

# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

186,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index  
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?  
Contact [book.department@intechopen.com](mailto:book.department@intechopen.com)

Numbers displayed above are based on latest data collected.  
For more information visit [www.intechopen.com](http://www.intechopen.com)



---

# Toxicology of the Bioinsecticides Used in Agricultural Food Production

---

Neiva Knaak, Diouneia Lisiane Berlitz and Lidia Mariana Fiuza

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/52070>

---

## 1. Introduction

As populations grow in numbers, the demands for food production increase and are generally met by the intensification of livestock breeding and the increase of agricultural activities. This in turn increases the quantity of chemical pesticides required to control the losses in production caused by insect pests preying on the food plants and disturbing the animals. Once applied these pesticides may cause resistance to the synthetic molecules, contaminate biotic and abiotic components like plants, soil, water and/or the local water network and can also effect non-targeted organisms, such as fish, small mammals, birds and so on.

Contamination of rivers with chemical pesticides is almost always due to excess material being carried away by rain or irrigation waters or by erosion of contaminated soil particles (1, 2). Therefore, as this generalized contamination of the environment increases, researchers are seeking alternative methods for controlling the pests but which cause less damage than the chemical products (3).

In the biological control system, the bacteria of the *Bacillus* genus have considerable potential for use as control agents, because, as well as being lethal to the insect pests, they remain viable for long periods in storage (4). Amongst these bacteria, *Bacillus thuringiensis* has achieved commercial-scale success in controlling various insect pests, plant pathogens, nematodes and mites, mainly because this micro-organism has a high specificity to the pest-targets (5).

In addition to the use of microorganisms, various botanical pesticides are being studied and can be associated with Integrated Pest Management (IPM). The products originating in plants refer to plant species that, over a long time developed defense mechanisms against herbivores, pathogens and other stress agents. Among the toxins produced by these plants are found nitrogenous substances such as non-protein amino acids, cyanogenic glycosides,

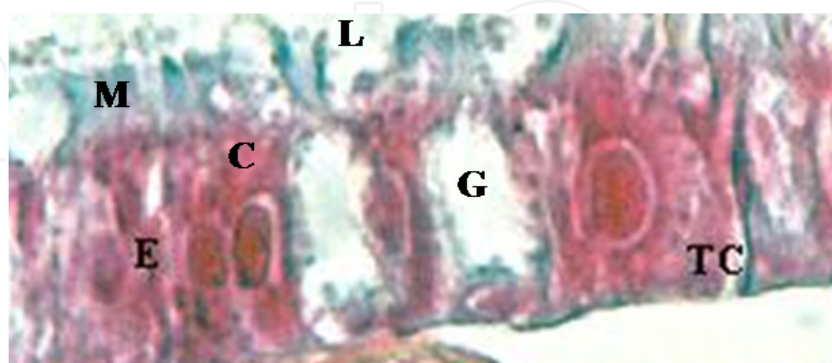
certain peptides and proteins, and several alkaloids. The toxicity of a substance is related to the dose taken by the insect, its age, the absorption mechanism and the manner of excretion (6).

In this chapter we address the toxicological aspects of microbial and botanical biopesticides that act by ingestion, with emphasis on histopathological analysis of tissues and cells in the alimentary channel of the Lepidoptera as well as the specificity of the *B. thuringiensis* bacteria, aqueous extracts and oil essential of medicinal and forest.

## 2. Histology of lepidoptera

The alimentary channel of insects is composed of three main regions with different embryological origins: stomodeum or foregut, middle or midgut and hindgut or proctodeu. This canal represents a contact area between the insect and the environment and is the focus of much of the applied research concerning pest control (7, 8), especially the midgut region where the epithelial cells are involved in the processes of absorption and secretion of enzymes (columnar cells), ion homeostasis (goblet cells), endocrine function (endocrine cells) and the renovation of the epithelia (regenerative cells) (9, 10, 11). A defense mechanism in this region is the peritrophic membrane, which plays a fundamental role in the biology of the midgut, being positioned between the food contents and epithelial layer, performing the function of protecting the epithelium from mechanical damage, and in addition acts as a barrier against toxins and chemicals harmful to the insects (12, 13).

In Lepidoptera, the midgut epithelium is composed of four cell types (Figure 1) which are involved in the processes of absorption and secretion of enzymes (columnar cells), ion homeostasis (goblet cells), endocrine function (endocrine cells) and the renewal of the epithelium (regenerative cells) (14, 15). The regenerative cells are undifferentiated and are responsible for the renovation of the midgut epithelium, substituting the cells that wear out and are lost during the digestive process – they also make it possible for the alimentary channel to grow larger at each ecdysis. They are found at the base of the midgut, alone or in groups, and there are no differences in their abundance along the midgut (16, 17, 18).



**Figure 1.** Types of cells found in Lepidoptera. L=lumen; TC=connective tissue; E=epithelium, C=columnar cells; G=globet cells;

Changes in the alimentary canal, especially in the region of the midgut, can affect the growth and development of insects, as well as all the physiological events, because these

processes depend on adequate food, on its absorption and transformation in the alimentary canal (9, 11).

Among the insects, the Lepidoptera order causes the greatest economic losses in crops – in maize and rice which are attacked principally by *Spodoptera frugiperda*, in sugar-cane attacked by the stem-borer *Diatraea saccharalis* and in soybeans mainly by the Lepidoptera pest *Anticarsia gemmatilis*.

All regions of the epithelial layer of the alimentary canal of *S. frugiperda* caterpillars are coated with a single layer of cells, with a flat morphology in the stomodeum region and cubic morphology in the proctodeus (19). The muscles are arranged along the channel in a uniform manner (7). According to Cavalcante & Cruz-Landim (20) and Pinheiro et al. (16), in the midgut epithelium four cell types predominate: columnar, goblet, regenerative, and endocrine cells. These cells predominate along the *S. frugiperda* midgut epithelium and are considered responsible for the absorption of digested food, and demonstrate morphology similar to that of other Lepidoptera (8, 21). Studies by Harper & Hopkins (22) and Harper & Granados (23), indicate that these epithelial cells are also responsible for secretion of a micro fiber net soaked in a matrix of proteins and glycoproteins -denominated a peritrophic membrane – which performs various functions such as: the protection of the epithelium against chemical and mechanical damage caused by the alimentation, the creation of a physical barrier against micro-organisms and digestion division (18).

Pinto & Fiuza (24), analyzing the histology of the midgut of *A. gemmatilis* caterpillars with an optical microscope, observed that the alimentary canal is divided basically into three portions, which are: the anterior intestine or stomodeum, the middle intestine or mesentero, and the posterior intestine or proctodeus. The cells do not have a cuticle coating, the intestinal wall being constituted by an epithelium of approximately 40µm in height, which is separated from the haemolymph only by a thin layer of loose connective tissue. The cylindrical and goblet cells are evenly distributed along the intestine section. In the intestinal lumen is a thin membrane, called the peritrophic membrane, which surrounds the bolus of food, separating it from the epithelial cells of the midgut. The apical surface of the cylindrical cells has dense microvilli measuring 3µm in height. The goblet cells show invaginations of the intestinal lumen as far as the nucleus.

### 3. Microbial biopesticides

Among the microbial insecticides, the *Bacillus thuringiensis* bacterium is highlighted because it shows great promise for development in the line of organic products and in the area of genetically modified plants. Besides being a sporulent bacteria found naturally in the soil, it is distinguished by its production of protein inclusions with insecticidal activity against several orders of insects and phytonematodes (25, 26). According to Shelton et al. (27), the first biopesticide containing subspecies of *B. thuringiensis* were sold in France in 1930. In 1995 more than 180 products based on this bacterial species were recorded by the Environmental Protection Agency (EPA) in the United States of America. In a study by

Fiuza & Berlitz (28) they stated that *B. thuringiensis* formulas were then being marketed in Brazil. These are described in Table 1.

