EFFECTS OF PYRIPROXYFEN AND HEXAFLUMURON ON CELLULAR IMMUNITY OF *EPHESTIA KUEHNIELLA* ZELLER (LEPIDOPTERA: PYRALIDAE)

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Effects of two insect growth regulators, pyriproxyfen and hexaflumuron, were studied on cellular immune responses of *Ephestia kuehniella* Zeller against *Beauvaria bassiana* var. B3. Although the highest number of total hemocytes were found after 12 hours of post-injection, but statistical differences were found at intervals of 24 hours between control and treated larvae after treating by pyriproxyfen. While statistical differences were observed after 3-12 hours of post-injection in the treated larvae by hexaflumuron. Numbers of plasmatocytes were obtained to be statistical after 24 hours of post injection by pyriproxyfen but time intervals of 3, 12 and 24 showed statistical differences after treating by hexaflumuron. Number of granulocytes in the treated larvae by pyriproxyfen showed a statistical difference after 24 hours of post-injection. The highest numbers of nodules were observed 3 and 24 hours after injection by pyriproxyfen and 3 and 12 hours in the injected larvae by hexaflumuron. Although phenoloxidase activity showed no statistical differences in the larvae treated by pyriproxyfen, intervals of 1 and 3 hours post-injected by hexaflumuron demonstrated statistical differences between control and treated larvae. Results of the current study clearly showed that treating of larvae by pyriproxyfen and hexaflumuron decreased, not only numbers of total and differentiated hemocyte counts, but also nodulation and phenoloxidase activity. Since these IGRs are selective and safe for non-target organisms, their combination with entomopathogenic fungi improves efficiency of control procedures.

Key words: Pyriproxyfen, Hexaflumuron, Cellular immunity, *Beauveria bassiana*, *Ephestia kuehniella*.

INTRODUCTION

Insect growth regulators (IGRs) are the compounds that intervene in common physiological processes of insects like molting, cuticle formation, maturation and reproduction (Ishaaya & Horowitz, 1998). These compounds have high activity and selectivity against insects besides low toxicity to non-target wildlife (Dhadialla *et al*., 1998). Pesticides that act as IGRs are divided into several classes including juvenile hormone analogues, 20-hydroxyecdyson agonist and inhibitors of chitin synthesis (Ishaaya & Horowitz, 1998; Dhadialla *et al.*, 1998). In case of our study,

pyriproxyfen and hexaflumuron belong to the first and the third classes, respectively. Pyriproxyfen contains pyridyl oxyethylene in its structure that affects the hormonal balance of insects by mimicking of juvenile hormone leading to strong suppression of embryogenesis, metamorphosis, and adult formation (Koehler & Patterson, 1991). Hexaflumuron belongs to benzoylphenyl ureas chemicals that inhibit chitin synthesis, disrupt hormonal balance involved in the molting process and lead to growth disturbance and death (Oberlander & Silhacek, 1998).

When a pathogen introduces to insect hemocoel, it encounters several defensive phenomena known as immune responses. These responses are divided into two main series as cellular and humeral reactions. In cellular one, non-self-objectives are removed from hemolymph by phagocytosis, nodule formation and encapsulation caused by hemocytes (Lavine & Strand, 2002). Humeral responses refer to synthesis of antimicrobial peptides, lysozymes, activation of phenoloxidase and production of reactive intermediates of oxygen and nitrogen (Soderhall & Cerenius, 1998; Schmid-Hempel, 2005). Several factors could modulate immune responses of insects such as insecticides, hormones, environmental temperature, cations, etc. (Mandato, 1998). Temperature and cations affect interactions between pathogens and insects by activating of immune responses and increasing ability of hemocyte to remove pathogen from hemolymph. Hormones and insecticides, both synthetic and IGRs, intervene in the intermediary metabolism and immune capability of insects. Organophosphates and botanicals impose changes in hemocyte number, differentiation, phagocytosis. It has been shown that phenoloxidase cascade and malanization are affected by several insecticides. Meanwhile, synthetic insecticides increase oxidative stress, impact the synthesis of some antimicrobial peptides in insects (James & Xu, 2012). In our previous studies, effects of pyriproxyfen and hexaflumuron were determined on intermediary metabolism of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) (Sharifi *et al*., 2013; Delkash *et al*., 2014). So, the current study was conducted to find effects of these IGRs on cellular immunity of the insects by evaluating numbers of hemocytes, nodule formation and phenoloxidase activity in the larvae treated by IGRs and *Beauveria bassiana*.

