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Nematicidal effect of cell-free culture filtrates of EPN- symbiotic bacteria on *Meloidogyne javanica*

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ABSTRACT

Biocontrol of plant parasitic nematodes to decrease the chemical pesticides effects is one of the top priorities. Entomopathogenic bacteria, *Xenorhabdus* spp. and *Photorhabdus* spp., are important due to production of natural products with antibacterial and antifungal activity. The effect of the cell-free culture filtrates (CFF) of symbiotic bacteria species *Xenorhabdus nematophila*, *X. bovienii* and *Photorhabdus luminescens* isolated from entomopathogenic nematodes *Steinernema carpocapsae*, *S. feltiae* and *Heterorhabditis bacteriophora* on the egg hatching and mortality of the second stage juveniles of root-knot nematode *Meloidogyne javanica* was determined. Exposure of eggs of *M. javanica* to CF resulted in the reduced hatching of nematode eggs with higher recorded effect for *X. nematophila* CF. Analysis of mortality data for juveniles at 24, 48 and 72 h following exposure to CFFs indicated that *X. nematophila* and *P. luminescens* were more toxic than *X. bovienii* after 24 h. However, *X. bovienii* was more toxic in lower concentration after 48 and 72h post-exposure. Thus, these bacteria have a potential as biocontrol agents for the management of root-knot nematode.

Key words: biological control, root-knot nematode, *Xenorhabdus*, *Photorhabdus*.

INTRODUCTION

Root-knot nematodes (RKN, *Meloidogyne* spp.), recognized as one of the most economically important and complex group of plant parasitic nematodes, cause damage and high yield losses on most cultivated plants throughout the world especially in developing countries (Sasser 1980, Sasser and Carter 1985, Sasser and Freckmann 1987, Netscher and Sikora 1990). Different species of root- knot nematodes and their dispersal have been reported in Iran that *Meloidogyne javanica* (Treub) Chitwood is the most common and important root-knot nematode species (Akhiani *et al.* 1984).

Many attempts have been carried out to manage this nematode due to the economic damage. Nematicides (e.g., methyl bromide) can be used to manage nematode infestation, but they are prohibited in organic farming. Several control strategies such as sanitation, host plant resistance, organic fertilizers and biological control have been reported to control of root- knot nematodes (Nyczepir and Thomas 2009).

The Gammaproteobacteria *Xenorhabdus* spp. and *Photorhabdus* spp. have a mutualistic relationship with entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis*, respectively (Thomas and Poinar 1979, Boemare *et al*. 1993). These bacteria are vectored by third-stage juvenile nematodes (infective juveniles or IJs) that search for an insect host, penetrate it either through natural openings (mouth, anus or spiracles) or the cuticle. When inside the host hemocoel, IJs release symbiotic bacteria from their digestive tract. Once the infection has occurred, the bacteria multiply, produce toxins and natural products to kill the host within 24-48 h, and preventing the growth of other microorganisms. Infective juveniles feed on insect tissues and bacterial cells, develop into fourth- stage juveniles and adults, and produce through 1-3 generations (Burman 1982, Akhurst and Boemare 1990, Fallon *et*

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al. 2004,). Phenotypic characteristics allow the distinction of two stages in the life cycle of mutualistic bacteria. Phase I and II are separated by the production of pigments, catalase, lecithinase, lipase, bioluminescence (in case of *Photorhabdus*), pathogenicity and antibiotics (Han and Ehlers 2001).

Xenorhabdus and *Photorhabdus* secrete a wide variety of compounds including toxins, lipases, proteases, antibiotics and lipopolysaccharides into the culture medium (Wang *et al*. 2011). Some of the metabolites produced by these bacteria have been shown to be insecticidal (xenorhabdin 2, thiolutin, xenematide, rhabduscin, rhabdopeptides, phenylethylamides, tryptamides), fungicidal (xenocoumacin, glidobatin, PAX peptide), antibacterial (xenocomacins) and antimalarial activities (Bode 2009, Challinor and Bode 2015.

