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PHENOTYPIC DIVERSITY AMONG ISOLATES OF *Macrophomina phaseolina* AND ITS RELATION TO PATHOGENICITY *

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Abstract

Sixty isolates of *Macrophomina phaseolina*, the cause of charcoal rot, were isolated from different parts of Iran on various plants including cantaloupe, long melon, soybean, cucumber, apricot , rosemary and sesame. Phenotypic characteristics of the isolates were compared by growing on PDA at 35˚C. Colony appearance, growth rate, production and amount of sclerotia and also the relationship between growth rate at 35˚C and size of sclerotia were determined. In a greenhouse study, sclerotia of different phenotypes were mixed with the soil and soybean and cantaloupe plants were used for pathogenicity tests. After eight weeks root colonization was assessed. All treatments were arranged in a randomized complete block design with three replications. The data were analyzed using SPSS software. Isolates were grouped in four phenotypes: fluffy with abundant sclerotia, fluffy with few sclerotia, partially fluffy, and appressed growth. There was a significant difference between fluffy with few sclerotia and other phenotypes. Fluffy isolates with few sclerotia could not colonize soybean and cantaloupe roots. No significant differences were observed among other phenotypes on colonizing cantaloupe and soybean roots.

Keywords: Charcoal Rot, Phenotype, Pathogenicity, *Macrophomina phaseolina*.

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Introduction

Macrophomina phaseolina (Tassi) Goid. is a soil-borne fungus causing the charcoal rot disease on more than 500 plant species from more than 100 families (Mihail, 1992, Wyllie 1988) distributed worldwide .Despite its wide host range, the genus *Macrophomina* contains only one species ,*M. phaseolina* (Wyllie 1993). Recent molecular study using RFLP and RAPD technique showed no variations among isolates from different hosts in restriction patterns of DNA fragments amplified by PCR of ITS region, also confirmed that *M. phaseolina* constitutes a single species (Su *et al*. 2001*).* Variation in morphology and virulence has been reported among isolates of *M. phaseolina* from different geographical regions (Dhingra and Sinclair 1973b) and in various crops including soybean and bean(Dhingra and Sinclair 1973a; Jain *et al*.1973). Similarly, isolates of *M. phaseolina* obtained from different plant species differed in cultural and morphological characters and pathogenicity (Dhingra and Sinclair 1973c). Because of extreme variability, efforts to classify the isolates of *M. phaseolina* have been very difficult.

The generation of *nit* mutantas to determine vegetative compatibility groups in *M.phaseolina* has been attempted but without success (Cloud and Rupe 1991, Edraki, 2007). Media containing chlorate also have been used to differentiate isolates on the basis of their growth morphology (Pearson *et al.*1986). The differences in growth pattern on chlorate medium was correlated with the host from which was recovered and could be used as a marker for the identification of host specific isolates of *M. phaseolina* (Pearson *et al*.1986, 1987). In another study Cloud and Rupe (1991) reported that the growth patterns of *M. phaseolina* on chlorate medium from soil or their respective hosts remained constant throughout the experiment.

The molecular technique has been attempted to investigate the genetic differentiation of *M. phaseolina* in order to classify them into specific groups (Vandermark *et al.* 2000, Jana *et al*. 2005) .They constructed primer from repetitive sequences derived from host genome as molecular markers which could be used for assaying genetic variability in *M. phaseolina* populations.

Following colony morphology studies on PDA medium, Dhingra and Sinclair (1973c) classified the isolates into 3 groups: fluffy, appressed and partially fluffy but did not correlated with pathogenicity. This paper describes the relationship between PDA phenotypes and their pathogenicity irrespective of host origin.

Materials and methods

Fungal isolation

During 2006, root and collar samples were collected from various host plants including cantaloupe, long melon, soybean, cucumber; apricot, rosemary and sesame from different parts of Iran. Each sample was thoroughly washed, surface disinfested with 0.5% NaOCl solution for 1-2 min, rinsed in sterile distilled water (SDW) and blotted dry on sterile paper towel. Infected tissues were cut into 2-3 mm segments and plated on acidified potato dextrose agar (PDA) and incubated at 35^{o}C for 4-6 days. Pure cultures were obtained on water agar by hyphal tip method. Sterile toothpicks were incubated on pure culture grown on PDA for several days for the colonization by the fungus and formation of sclerotia, dried at room temperature under laminar airflow and stored at 4^{o}C (Edmunds 1969).

