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Environmental Risk Assessment for
3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 Maize

Assessment

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LIST OF ACRONYMS AND ABBREVIATIONS

Bt	*Bacillus thuringiensis*
Bti	*Bacillus thuringiensis* subsp. *israelensis*
CPB	Colorado potato beetle
CEW	corn earworm
EEC	estimated environmental concentration
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
mEPSPS	double mutated maize 5-enol pyruvylshikimate-3-phosphate synthase
MOE	margin of exposure
NOAEC	no observed adverse effect concentration
NTOs	nontarget organisms
PAT	phosphinothricin acetyltransferase
PMI	phosphomannose isomerase
TSH	trait-specific herbicide
US EPA	United States Environmental Protection Agency

Abbreviations for Maize Growth Stages (Abendroth *et al.* 2011)

V6	first six leaves collared
R1	silking
R4	dough
R6	physiological maturity (black layer)

1.0 EXECUTIVE SUMMARY

3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize was developed by Syngenta using conventional breeding techniques that combined seven individual transformation events. 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize produces five insecticidal proteins, Cry1Ab, Vip3Aa20, and Cry1F, which provide control of certain lepidopteran pests including the European corn borer (*Ostrinia nubilalis*) and mCry3A and eCry3.1Ab which provide control of certain coleopteran pests including Western corn rootworm (*Diabrotica virgifera virgifera*).

The purpose of this assessment is to summarize data on the environmental safety of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize and to draw conclusions about the potential environmental impact of its cultivation. The environmental safety of commercial cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize was considered in two parts: (1) evaluation of the risk that the cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize will harm nontarget organisms (NTOs), and (2) evaluation of the risk that 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize will become a serious weed of agricultural or nonagricultural habitats.

Cultivation of Bt11, MIR162, MIR604, TC1507, and 5307 maize separately have been determined to pose negligible risk to NTOs due to the absence of adverse effects of Cry1Ab, Vip3Aa20, mCry3A, Cry1F, and eCry3.1Ab at concentrations in excess of those to which the NTOs are likely to be exposed to in the field. The cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize will pose negligible risk to NTOs, provided the following two hypotheses are corroborated. First, there is no interaction among Cry1Ab, Vip3Aa20, mCry3A, Cry1F, and eCry3.1Ab that increases their potency such that the laboratory NTO studies of the individual proteins underestimate their no observed adverse effect concentrations (NOAECs) when they are combined. Secondly, the concentrations of Cry1Ab, Vip3Aa20, mCry3A, Cry1F, and eCry3.1Ab in 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize are not increased to levels that reduce previously determined margins of exposure achieved in ecotoxicological studies of these proteins to an extent that confidence in the prediction of lack of harm to NTOs in the field is significantly weakened.

Data from protein interaction studies corroborated the hypothesis of no interaction among Cry1Ab, Vip3Aa20, mCry3A, Cry1F, and eCry3.1Ab that increases their potency, and therefore the NOAECs of Cry1Ab, Vip3Aa20, mCry3A, Cry1F, and eCry3.1Ab for NTOs are unlikely to be lower in combination than separately. A comparative protein expression study corroborated the hypothesis of no increases in protein concentrations in 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize compared with cultivation of the single component events that erode margins of exposure to an extent that prediction of lack of harm to NTOs is compromised. Therefore, cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize poses no greater risk to NTOs than does the separate cultivation of Bt11, MIR162, MIR604, TC1507, and 5307 maize. Consequently, the risk to NTOs from the cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize is negligible.

Conventional maize is not found outside of cultivation. Data from an agronomic performance study corroborated the hypothesis that the expression of AMY797E, Cry1Ab, PAT, Vip3Aa20, PMI, mCry3A, Cry1F, eCry3.1Ab, and mEPSPS is highly unlikely to alter the dispersal or competitive ability of maize. Therefore, 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize is highly unlikely to be weedier than conventional maize or form persistent feral populations. The probability of spread of the transgenic proteins outside maize cultivation through volunteers and self-sustaining feral populations of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize is therefore low.

2.0 INTRODUCTION

Syngenta developed 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize (*Zea mays* L., corn) by combining seven individual transformation events using conventional breeding. This stacked-event maize variety provides enhanced bioethanol production along with control of certain lepidopteran and coleopteran insect pests, and tolerance to glufosinate-ammonium and glyphosate herbicides.

