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Reviewer: Zig Vaituzis Ph.D. Date: 3/16/99
Microbial Pesticides Branch
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DATA EVALUATION REPORT

STUDY TYPE: Determination of potential effects of *Bacillus thuringiensis* Cry1Ab toxin on developmental time and mortality of *Chrysoperla carnea* (green lacewing) larvae.

CITATION: Hilbeck*, A., Moar, W.J., Pusztai-Carey, M., Fillippi, A. And F. Bigler (1998). Toxicity of *Bacillus thuringiensis* CryIAb toxin to the predetor *Chrysoperla carnea* (Neuroptera. Chrysopidae), Environ. Entomol., in press.

Published as: Hilbeck, A., Moar, W.J., Pusztai-Carey, M., Fillippi, A. And F. Bigler.(Oct.)1998. Toxicity of *Bacillus thuringiensis* CryIAb Toxin to the Predetor *Chrysoperla carnea* (Neuroptera: Chrysopidae), Environ. Entomol.,27:1255-1263

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DP BARCODE: D250457 **CASE**: 062714

REG./FILE#: 65268-1 **CHEMICAL/BIOL#**: 006444 Attribute Insect Protected Sweet Corn

COMPANY/SPONSOR: A research report submitted by:
Novartis Seeds, Inc.
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Novartis Seeds, Inc. submitted to BPPD a copy of the above mentioned research report preprint to be reviewed for any potential impact on Event 176-derived transgenic B.t. corn ecological effects determination.

TEST MATERIAL: Cry1Ab protoxin from *B.thuringiensis kurstaki* HD-1 expressed as a

single gene product in *Escherichia coli*.

REVIEW CONCLUSION: The data have no impact on the Agency's risk assessment of Event 76-derived Bt corn to beneficial predatory insects.

ADEQUACY OF STUDY: Not a valid study

RECOMMENDATIONS: In order to validate claims of detrimental effects on *C.carnea* larvae the author should study the impact in a field test (in the presence of environmental influences).

STUDY OBJECTIVES: The specific objectives were to investigate the effects of Cry1Ab delta endotoxin on the mortality and development of *C.carnea* larvae and to determine the suitability of feeding tests using a paraffin encapsulated, artificial diet containing *B.thuringiensis* proteins. This research is a continuation of previous investigations [Angelika Hilbeck. September 1996. INVESTIGATIONS ON SIDE-EFFECTS OF TRANSGENIC BT-CORN ON BENEFICIAL INSECTS. Report for the Swiss National Science Foundation. (SSP Biotechnologie, Gesuch Nr. 5002-042598)] done (1) to determine the effects of consumption of prey fed on Bt corn on the survival and development of the green lacewing, and (2) to determine whether the observed effects were due to exposure to Bt toxin in the prey, or if the observed effects were due to the indirect influence of consumption of sick prey fed a suboptimal diet.

To further refine the cause of observed effects in the first study, the current study was performed using pure Cry1Ab delta endotoxin in a paraffin encapsulated form, rather than infected, moribund insects.

MATERIALS & METHODS:

Insects: The predatory green lacewing larvae (*Chrysoperla carnea*) were from the permanent laboratory colony maintained on pea aphids [*Acyrthosiphon. pisum* (Harris)] and *Ephestia kuehniella* eggs, without introduction of field-collected insects. Nonpredaceous adults were kept on a mixture of yeast, honey and water.

Neonate larvae of *Ostrinia nubilalis* (Hubner) were also obtained from a permanent laboratory colony that had been maintained on a meridic diet for several generations under conditions described above for *C. carnea.*

Rearing conditions: Temperature 22-25° C, relative humidity 70%, 16:8 light/darkness hr.

***B.thuringiensis* Proteins and Artificial Diet**: The Cry1Ab protoxin from *B.thuringiensis kurstaki* HD-1 was expressed as a single gene product in *Escherichia coli* (Masson et al. 1990). The inclusion bodies containing the Cry1Ab protoxin were dissolved, trypsinized and lyophilized. The artificial diet consisted of paraffin spheres approximately 1mm in diameter containing a liquid diet specifically developed for optimal nutrition of *C. carnea* larvae

(Schwenk and Tygges, STB control, Aarbergen, Germany). The Cry1Ab toxin was solubilized in double distilled water and added to the liquid diet before encapsulation to make a final concentration of 100 μg/ml of diet. The biological activities were assayed with *Ostrinia nubilalis* in 5 replicates.

