RESEARCH

PATHOGENIC AND GENETIC DIVERSITY AMONG IRANIAN ISOLATES OF *Macrophomina phaseolina*

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Charcoal rot, caused by *Macrophomina phaseolina* (Tassi) Goid., is an economically important disease of oilseed plants in Northern Iran. Seventy isolates of *M. phaseolina* were obtained from different hosts, including soybean (*Glycine max* L.), and sunflower (*Helianthus annuus* L.) in the northern oilseed planting regions of Iran. RAPD-PCR amplification profiles, by using of six random OPA primers (kit A) showed polymorphisms among the isolates. The primer OPA-13 (5'-CAGCACCCAC-3') amplified the genomic DNA of all isolates of *M. phaseolina* producing 4-12 bands of sizes between 0.25 and 2.3 kb. Unweighted pair-group method with arithmetic mean (UPGMA) analysis classified the isolates into the nine major groups with 64% similarity. Pathogenicity of isolates was evaluated at seedling stage of soybean, sunflower, and maize (*Zea mays* L.) plants under *in vitro* conditions. None of the isolates were pathogenic on corn, while all of the isolates infected soybean and sunflower seedlings. Isolates were more virulent on soybean than sunflower. The disease index on sunflower and soybean varied between 19-24 and 27-30 respectively. These results indicated a significant pathogenic and genetic variability within the Iranian isolates of *M. phaseolina*. Cultivation with crop rotation was probably tended to induce less diversity of the pathogen isolates.

Key words: Charcoal rot, oilseed plants, disease index.

Macrophomina phaseolina (Tassi) Goid. causes charcoal rot disease on more than 500 plant species throughout the world (Srivastava *et al*., 2001). The disease has caused economically important losses on oilseed plants, especially on bean (*Phaseolus vulgaris* L.), corn (*Zea mays* L.), cotton (*Gossypium herbaceum* L.), sesame (*Sesamum indicum* L.), sorghum (*Sorghum bicolor* [L.] Moench), soybean (*Glycine max* L.), and sunflower (*Helianthus annuus* L.). *Macrophomina phaseolina* is the main fungal pathogen affecting sunflower in Egypt (Purkayastha *et al*., 2006). Estimates of yield reduction due to charcoal rot in the USA were 1.98, 0.28, and 0.49 million metric tones in 2003, 2004, and 2005, respectively. Differences in soybean yield suppression due to charcoal rot among years are due to differences in the environment with yield suppression due to this disease increasing with drought (Wrather and Koenning, 2006). Although only one species is recognized within the genus *Macrophomina*, great variability in morphology and pathogenicity was recognized among isolates from different hosts (Fernandez *et al*., 2006). Subdivisions of this mono specific genus are often based on virulence to a particular set of differential host cultivars that vary in disease resistance. Although useful in phytopathology, these tests bear inherent problems including dependence on different host cultivars, influence of numerous variables such as cropping systems, tillage practices, temperature, irrigation, and drought stress (Kendig *et al*., 2000; Mayek-Pérez *et al*., 2002; Amusa *et al*., 2007; Wrather *et al*., 2008).

The genetic diversity of *M. phaseolina* could favor its survival and adaptation to variable environments, because significant morphological (Mayek-Pérez *et al*., 2001), physiological (Mihali and Taylor, 1995), pathogenic (Mayek-Pérez *et al*., 2001; Su *et al*., 2001), and genetic (Vandemark *et al*., 2000; Mayek-Pérez *et al*., 2001; Su *et al*., 2001; Almeida *et al*., 2003; Jana *et al*., 2003; Aboshosha *et al*., 2007) diversity has been reported. However, no clear evidence to suggest formae specials subspecies or physiological races has been reported, although Su *et al*. (2001) suggested host specialization in the genus. Little information is available on the genetic diversity of the Iranian isolates of *M. phaseolina* that infect soybean, sunflower, and sesame in northern Iran. Techniques such as RAPD markers are useful for measuring genetic relationships and variations within

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and among populations of *M. phaseolina*. RAPD analysis has been used to study genetic diversity in populations of *M. phaseolina* in different countries like Australia (Fuhlbohm, 1997), Brazil (Almeida *et al*., 2003), and India (Jana *et al*., 2003).

