ISJ 14: 73-84, 2017 ISSN 1824-307X

RESEARCH REPORT

Immune defense of *Pieris brassicae* larvae in challenged with *Heterorhabditis bacteriophora*, its symbiotic bacteria and metabolites

A Abdolmaleki[1], ZT Maafi[2], HR Dastjerdi[1], B Naseri[1], A Ghasemi[2]

[1]Department of Entomology, Agricultural Sciences Faculty, University of Mohaghegh Ardabili, Tehran, Iran
[2]Iranian Research Institute of Plant Protection, Agricultural Research Education and Extension Organization (AREEO), Tehran, Iran

Accepted March 23, 2017

Abstract

In this study, virulence of the culture broth of *Photorhabdus temperata* subsp. *temperata* and its aqueous and organic extracts were investigated for 3rd and 4th instars of *Pieris brassicae* (Lepidoptera: Pieridae) larvae. Virulent efficacy was found in all the treatments. However, the culture broth of bacteria showed greater mortality effect. Also, this study presents the response of *P. brassicae* immune system factors, phenoloxidase, lysozyme and hemocytes, to infection by *Heterorhabditis bacteriophora*, its symbiotic bacteria and their aqueous and organic extracts. Results showed that after injecting infective juveniles (IJs) with phenoloxidase, specific activity was suppressed after primary increase. However, the phenoloxidase specific activity suppression was observed earlier after injecting *P. temperata* subsp. *temperata*, aqueous and organic extracts. Also, results indicate more phenoloxidase activity of 4th instars larvae in comparison with 3rd instars larvae, which can show higher activity of phenoloxidase in the 4th larval stage. After injecting IJs into hemocel, the total hemocyte count was increased until five h post-injection and then decreased at seven hours post-injection. Differential Hemocyte Count (DHC) indicated granolucytes and plasmocytes as frequent hemocytes after treatment. Lysozyme consideration showed an increase after injecting living and heat-killed bacteria into hemocel.

Key Words: hemocyte; lysozyme; phenoloxidase; entomopathogenic nematode; *Photorhabdus temperata subsp. temperata*

Introduction

Pieris brassicae (Lepidoptera: Pieridae) is one of the most important pests of plants from the family brassicaceae. This pest has five larval stages of which the last two inflict the most damage on plants. Currently, the most reliable management of this pest is provided by chemical insecticides (Cartea *et al*., 2009). However, residues along with potential resistance development encourage the development of alternative measures. Biological control-as a way to control pests like *P. brassicae*-has been taken into consideration (Abdolmaleki *et al*., 2015).

Entomopathogenic nematodes (EPNs) are an efficient alternative to chemical pesticides. EPNs are from two families, Heterorhabditidae and Steinernematidae, including two genera, *Heterorhabditis* and *Steinernema* respectively. EPNs have a symbiotic relationship with bacteria from the Enterobacteriaceae family. Genera *Heterorhabditis* and *Steinernema* have a symbiotic relationship associated with bacteria from genera *Photorhabdus* and *Xenorabdus*, respectively (Ciche and Ensign, 2003; Martens *et al*., 2003).

The infective juveniles (IJs) actively seek out insect hosts and penetrate the insect's body, usually via natural openings. The symbiotic bacteria are released from the IJs into the hemocel. They proliferate and produce some metabolites causing septicaemia, which kills the host (Gaugler, 2002). Symbiotic bacteria produce antimicrobial metabolites, which help the EPNs proliferate and grow under monogenic condition and suppress microbial growth of secondary infections (Webster *et al*., 1998).

These IJs invade the hemocel. The symbiotic bacteria are then released from the nematode's gut and cause septicaemia, which kills the host (Akhurst, 1983; Lewis *et al.* 1993; Forst and Clarke,

Corresponding author:
Arman Abdolmaleki
Department of Entomology
Agricultural Sciences Faculty
University of Mohaghegh Ardabili, Tehran, Iran
E-mail: arman.abdolmaleki@uma.ac.ir

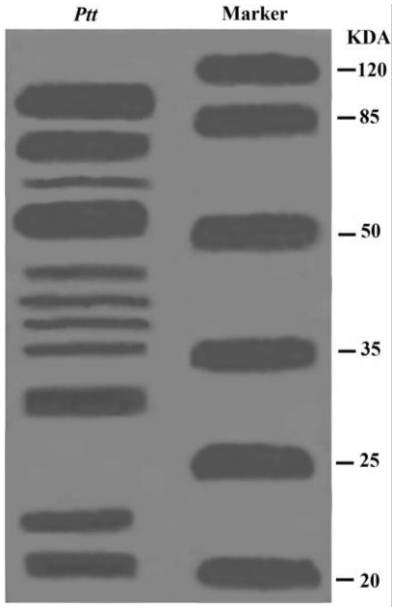

Fig. 1 Analysis of proteins in aqueous extracts of 48 h culture broths of *Ptt*.

2002). No adverse effects of EPNs have been proven on non-target insects, viz., predators and parasitoids (Mbata and Shapiro, 2010). *Heterorhabditis bacteriophora* is one of the most frequent and important EPNs in controlling insect pests. Virulence effect of this EPN was earlier approved in erstwhile studies on *P. brassicae* (Abdolmaleki *et al*., 2015).

Insects have varying immune strategies against pathogens and invaders. These strategies, including integument, cellular immune system, *e.g.,* hemocytes and phagocytes, and humeral immune system, viz., phenoloxidase and lysozyme, are mainly responsible for keeping insects safe. An important step for successful infection by a biological control agent and killing of the host is to overcome these insect immune strategies (Grewal *et al*., 2005; Beckage, 2008). Thus, considering virulence of biological control factors without investigating the response of the immunity system of the host seems irrational.

Several studies have been undertaken on the response of pests to EPNs and their symbiont bacteria. Several studies showed that infection of insects by EPNs cause hemocyte responses (Thurston *et al*., 1994; Wang *et al*., 1994, 1995; Steiner, 1996; Armer *et al*., 2004); Some indicated that EPNs and their symbiont bacteria are replete with different factors which they can suppress phenoloxidase activity and hemocytes (Dunphy and Webster, 1987; Yokoo *et al*., 1992; Brivio *et al*., 2002; Walter *et al*., 2008). Till date, different toxins, metabolites, and factors have been identified, which can have a suppressive effect on the host's immune systems and help EPNs kill the host (Kim *et al*., 2005; Balasubramanian *et al*., 2009).

