Research Journal of Recent Sciences ISSN 2277-2502
Vol. **2(ISC-2012),** 110-115 **(2013)** *Res.J.Recent.Sci.*

Genetic variability of *Macrophomina phaseolina* Affecting Sesame: phenotypic traits, RAPD markers and interaction with the Crop

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Available online at: www.isca.in
Received 04th August 2012, revised 26th January 2013, accepted 07th February 2013

Abstract

Macrophomina phaseolina is a fungus which affects more than 300 cultivated species. It is one of the most important biotic stresses on sesame (Sesamum indicum L.). A successful control strategy, especially plant resistance management, depends on comprehensive knowledge about genetic variability for both fungus and plant. To evaluate genetic diversity of M. phaseolina affecting sesame in the most important crop production region of Venezuela, seven isolates were characterized by means of phenotypic traits, and RAPD markers. Four of these isolates were used for evaluating the interaction with four sesame genotypes in two ways: interaction in vivo by inoculation, and effect of root and stem extracts on fungus growth. Variability for growth velocity (optical density ranged between 1.69 to 2.32 at 96 hours of growing) (P≤0.05) and microsclerotia production (18-56 in 100 µL) (P≤0.05), was observed. Ten primers used were able to amplify the DNA, generating 81 bands (100% polymorphic). Ordination of the seven isolates by means of principal coordinates analysis based on RAPD did not show a consistent relationship with phenotypic attributes or geographical origin. After inoculation, length lesion produced by the four isolates did not show statistical differences, but germination percentage did (P≤0.01). One of the fungus isolates reduced up to almost 70% average germination of the four sesame genotypes. Mycelial growth of four isolates was inhibited in 17 – 32 % as compared to the control. These results indicate it is difficult to manage charcoal rot by means of obtaining resistant cultivars because of the fungus variability found in all the levels evaluated..

Keywords: DNA, charcoal rot, sesame, inoculation, morphological traits.

Introduction

Charcoal rot is a disease caused by the fungus *Macrophomina phaseolina* (Tassi) Goid. On more than 300 cultivated species, causing obstruction at root tissue[1]. It is an important threat to sesame (*Sesamum indicum* L.) production in Venezuela[2]. Sesame season in Venezuela (November - April) is characterized by high temperatures and dry soils because of lack of rain, which are favorable conditions for charcoal rot[3]. In Venezuela, losses in sesame due to this fungus have been evaluated, resulting up to 65% of seed weight reduction for affected plants[4]. *M. phaseolina* survives in the soil by means of sclerotia, which have been quantified in Venezuelan soils of sesame production areas up to 200 g^{-1} of soil[5].

Suitable identification of plant pathogens is a key factor for establishing disease control strategies[6], for that, genetic and morphological characterization is needed. Characterization of individuals in a population is defined as the description based on phenotype or molecular traits[7], and both strategies are complementary according to the information provided[8]. One of the strategies for controlling the pathogen is the use of genetic resistance, but for using it, it is necessary a wide knowledge about genetic variability for both *Macrophomina phaseolina* and *Sesamum indicum*. Furthermore, knowledge about interaction between isolates of the fungus and sesame cultivars is required. Interaction host-pathogen could be studied *in vivo* and *in vitro*. Studies of interaction *in vivo* need an efficient inoculation protocol to ensure the pathogen reaches to the host, and the host is able to respond. Studies of interaction sesame-*M. phaseolina* has been previously reported (e.g.[9,10]). For *in vitro* studies, an approach used is to confront the pathogen to plant extracts and to evaluate the effect of this extract on pathogen growth. If extracts have some biochemical compounds which are toxic to the pathogen, the fungus will inhibit the growth. This kind of evaluation could have a huge potential. Number of plant species is about 500,000 but only few of them have been studied according to antimicrobial activity of extracts[11]. Effect of cultivated plant extracts (sunflower) on *M. phaseolina* have been reported (e.g.[12]), but never sesame extracts effect on this fungus has been evaluated.

