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Impact of two bisacylhydrazines on development of *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae) with respect to cuticular thickness and protein

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Abstract
Prototype (RH-5849) and Tebufenozide (RH-5992), two insecticides that imitate the action of ecdysone. Our study was conducted to assess the adverse effects of two different doses of these compounds; (LD_{50} and LD_{90}, RH-5849: 0.05 µg/insect; 0.18 µg/insect and RH-5992: 0.005 µg/insect; 0.05 µg/insect) were applied topically on newly ecdysed pupae. Cuticular thickness during metamorphosis and the quality of cuticular protein at apolysis were measured. Results had been shown that both analogues increased old cuticle thickness (pupal cuticle) in the first days, then they reduced it. Likewise, the RH-5992 induced the precocious apolysis and the early secretion of new cuticle (adult cuticle) and increased it at the fifth, seventh and ninth day. On another hand, the electrophoresis separation of cuticular proteins was shown that the RH-5849 provoked the absence of one protein band while the RH-5992 induced the absence of two protein bands. Thus, ecdysteroid agonists were found to interfere with cuticule secretion in *E. kuehniella* confirming the primary mode of action of the bisacylhydrazines insecticides.

Keywords: Prototype, tebufenozide, *Ephestia kuehniella*, cuticle proteins, electrophoresis

1. Introduction
The storage of cereals to supply population's needs for food promotes the appearance of many ravagers. *Ephestia kuehniella* is a very important cosmopolitan pest of stored products [1, 2]. Fighting against this pest is based on using conventional insecticides such as fumigants. However, the substantial and indiscriminate use of conventional pesticides leads to the appearance of resistance in target species [3-7], may affect both non-target species and the environment [8, 9].Also, these compounds are related to some serious health effects [10]. Insect growth regulators are better because of their selectivity and non-persistent proprieties. So, they are less toxic to both environment and mammals such us humans [11-14], which is compatible with their use in integrated pest management (IPM) programs [15, 16]. They are presented as alternative compounds against insect pests [17]. IGRs are potential compounds to control different insects by affecting the embryonic and post-embryonic development, reproduction, feeding, and behavior of treated insects [18-21].
The bisacylhydrazine, nonsteroidal ecdysteroid agonists, is a group of IGRs that includes chromafenozide, Tebufenozide, halofenozide and methoxyfenozide which are synthetic products interfering with the natural insect molting hormone (20-hydroxyecdysone) that controls several physiological and biochemical processes of growth and development by binding to the nuclear ecdysteroid receptor (EcR) as does the natural insect molting hormone, 20-hydroxyecdysone (20E) [14].
The molting is an important physiological phenomenon in insect's development cycles witch is activated by the 20-hydroxyecdysone [22, 23]. The hormone penetrates into the epidermal cells and stimulates genes related to molt. Then, it activates epidermal cells undergo mitosis. The existing cuticle (old) is separated from the epidermis cells; that is apolysis. Ecdysial space resulting between the cuticle and epidermal cells is filled with a molting liquid which contains inactive enzymes; these are activated after the secretion of a new epicuticle and begin to digest the old cuticle. The digested products are absorbed by epidermal cells and reused later in the production of the new cuticle.
The present study was undertaken to evaluate the effect of two bisacylhydrazine, the protype (RH-5849) and the Tebufenozide (RH-5992), on cuticles thickness, apolysis, secretion of new

cuticle during metamorphosis and on the quality of cuticular proteins at apolysis. The two compounds were applied topically to newly exuviate female's pupae of *E. kuehniella* with two doses (LD_{50}, LD_{90}).

2. Materials and methods

2.1 Insects

Last instars larvae of *E. kuehniella* were collected from flour infested and placed in plastic boxes, these last will be put then in a drying oven at a temperature of 27 °C and a relative humidity of 80%. Newly exuviate female pupae were collected every day and treated immediately. The duration of development of *E. kuehniella* females pupae is 10.02 days [18]. Pupae were taken at various times after pupal ecdysis (1, 3, 5, 7 and 9 days).

2.2 Insecticides and treatment

Technical products, RH-5849 (Prototype, Rohm & amp; Haas, Spring House PA, USA) and Tebufenozide (RH-5992, trade name: Mimic or Confirm, Dow Agro Sciences, USA) were kindly provided by Prof. G. Smagghe (Ghent University, Belgium). They were dissolved in acetone and administered by topical application on the ventral sternites of the female pupae newly exuviated using a micropipette to their LD_{50} and LD_{90} determined previously RH-5849: 0.05 µg/insect and 0.18 µg/insect; Tebufenozide: 0.005 µg/insect and 0.05 µg/insect [24]. Three groups of 10 pupae per dose were used. The compounds were easily diluted in acetone, allowing better diffusion of the active ingredient throughout the cuticle.

