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Midgut histopathology of resistant and susceptible *Plutella xylostella* exposed to commercial formulations of *Bacillus thuringiensis*

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Abstract

Plutella xylostella (L.) is a major pest of crops in the Brassicaceae. Pesticides based on *Bacillus thuringiensis* Berliner (*Bt*) are one of the most important tools in this pest control; however *P. xylostella* populations resistant to *Bt* toxins have been reported. This study tested the hypotheses that susceptible and resistant populations of *P. xylostella* exhibit different histopathology and histochemistry in the midgut upon exposure to *Bt*-based formulations. After bioassays to determine appropriate concentrations, groups of larvae from one susceptible population (SP) and one resistant population (RP) of the moth were exposed to Dipel® at 1.3 mg/L (SP) and 64.9 mg/L (RP) or XenTari® at 5.2 mg/L (SP) and 236.2 mg/L (PR). Midguts from fourth-instar larvae were collected at 0, 1, 6 and 12 hours after exposure and processed for analysis under light microscope. Regenerative cells were quantified through the application of dots using the ImageLab 2000 program. The insecticide Dipel® was more aggressive in the midgut regardless of population. Resistant populations exhibited dense granules (spherites) covering the surface of the columnar epithelial cells and mucus that may be involved in the preservation of larvae midgut resistant.

Key words: biological insecticide, diamondback moth, morphology, resistance.

Introduction

The diamondback moth *Plutella xylostella* (L.) (Lepidoptera Plutellidae) is considered the main pest in all regions that cultivate species of the family Brassicaceae (Castelo Branco and Gatehouse, 2001). Its control is characterized by the intense use of insecticides, with reports of as many as 16 applications per cultivation (Dias *et al*., 2004), potentially harming the health of farmers and the environment and selecting populations resistant to various chemical compounds such as pyrethroids and organophosphates (Vasquez, 1995; Castelo Branco and Gatehouse, 1997).

Pesticides based on *Bacillus thuringiensis* Berliner (*Bt*) are safe and effective alternatives to the synthetic products and, more recently, one of the most important tools in integrated pest management. *Bt* activity is usually attributed to Cry proteins, synthesized during the bacterium sporulation phase and accumulated as crystals in the spores periphery. Upon ingestion by susceptible insects, the crystals are solubilized in the intestinal pH and then processed by specific proteases. The resulting protein resistant to proteolysis irreversibly binds to specific receptors present on the apical microvilli of the epithelial cells in the midgut causing osmotic imbalance by forming pores in the cell membrane, disruption of intestinal integrity and consequent insect death (Copping and Menn, 2000, Herrero *et al*., 2001, Oestergaard *et al*., 2007).

The alimentary canal, a major area of contact between the insects and environment, has been the focus of many studies on pest control (Chapman, 1998; Levy *et al*., 2004). Alterations in the midgut of insects can affect growth, development and all physiological processes that depend on the adequate acquisition, absorption and transformation of nutrients (Mordue and Blackwell, 1993; Mordue and Nisbet, 2000). In Lepidoptera, the midgut epithelium is made up of four types of cells; these are involved in the absorption and secretion of enzymes (columnar cells), ionic homeostasis and detoxification (goblet cells), endocrine function (endocrine cells), and epithelium renewal (regenerative cells) (Terra *et al*., 2006; Pinheiro *et al*., 2008b, Gomes *et al.*, 2012).

Despite the largely known efficiency of *Bt* towards insects of different orders, field and laboratory resistant *P. xylostella* populations selected to *Bt* toxins have been reported (Tabashnik, 1994; Wright *et al*., 1997; Sayyed *et al*., 2000). The loss of toxins effectiveness in these populations has been attributed to changes in the membrane receptors structure due to a loss or reduction of toxin binding to the receptor (Ferré and Van Rie, 2002; Sayyed *et al*., 2004).

Due to the increased populations resistant to *Bt*, the aim of the present study was to test the hypotheses that susceptible and resistant populations of *P. xylostella* exhibit different histopathological and histochemical alterations in the midgut after exposure to commercial formulations containing *Bt*, and that the midgut of resistant insects shows recover capacity after exposure to the concentrations tested.

Materials and methods

Insect collection and maintenance

This study used *P. xylostella* populations either susceptible or resistant to two commercial formulations, Dipel®PM (*Bacillus thuringiensis* subsp. *kurstaki* HD-1) and XenTari®WDG (*Bacillus thuringiensis* subsp. *aizawai* ATTC SD-1372). The populations were collected from brassica crops in the counties of Chã

Grande (Susceptible) and Camocin de São Félix (Resistant) of the Pernambuco State, Brazil. The susceptible population has been maintained under laboratory conditions without insecticide selection since 1998. The resistant population, collected from an area with reported XenTari®WDG control failures, was brought to the laboratory where the moth larvae were fed on leaves of insecticide-free kale (*Brassica oleracea* var. *acephala*). The F_1 generation of those resistant moths was used for the susceptibility tests.

Susceptibility bioassays

For both moth populations, concentration-response curves were established with the insecticides Dipel®PM and XenTari®WDG through bioassays to estimate LCs, and the subsequent experiments were based on a concentration determined after fitting curves. For such bioassays, leaf discs of kale (5 cm in diameter, sanitized with 0.25% sodium hypochlorite) were immersed in seven increasing concentrations of each insecticide diluted in aqueous 0.01% Triton X-100 solution, then left to dry at room temperature. The concentrations for the susceptible population were 0.062, 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 mg/L of Dipel®, and 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 mg/L of XenTari®. For the resistant population, the concentrations were 12.5, 25, 50, 100, 200, 400, and 800 mg/L of Dipel®, and 50, 100, 200, 400, 600, 800, and 1000 mg/L of XenTari®. Aqueous 0.01% Triton X-100 solution was used as control. Petri dishes (60 × 15 mm) containing filter paper (5 cm) moistened with distilled water and the treated discs comprised the arenas to which 10 second-instar larvae were transferred. Each concentration was repeated three times and the bioassay redone at least once. The larvae were exposed for 72 hours inside a growth chamber at 25 ± 1 °C, 60 ± 10% relative humidity and 12-hour photoperiod. Mortality was assessed using the criterion of no larval movement at the touch of a fine smooth brush.

Estimated LC_{50}s (Lethal concentration in mg a.i.·L^{-1} of water) were used to evaluate the potential damage to the 4th-instar larvae midguts from the susceptible and resistant populations. Then, resistance ratios (the ratio of the LC_{50} estimates between resistant and susceptible populations) and their 95% confidence intervals were calculated according to the method of Robertson and Preisler (1992). Based on these resistance ratios, larvae from the Camocin de São Félix-PE population were 48-fold and 45.7-fold more resistant to Dipel and XenTari, respectively than larvae from the susceptible population (table 1).

