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Remya, S and Gokuldas, M

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RESEARCH ARTICLE

CHANGES IN THE TITRE OF ECDYSTEROIDS IN THE MANGO LEAF WEBBER, *ORTHAGA EXVINACEA* (HAMPSON) DURING DEVELOPMENT

Remya, S and *Gokuldas, M

Department of Zoology, University of Calicut, Kerala, India 673 635

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ABSTRACT

Investigations were carried out to estimate the qualitative and quantitative variation of ecdysteroids in the larval hemolymph and pupae of *Orthaga exvinacea*. The analyses were done by using HPLC and FTIR. HPLC separations were carried out using Shimadzu system with a reverse phase column (C18) of 250×4.6 mm i.d. The HPLC separations were carried out in binary gradient for 20 min. Acetonitrile 15% was used as solvent A, trifluoro acetic acid (TFA 0.1%) as solvent B. The eluents were monitored at 242 nm using a UV–visible detector. The main component found in the hemolymph and pupae was 20-ecdysteriod. The titre of ecdysteroid in the pupae showed a higher value (1.23 µg/pupae) than the ecdysteroid in the hemolymph of 6th (0.70 µg/10 µL equivalent) and7th (0.60 µg/10 µL equivalent) instar larvae. Structural characterisation of ecdysteroids in the samples were obtained from FTIR spectra of the samples and standard. The major steroid component in all the samples were found to be 20E. There were 3 major absorption peaks, viz., for OH, C=O and C=C functional groups.

INTRODUCTION

Insects, the hexapod arthropoda, are among the most diverse groups of animals on the planet. They include more than a million described species and represent more than half of all known living organisms. They play a remarkable role in the world of living things. Many species are extremely valuable to man. An endless variety of structural and physiological peculiarities and adaptations to different living conditions are found among these animals. For these reasons, insect provide an ideal model for conducting studies on almost all the problems in physiology. They have shorter life spans and require less time and money for maintenance.

The life cycles of insects vary but majority of insects hatch from eggs. Insects are provided with a non-elastic exoskeleton and the growth and development involves a series of moults. The immature stages can differ from the adults in structure, habit and habitat, and can include a passive pupal stage in those groups that undergo 4-stage metamorphosis. Insects that undergo 3-stage metamorphosis lack a pupal stage and adults develop through a series of nymphal stages.
Various biochemical and physiological process in insects are under the regulation of hormones secreted by endocrine glands. Ecdysteroids, a group of poly hydroxyl sterol compounds, are moulting hormones in both insects (Sehnal, 1989) and crustaceans (Chang and O'Connor, 1988). In insects, they play an important role during development, such as induction of ecdysis, termination of diapause and regulation of reproduction and embryonic development (Wang and Gong, 1997; Haegele and Wang, 2004). Evidences also show that ecdysteroids are involved in the regulation of yolk protein synthesis in insects (Bownes, 1989).

The main components of insect ecdysteroids are α-ecdysone (E) and 20-hydroxyecdysone (20E) (Tawfik *et al*, 1999; Cao and Jiang, 2002). They also contain compounds like 26-hydroxyecdysone (26E) and 2-deoxyecdysone (2dE). The hemolymph ecdysteroid titre at any instant of development of an insect reflects the balance between the entry of ecdysteroids into the hemolymph due to synthesis or release from storage, and their removal from the hemolymph by excretion, sequestration or uptake into storage. Such changes may also lead to changes in the relative proportions of different ecdysteroid molecules in the hemolymph (Gerstenlauer and Hoffmann, 1995).

Ecdysteroid titres decrease in hemolymph of *Drosophila melanogaster* after mating (Lawrence *et al*, 1999) which is thought to be due to the yolk protein uptake of ecdysteroids into developing vitellogenic oocytes as a consequence of male

*Corresponding author: **Gokuldas, M**
Department of Zoology, University of Calicut, Kerala, India 673 635

accessory gland protein stimulation of female oocyte maturation and yolk protein synthesis following mating. In this insect, both fat body and ovary synthesize yolk protein, and the fat body yolk protein gene transcription is stimulated by 20-hydroxyecdysone (Bownes, 1989). Ovarian and hemolymph ecdysteroid concentrations increases during ovarian development in the majority of the insects studied. During the last larval instar of *Gryllus bimaculatus*, the level of ecdysteroids in the hemolymph showed variation. The hemolymph ecdysteroid titre showed a small peak on day 1 and a large peak on day 7. Thereafter the titre declined drastically prior to the imaginal moult (Gerstenlauer and Hoffmann, 1995). Quantitative and qualitative differences in the ecdysteroid levels were also observed in other insects like *Pieris brassicae* (Lafont, 1975), *Bombyx mori (*Calvez *et al*, 1976*)*, *Heliothis zea* (Holman and Melola, 1978*)*, *Manduca sexta* (Bollenbacher, 1981), *H. armigera* (Zhu *et al*, 1986) and social bees (Pinto *et al*, 2002) during development.

