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INDUCE SYSTEMIC RESISTANCE AGAINST ROOT-KNOT NEMATODE, MELOIDOGYNE SPP.

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ABSTRACT: ***In this research, the ability of three rhizobacteria i.e. Serratia marcescens, Azospirillum lipoferum CRT1 and A. brasilense 245; one endophytic fungus Trichoderma harzianum as a spore suspension and as a talc powder formulation; one antioxidant Selenium at 25 and 50 ppm and one plant extract, Olive cake extract were used to induce systemic resistance toward Meloidogyne spp. in a split-root system. This research was carried out in two experiments. Results of both experiments revealed that all treatments reduced significantly all nematode parameters i.e. number of galls/half root system; number of egg masses/half root system; number of eggs /egg mass and number of juveniles/250 g soil when compared with plants treated with nematode alone. The percentage of reduction ranged between 17-87% in all nematode parameters in the first experiment, whereas it ranged between -0.49-88% in the second one. The highest reduction in nematode parameters in the first experiment was obtained with the antioxidant Selenium at 50 ppm followed by the rhizobacterium A. lipoferum CRT1 and the fungus Trichoderma spp. as a spore suspension respectively. The same results were also obtained in the second one. Results of both experiments revealed also that all treatments used enhanced the plant growth parameters i.e. fresh shoot and root weight, plant height as well as the chemical constituents i.e. phenoloxidase activity.***

Key words: ***Tomato Plants (Lycopersicon esculentum cv. Castle Rock); Rhizobacteria; Azospirillum spp.; Endophytic fungi, Trichoderma spp.; Antioxidant, Selenium; Plant extract.***

INTRODUCTION

Root-knot nematodes, *Meloidogyne*, is an important genus of plant parasitic nematodes that has a world-wide distribution especially in Egypt, extensive host ranges and is able to interact with other pathogens i.e. plant-parasitic nematodes, fungi, bacteria and virus to form complex disease syndromes (Agrios, 1988). Species of *Meloidogyne* cause severe damage to many crop plants, especially vegetable crops (Netscher and Sikora, 1990) as they reported that crop losses due to *Meloidogyne* exceed 32% on tomato according to one estimation by the International *Meloidogyne* Project (IMP).

Many different effective control methods were developed and used to manage plant-parasitic nematodes i.e. chemical, physical, cultural and biological methods. The most effective practical one of all these control methods was the chemical method followed by the biological control. Because of nematicides expensive costs, human, animal and environmental toxicity they are often not favorite to growers. Therefore, biological control is a safety alternative control method of plant-parasitic nematodes.

Induce systemic resistance is considered an effective technique against several pathogens attacking crop plants. Induced resistance is defined as an enhancement of the plants defensive capacity against a broad spectrum of pathogens and pests that is acquired after appropriate stimulation. The resulting elevated resistance due to an inducing agent to infection by a pathogen is called induce systemic resistance (ISR) or systemic acquired resistance (SAR) (Hammerschmidt and Kuc, 1995; Ramamoorthy *et al*., 2001).

The aim of this work is to evaluate some bio-control agents i.e. two rhizobacteria and one endophytic fungus; Selenium as antioxidant; and olive extract in controlling root-knot nematodes *Meloidogyne* spp. on tomato by using ISR technique.

MATERIALS AND METHODS

This research was designed as two experiments in split-root system as described by Hasky-Gunther *et al.*, (1998). Tomato plants (*Lycopersicon esculentum* cv. Castle Rock) grown in a three-pot system, each pot measuring 15 cm in diameter. Two weeks-old tomatoes were transplanted in the upper pot. Two openings at the bottom of the upper pot allowed the root to grow into the two bottom pots filled with mixture of sandy-clay soil (2:1 v/v) as shown in Fig. (1). The roots in the two bottom pots were spatially separated and treated individually.

In these two experiments two rhizobacteria i.e. *Serratia marcescens*, *Azospirillum lipoferum* CRT1 and *A. brasilense* 245; one endophytic fungus *Trichoderma harzianum*; one antioxidant Selenium and Olive cake extract were applied to manage root-knot nematodes.

