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Changes in cellular immune responses of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) due to pyriproxyfen treatment

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Abstract: The effects of pyriproxyfen were determined on the cellular immunity and phenoloxidase activity in the 4th instar larvae of *Chilo suppressalis* Walker. The bioassay results revealed the effective concentrations of: 10L : 18C, 30L : 72C and 50L : 190C μg · ml^{-1}. The sole effect of 18 and 72 μg · ml^{-1} concentrations at intervals of 1–3 h caused a higher number of total hemocytes in the treated larvae than the control, but the reverse results were observed after 6–24 h. The number of plasmatocytes was lower than that of the control for intervals of 3–24 h but the number of granulocytes was higher than the control after 1–3 h although no significant differences were observed at the other times. In the treated larvae, the activities of phenoloxidase were higher and lower than those of the control after 1–3 h and 6–24 h, respectively. The combined effects of pyriproxyfen and the entomopathogenic fungus, *Beauveria bassiana* isolate B3 caused higher numbers of total hemocytes, plasmatocytes, and granulocytes in the treated larvae by use of the three concentrations of pyriproxyfen, at intervals of 6 and 12 h. Although the numbers of nodules in the larvae treated with concentrations of 18 μg · ml^{-1} were higher than those of other treatments, the overall numbers were lower than those of the control. Finally, the activity of phenoloxidase in the treated larvae was higher than that of the control, at intervals of 6 and 12 h post-treatment. Findings of the current study indicate an intervening role of pyriproxyfen in the cellular immunity of *C. suppressalis* to entomopathogenic objects.

Key words: *Beauveria bassiana*, *Chilo suppressalis*, hemocyte, immune response, pyriproxyfen

Introduction

The juvenile hormones (JHs) are the products of corpora allata, in insects that belong to the family of acyclic sesquiterpenoids. Researchers have identified JHs in approximately 100 insect species from at least 10 insect orders (Goodman and Granger 2005). These compounds may be involved in the control of gonadal development, vitellogenin synthesis, regulation of metamorphosis, caste determination, behavior, diapause, and various polyphenisms (Nijhout 1994). Hence, the alteration in titer of JHs causes severe physiological and behavioral disruptions leading to death or malformation. The importance of JHs in the physiological processes of insects opened a new area to synthesis several compounds called JH analogs. Pyriproxyfen, 4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propyl ether, is a pyridine-based insecticide which acts as an insect growth regulator against cockroaches, fleas, lepidopteran, coleopteran, and other pests. Ishaaya and Horowitz (1992) suggested that pyriproxyfen mimics the action of the juvenile hormones on a number of physiological processes but it is a potent inhibitor of embryogenesis, metamorphosis, and adult formation.

Chilo suppressalis Walker (Lepidoptera: Crambidae) is a serious pest of rice in different regions. The larvae of this pest intensively feed on the stems. The three overlapping generations of the pest cause severe yield loss annually. Here in Iran, light traps and intensive spraying of pesticides such as diazinon, are the main control procedures to decrease population outbreaks of *C. suppressalis*. Resistance of *C. suppressalis* to diazinon in several areas of northern Iran was reported by Zibaee *et al*. (2009). Resistance to the pesticides used, and environmental concerns of chemical spraying, require changing the control procedures to safer ones. Recently, Ramzi and Zibaee (2014) determined the virulence of various entomopathogenic fungi against *C. suppressalis* larvae. The above-noted authors performed bioassays, biochemical analysis, and cluster analysis which indicated that isolate BB3 was the most effective fungus against the larvae. In another study, Zibaee and Malagoli (2014) studied the cellular immune responses of *C. suppressalis* in the presence of several entomopathogenic fungi; three isolates of *Beauveria bassiana*, *Metarhizium anisopliae*, *Isaria fumosoroseus*, and *Lecanicilium lecanii*. Prohemocytes, granulocytes, plasmatocytes, and oenocytoids were identified as the main circulating hemocytes in the hemolymph of larvae by the major roles of granulocytes and plasmatocytes. Also, the number of hemocytes, and the phenoloxidase activity, showed fluctuating results after injection of the entomopathogenic fungi. Although *C. suppressalis*, like other insects, could overcome entomopathogenic organisms via cellular and humeral responses, there are several factors which could