Maize plants derived from transformation Event 3272 contain the gene *amy797E*, which encodes the enzyme AMY797E, an alpha-amylase that catalyzes the hydrolysis of starch to soluble sugars, and the gene *pmi*, which encodes the enzyme phosphomannose isomerase (PMI). The synthetic gene *amy797E* was derived from three hyperthermophilic microorganisms of the archaean order *Thermococcales*. The increased thermostability of the AMY797E produced by 3272 maize enhances bioethanol production. The transgene *pmi* (also known as *manA*) was derived from *Escherichia coli* strain K-12. PMI enables transformed plant cells to utilize mannose as a primary carbon source; it was used as a selectable marker in the development of 3272 maize.

Maize plants derived from transformation Event Bt11 contain the transgene *cry1Ab*, which encodes the insecticidal protein Cry1Ab, and the transgene *pat*, which encodes the enzyme phosphinothricin acetyltransferase (PAT). The native, full-length Cry1Ab produced by the soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* is active against certain lepidopteran insect pests of maize, including *Ostrinia nubilalis* and *Sesamia nonagrioides*. The Cry1Ab produced by Bt11 maize is a truncated version of native Cry1Ab that retains activity against lepidopterans. The transgene *pat* was derived from the soil bacterium *Streptomyces viridochromogenes*. PAT acetylates glufosinate-ammonium, thus inactivating it and conferring tolerance to glufosinate-ammonium in herbicide products. PAT was used as a selectable marker in the development of Bt11 maize.

Maize plants derived from Event MIR162 contain the transgene *vip3Aa20*, which encodes the insecticidal protein Vip3Aa20, and the transgene *pmi*. Vip3Aa20 is a variant of the native Vip3Aa1 protein from the soil bacterium *B. thuringiensis* strain AB88, and is active against certain lepidopteran pests of maize, including *Spodoptera frugiperda* and *Helicoverpa zea.* PMI was used as a selectable marker in the development of MIR162 maize.

Maize plants derived from Event MIR604 provide control of certain coleopteran insect pests. Event MIR604 maize plants contain the transgene *mcry3A*, which encodes the insecticidal protein mCry3A, and the transgene *pmi*, which encodes the enzyme PMI. The native Cry3A from the soil bacterium *B. thuringensis* subsp. *tenebrionis* is active against certain coleopteran pests of maize. The mCry3A produced by MIR604 was modified to have enhanced activity against the *Diabrotica virgifera virgifera* and other related coleopteran pests. PMI was used as a selectable marker in the development of MIR604 maize. PMI expressed in MIR604 maize differs from the *E.coli* PMI by two amino acids and has been designated MIR604 PMI.

Maize plants derived from transformation Event TC1507 contain the gene *cry1F* which encodes the insecticidal protein Cry1F, and the gene *pat.* The native, full-length Cry1F produced by *B. thuringiensis* var. *aizawai* is active against certain lepidopteran insect pests of maize, including *Ostrinia nubilalis* and *S. frugiperda*. The Cry1F produced by TC1507 maize is a truncated version of the native Cry1F that retains activity against lepidopterans. PAT was used as a selectable marker in the development of TC1507 maize.

Maize plants derived from Event 5307 provide control of corn rootworm (*Diabrotica* spp.). Event 5307 maize plants contain the gene *ecry3.1Ab,* which encodes the insecticidal protein eCry3.1Ab, and the gene *pmi*. The engineered protein eCry3.1Ab is a chimera of mCry3A and Cry1Ab. The portion of Cry1Ab included in eCry3.1Ab has not preserved the activity of Cry1Ab against lepidopterans. The mCry3A protein provides enhanced activity against Western corn rootworm (*D. virgifera virgifera*) and other related coleopteran pests. PMI was used as a selectable marker in the development of 5307 maize.

Maize plants derived from transformation Event GA21 contain the transgene *mepsps,* which encodes the enzyme double-mutated 5-enol pyruvylshikimate-3-phosphate synthase (mEPSPS). The native 5-enol pyruvylshikimate-3-phosphate synthase (EPSPS) from *Z. mays* is involved in synthesis of aromatic amino acids and is inhibited by glyphosate. The double-mutated mEPSPS produced by GA21 maize has low affinity for glyphosate compared to the native EPSPS, thus conferring tolerance to glyphosate in herbicide products.