Morphological and cultural characters

To study cultural characters of each isolate, Petri plates containing PDA were inoculated with a 5 mm agar plug from a 7-day-old culture and incubated at 35ºC in the dark. The growth pattern and amount of sclerotia of each isolate was determined after 7 days. Colony diameter of each isolate was recorded from the second day till the growth completely covered the plate .The dimension of 50 sclerotia of each isolate was recorded . The relationships between sclerotial size, daily growth rate of colony and colony pattern were analyzed using SPSS software (Anova test,$P \leq 0.05$).

Pathogenicity tests

On the basis of phenotypes on PDA, four isolates were selected for pathogenicity test under greenhouse conditions. Sclerotia of the pathogen were produced, using potato dextrose broth (PDB) as reported earlier (Short and Wyllie 1978). A 6 mm agar transferred to 100ml PBD in 50x30 x10 cm sterile plastic container and incubated in the dark at room temperature for 15 days. The growing fungal mats were separated from the culture on

filter paper in Buchner funnel, washed several times with SDW and dried at 40° C for seven days. The dried sclerotia were further crushed using mortar and pestle and passed through 45-53µm screen and sclerotial powder was stored at 4° C until used. The viability of sclerotia was assessed by germination on PDA. A small sample of sclerotia was weighed and suspended in few ml of SDW and the numbers /ml was determined using haemocytometer and converted per gram of sclerotial powder. Local cantaloupe and soybean seeds were surface disinfested for 2-3 min in 0.5% NaOCl, rinsed in SDW and incubated between sterilized moist cheese cloth and incubated at 25°C until germination was resumed. Virgin soil (free from soil borne plant pathogens) was collected from non-cultivated slope in Bajgah (15 km north of Shiraz, clay loam, 2.2% OM, pH=7.9, EC=0.83 ds) was mixed with viable sclerotia at the rate of 50gram dry soil. The final population of the pathogen in infested soil was further assessed using serial dilution on acidified PDA supplemented with TMN (Banihashemi & deZeeuw1969). One Kg of infested soil was apportioned in the bottom of each 1.5 Kg plastic pot and the rest was filled with virgin soil. Eight germinated seeds were sown in each infested and non-infested soils and incubated in greenhouse and irrigated frequently for seedling emergence.Ten days later the number of seedlings per pot was reduced to three and soil moisture was reduced Irrigation was resumed before wilting and greenhouse temperature maintained at 28-35 ° C.

Data collection

Eight weeks after sowing seeds, all plants were carefully removed from the soil blocks and the roots were washed to separate adhering soil particles. Since no visible aerial symptoms could be observed under greenhouse conditions, root colonization was assessed (Cloud & Rupe 1991). Roots were randomly selected, surface disinfested with 0.5% NaOCl for 2-3 min and rinsed with SDW, blotted dry on sterile paper towel. One hundred (from each host×isolate) randomly selected 3-4 mm root segments were plated on acidified PDA supplemented with 500ppm surfactant TMN (Banihashemi & deZeeuw 1969). After 5 days incubation at 35 °C the number of root segments colonized by *M. phaseolina* was recorded. All greenhouse studies were conducted in a randomized complete block design with three replications. Data were analyzed using SPSS software(Anova test, $P \leq 0.05$).

Result

Fungal isolates

In this study,60 isolates of *M. phaseolina* were isolated from different parts of Iran from various plants including cantaloupe (*Cucumis melo* var. *cantaloupensis*), longmelon (*C. melo* var *inodorus*), soybean(*Glycine max*), cucumber (*C. sativus*); apricot (*Prunus armenica*), rosemary (*Rosmarinus officinalis*)and sesame (*Sesamum orientale* (Table 1). In Iran longmelon due to its longer growth period until harvest is more prone to the pathogen due to predisposition by drought and higher summer temperature than cantaloupe which are harvested much earlier. Of sixty isolates recovered in this study, 49 were isolated from cucurbits mainly long melon in Southern parts of Fars Province. Non-cucurbit isolates had been supplied from other sources.

