Ebrahimi et al. Egyptian Journal of Biological Pest Control (2018) 28:12
DOI 10.1186/s41938-017-0016-x

Egyptian Journal of Biological Pest Control

RESEARCH Open Access

Effect of entomopathogenic nematode, *Steinernema feltiae*, on survival and plasma phenoloxidase activity of *Helicoverpa armigera* (Hb) (Lepidoptera: Noctuidae) in laboratory conditions

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Abstract

Lethal effect of the entomopathogenic nematode, *Steinernema feltiae*, against prepupae of *Helicoverpa armigera* (Hb) (Lepidoptera: Noctuidae) in soil was evaluated. Plasma phenoloxidase activity of *H. armigera* prepupae against *S. feltiae* also was evaluated. In brief, 20–25 infective juveniles (IJs) of *S. feltiae* in 20 μl Ringer's solution were injected into the hemolymph of *H. armigera* prepupae in different time intervals. In order to evaluate the effect of the Ringer's solution, it was also injected into the larvae in a separate experiment as positive control. A third group of non-injected insects was used as negative control. Hemolymph of the insects was collected in different time intervals (0 to 24 h post injection). Phenoloxidase (PO) activity of *H. armigera* hemolymph was determined spectrophotometrically, using L-Dopa as substrate. The LC_{20}, LC_{50}, and LC_{80} values were 4.5, 19, and 76 IJs per insect, respectively. PO activity assay demonstrated higher levels of PO unit in nematode-injected insects compared to control groups. Nematode-injected insects showed the highest plasma PO activity 8 h post-injection.

Keywords: Cotton bollworm, Immunity, LC_{50}, Phenoloxidase

Background

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a polyphagous pest which attacks numerous crops, including cotton, soybean, corn, tomato, sorghum, and groundnuts (Fitt 1989). This pest is one of the most significant agricultural pests in worldwide (Tay et al. 2013). The main approach for controlling *H. armigera* rely on chemical pesticides, and outbreaks of this pest are partly due to the development of resistance to commonly used insecticides (Torres-Vila et al. 2003).

Entomopathogenic nematodes (EPNs) (Rhabditida: Heterorhabditidae and Steinernematidae) are generalist pathogens of insects, present in soils of many ecosystems around the world. Their symbiotic bacteria which have the key role in their pathogenicity belonging to the genera *Photorhabdus* and *Xenorhabdus* respectively, are vectored into insect s by the infection stage juveniles, respectively (Dunphy and Hulbert 1995; da Silva et al. 2000; Dowds and Peters 2002). EPNs can be effective tools to manage the insect pests especially that are spending a part of their life cycle inside the soil or cryptic habitats (Lacey and Georgis 2012).

Innate immune response of insects is traditionally divided into two main group factors including the following: (i) humoral factors (i.e., melanization, clotting of the hemolymph, and the synthesis of antimicrobial peptides) and (ii) cellular defense reactions (i.e., nodule formation, phagocytosis, or encapsulation by hemocytes) (Vilmos and Kurucz 1998). Many humoral factors regulate hemocyte activity, and hemocytes are the main sources of many humoral defense molecules (Strand 2008).

Prophenoloxidases (proPO) are the inactive form of phenoloxidases (PO). PO is synthesized primarily by hemocytes of the insects (Kanost and Gorman 2008).

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Activation of prophenoloxidases occur immediately against invading microbes in the hemolymph of insects. Insect phenoloxidases play a key role in initiating the humoral and cellular immune reaction against pathogen and parasites (Marmaras et al. 1996). During melanogenesis, the main role of PO is converting phenols to quinones, which subsequently polymerized to form melanin and generating toxic radicals (Shelby and Popham 2006; González-Santoyo and Córdoba-Aguilar 2012).

In the present study, susceptibility and PO activity of *H. armigera* prepupae against S. *feltiae* was investigated. Due to the life cycle of *H. armigera* which spent the prepupa and pupa stages inside the soil, therefore using EPNs as a part of its integrated management program can be promising, a little information about plasma PO activity of *H. armigera* and the effect of nematode infection on it.

Materials and methods

Lethal effect of *S. feltiae* against *H. armigera*

Nematode

Steinernema feltiae was collected from soil of Tabriz, Iran. Nematodes were cultured using the last instar greater wax moth larvae, *Galleria mellonella* (Woodring and Kaya, 1988). Infective juveniles were stored in distilled water at 5 °C and used in all the experiments within 30 days of emerging from the host. Before use, the nematodes were kept at 25 °C for 20–30 min for acclimatization.

