Impact of *Bacillus subtilis*, *Trichoderma* spp. and the Bioproduct Top Perfect on *Meloidogyne incognita* Infecting Pepper Plants

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ABSTRACT

Root-knot nematodes are considered one of the severe plant pathogens that can be managed effectively with chemical nematicides in spite of their hazards to humans and the environment. Our work focus on the efficacy of *Bacillus subtilis*, *Trichoderma* spp. culture filtrates, the bioproduct Top perfect and the nematicide Vydate® L 24% for controlling *Meloidogyne incognita* infecting pepper plants cv. Balady. Under laboratory and greenhouse conditions, treatments with 0.025 and 0.05 µl of Vydate® L 24%/ml distilled water caused the highest reductions ranging from 83.9-99% in nematode egg-hatching and increased 2*nd* stage juveniles' mortality; the number of root galls and egg masses, followed by treatments with the (100 µl/ ml, 10 ml/200 cc soil and 50 ml/ kg soil) of *B. subtilis*, *Trichoderma* culture filtrates, and the bioproduct Top perfect which showed 60.4-79.8 % inhibition. Meanwhile, treatments with the low doses (50 µl/ ml, 5 ml/200 cc soil and 25 ml/ kg soil) of *B. subtilis*, *Trichoderma* culture filtrates and the bioproduct, Top perfect resulted in 23.5-59.6 % inhibition in nematode parameters compared with the check treatment. In comparison to check treatment under greenhouse conditions application of Vydate® L 24% showed a 60.2-77.8% increase in growth parameters of pepper plants, followed by both treatments with high and low doses of *B. subtilis*, *Trichoderma* culture filtrates, and the bioproduct Top perfect which showed 43.1-58.8% and 29.7-42.0% increase, respectively. Tested biocontrol agents could achieve various degrees of root-knot nematode control on pepper plants infected with *M. incognita* under laboratory and greenhouse conditions.

Key words: biological control, *Solanaceae* plants, microorganism, root-knot nematodes, antagonists.

INTRODUCTION

Pepper, *Capsicum annuum* L is a vital vegetable crop that is cultivated extensively all over the world (Zayeda *et al.,* 2013). The total cultivated area in the open field and under plastic-houses during 2019-2020 reached almost 65240 Faddans in Egypt with a total production of 39000 tons (Helaly and EL-Bauome, 2020).

Among all plant-parasitic nematodes; root-knot nematode (RKN) considered one of the most seriously damaging genera. *Meloidogyne incognita* considered the most serious and the highest distribution proportion on different crops (Mukhtar, 2018).

Disease development as a result of RKN infection requires taking a quick curative action to stop epidemic infection for crops using different chemical nematicides (Gugino *et al.,* 2006). Because of, safety and environmental concerns, alternative low-impact methods of nematode control are highly recommendable (Molinari, 2011).

Over the past twenty years there has been great interest in the application of microorganisms against wide range of plant pathogens (Vega, 2018).

Numerous *Bacillus* species especially *B. subtilis* can produce lytic enzymes, cyclic lipopeptides which are able to inhibit *Meloidogyne* reproduction and multiplications and enhance plant growth (Sohrabi *et al.,* 2018).

Trichoderma is used frequently as a biological control agent as a qualified microorganism in controlling different plant pathogens (Woo *et al.,* 2014). *Trichoderma* has the ability in parasitism of different life forms of root-knot nematodes (Sharon et al., 2007). Modern studies indicated that *Trichoderma* as a bio-control agent is able to activate plant defence systems (Shoresh *et al.,* 2010).

Growing *Simmondsia chinensis* L. or using its essential oil received a great attention as an effective control method which can suppress the population of RKN on susceptible crops (Abd-El-Maksoud, 2014; El-Saedy *et al.,* 2015).

The goal of this research study was to evaluate; the efficacy of *B. subtilis* and *Trichoderma* culture filtrates; the bioproduct Top perfect, which is a combination of *Trichoderma* extracts and Jojoba oil for controlling RKN compared with the synthetic nematicide, Vydate® L 24%, on egg-hatch and 2*nd* stage juveniles' mortality under laboratory condition and on *M. incognita* reproduction on pepper plant cultivar Balady under greenhouse condition.

