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ASSOCIATED IMPACT OF MYCORRHIZAL FUNGI AND *Pasteuria penetrans* ON ROOT-KNOT NEMATODE *Meloidogyne javanica* MANAGEMENT ON TOMATO

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ABSTRACT

This study was carried out to evaluate the efficacy of two bio-control agents, mycorrhizal fungi and the endoparasitic bacterium *Pasteuria penetrans* in managing development of root-knot nematode, *Meloidogyne javanica* on tomato. Use of both bio-agents *P. penetrans* and mycorrhizal fungi, either alone or combined, reduced significantly all related nematode parameters on tomato plants infected with *M. javanica*. The reduction in galls, egg masses, females/root system and eggs/egg mass as well as juveniles in soil was highly significant when both bio-agents inoculated together as reached 76; 68; 74; 47 and 73%, respectively. Consequently, nematode infected plants that were treated with both bio-agents showed improvement in vegetative plant growth characters as reflected by a significant increase in the fresh shoots and roots as well as dry shoot weights of tomato compared with untreated plants. Moreover the contents of Nitrogen, Phosphorus and Potassium in tomato plants treated with both bio-agents were also increased compared with those found in untreated nematode infected plants. The overall disease symptoms caused by nematode on tomato was markedly alleviated in the pots treated with the tested bio-agents.

Keywords: *Meloidogyne javanica;* Mycorrhizal fungi; *Pasteuria penetrans*; Tomato (*Lycopersicon esculentum* cv. Super streen).

INTRODUCTION

Root-knot nematodes, *Meloidogyne* spp. are one of the most important plant-parasitic nematodes worldwide, especially in the new reclaimed lands in Egypt. These nematodes are cosmopolitan in distribution and spread in tropic and subtropic environments (Whitehead, 1997). They can attack almost all soft, underground plant tissues especially the roots of annual crops and play an important role in limiting vegetable production especially in tomato as it causes losses ranged from 24-38% (Sikora and Fernandez, 2005).

A lot of studies investigated several control methods against plant-parasitic nematodes i.e. chemical, cultural and physical as possible methods to reduce losses caused by nematode. Biological control of nematodes has been studied as an alternative or complementary approach to physical or chemical control methods (Weller, 1988).

The obligate endospore forming parasite *Pasteuria penetrans* has been extensively tested for the control of plant-parasitic nematodes (Duponnois *et al*., 1999). This bacterium has been attributed to the partial control of root-knot nematodes *Meloidogyne* spp. in pot experiments (Mahdy, 2002) and in microplots using either spore-infested soil or powdered roots containing

spores (Trudgill *et al*., 2000). Spore concentrations $5x10^4$ and $5x10^5$ per gram of soil showed immediate control of root-knot nematode populations in the field (Chen, *et al.,* 1996).

Mycorrhizal fungi are one of the promising bio-control agents against a broad range of pathogens especially plant-parasitic nematodes. It limits the densities of plant-parasitic nematodes on a wide range of host plants and subsequently reduces the losses in the crop yields (Sikora, 1981 and Sitaramaiah and Sikora, 1982). Moreover, various studies have showed that mycorrhizal fungi increase host tolerance in some plant-nematode associations (Talavera, *et al.,* 2002).

In modern agricultural systems, especially in horticulture, abundant application of chemical nematicides now makes very severe troubles in agricultural production, food safety and environmental protection. So, an effective and friendly environmental bio-nematicide is expected to be developed through biological control research.

Very little is known about the beneficial effects of combined application of *P. penetrans* and mycorrhizal fungi to soils infected with populations of *Meloidogyne* spp. Therefore, this research aimed to investigate the effectiveness of mycorrhizal fungi and *P. penetrans* isolate Egypt on development of root-knot nematode *M. javanica* in tomato and study their impact on tomato growth under greenhouse conditions.