The objective of this investigation was to evaluate genetic diversity of *Macrophomina phaseolina* coming from Venezuelan sesame production, in four different ways by means of: a. phenotypic attributes, b. molecular markers (RAPD), c. ability to cause disease on four sesame genotypes, d. growth with sesame extracts.

Material and Methods

Fungus isolates: Tissue presenting the disease collected from plants in the field was obtained to get seven isolates (table 1).

Table-1
Location of collected material at Venezuelan sesame production area to get seven isolates of *M. phaseolina*

Isolate name	Collecting place	Latitude of collecting place	Longitude of collecting place	Altitude of collecting place (m.a.s.l.)	Collected material
2-2010	Acequioncito	9º12´41,01´´ N	68º56´47,8´´ W	100	Sesame tissue
C3-2010	In between Chorrerones and El Ají	9º07´43,18´´ N	69º01´44,54´´ W	114	Sesame tissue
2-2011	Road V	9º18´10,4´´ N	69º07´13,9´´ W	133	Bean tissue
30-2011	Road V	9º18´10,4´´ N	69º07´13,9´´ W	133	Sesame tissue
36-2011	El Gateao	9º 07´ 38,3´´ N	68º53´ 19,8´´ W	90	Sesame tissue
37-2011	El Playón	9º 06´34,1´´ N	69º 02´ 31,3´´ W	96	Sesame tissue
41-2011	Camino 8	9º09´4,9´´N	68º54´06´´W	100	Sesame tissue

Tissue was incubated in Petri dishes containing potato dextrose agar to promote mycelia growth, which was transferred many times to other Petri dish until getting typical, clean and pure *Macrophomina phaseolina* mycelia, in the same way used by Csöndes I. and et al[13]. Seven isolates were obtained by this methodology

Phenotypic characterization: Two attributes were evaluated for the phenotypic characterization: growth velocity and number of microsclerotia. For number of microsclerotia, a completely randomized design with six replications was used, where the experimental unit was a Petri dish containing the fungus growing for two weeks. One gram of medium potato dextrose agar containing mycelium and microsclerotia was macerated in 10 mL distilled water From this volume, 100 µL were taken to count the number of microsclerotia; this was repeated 5 times, therefore the reported values for each experimental unit is the average of the 5 counted volumes. For growth velocity, 50 microsclerotia were obtained from a 14 days-old culture, and they were placed in an individual well of 96-well microplates used for ELISA, containing 200 µL of potato dextrose broth. It was repeated 64 times for each fungus isolate. Optical density of each individual well was measured each 12 hours by means of a spectrophotometer Multiskan FC (Thermo, Finland) at 450 nm. The more is the optical density, the more is the interference in each well caused by mycelia growth.

Molecular characterization: One hundred fifty milligrams of mycelium of each isolate growing in potato dextrose broth were used for DNA extraction with Dellaporta extraction buffer[14], containing 10 µL of mercapthoethanol and 10 µL of proteinase K (20 mg mL^{-1}). The homogenates were incubated for 20 m at 65°C, followed by addition of 500 µL of phenol:chlorophorm:isoamylic alcohol (25:24:1). Phases were separated by centrifugation for 10 min at 14000 rpm. Isopropanol (750 µL), and ammonium acetate 5 M (5 µL) were added to the aqueous phase. After 30 min samples were centrifuged at 14000 rpm for 10 min, pellet were washed twice with 70% ethanol, dried and dissolved in 50 µL of TE buffer. Additional step of cleaning with ammonium acetate and RNAse was done. DNA quantity and quality was determined by electrophoresis in a 0,8% agarose gel with lambda DNA standard. RAPD analysis for each combination isolate-primer was performed using 20 ng of DNA, 4 µL of PCR 5X buffer, 2 µL of $MgCl_2$ 25 mM, 4 µL of dNTPs 10 mM, 0,8 µL of gelatine 0,025% (w/v), 1 µL 2mM of one of 10 primers (OPA02, OPA03, OPA04, OPA05,OPA07,OPA09, OPA13, OPC04, OPC06 and OPC08), 1 U of Taq polymerase, in a final volume of 20 µL. The thermocycler program consisted of a first step at 93°C for 2 min, followed by 45 cycles consisting in 93°C for 1 min, 36°C for 1 min and 72°C for 1 min. Final step consisted in 72°C for 5 min. PCR products were resolved in agarose gel 1.4% w/v for 60 min at 60 V. Gels were stained in ethidium bromide and washed in water for visualizing band under UV light.