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modulate immunity. These factors include hormones, feeding status, developmental stages, and temperature. The determination of modulating factors on insect immunity could elucidate the ability or deficiency of an insect *vs.* non-self-objects (Zibaee *et al*. 2009). Juvenile hormone and its analogs are factors affecting the immunity of insects, mainly by altering the number of immunocytes (Gelbič *et al*. 2005; Franssens *et al*. 2006; Zibaee *et al*. 2012; Rahimi *et al*. 2013). Apart from immune responses, Mirhaghparast *et al*. (2014) found disturbance effects of pyriproxyfen on several enzymatic and non-enzymatic components engaged in intermediary metabolism of *C. suppressalis*. Regarding the impressive effects of pyriproxyfen on these processes, the positive or negative effects of the JH analogs (e.g. pyriproxyfen) on the cellular immunity of *C. suppressalis* larvae showing possible compatibility with biological control using *B. bassiana* must be made clear. Hence, the current study was conducted to find out how pyriproxyfen affects cellular immunity alone or in the presence of *B. bassiana* spores.

Materials and Methods

Chilo suppressalis rearing

Individuals of *C. suppressalis* were reared on the Hashemi variety of rice in the Department of Biological Control, the Iranian Institute of Plant Protection, Amol. Rice varieties were planted in buckets (height 40 cm, width 20 cm) under cages in greenhouse. After rearing for three generations, egg patches were transferred to a laboratory, where the conditions were: 25°C, 85% of relative humidity (RH) and 16L : 8D of photoperiod. These egg patches were then reared to 4th larval instars.

Beauveria bassiana culture

Based on Ramzi and Zibaee (2014), the BB3 isolate of *B. bassiana* showing the highest virulence on *C. suppressalis* was cultured at 25°C (±1°C) on Potato Dextrose Agar (pH 5.6) amended with 1% yeast extract. After 14 days, the conidia were washed off with a 0.01% solution of Tween 80 (Sigma Aldrich, USA), and a concentration of 10^4 spore · ml^{-1} was prepared for a combined exposure using pyriproxyfen (Zibaee and Malagoli 2014).

Bioassay of pyriproxyfen

Different concentrations of pyriproxyfen including 50, 100, 300, 500, and 1,000 µg · ml^{-1}, were prepared and 2 µl of each of the concentrations was topically exposed to 4th larval instars. The mortality of the larvae was recorded during the 72 h period. Then, the data were inserted in POLO-PC software to calculate the $LC_{10,}$ $LC_{30,}$ and LC_{50} values. The bioassay was done using three replicates containing 10 larvae in each, together with the control (N = 180).

Effect of pyriproxyfen on cellular immunity

Larvae of *C. suppressalis* (group I) were separately treated with 2 µl of pyriproxyfen concentrations (18, 72 and 190 µg · ml^{-1}) to find the sole effects of pyriproxyfen on hemocyte numbers and phenoloxidase activity. The control larvae treatment used acetone (number of individuals per treatment including the control was 30 larvae; N = 120). Group II larvae underwent a treatment that used 2 µl of pyriproxyfen concentration and then after 4 h, these Group II larvae were injected with 10^4 spore · ml^{-1} of *B. bassiana* var. BB3 to find the number of hemocytes, the nodule formation, and the phenoloxidase activity. The control larvae were treated by acetone and injected with distilled water (number of individuals per treatment including the control was 30 larvae, N = 120). After both experiments, the hemolymph of the larvae were collected from the first abdominal proleg at 1, 3, 6, 12, and 24 h intervals. The hemolymph was immediately diluted with an anticoagulant solution (0.01 M ethylenediamine tetraacetic acid, 0.1 M glucose, 0.062 M NaCl, and 0.026 M citric acid, pH 4.6) described by Azambuja *et al*. (1991). The preparation proportion was 1 : 4 of anticoagulant solution and hemolymph. Samples taken from different time intervals were loaded into a hemocytometer to count the number of total hemocytes, plasmatocytes, granulocytes, and nodules. Please note the number of nodules just evaluated after the combined effects of pyriproxyfen and *B. bassiana*.

