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HISTOPATHOLOGY OF *ANTICARSIA GEMMATALIS* HÜBNER (LEPIDOPTERA; NOCTUIDAE) TREATED WITH *NUCLEOPOLYHEDROVIRUS* AND *BACILLUS THURINGIENSIS* SEROVAR *KURSTAKI*

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ABSTRACT

The *Anticarsia gemmatalis* is responsible for the use of chemical insecticides in the soybean culture, causing a significant increase in the costs of farming and a great unbalance in the ecosystem. The use of microbial agents, like *Bacillus thuringiensis* serovar *kurstaki* (*Btk*) and *Anticarsia gemmatalis* nucleopolyhedrovirus (*Ag*NPV), they are an alternative to chemical control of the pest insects. In the interaction analysis of the entomopathogenic bacteria and virus it is considered important the *in vitro* action mode of these microbiology control agents. Therefore, the present study aims the histopathological analysis of the *A. gemmatalis* larvae digestive system after the interaction *in vivo* of the entomopathogenic *Btk* and *AgNPV*, represented the Dipel and *Baculovirus anticarsia* formulations, respectively. The evaluations were realized in larvae of 2nd instar, in which the mortality was evaluated daily, and a histopathology was done with collected larvae in time of 1, 3, 6, 12 and 24 hours after the treatments application. The results of the *in vivo* assays reveal that the treatment using the association of *Ag*NPV-*Btk* (98.68% of mortality) was more efficient than using *Ag*NPV isolatedly (81.28% of mortality), but the *Btk* when used isolatedly had a mortality of 100%. The treatments showed significant ($P<0.05$) differences between *Ag*NPV and *Btk*, *Ag*NPV and *Ag*NPV/*Btk*. The histopathological analysis of the *Ag*NPV and *Btk* in *A. gemmatalis* larvae suggests that the Dipel and *Baculovirus anticarsia* products were more efficient when they were used simultaneously, because the action of *Ag*NPV was intensified when used in association with *Btk*, causing changes in the larvae midgut after 6 hours of treatments. When the entomopathogens were used isolating the gut cells alterations were observed only 12 hours after the treatments.

Key words: virus, bacteria, biological control, Lepidoptera

INTRODUCTION

The velvetbean caterpillar *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) is an important insect pest of the soybean fields in Brazil. The larva cause serious damage by reducing the leaf area greatly and, consequently, reducing photosynthesis and productivity (18).

Among the alternatives to control this pest, the use of *Bacillus thuringiensis* has gained attention due to its efficiency and low impact on natural enemies (3). *B. thuringiensis* is a Gram-positive, aerobic or facultative anaerobic entomopathogenic bacterium found in soil, on plant surfaces, and in grain storage dust. During sporulation *B. thuringiensis* produce crystalline protein inclusions (i.e. crystal). When ingested by insects, this crystal is dissolved in the midgut, discharging proteins denominated delta-endotoxins or Cry proteins. Those proteins after hydrolysis have specific toxin activity for insects and other invertebrate, not causing harmful effects in other organism (25). In the midgut, the proteases activate these proteins that interact with the epithelial membrane, causing the insect death (6). In the field, as in the

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soybean farming's that agent of biological insects control is used in formulations as the Dipel product (Abbott Laboratories of *Brasil* Ltda).

In the microbial control context, the *Anticarsia gemmatalis nucleopolyhedrovirus* (*AgNPV*) stands out as the most important virus used in the insects biological control (5). In natural conditions, the curse is infected commonly when ingesting the contaminated food. After the ingestion, the bodies of polyhedral inclusion, finding alkaline conditions in the mesenterum are dissolved, liberating the virions. The virus begins multiplying itself in the nucleus cells of the insect, dispersing itself for the whole insect body, provoking death, usually from 6 to 8 days after the ingestion (12,24).

To understand the mode action of entomopathogenic bacteria and virus, it is important to knowledge of the insect histopatology. The insect digestive system's is one main physiochemical barriers against the pathogenic agents. In this way, the morphology and physiochemical aspects can support to understand of the insect defense mechanism's (16).