Inoculation on sesame plantlets: Plastic trays (10x19x5 cm) were filled of a mixture of sterilized substrate with a content of a Petri dish containing the fungus for two weeks on potato dextrose agar. Four trays were used for each of 4 isolates (2-2011, 36-2011, 37-2011 and 41-2011). For each tray, four sections were defined to sow 10 seed of each of 4 sesame cultivars (Maporal, UCLA295, 43x32 and India7), resulting in a split plot design with four replication. Isolates were the main plot, and sesame cultivar the subplot. After sowing, substrate was irrigated with 20 mL of distilled water. On each experimental unit the variables measured were germination percentage and length lesion on the stem.

Sesame extracts on fungus growth: Fifty seeds of each sesame cultivar (Maporal, UCLA295, 43x32 and India7) were germinated in a sterilized substrate. Three weeks later, roots were separated from stem. Each mass of root was homogenized in ethanol 80% in a ratio of 1 g of mass to 5 mL of ethanol, and kept at room temperature for 16 hours. Afterwards they were filtered and kept at 4°C until bioassays were established. Bioassays were performed in 96-wells microplates. Each well was filled with 200 µL of root extract; as control wells were filled with 200 µL of ethanol (without extracts). After 24 h (when ethanol was evaporated), 200 µL of potato dextrose broth containing 50 microsclerotia were poured in each well, including the control. This procedure was repeated eight times (eight wells) for each combination root extract – fungus isolate, for each sesame genotype. Fungus isolates used were 2-2011, 36-2011, 37-2011 and 41-2011. Optical density at 450 nm was recorded when bioassay was established, and each 12 hours during 120 hours.

Results and Discussion

Broad variation among fungus isolates was identified. Microsclerotia production ranged between 18 and 53, identifying the mean test that 2-2010, 30-2011 and C3-2010 had the highest production of microsclerotia, almost 3-times more than 2-2011 (table 2). Growth velocity also displayed variation among isolates (figure 1), and according Tukey test (data not shown) three groups were formed: the first one with the lowest velocity, conformed by C3-2010, 2-2011 and 37-2011, the second one with an intermediate growth velocity conformed by 30-2011, 36-2011 and 41-2011, and the third one with the highest growth velocity represented by 2-2010. Optical density as indicator of fungus growth shows the typical form of the curve in the figure 1: low growth the first 24 h, exponential growth from 24 h to 60 h, and decrease of growth rate from 60 h, therefore optical density measure is a reliable way to record *M. phaseolina* growth through the time. Other authors report growth velocity of *M. phaseolina*, but they did it in the conventional way (e.g.[15,16]), it is not possible to compare them to our results. Both microsclerotia production and growth velocity are attributes related to aggressiveness of pathogen isolates, therefore isolate 2-2010 seems to be the most aggressive because of the ability to grow quickly and produce high amount of inoculum. On the other hand, 2-2011 has the lowest aggressiveness, it grows slowly and produce low amount of microsclerotia.

Table-2
Number of microsclerotia of seven isolates of *M. phaseolina* counted in 100 µL of water after macerating 1 g of fungus tissue growing on potato dextrose agar during 2 weeks

Fungus isolate	Number of microsclerotia
2-2010	56.17 a
30-2011	53.67 a
C3-2010	53.00 a
36-2011	41.00 b
37-2011	23.77 c
41-2011	20.70 cd
2-2011	18.10 d

Means followed by the same letter are not statistically different (P<0.05) according to Tukey test

