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RESEARCH ARTICLE

Nematicidal activity of entomopathogenic bacteria against root-knot nematodes, *Meloidogyne incognita* in-vitro.

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Abstract

The in-vitro experiment was conducted to evaluate the nematotoxic activities of the cell-free conditioned media (CFCM) of entomopathogenic bacterium species *Photorhabdus luminescens* (strain: TT01), *Xenorhabdus budapestensis* (strain: AF 2013 or EMA) and *X. szentirmaii* (strain: EMC), isolated from entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema bicornutum* and *S. rarum*, respectively. The applied doses were 1, 2, 5, 10, 20, 40, 60 and 80 V/V %, respectively. The test organism was *Meloidogyne incognita* (J_2s) and the rate of mortalities were determined at the 6^{th}, 12^{th}, 24^{th} and 48^{th} hrs following exposure. Data revealed that the rate of larval mortality proved dose/dependent. Only the highest dose of TT01 CFCM resulted in 100% mortality rate after 48 hrs of exposure time. On the other hand, lower concentrations of EMA and EMC CFCM resulted in the same results. 6 hrs following application of 10 %, CFCM resulted in significantly the highest larval mortality rate 59.4%; in comparison with EMA (48 %) and TT01 (40%)

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INTRODUCTION

Plant-parasitic nematodes (PPN) are pathogens of almost all crops grown worldwide, reducing the yield and quality of crops. Worldwide, crop loss attributed to these pests could be estimated by 20.6 % (Sasser and Freckman, 1987). The root-knot nematode (RKN), *Meloidogyne incognita* is an important pest widely distributed all over the world causing yield losses. Such nematode is found in tropical regions and has been reported from different countries on different plant species. Unfortunately, most of effective chemicals used for controlling RKN are highly toxic, very expensive, and have negative environmental impacts. One of the possible effective alternative options is the utilization of biocontrol agents. Antagonistic bacteria have been successfully used for nematode management without causing environmental hazards. *Xenorhabdus* spp. and *Photorhabdus* spp. are symbiotic bacteria live in entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*, respectively. For over many years, entomopathogenic nematodes (EPNs) have been used as effective biological control agents against a wide spectrum of insect pests. Before 1996 entomopathogenic nematodes symbiotic bacteria were know to act only by means of nematodes. Different laboratories in the world, found out that symbiotic bacteria may have direct activity against pathogens and pests. Symbiotic bacteria (or their metabolites) have been used as insecticides, fungicides, antibacterial and anti-tumor. According to Han and Ehlers (2001), phenotypic characteristics allow the distinction of two stages in the cycle of mutualistic bacteria. Stage I is characterized by the production of pigments, catalase, lechitinase, lipase, bioluminescence and antibiotic substances. In stage II, which occurs after the food reserves of the dead insect are consumed, the bacteria lose these characteristics. Andalo et al. (2012) recorded that the nematode-bacteria complex has been commercially developed as an agent for the biological control of pests. Secondary

metabolites produced by *Xenorhabdus* spp. and *Photorhabdus* spp. attack the immune system of the insect (Forst and Nealson, 1996) and inhibit the growth of competing fungi and bacteria (Akhurst, 1982; Chen et al., 1994; Chen et al., 1996). Therefore, symbiont bacteria prevent corpse decomposition and enable the multiplication of nematodes and symbiont bacteria (Gaugler and Kaya, 1990; Bode, 2009).

Several researchers studied the effect of these bacteria against fungi and phytopathogenic bacteria (Hu and Webster, 2000; Ji et al., 2004; Hu et al., 1999 and Andalo et al., 2012). According to Ji et al. (2004), *Xenorhabdus nematophila* shows antibacterial activity in cultures of *Agrobacterium vitis*, *Pectobacterium carotovorum* subsp. *atrosepticum*, *P. carotovorum* subsp. *carotovorum*, *Pseudomonas syringae* pv. *tabacci* and *Ralstonia solanacearum*. Little attention has been given to study the biological activity of these bacteria against plant parasitic nematodes. However, in in vitro cultures, interactions with the host insects are excluded, and adequate culture medium and aeration may have altered the production of nematicide substances. For instance, Hu et al. (1999) observed that substances produced by *X. bovienii* and *Photorhabdus luminescens* bacteria were toxic to *M. incognita*. Although filtrates obtained from *X. bovienii* cultures after 48 to 120 h caused 100% mortality rates in the J_2s of *M. incognita* (Hu et al., 1999), filtrates obtained from bacteria cultured for 12 to 24 hours did not get the similar results.

