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*Full Length Research Paper*

# A study of the characteristics of fungus and native olive reaction in Golestan province of Iran

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**Diseased stem and root samples from 20 olive nurseries of Golestan province, northern Iran, Fifty six *Macrophomina phaseolina* isolates were recovered from different nurseries. The recovered *M. phaseolina* isolates were characterized for pathogenicity and colony phenotype on PDA and chlorate selective media. Inoculum density for all nurseies varied between 4 and 9 propagules per gram of air-dried soil with average 7.61±0.73. All the recovered isolates were pathogenic to the tested cultivars, Mary, Rooghany and Zard, as incited stem lesions ranged between 6.01 cm and 9.02 cm and plant death percent ranged between 56.7 and 78.6 in the pathogenicity test. One color phenotypes (brown colony color) on PDA and three growth patterns were recognized on chlorate media. Chlorate sensitive isolates divided into two classes. Isolates of the first class grew sparse with a feathery like pattern, being the most frequent (94%), and the secondary had a completely restricted radial growth. All the recovered *M. phaseolina* isolates tested were pathogenic to the tested olive cultivars (Mary, Rooghany, and Zard). Analysis of variance of the interaction between olive cultivars and isolates of *M. phaseolina* showed significant (*p*=0.05) effects of cultivar (Table 1) but nonsignificant effects (*p*=0.05) for isolate, and cultivar × isolate interactions for the tested parameters. Cultivar × isolate interactions were not important factor in determining the variation in root damage.**

**Keywords:** *Macrophomina phaseolina*, olive, Iran.

## INTRODUCTION

Olive (*Olea europaea* L.) is the most important and traditional woody crop that cultivated over a large areas in Iran. Olive cultivation has expanded during the last decade especially in Golestan province, the northern of Iran (Figure 1). In this province nearly 10,000 hectare of olive orchards are present, which represents about 20% of total national olive area (Anonymous, 2007). In the last decade most of new plantations in this region established with Rooghany, Zard and Mary cultivars, which are the native olive cultivars of Iran (Sanei *et al*., 2004). Commercial cultivars of olive are planted in Iran but wild olive are the important genetical sources of olive, that residue of them can be seen in the East of Golestan province, north of Iran (Sanei *et al.*, 2005).

Unfortunately, olive is subjected to be attacked with a variety of fungal pathogens which affect its health, yield and its oil quality (Sanei *et al*., 2012). Young olive especially Rooghany and Zard cultivars showing decline symptoms were observed in several greenhouse. This syndrome was associated with a severe root rot. Several fungal pathogens were consistently isolated from roots of symptomatic seedlings such as *Fusarium solani*, *Macrophomina phaseolina*, *Phytophthora megasperma* and *Pythium aphanidermatum* (Sanei *et al*., 2005, 2012). *Macrophomina phaseolina* (Tassi) Goid is the most fungal pathogens affecting olive cuttings in Golestan nurseries. The pathogen is an anamorphic and soil borne fungus and cause the charcoal-rot disease (Sergeeva *et al*., 2005), with a broad host range that includes 75 plant families and more than 500 host (Purkayastha *et al*., 2006), that is likely to become more important under climate change scenarios of increased heat and drought stress (Saleh *et al.,* 2010). Great variability in morphology and pathogenicity was recognized among isolates from different host species and between isolates from different parts of the same plant (Fernandez *et al*., 2006). Efforts

