UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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MEMORANDUM

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

SUBJECT: Review of the Insect Resistance Management (IRM) plan for SmartStax (MON 89034 x TC1507 x MON 88017 x DAS 59122-7) corn. EPA Reg No. 524-LIR. MRID#: 474449-11. Decision#: 394799. DP Barcode: 355691.

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Action Requested

BPPD[1] has been asked to review the insect resistance management (IRM) plan submitted by Monsanto Company to support registration of SuperStax Bt corn (EPA Reg. No. 524-LIR). SmartStax is a joint registration effort between Monsanto and Dow AgroSciences (Monsanto is the lead registrant) and contains the following previously-registered events: MON 89034 (Monsanto), TC1507 (Dow), MON 88017 (Monsanto), and DAS 59122-7 (Dow). The IRM plan is contained in a volume titled "Insect Resistance Management Plan for MON 89034 x TC1507 x MON 88017 x DAS 59122-7" (MRID# 474748-01).

Conclusions and Recommendations

1) Monsanto/Dow have submitted an acceptable IRM proposal for SmartStax corn, subject to the conclusions and recommendations described below. SmartStax corn is a stacked/pyramided Plant Incorporated Protectant (PIP) targeting two separate pest complexes (lepidopteran and corn rootworm). As such, separate IRM and refuge components for each pest complex have been considered. BPPD's conclusions on the proposed refuge for lepidoptera and corn rootworm are described below.

[1] The use of BPPD in this review refers to the BPPD IRM Team consisting of Alan Reynolds and Jeannette Martinez

2) Efficacy studies submitted by Monsanto/Dow demonstrated that the performance of SmartStax against lepidopteran and corn rootworm (CRW) pests is comparable to or better than the single trait products from which it is based. However, the implications for these studies to assess dose for CRW are summarized in #5 below. Other submitted data for cross resistance indicate that Cry3Bb1 and Cry34/35 are unlikely to share major binding sites in CRW.

3) The information submitted by Monsanto/Dow is sufficient to support the use of a 5% refuge with SmartStax corn for lepidopteran target pests. These pests include European corn borer (ECB), corn earworm (CEW), and southwestern corn borer (SWCB). SmartStax contains two toxins (Cry1A.105 and Cry2Ab2) that were registered in MON 89034, which also has a 5% lepidopteran refuge. The rationale for a 5% refuge for SmartStax is the same as that for MON 89034 (see BPPD 2008 for a discussion of the supporting data).

4) At the present time, BPPD cannot recommend the use of a 5% refuge for CRW for SmartStax. While Monsanto/Dow have made a good case for reducing the CRW refuge (from the 20% required for single trait CRW products), BPPD is concerned that the models used to assess the durability of 5% refuge included unrealistic dose estimates for the SmartStax toxins (Cry3Bb1 and Cry34/35).

5) The models submitted in support of SmartStax corn assumed high mortalities (mostly >99%) for the individual Cry3Bb1 and Cry34/35 toxins, based largely on a beetle emergence study (Huckaba and Storer 2008) that showed a high reduction in CRW production (after adjustment for density dependent effects) in Bt treatments relative to conventional corn. However, BPPD notes that other submitted studies conducted in greenhouse and field settings suggest that the actual toxin doses are less than those assumed in the models. Also, neither toxin has been considered to be a "high dose" toxin for CRW. BPPD is concerned that, when varied in the models, dose appeared to be a sensitive parameter and simulations with lower doses developed resistance more quickly than those with higher dose assumptions.

6) To address BPPD's concerns over CRW dose assumptions, Monsanto/Dow can either: i) provide additional information to explain why the current dose assumptions are justified, or ii) conduct additional model simulations using lower dose estimates. BPPD recommends dose levels of 85-95% for the single trait PIPs and 90-97% for the pyramid (SmartStax).

7) BPPD notes that there are several other areas that should also be addressed to provide additional support for the proposal. These areas include:

- Not all of the model simulations were conducted to compare 5% vs. 20% refuge for SmartStax; most simulations assumed a 5% refuge for MON 89034. As such it is difficult to assess the value (or risk) of 5% refuge relative to 20% refuge (or

other sizes). Additional simulations including 5, 10, and 20% refuges would be useful for comparative purposes.

- Recent selection experiments (i.e. Meihls et al. 2008) suggest that resistance could evolve quickly with non-recessive inheritance. Models could be adjusted to account for these and other similar findings. For example, Storer's stochastic model could include resistance allele frequencies > 0.001.

8) Should SmartStax ultimately be registered with two separate refuge requirements in the Corn Belt (5% for lepidoptera and 20% for CRW), a "common" refuge design will not be possible unless the refuge totals 20%. Separate 5 and 20% refuges would remain an option for growers planting SmartStax corn. In addition, BPPD notes that a 20% lepidopteran refuge will still be applicable in southern regions where cotton is also grown (CRW are not likely to be significant pests in most of these areas). This refuge was previously analyzed and approved for MON 89034 corn (see BPPD 2008).

9) Given the potentially different refuge strategies for lepidoptera and CRW, BPPD recommends that Monsanto/Dow submit a revised compliance plan specifically for SmartStax to address the various refuge requirements. Recent data have shown that refuge compliance for Bt corn has fallen in recent years.

10) Existing programs for resistance monitoring and remedial action that were established for MON 89034 (Cry1A.105 and Cry2Ab2), MON 88017 (Cry3Bb1), and Herculex Xtra (Cry1F and Cry34/35) should be applicable to SmartStax corn. However, a revised definition of "resistance" may be needed for the CRW monitoring and remedial action plans based on recent research and selection experiments.

Background

Monsanto Company and Dow AgroSciences have collaborated to develop a new Bt corn product with the trade name SmartStax. This product was developed through conventional breeding and is a combination of four previously registered Bt corn events. The components of SmartStax are summarized in table 1 below.

Table 1. Summary of the registered single gene components of SmartStax Bt corn.

Event	Toxin(s)	Company	First Registered	Target Pests[1]	Refuge Requirements
MON 88017	Cry3Bb1	Monsanto	2005	CRW	20%
DAS 59122-7	Cry34Ab1 Cry35Ab1	Dow	2005	CRW	20%
MON 89034	Cry1A.105 Cry2Ab2	Monsanto	2008	ECB, CEW, SWCB, FAW	5% (amended from original 20%)
TC1507	Cry1F	Dow	2001	ECB, CEW, SWCB, FAW	20%

[1] Pest legend: CRW = corn rootworm (*Diabrotica* spp.); ECB = European corn borer (*Ostrinia nubilalis*); CEW = corn earworm (*Helicoverpa zea*); SWCB = southwestern corn borer (*Diatraea grandiosella*); FAW = fall armyworm (*Spodoptera frugiperda*).

Since their original registration, the four single gene events in Table 1 have been pyramided to form multi-trait products targeting both lepidoptera and corn rootworm. Monsanto developed MON 89034 x MON 88017 (trade name YieldGard Triple) which was registered in 2008 with MON 89034. Dow previously registered TC1507 x DAS 59122-7 (trade name Herculex Xtra) in 2005. SmartStax functionally combines all of these traits into one product for control of lepidoptera (European corn borer, corn earworm, southwestern corn borer, and fall armyworm) and corn rootworm.

TC1507 (targeting lepidoptera) as well as MON 88017 and DAS 59122-7 (targeting corn rootworm) were all registered with a requirement to plant a 20% non-Bt corn refuge in the Corn Belt (TC1507 has an additional requirement for a 50% refuge in southern cotton-growing regions). MON 89034 was also initially registered with a 20% refuge requirement; although this refuge was applicable to all regions (a 50% refuge was not required in cotton regions). The stacked products containing these events were also registered with 20% refuge that could be planted either as a common lepidoptera/rootworm refuge or as two separate refuges. After MON 89034 was registered, Monsanto proposed an amendment to reduce the refuge in the Corn Belt. After review (BPPD 2008), EPA approved the amendment, reducing the lepidopteran refuge from 20% to 5%. However, the amount of refuge required for corn rootworm in the Corn Belt or lepidoptera in southern cotton-growing areas in MON 89034 x MON 88017 corn remained at 20%.

IRM Plan and Supporting Data for SmartStax Bt Corn

Monsanto/Dow's IRM proposal for SmartStax Bt corn is contained in a submitted volume titled "Insect Resistance Management Plan for MON 89034 x TC1507 x MON 88017 x DAS 59122-7" (MRID# 474748-01). This study includes information and data summaries covering confirmation of dose (expression levels of toxins *in planta* and efficacy data), protein mode of action, cross resistance, simulation modeling, and a proposed refuge strategy for SmartStax. The submitted IRM plan addresses both corn rootworm (CRW) and lepidopteran (ECB, CEW, SWCB, FAW) target pest complexes.

BPPD's review of the proposal will concentrate on the four major areas critical for resistance management of a pyramided Bt corn product: 1) dose; 2) cross resistance potential; 3) simulation modeling; and 4) refuge strategy. Other aspects of IRM including pest biology, resistance monitoring, refuge compliance, and grower education have been previously addressed for registered components of SmartStax (i.e. MON 89034, MON 88017, TC 1507, and DAS 59122-7 -- refer to the Biopesticide Registration Action Documents for each registration posted on-line at: http://www.epa.gov/oppbppd1/biopesticides/pips/pip_list.htm).

1. Dose Considerations for SmartStax

1.A. Lepidopteran Pests

SmartStax contains three toxins active against lepidopteran target pests: Cry1F, Cry1A.105, and Cry2Ab2. These proteins are effective against European corn borer (*Ostrinia nubilalis*, ECB), corn earworm (*Helicoverpa zea*, CEW), southwestern corn borer (*Diatraea grandiosella*, SWCB), and fall armyworm (*Spodoptera frugiperda*, FAW). Cry1F and Cry2Ab2 were derived from microbial *Bacillus thuringiensis* while the Cry1A.105 toxin is a "chimeric" protein containing domains I and II and the C-terminal from Cry1Ac and domain III from Cry1Fa. Cry2Ab2 and Cry1F are also registered in Bt cotton products targeting lepidopteran pests (Cry2Ab2 in Monsanto's Bollgard II and Cry1F in Dow's WideStrike).

Dose, defined as the amount of toxin expressed by the transgenic crop relative to the susceptibility of the target pest, is a critical variable in determining the ability of a pest to develop resistance to PIP toxin(s). A "high dose" has been defined as a level of toxin 25 times greater than is needed to kill all susceptible insects (models have shown that a high dose of toxin, coupled with a refuge to provide susceptible insects, is the most effective strategy for delaying resistance in Bt PIPs). Pyramided Bt crops containing two or more toxins can also be evaluated collectively to determine an "effective" high dose. In some examples, each toxin by itself may not supply a high dose, but in combination a sufficient control (>95% of heterozygotes) is provided to be considered high dose.

