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RESEARCH REPORT

Effects of *Steinernema carpocapsae* (Weiser) on immunity and antioxidant responses of *Glyphodes pyloalis* Walker

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Abstract

The effect of *Steinernema carpocapsae* (IRA18) infection was studied on the mortality, immunity and antioxidant responses of *Glyphodes pyloalis* Walker larvae. The LC_{50} value of 582.9 infective juvenile per mL with confidence limit of 359.1-811.5 was obtained via bioassay against the larvae. Injection of *S. carpocapsae* increased the number of total hemocytes after 1-6 h compared to intact and Ringer-injected larvae while the highest numbers of plasmatocyte and granulocytes were recorded after 1 and 3 h. Although intact larvae had a steady activity of phenoloxidase at different time intervals but those injected by *S. carpocapsae* showed the elevated enzymatic activity at 3-12 h. Nematode injection significantly increased the activities of superoxide dismutase and catalase compared to intact and Ringer-injected larvae, while no significant difference was observed in peroxidase activity. The injection with *S. carpocapsae* caused the highest activity of glutathione *s*-transferase using CDNB as reagent, but the enzymatic assay with DCNB showed no statistical differences among treatments. Also, activities of ascorbate peroxidase and glucose-6-phosphate dehydrogenase significantly increased in the nematode-injected larvae. Intact and Ringer-injected larvae showed no statistical differences in the concentration of malondialdehyde but the highest amount was recorded in nematode-injected larvae. Results of our study indicate that native isolate of *S. carpocapsae* cause mortality on the larvae of *G. pyloalis* and It interferes in the immune and antioxidant responses.

Key Words: *Steinernema carpocapsae*, *Glyphodes pyloalis*, immunity, antioxidant system

Introduction

Different varieties of Mulberry (*Morus* spp.) are the only source of silkworm feeding to produce high quality cocoons (Khosravi and Jalali Sendi, 2010). These varieties are attacked by different pests around the world but Lesser Mulberry Snout Moth, *Glyphodes pyloalis* Walker (Lepidoptera: Pyralidae), is a monophagous pest solely feed on mulberry leaves which has been widely distributed in USA, Mexico, India, Japan, Iran, Central Asia and Azerbaijan (Kanchaveli *et al.*, 2009). In 2002, a high population of *G. pyloalis* was reported thoughout mulberry orchards of northern Iran which caused severe damages not only on shortage of available leaves for silkworm but also to transmission of densoviruses and picornaviruses to silkworm as an alternative host (Watanabe *et al.*, 1988; Khosravi and Jalali Sendi, 2010). *G. pyloalis* has five generations per year and the fourth and fifth instar larvae cause the highest damages during cropping season. Extensive feed on mulberry and feces remnants are the main damages which significantly reduces quality of leaves (Khosravi and Jalali Sendi, 2010). Because of environmental concerns on wide spraying, control of *G. pyloalis* is based on mechanical and cultural tactics by removing infested leaves and ploughing of soil around mulberry trees in winter although spraying with Insect Growth Regulators (IGRs) are inevitable in some cases (Khosravi *et al.*, 2014).

Nematodes are the multicellular organisms which have different life styles in environment i.e. free-living, predator, pathogens of plants, animals and even human (Gaugler, 2002). Several nematodes may be found on insect orders but a few are able to kill insects. Nematode-insect associations are categorized as phoretic, commensalism, facultative or obligatory parasitism (Grewal *et al.*, 2006). Among nematode taxa showing entomopathogeic characteristics, Steinernematide and Heterohabditidae have been demonstrated as

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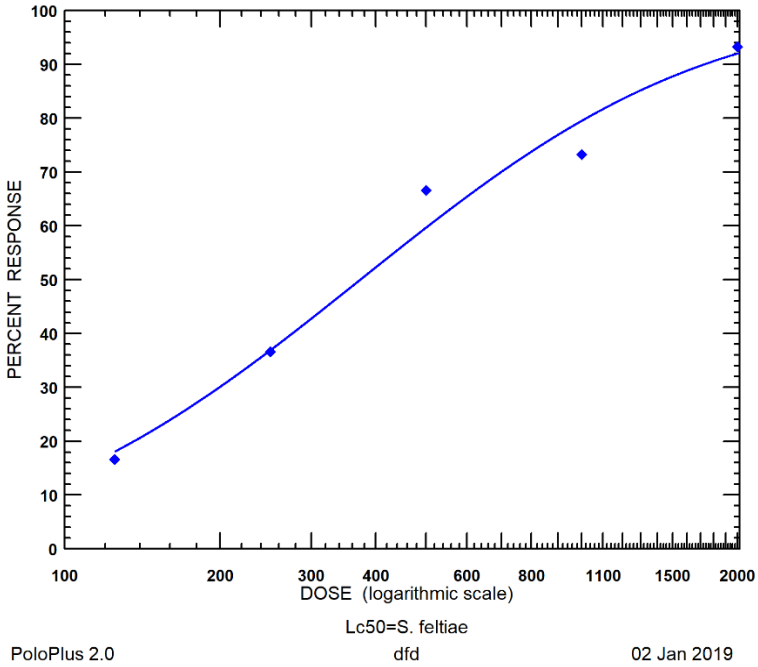