Morphological and cultural characters

All isolates of *M. phaseolina* showed four phenotypes when grown on PDA medium (Table 2): (a) fluffy with abundant sclerotia, (b) fluffy with few sclerotia, (c) partially fluffy and (d). appressed growth (Figs. 1&2).

Average of sclerotial dimesion and daily growth rate of 20 isolates were listed in Table 2. There was significant differences between fluffy isolates with few sclerotia with other phenotypes. Fluffy isolates with few sclerotia produced the smallest sclerotia and minimum growth rate at 35ºC. Most of the isolates had larger sclerotia with higher rates of growth at 35°C. All of the isolates from apricot from Marand in Azarbayejan a rather cold province had lowest numbers of sclerotia.

Pathogenicity tests

The results showed that fluffy isolates with few sclerotia could not colonize soybean and cantaloupe roots. So there was significant differences between fluffy isolates with few sclerotia with other phenotypes (Fig. 3). Under greenhouse conditions *M. phaseolina* hardly produces typical field symptoms. There was no apparent aerial symptomes in infested compared to non-infested soils. There were also different responses among phenotypes on root colonization in soybean and

Table1. Sources of Iranian isolates of *Macrophomina phaseolina* used

Isolate code	Host	Location	Province
BL1 to BL12	Long melon	Borazjan	Fars
LF1 to LF6	long melon	Fassa	Fars
CK1 to CK6	Cantaloupe	Kazeroon	Fars
CM1 to CM7	Cantaloupe	Mamasani	Fars
LD1 to LD2	Long melon	Darab	Fars
LM1 to LM3	Long melon	Maharlou	Fars
SJ1 to SJ2	Sesame	Jiroft	Kerman
RS	Rosemary	Shiraz	Fars
SG1 to SG3	Soybean	Ghaemshahr	Mazandaran
CJ1 to CJ2	Cucumber	Jahrom	Fars
AM1 to AM3	Apricot	Marand	Eastern Azarbayejan

cantaloupe but no significant differences were observed in each host plant.

Discussion

The present study demonstrated variation in morphology, cultural characters and pathogenicity among isolates of *M. phaseolina* recovered from various hosts and geographical regions. Based on morphological features and growth pattern on PDA , the isolates were grouped into four phenotypes. Three phenotypes on PDA medium as fluffy, partially fluffy, and appressed growth have been reported earlier (Dhingra and Sinclair, 1973c, Byadgi and Hedge1985) In the present study all three phenotypes were detected among isolates but the fluffy isolates were categorized further into 2 distinct phenotypic groups, fluffy with abundant sclerotia and fluffy with few sclerotia. Fluffy growth pattern with few sclerotia can be distinguished from other isolates in cultural characters and pathogenicity. They grow very slow on PDA medium and produce very small sclerotia. In addition they were not capable of colonizing soybean and cantaloupe roots. It was reported that isolates producing fewer sclerotia were less pathogenic on cluster bean (Purkayastha *et al.*2004). Chan and Sackston (1973) also reported non- pathogenic isolates of *M. phaseolina* without indicating their sclerotial production. Mihail and Taylor (1995) reported that isolates from the poacae were less pathogenic on some hosts, but did not mention their phenotypes on PDA. Dhingra and Sinclair (1973c) reported the relationship between growth rate and pathogenicity among isolates of *M. phaseolina* from soybean. On the contrary, Sobit and Bensal (1988) reported that there was no relationship between growth rate and pathogenicity among isolates from groundnut. Our results show that there is a relationship between growth rate, sclerotial production and pathogenicity among isolates from different hosts. Isolates with slow growth rate and few sclerotia are non-pathogenic to at least soybean and cantaloupe, a very sensitive plants to *M. phaseolina*.