Methods for establishing high dose have been established for lepidopteran pests of corn and cotton (see SAP 1998). However, for a pyramided product created by conventional breeding of previously-registered PIPs (like SmartStax) the dose profiles of the individual component toxins will have been determined during their original registration. Therefore, dose expression for the pyramided product is largely assessed through confirmatory studies. These studies typically include protein expression data (to confirm similar levels of protein in the single gene and pyramided products) and efficacy data (to confirm similar field level performance). To support SmartStax, Monsanto/Dow cited protein expression data for the pyramid and submitted the results of field efficacy trials against a broad range of lepidopteran corn pests.

The protein expression data cited by Monsanto (Stillwell and Silvanovich 2007) indicate that the protein levels of Cry1A.105 and Cry2Ab2 in SmartStax were equivalent to MON 89034 in various corn tissues (leaf, whole plant, root, pollen, and grain). A separate study for Cry1F also verified that the expression levels in SmartStax were very similar to Herculex corn (Phillips 2008). (Note: these two studies will be reviewed separately by BPPD as part of the product characterization assessment for SmartStax.)

Efficacy data (Head 2006) were previously developed and submitted to support the registration on MON 89034. BPPD's review of the data concluded that: (1) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provided essentially 100% control of ECB; (2) the Cry1A.105 protein in MON 89034 provided approximately 95% control

of SWCB, while the Cry2Ab2 protein provided 80-90% control; (3) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provided >95% control of FAW; and (4) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provided 90-95% control of CEW (BPPD 2007a). While these data clearly demonstrated a high level of control against the target pests (i.e. likely to kill >90% of susceptible insects), the data did not support a "high dose" under the definition put forth by the 1998 SAP (a level of toxin 25 times greater than needed to kill susceptible larvae; i.e. a dose greater than the LC_{99} of the pest). For Cry1F (TC 1507) as expressed Herculex and Herculex Xtra, it was previously determined to be high dose for ECB (see reviews in EPA 2001 and BPPD 2005a). Both hybrids also offered good protection against SWCB and FAW in field trials (though high dose was not formally determined).

Monsanto/Dow also provided data for two efficacy trials conducted with SmartStax. The first study (Vaughn et al. 2008) was sponsored by Monsanto, while the second (Neese 2008) was performed by Dow.

The first efficacy study (Vaughn et al. 2008) consisted of a series of trials conducted over a two year period (2006-2007) that evaluated product performance with lepidopteran and corn rootworm target pests (only lepidoptera are discussed in this section). Efficacy assessments were made for ECB, SWCB, CEW, FAW, Sugarcane borer (*Diatraea saccharalis*, SCB), and western bean cutworm (*Richia albicosta*, WBC) by determining pest damage with standardized scales. Trial locations were not well described in the methodology portion of the submission but apparently included sites in Alabama, Florida, Georgia, Illinois, Indiana, Iowa, Mississippi, North Carolina, Nebraska, Ohio, Tennessee, Texas, and Puerto Rico. Pest pressure was established either with natural infestation (SCB) or through artificial infestations to augment existing natural populations (ECB, SWCB, CEW, FAW, and WBC).

For ECB, feeding damage assessments (leaf damage and stalk tunneling) were made at two locations in Illinois. In all cases, MON 89034, TC 1507, and SmartStax incurred virtually no leaf damage (≤ 0.2 on the Guthrie scale) or stalk tunneling (0.0 cm). By comparison, the non-Bt control had a leaf damage rating of 5.2 and an average of 0.6 cm stalk tunneling. A second set of experiments at locations in Illinois (2 locations), Indiana, Iowa (2 locations), Nebraska, Ohio, and Tennessee evaluated stalk cavities created by corn borers (including ECB and SWCB). For these tests, only TC 1507 and SmartStax (as well as a non-Bt control) were compared. Both SmartStax and TC 1507 significantly reduced the number of ECB and SWCB cavities relative to the control group except at one location (Grant, NE) in which there was no significant difference between SmartStax (2.5 cavities/plant) and the control (3.0), though TC 1507 had significantly fewer cavities than both.

SWCB infestations were evaluated using a variety of criteria including leaf feeding, shank/stalk damage, stalk cavities, and larval presence in plants. Testing was conducted at four locations (Leesburg, GA, Union City, TN, Loxley, AL, and Leland, MS) with artificial infestation. In all of the experiments, SmartStax, MON 89034, TC 1507, and a non-Bt control variety were included in the test groups. The results of these tests were

consistent at almost all of the locations: SmartStax, MON 89034, and TC 1507 significantly reduced SWCB damage compared with non-Bt controls. In most cases, damage was reduced to minimal levels and there were no significant differences between the PIP test groups.

One test was conducted to evaluate efficacy against SCB at one location (Needville, TX) with high natural infestation. Similar to the trials for other stalk borers, SCB feeding cavities and the number of SCB larvae per plant were assessed for SmartStax, TC 1507, and a non-Bt control. Both SmartStax and TC1507 significantly reduced the number of cavities and larvae compared to the non-Bt hybrid. SmartStax had fewer cavities and larvae than TC 1507, though the difference was not statistically significant.

For FAW, experiments were conducted in the United States (7 locations in Alabama, Georgia, Illinois, and Tennessee) and Puerto Rico (2 locations) using artificial infestation to augment natural pest populations. Both leaf damage (0-9 scale) and ear damage were assessed for SmartStax, TC 1507, MON 89034, and non-Bt corn. In the leaf feeding studies, SmartStax treatments had significantly less damage than non-Bt corn. TC 1507 and MON 89034 also suffered less damage than non-Bt corn, though TC 1507 had high levels of damage (similar to the non-Bt control) in two trials with high FAW pressure in Puerto Rico. SmartStax generally had less leaf damage than TC 1507 and MON 89034, except for one trial in Monmouth, Illinois. Similar patterns were observed in the ear damage assessments with SmartStax showing significantly less tunneling than the non-Bt control. For the most part TC 1507 and MON 89034 also had less ear damage than non-Bt corn, although the differences were not always statistically significant.

CEW trials were conducted with artificial infestation to supplement natural pest levels at 17 locations in Alabama, Florida, Georgia, Illinois, Indiana, Iowa, Nebraska, North Carolina, Ohio, Tennessee, and Texas. A variety of parameters were measured including ear damage (tunneling), infestation (larvae per plant, percentage of plants infested), and kernel damage. In almost all trials, SmartStax significantly reduced the feeding damage and infestation levels due to CEW relative to non-Bt corn. In many cases, CEW ear damage in SmartStax treatments was close to zero. Where tested, MON 89034 also reduced CEW damage to levels similar to SmartStax. On the other hand, TC 1507 showed reduced efficacy against CEW with damage and infestation frequently not statistically different than the non-Bt control.

WBC was evaluated using natural pest populations at nine locations in Illinois, Indiana, Iowa, Nebraska, and Ohio. Infestation was assessed by tabulating the percentage of plants infested and the number of larvae per corn ear. SmartStax and a number of intermediaries (i.e. hybrids containing one or more of Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, and Cry34/35) were included in the tests. Due to low natural pest populations, there was little difference in larvae per ear among the treatments. Variable infestation also hampered the analysis of plant infestation, though the Bt corn treatments (SmartStax and intermediaries) had less infestation than the non-Bt control.

The second major efficacy study conducted by Dow (Neese 2008) was also conducted in the field during 2007. This study included assessments of WBC, CEW, FAW, and black cutworm (*Agrotis ipsilon*, BCW). In most of the trials, the treatments included SmartStax, TC 1507, MON 89034, and a non-Bt control (the single gene products were also typically stacked with a corn rootworm trait).

WBC was evaluated at two locations in Iowa and Nebraska using artificial infestation to measure pest abundance (percentage of ears with larvae and number of larvae per plant) and feeding damage (ear tunneling). The trials included SmartStax, TC 1507, MON 89034, and a non-Bt control (both TC 1507 and MON 89034 were also stacked with corn rootworm traits). All of the Bt corn varieties lowered WBC infestation relative to non-Bt corn with SmartStax showing the greatest reduction. Varieties containing TC 1507 performed significantly better than those with MON 89034 alone. Feeding damage followed a similar pattern with SmartStax and TC 1507 outperforming MON 89034. As with the infestation data, all of the Bt varieties had significantly less ear damage than the non-Bt control.

For CEW, experiments were conducted at four locations in Illinois, Indiana, Mississippi, and Wisconsin. As with WBC, artificial infestation was used and pest abundance (larvae per ear) and feeding damage to corn ears was tabulated. The results showed that treatments containing MON 89034 (including SmartStax) significantly reduced larvae per plant and ear damage compared to varieties with TC 1507 or non-Bt corn. In most cases, the infestation and damage was reduced to close to zero. TC 1507 provided some protection against CEW relative to non-Bt corn but not nearly at the levels of MON 89034 or SmartStax.

FAW was tested at two locations using artificial (Indiana) and natural (Mississippi) infestations to whorl stage corn. At each location, leaf feeding damage was assessed using the 0-9 Davis damage scale. The experiments showed that SmartStax significantly reduced FAW feeding damage (to ~ 1.0 on the Davis scale) compared to MON 89034, TC 1507, and a non-Bt control. MON 89034 and TC 1507 also suffered significantly less damage (~ 1.5 Davis scale) than the control (> 8 Davis scale), though they had somewhat more damage (but still statistically significant) than SmartStax.

Efficacy trials for BCW were conducted at a single location in Iowa using artificial infestation. Protection against BCW was measured by stand reduction (i.e. percentage of plants cut and fallen over) at five day intervals. By the end of the experiment (day 15), non-Bt corn had the greatest stand reduction (100%). All Bt treatments limited stand reduction relative to the non-Bt corn, though hybrids containing Cry1F (i.e. TC 1507 and SmartStax) provided the largest protection (20 -29% stand reduction) which was significantly better than MON 89034 alone (60%).

Based on these efficacy studies, Monsanto/Dow concluded that SmartStax should perform in an additive manner against lepidopteran target pests (i.e. exceed the performance of the individual MON 89034 and TC 1507 components). The companies asserted that field efficacy of SmartStax should be sufficient to kill heterozygous larvae

(i.e. those carrying one resistance allele) and justifies a reduced (5%) refuge for lepidopteran corn pests.

BPPD Review - Dose Considerations for Lepidopteran Corn Pests

Given that the components of SmartStax (MON 89034 and TC 1507) have been previously assessed for dose (as part of their initial registrations), the efficacy trials can serve as confirmatory tools -- i.e. to determine that the performance of the pyramid (SmartStax) is at least as high as the individual MON 89034 and TC 1507 components. These studies can also determine if there is an additive effect of combining multiple toxins (Cry1A.105, Cry2Ab2, and Cry1F) targeted at the same lepidopteran pest complex.

BPPD largely agrees with Monsanto that the performance (and expected dose) of SmartStax is equal to or (in many cases) or better than MON 89034 and TC 1507 alone. A wide range of lepidoptera was tested, including a number of secondary corn pests such as black cutworm, sugarcane borer, and western bean cutworm. As can be seen in Table 2 below, SmartStax appears to offer better control against a wider range of pest than the previously registered components. Data from the efficacy trials were also supported by protein expression data (reviewed separately) that confirmed the levels of Cry1A.105 and Cry2Ab2 protein in SmartStax were equivalent to MON 89034.

