EFFECT OF THURINGIENSIN ON CUTICLE DEVELOPMENT OF IMMATURE STAGES OF Tetranychus urticae Koch.
(ACARINA: TETRANYCHIDAE)¹

Efecto de thuringiensin en el desarrollo de la cutícula de estados inmaduros de Tetranychus urticae Koch. (Acarina: Tetranychidae)

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ABSTRACT

The effect of thuringiensin on the ultrastructure of Tetranychus urticae Koch cuticle was studied using an transmission electron microscope (TEM). Thuringiensin was found to affect epicuticle synthesis in larvae, protonymphs and deutonymphs. Its disruptive effects on larval cuticle formation were observed from 12 h onwards, indicating that the effect of the exposure time of mites to thuringiensin is related to the stage of cuticle development. The results suggest that the effects of thuringiensin occur in a relatively short time.

Key words: thuringiensin, β-exotoxin, Tetranychus urticae, cuticle.

RESUMEN

Los efectos de thuringiensin sobre la ultraestructura de la cutícula de Tetranychus urticae Koch fue estudiada usando un microscopio electrónico de transmisión (TEM). Thuringiensin afecta la síntesis de la epicutícula de larvas, protoninfas y deutoninfas. Este efecto disruptivo sobre la formación de la cutícula en larvas fue observado desde 12 h en adelante indicando que el efecto del tiempo de exposición de arañitas a thuringiensin está relacionado con el estado de desarrollo de la cutícula. Los resultados sugieren que los efectos de thuringiensin ocurren en tiempo relativamente corto.

Palabras clave: thuringiensin, β-exotoxin, Tetranychus urticae, cutícula.

INTRODUCTION

During development of pesticides, attention has been focused on their ability to inhibit moulting in arthropods. In particular, compounds like aminopterin, methotrexate, cyromazine, puromycin, cyclohexamine and several benzoylphenyl ureas have been reported to inhibit cuticle synthesis (Binnington and Retnakaran, 1991). Although thuringiensin, the β-exotoxin of Bacillus thuringiensis Berliner, was discovered in 1959 (McConnell and Richards, 1959) the reported research mostly relates to the biochemistry and toxicity (Sebesta et al., 1981; Beegle and Yamamoto, 1992). Information available indicate that thuringiensin has high

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potential to inhibit moulting in insects and mites, whereas only fecundity and longevity of adults are disrupted at high concentration (Sebesta et al., 1981; Royalty et al., 1990, 1991).

Thurigiensin belongs to a group of nucleic acid metabolism inhibitors. It acts by inhibiting ribosomal DNA-dependent RNA polymerase, one of the essential enzymes for transferring genetic information (Sebesta and Horská, 1968), by out-competing ATP for enzymatic binding sites, due to its structural analogy with ATP (Sebesta et al., 1981). Toxicity in arthropods is expressed when high growth rates and physiological processes (e.g., metamorphosis), which require higher rates of RNA synthesis than necessary for the comparatively slower growth of adults are occurring (Sebesta et al., 1981). Thurigiensin primarily disrupts epidermal activity because the epidermis is a major site of nucleic acid replication. Inhibition of nucleic acid metabolism could therefore affect cuticle formation and also the synthesis of enzymes, particularly chitin synthetase (Cohen, 1987a).

When studying cuticle synthesis in Tetranychus urticae Koch, Mothes-Wagner and Seitz (1981) and Mothes-Wagner (1986), found that the spider mite cuticle consists of a wax-containing epicuticle and a chitinous procuticle which reveals different layers when examined under high magnification. The outer cement layer, which is very thin and often ruptured, overlies a very thin wax layer. This sequence is clearer in regions with cuticular glands which may secrete the wax layer. Between the epicuticle and the endocuticle is the exocuticle, which contains mostly granular material that extends to the lobes. The endocuticle shows a lamellation due to the helicoidal arrangement of chitin fibrils in a protein matrix. The cuticular ridges, which are formed from the epicuticle and parts of the exocuticle, also extend to the lobes (Mothes-Wagner, 1986). The deposition of epicuticle occurs on top of small hypodermal microvilli, whereas procuticle deposition occurs in the spaces between the epicuticle and hypodermal cell surfaces during the time when old cuticle persists. Hypodermal cells are densely packed with many rough endoplasmic reticulum (ER) cisterns, and free ribosomes (Mothes-Wagner and Seitz, 1981).