MATERIALS AND METHODS

1- Preparation of *Meloidogyne javanica* inoculum:

Root-knot nematode, *Meloidogyne javanica* (Treub) Chitwood was used as inoculum in the experiment. Nematode inoculum was extracted from infected black nightshade roots (*Solanum nigrum* L.). Nematode stock culture was multiplied under greenhouse conditions at 25 ± 2^0C on tomato plants (*Lycopersicon esculentum* cv. Super streen) grown in 30 cm in diam. plastic pots filled with sand-soil mixture (2:1, v/v). The heavily galled tomato plants were uprooted and nematode eggs were extracted from roots by sodium hypochlorite technique (NaOCl) as described by Hussey and Barker (1973). Galled tomato roots were washed in water to remove soil debris. The roots were then cut into 1-2 cm pieces and macerated in a blender for 20 seconds. The macerates were filled into a flask containing 500 ml of 1.5% NaOCl solution and manually shaken for 3 minutes to free eggs from the gelatinous matrix. The eggs suspension was rinsed over a sieve combination of 250 µm, 100µm and 20µm to remove excess root debris. The eggs of 20µm sieve were gently washed several times to remove excess chlorine and collected under tap water. These eggs were passed again over the same sieve, recollected and hatched using a modified Baerman dish (Oostenbrink, 1960). The juveniles in the distilled water suspension were adjusted to 1000 J_2s/ml and used as inoculum.

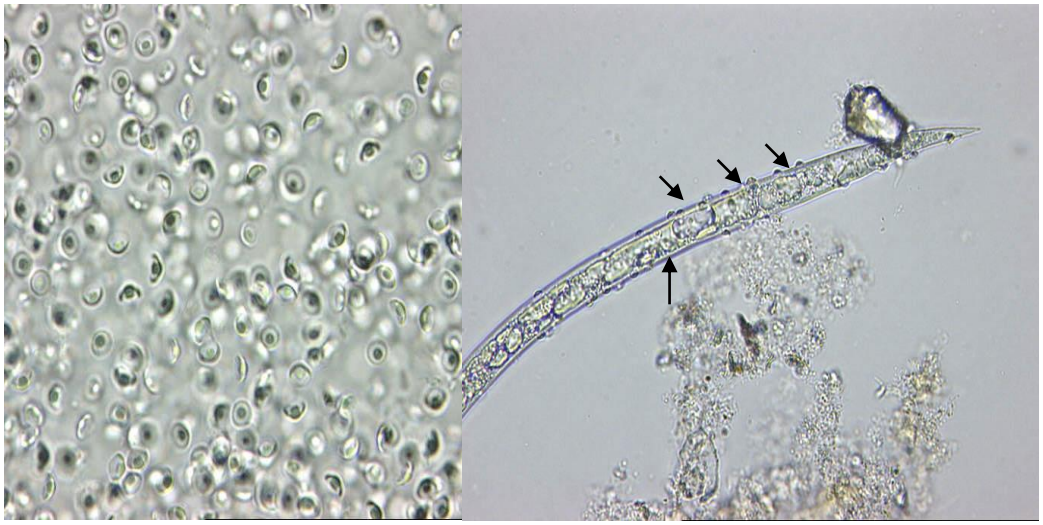
2- Preparation of spore suspension from *Pasteuria penetrans*:

The endoparasitic bacterium *P. penetrans* isolate Egypt (Pp EGY) was used to investigate its ability to attach to and control the root-knot nematode, *M. javanica* on tomato plants.

Spore suspension of *P. penetrans* isolate Egypt was prepared by adding 0.1 gram of Pasteuria root powder to small amount of distilled water and mixed using a pestle and mortar. After mixing thoroughly the root debris were removed by pouring the spore suspension through a 25µm sieve (Stirling and Wachtel, 1980). The concentration of the spore suspension (Fig. 1a) was counted with a hemicytometer slide and adjusted to $5x10^5$ spores/ml.

3- Attachment of *P. penetrans* spores to *Meloidogyne javanica* juveniles:

One thousand freshly second stage juveniles of *M. javanica* collected in 1 ml distilled water were added to 1 ml spore suspension of *P. penetrans* in test tubes and incubated for 24 hours at room temperature (25 ± 2^0C). After 24 hours, the attachment rate of *P. penetrans* was evaluated by counting the number of attached spores on 50 juveniles of *M. javanica* under Stereomicroscope (X600) (Fig. 1b).



(a) (b)

Fig. 1 (a) Spores of *Pasteuria penetrans* Isolate Egypt (Pp EGY).
(b) Attachment of Pp EGY spores to second-stage larva of *Meloidogyne javanica*.

