Soil amendments with *Streptomyces lydicus* WYEC108 and chitin against the northern root-knot nematode, *Meloidogyne hapla* Chitwood, on tomato

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Summary. Actinovate® SP and Actino-Iron®, two commercial formulations of the antagonist streptomycete strain *Streptomyces lydicus* WYEC108, were first tested singly or in combination with chitin against *Meloidogyne hapla* juveniles in a plant-less test. The best nematicidal treatment was the joint application of Actinovate and chitin at 1.0% (w/w), which significantly reduced by 95% the mean number of *M. hapla* as compared with the control. In two glasshouse bioassays, the joint application of Actinovate and chitin at 1.0% was again the best treatment and reduced the mean number of infective juveniles per pot by 73% (trial 1) and 98% (trial 2) compared with the control. This soil treatment also reduced the mean number of galls per pot by 81% (trial 1) and 99% (trial 2) compared with the control. However, the high variability and high costs of these soil treatments are major obstacles for them to become viable alternatives for the management of *M. hapla* on tomato.

Key words: Actino-Iron®, Actinovate® SP, biological control, chitin, *Solanum lycopersicon,* soil amendment.

In Quebec, the northern root-knot nematode *Meloidogyne hapla* is a nematode pest of organically grown tomato under glasshouse conditions. Currently, this production is small but it is rapidly expanding with the increased demand from consumers (Carrier, 2008). This industry cannot rely on pesticides but would be allowed to use organic amendments of all sorts to manage this pest. In some production system, organic soil amendments and biological control agents have been proposed as the main elements of integrated management of plant-parasitic nematodes (Oka *et al.*, 2000). Some successes in the biological control of plant-parasitic nematodes have been achieved with fungi *Paecilomyces lilacinus* strain 251 (Holland *et al.*, 1999; Schenck, 2004) and *Trichoderma harzianum* (Reddy *et al.*, 1996; Rao *et al.*, 1998; Sharon *et al.*, 2001) and with bacteria *Pasteuria penetrans* (Chen *et al.*, 1996, 1997; Jonathan *et al.*, 2000), *Streptomyces* spp. (Samac & Kinkel, 2001) and *Gluconacetobacter diazotrophicus* (Bansal *et al.*, 2005). A collagenolytic enzyme isolated from *Bacillus cereus* digested collagens extracted from intact cuticles of second-stage juveniles (J2) of the root-knot nematode *M. javanica* and thus proved its ability to damage nematode cuticles (Sela *et al.*, 1998). Similarly, *P. lilacinus* was cultured in liquid media wherein production of proteases and chitinases was induced by the addition of egg yolk and chitin, respectively. However, their capacity to degrade chitin-containing eggs and collagen-containing cuticles was not tested (Khan *et al.*, 2003). It is probable that chitinolytic behaviour of fungal strains isolated from cysts of *Heterodera glycines* was correlated with their capacity to parasitise eggs of *H. glycines* and *M. arenaria* (Godoy *et al.*, 1982).

Organic amendments were proposed as a way to increase nematicidal activity of soil microflora (Kerry, 2000). The rationale is to amend the soil with a molecule identical or structurally related to the one constituting the outer layers of a pathogen (Mitchell & Alexander, 1961). Such a strategy is believed to increase the proportion of soil microflora responsible for nematicidal activity. For example, galling of tomato roots caused by *M.*

javanica was sharply reduced in a collagen-amended soil (Galper *et al.*, 1990). Nematode control in chitin-amended soils has been studied more extensively (Spiegel *et al.*, 1986, 1987, 1988; Bell *et al.*, 2000). The effect of chitin amendment on the chitinolytic activity and diversity of soil microbial communities has also been analysed by classical microbiological methods (Hallmann *et al.*, 1999) and by molecular techniques (Metcalfe *et al.*, 2002).

The fungus *T. harzianum* has a nematicidal capacity but interestingly it was first considered for fighting plant pathogenic fungi (Elad *et al.*, 1982; Sivan *et al.*, 1984). The ability to degrade chitin may be one of the common links between both capacities (Elad *et al.*, 1982; Godoy *et al.*, 1982). Similarly, the streptomycete *Streptomyces lydicus* strain WYEC108 was considered for suppressing plant pathogenic fungi such as *Rhizoctonia solani* and *Pythium ultimum*, the fungae causing root rot and damping-off, respectively (Yuan & Crawford, 1995). Strain WYEC108 was chosen for a phytosanitary test against *M. hapla*, because it is also able to colonise pea roots and increase root nodulation frequency caused by *Rhizobium* spp. (Tokala *et al.*, 2002) and belongs to the *Streptomyces* genus known for its chitinolytic (Beyer & Diekmann, 1985; Mahadevan & Crawford, 1996) and nematicidal (Samac & Kinkel, 2000) abilities.

