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# Monitoring *Bacillus thuringiensis*-susceptibility in insect pests that occur in large geographies: How to get the best information when two countries are involved

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**Abstract**

The adoption of cotton producing insecticidal proteins of *Bacillus thuringiensis*, commonly referred to as Bt cotton, around the world has proven to be beneficial for growers and the environment. The effectiveness of this important genetically-modified crop can be jeopardized by the development of resistance to Bt cotton by pests it is meant to control, with the possibility that this phenomenon could develop in one country and spread to another by means of insect migration. To preserve the effectiveness of this agricultural biotechnology, regulatory agencies have developed plans to mitigate the development of resistance, and research institutions constantly monitor for shifts in Bt-susceptibility in important pests. If Bt-resistance is detected, this finding needs to be corroborated by an independent laboratory according to current regulatory requirements; a process that presents numerous challenges. We investigated the biological activity of Bt-incorporated diet on *Helicoverpa virescens* L. after it was stored for several days at different temperatures. Diet stored up to nine days at different temperatures (−14 to 27 °C) produced the same biological effect on *H. virescens* as freshly-prepared diet. Elevating the temperature of Bt stock solution to 76 °C as compared to 26 °C yielded significantly higher reading of apparent Cry1Ac concentration from MVP II, but not enough to elicit a significant biological response when these stock solutions were incorporated into insect artificial diet. These findings are important particularly when the confirmation of resistance is done at a distant location, such as Mexico, or when diet is shared between laboratories, and must be stored for later use, as in the case of international collaboration.
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*Keywords:* *Heliothis virescens*; Bioassays; Cry1Ac; Storage time; Temperature

## 1. Introduction

Insecticide resistance has been a common phenomenon in agricultural pests affecting growers and the environment around the world (Whalon et al., 2004). Well-implemented programs to prevent insecticide resistance in a region can be jeopardized by the influx of migratory pests that have acquired resistance somewhere else. Of particular importance would be the development of resistance in a pest in one country with subsequent migration of this pest into another; thus, establishing resistant alleles in the foreign local population. Cotton, *Gossypium hirsutum*, is an important crop in North America. The USA and Mexico plant large areas of cotton, that include nine of the 10 states that

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comprise their shared international border (except Nuevo León, Mexico). The areas planted with cotton in each country varies greatly, with about seven million ha in the US per year, while in Mexico this area has recently been only about 150,000 ha. Current adoption of genetically-modified cotton expressing *Bacillus thuringiensis* toxins (Bt cotton) in both countries has been quite high (> 50%); thus, exerting similar Bt-resistance selection pressure from this crop.

The use of Bt cotton has reduced the application of chemical insecticides to suppress lepidopterous pests in North America (Purcell and Perlak, 2004; Terán-Vargas et al., 2005; Fernandez-Cornejo and Caswell, 2006). Consequently, maintenance of insect susceptibility to Bt has been perceived to be in the public good and plans to mitigate the development of resistance to Bt have been implemented (Matten and Reynolds, 2003). The possibility of Bt-resistance developing in a pest in one country and then becoming established in another by migration, is especially feasible in known migratory noctuids such as *Trichoplusia ni,* (Huebner), *Spodoptera frugiperda* (J.E. Smith) *Helicoverpa zea* (Boddie) and/or *Heliothis virescens* L. (Johnson, 1995). A well-orchestrated effort between bordering countries can preserve Bt-susceptibility in important migratory pests of crops that are planted in proximity to their mutual international borders.