2.3 Histology

Histological procedures were performed following Martoja & Martoja (1967) [25]. Pupae aged 0, 1, 3, 5, 7 and 9 days were recuperated and fixed in alcoholic Bouin's solution for 48 h. Before carrying out the impregnation with paraffin, it is necessary initially carrying out successive passages of the pupae in alcohol baths of increasing order, as follows: (96 °, 100 °) then in butanol. The impregnation with paraffin is carried out in several paraffin baths. Then, one carries out the setting in block, in plastic cassettes, the blocks are cut by the microtome to a thickness of 5 µm. Sections were mounted on slides and stained using hematoxylin and eosin. After mounting with Canada balsam, the observations of the slides were made using a Leica DM500 microscope equipped with a Leica ICC50 HD camera.

2.4 Electrophoresis

2.4.1 Extraction: The samples were extracted at 4 °C with stirring for 24 hours. The homogenate is centrifuged at 5000 revolutions/min for 15 minutes. An aliquot (100 µl) is adapted to the quantification of protein and other fraction is lyophilized and used to study the electrophoretic protein.

SDS-gel electrophoresis: For electrophoretic studies 6 pupal cuticles of *E. kuehniella* were dissected to apolysis and conserved in phenyl methyl sulfonyl fluide or PMSF (45mg/ml ethanol) at 0.1% in distilled water. SDS–PAGE was accomplished in slab gels according to Laemmli (1970) [26]. Soluble proteins were mixed with the sample buffer, boiled at 60 °C for 4 minutes. Small amount (10 µg) of the boiled samples were cooled and loaded into 12% polyacrylamide gel. The gels were stained for 30 min in staining solution (0.025% Coomassie brilliant blue R 250 (Merck), 10% acetic acid, and 25% of 2-propanol). After 30 min, the gel was removed from the staining solution, rinsed with distilled water and destained in 10% acetic acid then placed in destaining solution contains (4.5% methanol and 10% acetic acid, 2.5% glycerol, 10% ethanol).

2.5 Statistical analysis

The results are represented as means ± standard deviation for n repetitions, the number of animals tested per series is given with the results using MINITAB Software (Version 16, PA, State College, USA).

3. Results

3.1 Effect of RH-5849 and RH-5992 on cuticles thickness

The secretion of the pupal cuticle begun from the transformation of larva to pupa and the thickness of cuticle peaking at the third day (apolysis) in controls and treated series with RH-5849 then started decreasing, while the secretion of the adult cuticle begun in fifth day. A comparison of the means cuticle thickness of the controls and treaties shown that the RH-5849 affected the thickness of the pupal cuticle only with DL_{90}. In fact, it increased significantly the thickness of the pupal cuticle in pupae in the 3rd day ($p = 0.002$), so that it significantly decreased in pupae in ninth day ($p= 0.025$), also the RH-5849 increased significantly ($p=0.028$) the thickness of the new cuticle in the 9 day (figure 1).

The results concerning the effect of Tebufenozide (RH-5992) after topical application in newly exuviate females pupae with two doses (LD_{50} and LD_{90}) on the cuticles thickness are shown in Figure 2. The RH-5992 increased significantly the thickness of the pupal cuticle in the first day with both doses ($p = 0.000$), in the third day with the LD_{50} ($p = 0.006$) and with the LD_{90} ($p = 0.000$). While in the fifth day the RH-5992 increased the thickness of the pupal cuticle significantly with both doses (LD_{50}: $p=0.002$ and LD_{90}: $p= 0.005$).In the ninth day the RH-5992 caused a significant decrease in thickness with the LD_{90} ($p= 0.07$). Thus, the compound inducing early apolysis coinciding with first day in the series treated with the highest dose (Figure 4) also caused the synthesis of a new cuticle in the third day, the administration of RH-5992 caused a significant increase in the thickness of the new cuticle ($p=0.000$).