The rhizobacterium *S. marcescens* was isolated from compost consists of animal wastes and rice straw (1:1) on nutrient agar medium (Difico, 1984) and was identified according to Bergy's Manual. The rhizobacteria *Azospirillum lipoferum* CRT1 and *A. brasilense* 245 were kindly supplied by UMR CNRS 5557 Ecologie Microbienne, Universite' Claude Bernard (Lyon 1), 43 bd du 11 Novembre, 69622 Villeurbanne Cedex, France. Bacterial inoculum of rhizobacteria was produced by fermentation in nutrient broth medium on a rotary shaker at 100 rpm for 24 hours at room temperature. The bacterial density was determined by serial dilution method and adjusted to optimum density (OD_{560}=2.0) by using spectrophotometer representing 10^9 and 10^7 cfu/ml, respectively (Abdul Munif, 2001). Each plant was inoculated with 5 ml of each rhizobacteria as a soil drench.

Figure (1): Split-Root System Technique

The endophytic fungus *Trichoderma harzianum*. was isolated from surface sterilized cotton seeds with 3% NaOCl for 3 minutes which was more effective in eliminating seed surface contaminants (Hallmann, 2001). After that, seeds were washed three times with distilled sterilized water, and then dried between 2 sterilized filter papers. The seeds were placed into sterilized porcelain dishes with a few drops of distilled sterilized water and then homogenized. One hundered microliter (100 µl) of seed suspension was inoculated on water agar and spread with a Drigaeski spatula to isolate the endophytic fungi. Petri dishes were incubated at 25°C for 3-4 days investigated daily and then the growing colonies were transferred onto Petri plates (9 cm in diameter) containing potato dextrose agar medium (PDA) supplemented with Streptomycin sulphate. *Trichoderma* spp. was the most frequent fungi in isolation and identified as *T. harzianum* according to Rifai (1969). Pure culture of *T. harzianum* was maintained on PDA medium and kept at 4^0C. *T. harzianum* was grown on potato dextrose broth (PDB) by inoculating equal disks of fungus into 500 ml flasks containing 200 ml PDB.

Flasks were incubated on a rotary shaker at 250 rpm at 28^{0}C for 2 weeks. Mixture of 10 gm of carboxymethyl cellulose (CMC) and 1kg of the talc powder were used to prepare the talc powder formulation as described by Vidhyasekaran and Muthamilan (1995). The fungal biomass (400ml) was added to the talc powder carrier (400ml/kg talc powder) and mixed well under sterilized conditions to form a pasta. The pasta was air dried in Laminar Flow Hood for 24 hours. The dried product was powdered using a blender, sieved and packed in polyethylene bags. One gram sub sample of *T. harzianum* was taken to count the colony forming unites (cfu/g) of fungal population by using dilution series method (Dhingra and Sinclair, 1995) on PDA medium. Five grams of *Trichoderma* powder (5g/pot) were incorporated thoroughly with soil pots. The spore suspension of *T. harzianum* was prepared by homogenizing the biomass of *Trichoderma* with the supernatant in a blender and then the colony forming units (cfu/ml) were determined and representing 6X10^5 cfu/ml. Five ml of the spore suspension was inoculated/plant as a soil drench.

The antioxidant Selenium was prepared at 2 concentrations 25 and 50 ppm and 10 ml of each one was applied as a soil drench/pot.

Olive cake extract was prepared by soaking 1kg of olive wet husk in 5 liter of distilled water, shaked overnight and then centrifuged at 4500 rpm for 10 minutes at 4^{0}C. Ten ml of olive cake extract were inoculated/pot as a soil drench. The different treatments were applied as follows:

In Experiment I:

Each treatment was applied in one side of the bottom pots and the nematode alone in the other one as shown in Fig. (2) to study the indirect effect of each treatment.

In Experiment II:

Both treatments and nematode were applied in one side of the bottom pots and nematode alone in the other one as shown in Fig. (3) to study the direct effect of each treatment.

T N T N

T = Treatment N = Nematode

Figure (2): Show the different treatments application system in experiment I.

T+N N T+N N T+N N

T+N =Treatment + Nematode N = Nematode

Figure (3): Show the different treatments application system in experiment II.

One week after the application of rhizobacteria, endophytic fungus, antioxidant and olive extract treatment, 2000 nematode eggs was applied to one and/or both bottom pots. Plants were received sterilized distilled water served as controls. Each treatment was replicated 3 times. Plants were watered daily and fertilized weekly with nutrient solution.

