



**Environmental Protection Agency**

**WHITE PAPER ON RESISTANCE IN LEPIDOPTERAN PESTS OF  
BACILLUS THURINGIENSIS (Bt) PLANT-INCORPORATED  
PROTECTANTS IN THE UNITED STATES**

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# **Resistance in Lepidopteran Pests to *Bacillus thuringiensis* (Bt) Plant Incorporated Protectants (PIPs) in The United States: EPA's Analysis of Scientific Uncertainties Related to Resistance Management and Options to Enhance the Current Insect Resistance Management (IRM) Program**

## **ABSTRACT**

Reports of lepidopteran resistance to Bt (*Bacillus thuringiensis*) traits of corn and cotton in the continental United States have been published by academic scientists in 2014 for the fall armyworm (*Spodoptera frugiperda*), in 2016 for the corn earworm (*Helicoverpa zea*), and in 2017 for the western bean cutworm (*Striacosta albicosta*). Likewise, a Bt corn registrant reported resistance for the southwestern corn borer (*Diatraea grandiosella*) in resistance monitoring reports to the United States Environmental Protection Agency (US EPA) in 2016. Based on information presented in these reports, the Agency concluded that the following were risk factors of resistance in these cases: 1) lack of available high dose traits; 2) use of single mode of action in Bt corn year-after-year; 3) use of corn seed blends in the southern US; 4) poor refuge compliance for Bt corn in southern states; 5) continuous selection with the same traits expressed in Bt corn and Bt cotton in a given year; 6) shortcomings in current EPA recommended methodological approaches with monitoring for resistant field populations; and 7) challenges with identifying resistance with current diet bioassay methods.

In this white paper<sup>1</sup>, the Biopesticides and Pollution Prevention Division (BPPD) within the Office of Pesticide Programs of US EPA (hereafter referred to EPA) discusses these major risk factors believed responsible for expediting lepidopteran adaptation to Bt toxins in the US and associated scientific uncertainties. Furthermore, the Agency discusses shortcomings of the current insect sampling methodologies, rearing and resistance testing of populations and the feasibility of successful mitigation when resistance is confirmed. In addition, EPA considers whether it is warranted scientifically to develop a resistance management plan for western bean cutworm.

EPA's overall goals as it considers the options in this document are to reduce resistance risks for lepidopteran pests, increase the longevity of currently functional Bt traits and future technologies, and improve the current insect resistance management program for lepidopteran pests of Bt corn and cotton.

Although resistance could only be confirmed for *D. grandiosella* using EPA's regulatory process, the agency's scientists agree with published reports by academic scientists that resistance to Bt has also evolved in *H. zea*, *S. frugiperda*, and *S. albicosta*. Given the identified risk factors and resistance occurrences, EPA is considering changes to EPA's IRM program to reduce the selection intensity on lepidopteran pests of Bt, improve resistance monitoring approaches to confirm resistance faster and

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<sup>1</sup> Contributors from the BPPD IRM Team include J.C. Martinez, A. Reynolds, K. Welch, E. Bohnenblust, and C. Blanco.

more reliably, and mitigate resistance where it is suspected or confirmed by academics. Management options could address the following to lower the risk of resistance to Bt PIPs:

- **Risk factor 1:** Single trait Bt corn in the presence of pyramided products in the landscape;
- Option: Limit or otherwise manage single trait commercial products;
- Option: Transition from single trait products to Bt corn pyramids.
  
- **Risk factor 2:** Limited number of highly effective Bt traits;
- Option: Adoption of intervening actions at “early warning resistance” in addition to actions when field failure occurs (see “molecular assay” option);
  
- **Risk factor 3:** The shift of dominance of resistance in ear-feeding pests of corn with exposure to Refuge-In-The-Bag (RIBs) corn products in the southern US;
- Option: Potential changes to the corn breeding program to move away from hemizygous parental corn lines, introduce pollen incompatibility between Bt and non-Bt cultivars, self-pollinating mechanism, etc.;
- Option: Manage the use of Bt corn RIB products in the southern US;
  
- **Risk factor 4:** Non-compliance with refuge plantings in the southern US;
- Option: The development of incentive programs to increase compliance with block refuge requirements for Bt corn in the southern US;
  
- **Risk factor 5:** Non-functional, non-high dose pyramided Bt products where individual traits control less than 95% of susceptible insect;
- Option: Implementation of best management practices (BMPs) consistent with the tenets of Integrated Pest Management (IPM) practices for fields with Unexpected Injury (UXI);
- Option: Manage the use of non-functional pyramids;
- Option: Increasing the amount of refuge;
  
- **Risk factor 6:** Resistance monitoring does not allow tracking the same populations year after year;
- Option: Use of sentinel plots to monitor the same populations from year to year;
  
- **Risk factor 7:** Lack of standardized UXI thresholds;
- Option: Development of such standards in corn and cotton would expedite insect collections, reporting to EPA, and early mitigation;
  
- **Risk factor 8:** Selection pressure on effective traits (e.g., Vip3A) in pyramids with less effective or compromised traits;

- Option: Focus on risk in Bt corn which also drive resistance risk to Bt cotton (discussion in section III. B. 2);
- **Risk factor 9:** Use of diet bioassay methods for non-high dose pests;
- Option: Development of molecular/DNA assays to monitor for “early warning” resistance that allows expeditious resistance confirmation followed by early implementation of mitigation actions;
- **Risk factor 10:** Mitigation of resistance when field failure occurs;
- Option: Establishing an early intervening threshold (e.g., “early warning resistance”) would create a greater likelihood of success for mitigation of resistance and to delay occurrences of field failure;
- Option: An additional option considered by EPA is the encouragement of IPM with IRM for lepidopteran pests of Bt corn and cotton at the onset of a new trait deployment and throughout its commercialization (see options in [Section IV](#)).
- **Risk factor 11:** Protracted mitigation in response to field failure;
- Options: *A priori* determined size for a Mitigation Action Area (MAA) based on scientifically defensible factors relying on noctuid dispersal propensity;
- Option: Implementing mitigation actions when field failure is apparent instead of after resistance is confirmed (i.e., practical resistance);
- Option: Use of best management practices (BMPs) consistent with the tenets of IPM to reduce UXI field occurrences and delay resistance development.

In addition to the listed options above, the agency is also seeking guidance on other approaches that would address the agency’s goals and the risk factors identified in this document.

A new framework for lepidopteran pests of Bt should encourage use of IPM with IRM before resistance is detected. Such IPM steps could include:

- Increased scouting for pest densities
- Use of alternate control methods when economic threshold on Bt crop is reached
- Increased tilling where possible
- Crop destruction methods
- Multi-year management plans to control lepidopteran pests including rotation of Bt pyramided products (different modes of action), rotation to conventional corn with insecticide use, etc.
- Use of standardized regulatory thresholds defining unexpected injury levels that trigger grower reporting to seed/trait providers
- Improved and timely communication by Bt registrants through notification to growers, state extension agents, and crop consultants of areas with early warning resistance, unexpected injury fields, and confirmed resistance cases.

Additionally, EPA considered whether it is warranted to design an IRM plan for *S. albicosta* given that resistance has evolved to Cry1F and only one other Bt trait, Vip3A (pyramided with other Bts), controls this pest in corn.

## DOCUMENT ORGANIZATION

This white paper is organized into the following sections: 1) background; 2) problem formulation; 3) scientific uncertainties with IRM for lepidopteran pests of Bt crops; and 4) EPA conclusions and options to reduce risks of resistance.

- The background section provides an overview of the resistance monitoring program for lepidopteran pests of Bt corn and Bt cotton (e.g., insect collection and diet bioassay methods), lists the current Bt toxins registered in corn and cotton, and summarizes life-history characteristics of the lepidopteran pests of concern (Section I);
- The problem formulation section presents EPA’s identified risk factors of resistance to Bt corn and Bt cotton in the continental US (Section II);
- EPA’s analysis in the “scientific uncertainties with IRM for lepidopteran pests and potential improvements to IRM for Bt crops” portion of the document (Section III) discusses each proposed risk factor with a focus on *H. zea*, though many also apply to other lepidopteran pests. Subsections include agency options to address the risk of resistance and identified uncertainties; and
- Lastly, EPA identifies major aspects of the IRM program and options for improvements that, if incorporated, could improve the durability and longevity of Bt crops in the southern US ([Section IV](#)).

### I. BACKGROUND

#### A. Pest of Bt corn and Bt cotton

The following pests of corn and cotton are the focus of this white paper (Table 1).

*Helicoverpa zea* (Lepidoptera: Noctuidae, CEW (corn earworm), CBW (cotton bollworm)) is a major economic, agricultural pest in North America, especially on sweet corn and cotton, engages in bi-directional migrations (South to North, and back), and has between 4-6 generations per year. The insect is highly polyphagous feeding on numerous wild hosts and cultivated crops, including corn and cotton. Females are able to lay up to 2,500 eggs in their lifetime and deposit their eggs in clusters on corn silks and cotton leaves. As neonates mature, they become cannibalistic, and typically one larva (occasionally two) establishes per corn ear. Once larvae have matured, they move to the soil where they pupate for approximately two weeks before they emerge as moths. Adults are nocturnal, live approximately 2-3 weeks, and feed on nectar. (Capinera 2000)

*Spodoptera frugiperda* (Lepidoptera: Noctuidae, fall armyworm) is a subtropical and tropical pest of the Americas, prefers to feed on wild and cultivated grasses, and migrates northward into temperate



North America during the growing season. In the US, it can have approximately 2 generations and in tropical regions up to 6 generations per year. Except for subtropical regions in south Florida and Texas, it does not overwinter in the US. Although *S. frugiperda* can cause severe damage when densities are high, it has been referred to as a sporadic pest of corn and cotton. Females are capable of laying up to 2000 eggs in their lifetime and deposit egg clutches on leaves. Once neonates hatch, they feed in whorls of corn plants and later move up the plant into the ears of corn. Larvae are less competitive than *H. zea* and may be cannibalistic occasionally. It is not atypical to find two or more larvae per corn ear. Late-instar larvae move to the soil where they pupate and later emerge as adults (approx. 2-3 weeks). Adults are nocturnal, capable of long-distance dispersal, and feed on plant nectar. (Purdue University Field Crops IPM 2009a)

*Diatraea grandiosella* (Lepidoptera: Crambidae, southwestern corn borer) is a pest found in northern Mexico and the southern US and prefers wild and cultivated grasses as hosts. It is a major pest of corn with two generations per year in the US. Females lay up to 400 eggs on upper and lower surfaces of leaves and stalks; clutches consist of 2-5 eggs. Larvae feed in whorls of plants first and later bore into the stalk. Mature larvae migrate to the base of the stalk where they pupate. Because of this behavior, typically only one pupa is found per plant. (Chippendale & Sorenson 2017)

*Striacosta albicosta* (Lepidoptera: Noctuidae, western bean cutworm) is native to North America and has recently undergone a range expansion from the west to the east of the US. It has one generation per year, feeds on reproductive parts of corn and legumes and is a sporadic pest where corn is grown on sandy soils. Females lay eggs on the upper surface of leaves near the whorls. Neonates consume their eggshells upon hatching. As they mature, they feed on leaves, anthers, pollen, and ultimately move on to kernels. Scouting for larvae is challenging because of the lack of evidence for egg hatch and lack of visible frass. Several larvae may be found on a corn ear since the pest is not cannibalistic. Kernel damage caused by larval feeding often leads to mycotoxin contamination of ears and, therefore, reduced quality of corn. Fully developed larvae drop off the ears and burrow into the soil where they overwinter and undergo pupation in spring. (Purdue University Field Crops IPM 2009b)

**Table 1. Lepidopteran Pests of Bt Corn and Bt Cotton at Risk of Resistance to Bt PIPs**

Scientific name	Common name	Abbreviation	Bt host	Feeding site	Origin
<i>H. zea</i>	Corn earworm, cotton bollworm	CEW, CBW	Corn, cotton	Corn ear, square, boll, flower	Native to the Americas
<i>S. frugiperda</i>	Fall army worm	FAW	Corn, cotton	Leaf, ear	
<i>D. grandiosella</i>	Southwestern corn borer	SWCB	Corn	Leaf, stalk	
<i>S. albicosta</i>	Western bean cutworm	WBCW	Corn	Leaf, ear	

## B. Current IRM Strategies and Requirements for Bt Corn and Cotton

Like other pesticides, Bt plant-incorporated protectants (PIPs) are at risk of resistance. However, Bt PIPs are likely at greater risk than conventional pesticides targeting the same insects because they are expressed at relatively high, though also variable levels throughout the life of the plant compared to conventional pesticides, which typically have shorter periods of efficacy and are applied when pests are likely to cause yield loss. Continuous exposure to Bt PIPs, often over multiple generations per season, exerts a significant selection pressure and facilitates pest resistance development (EPA 1998, Matten et al. 2013).

To mitigate the risk of resistance to Bt PIPs, the EPA has required insect resistance management plans for all commercially registered Bt PIPs as part of the terms and conditions of registration. The primary resistance mitigation strategy for Bt corn and cotton has been the use of non-Bt refuges, which serve as sources of Bt-susceptible insects that mate with any resistant individuals emerging from Bt fields (i.e. the high dose + refuge paradigm; US EPA FIFRA SAP 1998). The goal of the refuge strategy is to provide sufficient susceptible insects to reduce the frequency of resistance genes in the overall pest population. Under the high dose + refuge paradigm heterozygous resistant insects die when exposed to Bt, thus keeping the resistance allele frequency low in a population (US EPA FIFRA SAP 1998) (further discussed below).

Three different refuge strategies have been employed for Bt PIPs. A “structured” refuge consists of a portion of the crop that is planted to a non-Bt variety. These refuges are typically planted as discrete fields proximate to Bt fields or as blocks or strips within or adjacent to Bt fields. A second refuge strategy has been the use of seed blends, in which Bt and non-Bt seeds are comingled within the same seed bags. This approach, which has been used for Bt corn, has the advantage of ensuring grower refuge compliance, though there are concerns that durability could be reduced if larvae move readily between plants (SAP 2011) or if the pest is an ear-feeding insect and is exposed to a mosaic of Bt expression in kernels (Caprio et al. 2016). The third refuge type is referred to as “natural refuge” and includes naturally occurring non-PIP plants, such as weeds and other cultivated (non-PIP) crops that serve as sources of susceptible insects (SAP 2006a; Head et al. 2010; Matten et al. 2013). This type of refuge strategy is used for Bt cotton.

Refuge requirements for Bt corn and cotton PIPs are summarized in Table 2 below. For Bt corn PIPs, structured or seed blend refuges have been employed. The amount of refuge required is linked to the resistance risk: single toxin varieties have larger refuge requirements than pyramided varieties with multiple traits. Research has shown that pyramids of two traits with independent modes of action (i.e., no cross-resistance potential) and high activity (>95% of susceptible insects killed) can delay the evolution of resistance with a reduced refuge proportion relative to single trait varieties (Roush 1998; Zhao et al. 2003; Gould et al. 2006; summarized in Matten et al. 2013). Larger refuges have also been required for Bt corn products grown in cotton-growing regions due to resistance concerns with *H. zea*, a pest of both corn and cotton. In addition to the refuge size, structured refuges must be planted close enough to the Bt fields to increase the likelihood of random mating between susceptible insects (from

the refuge) and any resistant insects that might emerge from Bt fields. Refuges planted as discrete fields must be deployed within ½ mile of Bt fields, and refuges within Bt fields must be at least four rows wide. Insecticide use to control corn borers within the refuge is permitted provided pest pressure reaches an economic threshold for damage. Seed blends have been approved for pyramided Bt corn products, though a separate structured refuge must also be planted if these products are deployed in cotton-growing regions.

For Bt cotton, a detailed analysis was conducted that demonstrated sufficient natural refuge occurred in southeastern cotton-growing regions for resistance management (SAP 2006a; Head et al. 2010). In western cotton-growing regions, an ongoing pink bollworm (PBW) eradication program has supplanted the need for structured refuges (see SAP 2006b; USDA 2015).

Ideally, refuge is deployed in combination with PIPs that are highly efficacious (LC 99.99%) against the target pests. This so called “high dose + refuge strategy” (US EPA FIFRA SAP 1998; Matten et al. 2013) ensures that only resistant homozygote (*RR*) genotypes will survive exposure to Bt PIPs. Under this paradigm, resistance genes are assumed rare in the population, and thus, the likelihood for truly resistant survivors in Bt fields is low. The few resistant individuals that survive are likely to encounter susceptible insects from refuges which, assuming random mating, dilute the number of resistance genes in the overall population. High dose has been defined by the 1998 SAP as “25 times the protein concentration necessary to kill susceptible larvae”, or by a lethal dose of 99.99%. For Bt corn and cotton, the registered PIPs have generally been determined to be high dose for *Ostrinia nubilalis*, *Heliothis virescens*, and *Pectinophora gossypiella* but not for *H. zea*. Though high dose expression is preferable, for pests against which Bt is non-high dose, IRM can be practiced by including integrated pest management (IPM) approaches as well as proactive resistance monitoring. The concern with non-high dose PIPs, however, is that heterozygote genotypes (*RS*) survive exposure and increase the amount of resistance genes in a population much faster than under the high dose + refuge paradigm.

## Table 2. Refuge Requirements for Bt PIPs

<b>Crop</b>	<b>Bt PIP type</b>	<b>Refuge type</b>	<b>Refuge requirement</b>	<b>Proximity to Bt fields</b>
Corn	Single toxin	Structured	Corn Belt: 20% Cotton regions: 50%	<1/2 mile
Corn	Pyramid	Structured	Corn Belt: 5% Cotton regions: 20%	<1/2 mile
Corn	Pyramid	Seed Blend	5% (separate 20% structured refuge required in cotton regions)	N/A
Cotton (Southeastern region)	Pyramid	Natural refuge	Authorized only for southeastern cotton-growing regions	N/A
Cotton (Western region)	Pyramid	None	Under PBW eradication program	N/A

In addition to refuge, the Bt PIPs IRM plans include the following elements:

- A resistance monitoring program to sample target pests and detect potential shifts in susceptibility to the PIP toxin(s) as well as collecting and investigating insects from grower reported field failures;
- A remedial action strategy to mitigate resistance should it occur;
- A Compliance Assurance Program (CAP) for regions in which Bt corn PIP growers are required to plant a structured (non-Bt) refuge;
- Grower education activities to inform users about IRM concerns and appropriate mitigation strategies.

### C. Currently Registered Bt Corn Plant-Incorporated Protectants for Lepidopteran Pests

Table 3 below lists the currently registered Bt PIPs in corn for control of lepidopteran pests. These PIPs target major lepidopteran stalk boring and ear feeding pests, including European corn borer, corn earworm, and southwestern corn borer. Other lepidopteran pests, such as fall armyworm and western bean cutworm, may also be targeted.

Bt corn varieties are predominantly field corn, though several sweet corn and popcorn uses have also been registered. The registered PIP traits are bred into corn hybrids with genetic backgrounds that have been optimized for different corn-growing regions. Other traits, including herbicide tolerance, may also be stacked in the commercially available hybrids. The Bt PIPs have also been combined to create

“pyramided” varieties (i.e., two or more toxins targeting the same pest). Commercially available products include both single toxin and pyramided options.