BPPD notes that the data submitted for SmartStax do not address dose using the criteria established by 1998 SAP (see previous discussion). Therefore, it is not possible to conclude that SmartStax is a "high dose" for any of the target pests. Data previously submitted and reviewed for MON 89034 also did not establish high dose for either Cry1A.105 or Cry2Ab2 (BPPD 2007a). As part of the 2001 Bt crops reassessment (BPPD 2001), TC 1507 (Cry1F) was determined to be a high dose for ECB but not for CEW. While a formal "high dose" classification cannot be made for SmartStax, it is still possible to characterize performance against target pests as having high (i.e. >95% control), moderate, or low activity. Table 2 below summarizes the likely lepidopteran performance profile for SmartStax, MON 89034, and TC 1507 as determined by BPPD.

Table 2. Efficacy of SmartStax and registered components against major lepidopteran corn pests.

	Expected Target Pest Efficacy [1]				
	ECB	CEW	SWCB	FAW	WBC
SmartStax	High	Moderate	High	High	High
TC 1507 [2]	High	Low	High	Moderate	Moderate
MON 89034 [3]	High	Moderate	High	High	Low

[1] Efficacy is estimated from submitted performance data. "High" efficacy would be the equivalent of >95% control; "moderate" = 85 - 95% control; "low" < 85% control.
[2] Efficacy/dose for TC 1507 was evaluated in BPPD 2001 and data submitted to support SmartStax (MRID# 474449-11).

[3] Efficacy for MON 89034 was evaluated in BPPD 2007a and data submitted to support SmartStax (MRID# 474449-11).

1.B. Corn Rootworm

SmartStax contains two toxins that have been registered for use against corn rootworm (*Diabrotica* spp., CRW): Cry3Bb1 (registered in MON 863 and MON 88017) and Cry34Ab1/Cry35Ab1 (a binary toxin complex registered in DAS 59122-7). CRW has historically been a difficult insect to control with Bt toxins and both Cry3Bb1 and Cry34/35 are not considered "high dose" in currently registered PIPs using the established 25X definition (refer to the previous section for the specific definition) (BPPD 2005b, 2007b). Since dose expression of the two CRW components has been previously established in their initial registration, the goal for SmartStax is to confirm that the combination of the two proteins will provide as good (or better) control of the target pests to support the proposed IRM plan. As with the lepidopteran toxins, Monsanto/Dow have cited protein expression data and conducted a series of efficacy trials to address dose. In addition, Monsanto/Dow tested the possibility that the Cry3Bb1 and Cry34/35 proteins might have synergistic or antagonistic activity with CRW.

Protein expression data (reviewed by BPPD separately) indicate that the expression of Cry3Bb1 is SmartStax is comparable to its single gene component (i.e. MON 88017) and is expressed at high levels throughout the growing season, although the protein expression fell later in the season (Stillwell and Silvanovich 2007). Comparable protein expression was also demonstrated for Cry34/35 in SmartStax and Herculex Rootworm root tissues (Phillips 2008). Based on these results, Monsanto/Dow believes that the efficacy of Cry3Bb1 and Cry34/35 in SmartStax should be similar to efficacy of the single gene components.

To test the possibility of synergy or antagonism between Cry3Bb1 and Cry34/35, Monsanto/Dow designed an *in vitro* assay with southern corn rootworm (*Diabrotica undecimpunctata howardi*) (MacRae 2008). This study (also reviewed separately by BPPD) utilized diet bioassays to test larval response (mortality and growth) to treatments of Cry3Bb1, Cry34/35, and a combination of the two toxins. The results of these assays showed that Cry3Bb1 had less activity than Cry34/35, but the two toxins together provided an additive effect consistent with the predicted LC_{50} and GI_{50} (growth inhibition) for each toxin. Based on this work, Monsanto/Dow concluded that Cry3Bb1 and Cry34/35 do not exhibit synergism or antagonism.

Efficacy for CRW was evaluated using seedling assays (conducted in greenhouses) and field trials to measure adult emergence, estimate larval mortality, and determine larval root feeding damage. Monsanto/Dow also cited previous field work (Storer et al. 2006; not included in the SmartStax submission) that showed DAS 59122-7 both reduced adult emergence and caused larval mortality at levels greater than 95%. Another second study was cited for MON 88017 that also showed a purported 96 - 99% reduction in larval emergence, although the data have not been published nor made available in the SmartStax submission.

The seedling assay (Clark and Harrison 2008) was designed to calculate larval mortality and measure adult emergence from exposure to MON 88017, DAS 59122-7, and SmartStax corn lines. Two test systems were used in the experiments: a "single mat" system in which each container contains one plant that makes up the root mat and a "multiple mat" design consisting of containers with ten plants (of the same hybrid) contributing to the root mat.

To assess larval mortality, western corn rootworm neonate larvae were infested onto both types of root mats (50 larvae on V5/V6 plants for the single mat and 40 larvae on V2 plants for the multiple mat). Larvae were exposed for 6, 12, and 18 days (single mat) or 5, 10, 15, and 20 days (multiple mat) before collection by Berlese/Tullgren funnels. Growth was assessed by head capsule measurements and the weight of dried specimens. Larval recovery (data summarized in Table 3 below) for the single mat test systems was higher in conventional corn than any of the Bt corn treatments earlier in the observation period (day 6 and 12), while the opposite was true for the longer exposure period (day 18). For day 6 and 12, 50% and 70% (respectively) of larvae were recovered from conventional corn. This was a greater recovery rate than the approximately 16 - 22% (day 6) and 22 - 58% (day 12) for the Bt varieties (most comparisons were statistically significant). Generally, the Bt treatments containing MON 88017 had less larval recovery than DAS 59122-7, though the difference was significant only for day 12. By day 18 the trend reversed with larval recovery in the non-Bt control at about 5%, which was significantly lower than the observations for Bt corn. Again, more larvae were recovered from DAS 59122-7 (ca. 48%) than from MON 88017 (ca. 23%) or SmartStax (ca. 26%). For the multiple mat system, there were no statistically significant differences in larval recovery between non-Bt control and Bt treatments on days 5 and 10 (recovery was 30 - 42% on day 5 and 17 - 34% on day 10). In the later exposure periods (days 15 and 20), there were significantly fewer larvae (12 and 7%, respectively) recovered from conventional corn than Bt corn (similar to the single mat results). There were almost no significant differences in larval recovery between the Bt treatments on day 15 or 20. Monsanto/Dow attributed the general drop in larval recovery over the exposure periods (particularly in the non-Bt control) to the onset of pupation and mortality.

Table 3. Summary of larval recovery and beetle emergence data from Clark and Harrison (2008). Derived from data contained in the SmartStax submission (MRID# 474449-11)

Test System	Treatment	Larval Recovery (% of original infestation)[1]				Beetle Emergence (%)[2]
Single Root Mats		6 days	12 days	18 days		
	Non-Bt	50	70	5		67
	DAS 59122-7	22	58	48		44
	MON 88017	16	22	23		20
	SmartStax	21	28	26		21
Multiple Root Mats		5 days	10 days	15 days	20 days	
	Non-Bt	42	26	12	7	16
	DAS 59122-7	36	34	26	18	9
	MON 88017	30	17	21	16	6
	SmartStax	41	24	27	17	2

[1] Percent larval recovery approximated from Figures 1 and 7 in Clark and Harrison (2008).
[2] Beetle emergence approximated from Figures 4 and 10 in Clark and Harrison (2008).

Both measures of larval growth (larval dry weight and head capsule width) showed clear distinctions between the non-Bt and Bt test groups. Larvae from non-Bt mats (both single and multiple plant mats) weighed significantly more than those collected from Bt mats at comparable observation periods (only one comparison between non-Bt and DAS 59122-7 on day 5 for the multiple mat was not statistically significant). A similar pattern held for head capsule width: larvae recovered from conventional corn had larger head capsules than those from DAS 59122-7, MON 88017, or SmartStax. The differences were statistically significant except for a few comparisons late in the testing (day 18 and 20). In general, larvae exposed to SmartStax showed more growth reduction (weight and head capsules) than MON 88017 and DAS 59122-7, though the differences were not always statistically significant.

Adult beetle emergence from the mats was assessed by percent emergence (out of the total infested), cumulative emergence (time to complete emergence), and thorax width. Cumulative emergence can determine any delays in adult eclosion with the test groups and thorax width can establish physiological fitness costs due to treatment exposure. In both the single and multiple mat systems, beetle emergence was significantly lower for the Bt corn groups than for conventional corn (overall beetle emergence was higher in the single plant mats than the multiple plant mats). Among the Bt corn varieties, SmartStax had the lowest beetle emergence (21% for single mats and 2% for multiple mats). MON 88017 also had relatively low emergence (20% for single mats and 6% for multiple mats), while DAS 59122-7 was somewhat higher (44% and 9%) followed by non-Bt corn (67% and 16%). For cumulative emergence, beetles from non-Bt corn emerged sooner than those from Bt sources in both test systems. In the single mat tests, SmartStax delayed complete emergence the longest followed by DAS 59122-7 and MON 88017, while in the multiple plant mats there was little difference between the Bt corn types. A number of the emergence delays between non-Bt corn and SmartStax were substantial -- up to two weeks for 50% emergence in the single plant mat test. Beetle thorax widths were statistically smaller for both DAS 59122-7 and SmartStax relative to MON 88017 and non-Bt control in both test systems, with the size differences ranging from approximately 0.1 to 0.18 mm.

Field efficacy was investigated in three separate experiments (Huckaba and Storer 2008; Vaughn 2008; and Huckaba et al. 2008). These studies were conducted in 2006 and/or 2007 and measured CRW beetle emergence to determine comparative efficacy between SmartStax, DAS 59122-7, and MON 88017.

The first field trial (Huckaba and Storer 2008) was conducted during 2007 at 6 locations in Illinois (2 locations), Indiana, Iowa, Minnesota, and Nebraska. Treatments included SmartStax and its intermediates including MON 88017, DAS 59122-7, and DAS 59122-7 x MON 88017. An herbicide tolerance trait (NK 603) was included with some of the treatments and a non-Bt isoline was used as a control group. Test plots were selected with low natural infestation (mostly rotated cropland) to avoid confounding estimates of larval control. Western corn rootworm (*Diabrotica virgifera virgifera*, WCRW) eggs

were artificially infested into plots at a rate of 3300/meter. Adults emerging from test areas were collected in screen cages erected over each plot in 3-4 day intervals during the experiments. Larval feeding damage was also assessed by sampling plant roots with the Node Injury Scale (0-3) rating. Dose (essentially larval mortality) was determined using the methods of Storer et al. (2006) by dividing the observed number of emerging adults by the expected number and correcting for density dependent mortality.

Root damage ratings for the trials showed that all of the CRW-protected Bt treatments significantly reduced damage compared to the non-Bt control. There were no statistical differences between the Bt groups, although MON 88017 and SmartStax had somewhat less damage than DAS 59122-7. Adult emergence was also significantly lower for the Bt treatments relative to the control group. The median number of WCRW emerging from non-Bt plots was approximately 1000 compared to < 50/plot for almost all of the Bt treatments. Again, there were no statistically significant differences between the Bt treatment groups. Several outlier groups were excluded from the subsequent dose estimations presumably due to planting errors or faulty emergence cages; however, no verification of these experimental errors was reported in the submission.