In the USA and Mexico, research and regulatory agencies are interested in fast and reliable Bt resistance detection methods. Loss of Bt cotton as an effective pest management tool in any crop could potentially have serious adverse consequences for the environment to the extent that growers would shift to the use of foliar pesticides (Matten and Reynolds, 2003). Strategic plans to delay Bt-resistance and monitoring Bt-susceptibility shifts have been implemented in both countries and there is an increasing interest in monitoring target pests in areas that might act as a sink/source for these migratory insects. These research and regulatory agencies have coordinated with Bt cotton registrants in order to implement a feasible monitoring plan (Blanco et al., 2005). Advances in adapting a common protocol and the dissemination of results have progressed, but many logistical obstacles still await solution, particularly in the case of the confirmation of a potentially Bt-resistant insect(s). Current regulatory requirements stipulate that if Bt-resistance is 'suspected' after screening for Bt-resistance in a research laboratory, this finding should be confirmed by an independent laboratory in a series of steps (US EPA, 2001). If the need for Bt-resistance confirmation arises, this can be approached in at least three different ways: (a) a sample of the putative Bt-resistant insect strain can be shipped to one of preferably two laboratories to perform a common validation protocol, (b) a team of researchers can visit the laboratory where Bt-resistance is suspected and/or (c) a sample of ready-to-use diet can be exchanged between the laboratories to confirm the finding.

Each of these ways of confirming Bt-resistance by an independent laboratory poses different challenges. The movement of insecticide-resistant insects to another laboratory could jeopardize the integrity of these samples and create an unnecessary threat to the environment if resistant insects escape. The travel of researchers to other laboratories represents logistical challenges and expenses. In the case of the interaction between countries, an international trip is also not easily or quickly organized. Sharing diet might overcome some of these challenges because the common method already established for monitoring Bt-susceptibility is through bioassays (Patin et al., 1999; Blanco et al., 2004). Sharing ready-to-use Bt-treated diet manufactured by one laboratory could be a way to partially confirm Bt-resistance in other research institutions. However, the biological activity of the diet might be affected by the time required for delivery after shipment and the storage time required before the diet can be used. This might be especially important in the case of international shipments that might take longer because of customs inspections which can delay the delivery of ready-to-use diet.

We investigated the biological response of tobacco budworm colonies exposed to Cry1Ac-incorporated diet that was stored under different temperature and time regimes to determine if the different storage regimes affected a Bt-resistance confirmation protocol.

## 2. Materials and methods

### 2.1. Diet preparation

Lyophilized MVP II insecticide (Mycogen Corporation, San Diego, CA) containing ca 19.1% *B. thuringiensis* Cry1Ac protoxin was incorporated into wheat germ-based insect artificial diet (modified from Shaver and Raulston, 1971). Eight insecticide concentrations (0–1.50 μg of Cry1Ac/mL) were added to batches of prepared diet and dispensed (1.0 ± 0.15 mL) into 16 wells of a bioassay tray (C–D International®, Pitman, NJ) per treatment per replication. Trays were left to dry (≈0.5 h), covered with self-adhesive film (Glad® press'n seal, Oakland, CA) and inserted individually in plastic bags then stored under three different temperature regimes: 4.3 ± 1.5 °C (in refrigerators), −13.8 ± 2.7 °C (in freezers) and 27.3 ± 0.4 °C (in incubators). Three different refrigerators, freezers and incubators (storage devices) were used to serve as main-unit replicates. The stored times consisted of three, six and nine days of diet storage for the sub-unit. Diet was prepared at three different dates in order to be inoculated on the age-matched cohorts (< 6 h-old) neonates from two different laboratory *H. virescens* colonies (USDA-ARS, Stoneville, MS and Monsanto Company, Union City, TN) on the same day. Three sets of trays per insect colony containing the above described MVP II concentrations were prepared the day of the neonate inoculation to compare the different treatment to freshly prepared diet. This process was repeated three times at different dates. After larval inoculation, wells were covered with self-adhesive membrane (C–D International®) then stored in three different incubators at

$27 \pm 0.4$ °C, $75 \pm 10\%$ RH and 14:10 h (light: darkness) photoperiod. Environmental conditions in all storage devices were recorded with a Hobo® data logger (Onset Computer Corporation, Pocasset, MA). Insect evaluations were made 7 days later by recording growth inhibition (dead larvae or non-responsive to probing, and larvae that had not grown beyond first instar (molting-inhibiting concentration, MIC) (Siegfried et al., 2000). Weights of the >second instar live larvae in the lowest Bt-concentration (0.125 μg of Cry1Ac/mL of diet) were recorded.