Products	Companies	<i>B. thuringiensis</i> (Bt)	Target insects
Dipel	Abbott	<i>Bt kurstaki</i>	Lepidoptera
Thuricide	Sandoz	<i>Bt kurstaki</i>	Lepidoptera
Agree	Mitsui	<i>Bt aizawai</i>	Lepidoptera
Bactur	Milenia Agrociências	<i>Bt kurstaki</i>	Lepidoptera
Ecotech Pro	Bayer	<i>Bt kurstaki</i>	Lepidoptera
Bactospeine	Solvay	<i>Bt kurstaki</i>	Lepidoptera
Javelin	Sandoz	<i>Bt kurstaki</i>	Lepidoptera
Foray	Novo-Nordisk	<i>Bt kurstaki</i>	Lepidoptera
Biobit	Novo-Nodisk	<i>Bt kurstaki</i>	Lepidoptera
Foil/Condor	Ecogen	<i>Bt kurstaki</i>	Lepidoptera
Delfin	Sandoz	<i>Bt kurstaki</i>	Lepidoptera
Cutlass	Ecogen	<i>Bt kurstaki</i>	Lepidoptera
LarvoBt	Fermone	<i>Bt kurstaki</i>	Lepidoptera
Nubilacid	Radonja	<i>Bt kurstaki</i>	Lepidoptera
MVP	Mycogen	<i>Bt kurstaki</i>	Lepidoptera
Bac-control	Agricontrol	<i>Bt kurstaki</i>	Lepidoptera
XenTari	Abbott	<i>Bt aizawai</i>	Lepidoptera
M-One	Mycogen	<i>Bt san diego</i> <i>Bt tenebrionis</i>	Coleoptera
Di-Terra	Abbott	<i>Bt san diego</i> <i>Bt tenebrionis</i>	Coleoptera
Trident	Sandoz	<i>Bt san diego</i> <i>Bt tenebrionis</i>	Coleoptera
Novodor	Novo Nordisk	<i>Bt san diego</i> <i>Bt tenebrionis</i>	Coleoptera
M-One Plus	Mycogen	<i>Bt san diego</i> <i>Bt tenebrionis</i>	Coleoptera
Foil	Ecogen	<i>Bt</i> (recombinant)	Coleoptera

\*Table adapted by Fiuza & Berlitz (28)

**Table 1.** Products made from *Bacillus thuringiensis* for controlling agricultural pest\*

### 3.1. Case studies

A study by Berlitz & Fiuza (29) evaluated the toxicity of *B. thuringiensis aizawai* on *S. frugiperda* and demonstrated that, 6 hours after the application of the treatments, an increase in the cell volume occurred and the intestinal microvilli ruptured. Significant differences in the cell volume of the treatment as compared with that of the control caterpillars were observed 12 hours after the application.

In the histopathological analysis of the midgut of *S. frugiperda* caterpillars treated with the *B. thuringiensis thuringiensis* 407 (pH 408) strain, structural changes were observed six hours after application of the treatment (HAT), where there were cells in the intestinal lumen and elongation of the microvilli, as compared to the control. After nine applications of the treatment (HAT) the action was intensified with vacuolization of the cytoplasm, and the beginning of the degradation of the peritrophic membrane – this was entirely absent after 12 HAT.

Treatment with *B. thuringiensis kurstaki* HD-73 strain was similar, except that rupture of the microvilli (BBMV's) and vacuolization of the cytoplasm began at 12 HAT. The results of the histopathological analysis of the midgut of *S. frugiperda* caterpillars demonstrate that treatment with the *B. thuringiensis thuringiensis* strain was more efficient, because the degradations of the microvillousities started 9 hours after treatment application (HAT), while in the *B. thuringiensis kurstaki* the same effect was noticed only after 12 HAT (30).

Knaak and Fiuza (31) tested the nuclear polyhedrosis virus of *Anticarsia gemmatalis* (VPNAg) and *B. thuringiensis kurstaki* HD-1 (Dipel®) in 2<sup>nd</sup> instar caterpillars of *A. gemmatalis* (Lepidoptera, Noctuidae), and observed that when both entomopathogens are utilized simultaneously they are more efficient, because they caused alterations in the intestinal cells after 6 HAT while when used separately they produced the alteration only after 12 HAT.

If the dose of toxin administered does not cause the death of the insect, its cells are substituted permitting normal alimentation and the recuperation of the insect (32). Several studies report on the cell changes produced in the middle intestine of caterpillars intoxicated with Cry proteins from *B. thuringiensis* - for example: the increase in the volume of the epithelium cells, the rupture of the microvilli, cytoplasmic vacuolization, the changes in cytoplasmic organelles and cell hypertrophy (30, 33-35).

Studies of the mode action of the *B. thuringiensis* Cry proteins seek to clarify the mechanisms by which these proteins produce their entomopathogenic effects and to elucidate the specificity of the various toxins.

### 3.2. Mode action of the *Bacillus thuringiensis* Cry proteins

When the crystals of *B. thuringiensis* are ingested by susceptible insects they are solubilized under alkaline conditions in the middle intestine and then broken into smaller fragments by proteases (5, 36, 37). Binding occurs because of the association of the activated toxin with specific proteins located in the microvilli of the epithelial cells of the middle intestine (38) and is followed by the formation of the pore (39) – the ion flux from this pore leads to cell lyses and consequently, to the death of the susceptible insect (5).

The solubility of the protein and crystals in liquids with alkaline pH values such as those in the middle intestine of the insects liberates the protoxins of 130-140kDa for Cry1 and 70kDa for Cry2. This phase determines the specificity of the *B. thuringiensis* isolate to the target



species, both because of the alkalinity of the digestive system and because of the composition of the *B. thuringiensis* crystals.

1. The protoxins are activated by the digestive enzymes, forming toxic fragments of 60-65kDa. At this stage both the photolytic composition and the structure of the crystal protein are important. The toxins recognize specific receptors in the microvilli of the epithelial cells of the middle intestine of susceptible larvae to which they bind. Studies made with BBMV (*Brush Border Membrane Vesicles*) isolated from the larvae of Lepidoptera show that the strong binding affinity between the toxin and the receiver is considered an important factor in determining the insecticidal spectrum of the Cry proteins (40). Research data demonstrates that there is a positive correlation between binding, *in vitro*, of the toxin in the intestinal receptor and the toxicity, *in vivo*. On the other hand, other studies describe that while the recognition of the receptor is necessary it is not sufficient in itself to provoke the toxicity, which suggests the existence of other factors related to the mode of action of the Cry proteins. In 1994, Knight et al. (41) isolated from the BBMV of *Manduca sexta* (Lep., Sphingidae) larvae an aminopeptidase N implicated in the interaction of the toxin Cry1Ac. The receptor models now described demonstrate that an insect may present, in variable quantities, various kinds of receptors which could be recognized by different toxins. Research data show that these models may explain the specificity of the *B. thuringiensis* toxins.
2. After recognition of the receptor, the toxins induce the formation of pores in the cellular membrane of the intestinal epithelia.
3. The formation of pores in the cellular membrane provokes an ionic imbalance between the cytoplasm and the cell's external environment. Histopathological analysis performed after the intoxication of insects reveal destruction of the microvilli, epithelial cell hypertrophy, cytoplasmic vacuolization and cellular lyses which cause paralysis and death of the insect.

