RESEARCH REPORT

# Immunological and antioxidant responses of larval *Helicoverpa armigera* (Lepidoptera: Noctuidae) to gibberellic acid in the diet

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## Abstract

Gibberellic acid (GA3) is usually used as a plant growth regulator to enhance the quality and quantity of crop yield. Moreover, GA3 may also affect insects and other herbivores that rely on a particular plant for their living. Hence, in the present study the defensive mechanisms of *Helicoverpa armigera* (Hübner) fed on the diet contating GA3 have been considered. The larvae fed with GA3 significantly exhibited cellular and humoral inhibitory responses such as, reduced nodule formation and phenoloxidase activity. Antioxidant system was also affected; the lowest activity of peroxidase, catalase, superoxide dismutase, ascorbate peroxidise, glucose-6-phosphate dehydrogenase, and glutathione S-transferases was observed in control larvae while the highest activity was found in those larvae that were provided with a diet containing 800 µg/g GA3. The metabolites like triglyceride, glycogen, and cholesterol were reduced compared to the control. It is concluded that the use of plant growth regulators like GA3 not only does benefit plants for their growth and yield, but also can somehow help plants to withstand the impact of herbivores. Hence, studies covering direct field collected insects from crop plants treated with PGRs would be beneficial for further studies.

**Key Words**: cellular immunity; detoxifying enzymes; oxidative stress; phenoloxidase

## Introduction

*Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a cosmopolitan insect pest of various crops such as tomato, soybean, pea, cowpea, and chickpea (Sharma, 2005). Gibberellic acid (GA3) as a plant growth regulator is being used in certain crops to improve the quality and quantity of the yield. For example, tomato crop (Gelmesa *et al.*, 2012; Ning and Subroto, 2018), soybean, cowpea (Leite *et al.*, 2003; Emongor, 2007), chickpea, and pea (Bora and Sarma, 2006; Mazid, 2014).

In addition, the adverse impacts of GA3 use on insects have also been worked out in some studies. For example, a reduction in fecundity and fertility and shortening of the longevity has been reported in *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae) (Kaur and Rup, 2002; Kaur and Rup, 2003a). Abdellaoui *et al.* (2009) demonstrated that the use of GA3 in the diet led to higher mortality in *Spodoptera littoralis* F. (Lepidoptera, Noctuidae) and also in *Locusta migratoria* L. (Orthoptera, Acrididae).

The effects were particularly evident in discrepancy occurred in exuviations. The damages were also traced in target tissues of foregut and gastric caeca of *L. migratoria* (Abdellaoui *et al.*, 2009). A decrease in most of the stored energy by GA3 has been reported in the hemolymph of *Galleria mellonella* L. (Lepidoptera: Pyralidae) (Uckan *et al.*, 2011). Altuntaş *et al.* (2014) showed undulating titre in haemolymph free amino acids in *G. mellonella* larvae with different concentrations of GA3 used. Gibberellic acid also significantly reduced proteins, carbohydrates, and lipids in the ovaries of *L. migratoria*, as well as the amounts of ecdysteroid in ovaries and freshly laid eggs. In addition, GA3 significantly reduced both fecundity and fertility in *L. migratoria* (Abdellaoui *et al.*, 2015).

To the best of our knowledge, there is no report so far considering GA3 on *H. armigera* and specifically looking into the mode of action of this beneficial compound. Therefore, various aspects of its effect on *H. armigera* were investigated including life table parameters (Shayegan *et al.*, in press) and digestion (Shayegan *et al.*, in press), and here we report its effect on immunity and antioxidant system which has remained unexplored in the studied insects.

Immune responses in insects under the effect of various stresses including xenobiotics are depicted

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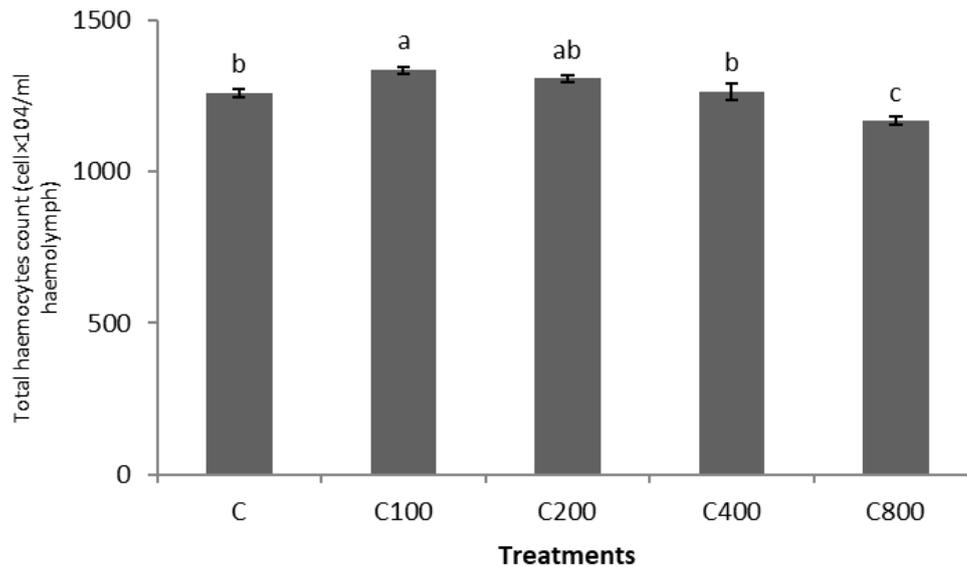
**Fig. 1** The total hemocyte numbers in the sixth instar *H. armigera* larvae fed on control and different concentrations of gibberellic acid (C= 0 µg/g diet - C800 = 800 µg/g diet). Statistical differences have been done within each treatments and marked by various letters (at $p < 0.05$, Tukey's test)

in the of form cellular and humoral responses. Cellular responses often involve hemocytes (particularly granulocytes and plasmatocytes) that are directly responsible for processes such as phagocytosis and nodule formation against foreign bodies entering hemolymph. The humoral responses include antimicrobial peptides and prophenoloxidase system that are responsible for eliminating the pathogens (Beckage, 2011). Antioxidants are compounds that inhibit oxidation and removes reactive oxygen species (ROS) which can damage the cells of any organism (Felton and Summers, 1995; Akbar *et al.*, 2012). Similarly, detoxifying enzymes like general esterases and glutathione S-transferase react against external stimulants and are also considered as a part of insect defense mechanism (Grant and Matsumura, 1989; Hemingway and Karunaratne, 1998; Zibaee and Bandani, 2010; Valizadeh *et al.*, 2013).