Therefore, the aim of this study was to evaluate the nematicidal activity of *Photorhabdus luminescens* (TT01), *X. budapestensis* (EMA) and *X. szentirmaii* (EMC) isolated from entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema* spp. at different concentrations on *Meloidogyne incognita* (J_2s) mortality after different exposure times.

Materials and Methods:

This study was carried out in the microbiology laboratory of Plant Protection Institute, Georgikon Faculty, Pannonia University, Hungary to determine the nematicide activity of filtrates of entomopathogenic bacteria *Photorhabdus luminescens* (TT01) and *Xenorhabdus budapestensis* (EMA) and *X. szentirmaii* (EMC) isolated from entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema* spp., respectively at different concentrations on *Meloidogyne incognita* (J_2s) mortality after different exposure times in- vitro.

Culture of entomopathogenic bacteria (EPB):

The bacteria *Photorhabdus luminescens* strain (TT01) isolated from entomopathogenic nematode *Heterorhabditis bacteriophora* as well as *Xenorhabdus budapestensis* strain DSM16342^T (EMA) and *X. szentirmaii* strain DSM16338^{T*} (EMC) isolated from *Steinernema bicornutum* and *S. rarum* were grown on indicator plates of LBTA (10 g trypton [Difco], 10 g yeast extract [Difco], 10 g NaCl, 15 g Bacto agar [Difco], 25 mg of bromthymol blue, 40 mg of 2,3,5-triphenyltetrazolium chloride, 1,000 ml of distilled water [pH 6.8]).

Preparation of CFCM (Cell free conditioned media):

Single colonies of each bacterium were removed from indicator plates and transferred to 5-ml of LB medium (10 g trypton [Difco], 10 g yeast extract [Difco], 10 g NaCl, 1,000 ml of distilled water [pH 6.8]) as an inoculum for 100 ml culture. The cultures were shaken for maximum aeration at room temperature overnight, and then transferred to 1000-ml Erlenmeyer flasks containing 400 ml of LB media, shaking (200 rpm, 5- d). The bacterial suspension was centrifuged at 13,000 rpm (10,000 g for 30 min) in 250 ml tubes, filtered through Millipore filter of 0.22 µm pore-size to remove the bacterial cells. These cell free culture filtrates were diluted with sterile distilled water to provide concentrations of 80, 60, 40, 20, 10, 5, 2 and 1%.

Nematode source and inocula:

Nematode inocula were obtained from a pure culture established from a single egg-mass of *M. incognita* that previously identified according to the characteristics of its perineal pattern (Taylor and Sasser, 1978), maintained and propagated on tomato plants c.v. Strain B in a greenhouse of Microbiology Dept., Plant Protection Institute, Georgikon Faculty, Pannonia University, Hungary.

Nematotoxic Activity Test:

Approximately one hundred newly hatched second stage juveniles (J_2s) of the root-knot nematode, *M. incognita* in 200 µL sterilized distilled water were poured into wells of 24-well tissue culture plates over 1800 µL of the tested bacterial filtrates and sterilized distilled water to adjust the concentrations. The plates were then covered with the lid and kept in an incubator at 25º C. A 2.0 ml of LB medium or sterilized distilled water containing nematode larvae served as control. The experiment had three replicates and was repeated once. The numbers of dead nematodes were recorded after 6, 12, 24 and 48 hrs. Specifically, J_2s without movement were touched with a fine needle to confirm mortality. Nematodes that appeared no realistic movement were considered as dead. The percentage mortality of the second stage juveniles was calculated and recorded. Mortality percentages were transformed to arcsine values just before statistical analysis. Statistically, the obtained data were subjected to analysis of variance (ANOVA) (Gomez and Gomez, 1984) followed by Duncan's multiple range test ($p<0.05$) to compare means (Duncan, 1955).

Results:

The bacterial CFCM of TT01, EMA and EMC and concentrations (80, 60, 40, 20, 10, 5, 2 and 1%) in comparison with LB media on mortality percentage of newly hatched juveniles of *M. incognita* after 6, 12, 24 and 48 hrs are depicted in table (1). In general, larval mortality percentages increased with increase in bacterial filtrates concentrations and exposure durations tested.