Assay of phenoloxidase activity

Phenoloxidase activities (PO) in the hemolymph of the control and treated larvae were assessed at different time intervals. They were assessed according to Willson *et al*. (2002). Briefly, 8 µl of hemolymph was added to 50 µl of ice-cold PBS (pH 7) and gently mixed. The samples were frozen at –20°C to disrupt hemocyte membranes. The enzymatic activities were assayed spectrophotometrically using 10 mM L-dopa (3,4-dihydroxyphenylalanine) as the substrate. Incubation was done for 5 min and the absorbance was then read at 492 nm during the linear phase of the reaction.

Protein assay

Protein concentrations were assayed according to the method described by Lowry *et al*. (1951). The method recruits the reaction of Cu^{2+}, produced by the oxidation of peptide bonds with Folin-Ciocalteu reagent. In the assay, 20 µl of the homogenised sample was added to 100 µl of the reagent, and incubation took place for 30 min prior to reading the absorbance at 545 nm (recommended by Ziest Chem. Co., Tehran-Iran).

Statistical analysis

All data from a complete randomised design were compared by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences between samples were statistically considered at a probability less than 5% and marked in the figures and tables. The treatment values were compared in the same time intervals.

Results and Discussion

Both pesticides and entomopathogenic organisms are considered to be efficient means for decreasing an insect population. Since Integrated Pest Management (IPM) is recommended as the rational and safe procedure to decrease the damage caused by pests, potential synergism or antagonism of the various control procedures must be determined. The effects of pesticides, mainly insect growth regulators (IGRs), must be determined to have a better background of IPM and to make way for the adaptability or inadaptability of the IGRs with other control tactics. In the current study, pyriproxyfen affected the numbers of hemocytes, the nodule formation, and the phenoloxidase activity in the *C. suppressalis* 4th instar larvae, in both sole exposure and combined with the entomopathogenic fungus, *B. bassiana* (isolate BB3). When pyriproxyfen, in concentrations of 18 and 72 μg · · ml^{-1}, was solely exposed to larvae, the number of the total hemocytes (after 1–3 h) was higher than that of the control (after 1–3 h). By increasing the incubation time, the number of total hemocytes in all the treated concentrations were lower than in the control (Fig. 1A). The combined treatment of pyriproxyfen and *B. bassiana* led to higher numbers of total hemocytes at intervals of 6–24 h post-treatment than in the control (except for the concentration of 190 μg · ml^{-1} after 24 h). But the number of cells in the treated larvae at time intervals of 1 and 3 h were lower than in the control, except for the concentration of 18 μg · · ml^{-1} (Fig. 1B). Sole treatment of pyriproxyfen decreased the number of plasmatocytes in all of the time intervals in comparison with the control, except for 1 and 3 h post-treatment. The number of plasmatocytes in the concentration of 18 μg · ml^{-1} tended to be higher or the same as the control (Fig. 2A). Plasmatocytes in combined effects with pyriproxyfen and *B. bassiana* at intervals of 6–24 h post-treatment, showed higher numbers than the control. However, the results were reversed in the two first, time-intervals (Fig. 2B). Although the numbers of granulocytes after the sole treatment of pyriproxyfen were higher than those of the control, the combined effects of pyriproxyfen and *B. bassiana* increased the number of these cells in comparison with the control, after 6 and 12 h of post-treatment (Fig. 3A, B). In other time intervals, the number of granulocytes were lower than for the control or no significant differences were observed (Fig. 3B). George and Ambrose (2004) found that treating *Rhynocoris kumarii* Ambrose and Livingstone (Hemiptera: Reduviidae) with organophosphate insecticides increased the total hemocyte counts, mainly granulocytes, while organochlorines decreased the total number of hemocytes, mainly granulocytes, but increased the numbers of prohemocytes and plasmatocytes. Botanical insecticides, like Azadirachtin and *Artemisia annua* L. (Asteraceae), decreased the number of plasmatocytes and granulocytes in *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) and *Rhodnius*