The agency has drafted a “Biopesticides Registration Action Document” (BRAD) for each registered Bt corn PIP in Table 3 below. These BRADs contain a summary of the EPA’s risk assessments for each PIP and have been updated periodically to reflect new scientific information and data. For IRM, assessments of dose, cross resistance, and simulation modeling are contained in these documents. All of these documents are available on-line (refer to the citations in Table 3).

**Table 3. Registered Bt Corn PIPs for Control of Lepidopteran Target Pests**

<b>Bt PIP (toxin)</b>	<b>Event</b>	<b>Registrant</b>	<b>Year Registered</b>	<b>BRAD Citation</b>
Cry1Ab	Bt11	Syngenta	1996	U.S EPA 2010a
Cry1Ab	MON 810	Monsanto	1996	US EPA 2010a
Cry1F	TC1507	Dow, Pioneer	2001	US EPA 2010a
Cry1A.105	MON 89034	Monsanto	2008	US EPA 2010b
Cry2Ab2	MON 89034	Monsanto	2008	US EPA 2010b
Vip3Aa20	MIR 162	Syngenta	2008	US EPA 2009

#### D. Currently Registered Bt Cotton PIPs for Lepidopteran Pests

Registered Bt PIPs for cotton are listed in Table 4 below. As with corn, these PIPs have been combined into pyramids. Unlike corn, commercial varieties are exclusively pyramids (no single trait products are currently commercially available). Table 4 also includes citations for BRAD documents that contain the agency’s complete IRM assessment for each registered PIP.

**Table 4. Registered Bt Cotton PIPs for Control of Lepidopteran Target Pests**

<b>Bt PIP (toxin)</b>	<b>Event(s)</b>	<b>Registrant</b>	<b>Year Registered</b>	<b>BRAD Citation</b>
Cry1Ac	MON 531, MON 15985	Monsanto	1995	US EPA 2001
Cry2Ab2	MON 15947	Monsanto	2002	US EPA 2003a
Cry1F	DAS-21023-5, 281-24-236	Dow	2004	US EPA 2005

<b>Bt PIP (toxin)</b>	<b>Event(s)</b>	<b>Registrant</b>	<b>Year Registered</b>	<b>BRAD Citation</b>
Cry1Ac	DAS-24236-5, 3006-210-23	Dow	2004	US EPA 2005
Vip3Aa19	COT102	Syngenta	2008	US EPA 2008
FLCry1Ab	COT67B	Syngenta	2008	US EPA 2008
Cry1Ab	T304-40	Bayer	2012	US EPA 2012
Cry2Ae	GHB119	Bayer	2012	US EPA 2012

### E. Current Resistance Monitoring and Mitigation Strategies for Bt Corn and Cotton

Through the terms of registration, Bt corn and cotton registrants are required to implement a resistance monitoring program for the major lepidopteran target pests. A primary goal of resistance monitoring is to detect shifts in the frequency of resistance genes (i.e., susceptibility changes) before the onset of resistance leads to widespread Bt crop failure (see EPA 2010a).

Monitoring for resistance has consisted of two main components: 1) investigation of unexpected field damage reports from growers, extension agents, consultants, or company agronomists, and 2) monitoring for changes in susceptibility through targeted population sampling and testing. Unexpected damage reports may reveal the occurrence of localized resistance (or hot spots) before the effects have spread. Resistance monitoring through targeted field sampling can reveal changes in susceptibility of geographically representative populations. If resistance is confirmed, a mitigation (remedial action) plan is triggered as defined by the terms of registration (see EPA 2010a). Reports covering each monitored pest and Bt PIP toxin must be submitted annually to the agency.

The resistance monitoring and mitigation plans for Bt corn and cotton are described below. The strategies are conceptually similar, though the target pests and resistance standards vary between the crops.

#### 1. Bt Corn – Resistance Monitoring and Mitigation

Resistance monitoring for Bt corn has been required for three lepidopteran pests: *O. nubilalis*, *D. grandiosella*, and *H. zea*. Monitoring for a fourth pest, fall armyworm, is required if an acreage threshold for Bt sweet corn is surpassed – i.e., 5,000 or more acres per growing season in an individual county that supports overwintering populations of FAW. To date, this acreage trigger has not been reached and routine monitoring for FAW has not been conducted since the 2000 growing season (EPA 2010a, BPPD 2015).

Resistance monitoring is required for each registered lepidopteran-active Bt toxin. The registered toxins include Cry1Ab, Cry1F, Cry1A.105, Cry2Ab2, and Vip3Aa20. Monitoring efforts for the Bt registrants have been coordinated by the Agricultural Biotechnology Stewardship Technical Committee (ABSTC)<sup>2</sup>.

*a. Bt Corn – Annual Population Sampling and Bioassays*

The terms of registration specify that Bt corn registrants must conduct annual sampling and monitoring of pest populations in corn-growing areas with the highest risk of resistance development. These regions include locations where lepidopteran active Bt traits are planted on a high proportion of the corn acres and the target species are regarded as key pests of corn. The number of populations to be collected is reflective of the regional importance of the pest, with specific sampling targets identified for each pest. For *O. nubilalis*, a minimum of twelve (12) populations across the sampling region must be targeted for collection at each annual sampling. For *D. grandiosella*, the target is a minimum of six (6) populations. For *H. zea*, the target is a minimum of ten (10) populations (EPA 2010f).

Collections are made from multiple corn-growing states to represent different geographies and agronomic conditions. To accomplish this goal, four sampling regions have been identified that encompass the Corn Belt. For *O. nubilalis*/*D. grandiosella* monitoring, Region 1 (*O. nubilalis* collections only) is defined as southwestern Minnesota, eastern South Dakota, southeastern North Dakota, and northwestern Iowa. The target is to sample 6-8 *O. nubilalis* populations from Region 1. Region 2 (*O. nubilalis* and *D. grandiosella* collections) covers southwestern Kansas and the Texas/Oklahoma panhandle. From this region, 4-6 *O. nubilalis* populations and 3-4 *D. grandiosella* (at least one from Texas) are targeted for sampling. Region 3 (*O. nubilalis* only) consists of central/southeastern Iowa and north-central Illinois with a sampling target of 4-6 *O. nubilalis* populations. Region 4 (*D. grandiosella* only) is focused on the Missouri bootheel, western Kentucky, western Tennessee, and the southern tip of Illinois (target sampling of 3-4 *D. grandiosella* populations). Individual sampling sites are determined by pest population size and are taken at distances greater than ½ mile from Bt cornfields, to minimize the effects of elevated resistance gene frequencies misrepresentative of the population average.

To obtain sufficient sensitivity to detect resistance alleles before they become common enough to cause measurable field damage, the goal of each population collection is 400 insect genomes. For each insect population to be sampled, either 200 larvae, 200 adults, 100 mated females, or 100 egg masses can be collected to reach the objective of 400 genomes per population (egg masses are assumed to have at least 4 genomes). However, a collection attempt can be considered successful with a minimum of 100 genomes since it may not be possible to collect the full target number due to factors such as

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<sup>2</sup> The Agricultural Biotechnology Stewardship Technical Committee (ABSTC) is an industry consortium of Bt corn registrants, including Monsanto, Dow, Pioneer/DuPont, and Syngenta.

natural fluctuations in pest density, environmental conditions, and area-wide pest suppression. In such cases, the minimum population sizes to be collected is 50 larvae, 50 adults, 25 mated females, or 25 egg masses. The specific life stage to be sampled depends on the insect and the practicality of the collection method. Most collections occur at the peak flights of second or later generations (first generation flights may not produce sufficient numbers for sampling).

Bioassays of the collected populations are conducted to assess susceptibility. For the lepidopteran corn pests, these assays have been conducted with artificial diet bioassays and Bt toxin overlays (e.g., Siegfried et al. 1995). Two bioassay approaches have been employed: dose-response testing to measure lethal ( $LC_{50}$ ) and sub-lethal ( $EC_{50}$ ) susceptibility, and diagnostic concentration assays with high levels of toxin that distinguish resistant individuals from susceptible ones (e.g., Marçon et al., 2000).

Any population that demonstrates unusually low sensitivity to a Bt toxin is further investigated to determine if the population has field-relevant resistance to the trait. The following investigative procedures and resistance determination criteria are required (taken from EPA 2010a):

- i. Step 1: Re-test progeny of the collected population to determine whether the unusual bioassay response is reproducible and heritable. If it is not reproducible and heritable, no further action is required.
- ii. Step 2: If the unusual response is reproducible and heritable, progeny of insects that survive the diagnostic concentration will be tested using methods that are representative of exposure to Bt corn under field conditions. If progeny do not survive to adulthood, any suspected resistance is not field relevant and no further action is required.

If insects survive steps 1 and 2, resistance is confirmed, and further steps will be taken to evaluate the resistance. These steps may include the following:

- a. Determining the nature of the resistance (i.e., recessive or dominant, and the level of functional dominance); estimating the resistance allele frequency in the original population;
  - b. Determining whether the resistance allele frequency is increasing by analyzing field collections in subsequent years sampled from the same site where the resistance allele(s) was originally collected;
  - c. Determining the geographic distribution of the resistance allele by analyzing field collections in subsequent years from sites surrounding the site where the resistance allele(s) was originally collected.
- iii. Step 3: Should field-relevant resistance be confirmed, and the resistance appears to be increasing or spreading, the registrant must consult with EPA to develop and implement a case-specific resistance management action plan.



### *b. Bt Corn – Investigation of Reports of Unexpected Damage*

In addition to the annual population sampling described above, Bt corn registrants are required to investigate grower reports of unexpected damage (UXD) or unexpected injury (UXI) (Andow et al. 2016) to Bt corn. EPA has established a two-step process to investigate these reports.

Once a UXD report is received, the registrant will confirm that the affected acreage was planted to a lepidopteran-active Bt corn variety with proper toxin expression in plant tissues. Provided the insect damage was not due to environmental (or other) reasons, the case is termed “suspected resistance.” The registrant must attempt to collect insects for bioassay testing while working with the affected grower to implement mitigation actions to minimize spread of the insect population (see “Resistance Mitigation” below). The terms of registration do not establish specific damage thresholds for UXD investigations; rather it is the responsibility of the registrant to determine the level of injury that constitutes significant field damage worthy of investigation.

As with the annual monitoring described above, dose-response and diagnostic concentration testing are used to determine resistance with UXD cases. If the following criteria are met, the UXD population is considered to have “confirmed resistance” (taken from EPA 2010a):

- There is >30% insect survival and commensurate insect feeding in a bioassay, initiated with neonate larvae, that uses methods that are representative of exposure to *Bt* corn hybrids under field conditions (*O. nubilalis* and *D. grandiosella* only).
- In standardized laboratory bioassays using diagnostic concentrations of the *Bt* protein suited to the target pest in question, the pest exhibits resistance that has a genetic basis and the level of survivorship indicates that there may be a resistance allele frequency of  $\geq 0.1$  in the sampled population.
- In standardized laboratory bioassays, the  $LC_{50}$  exceeds the upper limit of the 95% confidence interval of the  $LC_{50}$  for susceptible populations surveyed both in the original baselines developed for this pest species and in previous years of field monitoring.

### *c. Bt Corn – Resistance Mitigation*

If resistance is confirmed, either in populations collected from the annual sampling or from UXD reports, Bt corn registrants are required to implement a mitigation (remedial action) plan. The goal of resistance mitigation is to limit or contain (extirpate if possible) the spread of the resistant population and maintain the durability of the Bt trait in areas where it is still effective.

For resistant populations identified through the annual sampling, registrants are required to consult with EPA to develop and implement a case-specific resistance management action plan. For UXD cases, action levels have been established for “suspected” and “confirmed” resistance (EPA 2010a).

In cases of suspected resistance, registrants will work with growers to:

- Use alternative control measures in Bt corn fields in the affected region to control the target pest during the immediate growing season.
- Destroy Bt corn crop residues in the affected region within one (1) month after harvest with a technique appropriate for local production practices to minimize the possibility of resistant insects over-wintering and contributing to the next season's target pest population.

When resistance is confirmed, the following steps are required:

- EPA will receive notification within 30 days of resistance confirmation;
- Affected customers and extension agents will be notified about confirmed resistance within 30 days;
- Monitoring will be increased in the affected area and local target pest populations will be sampled annually to determine the extent and impact of resistance;
- If appropriate (depending on the resistant pest species, the extent of resistance, the timing of resistance, and the nature of resistance, and the availability of suitable alternative control measures), alternative control measures will be employed to reduce or control target pest populations in the affected area. Alternative control measures may include advising customers and extension agents in the affected area to incorporate crop residues into the soil following harvest to minimize the possibility of over-wintering insects, and/or applications of chemical insecticides;
- Unless otherwise agreed with EPA, stop sale and distribution of the relevant lepidopteran-active *Bt* corn hybrids in the affected area immediately until an effective local mitigation plan, approved by EPA, has been implemented;
- The registrant will develop a case-specific resistance management action plan within 90 days according to the characteristics of the resistance event and local agronomic needs. The registrant will consult with appropriate stakeholders in the development of the action plan, and the details of such a plan shall be approved by EPA prior to implementation;
- The registrant will notify affected parties (e.g., growers, consultants, extension agents, seed distributors, university cooperators, and state/federal authorities as appropriate) in the region of the resistance situation and the EPA approved action plan; and
- In subsequent growing seasons, maintain sales suspension and alternative resistance management strategies in the affected region(s) for the *Bt* corn hybrids that are affected by the resistant population until an EPA-approved local resistance management plan is in place to mitigate the resistance.

## 2. Bt Cotton – Resistance Monitoring and Mitigation

Resistance monitoring for Bt cotton follows the same approach as Bt corn, with annual population sampling and investigations of unexpected pest damage to Bt cotton fields. Three lepidopteran pests are routinely monitored: tobacco budworm (*H. virescens*), cotton bollworm (*H. zea*), and pink bollworm (*Pectinophora gossypiella*). *H. zea* is typically referred to as bollworm or cotton bollworm as a pest on cotton and as corn earworm when infesting corn. Monitoring for this pest also occurs in corn.

Registered Bt cotton PIP traits include Cry1Ab, Cry1Ac, Cry1F, Cry2Ab2, Cry2Ae, and Vip3Aa19. Resistance monitoring is conducted for the Cry1Ac, Cry1F, Cry2Ab2, and Vip3A. Given structural similarities between toxins, monitoring for Cry1Ac serves as a surrogate for Cry1Ab, while Cry2Ab2 testing also encompasses Cry2Ae. The summary of the cotton resistance monitoring strategy below was derived from the terms of registration for Bt cotton PIPs (Table 4) (see [Appendix B](#)).

### *a. Bt Cotton – Annual Population Sampling and Bioassays*

As required by the terms of registration, Bt cotton registrants must conduct annual monitoring of the primary lepidopteran target pests. Similar to Bt corn, registrants formed a consortium group, the Cotton Technology Stewardship Committee (CTSC), in 2015 to coordinate the annual sampling and bioassay activities for Bt cotton.

Registrants are required to focus collection sites for the annual monitoring in areas of high adoption of Bt cotton in the Southeastern US, with the goal of including all states where these insects are economic pests. The target is to collect 20 or more pest populations per growing season, with each population consisting of at least 200-500 third instar larvae, 100-200 adults, or 200 eggs. Sampling is focused on high risk areas, such as those with high Bt cotton adoption or low amounts of natural refuge.

Resistance detection bioassays have focused primarily on the use of diagnostic concentrations to measure survival to set doses of toxin. Some dose-response testing to determine LC<sub>50</sub> and EC<sub>50</sub> values has also been conducted for Cry1F monitoring. Assays have been conducted by Custom Bio-Products (*H. virescens*, *H. zea*).

### *b. Bt Cotton – Investigation of Reports of Unexpected Damage*

In addition to the annual sampling, registrants are required investigate reports of unexpected damage to Bt cotton fields. The investigative steps are similar to those for Bt corn and include confirmation that the affected plants are Bt-expressing varieties and that no other explanations (e.g., climatic, cultural, or other non-targeted pests) are responsible for the damage. Registrants will attempt to sample pest populations from the UXD fields for bioassay assessments of susceptibility (see Resistance Determination Criteria below). As with Bt corn, EPA has not established specific damage thresholds

for UXD investigations in the terms of registration. Each company is responsible for coordinating scouting and damage report responses for its products and determining whether UXD cases warrant further investigation.

Monsanto has also employed the use of “IPM-based monitoring” for its Bollgard cotton products. Crop consultants, cotton growers, and other commercial representatives normally scout for pest damage in cotton during the growing season. Monsanto works with these personnel to identify unusual pest survival or damage to Bt cotton and collect insects for further investigation.

### *c. Bt Cotton – Resistance Determination Criteria*

The criteria used to make resistance determinations have varied between registrants, but generally establish procedures for suspected (“putative”) and confirmed resistance cases. Resistance is suspected if populations collected through annual sampling or from a UXD field exhibit a statistically significant increase in survival relative to established baseline susceptibility data. To confirm resistance, companies conduct further study of the putative population to verify the initial bioassay results, evaluate heritability of the observed trait, assess potential field relevance (i.e., survival on Bt cotton plant tissue), and determine the resistance allele frequency. A population is considered resistant if the confirmatory studies show statistically significant survival (95% confidence interval) relative to the established LC<sub>50</sub> baseline susceptibility for *H. virescens* or *H. zea*.

### *d. Bt Cotton - Mitigation*

Mitigation strategies for Bt cotton essentially follow the same paradigm as those for Bt corn by establishing procedures for “suspected” and “confirmed” resistance.

For suspected resistance, registrants conduct additional field sampling for look for further signs of resistance, collect *H. virescens* or *H. zea* larvae for bioassays, and work with affected growers to implement alternate control measures and crop destruct techniques for the immediate growing season. For confirmed resistance, registrants follow the same mitigation steps developed for Bt corn (listed above).

## **II. PROBLEM FORMULATION**

The problem formulation identifies potential pathways of resistance among targeted insect pests from use of Bt plant-incorporated protectants. EPA determined a list of causal factors that may accelerate the risk of resistance development in lepidopteran target pests of Bt corn and cotton. Although the ‘Causal Factors of Resistance’ address mostly issues for *H. zea* and *S. albicosta*, the identified risk

factors also apply to other pests of Bt cotton and/or corn (e.g., *S. frugiperda*, *D. grandiosella*). Based on the identified risks and uncertainties, EPA discusses options for changes to the IRM strategies for lepidopteran pests targeted by Bt (see [Section III](#)), which are also summarized in [Section IV](#) of this document.

### A. Cases of Lepidopteran Resistance in the US

Resistance has been reported for *D. grandiosella*, *S. frugiperda*, *H. zea* (non-high dose pest), and *S. albicosta* in various parts of the US. With the exception of one report (*D. grandiosella*), resistance was determined by academic researchers and not through the monitoring activities conducted by registrants. It is important that the agency examine its current IRM program for lepidopteran pests of corn and cotton to ascertain that resistance is proactively detected and effectively mitigated by registrants. For a discussion of the resistance cases, see [Section III. A](#).