Midgut histochemistry and light microscopy analysis

In total, 100 fourth-instar larvae were assigned to one of five 24-hour-long feeding treatment groups: A) disks of kale leaves treated with Dipel® at 1.4 mg/L (susceptible population), or B) 64.9 mg/L (resistant population); C) disks of kale leaves treated with XenTari® at 5.2 mg/L (susceptible population), or D) 236.2 mg/L (resistant population); and E) larvae fed on disks of kale leaves treated with distilled water containing 0.01% Triton X-100 (control). Each larva corresponded to one replicate; five replicates/treatments/intervals were used.

After 24 hours, the first 5 randomly selected larvae were dissected for the removal of the alimentary canal (0 hour); the remaining larvae received untreated kale leaves until their alimentary canals were removed at 1, 6 and 12 hours, respectively, using the method described by Knaak and Fiuza (2005) with modifications i.e., after the fixation, in Bouin for 24 hours, tissues were submitted to dehydration in increasing graduation (70, 96 and 100%) of ethanol solutions, following by fast baths of xylol and impregnation in paraffin. The transversal histological cuts were accomplished to 5µm thickness in the histology laboratory. To remove the paraffin, the slide containing the tissue was passed through xylol and ethanol baths in decreasing order of graduation. To enable histological description of the midgut and quantification of regenerative cells, the material was fixed in 10% formaldehyde with Molling-Carson buffer 0.1 M pH 7.2 for 24 hours. Dehydration was performed in increasing concentrations of alcohol (70, 80 and 95%) for 10 minutes each. Infiltration was performed in Historesin Leica® for 24 hours. Sections of 4 µm obtained on a microtome (Leica® RM 2245) were stained with Toluidine Blue.

For the histochemical analysis, the material was fixed in aqueous Bouin solution for 24 hours, and then dehydrated with increasing concentrations of ethyl alcohol (70, 90 and 100%) for 10 minutes each. The samples were immersed in xylene (P.A.) for 10 minutes and heated in an oven at 60 °C for clearing. Infiltration was performed in paraplast diluted in xylene at proportions of 25, 50, 75 and 100% for 1 hour each and after the last wash. The sections obtained with the microtome (Minot, LEICA® RM 2035) adjusted to 7 µm were placed on slides previously coated with Mayer's albumin and kept in an oven at 37 ºC for 24 hours. Slides were stained with von Kossa, Mallory's trichrome and P.A.S (Periodic Acid Schiff) stain for identification of calcium, mucins and carbohydrate, respectively, following the methodology described elsewhere (Behmer *et al*., 1976; Junqueira and Junqueira, 1983; Michalany, 1990).

Table 1. Susceptibility of *P. xylostella* populations to Dipel®PM and XenTari®WDG.

Insecticide	Population	N [1]	Slope ± SE [2]	LC_{50} (95% CI) [3]	χ^2 (DF) [4]	RR (95% CI) [5]
Dipel®PM	Susceptible	213	1.30 ± 0.18	1.4 (1.0 - 2.1)	4.6	-
	Resistant	209	1.24 ± 0.17	64.9 (43.9 - 91.9)	4.3	48.0 (28.3-81.3)
XenTari®WDG	Susceptible	184	2.47 ± 0.33	5.2 (4.0- 6.6)	4.7	-
	Resistant	214	2.81 ± 0.29	236.2 (193.8 - 283.1)	3.7	45.7 (33.5-62.2)

[1] Total number of insects used in each bioassay; [2] Standard error; [3] Lethal concentration in mg a.i.·L^{-1} of water; [4] χ^2 test ($P > 0.05$) and degree of freedom; [5] Resistance ratio: ratio of the LC50 estimates between the resistant and susceptible populations, calculated according to Robertson and Preisler (1992) method and ratios 95% confidence intervals.

Quantification of regenerative cells

Regenerative cell numbers were estimated using five Toluidine Blue-stained sections/treatment/interval obtained from the midgut. Each section corresponds to an insect. The image was captured by a video camera (Sony®) connected to the microscope (Olympus® Bx50). Morphometry was performed through the application of dots associated to the ImageLab 2000 program for Windows 3x. For each replicate (slide with cut section), regenerative cells were counted throughout the midgut. The mean number of regenerative cells was subjected to analysis of variance at 3 × 4 factorial arrangement, with three treatments (control, Dipel® and XenTari®) and four evaluation intervals (0, 1, 6 and 12 hours) as the main

