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

OFFICE OF
PREVENTION, PESTICIDES, AND
TOXIC SUBSTANCES

MEMORANDUM

Date: September 3, 2008

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SUBJECT: EPA Review of Syngenta Seed's Insect Resistance Management Plan for Section 3 Full Commercial Registration of event MIR162 Maize (Bt11 x MIR162 x MIR604) [EPA Reg. No. 67979-RG, MRIDs 471372-12, 471374-07]

TO: Mike Mendelsohn, Regulatory Action Leader
Microbial Pesticide Branch
Biopesticides and Pollution Prevention Division (7511P)

FROM: Jeannette Martinez, Biologist
Microbial Pesticide Branch
Biopesticides and Pollution Prevention Division (7511P)

REVIEWED BY: Alan Reynolds, Entomologist
Microbial Pesticide Branch
Biopesticides and Pollution Prevention Division (7511P)

ACTION REQUESTED:

BPPD[1] has been asked to review Syngenta's Insect Resistance Management plan and supporting data for section 3 registration of Bt11(Cry1Ab)x MIR162(Vip3Aa20)x MIR604(mCry3A) Maize.

[1] The use of BPPD in this review refers to the BPPD IRM team.

I. CONCLUSIONS AND RECOMMENDATIONS

1. BPPD concluded the following about dose studies of Event MIR162 maize submitted for FAW, CEW, and ECB:

- Bt11xMIR162 expresses a high dose against FAW
- Bt11xMIR162 expresses a high dose against ECB
- Bt11xMIR162 expresses an effective high dose against CEW under verification method #4 only. Under verification method #1, Bt11xMIR162 expresses a probable effective high dose based on one replicate only (n=50). Based on what is known about CEW and its high variability in response to toxins, it is questionable whether such a result can be consistently replicated.

- MIR162 alone has no activity against ECB
- MIR162 does not express a high dose against CEW but may express a 'near high dose'
- MIR162 expresses a high dose against FAW

Table 1 BPPD's high dose determination for Bt11, MIR162, and their combined event Bt11xMIR162 against lepidopteran pests based on experimental data provided by Syngenta

Species	Method 1			Method 4		
	Bt11	MIR162	Bt11xMIR162	Bt11	MIR162	Bt11xMIR162
FAW	No high dose	High dose	High dose	Low activity	High dose	High dose
CEW	No high dose[1]	No high dose[1]	Probable effective high dose	No high dose	Near high dose	Effective high dose
ECB	High dose	No activity	High dose	High dose	No activity	High dose

Shaded fields indicate high dose determinations by BPPD for single toxins or stacked *Bt* product

[1] Control mortality was in excess of 10% and as high as 28%; thus the Agency's dose conclusions are more conservative and may differ from Syngenta's reported conclusions.

2. BPPD concurs with Syngenta based on the cross-resistance studies and background information available in the literature that the risk of cross-resistance is minimal between Vip3A and Cry1A toxins and Vip3A and Cry2Ab. 1) Vip3A does not bind to APN and cadherin-like proteins and to Cry2Ab2 non-specific binding sites, and thus, Vip3A does not share binding sites with Cry1A and Cry2Ab toxins; and 2) Vip3A pore channels formed in the midgut of insects are structurally and functionally distinct from Cry1A-type proteins.

3. Syngenta has proposed that a 20% refuge be used to manage insect resistance to Bt11xMIR162 in cotton growing areas rather than the current 50% structured refuge requirement for single gene lepidopteran-control products. The major pest of concern for Bt corn in continental U.S. cotton-growing areas is CEW.

   Syngenta commissioned Dr. Michael Caprio to evaluate the risk of resistance evolving to Bt11xMIR162 maize with a 20% refuge in cotton growing regions and in the presence of

other single gene cotton and corn products. When Bt corn refuge was reduced to 20% in the cotton growing region and no single-gene crop was present, resistance did not evolve to either Cry1Ab or Vip3A. The simulations further suggest that within 25 years, there is little risk of CEW resistance evolving to the Bt11xMIR162 stack whether 50% or 20% non-Bt corn refuge is planted in presence of other single gene cotton and corn products as well as VipCot cotton (Vip3Aa19 is very similar to Vip3Aa20 in MIR162).

Stable isotope analysis of pheromone trapped males from 1997-1999 support that CBW adults feed on a mix of C_3 (i.e. cotton) and C_4 plants (i.e. maize, sorghum and/or wild host) in the early season, while moths caught late in the season predominantly originate from C_4 hosts (Gould et al. 2002). In addition, host utilization data from the southern and southeastern U.S. (2002-2003) support that CBW larvae have been found predominantly on maize throughout the early and mid season and on soybean, tobacco, cotton, maize, and sorghum throughout the later season (Jackson et al. 2008). Authors comment that these alternate host crops provided a stable refuge during the years investigated with USGS/NASS data (1995-2002). Thus, CBW moths are produced on alternative hosts in cotton growing areas that may be available to mate with any putative resistant CBW moths and further dilute resistance. In addition, the cross-resistance data submitted by Syngenta demonstrates that the risk of cross-resistance is minimal between Vip3A and Cry1A toxins and Vip3A and Cry2Ab based on activation studies, receptor binding, competition binding, and ligand-blotting assays, as well as pore-forming studies. And finally, the dose studies show that Vip3A has good activity and that Event MIR162 maize expresses an effective high dose (under method 4) against CEW.

BPPD concludes that all the evidence from the host utilization, cross-resistance, binding, and dose studies supports that a 20% non-Bt corn refuge for Event MIR162 maize in the southern cotton growing areas should suffice to manage the risk of resistance evolution to Bt corn and Bt cotton products.

4. Under the established refuge strategy for stacked Bt trait corn with both lepidopteran and corn rootworm protection, growers can choose from two different planting options to fulfill IRM requirements. **The only refuge options that are acceptable for Bt11xMIR162xMIR604 maize are in-field and adjacent refugia** (common and separate for CRW).

5. Syngenta submitted a resistance monitoring program to the Agency for the MIR162 stack with Bt11 only. BPPD expects that monitoring for CRW will continue as outlined in the mCry3A BRAD (2007) and the terms of conditions of registration. Furthermore, BPPD recommends that Syngenta continue to consider sublethal bioassays (e.g. head capsule measurements) and molecular marker methods for CRW monitoring in addition to mortality assays.

   BPPD notes that Syngenta did not provide very much information about their collaborators and intended monitoring plans for Vip3Aa20 and SWCB, although it is unclear why SWCB monitoring is included here. Vip3Aa20 is not expected to have much if any activity at all against this pest should it behave anything like the ECB. A full

monitoring plan for Vip3Aa20 and Cry1Ab is required under the terms and conditions of the Bt11 registration that should address all of these issues.

BPPD recommends that Syngenta submit a complete monitoring program similar in structure to those established for other Bt corn registrations and for all the target pests as a condition of registration.

BPPD has the following recommendations for Syngenta: if a good amount of effort has been put into developing a discriminating or diagnostic concentration for Vip3A and CEW, and the diagnostic concentration cannot be achieved due to i.e. high-variability in response to the toxin, then a comparison in baseline susceptibility (i.e. LC_{50}s) may be a feasible approach to monitoring. Estimated LC_{50}s may serve well as a baseline monitoring tool for shifts in susceptibility to *Bt* toxins; however, the LC_{50} approach is not useful in discriminating resistant from susceptible individuals. Therefore, this approach must then be linked with follow-up testing of populations with elevated LC_{50}s relative to previously established baseline susceptibility. Other approaches may be feasible as well. Syngenta should describe the monitoring assays and protocols in a final resistance management plan submitted as a condition of registration.

6. In addition to Syngenta's proposed educational outreach in the Grower Education section of this review, BPPD recommends that Syngenta submit a copy of the grower agreement/stewardship documents and written description of a system assuring that growers will sign grower agreement within 90 days from product registration; 2) revise and expand as necessary its education program to take into account information collected through the compliance survey; and 3) maintain records of all signed grower agreements for Event MIR162 maize.

Deficiencies

1. Dose information for SWCB is not presented. In the past, BPPD has asked registrants to provide the Agency with confirmatory data for a new stacked product where its individual events were already registered. These confirmatory data are supposed to show that the stacked product has the same dose profile as its single Bt trait products. This deficiency can be resolved by providing BPPD with efficacy studies and a protein expression report for SWCB.

2. Syngenta did not provide a monitoring plan for FAW. BPPD notes that this deficiency can be corrected by addressing alternative ways to do monitoring (continental U.S.) since the situation for FAW is different from that of other lepidopteran pests. Such an approach could be focused on following up reports of unexpected pest damage.

