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Morphological and Pathogenic Variability among *Macrophomina phaseolina* Isolates Associated with Maize (*Zea mays*) in Punjab-Pakistan

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Abstract

Macrophomina phaseolina (Tassi) Goid. is a serious pathogen of charcoal rot disease in the maize plant recently in Punjab, Pakistan. In order to initiate monitoring of this pathogen, 24 isolates of *M. phaseolina* from four districts of Punjab province of Pakistan were collected to assess the variations in morphology and virulence on the maize plant. Regarding to the geographic origins, significant differences were observed in radial mycelial growth, sclerotial size, sclerotial population per unit area and virulence among the isolates. Thirteen isolates were rated as fast growing (77.10–88.10 mm), seven as medium growing (66.00–77.00mm), and the rest as slow growing (54.00–65.00 mm). Eight isolates produced large sized sclerotia (> 45µm), eleven produced medium sized (40–45 µm), and the remaining five isolates produced small sized sclerotia (< 40 µm). Three isolates exhibited abundant sclerotial population (91.10–108.10), six exhibited average sclerotial population (74.00–90.00), while the rest of the isolates exhibited low sclerotial population (56.00–73.00)/9mm disc of culture. Nine isolates of diverse origin proved to be highly virulent; eight isolates were regarded as moderately and seven fungal isolates appeared to be least virulent against maize cultivars. The discussion is about these morphological and pathogenic variations in various isolates of *M. phaseolina* as base line information for disease management and development of resistant cultivars against charcoal rot disease.

Keywords: Charcoal rot; Sclerotial size; Mycelial diameter; Virulence

Introduction

Maize is an important cereal crop of Pakistan (after wheat and rice) and is cultivated on an area of 0.96mha, producing 1.67 mt with an average yield 1730 kg ha^{-1}(GOP, 2013). The crop is grown in a wide range of agro-ecological zones. The average yield of maize in Pakistan is very low as compared to many other countries. The low yield of maize in Pakistan can be attributed to legions of biotic and abiotic constraints. Among biotic factors, diseases are the most dominant. Depending upon the crop variety, the losses due to diseases to cereals crops have been estimated to be as high as 44 percent (Bashir and Malik, 1988). Maize is vulnerable to about 112 diseases in the world (AGBIOS). Among these, charcoal rot caused by *Macrophomina phaseolina* (Tassi) Goid. is of prime importance in reducing crop yield. Charcoal rot infects plants at almost all growth stages and the symptoms appear as dark lesions first on the seed followed by the seedling death due to blockage of the xylem vessels. The pathogen produces red to brown lesions on roots and stems with production of dim mycelia and black micro-sclerotia, consequently defoliation, wilting (Abawi and Pastor-Corrales, 1990) and perishing of maize plants (Bashir and Malik, 1988). *M. phaseolina* is a soil and seed-borne pathogenic fungus, produces cushion shaped black sclerotia (Wheeler, 1975), and is found on more than 500 hosts including several legume and cereal plants (Dhingra and Chagas, 1981; Sinclair, 1982). Several host plants lend the great variability in the morphology and the pathogenicity among the fungal isolates and from different parts of the same plant (Beas-Fern'andez *et al*., 2006). Its prevalence can be enhanced by different physiological and ecological factors such as low moisture contents, high aerial temperature and soil heat (Papavizas, 1977; Dhingra and Sinclair, 1978). Disease severity is correlated with viable sclerotia present in the soil. Among the main management strategies, use of cultivars resistant to *M. phaseolina* has gained wide popularity and acceptance amongst farmers as application of fungicides is often intertwined with potential hazards to humans and the environment. Furthermore, resistant cultivars out strip fungicides in various aspects and thus emphasis are being laid on the development of new resistant germplasm. However, it has been observed that control measures against the pathogens has become complicated and even ineffective due to the variability among populations of the same pathogen in different areas.

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There are reports from different parts of the World that populations of *M. phaseolina* showed major morphological (Mayek-P´erez *et al*., 1997), physiological (Mihail and Taylor, 1995), pathogenic (Mayek-P´erez *et al*., 2001; Aboshosha *et al*., 2007) and genetic variations (Chase *et al*., 1994; Mayek-P´erez *et al*., 2001; Jana *et al*., 2005; Reyes-Franco *et al*., 2006; Farhana *et al*., 2013). These variations act as conducive tools to help the pathogen to adapt and survive in diverse environments. A thorough knowledge of pathogenic and morphological variability of *M. phaseolina* is thus essential to design disease management strategies for different areas of the country by breeding resistant cultivars. At present no information on the variability among *M. phaseolina* isolates is available from Pakistan. Hence, we investigated morphological and pathogenic variability among 24 isolates of *M. phaseolina* infecting maize, collected from four major maize growing districts of Punjab. Efforts were made to characterize the fungus population based on its pathogenic variability (Karunanithi *et al*., 1999) and morphological characteristics (Beas-Fern'andez *et al*., 2006). It has also been determined whether morphological variations among *M. phaseolina* isolates have any relationship with the pathogenic variability.