4- Preparation of mycorrhizal fungi inoculum:

The mycorrhizal fungi spores isolated from the rhizosphere of wheat field were used for inoculum propagation on sorghum plants. Mycorrhizal fungi spores were extracted from 250 gm soil from the rhizosphere of sorghum plants. The soil was taken and diluted in 1 liter tap water, suspended and then sieved using wet-sieving and decanting technique according to Jackson

(1958). Four sieves (400, 250, 150 and 75 µm) were used in this experiment. The 250, 150 and 75µm fractions were transferred into a glass bottle and diluted with tap water to give between 40-50 spores/ml. The numbers of spores were estimated by spreading certain volume of mycorrhizal spore suspension onto a grided filter paper or petri dish divided into squares from its base. The number was recorded using a binocular microscope according to Daft and Hograph (1983).

Cultures of mycorrhizal fungi were raised on sorghum seeds, which were surface sterilized with chlorox solution 0.01% and sown (5 seeds/pot) in clay pot (25cm in diam.) containing steamily sterilized sand soil. Fifty spores were layered at 2-6 cm depths. Thinning was done to one seedling/pot. After 120 days, plants were uprooted and spores of mycorrhizae were isolated from the pot soil using wet-sieve and decanting method. The roots were then stained and examined for mycorrhizal colonization. The roots colonized by mycorrhizal fungi were mixed with sand soil containing mixed spores of genera: *Glomus* spp.; *Gigaspora* spp. and *Acaulospora* spp. and added to the soil as inoculum at the rate of 1000 spores/ g. This inoculum (1000 spores/pot) was spread as a layer at a depth of 3-5 cm in the pot at the same time of tomato transplanting.

To estimate the root infection with mycorrhizal fungi; root samples were washed several times with tap water to remove remaining soil particles. Each sample was cut into small pieces (1cm long) and covered with 10% KOH in test tubes. Test tubes were autoclaved for 20 minutes at 121^{o}C according to the modified method by Kormanik *et al*., (1980). Depending on the age and size of the roots, the host cytoplasm and most of the cell nucleic acid were removed to allow penetration of stains to the roots. The roots were then washed with tap water and acidified with 1% HCL for 1-2 minutes, then the acid was poured off. Roots were submerged in the trypan blue stain (0.05%) in lactic acid and heated in water bath at 80-90^{o}C for 10-15 minutes according to Phillips and Haymann (1970). The percentage of mycorrhizal root infection was determined in 10 root sections from each mycorrhizal fungi treatment.

5- Treatment of tomato plants with bio-agents and nematode:

The experiment was carried out in 15 cm in diam. pots filled with mixture of soil/sand 1:2 (v/v). A hole is made in the soil and the mycorrhizal fungi inoculum (1000 spore/pot) was added into the hole. Three weeks old tomato seedlings cv. Super streen were transplanted in the same hole and then covered with pure sand. Three days later, 1000 freshly second-stage juveniles and 3 ml of 5x10^{5} Pp spores/ml of spore suspension were inoculated into 3-4 holes around plant roots.

In this study, five treatments were prepared; 1-plants treated with fresh healthy juveniles of *M. javanica* alone, 2- Freshly juveniles of *M. javanica* and mycorrhizal fungi. 3- Freshly juveniles of *M. javanica* and mycorrhizal fungi and *P. penetrans* spores. 4- Juveniles of *M. javanica* and *P. penetrans* spores, 5- completely untreated plants served as controls. Each treatment was replicated 3 times. The treatment 1 and 2 were repeated 6 times more to estimate the penetration rate of nematode into tomato roots in presence or absence of mycorrhizae at different intervals; 2, 4, 8, 24, 48 and 336 hours (3

replicates of each). Plants were watered daily and fertilized weekly with nutrient solution.

6- Evaluation of nematode parameters and plant growth conditions:

After two months of inoculation, tomato roots were carefully removed and washed with tap water. Number of galls/root system; number of egg masses/root system; number of eggs/egg mass; number of healthy and *P. penetrans* infected females/root system; nematode reproduction factor (RF); nematode final population (Pf); as well as number of juveniles in soil were recorded. Egg masses of *M. javanica* were stained by dipping the roots in 0.015% Phloxine B solution for 20 minutes as described by Daykin and Hussey (1985). Rate of nematode reproduction (Pf/Pi) was calculated according to Norton (1978) whereas:

*Reproduction Factor = Final population (Pf)/Initial population (Pi)

Females infected with Pp spores were also recorded by collecting all females of *M. javanica* by cutting the root system of each plant in 2 cm pieces and dipping the root pieces in a beaker full of tap water for 4 days at room temperature until they become soft (Ratnasoma and Gowen, 1996). Roots were washed with vigorous tap water stream through 500µm and 250µm sieves to separate the females from the root debris.