MATERIAL AND METHODS

INSECT REARING

E. kuehniella was reared on artificial diet containing wheat flour (43 gr), yeast (6 gr) and glycerine (20 ml) in plastic containers (17×9×5 cm) at 25 ± 1°C, 70% of humidity and 16L: 8D conditions (Lima *et al*., 2001).

BIOASSAY OF PYRIPROXYFEN AND HEXAFLUMURON

Different concentrations of pyriproxyfen (active ingredient 97%) and hexaflumuron (Active ingredient 98%) were prepared in acetone and 2 µl of each solution was topically exposed to fifth larval instars of *E. kuehniella*. Mortality was recorded after 24 hours and inserted in POLO-PC software to calculate LC_{50} concentration. Thirty larvae were used in each concentration and control larvae were treated by acetone alone (Sharifi *et al*., 2013; Delkash–Roudsari *et al*., 2014).

EFFECTS OF IGRs ON CELLULAR IMMUNITY AGAINST *B. BASSIANA*

Initially, larvae of *E. kuehniella* were treated separately by 2 µl of pyriproxyfen (45 µg/ml as LC_{50} value) and hexaflumuron (420 µg/ml as LC_{50}). After 4 hours, larvae were injected by 10^5 spore/ml of *B. bassiana* var. B3 to find their effects on hemocyte counts, nodule formation and phenoloxidase activity (N = 50 for each compound).

COLLECTION OF HEMOLYMPH AND DETERMINATION OF IGRs EFFECTS ON CELLULAR IMMUNITY

Hemolymphs of control and treated larvae of *E. kuehniella* were collected from the first abdominal proleg of larvae by a 50 µL sterile glass capillary tube (Sigma-Aldrich Co.) at intervals of 1, 3, 6, 12 and 24 hours. The hemolymph was immediately diluted by 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). Proportion of this preparation 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.