To our knowledge, no study has been performed on the effect of *H. bacteriophora*, its symbiont bacteria, *Photorhabdus temperata* subsp. *temperata* (*Ptt*), and aqueous and organic extracts of this bacterium on *P. brassicae* immune system factors. The major purpose of this study was to approach a comprehensive view on the role of *P. brassicae* immunity system challenged with the EPN species, *H. bacteriophora*. We selected this EPN due to its potential control of the cabbage butterfly. In this study, total hemocyte counts, differential hemocyte counts, phenoloxidase activity, and lysozyme concentration were measured in response to infection *H. bacteriophora*, living and heat-killed symbiont bacteria of *H. bacteriophora*, *Ptt*, and its aqueous and organic extracts.

Material and Methods

Preparation experimented insect

Pieris brassicae eggs were collected from the cabbage fields of Urmia (West Azerbaijan province, Iran). The larvae were fed fresh cabbage leaves cultivated in a research plot located at the Iranian Research Institute of Plant Protection, Tehran, Iran, until the 3rd and 4th instars larvae appeared. The cabbages received no doses of chemical or even biological insecticides.

Preparation of EPN and bacteria

Heterorhabditis bacteriophora strain HBIRI1, used in the experiments, was from the soils of Kurdistan province, Iran (Abdolmaleki *et al*., 2016). The nematode was maintained in the laboratory by passing through larval *Galleria mellonella*. Symbiotic bacteria, *Ptt*, strain PTIRI6 were isolated from surface-sterilized IJs of *H. bacteriophora* strain HBIRI1 (Abdolmaleki *et al*., 2016).

Preparation of bacterial metabolites

Aqueous and organic extracts of *Ptt* were prepared as per Shrestha and Kim (2010), with

slight modifications. The bacteria were cultured on LB (liquid broth) for 48 h. The culture broth was collected and for 20 min centrifuged at 7,000 rpm. The supernatant was collected and mixed with the same volume of hexane in a new tube and incubated at room temperature. After two h of incubation, the hexane fraction was collected and dried under N_2 gas and then re-suspended with dimethylsulfoxide (DMSO) to form an organic extract. The remaining culture broth was used as an aqueous extract. Aqueous and organic extracts were sterilized through filtering by a 0.22 µm pore size membrane and stored in a refrigerator at 4 °C until use.

Insecticidal activity

Bioassays were performed on the 3rd and 4th instars larvae of *P. brassicae* by injecting freshly cultured *Ptt*, and its aqueous and organic extracts into haemocoel. Ten µl (10^4 cell ml^{-1}) of *Ptt* and its metabolites were injected through proleg by a sterilized 25 µl Hamilton syringe (Hamilton Co., Reno, Nev.). Before injecting, the surface of the larvae was disinfected by immersing them in 1 % NaOH for 10 s and then rinsed with distilled water. Experiments were carried out at three replications and included 15 larvae. Median lethal times (LT_{50}) were calculated by counting dead larvae every 24 h for four days post-injection. After each injection, the syringe was rinsed three times with ddH_2O, three times with 70 % ethanol, and then three times again with sterile ddH_2O. Ten microliters of LB diluted in saline and LB were injected into hemocel through proleg using sterilized 25 µl Hamilton syringe as control for living bacteria, aqueous extract, respectively. However, ten microliters DMSO was used as organic extract.

Expression of bacterial protein extract

Aqueous extract was analysed by using 10 % sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (Hames and Rickwood, 1981). Proteins were stained with Coomassie brilliant blue as per the manufacturer's instructions.

Phenoloxidase activity measurement

Phenoloxidase activity and phenoloxidase specific activity of 3rd and 4th instars larvae of *P. brassicae* were considered when they were challenged by *H. bacteriophora*, living and heat-killed *Ptt*, aqueous and organic extracts of *Ptt* after one, three, six, 12, and 24 h after injection. In toto, 10 IJs per larvae, 10 µl of alive and heat-killed (10^4 cell ml^{-1}) *Ptt*, and 10 µl of aqueous and organic extracts were injected into hemocel of 3rd and 4th larval instars of *P. brassicae* through proleg. After injection, the larvae were reared under the same conditions and their behaviours and mortality were recorded. EPN injection was performed by insulin syringe; however, others were injected by Hamilton syringe. After each injection, the Hamilton syringe was rinsed three times with ddH_2O, three times with 70 % ethanol, and then three times again with sterile ddH_2O. However, insulin syringe was changed after each treatment and new one was used. As control, 10 µl of PBS was injected into hemolymph for EPN. However, 10 µl of LB mixed in saline was as control for living and heat-killed *Ptt* experiments. However, 10 µl of LB and DMSO were respectively injected as control for aqueous and organic extracts. Heat-killed *Ptt* was obtained by heating at 90 ºC for 30 min in a shaking water bath. The kill treatment was confirmed by plating 200 µl aliquots of the *Ptt* suppression on NBTA and incubating the plates for 48 h. No bacterial colony was observed on these plates. Experiments were replicated three times.

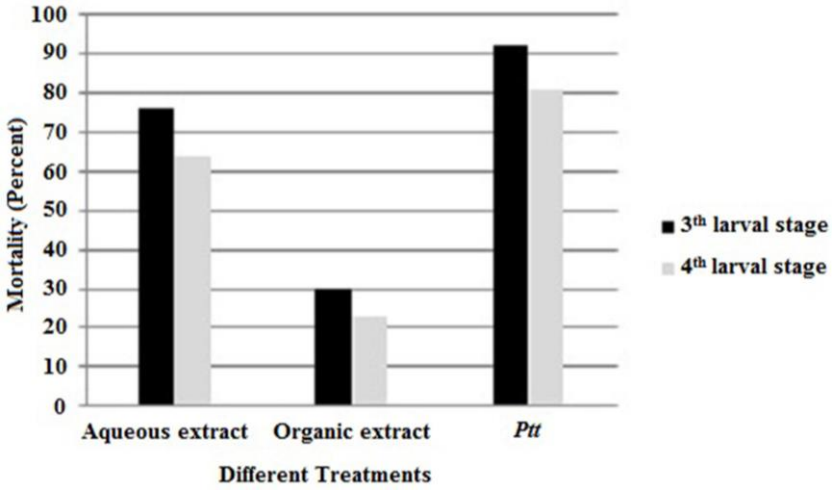

Fig. 2 Pathogenicity of *Ptt* and its aqueous and organic extracts on 3rd and 4th larval stages of *P. brasiicae.*

Table 1 Pathogenicity of *Ptt* and its aqueous and organic extracts against late 3rd and 4th instars larvae of *Pieris brassicae* by hemocelic injection.