Pasteuria infected females were distinguished by their opaque dull creamy white to amber colour compared to white, glistening females (Mankau and Prasad, 1977) as shown in Fig. (2). All collected females were crushed under a cover slips (3 females/slide) in a few drops of tap water and the presence of Pasteuria spores verified using the Stereomicroscope (X600) as shown in Fig. (1a).

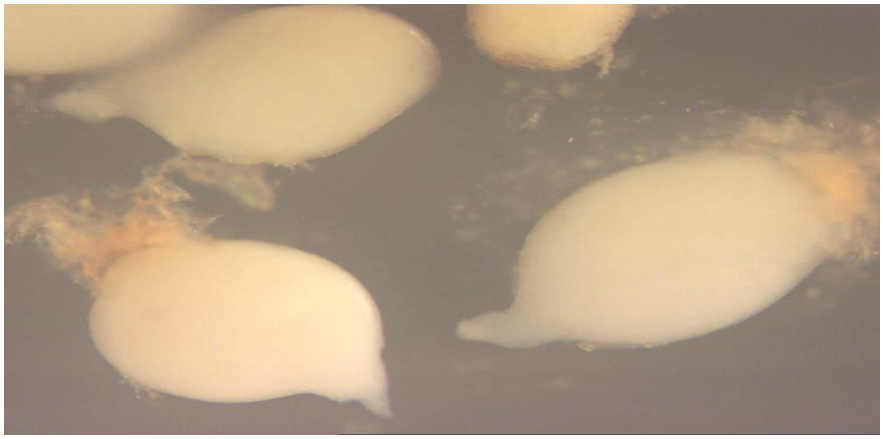


Fig. (2):Females of *Meloidogyne javanica* infected with *Pasteuria penetrans* isolate Egypt

The percentages of mycorrhizae infected roots after 30 and 60 days as well as the mean number of mycorrhizae spores in soil were also determined.

Vegetative plant growth parameters i.e. fresh shoot and root weights as well as dry shoot weight were also estimated. Chemical composition in plants

i. e. the percentages of nitrogen (N), phosphour (P) and potassium (K) contents were recorded.

All obtained data were statistically analyzed by using COSTAT-Computer program for statistics. The L.S.D. test at 5% level of probability was used to compare the means of the treatments according to Duncan (1955).

RESULTS

Data presented in table (1) revealed that the penetration rate of *M. javanica* juveniles into tomato roots inoculated with mycorrhizal fungi was increased over time either in presence or absence of mycorrhizal fungi. This increase was only significant after 336 hours of inoculation in pots treated with *M. javanica* alone as well as those treated with both *M. javanica* and mycorrhizal fungi.

Table (1): Effect of mycorrhizal fungi on the penetration rate of *Meloidogyne javanica* juveniles into tomato roots at different times.

Treatments	**Mean no. of penetrated juveniles after**						***Mean***
	2 hrs	**4 hrs**	**8 hrs**	**24 hrs**	**48 hrs**	**336 hrs**	
M. javanica + Mycorrhizal fungi	1.0	5.3	9.0	14.3	18.3	81.0	21.5 a
M. javanica alone	0.7	4.0	7.0	09.3	12.7	88.3	20.3 a
Mean	0.83 b	4.7 b	8.0 b	11.8 b	15.5 b	84.7 a	

Inoculation of both mycorrhizal fungi and *P. penetrans* either alone or together significantly reduced numbers of galls/root system, egg masses/root system and eggs/egg mass compared with plants treated with *M. javanica* alone. However, application of both bio-agents together was the effective treatment, as the percentage of reduction reached 76, 68 and 47% respectively (Table 2). Moreover, healthy females were lowered by 69% and 40% after application of mycorrhizal fungi and *P. penetrans,* respectively, and by 74% when applied together (Table 2). In this regard, reproduction factor was reduced by more than 50 % in plants treated with either individual or combined bio-agents.

Table 2. Effect of mycorrhizal fungi and *Pasteuria penetrans* on some nematode parameters.