Since there was variance in adult emergence between testing sites, a dose calculation was performed for each location. The percent reduction in adult emergence (relative to non-Bt corn) for the Bt treatments was at least 94% in all cases (the reduction in most of the treatments/locations exceeded 99%). Dose estimates (calculated by eliminating density dependent mortality in the control group) exceeded 99% for all Bt treatments and locations (the lowest estimated dose was 99.238%). The dose calculations for SmartStax treatments (MON 88017 x DAS 59122-7) were generally higher than the single gene component treatments (MON 88017 and DAS 59122 alone) and in almost all cases exceeded 99.9%.

The second field study for CRW efficacy was conducted by Vaughn et al. (2008) and also included trials for lepidopterans (reviewed in the preceding section). The rootworm experiments were conducted at nine total locations in Illinois, Indiana, and Iowa during 2006-2007 and relied on natural pest infestations. Feeding damage on corn roots (by WCRW) was compiled using the 0-3 Node Injury Scale (NIS) and adult emergence was determined by using cages over test plots to capture newly emerged beetles. Treatments included DAS 59122-7, MON 88017, MON 88017 x DAS 59122-7 (with no lepidopteran toxins), SmartStax, and non-Bt controls.

Data from the trials showed that both MON 88017 and DAS 59122-7 (single trait products) significantly reduced adult beetle emergence compared to non-Bt control groups. Both SmartStax and MON 88017 x DAS 59122-7 often reduced beetle emergence further than the single trait products alone. In many cases, the differences between treatment groups were statistically significant. Beetle emergence ranges for SmartStax and MON 88017 x DAS 59122-7 treatments were 0 - 19.5 adults/plot in 2006 (compared with 48.8 - 117.8 in non-Bt corn) and 0.5 - 18.0 adults/plot in 2007 (compared with 20.8 - 157.0 in non-Bt corn). These figures would correspond to percent reductions

in beetle emergence ranging from 100% (SmartStax treatment in Ames, IA location during 2006) to 72.1% (SmartStax treatment in Flatville, IL location during 2007).

Feeding (root) damage followed a similar pattern with the Bt treatments (single traits and pyramided lines) significantly reducing damage relative to non-Bt controls. In most cases there were no significant differences between the Bt treatments and node injury scores were typically less than 0.4 (NIS for control plants usually exceeded 1.0). Generally, DAS 59122-7 treatments suffered slightly more injury then test groups with MON 88017, though the differences were not statistically significant except in one case.

The final field trial (Huckaba et al. 2008) was conducted in 2007 at six locations in Illinois, Indiana, Iowa, and Nebraska. As with the other field trials, SmartStax and single trait intermediates (MON 88017 and DAS 59122-7) were included in the tests. Artificial infestation of WCRW was employed and efficacy was determined through NIS ratings. Root damage for the non-Bt control plants was highest (avg. 1.27) among the treatments and all Bt lines had NIS below 0.25 (no statistical differences between events). However, the results from three of the six locations were excluded because the NIS was less than 1.0 on non-Bt control plants.

Based on the submitted data, Monsanto/Dow concluded that Cry3Bb1 and Cry34/35 are each capable of reducing adult emergence by 97% and producing larval mortality of about 99.7%. SmartStax (with the combined traits) reduced emergence by 99% and caused morality at approximately 99.4%. Further, no synergism or antagonism was detected in any of the studies.

BPPD Review - Dose Considerations for Corn Rootworm

Overall, the efficacy data submitted by Monsanto/Dow demonstrate that both Cry3Bb1 and Cry34/35, either alone or together, significantly reduce both feeding damage and adult emergence compared with non-Bt corn. Generally, lines expressing both Cry3Bb1 and Cry34/35 (i.e. SmartStax) performed better than those expressing only a single trait suggesting a lack of antagonism between the proteins. In addition, protein expression data confirm that the protein levels in SmartStax are comparable to those expressed in the single toxin products. However, BPPD notes that the submitted data for Cry3Bb1 (Stillwell and Silvanovich 2008) showed a drop in protein expression later in the growing season. For example, dry weight expression of Cry3Bb1 in both SmartStax and MON 89034 root tissues at the R1 stage was less than half the level expressed at the V2-V4 stage. On the other hand, data for Cry34/35 (Phillips 2008) generally showed consistent expression in SmartStax and DAS 59122-7 throughout the growing season.

Although Cry3Bb1 and Cry34/35 are clearly efficacious against CRW, BPPD is not convinced that the activity (reduced adult emergence, larval mortality) meets or exceeds the 97 - 99% levels suggested by Monsanto/Dow. That conclusion appears to have been largely based on the Huckaba and Storer (2008) study. However, a second field study submitted by Monsanto/Dow (Vaughn et al. 2008) showed greater variability in adult emergence. In this study, significant beetle emergence was noted for both the single

traits (up to 60 beetles/plot for MON 88017 and up to 74 beetles/plot for DAS 59122-7) and the pyramided events (as many as 19 per plot for MON 88017 x DAS 59122-7 treatments). In some plots, the reduction in emergence relative to the control was less than 90% (as low as 72.1% in one case). BPPD notes that the Vaughn et al. study relied on natural infestation, so it is not possible to definitively calculate percent population reduction by tabulating adult emergence. Also, other adult emergence data (Storer et al. 2006) showed high reduction in adult emergence (close to 97%) from DAS 5122-7 plots. Still, BPPD is concerned that the actual level of control exerted by MON 88017 x DAS 59122-7 is less than 99% as claimed by Monsanto/Dow.

BPPD also notes that in the greenhouse seedling studies both CRW larvae and adults were recovered from root mats containing MON 88017 and DAS 59122-7. In all cases, Cry3Bb1 and Cry34/35 reduced the numbers of larvae and adults recovered (and also delayed emergence and decreased larval growth) relative to non-Bt corn. However, a sizable number of larvae (15 - 57%, depending on the test day) were retrieved from the Bt treatments, which seemingly indicates some degree of survival (though it cannot be assumed that all recovered larvae were exposed to the Bt proteins). Further, some beetle emergence was observed on Bt treatments (up to 44% in a single mat experiment for DAS 59122-7), though for most of the Bt test groups total emergence was less than 10%. While the seedling assay system is a simplified version of true agronomic conditions (and likely to underestimate actual field mortality), the results suggest that an assumption of at least 99% reduced beetle emergence and/or larval mortality for SmartStax is likely too high.

Previous dose classifications for both Cry3Bb1 (in MON 863 and MON 88017) and Cry34/34 (in Herculex Rootworm and Herculex Xtra) are "less than high dose" (see BPPD 2005a, b; BPPD 2007b), suggesting that the toxins exert less than 99% control at the levels expressed in Bt corn. BPPD also notes that data submitted for northern corn rootworm revealed mortality as low as 92.8% (BPPD 2005b). In consideration of these factors, BPPD recommends that a broader range of potential control levels be considered (e.g. 85 - 95%) for model simulations and other refuge analysis for SmartStax corn (refer to the modeling section later in this review).

2. Cross Resistance Potential for SmartStax Corn Toxins

Cross resistance is an important consideration for PIPs with pyramided toxins. The expression of two or more toxins against the same target pests can be beneficial for IRM provided that there is no cross resistance between the toxins (Roush 1998). On the other hand, if two toxins in a pyramid have significant cross resistance potential, the event could essentially be functional as a single trait PIP in which resistance to either toxin would compromise the product.

SmartStax contains multiple toxins for both lepidopteran and corn rootworm active pest complexes. These include Cry1A.105, Cry2Ab2, and Cry1F for lepidoptera and Cry3Bb1 and Cry34/35Ab1 for corn rootworm. Given the differences in mode of action, cross resistance has to be evaluated separately for each group of toxins.

2.A. Cross Resistance Potential for Lepidopteran Toxins

Monsanto/Dow's submission for SmartStax cited previously submitted data including Head (2006, 2008) and Schlenz et al. (2008) that were reviewed during the registration of MON 89034 (EPA Reg. No. 524-575). The registrants concluded that based on these data, there is evidence that Cry1A.105, Cry2Ab2, and Cry1F have three distinct modes of action with little or no cross resistance potential.

BPPD Review - Lepidopteran Cross Resistance Potential

As noted by Monsanto/Dow, cross resistance for the lepidopteran-active toxins (Cry1A.105, Cry2Ab2, and Cry1F) in SmartStax was largely assessed during the registration of MON 89034. In BPPD's assessment of the product, it was confirmed that Cry1A.105 and Cry2Ab2 had little cross resistance potential for the target pests based on protein structure, amino acid sequence homology, competitive midgut membrane binding assays (with ECB), and experiments with resistant colonies (BPPD 2007a).

Other toxins currently registered in Bt corn PIPs were also evaluated for cross resistance as part of the MON 89034 review including: Cry1F (also expressed in SmartStax), Cry1Ab, and Cry1Ac. BPPD's assessment of cross resistance is thoroughly discussed in the MON 89034 reviews (See BPPD 2007a and 2008) and is summarized in Table 4 below. Overall, low or no cross resistance potential is expected between Cry1A.105, Cry2Ab2, and Cry1F.

Table 4: Cross resistance potential of Cry1A.105 and Cry2Ab2 with previously registered Bt corn toxins (taken from BPPD 2008).

	Bt toxins in MON 89034	
Existing Bt toxins	**Cry1A.105**	**Cry2Ab2**
Cry1Ab	No cross resistance (ECB, SCB)	No cross resistance (ECB)
Cry1Ac	Cross resistance unlikely, but unverified experimentally	No cross resistance (TBW, PBW, CEW/CBW)
Cry1F (TC 1507)	Low level cross resistance (ECB, FAW)	No cross resistance (ECB, FAW)

[CEW/CBW = corn earworm/cotton bollworm; ECB = European corn borer, FAW = fall armyworm; PBW = pink bollworm; SCB = sugarcane borer; TBW = tobacco budworm]

While the low likelihood of cross resistance among the SmartStax proteins was determined during the registration of MON 89034, BPPD still has reservations about Cry1Ac, a protein registered in some Bt cotton PIPs. BPPD notes that Cry1A.105 (a chimeric protein) contains domains I and II and the C-terminal from Cry1Ac and that structural similarities between Bt toxins could lead to cross resistance. Monsanto (the registrant of MON 89034) has argued that Cry1Ac should be expected to behave like Cry1Ab due to a similar mode of action. Therefore, no experimental data (i.e. binding

studies or bioassays with resistant insect colonies) were provided either in the original MON 89034 IRM data (Head 2006) or the follow-up submission (Head 2008). BPPD's primary concern is that successive generations of CEW may feed on both corn and cotton during the same growing season, which could result in a potential "double" exposure to Bt cotton and Bt corn (including Cry1A.105). This could result in increased selection pressure for resistance, particularly if there is a risk of cross resistance between Cry1Ac and Cry1A.105. Because of this, a requirement remains as part of the terms and conditions of the MON 89034 registration for further analysis of possible cross resistance between Cry1Ac and Cry1A.105. Since SmartStax is derived from MON 89034, the need to fully assess cross resistance for Cry1Ac is also applicable to this product.