MATERIALS AND METHODS

Root-knot nematodes inoculum preparation:

The root-knot nematode *M. incognita* Kofoid and White (Chitwood) were obtained from laboratory of Plant Nematology, Department of

Plant Pathology, Faculty of Agriculture, Alexandria University. The root-knot nematode eggs and 2nd stage juveniles (J_2) were extracted from the infected tomato roots using sodium hypochlorite (NaOCl) solution (Hussey and Barker, 1973). Root-knot nematode eggs and the hatched J_2 were placed in sterile distilled water and used in all tests.

Preparation of *B. subtilis*, *Trichoderma* spp. culture filtration, Top perfect and the nematicide, Vydate® L

One isolate of *B. subtilis* (Ehrenberg) Cohn was obtained from Bacterial Plant Diseases and Molecular Bacteriology Lab., Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt. An isolate of *Trichoderma* spp. was obtained from Crop Plant Disease Lab, Faculty of Agriculture, Plant Pathology Department, Alexandria University, Egypt.

A conical flask filled with 250 ml nutrient broth (NB) medium was inoculated with a single colony of *B. subtilis* (Ramaley and Burden, 1970). Also, an isolate of *Trichoderma* was grown in a conical flask containing 250 ml of potato dextrose (PD) medium (Harman, 2006). Flasks were placed in an incubator at 28°C for 5 days. Later, *Bacillus* and *Trichoderma* culture filtrates was poured and the two doses of 50 and 100 µl of culture filtrate were used. The same doses of 50 and 100 µl of the bioproduct, Top perfect which is a combination of *Trichoderma* extracts and jojoba oil, were used for both laboratory and greenhouse experiments. The nematicide, Vydate® L 24% was tested at four doses (0.025-0.05 µl/ml) and (0.01-0.1 µl/200 cc soil) in laboratory experiments and at two doses of 0.25 and 0.5 ml/kg soil in the greenhouse experiment (Gugino *et al.,* 2006).

Laboratory experiment

Effect of *B. subtilis, Trichoderma* spp. culture filtrates, Top perfect and Vydate® L 24% on egg-hatching and J_2 activity of *M. incognita* (MI) under laboratory conditions

The effect of *B. subtilis*, *Trichoderma* spp. culture filtrates, the bioproduct, Top perfect and Vydate® L 24% on *M. incognita* egg-hatching and J_2 mortality were tested under laboratory conditions. Treatments were done in 24-well plates; each well received 2 ml of each treatment. A total of 70 *M. incognita*, eggs or J_2, suspended in 50 µl of water was added in each well. Culture filtrates of tested microorganisms were applied in two doses 50 and 100 µl/ ml distilled water. Also, two doses (0.025 and 0.05 µl of Vydate® L 24%/ml distilled water) and (50 and100 µl of the bioproduct; Top perfect /ml distilled water) were tested. Each treatment was replicated eight times. *M. incognita* eggs or J_2 added only in sterile distilled water were used to serve as a check treatment. Treatments were maintained at 25±2°C in an incubator. Observations were reported after 24 and 48 h after adding the nematode eggs or J_2 in each treatment.

Effect of *B. subtilis, Trichoderma* spp. culture filtrates, Top perfect and Vydate® L 24% on *M. incognita* on pepper seedlings planted under laboratory condition

Forty five plastic cups, 5 cm diameter, filled with 200 cc soil composed of sandy clay soil (2:1, v:v) were used in this experiment under the laboratory condition. Cups were transplanted with four-week-old pepper seedlings cv. Balady. One-week later cups were inoculated with *M. incognita* (350 eggs and J_2/200 cc soil). Cups were received culture filtrates of *B. subtills*, *Trichoderma* spp. in two doses of 5&10 ml culture filtrate/200 cc soil. The bioproduct, Top perfect and Vydate® L 24% were applied in two doses of (2 and 5 ml/200 cc soil) and (0.01 and 0.1 ml/200 cc soil), respectively. Treatments were applied at the same time of nematode inoculation and repeated five days later. Untreated cups received only *M. incognita* eggs and J_2 inoculum added in sterile distilled water were used to serve as a check treatment. Each treatment was replicated five times. Cups were irrigated daily and arranged in randomized complete block maintained under laboratory condition at 28±2 °C.

