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Application of *Metarhizium anisopliae* against *Meloidogyne javanica* in Soil Amended with Oak Debris

Mohammad Abdollahi

***Abstract*—**Tomato (*Lycopersicon esculentum* Mill.) is one of the most popular, widely grown and the second most important vegetable crop, after potatoes. Nematodes have been identified as one of the major pests affecting tomato production throughout the world. The most destructive nematodes are the genus *Meloidogyne*. Most widespread and devastating species of this genus are *M. incognita*, *M. javanica*, and *M. arenaria*. These species can cause complete crop loss under adverse growing conditions. There are several potential methods for management of the root knot nematodes. Although the chemicals are widely used against the phytonematodes, because of hazardous effects of these compounds on non-target organisms and on the environment, there is a need to develop other control strategies. Nowadays, non-chemical measures are widely used to control the plant parasitic nematodes. Biocontrol of phytonematodes is an important method among environment-friendly measures of nematode management. There are some soil-inhabiting fungi that have biocontrol potential on phytonematodes, which can be used in nematode management program. The fungus *Metarhizium anisopliae*, originally is an entomopathogenic bioagent. Biocontrol potential of this fungus on some phytonematodes has been reported earlier. Recently, use of organic soil amendments as well as the use of bioagents is under special attention in sustainable agriculture. This research aimed to reduce the pesticide use in control of root-knot nematode, *Meloidogyne javanica* in tomato. The effects of *M. anisopliae* IMI 330189 and different levels of oak tree debris on *M. javanica* were determined. The combination effect of the fungus as well as the different rates of soil amendments was determined. Pots were filled with steam pasteurized soil mixture and the six leaf tomato seedlings were inoculated with 3000 second stage larvae of *M. javanica*/kg of soil. After eight weeks, plant growth parameters and nematode reproduction factors were compared. Based on the results of our experiment, combination of *M. anisopliae* IMI 330189 and oak debris caused more than 90% reduction in reproduction factor of nematode, at the rates of 100 and 150 g/kg soil ($P \leq 0.05$). As compared to control, the reduction in number of galls was 76%. It was 86% for nematode reproduction factor, showing the significance of combined effect of both tested agents. Our results showed that plant debris can increase the biological activity of the tested bioagent. It was also proved that there was no adverse effect of oak debris, which potentially has antimicrobial activity, on antagonistic power of applied bioagent.

***Keywords*—**Biological control, nematode management, organic soil, *Quercus branti*, root knot nematode, soil amendment.

Mohammad Abdollahi is with Department of Plant Protection, Yasouj University, Yasouj, Iran (phone: +98-74-31006265; fax: +98-74-31006000; e-mail: abdollahi@ yu.ac.ir; mdabdollahi@gmail.com).

I. Introduction

NEMATODES are one of the major pests of vegetables throughout the world, particularly, in tropical and subtropical regions. The most destructive nematodes of vegetables are the genus *Meloidogyne*, the root-knot nematodes. Most widespread and devastating species of this genus are *M. incognita*, *M. javanica* and *M. arenaria* that can cause complete crop loss under unfavorable conditions [1]. There are many methods for management of these nematodes which can be grouped into chemical and non-chemical methods. Because of hazardous effects of chemicals on non-target organisms and on the environment, there is a need to develop other control strategies [2].

Biological control of nematodes is one of the most important approaches in nematode management and moving towards a sustainable agriculture [3]. Some soil inhabiting fungi has potential to controlling the nematodes [4]. Green muscardine, *Metarhizium anisopliae*, is a soil dwelling fungus with entomopathogenic characteristics, hence widely used in insect control program [5]. The effect of this fungus against *Rotylenchulus reniformis* [6], *Heterodera avenae* [7] have been reported. Biocontrol potential of *M. anisopliae* against some species of root knot nematodes has been shown [8]-[10].

Organic soil amendment can improve the fertility as well as the physical properties of soil [11]. Different kinds of waste materials have been used against plant parasitic nematodes. In a study, the composted agricultural waste materials were applied to the soil and reduced total number of root knot nematode [12]. In such organic amended soils, there are lots of beneficial microorganisms like fungi and bacteria which are able to parasitize or prey on nematodes [13]. Some of the soil amendments have nematocidal effects and suppress the nematode population. The licorice residue and spent compost of oyster mushroom (*Pleurotus ostreatus*) successfully were used against *M. javanica* [14]. Effect of cabbage leaf [15] and root bagasse of *Glycyrrhiza glabra* L. [16] on *M. javanica*, have been proved. The effect of debris of Iranian oak tree *Quercus branti*, on biological activity of *Pseudomonas fluorescens* and *Trichoderma vierns* against *M. javanica*, has been studied [17].