### B. Causal Factors of Resistance

#### 1. All Bt toxins are non-high dose for *H. zea* and expedite resistance development

All currently registered Bt toxins aimed at suppressing *H. zea* in corn and cotton express less than high dose (see Tables 3 & 4) with Vip3A being the most efficacious trait of all (Burkness et al. 2010). Overall, resistance can be expected to evolve faster under moderate or intermediate dose conditions (Comins 1977; Tabashnik & Croft 1982), especially with higher population growth rates as reported for *H. zea* (Caprio et al. 2009; Martinez et al. 2017). The pest has shown to have great genetic variability with respect to susceptibility to Bt toxins (Stone and Sims 1993; Ali et al. 2006). Populations that harbor a greater proportion of individuals with tolerance can be expected to evolve resistance to single as well as pyramided traits more rapidly. For an in-depth discussion of this issue, refer to [Section III. B.1](#).

#### 2. Bt toxins expressed in both corn and cotton exert continuous selection pressure on some pests

Most Bt toxins expressed in corn are also deployed in cotton products. This exerts continuous selection pressure on all traits for pests having multiple generations per year feeding on corn and cotton (e.g., *H. zea*, *S. frugiperda*). Currently, the most efficacious trait in corn and cotton is Vip3A. Given this trait scenario for both crops and recent resistance reports for *H. zea*, the risk of resistance to the most efficacious trait(s) in the southern U.S. is of concern. For further discussion of this topic, refer to [Section III. B.2](#).

### 3. Mosaic of single and pyramided traits undercuts pyramid durability

The southern US is a mosaic of single (corn) and pyramided Bt products that share the same traits. Resistance to single toxin Bt crops can undercut the durability of pyramid Bt crops (Tabashnik et al. 2008). Single traits serve as stepping stones for resistance to pyramids (Zhou et al. 2003) reducing the expected durability of the Bt technology below what could be expected if only pyramids were present in the landscape. For an in-depth discussion of this topic, refer to [Section III. B.3.](#)

### 4. Use of seed blends in the southern US expedite resistance in ear-feeding pests of corn

Planting of corn seed blends (Bt and non-Bt seed in the same field) creates four different pollination scenarios resulting in a mosaic of Bt expression in kernels. Two of these possible scenarios pose special IRM concern in the southern US: 1) maternal Bt  $\times$  paternal non-Bt and 2) maternal non-Bt  $\times$  paternal Bt. These particular pollination scenarios lead to a mosaic of Bt expressions with potentially sub-lethal toxic exposure allowing heterozygous resistant larvae to escape control while killing susceptible larvae only. This dynamic shifts the dominance of resistance towards higher values and can exacerbate resistance evolution in ear-feeding pests of Bt corn. For an in-depth discussion of this issue, refer to [Section III. B.4.](#)

### 5. Refuge non-compliance undercuts durability of pyramids

Publicly available data and data submitted to the agency as part of the IRM program for Bt corn products have shown that refuge compliance has historically been low in the southern US (Reisig 2017; BPPD 2017). Full compliance has been between 12-46%, while zero compliance (no refuge planted) ranged between 11-44% from 2011-2016. Surveys support a conclusion that knowledge of importance of available refuges does not increase refuge compliance among southern US corn growers. Lack of compliance increases the risk of resistance development. For an in-depth discussion of this issue, refer to [Section III. B.5.](#)

### 6. Methodology for sampling insects and diet bioassays delays resistance detection

EPA receives resistance monitoring reports from biotechnology companies that are based in part on random collections of insects from US corn and cotton growing areas. These types of samples provide a “snap shot” in time, do not track population susceptibility over time, and are reactive in nature.

Historical diet bioassay results for *H. zea* have been variable and difficult to interpret in part due to the random population sampling approach, the pest’s inherent genetic variability (Stone & Sims 1993; Ali & Luttrell 2007), lack of diagnostic concentrations for non-high dose toxins, fitness costs of resistant insects, and other methodological challenges. Given the current challenges, it is unlikely that resistance can be detected before it becomes visible as Bt field failure. For further discussions of this topic, refer to [Section III. B.6.](#)

#### 7. Lack of regulatory triggers for unexpected injury delays reporting of putative resistance

A lack of standardized, actionable regulatory thresholds of unexpected damage in Bt corn and cotton is likely leading to delayed reporting of problem fields and spread of resistance before mitigation can be initiated. While each registrant has its own thresholds for unexpected injury to corn and cotton, these are not expected to be uniform between companies and have not been shared with EPA. For an in-depth discussion of this topic, refer to [Section III. B.7.](#)

#### 8. Current definition of resistance delays mitigation actions

EPA's definition of pest resistance is based on heritability of the resistant trait, higher survival of resistant individuals compared to susceptible individuals on Bt crops, and visible economic damage caused to Bt crops in the field. This definition does not allow for proactive mitigation of resistance and is unlikely to achieve its intended goal of slowing the spread of resistance and maintaining the durability of Bt in surrounding areas. For an in-depth discussion of 'resistance' issue, refer to [Section III. B.8.](#)

#### 9. Cross-resistance between Bt toxins reduces durability of pyramids

Second generation, pyramided Bt crops produce two or more toxins active against the same pest and may delay the evolution of resistance through redundant killing (Comins 1986). However, cross-resistance can undermine redundant killing in pyramided crops and reduce the durability of the Bt technology. Cross-resistance occurs when selection for resistance to one toxin causes resistance to a second toxin. *H. zea* has shown documented resistance to first generation Cry1Ac and Cry1Ab Bt toxins, which are pyramided alongside other highly homologous Bt toxins. In cases where some insects survive on pyramids, even weak cross-resistance is expected to accelerate insect resistance evolution to additional toxins. For an in-depth discussion of cross-resistance, refer to [Section III. B.9.](#)

#### 10. Non-functional pyramids lead to faster resistance development

Bt resistance for multiple lepidopteran pests have been reported in the continental US (*H. zea*, *S. frugiperda*, *S. albicosta*, and *D. grandiosella*). Pyramided products expressing these traits have been compromised in some areas (<95% control by each toxin), yet are deployed with a relatively small refuge of 5% (the mandated refuge for pyramids). When one of the Bt components is compromised, then the reduced refuge will increase the risk of resistance to the second trait. For further discussion of this topic, refer to [Section III. B.10.](#)

#### 11. Mitigation of resistance occurs too late

Based on EPA's definition of resistance, mitigation tactics are not mandated until field resistance is confirmed (i.e. "practical resistance" and >50% of population carrying resistance genes, Tabashnik et al. 2014). The timing of mitigation is delayed and will complicate the success of the agency's goal to

extend the durability of Bt in unaffected regions and contain or reduce resistance in the affected areas. For further discussion of this topic, refer to [Section III. B.11](#).

## 12. *S. albicosta* resistance to Bt corn

### a. *Limited availability of Cry-toxins reduce longevity of Vip3A technology*

*S. albicosta* was identified as a sporadic pest of corn, and no IRM plan is currently in place, while only two registered Bt toxins showed activity against the pest at the time of product registration (Cry1F and Vip3Aa20). Since 2010, however, reduced susceptibility to Cry1F corn has been documented in corn growing areas. In 2017, control failure and resistance have been reported in Ontario, Canada, and several areas in the US (Smith et al. 2017; DiFonzo et al. 2016; Ostrem et al. 2016). Vip3Aa20 remains the only effective Bt trait available for western bean cutworm in these areas. For further information, see [Section III. B.12.a](#).

### b. *Low amount of refuge for single Bt trait reduces its durability*

Vip3A is pyramided with other toxins in Bt corn and deployed with a 5% refuge (external block or integrated) (US EPA 2010a). This trait is, therefore, deployed with a much lower refuge (5%) than would typically be required for single traits (20%) targeting major pests. This refuge scenario exerts a tremendous selection pressure on the only available Bt control tool for western bean cutworm. For further information, see [Section III. B.12.b](#).

### c. *Seed blends and risk resistance*

Larvae of *S. albicosta* feed in ears of corn, similar to *H. zea*. Sub-lethal expression to a mosaic of Bt kernels might favor survival of heterozygous resistant genotypes, while killing off susceptible insects, thereby effectively shifting dominance of resistance to higher values. Seed blends could, therefore, present another causal factor of resistance exacerbating resistance to Vip3A. For further information, see [Section III. B.12.c](#).

## III. SCIENTIFIC UNCERTAINTIES WITH IRM FOR LEPIDOPTERA PESTS AND POTENTIAL IMPROVEMENTS TO IRM STRATEGIES FOR Bt CROPS

### A. Lepidopteran Resistance Reports in the Continental US

Field resistance to three out of four Lepidoptera species has been reported by academic scientists in the continental US: *H. zea* (Dively et al. 2016), *S. frugiperda* (Huang et al. 2014), and *S. albicosta* (Smith et al. 2017). Resistance to these pests of Bt did not get reported through the regulatory process in place, likely because of methodological issues with sampling populations, confirmation of resistance, as well



as lack of uniform regulatory triggers (see earlier discussions). Nonetheless, the available evidence suggest resistance of these three pests has evolved to Bt in some parts of the continental US.

### 1. *H. zea* resistance and field failure reports

- a. Tabashnik et al. (2008a) suggested that *H. zea* field resistance to Cry1Ac cotton had evolved based on an analysis of laboratory diet bioassay data ranging from 1992 (pre-commercialization) to 2006 (post-commercialization). The authors reported resistance ratios from 1.2 up to 578. Moar et al. (2008) rebutted the reports of resistance by stating that the diet bioassay results from resistance monitoring activities needed to be tied to field performance of the traits as well as larval survival on the Bt plants. Tabashnik et al. (2008b) responded that progeny of USDA-collected adult insects were indeed from Bt and non-Bt cotton fields, and that reduced efficacy and increased larval survival had been measured in those Bt locations. Conversely, there was no reported field resistance in *H. zea* to any of the Bt toxins by Ali et al. (2006).
- b. Dively et al. (2016) conducted a 21-year and six-year field efficacy study for *H. zea* on Cry1Ab sweet corn and Cry1A.105+Cry2Ab2 sweet corn, respectively, in Maryland. The authors collected data on population abundance and damage observed in ears (Bt and non-Bt) as well as shifts in larval instar development across the years. The authors identified fitness cost in the resistant population when exposed to Bt, which resulted in prolonged larval and pupal development, lower pupal weight, and reduced survival to adulthood.

Across the years, infestation levels in the field were always high in non-Bt corn with >82% of ears damaged and >80% of larvae reaching late instar. The damage to Cry1Ab ears increased from >6% in 1996 to >85% in 2016. Mean instar size of surviving larvae in Bt also increased over the study period with the proportion of late instars getting increasingly larger compared to non-Bt corn. Between 1996-2006, fewer 4<sup>th</sup> – 6<sup>th</sup> instars were recorded. Between 2015-2016, 37% of surviving larvae reached 4<sup>th</sup> – 6<sup>th</sup> instar.

Trends in Cry1A.105+Cry2Ab2 field corn were similar to those observed for Cry1Ab sweet corn. Namely, damage in Bt increased significantly compared to non-Bt controls supporting a conclusion of reduced field efficacy (based on damage) of Bt plants. Between 2010-2012, >20% of Bt ears were damaged and >6% of larvae reached 4<sup>th</sup> instar. During 2015-2016, >59% of Bt ears were damaged with >70% of surviving larvae reaching 4<sup>th</sup> instar.

Assay results on the artificial diet without Bt leaf powder showed that the susceptible lab strain developed better (avg. weight 218 g) than the 2015 field collected strain (avg. weight 159 mg). Though the differences were not statistically significant ( $P$ -value = 0.099), this could indicate that the susceptible strain was better adapted to consuming the artificial diet than the field strain. When both strains were exposed to diet with incorporated Bt leaf powder at various concentrations (10-600 mg of Bt/25 ml diet), the weight gain of both colonies was reduced, but

the rate of decrease in weight was greater for the control colony than for the field colony ( $P$ -value  $< 0.05$ ).

The authors concluded that the rapid decrease in field efficacy and decreased susceptibility of *H. zea* in recent years provide strong evidence of field-evolved resistance to multiple Cry toxins (Cry1Ab, Cry1A.105, and Cry2Ab2).

- c. In a one-year study, Yang et al. 2017a<sup>3</sup> investigated the susceptibility to Bt toxins of *H. zea* with diet overlay assays. Populations were obtained from four areas of the southern US, such as Mississippi (1 population from a VT2P Bt corn), Louisiana (1 population collected on Bt cotton), Arkansas (1 population collected on non-Bt corn), and Tennessee (1 population each collected on Bt cotton and grain sorghum).

When the resistance ratios were obtained from LC<sub>50</sub> results, the range for the susceptible lab colonies (Benzon Research and USDA-ARS, Stonesville strain) was 1.0 – 4.9 for Cry1Ac and Cry2Ab2. The resistance ratios of populations collected from non-Bt hosts ranged from 1.6 – 48.3 (Cry1Ac) and 1.0 – 133.3 (Cry2Ab2) and on Bt hosts from 2.1 – 7.5 (Cry1Ac) and 2.4 – 133.4 (Cry2Ab2). The resistance ratios could not be calculated for Vip3A. When the resistance ratios were obtained from MIC<sub>50</sub> results, the range for the susceptible lab colonies (Benzon Research and USDA-ARS, Stonesville strain) was 1.0 – 5.1 for Cry1Ac and Cry2Ab2. The resistance ratios of field population resistance collected from non-Bt hosts ranged from 1.6 – 5.7 (Cry1Ac) and 1.0 – 133.4 (Cry2Ab2) and on Bt hosts from 9.5 – 48.3 (Cry1Ac) and 4.0 – 35.7 (Cry2Ab2). Any resistance ratios in excess of 10 were indicated to be significant.

- d. Two locations from which Yang et al. (2017)<sup>3</sup> collected population samples were subsequently reported by Monsanto Company (Monsanto) as problem performance sites for 2016 Bt cotton fields (Bollgard II, Cry1Ac + Cry2Ab2) in Tennessee and Louisiana (Berman et al. 2017). Monsanto did not collect insects in TN because 6% boll damage and possibly other factors did not meet their criteria for population collections. Likewise, in LA no collections were undertaken by the company because no insects were present after the problem field was sprayed. Reported boll damage was 19%. Monsanto reported that these results fell well into the range of the historical base data for *H. zea*.

Monsanto also reported that two additional UXI fields occurred in North Carolina and Texas (Berman et al. 2017). Both fields did not meet the criteria for insect collections to begin, although those criteria were not reported to the agency.

- e. Bayer CropScience (Bayer) submitted a report for four unexpected damage incidents in TwinLink Cotton (Cry2Ae + Cry1Ab) in three US locations (Texas, Louisiana, and Oklahoma) during the 2014 cotton growing season (Payne et al. 2017). The company was unable to obtain

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<sup>3</sup> EPA recognizes that these data have not been published yet and is treating them as a preliminary report of potential resistance.

insects in LA and OK, but collected two larval populations in Texas in 2015 and another population in 2016 all in excess of 100 individuals. The insects were reared and testing occurred with neonates of the F1 generation.

The LC<sub>50</sub> and MIC<sub>50</sub> (2<sup>nd</sup> and 3<sup>rd</sup> instar) for Cry2Ae and Cry1Ab were lower than the baseline monitoring data submitted to the agency (Payne et al. 2014). The field results were somewhat higher than the susceptible control colonies, however. Bayer concluded that the field-collected populations remained susceptible to the traits expressed in TwinLink Cotton.

### Uncertainties

EPA did not receive information about the larval survival of the field collected populations from Texas (UXD/UXI reports). It is unclear if potentially resistant larvae were killed during the transport to the lab and during the rearing process. This information is needed to judge the results in their entirety and context. The conclusion that the field populations were susceptible (yet caused calls about unexpected damage) is somewhat non-intuitive. If the unexpected damage fields had a high insect pressure year, this information should have also been submitted.

If heterozygous resistant individuals of *H. zea* behave much like their susceptible counterparts (Blanco et al. 2008), Bayer's diet bioassay with F1 generations would not detect resistance. Both *RS* and *SS* individuals could be expected to die from Bt exposure. An F<sub>2</sub> screen should have been conducted to obtain homozygous resistant survivors that then allow estimating the resistance allele frequency in the field sample.

### 2. *D. grandiosella* resistance reports

Pioneer Hi-Bred International, Inc. (Pioneer) submitted a Cry1F resistance monitoring report to EPA in which the company reported two unexpected damage incidents from Arizona (Cochise Valley) and one occurrence in New Mexico from Southwestern corn borer feeding in 2014 (MRIDs 497854-01 and 501749-01). The company was unable to collect population samples from the damaged fields in 2015 and obtained samples from three nearby locations in Arizona only but also obtained collections from outside the region (e.g., OK and TN for comparison purposes). Pioneer conducted diet incorporated bioassays on the offspring populations from Arizona field samples to estimate the EC/LC<sub>50</sub> and resistance allele frequency. Survivors of the diet bioassay study were reared to initiate an on-plant feeding experiment on Herculex (Cry1F) corn in the field.

The LC<sub>50</sub> could not be measured for the Arizona population. Pioneer reported that the LC<sub>50</sub> values were >31.0 µg/ml of diet than the highest concentration tested and ranged from 55.3-260 µg/ml. In contrast, the LC<sub>50</sub> of the susceptible lab colonies was 2.9 and 3.3 µg/ml of diet (MRID 497854-01).

Mortality for the Cochise Valley population at the highest concentration tested was 13.9% (68.6 µg of Cry1F/ml), while control mortality on non-Bt diet was 9.2% (0 µg of Cry1F /m). Pioneer concluded that the Cochise population had developed resistance to Cry1F based on the calculated resistance ratio (310) and the resistance allele frequency (0.97) (MRID 501749-01).

The infestation of Cry1F corn with eggs from survivors occurred over 3 days (20 eggs/day-plant). Plants were caged, larvae were collected from non-Bt and Bt plants, and survivors (larvae + pupae) were shipped in ethanol to the laboratory for measurements. The mean number of insects from UXD populations did not differ on Bt and non-Bt treatments (survivorship 89.8% and 100%, respectively). The instar distribution for the Arizona population was not significantly different on Bt and non-Bt plants. The lab colony's survival was 0.4% on Bt and 100% on non-Bt. In this case, the distribution of instars was significantly different on Bt compared to non-Bt (MRID 501749-01).

Pioneer concluded resistance had evolved in southwestern corn borer in the populations investigated. Because of the Cochise valley's isolated location, mitigation of SWCB resistance to Cry1F corn should be more likely attainable. Pioneer implemented southwestern corn borer resistance mitigation plan (planting of pyramided products only), best management practices (planting required refuge, early planting, scouting, use insecticides when needed, destroy crops post-harvest) in Cochise valley. Across Arizona, New Mexico and western Texas monitoring for resistance is increased and 5-7 insect collections total will be targeted yearly. Communication with key stakeholders in the regions was enhanced to inform them of the resistance to Cry1F and recommend best management practices when the economic thresholds were met (MRIDs 501749-01 and 501749-02).

#### Uncertainty:

EPA considers the mitigation approach taken by Pioneer reasonable. However, mitigation of resistance would ideally occur with an unrelated pyramided product or a three-gene containing pyramid expressing the compromised trait. It is unclear whether Pioneer has implemented mitigation with an unrelated pyramided product since resistance has been confirmed.

### 3. *S. frugiperda* resistance reports

In 2006, reports of Cry1F corn field failures caused by *S. frugiperda* were reported on the island of Puerto Rico and resistance confirmed later (Storer et al. 2010, 2012). Haplotype similarities between Puerto Rico and Florida insects suggested a mechanism of unidirectional migration from the island to the continental US mainland (Nagoshi et al. 2007), though no reduced Cry1F susceptibility was found in eastern *S. frugiperda* population collections obtained in 2010 and 2011 (Huang et al. 2011).