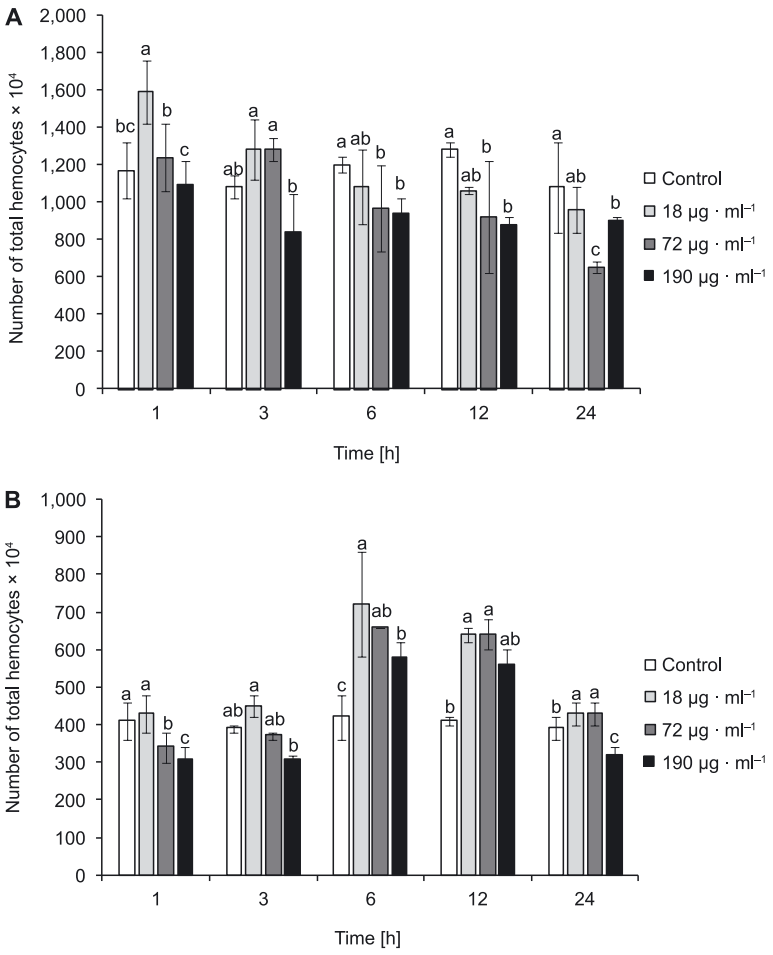

Fig. 1. Effects of pyriproxyfen (A) and pyriproxyfen + *Beauveria bassiana* (B) on total hemocyte counts of *Chilo suppressalis*. Statistical differences have been shown by various letters in each time intervals ($p \leq 0.05$, Tukey test)

