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COMBINED EFFECT OF THREE INSECT GROWTH REGULATORS ON THE DIGESTIVE ENZYMATIC PROFILES OF *CALLOSOBRUCHUS MACULATUS* (COLEOPTERA: BRUCHIDAE)

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Abstract

Insect growth regulators (IGRs) are insecticides that mimic insect produced hormones by regulating developmental process. Theyhave little or no mammalian toxicity, and are considered reduced-risk insecticides that are often exempt from tolerance requirements of regulatory agencies. IGRs, especially, chlorfluazuron, hydroprene and hexaflumuron (benzoylphenylurea) are currently studied because of possibility of using in stored products protection. Many of IGRs compounds used in insect pests control are known to affect digestive enzymes.

Chlorfluazuron, hydroprene and hexaflumuron were tested topically at doses of 0.25%, 0.5% & 1% for chlorfluazuron and hydroprene and 0.5, 1 & 2µg/ml of hexaflumuron to investigate its effects on the activities of the digestive enzymes protease, amylase and lipase in *Callosobruchus maculatus* larvae, which were affected by IGRs individually and in combination. When combined, the effect was more sever at low concentration. There were statistically significant differences ($P \leq 0.05$) in enzyme activities in combined and individual treatments. The combination three IGRs caused a two-fold decrease in enzyme activity even at reduced concentration. Clear dose-response relationships were established with respect to enzyme activity. A synergistic effect of IGRs was found by combination of low doses. These effects are most pronounced in early instars.

Key words: Insect growth regulators, stored pest, chlorfluazuron, hydroprene, hexaflumuron, *Callosobruchus maculates*, control.

Introduction

Insect growth regulators (IGRs) are produced naturally by insects to regulate the processes of molting and development from the egg to the adult stage. Although synthetic mimics of these chemicals have been tested and evaluate in stored products for more than 30 years (Oberlander *et al*, 1997), there is an increased emphasis and renewed interest in IGRs for insect control (Mondal and Parween, 2001). However, it is important to evaluate the product in a manner that is realistic for commercial application. Mondal and Parween (2001) tested IGRs to insect diet and that they did not reflect actual exposure of insects in storage facilities.

Callosobruchus maculatus, is a major insect pest of stored grains, in Africa and Asia with larval development inside seeds from several Leguminosae species

(Ouedraogo *et al*, 1996). *Vigna unguiculata* (Walp) was a major pest of plants (Janzen *et al*, 1977; Jackai and Daoust, 1986). In developing countries IGRs, reduced the load of synthetic chemical pesticides (Hall and Menn, 1990). Thus, plant vegetable oils were tested against several Bruchidae species (Gbolade and Adebayo, 1993; Seck *et al*, 1993; Don Pedro, 1996; Raja *et al*, 2001; Kellouche and Soltani, 2004; Kellouche *et al*, 2004). Laboratory and field studied evaluated the toxicity of several IGRs against stored pests (Mc Gregor and Kramer, 1975; Oberlander *et al*, 1975; Kamer and McGregor, 1979; Main and Mulla, 1982; Abo El-Ghar, 1992; Soltani-Mazouni, 1994; Soltani-Mazouni and Soltani, 1995; Soltani *et al*, 1996; Herbert *et al*, 1997; Peppuy *et al*, 1998).

The present study investigated the in vivo effect of 3 IGRs (chlorfluazuron, hydroprene and hexaflumuron) on activities of digestive enzymes protease, amylase and lipase in *Callosobruchus maculates*.

Materials and Methods

Stock cultures of *Callosobruchus maculates* were maintained in glass jars (18x11cm diameter) containing seeds of *Vigna unguiculate*. Insects were kept at 30±1˚C & 70±5% PH under continuous darkness (Keliouche and Soltani, 2004).

Insecticides (IGRs) and treatments: Chlorfluazuron, hydroprene and hexaflumuron (Tech. Grade 96.7%) was kindly supplied by Dow Elanco Specialist (USA). Serial dilutions were prepared in acetone (0.25, 0.5 &1.0 % for chlorfluazuron, hydroprene) and (0.5, 1.0 & 2µl/ml for hexaflumuron) and topically applied on newly emerged adults (<24 hrs-old). Acetone (1µl/individual) was used as control. The insecticidal assay was conducted with five replicates each of 50 adults (25 males & 25 females) in glass box (140cm x 2cm height) containing 50gm of *V. unguiculata* seeds.