The objective of this study was to investigate the efficacy of *S. lydicus* strain WYEC 108 and chitin amendment used alone or in combination for the control of *M. hapla* in a cup assay and also on tomato plants (*Solanum lycopersicon*) in two glasshouse trials.

MATERIAL AND METHODS

The two powdered commercial formulations of *S. lydicus* strain WYEC108, Actinovate® SP and Actino-Iron® Biological Fungicide, were obtained from Natural Industries Inc. (Houston, Texas). Chitin flakes produced from shells of northern shrimp (*Pandalus borealis*) were provided by Marinard Biotech (Rivière-au-Renard, Canada) and they ranged in size between 0.053 and 0.71 mm.

Cup assay. The test was conducted in 25-ml covered Solo® cups filled with approximately 26 g (dry weight) of a sandy and pasteurised soil passed through a 4.75-mm sieve and 7% water content. Egg masses of *M. hapla* were collected from infested roots of a celery field crop. The mean number of eggs per mass was 237 ± 117 as assessed from a sample of 60 masses deposited for hatching in a misting chamber for 2 weeks at 22°C (Seinhorst, 1950). Four egg masses were added to each cup and mixed in the soil to give an estimated 948 eggs per cup. Soil treatments were: control without amendment (PC), chitin (CT) at 1.0 g/100 g soil (dry weight), Actino-Iron (AI) at 2.2 x 10^4 spores/g soil (28.7 kg/m^3), Actinovate (AV) at 2.2 x 10^4 spores/g soil (2.87 kg/m^3), AI + CT and AV + CT. The treatments were made 48 h after adding the egg masses and mixed into the soil. All treatments were run in 15 replicates in a completely randomised design.

Cups were stored at 22 ± 2°C in total darkness for 4 weeks and surviving nematodes from each cup were extracted according to the modified Baermann pan method (Townshend, 1963). Nematode data were transformed using $\log_{10}$ (x + 2) before statistical analysis, but they were presented as back-transformed means in the figure. Statistical analysis was performed in SAS by the analysis of variance using the General Linear Model procedure (SAS Institute Inc., Cary, NC). Waller's test was used to compare treatments when the analysis of variance showed significant differences among means ($P \leq 0.05$).

Glasshouse bioassays. Two bioassays (trial 1 and 2) were conducted in a glasshouse at 22 ± 2ºC with a photoperiod of 16 h light and 8 h dark. Pots (12.5 cm diam.) were filled with 500 g (dry weight) of a soil composed by volume of 60% nematode-infested organic soil and 40% of a sandy pasteurised soil passed through a 4.75-mm sieve for trial 1. The sandy soil was pasteurised for 6 h at 70°C. This composite soil contained 23% organic matter (w/w) (pH = 5.6) and contained approximately 1,000 *M. hapla* J2 per pot as assessed by the modified Baermann pan method for trial 1. The same mix of soil was used for trial 2 but the organic soil was pasteurised because we inoculated the soil with egg masses directly. In trial 2, 10 egg masses of *M. hapla* were put into the soil in each pot to give an estimated 2,000 eggs per pot. A hatching test was performed by placing eight batches of 10 egg masses in a misting chamber for 2 weeks at 22°C (Seinhorst, 1950).

Soil treatments were: control without amendment (PC), chitin at 0.5 g/100 g soil (dry weight) (CT 0.5%), chitin at 1.0 g/100 g soil (CT 1.0%), Actinovate (AV) at 2.2 x 10^4 spores/g soil (2.87 kg/m^3), AV and CT 0.5%, and AV and CT 1.0%. Treatments were mixed directly in the soil previously infested or inoculated with nematodes. Soils were kept moist by watering as needed during the 2 week experimental period in order to keep microorganisms alive and active until transplantation of tomato plants. The experiment

was conducted in a randomised complete block design with six replicates per treatment in both trials.