The experimental design was a split plot where temperature regime was the main unit treatment. The experiment was repeated three times in order to replicate the three main unit treatments. The sub-unit treatment was a factorial arrangement of two colonies × three storage times. Three additional replications of each sub unit treatment within each storage device were conducted. For each experiment × colony × storage time × replication within each temperature regime, there were eight concentrations of the Cry1Ac protein in the diet. Probit analysis (based on the log transformation of dose) was performed on each replicate of each treatment combination and $MIC_{50}$ values were obtained. The log $MIC_{50}$ values were analyzed using an analysis of variance (ANOVA) for the split plot design. Means based on the log of the transformed values are geometric when transformed back to the original scale. Treatment mean comparisons were based on the least significant ratios ($P \leqslant 0.05$) from the ANOVA. The least significant differences (LSD) on the log scale become least significant ratios (LSR) when transformed to the original scale. Preliminary Probit analyses were performed on the mean of each treatment × dose, averaged over experiments and replicates, and the lack of fit was obtained by comparing the predicted to the observed values. The error associated with replication was estimated by ANOVA on the same data that was not averaged over experiments and replications, using dose as the treatment variable. Lack of fit from the Probit analysis was compared to the error from the ANOVA to determine if Probit trends correctly described the dose means. Analyses were performed using SAS version 9.1 (SAS Institute, 2001).

Logarithmically transformed average larval weights of each replication were analyzed by ANOVA for a randomized complete block design for each separate storage day using least significant ratios and a Cry1Ac concentration of 0.125 μg of Cry1Ac/mL. Analyses were performed using SAS version 9.1 (SAS Institute, 2001).

### 2.2. Measurement of diet water loss during storage

To provide information on diet water loss during storage, $1 \pm 0.15$ mL of control diet was dispensed into wells of a bioassay tray. Each diet tray was cut into eight 16-well sections (squares) and each square weighed ≈30 min after dispensing diet. The squares were covered with self-adhesive place inside a freezer bag, and stored in a storage device. A randomly chosen square was taken daily from the storage device, removed from the freezer bag, followed by removal of the self adhesive film. Condensation was removed from the diet square with a paper towel, then the square was weighed and discarded. This process was repeated for nine consecutive days.

### 2.3. Cry1Ac concentration detection

In order to quantify the potential Cry1Ac degradation over time, a separate study was conducted. Two sets of 30 mL MVP II stock solutions (0.50, 1.50 and 4.50 μg of Cry1Ac/mL of water) were prepared in 50-mL centrifuge tubes at four different dates in order to be evaluated simultaneously. One set of tubes with each concentration was maintained at 26 °C (un-heated) and the other set heated to 76 °C (heated) for 10 min in a water bath, simulating the maximum temperature at which the Cry1Ac stock solution is elevated when incorporated into the wheat germ diet protocol. After the temperature exposure, one mL of each concentration was pipetted into 2-mL microtubes and stored in the same storage devices used for bioassay trays for three, six and nine days. Cry1Ac concentrations in microtubes were assessed simultaneously using ELISA (QuantiPlate® kit for detection of Cry1Ab/Cry1Ac, Envirologix®, Portland, ME). Freshly prepared un-heated (26 °C) and heated (76 °C) MVP II solutions were also analyzed as a comparison. Methods used for quantifying the amount of Cry1Ac using ELISA were those described in the QuantiPlate kit's instructions. Briefly, samples were mixed well and 100 μl of each sample, negative controls, and calibrators were pipetted into respective antibody-coated assay wells provided with the kit. Samples were mixed thoroughly by rapidly moving the plate in a circular motion on a bench top for 20 s. Samples were then shaken in an orbital plate shaker for 15 min at room temperature. One hundred micro liters of the Cry1Ac antibody-enzyme conjugate was added to each sample followed by mixing and incubation at room temperature for 1 h in an orbital shaker. The wells were emptied at the end of the incubation period by vigorously shaking the contents of the wells into a sink. Wells were washed four times by filling them with 1× phosphate buffered saline (pH 7.4) containing 0.01% Tween 20 and then shaking the wash buffer into the sink. After the last wash, the remaining wash buffer was removed by tapping the inverted plate onto a paper towel. Hundred micro liters of substrate solution was then added to each well and they were incubated at room temperature for 30 min in an orbital shaker. Color development was stopped by adding 100 μl of stop solution (100 N Hydrochloric acid) and the plates were read immediately using a microtiter plate reader (Bio-Rad model 680, Hercules, CA) set to 450 nm. The negative control and calibration standards provided with the kit were used in triplicate in each plate to generate standard curves for sample quantification. Sample wells were covered with parafilm during all the incubation steps to prevent evaporation. Statistical