Changes in the hemolymph ecdysteroid titre throughout the life of an insect clearly indicate the correlation between moults and peaks in ecdysteroid titres. Generally massive peaks are observed during larval moults, displayed prior to moulting. In hemimetabolous insects the larval-adult moult like-wise display a single peak. Ecdysteroids in the hemolymph of the silk moth, *B. mori* closely paralleled that of the testes just after the 4th ecdysis, although the titres were much higher than the amount of ecdysteroids in testes. However, this level decreased rapidly by day 2 which increased gradually afterwards. An abrupt increase of ecdysteroids titre was observed during the wandering stage. After pupation, the level of ecdysteroid became increasingly detectable on day 3 of the pupal stage and it decreased rapidly thereafter (Fugo *et al*, 1996).

Ecdysteroids as well as juvenile hormones, the hormones that regulate the overall development and metamorphosis, collectively referred to as insect growth regulators (IGRs) are considered as prospective candidates for developing mimics for the control of insect pests. Any changes in the homeostasis of these hormones with exogenous sources of hormones or with synthetic analogues would result in the disruption or abnormal course of development and reproduction of the target insect (Hoffmann and Lorenz, 1998). In *M. sexta* larvae, administration of ecdysteroid analogue RH- 5849 showed an increase in the level of this compound in the hemolymph 6 h after administration and a slight decrease thereafter, which was stable over next 36 h. In addition to these effects, RH- 5849 was showing a powerful ecdysonergic activity by initiating premature lethal moults (Wing *et al*, 1988). In the spruce budworm *Choristoneura fumiferana* and *M. sexta*, a failure in endocuticle production was reported for the RH- 5992 treated larvae (Retnakaran and Oberlander, 1993). The strategy thus involves the use of steroid analogues to tamper with the hormonal balance in the insect there by interrupting normal moulting and growth and associated metabolic pathways leading ultimately to the death of the insect. Several steroidal and non-steroidal analogues have been attempted successfully for the control of pest insects (Chandler *et al*, 1992; Heller *et al*, 1992).

The mango leaf Webber *Orthaga exvinacea*, belonging to the family Pyralidae of Lepidoptera, is a serious and sporadic pest of mango throughout south India (Ayyar, 1940). *O. exvinacea* larvae club the leaves together with silken threads, feed on the leaves from inside (Berin *et al*, 2008). This holometabolous insect has 7 larval instars, pupal stage and adult stage. The life cycle of this insect is completed within 45 days.

In the present study, we investigated the qualitative and quantitative variation of ecdysteroids (20E) in the hemolymph of 6th and 7th instar larvae and pupae of *O. exvinacea* using HPLC, since the hormone play an important role throughout the development of the insect. Structural characterisations were also done using FTIR.

MATERIALS AND METHODS

Insect collection and rearing

Larvae of the mango leaf webber, *O. exvinacea* were collected from their natural habitat, i.e., mango trees and were brought and transferred to plastic troughs kept in the insectary. They were reared on mango leaves. The troughs were covered with cotton cloths and kept in large wooden insect cages of 14"×14"×14" with three side covered with aluminium gauze and the top with glass. There was a glass door for the cage on one side. The larvae were protected from ants by water barriers. The plastic troughs were cleaned on alternate days. The insect culture was maintained at a temperature of about 27 ± 2^{o}C and relative humidity of 70-80 %.

Collection of hemolymph from larvae

Hemolymph were pooled from 3-4 day old 6th and 7th instar larvae (5 to 10 nos.) by puncturing the prolegs, to get sufficient volume of the hemolymph. A volume of 35 µL each of both the hemolymph samples were drawn for analysis. Samples were transferred in to Eppendorf tubes containing 3 µL of insect ringer, kept on ice and were stored at 4^{o}C until use.