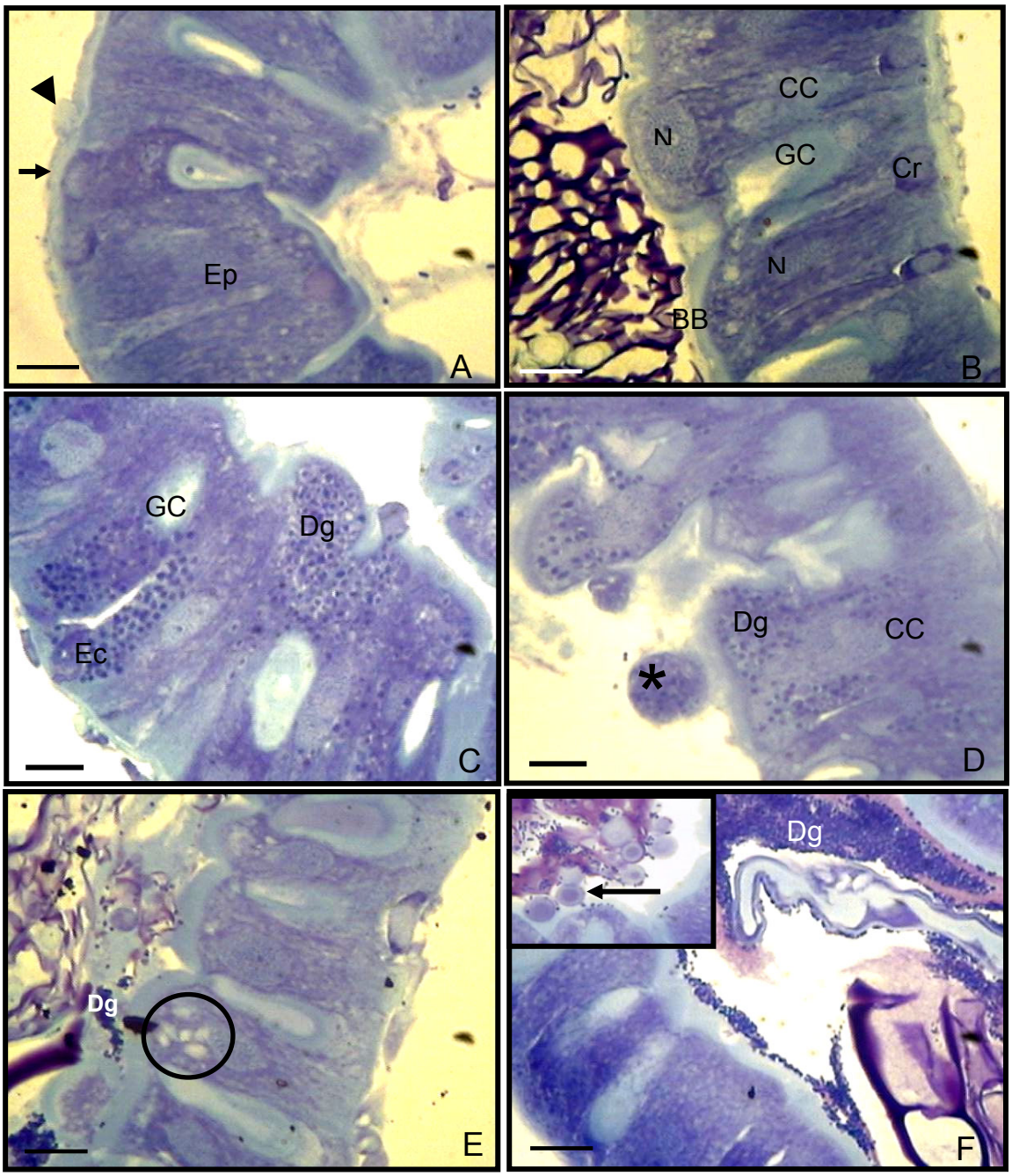

Figure 1. Midgut of susceptible *P. xylostella* larva unexposed to insecticides: (A) Simple epithelium supported by two layers of muscle; (B) Epithelial cells; (C) Dense granules in a goblet cell; (D) Note dense granules in the cytoplasm of columnar cells and fragments pointing to the lumen; (E) Columnar cells with prominent vacuoles. Midgut of resistant *P. xylostella* larva unexposed to insecticides: (F) Dense granules on the brush border and in contact with food. In detail, basophilic vesicles in the lumen; Ep: simple epithelium; arrowhead: longitudinal muscle; short arrow: circular muscle; CC: columnar cell; GC: goblet cells; BB: brush border; N: nucleus, Ec: endocrine cell; Dg: dense granules; Cr: regenerative cell; asterisk: cytoplasmic fragments; circle: vacuoles; black arrow: basophilic vesicle. Toluidine Blue staining. Bars = 25μm.
(In colour at www.bulletinofinsectology.org)

factors. In cases of a statistically significant response in the quantity of regenerative cells by the evaluation interval after treatment, the results were subjected to regression analysis for interpretation and the equations were selected based on their statistical significance (F and P) and higher coefficient of determination (R^2). The analyses were carried out using the SAS statistic program (SAS, 1999-2001).

Results

After both susceptible and resistant fourth-instar *P. xylostella* larvae unexposed to *Bt* formulations had been fed on kale leaves treated with distilled water for 24 hours, histological sections of their midguts revealed a simple epithelium supported by two layers of muscles, the inner muscle arranged circularly and the outer muscle arranged longitudinally (figure 1A). Four types of