BACKGROUND

Syngenta's Bt11xMIR162xMIR604 is a stacked transgenic corn trait that expresses the two registered crystal protein toxins Cry1Ab and mCry3A and incorporates the novel Vip3Aa20 Bt toxin (99.9% identical in amino acid sequence to the Vip3Aa19 produced in COT102). The Vip3A is different from Cry proteins as it is produced during vegetative growth of the bacteria, does not form parasporal crystal proteins, and is secreted (but not processed upon secretion) from the cell as a soluble protein. While its physical manifestations of intoxication resemble those of Cry proteins (gut paralysis and lysis of midgut epithelial cells) (Schnepf *et al.* 1998), activated Vip3A does not bind to the same receptors (APN and cadherin-like receptor). These two types of Bt proteins (Vip, Cry) do not appear to share binding sites. Lee et al. (2003) have investigated the mode of action of the Vip3A protein and determined that it involves a number of steps much like the mode of actions for the δ-endotoxins. Following ingestion by the lepidopteran target pest, the Vip3A protein becomes soluble in the gut and is then processed into four dominant bands (retaining activity). The authors propose that this processing is required for the bioactivity of the toxin (activation step). Interaction with the midgut epithelium is the next likely step in the mode of action of Vip3A. However, Vip3A does not bind to APN and cadherin-like glycoprotein receptors. Upon binding to midgut epithelial receptors, data support the existence of a pore-forming step that creates ion channels which are structurally and functionally distinct from those of Cry1Ab. Direct structural information is missing for Vip3A; however, preliminary data do not support the notion that the two proteins share similar domain organization or an α-helical bundle region.

In 2006, the Agency reviewed Syngenta's IRM plan for the stacked product of Bt11xMIR604 Maize and decided based on efficacy and protein expression studies that the IRM programs developed for the individual trait products should also be appropriate for the stacked product (i.e. 20% structured non-Bt refuge in corn growing areas, 50% structured non-Bt refuge in cotton growing areas), and in case of a combined refuge strategy for lepidopteran and coleopteran pests, some modifications should apply (BPPD, 2006c).

Syngenta received an Experimental Use Permit (EUP) to allow field testing of PIP Corn Event MIR162 and its combined trait hybrids, Bt11xMIR162 and Bt11xMIR162xMIR604, in 23 states to cover the period from March1, 2007 through February 29, 2008. Event MIR162 corn expresses the Vip3A insect control protein. The variant protein Vip3Aa20 produced in MIR162 has insecticidal activity against several lepidopteran pests of corn and specifically targets two major corn pests *Helicoverpa zea* (corn earworm, CEW) and *Spodoptera frugiperda* (fall armyworm, FAW) but is also effective against *Diatraea grandiosella* (SWCB). Vip3A does not have insecticidal activity against *Ostrinia nubilalis* (European cornborer, ECB). The toxin Cry1Ab expressed in Bt11 field corn is highly selective and very effective against ECB and SWCB. In addition, Bt11 is also effective against CEW and FAW. The modified toxin mCry3A as expressed in MIR604 has insecticidal activity against two major coleopteran pests of corn, *Diabrotica longicornis barberi* Smith and Lawrence (northern corn rootworm, NCRW) and *Diabrotica virgifera virgifera* (western corn rootworm, WCRW) but no activity against lepidopteran target pests.

The following IRM deficiencies were noted by BPPD and communicated to Syngenta on 8/07/2007. Syngenta submitted a letter in response (1/09/2008), and their rebuttal is provided for each deficiency:

Deficiency 1:
Syngenta has not provided sufficient data to determine the dose of the Cry1Ab toxin expressed in Bt11 maize and the Vip3Aa20 expressed in MIR162 maize, independently and combined as Bt11xMIR162 maize versus *Diatraea grandiosella* (southwestern corn borer). Both the Cry1Ab toxin and the Vip3Aa20 toxin are active against *D. grandiosella*. Expression levels of Cry1Ab in Bt11 maize and Bt11xMIR162 maize were noted as comparable by Syngenta although these data have not been reviewed by BPPD. Syngenta did not discuss the relevance of these expression data to dose. EPA requires data on the dose the plant provides for each of the plant-incorporated protectants (either singly or in combination) on all insect pests. Such data were not provided by Syngenta for Vip3Aa20 expressed in MIR162 maize or combined with Bt11 maize versus *D. grandiosella*, neither were they provided for Cry1Ab expressed in Bt11xMIR162 maize. A high dose is defined as 25 times the protein concentration needed to kill susceptible larvae and is determined by the use of one or more of five imperfect methods to demonstrate that a transgenic crop expresses a high dose of insecticidal protein. Such data are needed to complete BPPD's technical review of Syngenta's proposed IRM strategy for Bt11xMIR162 maize (and subsequently, MIR162xBt11xMIR604 maize).

Syngenta response 1:
...Scientific literature indicates that pyramiding a second plant-incorporated protectant with a currently registered PIP, both having independent activity against the same pest, will be beneficial for resistance management and can even allow for smaller refuge sizes than single protein events (Roush 1998; Caprio 1998; Zhao et al. 2003). Syngenta did not specifically discuss the dose of Vip3Aa20 in Bt11xMIR162 maize for D. grandiosella because no decrease in refuge size was requested below the currently approved 20% non-Bt corn refuge for D. grandiosella. There is no scientific evidence to suggest that by pyramiding an additional PIP with a currently registered PIP that expresses a novel protein which is active against the same pest that an existing refuge requirement will become unsuitable for managing resistance in that pest with the pyramided hybrid. Regardless of the presence of Vip3Aa20 produced by MIR162, Bt11xMIR162 maize has no systematic difference in Cry1Ab levels from Bt11 maize. Thus, the dose of Cry1Ab as produced by Bt11 for D. grandiosella is equivalent to that produced in Bt11xMIR162 maize for D. grandiosella. Consequently, Bt11xMIR162 maize will maintain the insecticidal activity and resistance management capabilities of Bt11 versus target pests and has the additional IRM benefit of producing Vip3Aa20 to further protect maize plants from other lepidopteran pests and resistance development.

Deficiency 2:
Simulation modeling did not consider *D. grandiosella* resistance to Bt11xMIR162 maize.

Syngenta response 2:
Simulation computer modeling results have consistently shown that pyramiding two insecticidal proteins in the same plant that are active against the same pest will be beneficial for resistance management. It is important to note EPA's summary of the primary literature on pyramiding

insecticidal proteins in its Review of Dow AgroSciences' Product Durability Plan in Support of the Section 3 Application for the Mycogen Brand Cry1F/Cry1Ac Construct 281/3006 Cotton; 2004. EPA states that... "Previous modeling efforts by Roush (1198), Caprio (1998), and Zhao et al. (2003), have predicted that the durability of a two-gene stack will always be greater than a single-gene insect control protein." Regardless of the dose of Vip3A20 expressed by Bt11xMIR162 maize, MIR162 will provide extra protection for delaying resistance when pyramided with Bt11 maize, Bt11 will provide extra protection for MIR162, and the existing 20% non-Bt maize refuge will suffice for delaying resistance development in D. grandiosella to Bt11xMIR162 maize nationwide. The following points support the conclusion that the information provided in Syngenta's IRM volumes (MRIDs 471374-07 and 471372-12) is sufficient to support the registrations of the Bt11xMIR162 and Bt11xMIR162xMIR604 and that the proposed IRM plan for each product is scientifically valid: 1) the EPA has already approved the IRM plan for Bt11 maize against D. grandiosella; 2) previous modeling data show that pyramids will always be more durable than single gene PIP's; 3) Bt11xMIR162 maize produces Cry1Ab protein at levels comparable to Bt11 maize; and 4) comparable levels of Cry1Ab in Bt11xMIR162 equate to a comparable dose of Cry1Ab in Bt11.

BPPD's Response to Syngenta's comments dated 1/09/2008:
SWCB is similar in biology to ECB, and therefore, for this particular registration request of the stacked product Bt11xMIR162 maize with 20% IRM refuge plan, BPPD assumes that the efficacy of the stack against SWCB is similar to its efficacy against ECB. No additional modeling is required at the moment since no reduction in refuge size is requested. If in the future, Syngenta requests a reduction in refuge for Bt11xMIR162, BPPD would require dose data as well as additional simulation modeling for SWCB. However, field efficacy data for SWCB and/or a protein expression report are still recommended, perhaps as a condition of registration.

