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Protein composition of *Erinnyis ello l.* (Lepidoptera:Sphingidae) haemolymph pre- and post-infection with *Bacillus thuringiensis*

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Abstract: A study of the effect of *Bacillus thuringiensis* (applied as Dipel) on the protein composition of haemolymph of 4th instar *Erinnyis ello* was conducted. The larvae of *E. ello* were treated with *B. thuringiensis* and haemolymph extracted daily over a 3-day period. Polyacrylamide gel electrophoresis (PAGE-SDS) was conducted on the haemolymph to determine changes in the protein concentrations before and after application of *B. thuringiensis*. The total haemolymph protein concentration as determined by Lowry's method on Days 1, 2 and 3 differed significantly from each other being highest on Day 3 (81.33 ± 1.25 µg/µl) and lowest on Day 2 (45.92 ± 1.25 µg/µl) post infection. There were a total of 13 protein bands present in the haemolymph of the untreated (control) larva, 15 in the haemolymph of the Day 1 treated larva, 15 in Day 2 treated larva and 10 in Day 3 treated larva. The viscosity and colour of the haemolymph on different days also varied.

Keywords: *Erinnyis ello,* haemolymph proteins, electrophoresis, *Bacillus* thuringiensis.

Introduction

The cassava hornworm, *Erinnyis ello* L. (Lepidoptera:Sphingidae) is a voracious pest of cassava (*Manihot esculenta* Crantz.) causing damage to foliage, tender stems and young buds which are consumed rapidly. It has been estimated that a single larva of this pest can consume 1100cm^2 of cassava leaves before it develops into an adult (Strobel, 1993). Apart from cassava, *E. ello* also feeds on 35 other plant species from 10 families, including several of economic importance (CAB International, 2007; Farias and Matlos, 2003). It is the only serious lepidopteran pest throughout the cassava-growing regions of the Neotropics (Farias and Matlos, 2003) which can cause complete plant defoliation, losses in bulk root production and reduced tuber quality. Farmers often react to severe attacks by excessive, wasteful applications of insecticides which may result in pest resurgence, development of resistance and ultimately, increased cost of production (Farias and Matlos, 2003). Globally, food security is increasingly becoming a more important issue as less food is available for an increasing population, most of which is in developing countries. More emphasis is therefore being placed on alternative sources of food, such as utilizing root crops more effectively since they are a major source of carbohydrates which can be substituted for rice and wheat flour. Both cassava and sweet potato (*Ipomoea batatas* (L.) Lam.) have high quantities of starch as well as other essential nutrients and are vital food sources especially in Africa, Asia, the Caribbean and South/Central America where both are frequently utilized by more than 500 million individuals (Strobel, 1993). Any decrease in the cost of cassava production including reduction of expensive, environmentally deleterious synthetic insecticides with less harmful alternatives will be a boon for farmers and *Bacillus thuringiensis* (Bt) is one such insecticide. Information on the mode of action of *B. thuringiensis* on lepidopterous larvae is readily available (Schünemann *et al*., 2014; Bravo *et al.,* 2007), however, there is no published information on the effect of *B. thuringiensis* on *E. ello* haemolymph protein composition. The objective of this study therefore was to evaluate the effect of pre- and post-application of *B. thuringiensis* (as Dipel® 2X) on the haemolymph protein composition of *E. ello* using SDS-PAGE.

Materials and Methods

Experimental site and larval collection: *Erinnyis ello* larvae were collected from a 1.6ha field of highly infested cassava located in Felicity, Chaguanas, Trinidad. Larvae of the same instar were collected to reduce any sources of error. The trial was conducted during the wet season and at the end of a drought period. Larvae were found mostly lying on the soil where they were gently picked up and placed in a transparent plastic container (8cm x 14cm x 20cm) with a mesh covered lid. This allowed for easy observations during the experiment. A maximum of four 4th instar larvae were placed in each container to prevent overcrowding. Fresh cassava leaves were placed in the container as a food source for the larvae *ad libitum*.