When selecting insecticidal proteins, synthesized by this bacteria, a preliminary *in vitro* analyzes of membrane receptors can facilitate a rapid determination of the range of action of Cry proteins against the target species. The evaluation of the toxicity *in vivo* can then be limited to those isolates which were pre-selected as active *in vitro*.

### 3.3. Detection of receptors in tissues of insects

#### 3.3.1. Preparation of the tissues of insects

The insect's digestive tubes are dissected and fixed for 24h in *Bouin Hollande Sublimé* 10%, washed in distilled water for 12 hours and dehydrated in an ethanol mixture increasing in strength gradually from 70 to 100% (42). The tissues are then impregnated in mixed baths (ethanol/toluene/Paraplast) and embedded in 100% Paraplast at 58°C. The longitudinal or transverse sections, 7-10 mm thick, prepared using LKB microtome are mounted on glass slides, tanadas with poly-l-lysine at 10% and stored at 4°C for subsequent analysis of the histology of the receptors.

### 3.3.2. Labeling of Cry proteins with biotin

Cry proteins can be biotinylated, according to the method described in Bayer & Wilcheck (43), where the incorporation of biotin at the N-terminal of the protein is done using BNHS (biotinyl-N-hydroxysuccinimide) in a buffer of sodium bicarbonate, pH 9.

The reaction product should be purified with Sephadex G-25 (Sigma), and the fractions biotinylated and identified by dot-blot, which uses a nitrocellulose membrane, the conjugate of streptavidin alkaline phosphatase diluted in TST buffer (Tris-Triton-Saline, pH 7.6) and the revelation substrate (BCIP and NBT in a Tris buffer, pH 9.5).

The concentration of biotinylated Cry proteins can be determined by the Bradford method (44) using BSA as the protein standard. The purity and integrity of the labeled proteins can be evaluated by western blot using a nitrocellulose membrane (Sigma) and Towbin buffer, pH 8.3 with 10% ethanol. The membranes can be developed using the same technique described in the dot-blot.

### 3.3.3. In vitro detection of membrane receptors

#### 3.3.3.1. Pre-treatment of tissues

The in vitro analyses of the receptors of Cry proteins of *B. thuringiensis* are performed with histological sections of the digestive systems of larvae being studied. The actual detection results from incubation with the tissue proteins, which have been previously dewaxed and rehydrated as shown in the drawing.

#### 3.3.3.2. Reactions with biotinylated proteins (45)

In the analyses with biotinylated Cry proteins, histological sections are incubated at room temperature for 1 hour with the biotinylated proteins. Proteins not bound to the receptor sites are removed with TST, pH 7.6.

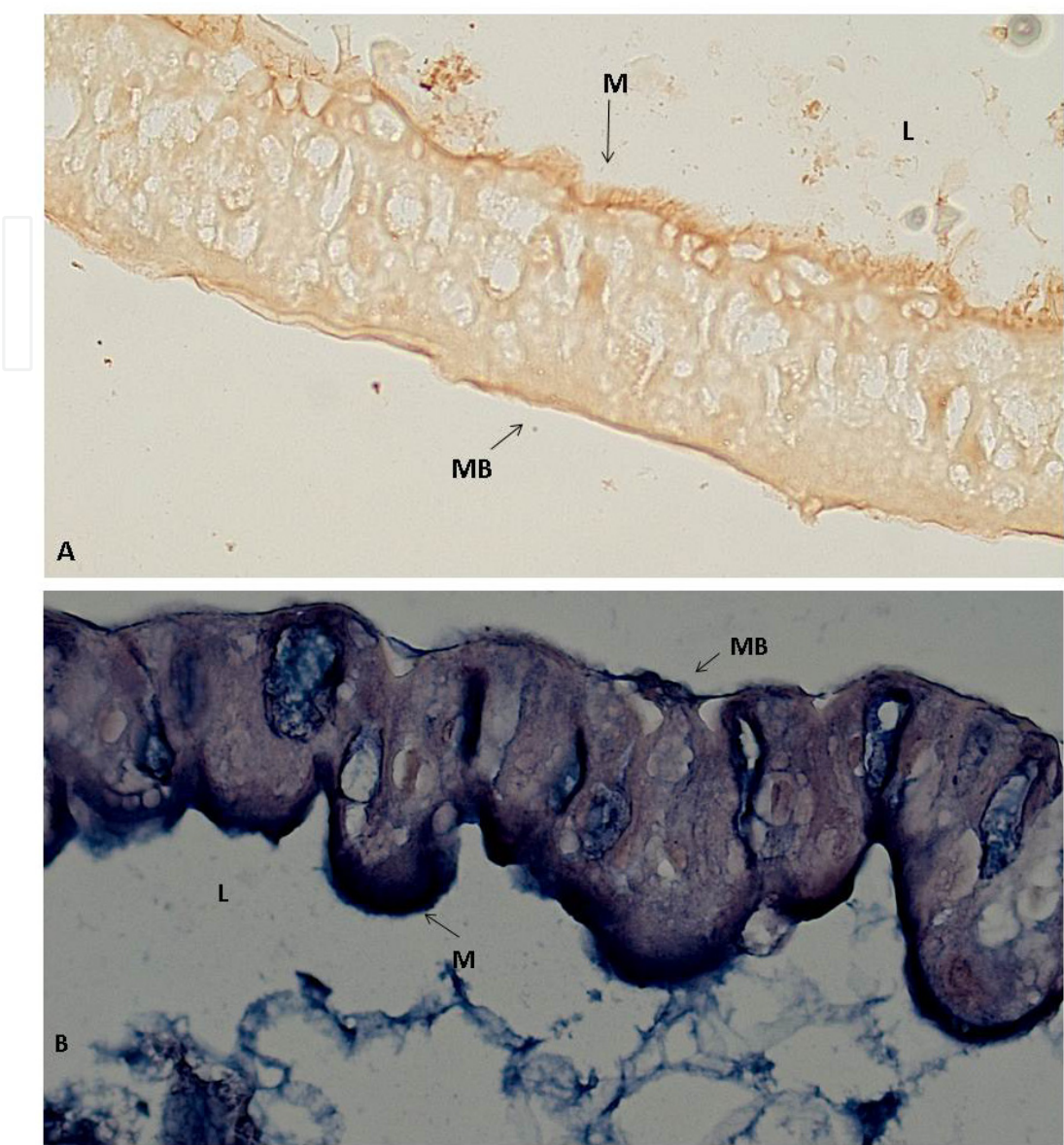
At the next step, the tissues are treated with streptavidin conjugated to an enzyme (peroxidase or alkaline phosphatase) or fluorochrome (fluorescein or phycoerythrin), diluted in a TST buffer. The resulting "protein-receptor" complex reaction, using the enzyme conjugate, can be developed with the DAB substrate for peroxidase (Figure 2A) and with BCIP/NBT for alkaline phosphatase (Figure 2B). The sections are fixed with Pertex mounting medium between the slide and the glass cover slip.

To develop tissues treated with fluorescein the histological sections are mounted with Mowiol and stored at 4°C for analysis by optical microscopy (OM - Figure 3A) or by laser scanning microscopy (LSM - Figure 3B).