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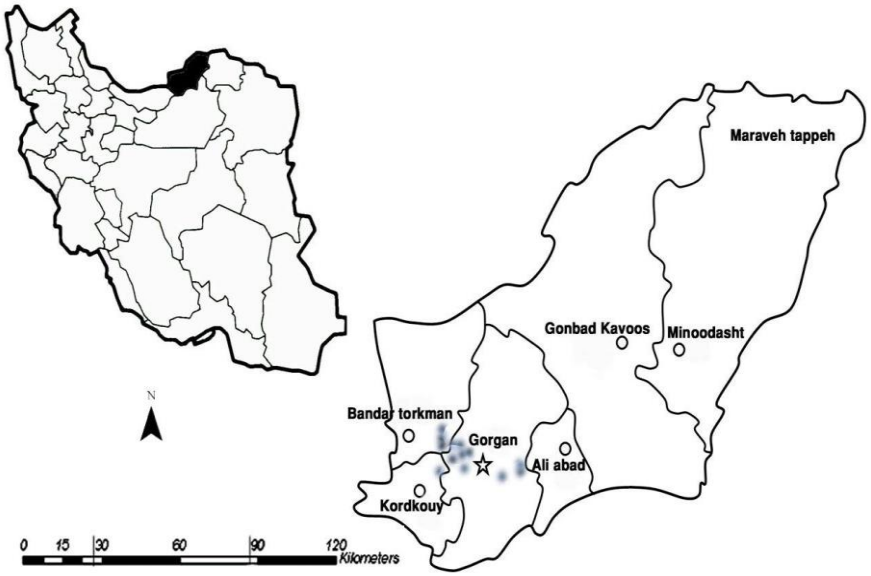


**Figure 1.** Iran map (left) and regional surveyed (Golestan province, right) in this study

were made to characterize the fungus population in different parts of the world. This was based on its pathogenic variability (Karunanithi *et al*., 1999), the morphological characteristics (Fernandez *et al*., 2006, Taliey *et al.*, 2007), as well as the molecular characteristics (Almeida *et al*., 2003; Jana *et al*., 2003; Purkayastha *et al*., 2006).

Charcoal disease of olive seedlings was first recorded in Iran in 2005 (Sanei *et al.*, 2005). Since then, the disease has spread with increase in olive nurseries. However, little is known about characteristics of *M*. *phaseolina* population in Iran. The present study therefore, was conducted to reveal characteristics of the fungus, fungus population and native olive reaction in Golestan olive nurseries which is a major area for olive propagation in Iran.

## MATERIALS AND METHODS

### Fungal isolates and inoculum production

Diseased stem and root samples from 20 nurseries of Golestan province were washed thoroughly with running tap water, air dried and cut into small pieces. Small infected portions in the surface were sterilized with 2% sodium hypochlorite solution for 2 min, rinsed in sterilized distilled water, dried in sterilized filter paper and plated on Potato Dextrose Agar (PDA) (Mihail and Taylor, 1995). Inoculated plates were then incubated at 28 ± 2ºC in darkness for 5 days and investigated for *M. phaseolina* colonies. Hyphal tips were obtained and transferred to fresh PDA plates. A substrate for growth of isolates was prepared in 250-ml Erlenmayer. Each bottle contained 50g of wheat grains and 40 ml of tap water. Contents of each bottle were autoclaved for 30 min. Isolate inoculum from one-week-old culture on PDA, was aseptically introduced into the bottle and allowed to colonize the substrate for three weeks. For estimation of *M*. *phaseolina* population in soil, the top 20 cm of soil was sampled from 20 nurseries in September. The soil was then air-dried and sieved through the 1 mesh diameter and used for soil samples. The number of propagules (viable microsclerotia) was assessed by soil plate method (Watanabe *et al.*, 1970). Melted potato dextrose agar kept at 50°C was freshly mixed with 0.13% solution of sodium hypochlorite and 100 ppm streptomycin sulfate in the final concentration. One sample weighting 100 mg of soil mixed with 100 ml medium and divided into 10 Petri dishes. From colonies developed on the plate after being incubated at 30°C for 10 days, number of *M*. *phaseolina* propagules was determined per gram of air dried soil.

### Colony characteristics of the recovered *M. phaseolina* isolates

All the recovered *M. phaseolina* isolates were characterized for colony color and growth pattern on PDA and Chlorate selective media (Pearson *et al*., 1986). Plates of the different media were inoculated with mycelia discs (0.5 mm in diameter) taken from the advancing

**Figure 2.** *Macrophomina* olive root rot causes dark brown to black (charcoal) discoloration and loss of feeder roots. Microsclerotia fill affected roots.

margin of 7-days-old PDA culture of the tested isolates. Five replicate plates were prepared for each isolate on each medium and incubated at 28 ± 2ºC for seven days in darkness. The developed colonies were characterized for the colony phenotype and the growth pattern according to Pearson *et al*. (1986) and Atiq *et al*. (2001). Growth rate of 56 isolates was recorded at 10, 15, 20, 25, 30, 35 and 37 ºC. Culture disks, 4 mm diameter, cut from the edge of a 4-day-old PDA culture of each isolate, grown at 25ºC, were transferred to center of 9 cm Petri dishes with 10 ml of PDA and incubated in the dark at different temperatures. Each treatment was replicated three times in a completely randomized design. The colony diameters were measured by 24 h intervals.

