0.05) in stand densities between the thick (white feathery) and the thin (black dense) isolates but showed significant difference in plant height compared to the control. Hypocotyls discoloration ratings were severe for thin isolates compared to the thick isolates and control. Hypocotyls discoloration ratings were significantly higher for MP23, MP29, and MP218 covering both isolate types. Plant height was significantly reduced in pots infested with MP23, and MP210 compared to all other isolates and control. All isolates were reisolated from diseased tissues of soybean plants and confirmed using Koch's postulate. The results from this study showed that the isolate cultural types of *M. phaseolina* did not vary in pathogenicity overall but some differences in individual isolates were evident.**

Keywords: ***Macrophomina phaseolina*, pathogenicity, isolates, stand densities, hypocotyls, discoloration, Koch's postulate.**

I. INTRODUCTION

Macrophomina phaseolina (Tassi) Goid is an anamorphic ascomycete that belongs to the family Botryosphaeriacease [1]. The fungi is a casual agent of charcoal rot disease (dry-weather wilt) of about 500 plant species worldwide, including row and horticultural crops such as soybeans (*Glycine max* L.) [2], and strawberry (*Fragaria ananassa* D.) [3]. Soybean charcoal rot disease is considered a problem in the southern United States of America and contributes to significant crop loss [4]. *M. phaseolina* is the major cause of significant economic losses to soybean producers in dry seasons [5]. Wrather et. al [6], [7] estimated that in the United States of America, the average annual yield soybean losses caused by charcoal rot in 2003-2009 range between 0.25- 1.98 million metric tons. *M. phaseolina* causes seed rot of many plant species including mungbean (*Vigna radiate* L.), and have the potential to survive on seeds for market [8]. Therefore methods (e.g. Identification of chemical signatures) to determine the presence of *M. phaseolina* in seeds and other crops such as sweet potato (*Ipomoea batatas* L.) become important in the agriculture industry.

M. phaseolina is a soil and seed borne polyphagous pathogen that produces pycnidial asexual structures and microsclerotia which can survive for several years in soil, and plant debris [9], [10]. The charcoal rot disease severity is directly related to viable sclerotia produced in soil [11]. The sclerotia serve as primary means of *M. phaseolina* survival [12]. The prevalence of the charcoal rot disease is linked to cultural practices and climatic conditions such as heat, and humidity [4], [12]. The symptoms of the charcoal rot disease include seedling blight, root and stem rot, pre-to post

damping off, wilting and death, and failure of seed germination [2], [12]. Plants infected by *M. phaseolina* often develop gray or brown discoloration on root and hypocotyls after flowering. Hypocotyls and root injury or damage is 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 [13]. At the seedling stage, the invasion is very fast and takes approximately two days for the fungi to be established in the host while at the cotyledon stage the seedlings get infected within 3-7 days [7]. Early infections of *M. phaseolina* impact plant survival and overall growth and yield greatly.

It has been established that fungi can produce unique sets of volatile organic compounds (VOCs) that could be used to distinguish between pathogenic and non-pathogenic isolates. Sun et. al [14] has discriminated between toxic and nontoxic strains of *Aspergillus flavus* using VOC profiles. Identification and discrimination of isolates of *M. phaseolina* using VOC profiles has not been studied. VOCs data of the eight isolates of *M. phaseolina* used in this study will be analyzed in a follow-up study to discriminate between these isolates in order to provide a method for identification.VOC profiles of cultured fungi depends largely on the duration of incubation, type of nutrient (growth media), temperature of incubation, and other environmental parameters [15].

The effect of cultural morphology of isolates of soil borne *Fusarium spp* (*Fusarium oxysporum* Schlecht. emend. Snyder & Hansen), *Fusarium solani* ((Mark) sacc) [16], [17] and *Alternaria spp* (*Alternaria brassicae* (Berk) sacc) [18] on pathogenicity have been studied. The results of these studies have shown that cultural morphology of some fungi has impact on pathogenicity. The cultural morphology variation of the thick (white feathery) and thin (black dense) forming mycelia isolate types of *M. phaseolina* on pathogenicity have not been studied. The main focus of this study is to determine whether two isolate types of *M. phaseolina* will show differences in pathogenicity on soybeans.