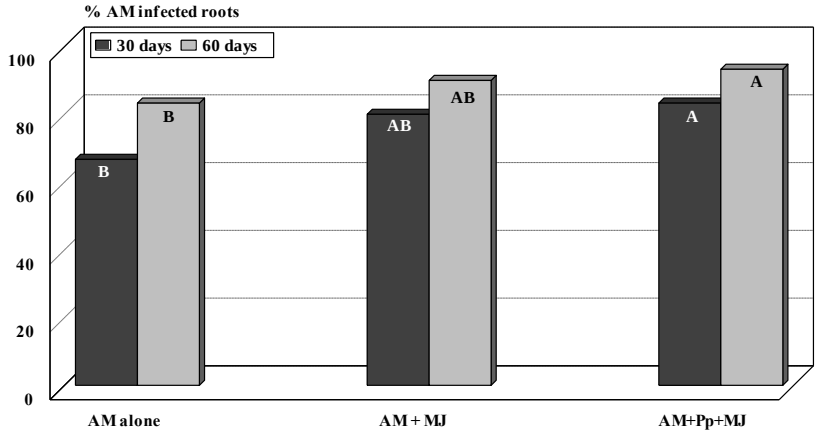
	Treatments*					
	Am-Mj	**Am-Mj-Pp**	**Mj-Pp**	**Mj**	**Am**	**Control**
Galls/root system	77.4 b	34 c	71.4 b	140 a	0.0 d	0.0 d
Egg masses/root system	17.4 b	13.4 b	14 b	41.4 a	0.0 d	0.0 d
Eggs/ Egg mass	169 b	117 c	153 b	219 a	0.0 d	0.0 d
Females	29.4 c	24.4 c	57.4 b	95.4 a	0.0 d	0.0 d
Pp. infected females	0.0 c	12 a	6.6 b	0.0 c	0.0 d	0.0 d
Reproduction factor	0.8 b	0.6 b	0.7 b	1.9 a	0.0 d	0.0 d
Number of Juveniles	$1.1x10^3$ b	$6.6x10^2$ b	$8.3x10^2$ b	$2.5x10^3$ a	0.0 d	0.0 d

*** AM= Arbuscular Mycorrhizae; MJ= *Meloidogyne javanica*; Pp= *Pasteuria penetrans***

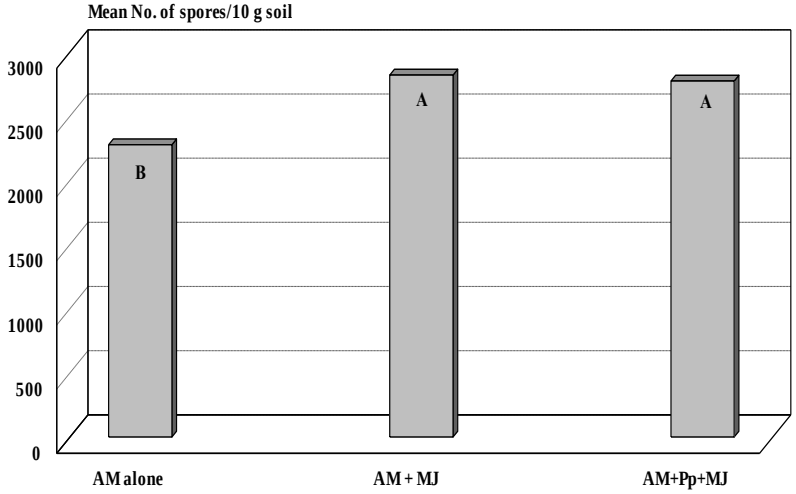
Data in table (2) also revealed that densities of juveniles per 250 g of soil were greater in pots treated with nematode alone than those treated with either mycorrhizae or *P. penetrans* or both bio-agents together. A significant increase in numbers of Pp infected females was also shown in the plants treated with mycorrhizae and Pp than those treated with Pp alone (table 2).

Results presented in Fig. (3 A and B) and illustrated in Fig. (4) revealed that the percentage of roots infected with mycorrhizal fungi and numbers of mycorrhizal spores in pots infected with *M. javanica* were significantly increased compared with those treated with mycorrhizal fungi alone. This increase in mycorrhizal infection was 17 % and 11% in presence of Pp and 20% and 14% in their absence after 30 and 60 days of inoculation, respectively. However, the percentage of mycorrhizal spores in rhizosphere of plants infected with nematode was almost similar in absence or presence of Pp as reached 18-19% after 60 days of inoculation.