The experiment was terminated 20 days after nematode inoculation. Number of nematode root galls/plant was determined.

Greenhouse experiments

Effects of *B. subtills*, *Trichoderma* spp. culture filtrates, Top perfect and the nematicide, Vydate® L 24% on *M. incognita* infected pepper seedlings cv. Balady under greenhouse conditions

Four-weeks-old pepper seedlings cv. Balady were transplanted in forty-five clay pots, 30 cm diameter, one seedling/pot. Pots were filled with 1k sandy clay soil (2:1, v:v) and inoculated with *M. incognita* (2000 eggs & J_2/pot). Pots were treated with two doses of 25 and 50 ml of *B. subtills*, *Trichoderma* spp. culture filtrates/kg soil. Also, two doses 10 and 20 ml of the bioproduct; Top perfect/kg soil were applied. The nematicide Vydate® L 24% was used at the rate of 0.25 and 0.5 ml/kg soil. Treatments were applied at the same time of *M. incognita* inoculation and repeated ten days later. Untreated pots received only *M. incognita* inoculum in sterile distilled water were used to serve as a check treatment.

Pots were arranged in randomized complete block design in a greenhouse with daily mean temperatures of 27±2°C. Pots were irrigated daily and fertilized every week with 2g/l of a complete soluble NPK. The experiment was terminated 45 days after nematode inoculation. Numbers of nematode root galls and egg-masses/plant were determined.

Statistical analysis

Data obtained were statistically analysed according to SAS software program (SAS, 1997). Data of the numbers of nematode root galls and egg masses were transformed to $\sqrt{x+1}$ before statistical analysis. Comparison among means was made via the least significant difference (LSD) ≤ 5% level of probability.

RESULTS

Data in Table (1) showed the effects of culture filtrates s of *B. subtilis* and *Trichoderma* spp., the bioproduct Top perfect and Vydate® L 24% on *M. incognita* egg-hatching and J_2 mortality %. The greatest reduction in egg hatching and increased J_2 mortality (85.4-99%) was achieved with Vydate® L 24% treatments. Also, treatments with high dose of *B. subtilis*, *Trichoderma* spp. and the bioproduct Top perfect caused (62.3-79.8%) inhibition, followed by treatments of low dose of same treatments which showed 27.5-59.6% inhibition in egg-hatching and increased J_2 mortality of *M. incognita* compared with the check treatments.

Data in Table (2) indicated that treatments with Vydate® L 24% gave the highest reductions (80.9 and 95.4%) in number of galls/roots. Also, treatments with the high dose of *B. subtilis* and *Trichoderma* spp. culture filtrates and the bioproduct, Top perfect showed 51.6-71.9% reduction, followed by treatments with low dose of same treatments which showed 23.5-47.0% reductions in number of galls/roots compared to the check treatment.

Data in Table (3) showed that treatments with Vydate® L 24% caused the highest reduction (89.7-98.3%) in number of nematode root galls and egg masses/root. Also, treatments with high dose of *B. subtilis, Trichoderma* spp. culture filtrates and the bioproduct, Top perfect showed (58.8-65.3%) reduction followed by treatments with low dose of same treatments which showed (42.2-55.1%) reduction in number of nematode root galls, egg masses/root compared to check treatment.

Data in Table (4) indicated that treatments with the two doses of Vydate® L 24% showed significant increases of 60.2-77.8% in dry weight of shoot and root systems. Meanwhile, treatments with high dose of *B. subtilis*, *Trichoderma* spp. culture filtrates and the bioproduct Top perfect caused (43.2-58.9 %) increases, followed by treatments with low dose of the same treatment which caused (29.7- 42.1%) increase in dry weight of shoot and root systems, compared to the check treatments.