II. MATERIALS AND METHODS

A. Preparation of cornmeal-sand-inoculums:

Eight isolates of *M. phaseolina* stored in the Mycological Culture Repository at Mississippi State University laboratory were subcultured on Potato Dextrose Agar (Difco, Fisher Sci, Pittsburgh, PA) (PDA) medium in Petri dishes (10×100 mm) and incubated in the dark at 35 ^{o}C for 7 days. Table 1 shows the plant hosts, geographical origins, cultural types and date of isolation of the eight isolates of *M. phaseolina* used in this study. The thick and thin isolates of *M. phaseolina* are shown in the figure 1.

TABLE 1: *M. phaseolina* isolates morphological types evaluated in a greenhouse study over a 28 days period.

Isolates	Host	Geographical Origin	Date of isolation	Cultural type*
MP120	Soybeans	Tupelo, MS	1999	Thick
MP30	Soybeans	North Greenwood, MS	2013	Thick
MP218	Soybeans	Leflore County, MS	2013	Thick
MP210	Soybeans	Leflore County, MS	1999	Thick
MP29	Soybeans	North Greenwood, MS	1999	Thin
MP32	Soybeans	North Greenwood, MS	1999	Thin
MP24	Soybeans	North Greenwood, MS	1999	Thin
MP23	Soybeans	North Greenwood, MS	1999	Thin

*Thick: White feathery or cottony tomentose when cultured on potato dextrose agar (PDA), Thin: Black dense or appressed mycelium when cultured on potato dextrose agar (PDA).

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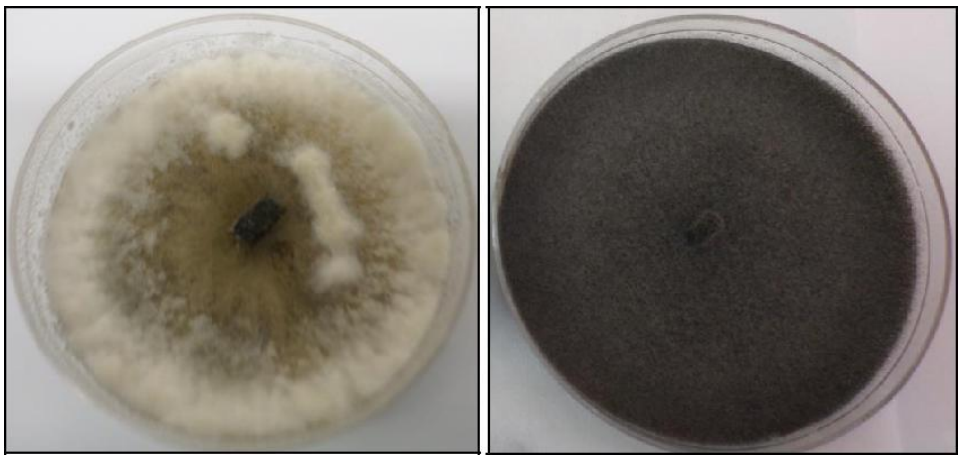

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Fig.1: Cultural morphology of *M. phaseolina*: (A) = Thick (White feathery or cottony tomentose), (B) = Thin (Black dense or appressed)

Cornmeal-sand-inoculums were prepared according to the procedure described by Baird et. al [19] with some little modifications. In a 250 ml Erlenmeyer flask, 6 g of cornmeal, 200 g of sand, and 26 ml of water were mixed. The cornmeal-sand was autoclaved for 1 h at 1kg/cm^2 (15 psi) and allowed to stand for 24 hours and autoclaved again to kill any possible surviving bacteria's. The cornmeal-sand was inoculated with 10 agar plug pieces of *M. phaseolina* isolates sectioned from the advancing edge of the mycelium of 7 days old cultures and incubated at 35^{o}C for 14 days. The flasks were shaken daily to ensure uniform distribution of the inoculums. Twelve inch plastic pots were soaked in 10-15% hypochlorite bleach for 1 h to kill any bacterial and fungus present. The pots were filled with 5000 ml of soil (Pro-Mix ®) (Premium Tech Horticulture, Quakertown, PA) (Canadian sphagnum peat moss (75-85%) containing: perlite-horticulture grade, vermiculite-horticulture grade, dolomitic and calcite limestone (pH adjuster), wetting agent and starter nutrient) followed by the addition of 15 ml of the cornmeal-sand-inoculums spread on the surface of 5000 ml Pro-Mix ® in the 54 pots.

