11/05/2002

DATA EVALUATION RECORD

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1/21/04

STUDY TYPE:	Expression of Cry1F and Cry1Ac proteins against tobacco budworm (TBW)
MRID NO:	45808417
DP BARCODE:	290936
TEST MATERIAL:	Cry1F/Cry1Ac Cotton
PROJECT STUDY NO:	GH-C 5580
TESTING FACILITY:	Regulatory Laboratories – Indianapolis Lab, Dow AgroSciences, LLC., 9330 Zionsville Road, Indianapolis, IN 46268
TITLE OF REPORT:	Investigations into high-dose expression of Cry1F and Cry1Ac proteins against the tobacco budworm in Bt cotton line MXB-13
AUTHORS:	Blanco, C., N. Storer, R. Herman
STUDY COMPLETED:	November 5, 2002

CONCLUSION:

Three methods (two laboratory and one field) outlined by USEPA's Scientific Advisory Panel were used to demonstrate that Dow AgroSciences's transgenic cotton line MXB-13 expresses a high dose of two Bt insecticidal proteins, Cry1F and Cry1Ac, to control TBW larvae. This dose is high enough to kill nearly all susceptible TBW, and therefore, is expected to cause low survival of neonates heterozygous for resistance alleles. Using Methods 1 and 2, MXB-7 expresses a high dose of Cry1Ac for control of tobacco budworm. Using Methods 1 and 2, MXB-9 expresses a not quite high dose of Cry1F for control of TBW. That is, the Cry1Ac component of the stack in MXB-13 is by itself a high dose, while the Cry1F component in MXB-13 is not. Methods 1 and 2 both show that the stack, MXB-13, produces a high dose to control TBW. Although Cry1F expression is not quite a high dose, neonate mortality is quite high, >90% based on results from Method 1 and >83% based on results from Method 2. The field experiments (Method 3) support that MXB-13 expresses a high dose against TBW. No larvae, other than 3 neonates compared to 679 larvae from the same sampling regime in the non-Bt control plots were found, a greater than 99.5% difference. There may be less certainty in determining a high dose for TBW using Method 2 than in using Methods 1 and 3 because of the variability associated with using leaf tissue. No statistical analysis was performed to quantitatively compare the data. Based on all of the data, MXB-7 and MXB-13 express a high dose of Cry1Ac and Cry1Ac combined with Cry1F, respectively. It is highly likely that resistance to MXB-13 will be functionally recessive, and thus evolve only very slowly in the presence of a structured refuge. Information from this study is used to support the product durability plan for MXB-13 to manage the evolution in TBW (MRID 458084-15) and is reviewed separately.

CLASSIFICATION:

Acceptable

RECOMMENDATION:

It is recommended that confirmatory statistical analyses, such as the chi-square test, be performed on the data to more quantitatively define the differences amongst the transgenic lines (MXB-13, MXB-7, MXB-9) and between the transgenic lines and the non-transgenic control line, PSC355 at all locations and amongst locations. Statistical analysis will help define the certainty in the conclusions.

GOOD LABORATORY PRACTICE: Non-GLP study

A. **STUDY PURPOSE:** Three methods (of five) recommended by the FIFRA SAP in 1998 were used to investigate the high-dose expression of Cry1F (MXB-9), Cry1Ac (MXB-7), and Cry1Ac/Cry1F Stack (MXB-13) proteins in Dow AgroSciences' cotton line against the tobacco budworm (TBW). Because MXB-13 expresses two insecticidal proteins, Cry1Ac and Cry1F, and because the expected durability of a stack of two proteins is in part dependent on the dose of the individual proteins then it is important to investigate the dose of each protein.

B. **MATERIALS and METHODS:**

Method 1: The purpose of Method 1 is to see whether the Cry1Ac and Cry1F proteins in MXB-13 are expressed at a level 25-fold that required to kill TBW neonates. A 25-fold dilution of MXB-13 tissues, lyophilized and mixed with artificial diet, should still cause high levels of mortality (SAP 1998). Lyophilized cotton leaf powder from plants grown in Mississippi or North Carolina and expressing insecticidal crystal proteins was used in the study (Table 1). Artificial insect diets were prepared, lyophilized, and mixed at a 1:24 ratio (equal to a 1:25 dilution or ~4%) with the cotton leaf powders. Approximately 100 mg of each dry diet mixture was placed in each of 32 wells of a 128-bioassay tray and 400 µL of water was added to hydrate the material. One neonate TBW was added to each well, and the wells sealed with vented covers. Mortality (measured as the failure to molt to second instar) and insect weight data were collected after six days.