Benzylideneacetone synthesized by *X. nematophila* and active compounds of *X. budapestensis* and *X. szentirmaii* were reported against some plant pathogenic bacteria (Ji *et al*. 2004) and *Erwinia amylovora* and *Phytophthora nicotianae* (Boszormenyi *et al*. 2009). The nematicidal metabolites, 3,5-dihydroxy-4-isopropylstilbene (ST) and indole isolated from *Photorhabdus luminescens*, and ammonia (Hu *et al*. 1995, 1996) produced by *Xenorhabdus bovienii* and *P. luminescens*, are toxic to *M. incognita* (Hu *et al*. 1999). Extracts of both *P. luminescens* strains showed nematicidal activity on *M. incognita* but had no effect on *Steinernema carpocapsae* and *Caenorhabditis elegans* (Orozco *et al*. 2016). The objective of the present investigation is to determine the the effects of culture filtrates of *Xenorhabdus nematophila*, *X. bovienii* and *Photorhabdus luminescens* at different concentrations on *Meloidogyne javanica* mortality of the second stage juveniles and egg hatching.

Material and Methods

Source and maintenance of nematodes

Entomopathogenic nematodes (EPNs) (*Steinernema feltiae*, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*) were obtained from Koppert company and *Galleria mellonella* larvae were reared on formulated diet (Eischen and Dietz 1990). Insect hosts were infected in petri dishes lined with filter paper to obtain insect cadavers. They were exposed to 100 IJs of each isolate. The petri dishes were incubated at 25 ^{o}C for 2 days, and infected cadavers were subsequently transferred to new petri dishes lined with dry filter paper for a further two days of incubation to allow the development of typical signs of EPN infection (Del Valle *et al*. 2013). Third-stage juveniles were harvested from modified White traps (Kaya and Stock 1997) and stored at 16 ^{o}C.

A culture of *Meloidogyne javanica* was maintained on tomato in glasshouse and eggs were collected by NaOCl-extraction (Hussey and Barker 1973). Hand- picked egg sacs of *M. javanica* were placed on a nylon screen immersed in shallow water in glass petri dishes, and hatched J_2 were washed out daily with distilled water.

Isolation of bacteria

Last instar larvae of *G. mellonella* infected with different strains of entomopathogenic nematodes were surface sterilized in 70% ethanol, transferred to clean tissue to dry in a laminar hood and a drop of hemolymph was streaked on NBTA (NA+ 0.0025% bromothymol blue + 0.004% triphenyltetrazolium chloride) agar. The plates were sealed with parafilm tape and incubated at 30^{o}C in the dark for 36 to 48 h. Primary form and single colonies were picked and streaked on NBTA agar. The process was continued until colonies of uniform size and morphology were obtained. Identity of all bacteria was confirmed through sequence analysis of 16SRNA (data not shown).

Preparation of cell-free filtrates (CFFs)

Seed cultures of each bacterial strain were prepared separately by adding single colony of 48 h old bacterial culture grown on NBTA plates to an Erlenmeyer flask (50

ml) containing 5 ml of LB medium. The flasks were shaken at 200 rpm on a gyratory shaker at 30°C for 24 h in darkness. After measuring OD_{600}, the equal amount of each bacteria of the seed culture was pipetted into each of three new flasks (250 ml) to an OD_{600} of 0.1 containing 20 ml LB broth, and the flasks were shaken for 5 days. The 5d-old broth cultures were centrifuged (13,000 g at 4°C for 20 min) separately and filter sterilized to obtain the CFFs. The CFF of each strain was then tested, diluted with sterile distilled water, and used for nematicidal bioassays. All experiments involving CFFs were conducted under standard, sterile conditions.

Analysis of bacterial compounds

For the first analysis of the presence of some compounds, *Xenorhabdus* and *Photorhabdus* strains were cultivated in 10 ml of LB media with 2% Amberlite^R XAD-16 (Sigma-Aldrich) with an overnight culture to an OD_{600} of 0.1. After 72 h of growth at 30°C with shaking at 200 rpm, Amberlite^R XAD-16 beads were extracted with one culture volume of MeOH for 1 h, as described previously (Brachmann *et al.* 2006). Following filtering and evaporation to dryness, extracts were re-dissolved in MeOH in 1/10 of the original culture volume and a 1:10 dilution was used for HPLC-UV/MS using a Dionex Ultimate 3000 system with a Bruker AmaZon X mass spectrometer as described previously (Tobias *et al*. 2017a).