The objective of this study was the determination of pathogenic and genetic variability within the isolates of *M. phaseolina*, obtained from different oilseed plants in the northern planting regions of Iran.

MATERIALS AND METHODS

Fungal isolates

Seventy samples were collected from the infected stems and roots of soybean and sunflower plants from Mazandaran Province in northern Iran, during 2006 to 2007 (Table 1). Each root or stem was thoroughly washed and dried at room temperature. Four small 0.3 cm epidermal section were excised from the each sample and sterilized in 0.8% NaOCl (1 min) and washed in sterile water for 1 min. Tissues were placed on potato dextrose agar (PDA) plate followed by incubation at 28 ± 1 °C in the darkness for 4 d. Purification was developed by single microesclerotium culture and maintained on PDA at 28 ± 1 °C.

Table 1. Characteristics of *Macrophomina phaseolina* isolates used in this study.

Isolates	Hosts	Geographic origin	Genetic group	Isolates	Hosts	Geographic origin	Genetic group
1	Soybean	Joybar	1	36	Soybean	Neka	3
2	Soybean	Joybar	7	37	Soybean	Neka	3
3	Soybean	Gaemshar	1	38	Soybean	Neka	3
4	Soybean	Gaemshar	7	39	Soybean	Neka	3
5	Sesame	Behshar	1	40	Soybean	Neka	3
6	Sesame	Behshar	5	41	Soybean	Neka	3
7	Soybean	Joybar	2	42	Soybean	Neka	3
8	Soybean	Joybar	5	43	Soybean	Neka	5
9	Soybean	Kiakola	1	44	Soybean	Neka	8
10	Sunflower	Gaemshar	9	45	Soybean	Neka	6
11	Soybean	Sari	1	46	Soybean	Neka	2
12	Soybean	Sari	1	47	Soybean	Neka	2
13	Soybean	Joybar	4	48	Soybean	Neka	2
14	Soybean	Kiakola	3	49	Soybean	Neka	2
15	Soybean	Joybar	3	50	Soybean	Behshar	3
16	Soybean	Kiakola	8	51	Soybean	Behshar	5
17	Soybean	Kiakola	4	52	Soybean	Behshar	5
18	Soybean	Sari	8	53	Soybean	Behshar	2
19	Soybean	Kiakola	2	54	sesame	Neka	4
20	Soybean	Sari	3	55	sesame	Neka	2
21	Soybean	Sari	2	56	Soybean	Behshar	1
22	Soybean	Sari	3	57	Soybean	Behshar	3
23	Soybean	Sari	2	58	Soybean	Behshar	1
24	Soybean	Sari	5	59	sesame	Neka	3
25	Soybean	Sari	2	60	Soybean	Behshar	1
26	Soybean	Sari	3	61	Soybean	Behshar	1
27	Soybean	Sari	2	62	Soybean	Behshar	1
28	Soybean	Sari	2	63	Soybean	Behshar	3
29	Soybean	Sari	3	64	Soybean	Behshar	6
30	Soybean	Sari	3	65	Soybean	Behshar	8
31	Soybean	Neka	3	66	Soybean	Behshar	6
32	Soybean	Neka	8	67	Soybean	Behshar	6
33	Soybean	Neka	3	68	Soybean	Behshar	3
34	Soybean	Neka	3	69	Soybean	Behshar	3
35	Soybean	Neka	3	70	Soybean	Behshar	6

DNA extraction

Isolates were grown in liquid potato-dextrose broth (PDB) at 28 °C for 10 d in the dark mycelia from PDB were harvested by centrifuging at 10 000 rpm for 15 min. The pellet was washed in sterile water, lightly squeezed in filter paper and lyophilized. Freeze dried mycelium was crushed in liquid nitrogen and treated with hexadecyltrimethyl-ammonium bromide (CTAB) extraction buffer (50 mM Tris-HCl, pH 8.0, 700 mM NaCl, 10 mM EDTA, 10% CTAB). Extracted genomic DNA was purified by the phenol-chloroform method and precipitated with ethanol. The quality of the genomic DNA was checked on 0.8% agarose gel and stored at -40 °C for further use.