TABLE 1. Plant code, protein expression, and source of cotton leaf powder for study.

Plant code	Protein expression	State
MXB-7	Cry1Ac	MS
MXB-7	Cry1Ac	NC
MXB-9	Cry1F	MS
MXB-9	Cry1F	NC
MXB-13	Cry1Ac/Cry1F Stack	MS
MXB-13	Cry1Ac/Cry1F Stack	NC
PSC355	Stack negative control	MS
PSC355	Stack negative control	NC

Data from page 11 of MRID 45808417

Method 2: The purpose of Method 2 is to see whether Cry1Ac and Cry1F in MXB-13 are expressed at a level 25-fold that required to kill TBW neonates, then an older larvae that is around 25 times more tolerant of these proteins should be killed on MXB-13 plant tissue at a high rate (>95%) (see SAP 1998). Fresh cotton leaves, at least 2.5 cm in diameter and field grown at Wayside, MS; Stoneville, MS; or Fresno, CA, were taken at random from plants expressing Cry1F (event 281-24-236), Cry1Ac (3006-210-23), the stack protein MXB-13, or from a non-transgenic parent (PSC355). The leaves were washed, allowed to air dry, and individually placed onto agar plates (except for leaves collected July 12, 2002 from Fresno CA where agar was not used). TBW, one neonate and one 2-day-old larva were weighed and

added to each plate. The plates were maintained for a period of 5-7 days (27 ± 3°C, 50-70% humidity, 14/10 light/dark) and mortality (inability of larvae to move after prodding) and growth inhibition were determined. At the end of the study, surviving larvae were weighed and recorded.

Method 3: The purpose of Method 3 is to survey for survival of TBW on large number of plants in the field. A high dose can be defined as the dose sufficient to cause at least 99.99% mortality in the field (see SAP 1998). Field plots of MXB-13 were infested with large numbers of neonates, and subsequently intensively sampled to recover any survivors. Field plots of cotton plants expressing the stack protein MXB-13 and non-transgenic parents (PSC355) surrounded by at least 12 rows of non-transgenic plants grown in Wayside MS, Macon Ridge LA or College Station TX were used in the study. Eight weekly infestations of hatched TBW neonates were released in each plot. The plots were evaluated weekly by random inspection for larvae and larval damage of TBW released the week prior. When this was completed, plant samples were taken and the plants shaken above a cloth for enumeration of larvae. All TBW found in plots of MXB-13 >2.5 cm in length were collected and placed in vials for confirmatory identification.

C. RESULTS:

Method 1: Diets containing leaves expressing Cry1F, Cry1Ac, or both had significant impacts on TBW development (Table 2). All surviving larvae grown on diets containing negative control plants developed to the 4th instar while those grown on leaf diets expressing Cry1 ceased feeding with most failing to develop to the 2nd instar. A very small number of larvae on diets containing Cry1F-expressing protein managed to survive to the 3rd instar.

TABLE 2. Mortality and growth of neonate TBW on negative control diets or diets containing a 25-fold dilution of plants expressing Cry1Ac, Cry1F, or both proteins

Test	State	Expression	% Mortality	% Larvae 2nd instar or smaller	Total weight of surviving larvae (mg)
1	MS	Neg. Control	0	0	4283.7
1	NC	Neg. Control	0	0	4220.6
1	MS	Cry1Ac/Cry1F Stack	100	100	4.5
1	NC	Cry1Ac/Cry1F Stack	100	100	7.6
2	MS	Neg. Control	0	0	2554.8
2	MS	Cry1F	ND	96.9	122.3
2	MS	Cry1Ac	100	100	4.5
2	NC	Neg. Control	0	0	2598.1
2	NC	Cry1F	ND	90.6	150.6
2	NC	Cry1Ac	100	100	4.1

