Journal of Phytopathology and Pest Management 2(2): 1-8, 2015
pISSN:2356-8577 eISSN: 2356-6507
Journal homepage: http://ppmj.net/

Effect of the Bio-agents (*Bacillus megaterium* and *Trichoderma album*) on Citrus Nematode (*Tylenchulus semipenetrans*) Infecting Baladi orange and Lime Seedlings

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Abstract

The effect of two commercial compounds namely Bioarc ™ (*Bacillus megaterium*) and Biozeid ™ (*Trichoderma album*) at different rates against *Tylenchulus semipenetrans* was examined under laboratory and greenhouse conditions. In laboratory experiment, both compounds Bioarc and Biozeid as bio-control agents found to be highly nematostatic agents against J2 of *T. semipenetrans*. The rates of 20, 25 and 30g/l gave more than 60% J2 mortality after 72h exposure time. The highest effect was achieved at 30 g/l whereas, Bioarc (90.5 %) exhibited the highest effect followed by Biozeid (88.3%) at 30g/l after 72h exposure time. In greenhouse experiment, the bio-agents were evaluated at rates of 20, 25 and 30 g/l compared to nematicide Nemathorin 10% G at recommended dose (12.5 kg/ feddan) on the development of *T. semipenetrans* infecting Baladi orange and Lime seedlings. Results showed significant differences between the tested rates of both bio-agents. At the higher rate (30.0g/l), Bioarc (89.0, 89.5%; 76.6, 82.9%) was found to be more effective than Biozeid (88.3, 89.0%; 72.0, 77.9%) in reducing number of J2/100g soil and females/g root of Baladi orange and Lime infected with *T. semipenetrans* respectively. Moreover, both Bioarc and Biozeid at 30.0g/l significantly increased plant growth of Baladi orange and Lime infected with *T. semipenetrans*.

Key words: Biological control, *Trichoderma album*, *Bacillus megaterium*, *Tylenchulus semipenetrans*, Nemathorin, Citrus.

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Introduction

The citrus nematode, *Tylenchulus semipenetrans* Cobb, causes slow decline disease, which name refers to the gradual development of symptoms in the host and slow rate of nematode population in newly replanted orchards. The citrus nematode was recorded as a pathogen attacking the roots of citrus trees in Egypt by Oteifa (1955) as well as many different countries of the world Duncan and Cohn (1990). Management of the citrus nematode remains difficult as no single tactic provides adequate control of the nematode, Verdejo and McKenry (2004). Therefore, alternative methods of controlling nematode in particular by the use of microorganisms as biocontrol agents are now being widely developed and several of them are already being produced commercially Montasser et al. (2012). Bioproducts contain microorganisms (bacterium, fungus, virus, protozoan or alga) as an active ingredient often referred to microbial pesticides. They are host specific and potential candidates with regard to integrated pest management Arora et al. (2000). Fungi and bacteria are among the most dominant soil-borne groups in natural soil ecosystem and some of them have shown great potential as biological control agents for root-knot nematodes Kerry (2000). The bacterial and fungal bio-control agents were tested against the nematode infection by many workers. *T. album* and *B. megaterium* have been used as biocontrol agents against soil borne, foliar and post-harvest phytopathogenic fungal pathogens Abou-Zeid and Zaid (2006). The soil fungi *Trichoderma* spp. are potential nematode bio-control agents on many food, vegetables and cash crops Dababat and Sikora (2007), Affokpon et al. (2011). Besides *Trichoderma* spp., *B. megaterium* is common soil beneficial bio-fertilizer belonging to plant growth promoting rhizobacteria have also been used for controlling root-knot nematode Padgham and Sikora (2007), Oliveira et al. (2009). The biological effects of the bio-active product of marine brown algae of *Ascophyllum nodosum* have been proved economic and eco-friendly approach in reducing root-knot nematode infestation Wu et al. (1998). In the past few decades, a number of commercial products based on microorganisms have been developed and marketed as bio-pesticides, bio-fertilizers and soil amendments. However, the use of imported foreign bio-agents, that are less adapted to local climates, conditions or target species, has lead to contribute a limited success and variability of results. It may therefore be necessary to isolate and identify locally suitable isolates for use in nematode management programs Stirling (1991). The ability of *B. megaterium* (Bioarc) and *T. album* (Biozeid) was tested for controlling *Meloidogyne incognita* on tomato by Radwan et al. (2012) and *Meloidogyne javanica* on sunflower by Hammad and Zaid (2007).