There are some works which explore the effect of GA3 on insects, including *L. migratoria* and *G. mellonella* (Uckan *et al.*, 2011; Altuntaş *et al.*, 2014; Abdellaoui *et al.*, 2015). It is a common belief that plant growth regulators (PGRs) like GA3 benefit plants in terms of their growth and yield. This compound can also somehow help plants to withstand the pressures implemented through herbivores. Our main aim in the present study was to explore the mechanisms indulged in reduction of insect immunity and make them susceptible to various environmental pressures.

**Materials and Methods**

*Insect rearing and experimental conditions*

The larvae of *Helicoverpa armigera* (Hübner) were collected from tomato farms at the University of Guilan in the city of Rasht in northern Iran (37°16′51″N 49°34′59″E). They were individually reared on artificial diet containing cowpea (204 g), yeast (30 g), wheat germ (30 g), ascorbic acid (3.5 g), sorbic acid (1.3 g), formalin (2.7 mL), refined sunflower oil (4 mL), agar (14 g), and distilled water (600 mL) (Shorey and Hale, 1965). The insects were reared on this diet for two generations and the third generation was provided with the same diet including gibberelic acid (Suvchem, Mumbai, Maharashtra, INDIA). The concentrations included 100, 200, 400, and 800 µg/g diet (denoted as C100, C200, C400, and C800, respectively). The rearing jars included transparent plastic containers (10×5×5 cm) maintained at 26 ± 1 °C, 65 ± 5% RH, and a photoperiod of 16:8 hours (L:D). The adult moths of both sexes were released into cages made of transparent PVC (14×9×20 cm), enclosed at the top with a fine-mesh net. A cotton wool soaked in 10% honey solution was provided to the moths. The hatched larvae were directly collected from the rearing boxes using a soft brush. They were reared on artificial diet including various concentrations of GA3. The desired concentrations of GA3 were made in distilled water and then were mixed with the diet. The controls received only equal amounts of distilled water in the diet. Thirty first instar larvae were individually reared on the diet containing different concentrations of GA3. They were maintained on their respective diet up to the sixth instar larval stage. Similarly, the controls were provided with their own diet. Totally, 150 larvae were used in these experiments.

*Total and differential hemocyte count (THC and DHC)*

The hemolymph of 48 hours sixth instar larvae (in all concentrations and controls) were collected from the first abdominal proleg. For THC a Neubauer
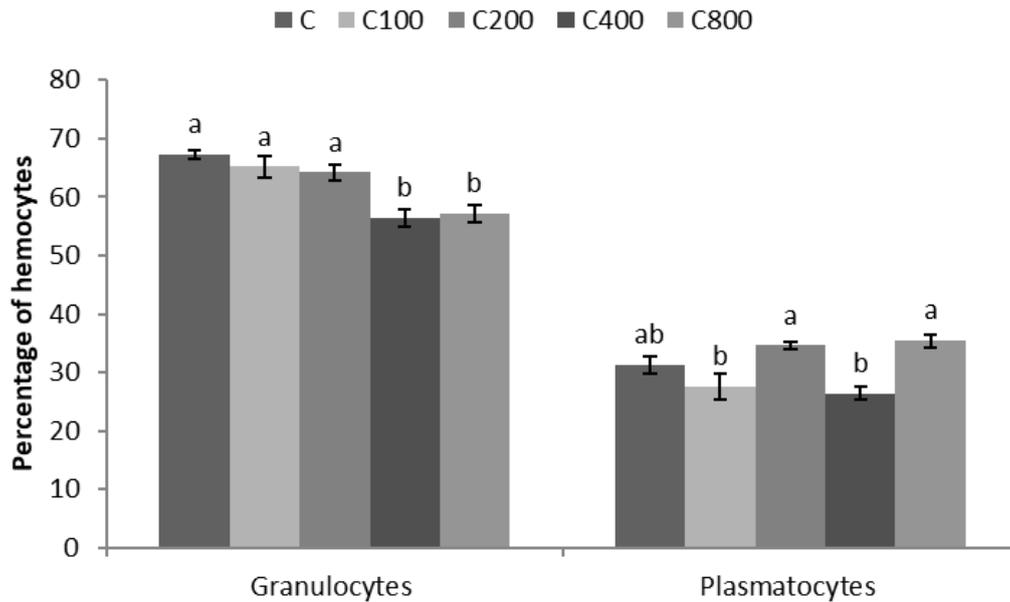
**Fig. 2** Percentage of granulocytes and plasmatocytes in the sixth instar *H. armigera* larvae fed on control and different concentrations of gibberellic acid (C= 0 μg/g diet - C800 = 800 μg/g diet). Statistical differences have been done within each treatments and marked by various letters (at *p*< 0.05, Tukey's test)

hemocytometer (HBG, Germany) was used. For this purpose, 10 μL hemolymph was mixed with 290 μL of anticoagulant solution (0.017 M EDTA, 0.041 M Citric acid, 0.098 M NaOH, 0.186 M NaCl, pH 4.5) (Gupta,1979; Amaral *et al.*, 2010). The DHC was counted using heat fixed larvae (larvae were immersed in hot distilled water (60 °C) for five min), after drying with blotting paper, the first abdominal proleg was excised and a drop of hemolymph was released on a clean slide and a smear was made using another slide. The air-dried smears were stained with 1 to 10 diluted stock Giemsa (Merck, Germany) and after 14 min the slides were washed by distilled water. The smears were dipped for 5 sec in saturated lithium carbonate (LiCO$_3$) for differentiation of cytoplasm and nucleus and then washed again in distilled water for a few minutes, dried at room temperature and then permanent slide was prepared in Canada balsam (Merck, Germany). The cells were identified based on the morphological characteristics observed under a microscope (Leica light-microscope) (Baghban *et al.*, 2018). Two hundred cells were randomly counted from four corners and central part of each slide (Wu *et al.*, 2016). Totally, 800 cells from four individuals were counted and percentage of each cell type was calculated. The number of cells in controls was also simultaneously recorded.