Larval bioassay: Four plastic containers were prepared and four 4th instar *E. ello* larvae were placed in each container together with fresh cassava leaves. *Bacillus thuringiensis* (as commercial insecticide Dipel® 2X) at the recommended rate of 0.2ml/100ml water was applied to clean cassava leaves by dipping into a suspension of Dipel® 2X, air dried and fed to larvae in the containers. Larvae in the control container were fed with untreated cassava leaves.

One-day post application of *B. thuringiensis,* haemolymph was extracted from one larva from each of the four containers. Larvae were held firmly using fingers, massaged gently and then pierced on the dorsal side in the region of the 3rd abdominal segment using a thin, sharp sterilized No. 3 insect mounting pin. The exuding

haemolymph was collected in Corning Pyrex® 20øl disposable micropipettes (Corning Science Products, USA) and then placed in 200øl Eppendorf tubes (Plate 1). This technique was repeated on Days 2 and 3. Pooled larval haemolymph samples from each day were kept in separate tubes. A small amount of the haemolymph from each sample was mixed separately with a sample buffer comprising 40% sucrose and bromophenol blue. A constant volume of 5øl of sample and 5øl of each marker was placed on a polyacrylamide gel on a glass plate and left to run for 45 minutes. Four markers were used in the experiment: - Lactalbumin (14.2kDa), Carbonic Anhydrase (29kDa), Albumin (45kDa) and Urease (545kDa). The gel was then removed from the glass plates, rinsed in distilled water then immersed in a protein stain comprising 0.2% Coomassie blue in 40% methanol and 10% acetic acid and left for 1h. At the end of the staining period the gel was de-stained in distilled water.

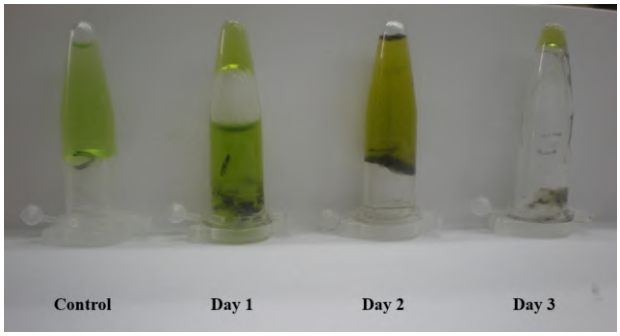

Plate 1:Haemolymph from 4th instar *Erinnyis ello* larvae infected with *Bacillus thuringiensis*

The total protein content in the haemolymph samples was determined using the Lowry method (Lowry *et al.,* 1951). Retention factor (R_f) values were calculated from the gel using the following formula:

These values were compared to those obtained using the markers and the molecular weights of the unknown proteins determined by plotting a graph of $\log_{10}$ molecular weight against R_f for standards and extrapolating the size of the unknown proteins. The results were subsequently tabulated and recorded.

Results and Discussion

There was a significantly lower ($F_{3,4} = 562.81$, $p<0.0001$) level of haemolymph proteins in the control (26.30 µ 0.63 µg/µl) compared to that in larvae post infection by *B. thuringiensis* (Table 1). The total haemolymph protein concentration on Days 1, 2 and 3 also differed significantly ($F_{2,3} = 319.04$, $p = 0.0003$) from each other with the highest haemolymph protein concentration occurring on Day 3 (81.33 µ 1.25 µg/µl) and lowest on Day 2 (45.92 ± 1.25 µg/µl) post infection (Table 1).

Table-1: Total protein content of haemolymph of *E. ello* 4th instar larvae 1, 2 and 3-days post *Bacillus thuringiensis* infection using Lowry's method

Days after treatment	Total larval protein in haemolymph (µ g/µ l) ± SE*
Day 1	53.00 µ 0.42[a]
Day 2	45.92 µ 1.25[b]
Day 3	81.33 µ 1.25[c]
Control	26.30 µ 0.63[d]

* Values followed by the same letter are not significantly different from each other using the Tukey- Kramer multiple comparisons test ($p< 0.05$).