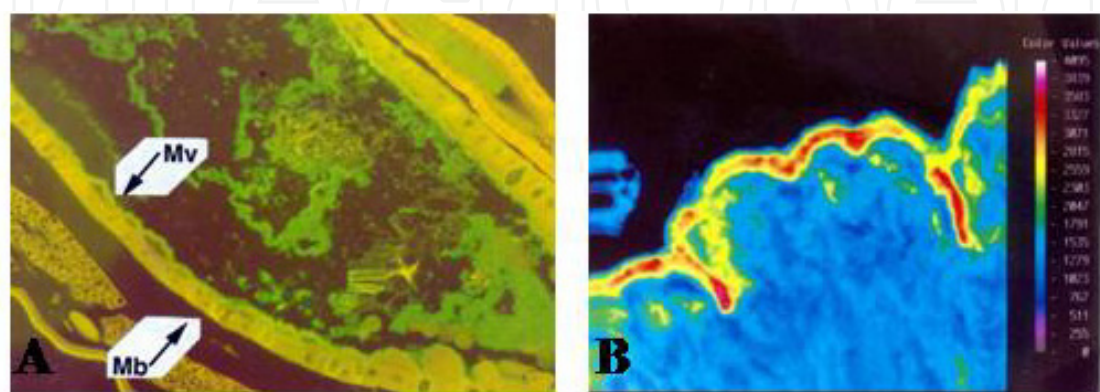
#### 3.3.3.3. Immune detection reactions of protein receptors

In immunohistochemical analyzes with unlabeled proteins (native preteins), the receptors are developed with the primary antibody (AC<sub>1</sub> specifically against the Cry protein) and secondary antibody (AC<sub>2</sub>, directed against the AC<sub>1</sub>) conjugated to an enzyme or fluorochrome, which are developed and assembled according to the method described above.





**Figure 2.** Cry proteins biotinylated receptor, in Lepidoptera insect tissue revealed with peroxidase (A) and alkaline phosphatase (B). (MB) Basal Membrane; (L) Lumen; (M) microvilli.



**Figure 3.** Receivers biotinylated Cry proteins, detected in the tissues of insects with fluorescein and observed in MO (A) and MVL (B) (45).

In immuno localization, the caterpillars are previously treated in vivo with the Cry proteins and afterwards the tissues and the immunohistochemical reactions are prepared.

In both methods, the controls are prepared by alternative omission of each step of the reaction, to eliminate the possibility of false-positive reactions. The samples developed with enzymes like peroxidase and alkaline phosphatase can be evaluated by optical microscopy.

#### 4. Plant bioinsecticides

Until quite recently the use of chemical insecticide has been the most widely used method of controlling insect pests. However, the products are expensive and in some cases inefficient and hazardous if used intensively and/or incorrectly. However, some success has been achieved in programs of Integrated Pest Management (IPM) with an alternative to chemicals extracted from various secondary metabolites present in roots, leaves and seeds of plants as an alternative to chemicals called "plant pesticides" (46-48).

The natural products obtained from vegetable raw materials present a wide variety of molecules, with great diversity in structure and biological activity (49). This wide range of new sites of action on target organisms can be considered another reason for the growing interest in phytotoxins, because, even if not commercially available, they may suggest lines for the synthesis of entirely new products (50). This is important if we consider the speed with which the insects and micro-organisms have developed resistance to chemicals commonly used as biological control agents of target species.

According to Mello & Silva-Filho (51), the components of insecticides can be divided into the following groups: (i) derivatives of chemical compounds (tannins, terpenoids, flavonoids, alkaloids, quinones, linomoides, phenols), (ii) molecules produced from the processing proteins (chitinase, lectins, inhibitors alpha-amylase and proteinase inhibitors) and (iii) volatile compounds of plants such as essential oil. In this case, the chemical components can be divided into two classes. The first, based on biosynthesis, in which are found the derivatives of the terpenoids, is formed via the mevalonic acetate, and the second, by derivatives which are located in the phenylpropanoid, aromatic compounds formed by way of shikimic acid (52).

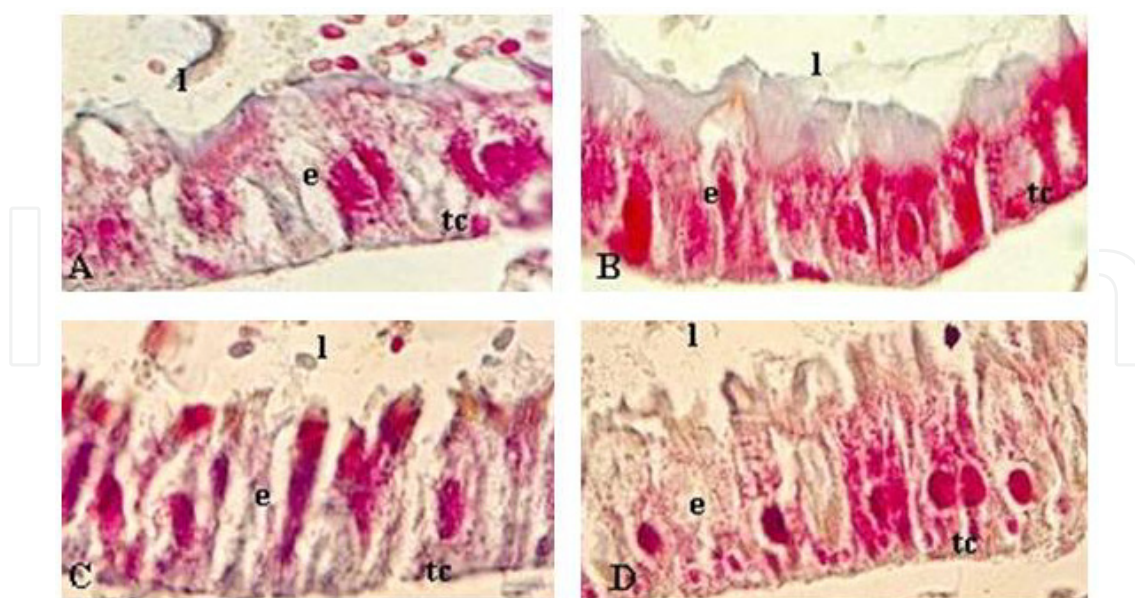
Normally, the oily essential in the leaves and resins contain some constituents in high concentration, and about 30-40 minor compounds at concentrations less than 1% (53). These substances are found in plants in the form of complexes, whose components are integrated and reinforce its action on the organism. Even when the plant has only one active principle, this has a beneficial effect superior to that produced by the same substance produced by chemical synthesis. However, the use of botanical insecticides depends on identification of the active compounds, their mode of action, production, formulation, stability, dose, action on natural enemies, field persistence, toxicity tests for record, among others (54).

The Laboratory of Microbiology and Toxicology at the UNISINOS University uses two models for histopathologic analysis:



1. Following treatment with plant extracts or oily essences 10 larvae of the insect target are collected in periods of 1, 3, 6, 9, 12, 24 and 48 hours after treatment (HAT). The specimens are fixed in aqueous paraformaldehyde and then dehydrated in ethanol solutions of increasing strength (50, 70, 90 and 100%). After dehydration, the larvae are embedded in resin (Leica Histoiresin) for 12 h. After that the specimens are put into molds of solid polypropylene using the same resin with a polymerize. The resin blocks are mounted on supports after polymerization, and cut into 3mm thick sections in a microtome with a glass blade. The histological sections are stretched out onto glass slides and stained with Schiff-Naphtol Blue Black periodic acid, dehydrated, and mounted between the glass slides and the cover slips with *Entellan*.
2. After application of the treatments with plant extracts or essential oil, 10 target insect larvae in each treatment, are collected in periods of 3, 6, 9, 12, 24 and 27HAT. After fixation in *Bouin Hollande Sublime-BHS* for 24 hours (38), the larvae are subjected to dehydration in increasingly strong ethanol solutions, followed by rapid baths of xylene and impregnation in paraffin. Longitudinal histological sections of the midgut are realized at a thickness of 5µm. Coloration is applied with *Azul de Heidenhain* (41), which differentiates the microvilli of the middle intestine by the presence of glycoproteins which can be observed by the system of comparative histology in an optical microscopy.