Feeding experiments: *C. carnea* larvae can use the encapsulated diet only in the 2nd instar when their mouthparts are sufficiently strong to penetrate the paraffin spheres. Therefore two different methods were used to rear the chrysopid larvae through the 1st instar. For method No.1, each 1st instar was supplied with a 0.5 mm polystyrene foam cube soaked in liquid diet with of without toxin added. For method No.2 Cry1Ab toxin was added at a concentration of 100 μg /ml of diet. Diet soaked foam cubes were replaced daily until the larvae reached instar 2. At instar 2, in treatments 3,4 and 5, the artificial diet was supplemented with, or consisted of only *Ephestia kuehniella* eggs.

Diet summary

Treatment	Bt exposure	Diet
1	- Bt	Artificial diet (AD) only (method 1)
2	+Bt	Artificial diet (AD) only (method 1)
3	-Bt	*Ephestia kuehniella* eggs/artificial diet (method 2)
4	+Bt	*Ephestia kuehniella* eggs/artificial diet (method 2)
5	- Bt	*Ephestia kuehniella* eggs only

The larvae were kept individually in open top plastic cups. Thirty one day old larvae were used per treatment. The experiment was replicated five times, resulting in 150 lacewing larvae per replicate (750 total larvae for treatments).

Sampling: Stage specific mortality and development time were monitored daily. The sampling took place up to adult eclosion - up to 37 days in some experiments.

Test Conditions: The experiments were carried out in an environmental chamber at fluctuating temperatures (25° C for 10 h during photophase and 20° C for the remaining 14 hours, averaging 22° C/day, at a relative humidity of 70%, and a photoperiod of 16:8 light/darkness hr.)

Observations: Stage specific development times were determined in number of days required to complete each instar. In addition, mortality and development time from 1st instar to adult was determined.

Bioassays of Cry1Ab with *O. nubilalis* Larvae: Three grams of the crushed test diet containing Cry1Ab toxin, or toxin free diet, was mixed with a 3 g standard meridic diet normally used for rearing of *O. nubilatis* larvae. Freeze-dried Cry1Ab toxin in distilled water was mixed at 100 μg/g of meridic diet as a reference standard. Distilled water was used as a negative control. Bioassays were replicated 5 times, resulting in using a total of 800 *O. nubilatis* larvae per assay. Dead larvae were scored in 4 or 5 days.

STATISTICAL METHODS:

Stage specific mortality: A logistic regression was carried out calculating the proportion of individuals that died during each instar and accounting for the binomial probability distribution of mortality data. Analyses were performed using the GENMOD procedure of the SAS statistical package, including a DSCALE and Type 1 and 3 statement producing the appropriate *F*-statistics. In addition, mean mortality and standard errors were determined and means were compared by the MEANS procedure (SAS Institute 1996).

Development times: A regular analysis of variance (ANOVA) was carried out testing for significant replication and treatment effects. *B. thuringiensis* and type of diet, and their interaction effect analyses were preformed using the general linear model (GLM) procedure of SAS, including LSMEANS statement.

REPORTED RESULTS:

Cry1Ab effects on mortality: Except during the first instar, overall mean mortality of *C. carnea* was slightly higher in Cry1Ab containing diet. The highest mortality occurred during the 2nd instar and the pupal stage. **Only the larvae receiving the toxin in an *artificial* diet after the 2nd instar showed a statistically significant total larval mortality**. Mortality of 43% and 57% was recorded for *C. carnea* continuously reared on a Cry1Ab containing *artificial* diet until pupation and adult eclosion respectively. (The toxin-free *artificial* diet showed a 21% pupation and 30% adult eclosion mortality). No significant mortality occurred during the 1st instar stage.

Larvae exposed to Cry1Ab in the *artificial diet (+eggs)* at the 2nd instar showed a mortality of 23% before pupation and 29% at adult eclosion with a (ca) 14% and 17% negative control mortality.

Cry1Ab effects on development: There were no significant differences in development time during the 1st instar stage in the presence of toxin. Significantly prolonged development time was observed during the 2nd and 3rd instar and the pupal stage in the presence of Cry1Ab toxin in the *artificial* diet. However, **for total development time until pupation and adult eclosion these differences were statistically insignificant.**

Diet effects on mortality: Except during the first instar, ***there was always a diet effect on mortality.*** Control *C. carnea* reared continuously on the *artificial* diet showed a significantly

increased mortality (30%). The lowest total mortality (8%) was observed in *C.carnea* raised exclusively on *Ephestia kuehniella* eggs. While the total immature mortality was intermediate (17%) in *C.carnea* provided with a combination of *Ephestia kuehniella* eggs and untreated artificial diet.

