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RESEARCH REPORT

Side effects of immune response of Colorado potato beetle, *Leptinotarsa decemlineata* against the entomopathogenic nematode, *Steinernema carpocapsae* infection

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Abstract

Entomopathogenic nematodes (EPNs) are lethal pathogens of agricultural insect pests. Little is known about their sublethal effects on the insect hosts. The lethal effects *of Steinernema carpocapsae* on fourth instar larvae of Colorado potato beetle (CPB), *Leptinotarsa decemlineata* were detected using soil infection and direct injection of the nematode into the hemocel. LC_{20} and LC_{80} values of 7.8 (3.0 - 13.4) infective juveniles (IJs) and 126.7 (91-206.7) IJs were obtained for the soil application method and 10.2 (8.7 - 11.4) IJs and 22.7 (19.73 - 28.0) IJs for direct injection, respecitvely. Sublethal effects of *S. carpocapsae* on last instar larvae and subsequent surviving adults and phenoloxidase (PO) activity in hemolymph of nematode-injected last instar larvae were investigated. Sublethal effects included adult cuticular discoloration, deformation of the wings, legs and antenna and decreased fertilized egg production in females. Considering cuticular discoloration in most treated insects, it is hypothesized that production of PO in the insect larvae infected with an entomopathogenic nematode, *S. carpocapsae* might have costs for surviving adult insects. PO specific activity in CPB against *S. carpocapsae* generally increased up to 48 h post injection. Here in, the sublethal effects are discussed as a potential tread-offs of PO production in nematode-injected insects.

Key Words: *Steinernema carpocapsae*; *Leptinotarsa decemlineata*; PO activity; cuticular discoloration

Introduction

Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Coleoptera: Crysomelidae) is one of the most destructive pests of potato foliage and prone to developing chemical insecticide resistance (Cutler *et al,.* 2005; Hitchner, 2007). The environmental hazardous effects of chemical insecticides have made the use of biocontrol agents necessary. Entomopathogenic nematodes (EPNs) are biological control agents of agricultural insect pests (Burnell and Stock, 2000). There are few studies on the sublethal effects of EPNs either on insect development or their immune system. *Heterorhabditis bacteriophora* and its symbiotic bacterium *Photorhabdus luminescens*, decreases larval feeding rate, wet weight gain, and frass production of *Schizura concinna* larvae (Milstead, 1980). Late instar *Spodoptera littoralis* larvae infected with *Steinernema riobrave* exhibit decreased rates of leaf consumption and eat fewer meals in comparison to control treatments (Alchanatis *et al.,* 2000). *Galleria mellonella* larvae infected with *Steinernema carpocapsae* showed reduced silk production (Simões *et al.,* 2000). Sublethal concentrations of *Heterorhabditis downesi* and *S. carpocapsae* caused no effect on feeding rate of adults of the large pine weevil, *Hylobius abietis* (Girling *et al.,* 2010). Wing and elytra deformation of adult CPBs were reported when infected with *S. feltiae* and *H. bacteriophora* (Ebrahimi *et al.,* 2011b). Awareness on entomopathogenic nematode s sublethal effects helps to increase their efficacy as biocontrol agents. In that, how sublethal effects of biological control agents like those of chemical insecticides may affect ecological fitness of the insects due to morphological, physiological or behavioral changes (Bao *et al.,* 2009; Girling *et al.,* 2010; Vidau *et al.,* 2011).

The cuticular phenoloxidase (PO) is a melanizing enzyme at the wound site which limits infection; PO in hemolymph is responsible for melanotic immune responses (Pham and Schneider,

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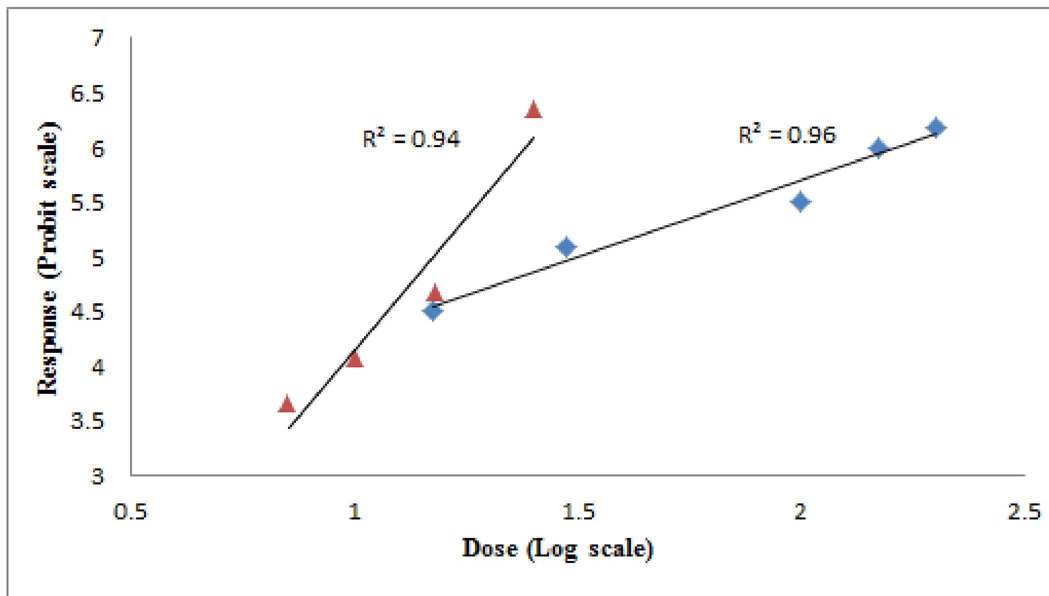

Fig. 1 Mortality (probit-transformed) of *L. decemlineata* prepupae injected with different doses of *S. carpocapsae* (solid triangles) and infected with different doses of *S. carpocapsae* in soil (solid squares).

2008); additionally, one PO type is common to both cuticle and hemolymph (Hiruma and Riddiford, 1988). The PO immune responses occur immediately against invading microbes in insects (Castillo *et al.*, 2011). PO involves the formation of short-lived toxic substances (*e.g.,* chemically reactive quinones) and long-lived products such as melanin (a brown-black pigment) both are deposited at wound sites and participating in antimicrobial responses including encapsulation and killing of microbial pathogens (Castillo *et al.,* 2011). The melanization cascade overlaps the humoral and cellular insect immune defenses (Castillo *et al.,* 2011).