PCR amplification

DNA from 70 isolates was amplified by the RAPD method (Williams *et al*., 1990), using six random primers, including OPA-09, OPA-10, OPA-11, OPA-12, OPA-13, and OPA-14. PCR reactions were carried out in final volumes of 25 μL containing 10 mM of Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 1.5 unit of Taq polymerase, 0.2 mM of each deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), 0.4 μM of primers and 30 ng of template DNA. Amplification was carried out using initial denaturation at 95 °C for 4 min followed by 45 cycles at 94 °C for 1 min, 35 °C for 1 min, 72 °C for 2 min and final extension step at 72 °C for 5 min. The amplified DNA fragments were separated by electrophoresis on agarose gel (1.4%) in 0.5 × TAE buffer, stained with ethidium bromide (0.75 μg mL^{-1}) and photographed under UV light (302 nm). All experiments were repeated at least three times. Only the bands that were presented in at least three replicates were scored and the others were omitted.

Data analysis

RAPD banding patterns were scored present (1) or absent (0) for each isolate. Polymorphism in *loci* with faint bands was not scored. Data analysis was performed with the NTSYSpc statistical software version 2.02e (Rohlf, 1997). Similarity coefficients for all possible pairs of isolates were estimated using the Jaccards coefficient based on their fingerprintings. A dendrogram was constructed from the similarity coefficient data by the unweighted pair-group method with arithmetic mean (UPGMA) clustering algorithm.

Pathogenicity study

Pathogenicity test of a subset of 24 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 randomized complete block design. Randomly about one third of the isolates from each genetically group (total 24 isolates) were selected for this test. Each treatment (isolate) had three replicates and each replicate 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-d-old colonies of each *Macrophomina* isolates on PDA plates and incubated at 30 °C in the dark. Pathogenicity was recorded 6 d after incubation, 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, and 5 = seed infected and not germinated.

The disease index was calculated by multiplying the number of seeds by the degree of disease severity (Manici *et al*., 1992). We used completely randomized design (CRD) for ANOVA, and the mean comparison also was Duncan's multiple range test (DMRT) and for statistical analysis we use Mstat-c software (Freed *et al*., 1991).

RESULTS AND DISCUSSION

RAPD markers were polymorphic among the 70 isolates studied. The primers OPA-13 and OPA-9 amplified the highest number of polymorphic *loci* (Figure 1). The highest number of polymorphic amplicons was obtained from the isolate 13. Data were analyzed with UPGM by using NTSYS ver. 2.02e, and a dendrogram was produced that could separate 70 isolates into nine groups at an arbitrary level of 64% similarity. Genetic similarity coefficients ranged from 0.52 to 1 (Figure 2).