Overall, the total haemolymph protein content of *B. thuringiensis* infected larvae was elevated 2-3 fold compared to that of uninfected (control) larvae (Table 1). These results were similar to that found by Tripathi and Singh (2002) who noted an increase in the total protein concentrations of *Spodoptera litura* F. (Lepidoptera:Noctuidae) larvae treated with *B. thuringiensis* var. *kurstaki* as well as Narayan and Jayaraj (1974) working on the citrus leaf caterpillar *Papilio demoleus* L. (Lepidoptera:Papilionidae). These authors concluded that this was a result of 'stimulated synthesis of protein producing factors in the insect as the protein requirement is increased for the germination of *B. thuringiensis* spores'. This data was also consistent with the findings of Sharma *et al.* (2008) who investigated the haemolymph protein concentration and midgut nitrate levels in mosquitoes and found that they were significantly higher than the control group as a result of large quantities being produced during the host defense and immunological reaction. Both Adamo (2004) and Seufi (2011) concluded that higher protein concentrations in bacteria infected haemolymph of Lepidoptera is in part due to production of bacteria induced proteins which are used for self-defense and development of immunity. Bacterial infection of the larvae of the silkworm, *Antheraea mylitta* Drury (Lepidoptera:Saturnidae) also resulted in faster synthesis of proteins and their release into the haemolymph (Singh *et al*., 2011).

The R_f values were calculated from the gel presented in Plate 2 using a graph of $\log_{10}$ molecular weight of the standards (Lact-albumin, carbonic anhydrase, albumin and urease) against their corresponding R_f values. The value of the molecular weights of the unknowns in the sample was subsequently determined from this graph. There was a total of 13 protein bands present in the haemolymph of the untreated (control) larvae, nine of which had molecular weights greater than that of albumin (45.4 kDa). There were a total of 15, 15 and 10 protein bands present in the haemolymph of the Days 1, 2 and 3 treated larvae respectively. The protein band with molecular weight 52.9kDa was in higher concentration in Days 1 and 2 treated larvae than in either the control or Day 3 treated larva (Plate 2). The protein band at 46.6kDa was in highest concentration in Day 3 treated larvae than in any

other including the control. The lowest molecular weight protein band was 10.9kDa and was present in higher concentrations in Days 1 and 3 treated larvae than in the control or Day 1 treated larva (Plate 2).

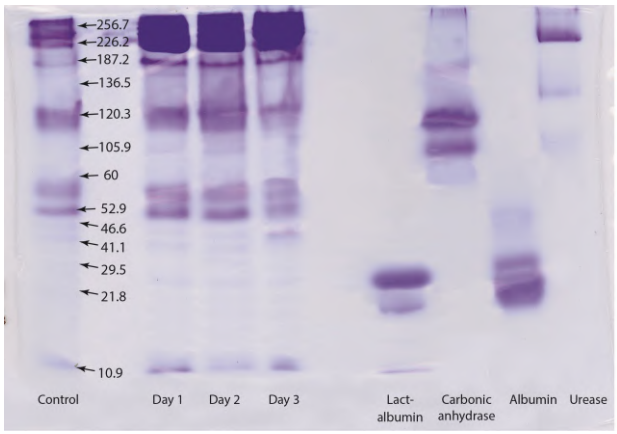

Plate 2: Electrophoresis gel of proteins present in haemolymph of *E. ello* before and after inoculation with *B. thuringiensis* (values are given in kDa, markers used were Lactalbumin, Carbonic anhydrase, Albumin and Urease)

The reduction in bands from 13 in the control to 10 on Day 3 treated larvae may be due to degradation of the proteins after a period of three days after the insecticide was present inside the larvae. These results are similar to that of El-Shershaby *et al* (2008) who also investigated the impact of *B. thuringieneisis* on protein content and enzyme activity of *Spodoptera littoralis* and also that of El-Moursy and Abdel-Rahman (1999) who examined the chemistry of the haemolymph of the corn borers *Chilo agamemnon* (Crambidae) and *Ostrinia nubilalis* (Pyraustidae) after treatment with *B. thuringiensis.* Results from their studies showed both positive and negative protein content in the treated larvae and concluded that *B. thuringieneisis* gradually suppresses protein synthesis after treatment and its greatest effect was after 5 days of treatment. *B. thuringieneisis* (Bt) causes substantial physiological changes in the vital systems during the course of infection. A summary of all the protein bands found in Days 1, 2 and 3 *B. thuringiensis* treated larvae is provided in Table 2.