Data in table (1) revealed that among all bacterial strains tested, EMC application obviously ranked first for the highest values of mortality percentages at all concentrations and exposure times tested; followed by EMA and TT01 as compared to LB media. Higher concentrations only from TT01 strain gave the highest mortality (100%) after 48 hrs of exposure time, whereas, the lower concentrations of EMC and EMA strains gave the same results after the same exposure period. It is evident that the larval mortality percentages were not affected by all bacterial strains at 1% after all exposure periods tested. Likewise, similar trend concerning the larval mortality percentages was recorded with application of 2% from TT01 at all exposure times, whereas, EMA at the same concentration gave 28.3 % mortality percentage after 48 hrs. Moreover, the same concentration from EMC gave considerable larval mortality percentages after 24 and 48 hrs of exposure times which amounted to 38.3 and 45 %, respectively.

When *M. incognita* J_2s exposed for 6 or 12 hrs to bacterial filtrates of all strains at 80%, EMC ranked first, followed by EMA then TT01 with mortality percentages averaged to 78.3 or 91.7 %, 71.7 or 90.0 % and 54.7 or 87.3 %, respectively. On the other hand, using EMC strain at 10 % resulted in significantly higher larval mortality percentage after 6 hrs than that of the other strains and control with value of 59.4% followed by EMA (48 %) and TT01 (40%).

Table (1): Effect of entomopathogenic bacteria on *Meloidogyne incognita* J_2 mortality

Treatments	Conc. %	Mortality%			
		6 h	12 h	24 h	48 h
TT01	80	54.7 $^{n-p}$	87.3 $^{c-e}$	91 $^{b-d}$	100 ^{a}
	60	45 ^{q}	74 $^{i-m}$	86.3 $^{c-f}$	100 ^{a}
	40	39 ^{qr}	71.7 $^{j-n}$	81 $^{e-i}$	100 ^{a}
	20	31.3 $^{q-t}$	47.7 ^{pq}	64.7 $^{l-o}$	83.3 $^{e-h}$
	10	40 ^{qr}	44.3 ^{q}	60.7 $^{m-o}$	76.7 $^{h-l}$
	5	28.7 $^{r-t}$	37.3 ^{qr}	54.7 $^{n-p}$	61.3 $^{m-o}$
	2	0.0 ^{u}	0.0 ^{u}	0.0 ^{u}	0.0 ^{u}
	1	0.0 ^{u}	0.0 ^{u}	0.0 ^{u}	0.0 ^{u}
EMA	80	71.7 $^{j-n}$	90 $^{b-d}$	94 ^{ab}	100 ^{a}
	60	59.3 $^{m-o}$	81.7 $^{e-i}$	90 $^{b-d}$	100 ^{a}
	40	53.3 $^{n-p}$	75 $^{i-m}$	83.7 $^{e-h}$	100 ^{a}
	20	50.7 ^{op}	60 $^{m-o}$	70 $^{k-o}$	100 ^{a}
	10	48 ^{pq}	50.7 ^{op}	69 $^{k-o}$	100 ^{a}
	5	30.3 $^{q-t}$	39.3 ^{qr}	65 $^{l-o}$	80 $^{f-j}$
	2	0.0 ^{u}	0.0 ^{u}	0.0 ^{u}	28.3 $^{q-t}$
	1	0.0 ^{u}	0.0 ^{u}	0.0 ^{u}	0.0 ^{u}
EMC	80	78.3 $^{g-k}$	91.7 ^{bc}	100 ^{a}	100 ^{a}
	60	73.3 $^{i-m}$	85 $^{d-g}$	100 ^{a}	100 ^{a}
	40	70 $^{k-o}$	81.7 $^{e-i}$	90 $^{b-d}$	100 ^{a}
	20	65 $^{l-o}$	70 $^{k-o}$	85 $^{d-g}$	100 ^{a}
	10	59.4 $^{m-o}$	61.7 $^{m-o}$	73.3 $^{i-m}$	100 ^{a}
	5	35 $^{q-s}$	45 ^{q}	68.3 $^{k-o}$	85 $^{d-g}$
	2	0.0 ^{u}	0.0 ^{u}	38.3 ^{qr}	45 ^{q}
	1	0.0 ^{u}	0.0 ^{u}	0.0 ^{u}	0.0 ^{u}
LB (Ck1)		0.0 ^{u}	0.0 ^{u}	0.0 ^{u}	0.0 ^{u}
N alone (Ck2)		0.0 ^{u}	0.0 ^{u}	0.0 ^{u}	0.0 ^{u}

*Each figure represents the mean of four replicates.