Twenty-five-day-old tomato seedlings (*Solanum lycopersicon* cv. Rutgers) were transplanted in soils amended 2 weeks earlier. Plants were fertilised weekly by adding 10 ml of a 20-20-20 fertilising solution (50 g in 1 l water). After growth periods of 63-day (trial 1) and 87-day (trial 2), plants were harvested. Root systems were washed and rinsed thoroughly under running tap water. Galls were counted on a 5-g subsample of moist roots for trial 1 and a 20-g subsample for trial 2 and expressed as a number of galls per pot. Soil nematodes were extracted from a mixed 50 cc soil subsample per pot for trial 1 and 100cc soil subsample for trial 2 using the modified Baermann pan method. In trial 1, aerial parts and roots were placed in paper bags and dried in an oven at 70°C for 3 and 1 days, respectively. Foliar and root dry weights were recorded.

In trial 1, three soil samples for each treatment were used to assess microbial populations at the end of the 63-day growth period by using the dilution plate method (Riis *et al.*, 1998). A 100-µl aliquot of a few dilutions was plated in duplicate on nutrient agar (Becton Dickinson and Company, Sparks, USA) 10% strength (NA 10%) with 1.5% Bacto agar, chitin agar (CA) and potato dextrose agar (Becton Dickinson and Company, Sparks, USA) acidified by adding 14 ml of filter-sterilised tartaric acid 10% (w/v) per 1 l (APDA) for obtaining viable counts of heterotrophic microorganisms, chitinolytic microorganisms and fungi, respectively. Chitin agar consisted (per 1 l) of K_2HPO_4 (4.5 g), $MgSO_4 – 7 H_2O$ (0.2 g), $FeSO_4 – 7 H_2O$ (0.01 g), $CaCl_2 – 2 H_2O$ (0.01 g), $(NH_4)_2SO_4$ (0.1 g), chitin flakes (10 g), Bacto agar (15 g) and 1 ml of Hopwood elements composed of $ZnCl_2$ (0.004%), $FeCl_2 – 6 H_2O$ (0.020%), $CuCl_2 – H_2O$ (0.001%), $MnCl_2 – 4 H_2O$ (0.001%), $Na_2B_4O_7$ (0.001%) and $(NH_4)_6Mo_7O_{24}$ (0.012%). Inoculated plates were incubated in the dark at 26°C for 6 days and viable counts were expressed as log CFU (colony-forming units) per g of dry soil.

All data were transformed using $\log_{10}$ (x + 2) before statistical analysis except the foliar and root dry masses. Nematode data are presented as back-transformed means in the tables. Statistical analysis was performed in SAS by the analysis of variance using the General Linear Model procedure (SAS Institute Inc., Cary, NC). Waller's test was used to compare treatments when the analysis of variance showed significant differences among means ($P \leq 0.05$).

RESULTS

Cup assay. The mean number of *M. hapla* per cup after 4 weeks in amended soils was significantly affected by the treatments ($F = 27.88$, $P \leq 0.0001$). Treatments with chitin, Actinovate, Actino-Iron + chitin and Actinovate + chitin were all significantly different from positive control and from Actino-Iron (Fig. 1). There was also a significant difference between chitin and Actinovate + chitin. The positive control was not significantly different from Actino-Iron (Fig. 1). The best treatments involved the application of chitin alone, Actinovate either singly or with chitin and Actino-Iron + chitin. The chitin and Actinovate used singly significantly reduced the survival of *M. hapla* by 79% and 86%, respectively, when compared with the control but not to the extent of the joint application of Actinovate + chitin, which decreased survival by 95% (Fig. 1). The Actino-Iron alone had no effect when compared with the control but the joint application of Actino-Iron + chitin significantly reduced the number of *M. hapla* per cup by 85% (Fig. 1).

Glasshouse bioassay (trial 1). Soil treatments had a significant effect on the number of J2 per pot extracted from soil ($F = 2.52$, $P \leq 0.0509$) (Table 1). The best treatments were Actinovate and Actinovate + chitin at 1.0%, which reduced the number of J2 per pot by 51% and 73%, respectively, when compared with the control. The number of galls per pot was not significantly influenced by the soil treatments ($F = 2.26$, $P \leq 0.0743$) (Table 1). Foliar dry masses are shown in Figure 2. The Actinovate treatment either alone or with chitin at 0.5 and 1.0% significantly increased the foliar dry mass of tomato when compared with the control ($F = 4.43$, $P \leq 0.0039$) (Fig. 2). Root dry masses ($F = 1.41$, $P \leq 0.2505$) and ratio of foliar dry masses over root dry masses ($F = 1.10$, $P \leq 0.3805$) were not affected by the amendments.