II. Objective

Considering the antimicrobial effects of oak trees and the hazards of the chemical pesticides, the main objective of this

research is to study the effects of oak trees debris and the biocontrol agent, *M. anisopliae*, on *M. javanica*.

III. Methodology

A. Preparation of Plant Debris

Rotten leaves of under canopy of oak trees were collected from the oak forest of Boyer-Ahmad, Kohgiluyeh and Boyer-Ahmad Province, Iran, with geographical coordinates of 30°41'26.35"N 51°35'36.43"E.

B. Inoculum Preparation

Eggs of *M. javanica* were extracted from infected roots of a susceptible tomato plant (*Lycopersicon esculentum* cv. Early Urbana) by grinding in NaOCl solution for three minutes. Second-stage juveniles emerging from eggs were daily collected on a 30 µm sieve, stored at 25 °C and used in experiments within 5 days [18].

C. Preparation of Metarhizium anisopliae

Green Muscle TC®, a biopesticide with spores of *M. anisopliae* var *acridum* (IMI 330189), purchased from the market and used in the experiments.

D. Raising Tomato Plants

Seeds of susceptible tomato cultivar (Early Urbana) were sown in plastic pots which filled with sterilized soil mixture. Three weeks after germination, uniform healthy seedlings of tomato were selected and transplanted to other plastic pots containing sterilized sandy clay-loam soil (60% sand: 40% mixture of silt and clay).

E. Experiment Designs

The combination effects of oak debris and *M. anisopliae* were studied under glasshouse condition. 16 treatments, each with four replicates were set up as follow:

T1. Uninoculated control without oak debris, without *Metarhizium* (Control)
T2. Uninoculated control without oak debris, with *Metarhizium* (F)
T3. Inoculated control without oak debris, without *Metarhizium* (N)
T4. Inoculated control without oak debris, with *Metarhizium* (N × F)
T5. Uninoculated, with 50g oak debris/kg soil, without *Metarhizium* (OD50)
T6. Uninoculated, with 50g oak debris/kg soil, with *Metarhizium* (OD50 × F)
T7. Inoculated, with 50g oak debris/kg soil, without *Metarhizium* (OD50 × N)
T8. Inoculated, with 50g oak debris/kg soil, with *Metarhizium* (OD50 × N × F)
T9. Uninoculated, with 100g oak debris/kg soil, without *Metarhizium* (OD100)
T10. Uninoculated, with 100g oak debris/kg soil, with *Metarhizium* (OD100 × F)
T11. Inoculated, with 100g oak debris/kg soil, without *Metarhizium* (OD100 × N)
T12. Inoculated, with 100g oak debris/kg soil, with *Metarhizium* (OD100 × N × F)
T13. Uninoculated, with 150g oak debris/kg soil, without *Metarhizium* (OD150)
T14. Uninoculated, with 150g oak debris/kg soil, with *Metarhizium* (OD150 × F)
T15. Inoculated, with 150g oak debris/kg soil, without *Metarhizium* (OD150 × N)
T16. Inoculated, with 150g oak debris/kg soil, with *Metarhizium* (OD150 × N × F)

In treatments with *M. anisopliae* var *acridum* IMI 330189, two centimeters of surface soil was pushed aside and a suspension of 2 g of biopesticide mixed in 20 ml of sterile distilled water was added to the soil (20 ml of distilled water was used in control treatments) and then covered with soil [19]. To meet the objectives of this study, four soil mixtures that varied in volumetric proportions of sand and oak debris but constant proportion of clay and silt, were prepared. In order to keep the soil texture uniform across all of the treatments, the coarse sand fraction was inversely varied with oak debris fraction (silt, clay, sand+oak debris with a proportion of 20:15:65 v/v, respectively). Plastic pots with 15 cm diameter were filled with one kg of sterile soil mixture. Six-leaf seedlings of tomato were transplanted to the pots and they were inoculated with 3000 J2/kg of soil, at the time of transplanting, then they were placed in a completely randomized arrange in the glasshouse and watered daily. Plants were grown under natural light conditions, relative humidity ranging from 55-68% and temperature ranging from 26-31 °C. Eight weeks after inoculation, plants were gently removed from pots and the growth factors of plant (length and weight of shoot and root) and the reproduction rates of the nematode (No. of galls/root, No. of egg masses/ root, No. of J2s/kg soil and reproduction factor) were determined [20].