Diet effects on development: Significant **differences in development time for most life stages were also seen between control larvae raised on different types of diet**. Larvae receiving untreated artificial diet after reaching the 2nd instar took 16 and 27.5 days until pupation and adult eclosion, whereas larvae raised on the *artificial* diet since the 1st instar took 28 and 37.5 days. Larvae raised exclusively on *Ephestia kuehniella* eggs required 12 and 23 days respectively for development.

Bioassays with *O. nubilalis:* The mortality of 1st instar larvae was 100% and 99% when crushed Cry1Ab toxin-paraffin spheres or purified, nonencapsulated Cry1Ab toxin solution was mixed into the meridic diet, respectively. The respective controls showed 24% and 26.5% mortality.

STUDY AUTHOR'S CONCLUSIONS:

Continuous exposure to Cry1Ab toxin resulted in significantly higher mortality of immature *C. carnea*. **Only the larvae receiving the toxin in an *artificial* diet after the 2nd instar showed a statistically significant total larval mortality**. This demonstrates a sensitivity of this species to Cry1Ab toxin at a concentration of 100 μg /ml of diet. Despite this higher mortality, only very small to no effects due to Cry1Ab toxin could be observed for the total developmental time to pupation or adult eclosion. **A slightly prolonged development time was observed only for *C.carnea* raised on Cry1Ab toxin containing *artificial* diet since the 1st instar**, but not on those exposed to Cry1Ab toxin later during larval development. Surviving, unaffected *C.carnea* developed at rates similar to those in the untreated control. From this **we concluded that total development time until pupation or adult eclosion is not an appropriate parameter for detecting Cry1Ab toxin effects.**

The prolonged control mortality of 30% during a 37 day trial (fed on an *artificial* diet) is acceptably low. But **it is acknowledged that a population suffering 30% mortality may be stressed, which may result in potentially enhanced responses**. (Total mortality of 8% was observed in *C.carnea* raised exclusively on *Ephestia kuehniella* eggs).

Further investigations are required to determine the reasons for greatest mortality during the 2nd instar stage. Also the possibility that the processing of Cry1Ab toxin within the herbivore host (target insect) makes it more toxic to *C.carnea* needs further study.

Previous reports showing no detrimental effects on *C.carnea* may have been flawed in that the diet presented to the larvae was inappropriate, such as insect eggs coated with the *B. thuringiensis* toxin. Due to the sucking feeding behavior of chrysopid larvae, it is likely that only little or no *B. thuringiensis* toxin actually was ingested in these trials. In addition, tests of short

duration (3-9 days) on only a portion of the larval period may have resulted in negative findings. Likewise presentation of normal diet after a 3-9 day exposure to the toxin may have given the larvae an opportunity to recover from potentially toxic long-term effects. Long term bioassays with natural enemies are more realistic indicators of possible population level effects in a system with transgenic plants.

"But obviously, **trials investigating** predation efficiency and predator **performance under field conditions are necessary before conclusions regarding the potential ecological relevance of the results presented in our paper can be drawn**." and "...**transgenic plants are still more environmentally friendly than most if not all chemical insecticides**."

QUALITY ASSURANCE MEASURES:

There is no QA statement indicating whether the study was conducted according to GLP.

DISCUSSION/REVIEWER'S COMMENTS:

The study reports significant mortality in the *artificial* diet test group and no significant mortality when the artificial diet was supplemented with *E.kuehniella* eggs. Therefore this study does not demonstrate any adverse effects to lacewing larvae under simulated field feeding conditions since the lacewing larvae have only natural diet (such as *E.kuehniella* eggs) in the field.

The concentration to which the larvae were exposed was massive (100 µg/ml of diet) and continuous, and therefore not reflective of Cry1Ab exposures that may occur under field conditions either by exposure to plant tissues, pollen or by consumption of exposed prey species, such as the European corn borer (ECB) larvae . The dosage used in these studies is at least 30 times that found in most corn tissues in the field. Also in nature the lacewing does not rely upon a single food source for development. The lacewing larvae have a choice of other insects or eggs performed to feed on. Therefore the field exposure will be intermittent, rather than continuous. Furthermore, in Bt corn fields intoxicated insects such as the ECB will not be available to the lacewings, since the ECB will be practically eliminated by the Bt toxin in corn plants. In addition, the surviving ECB larvae would normally be within the corn plant most of their larval life and not available for consumption by chrysopids.