Preparation of enzyme extract: Second to fifth instar larvae resulted from treated adults of treated *C. maculatus* were used to quantity the enzyme activities. Enzyme extracts were prepared after Applebaum (1964). Individuals were anaesthetized with 5x5mm², cotton pads soaked in ether and the entire digestive tract was dissected out in the ice-cold insect Ringers solution. The **malpighian tubules** adhering tissues, and gut contents were removed. The gut was split into regions (foregut, midgut and hindgut) and weighted and each region was homogenized for 3 min. at 4˚C in ice-cold citrate-phosphate buffer (PH.6.8) using at tissue grinder. Homogenized gut sections were suspended in ice-cold buffer and diluted to 1 ml. The homogenate was centrifuged at 500 rpm for 15 min. and supernatant was used as enzyme source.

Protease activity: The enzyme assays were carried out after Senthil Nathan *et al*. (2004). The reaction mixture of 1 ml of substracte (50 ppm bovine serum albumin), 1 ml of gut tissue extract and **0.1 ml solution of µg solution**, was incubated at 37°C, PH 11.7, for 1 hr. The control was 1 ml of heat-treated extract. The reaction was terminated by adding 1 ml of 50% trichloroacetic acid. The difference in

absorbance was measured at 600 nm, in spectrophotometer (Jascol Japan) UV/VIS Spectrophotometer Mode IV-570 Model).

Amylase activity: Amylase activity was determined after Bernfield (1955) modified by Ishaaya and Swirski (1970) who employed 3.5-dinitrosalicylic acid reagent. The reaction mixture consisted of 2 ml of 2% freshly prepared starch solution, 1 ml of 0.01µ phosphate buffer (PH 7.2), and 0.25 ml of enzyme extract. After incubating for 60 min at 37˚C, the enzyme activity was terminating by adding 0.4 ml of 3.5 dinitro-salicylic acid. The reaction mixture was maintained at 100°C for 5 min, sample absorbance was measured in optical density (OD) units at 550nm where the enzyme extract was replaced with deinonized water. The amylolytic activity was expressed as weight of the reducing sugars (glucose) produced by the enzyme action per unit weight of gut, per unit time, using glucose as the standard.

Lipase activity: The enzyme assays were carried out after Cherry and Crandall (1932). One milliliter of gut tissue extract (the control tube was placed in a boiling water bath for 15 min to destroy the enzyme activity and then cooled, 0.5 ml of phosphate buffer solution (PH 8.0) and 2 ml of olive oil (Riviera max. acidity 1%) emulsion were added, shaken well and incubated at 37˚C. After 24 hrs, 3 ml of 95% alcohol and 2 drops of 2% phenolphthalein indicator were added to each tube (control and experiment), tubes were titrated separately with 0.05N NaOH solution using Hamilton micro-burette, and the end point was marked by permanent pink color.

Statistical analysis: The effective concentration was calculated using Probit analysis (Finney, 1971), and values were expressed as means of five replicates with standard errors. Data from enzyme activity were subjected to analysis of variance (ANOVA of arcsine squar root transformed percentages). Differences between the treatments were determined by Tukey's multiple range test ($P<0.05$) (SAS, 2001).

Percentage reduction was calculated (Khazani, 1979).

$$\text{Reduction\%} = \frac{C - T}{C} X100$$

Where C = check experiment, T = treated experiment

Results

The results showed that IGRs affected the digestive enzymatic profiles of *C. maculates* at several doses. Tables 1-3 and figures 1-3demonstrate the efficacy and insecticidal activity of IGRs against digestive enzyme activities of the *C. maculates* hydroprene is unlikely to significantly enhance the activity of chlorfluazuron, when applied topically. Ec50 values of chlorfluazuron, hydroprene and hexaflumuron against *C. maculatus* are shown in Fig. 3.

Hydroprene was the most potent in all experiments with the lowest Ec50 third to fifth instars.

Differences in protease, amylase and lipase activities in the gut in the control and treated third instar larvae are shown in table 1. Treatment with hydroprene,

hexaflumuron and chlorfluazuron significantly decrease the activity of the digestive enzymes in individual and combined treatments. The maximal suppression of digestive enzyme activity was obtained by combinations of the three IGRs in all larval instars. Similarly, there was significant reduction in the activities of protease (maximum of 82%) amylase (maximum of 90%), and lipase (maximum of 92%) in the combination treatment. Digestive enzyme activity was considerably decreased when the insects were fed on seeds treated with all IGRs, compared to control treatment. Digestive enzyme activities significantly decreased with increasing concentrations of IGRs in all larval instars tested (Tabs. 1-3, Figs. 1-3).

