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ORIGINAL ARTICLE

Controlling the root-knot nematode, *Meloidogyne incognita* in cucumber plants using some soil bioagents and some amendments under simulated field conditions

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Abstract This study was carried out under simulated field conditions to evaluate the efficacy of some bioagents and soil amendments, as a single or combined treatments, in controlling root-knot nematode *Meloidogyne incognita* infecting cucumber. Each of the fungus *Verticillium chlamydosporium* and the symbiotic bacterium *Photorhabdus luminescens*, as single or joint treatments significantly reduced gall formation and other criteria on cucumber roots. Maximum reduction in gall formation, female numbers, egg-mass production, developmental stages and final population of juveniles in soil, was acquired by these treatments, *V. chlamydosporium* + *P. luminescens*, *P. luminescens* + compost (C) and *V. chlamydosporium* + *P. luminescens* + animal compost (AC), compared with the control and other treatments. Applications of all treatments significantly promoted plant growth i.e. length of shoot and root, fresh and dry weight of shoot and root, number of leaves, flowers, fruits and weight of fruits per each plant compared to control (infested plants with nematode only and healthy plants).

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Introduction

Plant-parasitic nematodes are recognized as major agricultural pathogens and are known to attack plants and cause crop losses throughout the world. Root-knot nematode is the most damaging plant-parasitic nematode (Barker, 1985). Biocontrol, when effective, usually is more enduring and safe in application with no toxic residues in food (Abd El-Moity, 1981).

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Entomopathogenic nematodes in the families Steinernematidae and Heteror-habditidae are biological control agents (Stock, 2005). These beneficial nematodes are parasites of insects, killing their hosts with the help of the associated symbiotic bacteria carried in their alimentary canals (steinernematids carry *Xenorhabdus* spp., whereas heterorhabditids carry *Photorhabdus* spp.) (Poinar, 1990; Adams and Nguyen, 2002). The nematode–bacterium team is capable of invading and killing the larval and adult stages of numerous insects (Akhurst and Boemare, 1990).

Both bacteria, *Xenorhabdus* and *Photorhabdus* spp. can be grown as free-living organisms on certain media under standard laboratory conditions. *In vitro* growth is probably supported by the rich nutrient supply of the growth media and the lack of competition that normally exists in the soil environment. As the bacteria enter the stationary phase of their growth cycle, they secret several extra-cellular products, including lipases, phospholipases, proteases and several different broad spectrum antibiotics (Akhurst, 1980; Akhurst and Boemare, 1990), that can be assayed in the culture media.

In contrast, *Verticillium chlamydosporium* Goddard was first recognized as a parasite of cyst nematodes after it had been isolated from the eggs of *Heteradera schachtii* Schmidt (Willcox and Tribe, 1974). *V. chlamydosporium* is a wide spread fungus that proliferates in the rhizosphere and parasitizes females and eggs of cyst and root-knot nematodes. The fungus has potential as a biocontrol agent root-knot nematodes (Viaene and Abawi, 2000). But it is very variable and only some isolates may have potential as commercial biological control agents (De Leij and Kerry, 1991).

The literature concerning suppression of plant-parasitic nematode densities by organic amendments is replete with both promising and inconsistent results (Muller and Gooch, 1982; Rodríguez-Kábana, 1986; Stirling, 1991; McSorley and Gallaher, 1995a,b; Hassan et al., 2010). Man has added organic and inorganic amendments to soil for centuries to improve soil fertility and increase crop yield. The nematicidal effect of some of these amendments has been recognized for some time, and several reviews on the subject have been published (Singh and Sitaramaiah, 1973; Muller and Gooch, 1982). However, the application of organic amendments for reducing plant-parasitic nematodes populations has met with both success and failure (Halbrendt, 1996).

The aim of the current work is to study the following objectives: (1) Potentiality of cell-free culture filtrates of *Photorhabdus luminescens* and chlamydospores of *V. chlamydosporium*; alone and in combinations to control *Meloidogyne incognita* under microplot conditions. (2) Effect of soil amendments (compost and animal compost) alone and in combinations with biocontrol agents to control *M. incognita* under microplot conditions.

Material and methods

Identification and propagation of pure cultures of root-knot nematode M. incognita

Galled roots of eggplants were carefully washed using gentle flow of water to remove the adhering soil particles. One egg-mass was collected by the aid of a needle specially adapted for this technique. The obtained culture was reared on tomato seedlings planted in pots filled with sterilized sand: clay media (1:1). Pots were kept under glasshouse conditions for 45–60 days to maintain the nematode inocula for further studies. A stock culture of the second-stage juveniles (IJ_2s) were obtained from the collected mature egg-masses after immersion in sterilized water for 7–10 days.