Fig. 1 Dose-response curve of *G. pyloalis* exposure to *S. carpocapsae*. Graph and data given were calculated by POLO-plus software.

the most successful nematodes in biological control (Grewal *et al*., 2006). Mortality on host is imposed due to presence of bacteria, *Xenorhabdus* spp. and *Photorhabdus* spp. which have mutualistic symbiosis with steinernematids and heterorhabditids, respectively (Poinar, 1990). Infection is initiated by entrance of Infective juveniles (IJs) though natural openings of body including mouth, anus, and spiracles. Then, symbiotic bacteria are released into hemocoel that leads to septicemia and kills host within 24–48 h. Almost thee generations are completed within host body, afterward IJs leave host cadaver and seeks for new hosts (Dowds and Peters, 2002).

The immune responses of insects include all processes that protect them against bacteria, viruses, fungi, and parasitoids (Lavine and Strand, 2002; Schmid- Hempel, 2005). These responses are divided into cellular and humoral immunities in which phagocytosis, nodule formation and encapsulation are devoted to cellular responses while production of reactive oxygen or nitrogen species, antimicrobial compounds and deposition of melanin though prophenoloxidase system are the features of humoral responses (Söderhäll and Cerenius, 1998; Siva-Jothy *et al*., 2005). These reactions in addition to evolution of pathogenic routes of entomopathogens contribute in success of microbial control or evasion of target pest from biocontrol agent.

Agricultural products are subjected to many pests requiring pesticide use to prevent severe damages although these chemicals impose production costs, human or wildlife health and environment pollution. In order to reduce risks of chemical pesticides, the use of biocontrol agents is of interest within organic agriculture. Entomopathogenic nematodes have been successful in recent decades due to their wide host range, growth capability on artificial media, stable pathogenicity and incorporation with some chemicals and fertilizers (Shapiro-Ilan *et al*., 2017). Since control of *G. pyloalis* though safe procedures is of importance due to environmental concerns, the current study was done to determine pathogenicity of a native entomopathogenic nematode of *S. carpocapsae* in addition to immune and antioxidant responses of *G. pyloalis* larvae following immune challenge. Understanding the physiological response of *G. pyloalis* to the pathogenic nematode and the level of infection on the larvae can provide an overview on the effectiveness of microbial control.

Material and methods

Insect rearing

Larvae of *Glyphodes pyloalis* were collected from infested mulberry trees in the campus of University of Guilan. Specimens were kept at 24 ± 2 °C, 75 ± 5 % of relative humidity and 16L:8D of photoperiod, fed on mulberry leaves within containers (18×15×7 cm) which have been sealed by cheesecloth. Leaves were daily replaced with new ones and remnants were cleaned to avoid potential infection. After pupation, males and females were discriminated based on morphology of distal abdomen and transferred to cages for mating. Some leaves which their petioles were covered with wet cotton were put in cages as the place of oviposition. Once eggs hatched, 1st instar larvae were transferred to rearing containers and provided with fresh leaves till 4th larval instars at the above-mentioned rearing condition (Khosravi and Jalali Sendi, 2010).