PO is responsible also in sclerotization and tanning of the cuticle (Arakane *et al.,* 2005). Insect cuticle is immunologically active in expressing PO as well as antimicrobial peptides supplementing its physiochemical passive antimicrobial properties (Brey *et al.,* 1993; Golkar *et al.,* 1993). Moreover, a correlation between increasing cuticular darkening and resistance to entomopathogens has been demonstrated in some insect species (Barnes and Siva-Jothy 2000; Wilson *et al.,* 2001; Armitage and Siva-Jothy 2005; Dubovskiy *et al.,* 2013). Cellular encapsulation and capsule melanization of the EPNs in CPB is documented (Thurston *et al.,* 1994; Armer *et al.,* 2004; Ebrahimi *et al.,* 2011a, b) Therefore, in the current study, lethal and sublethal effects of *S. carpocapsae* on CPB and the nematode interaction with PO activity of the hemolymph of the insect were investigated.

Table 1 $LC_{20,\ 50\ \text{and}\ 90}$ values for *S. carpocapsae* against *L. decemlineata* prepupae

Method of infection	Slope±SE	Chi-Square	LC_{20} (95% CL)	LC_{50} (95% CL[a])	LC_{90} (95% CL)	R^2	N
Soil infection	1.39±0.21	1.78	7.8 (3.0-13.4)	31.5 (20.4-43.0)	262.3 (168.6-555.2)	0.96	270
Direct injection	4.84±0.62	4.7	10.2 (8.7-11.4)	15.2 (13.7-17.1)	28.0 (23.5-36.6)	0.94	225

[a] Confidence limits

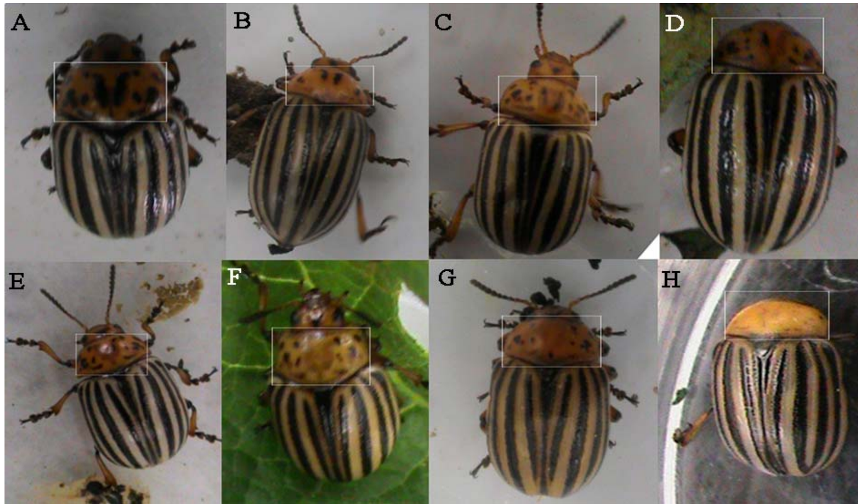

Fig. 2 Adult *L. decemlineata* infected with *S. carpocapsae* during the prepupal phase showing a continuum in the pronotum discoloration (white boxes): (A) control (injected with Ringer's solution), (B - H) injected with *S. carpocapsae.*

Materials and Methods

Nematodes

Steinernema carpocapsae was obtained from the Collection of Nematology Laboratory, University of Tabriz, Tabriz, Iran. Nematodes were cultured using the last instar greater wax moth larvae, *Galleria mellonella* (Woodring and Kaya, 1988). Infective juveniles (IJs) were stored in distilled water at 5 °C and used in all experiments within 30 days of emerging from the host. Before starting the experiments, nematodes were kept at 25 °C for 20-30 min.

Insects

Leptinotarsa decemlineata

Over-wintered adult Colorado potato beetles were collected from potato fields in Sarab (East Azarbaijan province) in June, 2012 and were stored in plastic boxes (19.3 cm length, 14.3 width, 3.6 cm height; 20 larvae per box) with ventilated lids (26 ± 2°C, 50 ± 5 % RH and 16:8 (L:D) photoperiod) and received fresh potato leaves daily. Offspring of the beetles were used for establishing the greenhouse colony (Ebrahimi *et al.,* 2011b). Fourth instar larvae develop into prepupae after three days and prepupae were used in all experiments.

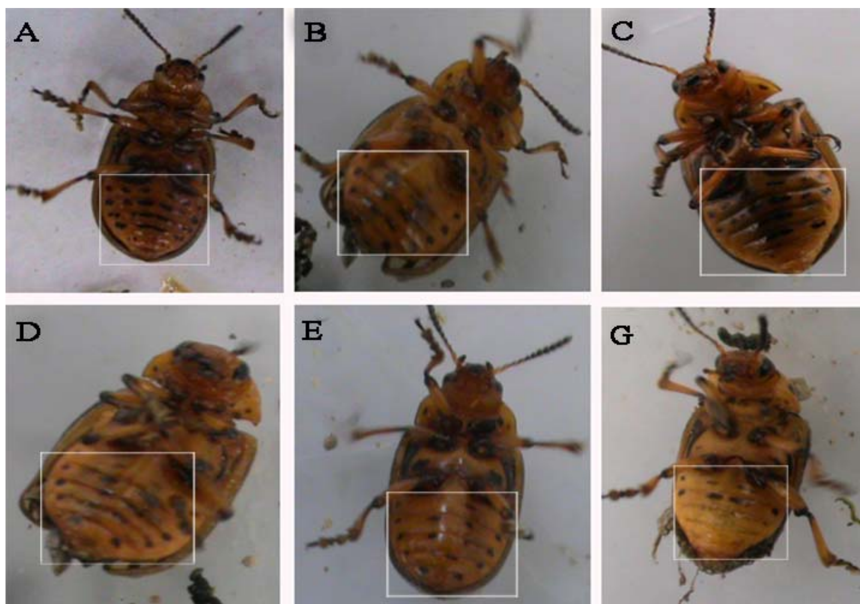

Fig. 3 Adult *L. decemlineata* infected with *S. carpocapsae* during the prepupal phase showing a continuum in the ventral abdominal discoloration (white boxes): A) control (injected with Ringer's solution), B - G) injected with *S. carpocapsae.*

Table 2 Percentages of adults of *L. decemlineata* showing the sublethal effects of *S. carpocapsae*

	Number of replicates	Total number of insects	Anatomical deformations (%) ±SE			
			coloring	Wings	legs	antenna
Direct injection						
Treated insects	3	65	44.62 ± 8.08^{a}	24.62 ± 8.08^{a}	21.54 ± 9.81^{a}	9.38 ± 5.53^{a}
Control	3	65	0^{b}	7.81 ± 1.56^{b}	0^{b}	0^{b}
Soil application						
Treated insects	3	47	52.92 ± 9.22^{A}	20.97 ± 10.5^{A}	19.31 ± 11.3^{A}	8.47 ± 3.5^{A}
Control	3	45	0^{B}	0^{B}	4.4 ± 7.6^{B}	0^{B}

No significant differences are present between the values marked with similar superscripts (either lower-case letters or capital letters) ($p < 0.05$)

Galleria mellonella

G. mellonella was reared under the same environmental conditions and in similar boxes as *L. decemlineata,* but on an artificial diet (1200 g wheat flour, 120 g beeswax, 300 g dried yeast, 600 g honey and 500 ml glycerol 99 %).