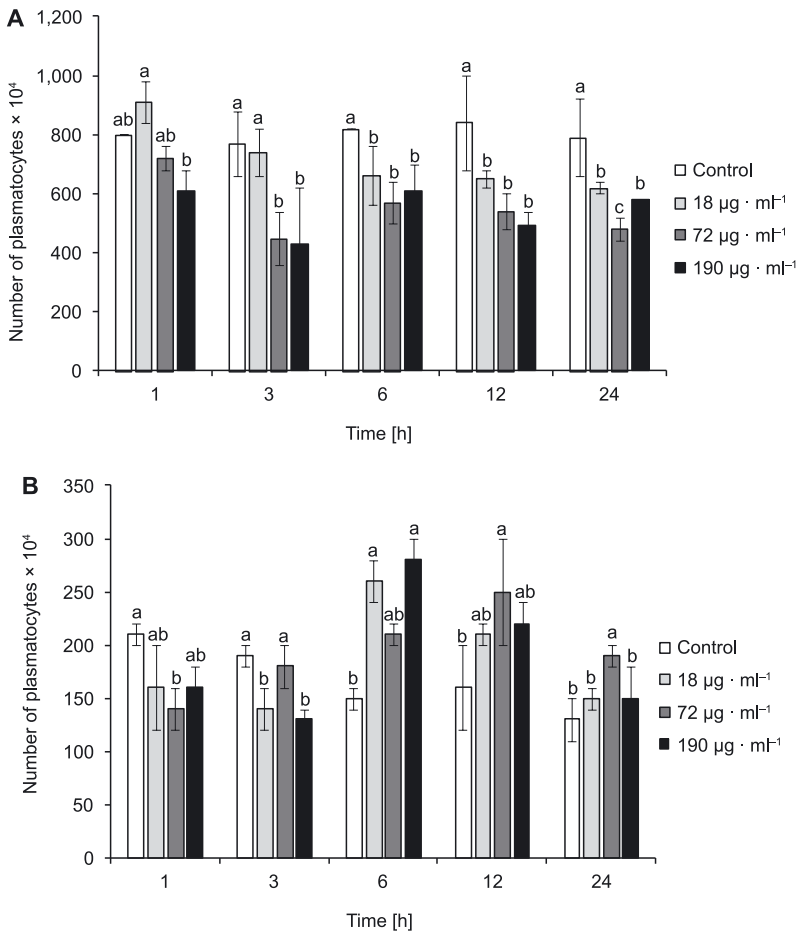

Fig. 2. Effects of pyriproxyfen (A) and pyriproxyfen + *Beauveria bassiana* (B) on plasmatocyte counts of *Chilo suppressalis*. Statistical differences have been shown by various letters in each time intervals ($p \leq 0.05$, Tukey test)

prolixus L. (Hemiptera: Reduviidae) in a dose-dependent manner (Azambuja *et al*. 1991; Zibaee and Bandani 2010). Insect hormones, like 20-hydroxyecdysone (20E) and JH, have shown two distinct effects on the cellular immunity of an insect. In the majority of studies, 20E caused proliferation of hemocytes, while JH and its analogs showed adverse results (James and Xu 2012). In their study, Gelbič *et al*. (2005) showed that the JH analog, metyrapone, reduced the number of granulocytes in *Spodoptera littoralis* Fabricius (Lepidoptera: Noctuidae), while it increased the number of plasmatocytes. Kim *et al*. (2008) reported an antagonistic effect of juvenile hormone on the numbers and the hemocyte-spreading behavior in *Spodoptera exigua* (Hübner). It was noted, in the study by Zibaee *et al*. (2012), that pyriproxyfen decreased the numbers of total hemocytes, plasmatocyte, and granulocyte in *E. integriceps* adults. Rahimi *et al*. (2013) demonstrated inhibitory effects of pyriproxyfen on the total hemocyte counts in addition to the numbers of plasmatocytes and granulocytes in the 4th instar larvae of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). Our overall results revealed that the sole treatment of pyriproxyfen decreased the number of total hemocytes, plasmatocytes, and granulocytes when taking into account statistical significance. There was an exception for the total hemocytes at the 1 h time interval at the lowest concentration. When the fungus, *B. bassiana*, was injected after the pyriproxyfen treatment, the highest number of hemocytes were observed after 6 and 12 h, which was higher than that of the control. The effects of pesticides on the hemocytes of insects are different and depend on the chemical nature of the pesticides. The negative effects of JH analogs, like pyriproxyfen on the hemocyte numbers, could be attributed to cytotoxic effects, to the inhibition of larval hematopoietic function, or to cell proliferation (Zibaee *et al*. 2012). Inhibition studies were done by Kim *et al*. (2008), regarding the differences of hemocyte numbers alone, and the combined effects of pyriproxyfen. They performed the studies to have a better inderstanding of the mechanism. They reported antagonistic effects of ethoxyzolamide; a known inhibitor of the JH membrane receptor on the plasmatocytes of *Spodoptera frugiperda* (J.E. Smith). They suggested that JH and maybe their analogs have a membrane action on the plasmatocyte. Hemocytes somehow showed a response to the JH treatment. Kim *et al*. (2008) concluded, though, that JH utilised membrane or cytosolic receptors to exert its effects on hemocytes rather than nuclear gene expression. Moreover, some other mediators, such as eicosanoids, are activated in response to pathogen infection so they caused the hemocytes' sensitivity at points of infection, increasing cellular responses such as phagocytosis and nodule formation (Kim *et al*. 2008).