Like this, the present study aimed the histopathological analysis of the *A. gemmatalis* larvae digestive system, after the interaction *in vivo* of the entomopathogenic *Bacillus thuringiensis kurstaki* and *Anticarsia gemmatalis nucleopolyhedrovirus*, representing the formulations Dipel (Abbott) and *Baculovirus anticarsia* (Embrapa-CNPSo), respectively.

MATERIALS AND METHODS

Insects

The *Anticarsia gemmatalis* were obtained from soybean fields in the *Rio Grande do Sul* state and maintained in laboratory in the *Ciências da Saúde* of the *Universidade do Vale do Rio dos Sinos* (UNISINOS). The insects were maintained in the laboratory at 70% relative humidity, photoperiod of 12 hours and 25ºC. Larvae were reared with artificial diet prepared according to Greene *et al.* (14).

Bioassay

The bioassays were accomplished in the same laboratory and on controlled conditions (25ºC, 70% of Relative humidity and 12 hours of photoperiod), using larvae of 2nd instar of *A. gemmatalis*, conditioned in acrylic mini-plates (35mm of diameter), in which was previously applied Greene's diet (14). In assays were used in the treatments *Anticarsia gemmatalis* nucleopolyhedrovirus (*Ag*NPV) and *Bacillus thuringiensis* serovar *kurstaki* (*Btk*), strain HD1. The products were obtained from *A. gemmatalis* Baculovirus (EMBRAPA/CNPSo) and Dipel (Abbott Laboratories of Brazil Ltda.), respectively. Were applied in the artificial diet the treatments *Btk*; *Ag*NPV; *Btk* + *Ag*NPV and witness (100 µL of NaCl 0.85% solution). Considering the treatments, to reach the wanted concentration (3.10^7 particles/mL; for *Ag*NPV and $2.5.10^7$ cells/mL; for *Btk*) dilutions were made and the exact number of bacterial cells and viral polyhedrons were determined in Neubauer chamber. After the treatments application, 20 insects were individually conditioned, in which four repetitions were accomplished by treatment. The mortality was daily evaluated until the 7th day after the treatments application and the corrected mortality was calculated according to Abbott (1). Data were subjected either to one-way analysis of variance (ANOVA), TUKEY 5% and factorial analysis of variance (FANOVA) depending upon the experimental design.

Insects' tissues

The *A. gemmatalis* larva were prepared according to the inclusion paraffin techniques. During the bioassay, 20 larvae were used for each treatment and 20 to witness, in which they were collected in periods of 1, 3, 6, 12 and 24 hours, after the treatments application. After the fixation, in Bouin Hollande Sublime (BHS) for 24 hours (7), the tissues were submitted to dehydration in ethanol solutions in growing order of graduation (70, 96 and 100%), following by fast baths of xylol and impregnation in paraffin. The longitudinal histological cuts were accomplished to 5µm thickness, in the histology laboratory (UNISINOS). To remove the paraffin, the slide containing the tissue passed by xylol and ethanol baths in decreasing order of graduation. The staining, of the tissues of *A. gemmatalis* was made with Heidenhain's Blue. The glasses were mounted with Etellan. The longitudinal sections of the gut tissues of the *A. gemmatalis* larvae were observed under direct optical microscopy were amplified 400 times.

RESULTS AND DISCUSSION

Pathogenicity *in vivo*

The evaluations of the pathogenicity were considering the *Anticarsia gemmatalis* corrected mortality medium, in which the treatment with *Anticarsia gemmatalis* nucleopolyhedrovirus (*Ag*NPV) was verified a mortality of 81.28%, in the association *Btk*/*Ag*NPV, the mortality was 98.68% and only *Bacillus thuringiensis* serovar *kurstaki* (*Btk*) was equivalent to 100%. The results, *in vivo*, reveal that regarding association treatment *Ag*NPV/*Btk* was more efficient than only *Ag*NPV, however only *Btk* caused a similar mortality. The *in vivo* toxicity obtained in this study using $2.5.10^7$ cells/mL of the *Btk* HD1 against the target species was similar to those obtained by Bobrowski *et al*. (8 and 9) using *Btk* HD73 and *Btk* UNI872 strains.