Insects

Helicoverpa armigera

H. armigera eggs and larvae were collected from soybean fields of Moghan, Iran. The stock culture was established using soybean fresh leaves in the laboratory at 26 ± 2 °C, 50 ± 5% RH and 16:8 (L: D) photoperiod.

Galleria mellonella

G. mellonella were reared on a modified artificial diet of Poinar Jr (1975) and incubated at 26 ± 2 °C, 50 ± 5% RH and 16:8 (L: D) photoperiod.

Bioassay

Late prepupae of *H. armigera* which stopped their feeding were used in bioassay experiment. The experiment was conducted in cylindrical plastic boxes (3-cm diameter and 5-cm height) which were filled with 15-g autoclaved sandy soil. Based on preliminary experiments, range of the appropriate nematode concentration which causes approximately 20 to 80% mortality was determined. Then, different nematode concentrations (i.e., 10, 20, 45, 95, and 200 infective juveniles (IJs) per larva) were added on to the surface of the wetted soil. In brief, 0.5 ml of distilled water was added to the soil; then, the nematode concentration was added in 1-ml distilled water. One prepupa of *H. armigera* was placed on the soil, and the boxes were covered with ventilated lids to avoid desiccation. Control boxes received 1.5-ml distilled water without nematode. Each nematode concentration and control was replicated three times, and insect mortality was recorded after 96 h. The boxes were incubated at 26 ± 2 °C, 50 ± 5% RH and 16:8 (L: D) photoperiod.

Plasma penoloxidase activity assay

Infective juveniles (20–25 IJs in 20 μl of Ringer's solution) of *S. feltiae* were injected into each of 50 larvae of *H. armigera*. The control groups consisted of 50 insects without injection and larvae injected with 20 μl of Ringer's solution. Prior to bleeding, insects were disinfected for 2 min in 70% ethanol and then placed on ice for 2–5 min. Five larvae were bled once at designated time intervals (0 min–48 h pi). Hemolymph samples were centrifuged at 4 °C and 12,000*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 (at an optical density (OD) wave length of 490 nm) (Biochrom WPA Biowave S2100 Diode Array Spectrophotometer) in samples incubated at 35 °C for 5 min in a water bath (Ebrahimi et al. 2014b). There were three replicates for each sample. The experiment was repeated twice.

Statistical analysis

LC_{20}, LC_{50}, and LC_{80} values were obtained by Probit analysis using SAS software (SAS Institute 2004). Analysis of variance was done, and the means were evaluated by Duncan's multiple-range test (SAS Institute 2004). Lethal experimental data was transformed into square root of $(x + 1)$ where needed, before analysis.

Results and discussion

Lethal effect

Regression analysis revealed a significant correlation between log dose and insect mortality ($P < 0.05$). Prepupae of *H. armigera* were found susceptible to infection by Iranian isolate of *S. feltiae*. The LC_{20}, LC_{50}, and LC_{80} values for *S. feltiae* on *H. armigera* were 4.5, 19, and 76 IJs per insect, respectively (Table 1). Because goodness-of-fit chi-square value was not significant for probit

Table 1 LC_{20}, LC_{50}, and LC_{80} values for *Steinernema feltiae* against *Helicoverpa armigera*

Slope ± SE	Chi-square	LC_{20}^{a} (95% CL)	LC_{50} (95% CL[b])	LC_{80} (95% CL)	R^2	*N*
1.37 ± 0.21	2.62	4.5 (2–8)	19 (12–26)	76 (72–196)	0.94	225

[a]LC values are based on IJs per insect
[b]Confidence limit

Table 2 Phenoloxidase units of injected nematode, Ringer's solution as compared to those of non-injected insects at different time intervals

Time intervals	Nematode-injected insects	Ringer's solution injected insects	Non-injected insects
15 min pi	0.093 ± 0.002^{g}	0.127 ± 0.001^{cd}	0.107 ± 0.001^{a}
30 min pi	0.198 ± 0.013^{d}	0.110 ± 0.008^{ef}	0.105 ± 0.001^{a}
1 h pi	0.123 ± 0.001^{e}	0.128 ± 0.005^{cd}	0.105 ± 0.001^{a}
1.5 h pi	0.067 ± 0.002^{h}	0.146 ± 0.013^{b}	0.107 ± 0.001^{a}
2 h pi	0.105 ± 0.006^{fg}	0.095 ± 0.006^{fg}	0.105 ± 0.000^{a}
4 h pi	0.119 ± 0.012^{ef}	0.084 ± 0.006^{g}	0.107 ± 0.001^{a}
6 h pi	0.119 ± 0.001^{ef}	0.138 ± 0.003^{bc}	0.104 ± 0.001^{a}
8 h pi	0.271 ± 0.026^{c}	0.180 ± 0.000^{a}	0.106 ± 0.001^{a}
10 h pi	0.299 ± 0.012^{b}	0.087 ± 0.009^{g}	0.104 ± 0.003^{a}
16 h pi	0.340 ± 0.009^{a}	0.093 ± 0.003^{g}	0.107 ± 0.000^{a}
24 h pi	0.298 ± 0.006^{b}	0.120 ± 0.002^{de}	0.103 ± 0.001^{a}