*Immune response assay*
The latex beads were used to stimulate the immune system (Borges *et al.*, 2008) of *H. armigera* in order to explore the interaction of gibberellic acid and its immunity response (i.e. nodulation process). Therefore, the larvae fed on GA3-mixed diet and control were injected with latex beads (10 distillate

latex beads with distilled water using 10 μL Hamilton syringe). The THC and DHC were counted at 3, 6, 12, 24, and 48-hour time intervals post injection as the method described above. Effect of latex beads on nodulation response was counted at 3, 6, 12, 24, and 48-hour time intervals post injection. For this purpose the hemolymph was collected from each larvae, then samples in four replicates were poured into the hemocytometer, and then the number of nodules were counted (Franssens *et al.*, 2006, Seyedtalebi, 2017).

*Phenoloxidase activity assay*
For measuring phenoloxidase activity, 10 μL of hemolymph and 90 μL of ice-cold sterile phosphate buffered saline (PBS) (0.13 M NaCl, 2.68 mM KCl, 8.1 mM Na$_2$HPO$_4$ and 1.47 mM KH$_2$PO$_4$, pH 7.4, autoclaved) were used. The L-DOPA (3,4-dihydroxyphenylalanine) (10 mM, Sigma-Aldrich Co., USA) was used as the substrate for assaying this enzyme using the method designed by Catalán *et al.* (2012), with some modifications. For this purpose, samples were centrifuged at 5,000 g at 4 °C for 5 min. Then 50 μL of hemolymph-buffer supernatant was added to 150 μL of L-DOPA. The activity measurement of phenoloxidase was read at 490 nm during the linear phase of the reaction. Calculation of specific activity was based on dividing absorbance with protein content in hemolymph using a microplate reader (Awareness Technology Inc., Florida, USA) (Khosravi *et al.*, 2014; Baghban *et al.*, 2018). Protein contents were quantified by the method of Lowry (1951) (using the manufacturer's procedure, Biochem Co., Iran). To compute the specific activity of all enzymes, the absorbance values were divided by protein content.
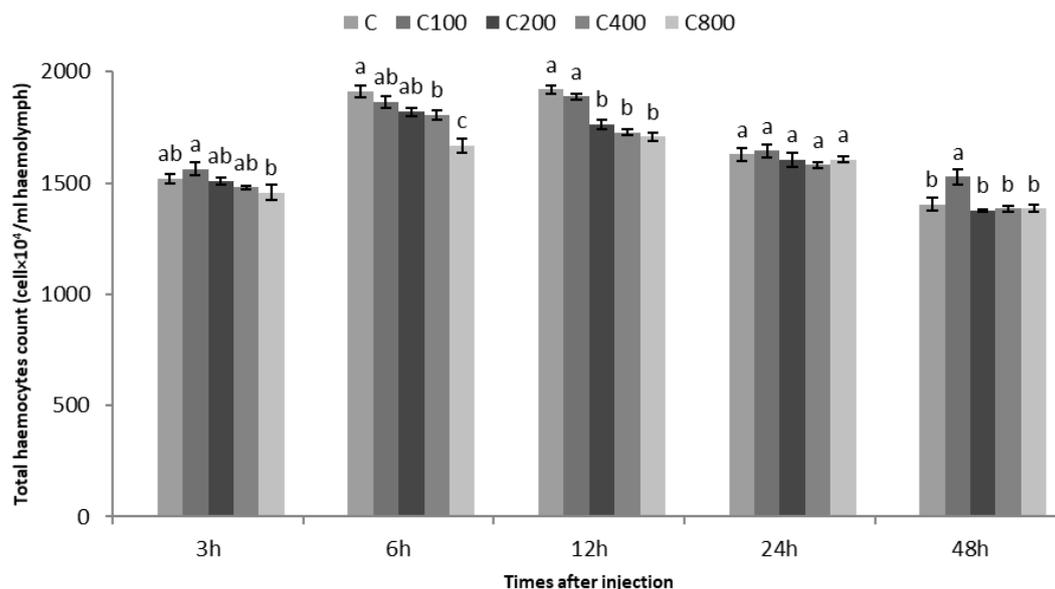
**Fig. 3** The effects of different concentrations of gibberellic acid (C= 0 µg/g diet - C800 = 800 µg/g diet) on total hemocyte numbers in the sixth instar *H. armigera* larvae after injection of latex beads. Statistical differences have been done within each time intervals and marked by various letters (at $p < 0.05$, Tukey's test)

*The antioxidant defense assays*
*Sample Preparation*

Nine larvae for every treated diet were randomly selected and the whole larvae were homogenized by a hand homogenizer in PBS (10 mM). Then, the homogenate was centrifuged at 28.600x*g* for 20 min at 4 °C. Supernatants were used to determine enzymes activities of *H. armigera* larvae. Protein contents were quantified by the method of Lowry (1951) (using the manufacturer's procedure, Biochem Co., Iran). To obtain the specific activity of all enzymes, the absorbance values were divided by protein content.

*Peroxidase assay*

The method described by Addy and Goodman (1972) was used for determination of the activity of this enzyme. Hence, 3 mL of pyrogallol buffer (0.05 M pyrogallol was mixed with 0.1 M PBS (pH 7.0)) were mixed with 500 µL of 1% $H_2O_2$. Then, 50 µL of sample was added. The absorbance was recorded every 30 sec for two min at 430 nm. Finally, the activity was calculated by an extinction coefficient of oxidized pyrogallol (4.5 liters/mol).

*Superoxide dismutase assay*

According to the method of McCord and Fridovich (1969), 80 µL of samples were added to 500 µL of reaction solution (70 µM of nitroblue tetrazolium and 125 µM of xanthine dissolved in PBS, and were mixed with 20 µL of xanthine oxidase solution containing 10 mg of bovine serum albumin). Then, 100 µL of xanthine oxidase (5.87 U/mL) (dissolved in 2 mL of PBS) was added to the reaction mixture and the reaction was initiated in darkness at 28 °C for 20 min. Absorbance was read at 560 nm.