Nematicidal activity bioassays

To evaluate any differential nematicidal effect on egg and J_2 of root-knot nematode, diluted CFFs (80, 60, 40, 20, 10, 5, 1, 0.5 and 0 %) of *X. bovienii*, *X. nematophila* and *P. luminescens* were prepared and tested against eggs and J_2s of *M. javanica*. Approximately 100 eggs and 100 J_2s were immersed in petri plates containing 1 ml of CFFs of bacterial strains at concentrations mentioned above. The plates were then sealed with parafilm and incubated at 26°C in darkness. After 10 days, the number of hatched juveniles was counted. The number of dead J_2s was recorded after 24, 48 and 72 h. Juveniles without movement were touched with a fine needle to confirm death. The factorial experiment was conducted based on completely randomized design with three replications.

Statistical analysis

Analysis of variance was carried out using SAS (v. 9.1) statistical software for the mortality and egg hatching inhibition data recorded in different treatments. The Duncan′s multiple range test was employed to test for significant difference among treatments at $P<0.05$. Values of LC_{50} were estimated using SAS software.

Results

Analysis of bacterial compounds

All bacteria used in this work were analyzed for their production of known natural products based on HPLC/MS analysis and comparison with an in-house database of natural products from entomopathogenic bacteria (Tobias *et al*. 2017b). From these comparisons *Photorhabdus* was identified as producer of anthraquinones (AQ), isopropylstilbene (IPS) and GameXPeptides, *X. nematophila* as producer of xenocoumacin II, xenortide, rhabdopeptide and nematophin and *X. bovenii* as producer of xenorhabdins (Table1).

Anthraquinones and xenorhabdins are responsible for orange-red pigmentation of *P. luminescens* and the yellow pigmentation of *X. bovenii* respectively (Bozhüyük *et al*. 2017).

Nematicidal activity bioassays

The effect of different concentrations of cell free filtrates of bacterial cultures (*X. nematophila*, *X. bovienii* and *P. luminescens*) on mortality of J_2 of *M. javanica* after 24, 48 and 72h has been shown in Table 2. Overall, the mortality percentage of J_2 was increased with increase of bacterial filtrates concentration and exposure time.

Table 1- Compounds detected from XAD extracts in strains by HPLC-MS

Bacteria	Compound	Retention time (min)	Height (mAU)
X. nematophila	xenocoumacin II	5.6	407.2
	xenortide A	7.1	410.2
	xenortide	7.1	449
	rhabdopeptide	7.7	574
	rhabdopeptide	8	588.4
	rhabdopeptide	8.1	673
	rhabdopeptide	8.3	687
	rhabdopeptide	8.6	701
	nematophine	8.7	273
X. bovenii	xenorhabdin	8	285
	xenorhabdin	8.6	299
P. luminescense	anthraquinone	7.3	271
	isopropylstilbene	9	255
	ɣ- X Peptide	9.6	215
	ɣ- X Peptide	9.9	229
	ɣ- X Peptide	10.2	552
	ɣ- X Peptide	10.3	586
	ɣ- X Peptide	10.6	566
	ɣ- X Peptide	10.7	600

The CFFs of *X. nematophila* and *P. luminescens* were more effective against *M. javanica* than *X. bovienii* after 24h. At the highest concentration, the mortality percentages with *X. nematophila*, *P. luminescens* and *X. bovienii* were 88, 94.7 and 67.7%, respectively. After 48h, CFFs of *X. bovienii* showed low mortality at concentration of 0.5-10%, whereas CFFs of *X. nematophila* and *P. luminescens* showed high mortality at concentrations of 60-80% and 40-80% respectively. Mortality percentages of nematodes in CFFs of *X. nematophila*, *X. bovienii* and *P. luminescens* (20-80% concentrations) after 72h were 82.3-100, 100, 90.3-100%, respectively (Table 2).

Table 2- The response of *M. javanica* J_2 to different concentrations of bacterial CCFs after 1, 2 and 3 days exposure.

