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Haemocytes immunity of rose sawfly, *Arge ochropus* (Hym.: Argidae) against entomopathogenic nematodes, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*

Hadi Sheykhnejad [a,c], Mohammad Ghadamyari [a], Vahid Ghasemi [b], Salar Jamali [a], Javad Karimi [c,*]

[a] Department of Plant Protection, College of Agriculture Sciences, University of Guilan, Rasht, Iran
[b] Department of Plant Protection, Faculty of Agriculture and Natural Resources, Higher Education Complex of Shirvan, Iran
[c] Biocontrol and Insect Pathology Laboratory, Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

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ABSTRACT

The rose sawfly, *Arge ochropus* (Gmelin), is one of the most destructive pests attacking rose bushes in northern Iran. In the present study, the haemocyte reactions of *A. ochropus* larvae were assessed against two entomopathogenic nematodes, *Steinernema carpocapsae* (Weiser) and *Heterorhabditis bacteriophora* Poinar. With injection of infective juveniles of *H. bactriophora* into *A. ochropus* larvae, the total haemocyte count showed alternating fluctuations at all time intervals. Encapsulation occurred 22 hours post injection (hpi) of *H. bacteriophora* infective juveniles into the rose sawfly larvae, while melanization was observed after 24 h. In the case of *S. carpocapsae,* initial attachment of the haemocytes was detected at 18 hpi and complete encapsulation at 24 hpi; no melanization was observed. In general, findings showed strong immune responses of the rose sawfly larvae against *H. bacteriophora*, while these reactions were weakened for *S. carpocapsae*. Therefore, it is concluded that *S. carpocapsae* has more ability to overcome the host defence system, and this research provides a new window on its potential as a biocontrol agent.

Introduction

The rose sawfly, *Arge ochropus* (Gmelin) is an important defoliator pest on rose bushes in the North of Iran (Sahragard and Heidari, 2001). This insect lays its eggs in the stems of rose bushes and causes hypertrophy scorch. The larvae are external leaf feeders and cause considerable damage and defoliation in the ornamental plants (Smith, 1989).

On rose bushes in urban areas, application of pesticides holds some special risks, as most pesticides are as harmful to humans as they are to the insects. So, a safe control method, such as a biocontrol approach, which is less hazardous to the environment and humans, is greatly needed.

Many studies have been carried out on the pathogenicity of entomopathogenic nematodes (EPNs) against leaf-feeder pests. For example, biological control of *Arge humeralis* (Beauvois) and some *Arge* species with entomopathogenic agents has been studied (Regas-Williams and Habeck, 1979; Yang et al., 2007). However, our knowledge about the pathogenicity of entomopathogenic nematodes against Hymenopteran species is still poor.

Insects have evolved an efficient immune system against invading foreign microbes, parasitoids and parasites. The innate immune system of insects can be classified as having humoral and cellular defence responses. Humoral defence involves production of phenoloxidase as well as secretion of antimicrobial compounds in response to invaders (Dunphy and Thurston, 1990; Ebrahimi et al., 2011). Cellular defences involve responses of haemocytes against pathogens such as phagocytosis, nodule formation and encapsulation. Encapsulation and melanization are relevant types of defence mechanism against entomopathogenic nematodes (Vega and Kaya, 2012). Encapsulation of *S. carpocapsae* in the Japanese beetle, *Popillia japonica* Newman, and the house cricket, *Acheta domestica* L., cannot prevent the death of the insects and bacterial propagation from the infective juvenile nematodes before capsule completion (Wang et al., 1994). *Steinernema glaseri* (Steiner) and *Steinernema scapterisci* (Nguyen and Smart, 1990) encounter no cellular immune responses in *P. japonica* and *A. domestica* and the death of the host occurs quickly (Wang et al., 1994). No report is available on the immune responses of *A. ochropus* to *S. carpocapsae* and *H. bacteriophora.* Therefore, the present study is conducted to compare the interaction of subjected entomopathogenic nematodes with the cellular immune responses of the rose sawfly larvae.

* Corresponding author.
E-mail address: jkb@ferdowsi.um.ac.ir (J. Karimi).

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Materials and methods

Rearing of nematodes

Steinernema carpocapsae and *Heterorhabditis bacteriophora*, which are isolated from the commercial products Capsanem® and Larvanem®, were used in the assays. Both products are provided by Koppert Biological Systems (Berkel en Rodenrijs, The Netherlands). The infective juvenile nematodes were reared on the last instar larvae of greater wax moth, *Galleria mellonella* (Woodring and Kaya, 1988) and stored in distilled water at 8 °C.

Rearing of insects

During September–November 2012, the different larval instars of *A. ochropus* were collected from rose bushes at the University of Guilan, northern Iran. The larvae were transferred to the laboratory and kept in a growth chamber at 25 ± 2 °C, 70 ± 10% relative humidity (RH) and with a photoperiod of 16:8 h (L:D). One-day-old fifth-instar larvae, which have a yellow head capsule, were used throughout the study. All larval instars of *A. ochropus* feed on leaves of rose bushes.

G. mellonella were reared on an artificial diet consisting of wheat flour (120 g), bran (260 g), yeast (100 g), wax (120 g), honey (150 ml), glycerin (200 ml) and water (500 ml) (Birah et al., 2008). The larvae were reared and maintained in a growth chamber at 25 ± 2 °C, 60 ± 10% RH with a photoperiod of 16:8 h (16 h light and 8 h dark).