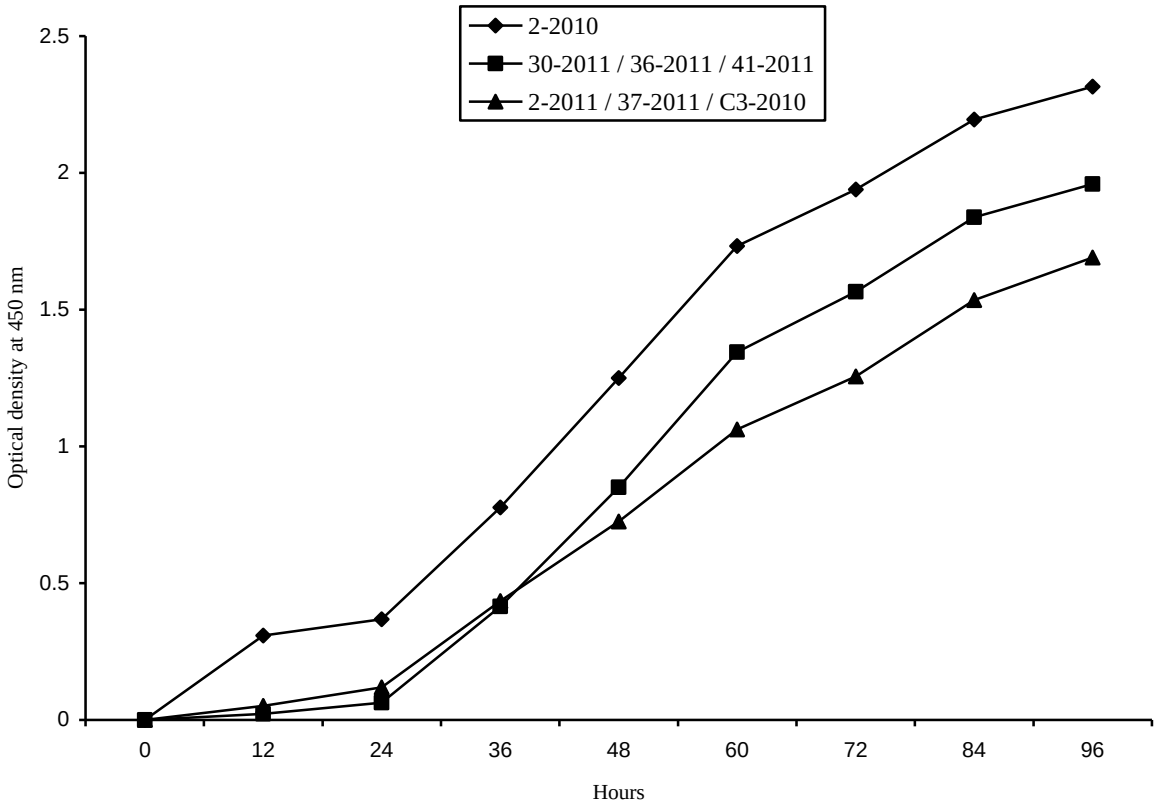

Figure-1
Growth velocity for seven isolates of *M. phaseolina* recorded in 96-wells microplates. The lowest and intermediate line indicate growth as average on the isolates which did not show statistical differences in growth