Table 1. Effect of *B. subtilis, Trichoderma* spp. culture filtrates, the bioproduct Top perfect and Vydate® L 24% on egg-hatching and J_2 mortality of *M. incognita* (MI) under laboratory condition

Treatment	After 24 hours					After 48 hours				
	Treated Eggs			Treated J_2		Treated Eggs			Treated J_2	
	H	RH[x]	I	No. of J_2	M	H	RH[x]	I[y]	No. of J_2	M
Check*	39.7 a	100	0.0	39.4 a	0.0	69.6 a	100	0.0	69.3 a	0.0
B. subtilis + MI										
50 µl/ ml	28.8 b	72.5	27.5	24.2 b	38.6	41.5 b	59.6	40.4	37.2 ab	46.3
100 µl/ ml	15.7 d	47.1	62.3	12.2 c	69.0	24.7 c	35.5	64.5	19.2 c	72.3
Trichoderma + MI										
50 µl/ ml	23.8 b	60.0	40.0	19.9 bc	49.5	36.8 bc	52.9	47.1	31.6 ab	54.4
100 µl/ ml	12.8 d	32.2	67.8	9.7 d	75.4	19.2 cd	27.6	72.4	15.8 cd	77.2
Top perfect + MI										
50 µl/ml	24.5 b	61.7	38.3	20.7 bc	47.5	30.8 bc	44.3	55.7	28.0 b	59.6
100 µl/ ml	13.3 d	33.5	66.5	8.9 d	77.4	17.2 d	24.7	75.3	14.0 d	79.8
Vydate® L 24% + MI										
0.025 µl/ml	5.8 e	14.6	85.4	3.4 e	91.4	6.9 e	9.9	90.1	4.2 e	93.3
0.05 µl/ml	2.6 e	6.5	93.5	0.9 f	97.7	2.9 e	4.2	97.1	0.7 f	99.0

*= *M. incognita* alone (MI). H= No. of hatched eggs, RH[x]= Relative hatching %= No. of hatched eggs in each treatment/No. of hatched eggs in check treatment×100. Nematode = 70 *M. incognita* eggs orJ_2/treatment. I = Inhibition % = 100 –RH, M= Mortality % = [No. of treated J_2 in check -No. of treated J_2 in treatment ×100]/No. of treated J_2 in check treatment. Data are averages of 8 replicates. Values of each column, followed by the same letter (s), are not significantly different at $P = 0.05$ of LSD test.

Table 2. Effect of *B. subtilis, Trichoderma* spp. culture filtrates, bioproduct Top perfect and Vydate® L 24% on number of *M. incognita* (MI) galls formed on pepper seedlings under laboratory condition

Treatment	No. of root galls/seedling	Reduction%
Check* (MI)	43.4 a	0.0
B. subtilis + MI		
5 ml/200 cc soil	33.2 b	23.5
10 ml/200 cc soil	17.2 d	60.4
Trichoderma + MI		
5 ml/200 cc soil	24.6 c	43.3
10 ml/200 cc soil	15.4 d	64.5
Top perfect + MI		
5 ml/200 cc soil	23.0 c	47.0
10 ml/200 cc soil	12.2 de	71.9
Vydate® L 24% + MI		
0.01 µl/200 cc soil	7.0 e	83.9
0.1 µl/200 cc soil	2.0 f	95.4

*= Dist. water + *M. incognita* alone (MI). Reduction% (control-treatment/control ×100)). Data are averages of 5 replicates. Values in each column, followed by the same letter(s), are not significantly different at $P = 0.05$.

Table 3. Effects of *B. subtills*, *Trichoderma* spp. culture filtrates, bioproduct Top perfect and the nematicide, Vydate® L 24% on *M. incognita* (MI) galls and egg masses/plant on infected pepper seedlings cv. Balady under greenhouse condition and reduction % (R)

Treatment	No. of galls/plant	R	No. of egg masses/plant	R
MI	514.8 a	0.0	510.2 a	0.0
B. subtills + MI				
25 ml/kg soil	297.4 b	42.2	286.8 b	43.8
50 ml/kg soil	202.6 d	60.6	194.6 cd	61.9
Trichoderma spp.				
25 ml/kg soil	264.6 bc	48.6	254.2 bc	50.2
50 ml/kg soil	192.0 d	62.7	176.8 d	65.3
Top perfect + MI				
25 ml/kg soil	242.6 bcd	52.9	229.2 bcd	55.1
50 ml/kg soil	212.0 cd	58.8	197.4 cd	61.3
Vydate® L 24%				
0.25 ml/kg soil	53.2 e	89.7	43.6 e	91.5
0.50 ml/kg soil	13.2 e	97.4	8.8 f	98.3

*= Dist. water + *M. incognita* alone. Reduction%= (control-treatment/control ×100). Data are averages of 5 replicates. Values in each column, followed by the same letter(s), are not significantly different at $P = 0.05$.