*Catalase assay*

Catalase assay was performed following the method of Wang *et al.* (2001) with slight modification. The reaction mixture containing 50 µL of the sample and 500 µL of Hydrogen peroxide (1%) was incubated at 28 °C for 10 min. The absorbance was read at 240 nm.

*Ascorbate peroxidase assay*

For ascorbate peroxidase assay, 750 µL of sample was added to 250 µL PBS (67 mM, pH 7). Then, 70 µL of ascorbic acid (2.5 mM) and 200 µL of hydrogen peroxide (30 mM) were added to the reaction mixture. The absorbance was read at 290 nm for 5 min (Asada, 1984).

*Glucose-6-phosphate dehydrogenase assay*

According to the method of Balinsky and Bernstein (1963), 100 µL of Tris-HCl (2-amino-2-(hydroxymethyl) propane-1,3-diol; hydrochloride) (100 mM, pH 8.2) was mixed with 30 µL of $MgCl_2$ (0.1 M), 50 µL of NADP (0.2 mM), 50 µL of water, 50 µL of the sample, and 100 µL of glucose-6-phosphate dehydrogenase (6 mM). The absorbance of the reaction mixture was read at 340 nm.

*The detoxifying enzymes assay*
*Sample Preparation*

Nine larvae for every treated diet were randomly selected and the whole larvae were homogenized by a hand homogenizer in PBS (10 mM) and the homogenate was centrifuged at 13000 rpm for 20 min at 4 °C. Supernatants were used for determining enzymes activities of *H. armigera* larvae. Protein contents were quantified by the method of Lowry (1951) (using the manufacturer's procedure, Biochem Co., Iran). To compute the

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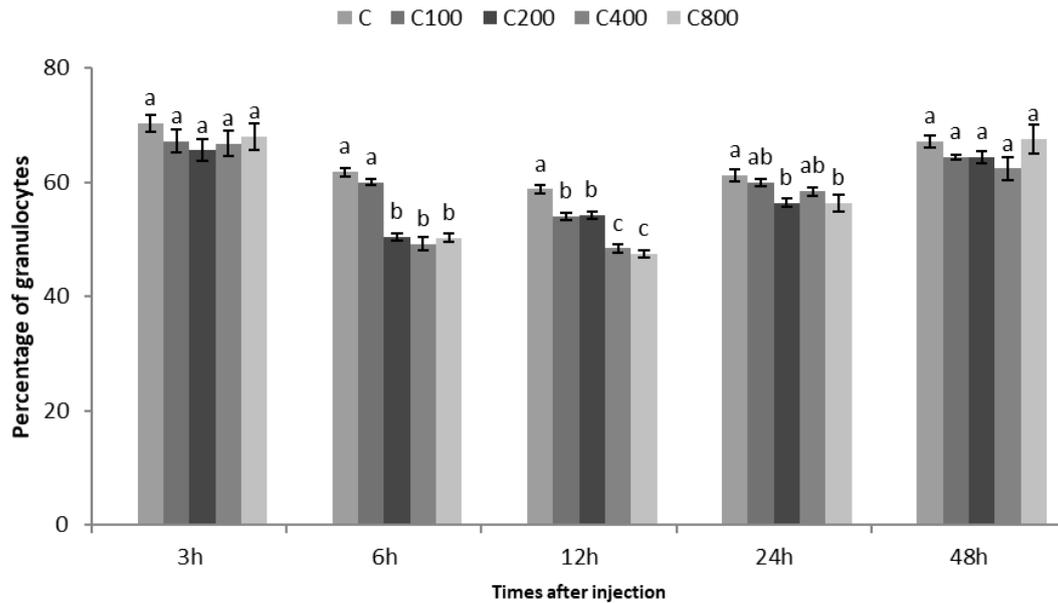
**Fig. 4** The effects of different concentrations of gibberellic acid (C= 0 μg/g diet - C800 = 800 μg/g diet) on percentage of granulocytes in the sixth instar *H. armigera* larvae after injection of latex beads. Statistical differences have been done within each time intervals and marked by various letters (at *p*< 0.05, Tukey's test)

specific activity of all enzymes, the absorbance values were divided by protein content.

*Esterase activity assay*

Esterase activity was measured by α-naphthyl acetate and β-naphthyl acetate (10 mM) as substrates, separately. Twenty μL of each substrate, 50 μL fast blue RR salt (1 mM), and 20 μL of enzyme solution were mixed and incubated for 1 min and then the absorbance was recorded at 450 nm (Han *et al.*, 1998).

*Glutathione S-transferase assay*

Twenty μL of CDNB (1-chloro-2, 4-dinitrobenzene, 20 mM) and DCNB (1, 2-dichloro-4-nitro-benzene, 40 mM) were separately added to 10 μL of the sample and 1 mM glutathione. The GST activity was read at 340 nm/min/mg protein after 5 min (Oppenoorth *et al.*, 1979).

*Amount of protein, glycogen, triglyceride, cholesterol, and glucose*
*Protein*

Protein contents of the treated-larvae and control were assayed according to the method of Lowry *et al.* (1951) (recommended by Biochem Co., Iran).

*Glycogen*

For glycogen assay, the fat body of the control and treated-larvae were plunged in tubes contained 1mL of lysis buffer (30% KOH with $Na_2SO_4$), at first. Then the samples of tubes were boiled for 20-30 min. Then the tubes were shaken and cooled in ice. For precipitation of glycogen from the samples, 2 mL of 95% EtOH was added and then was shaken

again and cooled in ice for 30 min. Then the samples were centrifuged done at 22,000 g for 30 min. The pellets (containing glycogen) were transported to new tubes and 1 mL of distilled water was added to the tubes and was shaken. The samples were mixed with 5% phenol and incubated in ice bath for 30 min. The absorbance of samples was read at 492 nm. A glycogen standard curve was used for calculating the glycogen concentration (Chun and Yin, 1998).