2.B. Cross Resistance Potential for Corn Rootworm Toxins

Although both the CRW-active toxins Cry3Bb1 (MON 863/MON 88017) and Cry34/35Ab1 (Herculex RW) have been registered by EPA, the cross resistance potential between the two has not been previously determined. For the SmartStax application, Monsanto/Dow have assessed cross resistance by analyzing two aspects of the proteins: 1) sequence and structural similarities (or lack thereof) and 2) midgut binding properties between the two proteins.

For structural comparisons, Monsanto/Dow cited existing protein characterization work for Cry3Bb1 and Cry34/35Ab1 (Galitsky et al. 2001; Ellis et al. 2002; Herman et al. 2002; Schenepf et al. 2005). Cry34/35Ab1 is a binary toxin with two components (14 and 44.3 kDas) that work together to produce CRW activity. On the other hand, Cry3Bb1 is a single peptide (65 kDa) Bt toxin with three domains that is structurally dissimilar to binary toxins. Other analysis of Bt protein domains and antibody detection responses have failed to reveal similarities between Cry3Bb1 and Cry34/35 that would suggest similar receptor site utilization in the midgut.

In addition to structural analysis, Monsanto/Dow initiated two experiments to test the midgut binding properties of Cry3Bb1 and Cry34/34Ab1. The first study (Li and Zhou 2008) was conducted by Monsanto to test the potential for cross resistance by antibody detection and comparative toxin binding in WCRW brush border membranes (BBM).

For the antibody detection assay, primary and secondary IgG antibodies to Cry3Bb1, Cry34Ab1, and Cry35Ab1 antigens were developed in test mammals (the Cry34/35 binary proteins were tested individually). Test preparations of the Bt proteins were incubated with the antibodies (the secondary antibodies were stained with infrared fluorescent dye for detection). The results showed that the antibodies bound with their matching antigens with no cross reactivity between proteins, though a weak interaction of the Cry34Ab1 antibody with Cry35Ab1 was noted at the higher concentrations tested.

The BBM binding analyses were conducted with ligand blotting and competitive ligand blotting experiments. SDS-PAGE was used to separate BBM of third instar larvae, which were then transferred to nitrocellulose (NC) membranes for treatment with Cry3Bb1, Cry34Ab1, or Cry35Ab1. Protein was allowed to interact with the NC

membranes under optimized binding conditions. Bound proteins were incubated with fluorescent dyed antibodies for detection by scanner (different color dyes were used for Cry3B1 and Cry34/35) similar to the antibody detection assay described above. The major BBM/toxin binding complexes were different for Cry3Bb1 and Cry34/35: Cry3Bb1 bound to 20, 100, and 250 kDa bands on the NC strip, while Cry34/35 bound to 35, 90, and 160 kDa bands. However, there appeared to be several similar minor bands between the two proteins on the NC strips including bands at approximately 50 and 115 kDa, suggesting that there may be some common binding sites in the WCRW midgut.

Competitive ligand blotting was conducted using similar procedures as above but with two protein exposure to the BBM NC membranes. One protein (either Cry3Bb1 or Cry34/35) was exposed to the membrane for a period of time followed by the second protein. Similar detection antibodies were used (as above) with red bands for Cry3B1, green bands for Cry34/35, and orange-red bands for common (shared) binding complexes. In trials where Cry3Bb1 was incubated followed by Cry34/35, only the 100 kDa Cry3Bb1 band was visible. On the other hand, the opposite incubation (Cry34/35 first, followed by Cry3Bb1) showed that all three Cry3Bb1 binding bands (20, 100, and 250 kDa) were visible in addition to the Cry34/35 bands (35, 90, and 160 kDa). Based on the results, Monsanto concluded that the 100 kDa band represents a unique binding site for Cry3Bb1 (no competition with Cry34/35) and that the other bands (20 and 250 kDa) are also likely unique Cry3Bb1 binding sites.

The second midgut binding experiment was coordinated by Dow (Zhuang 2008) using similar SDS-PAGE techniques as Li and Zhou (2008). WCRW midguts were dissected from larvae and homogenized and solubilized prior to overlay on nitrocellulose membranes and incubation with Bt cry proteins (Cry3Bb1, Cry34Ab1, and Cry35Ab1). Each protein was tested individually and Cry34Ab1 and Cry35Ab1 were also tested as a mixture. Detection was accomplished by treatment of the membranes with Cry3Bb1, Cry34Ab1, and Cry35Ab1 IgG antibodies and use of a chemiluminescence kit.

Gel results from the protein overlay assays showed that Cry34Ab1 had unspecific (and presumably weak) binding patterns, though bands were detected at 42, 60, 160, and 220 kDa. Stronger bands were detected at 65 and 70 kDa in the Cry35Ab1 assay. Both of these bands were also observed with the Cry34Ab1 and Cry35Ab1 mixture, suggesting that Cry35Ab1 was responsible for binding without interference from Cry34Ab1. Like Cry34Ab1, Cry3Bb1 was found to have non-specific binding patterns. However, one band was observed at 68-70 kDa that had a similar molecular weight as the 70 kDa band observed for Cry35Ab1. Dow concluded though that the band patterns were different and not likely representative of the same binding protein. Overall, the authors indicated that the binding cite analyses provide evidence that Cry3Bb1 and Cry34/35 are likely bind to different proteins in WCRW midgut.

BPPD Review - Corn Rootworm Cross Resistance Potential

After reviewing the data and analyses provided by Monsanto/Dow, BPPD concludes that Cry3Bb1 and Cry34/35 are unlikely (though not definitively) to have significant cross

resistance potential. BPPD agrees with Monsanto/Dow that there are key structural differences (e.g. single peptide vs. binary toxin) between Cry3Bb1 and Cry34/35 and that WCRW midgut binding analysis generally revealed dissimilar binding patterns for each protein.

On the other hand, BPPD notes that some of the binding site analysis appeared to be inconclusive. For example, both studies identified potential shared binding sites between Cry3Bb1 and Cry34/35. In Li and Zhou (2008), shared bands were observed at 50 and 115 kDa and at 68-70 kDa in Zhuang (2008), though the evidence for these bands was weaker than for other detected binding complexes. Each study also identified different patterns of banding for the same protein despite using similar experimental techniques. To illustrate, Li and Zhou (2008) showed Cry34/35 binding complexes with weights of 35, 90, and 160 kDa while, Zhuang (2008) detected Cry34/35 bands of 65 and 70 kDa (with weaker bands of 42, 60, 160, and 220 kDa). The reason for these discrepancies is unclear, as are any potential implications for cross resistance.

The potential for cross resistance can also be assessed by utilizing resistant colonies and testing their response to different toxins (as had been done for lepidopteran-active Bt toxins). BPPD notes that conducting bioassays with CRW in laboratory settings is difficult -- the life cycle and biology of the insects are not conducive to rearing or testing with artificial diets. However, in a recent study Meihls et al. (2008) were able to quickly select (within three generations) resistance to Cry3Bb1 with WCRW reared in greenhouses. If such a colony could also be tested with Cry34/35, further evidence of the cross resistance potential (or lack thereof) could be obtained.

3. Modeling

Monsanto/Dow note that previous modeling (Roush 1998) indicates that pyramided PIPs containing two or more toxins with high activity and no cross resistance that are targeted against the same pest complex can reduce the likelihood of resistance and the necessary refuge. For SmartStax, two separate pest complexes (lepidopteran stalk borers and corn rootworm) are targeted by multiple toxins. Modeling for the lepidopteran toxins Cry1A.105 and Cry2Ab2 was previously conducted for the registration of MON 89034 (see Gustafson and Head 2008a; reviewed in BPPD 2008) and was sufficient to support a 5% refuge. SmartStax also includes Cry1F as a third lepidopteran active toxin to further reduce the potential for resistance.

To address CRW, Monsanto/Dow conducted two modeling projects. The first of these was a deterministic model coordinated by Monsanto (Gustafson and Head 2008b) designed to evaluate various scenarios and include "realistic" and "worst case" parameters. The second effort (Storer 2008) was sponsored by Dow and included both a deterministic model and a stochastic spatially-explicit model to assess CRW adaptation to SmartStax corn.

3.A. CRW Model #1: Gustafson and Head (2008b)

A simulation model was developed by Monsanto (Gustafson and Head 2008b) to assess the potential use of a 5% refuge for CRW with SmartStax corn. It is similar in design to previously-designed models including those developed to support natural refuge for Bollgard II cotton and a 5% lepidopteran refuge for MON 89034 corn. The model structure is deterministic and incorporates two toxins (Cry3Bb1 and Cry34/35) both as single toxin PIPs (MON 88017 and DAS 59122-7) and pyramided together (SmartStax). A number of parameter assumptions were included in the model:

- Dose mortality for CRW: Both Cry3Bb1 and Cry34/35 are assumed to be 95-99% effective;
- Any resistance developing to Cry3Bb1 and/or Cry34/35 is complete (i.e. survival probability of heterozygote resistant individuals = 1) with no fitness costs. Resistance is assumed to be controlled by a single diallelic gene for each toxin;
- Heterozygotes' (i.e. individuals with one resistance allele) survival probability is two or five times that for homozygote susceptible insects (higher heterozygote fitness equates with less recessive resistance);
- No cross resistance is assumed between Cry3Bb1 and Cry34/35;
- Resistance alleles frequencies (initial) for Cry3Bb1 and Cry34/35 ranged from 0.005 to 0.01;
- Single gene PIPs (MON 888017 and DAS 59122-7) were assumed to have a refuge of 20%; SmartStax (MON 88017 x DAS 59122-7) was assumed to have a 5% refuge;
- CRW has no natural refuge (i.e. wild hosts or other cultivated crops that could serve as a source of susceptible insects) and has one generation per year on corn;
- A range of market share adoption values for MON 888017, DAS 59122-7, and SmartStax were included in the model simulations. MKT 1 = 100% SmartStax; MKT 2 = 50% SmartStax, 25% MON 88017, 25% DAS 59122-7; MKT 3 = 0% SmartStax, 50% MON 88017, 50% DAS 59122-7. No other registered CRW PIPs (i.e. MIR 604 expressing mCry3A) were included.

Monsanto believes that most of the assumptions for the parameters above are conservative estimates based upon the best available evidence for CRW and the PIP traits. Simulations were run to estimate resistance (R) allele frequency and the time to resistance (defined as R-allele frequency > 0.5) for a maximum of 30 years (the time limit of the model). The model included scenarios for each of the market share parameters described above. Within each market scenario, model runs were conducted for the variable dose (i.e. 95, 97, or 99% efficacy) and heterozygote survival (2x or 5x that of susceptible homozygotes) assumptions.