Knaak et al. (55) found that the toxicity of the extracts of *Petivesia alliacea*, *Zingiber officinale*, *Ruta graveolens*, *Malva silvestris*, *Baccharis genistelloides* and *Cymbopogon citratus* caused damage such as: vacuolization of the cytoplasm, disruption of microvilli, peritrophic membrane destruction and cell changes in the midgut of *S. frugiperda* (Figure 4). This study the changes mentioned in the previous work were not observed until 48HAT, which was the maximum time rated.

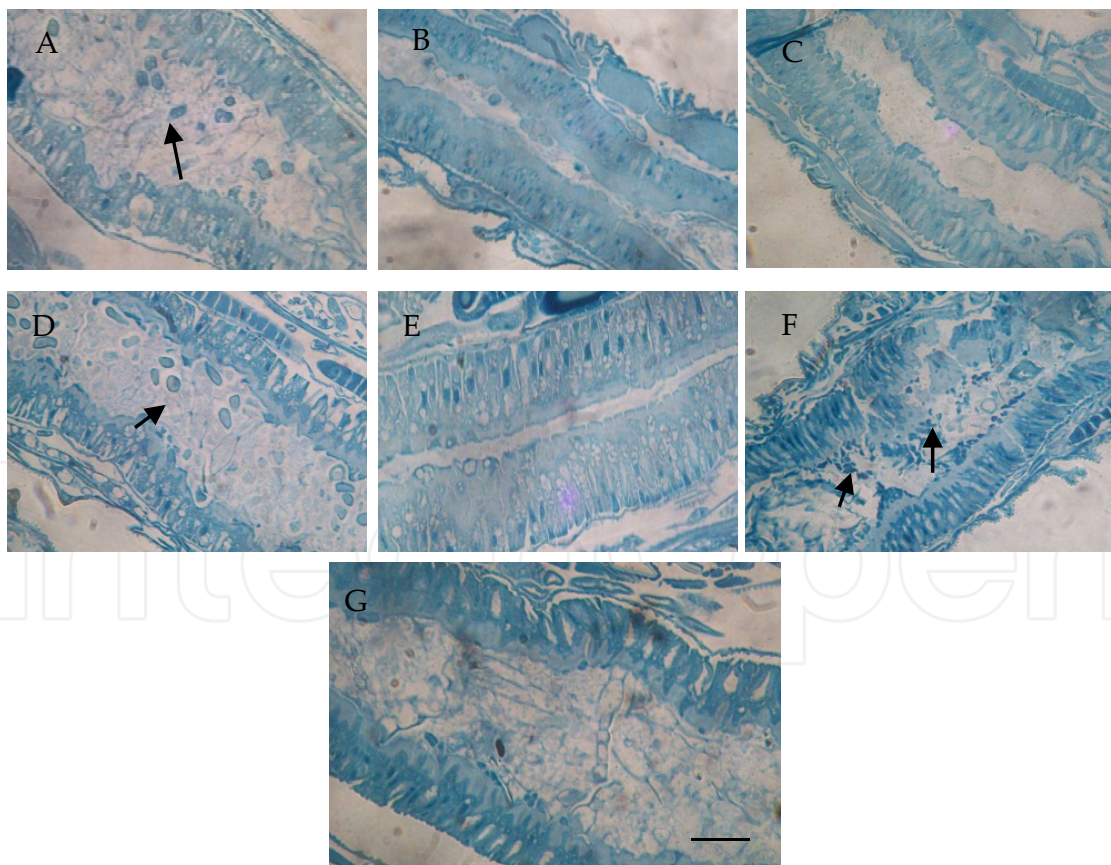


**Figure 4.** Longitudinal sections of the midgut of *Spodoptera frugiperda* caterpillars treated with plants extrats, (A) *Petivesia aliacea* (24 HAT), (B) *Petivesia aliacea* (24 HAT); (C) *Zingiber officinale* (6 HAT); (D) *Zingiber officinale* (27 HAT); (E) *Z. officinale* (6 HAT); (F) *Z. officinale* (24 HAT); (G) Controle. Increase 400X; bar=2.44µm; →=changes; TC=connective tissue; M=microvilli; E=epithelium; L= lumen.

The chemical compounds (tannins, terpenoids, flavonoids, alkaloids, quinones, linomoides, phenols), molecules produced from the processing of proteins (chitinase, lectins, alfa-amylase inhibitor and proteinase inhibitors) and volatile compounds from the plant [56, 57], present in plant extracts, undergo different changes according to the physico-chemical conditions along the digestive tract of insects.

In treatments with the essential oil of *R. graveolens* and *Malva* sp., cellular projections were observed in the intestinal lumen at 9 HAT, and the oil of *R. graveolens* caused cell elongation at 12 HAT. Thus, it appears that changes in the structure of the midgut of *S. frugiperda* may increase during the treatment with essential oils. The intensity of the pathological effects is dependent on time period and the concentration used (58).

In the treatments with essential oil of *Z. officinale* and *C. citratus*, was observed projections of epithelial cells in the lumen, at 3 and 6HAT respectively (Figure 5A, D and E). After 24 hours treatment with *C. citratus* (Figure 5B), we observed elongation of the microvilli, while in the treatment with *Z. officinale* cell elongation occurred (Figure 5G). At 24HAT with the *Z. officinale* oil several morphological changes occurred, such as: cellular disorganization, destruction of the epithelium, of the microvilli and the peritrophic membrane. It can still be observed that the changes are dependent on the time period and the concentrations used (56).



**Figure 5.** Longitudinal sections of the midgut of *Spodoptera frugiperda* caterpillars treated with essential oils, (A) *Cymbopogon citratus* (6 HAT) (B) *C. citratus* (24 HAT); (C) *C. citratus* (48 HAT); (D) *Zingiber officinale* (3 HAT); (E) *Z. officinale* (6 HAT); (F) *Z. officinale* (24 HAT); (G) Controle. Increase 400X; bar=2.44 $\mu$ m;  $\rightarrow$ =changes; TC=connective tissue; M=microvilli; E=epithelium; L= lumen.

Knaak et al. (55) evaluated the effect of the interaction of various plant extracts with Xentari®, *B. thuringiensis aizawai*, in the midgut of *S. frugiperda*, demonstrating that the histopathological effects of *Z. officinale*, *M. silvestris*, *R. graveolens* and *B. genistelloides*, in the midgut of *S. frugiperda* were more intense when compared to extracts of *P. alliacea* and *C. citratus*, which showed a positive interaction with Xentari®, accelerating the process of destruction of intestinal cells, which represents a reduction in the lethal time of the target species, *S. frugiperda*.

Some plants are outstanding, for instance, *Melia azedarach*, a plant similar to *Azadirachtina indica* which produces azadirachtine efficiently for more than 400 species of insects (58), its active ingredient is already a commercial product called Neemix 4.5®. This causes different reactions in insects, acting as an alimentary inhibitor retarding growth, reduces fertility, and causes morphogenetic and behavioral changes (59).