The extracted nematode was identified as *M. incognita* according to the scanning electron microscope image of the perineal patterns of the mature female. It illustrated the presence of a high, squarish dorsal arch, which contains a distinct whoral in the tail terminal area. The striae are smooth to wavy. Distinct lateral lines are absent, but breaks and forks in striae are obvious.

Isolation of bioagents

V. chlamydosporium was isolated from egg-masses of *M. incognita* according to the method of Zaki and Maqbool (1993), while *Photorhabdus luminescens* was isolated directly from the surface-sterilized infective juvenile stages of EPNs (Obtained from the Applied Center for Entomonematodes (ACE), Faculty of Agriculture, Cairo University), by the method of Caldas et al. (2002) and Cabral et al. (2004).

Propagation of the bioagent V. chlamydosporium

The fungal biocontrol agent *V. chlamydosporium* was grown on corn meal agar in 9-cm-diameter Petri plates at 25 °C. After 15 days of incubation chlamydospors were harvested. The teasted concentration of chlamydospores (10^7 clamydospores/ml) was determind by the aid of haemocytometer.

Preparation of cell-free filtrates of bacteria

The pure colonies of the isolated symbiotic bacteria *P. luminescens* was inoculated into 50 ml of tryptic soy broth contained in 100 ml Erlenmeyer flasks. Then allowed to multiply at the optimum temperature 28 °C for 24 h. The bacterial culture of was centerifugated at 3000 rpm, for 15 min. The supernatant broth solution which collected after centrifugated of bacterial cultures was sterilized by using the Filter Assembly Versapor® 0.2-μm-pore-diameter.

Preparation of microplots and soil treatment

Top soil (sand:loam 1:1 v/v), 0–30 cm depth, of each microplot 1 m^2 was sterilized by drenching with formalin solution (5%) and covered with polyethelene sheet for two weeks. The sheets were then removed and sterilized soil was thoroughly mixed and left for one week to allow the toxic evaporation of formalin residues (Ramses, 2001).

Soil treatments with compost (C/N = 2:16) and animal compost (C/N = 1:12.13) were incorporated into the top 15 cm of the beds of plots at the rate of 1 kg/plot before transplanting. Soil was treated with the biocontrol agents at transplanting time in the root zone at the rate of 20 ml of each bioagent/root. Treatment with the nematicide Vydate (Oxamyl) 24% L, was included for comparison after

48 h of infestation with nematode. Application was at the rate of 3 L/Fed as recommended. Check treatment without biocontrol agents or nematicide was included. Each treatment was replicated three times, each microplot contain nine plants. Treatments were deployment in a completely randomized design under microplots conditions. Plants were watered daily. Eight weeks after nematode inoculation the experiment was terminated and number of galls, number of egg-masses, females/root system, number of eggs/egg-mass and nematode population in 250 g soil were counted.

Assesment of nematode parameters

Egg-masses were stained by dipping the roots in 0.015% Phloxine B solution for 20 min as described by Daykin and Hussey, 1985. Stained roots were then washed with tap water to remove the residual Phloxine B. Number of eggs per egg-mass was determined by selecting 10 egg-masses randomly from each root system and shaking in 1% NaOCI solution for 3 min, the suspension of eggs was then sieved through 200 and 500 mesh (75 and 26 μm) with gentle tap water to remove the debris on the first sieve and collecting the eggs on the second one (Hussey and Barker, 1973). Released eggs were collected in 50 ml water suspension and number of eggs was counted in 1 ml by the aid of a light microscope (10×). Average number of eggs/egg-mass was calculated. Roots were stained by lactophenol acid foxin method to count the adult females/root system under a zoom steriscope (6×) (Franklin and Goodey, 1959).

Statistical analysis

All experimental data were subjected to analysis of variance with SAS software (SAS Institute, 1996) at the 5% level of probability and comparison of means by Duncan's Multiple Range Test.