In each column, similar lowercase letters in the same column are not significant ($P < 0.05$)

analysis, no heterogeneity factor was used in the calculation of confidence limits and LC_{20}, LC_{50}, and LC_{80} values.

Pathogenicity of different isolations and strains of EPNs against *H. armigera* were documented before (Glazer and Navon 1990; Glazer 1997; Navon et al. 2002; Kalia et al. 2014). The result of lethal experiment showed high susceptibility of *H. armigera* prepupae to *S. feltiae* which is agreed with the previous studies (Glazer and Navon 1990; Glazer 1997). Glazer and Navon (1990) reported a LC_{50} value equal to 54 IJs per insects for *S. feltiae* larvae. The present results with prepupae show either a greater virulence by the Iranian strain or increased pupal susceptibility, which lacking larval mobility and grooming behaviors, would be more easily infected by the IJs.

Plasma phenoloxidase activity

Variance analysis of plasma PO activity data showed significant difference between nematode injected and both control groups (F value = 628.36; df = 2; $P < 0.01$). Plasma PO activity was 0.184 ± 0.002 (OD/min) for nematode-injected insects while it was 0.119 ± 0.001 (OD/min) and 0.105 ± 0.00 (OD/min) for non-injected and Ringer's solution-injected control groups, respectively. All three insect groups showed significantly different PO activity from each other.

Po activity was significantly different among different time intervals (Table 2). PO was the lowest amount in 15 min pi which was increased significantly to the highest values until 16 h pi in nematode-injected insects. Subsequently, PO was decreased 24 h pi and insect death occurred. In Ringer's solution-injected control group, there was no regular trend in amount of PO during the time intervals. However, PO was the highest in 8 h pi and the lowest amount was recorded 10 h pi and 16 h pi. There were no significant differences between time intervals in non-injected control group (Table 2).

A few studies investigated the infection of EPNs on PO activity of host insects (Brivio et al. 2002; Walter et al. 2008; Ebrahimi et al. 2014a, 2014b; Kramarz et al. 2016). PO is the key component of insect humeral immune system. PO activation occurs upon different pathogens such as entomopathogenic nematode invasion in insect hemolymph. However, Brivio et al. (2002) reported *S. feltiae* inhibited PO activity of *G. mellonella* until 40 min pi compared to control larvae. They showed that the juveniles of *S. feltiae* induced the speedy suppression of phenoloxidase tend to avoid host humoral encapsulation. In overall, lepidopterans are susceptible to EPNs and *G. mellonella* is used as laboratory susceptible host for them. The results of PO activity assay demonstrated higher levels of PO unit in nematode-injected insect compared to both control groups (Table 2), which suggested *H. armigera* PO activation by nematode infection. Releasing the symbiotic bacteria and increasing the bacterial cells in hemolymph of *H. armigera* during the experimental time interval were reasonable explanation for increasing trend of PO activity in nematode-injected insects. While it is possible the nematode alone activated the enzyme, a GroEL-like toxin from *Xenorhabdus budapestensis* which activates PO in *G. mellonella* larvae. Yang et al. (2012) implies in *H. armigera*, *X. nematophilus* complex to activate the enzyme.

Conclusions

In conclusion, prepupae of H. armigera were found susceptible to infection by Iranian isolate of *S. feltiae*. Variance analysis of Plasma PO activity data showed

significant difference between nematode injected and both control groups. PO activation occurs upon different pathogens such as entonopatogenic nematodes invasion in insect hemolymph. The results showed *H. armigera* PO activation by nematode infection.The results of PO activity assay demonstrated higher levels of PO unit in nematode injected insect compared with both control groups, suggesting H. armigera PO activation by nematode infection.

Authors' contributions

L.Ebrahimi carried out the experiments. M.R. Shiri participated in the design of the study and performed the statistical analysis. L. Ebrahimi and G.B. Dunphy designated the study and conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Received: 17 June 2017 Accepted: 6 December 2017
Published online: 15 February 2018

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