No nodule was observed after the sole effects of pyriproxyfen. But the combined treatment of pyriproxyfen

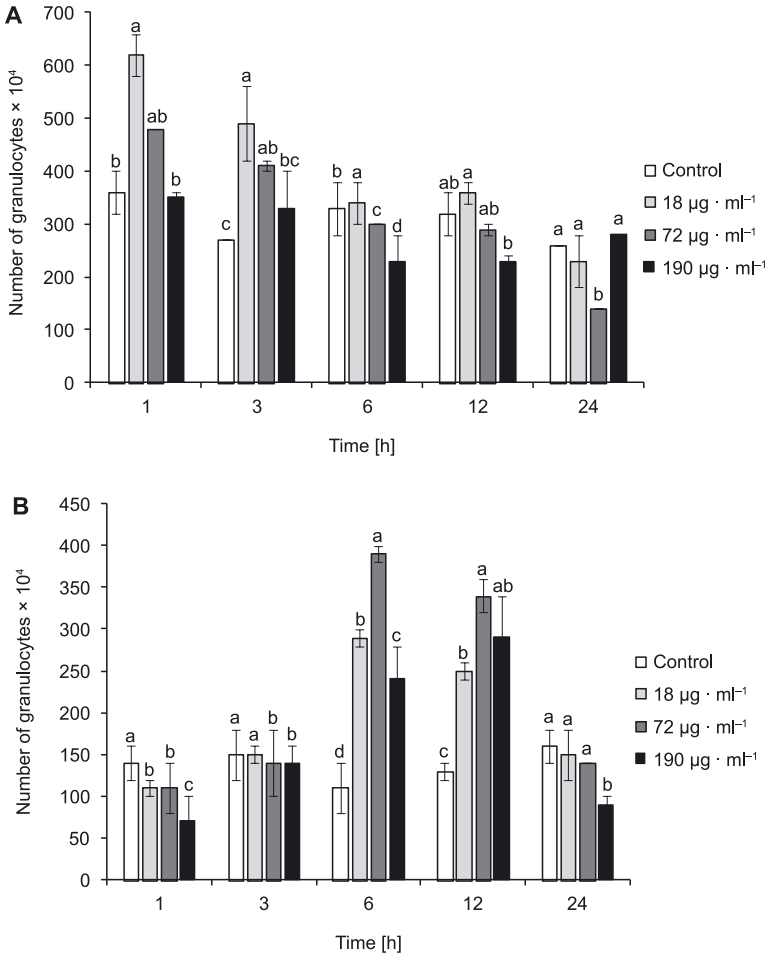

Fig. 3. Effects of pyriproxyfen (A) and pyriproxyfen + *Beauveria bassiana* (B) on granulocyte counts of *Chilo suppressalis*. Statistical differences have been shown by various letters in each time intervals ($p \leq 0.05$, Tukey test)