Heterotrophic microorganisms ($F = 1.57$, $P \leq 0.2420$) and fungi ($F = 2.37$, $P \leq 0.1027$) were not significantly influenced by the soil treatments. Chitinolytic microorganisms were significantly influenced by the soil treatments ($F = 3.46$, $P \leq 0.0362$). Chitinolytic microorganisms were 4.8 × more abundant in soil amended with Actinovate + chitin 1.0% than in the unamended positive control.

Glasshouse bioassay (trial 2). The hatching test showed that 550 J2 emerged from the 10 egg masses per pot. The number of J2 extracted from soil per pot was significant influenced by the soil treatments ($F = 10.02$, $P \leq 0.0001$) (Table 2). Chitin 1.0%, Actinovate, Actinovate + chitin 0.5% and Actinovate + chitin 1.0% were all significantly different from

Table 1. Mean number of second-stage juveniles (J2) and galls (G) per pot after bioassay for 63 days trial 1).[a]

Amendments	Parameters	
	J2	G
Control	8,750 a	4,241 a
Chitin 0.5%	4,716 ab	1,085 a
Chitin 1.0%	7,714 ab	3,974 a
Actinovate	4,271 b	1,996 a
Actinovate + Chitin 0.5%	5,583 ab	781 a
Actinovate + Chitin 1.0 %	2,360 b	800 a

[a] Means with different letters are statistically different using Waller's test for α = 0.05.

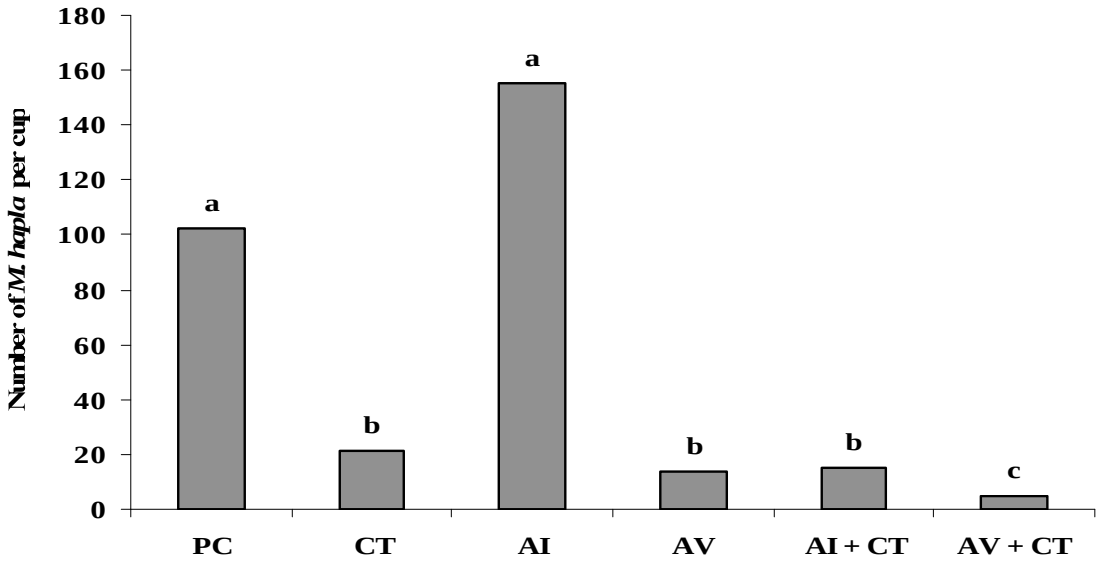

Fig.1. Mean number of *Meloidogyne hapla* per cup after 4 weeks in soil amended with the following treatments: control (PC), chitin (CT), Actino-Iron (AI), Actinovate (AV), AI + CT and AV + CT. Means with different letters are statistically different using Waller's test for α = 0.05.

