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Bio-management of Citrus Nematode, *Tylenchulus semipenetrans* and Dry Root Rot Fungi, *Fusarium solani* under Laboratory and Field Conditions

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ABSTRACT

Slow decline and dry root rot of citrus caused by *Tylenchulus semipenetrans* and *Fusarium solani*, respectively are serious diseases attacking many groves in Egypt. Efficacy of the bio-agents (*Trichoderma harzianum*, *Bacillus subtilis*, *Streptomyces griseus* and *Paecilomyces lilacinus*) against citrus nematode *T. semipenetrans* and their combination with Nemastop were studied *in vitro*. Such bio-agents and Nemastop were also studied under field conditions against both *T.semipenetrans* and *F. solani* infecting Washington Navel orange trees compared with control and Nemaphos. *In vitro* study revealed that all tested bio-agents had various degrees of effectiveness towards the juvenile's survival compared with control treatment. Meanwhile, Bio-Nematon achieved the highest percentage of mortality for *T. semipenetrans* (50.0%). The population and incidence of soil borne pathogens were examined after three, seven and twelve months *in vivo.* Results showed that all treatments led to clear significant reduction in disease incidence compared with control treatment. All bio-agents gave good effect on decreasing *T. semipenetrans* population and *F. solani* incidence for twelve months. Benefits, achieved by simultaneous application of bio-agents based on different mechanisms of actions, are discussed. A clear significant decrement in *T. semipenetrans* population was noticed with the combination of *T. harzianum* with Nemastop. Meanwhile, *B. subtilis* or *T. harzianum* reduced *F. solani* incidence.

Key words: *Bacillus subtilis*, *Streptomyces griseous*, *Trichoderma harzianum*, *Paecilomyces lilacinus*, dry rot and citrus nematode diseases

INTRODUCTION

Citrus (*Citrus* spp.) is one of the most important fruit crops grown in many tropical and subtropical countries. In Egypt, citrus has great attention due to its importance for local consumption or exportation to the European countries. Plant diseases caused by soil borne pathogens such as nematode and/or fungi have been considered as major problems in agricultural production throughout the world (Abd-ElGawad et al., 2009).

The citrus nematode, *Tylenchulus semipenetrans* (Cobb. 1913) is an important plant-parasitic nematode which is widely distributing in citrus growing regions worldwide where nematode population levels may affect fruit yield differently under various conditions causing citrus slow decline disease (El-Borai et al., 2002).

Likewise, dry root rot disease of citrus caused by *Fusarium solani* (Mart.) is a serious disease attacking citrus trees causing 39.6% loss in fruit yield (Catara and Polizzi, 1999). *Fusarium* spp. can be pathogenic on citrus roots alone (Nemec et al. 1989) or in combination with nematodes (Labuschange et al. 1989), which leads to the great destruction of the feeder roots. The loss of feeder roots results in increase drought stress and decrease uptake of soil nutrients, leading to chlorosis and loss of leaves. Affected trees do not die, but have an unthrifty appearance and yield fewer, smaller fruits than un- infested trees.

Controlling of nematode and soil borne diseases on citrus depends mainly on chemical applications. However, these chemical substances are always undesirable due to their high cost and their hazard potentials to the environment. Therefore, utilization of use bio-agents instead of the chemical forms of fungicides (Russo and Berlyn, 1990**)** or nematicides, has been regarded as effective and save tool**.**

For instance, *Paecilomyces lilacinus* is capable of parasitizing nematode eggs,and females causing reduction in soil population densities of plant parasitic nematodes *T. semipenetrans* **(**Atkins et al., 2005). Park et al. (2004) reported that *P. lilacinus* could produce leucino toxin and other nematicidal compounds.