Because the effect of thurigiensin on the ultrastructure of spider mite cuticle has not been studied, two experiments were performed. The first was designed to identify the structures in immature stages that were disrupted by thurigiensin, while the second aimed to trace the process of cuticle formation and to explain the higher toxicity of thurigiensin to older larvae (Vargas et al., 2001a). In both groups of experiments, changes in the ultrastructure of the cuticle were determined by examining sequential sections of treated and untreated mites by electron microscopy.

MATERIALS AND METHODS

Source of mites. All T. urticae used in this study were obtained from a colony that has been maintained on French dwarf bean (Phaseolus vulgaris L., cv. Tendergreen) in the laboratory at 21 ± 3 °C, and a 16:8 (L:D) photoperiod at the Department of Entomology, Lincoln University, Canterbury, New Zealand, since 1985, without exposure to pesticides.

Treatment of mites. An experimental formulation of thurigiensin (ABG-6320, 5% AI, aqueous suspension, Abbott Laboratories, North Chicago, Illinois, USA), was used in all experiments. Physical and chemical properties of thurigiensin have previously been reported by Sebesta et al. (1981). A Potter tower (Burkard Manufacturing Co. Ltd., Rickmansworth, UK) was used for applying thurigiensin in all experiments. Two milliliters of thurigiensin suspension was sprayed with the Potter tower on each occasion at 55 ± 5 kPa; a 10 s settling period was allowed, resulting in a wet deposit of 1.25 ± 0.01 mg cm\(^{-2}\).
For the first experiment whole French dwarf bean leaflets placed on damp cotton in 85 mm diameter Petri dishes were sprayed with three thuringiensiin concentrations (0.005, 0.05, 0.5 g Al L⁻¹), a water-treated control, with four replicates. After the residues had dried, at least 25 larvae, protonymphs or deutonymphs, were transferred to the leaflets; escapes were prevented by confining mites on 12 mm diameter arenas surrounded by a sticky insect trap adhesive (Davis Gelatine N.Z. Ltd., Christchurch). Leaflets were held 30 h at 23 ± 2 °C and a 16:8 (L:D) photoperiod, after which mites were removed, and fixed as indicated below. Twenty-five individuals were embedded per concentration and stage.

The second experiment was designed to investigate the reason for different responses by young (2-h) and old (12-h) larvae (Vargas et al., 2001b). To obtain sufficient numbers of larvae of uniform age, 200 females were allowed to lay eggs for 3-6 h on whole French dwarf bean leaflets placed on moist cotton in Petri dishes at 23 ± 2 °C and a 16:8 (L:D) photoperiod. Thirty 0.5-h-old larvae were transferred into arenas and exposed to thuringiensiin residues (0.005 g Al L⁻¹) for 6-30 h, after which they were removed and fixed every 6 h to arrest the moulting process. In addition, thirty larvae of two ages (2 and 12 h) were exposed for 6 h on residues (0.005 g Al L⁻¹) and transferred to residues-free leaves to be fixed when both groups were 24 h old. A water-only control treatment was set up for comparison. On each occasion, 25 larvae were embedded per treatment.

**Fixation.** The fixation technique used was based on that described by Rumpf et al. (1992). Immature stages were placed on double-sided sellotape and punctured on the back or head with an entomological pin (000) to allow fixing and embedding chemicals to penetrate into the tissues. Then they were fixed in 2.5% glutaraldehyde, buffered with 0.05M cacodylate at 4 ± 1 °C overnight and washed in cacodylate buffer. The specimens were then postfixed in reduced 1% Osmium tetroxide (OsO₄) for 2 h at 4 °C. This was followed by three separate washings in cacodylate and maleate buffer (30 min in each buffer). Specimens were then placed in 0.5% uranylacetate-maleate-buffer and left overnight. The final step involved dehydration in graded ethanol (50-100%).

**Embedding.** Specimens were embedded using Spurr's technique (Spurr, 1969), adapted by Rumpf et al. (1992). Total replacement of the ethanol was obtained using the following three-step infiltration process in a refrigerated chamber at 2 °C: i) constant rotation in 1:1 resin:ethanol mixture for 2 h; ii) constant rotation in 3:1 resin:ethanol mixture overnight; iii) constant rotation in pure resin for 8 h. The specimens were then transferred to embedding capsules and covered with pure resin after being oriented under a binocular microscope. The resin was polymerized at 75 °C for 24 h.

Approximately 100 serial cross sections per treatment of the episthosomal cuticle of larvae, protonymphs and deutonymphs were obtained by cutting with a diamond knife on a LKB ultratome (Hayat, 1970). The 20-40 nm thick sections, were examined at 80 kV with a Zeiss 902 transmission electron microscope (TEM) operated. All sections were examined in an unstained condition because lead-stained sections (Reynolds, 1963) were found to be inferior.