The aim of the present study was assess the effect of *Bacillus megaterium* and *Trichoderma album* against citrus nematode (*Tylenchulus semipenetrans*) infected Baladi Orange and Lime seedlings growth under laboratory and greenhouse conditions.

Materials and methods

Bio-agents: Two commercial compounds, namely Bioarc ™ (*Bacillus megaterium*, 2.5 x 10^7 colony forming unit (cfu/g) and Biozeid ™ (*Trichoderma album*, 1 x 10^7spores/g) used as bio-control agents against, *T. semipenetrans* in laboratory and greenhouse conditions. The tested compounds were obtained from the identification of microorganisms and biological control unit, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt.

***In viro* experiment:** Four rates (15.0, 20.0, 25.0 and 30.0 g/l), of both Bioarc and Biozeid were prepared and separately tested against each of one hundred freshly hatched juveniles of *T. semipenetrans* in Petri dishes (9 cm. diameter). The examination was followed according to schedule time of exposure (12, 24, 48 and 72 hours). Petri dishes containing distilled water served as control and each treatment was replicated four times, and incubation at 25 ± 2° C. Juveniles of *T. semipenetrans* exhibited no perceptible movement under stereomicroscope were considered as dead, counted and the corrected mortality (immobility stages) percentage was calculated according to Abbott formula, Abbott (1925).

$$\%\ \text{mortality} = \frac{\text{Number of juveniles killed}}{\text{Total number of juveniles}} \times 100$$

Greenhouse experiment: This work was undertaken in greenhouse of Plant Pathology Department, Faculty of Agriculture, Assiut University, Assiut, Egypt. Three months–old seedlings of citrus rootstocks; Baladi orange (*Citrus sinensis*) and Lime (*C. aurantifolia*) were transplanted singly in clay pot (40 cm diameter) filled with 4kg sterilized sandy-clay soil, then the pots were divided into treatments according to the following bio-agents rates, 20, 25 and 30 g/l of both *T.album* and *B. megaterium*, A volume of 30 cm^3 for each bio-agent was added to each of three pots (treatment). Nemathorin 10% G as a nematicide conc. of 12.5kg/ feddan (recommended dose) was used for comparison. After 10 days of application with either bio-agents or Nemathorin, each pot was inoculated with 5000 juveniles of *T. semipenetrans* obtained from the stock culture and added around the root system of each seedling. Pots were arranged in a completely randomized design for each treatment and watered. Plants were harvested 90 days after nematode inoculation. Data dealing with fresh shoot and root weight were recorded. Nematodes were extracted from 100gm soil according to sieving and modified Baermann technique Goodey (1957). Roots were stained with acid fuchsin in lactic acid Byrd et al. (1983) and counted for females/g root. Data was subjected to statistical analysis using ANOVA Gomez and Gomez (1984) and means were compared by Duncan's multiple range test Duncan (1955).

Results and Discussion

***In viro* experiment:** Data in Table 1 showed that the percentage of J2 mortality increased with increasing the rates and exposure periods. Both tested compounds were found to be a highly nematostatic against J2. Results showed that the rates of 20, 25 and 30 g/l gave higher mortality effect than 60% J2 mortality after 72 h exposure time. The two bio-agents, *B. megaterium* and *T. album* reached their highest activity at rate of 30g/l after 72h (90.5 and 88.3 respectively). Results also showed significant differences between them. Moreover, *B. megaterium* exhibited higher mortality effect than the *T. album*.

Table 1: Effect of different rates of *B. megaterium* and *T. album* on J2 mortality of *T. semipenetrans* under laboratory conditions.