Eight weeks after nematode inoculation the tomato plants were uprooted and the roots of each plant were washed by the tap water. Total number of galls and egg masses/half root system; number of eggs/egg mass as well as number of juveniles/250 g soil were recorded. Egg masses were stained by dipping the root system of each plant in 0.015% phloxin-B solution for 20 minutes (Daykin and Hussey, 1985). Vegetative parameters i.e. fresh shoot and root weights, plant height as well as phenoloxidase enzyme activity were also measured. Phenoloxidase enzyme activity in optimum density fresh weight (O.D/g) was determined in fresh leaf samples after 45 minutes and was extracted by the method of Broesh (1954).

Data were statistically analyzed by using Software Statgraphics version 3.1 for Windows. Duncan's Multiple Range Test was used to test for significant differences among means at P≤0.05.

RESULTS

Results of experiment I revealed that all treatments significantly reduced all nematode parameters i.e. number of galls and egg masses/half root system; number of eggs/egg mass as well as number of juveniles/250 g soil. The percentage of reduction ranged between 17 and 87%. The highest reduction was recorded with the antioxidant Selenium at 50 ppm followed by the rhizobacterium *Azospirillum lipoferum* and the endophytic fungus *T. harzianum* as a spore suspension.

Application of the antioxidant Selenium at 50 ppm reduced the percentage of galls and egg masses/half root system; eggs/egg mass and juveniles/250 g soil by 85; 86; 79 and 77%, respectively followed by *A. lipoferum* as 84; 87; 74 and 78% respectively and *T. harzianum* by 86; 80; 75 and 58% as shown in Fig. (4 A&B). The rhizobacterium *S. marcescens* came at the fourth rank in reducing the percentage of nematode parameters as it reached 57; 71; 75 and 79%, respectively.

The lowest percentage of reduction of all used treatments against *Meloidogyne* spp. was recorded with the rhizobacterium *A. brasilense* as shown in Fig. (4 A&B).

Results also revealed that all treatments encouraged markedly plant growth characters i.e. fresh shoot and root weight as well as the plant height as shown in Fig. (5 A&B). For the fresh root weight, results confirmed that Selenium at 50ppm, *A. lipoferum* and *T. harzianum* as a suspension were the best treatment followed by *S. marcescens*, whereas *A. brasiliense* the lowest one either in presence or absence the nematode as shown in Fig. (5A). All used treatments increased markedly the fresh shoot weight and plant height.

Treating the plants by *A. lipoferum* and Selenium at 50 ppm led to significant increase in plant height, whereas *A. brasiliense* the lowest one as shown in Fig. (5B).