Treatment	Larval stage	LT_{50}	Slope±SE	χ^{2a}	df
Ptt	3	38.84 (9.08-61-68)	3.63±0.51	3.85	2
	4	44.73 (38.08-51.24)	3.51±0.49	0.11	2
Aqueous extract	3	43.32 (37.55-48.96)	4.07±0.53	1.83	2
	4	53.86 (45.63-63.30)	2.94±0.48	1.65	2
Organic extract	3	49.89 (43.22-56.88)	3.64±0.51	2.88	2
	4	57.58 (49.61-67.08)	3.21±0.50	1.05	2

a: Pearson χ^2 of the slope.

Total protein determination

Protein concentrations were measured by using bovine serum albumin as a standard, in accordance with the method of Bradford (1976). Protein concentrations of 3rd and 4th larval instars of *P. brassicae* were measured after one, three, six, 12, and 24 h after injection by *H. bacteriophora*, living and heat-killed *Ptt*, aqueous, and organic extracts of *Ptt*. Experiments were replicated three times.

Lysozyme activity measurement

Lysozyme assay was performed in accordance with Hernández-Martínez (2010), with a little modification. Lysozyme concentrations of 4th instars larvae of *P. brassicae* were measured when they were challenged with living and heat-killed *Ptt*. For this approach, 10 µl of living and heat-killed (10^4 cell ml^{-1}) *Ptt* were injected into the hemocel through proleg by Hamilton syringe. After each injection, the syringe was rinsed three times with sterile ddH_2O, three times with 70 % ethanol, and finally three times again with sterile ddH_2O. For assessing lysozyme concentration in different treatments, 1 ml hemolymph was obtained by puncturing larval proleg. Petri dishes (diameter 8 cm, depth 1 cm) were filled with 20 ml phosphate buffer saline (PBS) containing 1 mg ml^{-1} of *Micrococcus luteus* Cohn (Schroeter) ATCC No. 4698 (Sigma Co., USA), with a final concentration of 1.5 % agar. After solidification of agar, the hemolymph was individually placed on it and incubated at 37 °C for 24 h. In each Petri dish, 10 samples were assessed. For the consistency of the experiments, different dilutions of standard lysozyme (0.3, 01, 0.03, and 0.01 mg ml^{-1}) (Roche Diagnostics SL, San Cugat del Vallés) were used as a control. Lytic activity in the hemolymph was estimated as the lytic zone diameter around the sample. Based on the halo diameters around the samples, the larvae were categorized into three groups ranging from 0 to 5 mm, from 5 to 10 mm, and from 10 to 15 mm. Experiments were replicated 20 times.

Hematology

For considering total hemocyte counts (THC) and DHC, 10 IJs by insulin syringe were injected into hemolymph of 3rd and 4th of *P. brassicae* through their prolegs. After one, three, six, 12, and 24 post-injection as interval times, hemolymph was collected by cutting the proleg. Hemolymph (1 µl) was diluted with anticoagulant buffer (1 g of ascorbic acid, 2 g of citric acid in 100 ml of Ringer's solution). The THC was determined by placing the diluted hemolymph on a Neubauer hemocytometer under a light microscope and reported as the total number of hemocytes per ml. For DHC, hemocytes monolayer was prepared and stained with 10 % Giemsa solution and examined by oil immersion light microscopy. In toto, 200 cells were counted randomly for each slide and hemocyte types were evaluated as a percentage of total cells (Gupta, 1979). After each treatment insulin syringe was changed and new one was used.

Statistical analysis

Probit programme was used to calculate LT_{50} (Raymond, 1985). Also, all means and variances of the treatments were analysed by one-way ANOVA using SPSS software Version 19 (IBM SPSS, 2010). All means were compared by Tukey's test (HSD) and it was used to determine significant differences among the treatments at the 0.05 level. The number of each hemocyte type number was reported as mean ± standard error (SE).

Results

Expression of bacterial protein extract

Figure 1 shows proteins detected in aqueous extract of *Ptt*.

Insecticidal activity

Pathogenicity of Ptt was investigated and reported in Figure 2 and Table 1. LT_{50} values show more efficacy of *Ptt* than its aqueous and organic extracts against 3rd and 4th larval instars of *P.*

Table 2 Total protein, phenoloxidase activity and phenoloxidase specific activity in *Pieris brassicae* when challenged with *Heterorhabditis bacteriophora*

Treatment	Larval Stage	Time (h)	Total Protein (mg)±SE	Total Activity (U)±SE	Specific Activity (U/mg)±SE
H. bacteriophora	3	1	16.30±0.29	13.09±0.43	0.99±0.00
		3	0.76±0.02	1.33±0.03	3.65±0.26
		6	0.77±0.08	1.90±0.07	1.93±0.09
		24	2.62±0.05	3.06±0.07	1.35±0.01
Control		1	0.46±0.04	1.42±0.04	3.09±0.20
		3	0.14±0.01	1.12±0.01	8.11±0.72
		6	0.37±0.11	1.33±0.09	4.40±1.39
		24	1.37±0.06	2.25±0.06	1.64±0.03
H. bacteriophora	4	1	16.30±0.29	15.90±0.27	0.97±0.00
		3	0.76±0.02	1.69±0.02	2.23±0.04
		6	0.77±0.08	1.69±0.07	2.24±0.15
		24	2.62±0.05	3.39±0.04	1.29±0.01
Control		1	1.99±0.03	2.81±0.02	1.41±0.01
		3	0.14±0.03	1.12±0.02	8.51±1.46
		6	0.13±0.01	1.11±0.01	8.56±0.29
		24	1.02±0.06	1.92±0.05	1.89±0.06

brassicae. The lowest LT_{50} was obtained from experiments carried out by organic extract. In comparison with two larval stages, more LT_{50} values were obtained in 4th instars. Mortality percentages of 3rd $F_{2, 6} = 127.73$, $p < 0.05$) and 4th ($F_{2, 6} = 89.89$, $p < 0.05$) larval instars treated by *Ptt* and its aqueous and organic extract show more lethality of bacteria than two extracts. In all experiments, 4th instars larvae proved to be more resistant than 3rd instars (Table 1, Fig. 2).

Phenoloxidase activity, phenoloxidase specific activity, and total protein determination

Results show an increase in phenoloxidase until three h after injection. Then, a decrease in specific activity was observed from six h post-injection of *H. bacteriophora* into the hemocel of larvae of *P. brassicae*. This increase and inhibition of phenoloxidase was observed in both treated larval instars of *P. brassicae*.