analysis was performed using SAS version 9.1 (SAS Institute, 2001).

### 2.4. Biological effect of elevating the temperature of stock solutions

A separate study to evaluate the biological effect of heating the MVP II stock solution to 76 °C on the resistance bioassay of tobacco budworm was conducted. Un-heated (26 °C) and heated (76 °C) MVP II insecticide stock solutions (1.5 μg of Cry1Ac/mL of water) were used to prepare a set of the 8 series dilution concentrations (0–1.50 μg Cry1Ac/mL of diet). Un-heated and heated series dilutions were incorporated into the Arthro Feeds diet (Stonefly® Industries Inc., Bryan, TX) following the manufacturer's instructions. Each concentration of prepared diet was scooped into 16-wells of a bioassay tray (≈1 mL/well). Wells were inoculated with ⩽16 h-old tobacco budworm neonates from the two previously mentioned laboratory colonies and the bioassays were evaluated as described before. This procedure was repeated four times at different dates. Probit Analysis was performed using SAS version 9.1 (SAS Institute, 2001).

## 3. Results

### 3.1. Diet biological activity

This experiment indicated that storage conditions ($F = 1.19$, $P = 0.31$, df = 2, 30.8) or storage time ($F = 0.48$, $P = 0.62$, df = 2, 33.7) did not affect the biological response of the tobacco budworm to Cry1Ac (MVP II)-incorporated wheat germ diet (Table 1). No significant differences were found in $MIC_{50}$ values for either colony ($F = 0.75$, $P = 0.61$, df = 6, 33.7) exposed to the different storage treatments for any period of time. The $MIC_{50}$ of USDA-ARS larvae fed diet stored in incubators for 6 days was significantly different ($P = 0.048$) from the $MIC_{50}$ of freshly made diet (Table 1). Significant differences in $MIC_{50}$ values were found between colonies ($F = 23.48$, $P < 0.0001$, df = 1, 33.7).

Average larval weights of USDA-ARS colony exposed to 0.125 μg of Cry1Ac/mL of diet stored for three ($P = 0.002$, $F = 17.45$, df = 6, LSD = 0.00056) and nine days ($P = 0.016$, $F = 7.83$, df = 6, LSD = 0.00073) were significantly different from larvae exposed to fresh diet at the same Cry1Ac concentration. Larval weights of the Monsanto colony exposed to 0.125 μg of Cry1Ac/mL of diet stored for six ($P = 0.024$, $F = 6.72$, df = 6, LSD = 0.00094) and nine days ($P = 0.003$, $F = 14.64$, df = 6, LSD = 0.00061) were significantly different from larvae exposed to fresh diet (Fig. 1).

### 3.2. Diet water loss

Control diet stored for up to nine days lost 2.4 ± 0.7% (in incubators), 1.0 ± 0.2% (in refrigerators) and 0.5 ± 0.1% (in freezers) water content.

### 3.3. Cry1Ac concentration

Significantly higher Cry1Ac concentrations were obtained when stock solutions were heated to 76 °C than when they were at 26 °C ($P < 0.0001$, $F = 76.87$, df = 1, 102). There were significant differences in Cry1Ac concentration between stock solutions ($P < 0.0001$, $F = 65.96$, df = 2, 102) and storage devices ($P = 0.0056$, F = 5.46, df = 2, 102). Storage time did not influence protein concentration ($P = 0.72$, $F = 0.32$, df = 2, 102) (Fig. 2).