F. Statistical Analysis

Data were tested for homogeneity of variance and normal distribution. The collected experimental data were organized and analyzed by using a one-way analysis of variance (ANOVA). General linear model procedures were used to perform the analysis of variance using SPSS 20 for Windows computer software package (SPSS Inc. Chicago, USA), where F-value was found to be significant, least significant difference (LSD) was used to compare the means at $P \leq 0.05$ levels of significance.

IV. Results and Discussion

Results of the both runs of the experiment have been shown in Figs. 1 and 2. Based on the results, by adding oak debris and the bioagent to the soil, fresh weight and length of shoot and root significantly increased in uninoculated treatments, which were at par with inoculated treatments, with 100 and 150 g/kg of oak debris plus *Metarhizium* ($P \leq 0.05$). Maximum length and also weight of shoot and root belonged to those uninoculated treatments that received more than 100 g of oak debris /kg of soil, with applying *Metarhizium* to the soil,

which were even more than the uninoculated control. Almost in all of the inoculated treatments, there was a significant increase in plant growth, as compared to inoculated control ($P \leq 0.05$). By increasing the amount of oak debris, up to 150 g/kg of soil, significant decrease in number of galls has been observed. In this regard, comparing the rate of effect, the role of *Metarhizium* in nematode inhibition was not that much prominent as individual use of this bioagent. Number of eggs/egg mass and number of J2s/kg soil significantly decreased by increasing the percentage of oak debris in the presence/absence of *Metarhizium*. The rate of decrease in number of eggs was not similar to decrease in J2s population in the soil. In this case, the important point was the effect of *Metarhizium* on eggs of nematode. When the fungus added to the soil, number of eggs significantly decreased ($P \leq 0.05$) (Table I). Minimum reproduction factor was recorded in treatments with 100 g and 150g of oak debris /kg of soil, as well as in the treatment of 50 g of oak debris with *Metarhizium* application ($P \leq 0.05$).

TABLE I
COMPARISON OF PERCENTAGE OF DECREASE IN NEMATODE-RELATED FACTORS ON TOMATO PLANTS INFECTED WITH *MELOIDOGYNE JAVANICA*, TREATED WITH DEBRIS OF OAK TREES AND *METARHIZIUM ANISOPLIAE*, AS COMPARED TO UNTREATED CONTROL

Factor \ Treatment	*Metarhizium*	50 g oak debris	50 g oak debris + *Metarhizium*	100 g oak debris	100 g oak debris + *Metarhizium*	150 g oak debris	150 g oak debris + *Metarhizium*
Galls/root	39%	35.49%	47%	77%	92%	129%	147%
Egg masses/root	88%	97.4%	105%	211%	223%	241%	258%
Eggs/root	355%	147.5%	425%	271%	556%	406%	691%
J2s/soil	144%	161%	183%	385%	409%	972%	1002%
RF	341.6%	147.67%	409.46%	273.35%	548.16%	414%	696.2%