Results - Gustafson and Head (2008b)

A truncated summary of the model output is contained in Table 5 below (without individual summaries for each efficacy scenario -- those data are included in Tables 5-8 of Gustafson and Head 2008b). Simulation results in the model were affected by the

varying parameters used for heterozygote fitness, initial resistance allele frequency, toxin efficacy, and market adoption.

Heterozygote fitness (set at either 2 or 5x the survival rate of susceptible homozygotes) appeared to have a large impact on years to resistance. In most of the scenarios with lower heterozygote survival (HF=2), resistance failed to evolve within the 30 year time horizon of the model. However, for the higher survival parameter (HF=5), resistance frequently developed in less than 30 years (in many cases, less than 15 years). These results were also influenced by the initial resistance allele frequency. In scenarios with the higher R-allele frequency (0.01), resistance developed sooner than in simulations with the lower frequency (0.005). Toxin efficacy also appeared to have a measurable effect on resistance. Many of the simulations in which efficacy was set at 99% were slower to develop resistance than those with 97 or 95% efficacy (in many cases, scenarios assuming 99% effectiveness failed to develop resistance within 30 years). Other than the initial efficacy setting, there appeared to be little difference between the toxins (Cry3 and Cry34/35) in the model output (i.e. both toxin appeared to perform the same under similar heterozygote survival and initial resistance allele frequency assumptions).

Market adoption of SmartStax and the corresponding single gene PIPs (MON 88017 and DAS 59122-7) also had an effect on the development of resistance. Generally (but not always), the addition of SmartStax to the model (in scenarios "MKT 1" and "MKT 2") increased the time to resistance (relative to "MKT 3" with no SmartStax). In some of the simulations with HF=5, the adoption of SmartStax in combination with single gene PIPs (MKT 2) resulted in a shorter time to resistance than comparable simulations with no SmartStax (MKT 3). Monsanto attributed this effect to less total refuge -- in "MKT 3" all PIPs have a 20% refuge, while in "MKT 2" the 50% SmartStax market share has a 5% refuge -- but noted that for the most part the "differences are trivial."

Overall, the simulations that assumed low heterozygote fitness (HF=2) and low initial resistance allele frequency (0.005) performed best in the model. In these cases, resistance failed to develop within 30 years, regardless of market adoption or toxin efficacy. Conversely, scenarios with the higher heterozygote fitness (HF=5) and resistance allele frequency (0.01) performed worst in terms of time to resistance. These simulations evolved resistance much more quickly (in some cases in as few as 13 years), though high toxin efficacy (99%) typically delayed resistance beyond 30 years. Both toxin efficacy and market adoption also had more of an impact in the model runs with the higher fitness and resistance allele parameters. However, in the submission Monsanto contended that these higher HF and R-allele assumptions represent "unrealistic, worst case scenario(s)."

Table 5: Results of Monsanto's model simulations for SmartStax (assuming 5% refuge) and single toxin products MON 88017 and DAS 59122-7 (assuming 20% refuge) expressed in years to resistance (30 year maximum). Results were pooled across all of the efficacy scenarios simulated. Derived from data reported in MRID# 474449-01.

Toxin		Market/HF scenario[1]					
		HF = 2			**HF = 5**		
		MKT 1	MKT 2	MKT 3	MKT 1	MKT 2	MKT 3
R-allele[2] = 0.01	Cry3	>30	28 - >30	26 - >30	16 - >30	14 - 29	13 - >30
	Cry34/35	>30	28 - >30	26 - >30	16 - >30	14 - 29	13 - >30
R-allele[2] = 0.005	Cry3	>30	>30	>30	20 - >30	16 - >30	15 - >30
	Cry34/35	>30	>30	>30	20 - >30	16 - >30	15 - >30

[1] Marketing scenarios included: MKT 1 = 100% SmartStax; MKT 2 = 50% SmartStax, 25% MON 88017, 25% DAS 59122-7; MKT 3 = 0% SmartStax, 50% MON 88017, 50% DAS 59122-7. HF = heterozygote fitness (survival relative to susceptible homozygotes).
[2] R-allele = initial resistance allele frequency.

3.B. CRW Model #2: Storer (2008)

A second modeling effort submitted to support SmartStax was developed by Dow AgroSciences (Storer 2008) and actually consists of two separate models with different approaches: 1) a deterministic, non-spatial model (not rootworm specific); and 2) a stochastic, spatially explicit model (rootworm specific). Both models were created to evaluate CRW resistance development to SmartStax in environments both with and without single toxin products (MON 88017 and DAS 59122-7).

Deterministic Model

The deterministic model was a general population genetics model for two toxins (i.e. two resistance loci) in a two patch (i.e. Bt and non-Bt) environment. Though not specifically designed for corn rootworm, the model was designed to compare dual and single gene PIPs with different doses and refuge sizes. Parameter assumptions in the model included the following:

- Complete random mating between Bt and non-Bt patches;
- No fitness costs for genotypes in refuges or homozygote resistant insects;
- No cross resistance between the two PIP toxins;
- Refuge size: 0 or 5% (pyramided PIP) and 20% (single gene PIP);
- Dose mortality: 90 - 99.9% (for each of the toxins);
- Functional dominance of resistance alleles: 0.01 - 1 (for each locus);
- Initial resistance allele frequency: 0.001 for each toxin;
- Allele frequency for resistance (i.e. model termination): 0.1.

Model simulations assessed changes in the resistance allele frequencies for the toxins up to 0.1 (assumed to be resistance) for a time period of up to 150 years. Results from the model runs showed that under all conditions, a 5% refuge with pyramided toxins evolved resistance more slowly than a single gene PIP with a 20% refuge. However, when varied both the dose and functional dominance of the resistance alleles had a measurable impact on the results -- resistance evolved in the pyramid more quickly for lower doses (e.g. 90%) and higher functional dominance (values closer to 1.0). Still, under the worst case assumptions (i.e. 90% dose and dominance = 1.0), resistance evolved more slowly for the pyramid than the single gene PIP. For scenarios with the highest dose (i.e. 99.7%), resistance did not develop in the simulations for the pyramid when the functional dominance was below 0.05 or lower (resistance developed to the single gene PIP in 20 generations at the 0.05 dominance level). With parameter values described by Dow as conservative estimates (97% dose mortality and 0.1 functional dominance), resistance evolved in 68 generations for the pyramid with 5% refuge compared to 15 generations for the single gene PIP (20% refuge). Only when the functional dominance was close to 1.0 were the pyramid and single gene PIPs comparable in time to resistance. Based on the model output, Dow concluded that the results demonstrated the better durability of two gene PIPs over single gene PIPs and confirmed similar model findings by Roush (1998).

Stochastic Model

A more complex stochastic model was also designed by Dow to evaluate the durability of SmartStax corn. This model incorporated a mosaic of single gene PIPs including MON 88017 (Cry3Bb1) and DAS 59122-7 (Cry34/35). Dow suggested that MIR 604 (a separate CRW-protected PIP expressing modified Cry3Aa protein) is functionally equivalent to MON 88017 if one assumes similar dose profiles and complete cross resistance between the two toxins. The parameter assumptions for the model included the following:

- No cross resistance between Cry3Bb1 and Cry34/34;
- Refuge: 20% for single gene PIPs (MON 88017 and DAS 59122-7), varied from 0 to 20% for SmartStax;
- Dose mortality (standard): 99.75% for single trait PIPs, 99.95% for pyramid (SmartStax);
- Dose mortality (worst case): 99% for single trait PIPs, 99.9% for pyramid (SmartStax);
- Fitness determined by genotype for each toxin -- homozygote resistant have complete resistance (fitness = 1.0), homozygote susceptible (fitness = 1.0 - toxin dose), heterozygotes have intermediate fitness;
- Susceptible adults emerging from Bt fields have a 7 day developmental delay; no developmental delay for resistant insects; heterozygote developmental delay calculated by functional dominance;
- Fecundity was artificially increased to prevent population extinctions in the simulations;
- Spatial design: 10 x 10 grid of 25 ha fields; crop types (single trait or pyramid PIPs) were either randomized each year or fixed;

- Toxin mosaic: 50% MON 88017, 50% DAS 59122-7 (baseline case); variable levels of pyramid (up to 100%) were simulated;
- Initial resistance allele frequency: 0.001 for each toxin;
- Allele frequency for resistance: 0.1.

The model was run to assess the time to resistance (generations until the resistance allele frequency exceeded 0.1) under the varying dose, refuge, and toxin mosaic scenarios described above (five model runs were conducted for each scenario). Resistance allele frequency was also measured after 10 generations in case populations went extinct during the simulation. The overall time horizon for the model was 50 years (generations).

Dow noted that in many of the simulations, population extinctions resulted, presumably due to the high effectiveness of the pyramided PIP assumed in the model. For the baseline case (i.e. 50% each of the single trait PIPs; dose = 99.75%), resistance (R allele > 0.1) was reached in 23.8 generations. Introducing the pyramided PIP to the landscape increased the time to resistance regardless of the refuge size. In all cases, the higher the proportion of the pyramid relative to the single gene PIPs, the longer it took to develop resistance to the toxins. CRW populations in simulations that included 80 or 100% adoption of the pyramid (with a 5% refuge) went extinct before 50 generations, while populations at other adoption levels (33 and 50%) evolved resistance in ca. 30-45 generations. A comparison between refuge sizes (0, 2, 5, 10, and 20%) for the pyramid at adoption levels of 33 and 50% showed only small differences in the number of generations to resistance. At 33% adoption of the pyramid, time to resistance ranged from ca. 35 - 39 generations, including the 0% refuge scenario. For 50% adoption, resistance developed in ca. 43 - 50 generations for all refuge sizes. Generally, larger refuges (i.e. 20%) increased the time to resistance; however, the differences were small and even 0% refuge provided similar protection to a 20% refuge in the simulations.

The "worst case" scenario (with lower dose mortalities for the toxins) produced somewhat different results. As with the standard dose simulations, when 80 or 100% of the landscape was devoted to the pyramided PIP the CRW populations went extinct within 50 generations. However, with no pyramid (i.e. 50% each of the single traits) resistance evolved in 11 generations and for lower adoptions of the PIPs (33 and 50%) resistance developed within ca. 15 - 20 years (compared to >35 years for the higher dose simulations). A separate simulation with crop fields fixed (i.e. fields remained single trait or pyramided PIP throughout the model run) revealed generally similar patterns as other scenarios: no resistance evolved when the pyramid adoption was 100%, while resistance evolved in ca. 22 to 48 generations for other adoption levels. Dow believes that a fixed field scenario is unrealistic with actual agricultural practices.

Dow (Storer 2008) concluded that their stochastic model simulations demonstrated the durability of a 5% refuge for SmartStax corn. Further, the results also appeared to indicate that refuges greater than 5% for SmartStax would not significantly increase durability, largely due to the larger (20%) refuges for the single trait PIPs in the landscape mosaic having a larger impact on resistance evolution. Dow believes this model represents a conservative approach to assessing the development of resistance by

assuming: 1) complete use of CRW-protected corn in the landscape, 2) complete resistance (i.e. resistant CRW have survival on Bt corn comparable to susceptible CRW on non-Bt corn), and 3) no fitness costs for resistant CRW.