Considering the lethal time, through the daily analysis of the bioassay, it was observed that the treatment with *Btk* on the 3rd day after the treatment (DAT) caused 96.05% of mortality, keeping like this until 7th DAT. As for the treatment with *Ag*NPV, in 3rd DAT there was a mortality of 25% that raised itself to

61.05% in 7th DAT. In the association *Btk*/*Ag*NPV, the mortality was of 45% in 2nd DAT, with a peak of 85% in 3rd DAT, causing 90.26% of mortality in 7th DAT (Fig. 1). The treatments showed significant ($P<0.05$) differences in *Ag*NPV and *Btk*, *Ag*NPV and *Ag*NPV/*Btk*.

These results can indicate that the natural resistance factor is very important at the moment of applying the biopesticide against *A. gemmatalis* larvae. Showing that the control's integrate method, using *Btk* HD1 (Dipel) and *Ag*NPV (Baculovirus), is a good alternative to reduce the problems concerning the resistance to the population of target pest. Janmaat *et al.* (15) mentioned that the continued use of the *Bt* microbial pesticide was directly correlated with the concentration's increase of *Bt* applied by treatment. According to Abot *et al.* (2), *A. gemmatalis* has a high potential of developing resistance to the *Anticarsia gemmatalis Ag*NPV in Brazil, where the virus occurs naturally and is extensively employed as a microbial pesticide in the soybean fields. In the United States, the potential for resistance interfering with a microbial insecticide based on *AgNPV* is lower. Resistant insects also had longer life spans, a lower rate of larval survival in rearing and lower pupa weights than susceptible insects (13).

Histopathology *in vitro*

The observations in optical microscopy analysis, of the midgut of *A. gemmatalis* larvae, when compared with the witness (Fig. 2A), show changes in the structure of the midgut, after 24 hours of the treatment with *Btk* (Fig. 2C), where was observed the desruption of microvilli and a part of the cells was already disorganized and lysed. *Bacillus thuringiensis* toxins thus appear to bind specifically on the apical membrane of the *Lymantria dispar* (Lepidoptera, Lymantriidae) larvae epithelial cells (21). According to this study, Raussel *et al*. (22) were observed the histopathology changes in the midgut of the 3rd instar of *Lymantria monacha* larvae (Lepidoptera, Lymantriidae) when treated with *B. thuringiensis* toxins, causing vacuolization of the cytoplasm and increase of the cellular volume. Similar effects were observed by Bobrowski *et al.* (10): disruption of microvilli and vacuolization of

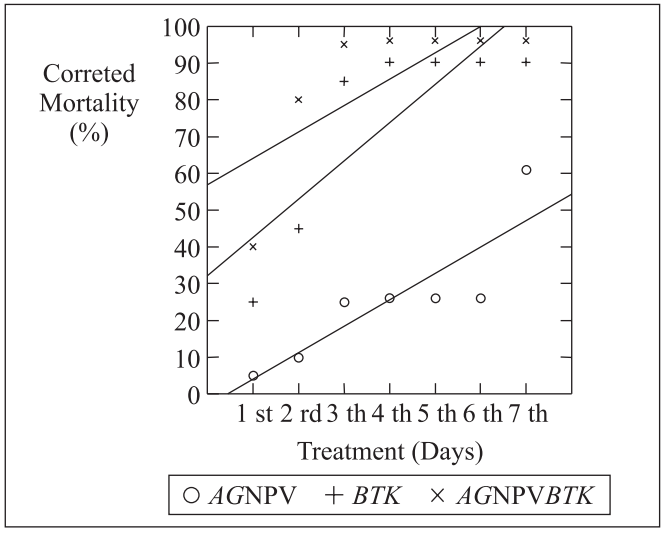

Figure 1. Lethal time of *Anticarsia gemmatalis* evaluated daily, after the application of the *nucleopolyhedrovirus* (*Ag*NPV) and *Bacillus thuringiensis* serovar *kurstaki* (*Btk*) treatments.