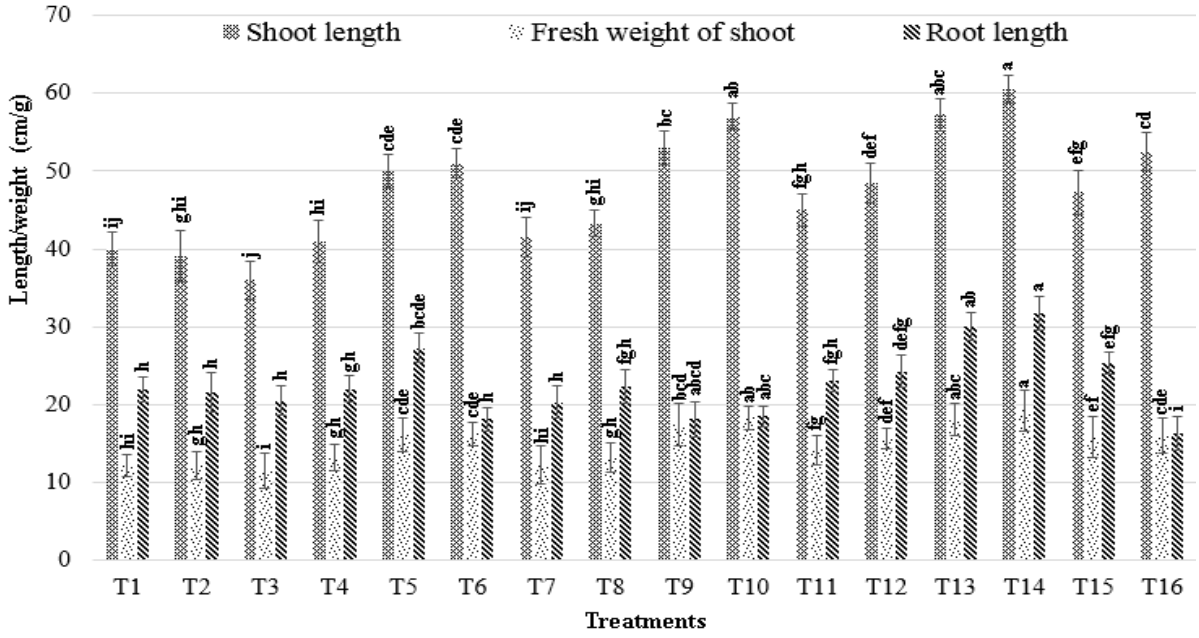

Fig. 1 (a) Effect of different rates of oak tree debris and *Metarhizium anisopliae* on growth parameters of tomato (shoot and root length, fresh weight of shoot), inoculated with *Meloidogyne javanica,* T1: Uninoculated control without oak debris, without *Metarhizium*; T2: Uninoculated control without oak debris, with *Metarhizium*; T3: Inoculated control without oak debris, without *Metarhizium*; T4: Inoculated control without oak debris, with *Metarhizium*; T5: Uninoculated, with 50g oak debris/kg soil, without *Metarhizium*; T6: Uninoculated, with 50g oak debris/kg soil, with *Metarhizium*; T7: Inoculated, with 50g oak debris/kg soil, without *Metarhizium*; T8: Inoculated, with 50g oak debris/kg soil, with *Metarhizium*; T9: Uninoculated, with 100g oak debris/kg soil, without *Metarhizium*; T10: Uninoculated, with 100g oak debris/kg soil, with *Metarhizium*; T11: Inoculated, with 100g oak debris/kg soil, without *Metarhizium*; T12: Inoculated, with 100g oak debris/kg soil, with *Metarhizium*; T13: Uninoculated, with 150g oak debris/kg soil, without *Metarhizium*; T14: Uninoculated, with 150g oak debris/kg soil, with *Metarhizium*; T15: Inoculated, with 150g oak debris/kg soil, without *Metarhizium*; T16: Inoculated, with 150g oak debris/kg soil, with *Metarhizium*