EFFECT OF IGRs ON PHENOLOXIDASE ACTIVITY

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

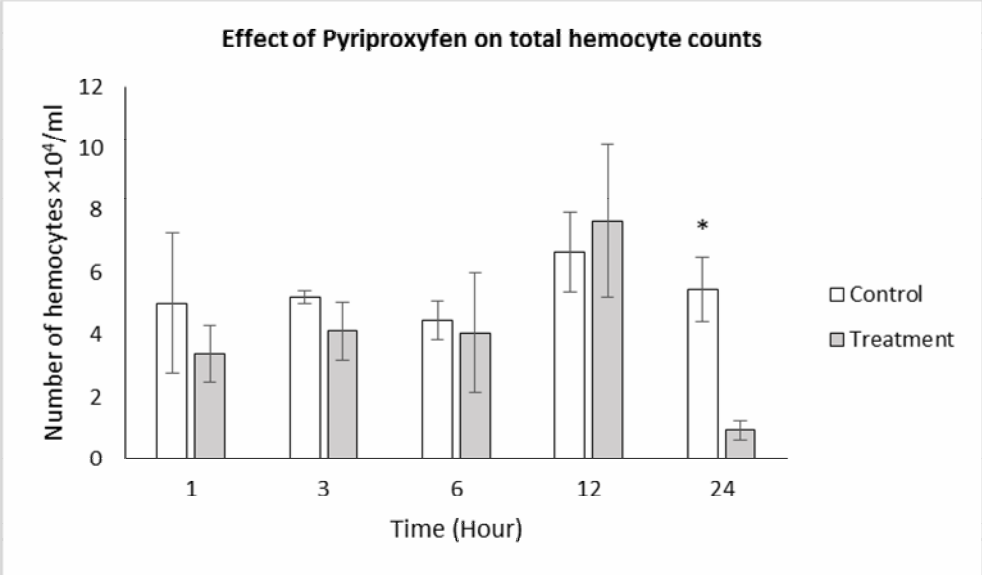
STATISTICAL ANALYSIS

All data obtained from a complete randomized design were compared by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences between samplings were considered statistically significant at a probability less than 5% and marked in figures.

RESULTS AND DISCUSSION

Results of the current study revealed interference of pyriproxyfen and hexaflumuron in cellular immunity of *E. kuehniella*. These results might be used to increase efficiency of entomopathogenic fungi since the given IGRs significantly disabled immune responses of *E. kuehniella* to *B. bassiana*. The highest numbers of total hemocytes were found 12 hours post-injection in both control and treated larvae by pyriproxyfen, but statistical differences were found 24 hours post-injection (Fig. 1). In case of hexaflumuron, the highest number of total hemocytes were observed 3-12 hours-post injection showing statistical differences (Fig. 1). Similar results were obtained in effects of pyriproxyfen and hexaflumuron on plasmatocyte numbers by indicating statistical differences at 24 hours of pyriproxyfen treated larvae and at 3, 12 and 24 hours in hexaflumuron treated larvae (Fig. 2). Statistical differences of granulocytes were obtained at 24 hours of treated larvae by pyriproxyfen and at 1-12 hours of treated larvae by hexaflumuron (Fig. 3).

Besides cellular immunity, hemocytes may also involve in metabolism and detoxification of xenobiotics. Gelbič *et al*. (2005) reported that metyrapone decreased the number of granulocytes in *Spodoptera littoralis* Fabricius (Lepidoptera: Noctuidae) while it increased the number of plasmatocytes. Nahla *et al.* (2010) demonstrated that sublethal concentrations of dimilin decreased the total hemocyte counts in *Agrotis ipsilon* Fabricius (Lepidoptera: Noctuidae). Also, the authors indicated a significant increase of plasmatocytes, granulocytes, and spherule cells as well as a decrease of prohemocytes (Nahla *et al*., 2010). Zhu *et al*. (2012) indicated that exposure of hexaflumuron to *S. litturalis* increased total and differentiate hemocyte counts. Zibaee *et al*. (2012) reported that pyriproxyfen reduced total hemocyte, plasmatocyte and granulocyte numbers in adults of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae). These negative effects on hemocyte numbers could be attributed to cytotoxic effects, inhibition of larval hematopoietic function or the cell proliferation (Zhu *et al*., 2012; Zibaee *et al*., 2012).