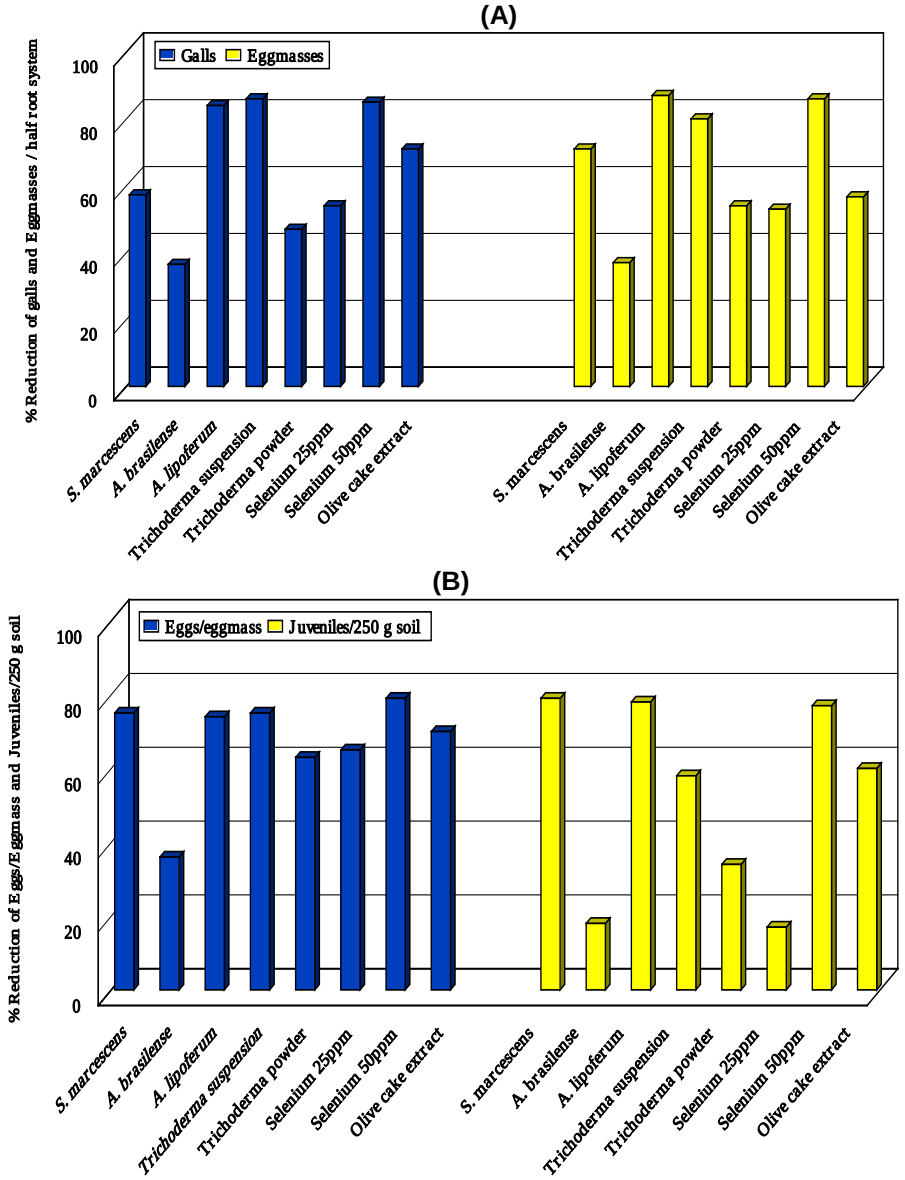


Figure (4): The percentage of reduction in number of galls; egg masses/half root system (A) and in number of eggs/egg mass and juveniles/250 g soil (B).