The toxicity of cinnamon is related to the presence of different compounds, such as salanalina, meliaterina and meliacarpinina E, from which additionally other derivatives can be obtained which show insecticidal action against the Coleopteran orders (Curculionidae, Tenebrionidae and Chrysomelidae) and Lepidoptera (60-62), amongst others.

Correia et al. (63) evaluated the histology of the alimentary canal of larvae of *S. frugiperda* treated with neem (Neemseto®) at concentrations 0.5 and 1.0%, on non-treated leaves. They found that regions of the stomodeum and proctodeus showed no morphological changes. However, the middle intestinal region, showed morphological changes that varied in intensity according to the exposure time and concentration (0.5 and 1.0%). After 48h and 96h of treatment, tissue changes were observed in the larvae treated with neem – the cells of the epithelial lamina became slender and elongated. It was not possible to distinguish cell types beyond reducing significantly reducing the secretion activity of the two concentrations studied, in comparison with the control.

## 5. Conclusion

In toxicological analysis of microbial biopesticides, with *Bacillus thuringiensis* as the object of analysis, the labeling detected in the region of the microvilli of the cells of the intestinal epithelial cells revealed the presence of membrane receptors of Cry proteins in the midgut of the larvae of the insects when compared to the controls representing the alternative omission of different components of the reactions, as can be seen by the absence of staining on the microvilli of intestinal epithelial cells of the insects used here as a model of this approach to the study. The immunodetections and the detection of biotinylated Cry proteins were used by various authors, for identifying the intestinal membrane receptors, in larvae of different species of Lepidoptera (33, 45, 64-65), Diptera (66) and Coleoptera (33, 67). These authors proved that the binding of the Cry proteins to the microvilli of the insect's midgut indicate the existence of a receptor specific to the cited protein in the target insect.

Considering therefore, the studies of receptors in vitro and the toxicological analyses in vivo, it can be confirmed that the methods for detection of membrane receptors can be



applied in selecting the toxins of *B. thuringiensis* that are active against insects, and that there is a positive correlation between the *in vitro* and the *in vivo* analysis. However, to determine the median lethal concentration (LC<sub>50</sub>) of a Cry protein a bioassay is necessary since the binding of proteins to the receptors may vary in concentration and affinity, as previously described by several authors for different insect species (64, 68, 69, 70). Other studies show that the Cry proteins that are toxic to insects correspond to those which bind irreversibly to the epithelial cell receptors of the target insects (71).

In the case of plant insecticides, chemical compounds, (tannins, terpenoids, flavonoids, alkaloids, quinones, phenols and linomoides), the molecules produced from the processing of proteins (chitinase, lectins, inhibitors alpha-amylase and proteinase inhibitors) and volatile compounds from plants, such as the oily essences present in plants (57) undergo different changes according to the physical and chemical conditions along the digestive tract of the insects.

In recent years, in the search for alternative methods to control agricultural pests without using artificial chemical substances, research was expanded to obtain greater knowledge on the mode action of microbial toxins and plant insecticides. Studies realized *in vitro* on the cells and tissues of the alimentary canal of insects, especially of Lepidoptera, have identified and located the receptors in the target species. Thus, for the application of plant and microbial toxins as biopesticides, it is fundamental to evaluate the range of action and the specificity of the active ingredient. In this context, Fiuza (72) mentions that the analysis *in vitro* of membrane receptors can be considered an indispensable tool due to the large number of botanical compounds plus the isolates, the strains and proteins of *B. thuringiensis* that have been identified, and which have considerable potential for pest management.

## Author details

Neiva Knaak, Diouneia Lisiane Berlitz and Lidia Mariana Fiuza

University of Vale do Rio dos Sinos, Laboratory of Microbiology and Toxicology, São Leopoldo, RS, Brazil

## 6. References

- [1] Cáceres, O. et al. (1981). Resíduos de pesticidas clorados em água das cidades de São Carlos e Araraquara. *Ciência e Cultura*, São Paulo, Vol. (33): 1622-1626.
- [2] Aguiar, L.H. & Moraes, G. (1999). Hepatic alanine and aspartic amino transferases of the freshwater teleost. *Bryconcephalus* (Matrinchã) exposed to the organophosphorous methyl parathion (folidol 600 registred), *Fish response to toxic-environments*, Kennedy, Canadá. pp. 145-152.
- [3] Carlini, C.R. & Grossi-de-Sá, M.F. (2002). Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. *Toxicon*, Vol. 40: 1515-1539.
- [4] Alves, S. B. (1998). *Controle microbiano de insetos*. 2.ed. Piracicaba: FEALQ, 1163p.



- [5] Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. & Dean, D.H. (1998). *Bacillus thuringiensis* and its pesticide crystal proteins. *Microbiology Molecular Biology Reviews*, Vol. (62): 775-806.
- [6] Saito, M.L. & Lucchini, F. (1998). Substâncias obtidas de plantas e a procura por praguicidas eficientes e seguros ao meio ambiente. Jaguariúna: EMBRAPA-CNPMA. 46p.
- [7] Chapman, R.F. (1998). *The insects: structure and function*, 4th ed., Cambridge, Cambridge University Press, 788p.
- [8] Levy, S.M., A.M.F. Falleiros, E.A. Gregório, N.R. Arrebola & L.A. Toledo. (2004). The larval midgut of *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae): light and electron microscopy studies of the epithelial cells. *Brazilian Journal of Biology*, Vol. (64): 633-638.
- [9] Mordue (Luntz), A.J. & Blackwell A. (1993). Azadirachtin: an update. *Journal of Insect Physiology*, Vol. (39): 903-924.
- [10] Chiang, A.S., Yen, D.F. & Peng, W.K. (1986). Defense reaction of midgut epithelial cells in the rice moth larva (*Corcyra cephalonica*) infected with *Bacillus thuringiensis*. *Journal of Invertebrate Pathology*, Vol. (47): 333-339.
- [11] Mordue (Luntz), A.J. & Nisbet A.J. (2000). Azadirachtin from the neem tree *Azadirachta indica*: its action against insects. *An. Soc. Entomol. Brasil*, Vol. (29): 615-632.
- [12] Lehane, M.J. (1997). Peritrophic matrix structure and function. *Ann. Ver. Entomol.*, Vol. (42): 525-550.
- [13] Terra, W.R. (2001). The origin and functions of the insect peritrophic membrane and peritrophic gel. *Arch. Insect Biochem. Physiol.*, Vol. (47): 47-61.
- [14] Terra, W.R. & Ferreira, C. (2005) Biochemistry of digestion. In *Comprehensive Molecular Insect Science* (Gilbert LI, Iatrou K & Gill SS, eds), Vol. 4, pp. 171-224. Elsevier, Oxford.
- [15] Pinheiro, D.O., Quagio-Grassiotto, I. & Gregório, E.A. (2008). Morphological regional differences of epithelial cells along the midgut in *Diatraea saccharalis* Fabricius (Lepidoptera: Crambidae) larvae. *Neotropical Entomology*, Vol. (37): 413-419.
- [16] Pinheiro, D.O., Silva, R.J., Quagio-Grassiotto, I. & Gregório, E.A. (2003). Morphometric study of the midgut epithelium in larvae Of *Diatraea saccharalis* Fabricius (Lepidoptera: Pyralidae). *Neotropical Entomology*, Vol. (32): 453-459.
- [17] Wanderley-Teixeira, V., Teixeira, A.A.C., Cunha, F.M., Costa, M.K.C.M., Veiga, A.F.S.L. & Oliveira, J.V. (2006). Histological description of the midgut and thepy loric valve of *Tropida criscollaris* (Stoll, 1813) (Orthopetera: Romaleidae). *Brazilian Journal of Biology*, Vol. (66): 104.
- [18] Martins, G.F., Neves, L.A.O. & Serrão, J.E. (2006). The regenerative cells during the metamorphosis in the midgut of bees. *Micron*, Vol. (37): 161-168.
- [19] Correia, A.A., Wanderley-Teixeira, V., Teixeira, A.A.C., Oliveira, J.V. & Torres, J.B. (2009). Morfologia do canal alimentar de lagartas de *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera:Noctuidae) alimentadas com folhas tratadas com Nim. *Neotropical Entomology*, Vol. (38): 1-9