However, Huang et al. (2014) reported that most of the populations from Georgia, Florida, Louisiana, North Carolina, and Texas, investigated during 2011-2013, had resistance ratios greater than 10 with the highest ratio exceeding 270. The authors concluded that Cry1F resistance had evolved in *S. frugiperda* and could be extensive in the southeastern US.

#### Uncertainty:

Currently, there are no IRM requirements for fall armyworm since the pest can only overwinter in a limited portion of the southern US. If the pest becomes established and overwinters in the US, this pest may need to be elevated to a primary pest of corn and require an insect resistance management plan.

#### 4. *S. albicosta* resistance reports

- a. Dyer et al. (2013) tested the survival of field collected *S. albicosta* from Nebraska at different concentrations of Cry1F and Cry1A toxins using toxin overlay assays. For Cry1A toxins, survival was not significantly different than from the negative control, and no concentration response was observed. Conversely, for Cry1F, no LC<sub>50</sub> could be determined due to low mortality at tested concentrations. An EC<sub>50</sub> (growth inhibition as evidenced by head capsule measurements) was estimated at approximately 731 ng/cm<sup>2</sup> after 14 days. Dose response assay results support that larval tolerance to Cry1F toxin was relatively high – even among neonates. At 2,500 ng/cm<sup>2</sup>, it was reported that larval mortality incurred was 46.9%; 100% mortality was never achieved across the range tested (250-25,000 ng/cm<sup>2</sup>). The authors concluded that field-collected larvae possessed genetic variation that allowed them to feed on Cry1F corn and finish development. The concentration response results were consistent with field reports of Cry1F corn damage.
- b. Ostrem et al. (2016) conducted a multi-year analysis for the purpose of developing a benchmark susceptibility of western bean cutworm to various Cry-toxins using (diet incorporated) bioassays. Insect samples were collected from cornfields in Nebraska (n = 5, 8) and Iowa (n = 3, 3) during 2003 and 2004 (respectively) as well as from cornfields in Nebraska (n = 5, 4), New Mexico (n = 1, 0), Texas (n = 2, 0), and Colorado (n = 0, 1) during 2013 and 2014 (respectively). Cry-toxin concentrations ranged from 0.5-100 µg/ml in 2003-2004 and 0.049-800 µg/ml of diet in 2013-2014. Most of the LC<sub>50</sub> values in 2003/4 were above the maximum concentration tested; extrapolation was not possible because the data did not fit the Probit model. For those populations where the LC<sub>50</sub> were measured and confidence limits could be calculated, the 2003 mean results were as low as 8.1 µg/ml (NE) and as high as 72.7 µg/ml (IA). In 2004, the lowest LC<sub>50</sub> that could be measured was 5.3 µg/ml (NE) and as high as 128.1 µg/ml (NE). In 2013/14 the concentration of Cry1F was increased to 800 µg/ml. A meta-analysis (based on all data – field and lab) calculated the mean LC<sub>50</sub> at 42.2 µg/ml (95% C.I. = 21.4-170.9) in 2003, 39.7 µg/ml (95% C.I. = 25.5-70.7) in 2004, 514.4 µg/ml (95% C.I. = 290.3-1156.4) in 2013, and 116.9

$\mu\text{g/ml}$  (95% C.I. =70.4 -172.4) in 2014. The authors identified a 5.2-fold increase in  $\text{LC}_{50}$  for the 2013 and 2014 populations compared to 2003 and 2004 results.

- c. Smith et al. 2017 investigated the damage caused by western bean cutworm to Cry1F expressing corn from 2011-2015 in Ontario, Canada. In addition, they reported on the susceptibility to Cry1F corn from 2015 field collections. The field trials were conducted on three farms with sandy loam soils. In 2015 western bean cutworm moths were collected from light traps and egg masses were obtained from naturally infested plots at their field trial locations. The moths were reared in the lab and diet bioassays were conducted with the resulting offspring as well as with neonates from obtained eggs using methods described by Dyer et al. (2013). The highest concentration tested was  $75 \mu\text{g/cm}^2$  (F1 neonates) and  $30 \mu\text{g/cm}^2$  (F0 neonates from field); mortality was assessed after 7 and 14 days, and weight of surviving larvae was also measured at that time.  $\text{LC}_{50}$  (lethal concentration) and  $\text{EC}_{50}$  (growth inhibition) were measured or estimated along with confidence limits.

The number of larvae found per corn ear were measured in field trials during 2013 and 2014. A comparison between numbers revealed that there was no statistically significant difference between densities from different treatment sites (Bt vs. non-Bt). Diet bioassays conducted at the highest concentration did not result in 100% mortality, but Smith et al. (2017) reported that mortality of WBCW increased with increasing concentration of Cry1F. Two of the populations had  $\text{LC}_{50}$  values that were higher than the highest concentration tested. All associated Chi-square values were significant, however, indicating that the model was a poor fit. The percent mortality at the highest concentration tested after seven days of exposure was available for two populations only. The reported efficacy was 51.4 and 76.0% for the F1-generation (14-day values not reported) and 40.3 – 84.7% for F0-generation.  $\text{EC}_{50}$  values (day 14) were 19.8 and  $12.9 \mu\text{g/cm}^2$  for F1 and ranged from 0.1 –  $4.9 \mu\text{g/cm}^2$  for the F0-generation.

The authors concluded a visible reduction in efficacy (similar to Ostrem et al. 2016) and raised the possibility of field-evolved resistance to Cry1F corn.

#### EPA options to address resistance concerns

EPA concludes that Cry1F resistance in *S. albicosta* has evolved in the western states of the US Corn Belt (Ostrem et al. 2016) and Canada (Smith et al. 2016). In light of only one Bt toxin available to control western bean cutworm (Vip3A) and additional Cry1F field failures across northern lake states in the US (DiFonzo 2016) and Canada, EPA considers whether it is warranted to develop an IRM plan for *S. albicosta*. IPM may need to be an integral part of managing this pest in Bt due to lack of availability of different traits (e.g., monitoring for adult densities, increased scouting for eggs in Bt corn and use of conventional insecticides) (Smith et al. 2017). A management plan for western bean cutworm may need to consider additional impacts on major lepidopteran pests in corn in the various regions of concern. To protect the durability of the only efficacious trait available for control which is

deployed with a 5% refuge, it may be necessary to use additional insecticide treatments for the early instar stage of this pest. Monitoring for flight of western bean cutworm may be necessary because of challenges associated with detecting early instar larvae.

EPA is considering whether sentinel plots in sweet corn could serve as a tool for resistance monitoring in western bean cutworm. Such a focused sampling procedure would allow keeping track of specific populations and could alert Bt registrants when early shifts in resistance to Vip3A occur. Sentinel plots would best be established in known areas of Cry1F resistance and where pest pressure is expected to be high based on environmental conditions.

### Uncertainties

For *H. zea* and *S. albicosta*, the only efficacious trait available (Vip3A) is planted with a 5% refuge because it is only available in pyramided varieties. This low refuge percentage exerts a large selection pressure for resistance when used with pyramided varieties containing Vip3A that are functionally equivalent to a single trait product in areas with resistance to the other traits in the pyramid. It is unclear whether IPM + IRM could significantly delay resistance development to Vip3A, especially for *H. zea*, since both corn and cotton express this trait in the south.

## B. Causal Factors of Resistance

### 1. Non-high dose traits and risk of faster resistance development

An inherent tolerance to Bt was reported for *H. zea* from various locations across the southern US before the initial Bt PIP commercialization. Underlying reasons for the observed tolerance are speculative and not well understood. One hypothesis is that *H. zea* may have increased types of detoxifying enzymes because of its polyphagous nature. This might also provide greater tolerance for Bt toxins (Stone & Sims 1993).

None of the registered Bt corn and cotton PIPs express a high-dose against *H. zea* (see [Section I.B.](#) for discussion of the high dose concept). In 2008, however, the agency determined that Vip3Aa20 (in corn) expressed “close to a high dose” for this pest (US EPA 2009). Its analog, Vip3Aa19 (in cotton), showed somewhat lower expression but was determined to be efficacious as well. Interestingly, even for the more efficacious Vip toxins, *H. zea* susceptibility (measured as failure to molt, MIC<sub>50</sub>) varied up to 59-fold (Ali & Luttrell 2011). The lack of available high dose PIPs in the southern US makes the stewardship of Bt traits with refuges more challenging and resistance development in *H. zea* more likely.

Single trait products pose a greater resistance risk than pyramids because pests have to overcome only one as opposed to multiple modes of action. If resistance evolves to single trait products, pyramids that

contain the compromised traits effectively become single trait products. Resistance to the second trait can then evolve more rapidly (Zhao et al. 2005) because of the lower percent refuge requirement.

If a single trait expresses less than high-dose, resistance evolves more rapidly than for a high dose product. The durability is lowest for traits that result in intermediate mortality and where heterozygous resistant genotypes are likely to survive Bt exposure (Comins 1977; Tabashnik & Croft 1982). The lowest durability occurs somewhere along the dose (susceptible mortality) range of 0.8 – 0.9 but can be shifted towards a higher durability for the trait if the pest has a low population growth rate (Martinez et al. 2017). Since *H. zea* has shown to have high population growth rates on corn and cotton (though somewhat lower on the latter) (Caprio et al. 2009), it is even more important that growers have access to traits that express a high dose for *H. zea*.

### EPA options to address risks of resistance

Development of new Bts or other novel traits expressing high dose activity against *H. zea* would greatly benefit the management of this economic pest in cotton. If these types of traits were incorporated into both corn and cotton, management options should focus more on corn since the pest funnels through that crop in the summer and spends two generations in corn in the southern US.

Other options for non-high dose single trait products are to use IPM with the deployment of single Bt products, limit single trait commercial corn products, and transition from single trait products to Bt corn pyramids.

### Uncertainties

Stadelbacher et al. (1983) proposed the hypothesis that *H. zea* could tolerate Bt better than other insects because of its polyphagous nature. Such a host adaptation could provide greater genetic variation for detoxifying enzymes. If true, then polyphagia may not be the only mechanism to allow pests to cope with Bt toxins. For example, corn rootworm (*Diabrotica virgifera* LeConte) has shown great tolerance for Bt and is far less polyphagous and primarily feeds on maize but also cucurbits and wheat (US EPA 2013). The causes for *H. zea*'s greater Bt tolerance (compared to other pests) need to be identified because this information may provide a key to developing a novel tool with which the natural tolerance to Bt could possibly be blocked or resistance may be reduced.

## 2. Toxins expressed in corn and cotton exert continuous selection pressure

With the exception of Cry1Ac and Cry2Ae, all toxins expressed in Bt cotton are also expressed in Bt corn products (see [Table 3](#) and [Table 4](#)). But because of documented cross-resistance between some of the toxins, it can be stated for simplicity that all toxins are expressed in both Bt crops. The presence of the same Bt traits in corn and cotton exerts a continuous selection pressure on the multiple generations of *H. zea* per year in the southern US and presents a resistance concern. Resistance management for



cross-resistance conditions across multiple pest generations per year has limited options to reduce selection pressure. The problem is further compounded with the limited availability of efficacious Bt toxins against *H. zea*. If such toxins are expressed in non-functional pyramids of corn and cotton, the durability of these traits could be reduced substantially.

### EPA options to address risks of resistance

Management of efficacious Bt traits that are expressed in corn and cotton should focus on the corn system in the south. *H. zea* prefers corn as its primary host in the summer, and hence, funnels through this crop with two generations per year. During the corn growing season, the relative population densities should be the highest on this crop compared to others in fall on Bt cotton. The subsequent two generations have a variety of equivalent hosts to choose from; those are cotton, sorghum, soy beans, and other cultivated crops and wild hosts. Appropriate and effective management options that primarily focus on Bt corn could prolong the lifetime of these traits in Bt cotton where *H. zea* is an economic pest of concern.

### 3. Mosaic of single and pyramided products undercuts durability of pyramids

Before pyramided Bt cotton (Cry1Ac × Cry1Ab) was introduced in Australia, the single Bt product (Cry1Ac) was limited to 30% of cotton acres (Mahon et al. 2007). This acreage limit was implemented to keep the Cry1Ac resistance allele frequency low in *Helicoverpa armigera* (i.e., old world bollworm) and to extend the durability of the later introduced pyramided product (Roush 1998). *H. armigera* is closely related to *H. zea* and is a less-than high dose pest of Bt also. In the US as well as Australia, both single Cry1Ac cotton products were subsequently removed after the introduction of the Bt cotton pyramid. In the US, this decision of removing single traits was reached to support the introduction of the natural refuge paradigm (EPA FIFRA SAP 2006a).

Conversely, Bt corn products are commercialized with single and pyramided traits with a block refuge, refuge strips, or seed blends. In the southern US, the risk of resistance development and establishment in populations of *H. zea* is greatest because the pest has multiple generations per year and successfully overwinters. Single Bt corn products in the south present a resistance risk to pyramided products (Zhao et al. 2005) in corn as well as cotton.

## EPA options to address risks of resistance

Single trait Bt corn planted concurrently with pyramided products reduce the lifetime of pyramids because the pest can evolve resistance to traits sequentially and does not incur ‘redundant mortality’ any longer on pyramided products. As such, reducing (or eliminating) single trait corn products will likely increase trait durability. All currently registered Bt cotton products for commercial use are pyramids, and the use of single trait cotton products were phased out to support the use of natural refuge for Bt cotton (Matten et al. 2013). However, management options to mitigate this resistance risk should focus on Bt corn because *H. zea* (major pest of cotton) has two generations in the southern US that funnel through corn in the summer.

## Uncertainties

EPA has concluded that *H. zea* developed resistance to Cry1 and Cry2 toxins in some areas of the US. The durability of Vip3A expressed in corn and cotton pyramids with such toxins will experience great selection pressure. The effect of mitigation on the durability of Vip3A is uncertain in light of these resistance cases.

### 4. Seed blends in the southern US and faster resistance development in ear-feeding pests of corn

#### *a. Mosaic of variable toxin expressions in kernels*

Seed blend refuges in southern regions are expected to increase the rate of adaptation to ear-feeding pests of corn, such as *H. zea* and *S. frugiperda* (US EPA 2012, Caprio et al. 2016). In a single Bt RIB cornfield consisting of 90% maternal Bt and 10% maternal non-Bt plants, corn ears could be pollinated by paternal Bt and paternal non-Bt (pollen) at a rate of 0.9 and 0.1, respectively. However, the actual cross-pollination rate can differ and may be affected by factors such as proximity of plant types, pollen shed timing, and wind (Burkness et al. 2011). Cross-pollination between the two types of plants results in a mosaic of Bt expression in kernels throughout the ears but is greatest in the ear tip area (Burkness & Hutchison 2012). Partially resistant pests may exploit such a mosaic of expression by tasting and rejecting toxic kernels and moving on to less toxic kernels, while truly susceptible insects die. Sub-lethal Bt exposure can provide a pathway of selection for heterozygous resistant larvae, which have a fitness advantage compared to susceptible individuals under this scenario. This mechanism of selection shifts (increases) the functional dominance of the resistance alleles and increases the risk of resistance development in ear-feeding pest. Yang et al. (2017) conducted a kernel feeding study using fall armyworm, and their results demonstrated that *RS* survival in RIBs was enhanced compared to *RS* survival in pure stand Bt because of cross-pollination. Conversely, early data by Burkness et al. (2011) did not support that survival of *H. zea* differed on kernels obtained from maternal Bt plants that were surrounded by either Bt or non-Bt plants. Even in a pure stand Bt field, a degree of mosaics can be encountered that could be exploit (further discussed below). Therefore, IRM for ear-feeding pests of

corn may have fundamental challenges that are not encountered for pests that exclusively feed on maternal tissue. In seed blends, however, the problem is further compounded because the within-field refuge is corrupted by Bt cross-pollination compromising the objective of the refuge.

*b. Pollination pathways, survival, and development:*

The pollination in RIBs is variable and can occur between i) Bt maternal  $\times$  Bt paternal, ii) Bt maternal  $\times$  non-Bt paternal, iii) non-Bt maternal  $\times$  Bt paternal, and iv) non-Bt maternal  $\times$  non-Bt paternal gametes. The first pollination scenario is the most likely to occur for Bt plants simply based on the fraction of Bt and refuge seed planted in seed blends (90:10 or 95:5). The second scenario will add to a reduction in toxin expression in kernels of Bt plants that are surrounded by refuge plants because one gamete lacks Bt genes. The third scenario is the more likely pollination outcome for non-Bt plants surrounded by a majority of Bt plants and introduces Bt exposure into refuge ears. The fourth scenario occurs at the lowest frequency and results in true refuge kernels.

*c. Maternal Bt plants:*

There are four major tissues in a kernel: the pericarp, a thin layer of maternal and diploid tissue around the embryonic tissue; the endosperm and aleurone, both triploid and embryonic tissues containing two maternal and one paternal gamete; and the embryo, a diploid tissue with one copy of a maternal and paternal gamete. The pericarp can be ignored for exposure purposes because it makes up a negligible amount of thin (Bt) tissue that a larva is exposed to when feeding on a kernel (Chilcut and Tabashnik 2004). The focus of this discussion is on the diploid embryonic tissue and its genotypes and frequencies that are possible from a cross of two Bt plants in a pure stand Bt plot for two hypothetical products, a three gene Bt pyramid (a.) and a single gene Bt product (b.).

Punnett Squares (a. and b.) show the genotypes resulting from the cross of a Bt plant and Bt pollen under the different trait scenarios in a pure stand Bt field where pollination occurs between Bt plants (for discussion of unlinked traits see Caprio et al. 2016).

- i. Mosaic of kernels with variable Bt expression for a three-gene pyramid planted to pure stands

			Paternal Gametes			
			Hypothetical, Linked Traits (A,B) X Trait C			
			++/++	++/-	--/+	--/-
Maternal Gametes	Hypothetical, Linked Traits (A,B) X Trait C	++/++	++/+, ++/++	++/-, ++/++	--/+, ++/++	--/-, ++/++
		++/-	++/+, ++/-	++/-, ++/-	--/+, ++/-	--/-, ++/-
		--/+	++/+, --/+	++/-, --/+	--/+, --/+	--/-, -/+
		--/-	++/+, --/-	++/-, --/-	--/+, --/-	--/-, --/-

Probabilities for embryonic genotypes for the three Bt traits:

- 1/16 of kernels expressing 0 copies of A\_B (linked traits) and 0 copies of C;
- 1/16 of kernels expressing 2 copies of A\_B and 2 copies of C;
- 4/16 of kernels expressing 1 copy of A\_B and 1 copy of C;
- 2/16 of kernels expressing 1 copy of A\_B and 0 copies of C;
- 2/16 of kernels expressing 0 copy of A\_B and 1 copies of C;
- 1/16 of kernels expressing 0 copy of A\_B and 2 copies of C;
- 1/16 of kernels expressing 2 copies of A\_B and 0 copies of C;
- 2/16 of kernels expressing 1 copy of A\_B and 2 copies of C;
- 2/16 of kernels expressing 2 copies of A\_B and 1 copies of C.

- ii. Mosaic of kernels with variable Bt expression for single trait products planted to pure stand

		Paternal Gamete		
		Hypothetical Single Trait (A)		
		+	-	
Paternal Gamete	Hypothetical Single Trait (A)	+	+, +	+, -
	-	-, +	-, -	

Probabilities for embryonic genotypes for the three Bt traits:

- 1/4 of kernels expressing 0 copies of A;
- 1/4 of kernels expressing 2 copies of A;
- 1/2 of kernels expressing 1 copy of A.