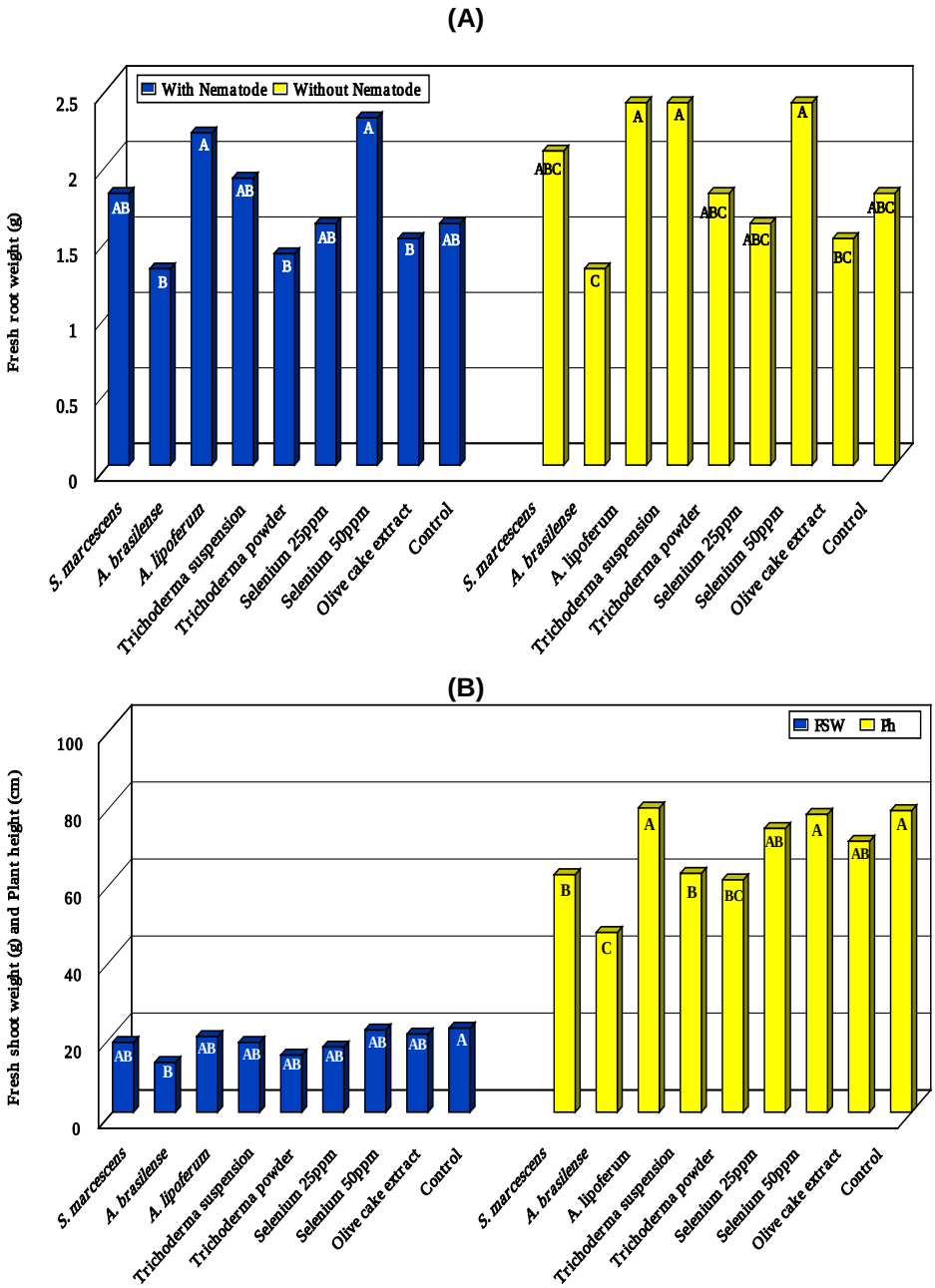


Figure (5): Effect of different treatments on fresh root (A); shoot weights and root length in plants infected with *Meloidogyne* spp. (B).

Results confirmed also in both experiments that adding some treatments significantly enhanced the activity of phenoloxidase enzyme. Selenium 50 ppm and *A. lipoferum* were the effective treatments in enhancing the phenoloxidase activity when compared to plants treated with nematode alone in Exp. I as shown in Fig. (6). Results found that also many treatments in Exp.II have positive effects in encouraging the activity of phenoloxidase enzyme when compared with treated plants with nematode alone. The effective treatments were *T. harzianum* suspension; *A. lipoferum*; Selenium 50 ppm and *A. brasiliense* as shown in Fig. (6). The lowest one in both experiments was olive cake extract treatment.