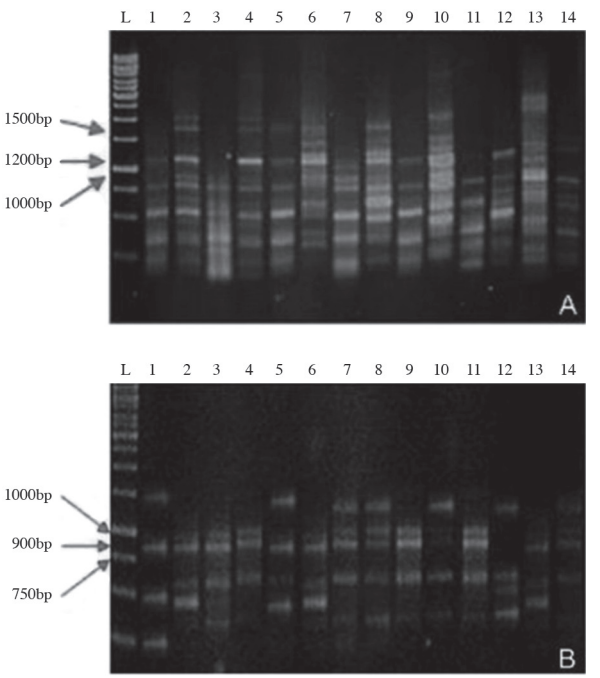

Figure 1. RAPD-PCR amplification profiles for the 14 isolates (lanes 1-14) of *Macrophomina phaseolina,* obtained by random primers OPA-13 (A) and OPA-9 (B). Lane L: 1 kb molecular weight ladder (FERMENTAS Inc.). PCR products were resolved on 1.4% agarose gel, in TAE (0.5 X) buffer.

The first group consists of 11 isolates, which was divided into two subgroups. There were two haplotypes (61 and 62) in this group and showed 100% similarity. The second group consisted of 13 isolates with two subgroups. Group 3, with 24 isolates and two haplotypes, was the largest that constituted by isolates originating from an area with crop rotation. Group 4 included three isolates (Table 1). Isolates from areas with crop rotation had limited genetic variation in comparison with the others, showing high similarity values and consequently formed the largest group. The minimum divergence was 36% within the groups in the clade and the subgroups were divided based on the similarity coefficient. Each group included the isolates from various areas that had single range genetic similarity. The pathogenicity of the selected 24 isolates of *M. phaseolina* was significantly different ($P < 0.01$) on the three plant species. All the isolates were pathogenic for soybean and sunflower. The isolates were highly virulent on soybean and virulent on sunflower (Table 2). The disease index on sunflower and soybean varied between 19-24 and 27-30, respectively.

Molecular techniques using RFLP, RAPD, AFLP, microsatellite and minisatellite fingerprinting have been used to identify and differentiate isolates of several filamentous fungi at population, genus, and species level (Sharma *et al*., 2003). RAPD markers has been a useful technique for detecting variation within *M. phaseolina* population in different countries (Fuhlbohm, 1997; Su *et al*., 2001; Almeida *et al*., 2003; Das *et al*., 2006). The results showed a low genetic diversity among most of the isolates of *M. phaseolina* inside each group. Low genetic variability is usually observed in population of fungi that do not reproduce sexually as occur with *M. phaseolina*. In the absence of sexual reproduction in *M. phaseolina*, genetic variability could occur by somatic recombination processes like fusion of cells or parasexual recombination. In areas where crop rotation was applied, the non host plants were cultivated, thus the number of propagules is reduced and lead to somatic recombinants limitation. In our study limited genetic variations observed among isolates in areas with crop rotation, this case was reported by the other workers for this fungus isolates (Almeida *et al*., 2003). Isolate 10 was the most genetically divergent and constituted a single group. It was isolated from a native area that was cultivated for the first time with sunflower. This study demonstrated the existence of genetic diversity among isolates of *M. phaseolina* in northern parts of Iran. The study also demonstrated that RAPD markers can be used for measuring genetic relatedness and detecting variation within and between *M. phaseolina* populations from different hosts. The pathogenicity test showed that soybean and sunflower plants are susceptible while maize plant is resistant to *Macrophomina*. Both of the isolates, from soybean and sunflower, were more pathogenic on soybean than sunflower. In addition of soybean and sunflower, *M. phaseolina* was reported as a pathogen on