		Mortality% ± SE		
Treatments	Concentration%	24h	48h	72h
X. nematophila	0.5	0.0±0.0^{h}	2.0±0.5^{fg}	3.6±0.6^{hi}
	1	0.0±0.0^{h}	2.0±0.5^{fg}	4.3±0.6^{h}
	5	3.3±0.3^{h}	7.0±1.5^{f}	8.3±1.4^{g}
	10	3.3±0.8^{h}	4.3±1.2^{fg}	12.6±2.1^{f}
	20	14.6±2.0^{g}	23.6±2.1^{e}	82.3±2.3^{d}
	40	40.6±4.4^{d}	61.0±3.7^{c}	94.3±1.8^{b}
	60	67.3±9.6^{b}	99.0±0.5^{a}	100±0.0^{a}
	80	88.0 ±4.0^{a}	98.3±0.8^{a}	100±0.0^{a}
X. bovenii	0.5	0.0±0.0^{h}	0.0±0.0^{g}	0.0±0.0^{i}
	1	0.3±0.3^{h}	2.6±1.3^{fg}	4.0±0.0^{hi}
	5	3.0±1.5^{h}	4.6±2.3^{fg}	6.0±1.0^{gh}
	10	18.0±3.5^{fg}	30.6±3.2^{d}	32.0±3.5^{e}
	20	28.0±4.0^{ef}	98.6±0.8^{a}	100±0.0^{a}
	40	34.3±5.2^{de}	100±0.0^{a}	100±0.0^{a}
	60	54.0±3.6^{c}	99.3±0.6^{a}	100±0.0^{a}
	80	67.6±11.1^{b}	100±0.0^{a}	100±0.0^{a}
P. luminescens	0.5	0.3±0.3^{h}	0.6±0.3^{fg}	0.0±0.0^{i}
	1	0.0±0.0^{h}	0.0±0.0^{g}	0.0±0.0^{i}
	5	0.3±0.3^{h}	0.3±0.0^{g}	3.3±0.8^{hi}
	10	3.6±1.6^{h}	4.3±2.3^{fg}	16.0±3.0^{f}
	20	16.6±1.7^{g}	64.6±7.2^{c}	90.3±0.8^{c}
	40	39.6±2.4^{d}	89.3±1.2^{b}	93.0±0.5^{bc}
	60	88.3±2.7^{a}	88.3±0.8^{b}	99.3±0.6^{a}
	80	94.6±1.7^{a}	96.3±0.8^{a}	100±0.0^{a}
Control (water, LB)	0	0.0±0.0^{h}	0.0±0.0^{g}	0.0±0.0^{i}

Mean values followed by the same letter are not significantly different at P<0.01 according to Duncan's Multiple Range test.

The results of toxicity bioassay of bacterial supernatants against J_2 of root-knot nematode are presented in Table 3. The LC_{50} values were reduced with increasing time of exposure in the treatments. The LC_{50} values of bacteria CFFs had no significant effect after 24h. However, the LC_{50} of *X. nematophila* indicated significant effect with *P. luminescens* after 48h.

There was a significant linear correlation between different concentrations percentage of bacteria CFFs and egg hatching inhibition percentage (Fig. 1).

The percentage of egg hatching of *M. javanica* decreased with increasing bacterial cell free concentrations (Table 4). The percentage of egg hatch was inhibited significantly at concentrations of 40-80% for *X. nematophila* and concentrations of 60-80% for *X. bovienii* and *P. luminescens*. Cell free filtrate of *X. nematophila* had a significant inhibitory effect on egg hatch at 5% concentration (52.7%). However, CFF of, *X. bovienii* and *P. luminescens* had inhibitory effect on egg hatch (50.4% and 72.7%) at 20% concentration, respectively.