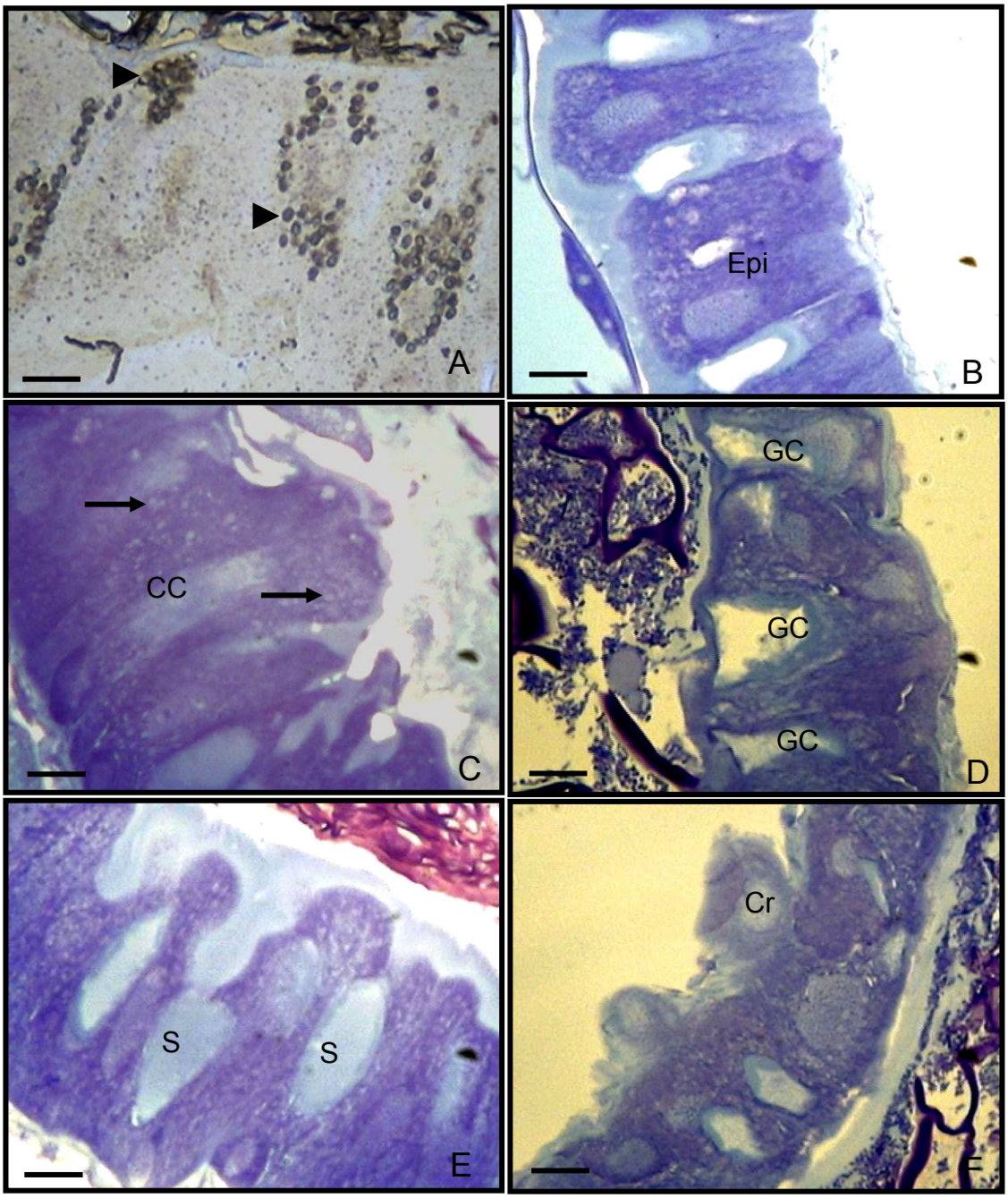

Figure 2. Midgut of resistant *P. xylostella* larva unexposed to insecticides: (A) Dense granules give positive reaction for von Kossa staining. Compare to midgut of susceptible *P. xylostella* larva exposed to XenTari® for 24 hours: (B) Area of intact epithelium; (C) Tall, richly vacuolated columnar cells; (D) Goblet cells with altered morphology; (E) Intense goblet cell secretion; (F) Hypertrophy of regenerative cells; arrowhead: Dense granules; Epi: intact epithelium; CC: columnar cell; GC: goblet cell; Cr: regenerative cell; arrow: vacuoles; S: secretion. Toluidine Blue staining. Bars = 25µm.
(In colour at www.bulletinofinsectology.org)

cells were identified throughout the epithelium: columnar, goblet, regenerative, and endocrine. The most frequent were columnar cells of varied size, with their characteristic striated border and centrally to apically located, very heterochromatic nucleus. Goblet cells had a typical cavity-shaped cup limited by projections of the plasma membrane with a basal nucleus. Regenerative cells with a central apical nucleus were arranged singly at the base of the epithelium (figure 1B). Endocrine cells were observed with elongated shapes and very dense granules that are typical of this type of cell (figure 1C).

In the susceptible population, some columnar cells had a large number of very dense and scattered cytoplasmic granules; these were also found in fragments of cytoplasm in the lumen (figure 1D). There were prominent vacuoles in the apical region of these cells and a few dense granules in the lumen (figure 1E). In the resistant population, such granules showed up in higher concentration and only located on the brush border surface and in contact with food, and there were basophilic vesicles

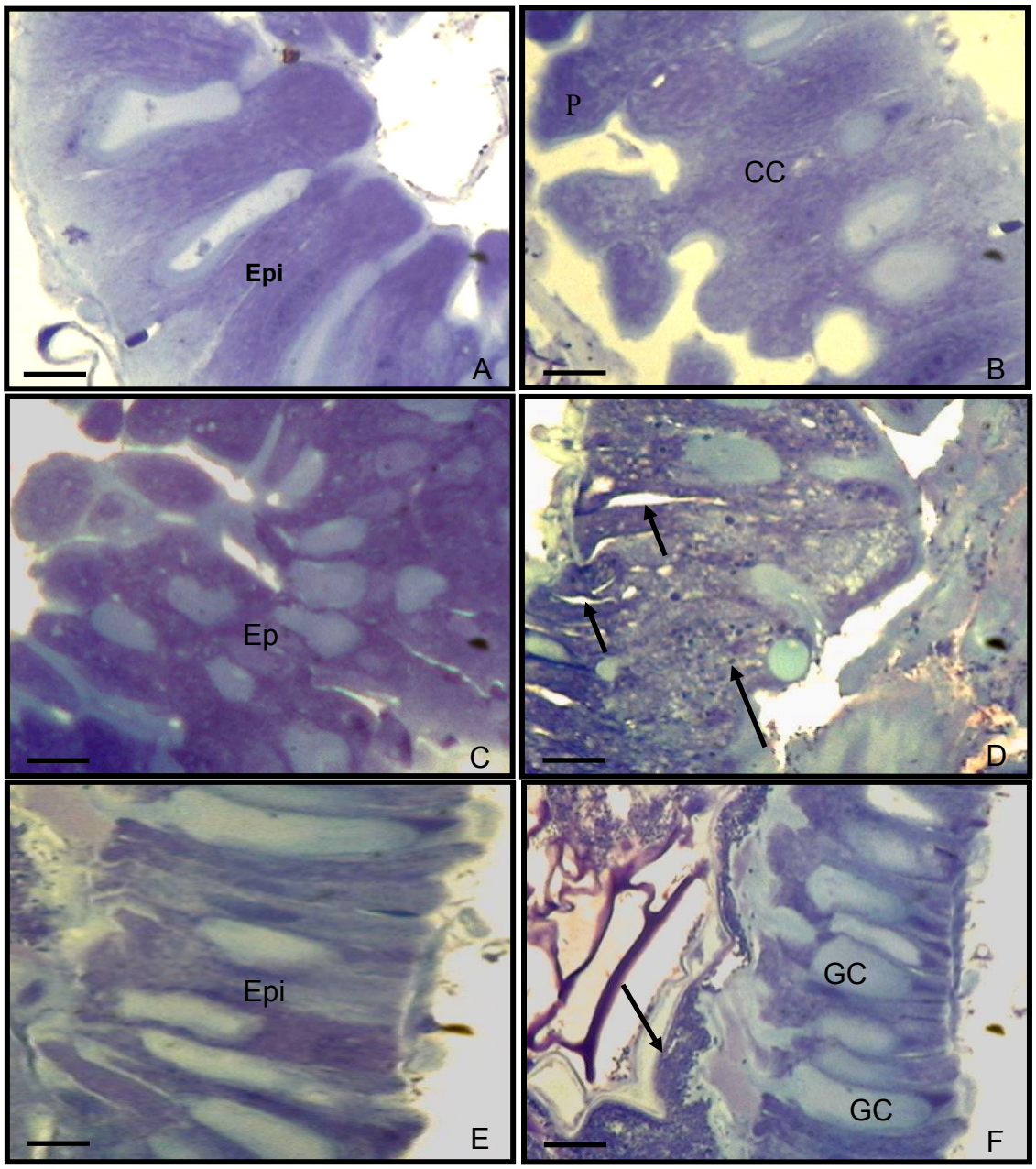

Figure 3. Midgut of resistant *P. xylostella* larva exposed to XenTari® for 24 hours: (A) Intact epithelium; (B) Tall columnar cells with protuberances. Compare to midgut of susceptible *P. xylostella* larva exposed to Dipel® for 24 hours: (C) Clutter and stratification of epithelium; (D) Distance of epithelial cells and presence of dense granules within the columnar cell. Compare to midgut of resistant *P. xylostella* larva exposed to Dipel® for 24 hours: (E) Intact epithelium; (F) Abundance of goblet cells and dense granules; Epi: intact epithelium; Ep: epithelium; CC: columnar cell; GC: goblet cell; P: protuberance; short arrow: distance between the epithelial cells; long arrow: dense granules. Toluidine Blue staining. Bars = 25µm (A, B, C, D, F) or 50 µm (E).
(In colour at www.bulletinofinsectology.org)

in the lumen of the midgut (figure 1F). The dense granules showed a positive reaction for von Kossa staining, indicating the presence of calcium (figure 2A).