Table 1: Isolates of *Macrophomina phaseolina* collected from maize plants from different districts of Punjab

Isolates codes	District	Location
K-1	KASUR	Bheala
K-2		Garewala
K-3		Talwandi
K-4		Atari
K-5		Noor Pur
K-6		Khudian Khas
O-1	OKARA	Aktharabad
O-2		Ahmadabad
O-3		Basir Pur
O-4		Haviali Lakha
O-5		Hujra Shah Muqeem
O-6		Renala Khurd
S-1	SAHIWAL	Kassowal
S-2		Chak 42/12 L
S-3		Chak 21/11 L
S-4		Chak 44/12 L
S-5		Adde Pur
S-6		Bashera
P-1	PAKPATAN	Chak 17 SP
P-2		Jaman Bodla
P-3		Bunga Hayat
P-4		Malka Hans
P-5		Chak 50 SP
P-6		Chak 30 SP

Materials and Methods

Collection of Fungal Isolates

A total of 24 isolates of *M. phaseolina* were collected from four major maize producing districts (Table 1) of Punjab province of Pakistan. Samples of stems bearing micro-sclerotia of the fungus and characteristic symptoms of charcoal rot were collected from the farmers' fields. The diseased samples were first packed in paper bags and then in 15 × 20 cm polyethylene bags, properly labeled, brought to the laboratory and stored at 4ºC until processed for identification.

Isolation, Purification and Identification of *M. phaseolina*

The fungus was isolated from stem bark tissues of maize bearing fungal sclerotia and showing characteristic charcoal rot symptoms. The samples were cut into small pieces (5–10 mm) and surface sterilized with 1% sodium hypochlorite for 2 min and then rinsed thrice in sterilized distilled water. The pieces were placed on Chloroneb Mercury Rose Bengal Agar (CMRA) medium (Meyer *et al*., 1973) in petri dishes (90 mm dia.) and incubated in dark at 27±2ºC for 7 days. A small portion of the fastest growing mycelium of *M. phaseolina* was taken from the periphery of the petri dishes, spread onto other petri dishes containing glucose agar medium (glucose, 20 g; agar, 20 g; and water, 1 L) and incubated in the dark at 27± 2ºC for 7 days for sclerotial formation. A small portion of the colony having sclerotia was taken up into a drop of sterilized water and agitated with a sterilized needle to separate the sclerotia from the mycelia. Sclerotia were then transferred to 90mm diameter petri dishes containing CMRA medium. Colonies appearing from single sclerotium were again transferred to CMRA medium in 90mm petri plates, incubated as mentioned above and identified by following Barnett and Hunter (1972)

Storage of Pure Cultures of *M. phaseolina*

The purified culture (5mm disc) from each isolate growing on CMRA was transferred to 10 mL culture tubes and incubated in the dark at 27±2ºC for 6 days, until the slant of CMRA was covered with a dense sclerotial layer of the fungal culture. The culture tubes were labeled and stored at 4ºC.

Multiplication of *M. phaseolina*

Rice seeds were water-soaked overnight, air-dried under room temperature and placed in conical flasks (250 mL ca.). The mouth of each flask was plugged with cotton wool, wrapped with aluminum foil and autoclaved at 121ºC (15psi) for 20 min. After cooling, the seeds in flasks were inoculated with 4 mm mycelial plugs from a 7day old culture of *M. phaseolina* and incubated at 27±2ºC for 15 days. The flasks were shaken at alternate days for uniform colonization of the grains. The inoculum thus produced was used in a field trial to infest the soil.