AMY797E, PAT, PMI, and mEPSPS are not plant-incorporated protectants, and therefore their properties will not be considered in this risk assessment.

The purpose of this risk assessment is to summarize data on the environmental safety of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize and to draw conclusions about the potential environmental impact of its cultivation. The environmental safety of commercial cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize is considered in two parts: (1) evaluation of the risk that the cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize will harm nontarget organisms (NTOs), and (2) evaluation of the risk that 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize will become a serious weed of agricultural or nonagricultural habitats.

3.0 NONTARGET ORGANISM RISK ASSESSMENT

Cultivation of Bt11, MIR162, MIR604, TC1507, and 5307 maize separately have been determined to pose negligible environmental risk because it is unlikely that the insecticidal proteins produced during cultivation of these crops will have harmful effects on nontarget organisms (NTOs). The effects of the insecticidal proteins on NTOs were investigated in laboratory studies that expose representative surrogate NTOs to purified protein. The highest concentration of insecticidal protein that has no observed adverse effect on a particular surrogate NTO is called the no observed adverse effect concentration (NOAEC) for that species.

The concentration of insecticidal protein to which the taxonomic or functional group of organisms represented by that surrogate is likely to be exposed via cultivation of the crop

producing that protein is called the estimated environmental concentration (EEC). EECs are usually estimated using the worst-case (most conservative) assumption that the organisms consume a diet comprised solely of transgenic crop tissue. The diets of non-pest organisms are unlikely to be comprised of crop tissue only; therefore, refinements of the worst-case EEC may be made using conservative assumptions about the dilution of the insecticidal protein in the diets of NTOs in the field (Raybould *et al*. 2007, Raybould and Vlachos 2011).

The ratio of the NOAEC/EEC is the margin of exposure (MOE). If the margin of exposure for a certain protein and surrogate species is 1 or greater, the risk to the functional group represented by the surrogate from cultivation of the crop producing that protein may be deemed negligible. If a sufficiently representative set of surrogate species is determined to have margins of exposure of 1 or greater, the risk to NTOs posed by cultivation of the transgenic crop may be deemed negligible with sufficient confidence for decision-making. Such assessments and conclusions of negligible risk to NTOs have been reported for Bt11 (US EPA 2001 and 2010a), MIR162 (US EPA 2009) MIR604 (US EPA 2010b), TC1507 (US EPA 2010a), and 5307 (US EPA 2012) maize.

The cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize poses negligible risk to NTOs, provided two hypotheses are corroborated: first, that there is no interaction among the proteins that increases their potency such that the laboratory studies of the individual proteins underestimate their NOAECs when they are combined; and secondly, that the concentrations of the insecticidal proteins are not greater than in the respective single event maize, such that the margins of exposure achieved in ecotoxicological studies of these proteins are reduced to an extent that confidence in the prediction of lack of harm to NTOs in the field is significantly weakened (Raybould *et al*. 2012).

3.1 Interaction Studies

3.1.1 Introduction to the approach

A FIFRA Scientific Advisory Panel stated it was "not aware of any instances where a 'new' toxin has been created by unexpected interaction between two known proteins" (US EPA 2005). This statement was also corroborated more recently via an extensive literature review (Walters *et al.* 2018).

Similarly, Syberg *et al.* (2009) concluded that in mixtures of chemicals "interactions seldom occur at concentrations below the individual chemical no observable effect concentration". Two important inferences for NTO risk assessment of crops with stacked insect-control traits may be drawn from these conclusions: first, if the insecticidal proteins individually have no adverse effects on NTOs at high margins of exposure, mixtures of the proteins at field concentrations are unlikely to result in increased potency of the components (i.e., synergism) such that they would have adverse effects on NTOs; and secondly, if tests of the hypothesis of no synergism are required to increase confidence in the NTO risk assessment, the most rigorous tests would use species that are sensitive to at least one of the components.

Literature corroborates that synergistic interactions are rarely found between Cry1, Cry3, and Vip3A insecticidal proteins, and specifically, supra-additive interactions are unlikely to occur for cross-class protein mixtures where the activity spectrums do not overlap (Walters *et al.*

2018). Studies of the effects of a combination of proteins in sensitive species can, therefore, be considered worst-case for the purposes of NTO risk assessment for the cultivation of crops with stacked insect-control traits, and be regarded as tier I effects tests for mixtures of proteins that separately have no effect on NTOs. Studies of the mixture in insensitive NTOs should be necessary only if increase potency is revealed in the tests using sensitive species, and the magnitude of the increase in potency is such that the margins of exposure in effects tests on the separate proteins are considered to be reduced below those required to conclude with confidence that risks to NTOs are acceptable.