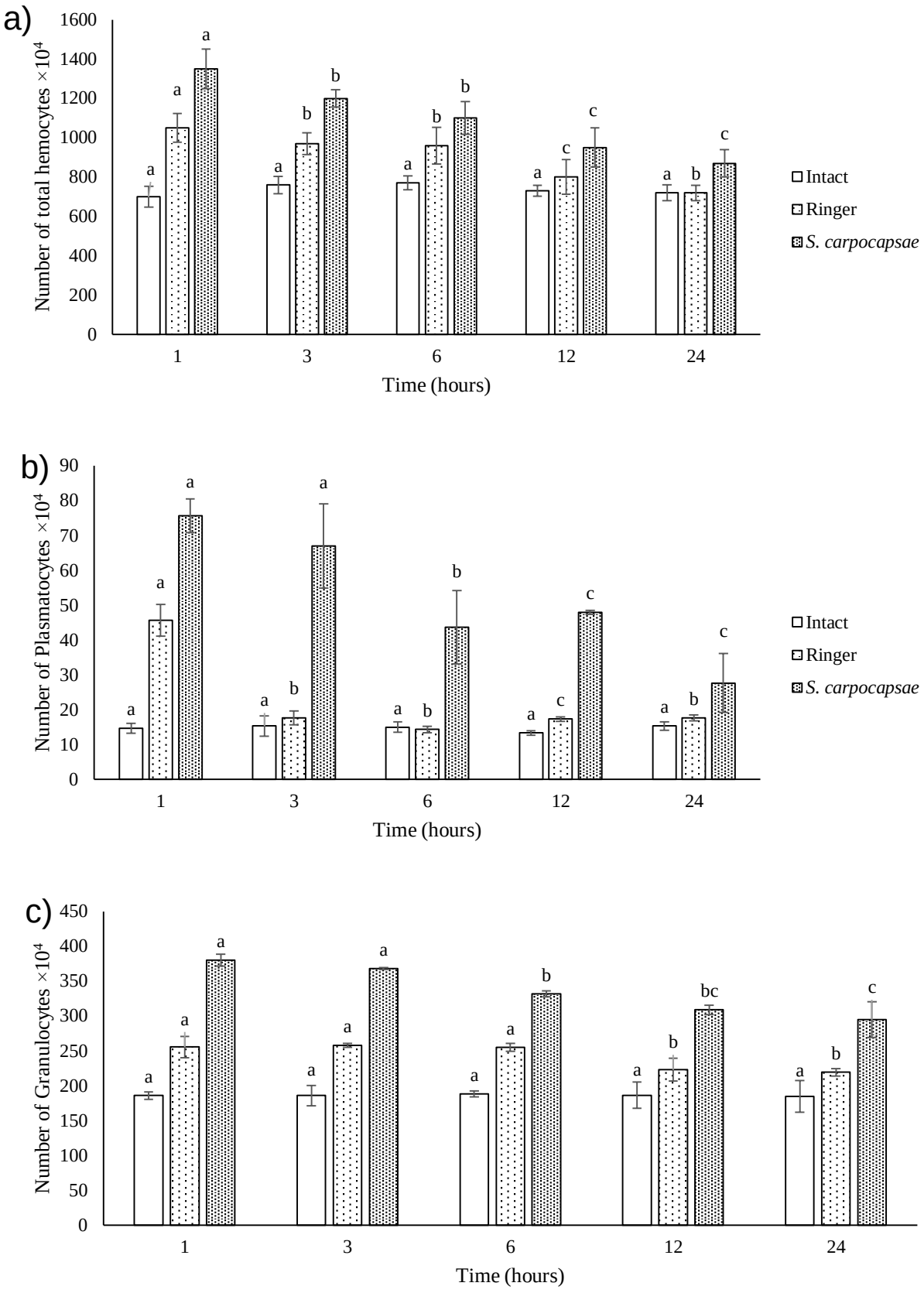

Fig. 2 Effect of *S. carpocapsae* injection on the numbers of total hemocytes (a), plasmatocytes (b) and granulocytes (c) of *G. pyloalis*. Statistical differences have been marked with different letters within each time interval (Tukey test, $p \leq 0.05$).

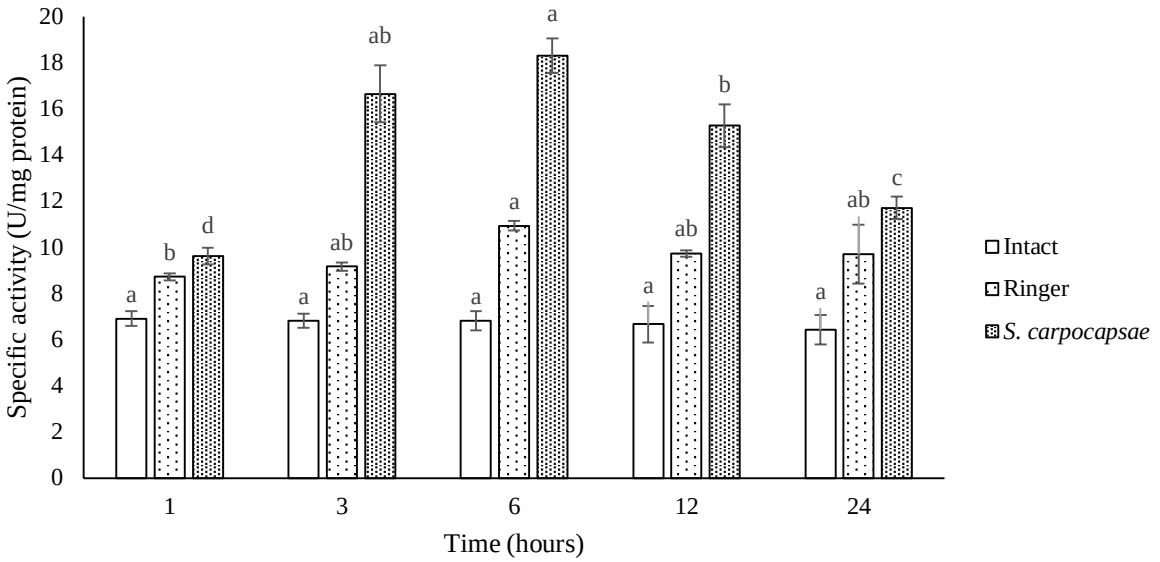

Fig. 3 Effect of *S. carpocapsae* injection on phenoloxidase activity of *G. pyloalis* larvae. Statistical differences have been marked with different letters within each time interval (Tukey test, $p \leq 0.05$)

Nematode Rearing

The Iranian isolate of *Steinernema carpocapsae* (IRA118) was generously provided from Insect Pathology Laboratory of Shahid Madani University and reared following inoculation on *Galleria mellonella* Fabricius larvae at 25 °C for emergence of IJs. Emerged IJs were kept at 15 °C for two weeks before main experiments.