*Triglyceride*

A diagnostic kit (Pars Azmoon Co., Iran) was used for triglyceride assay. The method was based on the method of Fossati and Prencipe (1982), the reaction mixture included 10 μL of sample and 70 μL of reagent containing phosphate buffer saline (50 mM, pH 7.2), 4-chlorophenol (4 mM), adenosine triphosphate (2 mM), Mg2+ (15 mM), glycerokinase (0.4 kU/L), peroxidase (2 kU/L), lipoprotein lipase (2 kU/L), 4-aminoantipyrine (0.5 mM), and glycerol-3-phosphate-oxidase (0.5 kU/L). The reaction was done for 15 min at 25 °C. The samples and reagent absorbance were read at 546 nm. To obtain of the amount of triglyceride, the following equation was used:

Mg/dl= (OD of sample)/ (OD of standard) ✕ 0.01126

*Cholesterol*

Cholesterol was measured based on Richmond's method (Richmond, 1973) and by utilizing a total cholesterol assay kit (Pars Azmoon Co., Iran). This method is based on hydrolyzing cholesterol esters with cholesterol oxidase, cholesterol esterase and peroxidase.
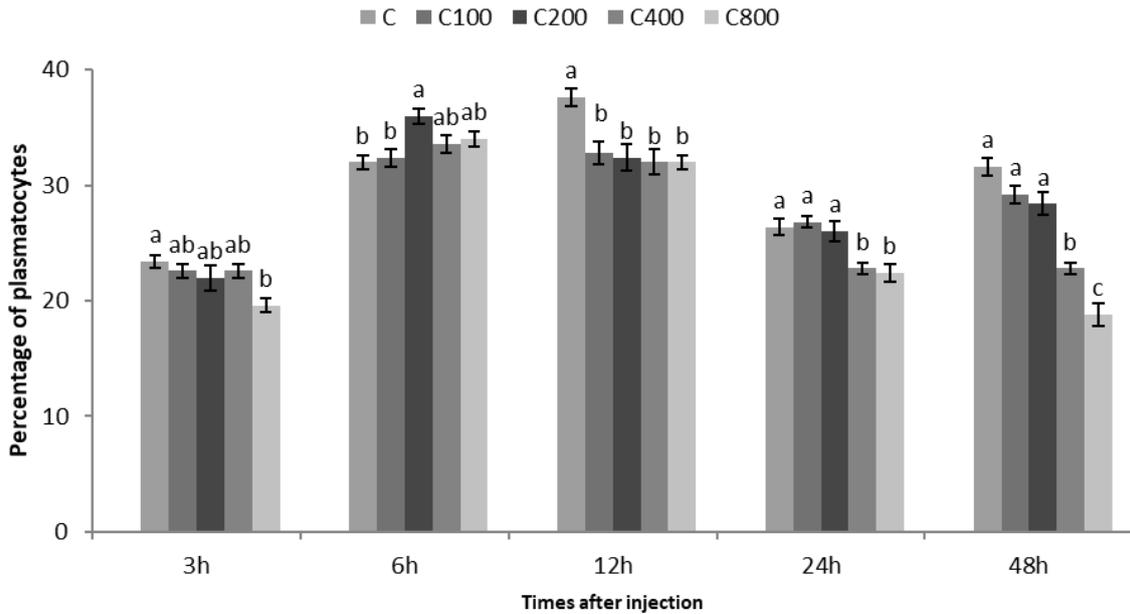
**Fig. 5** The effects of different concentrations of gibberellic acid (C= 0 µg/g diet - C800 = 800 µg/g diet) on percentage of plasmatocytes in the sixth instar *H. armigera* larvae after injection of latex beads. The Statistical differences have been done within each time intervals and marked by various letters (at *p*< 0.05, Tukey's test)

*Glucose*

Glucose assay was performed by a diagnostic kit (Pars Azmoon Co., Tehran, Iran) based on the procedure of Siegert (1987). Whole body (100 µL) added to 500 µL of 0.3 N perchloric acid and centrifuged at 12000g for 10 min. For determination of glucose concentrations, the supernatants were used.

*Statistical analysis*

The data obtained are presented as means ± standard error. All data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett's test). The data were compared by one-way analysis of variance (ANOVA). Differences between the treatments were determined by Tukey's family error rate by Minitab® statistical software. The statistical differences were considered at a probability less than 5%.

**Results**

*The reaction of cellular immunity of* H. armigera *against GA3*

Effects of GA3 on total hemocyte count (THC) are shown in figure 1 (F= 14.98; df= 4, 19; p< 0.05). As depicted in figure 1 the lowest THC are observed in those larvae that were reared on C800 diet. Differential hemocyte counts (DHC) in the highest concentration of GA3 resulted in significant reduction of granulocytes (Fig. 2) (F= 11.47; df= 4, 19; p< 0.05). This reduction was also evident (56.4% and 57.2%) for C400 and C800 diets, respectively. However, amount of 67.2% was recorded for granulocytes in control. Percentage of plasmatocytes in any of the treatments did not significantly alter (Fig. 2).