The test for protein interaction between Cry1Ab, Vip3Aa20, mCry3A, Cry1F, and eCry3.1Ab was previously examined to support the risk assessment of Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize (Graser *et al.* 2017). 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize does not include any additional insecticidal proteins compared to Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize; therefore the same data can be used to assess protein interactions of insecticidal proteins in 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize.

The hypothesis that there is no increase in the potency of the insecticidal proteins in 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize when in combination was tested in 3 stages:

1. A test for synergism among the proteins active against Lepidoptera (i.e., Cry1Ab, Vip3Aa20 and Cry1F)
2. A test for synergism among the proteins active against Coleoptera (i.e., mCry3A and eCry3.1Ab)
3. A test for synergism between Lepidopteran-active (Cry1Ab + Vip3Aa20 + Cry1F) and Coleopteran-active (mCry3A and eCry3.1Ab) proteins

This testing strategy was adopted as a means to adequately assess the potential for increased potency of Cry1Ab + Vip3Aa20 + Cry1F + mCry3A + eCry3.1Ab without testing for synergism between all pairs of proteins. If the hypotheses of no synergism among Cry1Ab, Vip3Aa20, and Cry1F, and between mCry3A and eCry3.1Ab are corroborated, the effects of mixtures Cry1Ab + Vip3Aa20 + Cry1F and of mCry3A + eCry3.1Ab on NTOs will be no greater than predicted from the effects of the proteins when tested separately; in other words, these mixtures are unlikely to have adverse effects on NTOs at concentrations of the components at least as high as those in previous effects tests with the individual proteins.

These conclusions hold for the mixture Cry1Ab + Vip3Aa20 + Cry1F + mCry3A + eCry3.1Ab, provided that the mixture mCry3A + eCry3.1Ab does not increase the potency of the mixture Cry1Ab + Vip3Aa20 + Cry1F, and that the mixture of Cry1Ab + Vip3Aa20 + Cry1F does not increase the potency of the mixture of mCry3A + eCry3.1Ab. The hypothesis of no increase in the potency of Cry1Ab + Vip3Aa20 + Cry1F may be tested by exposing a species sensitive to a mixture of Cry1Ab + Vip3Aa20 + Cry1F in the presence and absence of mCry3A + eCry3.1Ab; likewise, the hypothesis of no increase in the potency of mCry3A + eCry3.1Ab may be tested by exposing a species sensitive to a mixture of mCry3A + eCry3.1Ab in the presence and absence of Cry1Ab + Vip3Aa20 + Cry1F. All study designs are able to detect antagonism as well as synergism; however, a finding of antagonism would

not indicate greater risk to NTOs from cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize than from cultivation of the single component events.

3.1.2 Bioassays for interaction among Cry1Ab + Vip3Aa20 + Cry1F

Tests for synergism or antagonism among Cry1Ab, Vip3Aa20, and Cry1F were conducted in two bioassays (Fan *et al*. 2010): one using ECB, which is sensitive to Cry1Ab and Cry1F, and one using fall armyworm (*S. frugiperda*; FAW), which is sensitive to Vip3Aa20 and Cry1F.

In both bioassays, freshly hatched larvae were exposed to several treatments via a commercial insect diet prepared according the manufacturer's instructions. The treatments were placed into 24-well plates, with 100 µl diet per well. A single, randomly selected larva was placed in each well.

The treatments were various concentrations of Cry1Ab, Vip3Aa20, Cry1F (individually) and mixtures of Cry1Ab + Vip3Aa20 + Cry1F (= Mixture A for ECB; Mixture B for FAW). Each protein was supplied as a microbially produced test substance. The concentrations of the proteins were chosen to give a range of predicted responses to the mixtures of Cry1Ab + Vip3Aa20 + Cry1F based on preliminary experiments and historical data (e.g., Lee *et al.* 2003, Mostafa *et al.* 2003, Wolt *et al*. 2005). The concentrations used in the ECB bioassay are given in Table 1 and those used in the FAW bioassay in Table 2. Both bioassays included controls to assess the effect of the buffers used to dilute the proteins.