Five soybeans (Viking 2265 (OG)) seeds were sown about 6 to10 cm apart in pots containing infested soil and noninfested (control) soil. The trial containing eight isolates treatment and control pots were placed in a randomized block design using six replicates per treatment. Seeds were watered daily until saturated to ensure proper germination and growth. Soil temperatures within the pots were recorded daily using an electronic thermometers.

B. Pathogenicity Test:

The study was conducted from November 13 to December 12, 2013. Pathogenicity of *M. phaseolina* on soybeans was evaluated based on plant stand germination and survival, hypocotyls discoloration, and plant height. The plant stands were recorded at 9, 14, 22 and 28 days after planting. Hypocotyls discoloration ratings were determined on the 28th day using a scale of 1-4 (1 ≤ 5%, 2 = 2 to 10%, 3 = 11 to 50%, and 4 ≥ 50%), where 1, 2, 3, and 4 indicates normal (N), slight (Sl), moderate (Mod), and severe (Sev) symptoms of hypocotyls discoloration respectively. At the end of the 28th day growth period, the plants were carefully removed from each pot, and the roots and hypocotyls were washed with running tap water for five minutes. The plants heights were measured on the 28th day using a ruler.

To confirm the pathogenicity for each isolates, symptomatic plant tissue (pieces of plant tissues from lesions and wilted parts of the soybean plants) were surface disinfected with 70% ETOH and plated on PDA medium in petri dishes and incubated at 35 ^{o}C for 7 days.

C. Statistical Analysis:

The pathogenicity test data were subjected to analysis of variance (ANOVA) using the general linear models (GLM) procedure of SAS. Means were separated using Fisher's least significant difference (LSD) (P≤0.05) Temperatures in all the six replicates were compared by ANOVA using R software (www.r-project.org).

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III. RESULTS AND DISCUSSIONS

One important parameter that was monitored in this study is temperature. The pathogenicity of *M. phaseolina* is temperature dependent [20]. Temperatures measured in pots across all the replicates were not significantly different (P≥0.05) according to analysis of variance (ANOVA) (Table 2). The disease severity is greatest at higher temperatures. Charcoal rot disease can be severe when soil temperatures ranges between 28 to 35 ^{o}C [4], [15]. One reason associated with low infection levels than expected particularly on roots and hypocotyls in this study is due to low temperatures measured in pots. Also *M. phaseolina* isolates stored at -80 ^{o}C in the Mycological Culture Repository could have also caused the isolates to lose their pathogenicity.

TABLE 2: Soil temperatures across six replicate treatments of eight isolates of *M. phaseolina* in a greenhouse study

Replicate*	Maximum (^{o}C)	Minimum (^{o}C)	Average(^{o}C) **
I	25.8	11.7	17.5 ± 3.92
II	25.6	10.4	17.3 ± 4.01
III	25.4	12.4	17.4 ± 3.97
IV	25.8	10.6	17.7 ± 3.76
V	26.9	12.6	19.4 ± 4.39
VI	26.3	12.1	18.1 ± 4.16
ANOVA(P=0.05)			0.87

*Maximum, minimum, and average soil temperatures across six replicate treatments. Soil temperatures recorded randomly in each replicate for 28 days. ** Mean temperatures across the six replicates were not significantly different (P≥0.05) according to analysis of variance (ANOVA).

Plant stands during the four sampling dates for thick and thin morphological types of *M. phaseolina* were not significantly different (P≥0.05) compared to the control (Table 3) but six plants were found dead in pots treated with the thin isolates. Among the eight isolates, MP24 showed the greatest reduction in plant stands after 9, 14, 22, and 28 days of planting and with more dead plants (Table 4). This shows that MP24 appeared to be the most virulent isolates on soybean compared to the other isolates used in this study.