and *B. bassiana* revealed a lower numbers of nodules in comparison with the control at all the time intervals (Fig. 4). In the two first time-intervals, the activities of phenoloxidase were higher than those of the control after pyriproxyfen treatment. However, the enzymatic activities decreased in other time intervals (Fig. 5A). The activities of the enzyme were found to be higher than those of the control, at time intervals of 6–12 h for the combined treatment and mainly for the concentrations of 72 and 190 µg · · ml^{-1}. Adverse results were observed, though, in the two, first time-intervals (Fig. 5B). Rantala *et al*. (2003) demonstrated suppressive effects of JH on both PO and encapsulation in *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). It was reported by Rahimi *et al*. (2013) that the highest number of nodules were found at 6–12 h post injection of *E. kuehniella* larvae by *B. bassiana*, for both the control and the pyriproxyfen-treated larvae, yet the number of nodules in the treated larvae were significantly lower than those in the control. The authors found no significant differences in the case of phenoloxidase activity (Rahimi *et al*. 2013). It was observed by Franssens *et al*. (2006) that both JH and its analogs affected the nodulation in the larvae of *N. bullata* by reducing the ability of the larvae to form nodules in response to laminarin, along with a down regulation of phenoloxidase activity. The lower numbers of formed nodules in the pyriproxyfen-treated, *C. suppressalis* larvae might be because of the inhibitory effects of the insecticide on the cell proliferation, and the differentiation into macrophages. Also, since phenoloxidases are synthesised as zymogen by hemocytes, one of the reasons for the lowering of phenoloxidase activity might be the reduced numbers of hemocytes caused by pyriproxyfen. The second reason has been suggested by the works of Mirhaghparast and Zibaee (2013) and Delkash-Roudsari *et al*. (2015) on *in vitro* effects of pyriproxyfen on phenoloxidase of *C. suppressalis* and *E. kuehniella*. The results revealed significant inhibition by both extracted and purified phenoloxidase due to *in vitro* treatment using pyriproxyfen. So, it might be concluded that pyriproxyfen could decrease the phenoloxidase activity of insects by not only reducing the number of hemocytes but also by direct inhibition of phenoloxidase activity.

The results of the current study revealed two different effects of pyriproxyfen after a sole treatment or after a pyriproxyfen-treatment in combination with *B. bassiana* on the 4th instar larvae of *C. suppressalis*. The results revealed that a sole treatment of pyriproxyfen decreased the number of hemocytes and the nodule formation, and any positive effects would be expected in the earlier time intervals. The activity of phenoloxidase even showed a negative correlation by the effects of pyriproxyfen on the hemocytes. These finding and those of a previous study on intermediary metabolism, might indicate that there are intervening roles of this compound in the physiologi-