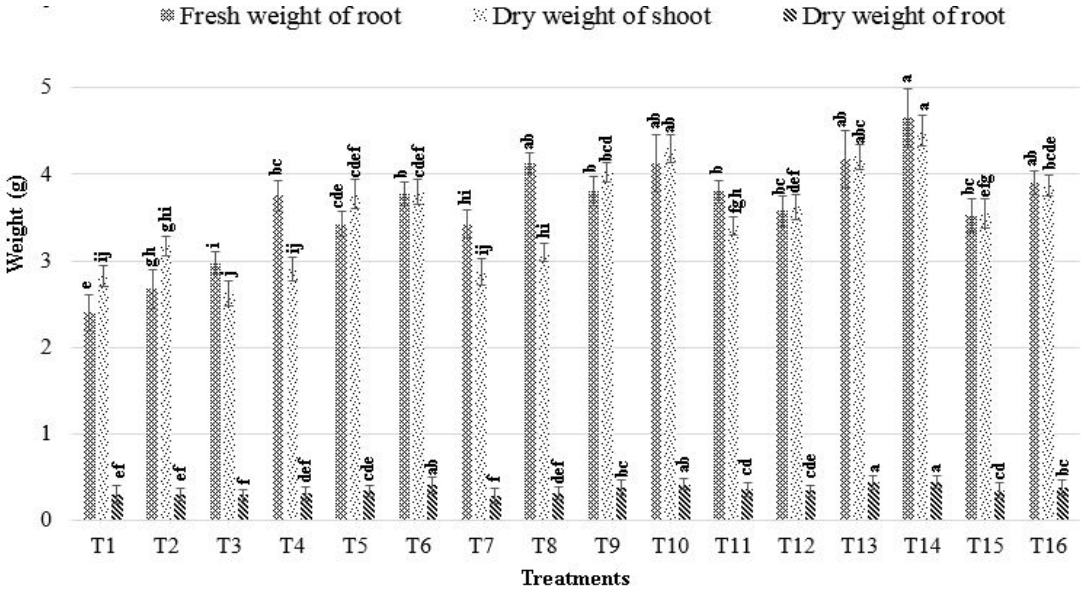

Fig. 1 (b) Effect of different rates of oak tree debris and *Metarhizium anisopliae* on growth parameters of tomato (fresh weight of root, dry weight of shoot and root), inoculated with *Meloidogyne javanica.* T1: Uninoculated control without oak debris, without *Metarhizium*; T2: Uninoculated control without oak debris, with *Metarhizium*; T3: Inoculated control without oak debris, without *Metarhizium*; T4: Inoculated control without oak debris, with *Metarhizium*; T5: Uninoculated, with 50g oak debris/kg soil, without *Metarhizium*; T6: Uninoculated, with 50g oak debris/kg soil, with *Metarhizium*; T7: Inoculated, with 50g oak debris/kg soil, without *Metarhizium*; T8: Inoculated, with 50g oak debris/kg soil, with *Metarhizium*; T9: Uninoculated, with 100g oak debris/kg soil, without *Metarhizium*; T10: Uninoculated, with 100g oak debris/kg soil, with *Metarhizium*; T11: Inoculated, with 100g oak debris/kg soil, without *Metarhizium*; T12: Inoculated, with 100g oak debris/kg soil, with *Metarhizium*; T13: Uninoculated, with 150g oak debris/kg soil, without *Metarhizium*; T14: Uninoculated, with 150g oak debris/kg soil, with *Metarhizium*; T15: Inoculated, with 150g oak debris/kg soil, without *Metarhizium*; T16: Inoculated, with 150g oak debris/kg soil, with *Metarhizium*

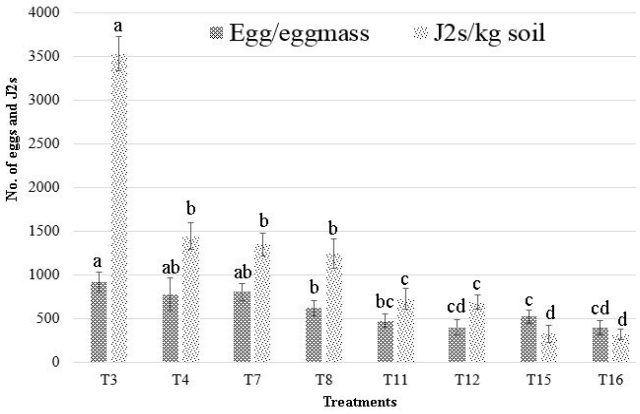

Fig. 2 (a) Combined effect of different rates of oak tree debris and *Metarhizium anisopliae* on reproduction of *Meloidogyne javanica* (eggs in egg mass and J2 in kg of soil) in tomato. T3: Treated without oak debris, without *Metarhizium* (control 1); T4: Without oak debris, with *Metarhizium* (control 2); T7: Treated with 50g oak debris/kg soil, without *Metarhizium*; T8: Treated with 50g oak debris/kg soil, with *Metarhizium*; T11: Treated with 100g oak debris/kg soil, without *Metarhizium*; T12: Treated with 100g oak debris/kg soil, with *Metarhizium*; T15: Treated with 150g oak debris/kg soil, without *Metarhizium*; T16: Treated with 150g oak debris/kg soil, with *Metarhizium*