### Interaction between olive cultivars and isolates of *M. phaseolina*

Pathogenicity and pathogenic variability tests were conducted on 3 olive cultivars to charcoal-rot disease. The tested cultivars were Mary, Rooghany and Zard, as the native olive for Iran. The nine-month-old olive plants in 10-cm-diameter pots with autoclaved soil, were stem inoculated with the tested isolates in the on lower stem (2 cm above crown) using the stem-tape inoculation technique of Zazzerini and Tosi (1989). Three isolates of *M. phaseolina* (one sensitive and two resistant to chlorate) were used in this study. 20 days after inoculation, developed lesions were measured in cm as the longitudinal bark necrosis below and above the site of inoculation. Percentage of plant death was also determined 30 days after inoculation. Re-isolation was conducted to ensure the association of the tested isolates with the developed disease. The experiment was conducted in a greenhouse with supplemental light provided by fluorescent tubes for 14 h per day. The air temperature during the experiment fluctuated between 18°C and 27°C. Plants were watered as needed and treated according to the normal agricultural practices. There were eight pots (replicates) for each treatment. Damages were recorded 30 days after planting.

### Statistical analysis of the data

The experimental design of the present study was a randomized complete block with five replicates. Analysis of variance (ANOVA) of the data and correlations were performed with the MSTAT-C Statistical Package. Duncan's student test was used to compare between isolate characteristics and cultivars reaction.

## RESULTS

The plant stem was brown without any type of black spots or streaks on bark of stem, but the collar region of the stem was black. On dissecting the main root, scattered black microsclerotia of *M. phaseolina* were seen. A partial destruction of secondary and tertiary roots was common. Root systems of the plants were totally destroyed (Figure 2). The root tissues of diseased plants are filled with microsclerotia of the fungus, giving it a grayish

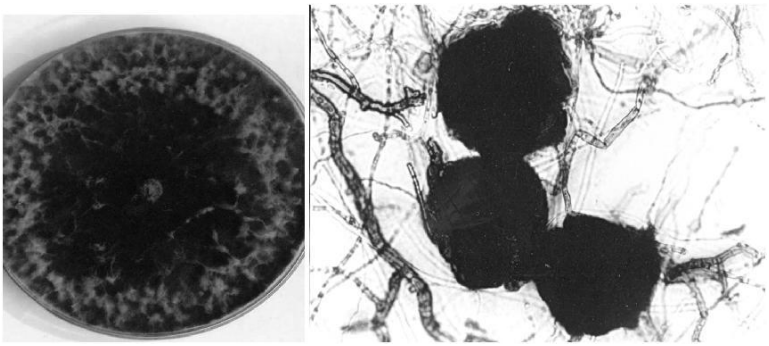

**Figure 3.** Colony of *Macrophomina phaseolina* recovered from olive plants collected from olive nursery in Gorgan region, Golestan province (left) and the microsclerotia under compound microscope (right, 40X).

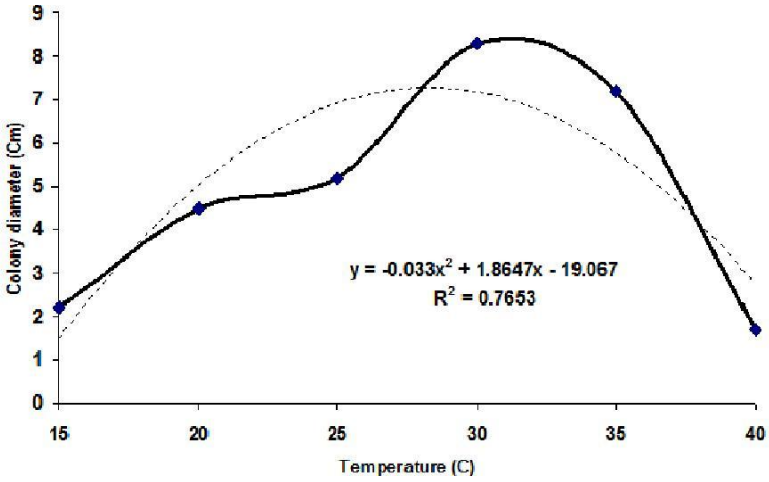


**Figure 4.** Experimental (solid line) and model (dash line) growth rate values of 56 isolates of *Macrophomina phaseolina* at different temperature in 5 days. Values are the mean of three replications.