II. PEST BIOLOGY AND ECOLOGY

A Summary of the biology and ecology for two major *Bt* corn lepidopteran target pests, the European corn borer and corn earworm, can be found in the IRM section in the Agency's *Bt* crop reassessment document (EPA 2001) at
http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm.
In 2001, limited pest biology was available for the south-western corn borer (SWCB), fall armyworm (FAW), western corn rootworm (WCRW), and northern corn rootworm (NCRW). The BPPD IRM team reports additional biological information for these lepidopteran and coleopteran pest species.

Biology and Ecology of Southwestern Corn Borer

Host Range: Primary: Corn

Life Cycle: SWCB is multivoltine occurring in the south central U.S. Two generations per year are typically reported; three generations are sometimes possible. The active season for SWCB extends from May through harvest. This insect overwinters in

its larval stage by tunneling into the base of the corn stalk. Pupation occurs with warming temperatures in spring. In the northern regions of its habitat, SWCB does not overwinter particularly well. In these cases, the first generation of SWCB will often be small followed by a larger second generation. Some dispersal by migration (older females) is thought to occur and contribute to periodic extensions of SWCB habitat. The life cycle mirrors another stalk-boring Lepidopteran, the European corn borer.

Larval: For the most part, SWCB larvae remain on their host plant with little interplant movement within the field.

Feeding: The feeding behavior of SWCB is substantially similar to ECB. First generation larvae feed inside the whorl on foliage and can cause the "dead heart" injury. This destruction of the whorl can cause total loss of yield for the plant. Older larvae move down the plant and tunnel into the bottom ⅔ of the stalk, similar to ECB. Second generation larvae cause the most severe damage due to both population dynamics and feeding behavior. As mentioned above, the second generation larvae feed in the leaf axils but also will feed on the primary ears between husks. Older larvae will move to the bottom of the plant in preparation of overwintering and tunnel into the stalk often girdling the plant at the base. This damage is quite destructive and readily causes stem breakage.

Mating: Similar to ECB (see discussion in the Agency's 2001 Bt Crop Reassessment document)

Oviposition: Eggs are laid singly or in groups of 2-5 on upper and lower leaf surface

Biology and Ecology of Fall Armyworm

(Nagoshi & Meagher 2004)

Host Range: Primary: Corn (sweet, field), sorghum, rice, grasses

Life Cycle: FAW is multivoltine throughout most of the U.S. and has 2-6 generations per year throughout the Corn Belt.

The active season for FAW on corn is later in the season from mid June until harvest. The insect overwinters most commonly in the pupal stage in the soil about 20 mm underground, although other life stages such as the larva and adult may also overwinter. FAW pupae are not cold resistant, and in most winters only Southern most populations in the Gulf Coast States survive winter. Populations north of the Gulf Coast are reestablished annually through progressive migrations of overwintering southern adults. Due to the nature of migration, FAW often do not arrive until later in the summer where it can pose threat to late plantings of corn and sorghum.

Larval: After larvae hatch, they feed gregariously on the remnants of the egg mass and then disperse within several days. All larvae are mobile and will readily move to other plants in search of food. Older larvae may move *en masse* to other fields if they are in need of host plants. FAW larvae will tolerate the presence of other larvae on the same host, and multiple larvae on the same plant are not uncommon.

Feeding: Hatching larvae feed on the egg mass remains before dispersing within the plant or to other suitable plants. Small larvae on corn typically move to the whorl and feed on emerging foliage

Mating: Pheromones may play a role in female mate selection. However, temporal partitioning could lead to assortative mating between strains of different host plants as well (i.e. corn-strain females call earlier than rice-strain females). In addition, strain specific mating has been observed to occur at opposite times of the night with no overlap.

Oviposition: Females are attracted to grasses in and about corn fields and to young pre-tassel stage corn plants. Eggs are laid in clusters of 50-100 on usually the underside of leaves. Anywhere from 1000-1500 eggs can be oviposited by a single female. Emerging females often fly for miles before locating a site suitable for ovipositing.

Biology and Ecology of Corn Rootworm (McCaffery et al. 2005; http://www.ipm.uiuc.edu/fieldcrops/insects/corn_rootworm/factsheet.html, http://ianrpubs.unl.edu/insects/ec1563.htm, http://www.cropsci.uiuc.edu/faculty/mgray/publications/2001_Transgenic_Insecticidal_Cultivars.pdf)

Host Range: Primary: Corn, some grasses

Life Cycle: Western corn root worm and Northern corn rootworm have similar life cycles. Insects are univoltine with larvae present from May through July. Adults are abundant from July through September. Rootworm larvae can complete development on corn and a few other grassy species only.

Larval: Mature larvae of the WCRW are approximately ½ inch in length, while larvae of the NCRW are approximately ¼ inch in length. Larvae of both species generally hatch in May, but hatching may vary due to temperature differences and occurs later in northern latitudes as compared to southern latitudes of the U.S. (note that mean emergence of WCRW adults appears to be delayed by about 6 days in MIR604 corn as compared to non-treated corn.)

Feeding: After larvae hatch they begin to feed on root hair of corn plants and later tunnel inside roots. Larvae go through three instars before they begin pupation. Adult

CRW feed on pollen and green silk of later planted cornfields and pollen of soy beans and alfalfa.

Mating: Females remain in the fields from which they emerged, while a small portion of males has been shown to leave native patches; mating occurs primarily within fields rather than between fields. Males emerge three to four days before females, and mating occurs shortly after females are present. Limited long-distance dispersal in adult females can occur but mostly in mated and pre-ovipositional females.

Oviposition: WCRW females need to feed for approximately 2 weeks before they are able to lay eggs. During late summer, they oviposit an average of 500 eggs over several weeks in clutches of approximately 80 eggs in upper soil layers (oviposition ranges from 6" – 12" in depth). This has been found to occur in corn fields but also soybeans in east-central Illinois for WCRW only. Females of the NCRW are less likely to lay there eggs below an 8 inch depth. Both NCRW and WCRW overwinter in the egg stage. Some eggs can remain dormant up to several years which may render crop rotation less effective as a tool to control CRW.

III. DOSE

The determination of dose or the amount of toxin expressed by the transgenic crop relative to the susceptibility of the target pests is a critical component of IRM. Models have shown that a high dose of toxin coupled with a non-transgenic refuge to provide a supply of susceptible insects is the most effective strategy for delaying resistance in *Bt* crops. The high dose/refuge strategy assumes that resistance to Bt is recessive and is conferred by a single locus with two alleles resulting in three genotypes: susceptible homozygotes (SS), heterozygotes (RS), and resistant homozygotes (RR). The high dose/refuge strategy also assumes that there will be a low initial resistance allele frequency and extensive random mating between resistant and susceptible adults. In practice, a high dose PIP should express sufficient quantities of toxin to kill all susceptible insects (SS) as well as heterozygous insects with one resistance allele (RS). Lower dose PIPs might allow for survival of insects with at least one susceptibility allele (SS or RS), and effective IRM may still be possible with a suitable refuge strategy. To be able to demonstrate high dose, it is recommended that registrants generate data by at least two of the five laboratory and field approaches as outlined by the SAP (1998) and described by the Agency in the 1998 Bt Plant-Pesticides and Resistance Management document (US EPA, 1998) and 2001 Biopesticide Registration Action document (US EPA, 2001). For procedures of high dose determination, see Appendix A at the end of this review.

It must be noted that both the high dose definition and verification techniques were developed in 1998 when all of the registered Bt crops were single toxin products targeted against lepidopteran pests. In recent years, PIPs in Bt cotton have been approved that contain two genes targeted at the same insect pest. These "pyramided" products can be beneficial for IRM since target pests must overcome two toxins to develop field resistance to the PIP. The benefits are greatest for

two toxins with unrelated modes of action (i.e. binding to different Bt receptor sites in the midgut) that are expressed at high doses in the plant (Roush 1994; Roush 1998).

For pyramided products, the dose of each toxin should be evaluated separately. This can be easily accomplished if the pyramided product is created through conventional breeding -- in this case, the dose of the single toxin products has already been established and the combined dose in the pyramided PIP can be determined with comparative efficacy studies. However, for pyramids created by non-conventional breeding (e.g. recombinant DNA techniques), defining the dose can be more complicated since single toxin lines may not be available (or commercialized) for comparisons. The dual 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 and can be considered high dose.

To evaluate dose, Syngenta conducted laboratory and field studies to demonstrate the dose status of Event MIR162 maize and its components Bt11 maize and MIR162 maize. Two sets of experiments were conducted for FAW, CEW, and ECB: 1) bioassays with the single proteins expressed in lyophilized plant material and both proteins expressed in lyophilized plant material and combined as Bt11xMIR162 to determine target pest susceptibility, and 2) field tests on Bt11, MIR162, Bt11xMIR162 plants, and control plants using controlled artificial infestation techniques during the 2006 growing season.