Figure 3 demonstrates the effects of GA3-diet treated diet on THC following injection of latex beads. A significant decrease in THC was detected six hours (F= 13.62; df= 4, 19; p< 0.05) and 12 hours (F= 32.01; df, 19= 4; p< 0.05) after injection of latex beads to the larvae fed on C400 and C800 diets, while there were no significant changes in these treatments at 3, 24, and 48 h after injection. Percentage of granulocytes in C200, C400 and, C800 treatments did not change significantly after three hours, but significantly decreased after six hours (Fig. 4) (F= 57.69; df= 4, 19; p< 0.05). Percentage of these cells was lower than control in all treatments after 12 hours (F= 49.79; df= 4, 19; p< 0.05), while after 24 hours the percentage of cells was significantly lower only in C200 and C800 treatments (F= 4.86; df= 4, 19; p< 0.05). After 48 hours, the percentage of granulocytes did not change significantly in different treatments (Fig. 4). On the other hand, after the latex beads infusion into larvae reared on different GA3 treatments, significant changes were observed in the percentage of plasmatocytes (Fig. 5). After three hours, all treatments except C800 showed no significant differences with the control (F= 3.99; df= 4, 19; p< 0.05), while after six hours, the control showed lower levels than treatments above C100 (F= 5.34; df= 4, 19; p< 0.05). Twelve hours after the latex beads injection, the percentage of plasmatocytes in the control larvae was significantly higher than those in the larvae fed with GA3 diet (F= 6.28; df= 4, 19; p< 0.05). After 24 and 48 hours, these cells were lower only in C400 and C800 treatments (F= 9.23; df= 4, 19; p< 0.05 and F= 39.72; df= 4, 19; p< 0.05, respectively).
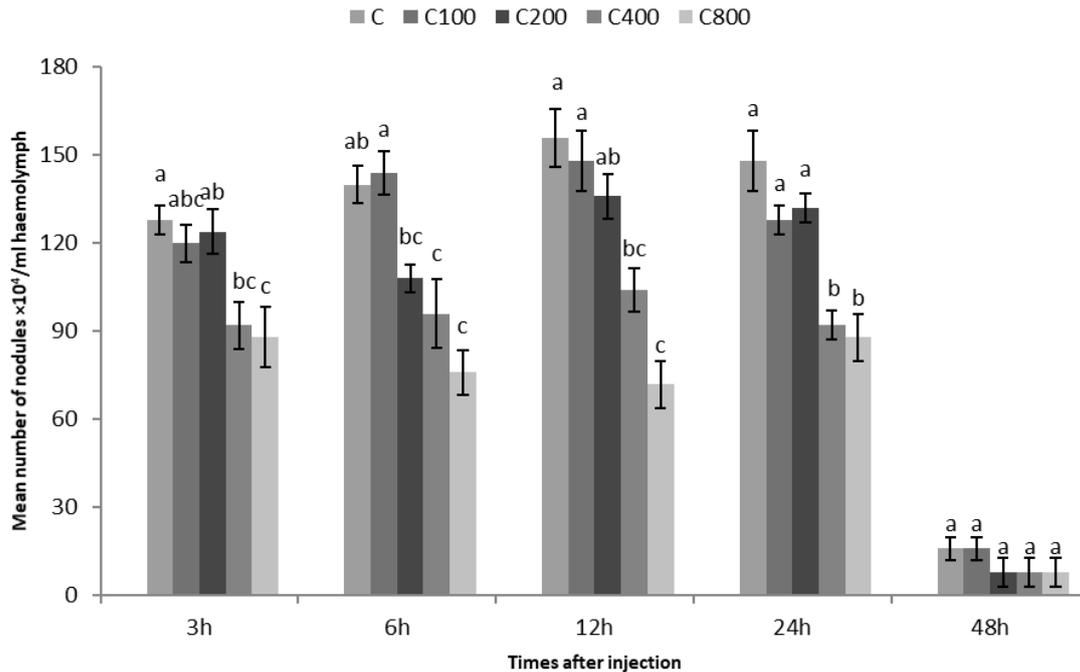
**Fig. 6** The effects of different concentrations of gibberellic acid (C= 0 μg/g diet - C800 = 800 μg/g diet) on nodule formation in the sixth instar *Helicoverpa armigera* larvae after injection of latex beads. Statistical differences have been done within each time intervals and marked by various letters (at *p*< 0.05, Tukey's test)

The larvae fed on the diet containing GA3 significantly inhibited the nodule formation after injection of latex beads (Fig. 6). In all time intervals except for 48 hours, the number of nodules in the larvae fed by C400 and C800 diet was lower than control (Fig. 6). In all treatments, 24 and 48 hours after injection the phenoloxidase activity was enhanced. The larvae feeding on the highest concentration of GA3 (C400 and C800) demonstrated the lowest phenoloxidase activity in all time intervals (Fig. 7).

*Antioxidant systems of* H. armigera *against GA3*

Feeding on the diet containing GA3 caused different effects on antioxidant enzymes of *H. armigera* (Table 1). The lowest activity of peroxidase and catalase was observed in the control larvae (0.063 and 0.794 U/ mg protein, respectively), while the highest activity was found in those larvae feeding on C800 diet (0.120 and 1.892 U/ mg protein respectively) (F= 26.48; df= 4, 44; p< 0.05 and F= 14.08; df= 4, 44; p< 0.05, respectively). The activity of superoxide dismutase, ascorbate peroxidase and glucose-6-phosphate dehydrogenase was the lowest in the control larvae, but it increased in GA3-diet fed larvae in a dose-dependent manner (F= 148.03; df= 4, 44; p< 0.05, F= 615.48; df= 4, 44; p< 0.05 and F= 36.23; df= 4, 44; p< 0.05, respectively) (Table 1).

*The reaction of the detoxifying enzymes of* H. armigera *against GA3*

The larvae bred on higher concentrations of GA3 depicted a statistically significant raise in

esterase activities only when β naphtyl acetate was used as a substrate (F= 40.06; df= 4, 44; p< 0.05) (Table 2). In the case of glutathione S-transferase, enzymatic activities of larvae fed on C800 diet were significantly higher than those of control.

*Effect of GA3 on protein, glycogen, triglyceride, cholesterol, and glucose content of* H. armigera

Effect of GA3 on macromolecules in the body of *H. armigera* larvae are shown in Table 3. No significant differences were found in the amount of protein and glucose, while the lowest amount of triglyceride was observed in larvae fed on GA3 diet compared to the control (F= 32.40; df= 4, 44; p< 0.05). The larvae fed on C800 had the lowest amount of glycogen and cholesterol (F= 11.21; df= 4, 44; p< 0.05 and F= 43.48; df= 4, 44; p< 0.05, respectively).