B

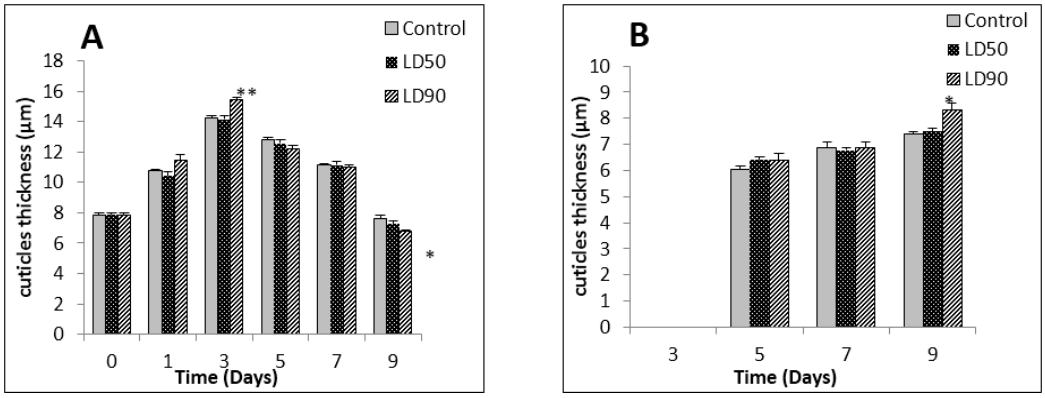

Fig 1: Effects *in vivo* of the RH-5849, administered by topical application in the newly exuviated females pupae of *E. kuehniella* on the cuticles thickness (µm) during pupal development of old (A) and new cuticle (B).

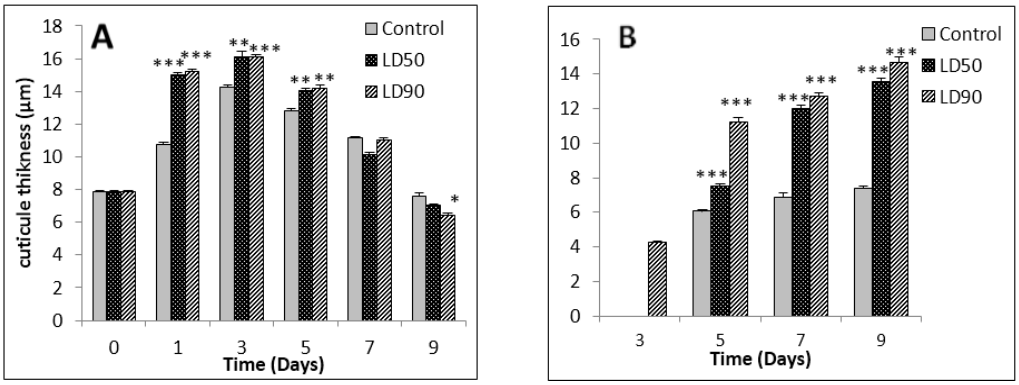

Fig 2: Effects *in vivo* of the RH-5992, administered by topical application in the newly exuviated females pupae of *E. kuehniella* on the cuticles thickness (µm) during pupal development of old (A) and new cuticle (B).