Comparison of two procedures for nematode infection; IJs in soil and direct injection of the nematodes for study of lethal effects

Both soil infection of the CPB with nematodes and direct manual injection of nematodes into the insects were used to determine the $LC_{20,\ 50}$ and $_{80}$ values of the two methods and to establish LC values for examining sublethal effects on surviving insect from both methods. The soil infection experiments were conducted in plastic cylindrical boxes (3 cm diameter and 5 cm height) filled with 30 g autoclaved sandy soil (85 % sand, 10 % silt and 5 % clay w/w). Distilled water (1ml) was added to each of the boxes and *S. carpocapsae* IJs were added at selected concentrations in 1 ml of water to the surface of the soil. The nematodes were used at rates ranging between 15 and 200 IJs per host prepupa (*i.e.*, 15, 30, 100, 150 and 200 IJ per prepupa). One prepupa was placed on the soil and the boxes were covered with ventilated lids to reduce desiccation. Control boxes received 2 ml distilled water without nematodes (water content of the soil was 10 % (w/w)). 15 boxes were used for each nematode concentration and the experiment was replicated three times. Mortality was recorded when the adults began to eclose in the control groups. All dead insects were dissected to ensure the presence of nematodes. An insulin syringe needle gauge (1 ml, SOHA, Iran) was used to inject different concentrations of nematodes (*i.e.*, 7, 10, 15 and 25 IJs in 20 µl Ringer's solution per prepupa) into the hemocoel of 15 prepupae, through the dorsal cuticle, the segment behind the pronotum. The IJs were counted in 20 µl aliquots to ensure the numbers were accurately delivered.

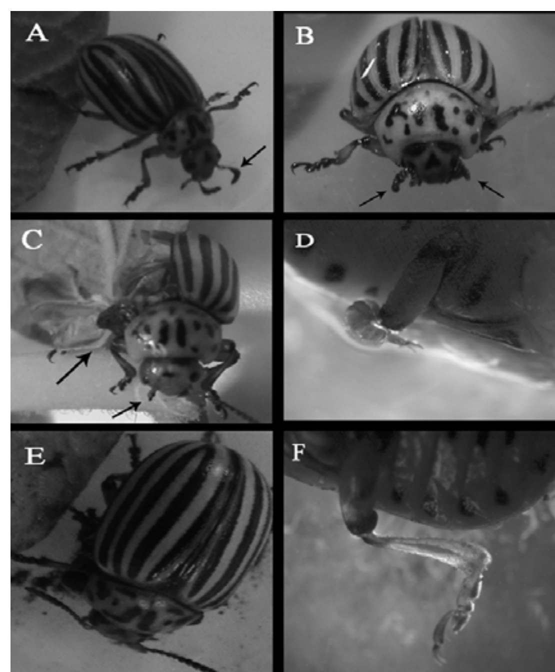

Fig. 4 Deformation in the adult beetles infected with *S. carpocapsae*: A-B) deformed antenna, C) deformed antenna and wings, D) deformed leg, E) control for antenna and wings and F) control for leg.

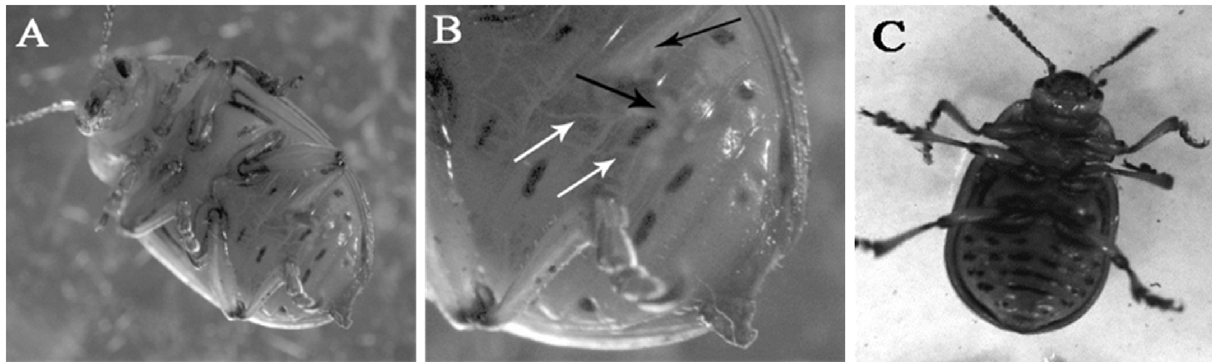

Fig. 5 A, B) Thinning of the cuticle in color defected *L. decemlineata* adults; white and black arrows were used to show trachea and fatty tissue under cuticle, respectively. C) control.

Control groups received 20 µl Ringer's solution. The experiment was replicated three times. After injection, the insects were transferred to soil contained in plastic boxes moistened with 2 ml distilled water as previously alluded. Mortality was recorded when the adults began to eclose in the control. All dead insects were dissected to ensure the presence of nematodes.

Sublethal effects

Direct injection method was chosen in the experiment on sublethal effects because of insertion of certain numbers of the nematode into the insect hemocoel. In addition, preliminary experiments showed the same kind of sublethal effects for both soil application and direct injection methods (which was reported in the results). Each of 25 prepupae were injected with 11 IJs (LC_{20}) in 20 µl Ringer's solution, the control group received the same volume of Ringer's solution. The experiment was replicated three times. Morphological deformations and changes in cuticle coloring of emerged adult insects were recorded and digital images were recorded using a LEICA MZ 125 stereomicroscope provided with a camera 2 - 3 h after emerging from the soil. Adult insects that emerged from control and treated boxes were transferred to new chambers and fed with fresh potato foliage for two weeks. One male and one female were kept in separate chambers and numbers of the eggs per female and numbers of the hatched eggs were recorded.