Because in the field setting the lacewings have a choice of natural diet, the field situation is analogous to the laboratory data obtained with the larvae exposed to Cry1Ab in the artificial diet (+*eggs*). Here the data show a 29% mortality at adult eclosion with a (ca) 17% negative control mortality. This was not a statistically significant increase in mortality over the control. **Therefore the data show that in a laboratory test where the diet is similar to that in a field setting there is no significant mortality to lacewing larvae**. Only larvae fed an *artificial* diet (shown to have its'own detrimental effects) had a statistically significant (57%) mortality of approximately 27% above the mortality in the negative control. Thus **a switch to the artificial**

diet resulted in a much larger effect than adding a high concentration of Cry1Ab protein to a diet supplemented with *Ephestia kuehniella* eggs.

Control *C. carnea* reared continuously on the artificial diet without Bt toxin showed a significantly increased mortality (30%). The lowest total mortality (8%) was observed in *C. carnea* raised exclusively on *Ephestia kuehniella* eggs. Significant differences in development time for most life stages were also seen between larvae raised on different types of diet. Only the larvae receiving *artificial* diet after the 2nd instar showed a statistically significant total larval mortality. Thus confirms the Agency's assessment of the author's previous study [Angelika Hilbeck. September 1996. Investigations on Side-effects of Transgenic Bt-corn on Beneficial Insects. Report for the Swiss National Science Foundation. (SSP Biotechnologie, Gesuch Nr. 5002-042598)] that a suboptimal diet was the probable cause of the reported mortality in the initial experiments, and appears to remain the cause of significant mortality both in the negative controls and the test system in the present study.

In the Agency's review of the previous study it was noted that the lack of quantitation of Bt consumption by the larvae makes it impossible to determine correlation between exposure to Bt and the observed responses. There no data were presented to show the amount of prey consumed by each test group to make an independent assessment of unpalatable and sick prey effects like starvation effects due to food avoidance. This same problem exists in the present study, in that it is not reported how much of a reduction in consumption of Bt toxin occurred in the replicates receiving a choice in diet. However, this does not negate the detrimental effects of the artificial diet alone, since data is presented showing an increase in mortality and development time in larvae reared exclusively on an artificial diet.

In addition, in the previous study the lacewing larvae were not given a choice between Bt-exposed and unexposed prey species throughout their development, which they have in a field setting. As a result, that study did not account for the effects of a suboptimal/starvation diet (consisting of sick and dying larvae) which appeared to have been unpalatable to the lacewings and therefore of limited nutritional value. The experimental design in the present study does permit a distinction between a direct effect due to the Bt toxin versus an indirect effect of consuming a suboptimal, synthetic diet. The present study has addressed these issues by presenting the larvae with a measured amount of pure toxin in a diet supplemented with the predators' natural diet (*E.kuehniella* eggs). Under these test conditions there were no statistically significant mortality or developmental effects. The data show that detrimental effects (including high negative control mortality) occurred only in those portions of the test system which included an artificial (suboptimal) diet. **Therefore there is no unequivocal support for the conclusion that the Bt toxin was directly responsible for the observed differences in lacewing mortalities.** Since predators in the field have a choice of a variety of species, eggs and pollen to feed on, the detrimental effects the author mentions are not expected to take place in a field setting.

The deleterious effect of the artificial diet can also be seen from the data. Control *C. carnea*

reared continuously on the artificial diet showed a significantly increased mortality (30%). The lowest total mortality (8%) was observed in *C.carnea* raised exclusively on *Ephestia kuehniella* eggs. Thus, **the high lacewing negative control mortality (30%) with the artificial diet shows a less than optimal (unhealthy) test system**

Environmental influences were also not taken into account when speculating that Bt corn may pose a risk to beneficial insects. In a field setting it is highly improbable that Chrysopid larvae will mature exclusively on a diet of Bt toxin containing larvae. Therefore it is highly unlikely that in the field the lacewings, or other beneficial insects, will ingest the amounts of Bt that the larvae were forced to consume in the laboratory study (i.e. there is a very low field exposure to the BT toxin). **So the reported laboratory findings are not representative of the feeding environment by predatory insects in the open ecosystem. Actual field studies are necessary to ascribe practical significance to adverse effects observed *in controlled* laboratory feeding experiments** in order to obtain a definitive assessment of the ecological relevance of the reported single species laboratory findings.