*Means in each column followed by the same letter did not differ at P< 0.05 according to Duncan's multiple range tests.

N= 100 *M. incognita* J_2

Discussion:

Entomopathogenic nematode / bacterium symbioses have been studied for a long time, and the latest results have excellently reviewed by Murfin et al. (2012). Toxic compounds of large molecular diversity play a significant role both in pathomechanisms and competition. The symbiotic complexes of *X. budapestensis* and *S. bicornutum* as well as of *X. szentirmaii* and *S. rarum* have been discovered and analyzed in our laboratory Lengyel et al. (2007). Several of the active compounds of the symbiotic bacteria have also been identified (Brachmann et al., 2006; Fodor et al., 2007 and 2012; Furgani et al., 2008; Nollmann et al., 2012; Ohlendorf et al., 2011; Xiao et al., 2012; Yang et al., 2012).

Hu et al. (1999) demonstrated that these bacteria produce metabolites that act as nematicides toward a large number of nematodes, including some phytonematodes. Filtrate obtained from EMC at 80 and 60 % achieved the highest percentage of M. incognita mortality (100%) after 24 hrs of exposure. These results are on par with Samaliev et al. (2000) who demonstrated that *X. nematophila* completely inhibited the hatching of *M. javanica* and paralyzed the emergence of J_2. In addition, a 74% reduction in hatching was observed when the eggs were removed from contact with the bacterial substrate, washed and stored in distilled water for 10 days. On the other hand, Fallon et al. (2004) observed that the *Steinernema feltiae-Xenorhabdus bovienii* complex could not suppress the development of *M. javanica* and penetration of the parasite into the roots of the host.

Insecticidal toxic proteins from *P. luminescens* strain W-14 different from that of TT01 consists of high molecular weight complexes that include toxin A and toxin B. These toxin complexes have been well-characterized both at a biochemical level and by identifying their genes (Bowen et al., 1998; Guo et al., 1999). Apparently, *Meloidogyne incognita* J_2s mortality percentages increased as the concentrations of bacterial filtrates and exposure times tested increased in comparison with LB or N alone controls. Moreover, bacterial filtrates of EMC surpassed all other strains of entomopathogenic bacteria tested in increasing larval mortality percentages, especially with low concentrations and little times for exposure with values of 35.0, 45.0, 68.3 and 85 % for only 5% of filtrate after 6, 12, 24 and 48 hrs of exposure time, respectively. The present findings are in accordance with those reported by Furgani et al. (2008) who found that EMA proved much more efficient against *Campylobacter* and *Salmonella* than EMC. But in other tests the EMC proved also effective indicating that the difference was quantitative rather than qualitative. The present investigation also indicated the possible use of either EMC or EMA or TT01 at 10% after 48 hrs of exposure time which achieved reliable larval mortality percentages with values of 100 % for the first two bacteria and 76.7 % for the last one.

The presence of natural nematicidal activity of either *Xenorhabdus* or *Photorhabdus* was confirmed with insufficient larval mortality percentages in this study, a situation which can explain the reasons of applying such components against *M. incognita* J_2s in-vitro as well as in-vivo in the future. These findings are agreed with Kaya and Gaugler (1993) who mentioned that *Xenorhabdu*s and *Photorhabdus* produce several agents with nematicide and antimicrobial activity. These antimicrobial agents include non-protein indoles, stilbene derivatives (Hu et al., 1998, 1999; Li et al., 1995), xenorhabdins, xenocoumacins (McInerney et al., 1991), proteinaceous chitinases (Chen et al., 1996) and bacteriocins (Thaler et al., 1995). A number of substances are produced by bacteria, and many metabolites or associations among compounds may produce nematicide effects.

Conclusion:

In general, we can said that highest percentages of *M. incognita* J_2 mortality were obtained from lower concentration of *Xenorhabdus* as well higher concentration of *Photorhabdus* after 48 hrs of exposure time which amounted to 100%. Finally, we can conclude from these results that those entomopathogenic bacteria could be effectively used to suppress phytonematodes including root-knot nematodes.

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