Percent mortality of the larvae was recorded for each treatment or control at 96, 120, and 144 hours after insects were placed on the diet. The ECB and FAW bioassays were conducted independently in triplicate on different days using fresh treatments and different batches of freshly hatched insects.

The presence of synergism or antagonism among Cry1Ab, Vip3Aa20, and Cry1F was assessed by comparing the observed mortality on diets containing protein mixtures with the predicted mortality based on bioassays of each protein individually. The mortality observed for each treatment was corrected to allow for the mortality observed in the negative control using Abbott's formula (Abbott 1925). Predicted responses were calculated based on an assumption of independent action, using an extension of the Colby method (Colby 1967): if Cry1Ab alone gives x% mortality, Vip3Aa20 alone gives y% mortality, and Cry1F alone gives z% mortality, then under the assumption of independent action, the predicted response to the mixture of Cry1Ab + Vip3Aa20 + Cry1F is [x + y + z – (xy + xz + yz)/100 – xyz/10000]; thus, if particular concentrations of Cry1Ab, Vip3Aa20, and Cry1F each caused 25% mortality, a mixture of the proteins at those concentrations is expected to give *ca*. 55% mortality (= 75 – (1875/100) – 15625/10000)%), and this expectation is compared with the observed mortality on that mixture.

If, over a series of mixtures, there is a tendency for higher observed mortality than expected, synergism among the components is inferred; similarly, a tendency for lower observed mortality than predicted implies antagonism. There is no formal statistical test for synergism or antagonism under the Colby method, and conclusions are derived from inspection of the

data. To ease interpretation of the data, the observed minus the expected mortality was plotted for each bioassay at each assessment time (Figures 1 and 2.).

TABLE 1 Concentrations of Cry1Ab + Vip3Aa20 + Cry1F mixtures used for ECB bioassays

Mixture	Protein concentration (ng/ml diet)		
	Cry1Ab	Vip3Aa20	Cry1F
A	200	100	400
A/2	100	50	200
A/4	50	25	100
A/8	25	12.5	50
A/16	12.5	6.3	25

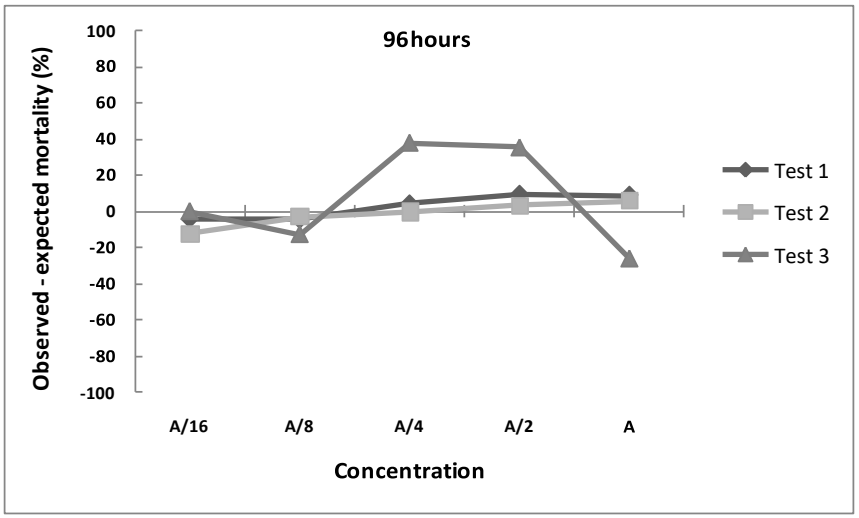
TABLE 2 Concentrations of Cry1Ab + Vip3Aa20 + Cry1F mixtures used for FAW bioassays