In general the reported laboratory findings do not show significant detrimental effects and do not provide data that show a risk to beneficial insects in a field use situation. The author, A. Hilbeck, agrees with this by stating that "...**trials investigating** predation efficiency and predator **performance under field conditions are necessary before conclusions regarding the potential ecological relevance of the results presented in our paper can be drawn."**

However, there already are published and in-house field studies on the effects of Bt crops on beneficial insect predators showing no significant differences in the density of beneficial insect populations (1,2,3). The published field testing results and field test data submitted to EPA show minimal to undetectable changes in the beneficial insect populations. Some actually report the densities of predatory and non-target insects as generally higher on transgenic than non-transgenic crops. To date the available field test data show that compared to crops treated with conventional chemical pesticides, the transgenic crops have no detrimental effect on beneficial insect populations.

In contrast, crops treated with conventional chemical pesticides practically eliminate all beneficial insects from the treated fields. In addition, the replacement of conventional chemical pesticides by Bt crops has an added benefit to the environment, ground water and toxic pesticide residues in human food and animal feed. These issues have to be taken into account when performing the risk-benefit analysis for Bt crops as mandated by FIFRA.

In summary, the data demonstrate that there is no significant effect on lacewing larvae when these are given a choice in diet. When laboratory data show toxic effects on single species, any conclusion of detrimental effects on the predatory insect populations can be demonstrated only by a field study. All available in-house and published data do not show significant detrimental effects due to Bt delta endotoxin on the beneficial insects studied (e.g., no significant effect on lacewing larvae fed 16.7 ppm pure CryIA(b) protein for seven days).

Therefore, based on the available laboratory data in the Agency's files and the lower field exposure to Bt relative to the concentrations used in this study, there should be no noticeable effect on the lacewing populations from the use of Bt crops. **The submitted study does not supply data that would be useful in changing the Agency's risk assessment at the present time.** In addition, the alternative to the use of Bt corn (i.e. the use of conventional chemical pesticides) would pose a much greater hazard by practically eliminating the beneficial insects from the corn fields and increasing the toxic chemical residues in ground water, animal feed and food products. In the analyses to date it is clear that Bt crops are a way to protect beneficial organisms, since in the non-Bt crop fields non-target organisms are decimated along with the target pests by the use of conventional chemical pesticides .

Therefore, the submitted data do not provide information that would alter the Agency's risk assessment on the effects to green lacewing populations or to beneficial insect communities from Cry1Ab producing corn.

BPPD agrees with the author, also with the Novartis seeds review, that no conclusions can be made from this study with respect to lacewing larval effects when exposed to Cry1Ab producing corn in a field setting.

Many of the author's other conclusions and projections for further research are not based on the data presented in the study, rather on conjecture or results based on feeding the larvae a synthetic diet, which is not present in the field setting. The recommendation that a paraffin encapsulated artificial diet containing *B.thuringiensis* proteins is suitable for studies on non-target insects effects testing is not borne out by **the presented data** which **show that the observed detrimental effects are diet-related.**

Adequacy of the Study:

1. **Validation Category**: Invalid

2. **Rationale**: This study was not performed according to EPA Guidelines, the negative control mortality is unacceptably high and there are other major flaws in the study design with an unjustified emphasis on the significance of very low mortality in an artificial setting.

This study was not required, since Tier 1 maximum hazard dose nontarget beneficial insect testing data on file with the Agency showed no significant adverse effects to the green lacewing larvae.

References cited:

1. Obrycki, J. (1997) Effects of Cry9C Corn on Predatory Non-Target Beneficial Insects and Endangered Species; Determination of Predatory Non-Target Beneficial Insect

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2. Orr, D.B., and D.A. Landis. 1997. Oviposition of European Corn Borer (Lepidoptera: Pyralidae) and Impact of natural Enemy Populations in Transgenic Versus Isogenic corn. J. Econ. Entomol. 90(4): 905-909.

3. Pilcher, C.D., M.E. Rice, J.J. Obrycki and L.C. Lewis. 1997. Field and Laboratory Evaluations of Transgenic *Bacillus thuringiensis* Corn on Secondary lepidopteran pests (Lepidoptera: Noctuidae). J. Econ. Entomol. 90(2): 669-678.