Table 4. Effects of *B. subtills* and *Trichoderma* spp. culture filtrates, the bioproduct Top perfect and the nematicide, Vydate® L 24% on growth parameters of pepper seedlings cv. Balady infected with *M. incognita* under greenhouse condition

Treatment	Length(cm)				Fresh weight(g)				Dry weigh(g)			
	Shoot	I	Root	I	Shoot	I	Root	I	Shoot	I	Root	I
Check* (MI)	9.5 c	0.0	6.9 c	0.0	10.9 c	0.0	5.1 d	0.0	4.4 c	0.0	1.4 d	0.0
B. subtills+ MI												
25 ml/kg soil	14.7 b	35.4	10.9 b	36.7	15.5 b	29.7	8.2 c	37.8	7.0 b	37.1	2.2 c	36.4
50 ml/kg soil	16.7 b	43.1	13.7 ab	49.6	19.2 b	43.2	10.2 bc	50.0	8.6 b	48.8	2.9 bc	51.7
Trichoderma+ MI												
25 ml/kg soil	17.4 ab	38.3	10.5 b	34.3	17.6 ab	38.1	8.4 c	39.3	7.4 b	39.7	2.0 c	33.3
50 ml/kg soil	19.8 ab	52.0	13.8 ab	50.0	23.9 ab	54.4	12.2 ab	58.2	10.2 ab	56.9	3.4 ab	58.8
Top perfect + MI												
25 ml/kg soil	16.0 ab	40.6	11.5 ab	40.0	18.7 ab	41.7	8.8 b	42.0	7.6 b	42.1	2.3 b	39.1
50 ml/kg soil	22.4 ab	57.6	16.0 ab	56.9	24.5 ab	55.5	12.3 ab	58.5	10.7 ab	58.9	3.3 ab	57.6
Vydate® L + MI												
0.25 ml/kg soil	25.0 a	62.0	17.9 ab	61.5	27.4 a	60.2	14.1 a	63.8	12.7 a	65.4	6.0 a	76.7
0.50 ml/kg soil	24.0 a	60.4	19.9 a	65.3	28.3 a	61.5	16.3 a	68.7	13.4 a	67.2	6.3 a	77.8

*= Dist. water + *M. incognita* alone. I %= Increasing %= (Treatment-Control/treatment×100). Data are averages of 5 replicates. Values in each column, followed by the same letter(s), are not significantly different at $P = 0.05$.

DISCUSSION

The present study revealed that treatments with *B. subtilis* and *Trichoderma* culture filtrates, the bioproduct Top perfect resulted in a significant reduction in nematode reproduction and enhanced plant growth parameters compared to Vydate® L 24% under laboratory and greenhouse conditions. Similar results were obtained with (Mokbel and Alharbi, 2014; Xiong *et al.,* 2015; D'Errico *et al.,* 2019).

Most review studies focused on effectiveness of oxamyl application for controlling *M. incognita* which showed a remarkable reduction % in nematode root galls and egg-masses (Khalil e*t al.,* 2012).

Huang *et al.* (2010) reported that the genus *Bacillus* produced a volatile product which have a nematecidal effect against *Meloidogyne* juveniles. Abd-El-Khair *et al.* (2019) reported that application of *B. subtilis*, *B. pumilus*, and *P. fluorescens* against *M. incognita* on cowpea in a greenhouse study significantly reduced nematode multiplication and increased plant growth. Also, Yang *et al.* (2012) indicated that application of *Bacillus* spp. as biocontrol agents are promising, but their activity should be evaluated under field conditions. Lee *et al.* (2016) reported that *B. subtilis* produced both protease and chitinase enzymes, which were the main causes for its root-knot nematode antagonistic characteristics.