PO specific activity and total protein quantification

Infective juveniles (28 IJs as LC_{80} and 11 IJs as LC_{20} in 20 µl of Ringer's solution) were injected into the hemocoel of each of 50 prepupae. Prior to bleeding, prepupae were disinfected for 2 min in 70 % ethanol, and then placed on ice for 2-5 minutes. Control insects (n = 50) were injected with 20 µl of Ringer's solution. Other control group consisted of 50 prepupae wounded with needle of injection syringe. Five prepupae were bled once at designated time intervals (0 min - 48 h pi) by puncturing dorsal posterior of the abdominal cuticle. The blood was collected directly into a chilled (4 °C) 1.5 ml microcentrifuge tube. Samples were kept at -80 °C until use. For the PO activity assay, hemolymph samples were centrifuged at 4 °C and 12000x*g* for 10 min to pellet cell debris. Aliquots (5 µl) of hemolymph supernatant were added to reaction buffer (195 µl 10 mM L-DOPA and 600 µl 10 mM Tris-HCl) and dopachrome formation followed spectrophotometrically (490 nm) (Biochrom WPA Biowave S2100 Diode Array Spectrophotometer) in samples incubated at 35 °C

Table 3 Number of the produced and hatched eggs in nematode-injected surviving and control adults of *L. decemlineata* in the sublethal experiment

	Number of Insects	Number of Eggs per female	Hatching(%)±SE	%Eggs in cluster
Nematode-injected insects	12	$48.3±22.3^{a}$	$24.8±21.6^{b}$	$21.8±5.5^{b}$
Control	12	$63.6±30.8^{a}$	$81.3±6.0^{a}$	$89.6±0.5^{a}$

No significant differences are present between the values marked with similar superscripts ($p < 0.05$)

for 5 min in a water bath. There were three replicates for each sample. The experiment was repeated twice. To calculate PO specific activity, the concentration of the total protein was determined using the Bradford protein assay (Bradford, 1976) with Bovine serum albumin as the standard. Hemolymph (5 µl) from each sample mixed with 795 µl distilled water was incubated with 200 µl of Bradford reagent and the absorbance measured spectrophotometerically (595 nm, 10 min after mixing). PO activity unit was defined as the change in optical absorbance unit at 490 nm per min. The specific activity was calculated as the units of enzyme activity per mg total protein.

Statistics

LC_{20}, LC_{50} and LC_{80} values for both soil infection and direct injection of the nematodes for lethal effects were obtained by Probit analysis using SAS software (SAS Institute, 2004). Data were analyzed by analysis of variance and, when appropriate, means were evaluated by Duncan's multiple-range test (SAS Institute, 2004). Lethal and sublethal experimental data were transformed into square root of (x+1) where needed, before analysis.

Results

Comparison of soil infection and direct injection of the nematodes methods for lethal effects

LC_{20}, LC_{50} and LC_{80} values for the two assays revealed a significant relationship between log dose and insect mortality of injected and soil infected insects ($p < 0.05$, Fig 1, Table 1), LC_{50} and LC_{80} values for the injection method being significantly lower than for the soil infection assay (about 2.1 fold and 5.7 fold, respectively), whereas LC_{20} values overlapped for the two infection methods.

Sublethal effects

The most frequent significant ($p < 0.05$) sublethal effect of *S. carpocapsae* injection on surviving injected adult beetles of *L. decemlineata* was cuticular discoloration; the black spots on the pronotum and abdominal sternites were not formed completely and the pronotum and abdomen exhibited a yellow appearance (Figs 2, 3; Table 2). Also, deformation in wings, legs and antennae of surviving adults was observed (Table 2, Fig. 4). Thinning of the cuticle or increased transparency occurred in color defective adults resulting in visible trachea and other internal tissues (Fig. 5). All mentioned sublethal effects were observed also in surviving insects from all five concentrations of the nematodes used in soil experiments (Table 2).

Numbers of deposited eggs were not found significantly different between control and nematode-injected insects, while the egg hatching percentages were significantly lower in nematode-injected insects ($p < 0.05$, Table 3). *Leptinotarsa decemlineata* deposits most of the eggs in clusters, but nematode-injected surviving beetles deposited most of their eggs individually rather than in the clusters (Table 3).

PO activity and total protein quantification

Significant ($p < 0.01$) differences occurred in PO units, total protein concentration and PO specific activity in the hemolymph among nematode-injected, Ringer's solution-injected and wounded insects over time. In the overall, PO unit mean was the highest for LC_{80} injected insects and the lowest for wounded insects ($F = 75.42$, df = 3, $p < 0.01$, Table 4). PO increased significantly to highest values from 24 - 48 h pi in nematode (LC_{20} and LC_{80}) injected insects. PO activity was significantly higher for LC_{80} nematode-injected insects at 24 and 48 h pi than all other treatments (Fig. 6).

Total protein values were lower in the hemolymph of the nematode-injected insects and Ringer's solution-injected than wounded insects ($F = 45.57$, df = 3, $p < 0.01$; Table 4). In overall, total protein values were the highest at 48h pi for all groups which showed slight decreasing compared with 0 min pi in nematode (LC_{20}) injected and Ringer's solution-injected insects (Fig. 7).

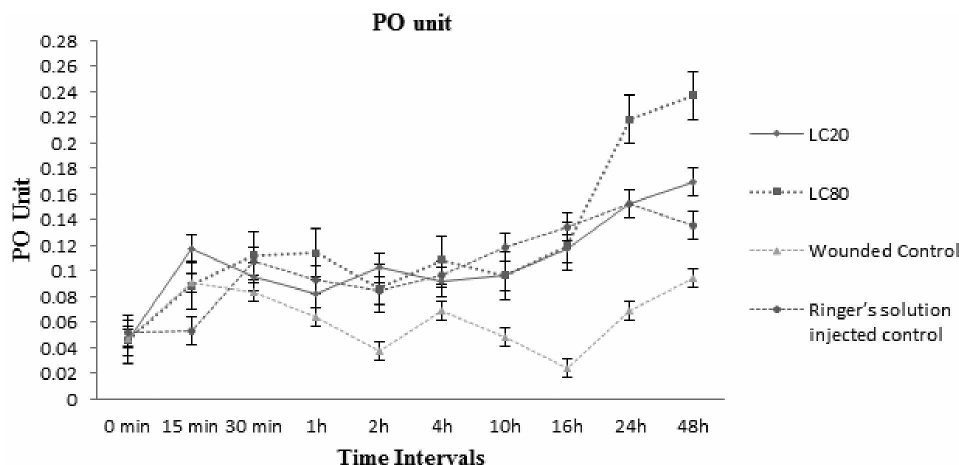

Fig. 6 Phenoloxidase Units of nematode-injected, wounded and Ringer's solution injected insects in different time intervals. The results are expressed as mean ± SD. The data were statistically analyzed using ANOVA and Duncan's multiple range tests with a specified significance level of $p < 0.05$.

Table 4 Mean comparison of Phenoloxidase Units, total protein concentration (mg/ml) and phenoloxidase specific activity among nematode-injected, wounded and Ringer's solution injected insects

Treatment	Total protein	PO Unit	PO specific activity
LC_{20} injected	$5.15±0.33^b$	$0.106±0.01^b$	$4.35±0.28^b$
LC_{80} injected	$5.13±0.27^b$	$0.122±0.01^a$	$4.74±0.44^a$
Wounded control	$6.28±0.17^a$	$0.062±0.00^c$	$2.01±0.07^d$
Ringer's solution injected	$5.15±0.27^b$	$0.102±0.01^b$	$3.92±0.24^c$

No significant differences are present between the values marked with similar superscripts ($p < 0.05$); N = 100 insects for each group

In the overall, PO specific activity was the highest in the hemolymph of LC_{80} nematode-injected insects and was the lowest in wounded insects (F = 232.61, df = 3, $p < 0.01$; Table 4). PO specific activity in the hemolymph of the nematode-injected insects was significantly higher than wounded control and Ringer's solution injected insects from 30 min - 16h pi (Fig. 8). PO specific activity was the highest at 24 h pi and 48 h pi for LC_{20} and LC_{80} injected insects, respectively and at 24 h pi Ringer's solution injected insects (Fig. 8).