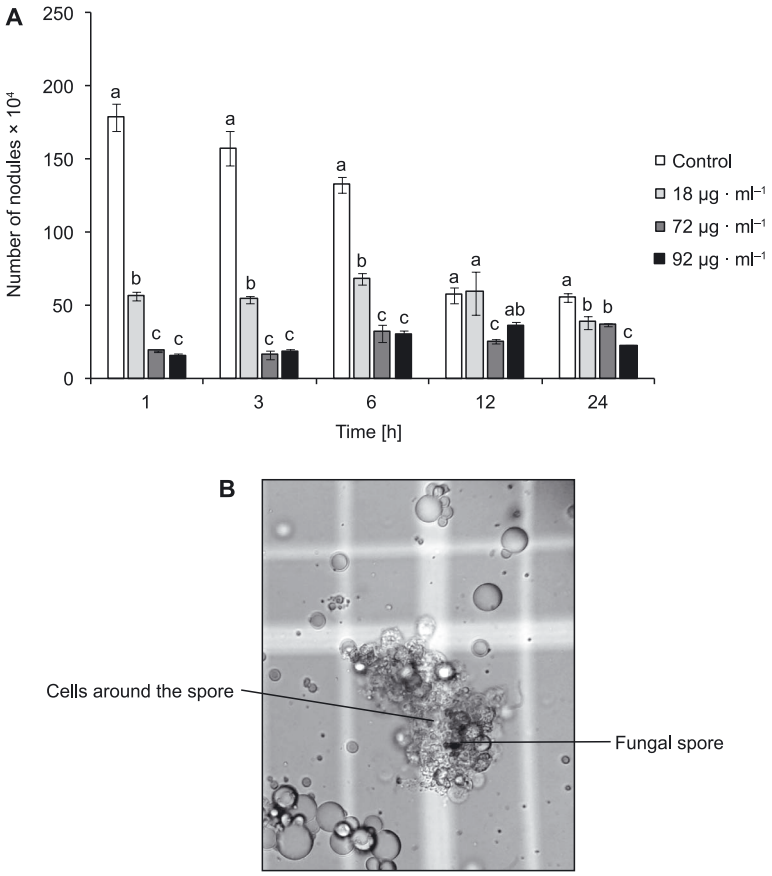

Fig. 4. Effects of pyriproxyfen + *Beauveria bassiana* (A) on number of nodules in *Chilo suppressalis*. A nodule found in the injected larvae of *Ch. suppressalis* (B). Statistical differences have been shown by various letters in each time intervals ($p \leq 0.05$, Tukey test)

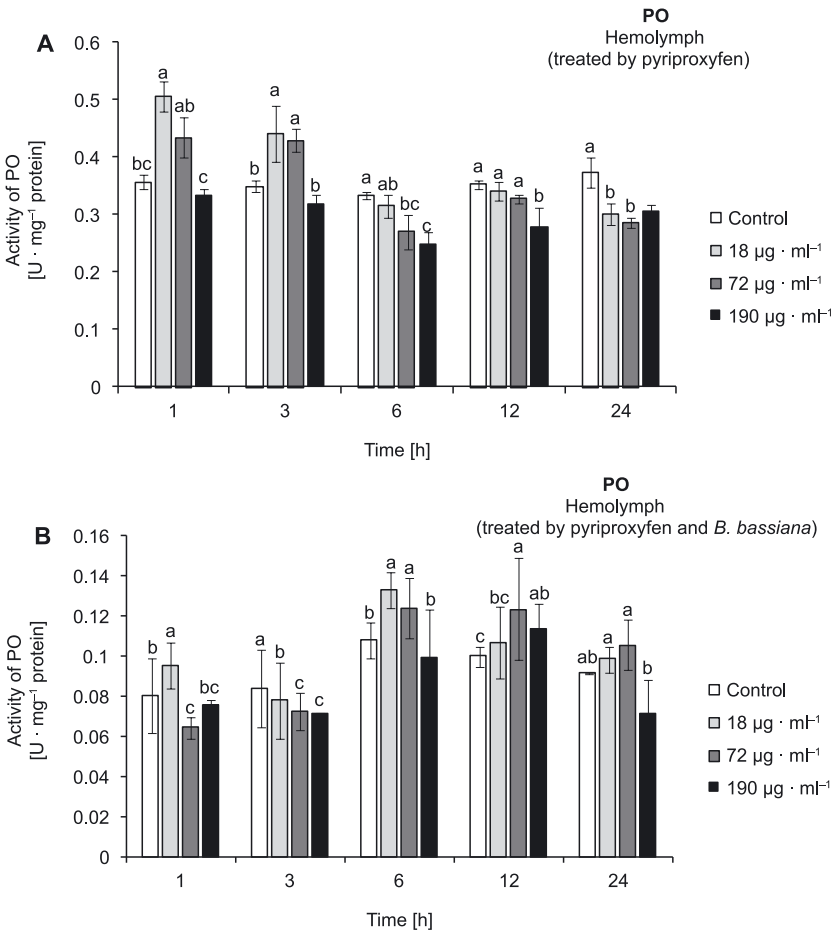

Fig. 5. Effects of pyriproxyfen (A) and pyriproxyfen + *Beauveria bassiana* (B) on activity of phenoloxidase (PO) in *Chilo suppressalis*. Statistical differences have been shown by various letters in each time intervals ($p \leq 0.05$, Tukey test)

cal processes of *C. suppressalis* even after the increased activities of general esterases as the major hydrolysing enzymes of JH and its analogues (Mirhaghparast *et al*. 2014). Hence, pyriproxyfen or other insect growth regulators could be considered as alternatives of organophosphorous insecticides against *C. suppressalis*. Pyriproxyfen or other insect growth regulators show that selective activity on pests do not cause environmental pollution and have the lowest effects on biocontrol agents.

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