- [20] Cavalcante, V.M. & Cruz-Landim, C. (1999). Types of cells present in the midgut of the insects: a review. *Naturalia*, Vol. (24): 19-39.
- [21] Levy, S.M., Falleiros, A.M.F., Gregório, E.A., Arrebola, N.R. & Toledo, L.A. (2004). The larval midgut of *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae): light and electron microscopy studies of the epithelial cells. *Braz J Biol*, Vol. (64): 633-638.
- [22] Harper, M.S. & Hopkins, T.L. (1997). Peritrophic membrane structure and secretion in European corn borer larvae (*Ostrinia nubilalis*). *Tissue Cell*, Vol. (29): 463-475.
- [23] Harper, M.S. & Granados, R.R. (1999). Peritrophic membrane structure and formation of larval *Trichoplusia ni* with an investigation on the secretion patterns of a PM mucin. *Tissue Cell*, Vol. (31): 202-211.
- [24] Pinto, L.M.N.P. Histologia do intestino médio de lagartas de *Anticarsia gemmatalis* Hübner 1818 (Lepidoptera, Noctuidae). *Acta Biologica Leopoldensia*, Vol. (22): 89-95.
- [25] Crickmore, N., Zeigler, D.R., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Bravo, A. & Dean, D.H. (2005). *Bacillus thuringiensis* toxin nomenclature. [Online.] <http://www.lifesci.su>
- [26] Marroquin, L.D.; Elyasnia, D.; Griffiths, J.S.; Feitelson, J.S. & Aroian, R.V. (2000). *Bacillus thuringiensis*: toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. *Genetics*, Vol. (155): 1693-1699.
- [27] Shelton, A. M., Ahao, J.Z. & Roush, R.T. (2002). Economic, ecological, food safety and social consequences of the deployment of Bt transgenic plants. *Annual Review of Entomology*, Vol. (47): 845-881.
- [28] Fiuza, L.M. & Berlitz, D.L. (2009). Produtos de *Bacillus thuringiensis*: registro e comercialização. *Biotecnologia Ciência & Desenvolvimento*, Vol. (38): 58-60.
- [29] Berlitz, D.L. & Fiuza, L.M. (2004). Avaliação toxicológica de *Bacillus thuringiensis aizawai* para *Spodoptera frugiperda* (Lepidoptera: Noctuidae), em laboratório. *Biociências*, Vol. (12): 115-119.
- [30] Knaak, N., Franz, A.R., Santos, G.F. & Fiuza, L.M. (2010). Histopathology and the lethal effect of Cry proteins and strains of *Bacillus thuringiensis* Berliner in *Spodoptera frugiperda* J.E. Smith Caterpillars (Lepidoptera, Noctuidae). *Brazilian Journal Biology*, Vol. (70): 677-684
- [31] Knaak, N. and Fiuza, L.M., 2005. Histopathology of *Anticarsia gemmatalis* Hübner (Lepidoptera; Noctuidae) treated with *Nucleopolyhedrovirus* and *Bacillus thuringiensis* serovar *kurstaki*. *Brazilian Journal of Microbiology*, vol. 36, no. 2, p. 195-199.
- [32] Spies, A.F. & Spence, K.D. (1995). Effect sublethal *Bacillus thuringiensis* crystal endotoxin treatment on the larval midgut of a moth, *Manduca sexta*. *Tissue and Cell*, Vol. (17): 394-397.
- [33] Bravo, A., Hendrickx, K., Jansens, S. & Peferoen, M. (1992). Immunocytochemical analysis of specific binding of *Bacillus thuringiensis* insecticidal crystal proteins to lepidopteran and coleopteran midgut membranes. *Journal of Invertebrate Pathology*, Vol. (60): 247-253.
- [34] Griego, V.M., Fancher, L.J. & Spence, K.D. (1980). Scanning electron microscopy of the disruption of tobacco horn worm, *Manduca sexta*, midgut by *Bacillus thuringiensis* endotoxin. *Journal of Invertebrate Pathology*, Vol. (350): 186-189.

- [35] Mathavan, S., Sudha, P.M. & Pechimuthu, S.M. (1989) Effect of *Bacillus thuringiensis* on the midgut cells of *Bombyxmori* larvae: A histopathological and histochemical study. *Journal of Invertebrate Pathology*, Vol. (53):217-227.
- [36] Shao, Z., Cui, Y., Yi, H., Ji, J. & Yu, Z. (1998). Processing of  $\Delta$ -endotoxin of *Bacillus thuringiensis* subsp. *Kurstaki* HD-1 in *Heliothis armigera* midgut juice and the effect of proteases inhibitors. *Journal of Invertebrate Pathology*, Vol. (72): 73-81.
- [37] Tojo, A. & Aizawa, K. (1983). Dissolution and degradation of *Bacillus thuringiensis*  $\delta$  endotoxin by gut juice protease of the silkworm *Bombyx mori*. *Appl. Environ. Microbiology*, Vol. (45): 576-580.
- [38] Schwartz, J.L., Juteau, M., Grochulski, P., Cygler, M., Prefontaine, G., Brousseau, R. & Masson, L. (1997). Restriction of intramolecular movements within the Cry1Aa toxin molecule of *Bacillus thuringiensis* through disulfide bond engineering. *FEBS Lett.*, Vol. (410): 397-402.
- [39] Masson, L., Tabashnik, B.E., Liu, Y.B. & Schwartz, J.L. (1999). Helix 4 of the *Bacillus thuringiensis* Cry1Aa toxin lines the lumen of the ion channel. *Journal of Biology Chemical*, Vol. (274): 31996-32000.
- [40] Fiuza, L. M., Nielsen-Leroux, C., Goze, E., Frutos, R. & Charles, J.F. (1996). Binding of *Bacillus thuringiensis* Cry1 toxins to the midgut brush border membrane vesicles of *Chilosuppressalis* (Lepidoptera, Pyralidae): evidence of shared binding sites. *Applied Environmental Microbiology*, Vol. (62): 1544-1549.
- [41] Knight, P., Crickmore, N. & Ellar, D. (1994). The receptor for *Bacillus thuringiensis* CryIAC delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. *Molecular Microbiology*, Vol. (11): 429-436.
- [42] Brandtzaeg, P. (1982). Tissue preparation methods for imunocytochemistry. In: Bullock, G. & Petruz, P., *Techniques in imunocytochemistry*. Academic Press, London. pp. 49-51.
- [43] Bayer, E. & Wilcheck, M. (1990). Protein biotinylation. *Methods in Enzymology*, Vol. (184): 138-159.
- [44] Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, Vol. (72): 248-254.
- [45] Fiuza, L.M. (1995). Etude des sites récepteurs et de la toxicité des delta-endotoxines de *Bacillus thuringiensis* Berliner chez les larves de la Pyrale du riz, *Chilo suppressalis* Walker. *Thèse de doctorat en Sciences Agronomiques*, ENSA-M, Montpellier, France. 180p.
- [46] Schmutterer, H. (1990). Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. *Annu. Rev. Entomology*, Vol. (35): 271-297.
- [47] Roel, A.R., Vendramim, J. D., Frighetto, R.T.S. & Frighetto, N. (2000) Atividade tóxica de extratos orgânicos de *Trichilia pallida* Swartz (Meliaceae) sobre *Spodoptera frugiperda* (J.E. Smith). *An. Soc. Entomol. Brasil*, Vol. (29): 799-808.
- [48] Roel, A.R. (2001). Utilização de plantas com propriedades inseticidas: uma contribuição para o desenvolvimento rural sustentável. *Ver Int Desenv Local* Vol. (1): 43-50.
- [49] Reigosa, M. & Pedrol, N. (2002). *Allelopathy from molecules to ecosystems*. Plymouth: Science Publishers, 316p.