Other workers classified isolates based on size and shape of sclerotia, cultural characteristics,

Table2. Dimension of sclerotia, PDA phenotypes and daily growth of 20 isolates of *Macrophomina phaseolina*

Isolate code	Growth at 35ºC (mm/day)	Dimension of sclerotia (µm)	PDA phenotype	Host
LF4	2	80-115	Fluffy with few sclerotia	Longmelon
CK16	1.6	85-102		Cantaloupe
AM1	1.5	84-95		Apricot
AM2	1.2	90-102		Apricot
AM3	1.3	62-85		Apricot
CM7	5.2	187-220	Appressed growth	Cantaloupe
LB2	5.1	145-187		Longmelon
SG3	2.1	93-122		Soybean
CJ1	3.7	107-126		Cucumber
LB1	3.9	87-147		Longmelon
LF6	3.9	130-180	Partially fluffy	Long melon
CK7	2.1	105-145		Cantaloupe
LD1	4.5	104-127		Longmelon
LF1	4.3	109-140		Longmelon
SG2	2.7	110-140		Soybean
LF3	5.5	142-167	Fluffy with abundant sclerotia	Long melon
SJ1	5.1	125-136		Sesame
CK2	3.4	110-112		Cantaloupe
SJ2	٥.0	125-127		Sesame
BL9	2.9	145-156		Longmelon

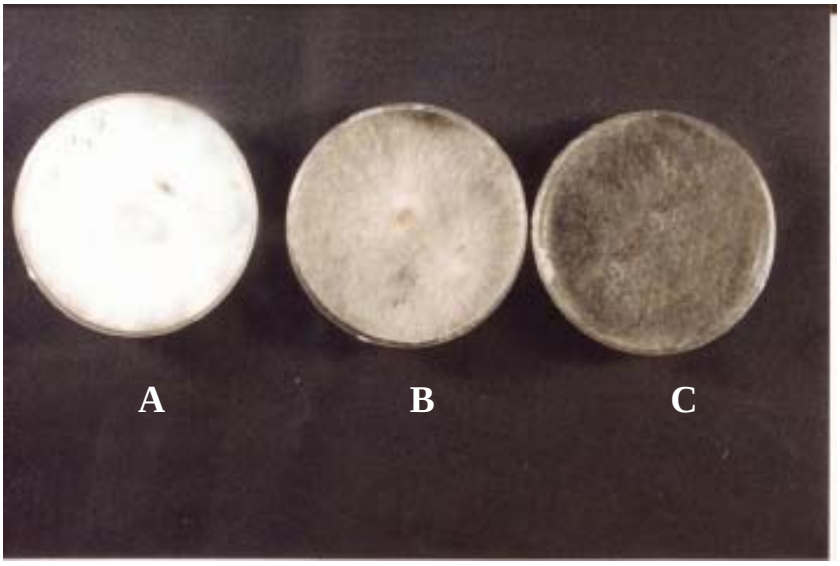

Figure 1. Phenotypes of *Macrophomin. phaseolina* isolates on PDA medium A. Fluffy B.Partially fluffy. C. Appressed growth

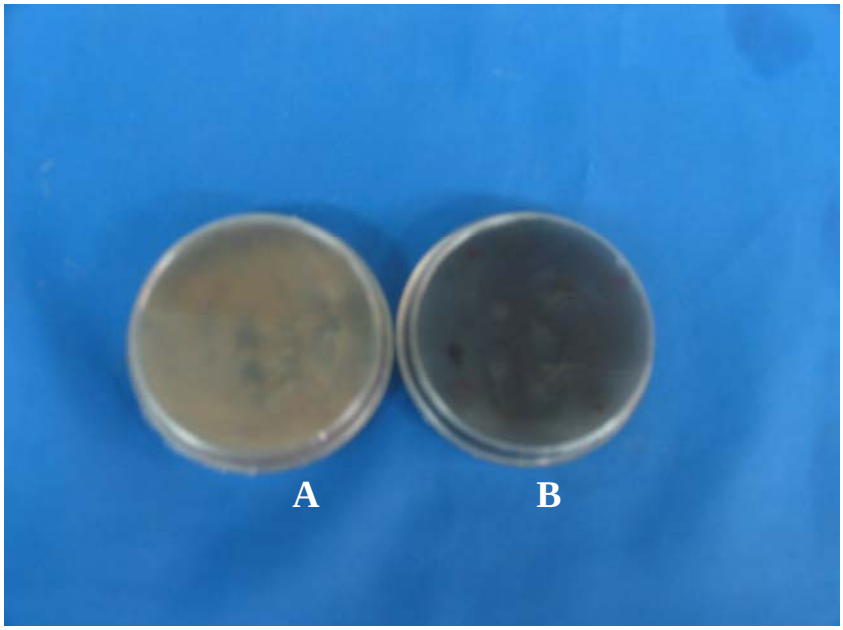

Figure 2. Fluffy phenotypes of *Macrophomina phaseolina* A. With few sclerotia B. With abundant sclerotia.