Several combinations of exposure times, diaphragm aperture settings, magnifications and photographic papers were tested to obtain the best resolution in micrographs. An enlarger (De Vere 504) and an automatic print processor (Agfa Rapidoprint DD 1437) were used for all processing carried out in the photographic laboratory of Landcare Research New Zealand Ltd., Canterbury.
RESULTS

Untreated Immature Stages. The fine structure of the intermoult cuticle of larvae at 6 and 12 h is shown in Figures 1 and 2. At these magnifications it is possible to observe ribosomes (R) and rough endoplasmic reticulum (rER) cisterns very close to the old cuticle (OC). At these ages, the old cuticle has not separated from the epidermis, the plasma membrane projections or microvilli (MV) are not visible and the new cuticulin layer has not been deposited. The procuticle (PC), laying immediately over the external surface of the epidermis, is distinguished from the epicuticle (EC) by its different electron density.

By 18 h (Figure 3) larvae have developed an extracellular space, usually called the exuvial space (ES), between the epidermis and the old cuticle. Deposition of the cuticulin (CU) layer of the second instar cuticle is evident in some areas. The cytoplasm contains rough endoplasmic reticulum and many free ribosomes (R).

The electron dense appearance is due to the close packing of free ribosomes in the cell. Ecdysial droplets are not visible within the exuvial space, however, coated vesicles (CO) with ecdysial droplets are located at the apical border of the epidermis.

At about 24 h, deposition of the new epicuticle layer is conspicuous throughout the section (Figure 4). Nuclei (N) of the epidermal cells are relatively large and irregular, and contain one or more nucleoli (NI). In Figure 5, it is possible to observe that cuticulin of the new cuticle (NC) appears in a narrow electron-dense line over the surface of the microvilli. Beneath the cuticulin, an electron dense region similar to the inner region of the cuticulin layer of the old cuticle occurs. This suggests that part of the dense layer (DL) may be secreted concurrently with the cuticulin, rather than sequential deposition of cuticulin followed by the dense layer (Filshie, 1970).

Figure 1. Transverse section of the larva cuticle 6 h after hatching, showing the entire old cuticle (OC), epicuticle (EC), procuticle (PC), rough endoplasmic reticulum (rER), ribosomes (R), and mitochondria (M). Transmission Electron Microscopy (TEM) x 87,000.

Figura 1. Sección transversal de la larva de 6 h, muestra la cutícula antigua (OC), epicutícula (EC), procutícula (PC), retículo endoplásmico rugoso (rER), ribosomas (R) y mitocondria (M). Microscopía Electrónica de Transmisión (TEM) x 87,000.

Figure 2. Transverse section of the larva cuticle 12 h after hatching, showing the entire old cuticle (OC), epicuticle (EC), procuticle (PC), rough endoplasmic reticulum (rER), ribosomes (R), and mitochondria (M). (TEM) x 97,000.

Figura 2. Sección transversal de la cutícula de la larva de 12 h, muestra la cutícula antigua (OC), epicutícula (EC), procutícula (PC), retículo endoplásmico rugoso (rER), ribosomas (R) y mitocondria (M). (TEM) x 97,000.
Figure 3. Transverse section of the larva cuticle 18 h after hatching, showing the entire old cuticle (OC), exuvial space (ES), new cuticle (NC), microvilli (MV), cuticulin (CU), ribosome (R), rough endoplasmic reticulum (rER), and coated vesicle (CO). (TEM) x 40,000.

Figura 3. Sección transversal de la cutícula de la larva de 18 h, muestra la cutícula antigua (OC), espacio exuvial (ES), cutícula nueva (NC), microvilli (MV), cuticulina (CU), ribosoma (R), retículo endoplásmico rugoso (rER), y vesícula cubierta (CO). (TEM) x 40,000.

Figure 4. Transverse section of the larva cuticle 24 h after hatching, showing old cuticle (OC), exuvial space (ES), new cuticle (NC), microvilli (MV), cuticulin (CU), ribosome (R), rough endoplasmic reticulum (rER), nuclei (N), mitochondria (M), and nucleoli (NI). (TEM) x 12,000.

Figura 4. Sección transversal de la cutícula de la larva de 24 h, muestra la cutícula antigua (OC), espacio exuvial (ES), cutícula nueva (NC), microvilli (MV), cuticulina (CU), ribosoma (R), retículo endoplásmico rugoso (rER), núcleo (N), mitocondria (M) y nucleolo (NI). (TEM) x 12,000.