appearance. Inoculum density for all nurseries varied between 4 and 9 propagules per gram of air-dried soil with average 7.61±0.73.

## Colony characteristics of the recovered *M. phaseolina* isolates

Colonies phenotype of the recovered isolates of *M. phaseolina* were variable on the PDA only for aerial mycelium. Colonies of the tested isolates were dense or light dense and the colour were mostly black, or brown (Figure 3). In 7-day-old cultures, microsclerotia ranged in size from 55 to 190 µm long by 45 to 120 µm wide (average 105×74 µm) (Figure 3). A temperature of 30ºC was optimal for 20 isolates. The mean of colony diameter at 15ºC and 40ºC were 2.2 and 1.7, respectively, after 5 days (Figure 4). For 15-30ºC, the growth rate increased with a positive and significant linear relationship ($r= 0.874$, $p< 0.01$), but the predicted line $Y= -0.033x^2 + 1.8647x - 19.067$ with $p<0.001$ obtained from regressional analysis for all data (Y= Colony diameter, X= Temperature, Figure 4).

All *M. phaseolina* isolates had dense growth when they were grown on the minimal medium without chlorate and could not be differentiated. 56 *M. phaseolina* isolates from plant samples collected from different regions of Golestan province were examined for their chlorate phenotype. Three various growth patterns (feathery spreading growth, restricted growth and dense growth)

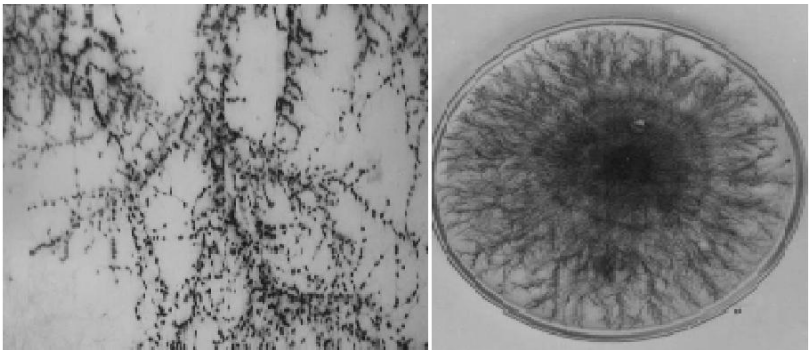

**Figure 5.** Growth patterns of *Macrophomina phaseolina* of colony (right) and microsclerotia (left) on a minimal medium containing 120 mM potassium chlorate.

**Table 1.** Pathogenicity and pathogenic variability of *Macrophomina phaseolina* isolates, recovered from olive seedlings tested on olive cv. Mary, Rooghany and Zard in pot experiment

| Olive cultivar | Lesion Size (cm)[1] | % Plant Death[2] |
|---|---|---|
| Mary | 6.01 a | 56.7 a |
| Rooghany | 9.02 b | 78.6 b |
| Zard | 8.97 b | 70.7 b |

Eight replicate plants were inoculated with each tested isolate. M = mean [1] measured 10 days after inoculation. [2], [3]estimated 21 days after inoculation of the non-inoculated control, inoculation was conducted in 60-day-old plants. Means in each single column sharing the same letter are not significantly different at 0.05 of probability

were observed when the isolates were grown on the minimal medium containing 120mM potassium chlorate. Among the isolates, feathery like pattern (Figure 5), were much more abundant (94%) than either restricted or dense isolates. There are no significant differences between sensitivity to potassium chlorate between isolates ($p< 0.01$) and also there is no correlation between cultivar origin of isolates and types of colony chlorate phenotypes ($p< 0.01$).