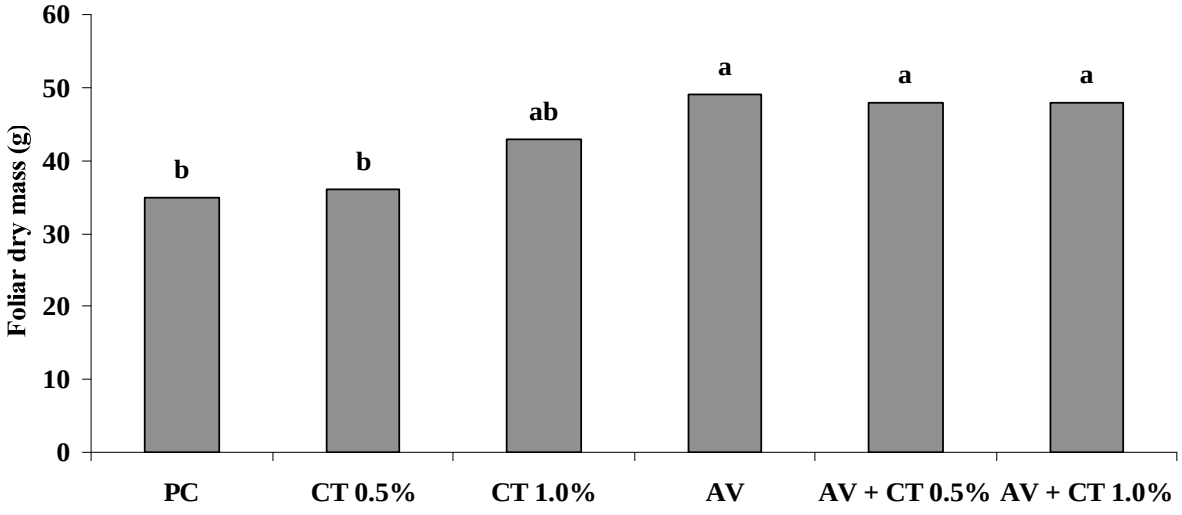

Fig. 2. Mean foliar dry masses after a 63-day culture in soil amended with the following treatments: control (PC), chitin 0.5% (CT 0.5%), chitin 1.0% (CT 1.0%), Actinovate (AV), AV + CT 0.5%, and AV + CT 1.0%. Means with different letters are statistically different using Waller's test for α = 0.05.

Table 2. Mean number of second-stage juveniles (J2), galls (G) and eggs (E) per pot after bioassay for 87 days (trial 2).[a]

Amendments	Parameters		
	J2	G	E
Control	711 a	1,411 a	28,230 a
Chitin 0.5%	565 a	2,440 a	63,073 a
Chitin 1.0%	37 b	957 a	10,790 ab
Actinovate	12 b	292 a	4,717 a
Actinovate + Chitin 0.5%	11 b	1,173 a	33,746 ab
Actinovate + Chitin 1.0 %	15 b	20 b	390 b

[a] Means with different letters are statistically different using Waller's test for $\alpha = 0.05$.

the positive control (Table 2). All these treatments reduced the number of J2 in the soil by 95% to 98% when compared with the control. There was no significant difference between the chitin 0.5% and the control (Table 2).

The numbers of galls (F=3.67, $P \leq 0.0105$) and eggs (F=2.85, $P \leq 0.0318$) per pot were also significantly influenced by the soil treatments (Table 2). Actinovate + chitin 1.0% significantly reduced the number of galls and eggs per pot when compared with the control and Actinovate alone.

DISCUSSION

The cup assay confirmed the nematicidal potency of chitin amendment and Actinovate applied singly or in combination. Our results also showed that the joint application of chitin with *S. lydicus* increased the effectiveness of chitin amendment for the control of nematodes. These results support the data of Mittal *et al.* (1995) and Tian *et al.* (2000), which showed that the efficacy of chitin amendment could be increased by the addition of chitinolytic fungi and bacteria. *Streptomyces lydicus* WYEC108 is known for its chitinolytic activity because it produces many extracellular chitinases (Beyer and Diekmann, 1985; Mahadevan & Crawford, 1996) and also for its nematicidal ability (Samac & Kinkel, 2000). The breakdown of chitin by chitinases can cause premature hatch, resulting in fewer viable J2 (Mercer *et al.*, 1992). It is suggested that chitin has stimulated the activity of *S. lydicus*. Chitin is used as a food source by *S. lydicus* and any other nematicidal and chitinolytic microbial species in soil could have contributed to lessen the competition offered by microbial species unable to degrade chitin. The higher number of chitinolytic microorganisms in the Actinovate plus chitin (1.0%) treatment supports also this hypothesis.