TABLE 3: Effect of thick and thin types of *M. phaseolina* isolates on soybean stand count (5 seeds sown) **

Mycelium*	Nov 22	Nov 27	Dec 5	Dec 11	Dead plants***
	(Day 9)	(Day 14)	(Day 22)	(Day 28)	
Thick	3.50 a****	3.83 a	4.00 a	4.00 a	0
Thin	2.87 a	3.83 a	3.88 a	3.71 a	6
Control	3.50 a	4.17a	4.17 a	4.17 a	0
ANOVA(P=0.05)	0.89	0.68	0.77	0.74	

*Thick: MP120, MP30, MP218, and MP210, Thin: MP29, MP32, MP24, and MP23. **Plant stands counts (5 sown seeds) taken in November 22 (9th day after planting, DAP), November 27 (14 DAP), December 5 (22 DAP), December 11 (28 DAP). *** Total number of dead plants from 13 November (day of planting) to December 11 (last day of stand count). ****Comparisons of plant stand means between thick and thin *M. phaseolina* and control. Numbers not followed by the same letter are significantly different (P≤ 0.05) according to Fisher least difference (LSD) procedure. The plant stand indicates the average total number of plants that emerged in pots infected with each isolate. Six replicates per treatment

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TABLE 4: Effect of the eight isolates of *M. phaseolina* on soybean stand count (5 seeds sown)

MP isolates*	Nov 22	Nov 27	Dec 5	Dec 11	Dead plants**
	(Day 9)	(Day 14)	(Day 22)	(Day 28)	
MP120	4.17a***	4.17a	4.50 a	4.50 a	0
MP30	3.17 ab	3.67 ab	3.83 a	3.83 a	0
MP218	3.33 ab	3.83ab	3.83 a	3.83 a	0
MP210	3.33 ab	3.67 ab	3.83 a	3.83 a	0
MP29	3.33 ab	4.17 a	4.33 a	4.17 a	1
MP32	2.67 b	4.00 ab	4.00 a	3.67 a	2
MP24	2.33 b	3.17 b	2.83 b	2.66 b	3
MP23	3.17 ab	4.00 ab	4.33 a	4.33 a	0
Control	3.50 ab	4.17 a	4.17 a	4.17 a	0
ANOVA(P=0.05)	1.25	0.96	0.99	0.93	

*MP isolates: Thick: MP120, MP30, MP218, and MP210, Thin: MP29, MP32, MP24, and MP23. **Plant stand taken in November 22 (9 d after planting, DAP), November 27 (14 DAP), December 5 (22 DAP), December 11 (28 DAP). ***Comparisons of mean plant stand between thick and thin *M. phaseolina* and control. Numbers not followed by the same letter (s) are significantly different (P≤ 0.05) according to Fisher least difference (LSD) procedure. The plant stand indicates the average number of plants that emerged in pots. Six replicates per treatment.

At the end of the 28 days of planting, the roots and hypocotyls of the soybean plants were examined for necrotic or blacken tissue symptoms of *M. phaseolina* infections. However, the roots were never found to have symptoms of *M. phaseolina*. Distribution of inoculums on the surface of the soil could be the reason for hypocotyls discoloration rather than the root.

TABLE 5: Effect of thick and thin isolate types of *M. phaseolina* on soybean plants hypocotyls discoloration.**

Mycelium*	N	Sl	Mod	Sev	Index	Plant height (cm) ****
Thick	2.29 b***	1.42 a	0.71 b	0.13 a	1.23 ab	9.04 b
Thin	1.29 c	1.17 a	0.92 a	0.50 a	1.68 a	9.21 b
Control	3.83 a	0.33 b	0.00 b	0.00 a	0.90 b	9.78 a
ANOVA(P=0.05)	0.92	0.71	0.71	0.64	0.58	0.43

*Thick: MP120, MP30, MP218, and MP210, Thin: MP29, MP32, MP24, and MP23. ** Root-hypocotyls discoloration rated on a scale of 1-4, where, 1 ≤ 5%, Sl = 2 to 10%, 3 = 11 to 50%, and 4 ≥ 50% on the 28th of planting. Index = ((N·1) + (Sl·2) (Mod·3) + (Sev·4))/5. N = normal, Sl = slight, Mod = moderate, and Sev= severe. Numbers under each hypocotyls discoloration rating scale indicates the average number of plants with that symptom. Disease index consider all symptoms together. ***Comparisons of mean hypocotyls discoloration rating. Numbers not followed by the same letter (s) are significantly different (P≤0.05) according Fisher least difference (LSD). ****Plant height measured on the 28th day of planting using ruler.