BPPD Review - Modeling

BPPD agrees with Monsanto/Dow that pyramided PIPs containing two or more toxins targeted at the same pest complex offer potential as a superior resistance management tool (assuming high toxin efficacy and no cross resistance) and could provide a rationale for reducing refuge sizes originally designed for single gene PIPs. Both the Monsanto and Dow models suggest that, under certain circumstances, CRW resistance can be delayed for SmartStax corn with the use of a 5% structured refuge. However, BPPD is concerned that some of the parameter assumptions (particularly those for dose mortality) in the models may be unrealistic and could have influenced the conclusions about durability from the simulations.

Monsanto's model (Gustafson and Head 2008b) and Dow's deterministic model (Storer 2008) simulated a range of potential "doses" for the CRW toxins in SmartStax. These dose mortalities included a range of 95 - 99% (Monsanto model) and 90 - 99.9% (Dow deterministic model) efficacy for each of the toxins (Cry3Bb1 and Cry34/35Ab1). Dow's stochastic model included doses of 99% ("worst case") and 99.75% for the single trait PIPs and 99.9% ("worst case") and 99.95% for the pyramided SmartStax PIP. In all of the models, Dow/Monsanto indicated that the higher dose estimates (i.e. 97% or greater) are realistic based on field data. The model simulations run with >99% dose values (e.g. Dow's stochastic model) essentially assumed that each trait was at or close to high dose levels, though neither Cry3Bb1 (in MON 88017) nor Cry34/35 (in DAS 59122-7) have been considered "high dose" as defined by the 1998 SAP.

The dose assumptions used in the models (particularly those developed by Dow) are derived from the reduction in CRW adult emergence due to Cry3Bb1 and Cry34/35 that were observed in Huckaba and Storer (2008) (reviewed earlier in this document). These data showed high larval reduction and, when corrected for density dependent mortality in control plots, estimated "doses" for Cry3Bb1, Cry34/35, and SmartStax (Cry3Bb1 + Cry34/35) exceeded 99%. Other published work (Storer et al. 2006) and data submitted to support initial registration of DAS 59122-7 (reviewed in BPPD 2005b) also noted similar reduction in larval emergence due to Cry34/35. By itself, this method of estimating CRW dose (field emergence data corrected for density dependent effects) appears to indicate that dose mortalities of 99% would be reasonable for simulation modeling. However, BPPD notes that other data submitted to support SmartStax suggest that the actual efficacy due to the toxins may be lower.

Field studies conducted by Vaughn et al. (2008) showed varied adult emergence relative to isoline corn. In some plots beetle emergence was reduced 100% (e.g. SmartStax treatment in Ames, IA location during 2006). On the other hand, significant number of CRW emerged from Bt treatments in other test plots. For both MON 88017 and DAS 59122-7, adult emergence exceeded 30% of the levels in control corn at three locations in

2007 (with a high of 48% for DAS 59122-7). During 2006 tests, the percent adult emergence on MON 88017 relative to emergence on conventional corn at the five test locations was 7.8, 15.2, 19.3, 28.7 and 60%. For DAS 59122-7, percent relative emergence was 4.1, 9.1, 18.5, 21.6, and 40.9% at the same locations. Beetle emergence on SmartStax treatments (Cry3Bb1 + Cry34/35) were generally less than 10% relative to control corn, although five locations tested in 2006 and 2007 exceeded that level with 11.5 to 27.9% beetle emergence compared to isoline corn. BPPD recognizes that the Vaughn et al. study was conducted with natural infestation and that dose estimates (like those conducted in Huckaba and Storer 2008) are not possible with this data set. The data were also variable by location and year. Nevertheless, the significant adult production observed in this study could indicate that the mortality due to MON 88017 or DAS 59122-7 is lower than the 97 - 99% level assumed by Monsanto/Dow.

A second efficacy study submitted for SmartStax also suggests that the dose mortality for Cry3Bb1 and Cry34/35-expressing corn may be less than the model assumptions. This greenhouse study utilized corn seedling "root mats" to calculate larval mortality and measure adult emergence from exposure to MON 88017, DAS 59122-7, and SmartStax corn lines. As with the field studies, Cry3Bb1 and Cry34/35 corn reduced the numbers of larvae recovered and adults emerged relative to non-Bt corn (and also delayed emergence and decreased larval growth). However, a sizable number of larvae (15 - 57%, depending on the test day) were retrieved from the Bt treatments which seemingly indicates some degree of survival (though it cannot be assumed that all recovered larvae were exposed to the Bt proteins). Further, some beetle emergence was observed on Bt treatments (up to 44% in a single mat experiment for DAS 59122-7), though for most of the Bt test groups total emergence was less than 10%. While the seedling assay system is a simplified version of true agronomic conditions (and likely to underestimate actual field mortality), the results suggest that a dose assumption of 97 to 99% for MON 88017 or DAS 59122-7 may be too high.

BPPD recognizes the difficulty in assessing dose or larval mortality for CRW targeted Bt toxins. Laboratory bioassays are difficult due to unsuitable artificial diets and the biology of the insect. Direct assessments of larval mortality are complicated by soil - root environments that are difficult to observe. Field studies must take into account factors such as abiotic conditions, environment/climate, agronomic circumstances, natural pest infestations, and density dependent responses. Moreover, at present there is still uncertainty with regard to the mode of action of Bt toxin in CRW PIPs, with some evidence that repellency/deterrence may be more important than acute toxicity. Differential toxin expression in roots could also affect the dose encountered by feeding larvae.

BPPD agrees with Monsanto/Dow that Cry3Bb1 and Cry34/35 provide strong protection against CRW, whether alone or in the SmartStax pyramid. Some evidence exists that the level of control of each protein is at least 97%. However, other studies (referenced above) suggest that the level of control is likely lower. Further, data have generally been lacking for other CRW species (notably northern corn rootworm); although data previously submitted for Cry34/35 indicated that the mortality for northern corn

rootworm may be slightly lower than WCRW (BPPD 2005b). In light of the factors discussed above, BPPD at present considers the true dose profiles of Cry3Bb1 and Cry34/35 to be uncertain. Therefore, BPPD cannot definitively conclude that the dose mortality due to Cry3Bb1 (as expressed in single trait PIP MON 88017) and Cry34/35 (as expressed in DAS 59122-7) exceeds 99% as assumed in the modeling (i.e. Storer 2008).

BPPD is particularly concemed about dose for SmartStax because it appears to be one of the more sensitive parameters in the model simulations. Monsanto's model (Gustafson and Head 2008b) included scenarios with toxin doses of 95, 97, and 99%. In scenarios that assumed 95% dose effectiveness for the two toxins, the time to resistance was often much lower than in scenarios with 97 or 99% dose assumptions. These differences were more drastic in the simulations in which the initial resistance allele frequency and/or heterozygote fitness were set to higher values (0.01 and 5x, respectively). Depending on the market scenario, resistance evolved in 14 to 16 years, compared with 27+ years for simulations with 99% dose parameters. Under these R-allele frequency and heterozygote fitness assumptions, even the simulations with 97% dose evolved resistance much more quickly than the 99% cases (16-18 years vs. 28+ years). BPPD notes, however, that in the simulations with a lower R-allele frequency (0.005) and heterozygote fitness (2x), dose had little impact on time to resistance. Both of Dow's models (Storer 2008) also revealed impacts of dose on the output. The deterministic model included a range of doses (90 to 99.9%) for the pyramid. Simulations run with these doses and assuming a 5% refuge for the pyramid showed that resistance could evolve more quickly for the lower doses (90 and 97%) depending on the functional dominance of the resistance trait (there was little difference between doses > 99%). The stochastic model included two higher dose scenarios: 1) 99.75% (single traits) and 99.95 (pyramid); and 2) 99.0% (single traits) and 99.9 (pyramid). In the higher dose simulations, when adoption of the pyramid was at least 33%, the time to resistance was at least 30 years (resistance did not evolve when adoption was > 80%). On the other hand, when the dose was slightly lowered (dose scenario 2), the time to resistance dropped to ca. 15-20 years for 33 and 50% pyramid adoption scenarios (as with the first dose scenario, resistance did not evolve at the higher >80% adoption levels).

Given the uncertainty with CRW toxin dose calculations and the importance of the dose parameters in the models, BPPD recommends that additional information on dose be provided to justify the assumptions used in the modeling. Alternatively, Monsanto/Dow could include a broader range of dose assumptions in the model to investigate the impact of lower doses on the evolution of resistance. By including a broader range of doses in the models, the registrants could add more conservatism to the analysis in lieu of generating additional dose studies that may be difficult to conduct. BPPD recommends including dose ranges of 85 - 95% for the single traits and doses as low as 90% for the two trait pyramid. It is assumed that lower dose values will decrease the time to resistance, though it would be useful to determine the magnitude of any such loss in durability due to lower dose estimates.

BPPD has also noted recent selection experiments with Cry3Bb1 (Meihls et al. 2008) in which tolerance to Cry3Bb1 was selected relatively quickly (within 3 generations). [A

separate selection study for Cry34/35 (Lefko et al. 2008) resulted in low level survival to the toxin within 9 generations, although the trait could not be fixed and was not considered to be "major" resistance.] Meihls et al. Cry3Bb1 research also suggested that the resistance trait could have non-recessive inheritance. Although more work is needed to further characterize potential resistance to these two toxins, these studies could have implications for determining resistance allele frequencies and heritability of resistance. Monsanto's model (Gustafson and Head 2008b) simulated two frequencies (0.01 and 0.005) that are reasonably conservative. The initial resistance allele frequency assumed in Dow's models (Storer 2008) was lower (0.001). BPPD is concerned that the quick selection of resistance observed by Meihls et al. could be indicative of resistance alleles that are more common in CRW populations than previously assumed (though the researchers did not determine a putative resistance allele frequency for their selected colony). Further, the ease with which resistance was selected does not suggest that Cry3Bb1 is expressed at "high dose" levels (such as those used in the Storer models). To the extent possible, BPPD recommends including findings such as Meihls et al. (2008) into the analysis as appropriate.

In addition to the discussion of the dose parameters above, BPPD has further comments on the Monsanto and Dow models.

BPPD generally agrees with Monsanto that conservative assumptions were used in the Gustafson and Head (2008b) model. Simulations included conservative estimates of resistance allele frequency (0.01) and heterozygote fitness (5x survival of homozygous susceptibles) as well as realistic assumptions for natural refuge (none assumed), resistance (no fitness costs for resistant individuals), and cross resistance (none assumed between Cry3Bb1 and Cry34/35). However, BPPD notes that several of the parameters could have been expanded or could have included an additional degree of conservatism or refinement to improve the model analysis. For example, the model assumed only a 5% refuge for SmartStax -- a range of potential refuges (i.e. 5 to 20%) were not considered. Separate simulations with 5% to 20% refuges for the pyramid would have been useful for comparative purposes. In all likelihood, the time to resistance would be increased for larger refuges, although the magnitude of such an increase is unknown (particularly in the scenarios with the higher R-allele and fitness assumptions). If the differences between refuges sizes been small, it could be argued that there is little value gained in having a 20% refuge versus a 5% refuge. Also, mCry3A (registered as MIR 604 corn) was not included in the market adoption scenarios, although it could be argued (as was done by Dow) that this toxin could be functionally the equivalent of Cry3Bb1 is complete cross resistance is assumed.