Bioassay of nematode

The 4th instar larvae of *G. pyloalis* were used to determine virulence of *S. carpocapsae*. This is an Iranian native nematode collected from Meshkin Shah, East Azerbaijan with the collection code of IRA18. After preliminary assay, the concentrations of 250, 500, 1000 and 2000 IJs per mL were prepared in Ringer solution (121.5 mM NaCl; 10 mM KCl; 2.1 mM NaH_2PO4; 10 mM $NaHCO_3$; 0.7 mM $MgCl_2$; 2.2 mM $CaCl_2$; pH 6.8). Then, 500 µL of the solution containing nematodes was separately pipetted onto filter paper (Watman No.1) fitted into a glass petri dish (10 cm). The experiment was done by 150 larvae which 30 larvae were devoted to each concentration in thee replicates including control larvae which received only Ringer solution. All petri dishes were kept at 25 °C and the filter papers were received 500 µL of sterile distilled water to keep moisture. Mortality of larvae was recorded from 24 h following experiment initiation and prolonged for a week.

Immune challenge

Initially, the third thoracic segment of *G. pyloalis* larvae was sanitized with ethanol solution (70 %), and injected with 1 µL of a solution containing LC_{30} concentration of nematode prepared in Ringer solution which has been calculated based on bioassay experiment. Control larvae were injected by Ringer only and a group of larvae leaved without any challenged named as intact group. Thirty larvae were separately devoted to each treatments including intact, Ringer- and *S. carpocapsae* injected in thee replicates. After time intervals of 1, 3, 6, 12 and 24 h post-treatment, hemolymph was collected and immediately diluted in an anticoagulant solution prepared based on Azambuja *et al*. (1991) containing 0.01 M, ethylenediamine tetraacetic acid; 0.1 M, glucose; 0.062 M, NaCl; 0.026 M, citric acid (pH 4.6). Then, 100 µL of the sample was pipetted onto a hemocytometer and the numbers of total and differentiated hemocytes- plasmatocytes and granulocytes- were counted by direct observation under light microroscopy. The experiment was done at laboratory conditions of 25 ± 2 °C, 70 % of relative humidity and 16:8 (L:D) h.

Phenoloxidase assay

Phenoloxidase activity was assayed in all treatments according to a method described by Wilson *et al*. (2002). Briefly, 10 µL of hemolymph was transferred to a plastic tube (1.5 mL), then 100 µL of ice-cold phosphate buffered saline (20 mM, pH 7) was added and the samples were frozen to disrupt hemocytes. To determine phenoloxidase (PO) activity in the defrosted solution, samples were poured into each well of a plate containing 20 mL of 10-mM L-dopa (3,4- dihydroxyphenylalanine) as a substrate. After 5 minutes of incubation at room temperature, the absorbance was measured at 492 nm.

Antioxidant Assays

Sample Preparation

Three groups including 20 larvae were considered as intact, Ringer and *S. carpocapsae* injected (LC_{30}). After 24 hours, the larvae separately selected and homogenized in distilled water using a

glass pestle. The homogenated samples were centrifuged at 20000 *g* for 20 min at 4 °C. The supernatants were stored at -20 °C for subsequent analyses.

Catalase assay

The reaction mixture contained 50 µL of sample and 500 µL of hydrogen peroxide (1 %) which were incubated for 10 min at 28 °C before recording absorbance at 240 nm (Wang *et al*., 2001).

Superoxide dismutase assay

Briefly, 50 µL of sample and 500 µL of reaction solution containing 70 µM of NBT (Nitro blue tetrazolium), 125 µM of xanthine, both dissolved in phosphate buffer (20 mM, pH 7.1) were mixed thoroughly and gently shaken. Then, 100 µL of xanthine oxidase (5.87 units/ml) dissolved in 2 mL of phosphate buffer were added to the initial mixture and incubation was initiated at darkness for 20 min at 28 °C. Afterward, absorbance was recorded at 560 nm (McCord and Fridovich, 1969).

Peroxidase assay

Thee component of reaction mixture including 50 µL of sample, 250 µL of buffered pyrogallol (0.05 M pyrogallol in 0.1 M phosphate buffer) and 250 µL of H_2O_2 (1 %) were mixed thoroughly prior to read absorbance at 430 nm every 30 s for 2 min. An extinction coefficient of oxidized pyrogallol (4.5 liters/mol) was used to activity calculation (Addy and Goodman, 1972).

Glutathione S-transferase assay

The activity of glutathione *S*-transferase (GST) was assayed based on a method described by Habig *et al*. (1974). Briefly, 20 µL of CDNB (1-chloro-2,4-dinitrobenzene, 20 mM) and DCNB (1,2-dichloro-4-nitro-benzene, 20 mM) were separately added into 50 µL of reduced glutathione solution (20 mM), then 10 µL of enzyme solution was added and the absorbance was read at 340 nm after 5 min of incubation.