Rose sawfly larvae inoculation with EPNs

To adapt the nematodes to laboratory temperature, they were kept at 25 °C for 10–15 min. According to a bracketing test, an LC_{30} value equal to approximately 12 nematodes per larvae was used for inoculation. Infective juveniles of each nematode species (12 IJs in 10 µl Ringer's solution) were carefully injected into the haemocoel of *A. ochropus* and *G. mellonella* larvae by a sterile insulin syringe through the base of the third sternal leg. The syringe was inserted parallel to the body wall of the last instar larvae to avoid injuring the digestive system. Control larvae of both insects were injected with 10 µl of distilled water. Treated larvae were kept in 9 cm diameter petri dishes and fed equally. Interaction of *S. carpocapsae* and *H. bacteriophora* nematodes with *A. ochropus* larvae was compared in the characteristics of free-moving, encapsulation and melanized nematodes at three different times (18 h, 22 h and 24 h after treatment). The insects were dissected in different time intervals and the process of cell reaction inoculation was grouped into three stages: primary attached, complete capsulation and melanization. All dark-coloured and brown nematodes were classified as melanized, including nematodes with any degree of encapsulation. Investigation and dissection of encapsulated nematodes were carried out under a stereomicroscope (Olympus SZX.ILL K200). Photographs of different processes were taken by a Dino-lite AM423X camera.

Total haemocyte counts (THC)

For THC, 1 µl of haemolymph of treated larvae was diluted with 9 µl of Tyson solution (NaCl 2.72 mM; Na_2SO_4 8.96 mM; glycerol 43.68 mM; methyl violet 0.061 mM). The treated larvae were heated at 60 °C for 1–2 min before bleeding. THC were conducted with a standard Neubauer haemocytometer (HBG, Germany). The cells were counted using a light microscope, and the number of total haemocytes per cubic millimetre (mm^3) was calculated using the formula by Jones (1962). This experiment was repeated seven times for seven specific time intervals. For all tests, corresponding control was used.

Statistical analysis

The percentages of melanization, encapsulation and free-moving nematodes were calculated based on the number of nematodes recovered against the number injected. The infective juveniles completely encased with haemocytes, even with a single layer, were considered as encapsulated nematodes.

Data were analysed using the SPSS program version 19.0 for analysis of variance (ANOVA); the means were grouped using Tukey's test ($p < 0.05$). Also, statistical comparison between treatment and control at each time interval was performed using Student's *t*-test.

Results

Significant differences were observed in the *A. ochropus* immune responses 24 h post injection (hpi) for both EPN species, *S. carpocapsae* and *H. bacteriophora*. Encapsulation was observed on *S. carpocapsae* surface 24 hpi (Fig. 2), and for *H. bacteriophora* primary attachment of haemocyte was observed 30 minutes post injection (Fig. 1) and encapsulation and melanization occurred at 18 h and 24 h (Table 1). For both EPN species, the injected larvae had died after this time.

In *H. bacteriophora*, 30 min before 18 hpi the haemocytes were attached to the nematodes' body surface, except the anterior and caudal parts; 22 hpi haemocytes surrounded the entire body of the nematode. The results showed thick layers of haemocyte around the IJs 24 hpi (Fig. 4). The thick layers of haemocyte were brown in colour and thus appeared melanized. The melanization occurred mainly in the middle part of the nematode's body and the inner cell layers attached to the nematode.

The intensity of immune responses was determined by both species of nematode and insect hosts. Haemocytes from the rose sawfly reacted to nematodes differently: *H. bacteriophora* had a large number of attached haemocytes while *S. carpocapsae* were moving freely in the haemolymph. *G. mellonella*, as an insect model, can be successfully infected by both nematodes but infectivity of *S. carpocapsae* was higher in *A. ochropus* than *H. bacteriophora* (LC_{50} in *S. carpocapsae* was 21 nematodes per larvae, but for *H. bacteriophora* this grew to 32 nematodes per larvae) (Sheykhnejad et al., 2014). The potential for encapsulation might be affected by the number of invading nematodes as well as the function of time. The encapsulation percentages of *H. bacteriophora* infective juvenile by 18, 22 and 24 hpi were, respectively, 38.5% ($F = 256$, $df = 6$, $n = 4$, $P \leq 0.01$), 29.3% ($F = 8.3$, $df = 6$, $n = 4$ and $P \leq 0.01$) and 25.08% ($F = 13.5$, $df = 6$, $n = 4$ and

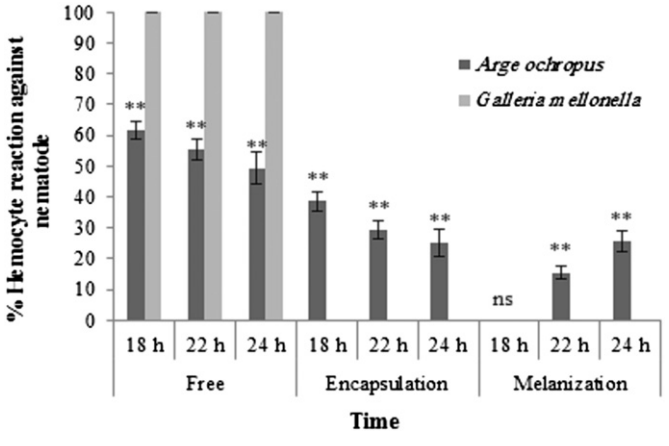

Fig. 1. Haemocytes reactions of *Arge ochropus* and *Galleria mellonella* larvae to *Heterorhabditis bacteriophora* 18–24 hpi of EPNs. Percentages (mean ± SEM) of melanized, encapsulated and free-moving nematodes were calculated from observation data after dissections of host insect. Means were compared pairwise for each time point between *G. mellonella* and *A. ochropus* by t-test. Statistically significant differences are denoted with * ($p < 0.05$) or ** ($p < 0.01$). ns: no significant difference.