The hypocotyls injury caused by the thick and thin isolates was greater than the control which suggests the reason for the greater reduction in plant height by the two isolate types (Table 5). Among the eight isolates, MP23 and MP29 showed the highest hypocotyls discoloration and disease index compared to all other isolates (Table 6). The hypocotyls discoloration of some plants attached by MP23 is compared to the control as shown in figure 2 below. The high disease index of MP23 reflected on the greater reduction (P≤0.05) of the plant height compared to all other isolates and control [21]. MP210 also showed a greater reduction in plant heights (figure 3) compared to the control and all other isolates except MP23. Since many plant died with MP24, the hypocotyls percent injury may not be a good indicator of plant height reduction.

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Fig 2: Hypocotyls discoloration (shown by arrows) caused by *M. phaseolina* inoculums of soybeans plants in a greenhouse study: (A) = Pots infested with MP23, plants rated normal, moderate, and severe from left to right, (B) = Non-infested pots (control), all plants rated normal.

TABLE 6: Effect of different isolates of *Macrophomina phaseolina* on soybean plants hypocotyls**

MP isolates*	N	Sl	Mod	Sev	Index	Plant height (cm) ***
MP120	4.00 a****	0.50 bc	0.00 b	0.00 b	1.00 cd	9.47 ab
MP30	2.00 b	1.67 a	0.00 b	0.00 b	1.07 cd	9.30 ab
MP218	1.67 bc	1.83 a	0.00 b	0.50 b	1.47 bc	9.17 abc
MP210	1.50 bcd	1.67 a	0.67 ab	0.00 b	1.37 bcd	8.67 bc
MP29	1.83 bc	0.67 bc	1.33 a	0.33 b	1.70 b	9.49 ab
MP32	1.67 bc	1.33 ab	0.67 ab	0.00 b	1.27 bcd	9.77 a
MP24	0.67 d	1.33 ab	0.33 b	0.00 b	0.87 d	9.31 ab
MP23	1.00 cd	1.33 ab	1.33 a	1.67 a	2.87 a	8.40 c
Control	3.83 a	0.33 c	0.00 b	0.00 b	0.90 d	9.78 a
ANOVA(P=0.05)	0.92	0.71	0.71	0.64	0.58	0.43

*MP isolates: Thick: MP120, MP30, MP218, and MP210, Thin: MP29, MP32, MP24, and MP 23. ** Hypocotyls discoloration rated on a scale of 1-4, where 1 ≤ 5%, 2 = 2 to 10%, 3 = 11 to 50%, and 4 ≥ 50% on the 28th d of planting. Index = ((N·1) + (Sl·2) + (Mod·3) + (Sev·4))/5. Numbers under each hypocotyls discoloration rating scale indicates the average number of plants with that symptom. Disease index consider all symptoms together. ***Plant height measured on the 28th d of planting using ruler. ****Comparisons of mean hypocotyls discoloration rating. Numbers not followed by the same letter (s) are significantly different (P≤0.05) according Fisher least difference (LSD).

Fig. 3: Comparison of soybeans stand count and height after 28 days of planting: (A) = Pot infested with *M. phaseolina* isolate MP210, (B) = Non-infested pot (control).

IV. CONCLUSION

Pathogenicity of eight isolates of *M. phaseolina* was successfully studied by evaluating the plant stands, hypocotyls discoloration, and plant height. Among the eight isolates used in this study, MP24 was highly virulent or pathogenic on the soybeans plants. MP24 showed the highest isolation frequency and greatest reduction in plant stand. The high disease index of MP23, MP29, and MP218 suggest that these isolates are also pathogenic on soybeans. Other isolates, MP30, MP210, MP32, and MP120 showed minor symptoms of the charcoal rot disease. The thick and thin isolates did not vary in pathogenicity overall but individual isolates showed differences in pathogenicity. The low temperatures measured in pots (10.4 to 26.9 ^{o}C) compared to normal (28-35 ^{o}C) could have impacted the disease levels of *M. phaseolina* isolates used in this study.

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