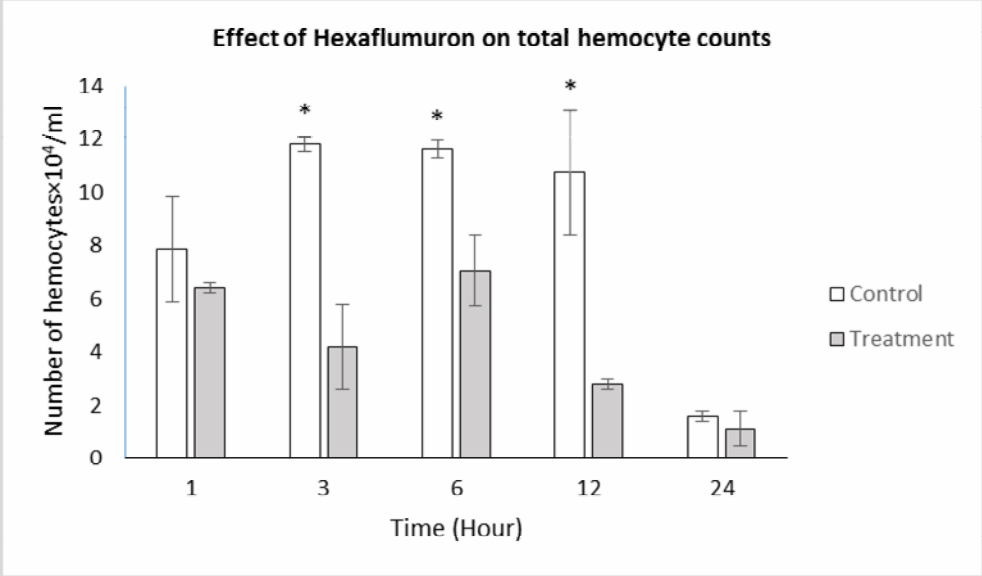
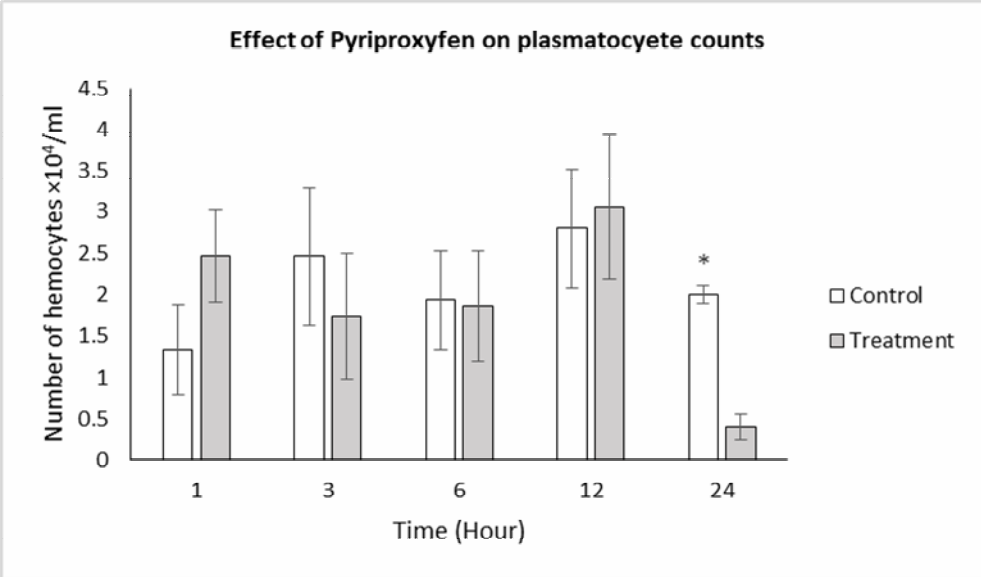


Fig. 1. Effects of pyriproxyfen and hexaflumuron on total hemocyte counts of *E. kuehniella*. Statistical analyses have been made by t-test between control and treatment groups in each time intervals and they have been marked by asterisks ($p \leq 0.05$).