Comparison between specific activities of phenoloxidase of treated larvae by *H. bacteriophora* with control shows significant differences ($F_{2, 32} = 56.61$, $p < 0.001$). Also, significant differences were observed among specific activities of phenoloxidase in different times after injecting *H. bacteriophora* ($F_{3, 32} = 54.33$, $p < 0.001$) (Table 2).

Phenoloxidase specific activity in 3rd and 4th larval stages treated with *Ptt* showed the same pattern as when treated with *H. bacteriophora,* albeit with some differences. In both treatments by living and heat-killed bacteria, inhibition was observed in specific activity of *P. brassicae* larvae. In comparison with *H. bacteriophora* treatments, preliminary increase of phenoloxidase specific activity was not demonstrated when larvae were treated by the bacteria. Results showed significant differences among phenoloxidase specific activities in treated larvae by living and heat-dead bacteria and control ($F_{4, 48} = 67.77$, $p < 0.001$). However, comparison of phenoloxidase specific activities showed significant differences in different interval times ($F_{3, 48} = 45.96$, $p < 0.001$) (Table 3).

Suppression effect pattern of aqueous and organic extracts on phenoloxidase specific activities were similar to the pattern when the larvae were injected by the bacteria. These results show significant differences among different treatments ($F_{6, 64} = 47.61$, $p < 0.05$). Also, Results showed significant differences among phenoloxidase specific activities among different interval times ($F_{3, 64} = 15.34$, $p < 0.001$) (Table 4).

Lysozyme activity

Determination of the lysozyme activity of 4th instars larvae of *P. brassicae*, when challenged with living and heat-killed *Ptt*, showed lyctic activity. Results of lyctic activity were reported in two ways-lyctic zone diameters and lysozyme concentrations. Lyctic zone diameters showed that by passing post-injection time frequency of samples with high diameter zone increased (Fig. 3). Results of treated larvae with living and heat-killed bacteria showed significantly higher lysozyme activity than controls ($F_{2, 171} = 84.94$, $p < 0.001$). Also, results show non-significant differences between lysozyme activities of larvae treated by living and heat-killed bacteria. However, significant differences were demonstrated among results of different interval times ($F_{2, 171} = 45.46$, $p < 0.001$) (Table 5).

Table 3 Total protein, phenoloxidase activity and phenoloxidase specific activity in *Pieris brassicae* when challenged with living and heat-killed *Ptt*

Treatment	Larval Stage	Time (h)	Total Protein (mg)±SE	Total Activity (U)±SE	Specific Activity (U/mg)±SE
Living *Ptt*	3	1	1.89±0.06	2.72±0.05	1.44±0.02
		3	6.20±0.27	6.67±0.25	1.07±0.01
		6	8.75±0.17	8.99±0.15	1.03±0.00
		24	12.43±0.03	12.36±0.02	0.99±0.00
Heat-Killed *Ptt*		1	0.68±0.04	1.62±0.03	2.37±0.08
		3	0.37±0.02	1.33±0.02	3.60±0.14
		6	0.59±0.09	1.53±0.09	2.72±0.35
		24	1.60±0.05	2.46±0.05	1.54±0.02
Control		1	0.46±0.04	1.42±0.04	3.09±0.20
		3	0.14±0.01	1.12±0.01	8.11±0.72
		6	0.37±0.11	1.33±0.09	4.40±1.39
		24	1.37±0.06	2.25±0.10	1.64±0.03
Living *Ptt*	4	1	2.13±0.05	2.94±0.05	1.38±0.01
		3	6.44±0.13	6.88±0.12	1.07±0.00
		6	8.98±0.17	9.20±0.15	1.02±0.00
		24	12.66±0.02	12.57±0.02	0.99±0.00
Heat-Killed *Ptt*		1	0.92±0.04	1.84±0.04	1.99±0.05
		3	0.62±0.02	1.56±0.02	2.53±0.06
		6	0.85±0.01	1.77±0.01	2.09±0.02
		24	1.86±0.01	2.69±0.01	1.45±0.01
Control		1	1.99±0.03	2.81±0.03	1.41±0.01
		3	0.14±0.03	1.12±0.03	8.51±0.46
		6	0.13±0.01	1.11±0.00	8.56±0.29
		24	1.02±0.09	1.93±0.05	1.89±0.06

Hematology

THC values showed an increase early after injecting IJs into hemocel. *P. brassicae* larvae, challenged with IJs of *H. bacteriophora*, showed significant THC differences in different times post-injection (F). THC values showed an increase until five h post-injection, though seven h post-injection showed a decrease (Fig. 4). Investigation by DHC showed that more frequent hemocytes were granulocytes and plasmocytes. Results of granulocyte counts show an increase through post-injection times and significantly count more than control (F_7 = 113.05, p < 0.001). Plasmocytes showed a gradual increase in passing time and showed significant differences with results of control (F_7 = 372.31, p < 0.001) (Fig. 5).

Discussion

Results of LC_{50} were in concurrence with LT_{50} values show high pathogenicity of *Ptt* than its aqueous and organic extracts. Also, aqueous

Table 4 Total protein, phenoloxidase activity and phenoloxidase specific activity in *Pieris brassicae* when challenged with aqueous and organic extracts of *Ptt*