(A)



(B)



AM= Arbuscular Mycorrhizae; MJ= *Meloidogyne javanica*; Pp= *P. penetrans*

Fig. (3): Effect of *Meloidogyne javanica* on % of mycorrhizal fungi infection after 30 and 60 days of nematode inoculation (A), and numbers of AM-spores in 10 g soil (B).

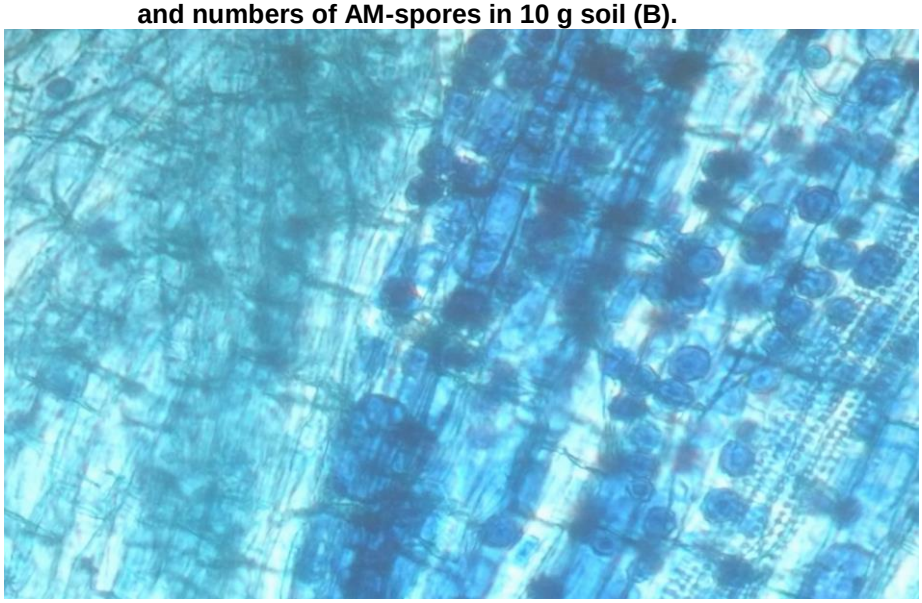
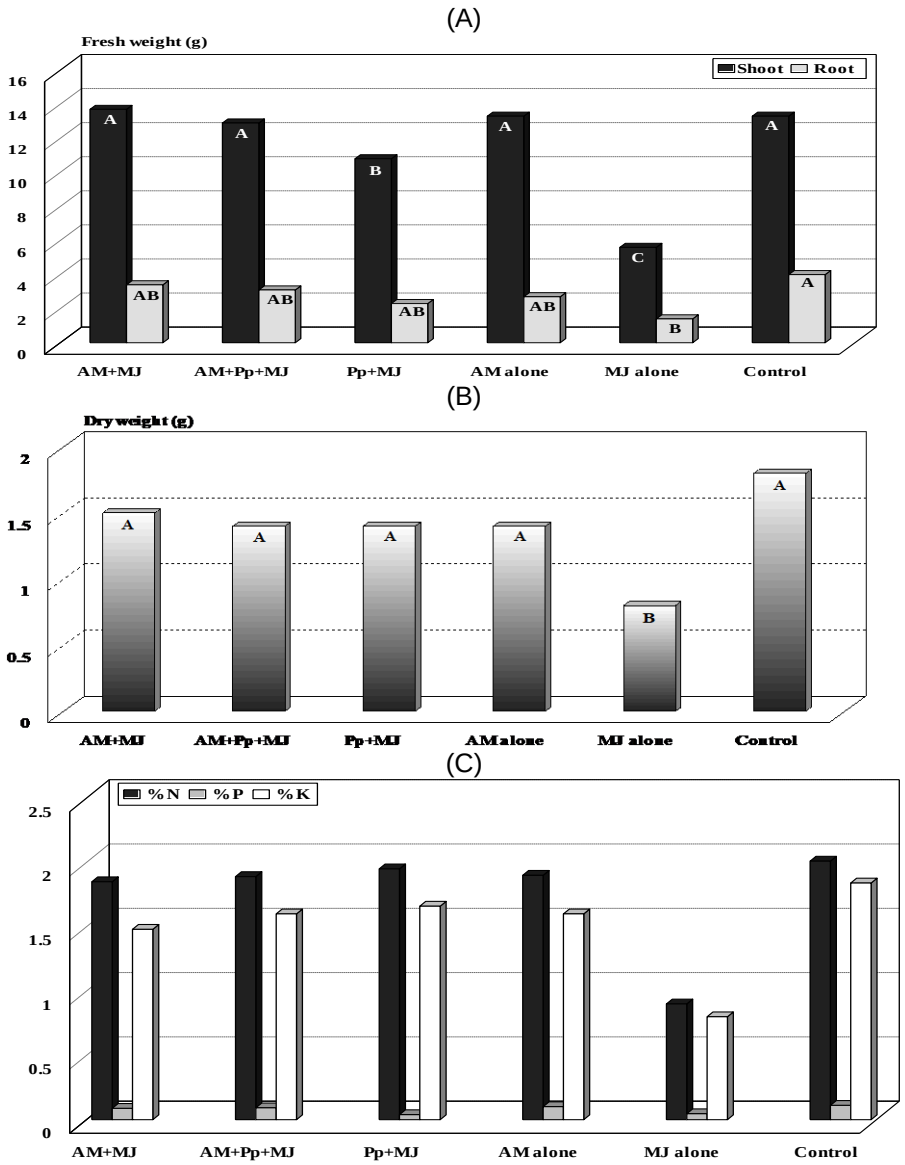