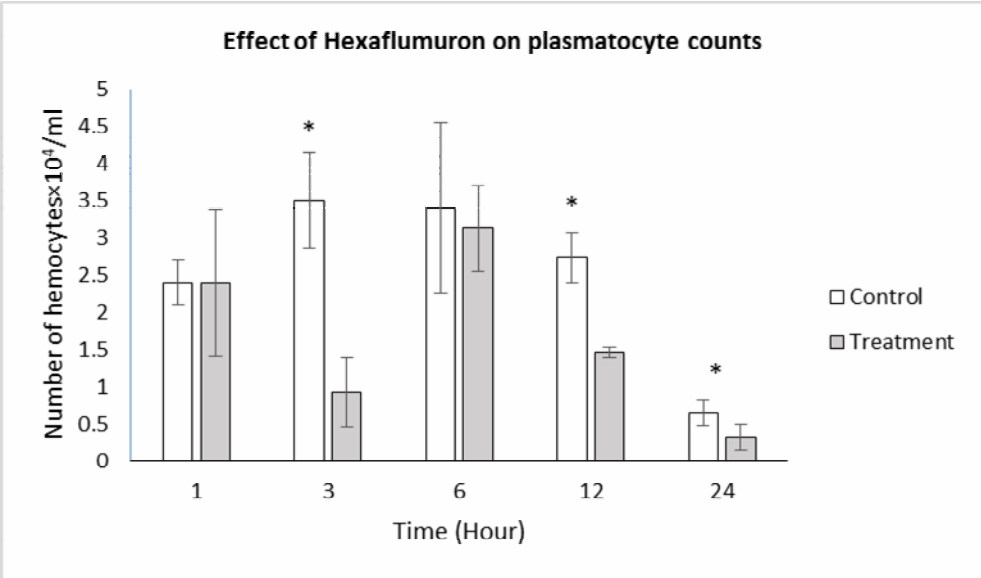
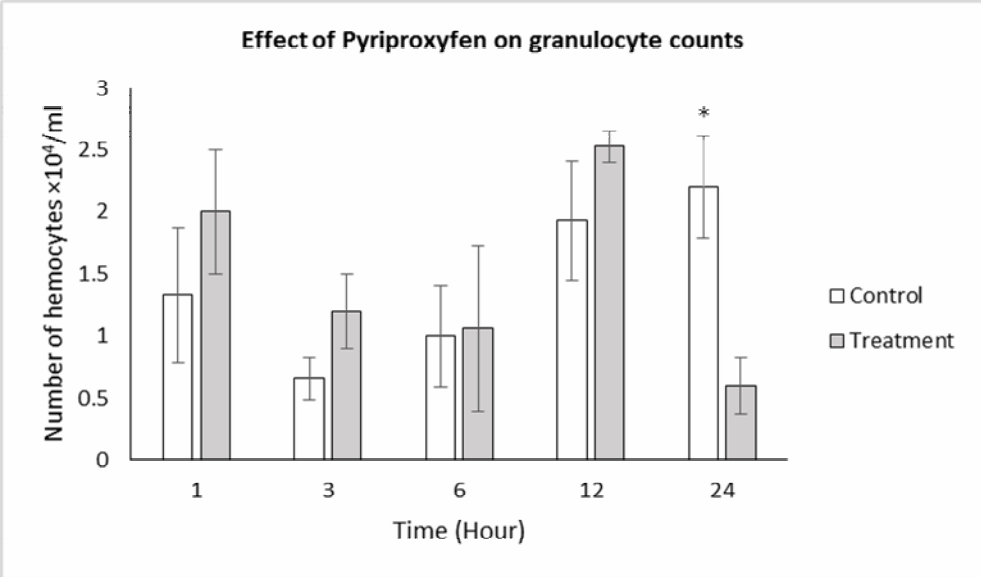


Fig. 2. Effects of pyriproxyfen and hexaflumuron on plasmatocyte counts of *E. kuehniella*. Statistical analyses have been made by t-test between control and treatment groups in each time intervals and they have been marked by asterisks ($p \leq 0.05$).