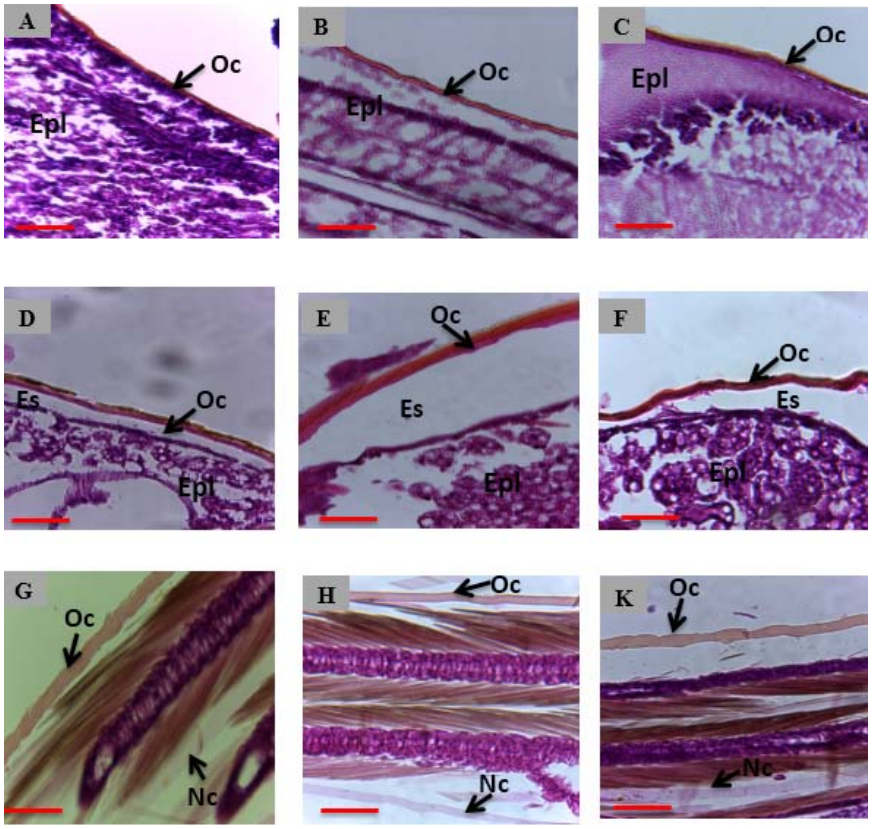

Fig 3: Transversal section of the integument of *Ephestia kuehniella* after topically application of RH-5849 with two doses in newly exuviated pupae at different aged (A: control at 1 day; B: treated with DL_{50} at1 day. C: treated with DL_{90} at 1 day; E: control at 3 day; F: treated with DL_{50} at 3day. C: treated with DL_{90} at 3 day; G: control at 9 day: treated with DL_{50} at 9 day. K: treated with DL_{90} at 9 day). Es: ecdysial space, Oc: Old cuticle, Nc: New cuticle, Epl: Epidermal Cell. Scale bars ( ):160 µm

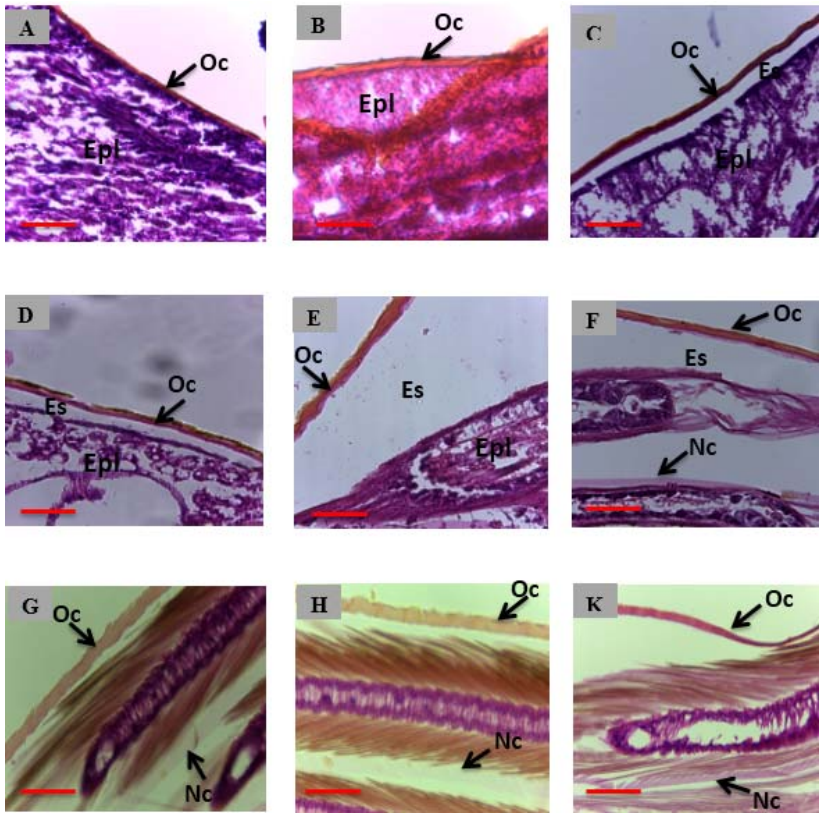

Fig 4: Transversal section of the integument of *Ephestia kuehniella* after topically application of RH-5992 with two doses in newly exuviated pupae at different aged (A: control at 1 day; B: treated with LD_{50} at 1 day. C: treated with LD_{90} at 1 day; E: control at 3 day; F: treated with LD_{50} at 3day. C: treated with LD_{90} at 3 day; G: control at 9 day: treated with LD_{50} at 9 day. K: treated with LD_{90} at 9 day). Es: ecdysial space, Oc: Old cuticle, Nc: New cuticle, Epl: Epidermal Cell. Scale bars ( ):160 µm exceptionally F, K Scale bars: 190 µm

3.2 Effect on the quality of cuticular proteins

Two compounds were administered topically with both doses LD_{50} and LD_{90} to evaluate their effects on the quality of cuticular proteins. The separation of protein bands of controls and treated series are presented in figure 5. The RH-5992 (LD_{50} and LD_{90}) caused the absence of two bands, the third and the sixth, while the RH-5849 caused the lack of the sixth one.