Ascorbate peroxidase assay

A reaction mixture containing 50 µL of sample, 150 µL potassium phosphate buffer (67 mM, pH 7.0), 70 µL ascorbic acid (2.5 mM) and 200 µL H_2O_2 (30 mM) was prepared based on Asada (1992). Absorbance was recorded at 290 nm for 5 min in continuous manner.

Glucose-6-phosphate dehydrogenase assay

The assay was done using 100 µL of Tris-HCl (100 mM, pH 8.2), 50 µL of NADP (Nicotinamide adenine dinucleotide phosphate 0.2 mM) and 30 µL of $MgCl_2$ (0.1 M). Afterward, 50 µL of water, 50 µL of the sample and 100 µL of GPDH (6 mM) was added to the initial mixture before recording absorbance at 340 nm (Balinsky and Bernstein, 1963).

Malondialdehyde assay

The concentration of Malondialdehyde (MDA) was determined based on Bar-Or *et al*. (2001) in which 100 µL of 20 % trichloroacetic acid and 50 µL of the sample were initially mixed and centrifuged at 15000 *g* for 10 min at 4 °C. Then, supernatant was mixed with 100 µL of 0.8 % thiobarbitoric acid (TBA) reagent and re-incubated at 100 °C for 60 min prior to reading absorbance at 535 nm. MDA concentration is defined as the amount of MDA produced per mg protein with a molar extinction coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$.

Protein assay

The amount of total protein was assayed by the method of Lowry *et al*. (1951) using a commercial kit manufactured by ZiestChem company (Tehan, Iran).

Statistical Analysis

A complete randomized design was adopted for the experiments. Data of bioassay were inserted in POLO-plus software to calculated LC values. Means were analyzed using Tukey's test and the statistical differences were considered at a probability less than 5 % and marked with different letters.

Results

The used native isolate of *S. carpocapsae* imposed significant mortality against fourth instar larvae of *G. pyloalis*. Figure 1; shows that the used concentrations led to 10-90 % mortality against the larvae with the LC_{50} value of 582.9 IJs per mL, confidence limit at 95 % of 359.1-811.5 and slope of 2.33 ± 0.54 2 ($p \leq 1.537$, df=2).

Figure 2; shows the effect of *S. carpocapsae* at LC_{30} concentration on the numbers of total and differentiated hemocytes of *G. pyloalis* larvae. Injection of the nematode augmented the number of total hemocytes at the time intervals of 1-6 h compared to intact and Ringer-injected larvae, but the total hemocyte count sharply decreased at the two other time intervals (Figure 2a). Although injection of Ringer solution only increased the number of plasmatocytes after 1 h, but nematode induced proliferation of plasmatocytes at all time intervals compared to other treatments with the highest number after 1 and 3 h (Figure 2b). The highest number of granulocytes was obtained after 1 and 3 h post-injection while it decreased after these intervals (Figure 2c).

The larvae of *G. pyloalis* exposed to experimental treatments showed significant differences in phenoloxidase activity (Figure 3). Although intact larvae had a steady activity of phenoloxidase at different time intervals but those injected by *S. carpocapsae* showed an elevated enzymatic activity after 3-12 h post-injection (Figure 3). Although similar trend was found in the larvae injected by Ringer solution, but phenoloxidase activity was significantly lower than nematode-injected larvae (Figure 3).

Figure 4 and 5; show the effect of *S. carpocapsae* injection on the antioxidant enzymes of *G. pyloalis* larvae compared to intact and Ringer-injected ones. Nematode injection significantly increased activities of superoxide dismutase and catalase compared to intact and Ringer-injected larvae while both nematode and Ringer injections