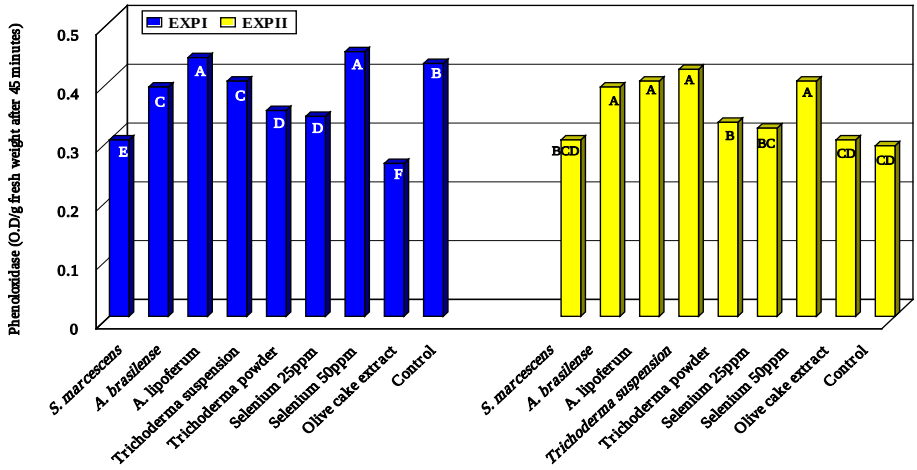
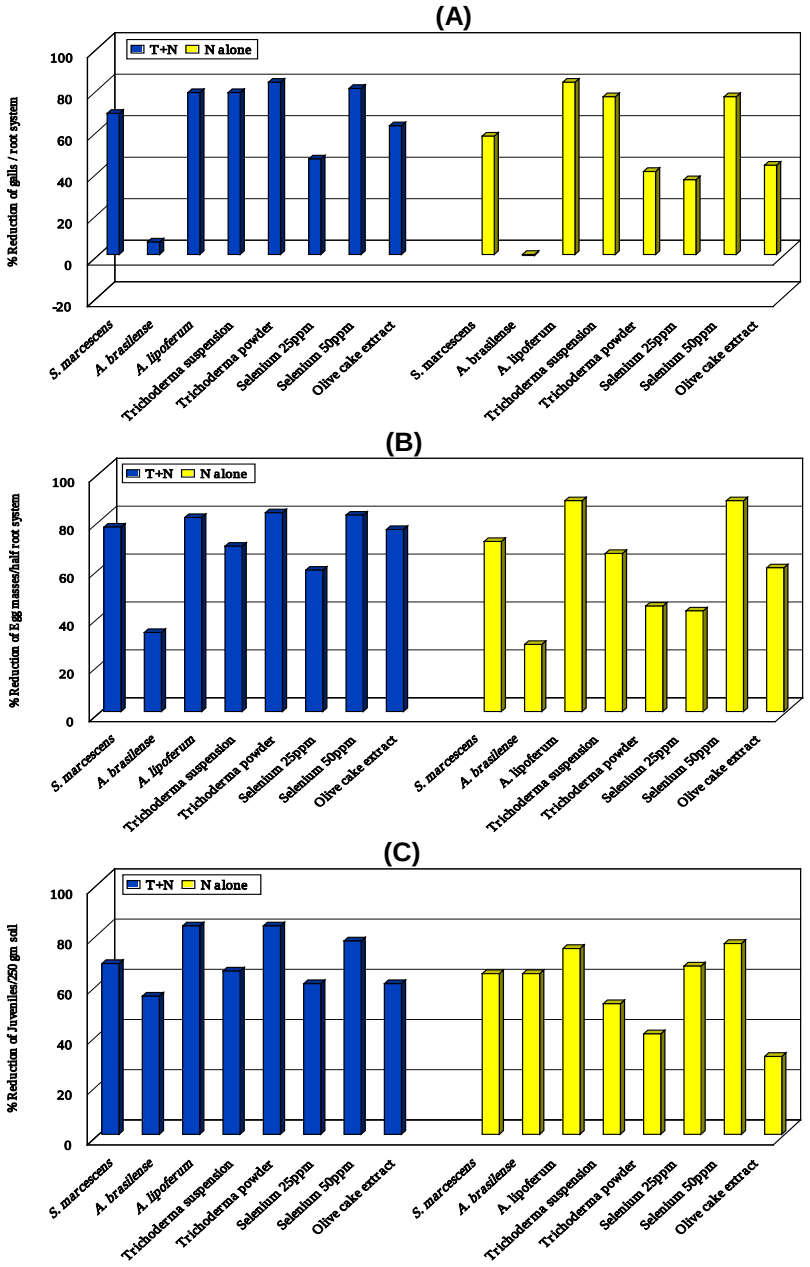


Figure (6): Effect of different treatments on phenoloxidase enzyme activity after 45 minutes in healthy and infected plants with *Meloidogyne* spp in both two experiments.

Results of experiment II clear also that all treatments reduced markedly all nematode parameters compared with plants treated with nematode alone. Results of nematode parameters showed the same trend of experiment I except the rhizobacterium *A. lipoferum* as it came at the first rank followed by the antioxidant Selenium at 50 ppm and *T. harzianum* as a spore suspension, respectively.