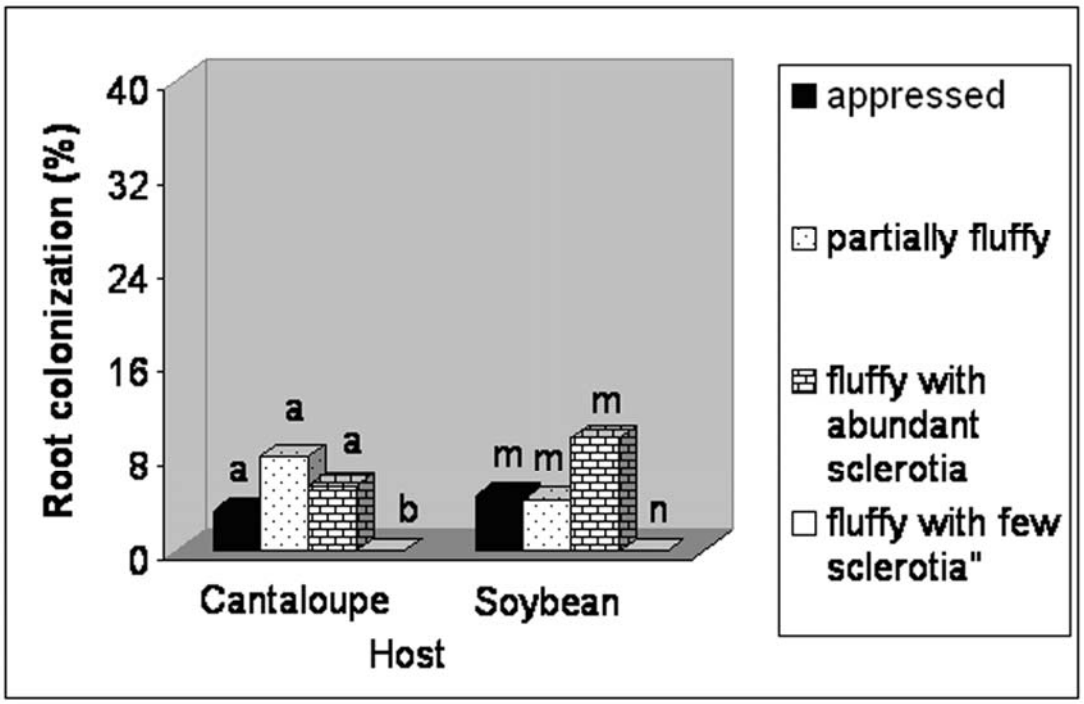

Figure 3. Effect of PDA phenotypes on root colonization of soybean and cantaloupe by *Macrophomina phaseolina.* Letters above bars indicate significance (P≤*0.05*) according to Anova test. Means with the same letter do not differ.

pathogenicity, growth on chlorate medium and molecular criteria without mentioning sclerotial production(Cloud and Rupe 1991, Dhingra and Sinclair 1973c, Jana *et al*. 2005,Pearson *et al.* 1986,1987, Su *et al*.2001). Most of our isolates were obtained from melon roots and collar regions. We found various growth pattern and virulence among the isolates. All of the apricot isolates were non-pathogenic on soybean and melon but majority of the isolates recovered from various hosts were capable to colonize soybean and cantaloupe that considered to be susceptible to the pathogen. *M. phaseolina* is not a pathogen of trees and most perennial plants. It was not surprising that apricot isolates could not colonize roots of melon and soybean. Our results showed that host is the determinant factor on the variability of the fungus. Of 49 isolataes of *M. phaseolina* recovered from melons only two isolates were not pathogenic but most were highly pathogenic and produce larger sclerotia and higher growth rate at 35^{o} C.

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