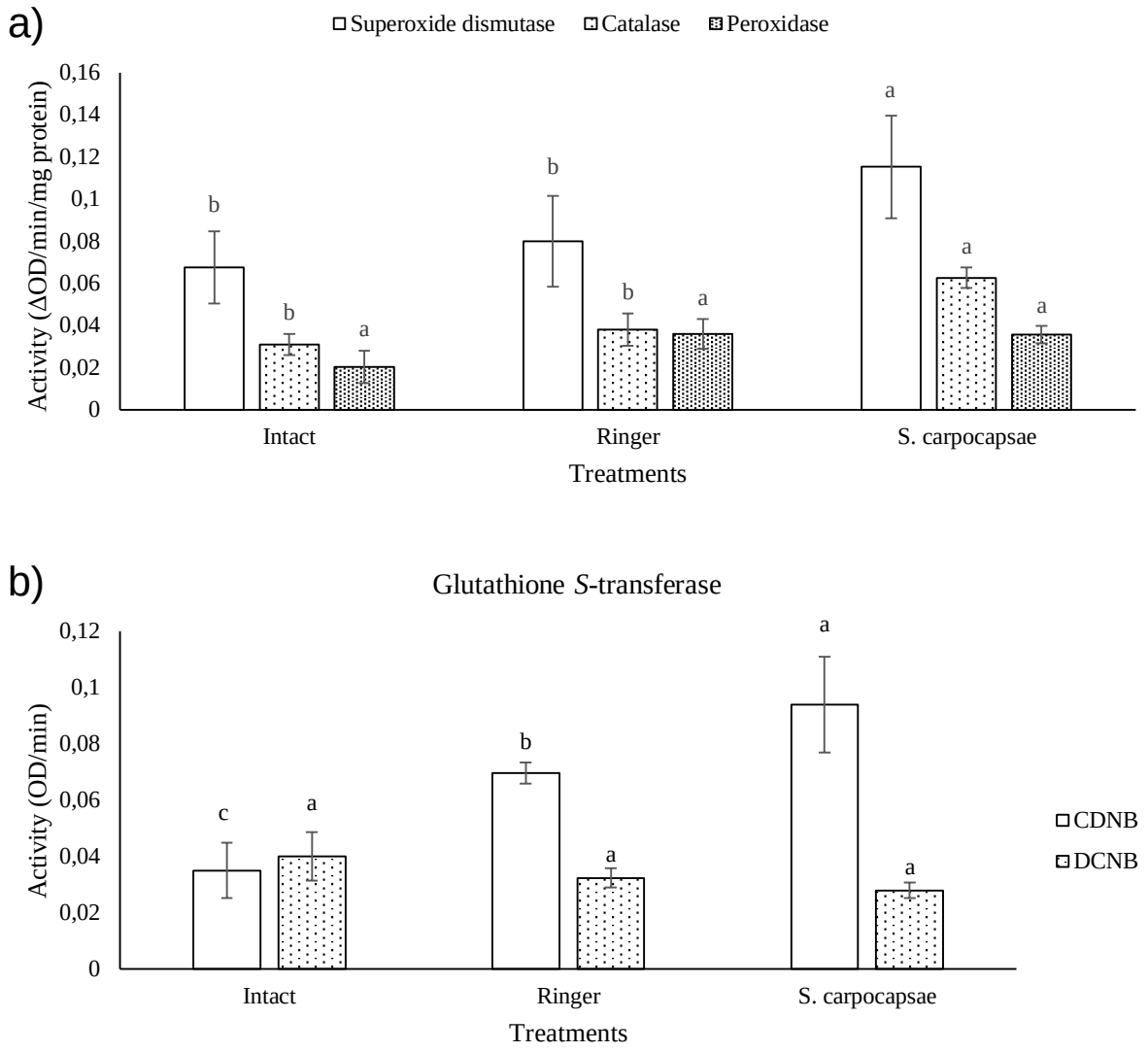

Fig. 4 Effect of *S. carpocapsae* injection on a) superoxidase dismutase, catalase, peroxidase and b) glutathione-*s*-transferase activities in *G. pyloalis*. Statistical differences have been marked with different letters within treatments (Tukey test, $p \leq 0.05$).

led to an equal elevation of peroxidase activity compared to intact larvae (Figure 4). Although *S. carpocapsae* injection caused the highest activity of glutathione *s*-transferase activity using CDNB as reagent, but it showed no statistical differences among intact, Ringer- and nematode injected larvae using DCNB (Figure 4). Moreover, activities of ascorbate peroxidase and glucose-6-phosphate dehydrogenase significantly elevated in the larvae injected by *S. carpocapsae* but no statistical difference was found in ascorbate peroxidase between Ringer- and nematode-injected larvae (Figure 5). Finally, intact and Ringer-injected larvae showed no statistical differences in the concentration of malondialdehyde but nematode injection significantly led to the highest level (Figure 5).

Discussion

Entomopathogenic nematodes mainly the members of Steinernematidae and Heterohabditidae have gained significant attentions because of their efficient potential in biological control of insect pests (Shapiro-Ilan *et al*., 2017). This capability results from some features including rapid kill of hosts, searching ability, ease of application, potential long-term effects due to environmental persistence, safety to non-target organisms and compatibility with several chemical insecticides (Koppenhöfer and Kaya, 2002; Vashisth *et al*., 2013; Shapiro-Ilan *et al*., 2017). Previous studies have shown significant variations of host mortality after treatment with entomopathogenic nematodes which has been attributed to host