Dow's stochastic model (Storer 2008) provides a more complex simulation analysis of SmartStax with an added spatial dimension. This model showed that, if the critical dose assumptions hold, the SmartStax pyramid should have strong durability with a 5% refuge and market adoption above 50%. The model also suggests that durability is only slightly affected by refuge size; there was little difference between 0 and 20% refuges for 33 and 50% market adoption of the pyramid (again, assuming that the high dose mortality assumptions are realistic). Several other parameters (no fitness costs for resistance and

developmental delays on Bt corn) also add some conservatism to the model. However, the initial R-allele frequency (0.001) was "arbitrarily" selected; in light of recent selection experiments (i.e. Meihls et al. 2008) a more conservative value may be warranted. BPPD notes that Monsanto selected higher frequencies (0.005 and 0.01) for their deterministic model. BPPD recommends conducting simulation runs with Dow's model and higher R-allele frequencies to determine any effects on the model output.

BPPD has summarized the major parameter values for each of the models submitted for SmartStax in Table 6 below.

Table 6. A comparison of the parameters and assumptions used in the simulation models to support SmartStax (Gustafson and Head 2008b and Storer 2008)

Parameter	Monsanto (Gustafson and Head 2008b)	Dow #1 (Storer 2008)	Dow #2 (Storer 2008)	BPPD comments
Model type	Deterministic	Deterministic	Stochastic, spatially-explicit	
PIPs toxins included	Cry3Bb1, Cry34/35, Smart Stax (Cry3Bb1 + Cry34/35)	Cry3Bb1, Cry34/35, Smart Stax (Cry3Bb1 + Cry34/35)	Cry3Bb1, Cry34/35, Smart Stax (Cry3Bb1 + Cry34/35)	mCry3A (MIR 604) not specifically included but was assumed to be comparable to Cry3Bb1 in Dow's models
Marketing scenarios	MKT 1 = 100% SmartStax; MKT 2 = 50% SmartStax, 25% MON 88017, 25% DAS 59122-7; MKT 3 = 0% SmartStax, 50% MON 88017, 50% DAS 59122-7.	Not specified	0, 33, 50, 80, 100% adoption of the pyramid were included	BPPD assumes that Dow model #1 has equal market adoption of CRW PIPs
Refuge	Single trait PIPs: 20% Pyramid: 5%	Single trait PIPs: 20% Pyramid: 0 or 5%	Single trait PIPs: 20% Pyramid: varied from 0 to 20% in some runs; most runs assume 5%	A broader range of refuges for the pyramid may need to be considered (i.e. 5 to 20%)
Cross resistance	No cross resistance assumed between Cry3Bb1 and Cry34/35	No cross resistance assumed between Cry3Bb1 and Cry34/35	No cross resistance assumed between Cry3Bb1 and Cry34/35	This is a reasonable assumption based on submitted data

Parameter	Monsanto (Gustafson and Head 2008b)	Dow #1 (Storer 2008)	Dow #2 (Storer 2008)	BPPD comments
Dose mortality	Simulations with 95, 97, or 99% dose mortality (for each toxin) were included	Varied from 90 to 99.9% in one simulation; other simulations assumed >97% dose (single traits)	Scenario 1: 99.75% (single traits) and 99.95 (pyramid); Scenario 2: 99.0% (single traits) and 99.9 (pyramid)	A broader range of dose mortalities should be considered (i.e. 85 - 95% for single traits, 90 - 99% for pyramid)
Initial resistance allele frequency	0.01 or 0.005	0.001	0.001	Monsanto's model provides a more conservative R-allele frequency assumptions
Fitness	Resistance is complete with no fitness costs; heterozygotes assumed to have 2x or 5x survival of homozygote susceptibles	Resistance is complete with no fitness costs	Homozygote resistant have complete resistance (fitness = 1.0), homozygote susceptible (fitness = 1.0 - toxin dose), heterozygotes have intermediate fitness	BPPD agrees complete resistance with no fitness costs is a conservative assumption
CRW biology assumptions	1 generation per year; no natural refuge	1 generation per year; random mating between Bt and refuge; infinite population size	1 generation per year; susceptible adults emerging from Bt fields have a 7-day developmental delay; fecundity was artificially increased to prevent population extinctions	Assumptions of random mating and no natural refuge are reasonable
Model time limit and R-allele frequency considered "resistance"	30 years; R-allele > 0.5	150 years; R-allele > 0.1	50 years; R-allele > 0.1	

BPPD Review - Overall Proposal to Reduce Refuge and Other IRM Considerations

Monsanto/Dow have proposed a significant reduction (by 75%) in the amount of refuge to manage potential CRW resistance to Cry3Bb1 and Cry34/35 (from 20% structured refuge to 5%). The major basis for this reduction is the use of both the Cry3Bb1 and Cry34/35 toxins in a pyramid to target the same pest complex (CRW). Modeling by

Roush (1998) and Storer (2008; deterministic model) has shown that two toxins deployed in a pyramided PIP can reduce the amount of refuge needed to manage potential resistance. These models predict that a pyramided PIP is superior than single trait PIPs provided that the two toxins have high efficacy (i.e. 95% mortality against homozygous susceptibles and 70% for heterozygotes) and do not have cross resistance.

BPPD agrees with Monsanto/Dow that cross resistance is not likely between Cry3Bb1 and Cry34/35. As discussed in section 2 of this review, available evidence on protein structure and binding sites suggest that Cry3Bb1 and Cry34/35 act independently. However, BPPD cannot definitively rule out potential cross resistance since there were some minor shared binding sites in some of the midgut analyses.

Efficacy (dose) presents a more complex issue for SmartStax. Monsanto/Dow's conclusions from the models (i.e. demonstrating the high durability of SmartStax) were largely derived from simulations run with dose assumptions of 97% or higher for each of the toxins. There is some evidence from field studies to support these assumptions; however, BPPD is concerned that other studies suggest the actual doses may be lower (see the discussion in the previous section). Neither Cry3Bb1 nor Cry34/35 was considered to be a "high dose" toxin when initially registered (2005b, 2007b). Further, expression data submitted by Monsanto (Stillwell and Silvanovich 2008) for Cry3Bb1 showed that protein expression is lower later in the season (i.e. R1 stage) compared to earlier growth stages (i.e. V2-V4). In the models submitted for SmartStax, the dose parameter appeared to be sensitive to variation -- simulations with lower assumed doses typically evolved resistance sooner (in some cases significantly) than runs with higher doses.

In addition to the issue of efficacy, recent research has shown that tolerance to Cry3Bb1 (Meihls et al. 2008) and Cry34/35 (Lefko et al. 2008) can develop relatively quickly in greenhouse and laboratory selection experiments (within three generations for Cry3Bb1 and nine generations for Cry34/35). The tolerance observed in these studies led to moderate levels of increased survival for the selected groups, possible due to incomplete resistance or a minor (tolerance) gene (as described by Lefko et al. for Cry34/35). [It should be noted that Monsanto and Dow's models assumed complete resistance with no fitness effects.] Meihls et al. research also suggested that the resistance trait could have non-recessive inheritance. Monsanto's model considered two fitness levels for heterozygotes (higher fitness indicates less recessive resistance) -- the findings by Miehls et al. may indicate that the simulations with the higher level (5x survival) are more realistic for Cry3Bb1. Implications for selection under field conditions are still unclear (greenhouses are more optimal for CRW rearing), but the studies do reinforce the need for adequate refuges to mitigate potential resistance.

Overall, Monsanto/Dow have made a good case for a 5% refuge for SmartStax corn. The proposal is supported by the development of a two toxin pyramid, data that indicate low cross resistance potential, and simulation modeling that predicts (under certain conditions) high durability of the product. However, as discussed earlier in this review, BPPD is concerned that the supporting models may have used overestimates for dose, a

critical parameter in the simulations. To address this concern, Monsanto/Dow can either: 1) provide additional information or data to justify the use of high dose parameters (> 99%); 2) conduct model simulations using lower dose estimates (i.e. 85-95% for the single trait PIPs and 90-97% for the pyramid).

BPPD also notes that there are several other areas that should be addressed to provide additional support for the proposal. These areas include:

- Not all of the model simulations were conducted to compare 5% vs. 20% refuge for SmartStax; most simulations assumed a 5% refuge for MON 89034. As such it is difficult to assess the value (or risk) of 5% refuge relative to 20% refuge (or other sizes). Additional simulations including 5, 10, and 20% refuges would be useful for comparative purposes.
- Recent selection experiments (i.e. Meihls et al. 2008) suggest that resistance could evolve quickly with non-recessive inheritance. Models could be adjusted to account for these and other similar findings. For example, Storer's stochastic model could assume resistance allele frequencies above 0.001.

Given the magnitude of the proposed refuge reduction (75%), BPPD believes that the supporting evidence for the proposal must be rigorous and scientifically sound. Until the dose issues described in this review are sufficiently addressed, BPPD cannot recommend a 5% refuge for SmartStax corn. The existing 20% refuge paradigm for CRW should be maintained until evidence exists that warrants lower levels. Should Monsanto/Dow satisfactorily address the dose issue, BPPD can consider a 5% refuge for SmartStax corn. Provided that SmartStax is registered, BPPD recommends implementing the appropriate terms and conditions of registration for resistance monitoring (Cry1A.105, Cry2Ab2, Cry1F, Cry3Bb1, and Cry34/35Ab1), grower education, compliance monitoring and assurance, and annual reporting as detailed in the registrations for the relevant single trait PIPs. BPPD also notes that should SmartStax ultimately be registered with two separate refuge requirements (5% for lepidoptera and 20% for CRW), a "common" refuge design will not be possible unless the refuge totals 20%. Separate 5 and 20% refuges would remain an option for growers planting SmartStax corn in the Corn Belt.

In addition to the considerations above, BPPD notes that a 20% lepidopteran refuge will still be applicable in southern regions where cotton is also grown (CRW are not likely to be significant pests in most of these areas). This refuge was previously analyzed and approved for MON 89034 corn (see BPPD 2008).

Monsanto/Dow intend to largely rely on the previously developed programs established for MON 89034 and other Bt corn registrations. The existing monitoring program and remedial plan for MON 89034 (Cry1A.105 and Cry2Ab2), MON 88017 (Cry3Bb1), and Herculex Xtra (Cry1F and Cry34/35) should be applicable to SmartStax. However, BPPD notes that a revised definition of "resistance" may be needed for CRW based on recent research and selection experiments. BPPD is also concerned about compliance for Bt corn PIPs approved with lower refuges. Overall refuge compliance has declined (registrant data, not yet formally reviewed) in recent years and BPPD believes significant

non-compliance could compromise the durability of IRM plans. Given the different refuge strategies for lepidoptera and CRW, BPPD recommends that Monsanto/Dow submit a revised compliance plan specifically for SmartStax to address the various refuge requirements.

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