Ecdysteroid extraction from larval hemolymph and pupae

Ecdysteroids was extracted as detailed by Haegele and Wang (2004). Hemolymph (35 µL), collected from the 6th and 7th instar larvae were mixed with 1.0 mL 70% methanol. The samples were then heated to 60^{o}C for 10 min, cooled to room temperature and centrifuged at 10000 g for 10 min. Supernatants were collected and the pellets re-extracted twice with 70% methanol. Combined supernatants were dried under reduced pressure and the dried residues partitioned between 300 µL 70% methanol and 300 µL hexane to remove apolar lipids (Dinan and Rees, 1981). The hexane phase was discarded and the lower methanolic phase was desiccated, re-dissolved in 500 µL 70% methanol and taken for FTIR and HPLC analysis.

Since the hemolymph was not accessible from the pupae, the whole pupae were used for the extraction of ecdysteroids. Pupae (5nos.) were homogenized in 1.0 mL 70% methanol and

extraction of ecdysteroid was done by using the same procedure used for the larval hemolymph.

Analysis of ecdysteroids using HPLC

Aliquots (100 µL) of the extracts prepared as described above were diluted with 100 µL each of 70% methanol. The samples were filtered using a filtration unit (Millipore, USA) with a filter of 0.45 µm pore size. Samples (20 µL) drawn from the diluted extracts were further mixed with 60 µL each of 70% methanol. Used 20 µL each of this diluted samples for injection into the HPLC instrument (Shimadzu, LC 20AD, LC 20AD, SPD 20A) using a Hamilton micro-syringe. High performance liquid chromatographic (HPLC) separations of all the samples and standard were carried out with a reverse phase column (C18) of 250×4.6mm i.d., in a binary gradient in 20 min at a flow rate of 1.0 mL/min. Acetonitrile 15% was used as solvent A and trifluoro acetic acid (TFA 0.1%) as solvent B. All the solvents were filtered in Millipore filter of pore size 0.45 µm. The eluents were monitored at 242 nm using a UV–visible detector. The chromatograms were exported in to Microsoft Word file.

The standard ecdysteroid (20E) was a gift from Central Silk Board, Madivala, Bangalore, India. Samples of the standard were run on HPLC for purity check. The single large peak with retention time of 3.4 min confirmed 100%purity. Known concentration of the standard (20 µL equivalent to 10.6 µg) was injected in to the HPLC instrument maintained in the same condition as for the samples. Similarity of retention time of any materials (appearing as peak) of the extracts of *O. exvinacea* with that of standard ecdysone was confirmed by overlying this profile with that obtained for standard ecdysone. HPLC run was repeated for 3 samples and the peak area calculated were almost same for all the runs.

Sample preparation for FTIR

Samples (10 µL each) for FTIR analysis were taken from the extracts prepared from larvae and pupae of *O.exvinacea* as described earlier. The secondary structural characterisation of the ecdysteroid samples were done by using JASCO FTIR-4100 instrument (USA). To obtain the infrared spectrum, the samples were placed in the sample holder between a pair of KBr plates, referred to as salt plates. When the plates were squeezed gently, a thin liquid film formed between the plates. The pair of plates was then inserted into the holder that fits into the spectrometer. The frequency of absorption selected was in the region of 4000 to 400cm^{-1} at room temperature. Infrared spectra were recorded for all the samples, each from32 scans per samples with resolution of 4cm^{-1}. Same procedure was employed for analysing the standard ecdysteroid.

RESULTS

Qualitative analysis of ecdysteroids

Ecdysteroids extracted from the hemolymph of 6th and 7thlarval instars and pupae of *O. exvinacea* were separated on HPLC. An obvious peak and some small peaks were found in all samples. The main peak had the retention time of 3.2 min which was similar to that of standard 20E (Fig. 1-4). This shows that the main ecdysteroid present in the extract derived from 6th and 7th instar larval hemolymph and pupae of *O. exvinacea* is 20-ecdysteroid.