### 3.4. Biological effect of elevating the temperature of stock solutions

Heated (76 °C) or un-heated (26 °C) stock solutions incorporated into the Arthro Feeds diet did not produce a significantly different $MIC_{50}$ ($t = 0.05$) within insect colo-

Table 1
Molt-inhibiting concentrations ($MIC_{50}$) values differences of two *H. virescens* colonies exposed to Cry1Ac-incorporated diet stored under different temperature and time regimes

| Storage time | Fresh | Freezer | Refrigerator | Incubator | Average | LSR[1] comparing treatments |
|---|---|---|---|---|---|---|
| USDA-ARS colony | | | | | | |
| 0 Days | 0.129 | — | — | — | — | — |
| 3 Days | — | 0.088 | 0.176 | 0.144 | 0.130 | 3.157 |
| 6 Days | — | 0.336 | 0.237 | 0.487* | 0.341 | 3.157 |
| 9 Days | — | 0.201 | 0.148 | 0.193 | 0.179 | 3.157 |
| Average | — | 0.182 | 0.184 | 0.238 | | 2.649 |
| LSR[1] comparing storage time | — | 4.435 | 4.435 | 4.435 | 4.260 | |
| Monsanto colony | | | | | | |
| 0 Days | 0.332 | — | — | — | — | — |
| 3 Days | — | 0.420 | 0.398 | 0.387 | 0.402 | 1.434 |
| 6 Days | — | 0.379 | 0.369 | 0.358 | 0.369 | 1.434 |
| 9 Days | — | 0.456 | 0.323 | 0.328 | 0.365 | 1.434 |
| Average | — | 0.417 | 0.362 | 0.357 | | 1.319 |
| LSR[1] comparing storage time | — | 1.681 | 1.681 | 1.681 | 1.663 | |

* Significantly different from fresh diet ($P = 0.048$), not significantly different from all other means shown above.
[1] Least significant ratio.