Data from page 23 of MRID 45808417
ND = No data

Method 2: The percent mortality of neonates and 2-day-old larvae fed each genotype of cotton plant grown in CA or MS are given in Table 3. The highest percent mortality within the same development stage was from transgenic cotton plants grown in MS. Slightly lower mortality was found on transgenic plants grown in CA, however, the mortality of 2-day-old larvae was ~90% for the stacked transgenic proteins and ~95% for the neonates. Across all test sites, mortality of neonates and 2-day-old larvae were similar, indicating that the larvae

that are 25-fold more tolerant, nevertheless develop poorly on genetically-modified Bt-cotton lines. Since 2-day-old larvae are surrogates for neonate heterozygous insects, the data suggests that heterozygous resistance alleles to the transgenic proteins will not significantly affect survivability compared to homozygous alleles. In addition, plants containing the Cry1Ac protein caused similar levels of mortality to both neonate and 2-day-old TBW larvae, indicating that this component of the stack is in itself a high-dose.

TABLE 3. Mortality of neonate and 2-day-old TBW on cotton leaves grown in three locations

Location	Genotype	% Mortality (neonates)	% Mortality 2-day-old larvae
Wayside MS	Neg Control	20.6	36.6
	Cry1F	92.6	93.3
	Cry1Ac	97.0	99.0
	Cry1Ac/Cry1F Stack	93.0	97.3
Fresno, CA	Neg Control	15.0	15.0
	Cry1F	83.0	76.0
	Cry1Ac	81.0	89.0
	Cry1Ac/Cry1F Stack	96.0	91.0
Stoneville MS	Neg Control	8.5	3.7
	Cry1F	95.0	82.5
	Cry1Ac	96.6	88.7
	Cry1Ac/Cry1F Stack	96.7	90.0
Across Locations	Neg Control	12.2	14.1
	Cry1F	89.7	85.7
	Cry1Ac	91.9	92.1
	Cry1Ac/Cry1F Stack	94.6	93.0

Data from Table 3, page 24 MRID 45808417

Method 3: Shown in Table 4 are the number of neonates inoculated per plant per week at sites in LA, MS, and TX. More eggs were available for inoculation in LA than at the other two sites. In addition, TX trial was located in a zone undergoing Phase II boll weevil eradication and had repeated applications of malathion made to the plot.

Also shown in Table 4 are the number of TBW larvae found on cotton plants expressing the Cry1Ac.Cry1F Stack protein and on negative control plants in field plots at three locations. Weekly evaluation for the presence of TBW larvae beginning one week after the first inoculation showed that through the season, ~2.4% of the 160 Cry1Ac/Cry1F Stack squares showed damage compared to ~28.7% for the negative control squares. No TBW were recovered from 396 beat cloth samples from genetically modified cotton No live TBW larvae were found on MXB-13 following inspection of 160 bolls/location or following inspection of 1000 first-position bolls/treatment site.

TABLE 4. Number of neonates released and larvae recovered weekly from Cry1Ac/Cry1F Stack and Negative control cotton plants grown in three locations[a]

Week	No. Inoculated/Plant/Week			MXB-13/PSC355[b]			MXB-13/PSC355[c]			MXB-13/PSC355[d]		
	LA	MS	TX	LA	MS	TX	LA	MS	TX	LA	MS	TX
1	3.02	1.81	0.99	0/1	0/6	0/1	0/2	0/3	0/1	–	–	–
2	1.56	2.17	1.06	0/4	0/14	ND	0/0	0/48	ND	–	–	–
3	1.88	1.79	1.07	1/5	0/8	ND	0/5	0/8	ND	0/1	0/4	ND
4	3.54	1.61	0.55	0/5	0/8	0/2	0/20	0/11	0/10	0/9	0/8	0/3
5	4.79	1.20	1.18	ND	0/16	1/12	0/28	0/12	0/1	ND	0/15	0/16
6	5.00	2.32	1.10	0/3	0/6	0/4	0/42	0/12	0/9	0/7	0/6	0/14
7	2.29	2.38	0.83	0/3	0/16	0/3	0/16	0/20	0/1	0/5	0/10	0/8
8	1.88	1.87	1.06	1/9	0/4	0/0	0/6	0/4	0/1	0/28	0/2	0/6
9	-	-	-	0/11	0/0	0/0	0/2	0/6	0/1	0/19	0/9	0/0
Total Neonates	138,000	87,183	45,158						Final[e]	0/8	0/63	0/28

Data from Tables 1-2, pages 25-28, MRID 45808417

[a]Data presented as Cry1Ac/Cry1F Stack (MXB-13)/Negative control (PSC355)