Furthermore, *Trichoderma* spp. play major roles in controlling plant diseases in roots and soil. The antagonistic activities of *Trichoderma* spp. have been documented as effective biological control agents for plant diseases caused by soil borne fungi and nematodes (Mclean et al., 2004). The hyphae of *Trichoderma* could penetrate nematode eggs and juvenile's cuticle by dissolving the chitin layer through enzymatic activity. Thus, the enzymes produced by *Trichoderma* spp. such as chitinases, glucanases and proteases seem to play an important role in parasitism (Haran et al., 1996 and Sharon et al., 2001).

Although *Bacillus subtilis* was reported as a bio-agent against soil borne fungi (Ali, 2013) some strains of *B. subtilis* exhibited enormous potential as bio- agent in the management of nematodes (Huang et al., 2005). *B. subtilis* would act through producing a number of antibiotics as bacterocin and subitisin (Khan et al., 2002 and Huang et al., 2009).

Streptomycetes are the major group of actinomycetes producing secondary metabolites that could decrease the invasive juveniles of root-knot nematodes. Streptomyces is known for its chitinoliytc activity which produces more extracellular chitinase (Mahdevan and Crawford, 1997). Some species of streptomycetes release compounds like antibiotic that inhibit the growth of plant-pathogenic fungi (Nemec et al., 1996) and plant parasitic nematodes (Dicklow et al., 1993).

Nemastop (natural oils) as commercial nematicide, play very important role in controlling nematodes**.** The effect of Nemastop on the nematode might be due to alkyl cysteine sulphoxides which released a mixture of volatile alkyl thiols and sulphides (Coley-Smith, 1976). Whereas, Nemaphos belonging to organophosphate group (Giannakou et al., 2005) showed a highly performance systemic nematicide.

The aim of the present work, was an attempt to increase the efficacy of Nemastop through the integration with four different bio-agents against *T. semipentrans* and *F. solani* on citrus trees.

MATERIALS AND METHODS

Tested bio-agents

Four bio-agents i.e. *B. subtilis, T. harzianum, S. griseus* and *P. lilacinus* were used for laboratory and field experiments.

Bio-agents and nematocide source

B. subtilis, *T. harzianum*, *S. griseus* and Nemastop were kindly obtained from central lab of Organic Agric. Agriculture Research Center, Giza, Egypt. Bio-Nematon (a commercial product of *Paecilomyces lilacinus*) and Nemaphos were obtained from Nematode Department, Plant Pathology Institute, Agric. Research Center, Giza, Egypt.

Bio-agents culture

B. subtilis was grown on nutrient glucose broth (NGB) suggested by Dowson (1957), *T. harzianum* was grown in liquid gliotoxin fermentation medium (GFM) (Brian and Hemming 1945). *S. griseus* was grown in Starch nitrate medium (Waksman 1959).

Chemical nematicide

Nemaphos is a commercial name, which belongs to organophosphate. The active ingredient is Fenamiphos which contains phosphoramidate ester.

Laboratory experiment (*in vitro*):

Inhibition effect of Nemastop and different bio-agents, on *T. semipenetrans* under laboratory conditions.

The effect of Nemastop (natural oils) and four bio-agents *i.e., B. subtilis, T. harzianum, S. griseus, P. lilacinus* were tested on survival of *T. semipenetrans* juveniles under laboratory conditions. Fifty active nematode juveniles were pipetted in vials (5cm-diameter) then bio-agent's suspensions and Nemastop were separately added at the rate of one ml/ vial to detect their effect on the juvenile's mortality. The same number of juveniles received distilled water and served as control at room temperature. Each treatment was applied in four replicates. The percentage of juvenile's mortality was recorded after 48 hours under a stereoscopic microscope.

Field experiment:

The present work was carried out in a private orchard at Menia EL-kamh, Sharkia governorate during 2017and 2018 seasons on 10 years old Washington Navel orange trees, (*Citrus aurantium* L.) naturally infested with *T. semipenetrans*. The trees were spaced 5×5 m apart and an experimental area was irrigated by flood irrigation system. Three replicates were used for each treatment, each replicate contains ten trees. Different treatments were added in March 2017 as soil drench at the rate of 500 ml/tree. Nemastop was added in soil with concentrations 1L: 50 L of water.