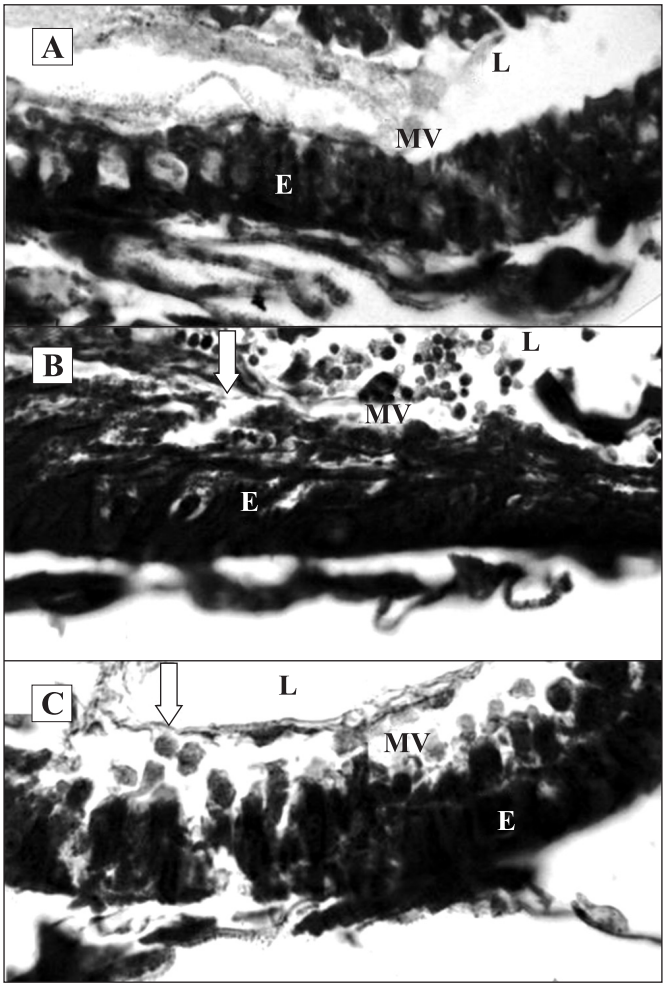

Figure 2. Longitudinal sections of the midgut Anticarsia gemmatalis larvae treated with: (A) Control, not treated; (B) 24 hours after the treatment with Baculovirus anticarsia and Bacillus thuringiensis serovar kurstaki; (C) 24 hours after treatment with Bacillus thuringiensis kurstaki; (E) Epithelium; (L) Lumen; (MV) Microvilli; (⇩) Effects caused by the treatments. The slides were amplified 400x.

cytoplasm began 6 hours after *B. thuringiensis* ingestion by *A. gemmatalis* larvae. Despite the lower toxicity of Cry1C, synthesized by *B. thuringiensis* serovar *kurstaki* HD1 strain used in the Dipel biopesticide, which lacks Cry1C, it increased toxicity to *Spodoptera exigua* (Lepidoptera, Noctuidae). These indicate the half of Cry1C was synthesized effectively in this strain, a finding that may increase these strains commercial utility (19). According to Aranda *et al.* (4), 15 minutes after the application of Cry 1C and Cry 1D in *S. frugiperda*, it was already possible to observe changes in their cells, however the vacuolization of the cytoplasm, the degradation of the membrane and the destruction of the cells are observed 1 hour after the treatments.

An increase of the cellular volume, vacuolization of the cytoplasm, ejection of a great number of cells in the midgut and disruption on the microvilli were observed in the application of *Ag*NPV (Fig. 2B), after 24 hours of the treatment. Similar results were described by several researches. According to Matos *et al.* (17) although the virus were not found in the nuclei of columnar cells until late on infection, it is believed that these cells are the primary sites of infection and replication. This fact can be explained by the continuous regeneration of the midgut epithelium. Besides, the infection may be occurring in isolated cells. Pombo *et al.* (20) observed that 12 hours post infection of *A. gemmatalis* with *AgMNPV*, the cells became round and exhibited a decrease in the number of cytoplasmic projections. The authors described that by 24 hours it was possible to detect a virogenic stroma inside the cell nucleus and after 48 hours, polyhedral inclusion bodies were observed. According to this present study, the Castro *et al*. (11) evaluated infection of the permissive *A. gemmatalis* cell line with *Ag*NPV showed pronounced cytopathic effect by 48 hours. These cells were rounded and developed nuclear hypertrophy. Same data was observed by Sciocco-Cap *et al.* (23), showing in the histological analysis of the 4th instar of *Epinotia aporema* larva (Lepidoptera, Tortricidae) infected with *Baculovirus* that were presented the same phases of cellular disruption.