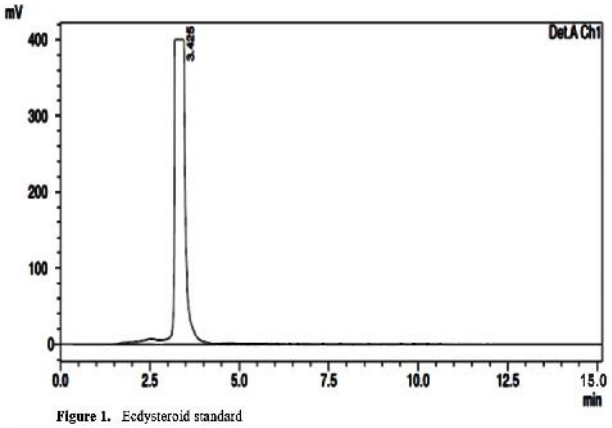

Figure 1. Ecdysteroid standard

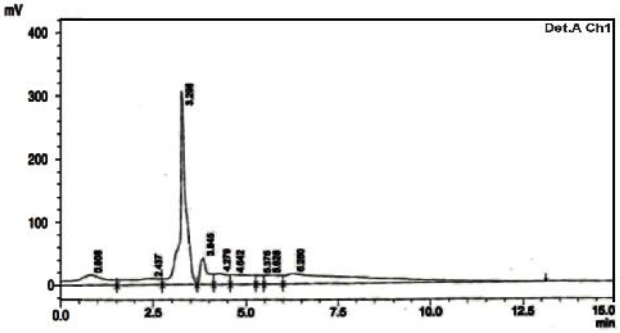

Figure 2. 6th instar hemolymph ecdysteroid

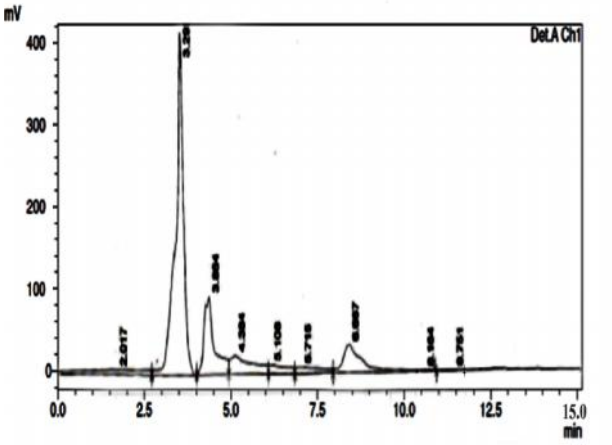

Figure 3. 7th instar hemolymph ecdysteroid

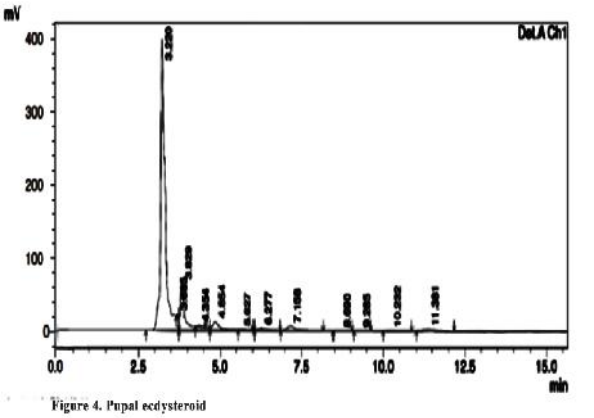

Figure 4. Pupal ecdysteroid

Quantitative analysis of ecdysteroids

Concentrations of ecdysteroid present in the samples were calculated by using the area percent.

$$\text{Concentration of the sample} = \text{Concentration of the standard} \times \frac{\text{Area of sample}}{\text{Area of standard}}$$

The titre of ecdysteroid in the pupae showed a higher value (1.23 µg/pupae) compared to the titre of ecdysteroids present in the hemolymph of both the 6th and 7th instar larvae. The ecdysteroid in the hemolymph of 6th instar larval showed a titre (0.70 µg/10 µL equivalent), which was a little bit higher than the titre of ecdysteroids present in the 7th (0.60 µg/10 µL equivalent) instar larval hemolymph.

Ecdysteroid structural characterization using FTIR

The secondary structural characterisation of the ecdysteroid samples were done by using JASCO FTIR-4100 instrument (USA) by placing a drop of ecdysteroid sample with KBr. The frequency of absorption was in the region of 4000 to 400cm^{-1} at room temperature. Infrared spectra were recorded from 32 scans per samples with resolution of 4cm^{-1}.

Standards

Ecdysteroid standard shows three major peaks (Fig. 5). The presence of a number of hydroxyl groups on most ecdysteroids ensures strong absorption in the infrared (IR) spectrum in the region of 3340 cm^{-1} – 3500 cm^{-1}. The standard showed absorption peaks of OH at 3452.92 cm^{-1}, carbonyl (C=O) at 1637.27cm^{-1}. Normally C=O in the range of 1640cm^{-1} – 1670 cm^{-1}. Here, the small decrease shown in frequency is due to the presence of adjacent C=C bond in the structure of ecdysteroid. A strong absorption peak at 1407.78 cm^{-1}was also observed. This is due to the presence of C=C in the structure.