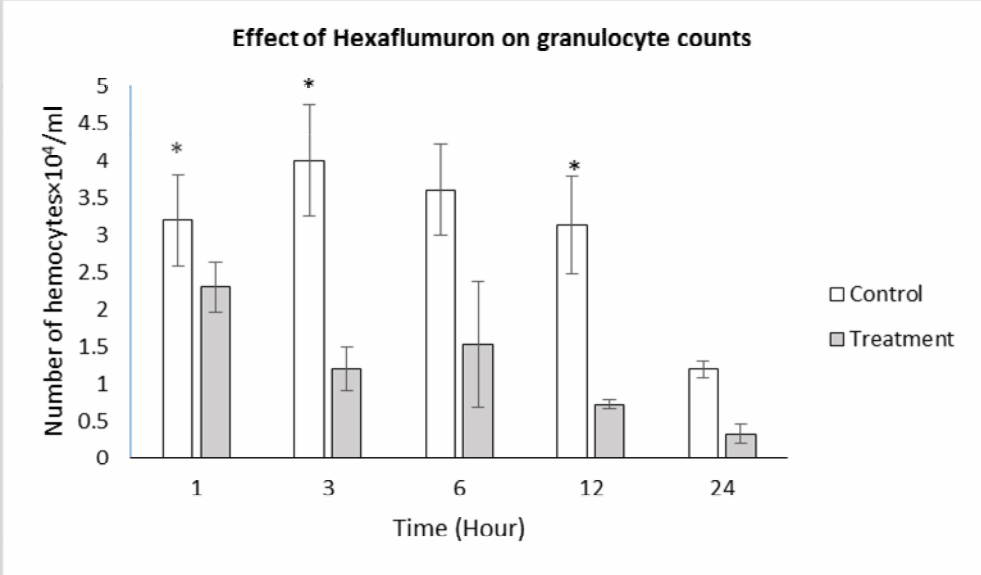
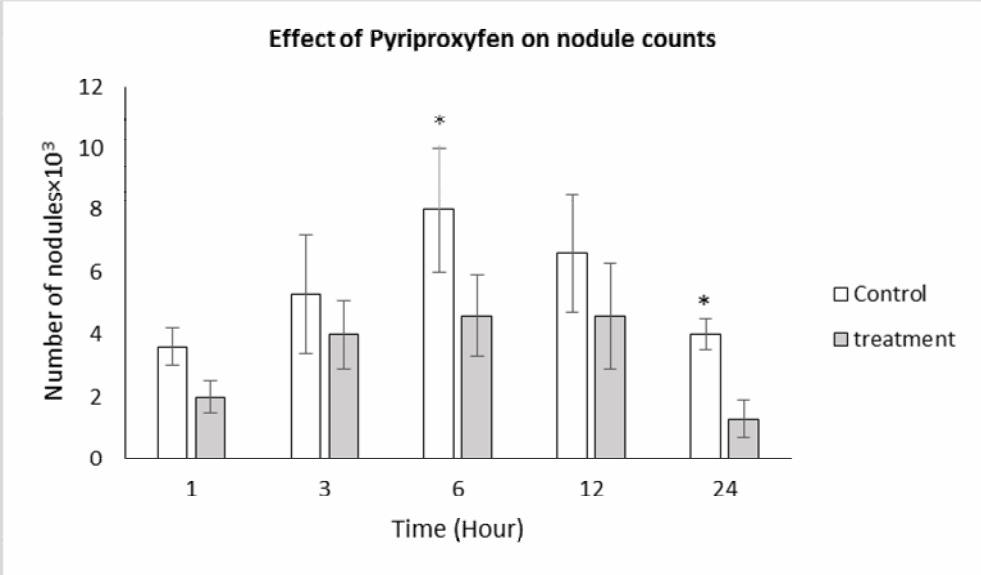


Fig. 3. Effects of pyriproxyfen and hexaflumuron on granulocyte counts of *E. kuehniella*. Statistical analyses have been made by t-test between control and treatment groups in each time intervals and they have been marked by asterisks ($p \leq 0.05$).

Although the highest number of nodules were found 3-12 hours post-injection in control larvae, the numbers in the larvae treated by pyriproxyfen and hexaflumuron are significantly lower than in the control at time intervals of 6 and 24 h for pyriproxyfen as well as 3 and 12 h for hexaflumuron treated larvae (Fig. 4). Since plasmatocytes and granulocytes are the immunocytes involved in cellular responses of insects, alterations in their numbers do change phagocytosis and nodule formation. In our results, a decreased number of nodules corresponds to the lowering numbers of plasmatocytes and granulocytes. These results have been reported when adults of *E. integriceps* were treated by pyriproxyfen (Zibaee *et al*., 2012).