*d. Cross-pollination of refuge ears in RIBs:*

Cross-pollination between maternal non-Bt and paternal Bt plants in RIBs can be expected to reduce the available amount of ‘true’ refuge kernels. Burkness et al. (2011) found that maternal non-Bt plants pollinated by paternal Bt (Cry1Ab) plants caused intermediate survival. In their preliminary study, survivorship on non-Bt plants was determined for two ear-feeding lepidopteran pests of corn (*Ostrinia nubilalis* and *H. zea*). Both pests experienced some mortality on refuge plants in the field that were pollinated by surrounding paternal Bt plants. Actual field survival was reported at 43% and 63% for *O. nubilalis* and *H. zea*, respectively, compared to survival on pure stand non-Bt corn, though the authors acknowledged that other factors could have contributed to observed mortality. In the lab, *O. nubilalis* survival on cross-pollinated kernels was higher, for example (60%). A second, more extensive study focused on the larval movement, development, and survival of *H. zea* in a seed mixture (Cry1F × Cry1Ab × Vip3A). The authors reported early instar inter-plant movement (2-16%), which may have been underestimated, and delayed development and lower survival on refuge plants in RIBs compared to structured refuges (Burkness et al. 2011, 2015).

Similarly, Yang et al. (2017b) estimated the contamination of refuge kernels to be 87% (5% RIB) and 66% (20% RIB) based on two trial locations. Survival to adulthood on cross-pollinated refuge kernels

was 42.3 – 50% and 8.7 –10% for heterozygous resistant and susceptible genotypes, respectively (Yang et al. 2017bc). Survival on pure stand refuge ears was similar for all genotypes and ranged from 62.5 – 73.7%. In addition, pupal development of SS and RS pupa was significantly delayed on seed blend refuge plants compared to a pure refuge stand.

#### EPA options to address risks of resistance

EPA has concluded that the risk of resistance for ear-feeding pests of Bt in RIB corn needs to be addressed in the southern US where *H. zea* is a major pest, has multiple generations, and overwinters. This risk of resistance posed by RIBs for pests like *H. zea*, will likely compromise EPA's goals for IRM, which are to prolong the lifetime of non-high dose traits in cotton and corn, especially in areas where the technology is still effective, and protect the longevity of still functional and efficacious Bt trait(s). An option would be to limit the use of Bt corn RIB products in the southern US. Though recent modeling proposes that RIBs for *H. zea* may be equally if not more durable than with block refuge deployment when incorporating grower refuge non-compliance (Onstad et al. 2018).

Seed blends could become a viable option for resistance management in the southern US if the Bt mosaic and corruption of refuge kernels in seed blends can be eliminated or substantially reduced. A potential solution for maternal Bt plants is to construct parental lines that are no longer hemizygous for Bt traits and contain matching sets of Bt genes on both chromosomes. Changes to the corn breeding program could create possible solutions for RIBs, however: 1) Move away from hemizygous parental lines; and 2) introduce pollen incompatibilities between Bt and non-Bt cultivars, and self-pollinating mechanisms through advances in gene editing, which create kernels with homozygous maternal traits.

#### Uncertainties

It is currently unknown whether kernels expressing one copy of the Bt gene express less protein than a kernel with two copies of the gene. This information would be useful when more thoroughly discussing and assessing the risk of resistance from hemizygous Bt plants.

If self-compatible refuge corn hybrids were developed for seed blend products, the integrity of the refuge could be maintained for ear-feeding pests. It is unclear whether the cost of developing self-compatible refuge plants with matching genes on both chromosomes would be economically profitable or whether it would exceed the cost of developing an incentive program that assures grower compliance with structured refuges.

Similarly, Bt hybrids could be developed that are only compatible with Bt pollen. Since Bt plants are surrounded by mostly other Bt plants (95% for pyramided products), this would assure sufficient pollen donors for Bt ears.

## 5. Refuge non-compliance in the southern US undercuts durability of pyramids

Full grower compliance with refuge requirements (meaning they planted the correct percentage of refuge corn) in the cotton region of the US has historically been lower (7-46%) than in the northern Corn Belt (68-73%, not shown in Table 5). The percent of growers being partially compliant, meaning they planted a refuge but less than the mandated percentage, remained variable over the course of seven years and highest in 2017. Non-compliance more than doubled from 2015 – 2017 (BPPD 2014, BPPD 2017, MRID 505101-01). Zero compliance (no refuge at all) was high, very similar over the seven years reported, and lowest in 2017.

EPA concluded (BPPD 2017) that refuge compliance in the southern US had further decreased over the past several years. This increase in non-compliance is likely caused by multiple factors, some of which are 1) seed blend adoption in that region; 2) the mandate for a greater refuge requirement in the south for single trait and pyramided products (50% and 20%, respectively) compared to the northern Corn Belt; 3) lack of desired hybrids in refuge products (Reisig 2017); and 4) other factors driven by the market and pest complexes in these areas.

**Table 5. Grower Compliance with Refuge Requirements for Bt Corn in the Southern US**

<b>Southern Growers Surveyed who:</b>	<b>2011</b> (n =122)	<b>2012</b> (n =112)	<b>2013</b> (n =95)	<b>2014</b> (n =100)	<b>2015</b> (n =95)	<b>2016</b> (n =48)	<b>2017<sup>1</sup></b> (n =105)
Met refuge size requirement	42%	42%	46%	45%	34%	7%	19%
Partially met refuge size requirement	26%	24%	25%	18%	25%	47%	55%
Planted no refuge acres	32%	34%	29%	37%	41%	46%	26%

ABSTC survey data submitted to EPA before 2011 did not provide the same detail of information and, thus, was excluded from this table.

<sup>1</sup> Survey results were not separated for below and above-ground pests in 2017 but reported as a combined percentage of compliance.

An independent survey conducted in North Carolina in 2014 and 2016 assessed growers' knowledge of IRM and the willingness to plant a refuge for Bt corn (Reisig 2017). Some conclusions based on the data were that owners of small farms were less likely to plant a refuge. The opposite was the case for growers of large farms who planted corn and soybeans. Communicating the importance of refuges did not increase growers' intentions to plant a refuge.

### EPA options to address concerns

Growers likely favor the planting of RIB products because Refuge-In-the Bag products expedite the planting process and reduce labor inputs. Likewise, RIB products could be favored by industry because they guarantee that refuges for Bt corn are planted and growers remain compliant with refuge

requirements. It is, therefore, not surprising that RIBs are viewed as the solution to grower refuge non-compliance in southern states. At this point, however, there are several lines of evidence supporting the conclusion that RIBs are less successful IRM strategies for ear-feeding pests of Bt corn (e.g., *H. zea*, *S. frugiperda*, etc.) than block refuges, especially in areas where pests have multiple generations per year and overwinter. EPA's refuge requirement for single trait RIB products is lower than for the equivalent technology deployed with a block refuge (10% vs. 50% in the south) - though an accompanying structured refuge of 20% is also required in south. Preliminary modeling results suggest that the addition of a structured refuge with seed blends (if planted) may not adequately delay resistance evolution (Caprio and Martinez 2013). The agency considers options to increase grower compliance with planting block refuges through, for example, grower incentives or reward programs, that assist in reducing resistance occurrences in Bt crops. More research is needed into the human factors underlying growers' behaviors that lead to non-compliance.

### Uncertainties

It is uncertain whether industry will be able to successfully increase refuge compliance among southern growers to delay resistance development sufficiently in *H. zea*. Delaying resistance will also depend on the Bt trait of concern, the respective resistance levels present in the pest, whether cost to resistance exists in *H. zea*, and the resistance management options implemented in Bt corn. This demonstrates that resistance management in the southern US is complex; the ultimate resistance outcome (measured as durability of trait) depends on multiple factors acting together in favor of extending the Bt lifetime. However, for non-compliance with refuge requirements to decrease, a greater number of on-farm visits may be needed. In addition to industry efforts, outreach to growers through the National Corn Grower Association, grower consulting groups, and University extension experts may be needed to communicate the importance of planting block refuges. Furthermore, a greater focus on on-farm assessments could be coupled with a rewards program that motivates growers to comply with requirements. Assessing the success (gain in years before resistance evolves) of increased refuge compliance in the south can be simulated with complex theoretical models that incorporate grower behavior, landscape, diverse cropping system, cost and benefits of compliance, ecology, and other parameters. Increasing the refuge compliance should extend the life-time of Bt traits compared to seed blends (Carrière et al. 2016 and others). Increasing the refuge compliance would lead to a greater proportion of non-Bt corn in the landscape, which may not necessarily elicit a linear increase in durability gains for pyramided traits (Ives et al. 2011). In addition, these models show that pest population dynamics (e.g., density dependence, growth rate) interact with the dose of Bt and which results in an associated increase or decrease in the expected gain in years before resistance evolves based on the amount of available refuge (Martinez et al. 2017).



## 6. Methodology for insect collections and diet bioassays delays resistance detection

### *a. Current Resistance Monitoring and Mitigation Strategies for Bt Corn and Cotton*

Through the terms of registration, Bt corn and cotton registrants are required to implement a resistance monitoring program for the major lepidopteran target pests. A primary goal of resistance monitoring is to detect shifts in the frequency of resistance genes (i.e., susceptibility changes) before the onset of resistance leads to widespread Bt crop failure (see EPA 2010a).

Monitoring for resistance has consisted of two main components: 1) investigation of unexpected field damage reports from growers, extension agents, consultants, or company agronomists, and 2) monitoring for changes in susceptibility through targeted population sampling and testing. Unexpected damage reports may reveal the occurrence of more localized resistance before the effects might establish regionally. Resistance monitoring through targeted field sampling can reveal changes in susceptibility of geographically representative populations. In the event that resistance is confirmed, a mitigation (remedial action) plan is triggered as defined by the terms of registration (see EPA 2010f). Reports covering each monitored pest and Bt PIP toxin must be submitted annually to the agency. A summary of the data submitted for *H. zea* is detailed in the section below.

The resistance monitoring and mitigation plans for Bt corn and cotton were previously described. The strategies are conceptually similar, though the target pests and resistance standards vary between the crops.

### *b. Summary of H. zea Bioassay Results*

Historically, insect resistance monitoring for conventional pesticides has been conducted with diet bioassays using dose/response curves to make comparisons between LD/LC<sub>50</sub>s of reference (susceptible) strains and field-collected populations. Roush and Miller (1986) concluded that this type of comparison was adequate if resistance allele frequencies in a population were high but that it was not an effective method for early resistance detection. They proposed that a diagnostic assay approach, in which susceptible individuals were killed but resistant individuals survived, would be more efficient when monitoring for resistance. However, the authors also noted that even with diagnostic tests, the required sample size needed to detect a resistance allele frequency of 0.01 could be very large.

Both susceptibility (LC and EC) comparisons and diagnostic assays have been incorporated into the currently mandated IRM program for Bt PIPs to identify potentially resistant populations. For Lepidoptera pests (e.g., ECB, SWCB), diagnostic concentrations have been established. For *H. zea*, diagnostic concentrations for mortality were never achieved and a shift to measuring sub-lethal effects was undertaken. Data for *H. zea* are summarized below in Tables 6 (Bt corn PIP toxins) and Table 7 (Bt cotton PIP toxins).

The annual corn and cotton monitoring reports have provided no information about the sampling locations of insect populations that served to assess susceptibility to Bt traits. Inconsistent reporting of laboratory control susceptibility for the cotton monitoring reports makes it challenging to place the field results in context. Additionally, insufficient description about the tested generation(s) in bioassays does not allow calculating the number of genomes and quantifying potential non-segregation of homozygous resistant alleles in larvae. Homozygous susceptible (*SS*) and heterozygous (*RS*) larvae have shown to have similar responses when exposed to Cry1Ac (Blanco et al. 2008), and only homozygous resistant larvae survived and developed on diagnostic concentrations. This leads to an underestimation of resistance levels, a delay in concluding resistance has evolved, and thus, a delay in initiating mitigation of resistance.

The bioassay methodology was changed over the years to address the need for better detection methods. Sub-lethal endpoints were adopted because of inherent Bt tolerance by *H. zea*. Diagnostic concentrations have been available for Cry1Ac and Cry2Ab2 since 2009 using endpoints, such as failure to molt. The results in Table 7 support a conclusion that suppression of third instar larvae was achieved (> 98%) from 2009-2015 using a concentration of 10 µg/m<sup>2</sup> for Cry1Ac and 10 µg/ml of diet for Cry2Ab2. These two toxins serve as surrogates for all registered Cry1A and Cry2A toxins because of cross resistance and protein structure similarity. Cry1F's highest concentration tested (4,000 µg/cm<sup>2</sup>) is not a diagnostic concentration, however; only approximately 80% of third-instar larvae failed to molt. A more or less consistent trend of *H. zea* susceptibility to the three Bt proteins is apparent.

Laboratory (negative) control colonies were not used in the early years of the IRM program (Tables 6 & 7). This makes it difficult to put the field results in context. All that can be concluded for those results is that *H. zea* has variable responses to the Bt toxins tested. When negative control colonies were used, no clear pattern was apparent for field populations. Sometimes, the sampled populations showed lower or higher susceptibility than the lab colonies. Since the sample collections did not track the same populations over time, this should not be too surprising. Also, it is possible that the lab reference strains did not represent a truly susceptible field population since the insects that had been reared in the lab for generations, may be genetically less diverse and able to deal with the lab exposure/diet better than their natural counterparts. Typically, reference strains go through a genetic bottleneck when established, and it is unclear whether occasional outcrossing occurs to maintain genetic variability and relevance compared to wild populations. EPA also notes that when the field collections turned out to be less susceptible than the lab reference strains, no follow up investigations occurred to test for resistance in survivors (see Table 6, year 2006, EC<sub>50</sub> for Cry1F).

EPA provides options to address uncertainties with insect sample collections and difficulty with interpretation of diet bioassay results in the following sections below.

**Table 6. Susceptibility of Field-Collected *H. zea* Populations to Bt Corn PIPs (2001 – 2015) (Compiled from studies listed in Appendix A)**

		Cry1Ab		Cry1F		Vip3A		Cry1A.105	
Year	Population	EC <sub>50</sub> (ng /cm <sup>2</sup> )	Disc. Dose (% mort.) <sup>a</sup>	EC <sub>50</sub> (ng /cm <sup>2</sup> )	Disc. Dose (% mort.) <sup>b</sup>	EC <sub>50</sub> (ng /cm <sup>2</sup> )	Disc. Dose (% mort.) <sup>c</sup>	EC <sub>50</sub> (ng/cm <sup>2</sup> )	Disc. Dose (% mort.) <sup>d</sup>
2001	Field	0.14 – 0.31	≥99	43.2 – 229.1	94.0 – 100	---	---	---	---
	Lab	NT	NT	NT	NT	---	---	---	---
2002	Field	0.10 – 0.35	97.8 – 100	39.2 – 173.5	85.3 – 100	---	---	---	---
	Lab	NT	NT	NT	NT	---	---	---	---
2003	Field	0.20 – 0.46	90.8 – 100	32.1 – 96.9	82.9 – 99.0	---	---	---	---
	Lab	0.14	98.4	74.2	95.5	---	---	---	---
2004	Field	0.08 – 0.28	99.4 – 100	12.6 – 120.6	99.1 – 100	---	---	---	---
	Lab	0.49	99.7	182.4	100	---	---	---	---
2005	Field	0.10 – 0.42	97.5 – 100	19.6 – 118.7	62.2 – 96.1	---	---	---	---
	Lab	0.18	99.9	31.7	80.7	---	---	---	---
2006	Field	0.13 – 0.47	99.6 – 100	8.2 – 96.0	87.5 – 100	---	---	---	---
	Lab	0.21	100	34.7	94.0	---	---	---	---
2007	Field	0.47 – 0.88	99.4 – 100	42.2 – 91.5	48.7 – 99.6	---	---	---	---
	Lab	1.35	89.0	95.9	52.1	---	---	---	---
2008	Field	0.12 – 1.10	96.7 – 100	12.6 – 184.5	69.0 – 100	---	---	0.0021 – 0.0447	NT
	Lab	0.57	99.7	472.9	65.8	---	---	0.0034	NT
2009	Field	0.09 – 1.24	99.4 – 100	12.0 – 142.3	84.5 – 100	---	---	0.0007 – 0.0095	NT
	Lab	0.47	97.2	200.8	68.2	---	---	0.0064 – 0.0096	NT
2010	Field	0.12 – 0.41	99.3 – 100	9.5 – 76.8	80.6 – 99.7	---	---	0.0016 – 0.0133	100

		Cry1Ab		Cry1F		Vip3A		Cry1A.105	
Year	Population	EC <sub>50</sub> (ng /cm <sup>2</sup> )	Disc. Dose (% mort.) <sup>a</sup>	EC <sub>50</sub> (ng /cm <sup>2</sup> )	Disc. Dose (% mort.) <sup>b</sup>	EC <sub>50</sub> (ng /cm <sup>2</sup> )	Disc. Dose (% mort.) <sup>c</sup>	EC <sub>50</sub> (ng/cm <sup>2</sup> )	Disc. Dose (% mort.) <sup>d</sup>
	Lab	0.74 – 0.81	94.9 – 95.7	166.5	67.4	---	---	0.0145 - 0.0214	100
2011	Field	0.14 – 0.68	96.9 – 100	16.4 – 97.6	78.3 – 100	---	---	0.0012 – 0.0219	100
	Lab	1.13 – 2.65	57.1 – 83.3	153.7 – 2764.0	77.7	---	---	0.0540 – 0.0741	100
2012	Field	0.14 – 0.65	99.3 – 100	1.3 – 115.8	42.3 – 99.4	---	---	0.0018 – 0.0092	100
	Lab	1.34	95.8	646.7	28.9 – 64.0	---	---	0.0200 – 0.0269	100
2013	Field	0.15 – 0.51	93.5 – 100	3.9 – 364.1	27.4 – 99.7	NT	100	0.0016 – 0.0159	100
	Lab	2.79 – 3.27	37.6 – 72.6	719.5 – 2704.5	5.1 – 8.3	NT	100	0.0400	58.3
2014	Field	0.11 – 0.59	91.2 – 99.9	15.8 – 448.1	58.3 – 99.4	NT	100	0.0014 – 0.0327	NR
	Lab	2.45 – 3.33	37.1 – 78.1	> 2025	15.8 – 18.8	NT	100	0.0980 – 0.2600	NR
2015	Field	0.09 – 6.43	84.2 – 99.6	16.5 – 228.8	53.6 – 95.8	NT	100	0.0013 – 0.0084	NR
	Lab	0.10 – 7.83	36.0 - 100	34.4 – 77.3	2.1 – 97.6	NT	100	0.0010 – 0.5000	NR

<sup>a</sup> Cry1Ab discriminating concentration 40 ng/cm<sup>2</sup> (2001-3); 80 ng/cm<sup>2</sup> (2004-10)

<sup>b</sup> Cry1F discriminating concentration of 5000 ng/cm<sup>2</sup> (2001); 7500 ng (2002-3); 54,675 ng/cm<sup>2</sup> (2004); high concentration 4,000 ng/cm<sup>2</sup> (2005-10)

<sup>c</sup> Vip3A discriminating concentration = 10 µg/cm<sup>2</sup>

<sup>d</sup> Cry1A.105 discriminating concentration = 5 µg/cm<sup>2</sup>

**Table 7. Susceptibility of Field-Collected *H. zea* Populations to Bt Cotton PIPs (2003-2015)  
(Compiled from studies listed in Appendix B)**

Year	Control Mortality	Percent Failure to Molt to 3 <sup>rd</sup> Instar							% Larval Mortality
		Cry1Ac/cm <sup>2</sup>		Cry1F/cm <sup>2</sup>		Cry2Ab2/ml diet			Cry1Ac/ml diet
		10 µg	30 µg	400 µg	4000 µg	10 µg	30 µg	100 µg	100 µg
2003									75.5
2004								82	76.1
2005								67.2	89.9
2006									57.8
2007								83.2	74.2
2008					80.8			78.9	83.5
2009	6.9	0.4	0	55	16.6	83.3	0.6		98
2010	3.2	0	0	59.7	9.2	0.3	0		
2011	8.7	0	0	51.5	10.9	0.3	0		
2012	7.0	0	0	41.7	2	0.3	0		
2013	3.9	0	0	54.4	11.4	0	0		
2014	11.2	0	0	56.6	20.4	0.1	0		
2015	2.3	0.4	0.3			0	0		

EPA identified options to improve current methodologies

The effectiveness of the monitoring program to proactively detect resistance is largely dependent on how population sampling is conducted. To date, insect samples have been collected in a more or less random fashion from non-Bt hosts (e.g., refuge corn or wild hosts). This type of sampling has been used to determine whether a change has occurred in the average susceptibility of *H. zea*. Because the populations are selected randomly, however, this approach does not track susceptibility of individual populations (or populations within discrete geographic locations) over time. Since the samples stem from non-Bt hosts, it is also a less proactive sampling method. EPA is, therefore, considering the adoption of sentinel plots in major cotton growing areas for resistance monitoring purposes instead of continuing with the random sampling approach for *H. zea*. Such a targeted approach of collecting insects would allow, in theory, to detect shifts in susceptibility more proactively. Such sentinel plots may need to be set up in areas or regions with high risk factors of resistance, taking into consideration greater Bt adoption, fields with use of same trait year-after-year, areas with low refuge compliance for Bt corn, and high *H. zea* pest pressure. An analogous approach may need to be adopted for other lepidopteran pests and their IRM plans. The primary objective of this proposed change is to actively target areas of resistance concern based on high risk factors, gain insight into the resistance development in the same populations year after year, and thereby identify resistance problems more proactively.