### Interaction between olive cultivars and isolates of *M. phaseolina*

All the recovered *M. phaseolina* isolates tested were pathogenic to the tested olive cultivars (Mary, Rooghany, and Zard). Analysis of variance of the interaction between olive cultivars and isolates of *M. phaseolina* showed significant ($p$=0.05) effects of cultivar (Table 1) but nonsignificant effects ($p$=0.05) for isolate, and cultivar × isolate interactions for the tested parameters. Cultivar × isolate interactions were not important factor in determining the variation in root damage.

## DISCUSSION

Charcoal rot is a serious threat for different crops and trees around the globe, especially in the temperate regions (Dhingra and Sinclair, 1978, Macini *et al.*, 1995). On the basis of this study, *M. phaseolina* isolates to adapt to the higher temperature in nurseries than in other warm areas (Figure 4). The disease exhibited with wide range of variation in disease severity (Kaunanithi *et al.*, 1994). Due to high degree of genetic variation in the pathogen, cultivation of resistant varieties is the most economical and practical approach. Other remedies of the disease are either uneconomical or cannot be applied under farmer field conditions.

Under field conditions *M. phaseolina* establishes itself during early stages of plant growth but severe symptoms of the disease occur when plant contains lower level of moisture percentage (Saleh *et al*., 2010). Appearance of typical symptoms at crop maturity suggests the possibility of latent quiescent infection because plant showing good growth and high vigor during early stages of its growth shows severe disease symptoms at maturity (Meyer *et al.,* 1974). The infected plants show different visibility of

the symptoms depends upon the severity of infection. The fungus primarily invades secondary and tertiary roots then travels to primary root (Ahmad and Burney, 1990).

Variations were not identified for the olive isolates colony phenotypes as the others report for colour phenotypes and growth patterns on the PDA (Clude and Rupe, 1991; Mihail and Taylor, 1995, Aboshosha *et al*., 2007). The use of the chlorate selective medium to differentiate strains of *M. phaseolina* was suggested (Pearson *et al*., 1986). The reduction of chlorate, an analog of nitrate, to chlorite in fungi, where the nitrate reductase pathway was functional can result in toxicity, which differentiate fungal isolates to sensitive and resistant strains. Soybean and sunflower isolates were chlorate–sensitive and showed either a restricted or feathery growth patterns. These results were same as the former reports (Meyer *et al*., 1974; Pearson *et al*., 1986; Su, *et al*., 2001). With studies on nitrogen source utilization by chlorate–sensitive and chlorate–resistant *Macrophomina* isolates, some authors found that the isolates of soybean were having a high level of nitrate reductase (Pearson *et al*., 1986). It is known that the amino acids compositions can be differ widely between types of crops and the chlorate–sensitive isolates of *M. phaseolina* do not utilize all the same nitrogenous compound as the chlorate–resistant isolates. Furthermore, nitrate reductase may function as an allosteric enzyme, based on fluctuations of its activity in the presence of amino acid contains (Clude and Rupe, 1991). Reasons for the phenotype shifts have not cleared yet. They may be associated with the heterogeneous, possibly polygenic nature of microsclerotia. But the fact that no sectors were observed on media amended with chlorate, argues against this explanation. More works need to be done to determine the mechanisms of chlorate assimilation in *M. phaseolina*. There are limited reports for chlorate–sensitivity in olive isolates of *M. phaseolina* (Taliey *et al*., 2007) but the result for 56 isolate with feathery form, show that they are essentially chlorate–sensitive.

Evaluate the reactions commercial Iranian olive to infect by *M. phaseolina* showed that most traits of the tested cultivars severely deteriorated in infested soil. True specificity implies that genetic variation in the host and the pathogen are correlated and may affect the durability of host resistance to the pathogen (Aboshosha *et al*., 2007). Lack of a significant interaction is taken to indicate that association is non-differential (horizontal),

implying that differences in cultivar susceptibility are consistent relative to one another, regardless of pathogen isolates. In any host-pathogen relationship the two types of resistance may act together in determining the outcome of the association between the host and the pathogen (Saleh *et al*., 2010). The result show that although no nematicide treatment threshold has been established for olive seedlings in this province, the infected soil and the susceptibility of native olive to Charcoal disease warrant further investigations.

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