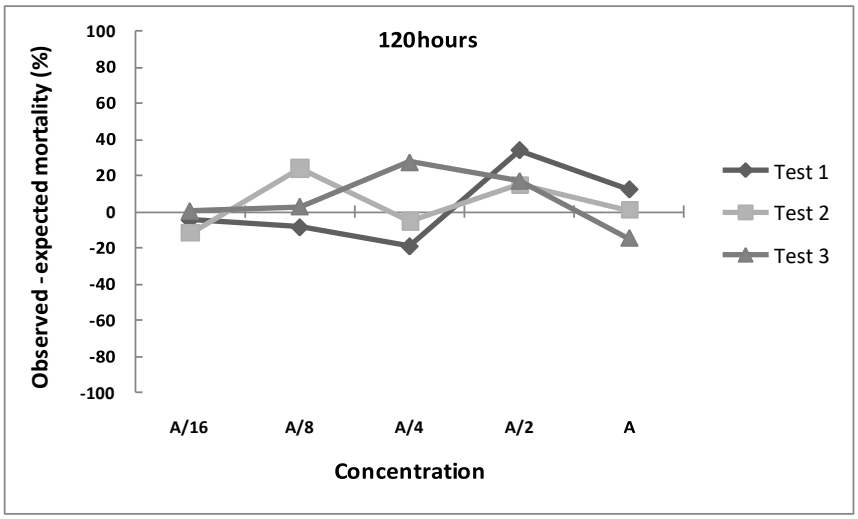
Mixture	Protein concentration (ng/ml diet)		
	Cry1Ab	Vip3Aa20	Cry1F
B	200	400	2000
B/2	100	200	1000
B/4	50	100	500
B/8	25	50	250
B/16	12.5	25	125

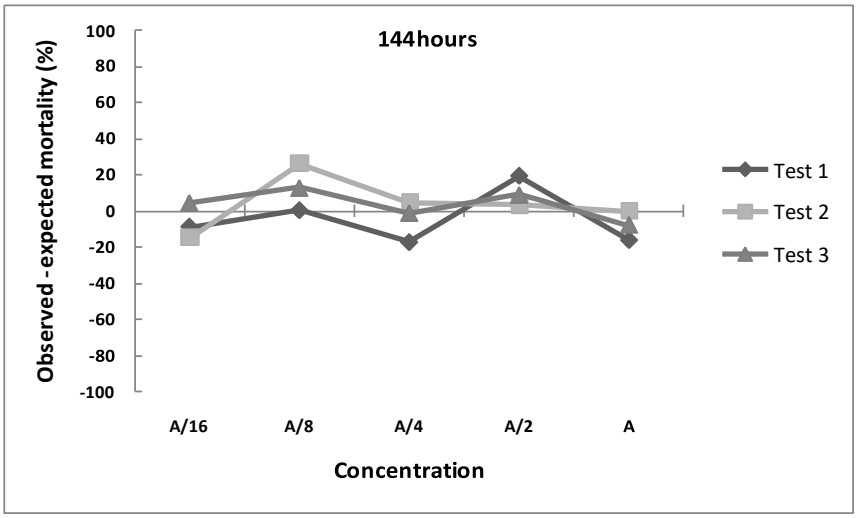
3.1.2.1 Results of the ECB bioassay

Plots of observed – expected mortality are displayed in Figure 1. There is no tendency for an excess or deficit of observed mortality in any test at any time; therefore the results of the ECB bioassay corroborate the hypothesis that there is no synergism or antagonism among Cry1Ab, Vip3Aa20, and Cry1F.

FIGURE 1 Comparison of observed and expected mortality of ECB on diet containing mixtures of Cry1Ab + Vip3Aa20 + Cry1F, 96, 120, and 144 hours after treatment