Insect phenoloxidases are available as zymogens and they are activated upon wounding or infection as a part of immune response (Kanost & Gorman, 2008). Phenoloxidases hydroxylate tyrosine to form L-dihydroxyphenylalanine, and oxidize o-diphenols to form quinones (Gorman *et al*., 2007). Finally, quinones are processed to form melanin, then it is deposited on the surface of encapsulated parasites, hemocyte nodules and wound sites (Kanost & Gorman, 2008). In our study, no statistical differences were obtained in phenoloxidase activity of the larvae treated by pyriproxyfen, but hexaflumuron affected the enzymatic activity after 1-3 hours of post-injection (Fig. 5). Although, lower activity of the enzyme could be attributed to the decrease of hemocyte number, but it may be due to direct effect of hexaflumuron on enzymatic inhibition. Mirhaghparast & Zibaee (2013) revealed that pyriproxyfen and hexaflumuron directly decreased the activity of the purified phenoloxidase in *Chilo suppressalis* Walker (Lepidoptera: Crambidae). The inhibition was pH and temperature dependent and led to the calculation of IC_{50} values.

Along with our previous studies that revealed negative effects of pyriproxyfen and hexaflumuron on intermediary metabolism of *E. kuehniella*, the current study confirms disruption of cellular immunity of the larvae against *B. bassina*. These results depict that combination of using these IGRs and *B. bassiana* may increase efficiency of microbial control against the pest. Finally, effects of these compounds must be carried out on humoral responses to have a better perspective in case.



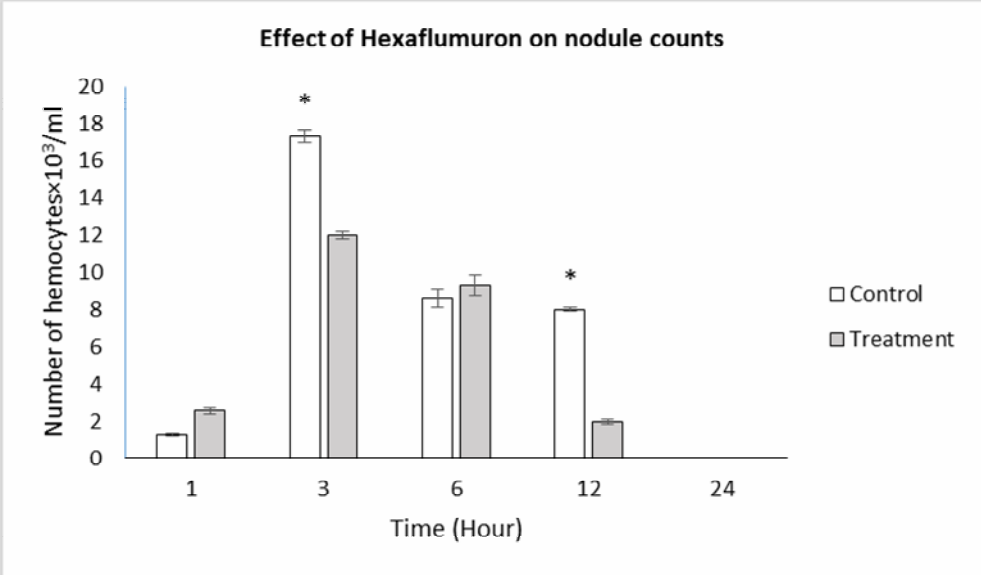
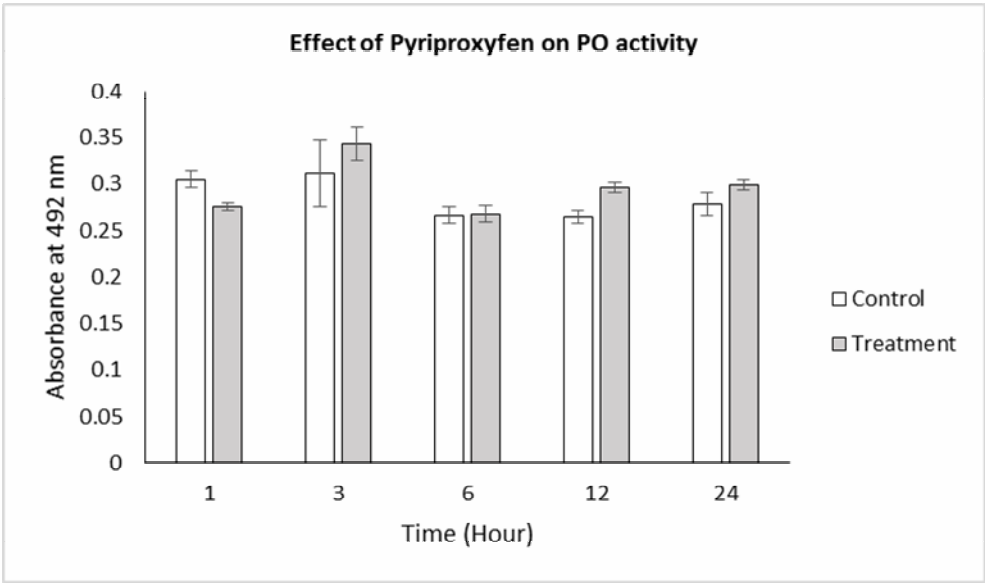