Bio- Nematon 1 % WP contains 2 x 10^4cfu/mg of fungus (*P. lilacinus*) was used according to the recommended dose 4kg/ fed. Nemaphos was used at the rate of 6ml /100L water twice with 15 days interval. The Bio-agents were added as single treatments or integrated with Nemastop to study their effect on *T. semipenetrans* and

F. solani. The population counts of both in soil were obtained before application and after, 3, **7**, and 12 months.

The method developed by Louw and Webely (1959) for studying the soil micro-flora was used to isolate soil borne fungi. The developed fungi were purified using single spore technique (Dhingra and Sinclair, 1995) and identified according to microscopic characteristics according to Booth (1971) for *Fusarium* spp. The percentage of reduction in pathogen density during different months was recognized, to figure out any correlation between pathogens and dominant climatic conditions during growing season.

Extraction of nematode from the soil and microscopic examination:-

Soil samples for nematode assay of each treatment were collected once before the treatment's application as zero day, after that all treatments were applied and sampled 3, 7 and 12 months after applications. *T. semipenetrans* was extracted with a modified sieving and centrifugation technique (Grewal et al., 1999) and counted using a compound microscope. Citrus nematode counts were expressed as number of second-stage juveniles (J_2) in soil, number of females and eggs/egg mass in root. Percentages of nematode reduction in either soil or roots (% efficiency) were determined.

Statistical Analysis:

Data obtained were subjected to statistical analysis of variance for completely randomized design (CRD) and randomized complete block design (RCBD) as outlined by Steel and Torrie (1980).

RESULTS AND DISCUSSION

The effect of different bio-agents singly or integrated with Nemastop on *T. semipenetrans* survival was determined *in vitro* (Table 1). All tested bio-agents had various degrees of effectiveness toward the juvenile's survival compared with control treatment. Bio-Nematon (50.0%) and Nemastop (48.0%) gave the best mortality percentage followed by *T. harzianum* (46.0%).

Table 1. Mortality percentages of *Tylenchulus semipentrans* using different bio-agents singly or integrated with Nemastop *in vitro.*

Treatments	**Nematode (j_2) after 48 h**		**Mortality %**
	Alive	Dead	
Trichoderma harzianum	27.0^{e}	23.0^{f}	46.0
Streptomyces griseus	37.0^{c}	13.0^{h}	26.0
Bacillus subtilis	46.0^{b}	4.0^{i}	8.0
Nemastop	16.0^{h}	24.0^{e}	48.0
Bio-nematon*	25.0^{f}	25.0^{d}	50.0
Trichodermaharzianum+ Nemastop	10.0^{i}	40.0^{a}	80.0
Streptomyces griseus + Nemastop	16.0^{h}	34.0^{b}	68.0
Bacillus subtilis + Nemastop	33.0^{d}	17.0^{g}	34.0
Bio-nematon + Nemastop	19.0^{g}	31.0^{c}	62.0
Control	50.0^{a}	0.0^{j}	0.0

N= 50 second stage of *Tylenchulus semipentrans* (J_2).*Bio-nematon =*P.lilacinus*

Mortality % = (number of dead nematode juveniles in treatment / (number of live nematode juveniles in treatment + number of dead nematode juveniles in treatment) × 100