After susceptible *P. xylostella* larvae had been exposed to XenTari® for 24 hours, the midgut examination revealed some areas of intact epithelial lamina (figure 2B) and other areas with alterations characterized by the presence of very tall, well-vacuolated columnar cells (figure 2C), as well as large amounts of granules in the lumen and modifications in the morphology of some goblet cells, with the lumen of completely filled with secreted material (figures 2D and 2E); hypertrophy of the regenerative cells also was observed (figure 2F). In larvae of the resistant population exposed to the same insecticide for the same length of time, the midgut epithelium was intact, with tall columnar cells and goblet cells of normal morphology (figure 3A). However, some columnar cells exhibited a large cytoplasmic protuberance (figure 3B).

Dipel® treatment caused more pronounced alterations

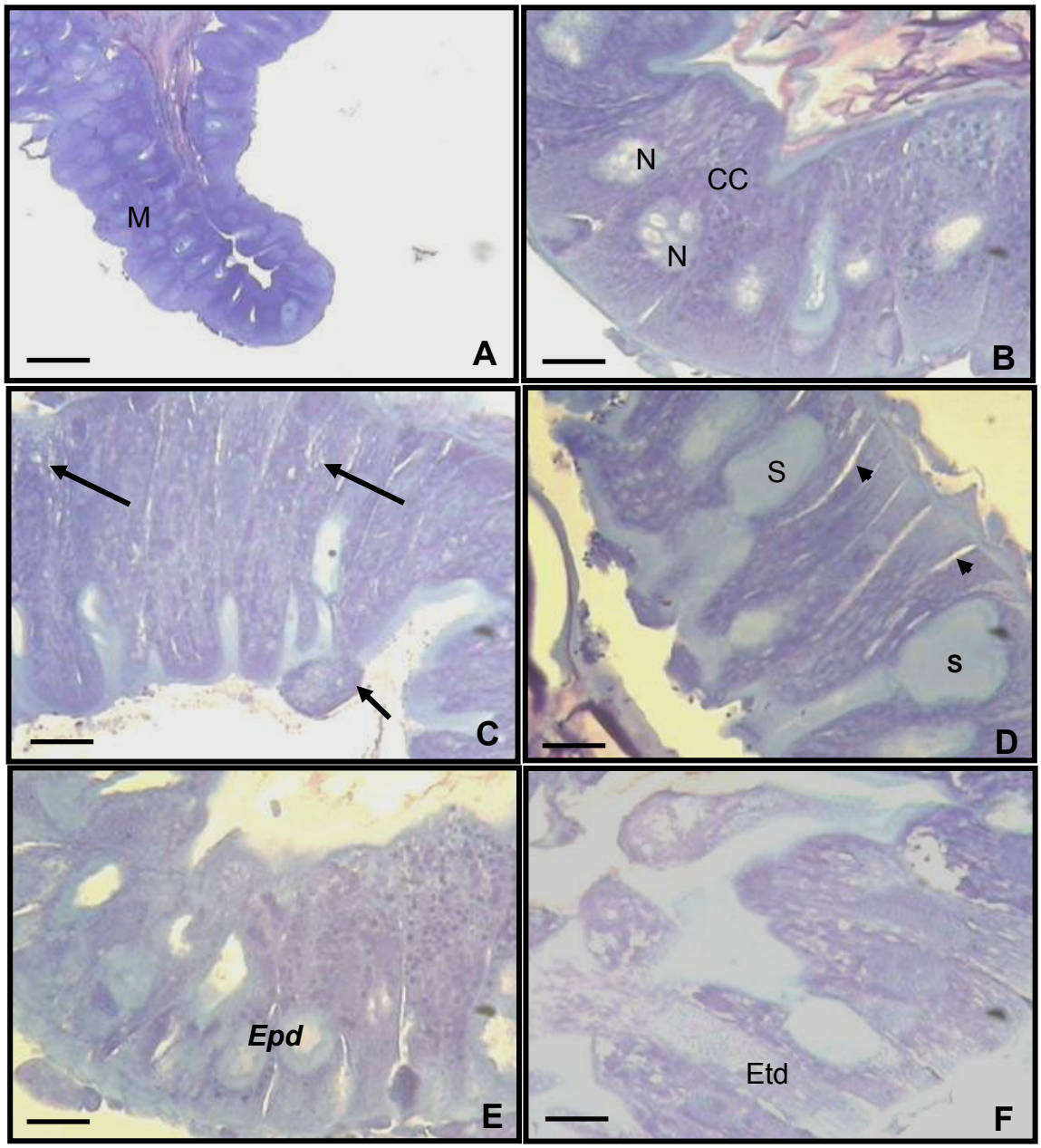

Figure 4. Midgut of susceptible *P. xylostella* larva exposed to XenTari® for 24 hours: At 1 hour, (A) metaplasia and (B) vacuolated nucleus, Bar = 100µm in diameter; At 6 hours, (C) vacuolation and protuberances in columnar cells and (D) intense secretion of goblet cells and distance between cells, Bar = 25µm; At 12 hours, (E) partial degradation of midgut, Bar = 25µm. Compare to midgut of susceptible *P. xylostella* larva exposed to Dipel® for 24 hours: At 1 hour, (F) total degradation of midgut, Bar = 25µm; M: metaplasia; N: Nucleus; Epd: partially degraded epithelium; Etd: totally degraded epithelium; CC: columnar cell; S: secretion; Arrowheads: separation of epithelial cells; Long arrow: vacuoles; Short arrow: protuberance. Toluidine Blue staining.
(In colour at www.bulletinofinsectology.org)

in the midgut of susceptible larvae, producing complete epithelial tissue disorganization with areas of stratification (metaplasia) (figure 3C); there was also distancing of the cells. Dense granules remained in the interior of the columnar cells (figure 3D). However, in the midguts analyzed from resistant larvae, the epithelium remained intact with tall columnar cells and many goblet cells. The dense granules were only on the surface of the cell and in contact with food (figure 3E and 3F).