In nematode-injected insects released bacteria were observed 10 h pi and bacteria concentration was increased with increasing the time. Tissue damage was observed after 16 h pi. Fat body lysis was the first obvious sign of tissue damage due to bacteria releasing and nematode activity.

Discussion

Comparison of insect infection with nematodes by the soil infection and direct nematode injection methods on lethal effect showed that slope of direct injection method was about three fold more than soil infection method. Injecting the nematodes into the hemocel allows straightforward pathological expression of nematodes in the site of action, whereas in soil application, nematodes spend time navigating different barriers such as distances in the soil environment, penetrating of the host while dealing with insect behaviors and/or structural defenses to reach the site of action in hemocel (Koppenhöfer *et al.,* 2000; Toubarro *et al.,* 2009). Nevertheless, in the soil method some of the applied nematodes penetrate into the hosts, therefore injection method was chosen for sublethal and PO activity experiments.

In the overall, surviving CPB adults sublethal effects including adult discoloration and thinning of the cuticle in color defective adults, anatomical deformations in wings, antenna and legs and decreased fertilized eggs when infected with low concentration of the nematodes in prepupa stage. None of these sublethal effects have been mentioned and documented in nematode-infected insects.

The sublethal defect on CPB adult coloring is common here in, however there is no similar finding in the published studies related to nematode-infected insects. Cuticular sclerotization is the process in which the cuticle is stabilized by incorporating phenolic compounds resulting in the formation of brownish colors of varying intensities, but nearly colorless and completely black cuticles can also be formed (Andersen, 2010). Black cuticles contain melanin which in some species is present in granules and in others the pigment is diffusely distributed (Andersen, 2010). It should be noted that a phenotypic correlation has been demonstrated between increasing cuticular colour and insect resistance to entomopathogenic viruses in *Spodoptera exempta* (Reeson *et al.,* 1998) and entomopathogenic fungi the coleopteran *Tenebrio molitor* (Barnes and Siva-Jothy 2000; Armitage and Siva-Jothy 2005), and the two lepidopteran insects *Spodoptera exigua* (Wilson *et al.,* 2001) and *G. mellonella* (Dubovskiy *et al.,* 2013). Therefore, defect in adult coloring and formation of lighter cuticle compared with control insects may impose the physiological cost of increased susceptibility against entomopathogens.

In the sublethal experiments infective juveniles of nematodes were injected into the hemocoel in prepupa stage and two or three days later metamorphosis of the insects began. Perhaps, secreting proteases by the nematodes or their symbiotic bacteria (Hwang *et al.,* 2003; Cho and Kim 2004; Hinchliffe *et al.,* 2010) induces apoptosis or less pronounced tissue damage on metamorphosing tissues such as the wings, antenna and legs of the beetles leading to such deformations. On the other hand, *Photorhabdus luminescens* TT01 genome sequence revealed two loci with high similarity to the Juvenile Hormone Esterase of CPB; the esterase regulates metamorphosis by inactivating juvenile hormones which maintain the larval stage (Hinchliffe *et al.,* 2010). It is possible that analogs to CPB juvenile hormone esterase produced by the symbiotic bacteria disrupt adult organogenesis during metamorphosis. Additional experiments are needed to interpret the deformations.

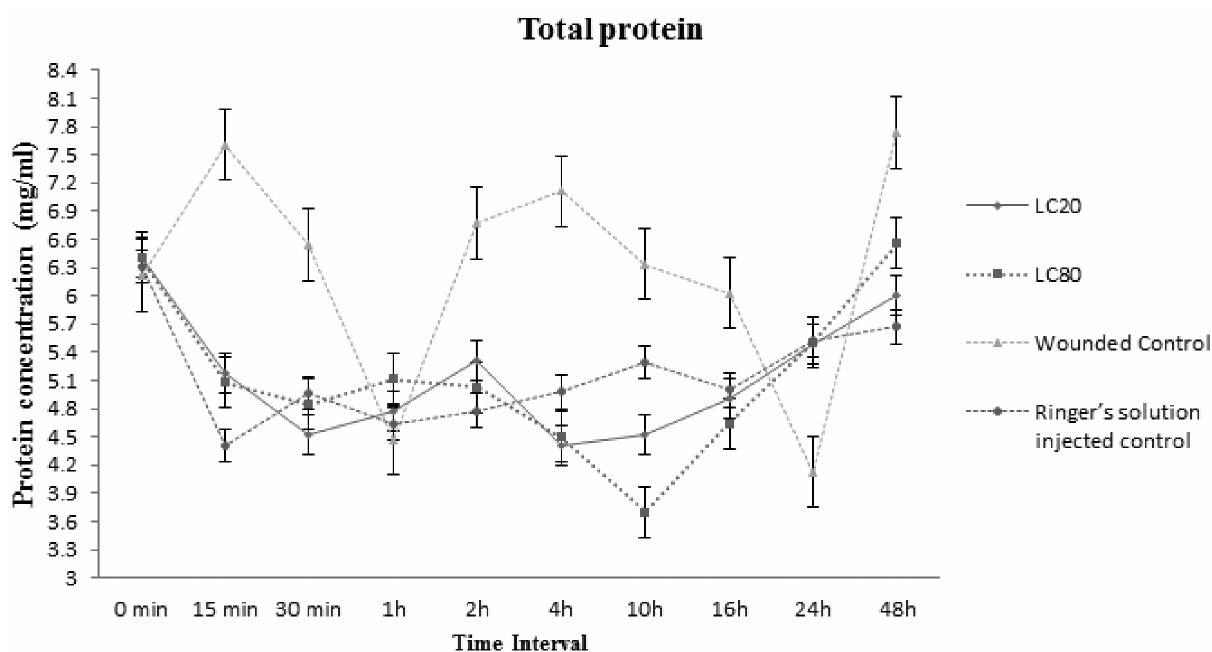

Fig. 7 Total protein concentrations (mg/ml) of LC_{20} and LC_{80} nematode-injected, wounded and Ringer's solution injected insects in different time intervals. The results are expressed as mean ± SD. The data were statistically analyzed using ANOVA and Duncan's multiple range tests with a specified significance level of $p < 0.05$.

Decreasing fertilized eggs in surviving adults was the other sublethal effect of *S. carpocapsae* on CPB. Deposition the eggs individually rather than in cluster could be a behavioral disorder that occurred in nematode-injected surviving adults; however immune activity and reproduction areoften negatively correlated in insects (Siva-Jothy *et al.,* 2005; Lawniczak *et al.,* 2007) and reduced reproduction have been noticed as a trade-off for immune function against pathogens in insects (Calleri *et al.,* 2006).