EPA is considering the use of diagnostic molecular assays for Cry1A, Cry1F, Cry2A, and Vip3A toxins targeting *H. zea*. Because of the speed of detection with such available approaches and cost to resistance concerns that could lead to false negatives with diet bioassays, the agency considers molecular tools

superior since they allow the use of in-field collected insects rather than their offspring generations (Banerjee et al. 2017). This type of approach would expedite the detection of resistance, allow monitoring for early resistance and effective implementation of mitigation, increase the accuracy of resistance monitoring data, and improve EPA's overall resistance monitoring program for *H. zea*. In addition, the development of a resistant reference colony, against which field results could be compared, should further enhance the interpretation of monitoring results. Likely, significant research efforts will be needed to develop such tools.

For other lepidopteran pests where diagnostic assays are available, EPA considers whether a shift to F<sub>2</sub> screens would be useful to identify resistant populations (Andow and Alstad 1998). This option is based on observations in the literature that *RS* and *SS*-genotypes can behave similarly in diet bioassays, which underestimates resistance (Blanco et al. 2008). At a minimum, 400 genomes may be needed for this approach. F<sub>2</sub> screens have the advantage that they express homozygous resistant offspring, which survive (unlike *RS*-genotypes behaving like *SS*-genotypes). With an appropriate sample size, the resistance allele frequency in the population can be estimated and confidence intervals calculated.

### Uncertainties

There may be additional benefit in supplementing the focused sampling approach in southern areas of the US with sampling from northern and non-overwintering areas (*H. zea*) of the Corn Belt. Comparing the Bt susceptibility of *H. zea* in southern populations to more northern states might provide additional insights into how quickly resistance can establish on a continent scale.

It is unclear what the appropriate number of population samples would have to be in the southern US for early resistance monitoring purposes. Historically, sampling targets (total populations and number of genomes) have been based on a presumed resistance allele frequency. For example, if the phenotypic frequency of resistance was assumed to be one in 1,000 (0.001), it was suggested that more than 3,000 individuals needed to be sampled to have a 95% likelihood of detecting one resistant individual (Roush & Miller 1986). For *H. zea* and most Bt toxins, the frequency of the resistance alleles is expected to be higher since Bt PIPs do not express high dose levels. Given these circumstances, the genome sample size should be lower for this pest. In absence of reliable resistance allele frequency estimates, however, it will be challenging to propose a sufficiently accurate sample size needed from each population to conduct a meaningful F<sub>2</sub>-test.

It is uncertain whether a proper sample size can feasibly be collected for *H. zea* to assess 400 genomes in an F<sub>2</sub>-test. For example, more than 50% of field-collected larvae could die before becoming adults. This reduction of genomes can further increase if mating success and viable egg production are low during the breeding process. This additional mortality reduces the gene pool in bioassays.

Given the knowledge about *H. zea*'s high genetic variability and susceptibility to Bt, it is surprising not to see any evidence of variability in the assay results for cotton monitoring (Table 7). The reasons for this lack of variability are unclear, but EPA hypothesizes that contributing factor may be the elimination of resistant genotypes during the rearing process. Typically, companies do not report mortality of field

collected individuals prior to starting diet bioassays; this information would be needed to further understand the lack of variability seen in assay results. Also, it is unclear what the methodological differences were between assays leading to results listed in Tables 6 and 7.

#### 7. Lack of thresholds for unexpected injury delays reporting of putative resistance

An unexpected injury (UXI) threshold serves as an alert that a pest may have evolved resistance to Bt. Such injury (typically known as Bt failure) warrants further investigation by the company whose product was affected. The UXI threshold is not to be mistaken with “economic injury level” (EIL) (i.e., lowest population density that will cause economic damage) or “economic threshold” (ET) (i.e., amount of injury that justifies the cost of artificial control) (Stern et al. 1959), which are terms and tools of integrated pest management (IPM) options. The threshold for UXI should be higher than EIL and ET.

##### a. Lack of regulatory UXI thresholds

As described in the Background section, the registration terms for Bt PIPs do not specify lepidopteran UXI levels that would trigger investigative actions. Without established regulatory threshold triggers for unexpected Lepidoptera injury in Bt corn and Bt cotton, it is unlikely that timely collections of relevant insect samples and follow-up bioassays can occur. Resistance is, therefore, provided an opportunity to spread unchecked and before mitigation actions can be initiated in a timely manner.

Likely all companies have their own thresholds for unexpected injury in corn and cotton, though these have not been shared with EPA and may not be uniform across industry. The terms of Bt registration simply state that each company needs to conduct follow up investigations when unexpected damage occurs. The terms do not prescribe what the particular thresholds should be.

##### b. Criteria for UXI thresholds

UXI thresholds may be toxin, pest, and/or crop specific. Damage caused in Bt cotton and corn by the same pest likely has distinct thresholds simply based on the different plant tissues that are affected. A non-high dose trait may have a higher threshold than a high dose toxin because some damage should always be expected for the former.

##### *Cotton*

A uniform regulatory threshold needs to be established for unexpected crop injury in Bt cotton; such a threshold has to be ‘stand-alone’ since no refuge cotton is planted and can serve as an infestation and damage comparator (natural refuge paradigm for pyramided Bt cotton). Currently, 6 - 7% damage to bolls is considered unexpected Bt injury in some southern US areas (Porter and Reising, 2017 *personal communication*).

## *Corn*

Likewise, there is a need for a regulatory and uniform threshold for UXI in Bt corn. Field entomologists compare the damage in Bt cornfields to that observed in the refuge fields, since high pest pressure could lead to false positives based on UXI in Bt alone (resistance suspected, yet low resistance allele frequency in high density population). Conversely, low pest pressure could result in missing resistance development (false negative, yet high resistance allele frequency in population). The comparison to a check (i.e., refuge) should in theory account for pest pressure.

Growers may not scout for *H. zea* damage in field corn, however, since they do not consider it a major pest (Storer, 2017 *pers. comm.*). Given the lack of concern about *H. zea* damage in field corn of the southern US, a resistance problem to Bt trait may not become apparent until the resistant population moves into Bt cotton and UXI becomes apparent there.

### EPA options to address concerns

EPA is considering the adoption of uniform unexpected injury thresholds in Bt corn and Bt cotton based on percent damage in ears and stalks (corn) and squares and/or bolls (cotton) for the various major lepidopteran pests. When those thresholds are triggered, insects will need to be collected for resistance testing. For Bt corn, additional information about refuge damage should be collected to estimate pest pressure representative of a resistant problem. If it is impractical to establish thresholds for *H. zea* in Bt corn because growers do not scout for this pest, monitoring for pest abundance may need to occur with adult traps. Such trap samples could be utilized to screen for resistance frequencies in populations when adult number triggers are exceeded.

### Uncertainties

Depending on the trait and its effectiveness against a target pest (intermediate vs. high dose), thresholds may differ between traits and require flexibility and adjustment. In addition, the same trait may have different expression in cotton than in corn (e.g., Vip3A). In such a case, thresholds may need to be crop specific.

For cotton, damage to bolls incurred may be of economic significance but may not be the best measure for unexpected damage. Bolls that have incurred early damage are aborted by the cotton plant (Reisig, 2017 *pers. comm.*). Hence, an UXI threshold based on boll damage alone would underestimate actual damage and may not be proactive enough. It may, therefore, be more relevant to scout for square damage when looking for Bt performance in cotton.

If growers do not typically scout for *H. zea* damage in field corn, the UXI approach may not be practical, and an alternate approach would be needed. Not scouting for *zea* damage in field corn will delay resistance detection and puts additional risk on the durability of Bt cotton.



## 8. Current regulatory definition of resistance delays mitigation actions

The Food and Agricultural Organization of the United Nations (FAO 1979) defined resistance as “the inherited ability in a strain of a pest to tolerate doses of toxicant that would prove lethal to a majority of individuals in a normal population” and further stated that lab resistance did not necessarily equal loss of efficacy in the field. The National Research Council of the National Academy of Sciences (1986) defined resistance broadly as “any heritable decrease in sensitivity to a chemical within a pest population” but specified that it could occur on a spectrum of slight to severe as well as locally rare to widespread. Tabashnik et al. (2009) elaborated on field-evolved resistance with respect to Bt crops and explained that it did not always imply economic loss. Tabashnik et al. (2014) presented five categories of Bt resistance with each being successively more severe and having an increasing proportion of resistant individuals in the pest population. These categories are:

- 1) *Incipient resistance*: a statistically significant increase in resistance allele frequency (Downes et al. 2010), and <1% of individuals in a population are resistant;
- 2) *Early warning resistance*: a statistically significant increase in resistance allele frequency (Zhang et al. 2011), and 1-6% of individuals in a population are resistant (warrants consideration to manage resistance);
- 3) *>6-50% of the population resistant*;
- 4) *>50% of the population resistant*: reduced efficacy is expected but not reported (Tabashnik et al. 2012); and
- 5) *Practical resistance*: >50% of the population resistant, and reduced efficacy is reported with practical implications for pest control (Tabashnik et al. 2012).

The US EPA’s definition for Bt resistance includes three criteria that have to be met before resistance is confirmed and mitigation initiated. The following needs to be demonstrated scientifically:

1. Higher survival of resistant individuals on Bt compared to susceptible individuals;
2. Heritability of the resistant trait; and
3. Field relevance (i.e., economic damage to Bt crops).

The agency’s definition of Bt resistance describes the most severe state of field-evolved resistance described by Tabashnik et al. (2014), namely “*practical resistance*”.

EPA notes that if “confirmed field-resistance” is used as the trigger for initial mitigation, then the likelihood of success is greatly diminished. Once >50% of individuals in the population are resistant, it will be exceedingly difficult to mitigate effects of resistance, especially for lepidopteran species with great dispersal abilities (e.g., Noctuidae, Crambidae). For EPA’s IRM program to achieve its intended goal (to contain, reduce, or delay the spread of resistance and extend durability of Bt in non-affected areas), a new mitigation trigger (other than confirmed resistance) should be considered that allows for timely and effective implementation of mitigatory strategies (also see discussion in Section III. A.11). Monitoring for resistance can be an effective stewardship tool if mitigation of putative resistance is initiated at lower resistance allele frequencies (e.g. category 2 “*early warning resistance*”).

## EPA options to address concern

EPA is considering the adoption of two triggers for mitigation of resistance. The first trigger (“early warning resistance”) would result in proactive mitigation actions that delay the development and spread of resistance and maintain the Bt trait longevity in areas where the insects are still susceptible. The second trigger (UXI, field failure, etc.) would initialize immediate actions based on best IPM practices before resistance was confirmed in the lab. The goals of this immediate intervention when field failure is visible is to reduce the spread of resistant individuals in the landscape by managing the population densities. Once resistance is confirmed, a lepidopteran-specific mitigation plan would be implemented and a mitigation action area established.

An additional option considered by EPA is the encouragement of IPM with IRM for lepidopteran pests of Bt corn and cotton at the onset of a new trait deployment and throughout its commercialization (see options in [Section IV](#)).

## Uncertainties

It may not be expeditious to employ diet bioassays on insects for investigating field failures and making early resistance determinations. Insect rearing takes time, especially with EPA’s proposed option to adopt the more resource intensive F<sub>2</sub> screens ([Section III. B. 6](#)). By the time, EPA receives a resistance report from industry, at least one growing season will have passed and official mitigation cannot begin until year two. While developing the molecular tools to conduct quick assays for resistance screening of field collected insects may be laborious, it would ultimately speed up the resistance determination and subsequent implementation of mitigation. Thus, it may be of benefit to abandon the diet bioassay methods in the long run and consider a shift to more rapid and more advanced analytical tools.

Mitigation of lepidopteran resistance is unlikely to succeed with a trigger for mitigation relying on “practical resistance”. Noctuid moths, specifically *H. zea*, engage in migratory flight behavior on a continental scale (Beerwinkle et al. 1995) as well as substantive inter-field dispersal. Computer simulations for a pest with more limited dispersal propensities than *H. zea* have shown that once resistance is widespread, regional mitigation of resistance may have minimal impact on slowing resistance from increasing and extending the lifetime of the compromised Bt trait (Martinez and Caprio 2016). By extrapolation, resistance should spread even faster through a landscape for a more mobile pest like *H. zea*. If practical resistance from UXI cases is confirmed and resistance is widespread, it may be useful to rename the regulatory action from “mitigation of resistance” to “management of resistance”.

## 9. Cross-resistance between Bt toxins reduces durability of pyramids

Cross-resistance is more likely to occur between Bt crystalline (Cry) toxins with high amino acid sequence similarity, based on the predominant Bt mode of action that presumes such toxins share binding sites in the insect midgut (Tabashnik et al. 1996; Hernández-Rodríguez et al. 2013; Adang et al. 2014; and

Carrière et al. 2015). For Cry1Ac, Welch et al. (2015) confirmed that sequence homology within domain II, the domain linked with receptor binding, was more closely associated with cross-resistance than similarity of domains I and III in a resistant colony of *H. zea*. A comprehensive review corroborated this trend for cross-resistance among Cry toxins homologous at domain II in 10 major pests of Bt crops (Tabashnik et al. 1996; Carrière et al. 2015).

Some studies have observed shared binding sites among Cry toxins expressed in Bt crops for *H. zea* and *H. armigera*. The six toxins currently expressed by Bt crops with activity against *H. zea* include Cry1Ac, Cry1A.105, Cry1Ab, Cry1F, Cry2Ab, and Vip3Aa. Starting with Cry1A.105 these toxins respectively share 99, 99, 51, 15, and 0 percent sequence similarity to Cry1Ac at domain II (Carrière et al. 2015). Competitive binding data show that Cry1Ac and Cry1F share a receptor in *H. zea* and *H. armigera* (Hernández and Ferré 2005) and Cry1Ab and Cry1Ac share a receptor in *H. armigera* (Estela et al. 2004). Additional binding studies found Cry1A.105 shares a receptor with Cry1Ab, Cry1Ac, and Cry1F in *O. nubilalis* and *S. frugiperda* (Hernández-Rodríguez et al. 2013). These results support that cross-resistance among Cry1Ac, Cry1A.105, Cry1Ab, and Cry1F may be explained by reduced binding to a shared receptor.

Cross-resistance is likely an important factor predicting the rate of resistance evolution in *H. zea* given observed Cry1Ac and Cry1Ab “practical resistance” (Tabashnik et al. 2008, but also see Moar et al. 2008; Reisig and Reay-Jones 2015; Dively et al. 2016) and non-functional pyramid crops (<95% mortality of susceptible insect, Roush 1998). Since no Bt toxin expresses a high dose for *H. zea*, even weak cross-resistance is expected to greatly accelerate resistance evolution.

Five studies report varying degrees of cross-resistance in Cry1Ac-selected *H. zea* strains (Burd et al. 2003; Anilkumar et al. 2008; Caccia et al. 2012; Brévault et al. 2013; Welch et al. 2015). The literature indicates consensus regarding cross-resistance between Cry1Ac and Cry1Ab (Anilkumar et al. 2008; Caccia et al. 2012; Welch et al. 2015). Cross-resistance between these two toxins is expected based on the mechanism outlined above as these proteins share more than 99% amino acid sequence similarity at domain II and may share a midgut receptor. However, results vary concerning other toxins.

Limited evidence of strong cross-resistance has been found between Cry1Ac and Cry1A.105, the latter being used in many Bt corn pyramids. Cry1A.105 is a chimeric protein with domain II and most of the C-terminus taken from Cry1Ac (Biosafety Clearing-House 2008). One study has assayed a MVP II (a formulation 98.5% identical to Cry1Ac) selected strain of *H. zea* against Cry1A.105 and found high levels of cross-resistance (Welch et al. 2015). This is not surprising because of the high sequence homology between Cry1Ac and Cry1A.105.

Cry2Ab is found in both corn and cotton pyramids. Cry1Ac and Cry2Ab exhibit relatively low sequence homology at domain II (15%). Cry1Ac-selected strains of *H. zea* have been found to have weak cross-resistance to Cry2A group toxins (Burd et al. 2003; Welch et al. 2015) but not in all cases (Anilkumar et al. 2008; Brévault et al. 2013). Responses to Cry1Ac and Cry2Ab were positively correlated in two studies of field-collected populations of *H. zea* (Jackson et al. 2006; Ali and Luttrell 2007). Higher

survivorship of Cry1Ac resistant individuals has been found in plant bioassays with Cry1Ac + Cry2Ab cotton as well (Brévault et al. 2013). Laboratory assays in conjunction with consistent field results indicate that selection of *H. zea* with Cry1Ac causes weak cross-resistance to Cry2A toxins.