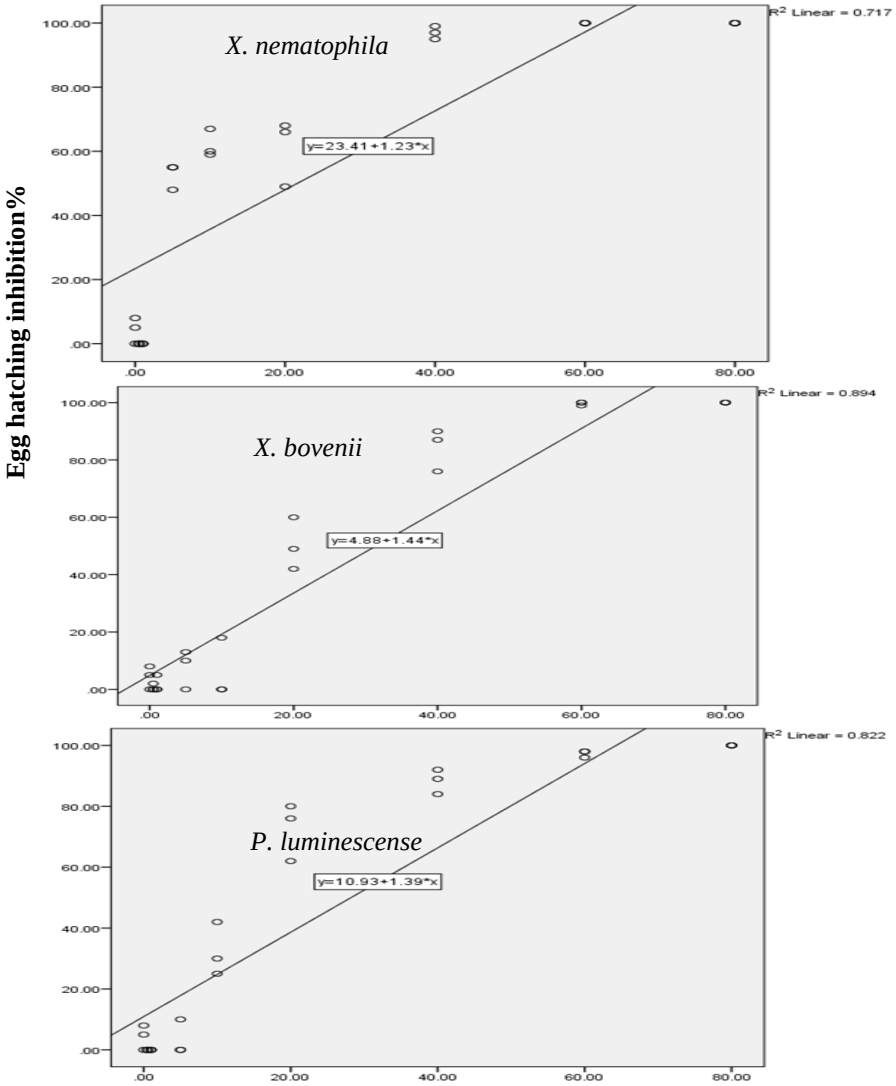

Fig 1- The relationship between different concentrations % of bacteria supernatants and egg hatching inhibition%. Dotted lines show 95% confidence interval.

Table 3- Toxicity of bacterial filtrates against J_2 of *M. javanica*

Cell-free filtrates of bacteria	Number of root-knot nematode	Time of exposure (h)	Slope ± *SE*	□□	LC_{50} (95% FL)
X. nematophila	300	24	4.1 ± 0.4	96.4	45.8 (40.7-50.6)
		48	5.3 ± 0.4	123.7	31.6 (28.6-34.4)
X. bovenii	300	24	1.8 ± 0.2	52.0	55.3 (44.24-74.01)
		48	6.9 ± 28.5	0.06	11.8
P. luminescens	300	24	5.1 ± 0.6	59.4	40.3 (35.3-44.52)
		48	3.6 ± 0.3	88.4	21.8 (18.23-25.60)

lethal concentration 50 and 95% fiducial limits (FL) were estimated using logistic regression (SAS Institute, 2002).

Table 4- Effect of bacterial filtrates on egg hatching inhibition of *M. javanica*

Treatments	Concentration %	egg hatching inhibition % ± SE
X. nematophila	0.5	0.0±0.0^{g}
	1	0.0±0.0^{g}
	5	52.6±2.3^{e}
	10	62.0±2.5^{d}
	20	61.0±6.0^{d}
	40	96.6±1.1^{a}
	60	100±0.0^{a}
	80	100±0.0^{a}
X. bovenii	0.5	0.6±0.6^{g}
	1	01.6±1.6^{g}
	5	07.6±3.9^{g}
	10	03.3±6.0^{g}
	20	50.3±5.2^{e}
	40	87.3±4.2^{b}
	60	99.6±0.3^{a}
	80	100±0.0^{a}
P. luminescens	0.5	0.0±0.0^{g}
	1	0.0±0.0^{g}
	5	3.3±3.3^{g}
	10	32.3±5.0^{f}
	20	72.6±5.4^{c}
	40	88.3±2.3^{b}
	60	97.3±0.6^{a}
	80	100±0.0^{a}
Control	0	4.4±2.3^{g}

Mean values followed by the same letter are not significantly different at P<0.01 according to Duncan's Multiple Range test.