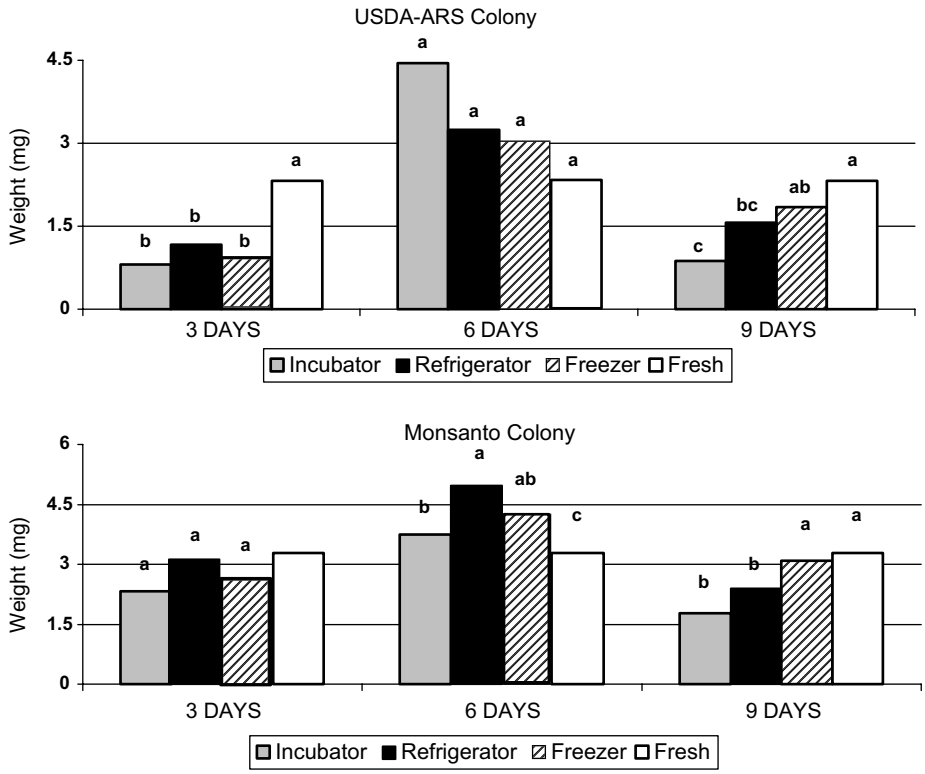


Fig. 1. Differences on average larval weights (mg) in two different *H. virescens* colonies exposed to 0.125 μg of Cry1Ac/mL-incorporated into insect artificial diet that was stored under different temperature and time regimes.

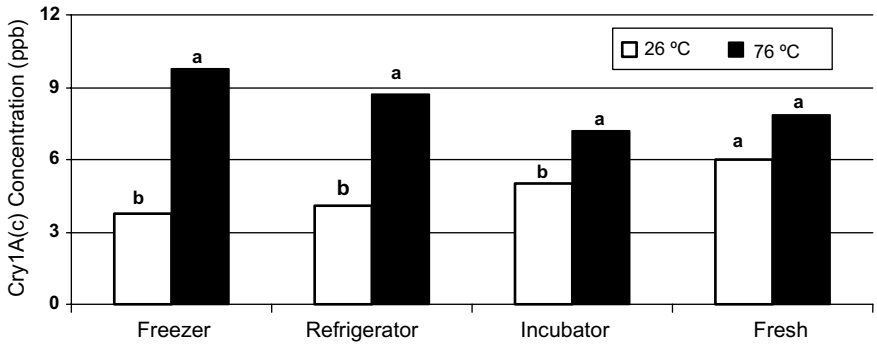


Fig. 2. Average Cry1Ac concentration in two stock solutions exposed to different temperature treatments stored at three different temperature regimes for nine days.

Table 2
Molting-inhibitory concentration ($MIC_{50}$) response of two different *H. virescens* colonies to stock solutions treated at different temperatures incorporated into the Arthro Feeds diet

| Stock solution (°C) | Slope ± SE | Significance of slope | | MIC50 (μg/mL of diet) | | Goodness of FIT | |
|---|---|---|---|---|---|---|---|
| | | $\chi^2$ | Probability | Dose | 95% FL | $\chi^2$ | Probability |
| USDA-ARS colony | | | | | | | |
| 26 | 0.713 ± 0.11 | 38.76 | <0.0001 | 0.205 | 0.11–0.31 | 30.24 | <0.0001 |
| 76 | 0.549 ± 0.04 | 166.51 | <0.0001 | 0.207 | 0.17–0.24 | 2.57 | 0.76 |
| Monsanto colony | | | | | | | |
| 26 | 0.754 ± 0.08 | 84.87 | <0.0001 | 0.609 | 0.46–0.82 | 9.85 | 0.07 |
| 76 | 0.699 ± 0.13 | 27.86 | <0.0001 | 0.657 | 0.40–1.30 | 28.47 | <0.0001 |

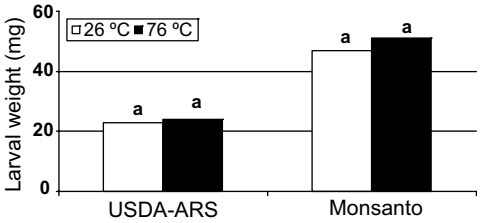


Fig. 3. Average larval weights (mg) of two different *H. virescens* colonies exposed to 0.125 μg of Cry1Ac/mL from stock solutions treated at different temperatures and incorporated into Arthro Feeds diet.

nies. Significant $MIC_{50}$ differences ($t \leqslant 0.05$) were observed between insect colonies (Table 2). Larval weights were not significantly different (ARS colony $P = 0.90$, $t = -0.13$, $df = 3$, Monsanto colony $P = 0.60$, $t = -0.58$, $df = 3$) when exposed to 0.125 μg of Cry1Ac/mL incorporated diet made with Cry1Ac stock solutions heated to 76 or unheated at 26 °C (Fig. 3).