Results and discussion

Influence of soil treatment with fungal and bacterial bioagents on the infection of cucumber plants with M. incognita in autumn-2010 under simulated field conditions

The influence of soil treatment under micropolt conditions with fungal and bacterial bioagents (*V. chlamydosporium* and *P. luminescens*) alone or in combination compared to nematicide treatments were studied. Results in Table 1 indicated that, all treatments with the fungal, bacterial and nematicide significantly reduced nematode infection compared with control. They showed the least numbers of galls, females, egg-masses and developmental stages per root system, number of eggs per egg-mass and final population of IJ2s/250 g soil. Treatment with filtrate of *P. luminescens* and combined effect between *V. chlamydosporium* and *P. luminescencs* showed the highest effect on the numbers of egg-masses and females. These results were agreement with Grewal (1999), Shapiro et al. (2002), De Nardo et al. (2006) and Vyas et al. (2008), whose found that, Application of formulated bacterial cell-free filtrate of *Xenorhabdus* sp. temporarily suppresed *M. incognita* penetration into tomato and groundnut roots in the greenhouse trials. The short-term effects of cell-free bacterial filtrates, namely toxicityand repellency, were almost entirely due to ammonium. Indeed, both of *Xenorhabdus* and *Photorhabdus* bacteria produce large amounts of ammonium in culture, which have been shown to be toxic to the second-stage juveniles of *M. incognita* (Hu et al., 1999; Grewal, 1999). Moreover De Leij and Kerry (1991) reported that, *V. chlamydosporium* as biological control agents against *M. arenaria* on tomato plants under glasshouse conditions resulted in population reductions of nematode of less than 80% after the first nematode generation. The fungus did not invade the root cortex and there were no adverse effects of the fungus on plant growth. In a microplot experiment on sandy loam soil, *V. chlamydosporium* controlled population of *M. hapla* on tomato plants by more than 90% reduction

Table 1 Influence of soil treatment with fungal and bacterial bioagents on the infection of cucumber plants with *Meloidogyne incognita* in fall season of 2010 under simulated field conditions.[A]

Treatments	Roots					Soil
	No. galls[B]	No. Egg-masses[C]	No. eggs[D]	No. females[E]	No. developmental stages[F]	No. IJ_{2s}[G]
Verticillium chlamydosporium (10^7)	55^b	46^b	271^a	38^b	50^a	456^b
Photorhabdus luminescens (CF 100%)	44^b	21^c	268^{ab}	11^c	34^b	267^b
Verticillium chlamydosporium (10^7) + *Photorhabdus luminescens* (CF 100%)	42^b	20^c	262^b	8^c	32^b	228^b
Oxamyl 24% SL (Control treatment)	50^b	38^b	266^{ab}	27^b	37^b	439^b
Untreated (Check)	77^a	71^a	270^a	64^a	58^a	788^a

Each treatment was represented by three replicates, each with nine plants.
All results were recorded after 45 days post treatment.
Numbers with the same letter within the same column are not significantly different ($P < 0.05$ using Duncan's Multiple Range Test in SAS-statistical program).
[A] Soil was infested with 2000 IJ_{2s}/plant.
[B] Mean number of galls/root system.
[C] Mean number of egg-masses/root system.
[D] Mean number of eggs for 10 egg-masses/root system.
[E] Mean number of females/root system.
[F] Mean number of developmental stages/root system.
[G] Mean number of the second stage juvenile/250 g. of soil.

(De Leij et al., 1993). *V. chlamydosporium* is one of the most promising natural enemies with potential as a biological control agent so far tested against root-knot nematodes under field conditions (Kerry and Bourne, 1996; Viaene and Abawi, 2000). *V. chlamydosporium* also has been shown to be capable of preventing egg hatching of *M. arenaria* and *M. javanica* and to colonize eggs by hyphal penetration (Morgan-Jones et al., 1993; Erma and Shahzad, 1998). Secretion of pro-

Table 2 Influence of soil treatment with fungal and bacterial bioagents on vegetative growth of cucumber plants infected with *Meloidogyne incognita* in fall season of 2010 under simulated field conditions.[A]

Treatments	Length (cm.)		Fresh weight (g.)		Dry weight (g.)		No. leaves	No. flowers	No. fruits	Weight of fruits
	Shoot	Root	Shoot	Root	Shoot	Root				
Verticillium chlamydosporium (10^7)	141.9^{bc}	41.9^{b}	60.8^{ab}	4.9^{b}	16.7^{b}	2.5^{a}	28^{c}	27^{bc}	16^{a}	1116.7^{b}
Photorhabdus luminescens (CF 100%)	150.5^{ab}	50.2^{a}	67.8^{ab}	7.9^{a}	18.8^{ab}	1.5^{bc}	33^{b}	29^{b}	15^{b}	1075.0^{b}
Verticillium chlamydosporium (10^7) + *Photorhabdus luminescens* (CF 100%)	158.9^{a}	53.1^{a}	80.3^{a}	9.5^{a}	21.9^{a}	2.2^{ab}	35^{a}	32^{a}	16^{a}	1229.2^{a}
Oxamyl 24% SL (Control treatment)	131.9^{c}	33.9^{c}	49.8^{bc}	4.4^{b}	10.7^{c}	2.2^{ab}	26^{d}	25^{c}	14^{bc}	989.3^{c}
Untreated (Check)	61.1^{e}	30.9^{c}	37.8^{c}	3.2^{b}	6.5^{d}	1.1^{c}	22^{e}	15^{d}	2^{d}	158.7^{d}
Healthy plants	92.3^{d}	32.9^{c}	47.8^{bc}	3.5^{b}	10.5^{c}	1.6^{bc}	26^{d}	26^{c}	13^{c}	967.0^{c}