Figure 5. Transverse section of the larva cuticle 24 h after hatching, showing old cuticle (OC), new cuticle (NC), microvilli (MV), cuticulin (CU), ribosome (R), and dense layer (DL). (TEM) x 115,000.

Figura 5. Sección transversal de la cutícula de la larva de 24 h, muestra la cutícula antigua (OC), cutícula nueva (NC), microvilli (MV), cuticulina (CU), ribosoma (R), y capa densa (DL). (TEM) x 115,000.

After 30 h (Figure 6), further secretion of cuticulin causes lateral extension and fusion of neighbouring areas until the membrane is complete. The dense layer is, at this time, in an advanced stage of development. From 30 h (Figure 7) until ecdisis, the cuticle continues to increase in thickness, and the old endocuticle becomes separated from the new epicuticle. The formation of the epicuticle will be completed and the new procuticle will be deposited beneath the epicuticle.

With protonymphs and deutonymphs (Figure 8 and 9), after 24 h the extracellular space has developed between the old and the new cuticle, as was observed in larvae. At this time deposition of the new cuticulin layer is evident. Cuticulin appears in electron dense areas over the surface of the microvilli. At 24 h protonymphs and deutonymphs show structures similar to those found in larvae.
Figure 6. Transverse section of the larva cuticle 30 h after hatching, showing old cuticle (OC), new cuticle (NC), exuvial space (ES), microvilli (MV), cuticulin (CU), and dense layer (DL). (TEM) x 40,000.

Figura 6. Sección transversal de la cutícula de la larva de 30 h, muestra la cutícula antigua (OC), cutícula nueva (NC), espacio exuvial (ES), microvilli (MV), cuticulina (CU), y capa densa (DL). (TEM) x 40,000.

Figure 7. Transverse section of the larva cuticle 36 h after hatching, showing old cuticle (OC), new cuticle (NC), exuvial space (ES), epicuticle (EC), with dense layer (DL), and procuticle (PC). (TEM) x 67,000.

Figura 7. Sección transversal de la cutícula de la larva de 36 h, muestra la cutícula antigua (OC), cutícula nueva (NC), espacio exuvial (ES), epicutícula (EC), con capa densa (DL), y procutícula (PC). (TEM) x 67,000.

Figure 8. Transverse section of the protonymph cuticle 24 h after emerging, showing old cuticle (OC), new cuticle (NC), exuvial space (ES), epicuticle (EP), microvilli (MV), rough endoplasmic reticulum (rER), mitochondria (M), and nucleus (N). (TEM) x 40,000.

Figura 8. Sección transversal de la cutícula de protonífa de 24 h, muestra la cutícula antigua (OC), cutícula nueva (NC), espacio exuvial (ES), epicutícula (EP), microvilli (MV), retículo endoplásmico rugoso (rER), mitocondrias (M), y núcleo (N). (TEM) x 40,000.

Figure 9. Transverse section of the deutonymph cuticle 24 h after emerging, showing old cuticle (OC), new cuticle (NC), exuvial space (ES), cuticulin (CU), rough endoplasmic reticulum (rER), and mitochondria (M). (TEM) x 24,000.

Figura 9. Sección transversal de la cutícula de deutonífa de 24 h, muestra la cutícula antigua (OC), cutícula nueva (NC), espacio exuvial (ES), cuticulina (CU), retículo endoplásmico rugoso (rER), y mitocondrias (M). (TEM) x 24,000.
Treated Immature Stages. Figure 3 shows that 18-h-old larvae have started cuticle formation and secretion of the new cuticle is at the first phase. However when immature stages were exposed to thuringiensin residues on French dwarf bean leaflets the cuticle formation process was disrupted. This effect on cuticle formation is perceivable only in treated larva after 18 h, when no cutulin envelope is observed (Figure 10).

At 24 and 30 h, inhibition of cuticle synthesis is more evident, microvilli are not visible, mitochondria (M), endoplasmic reticulum and ribosomes have an abnormal shape (Figure 11 and 12). Therefore, in treated larvae, the cutulin (or outer epicuticle) and its component lipids and proteins were not synthesized, and subsequently the inner epicuticle or dense protein layer and procuticle also were not formed.