Preparation of Toothpicks

Tooth picks were boiled in water for two h to remove toxic substances that may inhibit the fungal growth. After boiling, they were washed thoroughly in fresh tap water. When toothpicks became dry, about ten of them were placed in each 100 mL flask and was sterilized at 1.1 kg/cm² for 20 min. The potato broth was sterilized at 1.1 kg/cm² for 15 min in 100 mL flasks. After cooling, respective *M. phaseolina* isolates were inoculated in two 100 mL flasks and a rich suspension was made. This suspension was poured into two tooth pick flasks to cover lower one third of the toothpicks (Young, 1943).Thus, for each isolate, two separate sets of tooth picks were prepared. The flasks were incubated for seven days at 30°C, allowing the tooth picks to be covered with the fungal growth and these were then ready for inoculation. Tooth picks were introduced at the second internode 15 days after flowering during drought stress conditions.

Determination of Morphological Variability

Morphological variability among 24 isolates of *M. phaseolina* was studied on the basis of the following parameters

Radial Growth

For studying variability in radial growth, the isolates were grown on Potato Dextrose Agar (PDA) medium. The autoclaved PDA (15 mL) was poured in petri plates, allowed to solidify and inoculated in the center of petri dishes with a 5mm plug from the actively growing culture of each isolate of the fungus. The plates were incubated at 27±2ºC for 7 days. Each isolate was replicated three times. After the stipulated period, the growth of each isolate was measured in terms of colony diameter and their means were computed. On the basis of radial growth, the isolates were categorized as fast (77.10–88.10 mm) medium (66.00–77.00 mm) and slow (54.00–65.00 mm) growing.

Sclerotial Size

Slides from 7dayold pure cultures of *M. phaseolina* isolates were prepared and examined under a microscope with ocular micrometer. Size of 10 randomly selected sclerotia was measured and means were calculated. The isolates were classified as large (>45μm), medium (40–45 μm) and small (<40μm) sized sclerotia.

Sclerotial Population

Sclerotial population in different isolates was calculated by observing the 9 mm disc of seven day old culture of *M. phaseolina* under microscope. The isolates were classified as abundant (91.10–108.10), average (74.00–90.00) and low (56.00–73.00) sclerotial population.

Determination of Pathogenic Variability

All the 24 isolates were inoculated to a popular maize cultivar MMRI-yellow. The completely randomized design with three replications for each of the twenty four isolates was adopted. In the field experiment, care was taken to see that the area chosen was homogenous for soil factors e.g. soil moisture, soil temperature and soil fertility. Each of the isolates was inoculated by toothpick method at the second internode, 15 days after flowering. Observations were recorded after 110 days of sowing.

Statistical Analysis

Analysis of variance (ANOVA) technique was used for data analysis with GenStat package 2009 version 12.1.0.3278 (http://www.vsni.co.uk). Means were compared by Fisher's protected least significant difference test at $P \leq 0.05$.

Results

Morphological Variability among *M. phaseolina* Isolates

Significant variations were observed in the morphological parameters among 24 isolates of *M. phaseolina* collected from four districts of Punjab.

Radial Growth

Significant differences among 24 isolates of *M. phaseolina* collected from four districts were observed on the basis of radial growth. The individual radial growths of all the isolates are shown in Table 2. The individual average radial growth of 24 isolates of *M. phaseolina* ranged from 59.00 to 88.00 mm 7 days after incubation. Maximum colony diameters of 88.00 and 87.00 mm were observed in case of isolate O-5 (Hujra Shah Muqeem) and O-3 (Basir Pur) graded to be the fast growing, while isolates P-1 (Chak 17 SP), S-5 (Adde Pur) and S-6 (Bashera) showed the minimum radial growth and were rated as slow growing. Thirteen isolates showed radial growth between 77.01–88.01 mm and were rated as fast growing while the growth of four isolates were found between 55.01–66.01mm and were categorized as slow growing. The rest of the isolates showed growth between 66.01–77.01 mm and hence were classified as medium growing.

Sclerotial Size

Significant variations were also observed among these isolates regarding the size of their sclerotia. Maximum sclerotial size was observed in case of isolates O-5 and S-4 with 55.00 and 51.00μm diameter, respectively, while the sclerotia of isolates O-4 and P-6 were found to be the smallest in size. The individual average sclerotial size of isolates ranged from 37.00 to 55.00 μm (Table 2). The size