## 4. Discussion

The Bt-resistance conformation process can have additional complications if two countries are involved. Logistical constraints associated with scientists' foreign travel and shipping live insects across international borders may retard the confirmation process; thereby, delaying the response of regulatory agencies, growers, and Bt cotton registrants for developing and implementing a remedial action plan. The exchange of a batch of Bt-treated diet made by a laboratory reporting Bt-resistance and another batch from an independent laboratory could allow for comparison of the response of an insect colony to the two different diets. It could also be used to study the response of two insect strains to the same diet. The Cry1Ac-incorporated diet suffered no negative biological effects when stored at different temperature conditions for up to nine days. This showed the reliability of the diet for use in the exchange of ready-to-use diet between different laboratories to confirm Bt-resistance. The only negative effect of this diet was observed with the USDA-ARS colony fed on diet stored at 27 °C (in incubators) for six days when compared with the same colony fed on fresh diet (Table 1). Since this only occurred at one date with one colony, we think it was a condition caused by experimental error, and was not a real adverse biological effect. Overnight delivery of the same type of diet had no detrimental biological effect using an international commercial carrier (Blanco et al., 2005). Our findings are corroborated by a similar study that utilized another Bt insecticide (Javelin) incorporated into diet maintained at 27 °C for 10 d or at 5 °C for up to 14 d retaining its effectiveness against diamondback moth larvae (Perez et al., 1997).

Weight of larvae exposed to stored diet offered another indicator of the preservation of the diet's biological activity. Although larvae in our experiment tended to be heavier in fresh diet as compared to refrigerated diet or diet placed in incubators, this trend was not consistent for the two insect colonies or for all the temperature regime days (Fig. 1). Very little humidity ($\leqslant$2.5%) from the diet was lost after nine days of exposure, offering another parameter to ensure the diet's effectiveness.

Unexpectedly, more Cry1Ac was detected when the MVP II preparation was heated to 76 °C than when maintained at 26 °C (Fig. 2). A possible explanation for this finding could be due to the disruption of *Pseudomonas flourescens* cell walls. MVP II is a highly-stable insecticide produced by a fermentation process in which dead *P. flouresens* that express Cry1Ac are encapsulated in cross-linked cell wall components which protect the toxin crystal (Tomlin, 1997). This process (CellCap®) is believed to create the stability of the active ingredient (Soares and Quick, 1992). Disrupting these cells during the heating the preparation to 76 °C might have released a higher amount of Cry1Ac. This Bt toxin concentration difference led to further testing of heated and un-heated stock solutions which were incorporated into an insect artificial diet that did not require hot water in the process. $MIC_{50}$ responses in both colonies were not affected by the two types of temperature regimes in stock solution (Table 2). Perhaps the Cry1Ac concentration difference (≈25%) released by the heated stock solution is not high enough to elicit a biological response at the low protein concentration used in our bioassays. Larval weights on diet with heated and un-heated stock solution showed the same, not statistically significant, trend (Fig. 3).

Sharing prepared diet among laboratories could be a feasible Bt-confirmation process. The manufacture of this diet with a reference Bt protein batch and a validated protocol, plus the biological response of this diet utilizing a known reference insect strain, are basic requirements that are recommended for the Bt-resistance confirmation process. A well coordinated effort between laboratories in two countries, knowledge, as well as coordination with custom authorities and shipment carriers will increase the rapid delivery of diet across international borders and the accuracy of results.

## Disclaimer

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## References


Blanco, C.A., Adams, L.C., Gore, J., Hardee, D., Mullen, M., Bradley, J.R., Van Duyn, J., Ellsworth, P., Greene, J., Johnson, D., Luttrell, R., Studebaker, G., Hebert, A., Karner, M., Johnson, D., Luttrell, R., Studebaker, G., Herbert, A., Leonard, R., Lewis, B., Lopez, J.D., Parker, D., Williams, M., Parker, R.D., Roof, M., Sprenkel, R., Stewart, S., Weeks, J.R., Carroll, S., Parajulee, M., Roberts, P., Rubseron, J., 2004. *Bacillus thuringiensis* resistance monitoring program for tobacco budworm and bollworm in 2003. In: Proc. Beltwide Cotton Conf.. National Cotton Council, Memphis, TN, pp. 1327–1331.