Cry1F is expressed in single toxin corn as well as pyramid corn and cotton. Selection to Cry1F in single toxin corn favors resistance development to other similar Cry toxins in pyramided crops. Cry1Ac shares approximately 50 percent sequence homology at domain II with Cry1F. One study reports weak, non-significant cross-resistance between Cry1Ac and Cry1F in *H. zea* based on conservative criterion of non-overlapping 95% fiducial limits with associated LC<sub>50</sub>s between selected and susceptible reference strains (Welch et al. 2015). This report likely underestimates cross-resistance between the toxin pairing due to low susceptibility to the non-high dose trait Cry1F in the unselected control strain. Cross-resistance between Cry1F and either Cry1Ac or Cry1b was significant in 16 documented cases of a total of six different pest species (Carrière et al. 2015).

Cross-resistance is not expected between Vip3Aa and Cry1Ac because there is no sequence overlap between the two families of toxins. As expected, Anilkumar et al. (2008) reported no cross-resistance between these two Bt toxins. Non-significant cross-resistance was, however, detected by Welch et al. (2015) based on the conservative criterion of non-overlapping 95% fiducial limits for the LC<sub>50</sub> measures of the resistant and susceptible reference strains. However, there was higher survivorship in the Cry1Ac resistant strain when assayed against Vip3Aa than anticipated (Welch et al. 2015). Studies with *H. armigera*, *Trichoplusia ni* (cabbage looper), and *H. virescens* have also reported weak cross-resistance to this toxin pairing (Carrière et al. 2015). Based on the sum of these observations, weak cross-resistance cannot be ruled out between Cry1Ac and Vip3Aa (Welch et al. 2015; Carrière et al. 2015). Any cross-resistance between these two unrelated toxins implies that other factors could be responsible that are not typically considered under the prevailing view of Bt mechanism of action.

#### EPA options to address concern

For pests with low susceptibility to Bt toxins, even weak cross-resistance can accelerate the evolution of resistance. To protect the remaining most efficacious Bt trait(s), managing non-high dose single toxin products in Bt corn should be the focus, which should also reduce the risk of resistance to pyramided Bt cotton in the southern US.

Under ideal conditions, resistance genes for all toxins in a pyramid should be rare at the time of commercialization. While this is not always achievable, resistance management is more effective with a high dose profile and low resistance allele frequencies. PIP registrants as well as growers should benefit from the development of high dose traits against lepidopteran pests of corn and cotton and maximize the durability of such products.

Companies that are developing new traits should avoid pyramiding Bt proteins that share high sequence homology or chimeric toxins composed of first generation proteins like Cry1A.105. Registrants should also consider including full-length protoxins in lieu of activated toxins in Bt crops (Tabashnik et al. 2015;

Soberón et al. 2016). For best outcomes, developing transgenic plants with novel traits would improve the options and success of IRM in a challenging and diverse cropping area such as the southern US.

### Uncertainties

Cross-resistance is expected to accelerate the rate of resistance to Bt toxins in *H. zea*, however, there are several uncertainties that may undermine this claim. It is generally agreed that cross-resistance is less likely between toxins that differ markedly in structure and target sites (Ferré and van Rie 2002). However, the molecular mechanisms resulting in cross-resistance are still somewhat uncertain and may be irrelevant depending on the course of resistance evolution in field scenarios. Resistance to Bt crops can develop through numerous pathways, not all of which may be linked to receptor binding affinity. Not all Bt-resistant *H. zea* with known mechanisms of resistance showed reduced binding of Cry1Ac as an important factor contributing to resistance (Karim et al. 2000; Anilkumar et al. 2008). Since weak cross-resistance has been documented for Vip3Aa and Cry1Ac (Welch et al. 2015) despite the fact that they do not share a receptor, cross-resistance between these toxins is caused by other mechanisms not currently explored in the literature. Bt toxins may share low-affinity receptors (Hernández-Rodríguez et al. 2008), which could affect the development of weak cross-resistance between Bt toxins even in strains where reduced binding affinity does not fully explain the mechanism of resistance.

The source of toxins used in laboratory selection and bioassays may skew research results. Bt crops express activated Bt proteins, yet studies published in the scientific literature used either full length protoxins or activated toxins for selection and comparison. Many laboratories select *H. zea* with MVP II because it is readily available in large quantities (Brévault et al. 2013; Welch et al. 2015). MVP II is a formulation containing a hybrid protoxin that is identical to Cry1Ac in the active portion of the toxin and 98.5% identical overall. Protoxins, however, are more effective against lepidopteran pests than their activated counterparts (Tabashnik et al. 2015; Soberón et al. 2016), which implies that studies using protoxin selected *H. zea* strains may have overestimated the magnitude of cross-resistance potential.

There are conflicting reports of fitness costs among Bt selected *H. zea* strains (Storer et al. 2003; Orpet et al. 2015). This is discussed in more detail in [Section III. B. 12. d](#). However, any mechanism that undermines the fitness of resistant individuals compared to susceptible individuals will delay resistance evolution (Gassmann et al. 2009), whether on Bt or non-Bt, and may negate the consequences of cross-resistance.

### 10. Non-functional pyramids lead to faster resistance development

If individual Bt traits in pyramids each achieve  $\geq 95\%$  mortality of susceptible (*SS*) target genotypes, Roush (1998) proposed that a non-Bt refuge could be as low as 10% and offer “superior delays in resistance” compared to sequential release of the single toxins with larger refuges. However, the modeling results also showed that reducing the refuge could still carry a significant risk for resistance. Hence,

Roush concluded that refuges should be kept as large as could be justified economically to avoid reducing a pyramid's lifetime. EPA scientists refer to pyramids as "functional" when they meet the mortality standard set by Roush (1998) and "non-functional" if they do not.

Historically, Bt corn traits were first commercialized as single Bt products. Subsequently, these traits were pyramided with each other in various combinations. Thus, target pests were offered an initial opportunity to adapt to Bt traits before being exposed to their respective pyramids. Such a commercialization strategy effectively lowers a pyramid's efficacy or lifetime and is especially problematic for non-high dose pests such as *H. zea*. In the southern US, the corn refuge for pyramids was set to 20% (compared to 5% in the Corn Belt), however, to mitigate resistance concerns for *H. zea*.

#### EPA options to address concern

Theoretical analyses may need to explore new management options for non-functional Bt corn pyramids (less than 95% mortality provided by individual traits), including cost to resistance, community block refuges (in light of grower non-compliance with requirements), bigger refuges, grower incentive programs to plant refuges, etc.

#### Uncertainties

It is unclear by how much new management strategies in Bt corn and greater compliance with block refuges would delay product failure for compromised pyramids in cotton. Density dependent effects in a structured refuge coupled with high pest growth rates (as documented for *H. zea*) may result in lower than expected durability gains (Martinez et al. 2017).

### 11. Mitigation of resistance occurs too late

Lepidopteran resistant cases to Bt have increased in the US over the past decade (see [Section III. A.](#)). This phenomenon could generally be addressed by implementing 1) effective and proactive strategies that delay the onset of product failure (see [Section III. B. 8](#) & [Section IV](#)) and 2) reactive strategies that allow farmers to manage pest densities in wide-spread product failure scenarios.

#### *a. Goal of Mitigation*

From a scientific perspective, the goal of mitigation is to implement a series of effective management actions that substantially reduce insect densities in the sites of concern and limit the spread of resistance (Andow et al. 2016). If subsequent recolonization into the previously identified resistant sites occurs with primarily susceptible individuals, then overall resistance levels are likely mitigated (Comins 1977). Depending on the population dynamics, ecology, and biology of the organism, however, extirpation of

localized resistance may or may not be an option and likely depends heavily on the timing of resistance detection and mitigation.

EPA's regulatory goal for mitigating Bt resistance is to reduce resistance in the site of concern and maintain the durability of those traits in areas where they are still effective (US EPA 2013). For mitigation actions to make a significant impact (e.g. extend overall trait durability in area), several life-history parameters should be known and certain methodological tools need to be in place. Some of these biological parameters are the spatial scale occupied by the resistant population (e.g. random mating unit) and the typical fraction of individuals in a population engaging in non-trivial dispersal (i.e., leaving natal habitat despite availability of food) (Southwood 1962). The first factor aids in delineating a potential Mitigation Action Area (MAA) for emerging resistance in a particular location (i.e., hot spot). The second factor allows for estimating the rate with which resistance spreads from a resistant site. In addition, reliable, high resolution, and rapid resistance confirmation tools and uniform regulatory threshold triggers for unexpected damage have to be in place to allow timely implementation of mitigation.

#### *b. Uniform Regulatory Threshold Triggers for Mitigation*

The risk of having a late mitigation trigger (e.g., at field failure) is that resistance can spread and develop to high levels in the landscape before it is detected. This is especially of concern for Noctuid moths, which have a great dispersal propensity (Beerwinkle et al. 1995; Westbrook 2008). Theoretical work suggests that once resistance is widespread ( $r$ -frequency = 0.1), it would be difficult to extend the durability of Bt sufficiently even with 100% of fields mitigated in an affected areas and limited pest dispersal (Martinez & Caprio 2016). Thus, early mitigation of resistance should be the primary focus of a resistance management program to delay the onset of Bt field failure or practical resistance. Pest management rather than mitigation of resistance will likely be the strategy when practical resistance is widespread.

Proactive mitigation needs to be initiated when an early warning threshold for resistance is met; such a threshold would be independent of field performance or plant damage and solely rely on resistance levels in populations. Furthermore, this threshold would preferably be combined with high resolution, molecular tools and set at a low level of resistance (1-6% in a population).

Since trait failure is unlikely to be avoided and resistance to Bt will eventually evolve, a second and uniform regulatory threshold is needed. Such a trigger would initiate sample collections and assay investigations to confirm resistance, while at the same time triggering best management practices (IPM) to control pest densities. This particular threshold should rely on plant damage or Bt plant performance.

#### *c. Use of diagnostic assay tools*

*i)* Where diagnostic concentration assays are available for high dose lepidopteran pests (e.g. *Heliothis virescens*, *D. grandiosella*), and *RS*-genotypes survive like *RR*-genotypes, an  $F_1$  screen may suffice. If *RS*-genotype survival is similar to that of *SS*-genotypes, an  $F_2$  screen is recommended. Two independent tests should be used to measure and determine resistance levels of field-collected insects (e.g., percent

mortality at the highest Bt concentration tested (positive and negative control and field collections); sub-lethal measure comparisons on Bt and non-Bt leave tissue for the same population (positive and negative control and field collection) (see BPPD 2013). When between 1-6% of individuals in any population tested exhibit resistance (early warning threshold), then an agreed upon, early mitigation plan should be implemented. The agency considers resistant (positive control) colonies to be an imperative tool to improve interpretation of these assays in addition to susceptible (negative control) colonies.

ii) Where diagnostic concentration assays are not available and lepidopteran species are non-high dose pests of Bt (e.g., *H. zea*), the goal to detect *early warning resistance* is unattainable with the current bioassay methods (and resistance is likely much higher already). In addition, cost to resistance (Dively et al. 2016) will hamper efforts to detect resistance proactively with diet bioassays. For this reason, EPA proposes that molecular (e.g., microsatellites, SNPs, etc.) assays be developed to identify resistance genes in such non-high dose pests, possibly analogous to work done for fall armyworm (Banerjee et al. 2017). Such tools could be used on the collected field insects rather than having to go through the mating and rearing process.

Until such tools are available for *H. zea*, focusing on the following should aid in reducing resistance reports: 1. use of IPM with IRM (e.g. increased scouting, alternate control tools, etc.; see [Section IV.A](#)), 2. use of alternate pesticides when economic thresholds on Bt have been reached, and 3. immediate mitigation of field incidents where growers report to have had high pest pressure and crop injury by managing population density and switching to other Bt products the following year. In the absence of confirmed resistance, best management practices relying on the tenets of IPM should be used around the field and encompassing area (e.g. preferably the size of random mating population) to proactively manage such incidents (see EPA Options Section below).

If molecular tools and methodologies can be developed to reliably estimate resistance allele frequencies in *H. zea* populations, there is sufficient evidence to support that EPA's IRM program could benefit from adopting monitoring for "early warning resistance" (likely applicable for new technologies). Such a change will initiate mitigation actions when a shift in susceptibility becomes apparent and much earlier than field failure will occur. Early mitigation actions do not presume that Bt products are compromised but rather alert stakeholders and growers in such areas that these stewardship steps are aimed at further extending the durability of these traits.

#### *d. Fitness cost and benefits for mitigation of resistance*

Evolutionary trade-offs arising from the development of pesticide resistance may be key to slowing or possibly reversing resistance in pest populations (Gould et al. 2006; Gassmann et al. 2009). Fitness costs occur when resistant individuals have reduced fitness on Bt (incomplete resistance) or in the absence of selection (cost-to-resistance). The focus hereafter is on fitness costs that occur for resistant insects in absence of selection.



#### *e. Cost to resistance*

If cost to resistance is present in resistant populations, theoretical simulations have shown that resistance levels can be reduced when single traits were removed from the landscape and only pyramids remained (Gould et al. 2006). When all toxins were high dose, initial resistance allele frequencies were low, susceptible insects from refuges mated at random, and fitness costs were associated with resistance, then resistance did not evolve. Even with moderate Bt dose, the presence of fitness costs in resistant populations reduced the resistance allele frequency in these modeling scenarios. When just a small proportion of single toxin plants were in close proximity to pyramid crops, however, cost to resistance became ineffective and resistance evolved (Gould et al. 2006).

The presence and magnitude of fitness costs in field collected and/or laboratory-selected strains is further discussed for key lepidopteran pests of Bt and how these pest attributes may influence the development of and monitoring for resistance.

#### Examples of fitness cost

##### *H. zea*

Fitness costs associated with resistance to Cry1Ac have been found in most cases for *H. zea* (Orpet et al. 2015; Moar et al. 2010; Anilkumar et al. 2008; Jackson et al. 2006; Jackson et al. 2004; Burd et al. 2003; but see Brévault et al. 2013). Studies comparing other Cry1Ac laboratory selected strains to the unselected strains demonstrated reduced larval weight, larval duration, pupal weight, and pupal duration, along with an increase in percentage of malformed adults and percentage reversion to susceptibility (Anilkumar et al. 2008; Moar et al. 2010; Orpet et al. 2015). Field collected family lines resistant to both Cry1Ac and Cry2A toxins demonstrated fitness costs as well as slower growth rates than expected (Burd et al. 2003; Jackson et al. 2004; Jackson et al. 2006). In conclusion, fitness costs appear to be prevalent amongst *H. zea* resistant strains.

The magnitude of fitness costs differed between artificial diet and on-plant bioassays (Bird and Akhurst 2004) for *H. armigera* (a close relative of *H. zea*). In on-plant assays, there was a measurable developmental delay between resistant and susceptible individuals on non-Bt cotton plants but none was measured on artificial diet (Bird and Akhurst 2004) whereas with other colonies, fitness costs were found with both on plant assays and artificial diet with higher costs associated with higher levels of resistance (Liang et al. 2008). Fitness costs were higher for some host plants than others, though these observations were not necessarily linked to nutritional suitability. For example, susceptible *H. armigera* had equivalent fitness among cotton, pigeon pea, and sorghum; however, Bt resistant individuals had more dominant fitness costs on cotton and sorghum (Bird and Akhurst 2007). These results indicate that laboratory bioassays may sometimes underestimate the costs of resistance, while the magnitude of fitness costs measured via on-plant assays may change depending on the host plant selected.

## *S. frugiperda*

Numerous studies have found fitness costs in *S. frugiperda* associated with Cry1F (Horikoshi et al. 2016; Dungal et al. 2015; Jakka et al. 2014), Vip3A (Bernardi et al. 2016), and pyramid corn containing Cry1F, Cry1A.105, and Cry2Ab (Bernardi et al. 2017). Fitness costs in *S. frugiperda* included longer development time, reduced survival, lower reproductive capacity, reduced larval weight, and skewed sex ratio (Bernardi et al. 2017; Bernardi et al. 2016; Horikoshi et al. 2016; Dungal et al. 2015; Jakka et al. 2014). Some field collected strains had no significant fitness costs associated with Cry1F resistance in laboratory bioassays (Santos-Amaya et al. 2017) or on non-Bt corn leaves (Leite et al. 2016). One Cry1F resistant strain demonstrated heterosis (Velez et al. 2014), which may accelerate resistance evolution or be a result of a laboratory artifact of increased diversity when crossing strains. *S. frugiperda* has more variable responses to developing fitness costs on Bt diets than *Helicoverpa spp.*

### EPA options to address concerns with delayed mitigation of resistance

#### *Early Warning Resistance and Mitigation Strategies in Corn*

As discussed in an earlier section of this document, resistance development for *H. zea* is driven by corn since the pest funnels through this crop in summer (two generations). Hence, any mitigation targets discussed here should focus on this crop. Not all proposed options would be equally effective at mitigating resistance, and it is unclear what is currently incorporated already. In addition, some may be preferred financially, while others may have more scientific support and environmental benefits. An early resistance mitigation program could incorporate the following:

- Addressing the risk of single traits and seed blends in the southern US to extend the durability of pyramids: such actions would reduce the number of field failures requiring mitigation and help delay the onset of resistance to still functional traits. For areas where pyramids remain partially efficacious, addressing these risks should also benefit growers and decrease the number of problem fields, though it may be necessary to incorporate additional control tools. In areas with widespread resistance to numerous toxins, rotating to other traits may not be a viable strategy and management of pest densities may be the primary focus of ‘mitigation’ efforts.
- Alternate control measures. These are:
  - Mixture strategy for chemical pesticides on conventional corn: this is analogous to pyramiding Bt traits. If no high dose, chemical pesticides are available, this option would provide another tool with more than one chemistry to control a pest;
  - Rotation of pyramids: this strategy works if rotated products have no cross resistance and if at a minimum two functional traits are expressed in plants;
  - Rotation of traits with rotation of mixture strategies: a combination of pyramided Bt and pyramided chemistries could be an option for pest outbreaks or other unusual density phenomena;
  - Rotation of traits with conventional (non-Bt) corn, where growers would use chemical control tools (etc.) when the economic threshold is reached;

- Pheromone strategies: this option is costly but could be used with other density management approaches; and
- Planting of trap crops.
- Addressing mechanisms to increase refuge compliance for Bt corn in the southern US. To further quantify the potential durability gains from higher refuge compliance, EPA recommends that more complex probabilistic modeling be conducted for the southern corn growing areas incorporating pest density dependence, delayed adult emergence from Bt compared to refuge fields, etc.
- Improving communication between industry, seed dealers, growers, extension entomologist, and crop consultants will assure that the latest field observations and performance issues are shared. Growers will then be able to make informed decisions about what to anticipate and how to prepare for next season.

### *Field Failure and Additional Mitigation*

When the threshold for unexpected injury is met, insects should be collected and tested for resistance, preferably from the generation causing the injury. Since UXI could be an early indication of field resistance, EPA is considering mitigation options, which would be implemented on the affected farm(s) even before resistance investigations are concluded (e.g., adulticide spraying to avoid spread of resistance, rotation of control strategies the following year, and other mitigatory measures designed to limit the spread of the population and reduce selection pressure for further resistance).