		J2 mortality after (hours)				
Treatment	Rate g/l	12	24	48	72	Mean
B. megaterium	15	32.3 r	35.3 q	38.0 p	40.8 o	36. 60 g
	20	51.3 m	55.0 l	59.5 k	62.3 ij	57.03 e
	25	60.0 jk	63.5 hi	66.3 g	68.0 g	64.45 c
	30	80.3 de	84.0 c	87.0 b	90.5 a	85.45 a
T. album	15	20.3 u	25.0 t	28.3 s	32.8r	26.60 h
	20	49.3 mn	50.8 mn	58.0 k	62.5 l	55.15 f
	25	59.0 k	60.3 jk	63.0 i	65.8 gh	62.03 d
	30	77.5 f	80.0 e	82.5 cd	88.3 ab	82.08 b
Control	0	1.0 v	1.0 v	2.0 v	2.0 v	1.50 i
Mean		47.89 d	50,54 c	53.84 b	57.0 a	-

- Each value presented the mean of four replicates.
- Means in each column followed by the same letter are not significantly different at $p < 0.05$ according to Duncan multiple range test.

Greenhouse experiment: Data in Table 2 represent the percentage of reduction in number of J2/100g soil, number of females/g root and final population of *T. semipenetrans* that is obviously increased with increasing bio-agents rates. The effect of *B. megaterium* and *T. album* at 30g/l showed no significant difference in previous criteria on both Baladi orange and Lime. *B. megaterium* was found to be more effective than *T. album* in reducing number of J2 and females. The commercial products of *B. megaterium* (89.0, 89.5%) and *T. album* (88.3, 89.0%) at the rate of 30g/l gave the highest % of reduction in number of J2 of *T. semipenetrans* on Baladi orange and Lime respectively. Meanwhile the highest % of reduction in number of females/g root recorded with *B. megaterium* (76.6, 82.9 %) and *T. album* (72.0, 77.9% %) on Baladi orange and Lime respectively. The highest reduction in final population was obtained with *B. megaterium* on Baladi orange (88.3 %) and Lime (89.1%).

Table 2: Effect of *B. megaterium* and *T. album* applied at different rates on population density of *T. semipenetrans* under greenhouse conditions.

		Baladi orange						Lime					
		Nematode population						Nematode population					
Treatment	Rate g./l.	J_2/100 g soil	% R	Females/g. root	% R	Final population	% R	J_2/100 g soil	% R	Females/g root	% R	Final population	% R
B. megaterium	20	230.3^{d}	78.5	31.0^{d}	52.5	261.3^{d}	77.0	142.0^{g}	86.4	16.3^{gh}	73.0	158.3^{g}	85.7
	25	150.0^{f}	86.0	18.7^{fg}	71.4	168.7^{f}	85.2	119.3^{hi}	88.6	12.7^{hi}	78.9	132.0^{ij}	88.1
	30	118.3^{hi}	89.0	15.3^{gh}	76.6	133.6^{ij}	88.3	109.7^{j}	89.5	10.3^{ij}	82.9	120.0^{k}	89.1
T. album	20	252.0^{c}	76.5	35.3^{c}	45.9	287.3^{c}	74.8	153.7^{f}	85.3	20.3^{ef}	66.3	174.0^{f}	84.3
	25	170.4^{e}	84.1	23.7^{e}	63.7	194.1^{e}	82.9	123.3^{h}	88.2	15.7^{gh}	74.0	139.0^{hi}	87.4
	30	125.3^{h}	88.3	18.3^{fg}	72.0	143.6^{h}	87.4	115.0^{ij}	89.0	13.3^{hi}	77.9	128.3^{j}	88.4
Nemathorin	Rec.	50.3^{k}	95.3	7.3^{j}	88.8	57.6^{l}	94.9	50.8^{k}	95.1	8.0^{j}	86.7	58.8^{l}	94.7
Control	0	1073^{a}	-	65.3^{a}	-	1138.3^{a}	-	1045^{b}	-	60.3^{b}	-	1105.3^{b}	-

- Each value presented the mean of three replicates.
- Means in each column followed by the same letter are not significantly different at $p < 0.05$ according to Duncan multiple range test.