In the *Btk*/*Ag*NPV association, it was observed that after 6 hours of the application, it had begin disintegrate of the peritrophic membrane and brush border membrane. The cellular lyse was greater and the lumen presented amount of cells after 12 hours of the treatments and this one was intensified after 24 hours. Those observations demonstrated that, in the individual treatments with *Btk* and *Ag*NPV, until 6 hours after application of the treatments, there were no alterations in the intestinal cells of the *A. gemmatalis* larva. However, *Btk* and *Ag*NPV in association, after 6 hours already presented intense vacuolization of the cytoplasm, causing cellular disorganization. After 12 and 24 hours all treatments revealed the alterations (i.e. increase of the nucleus) in the midgut cellular structure of the velvetbean caterpillar.

The *in vitro* interaction between *Ag*NPV and *Btk* showed a difference significant (P <0.05) when compared whit the treatments isolated. The pathogenicity analysis of *AgNPV* and *Btk* in *A. gemmatalis* larvae suggests that the Dipel and *Baculovirus anticarsia* products were more efficient when used simultaneously, because the action of *Ag*VPN was intensified when used in association with *Btk*, causing changes in the larvae midgut after 6 hours of treatments. Only 12 hours after the treatments, and when the entomopathogens were used isolating, were observed the gut cells alterations.

RESUMO

Histopatologia de *Anticarsia gemmatalis* Hübner (Lepidoptera; Noctuidae) tratadas com *Virus de Poliedrose Nuclear* e *Bacillus thuringiensis* sorovar *kurstaki*

A *Anticarsia gemmatalis* é responsável pelo uso de inseticidas químicos na cultura da soja, ocasionando um significativo aumento nos custos das lavouras e um grande desequilíbrio no ecossistema. O uso de agentes microbianos, como *Bacillus thuringiensis* sorovar *kurstaki* (*Btk*) e *Vírus de Poliedrose Nuclear* de *Anticarsia gemmatalis* (*VPNAg*), é uma alternativa para o controle químico de insetos-praga. Na análise da interação de bactérias e vírus entomopatogênicos, considera-se importante o modo de ação *in vitro* desses agentes de controle microbiano. Assim, o presente trabalho objetiva a análise histopatológica do sistema digestivo das lagartas de *A. gemmatalis*, após a interação dos entomopatógenos *Btk* e *VPNAg*, representados nas formulações Dipel e *Baculovirus anticarsia*, respectivamente. As avaliações foram realizadas com lagartas de 2º ínstar, onde a mortalidade foi avaliada diariamente, e a histopatologia foi realizada com lagartas coletadas nos períodos de 1, 3, 6, 12 e 24 horas após a aplicação dos tratamentos. Os resultados dos ensaios *in vivo*, revelam que o tratamento referente à associação VPN*Ag/Btk* (98.68% de mortalidade*)* foi mais eficiente que VPN*Ag* (81.28% de mortalidade*)* isoladamente, porém o *Btk* isoladamente causou 100% de mortalidade. Os tratamentos mostraram diferenças significativas (P <0,05) entre *Ag*NPV e *Btk*, *Ag*NPV e *Ag*NPV/*Btk*. As análises de patogenicidade do *VPNAg* e *Btk* em lagartas de *A. gemmatalis* sugerem que os produtos Dipel e *Baculovirus anticarsia* foram mais eficientes, quando utilizados simultaneamente, pois a ação do VPN*Ag* foi intensificada quando utilizada em associação com *Btk*, provocando alterações no intestino médio das lagartas a partir de 6 horas após os tratamentos. Quando os entomopatógenos foram utilizados isoladamente, as alterações das células intestinais foram observadas apenas 12 horas após a aplicação dos tratamentos.

Palavras-chave: vírus, bactéria, controle biológico, Lepidoptera

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