Verification Method#1, Results and Discussion:

1. <u>Fall Armyworm (FAW):</u>

<u>A) Bt11 high dose methodology and results</u>
Tests were performed at two Syngenta laboratories, Syngenta Seeds, Inc. Research Center in IA and MN. Seed sources used in the assays were the same across both locations and all three insect species. Three transgenic maize hybrids (Bt11, MIR162, and Bt11xMIR162; 42-45 plants each) and a non-transgenic negative control were green-house grown at each location and provided the leaves for lyophilization.

One negative control and three trials with different concentrations (4% by weight = 25 fold dilution, 2% by weight = 50 fold dilution, 1% by weight = 100 fold dilution) per transgenic treatments were established in commercially available FAW meridic diet. Samples sizes ranged from 40 to 60 neonate larvae (1 larva per well); three total experiments were conducted over time to ensure repeatability of results. Dead larvae were recorded starting between day 10 and 12 and then every two to four days until all larvae were dead or no more mortality occurred in the 25X dilution wells. If mortality did not reach 100% in transgenic treatments, mortality in the transgenic treatments was corrected using Abbott's method.

BPPD notes that Bt11 does not express a high dose with this method and has very little activity against FAW as is apparent by % mortality reported under method #1; mean (corrected) mortality ranges from 1.4% at the 100X dilution to 5.7% at the 25X dilution.

Table 2 Bt11 mortality results for FAW using lyophilized tissue bioassays

Test Material	Lyophilized Dilution	Mean Mortality % (Test 1)	Mean Mortality % (Test 2)	Mean Mortality % (Test 3)	Mean Observed or Corrected Mortality %
Negative control	25X	2.3	0	4.2	2.1
Bt11	25X	17.0	2.1	4.1	5.7[1]
Bt11	50X	5.8	2.1	2.0	1.2[1]
Bt11	100X	4.4	0	0	1.4

[1] mean corrected mortality

B) MIR162 high dose methodology results

For methodology, refer to procedures used for Bt11 and FAW above.

BPPD agrees with Syngenta's conclusion: results support that MIR162 expresses a high dose against FAW under method #1; mean (corrected) mortality ranges from 80.9% at the 100X dilution to 100% at the 25X dilution.

Table 3 MIR162 mortality results for FAW using lyophilized tissue bioassays

Test Material	Lyophilized Dilution	Mean Mortality % (Test 1)	Mean Mortality % (Test 2)	Mean Mortality % (Test 3)	Mean Observed or Corrected Mortality %
Negative control	25X	2.3	0	4.2	2.1
MIR162	25X	**100**	**100**	**100**	**100**
MIR162	50X	100	91.6	91.8	94.2[1]
MIR162	100X	88.6	79.2	76.6	80.9[1]

[1] Mean corrected mortality

C) Bt11xMIR162 high dose methodology results

For methodology, refer to procedures used for Bt11 and FAW above.

BPPD agrees with Syngenta's conclusion: results support that Bt11xMIR162 expresses a high dose against FAW under method #1; mean (corrected) mortality ranges from 88.2% at the 100X dilution to 100% at the 25X dilution.

Table 4 Bt11xMIR162 mortality results for FAW using lyophilized tissue bioassays

Test Material	Lyophilized Dilution	Mean Mortality % (Test 1)	Mean Mortality % (Test 2)	Mean Mortality % (Test 3)	Mean Observed or Corrected Mortality %
Negative control	25X	2.3	0	4.2	2.1
Bt11xMIR162	25X	**100**	**100**	**100**	**100**
Bt11xMIR162	50X	97.7	98.2	100	98.6[1]
Bt11xMIR162	100X	83.0	96.0	86.0	88.2[1]

[1] Mean corrected mortality

2. Corn Earworm (CEW):

A) Bt11 high dose methodology and results

Tests were performed at Syngenta laboratories, Syngenta Seeds, Inc. Research Center in IA. Seed sources used in the assays were the same across both locations and all three insect species.

Three transgenic maize hybrids (Bt11, MIR162, and Bt11xMIR162; 330-440 plants each) and a non-transgenic negative control were green-house grown at each location and provided the silk material for lyophilization.

One negative control and three trials with different concentrations (4% by silk weight = 25 fold dilution, 2% by silk weight = 50 fold dilution, 1% by silk weight = 100 fold dilution) per transgenic treatments were established in commercially available FAW meridic diet. Sample sizes were 50 wells per treatment with one neonate larva per well; three total experiments were conducted over time to ensure repeatability of results. Dead larvae were recorded daily until all larvae were dead or no more mortality occurred in the 25X dilution wells. If mortality did not reach 100% in transgenic treatments, mortality in the transgenic treatments was corrected using Abbott's method.

BPPD agrees with Syngenta's conclusion: results support that Bt11 does not express a high dose against CEW under method #1; mean (corrected) mortality ranges from 19.1% at the 100X dilution to 64.3% at the 25X dilution. There is a relatively large mortality in the control treatment which indicates the presence of some non-controlled effects.

Table 5 Bt11 mortality results for CEW using lyophilized tissue bioassays

Test Material	Lyophilized Dilution	Mean Mortality % (Test 1)	Mean Mortality % (Test 2)	Mean Mortality % (Test 3)	Mean Observed or Corrected Mortality %
Negative control	25X	27.3	28.0	24.0	26.6
Bt11	25X	75.5	70.0	76.0	64.3[1]
Bt11	50X	64.0	52.0	68.0	47.3[1]
Bt11	100X	44.0	28.0	50.0	19.1[1]

[1] Mean corrected mortality

B) MIR162 high dose methodology results

For methodology, refer to procedures used for Bt11 and CEW above.

There is high mortality in the negative controls ranging from 24% to 28%, which implies that the mortality observed in MIR162 transgenic treatments is not caused by treatment effects alone and is confounded by other non-controlled effects. Mean mortality (at 25X dilution) reported by the three independent tests ranges from 66%-82%. BPPD notes that due to higher than preferred control mortality ($\leq$ 28%), MIR162 appears to be less efficacious against CEW than reported by Syngenta. Regardless of control mortality, this method did not demonstrate high dose for MIR162 and CEW.

Table 6 MIR162 mortality results for CEW using lyophilized tissue bioassays

Test Material	Lyophilized Dilution	Mean Mortality % (Test 1)	Mean Mortality % (Test 2)	Mean Mortality % (Test 3)	Mean Observed or Corrected Mortality %
Negative control	25X	27.3	28.0	24.0	26.6
MIR162	25X	66.0	92.0	82.0	72.7[1]
MIR162	50X	52.0	80.0	66.0	53.7[1]
MIR162	100X	50.0	48.0	62.0	36.4[1]

[1] Mean corrected mortality

C) Bt11xMIR162 high dose methodology results
For methodology, refer to procedures used for Bt11 and CEW above.

There is a higher than preferred mortality in the negative controls ranging from 24% to 28%, which implies that the mortality observed in the MIR162 stacked treatments is not caused by treatment effects alone and is confounded by other non-controlled effects. BPPD concludes that Bt11xMIR162 likely expresses an effective high dose for CEW.

Table 7 Bt11xMIR162 mortality results for CEW using lyophilized tissue bioassays

Test Material	Lyophilized Dilution	Mean Mortality % (Test 1)	Mean Mortality % (Test 2)	Mean Mortality % (Test 3)	Mean Observed or Corrected Mortality %
Negative control	25X	27.3	28.0	24.0	26.6
Bt11xMIR162	25X	**100**	**100**	**100**	**100**
Bt11xMIR162	50X	78.0	100	100	90.5[1]
Bt11xMIR162	100X	68.0	64.0	66.0	55.8[1]

[1] Mean corrected mortality

3. European Corn Borer (ECB):

A) Bt11 high dose methodology and results
Tests were performed at Syngenta laboratories, Syngenta Seeds, Inc. Research Center in IA. Seed sources used in the assays were the same across both locations and all three insect species. Three transgenic maize hybrids (Bt11, MIR162, and Bt11xMIR162; 330-440 plants each) and a non-transgenic negative control were green-house grown at each location and provided the leaves for lyophilization.

One negative control and three trials with different concentrations (4% by weight = 25 fold dilution, 2% by weight = 50 fold dilution, 1% by weight = 100 fold dilution) per transgenic treatments were prepared in General Lepidoptera diet from BioServ. Sample sizes were 10 plates with five neonate larvae each; three total experiments were conducted over time to ensure repeatability of results. Dead larvae were recorded daily until all larvae were dead or no more mortality occurred in the 25X dilution wells. If mortality did not reach 100% in transgenic treatments, mortality in the transgenic treatments was corrected using Abbott's method.