Blanco, C.A., Ali, I., Luttrell, L., Sivasupramaniam, S., Martínez-Carrillo, J.L., 2005. Susceptibility of four *Heliothis virescens* and *Helicoverpa zea* reference colonies to a homogeneous Cry1Ac-incorporated insect diet: Implications for an area-wide monitoring program. In: Proc. Beltwide Cotton Conf.. National Cotton Council, Memphis, TN, pp. 1226–1233.

Fernandez-Cornejo, J., Caswell, M., 2006. The first decade of genetically engineered crops in the United States. United States Department of Agriculture, Economic Research Service. Economic Information Bulletin Number 11, April 2006.

Johnson, S.J., 1995. Insect migration in North America: Synoptic-scale transport in a highly seasonal environment. In: Drake, V.A., Gatehouse, A.G. (Eds.), Insect Migration. Cambridge University Press, Cambridge, UK, pp. 31–66.

Matten, S.R., Reynolds, A.H., 2003. Current resistance management requirements for Bt cotton in the United States. J. New Seeds 5, 1317-178.

Patin, A.L., Dennehy, T.J., Sims, M.A., Tabashnik, B.E., Liu, Y.B., 1999. Status of the pink bollworm susceptibility to Bt in Arizona. In: Proc. Beltwide Cotton Conf.. National Cotton Council, Memphis, TN., pp. 991–996.

Perez, C.J., Tang, J.D., Shelton, A.M., 1997. Comparison of leaf-dip and diet bioassays for monitoring *Bacillus thuringiensis* resistance in field populations of diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 90, 94–101.

Purcell, J.P., Perlak, F.J., 2004. Global impact of insect-resistant (Bt) cotton. AgBioForum 7, 27–30.

SAS Institute. 2001. Version 9.1. SAS Institute, Cary, NC.

Shaver, T.N., Raulston, J.R., 1971. A soybean-wheat germ diet for rearing tobacco budworm. A. Entomol. Soc. Am. 64, 1077–1079.

Siegfried, B.D., Spencer, T., Nearman, J., 2000. Baseline susceptibility of the corn earworm (Lepidoptera: Noctuidae) to the Cry1Ab toxin from *Bacillus thuringiensis*. J. Econ. Entomol. 93, 1265–1268.

Soares, G.G., Quick, T.C. 1992. MVP, a novel bioinsecticide for the control of diamondback moth. Second International Workshop, Tainan, Taiwan, Dec. 10–14, 1990.

Terán-Vargas, A.P., Rodríguez, J.C., Blanco, C.A., Martínez-Carrillo, J.L., Cibrián-Tovar, J., Sánchez-Arroyo, H., Rodríguez-del-Bosque, L.A., Stanley, D., 2005. Bollgard cotton and resistance of tobacco budworm (Lepidoptera: Noctuidae) to conventional insecticides in Southern Tamaulipas, Mexico. J. Econ. Entomol. 98, 2203–2209.

Tomlin, C.D.S., 1997. The Pesticide Manuall, Eleventh ed. British Crop Protection Council.

US Environmental Protection Agency (US EPA). 2001. Biopesticides Registration Action Document—*Bacillus thuringiensis* Plant-Incorporated Protectants (October 16, 2001) http://www.epa.gov/oppbppd1/biopesticides/pips/btbrad.htm (last updated May 2, 2006).

Whalon, M.E., Bills, P.S., Mota-Sanchez, D., Hollingworth, R., Thompson, G.D., Ehnis, D., 2004. Arthropods Reported to Be Resistant to Pesticides. In: Management of Pest Resistance: Strategies Using Crop Management, Biotechnology, and Pesticides. Council for Agricultural Science and Technology, Ames, Iowa. Special Publication No. 24 June 2004, pp. 9–11.