The effects of thuringiensin on protonymphs and deutonymphs were similar to those on larvae.
Figures 13 and 14 show protonymph and deutonymph stages that were exposed to residues for 24 h and where cuticle formation was disrupted and epicuticle was only partially deposited. In the water-only control treatment, the cuticulin envelope was formed as the first layer of new cuticle, where it arises from the tips of the plasma membrane plaques and defines the outer limit of the cuticle (Figures 8 and 9).

Two-h-old larvae exposed to residues for 6 hours were not affected by thuringiensin, and the synthesis of cuticle proceeded normally (Figure 15). However, 12-h-old larvae that remained for the same time on thuringiensin residues were unable to form new cuticular material (Figure 16).
DISCUSSION

The principal objective for studying the effects of thuringiensin on the ultrastructure of *T. urticae* was to determine how and when it inhibited cuticle formation. By understanding these aspects, more efficient use of thuringiensin may result.

Because the effects of thuringiensin on cuticle formation in spider mites have not yet been reported, an experimental approach for the motile stages of *T. urticae* was developed. Of these stages, larval stage was chosen as it represented the most susceptible stage to thuringiensin. Additional benefits of using larvae was their uniformity of age and short developmental period. However, their small size and lack of information available on cuticle structure presented problems for the study. The success of this study was dependent on the use of an appropriate thuringiensin concentration and exposure period to disrupt moulting without killing mites at any time during their development. This was achieved by trial and error.

The external manifestation of moult disruption was similar to those observed with other cuticle inhibitors, i.e., juvenile individuals were unable to reach the succeeding chrysalid stage. However, the biochemical processes involved and the structures disrupted differ between inhibitors (Fogal and Fraenkel, 1969; Oberlander *et al.*, 1980; Chen and Riddiford, 1981; Sebesta *et al.*, 1981; Mothes-Wagner and Seitz, 1982; Hajjar, 1985; Retnakaran *et al.*, 1985; Cohen 1987a, b). From the observations of this study the synthesis of cuticulin was consistently disrupted and consequently no epicuticle was formed in *T. urticae* immature stages when they were exposed to thuringiensin. In contrast, nikkomycin, another inhibitor of cuticle synthesis, has been shown to affect chitin synthesis of the procuticle of *T. urticae* (Mothes-Wagner and Seitz, 1982). Because our results showed that thuringiensin mainly affected synthesis of epicuticle, a non-chitinous cuticle layer that is composed of lipids and proteins (Neville, 1975), they support that thuringiensin is an efficient inhibitor of the moulting process at the nucleic acid level by blocking ribosomal-protein synthesis (Sebesta *et al.*, 1981; Cohen, 1987b). Furthermore, because cuticulin is the first layer formed of the new cuticle (Binnington and Retnakaran, 1991), it may be assumed that thuringiensin blocks the formation of new cuticle earlier than other cuticle inhibitors.

Cuticle formation was seen to be disrupted gradually and larvae were affected from 12 h onwards. Thus, when 12-h-old larvae remained on residues for 6 h they were unable to synthesize cuticular material. Because larvae younger than 12-h-old were not affected, it may be inferred that the higher susceptibility to thuringiensin may be related to the low rate of ATP metabolism (Sebesta and Horská, 1968) during early stages of development.

Although protonymphs and deutonymphs required higher thuringiensin concentrations to cause similar moulting disruption effects, metabolic reasons could explain such a response.
According to Sebesta et al. (1981), thuringiensin achieves its effect by out-competing ATP for enzymatic binding sites, and the inhibition becomes reversible depending on the ratio of ATP to thuringiensin (Sebesta et al., 1969). Therefore, it could be hypothesized that protonymphs and deutonymphs have higher amounts of ATP and consequently, a higher amount of thuringiensin would be necessary to out-compete ATP for enzymatic binding sites.

This study has shown that it is possible to identify the effect of thuringiensin at the ultrastructural level by comparing treated and untreated immature T. urticae. The study has also shown that the time of exposure to thuringiensin is critical and that the higher susceptibility of older larvae is related to the stage of cuticle development. The results also suggest some important practical consequences for toxicological studies, particularly with respect to appropriate exposure times for mites to moul inhibitors as thuringiensin.

Further ultrastructural studies are necessary to determine the exact time that epicuticle synthesis in protonymphs and deutonymphs is disrupted. Also, it may be important to study the reversibility of the inhibition of ribosomal DNA-dependant RNA polymerase by thuringiensin and its consequences for spider mite development.

The high toxicity of thuringiensin to immature spider mites stages and the sublethal effects on females suggest that thuringiensin may successfully control field populations and the effect of host plant and temperature may enhance the effectiveness of thuringiensin.

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