Trichoderma species are commonly found in root ecosystems, which develop symbiotic relationships with different kinds of crops, by colonizing root surfaces and enter the epidermis to enhancing root growth, increasing production and improved nutrients uptake (Kiriga *et al.,* 2019). *Trichoderma* have provided excellent control method against root-knot nematodes in previous studies (Sonkar *et al.,* 2018). Al-Hazmi and Javeed (2016) reported that *T. harzianum* and *T. viride* suppressed nematode multiplication and root galling and increased growth of tomato plants. Many biocontrol compounds and activators were obtained from *Trichoderma* spp. such as trichodermin and trypsin-like protease (Yang *et al.,* 2012). Also, *Trichoderma* produce conidia that can attach to different nematode life stages e.g., eggs and J_2 by formation of fungal coiling and appressorium-like structures which resulting in reducing nematode reproduction and increasing plant growth parameters (Sharon *et al.,* 2007; Akladious and Abbas, 2014).

Considering the importance of economic losses caused by root-knot nematodes, future studies should focus on multidisciplinary strategies that can fill the gaps in single-sided management methods. Future attempts should focus on important factors such as synergism between nematode antagonists, environmental conditions, sustainability, and association of individual plants with nematode antagonists of interest.

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تأثير استخدام كل من راشح مزارع البكتيرة *Bacillus subtilis* وفطر الـــ *Trichoderma* والمنتج الحيوي Top Perfect على نيماتودا تعقد الجذور *Meloidogyne incognita* التي تصيب نباتات الفلفل

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تعتبر نيماتودا تعقد الجذور واحدة من أهم مسببات الأمراض النباتية التي تؤثر سلباً على معدل نمو وإنتاجية النباتات المختلفة، وللحد من أضرار الإصابة النيماتودية يتم تطبيق مختلف المبيدات النيماتودية الكيميائية وذلك على الرغم من مخاطرها على كل من صحة الإنسان والبيئة. يهتم العمل الحالي بدراسة مدى فعاليةكل من راشح مزارع بكتيريا الـــ *Bacillus subtilis*، وفطر الـــ *Trichoderma* spp.، والمنتج الحيوي Top perfect مقارنة بالـــمبيد Vydate® L 24٪ وذلك لمقاومة نيماتودا تعقد الجذور *Meloidogyne incognita* والتي تصيب نباتات الفلفل وذلك تحت ظروف الظروف المعملية وظروف الصوبة الزجاجية. واتضح من النتائج أن استخدام التركيزين ٠,٠٢٥ و٠,٠٥ ميكرولتر من المبيد Vydate® L 24٪ أدى الى حدوث أعلى نسبة خفض معنوى ٨٣,٩–٩٩٪ في كل من معدل فقس البيض، زيادة فى معدل موت اليرقات وخفض معنوى فى عدد العقد الجذرية وكتل بيض النيماتودا، تلى ذلك استخدام تركيز (١٠٠ ميكرولتر/مل، ١٠ مل/٢٠٠ سم^3 تربة، ٥٠ مل/ كجم تربة) من راشح مزارع البكتيريا *Bacillus subtilis*، وفطر *Trichoderma* spp.، والمنتج الحيوي Top perfect الى حدوث تثبيط مقداره ٦٠,٤ – ٧٩,٨٪. أدى كذلك أستخدام جرعات منخفضة من نفس المعاملات (٥٠ ميكرولتر/مل، ٥ مل/٢٠٠ سم^3 من التربة، ٢٥ مل/ كجم من التربة) إلى حدوث تثبيط معنوى تراوح بين ٢٣,٥–٥٩,٦٪ في كل منى أعداد العقد الجذرية النيماتودية وأكياس البيض مقارنة بالكنترول. أدى أستخدام المبيد Vydate® L 24٪ الى حدوث زيادة معنوية بنسبة ٦٠,٢–٧٧,٨٪ في معدل نمو نباتات الفلفل، تليها المعاملة براشح مزارع بكتيريا الـــ *Bacillus subtilis*، وفطر الـــ *Trichoderma* spp.، والمنتج الحيوي Top perfect والتي أظهرت ٢٩,٧–٥٨,٨٪ زيادة في معدل نمو نباتات الفلفل مقارنة بالكنترول. اتضح مما سبق كفاءة عوامل المكافحة الحيوية المختبرة فى خفض معدلات الإصابة بنيماتودا تعقد الجذور *M. incognita* تحت ظروف المختبر والصوبة الزجاجية.