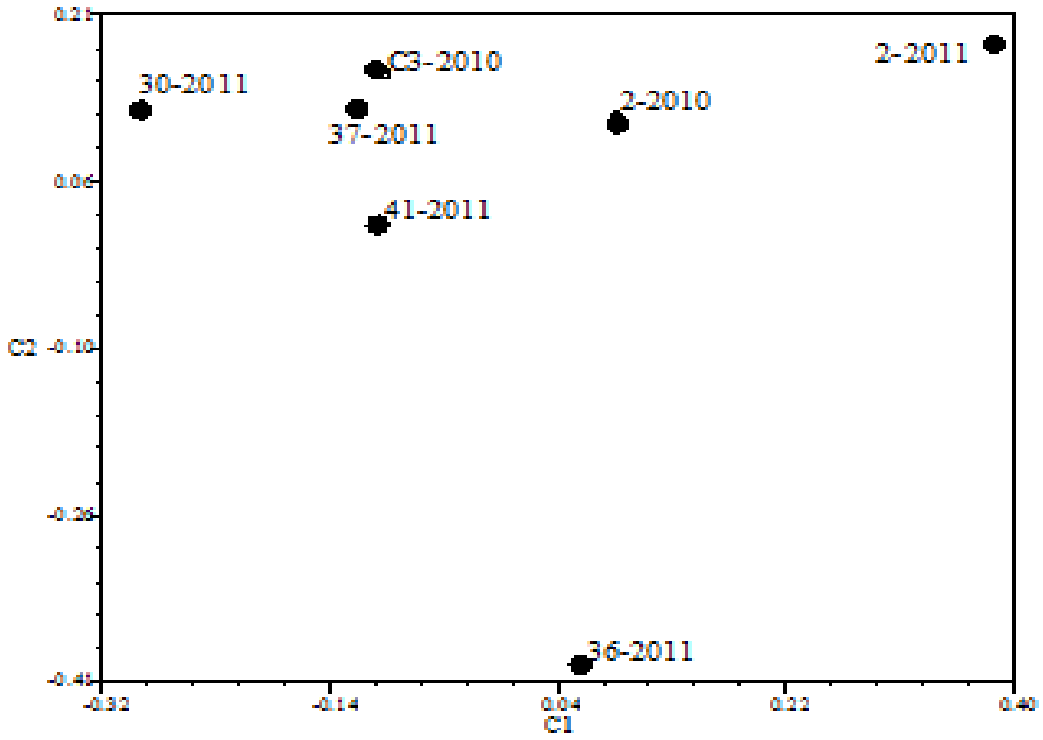

Figure-2
Biplot from principal coordinates analysis showing ordination of seven *Macrophomina phaseolina* isolates based on 81 RAPD bands

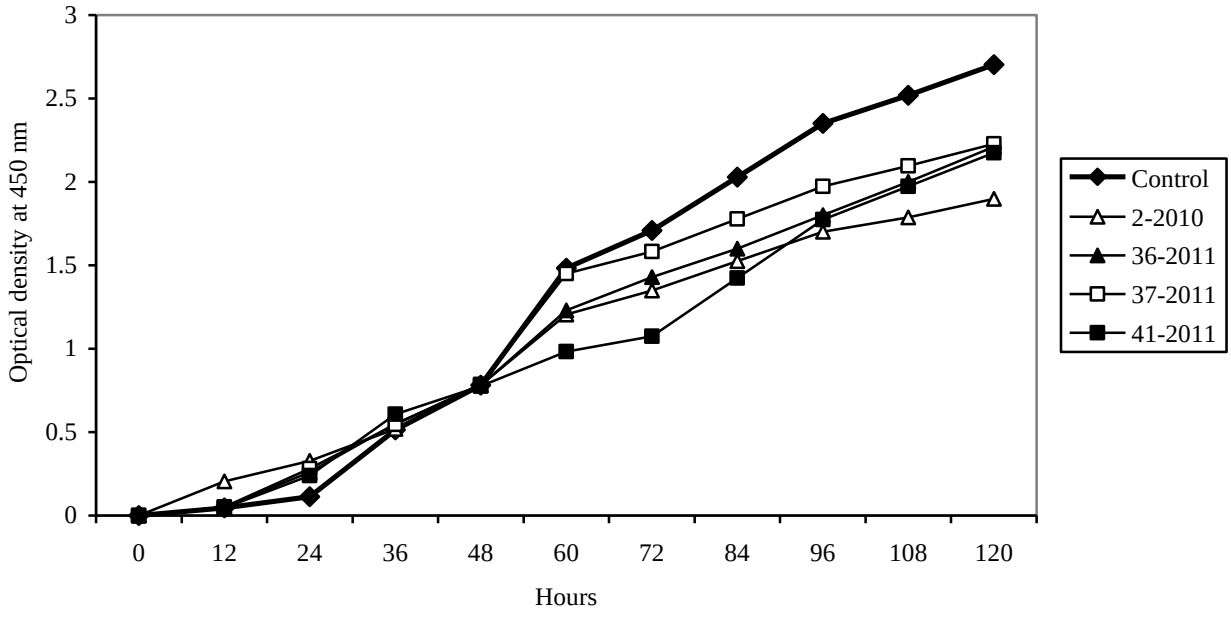

Figure-3
Optical density in wells of microplates as indicator of growth velocity for four isolates of *M. phaseolina* growing with sesame root extracts