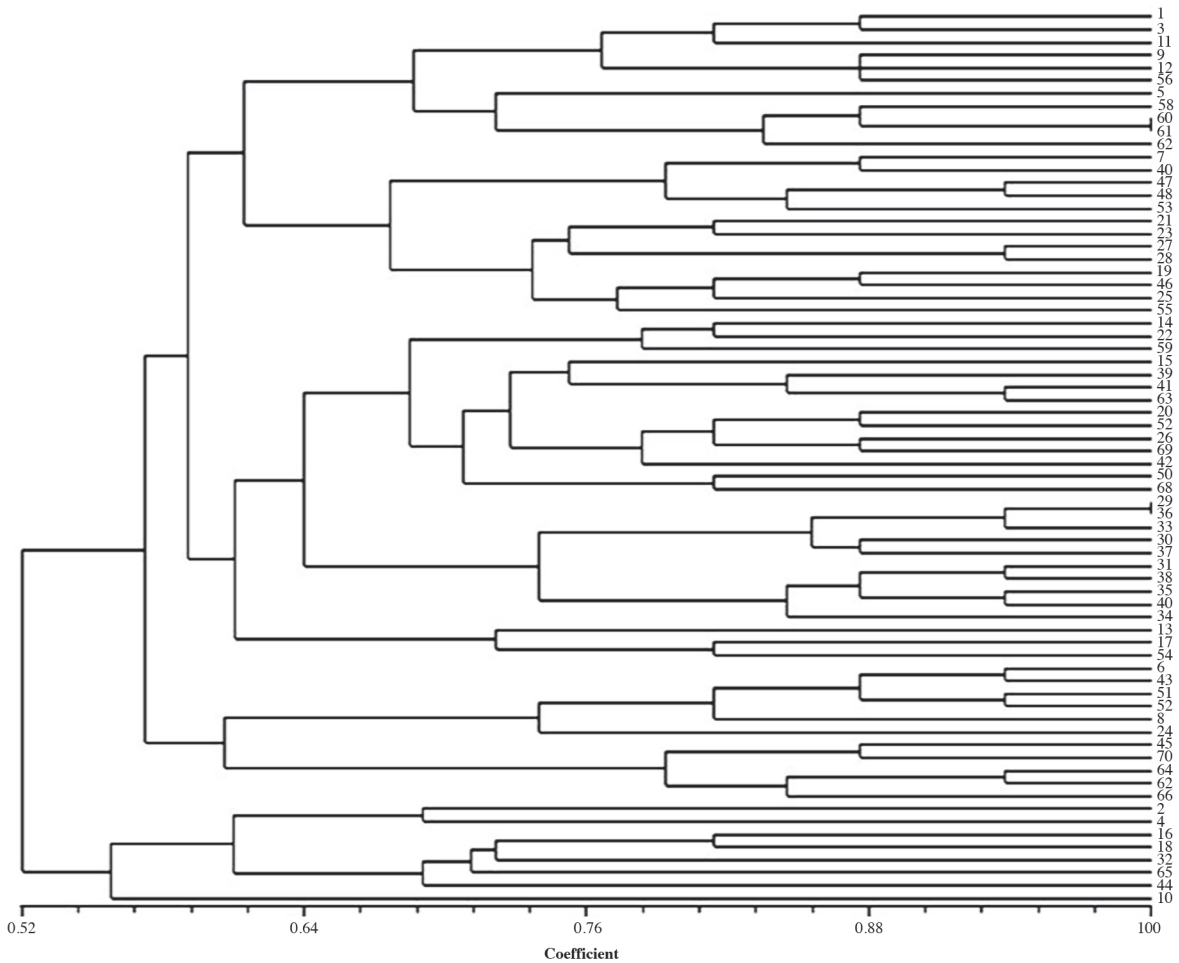

Figure 2. Dendrogram obtained from 70 isolates of *Macrophomina phaseolina* with Unweighted pair-group method with arithmetic mean (UPGMA) clustering method based on Jaccards coefficient. Distance is based on genetic similarity coefficient.