[b]TBW larvae found in 160 randomly inspected squares

[c]TBW larvae found in 396 beat cloth samples

[d]TBW larvae found in 160 randomly inspected bolls

[e]Data taken from 1000 first position bolls/treatment three weeks after final infestation

ND - No data

D. DISCUSSION AND CONCLUSIONS:

U.S. EPA has determined based on two Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel Subpanel's (SAP) (1998, 2000) that a key step in devising an effective and practical insect resistance management (IRM) strategy is investigating whether or not Bt cotton expresses a high dose against the key target pests. For target pests against which Bt cotton expresses a high dose, the high dose plus structured refuge strategy is appropriate. However, for target pests against which Bt cotton doesn't express a high dose, additional information is needed to establish an optimal IRM strategy. The 1998 and 2000 SAPs defined high dose for lepidopteran-resistant plant incorporated protectants as 25 times the dose required to kill 99% of susceptible insects (SAP 1998, 2000; EPA 2001). High dose can also be defined as that dose which is sufficient to kill 99.99% of insects in the field, or sufficient to cause high mortality (>95%) of instars that are around 25 times more tolerant of the protein than are neonates. The later instar is a surrogate for a heterozygote. The 1998 SAP described five imperfect methods for demonstrating high dose and suggested that using at least two of the five methods would provide a reasonable assurance of high dose. Dow AgroSciences has employed two laboratory-based and one field-based method to demonstrate high dose. Because MXB-13 expresses two insecticidal proteins, Cry1Ac and Cry1F, and because the expected durability of a stack of two proteins is in part dependent on the dose of the individual proteins then it is important to investigate the dose of each protein.

Method 1. Artificial diet containing 4% concentration lyophilized leaf material expressing Cry1F or Cry1Ac or both had a significant impact on the development of the tobacco budworm (lower weight gain and % mortality) compared with the same concentration of lyophilized leaf material not expressing the proteins (Table 2.) The expression of Cry1Ac alone in Bt cotton event 3006-210-23 and the expression of Cry1Ac and Cry1F combined in MXB-13 is at least 25-fold that required to kill susceptible neonates (a high dose). However, expression of Cry1F alone in Bt cotton event 281-24-236 is slightly lower than a high dose, 96.9% in MS and 90.6% in NC, as defined by the 1998 and 2000 SAPs. No statistical analysis was performed to quantitatively compare the data.

Method 2. This study is a laboratory study using freshly harvested young leaves from field-grown plants. Across all tests, mortality of neonates and 2-day old larvae were very similar for all of the transgenic cotton lines, MXB-13 (Cry1Ac/Cry1F), MXB-7 (Cry1Ac alone), MXB-9 (Cry1F alone), although mortality was not 100% for either neonates or 2-day old larvae. Mortality of the 2-day old larvae was approximately 7-12% lower than neonate mortality at Stoneville, MS. At Fresno, CA, there were some problems with the non-transgenic control cotton, PSC355. Mortality for the 2-day old larvae and neonates was virtually identical in the Wayside, MS trial. Across all three locations, the mortality of 2-day old larvae was greater than 95% relative to mortality of neonates indicating that larvae that are 25-fold tolerant of the toxins are extremely unfit on the Bt cotton lines and were much higher than the non-transgenic control cotton, PSC355 (Table 3). Since the 2-day old larvae is a surrogate for heterozygotes, the data suggest that insects that are heterozygous for resistance alleles to the Bt proteins will not exhibit significantly higher survival compared to susceptible insects.

During this bioassay, one would not necessarily expect all the insects to actually be dead by the end of the 5- to 7-day bioassay period based on the relatively slow action of the Bt proteins once ingested. Mortality in this assay is assumed when the larvae failed to respond when prodded by a probe, while what is relevant in the field, is the ability to develop to a fertile adult. Weight gain