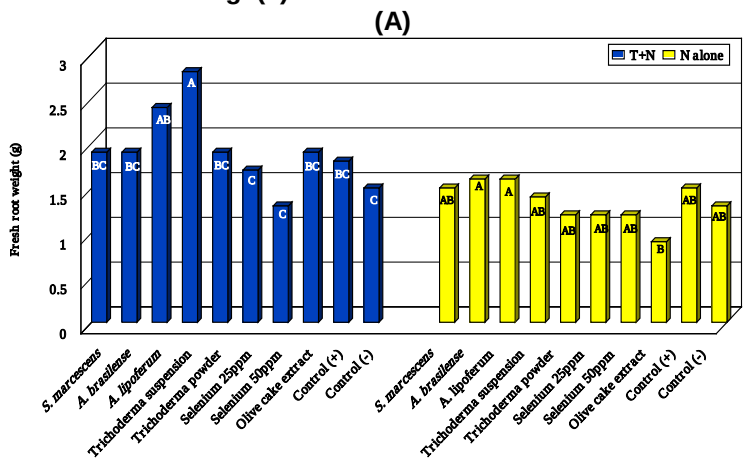
Results revealed that Selenium at 50 ppm led to the best result in reducing the percentage of galls and egg masses/root system; eggs/egg mass and juveniles/250 g soil at both bottom treated pots either in the pot treated with Selenium or the another pot treated with nematode alone without Selenium as shown in Fig. (7 A, B&C). The percentage of reduction were 80 and 76%; 82 and 88%; 73 and 79% as well as 77 and 76% respectively either in presence or absence Selenium 50 ppm followed by *A. lipoferum* by 78 and 83%; 81 and 88%; 72 and 62% as well as 83 and 74%, respectively as shown in Fig. (7 A, B&C).



T+N= Treatment + Nematode **N alone= Nematode alone**

Figure (7): The percentage of reduction in number of galls (A); egg masses/ root system (B) and juveniles /250 g soil (C).

Results showed also that all treatments were promoted the plant growth characters compared with plants treated with nematode alone as shown in Fig (8 A&B). The same results referred that all treatments enhanced significantly the phenoloxidase enzyme activity compared to plants treated with nematode alone. The highest increase in phenoloxiodase enzyme activity in Exp.I was recorded with the antioxidant Selenium at 50 ppm followed by the rhizobacterium *A. lipoferum* as shown in Fig. (6). The obtained results in Exp.II showed that the endophytic fungus *T. harzianum* as a spore suspension came in the first rank followed by the antioxidant Selenium at 50 ppm and there is no significant differences between both treatments as shown in Fig. (6).



T+N= Treatment + Nematode **N alone= Nematode alone**

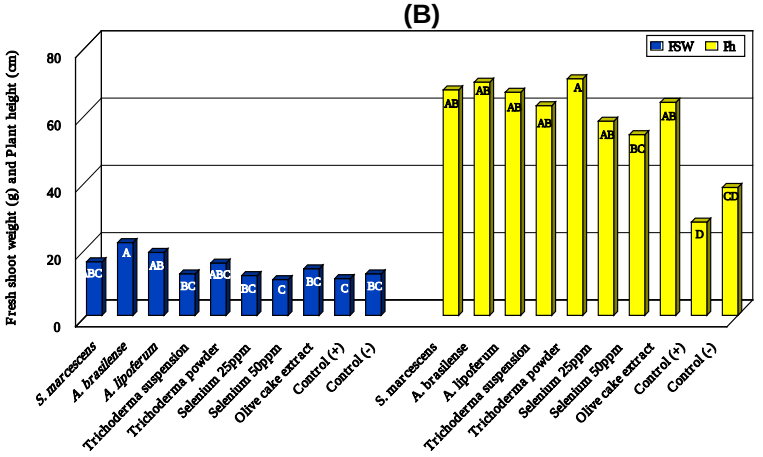


Figure (8): Effect of different treatments on fresh root (A); shoot weights and root length in plants infected with *Meloidogyne* spp. (B).

DISCUSSION

Our results revealed that all used treatments decreased all nematode parameters i.e. number of galls and egg masses/half root system; number of eggs/egg mass as well as number of juveniles/250 g soil. Results found that also the antioxidant Selenium at 50 ppm; the rhizobacterium *A. lipoferum* and the endophytic fungus *T. harzianum* either as a suspension or as a powder were the most effective treatments.