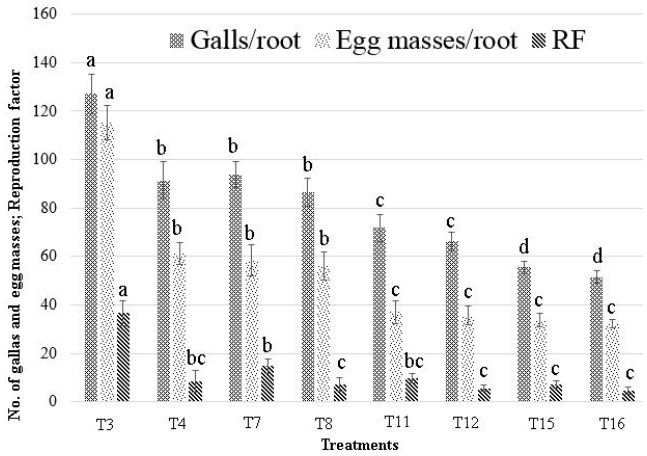

Fig. 2 (b) Combined effect of different rates of oak tree debris and *Metarhizium anisopliae* on reproduction of *Meloidogyne javanica* (galls and egg masses in root and reproduction factor) in tomato. T3: Treated without oak debris, without *Metarhizium* (control 1); T4: Without oak debris, with *Metarhizium* (control 2); T7: Treated with 50g oak debris/kg soil, without *Metarhizium*; T8: Treated with 50g oak debris/kg soil, with *Metarhizium*; T11: Treated with 100g oak debris/kg soil, without *Metarhizium*; T12: Treated with 100g oak debris/kg soil, with *Metarhizium*; T15: Treated with 150g oak debris/kg soil, without *Metarhizium*; T16: Treated with 150g oak debris/kg soil, with *Metarhizium*

In our experiment, we successfully used the fungus *M. anisopliae* against the root knot nematode, *M. javanica*.

Results showed that application of oak debris can significantly reduce the rate of nematode reproduction and improve the growth of tomato plants as well. In any treatment with the fungus, the growth of plant was improved, and the reproduction rate of the nematode has been decreased as comparing to the healthy plants (Control) ($P \leq 0.05$). Maximum shoot and root length, as well as fresh and dry weight of shoot and root was obtained in treatment in interaction of the fungus and oak debris ($OD_{150} \times F$) which was significantly above the normal growth in control plants ($P \leq 0.05$). Inoculation with nematode did not cause a significant decrease in growth parameters, as compared to uninoculated control, but application of oak debris or/and the fungus *M. anisopliae*, had promotive effects on plant growth and it was at the highest level for interaction of $OD_{150} \times F$. In case of inoculated tomatoes, best results were taken in treatments with 150 g of oak debris. Comparing to treatments with the same rate of oak debris, the interaction of $OD_{150} \times N \times F$ was more successful in grown promotion, than the interactions $N \times F$ and $OD_{150} \times N$, which is showing the enhancement of the bioactivity of the fungus in the presence of oak debris.

Some microorganisms are able to promote the growth of some plants. The capability of microorganism to colonizing the roots of plant is an important factor to have the promoting power [21]. According to previous study [22], an increase in shoot weight is due to health of roots that can have a good uptake and transport of water and nutrients. Some species of *Metarhizium* are attracted to roots of certain plant species and has root colonization ability [23]. Bio-priming effects of *M. anisopliae* on germination and seedling growth of flax seed have been shown earlier [24]. Based on their study, bio-priming treatments improved germination characteristics of flax seed as well as the seedlings vigor. In current study, all of growth-related factors of tomato plants were improved by soil amending, using oak debris. By adding the oak debris (OD; $OD \times F$; $OD \times N \times F$), growth related parameters had significantly increased, showing the significance of the interaction of oak debris with fungus in improving the rate of plant growth ($P \leq 0.05$).

Reduction in nematode population varied in different treatments. As compared to inoculated control (N), interaction of nematode with fungus ($N \times F$) significantly decreased the nematode related factors. The rate of eggs/root and the reproduction factor most affected than the other factors (more than three times reduction). According to research on *M. anisopliae* parasitism [7], the fungus produces sticky conidia that attach to nematode cuticle. The conidia germinate, parasitize and kill the cadaver, by direct penetration and producing the infective hyphae inside the nematode body. The fungus produces some cyclopeptides and destruxins which may play an important role in its pathogenicity [25]. The lethal effect of *M. anisopliae* culture extract has been also reported [26], [27].