The effect of Nemastop on nematode may be due to alkyl cysteine sulphoxides which released a mixture of volatile alkyl thiols and sulphides, these volatile compounds override the inhibitory effect on parasitic nematode (Coley-Smith, 1976). Also, the mode of action of Bio-Nematon (*P. lilacinus*) may due to direct penetration of fungal hypha to the female cuticle of *Meloidogyne* spp. and infested eggs then destroys the embryos within 5 days because of simple penetration of the egg cuticle by individual hypha aided by mechanical and/or enzymatic activities. Otherwise, it could produce leucino toxin and other nematicidal compounds, these was interpreted in multitude investigations (Jatala, 1986; Jatala et al.,1985; Bonants et al., 1995; Khan et al., 2004; Park et al., 2004; Khan et al., 2006; Khalil, 2013 ; Soares et al. 2015; Abd-Elgawad, 2016). *T. harzianum* results are compatible with other studies showing the ability of *Trichoderma* inoculum densities suppressed the nematode reproduction and root galling compared to control (Al-Hazmi and Javeed, 2016). Meanwhile, B. subtilis was the least one (8.0%). Combination between *T. harzianum* and Nemastop recorded the highest effect on *T. semipenetrans* mortality (80.0%) followed by *S. graseius* + Nemastop (68.0%). These results are compatible with those of Qingfei et al. (2013) who stated that *Streptomyces* spp. produce lytic enzymes and nematicidal compounds and can be one of candidates for bio-agents against nematodes. While, *B. subtilis* combined with Nemastop occupied the last rank (34.0%).

Effects of bio-agents singly or combined with Nemastop on controlling citrus nematode, *T. semipenetrans* and root rot disease induced by *F. solani* in orange trees were studied to figure out the most effective treatment to reduce nematode population and root rot incidence. Data in Table (2) show that population density of *T. semipenetrans* juveniles drastically decreased after three months from application. Among single application, *T. harzianum* (42.9%) significantly (P=0.5) suppressed total nematode population. However, the greatest reduction was recorded with soil receiving the dual application of *T. harzianum* and Nemastop (62.2%) relative to control plants. All bio-agents either in single form or in mixture led to significant reduction in *F. solani* incidence compared with control.

The most effective treatment was induced with *B. subtilis* (85.0%) singly or plus Nemastop (80.0%). Some investigators explain this positive effect as antibiosis action occurred by *B. subtilis*. Isolates producing different antibiotic and antifungal substances (Singh et al., 2008). Application of *T. harzianum* (80.0%) singly or with Nemastop (75.0%) occupied the second rank. It was recorded that, *T. harzianum* could cause reduction in different pathogenic fungi and this is due to antifungal substances produced by the fungus as gliotoxin and Trichodermin (Abd-El-Motiy, 1985).

Combining the previously mentioned bio-agents plus Nemastop showed nematocidal activity against citrus nematode juveniles after seven months as shown in Table (2). Also, single application of *T. harzianum* (50.3%) achieved the greatest suppression in total nematode population. Meanwhile, the application of Bio-Nematon (11.7%) was the lowest one. Application of *T. harzianum* (57.7%) integrated with Nemastop and *B. subtilis* plus Nemastop (42.3 %) were the greatest and the lowest applications, respectively. Nevertheless, total nematode population was significantly suppressed with nemaphos application with percentage of reduction amounted to 39.9%.

Table 2. Effect of some bio- agents applied singly or integrated with nemastop on controlling *Tylenchulus semipentrans* and *Fusarium solani* after three, seven and twelve months of treatments under field conditions.

Treatments	**After three months**			**After seven months**			**After twelve months**		
	Nematode Final population	Percentage reduction	% Reduction of *F.solani* incidence	Nematode Final population	Percentage reduction	% Reduction of *F.solani* incidence (c.f.u)	Nematode Final population	Percentage reduction	% Reduction of *F.solani* incidence (c.f.u)
Trichoderma harzianum	5720.0	42.9	80.0^{b}	3730.0	50.3	83.0c	3080.0	35.8	76.0^{c}
Streptomyces griseus	7840.0	21.8	70.0^{d}	5060.0	32.5	74.0e	3490.0	27.3	75.0^{d}
Bacillus subtilis	9030.0	9.9	85.0^{a}	5720.0	23.7	87.0a	4360.0	9.2	88.0^{a}
Nemastop	6842.0	31.7	0.0^{e}	5760.0	23.2	0.0g	4280.0	10.8	0.0^{g}
Bio-nematon	9480.0	5.4	0.0^{e}	6620.0	11.7	0.0g	4740.0	1.3	0.0^{g}
T.harzianum+ Nemastop	3790.0	62.2	75.0^{c}	3175.0	57.7	78.0d	2320.0	51.7	73.0^{e}
S.griseus + Nemastop	4837.0	51.7	70.0^{d}	4170.0	44.4	72.0f	3995.0	16.8	71.0^{f}
B.subtilis + Nemastop	5940.0	40.7	80.0^{b}	4330.0	42.3	85.0b	4606.0	4.0	81.0^{b}
Bio-Nematon + Nemastop	6260.0	37.5	0.0^{e}	4290.0	42.8	0.0g	4556.0	5.1	0.0^{g}
Nemaphos	6240.0	37.7	0.0^{e}	4510.0	39.9	0.0^{g}	3213.0	33.1	0.0^{g}
Control	10020.0	0.0	0.0^{e}	7500.0	0.0	0.0^{g}	4800.0	0.0	0.0^{g}