Treatment	Larval Stage	Time (h)	Total Protein (mg)±SE	Total Activity (U)±SE	Specific Activity (U/mg)±SE
aqueous extract	3	1	0.94±0.04	1.85±0.04	1.99±0.09
		3	1.95±0.06	2.78±0.06	1.42±0.02
		6	5.97±0.19	6.46±0.18	1.08±0.01
		24	12.38±0.37	12.31±0.34	0.99±0.00
organic extract		1	6.76±6.08	7.18±0.98	1.91±0.47
		3	1.32±0.09	2.19±0.08	1.67±0.05
		6	5.59±0.07	6.10±0.07	1.09±0.00
		24	10.61±0.03	10.69±0.03	1.01±0.00
control (LB)		1	0.86±0.06	1.78±0.05	2.08±0.08
		3	0.96±0.04	1.88±0.04	1.95±0.04
		6	1.41±0.03	2.29±0.03	1.62±0.01
		24	2.38±0.02	3.17±0.02	1.33±0.00
control (DMSO)		1	0.14±0.03	1.12±0.03	9.02±1.98
		3	0.09±0.03	1.08±0.02	14.51±4.39
		6	0.08±0.01	1.07±0.01	14.16±1.91
		24	1.28±0.06	2.17±0.05	1.69±0.03
aqueous extract	4	1	1.37±0.01	2.25±0.01	1.64±0.01
		3	2.19±0.06	3.00±0.05	1.37±0.01
		6	4.16±0.27	4.80±0.25	1.15±0.01
		24	11.02±0.19	11.07±0.17	1.00±0.00
organic extract		1	0.75±0.04	1.68±0.04	4.89±0.76
		3	1.89±0.02	2.73±0.02	1.49±0.01
		6	3.02±0.25	3.76±0.39	1.22±0.02
		24	9.89±0.05	10.04±0.08	1.03±0.00
control (LB)		1	0.75±0.04	1.96±0.03	1.85±0.03
		3	1.89±0.02	2.08±0.03	1.75±0.02
		6	3.02±0.25	2.60±0.02	1.48±0.01
		24	9.89±0.05	3.86±0.03	1.23±0.00
control (DMSO)		1	0.16±0.02	1.14±0.02	7.19±0.76
		3	0.11±0.01	1.09±0.01	10.56±1.19
		6	0.09±0.02	1.08±0.02	12.56±2.66
		24	1.76±0.04	2.61±0.04	1.48±0.01

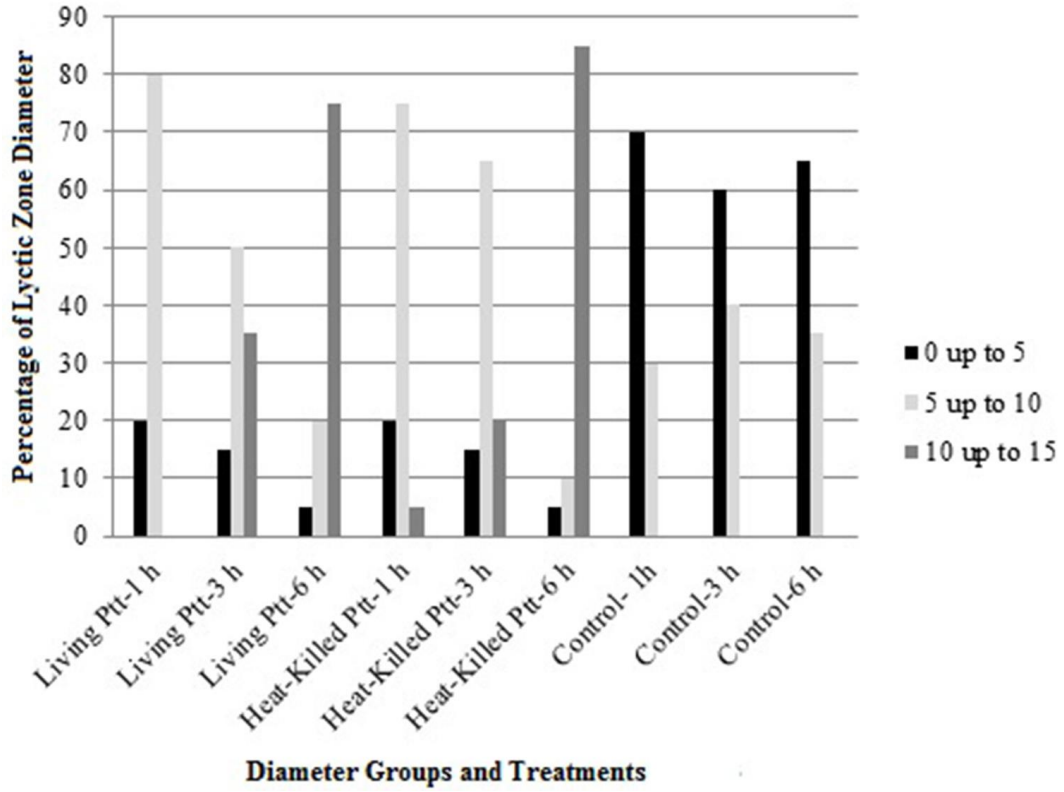

Fig. 3 Changes on the larvae distribution according to their lytic zone values observed for the different treatments and interval times post-injection. For each treatment and interval time, three groups of lysozyme concentration could be distinguished according to the diameter of the inhibition halo obtained in the lytic zone assays.

extracts had more virulent effect than organic extract on 3rd and 4th instars larvae. These results are in agreement with the results of Shrestha and Kim (2010), who found higher pathogenicity of aqueous extract than organic extract of *Ptt* on *Tribolium castaneum* larvae. Several reasons could affect higher pathogenicity of aqueous extract of *Ptt* than organic extract. On the one hand, the effect of higher suppression of aqueous extract than organic extract found in the current study could be an important reason for this difference between aqueous and organic extracts. On the other hand, inhibiting eicosanoid biosynthesis by aqueous extracts, as found by Shrestha and Kim (2010), could be another reason for higher virulence of this extract than organic extract. Also, higher virulence of *Ptt* than its extract could be due to the simultaneous effect of both extracts in the infected host. However, more studies are required to find all the exact reasons.

Between the successful infection by EPNs and host immune response is a close relationship (Li *et al*., 2007). Therefore, investigation of their virulence without considering the host's immune response seems irrational. Insects usually respond to bacterial or parasite infections with humoral and cellular immune reactions (Dunphy and Thurston, 1990; Feldhaar and Gross, 2008). Several types of hemocytes carry out the cellular immune responses, including phagocytosis, encapsulation, and nodule

Table 5 Lysozyme concentration of (mg/ml) 4th larval instars of *Pieris brassicae* when challenged with living and heat-killed *Ptt*

Treatments	Time after Injection (h)		
	1	3	6
Living *Ptt*	0.09 ± 0.01^{de}	0.18 ± 0.02^{b}	0.29 ± 0.03^{a}
Heat-Killed *Ptt*	0.11 ± 0.02^{bd}	0.16 ± 0.02^{bd}	0.31 ± 0.02^{a}
Control	0.03 ± 0.01^{e}	0.03 ± 0.01^{e}	0.03 ± 0.01^{e}

Different letters are significant treatments at $P<0.05$, according to Tukey's test.