Table 2: Morphological variations among different isolates of *M. phaseolina*

Isolates code	Radial growth (mm)	Sclerotial size (μm)	Sclerotial Population (7 days after incubation)
K-1	81.33 A-E	40.00 E-J	69.00 EFG
K-2	82.33 A-D	45.33 B-F	72.00 DE
K-3	84.33 ABC	41.00 D-J	64.00 F-I
K-4	86.00 AB	45.00 B-F	79.00 BCD
K-5	87.00 A	43.00 C-H	75.00 CDE
K-6	81.00 A-E	47.00 BCD	72.00 DE
O-1	81.00 A-E	43.00 C-H	72.00 DE
O-2	80.33 A-F	41.00 D-J	71.00 EF
O-3	87.00 A	47.00 BCD	72.00 DE
O-4	83.00 ABC	38.33 G-J	68.00 E-H
O-5	88.00 A	55.00 A	64.00 F-I
O-6	86.00 AB	49.33 ABC	85.00 B
S-1	70.33 GH	35.67 IJ	61.00 HI
S-2	72.33 E-H	42.00 D-I	85.00 B
S-3	73.33 D-H	35.00 J	101.00 A
S-4	77.33 B-G	46.00 B-E	82.00 BC
S-5	60.00 IJ	51.00 AB	59.00 I
S-6	64.33 HIJ	41.33 D-J	68.00 E-H
P-1	59.00 J	46.00 B-E	58.00 I
P-2	64.00 HIJ	44.00 C-G	82.00 BC
P-3	69.33 GHI	42.33 D-H	72.00 DE
P-4	76.00 C-G	39.00 F-J	103.00 A
P-5	69.00 GH	42.33 D-H	108.00 A
P-6	71.33 FGH	37.00 HIJ	63.00 GHI
LSD (5%)	9.51	6.616	7.231

Means sharing similar letter in a column are statistically non-significant (P>0.05).

a) Categorization of *M. phaseolina* isolates on the basis of radial growth

Category	Number	Isolates
Fast growing (77.10-88.10 mm)	13	K-1, K-2, K-3, K-4, K-5, K-6, O-1, O-2, O-3, O-4, O-5, O-6,S-4
Medium growing (66.00–77.00 mm)	7	S-1, S-2, S-3,P-4,P-3,P-5, P-6
Slow growing (54.00-65.00 mm)	4	S-5, S-6, P-1, P-2,

b) Categorization of *M. phaseolina* isolates on the basis of size of sclerotia

Category	Number	Isolates
Large sized >45 µm	8	K-2, K-6, O-3, O-5, O-6, S-4, S-5, P-1
Medium sized 40-45 µm	11	K-1, K-3, K-4, K-5, O-1, O-2, S-2, S-6, P-2, P-3, P-5
Small sized <40 µm	5	0-4, S-1, S-3, P-4, P-6

c) Categorization of *M. phaseolina* isolates on the basis of number of sclerotia

Category	Number	Isolates
Abundant population (91.10-108.10)	3	S-3, P-4, P-5
Average population (74.00-90.00)	6	K-4,K-5,O-6,S-2,S-4,P-2
Low population (56.00-73.00)	15	K-1,K-2,K-3,K-6,O-1,O-2,O-3, O-4, O-5, S-1, S-5, S-6, P-1,P-3, P-6

of sclerotia of 8 isolates were above 45μm and classified as large sized while 5 isolates with sclerotial size less than 40μm were rated as small sized. The remaining 11 isolates ranged between 40 and 45 μm in sclerotial size and were categorized as medium sized (Table 2 a, b, c).

Sclerotial Population

The number of sclerotia per 9 mm disc varied from 58 to 108, three days after incubation. Among the isolates, S-3, P-4 and P-5 isolates produced comparatively large number of sclerotia, while the minimum number was observed in S-5 and P-1 (59 and 58, respectively).

Pathogenic Variability among *M. phaseolina* isolates

Highly significant differences were observed among isolates, varieties and their interactions. Significant variations in pathogenicity were found among 24 isolates of the fungus when tested against maize cultivar. Nine isolates, namely, K-1, K-2, K-4, K-5, K-6, O-2, O-4, P-2 and P-4, were designated to be highly virulent against MMRI yellow with an average lesion length of spread ranging from 10.00 to 15.43, while seven isolates, namely, O-3, S-1, S-4, S-5, P-1, P-5 and P-6, showed least pathogenic reaction against MMRI yellow with average length of spread ranging from 1.43 to 5.33 and the remaining 8 isolates proved to be intermediate in their virulence (Table 3).