**Discussion**

Exogenous compounds could affect the number of circulating hemocytes (Perez and Fontanetti, 2011). Our results indicate that feeding on diet containing gibberellic acid affect the number of circulating hemocytes, especially granulocytes. It is inferred from the reducing number of circulating hemocytes, that the hemocytes might have migrated to damaged tissues in order to phagocytize the remains of the damaged tissues (Pipe and Coles, 1995). We have noticed damages to the midgut under the effect of GA3 feeding (unpublished data). Reports by Abdellaoui *et al.* (2009; 2013) also reported damages implicated on the digestive system, which support our own results. The THC and
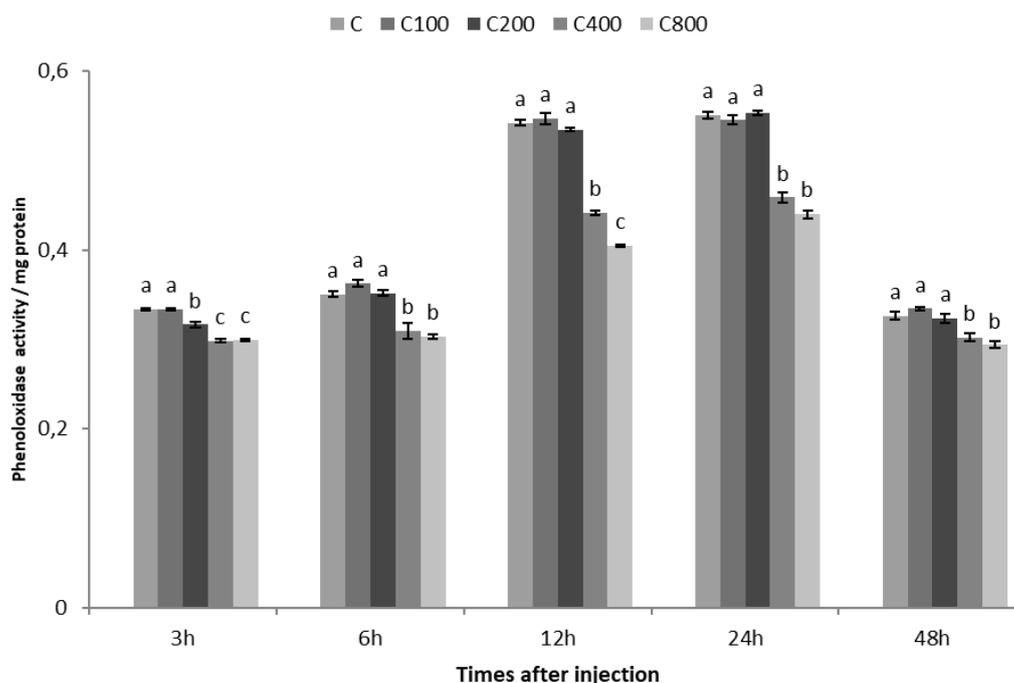
**Fig. 7** The effects of different concentrations of gibberellic acid (C= 0 µg/g diet - C800 = 800 µg/g diet) on the phenoloxidase activity in the sixth instar *H. armigera* larvae after injection of latex beads. Statistical differences have been done within each time intervals and marked by various letters (at *p*< 0.05, Tukey's test)

DHC (granulocytes and plasmatocytes) reduction have been shown in *G. mellonella* under the effect of abscisic acid, another PGR (Er and Keskin, 2016). Paradoxically, there is a report showing an increased THC in *G. mellonella* larvae fed on diet treated with gibberellic acid. However, DHC in granulocytes and plasmatocytes did not change significantly (Altuntaş *et al.*, 2012).

Insects show cellular immune defense by phagocytosis, encapsulation, or nodulation against invaders (Er and Keskin, 2016). In order to confirm the hypothesis that GA3 weakens the immune system, we undertook the experiment of injecting latex beads. A trend of reducing hemocytes confirms that the immunity of our insect has been

affected by feeding on GA3 treated-diet. The decrease in insect's ability to confront foreign bodies is similar to the reports by Altuntas *et al.* (2012) when using GA3 in the diet of *G. mellonella*. The mode of action of GA3 is rather variable and one of the possible modes could be that this hormone might act the way juvenile hormone (JH) does to the insect (Kaur and Rup, 2002; Abdellaoui *et al.*, 2015). Structural similarity of PGRs to insect indigenous JH might reflect similar mode of action. Several studies have shown that juvenile hormone and their analogues reduce the ability of insect larvae to form nodules by inhibiting cell proliferation (Rantala *et al.*, 2003; Franssens *et al.*, 2006; Zibaee *et al.*, 2012).

**Table 1** Antioxidant enzyme activities of sixth instar *H. armigera* larvae provided with different concentrations of gibberellic acid in their diet

| Enzymes | Specific activity of enzymes (U/ mg protein) | | | | |
|---|---|---|---|---|---|
| | C | C100 | C200 | C400 | C800 |
| **Peroxidase** | 0.063±0.003[c] | 0.097±0.004[b] | 0.111±0.002[a,b] | 0.108±0.002[a,b] | 0.120±0.006[a] |
| **Superoxide dismutase** | 0.073±0.002[d] | 0.292±0.033[c] | 0.408±0.011[b] | 0.430±0.005[b] | 0.606±0.003[a] |
| **Catalase** | 0.794±0.140[c] | 0.965±0.115[b,c] | 1.643±0.119[a] | 1.366±0.089[a,b] | 1.892±0.136[a] |
| **Ascorbate peroxidase** | 0.232±0.014[d] | 0.732±0.038[c] | 1.207±0.018[b] | 1.234±0.032[b] | 1.944±0.013[a] |
| **Glucose-6-phosphate dehydrogenase** | 0.199±0.003[d] | 0.269±0.004[c] | 0.366±0.010[b] | 0.383±0.003[b] | 0.502±0.021[a] |

Means with the same letters in a row are not significantly different at *p* < 0.05 (Tukey's test)

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**Table 2** Detoxifying enzyme activities of sixth instar *H. armigera* larvae provided with different concentrations of gibberellic acid in their diet

| Enzymes | Enzyme activity (OD/ min) | | | | |
|---|---|---|---|---|---|
| | C | C100 | C200 | C400 | C800 |
| **General esterases (α-naphtyl acetate)** | 0.054±0.007[a] | 0.053±0.001[a] | 0.055±0.002[a] | 0.055±0.005[a] | 0.056±0.004[a] |
| **General esterases (β-naphtyl acetate)** | 0.077±0.004[c] | 0.101±0.007[b] | 0.095±0.012[b,c] | 0.127±0.004[a] | 0.138±0.001[a] |
| **Glutathione S-transferases (CDNB)** | 0.163±0.008[c] | 0.173±0.013[c] | 0.193±0.020[c] | 0.303±0.012[b] | 0.450±0.017[a] |
| **Glutathione S-transferases (DCNB)** | 0.270±0.017[b] | 0.320±0.028[b] | 0.486±0.044[b] | 0.486±0.046[b] | 1.273±0.093[a] |