Once resistance has been confirmed, strategies that could be employed are listed above in the “*Early Resistance and Mitigation*” section. In addition, planting an increased amount of non-Bt expressing plants may assist by reducing resistance allele frequencies in areas if cost to resistance is present. Likewise, if a pyramid is chosen to mitigate resistance, and it contains the compromised trait, then the associated refuge size may need to be reconsidered. Possible new mitigation options in addition to the ones in place could be:

- Limit the use of the compromised trait in the affected area
- Planting a greater block refuge: this could be challenging in the southern US given the historical grower non-compliance;
- Adjusting planting time of Bt crop to reduce egg laying by pest of concern;
- Use of trap crop;
- If compromised traits are part of the pyramids, or each trait is less than 95% effective, or there is cross resistance between pyramided traits, the refuge may need to be increased to protect the still functional trait(s).

If a non-related pyramid is planted, yet one or more traits do not result in  $\geq 95\%$  mortality, then the refuge size may need to be reconsidered.

### *Fitness Costs*

Fitness costs have the potential to delay field-evolved resistance to Bt crops in lepidopteran pests but not in the presence of single toxin Bt (Gould et al. 2006). In the case of *H. zea*, due to low inherent susceptibility to Bt toxins, documented field-evolved resistance (Tabashnik et al. 2013), and often lack of structured refuges in southern corn growing areas, the presence of fitness costs may not be enough to extend durability of Bt crops significantly.

Developing Bt or other new technology crops with high toxicity to pests like *H. zea* that have shown to have low inherent susceptibility to Bt, would provide options to exploit fitness costs and delay resistance (Gould et al. 2006; Carrière et al. 2010). EPA is considering addressing the risk posed by single toxin Bt corn to pyramided Bt and lack of available non-Bt refuges. However, differences in density dependent survival in refuge and Bt fields have shown to reduce the expected durability gain of pyramids (Ives et al. 2012), especially when the pest growth rate is high, as for *H. zea* (Caprio et al. 2009; Martinez et al. 2017).

Fitness costs causing mortality during the rearing process affect the ability to detect shifts in susceptibility using diet bioassays as monitoring tools and, thus, produce results that are not necessarily reflective of field conditions. Prior to rearing, the collections of insects exposed to non-Bt corn diets during the transport to the lab could also remove resistant individuals from the samples. These are examples of how the resistance allele frequency and resistance levels can be underestimated in the presence of fitness costs. As described in [Section III.B.6](#), EPA considers other options than diet bioassays that would allow resistance assessment on field-collected insects.

The mechanism by which resistance evolves may differ from selection under field conditions versus laboratory condition (US EPA FIFRA SAP 2009). If fitness costs are linked to the mechanism of resistance, the results of such studies may not be applicable to field scenarios. For example, poor sperm competition affecting mating success in Cry1Ac-resistant *H. armigera* fed on non-Bt diet was related specifically to cadherin mutations leading to Bt resistance (Zhang et al. 2014). Bt resistance related to cadherin mutations, and thus gut permeability, may be more likely to incur fitness costs in non-Bt arenas due to increased susceptibility to host plant defenses (Carrière et al. 2010). If resistance evolves by another mechanism in field scenarios, such results may not be relevant.

### *Threshold Triggers*

Field failure is a function of pest pressure and resistance levels. When using thresholds triggers for determining UXI in corn, a subsequent comparison between Bt and non-Bt damage should always be undertaken after a threshold has been met. High pest densities can lead to field failure with lower resistance allele frequencies. Conversely, no field failure may be observed at higher resistance levels if

the pest densities are low. This uncertainty supports the option to shift mitigation of resistance to an earlier point in the pest's evolutionary time, namely when 'early warning resistance' is identified.

### *Life-History*

Prolonged development of resistant field populations on artificial diet with Bt (Dively et al. 2016) may reflect a possible delay in emergence for *H. zea* coming out of Bt fields compared to non-Bt fields. It is unclear if and how much assortative mating takes place in the field; no such data are available. This information is needed to explore theoretically whether a greater refuge size could be an effective mitigatory tool.

Moths in the family Noctuidae show great propensity for movement and are able to engage in long distance migration on the scale of the North American continent (Westbrook 2008). Flight mill studies support that male Noctuid moths are capable of uninterrupted flight lasting three hours. This duration is estimated to be an equivalent of 18.8 km (n = 132) (Beerwinkle et al. 1995). Given this long-distance dispersal potential, it needs to be determined what fraction of a typical *H. zea* population engages in this behavior. These data are currently not available but would be needed to estimate the spread of resistance genes out of a resistant site and the establishment of resistance in the landscape.

## 12. Risk factors for *S. albicosta* resistance in Bt corn

Hutchison et al. (2011) identified several underpinning factors that seem to have contributed to the rapid range expansion of western bean cutworm across the northern Corn Belt. Some of those are reduced tillage leading to greater overwintering survival, reduced chemical pesticide use with expanding Bt corn adoption, the pest's propensity for long distance dispersal, and possibly climate change facilitating the expansion. Increased Bt corn adoption contributes to greater risk of resistance development in *S. albicosta*. Several factors already discussed for *H. zea* are further contributors to greater resistance risk. Those are lack of high dose Bt traits, 5% refuge for single trait acting on *S. albicosta*, and possibly seed blends.

### a. *Availability of Cry-toxins is limited*

Cry1F has never fully controlled *S. albicosta* but was added to the label in 2003. EPA concluded at the time of review that Cry1F at best suppressed the pest (US EPA 2003b). Since *S. albicosta* has historically not been considered a major pest of Bt but a sporadic pest, registrants may not have developed Bt toxins with this pest in mind. This would explain why there are so few Bt tools available today.

Vip3A was developed by Syngenta to control Lepidoptera pest of corn and cotton and registered by the EPA in 2009. This particular toxin has shown to have good activity against *S. albicosta*. It is currently the only Bt PIP tool available to reduce larval densities in corn.

### b. *Percent refuge for single Vip3A trait reduces its durability*

Pyramided Bt corn products are deployed with a 5% refuge in the northern Corn Belt (external block and seed blends) (US EPA 2010) and where *S. albicosta* has developed a tolerance towards Cry1F (Smith et

al. 2017), one of only two Bt toxins that has historically shown some efficacy against this pest. Vip3A is pyramided with other lepidopteran Bt traits in corn, none of which suppress or control *S. albicosta*. Therefore, this toxin is essentially deployed as a single trait product with a much lower refuge (5%) than is typically required (20%). This exerts a tremendous selection pressure on the only available Bt PIP tool for western bean cutworm.

*c. Seed blends and resistance risk*

*S. albicosta* feeds in ears of corn with weak intra-specific density dependence (Ostrem et al. 2016). The resistance risk to Vip3A may be higher in *S. albicosta* because only one viable trait is expressed in the maternal tissue. In a seed blend environment, kernels have a 25% probability of expressing both genes or no genes and a 50% probability of expressing one Bt gene. Thus, sub-lethal exposure to a Bt mosaic may present a resistance risk for this pest, as was described for *H. zea*.

EPA options to address risks of resistance

EPA is considering whether western bean cutworm should be viewed as a primary pest of corn and if a Bt corn IRM plan is warranted for the insect. Such a plan should consist of the following post-registration requirements: monitoring for and mitigating resistance (insect collections, assay development, and mitigation strategies) and grower education. EPA notes that IPM with IRM is another option that would delay resistance development in *S. albicosta*.

Uncertainties

Since western bean cutworm feeds on maternal corn tissue first as a young instar larva, it should, therefore, always be exposed to Bt expressed in maternal tissue after hatching. Therefore, it could be argued that selection occurs early and on the leaf tissue rather than the kernels and that the potential for sub-lethal expression in a mosaic environment may not be relevant. Seed blends would then not pose an additional resistance risk to western bean cutworm.

If western bean cutworm is elevated to a primary pest of corn, then refuge proportion may need to be reconsidered for pyramided Bt products that express only one functional toxin for the control of this insect. Modeling analyses may be necessary to assist with such an option.

#### **IV. EPA CONCLUSIONS AND OPTIONS TO REDUCE RESISTANCE RISKS**

It has been reported that four lepidopteran species developed resistance to Bt in the continental United States over the last decade. Four academic publications found resistance in corn earworm, fall armyworm, and western bean cutworm (Dively et al. 2016; Huang et al. 2014; Ostrem et al. 2016; Smith et al. 2017). A PIP registrant reported to the US EPA that resistance was found in southwestern corn borer (MRIDs 497854-01 and 501749-01). EPA agrees with all four resistance claims, although only one was confirmed using the regulatory process in place. Based on scientific concerns for risks of resistance development in

non-high dose pests of Bt corn and Bt cotton, EPA is proposing that changes be adopted into EPA's IRM program for lepidopteran pests of Bt to reduce the selection intensity and improve the resistance monitoring approach and success of mitigation of resistance.

#### A. Options to Reduce Resistance Risks:

Potential options for developing an improved IRM program would have to address the following identified risk factors (RF):

- **Risk factor 1:** Single trait Bt corn in the presence of pyramided products in the landscape;
- Option: Limit or otherwise manage single trait commercial products;
- Option: Transition from single trait products to Bt corn pyramids.
  
- **Risk factor 2:** Limited number of highly effective Bt traits;
- Option: Adoption of intervening actions at "early warning resistance" in addition to actions when field failure occurs (see "molecular assay" option);
  
- **Risk factor 3:** The shift of dominance of resistance in ear-feeding pests of corn with exposure to Refuge-In-The-Bag (RIBs) corn products in the southern US;
- Option: Potential changes to the corn breeding program to move away from hemizygous parental corn lines, introduce pollen incompatibility between Bt and non-Bt cultivars, self-pollinating mechanism, etc.;
- Option: Manage the use of Bt corn RIB products in the southern US;
  
- **Risk factor 4:** Non-compliance with refuge plantings in the southern US;
- Option: The development of incentive programs to increase compliance with block refuge requirements for Bt corn in the southern US;
  
- **Risk factor 5:** Non-functional, non-high dose pyramided Bt products where individual traits control less than 95% of susceptible insect;
- Option: Implementation of best management practices (BMPs) consistent with the tenets of Integrated Pest Management (IPM) practices for fields with Unexpected Injury (UXI);
- Option: Manage the use of non-functional pyramids;
- Option: Increase the amount of refuge;
  
- **Risk factor 6:** Resistance monitoring does not allow tracking the same populations year after year;
- Option: Use of sentinel plots to monitor the same populations from year to year;
  
- **Risk factor 7:** Lack of standardized UXI thresholds;

- Option: Development of such standards in corn and cotton would expedite insect collections, reporting to EPA, and early mitigation;
- **Risk factor 8:** Selection pressure on effective traits (e.g., Vip3A) in pyramids with less effective or compromised traits;
- Option: Focus on risk in Bt corn which also drive resistance risk to Bt cotton;
- **Risk factor 9:** Use of diet bioassay methods for non-high dose pests;
- Option: Development of molecular/DNA assays to monitor for “early warning” resistance that allows expeditious resistance confirmation followed by early implementation of mitigation actions;
- **Risk factor 10:** Mitigation of resistance when field failure occurs;
- Option: Adoption of an early intervening threshold when resistance allele frequencies are still low (e.g., “early warning resistance”);
- **Risk factor 11:** Protracted mitigation in response to field failure;
- Options: *A priori* determined size for a Mitigation Action Area (MAA) based on scientifically defensible factors relying on noctuid dispersal propensity;
- Option: Implementing mitigation actions when field failure is apparent instead of after resistance is confirmed (i.e., practical resistance);
- Option: Use of best management practices (BMPs) consistent with the tenets of IPM to reduce UXI field occurrences and delay resistance development.

#### B. Additional Options to Reduce Resistance Risks:

A new framework for lepidopteran IRM could further benefit from the use of IPM with IRM and increased communication between stakeholders and other proactive actions such as:

- Increased scouting of pest densities
- Use of alternate control methods in addition to Bt when economic threshold is reached
- Increased tilling where possible and crop destruct
- Multi-year management plan to control major pests including rotation of Bt pyramided products (different modes of action), Bt corn with conventional corn and insecticide use, etc.
- Use of standardized regulatory thresholds defining unexpected injury levels that trigger insect population investigation (collections, BMPs, and assay follow up to test for resistance)
- Implementation of best management practices (consistent with tenets of IPM) when unexpected injury levels are triggered



- Improved and timely communication through notification to inform growers, state extension agents, and crop consultants of areas with early warning resistance, unexpected injury fields, and confirmed resistance cases.

### C. IRM for *S. albicosta*

The agency is considering whether an insect resistance management plan should be developed for *S. albicosta* and Bt corn incorporating the options in the previous two sections including the use of IPM with IRM (see IV. 1 & 2.).

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**VI. APPENDIX A. Bt CORN RESISTANCE MONITORING STUDIES SUBMITTED TO EPA FOR *H. ZEA***

<b>Year</b>	<b>Toxin(s)</b>	<b>MRID# or Citation</b>
1997	Cry1Ab, Cry1Ac	MRID#s 444754-01, 444756-01
1998	Cry1Ab	MRID# 447753-01
1999	Cry1Ab	MRID# 450568-01
2000	Cry1Ab	No report submitted
2001	Cry1Ab	Custom Bio-products, 2002a. Monitoring Bt susceptibility of <i>H. zea</i> to Cry1Ab: 2002 Final Report. (Report for 2001 season.) Unpublished studies submitted to EPA - No MRID #.
2001	Cry1F	Custom Bio-products, 2002b. Monitoring Bt susceptibility of <i>H. zea</i> to Cry1Fa: 2002 Final Report. (Report for 2001 season.) Unpublished studies submitted to EPA - No MRID #.
2002	Cry1Ab	Custom Bio-products, 2002c. Monitoring Bt susceptibility of <i>H. zea</i> to Cry1Ab: 2002 Collections and Assays. (Report for 2002 season.) Unpublished studies submitted to EPA - No MRID #.
2002	Cry1F	Custom Bio-products, 2002d. Monitoring Bt susceptibility of <i>H. zea</i> to Cry1Fa: 2002 Collections and Assays. (Report for 2002 season.) Unpublished studies submitted to EPA - No MRID #.
2003	Cry1Ab	Custom Bio-products, 2003a. Monitoring Bt susceptibility of <i>H. zea</i> to Cry1Ab: 2003 collections and assays. Unpublished study submitted to EPA - No MRID #.
2003	Cry1F	Custom Bio-products, 2003b. Monitoring Bt susceptibility of <i>H. zea</i> to Cry1Fa: 2003 collections and assays. Unpublished studies submitted to EPA - No MRID #.
2004	Cry1Ab	Custom Bio-products, 2004a. Monitoring Bt susceptibility of <i>H. zea</i> to Cry1Ab: 2004 collections and assays. Unpublished study submitted to EPA - No MRID #.
2004	Cry1F	Custom Bio-products, 2004b. Monitoring Bt susceptibility of <i>H. zea</i> to Cry1Fa: 2004 collections and assays. Unpublished studies submitted to EPA - No MRID #.
2005	Cry1Ab, Cry1F	MRID# 468749-01
2006	Cry1Ab, Cry1F	MRID# 471184-01
2007	Cry1Ab, Cry1F	MRID# 474139-01
2008	Cry1Ab, Cry1F	MRID# 478418-01
2009	Cry1Ab, Cry1F	MRID# 482082-01
2009	Cry1A.105	MRID# 482074-01

<b>Year</b>	<b>Toxin(s)</b>	<b>MRID# or Citation</b>
2010	Cry1Ab, Cry1F	MRID# 485938-01
2010	Cry1A.105	MRID# 485888-01
2011	Cry1Ab, Cry1F	MRID# 489261-01
2011	Cry1A.105	MRID# 489232-01
2011	Vip3A	MRID# 485912-01
2012	Cry1Ab, Cry1F	MRID# 492070-01
2012	Cry1A.105	MRID# 492046-01
2012	Vip3A	MRID# 492064-01
2013	Cry1Ab, Cry1F, Vip3A	MRID# 494600-01
2013	Cry1A.105	MRID# 495010-01
2014	Cry1Ab, Cry1F, Vip3A	MRID# 497848-01
2014	Cry1A.105	MRID# 497463-01
2015	Cry1Ab, Cry1F, Vip3A	MRID# 503241-01

**VII. APPENDIX B. Bt COTTON RESISTANCE MONITORING STUDIES SUBMITTED TO EPA FOR *H. ZEA***

Year	Toxin(s)	MRID# or Citation
1996-1998	Cry1Ac	MRID# 448633-01
1999-2000	Cry1Ac	Public literature reviewed in: EPA, 2001. Bt Plant-Incorporated Protectants Biopesticides Registration Action Document. October 15, 2001. Available at: <a href="http://www3.epa.gov/pesticides/chem_search/reg_actions/pip/1-overview.pdf">http://www3.epa.gov/pesticides/chem_search/reg_actions/pip/1-overview.pdf</a>
2001	Cry1Ac	MRID# 456863-01
2002	Cry1Ac	Hardee, D.R., et al., 2003. Monitoring for Cry1Ac susceptibility among field populations of cotton bollworm and tobacco budworm during the 2002 growing season. Unpublished study submitted to EPA. No MRID# assigned.
2002-2003	Cry2Ab2	MRID# 462720-02
2003	Cry1Ac, Cry2Ab2	MRID# 462720-03
2004	Cry1Ac, Cry2Ab2	MRID# 465476-01
2005	Cry1Ac, Cry2Ab2	MRID# 468266-01
2005	Cry2Ab2	MRID# 468266-02
2005	Cry1F	MRID# 469387-01
2006	Cry2Ab2	MRID# 471664-01
2006	Cry1Ac, Cry2Ab2	MRID# 471664-03
2006	Cry1F	MRID# 472235-01
2007	Cry1Ac, Cry2Ab2	MRID# 474608-01
2007	Cry1Ac, Cry1F	MRID# 474622-01
2008	Cry1Ac, Cry2Ab2	MRID# 477913-02
2008	Cry2Ab2	MRID# 477913-03
2008	Cry1Ac, Cry2Ab2, Cry1F	MRID# 477913-04
2008	Cry1Ac, Cry1F	MRID# 477911-01



<b>Year</b>	<b>Toxin(s)</b>	<b>MRID# or Citation</b>
2009	Cry1Ac	MRID# 481673-03
2009	Cry2Ab2	MRID# 481673-04
2009	Cry1Ac, Cry1F	MRID# 481714-01
2010	Cry1Ac	MRID# 485241-03
2010	Cry2Ab2	MRID# 485241-04
2010	Cry1Ac, Cry2Ab2	MRID# 485241-05
2010	Cry1Ac, Cry1F	MRID# 486241-01
2011	Cry1Ac	MRID# 488789-03
2011	Cry2Ab2	MRID# 488789-04
2011	Cry1Ac, Cry1F	MRID# 489571-01
2011	Vip3A	MRID# 485912-01
2012	Cry1Ac	MRID# 491632-03
2012	Cry2Ab2	MRID# 491632-04
2012	Cry1Ac, Cry1F	MRID# 492250-01
2012	Vip3A	MRID# 492064-01
2013	Cry1Ac	MRID# 494195-03
2013	Cry2Ab2	MRID# 494195-04
2013	Cry1Ac, Cry1F	MRID# 494778-01
2013	Cry1Ab, Cry1F, Vip3A	MRID# 494600-01
2014	Cry1Ac	MRID#s 496603-03, 497102-01
2014	Cry2Ab2	MRID#s 496603-04, 497102-01
2014	Cry1Ac, Cry1F	MRID# 497242-01
2014	Vip3A	MRID# 497848-01
2015	Cry1Ac, Cry2Ab2	MRID# 499587-01
2015	Cry1Ac, Cry1F	MRID# 500345-01