Table 2. Pathogenicity of 24 isolates of *Macrophomina phaseolina* on three plant species.

Species	Cultivars	Average
Glycine max	Willyams	28.56
Helianthus annuus	Master	22.48
Zea mays	302	0

the other crops, same as sesame, strawberry, and cannabis in northern Iran (Ershad, 1995). Cultivation of resistant species such as maize and other cereals in crop rotation in northern Iran, enable to reduce soil infection and incidence of diseases.

CONCLUSIONS

The pathogenicity of 24 isolates of *Macrophomina phaseolina* was different ($P < 0.1$) on three plant species. None of the isolates were pathogenic on maize and all were pathogenic on soybean, and sunflower. Isolates were highly virulent on soybean and virulent on sunflower. The disease index on sunflower and soybean varied between 19-24 and 27-30 respectively. The most aggressive isolates originated from North of Mazadaran province were mainly isolated from soybean plants.

PCR-RAPD markers based on six random primers of kit A (OPA) on 70 isolates showed a degree of polymorphism in different isolates. The primers OPA-13 (5'-CAGCACCCAC-3') and OPA-9 (5'-GGGTAACGCC-3') amplified the highest number of *loci*. The primer OPA-13 amplified the genomic DNA of all isolates of *M. phaseolina* producing 4-12 bands of sizes between 0.25 and 2.3 kb. Nearly all isolates of *M. phaseolina*, irrespective of their host and geographical origin, exhibited a common band of 1.2 kb, except isolates 3, 7, 11, 15, 21, 34, 39, 56, 69. Isolates from the same locations showed a tendency to group closer, substantiating closer genetic relatedness. Results showed that more divergent isolates originated from areas with a single crop. Isolates from areas with crop rotation were less divergent, showing high similarity values and consequently formed the largest group.

The study demonstrated that RAPD markers are suitable for measuring genetic relatedness and detecting

variation within and between *M. phaseolina* populations from different hosts. Providing tolerant cultivars by pathogenicity test to different isolates of *Macrophomina phaseolina* and crop rotation are useful to control this disease.

Diversidad patogénica y genética entre aislamientos iraníes de *Macrophomina phaseolina*. Pudrición carbonosa, causada por *Macrophomina phaseolina* (Tassi) Goid., es una enfermedad de importancia económica de las plantas de semillas oleaginosas en el norte de Irán. Setenta aislamientos de *M. phaseolina* se obtuvieron de diferentes hospederos, incluyendo soya (*Glycine max* L.) y girasol (*Helianthus annuus* L.) en las regiones de plantación de semillas oleaginosas del norte de Irán. Perfiles RAPD-PCR de amplificación, utilizando primers aleatorios de seis OPA (kit A) mostraron polimorfismos entre los aislamientos. El primer OPA-13 (5'-CAGCACCCAC-3') amplificó el ADN genómico de todos los aislamientos de *M. phaseolina* produciendo 4 a 12 bandas de tamaños entre 0,25 y 2,3 kb. El análisis UPGMA (unweighted pair-group method with arithmetic mean) clasificó los aislamientos en nueve grupos principales con 64% de similitud. La patogenicidad de los aislamientos se evaluó en estado de plántula de soja, girasol y maíz (*Zea mays* L.) en condiciones *in vitro*. Ninguno de los aislamientos fue patogénico en maíz, mientras que todos los aislamientos infectaron plántulas de soya y de girasol. Los aislamientos fueron más virulentos en soya que en girasol. El índice de enfermedad en girasol y soya varió entre 19-24 y 27-30, respectivamente. Estos resultados indican una gran variabilidad genética y patogénica significativa dentro de aislamientos iraníes de *M. phaseolina*. Cultivo con rotación de cultivos probablemente tendió a inducir una menor diversidad de los aislamientos patógenos.

Palabras clave: Pudrición carbonosa, plantas oleaginosas, índice de enfermedad.

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