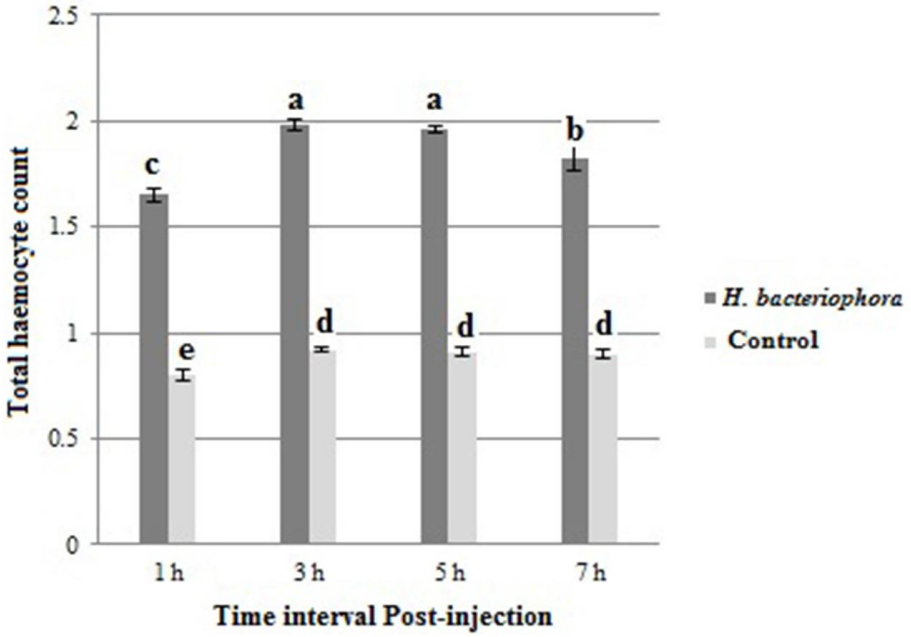

Fig. 4 Changes in haemocyte populations of *Pieris brassicae* larvae treated with *Heterorhabditis bacteriophora*. Means ± SE are given. Different letters in each time post-injection are significant at $p < 0.05$, according to Tukey's test.

formation to pathogens and parasites (Strand, 2008). Also, humeral factors, such as phenoloxidase and lysozyme, play an undeniable role in the immunity of insects. The purpose of this study was to consider the cellular and humeral responses of 3rd and 4th instars larvae of *P. brassicae* when challenged with IJs of *H. bacteriophora*, its symbiont bacteria, and metabolites.

Results of phenoloxidase specific activity showed that all the treatments-*H. bacteriophora*, *Ptt* and aqueous and organic extracts-caused suppression in both experimented instars of *P. brassicae*, though there were some differences in pattern. Phenoloxidase specific activity values of 3rd and 4th instars larvae of *P. brassicae* treated by *H. bacteriophora* showed a decrease in six h post-injection after a preliminary increase. This increase could be due to the recognition of an invader; however, subsequent decrease might be related to release, proliferation, activity of symbiont bacteria and their metabolites production. In the study performed by Rahatkhah *et al*. (2015), after injecting *S. feltiae* and *H. bacteriophora* into hemolymph of *Agriotes lineatus* and *G. mellonella*, phenolooxidase inhibition was observed 16 h post-injection. Also, results of Yokoo *et al*. (1992) showed the inhibition effect on prophenoloxidase hemolymph of *Agrotis segetum* larvae challenged with living and heat-killed *Steinernema carpocapsae.*

Remarkable results were observed when *P. brassicae* larvae were challenged with living and heat-killed bacteria. Both living and heat-killed *Ptt* caused phenoloxidase specific activity inhibition in 3rd and 4th instars larvae of *P. brassicae*. However, in larvae treated by living and heat-killed *Ptt*, suppression effect was observed early after living and heat-killed bacteria injection. In concurrence with this study, Yokoo *et al*. (1992) found that living and dead *Xenorhabdus nematophilus* inhibited phenoloxidase activity. It suggested that the suppression of phenoloxidase by both living and killed *S. carpocapsae* and its symbiotic bacteria was probably due to a factor in the living or dead responsible for the masking of the recognition protein that binds to laminarin. Also, suppression by both living and heat-killed bacteria was possibly due to another factors present in living and heat-killed bacteria. For instance, Dunphy and Webster (1988a) considered *X. nematophilus* in *G. mellonella* hemolymph, and found that activation of prophenoloxidase was suppressed.

The effect of aqueous extract, organic extract, and bacteria on phenoloxidase activity had the same pattern. In all these treatments, phenoloxidase activities were reduced early after treating. However, aqueous extract had more inhibition on phenoloxidase activity than organic extract. Differences in inhibiting phenoloxidase activity may be due to the presence of different protein contents between two extracts. Identifying protein contents of these extracts indisputably are necessary to determine the main reasons of difference between phenoloxidase activity inhibition of aqueous and organic extracts.

A comparison of phenoloxidase activity revealed this truth: injecting *H. bacteriophora* IJs caused delayed inhibition on phenoloxidase activity. However, results showed phenoloxidase suppression early after injecting *Ptt* or its extracts. In