To determine whether there was recovery of epithelial lamina, larvae from both populations that had been treated with the insecticides for 24 hours and subsequently fed on untreated kale leaves for 1, 6, or 12 hours prior to analysis were examined. In the susceptible population exposed to XenTari®, metaplasia was apparent at 1 hour. However, some columnar cells exhibited vacuolated nuclei, indicating a degenerative process (figures 4A and 4B). At 6 hours, there was intense secretion of goblet cells, vacuolization and formation of protuberances in the columnar cells, and intense distancing between these

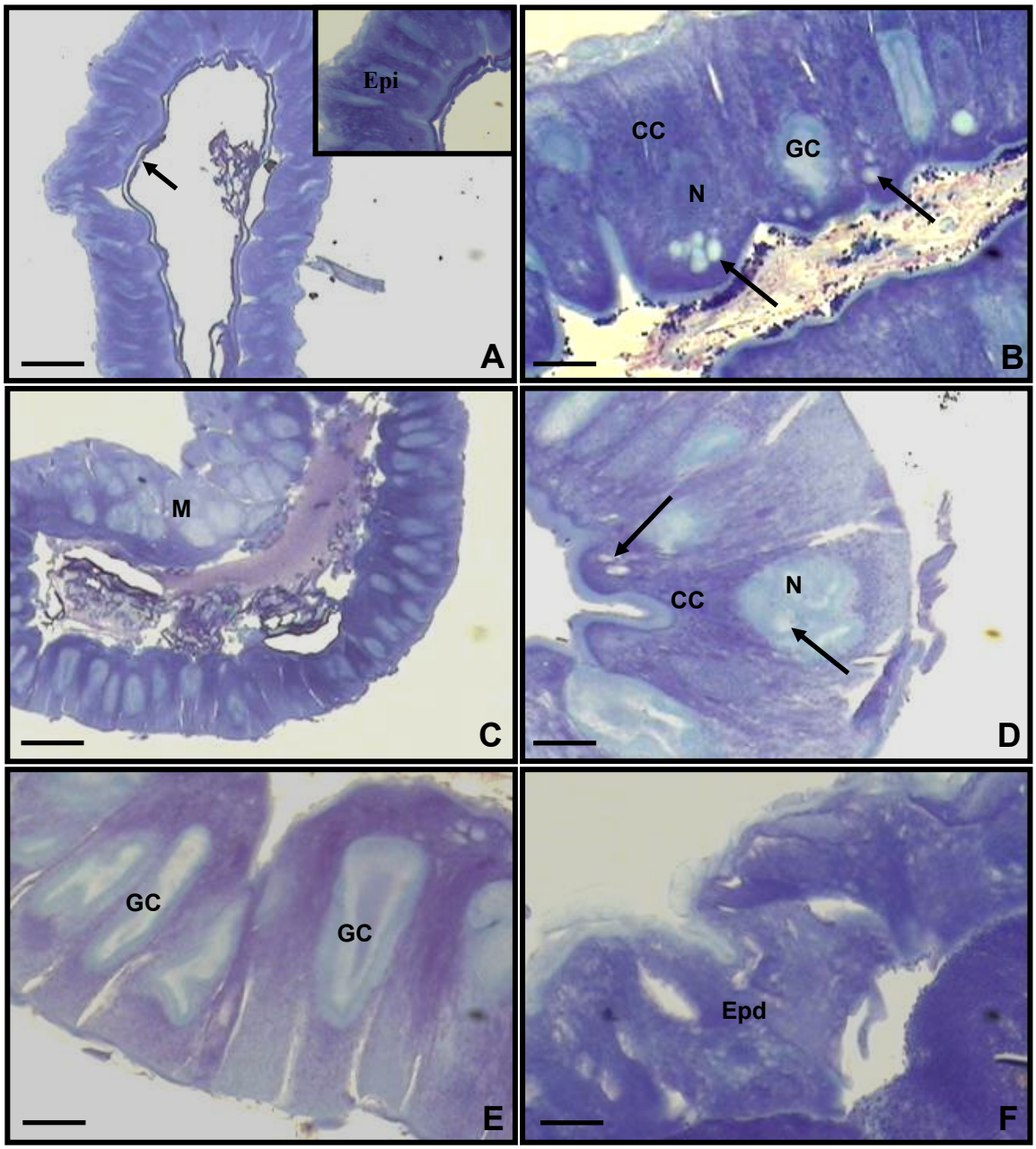

Figure 5. Midgut of resistant *P. xylostella* larva exposed to XenTari® for 24 hours: At 1 hour, (A) epithelial layer coated with dense granules; in detail, intact epithelium; (B) Hypertrophy of columnar cell and prominent vacuoles. Compare to midgut of resistant *P. xylostella* larvae exposed to Dipel® for 24 hours: At 1 hour, (C) Epithelial metaplasia,; (D) Columnar cell with vacuolated nucleus and cytoplasm; (E) Secretion of goblet cell; At 12 hours, (F) degradation of midgut; Short arrow: dense granules; Long arrows: vacuoles; M: metaplasia; N: nucleus; Epi: intact epithelium; CC: columnar cell; GC: goblet cell; Epd: degraded epithelium. Toluidine blue staining. Bars = 100µm (A, C) or 25µm (B, D, E, F).
(In colour at www.bulletinofinsectology.org)

cells (figures 4C and 4D). At 12 hours, there was partial midgut degradation (figure 4E). The treatment with Dipel® was extremely aggressive with complete degeneration of the epithelium within 1 hour (figure 4F).

At 1 hour after resistant larvae having been treated with XenTari®, their midgut epithelial lamina were still covered with dense granules (figure 5A) and the epithelium was intact, despite the prominent vacuolization, hypertrophy of columnar cells and changes in some goblet cells in certain areas (figure 5B). These characteristics also remained at 6 and 12 hours. With Dipel®, the epithelial lamina of resistant larvae exhibited areas of epithelial metaplasia (figure 5C), columnar cells with vacuolated cytoplasm and nucleus (figure 5D) and secretion in goblet cells (figure 5E) at 1 and 6 hours; the midgut degradation was evident at 12 hours (figure 5F).

Histochemically, the susceptible and resistant larvae with and without exposure to insecticides for 24 hours had a positive reaction to Mallory's trichrome stain only on the surface of the epithelial lamina; this was also observed in the larvae of the susceptible population at the varied time intervals studied (figures 6A and 6B). However, similar secretion in goblet cells was observed at all-time intervals in susceptible and resistant larvae (fig-