PO is a key component in the immune system of insects and the main role of PO in melanogenesis is converting phenols to quinones, which subsequently polymerize to form melanin (González-Santoyo and Córdoba-Aguilar, 2012). Some species of entomopathogenic nematodes including *S. carpocapsae* are encapsulated and melanized in CPB (Thurston *et al.,* 1994; Armer *et al.,* 2004; Ebrahimi *et al.,* 2011a, b) as a part of the CPB immune system that displays some degree of resistance against the entomopathogenic nematodes. In the present study, PO activity of nematode-injected CPB increased in the hemocel in a nematode dose-dependent manner compared to injection of Ringer's solution. Increasing nematode concentration led to increased PO activity which was coincident with the appearance of symbiotic bacteria in the hemolymph of the insects. Yang *et al.* (2012) identified an insecticidal protein from *Xenorhabdus budapestensis* which extensively activated the PO cascade in *G. mellonella* which potentially caused larval *G. mellonella* death (Yang *et al.,* 2012). Despite suppression of PO activity by symbiotic bacteria of entomopathogenic nematodes in *Plutella xylostella* (Song *et al.,* 2011; Seo *et al.,* 2012) and *Manduca sexta* (Eleftherianos *et al.,* 2007, 2010), PO activation in nematode-injected CPB larvae is expected since cellular encapsulation and capsule melanization is a common response of CPB against EPNs (Thurston *et al.,* 1994; Armer *et al.*, 2004; Ebrahimi *et al.,* 2011a, b) which implies the IJ and its symbiont may overcome the insect defense system.

The cuticular pro-phenoloxidases (proPO) are, at least for some species, derived from the pool of hemolymphal proPO (Andersen, 2010). The epidermal cells in *Bombyx mori* larvae can transport proPO from hemolymph to cuticle (Asano and Ashida, 2001a, b). The cost of production and maintenance of the PO system (including the proPO-activating system) is likely to be high because melanin production, the final product of proPO-activating system, is nitrogen-rich, requiring substantial nitrogen or protein investment for its synthesis (Blois 1978; Lee *et al.,* 2008). Producing the regulatory proteins of the proPO-activating system uses energy. The suppressive effect of immune activation on reproduction in insects is usually thought to be due to competition for resources between these two energetically expensive functions (Adamo, 2008). Perhaps, decreasing fertilized eggs in surviving adults is an indicator for such energy and nitrogen investments; egg production being dependent to proteins stored in the insect (Wheeler *et al.*, 2000).

In present study, PO specific activity of CPB against *S. carpocapsae* was generally increased

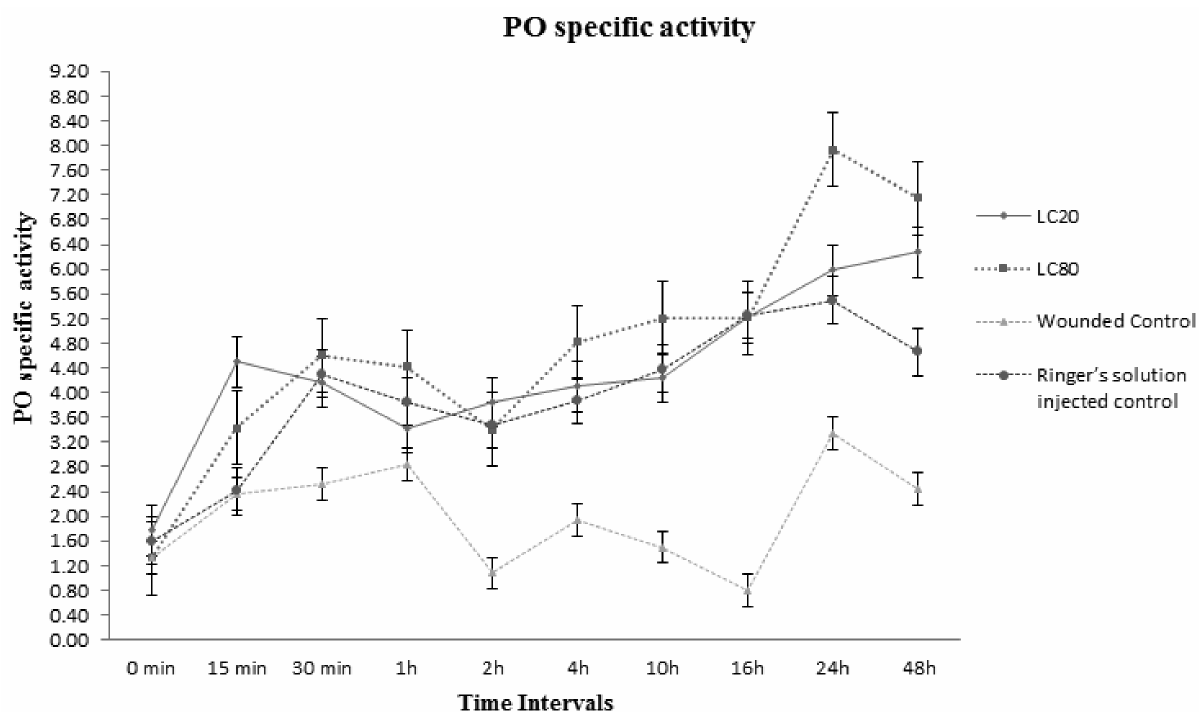

Fig. 8 Phenoloxidase specific activity of LC_{20} and LC_{80} nematode-injected, wounded and Ringer's solution injected insects in different time intervals. The results are expressed as mean ± SD. The data were statistically analyzed using ANOVA and Duncan's multiple range tests with a specified significance level of $p < 0.05$.

up to 48 h pi. PO specific activity against entomopathogenic nematodes was investigated in *G. mellonella*. Brivio *et al.* (2002) reported *Steinernema feltiae* inhibited enzyme activity until 40 min pi compared with the control larvae. The juveniles of *S. feltiae* induced speedy suppression of phenoloxidase avoiding host humoral encapsulation. Regardless of the nematode species, CPB shows effective but dose dependent immune defenses against entomopathogenic nematodes (Dunphy and Thurston 1990; Armer *et al.*, 2004; Ebrahimi *et al.,* 2011b), whereas *G. mellonella* is a susceptible host for the nematodes, so these differences are acceptable. Walter *et al.* (2008) showed that live *S. carpocapsae* axenic infective juveniles and their exudates did not activate PO in larvae of the lepidopteran *G. mellonella* and *Malacosoma disstria.* As mentioned above, probably, insecticidal proteins of symbiotic bacteria are responsible for activation of PO cascade in present study, while Walter *et al.* (2008) used axenic nematodes. Neither Brivio *et al.* (2002) nor Walter *et al.* (2008) noticed sclerotization of the infected insect's cuticles and melanin formation.