Fig. (4): Endomycorrhizal fungus vesicles and arbuscules of *Glomus* spp. within tomato root tissues (60 days age) infected with *Meloidogyne javanica*.

Results in Fig. (5) showed that all bioagents treatments either alone or combined significantly improved the fresh weight of shoot and root (Fig. 5 A) as well as dry weight of shoot (Fig. 5 B) of plant infected with nematode compared with those treated with nematode alone. This improvement in these characteristics reached 57%, 55% and 43% respectively, in plants treated with combined bio-agents. Moreover, the percentages of nitrogen (N), phosphour (P) and postassium (K) were also enhanced with the use of all bio-agents either individual or combined when compared with plants treated with nematode alone as shown in Fig. (5 C). A high increase in NPK contents (more than 50%) in plants infected with nematodes was shown when treated with both bio-agents together compared with those treated with nematode alone (Fig 5 C).



AM= Arbuscular Mycorrhizae; MJ= *Meloidogyne javanica*; Pp= *P. penetrans*

Fig. (5): Effect of used bio-agents either alone or combined on; fresh shoot and root weights (A), shoot dry weight (B) and % of NPK contents of tomato (C) infected with *Meloidogyne javanica*.

DISCUSSION

Inoculation of mycorrhizal fungi three days earlier than *M. javanica* did not retard penetration of nematode juveniles into tomato roots. Moreover, the penetration rate of juveniles was increased over time and became significant

after 2 weeks of nematode inoculation either in presence or absence of mycorrhizae. This might be due to the short period between application of mycorrhizae and nematodes and the weak early colonization of mycorrhizae. In this regard, Talavera et al., (2001), found that inoculation of mycorrhizae (*Glomus* sp.) two weeks earlier than *M. incognita* allowed high colonization proportion by mycorrhizae and reduced damages caused by nematodes.

On the other hand, numbers of mycorrhizal spores in soil and % of mycorrhizae infected roots was increased after 30 and 60 days in plants infected with nematode compared with those treated with mycorrhizae alone. Similar observation was shown by Bagyaraj *et al*., (1979), they found that inoculation of tomato roots with root-knot nematodes, *M*. *incognita* and *M. javanica*, enhanced infection and spore production by mycorrhizae (*Glomus fasciculatus*). In view of this, fast invasion of nematodes may facilitate penetration of mycorrhizal fungi into tomato roots and enhance colonization process.

The actual action of mycorrhizal fungi in reducing vegetative plant growth parameters caused by nematodes and other pathogens is not well known. The proposed hypotheses include improving the availability of phosphorus as well as other nutrients within the host and/or by antagonistic interactions with the pathogens (Siddiqui and Mahmood, 1995), or by inducing local and systemic resistance against *Meloidogyne* species Cordier *et al*., (1998), that might contribute to limit further development of *M. incognita* within roots (Diedhiou *et al*., (2003), and production and accumulation of compounds such as phenols, phytoalexins and hormones within mycorrhizal root system that affect nematode feeding (Talavera *et al*., 2002).