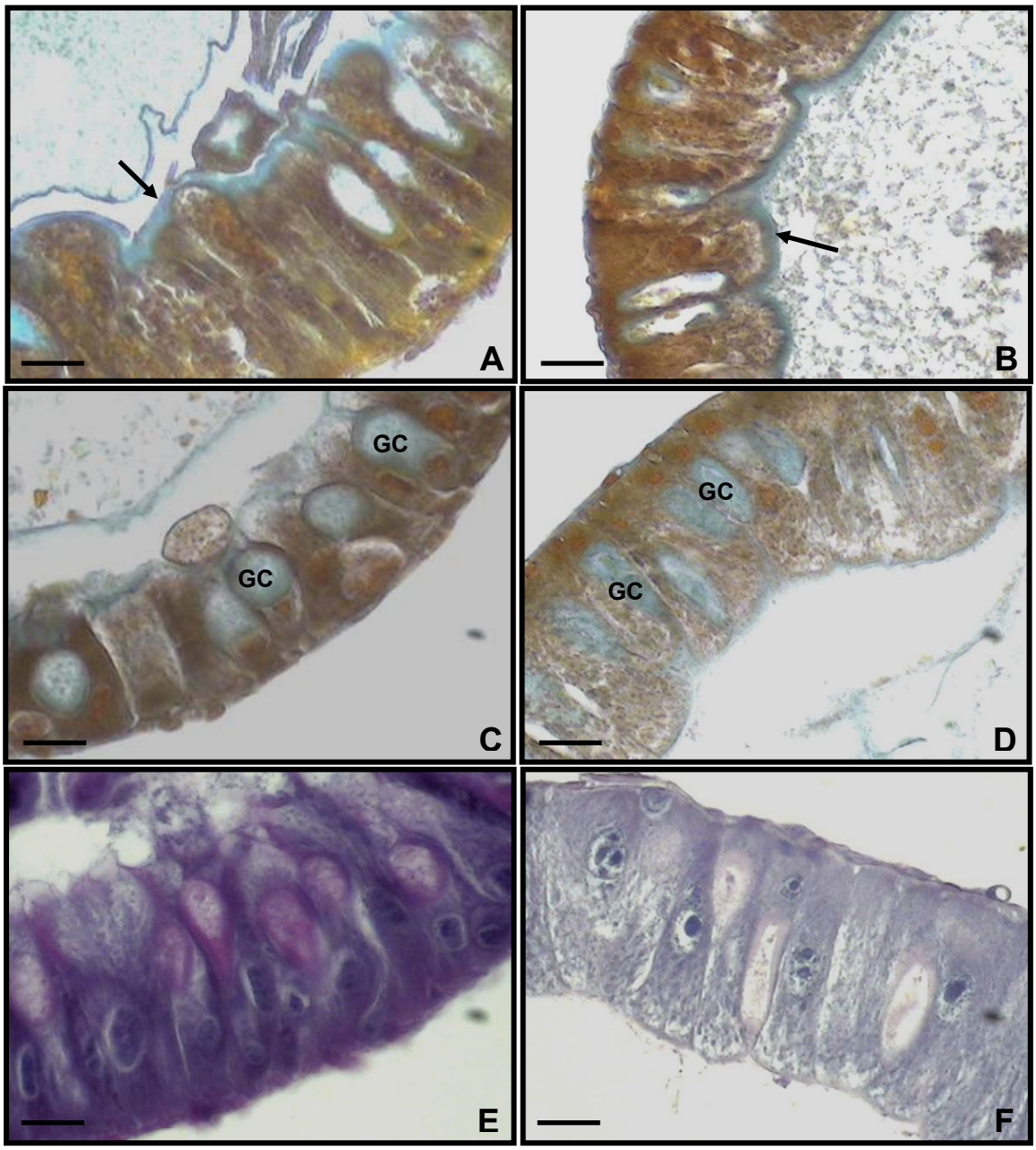

Figure 6. Histochemistry of midguts of (A) susceptible and (B) resistant *P. xylostella* larvae unexposed to insecticides. Observe in both the presence of mucus on the epithelial surface. Compare midguts of (C) resistant *P. xylostella* larva exposed to Dipel® at 1 hour and (D) resistant *P. xylostella* larva exposed to XenTari® at 6 hours. Note mucous secretion in goblet cells. (E) Note positive reaction with P.A.S. in midgut of unexposed susceptible larva and (F) negative reaction in the midgut of unexposed resistant larva; GC: goblet cells; Arrows: mucus. Bars = 25 µm.
(In colour at www.bulletinofinsectology.org)

ures 6C and 6D). The P.A.S. reaction was more strongly positive in the susceptible larvae in all treatments and at all times compared to that of the resistant larvae (figures 6E and 6F).

Quantitative analysis revealed that the treatment with Dipel® caused a statistically significant reduction in the regenerative cells of the susceptible larvae at 1 hour (figure 7). In the larvae of the resistant population that were treated with Dipel®, there was statistically significant reduction in these cells only at 6 hours; with XenTari® such reduction was only observed at 12 hours (figure 8).

Discussion

Histologically, the midgut of fourth-instar *P. xylostella* larvae of both populations without exposure to *Bt* formulations exhibited the same characteristics that others have described for insects of the order Lepidoptera (Cristofoletti *et al*., 2001; Pinheiro *et al*., 2003; Sousa *et al*., 2009). Columnar, goblet, regenerative, and endocrine cells (closed type) were evident in the epithelium (Cavalcante and Cruz-Landim, 1999); the latter were not easily identified under light microscopy using routine techniques, observation that corroborates the findings of Pinheiro *et al*. (2008b) and Sousa *et al*. (2009), who cite the need for ultrastructural and immunohistochemical analyses.

The dense granules observed in the cells and lumen of the midgut resemble structures called spherites that have been described in cells of different organs in invertebrates, including insect midguts (Cruz-Landim, 2000; Serrão and Cruz-Landim, 2000). A number of authors (e.g. Lipovsek *et al*., 2002; 2004) report that these granules are associated with cell detoxification and excretion of ions (mainly calcium and phosphorus). Some studies report the release of spherites into the intestinal lumen (Serrão and Cruz-Landim, 1996). Spherites are also involved in the rapid transport of fluids and excretion of heavy metals, as well as both organic and inorganic materials (Hazelton *et al*., 2001). Thus, one may assume that the permanence of the dense granules covering the surface of the epithelial lamina was promoting protection from XenTari® in the larvae of the resistant population, possibly serving as site for accumulation of toxic waste that cannot be metabolized (Lipovšek *et al*., 2012). This finding corroborates the results described by Pinheiro *et al.* (2008a), who found that spherites were more numerous in the midgut of *Diatraea saccharalis* F. (Lepidoptera Pyralidae) larvae parasitized by *Cotesia flavipes* (Cameron) (Hymenoptera Braconidae) than in those non-parasitized.

Some columnar cells exhibited rich vacuolization when exposed to *Bt*. This bacterium is known to cause changes in the cell membrane, a common manifestation of cell degeneration (Cavados *et al*., 2004).