Each treatment was represented by three replicates, each with nine plants.
Values are means of 18 plants of each treatment.
Numbers with the same letter within the same column are not significantly different ($P < 0.05$ using Duncan's Multiple Range Test in SAS-statistical program).
[A] Soil was infested with 2000 IJ_{2s}/plant.

Table 3 Influence of soil treatment with animal compost and compost alone or in combinations with some bioagents on the infection of cucumber plants with *Meloidogyne incognita* in spring season of 2011 simulated field conditions.[A]

Treatments	Roots					Soil
	No. galls[D]	No. egg-masses[E]	No. eggs[F]	No. females[G]	No. developmental stages[H]	No. IJ_{2s}[I]
Verticillium chlamydosporium (10^7) + C[B]	72^{bc}	48^{bc}	279^{ab}	32^{b}	65^{bc}	317^{bc}
Verticillium chlamydosporium (10^7) + AC[C]	58^{cd}	36^{bcd}	271^{abc}	22^{bcd}	57^{cd}	267^{bc}
Photorhabdus luminescens (CF 100%) + C	69^{bcd}	47^{bc}	273^{abc}	30^{bc}	63^{bc}	311^{bc}
Photorhabdus luminescens (CF 100%) + AC	53^{de}	31^{cd}	265^{c}	19^{cd}	53^{de}	244^{bc}
Verticillium chlamydosporium (10^7) + *Photorhabdus luminescens* (CF 100%) + C	68^{bcd}	45^{bc}	272^{abc}	29^{bc}	63^{bc}	399^{bc}
Verticillium chlamydosporium (10^7) + *Photorhabdus luminescens* (CF 100%) + AC	43^{e}	24^{d}	268^{bc}	16^{d}	49^{e}	217^{c}
C + Mi	74^{b}	50^{b}	270^{abc}	34^{b}	66^{b}	328^{b}
AC + Mi	64^{bcd}	43^{bc}	266^{c}	26^{bcd}	61^{bc}	289^{bc}
Untreated (Check)	158^{a}	145^{a}	279^{a}	133^{a}	120^{a}	839^{a}

Each treatment was represented by three replicates, each with nine plants.
All results were recorded after 45 days post treatment.
Numbers with the same letter within the same column are not significantly different ($P < 0.05$ using Duncan's Multiple Range Test in SAS-statistical program).
[A] Soil was infested with 2000 IJ_{2s}/plant.
[B] Compost.
[C] Animal compost.
[D] Mean number of galls/root system.
[E] Mean number of egg-masses/root system.
[F] Mean number of eggs for 10 egg-masses/root system.
[G] Mean number of females/root system.
[H] Mean number of developmental stages/root system.
[I] Mean number of the second stage juvenile/250 g. soil.

Table 4 Influence of soil treatment with animal compost and compost alone or in combinations with some bioagents on some vegetative growth of cucumber plants infected with *Meloidogyne incognita* in spring season of 2011 under simulated field conditions.[A]