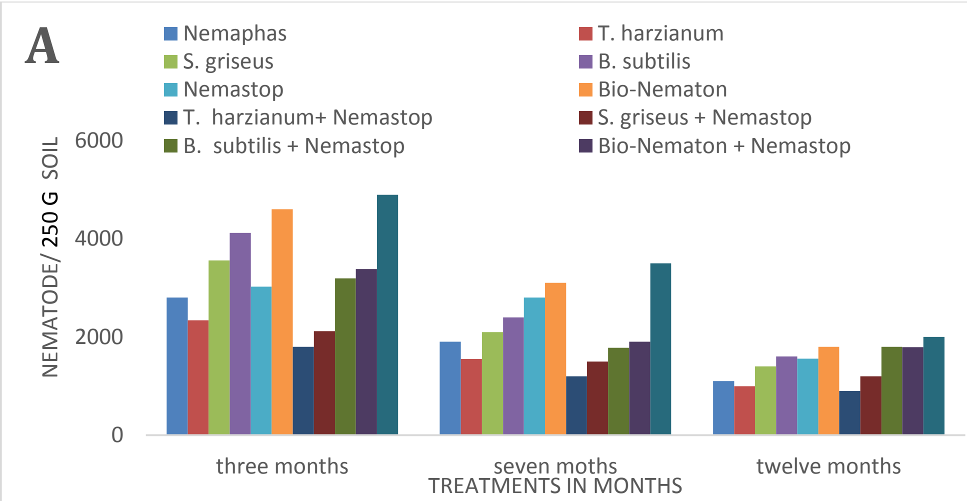
*Means in each column followed by the same letter(s) did not differ at P≤ 0.05 according to Duncan`s multiple range test

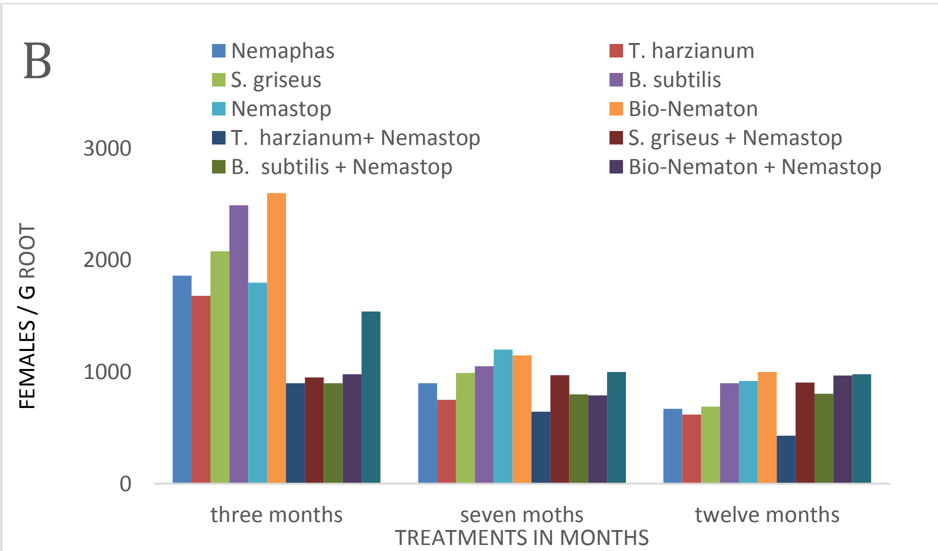
Data also showed that, there is no differences in the efficiency of *B. subtilis* when used as single treatment (87.0%) or plus Nemastop (85.0%) and gave the highest reduction in *F. solani* incidence compared with any other treatment (Table 2). This increment is due to the establishment of the antagonist and that, *B. subtilis* produces a huge number of antibiotics and antifungal substances (Attia et al., 2011). *T. harzianum* followed *B. subtilis* as an effective bio-agent in reducing *F. solani* incidence. Meanwhile, *S. griseus* alone (74.0%) or integrated with Nemastop (72.0%) was the least bio-agent in reducing *F. solani* incidence. Golinska and Dahm (2011) found that most isolated strains of *Streptomyces* spp. have enzymatic activity (chitinolytic, proteolytic, pectolytic and cellulolytic). Most strains produce chitinases, catalyzing the degradation of chitin, the main component of fungal cell walls including pathogenic fungi.