There is a higher mortality in the negative controls (10% to 12%) than is preferred by the Agency, which may imply that the mortality observed in Bt11 transgenic treatments may not be caused by treatment effects alone and is confounded by other non-controlled effects. However, 100% mortality at the 25X dilution provides strong evidence for a high dose in Bt11 against ECB.

Table 8 Bt11 mortality results for ECB using lyophilized tissue bioassays

Test Material	Lyophilized Dilution	Mean Mortality % (Test 1)	Mean Mortality % (Test 2)	Mean Mortality % (Test 3)	Mean Observed or Corrected Mortality %
Negative control	25X	10.0	9.8	12.0	10.6
Bt11	25X	**100**	**100**	**100**	**100**
Bt11	50X	68.0	72.0	60.0	54.6[1]
Bt11	100X	50.0	50.0	36.0	25.5[1]

[1] mean corrected mortality

B) MIR162 high dose methodology results

ECB:

For methodology, refer to procedures used for Bt11 and ECB above.

BPPD agrees with Syngenta that MIR162 does not express a high dose and has very little efficacy against ECB. Furthermore, control mortality in the experiments is slightly higher than desirable, which suggests that mortality in MIR162 transgenic treatments may be confounded by other non-controlled effects and actual efficacy of MIR162 against ECB may be lower than results suggest.

Table 9 MIR162 mortality results for ECB using lyophilized tissue bioassays

Test Material	Lyophilized Dilution	Mean Mortality % (Test 1)	Mean Mortality % (Test 2)	Mean Mortality % (Test 3)	Mean Observed or Corrected Mortality %
Negative control	25X	10	8.0	12.0	10.0
MIR162	25X	10.0	12.0	10.0	10.7
MIR162	50X	4.0	0	10.0	4.7
MIR162	100X	4.0	12.0	4.0	6.7

[1] Mean corrected mortality

C) Bt11xMIR162 high dose methodology results

ECB:

For methodology, refer to procedures used for Bt11 and ECB above.

There is a slightly higher mortality in the negative controls (10% to12%) than is preferred by the Agency, which implies that the mortality observed in Bt11xMIR162 transgenic treatments may not be caused by treatment effects alone and is confounded by other non-controlled effects. However, 100% mortality at a 25X dilution provides sufficient evidence for a high dose determination in Bt11xMIR162 against ECB.

Table 10 Bt11xMIR162 mortality results for ECB using lyophilized tissue bioassays

Test Material	Lyophilized Dilution	Mean Mortality % (Test 1)	Mean Mortality % (Test 2)	Mean Mortality % (Test 3)	Mean Observed or Corrected Mortality %
Negative control	25X	10.0	9.8	12.0	10.6
Bt11xMIR162	25X	**100**	**100**	**100**	**100**
Bt11xMIR162	50X	80.0	80.0	68.0	68.8[1]
Bt11xMIR162	100X	58.0	54.0	48.0	39.3[1]

[1] Mean corrected mortality

Verification Method#4, Results and Discussion:

In 2006, each pest was tested in separate trials at two locations (IA, MN). At each location and within each trial, one non-replicated block of four treatments was grown (Bt11, MIR162, Bt11xMIR162, and control); between 50 and 655 plants were grown for controls and transgenic treatments. FAW eggs were all provided by Syngenta Seeds, Inc., MN; CEW larvae were provided by two labs, Syngenta Seeds, Inc., in IA and MN; and ECB eggs were provided by one lab, Syngenta Seeds, Inc., IA. The number of neonate larvae applied to plants was constant within but not across trials and locations; 75 and 77 neonates FAW/plants , 20 and 20 neonates CEW/plant, and 163 and 210 neonates ECB/plant in IA and MN, respectively. Leaf damage and larval survival for FAW were assessed as early as 10 days after the final infestation to prevent significant plant-to-plant migration; ear damage and survivors for CEW were assessed as early as 19 days after the infestation before larvae exited ears to pupate; ECB ear and stalk damage and survivors were assessed as early as 49 days after the infestation.

1. Fall Armyworm (FAW):

Control: A minimum of fifty random samples of plants were evaluated for FAW larvae at both locations. Number of insects observed on control plants in IA and MN were 67 and 222, respectively. The number of survivors per plant was much greater in MN than in IA.

Bt11: A minimum of fifty random samples of plants were evaluated for FAW larvae at both locations because very little activity against FAW was expected by Bt11. Number of insects observed on control plants in IA and MN were 47 and 39, respectively. The number of survivors per plant was greater in MN than in IA.

MIR162: Total number of plants assessed in IA and MN were 604 and 638; at both locations, no survivors were found. The results suggest that MIR162 expresses a high dose against FAW under method #4.

Bt11xMIR162: Total number of plants assessed in IA and MN were 607 and 655; at both locations, no survivors were found. The results suggest that Bt11xMIR162 expresses a high dose against FAW under method #4.

2. Corn Earworm (CEW):

Control: A random sample of approximately 100 plants each was evaluated for CEW larvae at both locations. Number of insects observed on control plants in IA and MN were 184 and 102. The number of survivors/plant appears to be similar in both locations.

Bt11: Total number of plants assessed in IA and MN were 403 and 100, respectively. Number of larvae observed was 424 and 26. The results suggest that Bt11 has some activity but does not express high dose against CEW under method #4.

MIR162: Number of plants assessed in IA and MN were 348 and 426, respectively. Number of larvae observed was 10 and 2. The results suggest that MIR162 has very good activity, at least near high dose, against CEW under method #4.

Bt11xMIR162: Total number of plants assessed in IA and MN were 409 and 440; at both locations, no survivors were found. The results suggest that Bt11xMIR162 expresses an effective high dose against CEW under method #4.

3. European Corn Borer (ECB):

Control: A random sample of 100 and 50 plants was evaluated for ECB larvae at both locations. Number of insects observed on control plants in IA and MN were 75 and 125. The number of survivors/plant is higher in MN (2.5/plant) than in IA (0.75/plant).

Bt11: Total number of plants assessed in IA and MN were 501 and 600; at both locations, no survivors were found. The results suggest that Bt11 expresses a high dose against ECB under method #4.

MIR162: Total number of plants assessed in IA and MN were 100 and 50, respectively; number of survivors found was 85 and 90 and compares to the number of survivors found on control plants. Results indicate that MIR162 not have any activity against ECB.

Bt11xMIR162: Total number of plants assessed in IA and MN were 650 and 601; at both locations, no survivors were found. The results suggest that Bt11xMIR162 expresses a high dose against ECB under method #4.

BPPD's Conclusions on High dose:

To be able to demonstrate high dose, registrants are required to provide data generated by **at least two** of the five laboratory and field approaches as outlined by the SAP (1998) and described by the Agency in the 1998 *Bt* Plant-Pesticides and Resistance Management document (US EPA, 1998; US EPA 2001). The BPPD IRM team's conclusions regarding the activity of the stack Bt11xMIR162 are based on the review of 'dose' data from verification methods #1 and #4 submitted in Syngenta's IRM chapter (MRID 471374-07) and are summarized below. For BPPD's high dose conclusion with respect to single events and verification methods, Table 10 can also be consulted.

- Bt11xMIR162 expresses a high dose against FAW
- Bt11xMIR162 expresses a high dose against ECB
- Bt11xMIR162 expresses an effective high dose against CEW under verification method #4 only. Under verification method #1, Bt11xMIR162 expresses a probable effective high dose based on one replicate only (n=50). Based on what is known about CEW and its high variability in response to toxins, it is questionable whether such a result can be consistently replicated.

- MIR162 alone has no activity against ECB
- MIR162 does not express a high dose against CEW but may express a 'near high dose'
- MIR162 expresses a high dose against FAW

The activity and efficacy of Bt11 against some major pests has already been assessed previously (US EPA 2001). However, Bt11 is one of the events in the MIR162 stack, new efficacy data had to be submitted for this sec (3) registration. BPPD's conclusions about these data are listed here:
Bt11 has low activity against FAW
Bt11 does not express a high dose against CEW
Bt11 expresses a high dose against ECB

Table 11 BPPD's high dose determination for Bt11, MIR162, and their combined event Bt11xMIR162 against lepidopteran pests based on experimental data provided by Syngenta

Species	Method 1			Method 4		
	Bt11	MIR162	Bt11xMIR162	Bt11	MIR162	Bt11xMIR162
FAW	No high dose	High dose	High dose	Low activity	High dose	High dose
CEW	No high dose[1]	No high dose[1]	Probable effective high dose	No high dose	Near high dose	Effective high dose
ECB	High dose	No activity	High dose	High dose	No activity	High dose

Shaded fields indicate high dose determinations by BPPD for single toxins or stacked *Bt* product
[1] Control mortality was in excess of 10% and as high as 28%; thus the Agency's dose conclusions are more conservative and may differ from Syngenta's reported conclusions.