The activity of glutathione S-transferase using CDNB (1-chloro-2, 4- dinitrobenzene) and DCNB (1, 2-dichloro-4-nitro-benzene) as reagents and the activity of general esterases using α-naphtyl acetate and β-naphtyl acetate as substrates. Means with the same letters in a row are not significantly different at $p < 0.05$ (Tukey's test)

Humoral immunity in insects is considered as complementary to cellular immunity by production of antimicrobial peptides and phenoloxidases (Lavine and Strand, 2002; Beckage, 2011). The phenoloxidases are also a complementary part of cellular immunity in the last stage of encapsulation and nodulation processes, forming melanisation of both. The precursors of phenoloxidases are the prophenoloxidases which are maintained in hemocytes (mostly oenocytoids) (Lavine and Strand, 2002; Beckage, 2011). We assume that the reduced activity of phenoloxidases might be due to reduction in cell number. Although while counting DHC we did not count the oenocytoids and instead focused on immunocytes (i.e. granulocytes and plasmatocytes). However, THC reduction gives a clear picture of what was expected under the impression of GA3. The similarity in structure of GA3 to insect JH brings it to the mind that the inhibition of phenoloxidase activity is the same as what JH does. The JH has been shown to have inhibitory effect on phenoloxidase activity (Hiruma and Riddiford, 1988; Rolff and Siva-Jothy, 2002; Rantala *et al.*, 2003).

The antioxidant system enables the insect to inhibit oxidation reactions that produce free radicals damaging the cells (Dkhil *et al.*, 2015). Increasing activity of peroxidase, catalase, superoxide dismutase, ascorbate peroxidise, and glucose-6-phosphate dehydrogenase can be explained since this insect has faced exogenous stress, here GA3 (Lyakhovich *et al.*, 2006, Altuntas, 2015; Sezer and Ozalp, 2015).

Glutathione S-transferase is a detoxifying and antioxidant enzyme that eliminates lipid peroxidation products or hydroperoxides of cells (Dubovskiy *et al.*, 2008). This enzyme also plays an important role in detoxification by increasing the solubility of toxic substances (Grant and Matsumura, 1989). Esterases are important detoxifying enzymes which hydrolyse esoteric bonds in synthetic chemicals (Hemingway and Karunaratne, 1998). Increasing activity of glutathione S-transferases and esterases is an important indication of the unpleasant effects of GA3 on the insect. Mirhaghparast *et al.* (2016) reported increased activity of these two enzymes in *C. suppressalis* larvae under the influence of pyriproxyfen. The detoxifying enzymes and oxidative defense system work in coordination to increase adaptation of insects by deactivating and eliminating harmful compounds (Felton and Summers, 1995; Hemingway and Karunaratne, 1998; Büyükgüzel *et al.*, 2010; Aslanturk *et al.*, 2011).

In assessing the effect of gibberellic acid on protein and glucose in the insect body no significant changes were observed. Contrary to our report Uckan *et al.* (2011) showed higher protein levels in all concentrations (50 to 2000 ppm) of GA3 in the larvae of *G. mellonella*. However, hemolymph free amino acids in *G. mellonella* larvae after using GA3 undulated among dosages (Altuntaş *et al.*, 2014). Also, carbohydrate levels of larval *G. mellonella* decreased significantly after GA3 use. Moreover, the amount of glycogen in *Zaprionus paravittiger* (Godbole and Vaidya) (Diptera: Drosophilidae) and *B. cucurbitae* was reduced after using gibberellic acid (Rup *et al.*, 1998; Kaur and Rup, 2003b). Triglycerides and cholesterol reduction was similar to what has been reported in other insects (Rup *et al.*, 1998; Uckan *et al.*, 2008). The lower level of total lipid, as well as triglyceride lipid biosynthesis in the experimental larvae, emphasizes the need of this macromolecule for energy demands due to induced stress by GA3. Furthermore, the reactive oxygen species also cause cell death, mutation, and even death through degradation on macromolecules such as lipids (Ryter *et al.*, 2007).

**Conclusion**

The PGRs can be used to increase the crop yield quality and quantity. However, we should be cautious about the interaction between the GA3,

**Table 3** Amount of protein, glycogen, triglyceride, cholesterol, and glucose in sixth instar *H. armigera* larvae provided with different concentrations of gibberellic acid in their diet

| Macromolecules (mg/ml) | Treatments | | | | |
|---|---|---|---|---|---|
| | C | C100 | C200 | C400 | C800 |
| **Protein** | 3.560±0.061[a] | 3.681±0.046[a] | 3.656±0.070[a] | 3.808±0.066[a] | 3.712±0.063[a] |
| **Glycogen** | 0.251±0.004[a,b] | 0.242±0.007[a,b] | 0.232±0.012[b,c] | 0.270±0.004[a] | 0.207±0.001[c] |
| **Triglyceride** | 0.171±0.001[a] | 0.166±0.001[b] | 0.166±0.005[b] | 0.158±0.009[c] | 0.154±0.001[c] |
| **Cholesterol** | 1.260±0.018[b] | 1.454±0.048[a] | 1.107±0.047[b,c] | 0.990±0.043[c] | 0.763±0.032[d] |
| **Glucose** | 0.172±0.003[a] | 0.165±0.002[a] | 0.171±0.002[a] | 0.165±0.002[a] | 0.163±0.001[a] |

Means with the same letters in a row are not significantly different at $p < 0.05$ (Tukey's test)

insect, and the crop plant. In the present and previous reports we demonstrated this effect which aimed at digestion, reproduction, and now the immunity. Further studies concerning insects collected directly from treated and untreated crops would help for planning on the use of various PGRs.

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