Discussion

M. phaseolina, a soil as well as seed-borne fungus, induces charcoal rot in different crops including maize. In the present studies, 24 isolates of *M. phaseolina* from different districts of Punjab province of Pakistan showed variations in different morphological traits such as radial growth, sclerotial size and

Table 3: Differential response of selected maize cultivar against various isolates of *M. phaseolina*

Isolates	MMRI yellow Lesion Length (cm)
K1	12.13 B
K2	12.03 B
K3	6.20 GH
K4	10.73 CD
K5	15.43 A
K6	10.40 CD
O1	7.47 F
O2	10.23 D
O3	4.50 IJ
O4	12.23 B
O5	6.40 FGH
O6	8.80 E
S1	3.40 J
S2	6.47 FGH
S3	7.30 FG
S4	5.33 HI
S5	3.50 J
S6	6.00 H
P1	5.33 HI
P2	10.00 D
P3	6.30 GH
P4	11.47 BC
P5	1.43 K
P6	3.97 J
LSD (5%)	1.145

population as well as in virulence. The variations in morphology might be due to difference in the

temperature, moisture, soil types and other edaphic factors of various districts of Punjab. Morphological variability has also been reported by many workers in terms of growth, color, sclerotial production and chlorate sensitivity among different isolates of *M. phaseolina* on different hosts (Dhingra and Sinclair, 1973; Dhingra and Sinclair, 1978; Riaz *et al*., 2007) which corroborated our findings. Similarly, variations in morphology and pathogenicity amongst *M. Phaseolina* isolates taken from diverse hosts as well as from different parts of the same host have also been observed by (Beas-Fernández *et al*., 2006). However, in the present studies, no relationship was found among the morphological characters and pathogenicity of the isolates. Among the highly virulent isolates of *M. phaseolina*, namely, K-1, K-2, K-4, K-5, K-6, O-2, O-4, P-2 and P-4, against maize, not all the isolates were fast growing (radial growth 77.10-88.10 mm) or large sized sclerotia (<45μm) or abundant sclerotial population (< 80).Of these highly virulent isolates, seven (K-1, K-2, K-4, K-5, K-6, O-2 and O-4) were the fast growing, P-2 and P-4 were slow and medium growing, respectively. Similarly, isolates K-2and K-6 produced large sized sclerotia, while K-1, K-4, K-5, O-2 and P2 produced medium sized sclerotia, P-4 and O-4 small sized sclerotia. Likewise, P-4 had the abundant population of sclerotia while K-5, K-4 and P-2 had average population and K-1, K-2, K-3, O-2 and O-4 was low population of sclerotia. Similar pattern was observed in moderately and least virulent isolates. Confirmatory and contradictory findings in this regard have also been reported by others. A close linkage between virulence and growth was reported by Rayner (1991). Purkayastha *et al*. (2004) also found relationship between morphological variations and pathogenicity. On the other hand Dhingra and Sinclair (1978) and Beas-Fern'andez *et al*., (2006) reported that pathogenicity had no relation with size and sclerotial population. The pathogenic fungus, *M. phaseolina*, has a broad host range and exists in two asexual forms which ensure its survival better (Dhingra and Sinclair, 1978; Cloud and Rupe, 1988; Mihail and Taylor, 1995). Some workers also related variability to the phenomena of host specialization and survival of fungus in different asexual forms. Su *et al*., (2001) found horde specialism in maize on the basis of pathogenic, genetic and physiological differences. Similarly, Cloud and Rupe (1988) analyzed host specialization in soybean. This mechanism takes long time to establish within a specific host. Mihail and Taylor, (1995) suggested that, due to heterogenic nature of *M. phaseolina*, categorization into distinct subgroups based upon pathogenicity and morphology could not take place. 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 (Fuhlbohm, 1997; Mayek-P´erez *et al*., 1997; Almeida *et al*., 2003; Jana *et al*., 2003; Reyes-Franco *et al*., 2006; Allaghebandzadeh *et al*., 2008). As the pathogen has no sexual phase, genetic diversity is produced either by fusion of vegetative cells or by parasexual recombination between nuclear genes (Carlile, 1986). In nature genetic variability improves survival of a fungus (Rajkumar *et al*., 2007).It is quite evident that variability in morphology, physiology, genetics, pathogenicity and so forth is imperative for the fungus to have better adaptation in response to diversified environmental conditions. It also leads to host plant resistance, development of resistant varieties of different crops against disease and implementation of new disease controlling strategies (Mayek-P´erez *et al*., 1997; Purkayastha *et al*., 2006).

Conclusion

The determination of variability among *M. phaseolina* isolates is fundamental to guide the development of appropriate strategies for disease management according to different districts. As there are no reports about the determination of morphological and pathogenic variability, the present studies for the first time provide information on the variability of *M. phaseolina* in major maize growing areas of Punjab. Results will be useful in developing integrated strategies for the management of charcoal rot problem.

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