IV. CROSS-RESISTANCE POTENTIAL

Bt11xMIR162 maize is the second Bt corn product with stacked lepidopteran active traits. There are also stacked lepidopteran-active products available in cotton already (i.e. Bollgard II®, VipCot™, and Widestrike®). While these stacks in cotton are for two different Cry proteins, Bt11xMIR162 maize expresses two completely unrelated insecticidal proteins, a crystal protein and a vegetative insecticidal protein. In its submission for the Bt11xMIR162xMIR604 registration request, Syngenta provided data and discussed the potential for cross-resistance for CEW since it is a pest of both corn and cotton in the US. Thus, cross-resistance between similar Cry toxins and Cry toxins and Vip3A is of concern. Cross-resistance potential for ECB was not addressed since the pest is not susceptible to Vip3A. SWCB has a similar biology as ECB and therefore, in absence of any dose data, BPPD assumes per this registration request for a 20% refuge that SWCB has a similar response to the two toxins as ECB. FAW is susceptible to Vip3A but does not show much susceptibility for Cry1Ab.

Analyses of resistance to *Bt* Cry proteins indicate that cross-resistance occurs most often with proteins that are similar in structure (Tabashnik, 1994; Gould et al., 1995). While direct structural information of the Vip3A protein is missing (Lee et al. 2003), this novel *Bt* protein does not share any sequence homology with the known *Bt* Cry protein genes, and the predicted secondary structure give no indication of a similar domain organization or α-helical bundle region within the polypeptide sequence of Vip3A as exists for the Cry proteins. Protein folding blasts reveal that Vip3A may be a pore forming protein that has a structure of β-barrels

(Syngenta unpublished data). In order to further investigate the potential for cross-resistance of Vip3A to Cry proteins, Syngenta examined the mode of action of Vip3A at selected steps critical to the mode of action of Bt Cry proteins: proteolytic activation, receptor binding, and pore forming.

Activation:
Vip3A protein activation studies have shown that proteolysis occurs in the midgut of both susceptible and non-susceptible insects. These data suggest that proteolytic activation is not a key factor in insect toxicity and specificity. Further studies have shown that there are similarities between how Vip3A, Cry1Ac, and Cry2Ab are processed; all three toxins are activated by trypsin or gut juice extracts (Lee et al. 2006). Therefore, a small but theoretical risk of cross-resistance between these toxins exists at this step.

Receptor binding:
Several studies (receptor binding, competition binding, ligand-blotting assays) in the tobacco hornworm (*M. sexta*), corn earworm (*H. zea*) and tobacco budworm (*H. virescens*) have shown that receptors for Vip3A are distinct from those of Cry1Ab, Cry1Ac, and Cry2Ab. In these studies, Vip3A did not bind to aminopeptidase-N (APN) and cadherin-like proteins which are known to be Cry1A receptors. Cry2Ab appears to have non-specific binding properties; nonetheless, in competition binding assays, results indicate that Vip3A does not share binding sites with Cry2Ab. BPPD concurs with Syngenta that the risk of cross-resistance should be minimal between Vip3A and Cry1Ac/b and Vip3A and Cry2Ab2 based on receptor binding studies.

Pore forming:
The pore forming properties of Vip3A are unique: the kinetics of Vip3A pore formation are more than 8 times slower than for equimolar Cry1Ab; pore channels are characterized by long open times and a predominantly open state; stable channels formed by Vip3A differ considerably in their conductance state and cation specificity from Cry1A protein. In addition, Domain I, modulated by Domain III interactions, has been considered responsible for the pore formation steps in the *Bt* Cry protein mode of action. Again, direct structural information is not available for the Vip3A protein, yet, available information gives no indication of a similar domain organization or α-helical bundle region within the polypeptide sequence as exists for the Cry proteins. BPPD agrees with Syngenta that the risk of cross-resistance between Vip3A and Cry1A proteins is minimal based on pore forming studies which show that channels formed by Vip3A are structurally and functionally distinct.

BPPD concurs with Syngenta based on the cross-resistance studies and background information available in the literature that the risk of cross-resistance should be minimal between Vip3A and Cry1A toxins and Vip3A and Cry2Ab. 1) Vip3A does not bind to APN and cadherin-like proteins and to Cry2Ab2 non-specific binding sites, and thus, Vip3A does not share binding sites with Cry1A and Cry2Ab toxins; and 2) Vip3A pore channels formed in the midgut of insects are structurally and functionally distinct from Cry1A-type proteins.

V. MODELING

EPA has used predictive models to compare IRM strategies for *Bt* crops. Because models cannot be validated without actual field resistance, models have limitations and the information gained from the use of models is only a part of the weight of evidence used by EPA in assessing the risks of resistance development. It was the consensus of the 2000 SAP Subpanel (SAP 2001) that models were an important tool in determining appropriate *Bt* crop IRM strategies. They agreed that models were "the only scientifically rigorous way to integrate all of the biological information available, and that without these models, the Agency would have little scientific basis for choosing among alternative resistance management options." They also recommended that models must have an agreed upon time frame for resistance protection. For example, conventional growers may desire a maximum planning horizon of five years, while organic growers may desire an indefinite planning horizon. The Subpanel recommended that model design should be peer reviewed and parameters validated. Models should also include such factors as level of Bt crop adoption, level of compliance, economics, fitness costs of resistance, alternate hosts, spatial components, stochasticity, and pest population dynamics.

Syngenta has proposed that a 20% refuge be used to manage insect resistance to Bt11xMIR162 in cotton growing areas rather than the current 50% structured refuge requirement for single gene lepidopteran-control products. The major pest of concern for Bt corn in the cotton-growing areas is CEW (also know as cotton bollworm when it feeds on cotton), although ECB, FAW, SCB (sugar cane borer) are also sporadic corn pests in cotton-growing areas. As outlined in the 2001 Bt-crop reassessment document (http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm), the cotton growing areas where the 50% structured non-Bt corn refuge is a requirement include the following states: Alabama, Arkansas, Georgia, Florida, Louisiana, North Carolina, Mississippi, South Carolina, and some counties in Oklahoma, Tennessee, Texas, Virginia, and Missouri (for specific county listing, the 2001 Bt crop reassessment can be consulted).

Syngenta commissioned Dr. Michael Caprio to evaluate the risk of resistance evolving to Bt11xMIR162 maize with a 20% refuge in cotton growing regions. In the next few paragraphs, BPPD summarizes the most important features and assumptions of the model, the scenarios modeled, and simulation results for CEW.

Dr. Caprio used a spatially explicit, stochastic population genetic model incorporating parameter uncertainty (max/min value, most likely value, assuming normal distribution) and interaction, two loci, heterogeneous habitats (wild hosts, Bt and non-Bt corn, Bt cotton) with different toxin expression levels in different parts of corn plants, and pest biology/ecology. The model assumed that there were two lepidopteran active Bt traits available for transgenic crops, a Vip3A trait and a Cry1Ab/c trait expressing a high dose for the Vip toxin and a moderate to high dose for Cry toxin in corn and cotton. Both Bt proteins were either expressed in a single gene or in a stacked product; Vip3A, Cry1Ab/c, and VipCot™ and Bt11xMIR162. Dr. Caprio's simulation model incorporated crop utilization data from several studies that indicate that in the south-central U.S., CEW larvae feed on non-crop hosts such as red clover and geranium in spring, the following two generations feed on corn, and the next 1-2 generations move on to cotton and other crop hosts such as soybean and sorghum before getting ready to overwinter.

Several scenarios were modeled and produced the following outcomes:

1) 20% sprayed *cotton* non-Bt refuge with 80% VipCot™, and 50% sprayed *corn* non-Bt refuge with 50% Bt11xMIR162; and
2) 20% sprayed *cotton* non-Bt refuge with 80% VipCot™ and 20% sprayed *corn* non-Bt refuge with 80% Bt11xMIR162; and
3) A series of single gene Bt *cotton* and Bt *corn* (Cry1A) planted along with VipCot™ cotton and Bt11xMIR162 corn stacks

Impact of reducing the non-Bt refuge in cotton growing regions:
When Bt corn refuge was reduced to 20% in the cotton growing region and no single-gene crop was present, resistance did not evolve to either Cry1Ab or Vip3A. The simulations further suggest that within 25 years, there is little risk of CEW resistance evolving to the Bt11xMIR162 stack whether 50% or 20% non-Bt refuge is planted in cotton growing regions.