Briefly, the level of total protein in the nematode as well as Ringer's solution injected insects was found lower than wounded control. As entomopathogenic nematodes (Simões and Rosa 1996; Toubarro *et al.,* 2009) and their symbiotic bacteria (Caldas *et al.,* 2002) induce proteases production inside the infected host insects, accordingly, it can be concluded that decline of total protein in the nematode treated insects in our study is the cost of the insects response against infection, however due to dilution of the hemolymph by Ringer's solution used in the experiment, decrease in total protein in nematode or Ringer's solution injected insects compared with wounded insects is expected.

In conclusion, sublethal effects of *S. carpocapsae* on surviving adult CPB include discoloration and thinning of the cuticle in color defective adults, uniquely deformation in wings, antenna and legs, and decreasing fertilized eggs when the insects in the prepupal stage are infected with low numbers of the nematodes.

Our results showed higher PO units and PO specific activity in nematode-injected insects than controls, which demonstrate the nematodes activate PO. LC_{80} injected insects showed the highest PO specific activity due to increased nematode numbers increasing immune stimulation. Increasing symbiotic bacterial levels over the time lead to increased PO activity. On the other hand, discoloration of the IJ-infected adults was the most prominent sublethal effect. It is possible that production of the enzyme in this stage leads to its shortage for sclerotization of the adult cuticles and melanin formation which can be, at least in part, responsible for nitrogen investments and a decrease in fertilized egg production.

References

Adamo SA. Bidirectional connections between the immune system and the nervous system in insects. In: Beckage NE (ed.), Insect Immunology, Academic Press, San Diego, CA, USA, pp 129-149, 2008.

Alchanatis V, Navon A, Glazer I, Levski S. An image system for measuring insect feeding effects caused by biopesticides. J. Agr. Eng. Res. 77: 289-296, 2000.

Andersen SO. Insect cuticular sclerotization: A review. Insect Biochem. Mol. Biol. 40:166-78, 2010.

Arakane Y, Muthukrishnan S, Beeman RW, Kanost MR, Kramer KJ. Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. Proc. Natl. Acad. Sci. USA 102: 11337-11342, 2005.

Armer CA, Rao S, Berry RE. Insect cellular and chemical limitations to pathogen development: the Colorado potato beetle, the nematode *Heterorhabditis marelatus*, and its symbiotic bacteria. J. Invertebr. Pathol. 87: 114-122, 2004.

Armitage SAO, Siva-Jothy MT.Immune function responds to selection for cuticular colour in *Tenebrio molitor*. Heredity 94: 650-656, 2005.

Asano T, Ashida M. Cuticular pro-phenoloxidase of the silkworm, *Bombyx mori*: purification and demonstration of its transport from hemolymph. J. Biol. Chem. 276: 11100-11112, 2001a.

Asano T, Ashida M. Transepithelially transported pro-phenoloxidase in the cuticle of the silkworm, *Bombyx mori*. Identification of its methionyl residues oxidized to methionine sulfoxides. J. Biol. Chem. 276: 11113-11125, 2001b.

Bao HB, Liu S, Gu JH, Wang XZ, Liang XL, Liu ZW. Sublethal effects of four insecticides on the reproduction and wing formation of brown planthopper *Nilaparvata lugens*. Pest Manag. Sci. 65: 170-174, 2009.

Barnes AI, Siva-Jothy MT. Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. Proc. R. Soc. Lond. B. Biol. Sci. 267: 1-6, 2000.

Blois MS. The melanins: their synthesis and structure. In: Smith KC (ed.), Photochemical and photobiological reviews, Vol. 3, Plenum Press, New York, NY, USA, pp 115-134, 1978.

Bradford MM. A dye binding assay for protein. Anal. Biochem.72: 248-254, 1976.

Brey PT, Lee W, Yamakawa M, Koizumi Y, Perrot S, Francois M, *et al.* Role of the integument in insect immunity: epicuticular abrasion and induction of cecropin synthesis in epithelial cells. Proc. Natl. Acad. Sci. USA 90: 6275-6279, 1993.

Brivio MF, Pagani M, Restelli S. Immune suppression of *Galleria mellonella* (Insecta,Lepidoptera) humoral defenses induced by *Steinernema feltiae* (Nematoda, Rhabditida): involvementof the parasite cuticle. Exp. Parasitol. 101: 149-156, 2002.

Burnell AM, Stock SP. *Heterorhabditis*, *Steinernema* and their bacterial symbiont- lethal pathogens of insects. Nematology 2: 31-42, 2000.

Caldas C, Cherqui A, Pereira A, Simões N. Purification and characterization of an extracellular protease from *Xenorhabdus nematophila* involved in insect immunosuppression. Appl. Environ. Microbiol. 68: 1297-1304, 2002.

Calleri DV, Rosengaus RB, Traniello JFA. Disease and colony establishment in the dampwood termite Zootermopsis angusticollis: survival and fitness consequences of infection in primary reproductives. Insectes Sociaux 53: 204-211, 2006.

Castillo JC, Reynolds SE, Eleftherianos I. Insect immune responses to nematode parasites. Trends Parasitol. 27: 537-547, 2011.

Cho S, Kim Y. Hemocyte apoptosis induced by entomopathogenic bacteria, *Xenorhabdus* and *Photorhabdus*, in *Bombyx mori*. J. Asia Pac. Entomol. 7: 195-200, 2004.

Cutler GC, Tolman JH, Scott-dupree DS, Harris CR. Resistance potential of Colorado potato beetle (Coleoptera: Chrysomelidae) to novaluron. J. Econ. Entomol. 98: 1685-1693, 2005.

Dubovskiy IM, Whitten MMA, Yaroslavtseva ON, Greig C, Kryukov VY. *et al.,* Can Insects Develop Resistance to Insect Pathogenic Fungi? PLoS ONE 8(4), e60248. doi:10.1371/journal.pone.0060248, 2013.

Dunphy GB, Thurston S. Insect immunity. In: Gaugler R, Kaya HK (eds), Entomopathogenic nematodes in biological control, CRC Press, pp 301-323, 1990.

Ebrahimi L, Niknam G, Dunphy GB. Hemocyte responses of *Leptinotarsa decemlineata* and *Galleria mellonella* to the entomopathogenic nematodes, *Steinernema feltiae* and *Heterorhabditis bacteriophora*. J. Insect Sci. 11, 76 available online: insectscience.org/11.76 2011a.

Ebrahimi L, Niknam G, Lewis EE. Lethal and sublethal effects of Iranian isolates of *Steiner-nema feltiae* and *Heterorhabditis bacteriophora* on the Colorado potato beetle, *Leptinotarsa decemlineata*. BioControl 56: 781-788, 2011b.