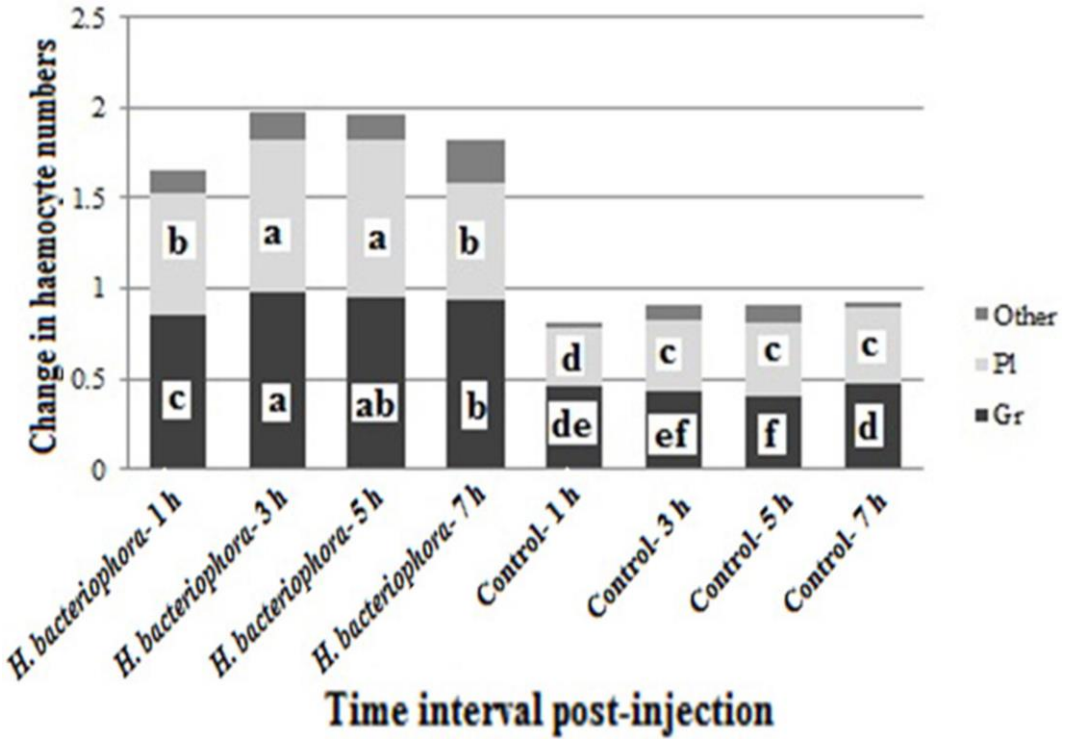

Fig. 5 Changes in differential haemocyte populations of *Pieris brassicae* larvae treated with *Heterorhabditis bacteriophora*. Means are given. Different letters in each time post-injection are significant at $p < 0.05$, according to Tukey's test. Pl: plasmatocyte, Gr: granulocyte.

the study by Dunphy and Webster (1988b), they found *Heterorhabditis heliothidis* released its symbiont bacteria four to five h after injecting the EPN. Besides, Wang *et al*. (1994) found that *S. carpocapsae* released its symbiont bacteria, *X. nematophilus*, and some four h after entering the host hemocel. The time required to release bacteria from IJs can reveal delayed phenoloxidase inhibition by EPN in comparison with symbiont bacteria and their extracts. Also, in all the treatments, 4th instars larvae showed higher phenoloxidase activity than 3rd instars larvae. This could be due to higher immune ability of 4th instars than 3rd instars larvae.

The most important immunity factor in the case of bacterial infection is lysozyme (Boman *et al*., 1991). Hence, the lysozyme response of 4th instars larvae was considered against *Ptt* infection. In the current study, an increase in lysozyme concentration was observed in response to living and heat-killed *Ptt* and significant difference with control. However, non-significant differences were observed between treatment by living and heat-killed bacteria at the same interval. These results show no suppression activity of *Ptt* on lysozyme activity. In concurrence with these results, Dunphy and Webster (1988b) found that *H. bactetriophora* and its symbiont bacteria, *P. luminescens*, do not inhibit lysozyme activity.

Hemocytes are one of the most important immune factors of insects against pathogens and invaders (Li *et al*., 2007; Strand, 2008). Studies abound about the response of hemocytes against EPNs (Alvandi *et al*., 2014; Rahatkhah *et al*., 2015). Their numbers rapidly increase during an infection, and they are responsible for several cellular defences. They can also take part in humoral reactions (Strand, 2008). In the current study, THC and DHC values of *P. brassicae* larvae were investigated when they were challenged with *H. bacteriophora*. The results show an increase of THC early after injecting IJs into hemolymph. The THC increasing and significant differences treatments with control can show immunological activation of host due to the presence of foreign bodies (Li *et al*., 2009). However, seven h post-injection of IJs THC were significantly decreased. This study is in concurrence with a study by Abu-Elmagd and El-Kifl (1993) that considered cellular response of *Spodoptera exigua* and *A. segetum* larvae challenged with *H. heliothidis*. Also, these results agree with data obtained by Rahatkhah *et al*. (2015) in *A. lineatus* against with *H. bacteriophora* and *S. feltiae*. Symbiont bacteria of EPNs have a detrimental effect on the health of host haemocytes, causing a decrease in the number of hemocytes (Bowen *et al*., 1998; Cho and Kim, 2004; Brivio *et al*., 2005). This fact-along with the time required to release bacteria from IJs-can reveal the reason for the decrease in hemocyte numbers after a primary increase (Dunphy and Webster 1988b; Wang *et al*., 1994).

In conclusion, our study indicates that several immune defenses in *P. brassicae* are engaged in the challenge against EPNs. A good understanding of insect host-pathogen relationships can help the progress of integrated pest management techniques. However, more study is necessary in this area for a deeper understanding of the immunity system of *P. brassicae* in its interaction with EPNs and its symbiont bacteria.

Acknowledgment

We would like to thank Prof. Lewis E (Department of Nematology, University of California, Davis, USA) and Prof. Dunphy GB (Department of Natural Resource Sciences, Montréal, Quebec, Canada) for their valuable advices.

References

Abdolmaleki A, Tanha Maafi Z, Rafiee Dastjerdi H, Lewis E. Potential efficacy of Iranian isolates of *Heterorhabditis bacteriophora* and *Steinernema feltiae* on *Pieris brassicae* (Lepidoptera:Pieridae). Russ. J. Nematol. 23: 91-97, 2015.

Abdolmaleki A, Tanha Maafi Z, Rafiee Dastjerdi H, Naseri B, Ghasemi A. Isolation and identification of entomopathogenic nematodes and their symbiotic bacteria from Kurdistan province in Iran. JCP 5: 259-271, 2016.

Abu-Elmagd AA, El-Kifl TAH. Cellular and humoral immune reactions of *Spodoptera littoralis* and *Agrotis ipsilon* larvae against the nematode *Heterorhabditis heliothidis*. J. Egypt. Ger. Soc. Zool. 12: 475-488, 1993.

Akhurst RJ. *Neoaplectana* species: specificity of association with bacteria of the genus *Xenorhabdus*. Exp. Parasitol. 55: 258-263, 1983.

Alvandi J, Karimi J, Dunphy GB. Cellular reactions of the white grub larvae, *Polyphylla adspersa*, against entomopathogenic nematodes. Nematology 16: 1047-1058, 2014.

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.

Balasubramanian N, Hao YJ, Toubarro D, Nascimento G, Simoes N. Purification, biochemical and molecular analysis of a chymotrypsin protease with prophenoloxidase suppression activity from the entomopathogenic nematode *Steinernema carpocapsae*. Int. J. Parasitol. 39: 975-984, 2009.

Beckage NE. Insect immunology. Academic, San Diego, USA, 2008.

Boman HG, Faye I, Gudmundsson GH, Lee JY, Lidholm DA. Cell-free immunity in Cecropia. A model system for antibacterial proteins. Eur. J. Biochem. 201: 23-31, 1991.