Impact of single gene events on the longevity of stacked events:
In 80% of the simulations, resistance evolved to Cry1Ab during a 25 year period when a single gene crop was planted. The more single gene crop was planted, the faster resistance evolved to Cry1Ab/c. When no single-gene crop was present, resistance did not evolve to either Cry1Ab or Vip3A.

Based on the simulation results with high dose assumptions for Bt11xMIR162, Dr. Caprio concludes that reducing the structured non-Bt corn refuge in cotton growing regions from 50% to 20% may not lead to increased risk of resistance in CEW to VipCot™ cotton and Bt11xMIR162 maize during the 25 year time frame of the model. BPPD notes that Syngenta's dose results warrant a near-high dose expression for Vip3A against CEW rather than a high dose but a probable 'effective high dose' for the MIR162 stacked product. It is not clear how sensitive modeling results are to the "dose parameter inputs" and how such a slight change in dose input parameter value in conjunction with a reduced refuge requirement in the cotton growing regions would affect CEW resistance.

In addition to Dr. Caprio's modeling efforts and results, further consideration needs to be given to 1) justification for the assumed crop patterns/host availability in the simulation model, 2) cross-resistance potential, and 3) dose for the single toxin and stacked product before a conclusion regarding reduced corn refuge in the cotton growing region can be warranted. Stable isotope analysis of pheromone trapped males from 1997-1999 support that CBW adults feed on a mix of C_3 (i.e. maize) and C_4 plants (i.e. sorghum and/or wild host) in the early season, while moths caught late in the season predominantly originate from C_4 hosts (Gould et al. 2002). In addition, host utilization data from the southern and southeastern U.S. (2002-2003) support that CBW larvae have been found predominantly on maize throughout the early and mid season and on soybean, tobacco, cotton, maize, and sorghum throughout the later season (Jackson et al. 2008). Authors comment that these alternate host crops provided a stable refuge during the years investigated with USGS/NASS data (1995-2002). Thus, CBW moths are produced on alternative hosts in cotton growing areas that may be available to mate with any putative resistant CBW moths and further dilute resistance. In addition, the cross-resistance data submitted by Syngenta demonstrates that the risk of cross-resistance is minimal between Vip3A and Cry1A toxins and

Vip3A and Cry2Ab based on activation studies, receptor binding, competition binding, and ligand-blotting assays, as well as pore-forming studies (see section V in this review). And finally, the dose studies show that Vip3A has good activity and that the MIR162 stacked product expresses an effective high dose (under method 4) against CEW (see section IV in this review).

BPPD concludes that all the evidence together from the host utilization, cross-resistance, binding, and dose studies supports that a 20% non-Bt corn refuge for Bt11xMIR162 in the southern cotton growing areas would be sufficient to manage the risk of resistance evolution to Bt corn and Bt cotton products.

VI. REFUGE STRATEGY

The size, placement, and management of the refuge are critical to the success of the high dose/structured refuge strategy to mitigate insect resistance to *Bt* proteins produced in corn (as well as cotton and potatoes). The 1998 SAP Sub-panel defined structured refuges to "include all suitable non-*Bt* host plants for a targeted pest that are planted and managed by people. These refuges could be planted to offer refuges at the same time when the *Bt* crops are available to the pests or at times when the *Bt* crops are not available." The 1998 Sub-panel suggested that a production of 500 susceptible adults in the refuge for every adult in the transgenic crop area (assuming a resistance allele frequency of 5×10^{-2}) would be a suitable goal. The placement and size of the structured refuge employed should be based on the current understanding of the pest biology data and the technology. The 2000 SAP Sub-panel echoed the 1998 SAP's recommendations that the refuge should produce 500:1 susceptible to resistant insects and that regional IRM working groups would be helpful in developing policies.

Syngenta submitted its reduced refuge request for Bt corn in cotton growing regions for Event MIR162 maize. Syngenta states that their refuge planting options include: separate fields, blocks within fields, and strips across fields. Generally, these refuge options are sufficient for Bt11xMIR162. However, since the final marketed product will be the MIR162 stack with MIR604, refuge options are driven by the requirements for CRW refugia. The only refuge options that are acceptable for Bt11xMIR162xMIR604 maize are in-field and adjacent (also common) refuge. No other refuge option will be permissible because there is evidence of non-random mating for CRW between non adjacent corn fields.

For clarity, BPPD restates the refuge planting options available to Syngenta for Bt11xMIR162xMIR604. These options are taken out of the mCry3A BRAD (20007), a separately registered Bt corn trait (http://www.epa.gov/oppbppd1/biopesticides/ingredients/tech_docs/brad_006509.pdf): under the established refuge strategy for stacked Bt trait corn with both lepidopteran and corn rootworm protection, growers can choose from two different planting options to fulfill IRM requirements. These options include one shared common refuge for both insect groups or separate refuges for each insect group and are briefly summarized below.

Agency approved common refuge option for CRW:

- 20% refuge of total corn acres

- Refuge planted directly next to or within stacked *Bt* corn field
- Refuge can be treated with soil insecticide to control root worm larvae

Agency approved separate refuge option for CRW:

- 20% refuge for corn rootworm planted immediately next to or within *Bt* trait corn; single *Bt* trait lepidopteran corn may be planted in refuge but total acreage is not to exceed 80% of *Bt* lepidopteran corn acres
- Rootworm refuge may be treated with non-*Bt* foliar insecticide for control of late season Lepidopteran pests. But if adult rootworms are present, *Bt* trait corn must be sprayed as well
- 20% refuge for Leptidopteran pests (50% in cotton growing regions); single *Bt* trait rootworm corn may be planted in refuge, but total acreage not to exceed 80% of *Bt* rootworm corn acres
- Lepidopteran refuge may be treated with non-Bt foliar insecticide if economic threshold for late season pests are met; the stacked Bt corn field would not have to sprayed under this option

BPPD recommends that these specific details be applied to MIR162 stacked corn.

VII. RESISTANCE MONITORING PROGRAM

Syngenta submitted a resistance monitoring program to the Agency for the MIR162 stack with Bt11 only. BPPD concludes that monitoring for CRW will continue as outlined in the mCry3A BRAD (2007). Furthermore, BPPD recommends that Syngenta continue to consider sublethal bioassays (head capsule measurements) and molecular marker methods for CRW monitoring in addition to mortality assays. Monitoring for the Cry1Ab toxin has been (and will continue to be) conducted under the Bt11 registration.

Syngenta will work with the USDA cotton pest resistance monitoring program to monitor for resistance and/or trends in increase to Vip3Aa20 in CEW. Syngenta has been working with Dr. Randy Luttrell since 2006 and 2007, respectively, to develop assay methods and baseline Vip3A susceptibility data. Syngenta mentions that it will monitor for resistance in SWCB but does not provide any information beyond that.

BPPD notes that Syngenta did not provide very much information about their collaborators and intended monitoring plans for Vip3A and SWCB. In order to facilitate future communication between BPPD and the registrant, the IRM team makes the following recommendations for monitoring procedures: Syngenta should use the diagnostic concentration (LC_{99}) for Vip3A if the approach has proven successful, and the pest is susceptible to toxin and population variance is small. In addition, follow-up testing of larval survivors needs to be conducted for all toxins where field population survivorship on a diagnostic concentration is significantly different from lab/reference colony's survivorship. Further, BPPD recommends that Syngenta submit a final Vip3Aa20 monitoring plan for the major target pests (CEW, SWCB, FAW) as a condition of registration.

BPPD has the following recommendations for Syngenta specifically for CEW (but not only): if a good amount of effort has been put into developing a discriminating or diagnostic concentration for CEW and Vip3A and the diagnostic concentration cannot be achieved due to i.e. high-variability in response to the toxin, then a comparison in baseline susceptibility (i.e. LC_{50}s) may be a feasible approach to monitoring. Estimated LC_{50}s may serve well as a baseline monitoring tool for shifts in susceptibility to *Bt* toxins; however, the LC_{50} approach is not useful in discriminating resistant from susceptible individuals. Therefore, this approach must then be linked with follow-up testing of populations with elevated LC_{50}s relative to previously established baseline susceptibility.