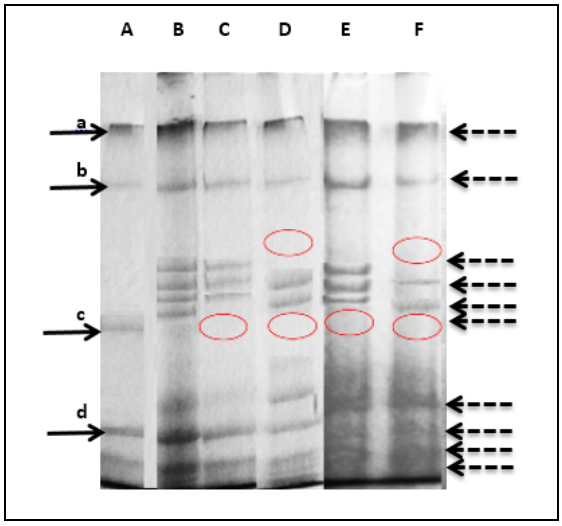

Fig 5: SDS–PAGE pattern of cuticular proteins to apolysis after topical application with RH-5849 and RH-5992 on newly ecdysed pupae of *E. Kuehniella*. A: protein markers (a: Phosphorylase b; b: Bovine serum albumin; c: carbonic hydrolase; d: soybean trypsin inhibitor); B: control; C: DL_{50} RH-5849; D: DL_{50} RH-5992; E: DL_{90} RH-5849; F: DL_{90} RH-5992.

4. Discussion

Insect growth and development is regulated by various hormones which are the two principal 20-hydroxyecdysone and juvenile hormone [14]. The morphological and ultrastructural changes that occur in the epidermis during insect growth and development are dependent upon the regulation of gene expression with different concentrations of 20E in the absence or presence of JH. Any interference by IGRs with a natural hormone action would result in the disruption or abnormal development of the target insect.

In the present work, the histological study of the cuticle to the pupa of *E. kuehniella* has summoning ages after treatment by topical application on newly ecdysed pupae with two nonsteroidal ecdysteroid agonists (RH-5849 and RH-5992) allowed to estimate the effect of products on the thickness of pupal and adult cuticle during the metamorphosis. In fact, according to our experimentation witch showed that the analogues accelerate the development *via* the acceleration of the cuticular cycle. They have also caused the increase of the thickness of the pupal cuticle during the first days then a reduction in treated compared with controls, thus the RH-5992 increased the thickness of the new cuticle. Similar results were obtained in the fourth-instar larvae of *C. pipiens* after treatment with halofenozide [27, 28].

Also according to our work the RH-5992 causes a precocious apolysis (detachment of the cuticle) and the synthesis of new cuticle. This is in accordance with other experiments, for example the secretion of a new cuticle in mealworms can be induced by 20-hydroxyecdysone [29] and with RH-0345 [30]. Similarly the treatment of *Tenebrio molitor* pupae with RH-0345 only or in combination with KK-42 with the renewal of the culture medium induced an early apolysis and secretion of a new cuticle of a thickness of about 3.6 microns [31]. Thus four benzylhydrazine (RH-5849, RH-5992, RH-0345 and RH-2485) tested *in vivo* on the last-instars of *Spodoptera littoralis* caused a premature molting leading to death and could initiate and sustain the evagination of isolated wing discs *in vitro* [32] and the Tebufenozide stimulated epidermal cells to undergo apolysis prematurely and to synthesize a new larval cuticle by imitating the activity of ecdysteroids [33]. Moreover the ecdysone agonists RH-5849 and Tebufenozide induced precocious molting, but without a surge of the ecdysteroid titers in last-instar larvae of *Spodoptera exigua* and *Leptinotarsa decemlineata* [34] and induced premature and lethal molts in *Plodia interpunctella* [35]. Similarly the Tebufenozide caused the detachment of the cuticle from the hypodermis, undistinguishable appearance of epicuticle and exocuticle in last instar nymphs of *Schistocerca gregaria* [36]. Another experiment showed that the RH-5992 caused the early apparition of exuvial space as well as the news epicuticle with distinct cuticulin and dense epicuticle layers in newly moulted sixth instar larvae of *Choristoneura fumiferana* [37]. Sundaram, showed that RH-5992 induced premature adult cuticle formation in *Choristoneura fumiferana* [38]. Also, treatment of *L. decemlineata* larvae by RH-0345, RH-5849, RH-5992 and the 20E, caused premature molts followed by inhibition of moulting [39].

Electrophoresis revealed that RH-5849 and RH-5992 affected softly the number of proteic band. Similar results were found with other molecules, pyriproxyfen did not affect the pattern of hemolypmh proteins on pupae of *T. molitor* [40], halofenozide and 20E tested *in vitro* on *T. molitor* do not affected the pattern of cuticular proteins [41, 42].

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