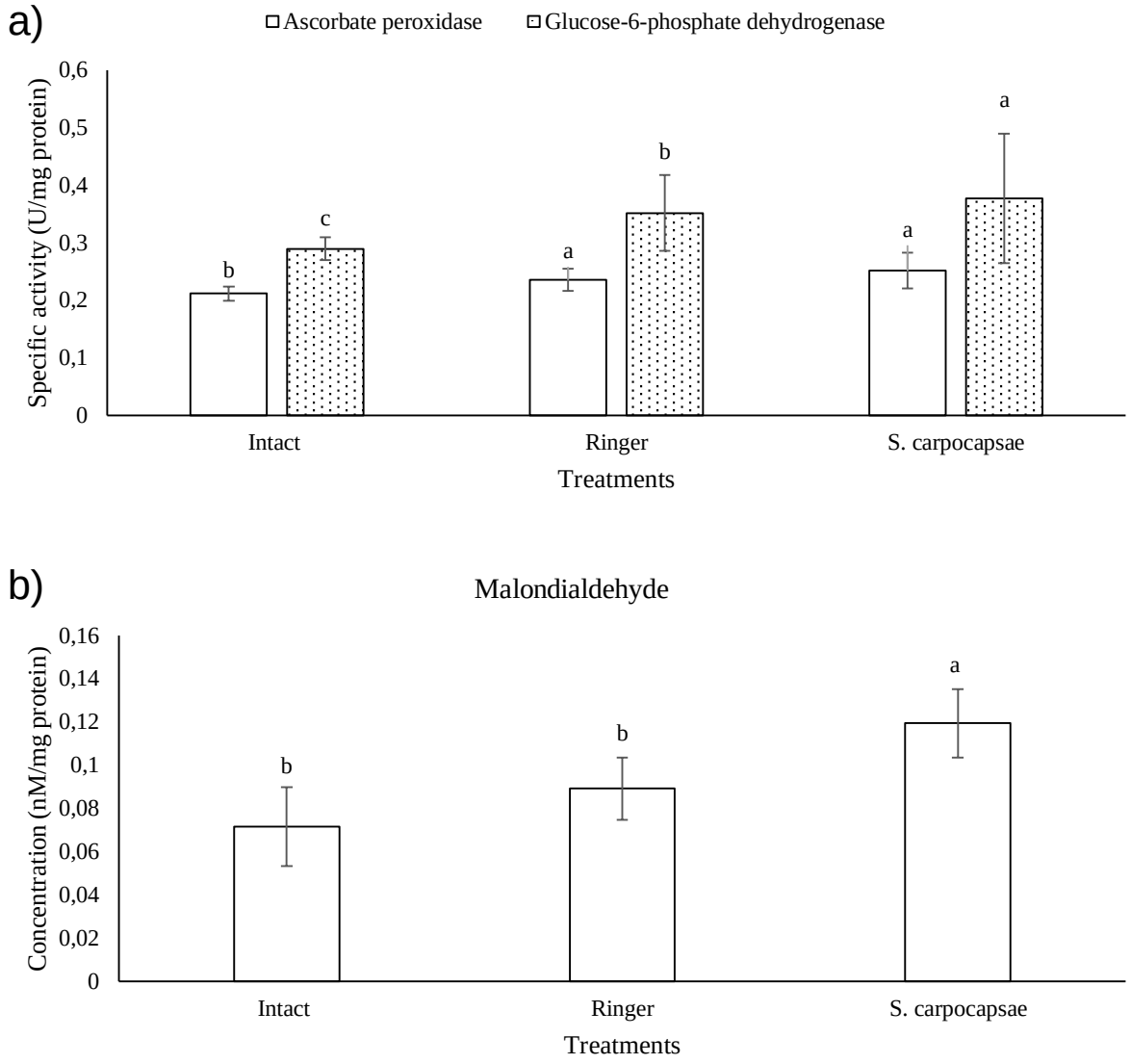

Fig. 5 Effect of *S. carpocapsae* injection on a) ascorbate peroxidase, glucose-6-phosphate dehydrogenase and b) malondialdehyde activities in *G. pyloalis*. Statistical differences have been marked with different letters within treatments (Tukey test, $p \leq 0.05$).

preference of species/isolates, environmental adaptability and pathogenic mechanisms (Shapiro-Ilan *et al.*, 2017). Nevertheless, it has been suggested to use indigenous isolates of entomopathogenic nematodes which have led to more efficiency against target insects because of their compatibility to native habitats (Griffin *et al.*, 2005; Lacey and Georgis, 2012). In the current study, a native isolate of *S. carpocapsae* caused significant mortality on the larvae of *G. pyloalis* within five days after treatment. Our findings showed larval mortality appeared a day post-injection or post-treatment for all concentrations. Finally, the LC_{50} concentration of 582.9 IJs/mL were calculated based on recorded data. Although these results should be compared with bioassays using other nematode species/isolates, but the mortality after 24 h and median lethal concentration found here may confirm efficiency of *S. carpocapsae* (IRA18) against *G. pyloalis*.

Results of the current study demonstrated induction of immune system of *G. pyloalis* larvae though proliferation of hemocytes and higher activity of phenoloxidase. Although the highest total hemocyte count was recorded one hour after nematode injection then it slightly decreased, plasmatocytes remained the highest number after 1 and 3 h while granulocytes kept the highest numbers for all time intervals. It has been found that releasing symbiont bacteria, *Xenorhabdus nematophila*, of *S. carpocapsae* is initiated 2 h after incubation in host hemolymph (Snyder *et al.*, 2007). So it may be

concluded that the initial increase of total hemocyte, plasmatocyte and granulocyte counts in *G. pyloalis* larvae may be due to recognition of *S. carpocapsae* within hemocoel and response of hemocytes to possibly encapsulate nematode. In contrast, reduction in numbers of hemocytes may be attributed to secretion of toxic effects of symbiont bacterial secretion against *S. carpocapsae*. The toxicity may be imposed by lipopolysaccharide, cytolysins, toxins and the pore-forming fimbrial subunit (Herbert and Goodrich-Biair, 2007). These components lead to actin polymerization, destabilizing of cytoskeleton architecture, inhibition of phospholipase A2 and hemocyte apoptosis (Kim *et al*., 2005; Li *et al*., 2009; Eleftherianos *et al*., 2010). Li *et al*. (2009) observed a significant decrease of total hemocyte count in *Helicoverpa armigera* Hubner (Lepidoptera: Noctuidae) following infection with *Ovomermis sinensis* after 4 and 8 h. Similarly, Lalitha *et al*. (2018) showed reduction of total hemocyte counts after 3 h of *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) injection with *H. indica* Poinar et al. due to releasing toxins by *Photorhabdus* spp.