In the same line, percentages of reduction in citrus nematode, *T. semipenetrans* and *F. solani* were determined at the end of the experiment after twelve months of application (Table 2). It is evident that all treatments whether single or integrated with Nemastop showed slight suppression rates in total nematode population compared with those obtained after 9 months. However, the solo (35.8%) or dual application of *T. harzianum* with Nemastop (51.7%) achieved the greatest suppression in total nematode population. In addition, *B. subtilis* gathering with Nemastop (4.0%) induced the least percentage in nematode population after twelve months of application.

A detectable significant effect was also noticed when *B. subtilis* was compared with *T. harzianum* or *S. griseus* in reducing *F. solani* incidence (Table 2). All used bio-agents gave good effect on decreasing *F. solani* incidence for twelve months. That's may be due to propagation of theses bio-agents through the year causing increase in number and secretion of enzymes and antibiotics (Ali, 2013 and Hammam et al., 2016).

By monitoring nematode population dynamics throughout the experimental periods, number of *T. semipentrans* juveniles/ 250g soil were significantly suppressed with all treatments whether singly or concomitantly in the three tested periods. However, best results were obtained with concomitant treatments especially *T. harzianum* + Nemastop and *S. griseus* +Nemastop after seven months of treatments (Fig.1A). Among single applications, numbers of females per gram of orange root increased, reaching their peak after three months of treatments and exceeded those of the control (Fig. 1B). Bio-Nematon and *B. subtilis* adversely affected numbers of females / root in March after three months of treatments although the proportional increase was lower in July (after 7 months) and December (after 12 months).