Fig. 4. Effects of pyriproxyfen and hexaflumuron on nodule counts of *E. kuehniella*. Statistical analyses have been made by t-test between control and treatment groups in each time intervals and they have been marked by asterisks ($p \leq 0.05$).



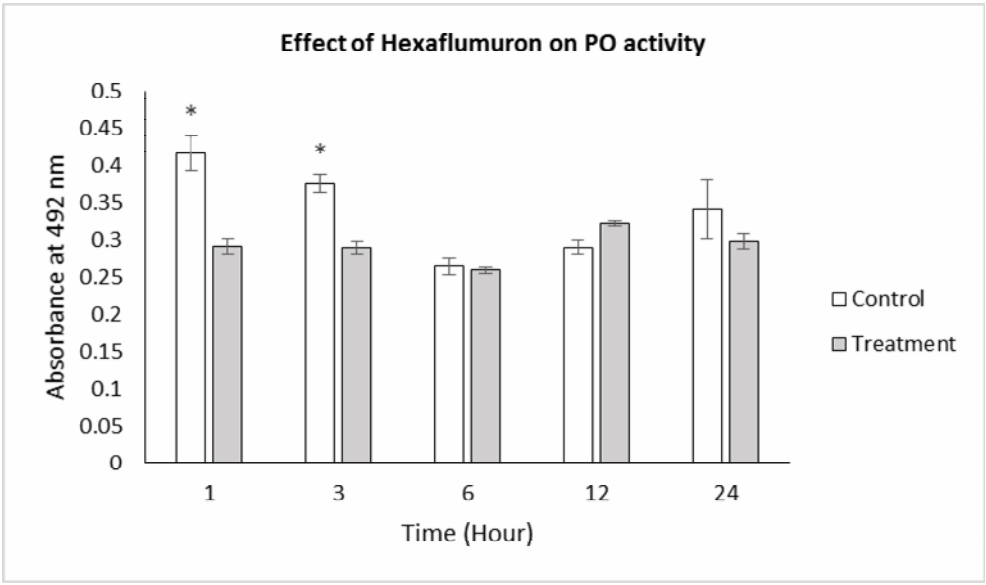


Fig. 5. Effects of pyriproxyfen and hexaflumuron on phenoloxidase activity of *E. kuehniella*. Statistical analyses have been made by t-test between control and treatment groups in each time intervals and they have been marked by asterisks ($p \leq 0.05$).

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