Molecular characterization based on RAPD generated 81 bands (100% polymorphic). Seventy five percent of the bands had a size range between 150 and 1000 base pairs (bp). Ratio number of bands per primer resulted in 8.1. A previous characterization of the fungus found a ratio of 3.36[17]. Primer OPA13 was reported as the one which amplified on all *M. phaseolina* isolates[18], however it did not occur for the seven isolates evaluated in this research. This is evidence that the value of some primers could vary depending on the population used, they are not necessarily valuable through all the population in a species. Figure 2 shows ordination of isolates by means of principal coordinates analysis. There was not a consistent relationship between grouping based on RAPD and phenotypic traits, or geographical origin. Furthermore, RAPD did not discriminate the isolate coming from bean. However there was a trend to separate isolates according to the margin of Acarigua River (main river of the zone) from where they were collected. There is little evidence which supports independent evolution of isolates, on the contrary, it seems there is a constant exchange of microsclerotia through sesame production area, which is consistent with the constant seed exchange among farmers.

Effect of the fungus isolates on four sesame genotypes was not different in length lesion, it ranged between 10.3 to 18.5 mm but they did not show significant differences (P<0.05); however, they were different when germination percentage was evaluated (table 3). When isolate 2-2010 was inoculated, germination resulted in only 30% as compared to the control. This result is consistent with the phenotypic attributes, which identifying isolate 2-2010 as the most aggressive. Variability among isolates of *M. phaseolina* on sesame seed germination has been previously reported 5.

Table-3
Length lesion and germination percentage caused by four isolates of *M. phaseolina* (values were averaged on the damage of four sesame genotypes)

Fungus isolate	Length lesion (mm)	Germination percentage
36-2011	10.3 a	85 a
37-2011	18.5 a	76 a
41-2011	14.1 a	72 a
2-2010	13.7 a	32 b

Means followed by the same letter are not statistically different (P<0.05) according to Tukey test

Sesame root extracts inhibited growth of the four isolates of *M. phaseolina*. Figure 3 show the optical density for each isolate during 120 h averaged on the four sesame cultivars used. The behavior for the four isolates was very similar, showing inhibition since hour 72; however 2-2010 was more inhibited than 37-2011; 2-2010 grew 68% as compared to the control, whereas 37-2011 did it 83%. There is no previous reports about effect of sesame root extracts on *M. phaseolina*, however, there is a previous report of effect of root extract of another cultivated plant (sunflower) on this fungus; these authors found a strong inhibitory effect 12.

Conclusion

Macrophomina phaseolina presented a broad genetic variation in the Venezuelan sesame production area in all the levels which were evaluated: phenotypic traits, molecular markers, effect on sesame cultivars and response to sesame root extracts. These results indicate it is difficult to manage charcoal rot by means of obtaining resistant cultivars because of the fungus variability. For generating this kind of cultivars could be necessary stratification of the area according to genetic variability of the fungus.

Acknowledgement

Authors want to express the acknowledgement to Consejo de Desarrollo Científico, Humanístico y Tecnológico (C.D.C.H.T.) of Universidad Centroccidental Lisandro Alvarado and to Internactional Foundation Sciences (IFS) for financial support of the project 033-AG-2009 and IFS C/4408-1 respectively, from which resulted this paper.