Bowen D, Rocheleau TA, Blackburn M, Andreev O, Golubeva E, Bhartia R, *et al.* Insecticidal toxins from the bacterium *Photorhabdus luminescens*. Science 280: 2129-2132, 1998.

Bradford MM. A rapid and sensitive method for the quantitating of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254, 1976.

Brivio MF, Mastore M, Pagani M. Parasite-host relationship: a lesson from a professional killer. J. Insect. Sci. 2: 41-53, 2005.

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

Cartea ME, Padilla G, Vilar M, Velasco P. Incidence of the major Brassica pests in Northwestern Spain. J. Econ. Entomol. 102: 767-73, 2009.

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

Ciche TA, Ensign JC. For the insect pathogen *Photorhabdus luminescens*, which end of a nematode is out? Appl. Environ. Microbiol. 69: 1890-1897, 2003.

Dunphy GB, Webster JM. Partially characterized components of the epicuticle of dauer juvenile *Steinernema feltiae* and their influence on hemocyte activity in *Galleria mellonella*. J. Parasitol. 72: 584-588, 1987.

Dunphy GB, Webster JM. Lipopolisaccharides of *Xenorhabdus nematophilus* (Enterobacteriaceae) and their haemocyte toxicity in non-immune *Galferia mellonella* (insect: Lepidoptera) larvae. J. gen. Microbiol. 134: 1017-1028, 1988a.

Dunphy GB, Webster JM. Virulence mechanisms of *Heterorhabditis heliothidis* and its bacterial associate, *Xenorhabdus lumirescens,* in nonimmune larvae of the greater wax moth, *Galleria mellonell*a. J. Parasitol. 18: 729-737, 1988b.

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

Feldhaar H, Gross R. Immune reactions of insects on bacterial pathogens and mutualists. Microbes Infect. 10: 1082-1088, 2008.

Forst S, Clarke D. Bacteria-nematode symbiosis, In: Gaugler R (ed.), Entomopathogenic nematology, CABI Publishing, London, UK, pp. 57-77, 2002.

Gaugler R. Entomopathogenic nematology, CABI Publishing, Wallingford, UK, 2002.

Grewal PS, Koppenhofer AM, Choo HY. Lawn, turfgrass and pasture application, In: Grewal PS, Ehlers RU, Shapiro-Ilan DI (eds), Nematode as biocontrol agant, CABI, London, pp 85-127, 2005.

Gupta AP. Haemocyte types: their structure, synonymies, interrelationships and taxonomic significance. In: Gupta AP (ed.), Insect haemocytes, Cambridge University Press, Cambridge, UK, pp 85-127, 1979.

Hames BD, Rickwood D. Gel electrophoresis of proteins: a practical approach. IRL Press Ltd., Oxford, UK, 1981.

Hernández-Martínez P, Naseri B, Navarro-Cerrillo G, Escriche B, Ferré J, Herrero S. Increase in

midgut microbiota load induces an apparent immune priming and increases tolerance to *Bacillus thuringiensis.* Environ. Microbiol. 12: 2730-2737, 2010.

IBM SPSS Inc. 2010. SPSS for windows user's guide release 6. SPSS Inc., Chicago, IL.

Kim D, Forst S. *Xenorhabdus nematophila*: mutualist and pathogen. ASM News. 71: 174-178, 2005.

Lewis EE, Gaugler R, Harrison R. Response of cruiser and ambusher entomopathogenic nematodes (Steinernematidae) to host volatile cues. Can. J. Zool. 71: 765-769, 1993.

Li Q, Sun Y, Wang G, Liu X. Effects of the mermithid nematode *Ovomermis sinensis* on the hemocytes of its host *Helicoverpa armigera*. J. Insect. Physiol. 55: 47-50, 2009.

Li XY, Cowles PS, Cowles EA, Gaugler R, Cox-Foster DL. Relationship between the successful infection by entomopathogenic nematodes and the host immune response. Int. J. Parasitol. 37: 365-374, 2007.

Martens EC, Heungens K, Goodrich-Blair H. Early colonization events in the mutualistic association between *Steinernema carpocapsae* nematodes and *Xenorhabdus nematophila* bacteria. J. Bacteriol. 185: 3147-3154, 2003.

Mbata GN, Shapiro-Ilan DI. Compatibility of *Heterorhabditis indica* (Rhabditida: Heterorhabditidae) and *Habrobracon hebetor* (Hymenoptera: Braconidae) for biological control of *Plodia interpunctella* (Lepidoptera: Pyralidae). Biol. Control 54: 75-82, 2010.

Rahatkhah Z, Karimi J, Ghadamyari M, Brivio MF. Immune defenses of *Agriotes lineatus* larvae against entomopathogenic nematodes. BioControl. 60: 1-11, 2015.

Raymond M. Presentation d'un programme d'analyse log-probit pour microordinateur. Cah. ORSTOM. Se. Ent. Med. Et. Parasitol. 22: 117-121, 1985.

Shrestha S, Kim Y. Differential pathogenicity of two entomopathogenic bacteria, *Photorhabdus temperata* subsp. *temperata* and *Xenorhabdus nematophila* against the red flour beetle, *Tribolium castaneum*. J. Asia Pac. Entomol. 13: 209-213, 2010.

Steiner WA. Melanization of *Steinernema feltiae* Filipjev and *S. kraussei* Steiner in larvae of *Otiorhynchus sulcatus* (F.). Fund. Appl. Nematol. 19: 67-70, 1996.

Strand MR. The insect cellular immune response. J. Insect. Sci. 15: 1-14, 2008.

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.

Wang Y, Campbell JF, Gaugler R. Infection of entomopathogenic nematode *Steinernema glaseri* and *Heterorhabditis bacteriophora* against *Popillia japonica* (Coleoptera, Scarabaeidae) larvae. J. Invertebr. Pathol. 66: 178-184, 1995.

Wang Y, Gaugler R, Cui L. Variations in immune response of *Popillia japonica* and *Acheta domesticus* to *Heterorhabditis bacteriophora* and *Steinernema* species. J. Nematol. 26:11-18. 1994.

Webster JM, Chen G, Li J. Parasitic worms: an ally in the war against superbugs. Parasitol. Today 14: 161-163, 1998.

Yokoo S, Tojo S, Ishibashi N. Suppression of the prophenoloxidase cascade in the larval haemolymph of the turnip moth, *Agrotis segetum* by an entomopathogenic nematode, *Steinernema carpocapsae* and its symbiotic bacterium. J. Insect. Physiol. 38: 915-924, 1992.