Discussion

This study demonstrates that the cell-free supernatant of *X. nematophila*, *X. bovienii* and *P. luminescens* possess nematicidal activity against *M. javanica*. Also, it indicates the inhibitory effect of CFFs of these entomopathogenic bacteria on egg hatching. Entomopatogenic bacteria are known to produce toxic metabolites (Akhurst 1982, chen *et al*. 1994), some of which have been identified (Li *et al*. 1995, Hu and Webster 2000, Ji *et al*. 2004, Lang *et al*. 2008, Bode 2009**,** Reimer *et al*. 2011, Kronenwerth *et al*. 2014, Bozhüyük *et al*. 2017, Engel *et al*. 2017). A number of these and other natural products might show nematicidal effects, which has not investigated in detail for most of these compounds (H.B Bode, unpublished results).

Our findings are in accordance with those reported by Hu *et al*. (1995) who found that CFFs of *X. bovienii* grown for 48-120 h at 50% concentration, caused

100% mortality of *M. incognita* J_2 but no effect was detected when a 12-24 h culture filtrate was tested (Fallon *et al.* 2004). According to Hu *et al*. (1999) entomopathogenic bacteria produce metabolites that act as nematicides toward a large number of nematodes including some phytonematodes. Further investigation demonstrated that an organic extract of the culture filtrate of *P. luminescence* showed nematicidal activity.

We observed CFFs of all strains exhibited nematicidal activity but metabolites with nematicidal activity in *X. bovienii* are different compared to the two other bacteria due to high mortality after 48h. CFFs of *X. nematophila* and *P. luminescens* also showed a same nematicidal effect after 24h. Orozco *et al*. (2016) reported extracts of *P. luminescens* strain CH35 caused >90% mortality of *M. incognita* J_2 at 40 µg/ml concentration and extracts of *P. luminescens* strain TT01 also caused an equally strong nematicidal effect but these extracts showed a low nematicidal effect on *C. elegans* at 20 µg/ml and had no effect on *S. carpocapsae*.

Photorhabdus and both of *Xenorhabdus* showed to produce metabolites that inhibited egg hatch of *M. javanica* and *X. nematophila* CFF was more toxic. The 15% concentration of *X. nematophilus* or *X. bovienii* cell-free filtrates caused 98-100% mortality on J_2 of *M. incognita* after 3 days exposure at 25^{o}C and delayed egg hatch after exposure for 7 days (Grewal *et al*. 1999). Samaliev *et al*. (2000) reported J_2 of *M. javanica* in contact with bacteria (*Xenorhabdus nematophilus* and *Pseudomonas oryzihabitans*) and their metabolites exhibited disorientation and convulsive movements.

At concentrations of 10^6 and 10^7 cells ml^{-1} mortality was 100% after 24 h exposure, *Xenorhabdus* being more toxic than *Pseudomonas*. In addition a 74% reduction in hatching was observed when the eggs removed from contact with the bacterial substrate, washed and stored in distilled water for 10 days. Andalo *et al*. (2007) used filtrates to evaluate the effect of metabolites on *M. incognita* and the eggs and juveniles of *M. javanica*, and demonstrated that high mortality rates were obtained after the nematodes were in contact for 24 h. 3,5-dihydroxy-4-isopropylstilbene (ST) and indole from the culture filtrate of *P. luminescens* MD, were shown to have nematicidal activity. ST caused mortality of *Aphelenchoides rhytium*, *Bursaphelenchus* spp. and *Caenorhabditis elegans* but had no effect on J_2 of *M. incognita*. Indole was lethal to *M. incognita*. Both ST and indole inhibited egg hatch of *M. incognita* (Hu *et al*., 1999). Ferreira *et al*. (2011) indicated IJs of *H. baujardi* LPP7, *P. luminescens* or its metabolites had no effect on the embryogenesis of *M. mayaguensis*, possibly due to low permeability of the eggs, whereas in eggs with active J_2, delay in hatching was observed, possibly due to the release of *P. luminescens* and to the concentration of metabolites in the medium. Also inhibition of egg hatch of *M.incognita* by *X. nematophila* and *P. luminescens* was reported (Grewal *et al*. 1999, Hu *et al*. 1999).

Researches by Hu *et al*. (1999), Andalo *et al*. (2012), Atif *et al*. (2012). have demonstrated the toxicity of entomopathogenic bacteria to J_2 of *M. incognita*.

In conclusion, the presence of natural nematicidal activity of either *Xenorhabdus* or *Photorhabdus* was confirmed with sufficient larval mortality percentages in this study, a situation which could be explained due to the production of different natural products by these bacteria. Further study will focus on the identification of these natural products and their mode of action in order to use them as potential treatment against plant parasitic nematodes.

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