References

1. Wyllie T. Charcoal rot, In: Compendium of soybean diseases. Third edition. J.B. Sinclair and P.A. Backman (eds). Pages 30-33. American Phytopathological Society, St. Paul, MN, USA, **(1989)**

2. Pineda J. Enfermedades del ajonjolí: algunas medidas de control. II Curso sobre producción de ajonjolí, soya y otras leguminosas, 114 pp. ASOPORTUGUESA, UCLA and FONAIAP, Venezuela **(2002)**

3. Odvody G. and Dunkle L., Charcoal stalk rot of sorghum: effect of environment in host-parasite relations, *Phytopathology*, **126,** 343-352 **(1979)**

4. *Pineda J. and Avila J., Alternativas para el control de* Macrophomina phaseolina *y* Fusarium oxysporum *patógenos del ajonjolí (*Sesamum indicum *L.),* Agronomía Tropical, ***38,** 79-84 **(1988)***

5. Pineda J., Nass H. and Rodríguez H. Efecto de la densidad de inóculo de (*Macrophomina phaseolina)* en la infección de plántulas de ajonjolí, *Agronomia Tropica*, **35,** 133-138 **(1985)**

6. Weiland J. and Sundsbak J. Differenciation and detection of sugar beet fungal pathogens using PCR amplification of actin coding sequences and the ITS Region of the Rrna. *Gene*, **84,** 475-482 **(2000)**

7. Laurentin H. Genética Agrícola. Editorial Académica Española. Saarbrücken, Germany **(2011)**

8. Karp A., Kresovich S., Bhat K., Ayady W. and Hodgkin T., Molecular tools in plant genetic resources conservation:

a guide to the technology, IPGRI Technical Bulletin No. 2. International Plant Genetic Resources Institute, Rome, Italy **(1997)**

9. El-Bramawy M. Field resistance of crosses of sesame (*Sesamum indicum* L.) to charcoal root rot caused by *Macrophomina phaseolina* (Tassi) Goid, *Plant Protection Science,* **42,** 66-72 **(2006)**

10. Simosa N. and Delgado M. Virulence of four isolates of *Macrophomina phaseolina* on four sesame (*Sesamum indicum*) cultivars, *Fitopatología Venezolana,* **4,** 20-23 **(1991)**

11. De Lucca A., Cleveland T. and Wedge D. Plant-derived antifungal proteins and peptides, *Canadian Journal of Microbiology,* **51,** 1001-1014 **(2005)**

12. Khan S., Ayub N. and Ahmad I., Inhibitory effect of extracts of plant parts of sunflower hybrids on sclerotia production of *Macrophomina phaseolina*, *Pak. J. Phytopathol,* **19**, 150-154 **(2007)**

13. Csöndes I., Cseh A and Taller J. Genetic diversity and effect of temperature and pH on the growth of *Macrophomina phaseolina* isolates from sunflower fields in Hungary, *Molecular Biology Reporter,* DOI 10.1007/s11033-011-1094-6 **(2011)**

14. Dellaporta S., Wood J. and Hicks J., A plant DNA minipreparation: versión II, *Plant Mol. Biol. Rep.,* **1,** 19-21 **(1983)**

15. Beas-Fernández R., De Santiago A., Hernández-Delgado S. and Mayec-Pérez N., Characterization of Mexican and non-Mexican isolates of *Macrophomina phaseolina* based on morphological characteristics, pathogenicity on bean seeds and endoglucanase genes, *Journal of Plant Pathology,* **88,** 53-60 **(2006)**

16. Mahdizadeh V., Safaie N. and Goltapeh E., Diversity of *Macrophomina phaseolina* based on morphological and genotypic characteristics in Iran, *Plant Pathology Journal*, **27,** 128-137 **(2011)**

17. Almeida A., Abdelnoor R., Arrabal C., Carvalho V., Jacoud D., Marin L., Benato M. and Carvalho C. Genotypic diversity among Brazilian isolates of *Macrophomina phaseolina* revealed by RAPD, *Fitopatología Brasileira*, **28**, 279-285 **(2003)**

18. Jana T., Sharma T., Prasad R. and Arora D., Molecular characterization of *Macrophomina phaseolina* and *Fusarium* species by a single primer RAPD technique, *Microbiological Research*, **158**, 249-257 **(2003)**