Bacillus thuringiensis affected the total protein content in the larvae and interfered with the activity of enzymes, which played a dominant role in the insect's metabolism (Salama et al, 1983). Dipel® 2X caused highest mortality 3-days post ingestion by *E. ello* larvae. Additionally, after three days the majority of treated larvae were dead which was expected as *B. thuringieneisis* produces spores when appropriate nutrients are present and crystalline inclusion bodies when required nutrients are absent. This crystal contains insecticidal proteins (endotoxins) which causes paralysis (Knowles, 1994).

The collected haemolymph from *E. ello* before and after infection with *B. thuringiensis* showed certain characteristics which seem to explain the above conclusions. The control sample was light green in colour and not very viscous. Terra *et al.* (1982) note that this

Table-2: Summary of molecular weights of proteins (kDa) found in untreated (control) and 1, 2 and 3-day *B. thuringiensis* treated *E. ello* 4th instar larvae.

Control	Day 1	Day 2	Day 3
256.7	249.6	246.5	227.7
226.2	241.4	226.8	201.0
187.2	236.5	191.9	191.9
136.5	184.1	162.5	169.4
120.3	169.4	116.4	111.6
105.9	149.5	98.5	86.9
60.0	98.5	90.6	46.5
52.9	90.6	73.6	37.7
46.6	70.5	44.6	30.6
41.1	46.5	38.5	8.7
29.5	37.8	30.6	
21.8	33.3	27.4	
10.9	28.2	25.0	
	18.6	20.2	
	8.4	8.9	

greenish-yellow colouration of the haemolymph of *E. ello* is due to isozeaxanthin which is bound to a protein. The Day 1 treated sample changed colour from light green (as in the control) to dark green, with deposits of black residues in the sample, possibly because of protein degradation by Dipel® 2X and also a result of general metabolic breakdown of gut cells, cells slough from basement cells, and cells lysis (Knowles, 1994). The haemolymph of Day 2 treated larvae changed from dark green (as on Day 1) to greenish · yellow and there were also black deposits in the haemolymph. The viscosity of the haemolymph also increased as compared to larvae in the control and Day 1 treatment and could be as a result of increase blood pH and potassium levels in the system after the ingestion of B. thuringiensis endotoxins, which disrupts the balance in the insect system (Terra et al., 1982; Knowles, 1994). The amount of sample obtained from Day 3 treated larvae was greatly reduced as a result of the high viscosity of the haemolymph making it difficult to extract haemolymph from Day 3 treated larvae and may be due to the coagulation of haemolymph proteins preventing its free flow (Singh et al., 2011). El-Shershaby et al. (2008) showed that the toxins from B. thuringiensis are responsible for the inhibition of protein synthesis by forming a protein complex resulting in change in colour and increase in viscosity of the haemolymph of infected Lepidoptera larvae. These, together with reductions in pH in the larval systems provide nutrients for spore proliferation (Knowles, 1994).

Conclusions

The total haemolymph protein concentration of *E. ello* 4th instar larvae infected with *B. thuringiensis* was substantially higher (as much as 2-3 fold) than that of the control. Additionally, the highest haemolymph protein concentration occurred on Day 3 and lowest on Day 2 post infection. The total number of protein bands in infected larvae also varied from the control with the most bands (15) occurring on Days 1 and 2 compared with the control (13 bands) and Day 3 (10 bands). Both the haemolymph protein concentration and number of bands was explained

in terms of stimulated synthesis of proteins and their faster release into the haemolymph as a result of bacterial infection.

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