When susceptible larvae were exposed to XenTari® for 24 hours, the histopathological changes that occurred in their midguts were similar to, but less pronounced than, those observed in studies on *Alabama argillacea* (Hubner) (Lepidoptera Noctuidae) after 20 minutes of ingestion of approximately 0.183 ± 0.077ng of Cry1Ac present in cotton *Bt* AcalaDTL/-90B (Sousa *et al*., 2010). The midgut remained intact in the resistant larvae exposed to XenTari®, despite the protuberances in columnar cells. According to Pinheiro *et al.* (2008b), these protuberances are the result of the process of apocrine secretion of digestive enzymes into the lumen. However,

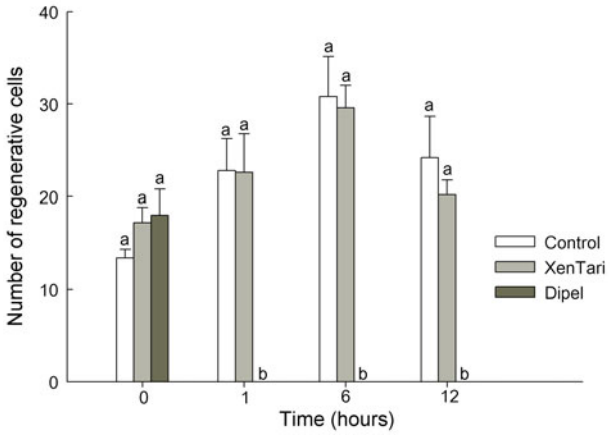

Figure 7. Mean number (± SE) of regenerative cells in the region of the midgut of susceptible larvae of *P. xylostella*; compare values for control group ($y = 15.595 + 4.660x - 0.330x^2$, $F = 5.40$, $P = 0.0153$, $R^2 = 0.38$) and for groups exposed to XenTari® ($y = 18.026 + 3.793x - 0.301x^2$, $F = 5.97$, $P = 0.01$, $R^2 = 0.41$) or to Dipel® ($y = 1.203 - 0.761x + 0.060x^2$, $F = 60.34$, $P < 0.0001$, $R^2 = 0.87$) in the intervals of 0, 1, 6, and 12 hours. Means with same letter over time did not differ statistically between treatments by Tukey's HSD test at 5% probability.

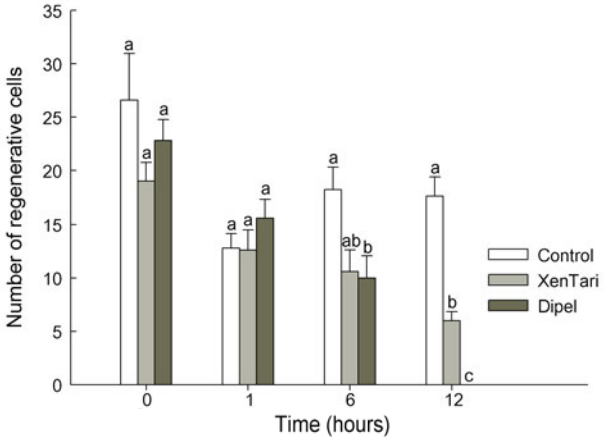

Figure 8. Mean number (± SE) of regenerative cells in the region of the midgut of resistant larvae of *P. xylostella*. Compare values for control group ($y = 26.600 - 17.573x + 3.989x^2 - 0.215x^3$, $F = 4.57$, $P = 0.0171$, $R^2 = 0.46$) and for groups treated with XenTari® ($y = 19.000 - 7.916x + 1.602x^2 - 0.086x^3$, $F = 10.02$, $P = 0.0006$, $R^2 = 0.65$) or with Dipel® ($y = 22.800 - 8.744x + 1.633x^2 - 0.088x^3$, $F = 33.34$, $P < 0.0001$, $R^2 = 0.86$) in the intervals of 0, 1, 6, and 12 hours. Means with same letter over time did not differ statistically between treatments by Tukey's HSD test at 5% probability.

Hakim *et al*. (2010) state that the protuberances may be associated with cell death, once when the midgut is infected with microbial agents, the columnar cells swell and form apical protuberances releasing their entire contents into the lumen.

Oliveira *et al*. (2009) have reported basophilic vesicles in the midgut of larvae of *Aedes aegypti* L. (Diptera Culicidae), *Aedes albitarsis* Lynch-Arribalzaga (Diptera Culicidae), and *Culex quinquefasciatus* Say (Diptera Culicidae) exposed or not to toxins from the 2362 and S1116 strains of *Bacillus sphaericus* (Neide). In the present study, however, these vesicles were only found in the lumen of the midgut of resistant larvae prior to exposure to the insecticides, which may be related to their greater contact time with the toxin.

Cell and nucleus hypertrophies also have been reported by Ruiz *et al*. (2004) and Pandey *et al*. (2009) in studies on the larvae of *A. aegypti*, *Anopheles albimanus* Wiedemman (Diptera Culicidae) and *C. quinquefasciatus* exposed to the toxin Cry11bB from *Bacillus thuringiensis* subsp. *medellin*, as well as in the larvae of *Spodoptera litura* F. (Lepidoptera Noctuidae) after ingestion of the delta toxin from *Bt*. According to Kumar *et al.* (2004), hypertrophy is an adaptive response from cells to such factors as increase in functional requirements, increase in hormonal stimulation, or infection by viruses, bacteria or fungi. Metaplasia was observed in the larvae of both populations after treatment with Dipel®. However, due to the degeneration of epithelial layer, there was evident reduction in the number of regenerative cells. This event was slower in the resistant larvae occurring only at 6 hours post-exposure. The significant reduction in regenerative cells in the larvae of the resistant population exposed to XenTari® at 12 hours may be related to the activity of columnar cells. Through immunohistochemical analysis, it has been determined that these cells are the main source of cell production differentiating factor in the midgut and that their levels increase in columnar cell cultures treated with *Bt* (Loeb *et al*., 2001; Hakim *et al*., 2010). Thus, it is possible that the columnar cells have released this factor, thereby promoting the differentiation of regenerative cells and leading to their reduced number.

The analysis by Mallory trichrome histochemistry revealed that in both populations, regardless of treatment, there was secretion of mucus-rich mucin (sialomucin and sulphomucins) (Behmer *et al*., 1976; Junqueira and Junqueira, 1983; Michalany, 1990). Mucins are polymeric glycoproteins, whose primary function is to protect the epithelium from chemical, physical and biological agents that may be present in the intestinal lumen (Deplancke and Gaskins, 2001; Myers *et al*., 2008). Thus, this secretion suggests that these mucins participate into the preservation of the epithelial layer. Concerning to P.A.S., the reaction was more intense in susceptible larvae demonstrating neutral carbohydrate reserves, this can be linked to the fact that insecticide resistance is usually associated with higher demands of energy in other insect species (Ahmad *et al*., 2006; Guedes *et al*., 2006; Lopes *et al*., 2010).

Based on the results of this study, both Dipel® and XenTari® caused histopathological changes of different intensities in the midgut of both populations of diamondback moth larvae, being more aggressive in those susceptible, mainly Dipel®. The presence of spherites covering the epithelial layer, cell hypertrophy columnar and mucus secretion may be involved in preservation of larvae midguts resistance, but these features were not sufficient to prevent the cell metaplasia and regenerative cells reduction.

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