Actino-Iron even at tenfold the recommended rate was ineffective in reducing the number of surviving nematodes. Spore concentration of *S. lydicus* in Actinovate is tenfold the concentration observed in Actino-Iron (i.e. 1 x 10^7 and 1 x 10^6 CFU/g, respectively). Soil application rates of Actinovate and Actino-Iron were 1.7 and 13.2 kg per m^2 respectively, to reach the same spore concentration of 2.2 x 10^4/g of soil. Thus**,** Actino-Iron formulation was applied at tenfold the recommended rate, along with its 21.9% iron content. It is suggested that these high levels of iron in soil may have disrupted the normal processes triggered by *S. lydicus*. The higher activity of Actino-Iron when applied with chitin supports this hypothesis. Chitin could also have adsorbed some of the iron and acted as a food source for *S. lydicus*. Further testing with Actino-Iron at the 1.7 kg rate is needed to narrow down these deleterious effects.

From the glasshouse trials, the joint application of Actinovate and chitin provided some efficiency in protecting tomato roots against *M. hapla*. In both trials, Actinovate and chitin were applied 2 weeks prior to tomato transplants to allow sufficient time for chitinolytic microorganisms to attack *M. hapla* eggs in the soil. In trial 2, the inoculum was made of egg masses only, whilst in trial 1 it was made of a mixture of egg masses and J2. Chitin is mainly found in the eggshells of plant-parasitic nematodes (Bird & McClure, 1976) and also in the gelatinous matrix of *Meloidogyne* spp. (Spiegel & Cohn, 1985). We could explain the difference in efficiency between both trials by the fact that chitinolytic microorganisms will act mostly on eggs rather than J2.

The chitin amendment caused no phytotoxicity on tomato plants during trial 1 even at the rate of 1.0%. These results contradict those found by Mian *et al.* (1982), Godoy *et al*. (1983) and Rodriguez-Kabana *et al*. (1984), which showed that chitin levels equal to or greater than 0.8% and 1% are phytotoxic to crop such as squash and soybean. Spiegel *et al.* (1986) have also showed that the addition of 0.3% (w/w) chitin to a sandy loam (0.8% organic matter) caused phytotoxicity on tomato plants, which are recognised to be sensitive to the release of ammonia during the breakdown of chitin. This discrepancy could be due to the soil type used

during the experiment. Their experiments were carried out in sandy loam with less than 1% (w/w) organic matter. By contrast, the soil used in our glasshouse trials was a sandy loam with 23% organic matter (w/w). Their composite soil was poorer in organic matter than the soil used in our trial. It is known that a higher organic matter content may adsorb the chitin and/or sustain a more abundant and active microflora capable to degrade the chitin, thus no longer phytotoxic.

Based on our results, a soil treatment with a commercial formulation of *S. lydicus* plus a high level of chitin will provide some level of control of *M. hapla* on tomato. However, our results were highly variable. The lack of reliability of biological control agents and costs remain major obstacles to their being widely adopted by the industry as a management tool. For the most efficient treatment of Actinovate plus chitin, we estimated the cost at 100 US$ per m^2 of treated area or 15,000 US$ per 300-m row of glasshouse, which is obviously uneconomic. Originally, this research work was set up to get some information on the potential of these formulations and amendment to control *M. hapla* in biologically certified tomato production. Based on the efficiency of these treatments and their costs, the industry will not pursue this avenue in the near future. As mentioned previously, organic tomatoes are grown in field soil substantially amended with compost and organic matter. As suggested by our study, organic matter will be an obstacle for the establishment and efficacy of many biological control agents, including current formulations of *S. lydicus*.

Much more research work has to be undertaken to understand the mechanisms involved in the efficacy of biological control agents against soil nematode pests, such as *M. hapla*.

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Резюме. Два коммерческих препарата Actinovate® SP и Actino-Iron® на основе антагонистических грибов-стрептомицетов *Streptomyces lydicus* штамма WYEC108 были впервые испытаны как в отдельности, так и в комплексном применении с хитином против личинок *Meloidogyne hapla* в тестах без растений. Наилучшим по эффективности оказалось совместное применение Actinovate и хитина в количестве 1.0% по весу, которое существенно снижало (на 95%) среднее количество *M. hapla* по сравнению с контролем. В двух тепличных экспериментах совместное применение Actinovate и хитина (1.0%) также показало наилучшие результаты и снижало среднее число инвазионных личинок на контейнер с растением на 73% (1-й эксперимент) и на 98% (2-й эксперимент) по сравнению с контролем. Такие обработки снижали также среднее число галлов, приходящееся на контейнер с растением на 81% (1-й эксперимент) и на 99% (2-й эксперимент) по сравнению с контролем. Отмечено, что значительная вариабельность достигаемой эффективности, а также высокая стоимость обработок представляют собой существенное препятствие к внедрению этого препарата для контроля *M. hapla* на томатах.