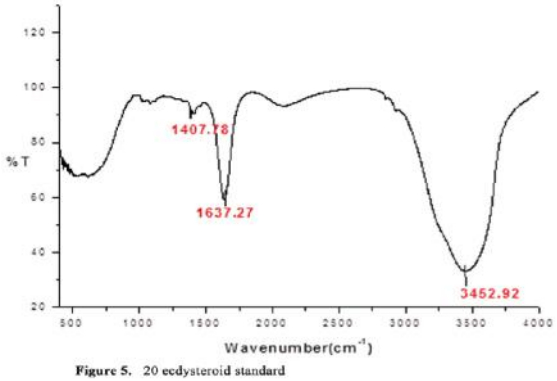

Figure 5. 20 ecdysteroid standard

Samples

The FTIR spectrum of the main eluting peaks for the ecdysteroids extracted from pupae and hemolymph of 6th and 7th instar larvae of *O. exvinacea* also showed a strong absorption with similarities in the finger print region of 20 ecdysteroid. In the case of ecdysteroid samples obtained from the hemolymph of 6th and 7th instar larvae, the infrared spectrum showed three major absorption peaks (Fig. 6&7) as in the case of the standard. The prominent absorption peak observed at 1634.86 cm^{-1}is due to the presence of C=O. Both samples showed OH absorption peaks, which was at 3445.69 cm^{-1} for 6th instar and at 3434.61 cm^{-1} for 7th instar. Absorption peak corresponding to C=C is at 1384.16 cm^{-1}in case of 6th instar and 1454.55 cm^{-1}in case of 7th instar.

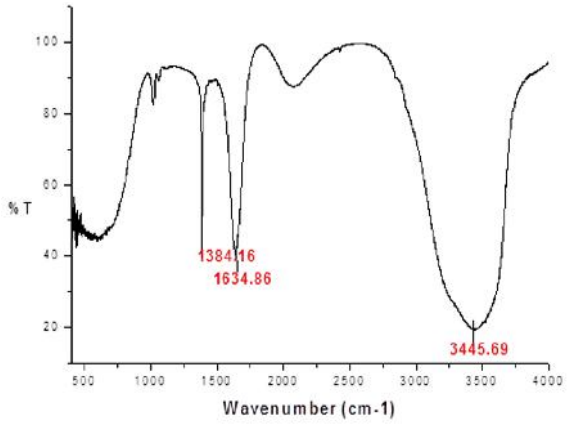

Figure 6. 20 ecdysteroid present in the 6th instar larval hemolymph

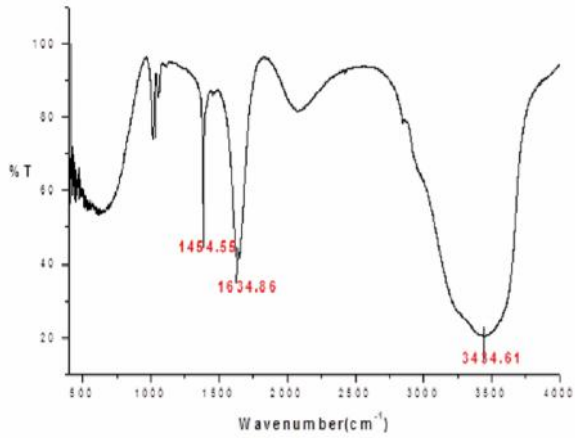

Figure 7. 20 ecdysteroid present in the 7thinstar larval hemolymph

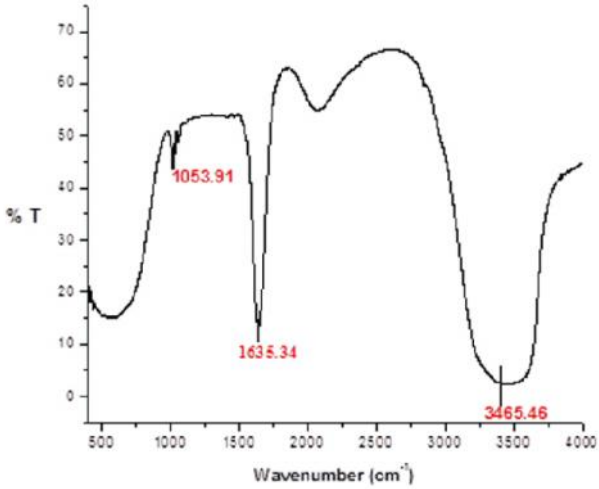

Figure 8. 20 Ecdysteroid present in the pupae

The FTIR spectrum of the main eluting peaks for the ecdysteroids extracted from pupae showed strong absorption (Fig. 8) at 1635.34 cm^{-1}for C=O group, 1053.91 cm^{-1}for C=C and 3465.46 cm^{-1}for OH group.