There were statistically significant differences ($P \leq 0.05$) in enzyme activities in individual and combined treatment. Our data demonstrated that suppression of the digestive enzyme activities among the symptoms of toxicity that were observed following exposure to these bio insecticides. Insects fed with 1µg/ml – of all IGRs showed maximum reduction in protease (74% in fourth instar and 83% in fifth instar), and amylase (90% in fourth and 83% in fifth instar), and lipase (88% in fourth instar and 86% in fifth instar), compared with controls. The combined treatment of the tested IGRs affected digestive enzyme activity of the third to fifth instar larvae were decreased considerably more after treatment with combination of the tested IGRs in a dose dependent manner.

Discussion

Generally, chlorfluazuron, hydroprene and hexaflumuron have been tested on the beet sugar weevil, *Aubeonymus mariaefranciscae* (Coleoptera: curculionidae), to know their mode of action in adults and its metabolism, the lower concentration recovered in the eggs slows down the embryonic development and inhibit their hatching (Farions *et al*, 1998). Otherwise, this IGRs proved to be also very toxic against the larvae of *Schystocerca gregaria* (Coppen and Jepson, 1996) and the subterranean termite (Isoptera: Rhinotermitidae) workers who contaminated themselves by trophalaxis and of which it also disrupts the chitin synthesis (Sheets *et al*, 2000).

Others reported the effect of other growth regulators of the same family that the hexaflumuron, against insect pests of stored products. The analogue of JH (1-4' methyl-phenyl-3, 7 dichloro-3, 7-dimethyl-acetone) caused a strong mortality of *C. maculates* larvae with dose of 1.5 &2% as well as pathologic lesions in ovarian cloths (Sareen *et al*, 1992).

Five growth regulators analogues of the JH (MV-678, RD-20458, CGA-45128 and the fenoxy crab in treatment of *V. unguiculata* seeds with different doses of (10-100 mg/kg), induce a significant reduction of oviposition and hatching, protein synthesis inhibition, (Abo-El-Ghar, 1992). The terpen extract of *Saussura lappa* Clarke roots analogous of the juvenile hormone (JH), reduce significantly the fertility, the hatching rate of the eggs and the number of descendants in *C. maculatus* with dose of 0.75 & 1.0µg/adult female (Singh, 1998).

Exposure of *C. maculatus* adults to sublethal doses of IGRs in our laboratory studies reduced digestive enzyme activity. Higher enzyme activity in the mid gut of control insects is most probably due to consumption and utilization of large quantities of food. Imbalance in enzyme substract complex and inhibition of peristaltic movement of the gut (Hori, 1969), might have inhibited the enzyme activity in the treated insects. Chapman (1985) reported that enzyme production is clearly related to the feeding behavior (amount of food that passes through the alimentary canal). The activity of the enzyme is related to the physiological conditions of the rice leaf folder and effects the absorption, digestion and positive transport of nutrients in the mid-gut. IGRs affect serine proteases under the alkaline conditions in the intestinal fluid. The damage to the mid gut caused a decrease in digestive enzyme activity (Egwchi *et al*, 1972; Mathavan *et al*, 1989; Smirle *et al*, 1996; Senthil Nathan *et al*, 2005).

Decreased levels of digestive enzymes at higher concentrations of hydroprene and hexaflumuron suggest reduced phosphorous liberation for energy metabolism, decreased rate of metabolism, decreased rate of transport of metabolites and may be due to the direct effects of the tested IGRs on enzyme regulation. In the present study, after hydroprene and hexaflumuron treatment, the biochemical parameters and enzymatic profiles were markedly affected, It is evident that exposure to IGRs in adult diet has significant effects on several enzyme activities found in the late instar larvae and adult of *C. maculates*. IGRs may interfere with the production of certain types of proteins. This activity is apparently strongest during pupation. Pupae were very susceptible after larval exposure of hydroprene.

In conclusion hydroprene and hexaflumuron had significant effects on larval *C. maculatus* and they act synergistically with chorfluazuron causing reduction of digestive enzyme activity. However, Johnon *et al*, (1990) made a study of protease activity in the mid guts of larvae of susceptible and resistant strains of *Plodia interpunctella* (Hubner) and results indicated that resistance was not due to obvious changes in larval mid gut protease activity.

According to Dalaire *et al.* (2004), tebufenozide interferes with various aspects of the reproductive biology of males and females of *Choristoneuta fumiferana* and *C. rosaceara* (Lepidoptera: Tortricidae). The high accumulation of three insect growth regulators diflubenzuron, flucycloxuron and halotenzide, in the reproductive system of females and males of *T. molitor* explained their strong reproductive effects (Chebira *et al*, 2006).

The present study suggests that chlorfluazuron, hydroprene and hexaflumuron have the potential activity against *C. maculatus* and the results agreed with Suman *et al*. (2010).

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