Treatments	Length (cm.)		Fresh weight (g.)		Dry weight (g.)		No. leaves	No. flowers	No. fruits	Weight of fruits
	Shoot	Root	Shoot	Root	Shoot	Root				
Verticillium chlamydosporium (10^7) + C[B]	150.7^{a}	52.1^{a}	78.2^{a}	9.9^{a}	21.8^{a}	2.3^{bc}	29^{ef}	28de	10^{e}	758.4^{d}
Verticillium chlamydosporium (10^7) + AC[C]	155.0^{a}	53.6^{a}	80.4^{a}	11.4^{a}	22.5^{a}	2.9^{abc}	35^{ab}	35^{ab}	16^{b}	1196.4^{b}
Photorhabdus luminescens (CF 100%) + C	151.8^{a}	52.2^{a}	78.7^{a}	10.7^{a}	21.9^{a}	2.5^{abc}	31d^{e}	29^{cd}	11de	831.4^{d}
Photorhabdus luminescens (CF 100%) + AC	157.8^{a}	55.5^{a}	82.8^{a}	11.5^{a}	22.9^{a}	3.0^{ab}	36^{ab}	36^{ab}	18^{b}	1277.5^{ab}
Verticillium chlamydosporium (10^7) + *Photorhabdus luminescens* (CF 100%) + C	153.1^{a}	52.4^{a}	79.2^{a}	11.2^{a}	22.2^{a}	2.7^{abc}	32^{cd}	31^{c}	12^{d}	904.4^{d}
Verticillium chlamydosporium (10^7) + *Photorhabdus luminescens* (CF 100%) + AC	162.5^{a}	55.8^{a}	83.4^{a}	11.8^{a}	23.2^{a}	3.2^{a}	37^{a}	37^{a}	19^{a}	1415.4^{a}
C + Mi	149.8^{a}	51.9^{a}	77.9^{a}	9.8^{a}	21.7^{a}	2.2^{c}	28^{f}	26^{ef}	8^{f}	612.4^{e}
AC + Mi	153.3^{a}	52.7^{a}	79.5^{a}	11.3^{a}	22.3^{a}	2.8^{abc}	34^{bc}	34^{b}	14^{c}	1050.4^{c}
Untreated (Check)	59.8^{c}	30.4^{b}	37.1^{b}	6.0^{b}	6.3^{c}	1.0^{d}	20^{h}	14^{g}	2^{g}	133.8^{f}
Healthy plants	91.0^{b}	32.4^{b}	47.1^{b}	6.6^{b}	10.3^{b}	1.5^{d}	24^{g}	25^{f}	11de	794.9^{d}

Each treatment was represented by three replicates, each with nine plants.
Values are means of 18 plants of each treatment.
Numbers with the same letter within the same column are not significantly different ($P < 0.05$ using Duncan's Multiple Range Test in SAS statistical program).
[A] Soil was infested with 2000 IJ_{2s}/plant.
[B] Compost.
[C] Animal compost.

tease VCPI from *V. chlamydosporium* has been immunolocalised to appressoria formed on *M. incoginta* (Segers et al., 1996).

Results in Table 2 indicated that, the combined effect between *V. chlamydosporium* and *P. luminescens* filtrate showed the highest improvement in all vegetative growth criteria (Length of shoot and root, fresh and dry weight of shoot and root, number of leaves, flowers and fruits and weight of fruits per plant).

Influence of soil treatment with animal compost and compost alone or in combinations with some bioagents on the infection of cucumber plants with M. incognita in spring-2011 under simulated field conditions

Different applications of the combinations between fungal and bacterial bioagents and soil amendments were applied under microplot conditions in order to controlling root-knot nematode *M. incognita* in cucumber plants. Results in Table 3 indicated that all treatments significantly reduced nematode infection compared with control. They showed the least numbers of galls, females, egg-masses and developmental stages per root system, number of eggs per egg-mass and final population of IJ2s/250 g soil. Treatment with *V. chlamydosporium* + *P. luminescens* + Animal compost (AC), resulted the highest reduction effect on all nematode infection criteria followed by the treatment with *P. luminescens* + AC. On the other hand, treatment with compost only resulted the lowest reduction on all nematode infection criteria.

Evidence from the present results indicated that, Incorporation of organic amendments into the soil enhance antagonistic microorganisms and thereby enhance biological control of plant-parasitic nematodes (Stirling, 1991; Wang et al., 2002). Generally, efficacy on nematode suppression by these materials depends on the C:N ratio and the state of decomposition (Stirling, 1991). Properly decomposed materials release mineral elements into soil solution, increasing osmotic potential of soil solutions (Bohn et al., 1985; Stirling, 1991). Miller and Wihrheim (1966), Miller et al. (1968) and Kirmani et al. (1975) varied the C:N ratio of organic amendmentsand found that when more nitrogen was available, nematode control was enhanced. Organic materials with C:N ratio less than 20:1 have higher degradation rates and often nematicidal activities (McSorley and Gallaher, 1995a,b).

Results in Table 4 indicated that all treatments resulted an improvement in all vegetative growth criteria (Length of shoot and root, fresh and dry weight of shoot and root, number of leaves, flowers and fruits and weight of fruits per plant) compared with control.

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