- [50] Duke, S.O., Dayan, F.E. & Rimando, A.M. (2000). Natural products and herbicide discovery. IN: Cobb, A.H.; Kirkwood, R.C. (Ed.). *Herbicides and their mechanisms of action*. Sheffield: Academic Press, pp.105-133.
- [51] Mello, M.O. & Silva-Filho, M.C. (2002). Plant-insect intections: na evolutionary arms race between two distinct defense mechanisms. *Brazilian Journal of Plant Physiology*, Vol. (14): 71-81
- [52] Strapazzon, J.O. (2004). Composição química e análise antimicrobiana do óleo volátil de *Annona squamosa* L. (Ariticum.) Chapecó, SC. Monografia de graduação. Universidade Comunitária Regional de Chapecó, 53 p.
- [53] Takabayashi, J., Dicke, M. & Posthumus, M. A. (1994). Volatile herbivore-induced terpenoids in plant-mite interactions: variation caused bybiotic and abiotic factors. *Journal of Chemical Ecology*, Vol. (20): 1329-1354. <http://dx.doi.org/10.1007/BF02059811>
- [54] Martinez, S.S. & Emdem, H.F. (2001). Redução do crescimento, deformidades e mortalidade de *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) causadas por *Azadiractina*. *Neotropical Entomology*, Vol. (30): 113-125.
- [55] Knaak, N., Tagliari, M.S. & Fiuza, L.M. (2010). Histopatologia da interação de *Bacillus thuringiensis* e extratos vegetais no intestino médio de *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Arquivos do Instituto Biológico*, São Paulo, Vol. (77): 83-89.
- [56] Knaak, N. 2011. Potencial dos óleos essenciais e proteínas vegetais, obtidos de plantas medicinais, no controle de pragas da cultura do arroz irrigado.Tese de Doutorado, Programa de Pós-Graduação em Biologia da Universidade do Vale do Rio dos Sinos. 107p.
- [57] Simões, C.M.O., Schenkel, E.P., Gosmam, G., Mello, J.C.P., Mentz, L.A. & Petrovick, P.R. (2003). Farmacognosia da Planta ao medicamento. 5ª ed., Porto Alegre, Universidade, 1102 p.
- [58] Martinez, S.S. (2002). O nim – *Azadirachta indica*: natureza, usos múltiplos, produção. Londrina, Instituto Agrônômico do Paraná, 142p.
- [59] Breuer, M.; Hoste, B.; De Loof, A. & Naqvi, S.N.H. (2003). Effect of *Melia azedarach* extract on the activity of NADPH-cytochrome c reductase and cholinesterase in insects. *Pestic. Bioch. Physiology*, Vol. (76): 99-103.
- [60] Huang, Y., Tan, J.M.W.L., Kini, R.M. & Ho, S.H. (1997). Toxic and antifeedant action of nutmeg oil against *Tribolium castaneum* (Herbst) and *Sitophilus zeamais* Motsch. *Journal of Stored Products Research*, Vol. (4): 289-298.
- [61] Bohnenstengel, F.I.; Wray, V.; Witte, L.; Srivastava, R.P. & Proksch, P. (1999). Inseticidal meliacarpins (C-seco limnoids) from *Melia azedarach*. *Phytochemistry*, Vol. (50): 977-982.
- [62] Carpinella, M.C.; Defago, M.T.; Valladares, G. & Palacios, S.M. (2003). Antifeedant and inseticide proprieties of a limnoid from *Melia azedarach* (Meliaceae) with potential use for pest management. *J. Agric. Food Chemistry*, Vol. (51): 369-374.
- [63] Correia, A.A., Wanderley-Teixeira, V., Teixeira, A.A.C., Oliveira, J.V. & Torres, J.B. (2009). Morfologia do Canal Alimentar de Lagartas de *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) Alimentadas com Folhas Tratadas com Nim. *Neotropical Entomology*, Vol. (38): 083-091.



- [64] Denolf, P., Jansens, S., Peferoen, M., Degheele, D. & Van Rie, J. (1993). Two different delta-endotoxin receptors in the midgut brush border membrane of the European corn borer, *Ostrinia nubilalis*. *Applied Environmental Microbiology*, Vol. (59): 1828-1837.
- [65] Estada, U. & Ferre, J. (1994). Binding of insecticidal crystals proteins of *Bacillus thuringiensis* to the midgut brush border of the Cabbage Looper, *Trichoplusia* and selection for resistance to one of the crystal proteins *Applied Environmental Microbiology*, Vol. (60): 3840-3846.
- [66] Ravoahangimalala, O., Charles, J-F. & Schoeller-Raccaud, J. (1993). Immunological localization of *Bacillus thuringiensis* serovar *israelensis* toxins in midgut cells of intoxicated *Anopheles gambiae* larvae (Diptera: Culicidae). *Research of Microbiology*, Vol. (144): 271-278.
- [67] Boets, A., Jansens, S., Denolf, P., Peferoen, M. Degheele, D. & Van Rie, J. (1994). Sequential observations of toxin distribution and histopathological effects of CryIIIA in the gut of intoxicated *Leptinotarsa decemlineata* larvae. *XXVIIth Annual Meeting of the Society for Invertebrate Pathology*, pp. 377.
- [68] Van Rie, J., Jansen, S., Höfte, H., Degheele, D. & VanMellaert, H. (1990). Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis*  $\delta$ -endotoxins. *Applied Environ. Microbiology*, Vol. (56): 1378-1385.
- [69] Lee, M.K., You, T.H., Young, B.A., Cotrill, J.A., Valatis, A.P. & Dean, D.H. (1996). Aminopeptidase N purified from Gypsy moth brush border membrane vesicles is a specific receptor for *Bacillus thuringiensis* Cry1Ac toxin. *Applied Environmental Microbiology*, Vol. (62): 2845-2849.
- [70] Hua, G., Masson, L., Jurat-Fuentes, J.L., Schwab, G. & Adang, M.J. (2001). Binding Analyses of *Bacillus thuringiensis* Cry delta-endotoxins using brush border membrane vesicles of *Ostrinia nubilalis* *Applied Environmental Microbiology*, Vol. (67): 872-879.
- [71] Liang, Y., Patel, S.S. & Dean, D.H. (1995). Irreversible binding kinetics of *Bacillus thuringiensis* Cry1A delta-endotoxins to Gypsy moth brush border membrane vesicles is directly correlated to toxicity. *Journal of Biology Chemical*, Vol. (270): 24719-24724.
- [72] Fiuza, L.M. (2009). Mecanismo de ação de *Bacillus thuringiensis*. *Biotecnologia Ciência & Desenvolvimento*, Vol. (38): 32-35.