Phenoloxidases are the significant components of insect immunity to complete nodulation/encapsulation responses, clotting of hemolymph, wound healing and production some toxic elements against invading microorganisms. Upon infection, a cascade of serine proteases is activated to initiate activation of proPhenoloxidase (zymogen) via proteolytic cleavage at enzyme polypeptide chain (Nappi and Chistensen 2005). Afterward, activated phenoloxidase finalizes nodulation/encapsulation of invading microorganisms by deposition of melanin in peripheral hemocytes (Cerenius *et al*., 2008). The highest activity of phenoloxidase in *G. pyloalis* was obtained 6 h following *S. carpocapsae* injection then it sharply decreased to 24 h. Similar to hemocyte counts, initial activation of phenoloxidase is related to signal transaction following recognition of nematodes within larval hemocyte which led to increase of hemocyte counts and secretion of phenoloxidase. In contrast, suppression of phenoloxidase at other time intervals may be due to inhibiting signal transaction for immune responses including phenoloxidase by symbiotic bacteria. This phenomenon has already shown by Brivio *et al*. (2002) who reported lipoxygenase-mediated ProPO activation pathway in *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae following *S. feltiae* infection.

Oxidative stress refers to production of reactive oxygen species (ROS) in response to environmental extremes like temperature, chemicals and microorganisms which should be overcome by host to prevent cell damage, protein denaturation, lipid peroxidation, inhibition of DNA replication and mutation (Felton and Summers, 1995; Zhang *et al*., 2015). In details, organisms, e.g. insects, utilize a set of components including catalases, peroxidases, superoxide dismutases, ascorbate peroxidases, glucose-6-phosphate dehydrogenase and malondialdehyde known as antioxidant components to prevent biological damages. The thee antioxidant enzymes, superoxidase dismutase, catalase and peroxidase, have a sequential activity in which the first one transforms superoxide anions to hydrogen peroxide and oxygen while the two other enzymes break down hydrogen peroxide in stressed tissue (Gaetani *et al*. 1996; Halliwell, 1999; Zelko *et al*. 2002). Ascorbate peroxidase destroys hydrogen peroxide though concurrent oxidation of ascorbate although glucose-6-phosphate dehydrogenase deals with oxidation-reduction and decontamination of oxidant agents though production of NADPH to neutralize ascorbate peroxidase productions (Asada 1984). Glutathione *S*-transferase is one of the critical enzymes in detoxifying mechanisms against xenobiotics that removes products of lipid peroxidation or hydroperoxides once oxidative stress is induced within host tissues (Rahimi *et al*., 2018). Malondialdehyde is a peroxidation product of unsaturated fatty acids from phospholipids so it is an index of cell membrane damages. In the current study, injection of *S. carpocapsae* caused induction of antioxidant enzymes in *G. pyloalis* except for peroxidase and glutathione *S*-transferase once DCNB was used as substrate. In details, no statistical difference in activity of peroxidase may be attributed to sufficient activity of catalase to remove hydrogen peroxide. Moreover, findings on glutathione *S*-transferase indicate involvement one of the isozymes in antioxidant function of the enzyme. Finally, induction of malondialdehyde in nematode injected *G. pyloalis* may refer to cell damaging function of secondary metabolites from symbiotic bacteria of *S. carpocapsae*. Lalitha *et al*. (2018) believe that production of ROS in infected insects with entomopathogenic nematodes is due to synthesis of secondary metabolites from symbiotic bacteria and immune function of host insects to limit microbial growth within hemocoel. These authors reported the highest activities of superoxide dismutase and catalase after 6 h, peroxidase after 24 h and glutathione *S*-transferase after 9 h following injection with *H. indica*. Krystyna *et al*. (2006) demonstrated that activity of superoxidase dismutase increased 12 h in *G. mellonella* larvae injected by *S. feltiae*.

Conclusions

Results of our study indicated that native isolate of *S. carpocapsae* could infect the larvae of *G. pyloalis* and interfered in immune responses and antioxidant system. Enhanced hemocytes number and phenoloxidase activity in the early hours following injection show a rapid diagnosis of nematodes in host hemolymph, but suppression recorded in these immune factors may be related to negative effects of the secondary metabolites produced by symbiotic bacteria of *S. carpocapsae*, which can also lead to oxidative stress and subsequent induction of antioxidant system of larvae. In general, this immunodeficiency and oxidative stress emboss the proper function of *S. carpocapsae* against *G. pyloalis*.

Acknowledgement

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