The effectiveness of *T. harzianum* might be due to different modes of action i.e. induced resistance to some plant pathogens, competition for nutrients and space; and microbial changes in the plant rhizosphere. Moreover *T. harzianum* increase the populations of beneficial microbes as observed by Levy *et al.* (2006). In this regard, Sharon *et al*. (2001) found that the fungus *Trichoderma harzianum* can act against the nematode juveniles and eggs. The fungal hyphae coil around juveniles and penetrate them as well as to nematode eggs as a direct parasitism. They revealed that also *T. harzianum* produced metabolites affect the juvenile's viability and egg hatching.

The positive effect of Selenium may be due to the several beneficial effects of Selenium in plants, although Selenium is not considered to be required by higher plants (Terry *et al*., 2000). Moreover, Tailin Xue *et al.* (2001) indicate that the antioxidant Selenium may exert a beneficial role in plants under stress. Hartikainen and Xue (1999) support that Selenium defends plants under stress. Selenium also protect plants against fungal infection and from herbivory as reported by Hanson *et al*., (2003) and plays an important role in the defense response in many plant species to pathogen attack (Omar Borsani *et al*., 2001). Antioxidants toxicity toward several pathogens has been reported as indicated by Galal and Abdu (1996) and Abd-El-Megid *et al*. (2004). Saleh *et al*. (2009) who found that the antioxidant increased oxidative enzyme catalase activity.

Oda Steenhoudt and Jos Vanderleyden (2000) reported that the genus *Azospirillum* comprises predominantly rhizosphere colonizing bacteria that can enter the root system. They found that also *Azospirillum* ranked as one of the best characterized genera among associative plant growth-promoting rhizobacteria and concerning stimulation of plant development. They revealed also that *Azospirillum* inoculation an alteration in root morphology as it produced plant growth regulating substances. *Azospirillum* can positively influence plant growth, crop yields and N-content of the plant. From these results it can be postulated that the rhizobacerium *Azospirillum* may has an indirect effect as it enhanced the plant growth by producing regulating substances. These substances alter the root morphology and consequently the root exudates and that it may be affect the nematode behavior i.e. movement, orientation towards the roots and invasion into roots, so reduced the galls and the egg mass numbers / half root system.

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المقاومة المستحثة لنيماتودا تعقد الجذور "جنس ميلودوجينا"

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الملخص العربى

فى هذا البحث تم استخدام ثلاثة انواع من الريزوبكتريا : ***Serratia marcescens*** ؛ ***Azospirillum lipoferum*** **CRT1** ؛ ***A. brasilense*** **245** ؛ وفطر الأندوفايت ***Trichoderma harzianum*** كمعلق جراثيم و كبودرة و مضاد الأكسدة سيلينيوم بتركيزين ٢٥ و ٥٠ جزء فى المليون و مستخلص بقايا نباتات الزيتون وذلك لدراسة قدرتهم على استحثاث المقاومة فى نباتات الطماطم ضد نيماتودا تعقد الجذور ***Meloidogyne spp.***. هذا و قد صمم هذا البحث فى تجربتين و قد اكدت نتائج كلا التجربتين حدوث انخفاض معنوى فى كل الصفات الخاصة بالنيماتودا مثل اعداد العقد النيماتودية و عدد اكياس البيض وعدد البيض/كيس بيض وكذلك عدد اليرقات/٢٥٠ جرام تربة وذلك عندما قورنت بالنباتات المعاملة بالنيماتودا فقط . هذا وقد تراوحت نسبة الأنخفاض فى الصفات النيماتودية بين ١٧ الى ٨٧% وذلك بالتجربة الأولى، بينما تراوحت النسبة بين ٤٩- . الى ٨٨% فى التجربة الثانية. وكانت اعلى نسبة انخفاض فى الصفات النيماتودية فى التجربة الأولى عند معاملة النباتات بمضاد الأكسدة السيلينيوم عند تركيز ٥٠ جزء فى المليون تلاها بكتريا ***Azospirillum lipoferum*** **CRT1** و فطر ***Trichoderma harzianum*** عندما اضيف فى صورة معلق جراثيم. نفس النتائج تم الحصول عليها فى التجربة الثانية. وجد كذلك من نتائج كلا التجربتين ان كل المعاملات المستخدمة قد شجعت من الصفات الخضرية لنباتات الطماطم مثل الوزن الطازج للمجموع الخضرى و الجذرى وطول النبات وكذلك قد شجعت هذه المعاملات من نشاط انزيم الفينول اوكسيديز.