information (Tables B4-B5) show that the transgenic lines have much lower weight gains than the non-transgenic control line (PSC355). In the field, lack of growth results in death (e.g., failure to reach adulthood). The goal of this study is not to show neonate mortality >99.9% rather the field study, Method 3, is the better way to show this. The goal of this study is to predict survival of heterozygote neonates, and the 2-day old larvae represent heterozygotes. Because this study can't be directly translated to field mortality, as noted above, survival of 2-days olds should be expressed relative to survival of neonates, remembering that the goal of a "high dose" is to assure high likelihood of functional dominance being <0.05 (i.e., 95% mortality of RS in the field). Using the across-study means (Table 3), it is reasonable to conclude from this study that Cry1Ac (MXB-7) and the stack (MXB-13) are at least a high dose of Cry1Ac and Cry1F combined with Cry1Ac to control TBW (RS relative mortality >95%, functional dominance < 0.05), respectively. MXB-9 does not express a high dose of Cry1F, but is close to a high dose (RS relative mortality is close to 95%, functional dominance is approximately equal to 0.04) to control TBW. There may be less certainty in determining a high dose for TBW using Method 2 than in using Methods 1 and 3 because of the variability associated with using leaf tissue. No statistical analysis was performed to quantitatively compare the data.

Method 3. *Across all three collection methods, 3,840 squares and 6,400 bolls were examined* and beat cloth samples of 9,900 plants were made from MXB-13 plants infested with a total of 270,341 neonates on MXB-13 plants over a period of 56 days at 3 different locations. No larvae, other than 3 neonates compared to 679 larvae from the same sampling regime in the non-Bt control plots were found, a greater than 99.5% difference. The field experiments support that MXB-13 provides a high dose against TBW. No statistical analysis was performed to quantitatively compare the data.

Conclusion

Three methods (two laboratory and one field) outlined by USEPA's Scientific Advisory Panel were used to demonstrate that Dow AgroSciences's transgenic cotton line MXB-13 expresses a high dose of two Bt insecticidal proteins, Cry1F and Cry1Ac, to control TBW larvae. This dose is high enough to kill nearly all susceptible TBW, and therefore, is expected to cause low survival of neonates heterozygous for resistance alleles. Using Methods 1 and 2, MXB-7 expresses a high dose of Cry1Ac for control of tobacco budworm. Using Methods 1 and 2, MXB-9 expresses a not quite high dose of Cry1F for control of TBW. That is, the Cry1Ac component of the stack in MXB-13 is by itself a high dose, while the Cry1F component in MXB-13 is not. Methods 1 and 2 both show that the stack, MXB-13, produces a high dose to control TBW. Although Cry1F expression is not quite a high dose, neonate mortality is quite high, >90% based on results from Method 1 and >83% based on results from Method 2. The field experiments (Method 3) support that MXB-13 expresses a high dose against TBW. No larvae, other than 3 neonates compared to 679 larvae from the same sampling regime in the non-Bt control plots were found, a greater than 99.5% difference. There may be less certainty in determining a high dose for TBW using Method 2 than in using Methods 1 and 3 because of the variability associated with using leaf tissue. No statistical analysis was performed to quantitatively compare the data. Based on all of the data, MXB-7 and MXB-13 express a high dose of Cry1Ac and Cry1Ac combined with Cry1F, respectively. It is highly likely that resistance to MXB-13 will be functionally recessive, and thus evolve only very slowly in the presence of a structured refuge. Information from this study is used to support the product durability plan for MXB-13 to manage the evolution in TBW (MRID 458084-15) and is reviewed separately.

RECOMMENDATION: It is recommended that confirmatory statistical analyses, such as the chi-square test, be performed on the data to more quantitatively define the differences amongst the transgenic lines (MXB-13, MXB-7, MXB-9) and between the transgenic lines and the non-transgenic control line, PSC355 at all locations and amongst locations. Statistical analysis will help define the certainty in the conclusions.

CLASSIFICATION: ACCEPTABLE

DATA EVALUATION RECORD

Mycogen Brand Cry1F(synpro)/Cry1Ac (synpro) Construct 281/3006 Cotton

STUDY TYPE: EXPRESSION OF Cry1F and Cry1Ac PROTEINS AGAINST TOBACCO BUDWORM

MRID 45808417

Prepared for

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R139660

Chemical:	Bacillus thuringiensis var. aizawai Cry1F (synpro) and the genetic material (from the insert of plasmid pGMA281) necessary for its production in cotton Bacillus thuringiensis var. kurstaki Cry1Ac (synpro) and the genetic material (from the insert of plasmid pMYC3006) necessary for its production in cotton
	PC Code: 006512 006513
HED File Code:	41400 BPPD IRM
Memo Date:	1/21/2004
File ID:	DPD290936
Accession #:	000-00-9001

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3/22/2007