In *P. penetrans* treatments, the significant reduction in numbers of egg masses/root system and eggs/egg mass suggests that the main effect of Pp was in reducing egg laying (Talavera, et al., 2002). In this regard, Chen *et al*. (1996) and Adiko and Gowen, (1999) reported that the reduction in *M. javanica* development and reproduction and by *P. Penetrans* is mainly due to reduced mobility and death of spore-encumbered juveniles, and reduced egg-production by females.

Application of *P. penetrans* or mycorrhizae alone to nematode infected plants, although it had a marked effect on nematode development, combined inoculation of both *P. penetrans* and mycorrhizal fungi significantly reduced all nematode parameters compared with all other treatments. Moreover, it partially compensated for growth reduction in tomato plants infected with *M. javanica* (Fig. 6). In addition to nematode suppression by both bio-agents, improving the biological, chemical and physical properties of the soil and improving the mineral elements uptake might be complementary reasons of enhancing the growth of nematode infected plants.

AM-Pp-MJ AM-MJ MJ alone

AM= Arbuscular Mycorrhizae; MJ= *Meloidogyne javanica*; Pp= *P. penetrans*

Fig. (6) Impact of inoculation with the used bio-agents on vegetative tomato growth characteristics as affected by root-knot nematodes.

Our results also showed a significant increase in % of mycorrhizae infected roots in presence of *P. penetrans,* and in numbers of females infected with *P. penetrans* in plants treated with mycorrhizae. These results were consistent with the finding of Rao and gowen (1998), as they noticed a greater attachment and infection of *Pasteuria* to juveniles of *M*. *incognita* in presence of mycorrhizae. All these observations suggest that the positive effect on plant growth conferred by combined application of used bio-agents may due to a synergistic interaction between *P. penetrans* and mycorrhizal fungi. It may also explain the superiority of application of both bio-agents together over the treatments with either organism alone.

The application of these two organisms appears to be compatible and should be considered as a nematode management strategy. However, further study is needed to reconfirm the effectiveness of these bio-agents in the open field.

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التأثير المرتبط لفطريات الميكروهيزا وبكتريا باستيريا بينترانس على مقاومة نيماتودا تعقد الجذور ميلودوجينيا جافنيكا على نباتات الطماطم

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تم إجراء هذه الدراسة بهدف تقييم تأثير نوعين من كائنات التضاد الحيوي ، فطريات الميكروهيزا والبكتريا المتطفلة إجباريا وداخليا باستيريا بينترانس على مقاومة نيماتودا تعقد الجذور ميلودوجينيا جافنيكا على نباتات الطماطم .

أظهرت النتائج أن استخدام كلا كائني التضاد الحيوي بكتريا الباستريا بينترانس وفطريات الميكروهيزا سواء كل منهم بمفرده أو معا قد قلل معنويا كل الصفات الخاصة بالنيماتودا على نباتات الطماطم المعداه بنيماتودا تعقد الجذور ميلودوجينيا جافنيكا .

فلقد لوحظ انخفاض معنوي في عدد العقد الجذرية وعدد أكياس البيض وعدد الإناث للمجموع الجذري وعدد البيض لكل كيس بيض بالإضافة إلى عدد اليرقات في التربة . حيث وصلت نسبة الانخفاض في هذه الصفات إلى ٧٦ ، ٦٨ ، ٧٤ ، ٤٧ ، ٧٣% على التوالي وذلك عندما تم تلقيح كلا كائني التضاد الحيوي معا .

وترتب على ذلك تحسن في صفات النمو الخضري لنباتات الطماطم المعداه بالنيماتودا والتي عوملت بكلا كائني التضاد الحيوي معاً ، حيث وجد زيادة معنوية في الوزن الطازج لكل من المجموع الخضري والمجموع الجذري بالإضافة للوزن الجاف للمجموع الخضري مقارنة بنباتات الطماطم الغير معاملة .

علاوة على ذلك أظهرت النتائج أيضا زيادة في محتوى النيتروجين والفوسفور والبوتاسيوم لنباتات الطماطم مقارنة بالنباتات المعداه بالنيماتودا فقط .

وبصفة عامة وجد أن الأعراض المرضية الناتجة عن النيماتودا على نباتات الطماطم قد قلت وظهر تحسن ملموس فى الأصص المعاملة بكائنات التضاد الحيوي .