Data on plant growth based on shoots, roots and whole plant fresh weight shown in Table 3 revealed that all tested treatments applied at different rates successfully improved the growth as compared to infected untreated Baladi orange and Lime seedlings. Significant differences between *B. megaterium* and *T. album* observed in plant growth of Baladi orange and Lime. A high percentage of increase in fresh shoot and root weight was recorded with *B. megaterium* and *T. album* at rate of 30g/l. However, rate 20g/l for both *B. megaterium* and *T. Album* caused the lowest increment in this respect. The same trend was obtained with total plant fresh weight as compared with nematode alone. In general all tested treatments as well as Nemathorin 10% G caused remarkable increase in the plant growth of Baladi orange and lime seedlings. Nemathorin showed superior effect than the two bio-agents, however the bio-agents were safer to human and animal health and the environment, as well as lower cost. These results are in agreement with those obtained by many reporters Hammad and Zaid (2007), Hallman et al. (2009), Affokpon et al. (2011), Radwan et al. (2012). Cherif and Benhamou (1990) recorded that *Trichoderma* spp. considered a good source of various toxins and may produce lytic enzymes, so it may play an important role in the inhibition of *T. semipenetrans* populations. Also Spiegel et al. (2005) concluded the direct fungal parasitism of the spores to nematode and diffusion of their lytic enzymes which stimulated with special genes. These enzyme genes were turned on during the interaction between the fungus and the nematodes. So, a significant enhancement of fungal parasitism on the nematode was done. Similar mode of action of *Bacillus* spp. was noticed by Niu-qiu Hong, et al. (2006), when extracted the crude extra-cellular protein from culture of bacteria.

Table 3: Plant growth response of Baladi orange and Lime seedlings infected with *T. semipenetrans* under greenhouse conditions.

Treatment	Rate g./l.	Baladi orange						Lime					
		Fresh weight (g)						Fresh weight (g)					
		Shoot	% Increase	Root	% Increase	Fresh wt. of the plant (g)	% Increase	Shoot	% Increase	Root	% Increase	Fresh wt. of the plant (g)	% Increase
B. megaterium	20	75.3^{cde}	32.8	92.7^{efg}	2.2	168.0^{def}	14.1	69.3^{f}	45.3	90.7^{fg}	1.6	160.0^{fg}	16.7
	25	79.3^{abc}	39.9	96.7^{de}	6.6	176.0^{cd}	19.2	76.3^{bcd}	60.0	97.3^{cde}	8.9	173.6^{cde}	26.6
	30	81.3^{abc}	43.4	106.3^{ab}	17.2	187.6^{ab}	27.1	79.3^{abc}	66.2	102.7^{bc}	15.0	182.0^{bc}	32.4
T. album	20	65.7^{fg}	15.9	90.3^{fg}	1.8	156.0^{gh}	5.6	60.7^{gh}	27.3	89.7^{g}	0.44	150.4 ^{h}	9.5
	25	69.3^{f}	22.2	95.7^{def}	5.5	165.0 ^{ef}	11.8	63.3^{g}	32.7	92.3^{efg}	3.4	155.6^{gh}	13.3
	30	71.3^{def}	25.7	102.7^{bc}	13.2	174.0^{cde}	17.8	69.7^{ef}	46.1	98.3^{cd}	10.1	168.0^{def}	22.5
Nemathorin	Rec.	84.7^{a}	49.4	110.3^{a}	21.6	195.0 ^{a}	32.1	82.3^{ab}	72.5	109.3^{a}	22.4	191.6^{a}	39.8
Control	0	56.7^{h}	-	90.7^{fg}	-	147.4^{h}	-	47.7^{I}	0	89.3^{g}	-	137.0^{i}	-

- Each value presented the mean of three replicates.
- Means in each column followed by the same letter are not significantly different at $p < 0.05$ according to Duncan multiple range test.

This extraction killed about 80% of the tested nematode within 24 h. They also found that the purified protease can hydrolyze several native proteinaceous substrates, including collagen and nematode cuticle. Beside this result the percentage increase in fresh weight of the whole plant which obtained by using bio-nematicide agent supported by Haseeb et al. (2005) & Khan et al. (2005). In conclusion, the bio-effectiveness of treatments with *T.album* and *B. megaterium* at higher rate of 30 g/l resulted an improvement in Baladi orange and Lime growth and reduced the reproduction rate of *T. semipenetrans* further experiments are need to test *B. megaterium* and *T.album* at rate 30 g/l per square meter in open field as a bio-nematicides.

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