Some small peaks were also seen, which are due to the presence of CH_3 groups in the structure of ecdysteroid and aromatic compounds present in the solvent.

DISCUSSION

Insect development is controlled by two types of hormones, ecdysteroids which drive moulting and metamorphosis, and juvenile hormone, which determines the nature of moult. Ecdysteroids produced by the prothoracic glands have an important role in insect life cycle (Sakurai, 1995).

In our study using HPLC and FTIR, we found that the main ecdysteroid component present in the larvae and pupae of *O. exvinacea* is 20-ecdysteroid (20E). Occurrence of 20E as one of the major ecdysteroid was reported in *Schistocerca gregaria* (Tawfik *et al*, 1999). Along with 20E, α-ecdysone also was reported to be a major component. Other compounds included 26E, 2dE and 20, 26-hydroxy ecdysone.

Analysis for the changes in the ecdysteroid titre during development, showed that the ecdysteroid titre was 0.70 µg/10 µL in the 6th instar larval hemolymph whereas it was 0.60 µg/10µL in the 7th instar larval hemolymph. In the case of pupae the ecdysteroid titre was higher (1.23 µg/pupae) compared to the ecdysteroids present in the larval hemolymph. The amounts of the hormone in the larval stages appear to be in the normal ranges reported in insects. The hormone titre maintained in the hemolymph meets the requirement for moulting during the larval stages. However, the pupal ecdysteroids show a higher range value than some of the reported values. In *Opogona sacchari*, for example, the titre of ecdysteroids in both male and female pupae reaches a peak of 80 ng/pupae which later dropped to 30 ng/ pupae. It was also seen that the content of ecdysteroids is higher in female pupae than in male pupae during development (Wang *et al*, 2006). Investigations on the sex difference in the titre of ecdysteroids was not attempted in our study. The high value for the pupal hormone titre obtained for this species of insect can only be explained in terms of the species difference.

Structural studies using FTIR showed 3 major absorption peaks, for OH, C=O, C=C functional groups present in the ecdysteroid structure. These functional groups are characteristic of ecdysteroids. Similar functional groups have been reported in some plants using FTIR. For example, in the caryophyllaceae plant, *Lychnis flos-coculi*, FTIR have shown the presence of functional groups characteristic of insect ecdysteroids which were identified as phytoecdysteroids present in those plants (Louden *et al*, 2001).

Ecdysteroids as well as juvenile hormones, the hormones that regulate the overall development and metamorphosis, are collectively referred to as insect growth regulators (IGRs). Insects are unable to develop resistance to molecules that mimic their own hormones. Compounds such as methoprene, hydroprene, kinoprene, pyriproxifen, RH-5992, diflubenzuron are found to be effective against many dipteran, coleopteran, homopteran and lepidopteran insect pests. Ketokonazole, a synthetic imidazole derivative, is known to inhibit the ecdysone-20 monooxygenase and is very effective in inhibiting the terminal hydroxylation steps of ecdysteroid biosynthesis in adult locusts and crickets (Jarois *et al*, 1994; Lorenz *et al*, 1995). The newest member, RH-2485 of the bisacylhydrazine class, are more effective than tebufenozide and act against a wide range of lepidopteran pests, with a high degree of safety with respect to non-target organisms. A high level of insecticidal activity of methoxyfenozide against a wide range of important caterpillar pests was reported by Carlson *et al* (2001). A reduction in the egg laying was observed in *Cydia pomonella* treated with RH-5992 and RH-2485 (Knight, 2000; Knight *et al*, 2001). All these non-steroidal ecdysone agonists manifest their effects through the interaction with the EcR/USP receptor complex. Designing analogues of the ecdysteroids will be challenging in the development of non-chemical insecticides, as these compounds are highly specific to insects, eliciting no reaction on other species of animals. Determining the dosage and formulation will be other areas in which investigations are to be extended. Attempts to control more pests including *O. exvinacea* are to be carried out in order to establish the effectiveness of the new compounds.

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