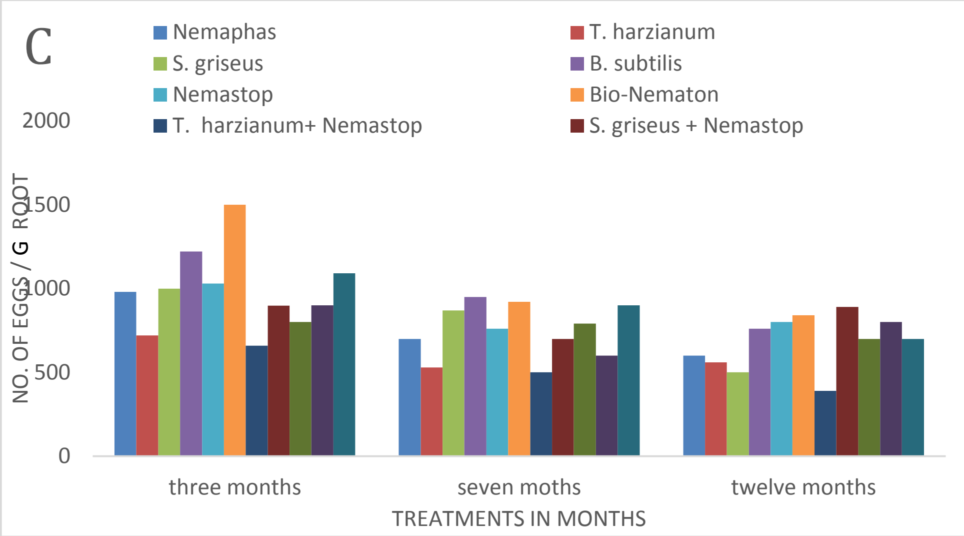


Fig. 1. Changes in population densities of *Tylenchulus semipenetrans* in citrus roots after 3, 7 and 12 months of treatments. A) Seasonal fluctuations of second-stage juveniles in soil. B) Seasonal fluctuations of females per g. of root. C) Seasonal fluctuations of eggs per gram of root.

Concomitant treatment of *Trichoderma* +Nemastop gave better results in reducing number of females/g root after 3, 7 and 12 months of treatments compared to those of control. It is worth noting that the proportional decrement in number of females/g root in check plants during the experiment period could be related to seasonal fluctuations (Fig.1B).Similar trend was noticed with numbers of eggs per gram of root, where with Bio-Nematon and *B. subtilis* they reached the peak after three months in July compared to that of check (Fig. 1C). There was no recognizable peak in egg production in December. These differences in patterns were most likely the reason that seasonal fluctuations greatly affected the number of eggs per gram of root, more than not effect of the treatments. After a year of application, *T. harzianum* + Nemastop was the best which significantly suppressed female fecundity. An inverse relation was found between the number of females per gram of root (900.0) and the number of eggs produced per female (660.0). This result support the findings of Ahmed et al. (2009) and in the same line with Deepa and Subramanian (2013).

In conclusion, *T. harzianum* singly or integrated with Nemastop induced significant suppression in *T. semipenetrans* population and *F. solani* incidence after 3, 7 and 12 months of treatments. However, seasonal fluctuations had adversely affected citrus nematode population in check and treated plants.

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الملخص العربي

المكافحة الحيوية لنيماتودا الموالح وفطر الفيوزاريوم تحت الظروف المعملية والحقل

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يعد مرض التدهور البطئ وتعفن جذور الموالح الناجم عن *Tylenchulus semipenetrans* و *Fusarium solani* على التوالي من الأمراض الخطيرة التي تهاجم العديد من البساتين في مصر. ستناقش هذه الدراسة كيفية تحسين فعالية الكائنات الحيوية

(*Trichoderma harzianum*, *Bacillus subtilis*, *Streptomyces griseus*, *Paecilomyces lilacinus*) على السيطرة على نيماتودا الموالح منفردة معمليا, حيث حققت المعاملة بالبيونيماتون أعلى نسبة موت لليرقات. وكذلك تأثيرها على نيماتودا الموالح و الفيوزاريوم سولانى التي تغزو أشجار برتقال واشنطن نافل من خلال اضافتها مع مركب نيماستوب تحت ظروف الحقل. تم تقدير تعداد مسببات الأمراض التي تنقلها التربة بعد ثلاثة وسبعة واثني عشر شهرا. ولقد أظهرت النتائج أن جميع المعاملات أدت إلى انخفاض كبير في درجة حدوث المرض مقارنة بالكنترول. ولقد أثرت جميع الكائنات الحيوية المستخدمة تأثيرًا جيدًا على تناقص أعداد *T. semipenetrans* و *F. solani* لمدة اثني عشر شهرًا. وتم مناقشة النتائج التي تحققت من خلال استخدام كائنات المقاومة الحيوية على أساس آليات المقاومة المختلفة. وقد لوحظ انخفاض كبير واضح في *T. semipenetrans* عند خلط *T. harzianum* مع النيماستوب. وفي الوقت نفسه *B. subtilis* أو *T. harzianum* خفضت تواجد *F. solani* .