It has been found that the interaction of "$OD_{150} \times N \times F$" was most effective treatment with seven times reduction of number of eggs/root and reproduction factor and ten times reduction in number of J2s/soil. Any increase in amount of oak debris, enhanced the nematocidal activity of the tested bioagent. Reductions in nematode population have been reported as a result of compost application in the soil [28]. As reported earlier, some changes in chemical and physical properties of the soil may induce plant response and also increase their tolerance against plant parasitic nematodes [29]. In our study, the greatest reduction in nematode-related factors was observed in those plants grown in soils which treated with 100 g of oak debris/kg of soil, with applying the bioagent, *Metarhizium*. Our results also showed that there is no significant increase in nematode control by adding more than 100 g of oak debris.

Our results on application of oak debris in the soil are similar to the findings of some researchers [30]. They have reported that plant parts of *Azadirachta indica* and *Melia azadirach* have nematicidal properties. In their experiments, development of *M. incognita* has been inhibited, when soil was amended with different parts of these plants. In a greenhouse experiment, application of neem cake at the rate of 1%, reduced the number of *Pratylenchus penetrans* and *M. hapla* in tomato roots, by 67% and 90%, respectively [31].

Organic amendments probably release ammonia with nematicidal properties related to increase of carbon dioxide and nitrogen levels [32]. Some phenolic compounds and terpenoids with nematicidal activity are also known in organic waste materials [33]. In our study, a good control of *M. javanica* has obtained by adding oak debris and/or *Metarhizium*. Some studies have been carried out to investigate the antimicrobial properties of oak. Analysis of the extract components of oak fruits has given [34]. According to their study, tannins and phenolic compounds could be responsible for antimicrobial activities. In a study on physical and chemical properties of debris of oak trees [35], it has proved that the decayed debris contains considerable amounts of organic carbon, total nitrogen, available phosphorus, absorbable potassium, EC, micro-nutrients such as iron, manganese and zinc. In their study, significant decrease in pH, calcium carbonate and copper in decayed debris, has been observed. In the time course of decomposition of plant debris, some substances such as phenol components or toxic materials including free ammonium gases, nitrate, sulfur gases and organic acids are produced. Such materials kill nematodes directly or reduce egg hatching. It may also make some chemical and physical changes in the soil and subsequently increase the amount of phosphorus, potassium and sodium of soil, improving the plant growth [36].

Soil condition is a main factor for enhancing the bioefficacy of useful microorganisms. It is necessary to provide a favorable environmental condition to enhance the effectiveness of bioagents. There is a positive correlation between the percentage of organic matter obtained from decomposition of plant debris in the rhizosphere and the population density of bioagents [37]. In our study, it has been observed that in amended soil, the tested fungus was more effective on nematode, than the unamended soil. Enhancing the biocontrol activity of *Pseudomonas fluorescens* and *Trichoderma vierns* against *Meloidogyne javanica*, by

application the oak debris in tomato, has been demonstrated [17]. Based on their study, soil amendment had significant effects on activity of tested bioagents. As compared to unamended soil, 56.3% reduction in root galling was observed in those soils that were amended with *T. virens*, and maximum increase in dry weight of root was respectively obtained by 68.2% and 56.1%, in treatments with *P. fluorescens* or *T. virens*.

V. Conclusion

In our studies, number of galls, egg masses and eggs were reduced by soil application of *M. anisopliae* spore suspension. So, it can be concluded that the tomato roots have been colonized by *M. anisopliae* and in this way, the rate of nematode penetration to the roots was declined. Soil amendment with plant materials such as chopped stem and leaf is a low cost method which can use in nematode management. Because this is a very safe and inexpensive method, it can be easily achieved by farmers. Co-application of two or more antagonistic agents can provide a good tool for use of different capabilities from different sources, against the target pathogen and make a broad spectrum of biocontrol by simulating the natural condition. It is suggested that providing a favorable condition for activating of antagonistic agents is required for having a successful biocontrol program against the plant pathogens. In our study, the role of plant debris in increasing the activity of the tested biopesicide has been shown. In our study, we did not observe any adverse effect of used antagonistic materials on plant growth. Complementary studies are required to investigate the effects of different rates of *Metarhizium* in combination with oak debris, and also, studying the effects of different environmental conditions on controlling the nematode is suggested.

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