Eleftherianos I, Boundy S, Joyce SA, Aslam S, Marshall JW, Cox RJ, *et al.* An antibiotic produce by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. Proc. Natl. Acad. Sci. USA 104: 2419-2424, 2007.

Eleftherianos I, Ffrench-Constant RH, Clarke DJ, Dowling AJ, Reynolds SE. Dissecting the immune response to the entomopathogen *Photorhabdus*. Trends Microbiol. 18: 552-560, 2010.

Girling RD, Ennis D, Dillon AB, Griffin CT. The lethal and sub-lethal consequences of entomopathogenic nematode infestation and exposure for adult pine weevils, *Hylobius abietis* (Coleoptera: Curculionidae). J. Invertebr. Pathol. 104: 195-202, 2010.

Golkar L, LeBrun RA, Ohayon H, Gounon P, Papierok B, Brey PT. Variation of larval susceptibility to *Lagenidium giganteum* in three

mosquito species. J. Invertebr. Pathol. 62: 1-8, 1993.

González-Santoyo I, Córdoba-Aguilar A. Phenoloxidase: a key component of the insect immune system. Entomol. Exp. Appl. 142: 1-16, 2012.

Hinchliffe SJ, Hares MC, Dowling AJ, Ffrench-Constant RH. Insecticidal toxins from the *Photorhabdus* and *Xenorhabdus* bacteria. Open Toxinol. J. 3: 101-118, 2010.

Hiruma K, Riddiford LM. Granular phenoloxidase involved in cuticular melanization in the tobacco hornworm: regulation of its synthesis in the epidermis by juvenile hormone. Dev. Biol. 130: 87-97, 1988.

Hitchner EM. Investigations of the integrated pest management of Colorado potato beetle, *Leptinotarsa decemlineata* (Say): host plant preference, development of semiochemical-based strategies, and evaluation of a novel insecticide, PhD thesis, Virginia University, 2007.

Hwang SY, Paik S, Park SH, Kim HS, Lee IS, Kim SP, *et al.* N-Phenethyl-2-phenylacetamide isolated from *Xenorhabdus nematophilus* induced apoptosis through caspases activation and calpain-mediated Bax cleavage in U937 cells. Int. J. Oncol. 22: 151-157, 2003**.**

Koppenhöfer AM, Grewal PS, Kaya HK. Synergism of imidacloprid and entomopathogenic nematodes against white grubs: the mechanism. Entomol. Exp. Appl. 94: 283-293, 2000.

Lawniczak MKN, Barnes AI, Linklater JR, Boone JM, Wigby S, Chapman T. Mating and immunity in invertebrates. Trends Ecol. Evolut. 22: 48-55, 2007.

Lee KP, Simpson SJ, Wilson K. Dietary protein-quality influences melanization and immune function in an insect. Funct. Ecol. 22: 1052-1061, 2008.

Milstead JE. Pathophysiological influences of the *Heterorhabditis bacteriophora* complex on fifth-Instar larvae of the Red Humped Caterpillar, *Schizura concinna*: changes in feeding rate, larval weight, and frass production. J. Invertebr. Pathol. 35: 260-264, 1980.

Pham LN, Schneider DS. Evidence for specificity and memory in the Insect innate immune response. In: Beckage NE (ed.), Insect immunology, Academic Press, San Diego, CA, USA, pp 97-128, 2008.

Reeson AF, Wilson K, Gunn A, Hails RS, Goulson D. Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. Proc. R. Soc. Lond. Ser. B Biol. Sci. 265: 1787-1791, 1998.

SAS Institute. SAS enterprise guide ver. 3.0, 2004.

Seo S, Lee S, Hong Y, Kima Y. Phospholipase A2 inhibitors synthesized by two entomopathogenic bacteria, *Xenorhabdus nematophila* and *Photorhabdus temperata* subsp. *temperata*. Appl. Environ. Microbiol. 78: 3816-3823, 2012.

Simões N, Caldas C, Rosa JS, Bonifassi E, Laumond C. Pathogenicity caused by high virulent and low virulent strains of *Steinermena carpocapsae* to *Galleria mellonella*. J. Invertebr. Pathol. 75: 47-54, 2000**.**

Simões N, Rosa JS. Pathogenicity and host specificity of entomopathogenic nematodes. Biocontrol. Sci. Tech. 6: 403-411, 1996.

Siva-Jothy MT, Moret Y, Rolff J. Insect immunity: An evolutionary ecology perspective. Adv. Insect Physiol. 32: 1-48, 2005.

Song CJ, Seo S, Shrestha S, Kim Y. Bacterial metabolites of an entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibit a catalytic activity of Phenoloxidase of the diamondback Moth, *Plutella xylostella*. J. Microbiol. Biotechnol. 21: 317-322, 2011.

Thurston GS, Yule WN, Dunphy GB. Explanations for the low susceptibility of *Leptinotarsa decemlineata* to *Steinernema carpocapsae*. Biol. Control 4: 53-58, 1994.

Toubarro D, Lucena-Robles M, Nascimento G, Costa G, Montiel R, Coelho A, *et al*. An apoptosis-inducing serine protease secreted by the entomopathogenic nematode *Steinernema carpocapsae*. Int. J. Parasitol. 39: 1319-1330, 2009.

Vidau C, Diogon M, Aufauvre J, Fontbonne R, Vigues B, *et al.* Exposure to sublethal doses of Fipronil and Thiacloprid highly increases mortality of honeybees previously infected by *Nosema ceranae*. PLoS ONE 6(6): e21550. doi:10.1371/journal.pone.0021550, 2011.

Walter NT, Dunphy GB, Mandato CA. *Steinernema carpocapsae* DD136: metabolites limit the non-self-adhesion responses of hemocytes of two lepidopteran larvae, *Galleria mellonella* (F. Pyralidae) and *Malacosoma disstria* (F. Lasiocampidae). Exp. Parasitol. 120: 161-174, 2008.

Wheeler DE, Tuchinskaya I, Buck NA, Tabashnik BE. Hexameric storage proteins during metamorphosis and egg production in the diamondback moth, *Plutella xylostella* (Lepidoptera). J. Insect Physiol. 46: 951-958, 2000.

Wilson K, Cotter SC, Reeson AF, Pell JK. Melanism and disease resistance in insects. Ecol. Lett. 4: 637-649, 2001.

Woodring JL, Kaya HK. Steinernematid and Heterorhabditid nematodes: a handbook of biology and techniques. Southern cooperative series bulletin 331. Arkansas Agricultural Experiment Station, Fayetteville, 1988.

Yang J, Zeng HM, Lin HF, Yang XF, Liu Z, Guo LH, *et al.* An insecticidal protein from *Xenorhabdus budapestensis* that results in prophenoloxidase activation in the wax moth, *Galleria mellonella*. J. Invertebr. Pathol. 110: 60-67, 2012.