VIII. GROWER EDUCATION

Syngenta proposes to use the following methods to educate growers which have already been established for other registered PIPs:

- Signing of grower agreement with purchase of Event MIR162 maize
- Grower agreement and/or stewardship documents referenced in the grower agreement will set forth terms of current IRM program and contractually bind grower to comply with IRM requirements
- Annual affirmation system for MIR162 maize growers to ensure they understand that they are contractually bound to comply with requirements
- Communication of IRM educational material to growers through written materials, in-person communication, and other media (i.e. internet)
- IRM requirement raining to sales personnel and seed distributors in order to provide another educational resource for growers
- Coordination of educational efforts with other organizations

In addition to Syngenta's proposed educational outreach, BPPD requests that Syngenta submit 1) within 90 days from product registration a copy of the grower agreement/stewardship documents and written description of a system assuring that growers will sign grower agreement; 2) revise and expand as necessary its education program to take into account information collected through the compliance survey; and 3) maintain records of all signed MIR162 maize.

BPPD concludes that the proposed grower education plan meets the Agency's requirement for Grower Education at this stage of the product registration process.

IX. GROWER COMPLIANCE PROGRAM

Grower compliance with refuge and IRM requirements is a critical element for resistance management. Significant non-compliance with IRM among growers may increase the risk of resistance for *Bt* crops. To minimize the effects of non-compliance, it is necessary to develop a broad compliance program as part of the IRM strategy. Such a program has to include 1) an understanding of the effect of non-compliance on IRM; 2) identification of compliance mechanisms to maximize adoption of IRM requirements; 3) measurement of the level of

compliance; and 4) establishment of an enforcement structure to ensure compliance and penalize non-compliance.

Syngenta has committed to implementing a compliance assurance program (CAP) designed to evaluate the extent to which growers of MIR162 stacked product are complying with the IRM requirements and take reasonable actions necessary to assure that non-compliant growers become compliant with those requirements. Consistent with the registration of other Bt corn PIPs, there are several key elements to the CAP that Syngenta commits to employ:

- Establish and publish a phased compliance approach that outlines instances of non-compliance to IRM terms and options of responding to non-compliant growers, such as denying access to MIR162 technology
- Annual survey conducted by third party will measure degree of compliance by growers in different regions where the MIR162 stacked product is grown
- Survey will obtain grower feedback on usefulness of educational tools and initiatives and provide understanding of any difficulties growers encounter with IRM requirements [2]
- Annual on-farm assessment followed by appropriate action consistent with the 'phased compliance approach' for non-compliant growers
- 'Tips and complaints' line with follow-up investigations and appropriate actions taken consistent with the 'phased compliance approach' for non-compliant growers

BPPD concludes that Syngenta has included the major requirements needed by a compliance program. Syngenta's proposed CAP resembles CAPs for other already registered Bt PIPs and meets the Agency's requirement at this stage of the product registration process. BPPD recommends that the compliance program for MIR162 corn be harmonized with the compliance plans already in place for previously registered Bt corn products.

X. REMEDIAL ACTION PLAN

Remedial action plans are a potential response measure should resistance develop to Bt crops. Since resistance may develop in "localized" pest populations, it may be possible to contain the resistance outbreak before it becomes widespread. A specific remedial action plan should clearly indicate what actions the registrant will take in cases of "suspected" resistance (i.e., unexpected damage) and "confirmed" resistance. The remedial action plan can also include appropriate adaptations for regional variation and the inclusion of appropriate stakeholders. To fully mitigate resistance, a critical element of any remedial action plan should be that once pest resistance is confirmed, sales of all *Bt* corns hybrids that express a similar protein or a protein in which cross-resistance potential has been demonstrated would be ceased in the affected region. (http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm)

[2] Syngenta proposes to revise and expand, as necessary, its compliance assurance program to take into account information collected through the compliance survey.

Syngenta states that it will take following steps if Cry1Ab and Vip3A resistance to any of the major target pests is suspected:

- Expression levels in damaged plants are measured to ascertain that they match expected levels for Cry1Ab and Vip3A
- Other reasonable causes for crop damaged will be investigated
- Instruct Growers in affected region to use alternate pest control measures for pest with suspected resistance and to destroy crop residues immediately after harvest

Syngenta states that it will take the following actions if Cry1Ab and/or Vip3A resistance to any of the major target pests has been confirmed:

- Notify the Agency within 30 days of resistance confirmation
- Notify affected customers and extension agents about confirmed resistance
- Direct affected customers and extension agents to employ alternative control measures
- Instruct customers and extension agents to incorporate crop residues into soil following harvest to minimize possibility of overwintering by resistant insects
- Cease sale and distribution of MIR162 maize in affected area
- Notify the Agency within 90 days of mitigation measures that were implemented
- Provide the Agency within 90 days with a proposed long-term resistance management action plan for the affected area including elements such as information exchange with customers and extension agents, increased monitoring of target pest, alternative measure to reduce or control target pest

BPPD concludes that the steps outlined in the remedial action plan and their depth of detail provided are similar to remedial action plans for other already registered Bt PIP products; Syngenta's Remedial Action Plan meets the Agency's requirement for this stage of the product registration process. BPPD recommends that the remedial action plan for MIR162 corn be harmonized with the plans already in place for other registered Bt corn products.

XI. REPORTING REQUIREMENTS

Syngenta commits to meeting with the EPA to discuss results from the grower survey, insect monitoring program, and other relevant IRM plan issues. In addition, Syngenta will provide the following by January 31st each year: 1) annual sales summed by state; 2) number of units of Bt11xMIR162 maize seed shipped/sold and not returned; 3) number of units sold to persons with signed grower agreements; 4) final written summary of survey results and plans for the following year; 5) annual report summarizing activities and results of their CAP; and IRM monitoring results.

BPPD concludes that at this stage of the Bt11xMIR162xMIR604 maize registration process, the Agency is satisfied with Syngenta's commitment to fulfill their reporting requirements.

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Appendix A. Procedure for High dose Determination

The 1998 SAP defined high dose as a level of toxin 25 times greater than is needed to kill all susceptible insects. The SAP also outlined five techniques to determine high dose: 1) Serial dilution bioassay with artificial diet containing lyophilized tissues of *Bt* plants using tissues from non-*Bt* plants as controls; 2) Bioassays using plant lines with expression levels approximately 25-fold lower than the commercial cultivar determined by quantitative ELISA or some more reliable technique; 3) Survey large numbers of commercial plants in the field to make sure that the cultivar is at the $LD_{99.9}$ or higher to assure that 95% of heterozygotes would be killed (see Andow & Hutchison 1998); 4) Similar to #3 above, but would use controlled infestation with a laboratory strain of the pest that had an LD_{50} value similar to field strains; and 5) Determine if a later larval instar of the targeted pest could be found with an LD_{50} that was about 25-fold higher than that of the neonate larvae. If so, the later stage could be tested on the *Bt* crop plants to determine if 95% or more of the later stage larvae were killed.

Appendix B. Cross-Resistance Models and Mechanisms

There are three models that have been proposed to explain the mode of action of Cry1A toxin mode of action (see discussion in Piggott and Ellar, 2007). The most accepted Bravo model proposes that both the cadherin and aminopeptidase (APN) receptors are required for full Cry1A toxicity. This model suggests that receptor binding is sequential: 1) ingestion of the protein inclusions by a susceptible insect larva, 2) solubilization of the protein in the insect midgut, 3) cleavage of the protoxin by host proteases and release of the active toxin, 4) binding of the active toxin to specific receptors on the midgut epithelieum, 5) oligomerization of toxin subunits to form pore structures that insect into the membrane, 6) passage of ions and water through the pores, resulting in swelling, lysis, and the eventual death of the host. Differences in any of these steps will reduce the probability of cross-resistance between any two Cry proteins. The more controversial Zhang model suggests that receptor binding activates a Mg^{+}-dependent signaling cascade that promotes cell death. The Jurat-Fuentes model suggests that cytotoxicity is due to the combined effects of osmotic lysis and cell signaling. The later two models are, at present, more speculative.

Resistance associated with modification of the binding site receptor has been the primary Bt resistance mechanism reported to date (reviewed in Ferré & Van Rie 2002). Other Bt resistance mechanisms have been reported that are based on alterations in the proteases that cleave the protoxin processing it into a smaller active toxin (Candas et al. 2003) and most recently, the discovery that esterases can bind and detoxify Bt toxins (Gunning et al. 2005). Only the binding reduction mechanism has a demonstrated causal link between the biochemical modification and resistance (Ferré and Van Rie 2002). Ferré and Van Rie (2002) indicate that in all cases of binding site modification, resistance is due to a recessive or partially recessive mutation in a major autosomal gene, and cross-resistance extends only to Cry proteins sharing binding sites. Cry proteins that do not share high levels of sequence similarity tend to have different binding sites and different modes of action.