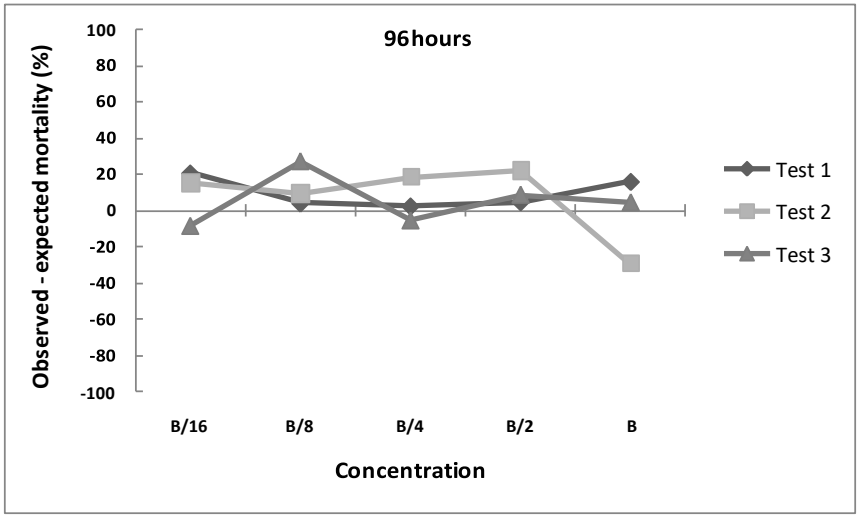


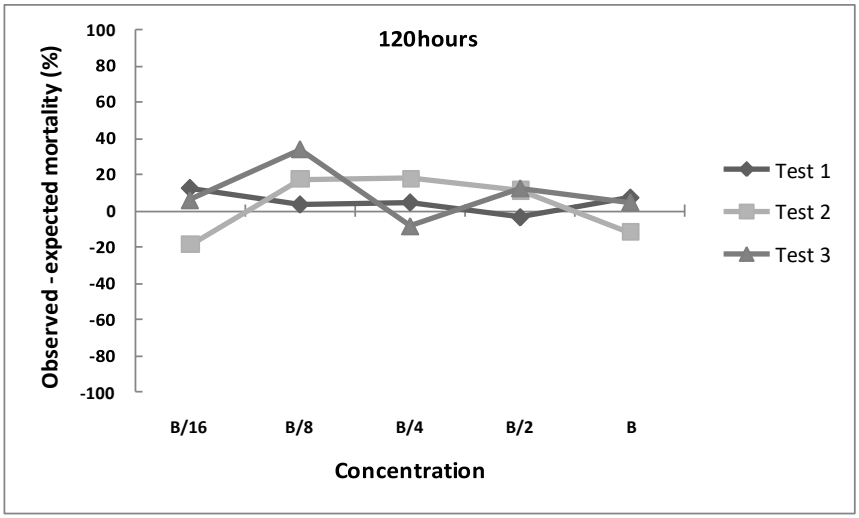


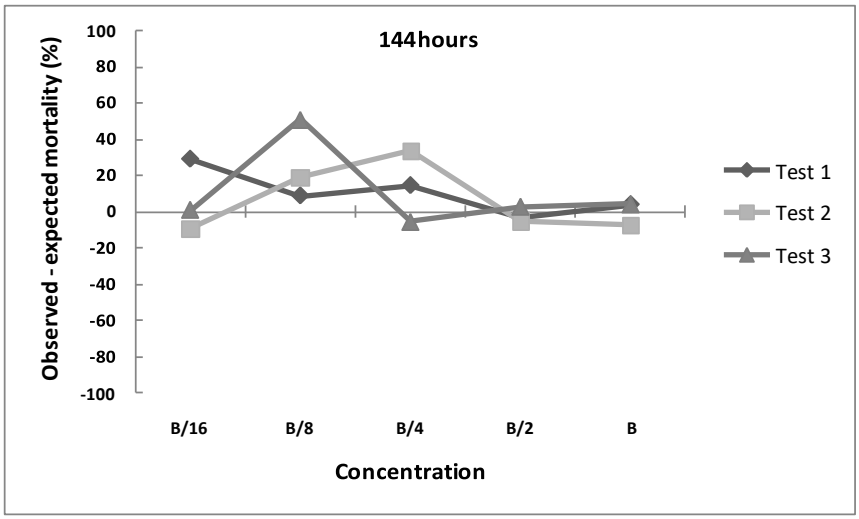
3.1.2.2 Results of the FAW bioassay

Plots of observed – expected mortality are displayed in Figure 2. There is no tendency for an excess or deficit of observed mortality in tests 2 and 3 at any time. In test 1, there is a suggestion of greater observed over expected mortality; however in many comparisons, the excess is small (< 5%) and synergism is not strongly suggested by these data. Overall, the results of the FAW bioassay corroborate the hypothesis that there is no synergism or antagonism among Cry1Ab, Vip3Aa20, and Cry1F.

FIGURE 2 Comparison of observed and expected mortality of FAW on diet containing mixtures of Cry1Ab + Vip3Aa20 + Cry1F, 96, 120, and 144 hours after treatment







3.1.2.3 Conclusions for interaction of Cry1Ab + Vip3Aa20 + Cry1F

Bioassays of first-instar ECB and FAW corroborated the hypothesis of no synergism or antagonism among Cry1Ab, Vip3Aa20, and Cry1F. These results suggest that the effects on NTOs of a mixture of Cry1Ab + Vip3Aa20 + Cry1F can be predicted from the effects of these proteins when tested separately.

3.1.3 Bioassays for interaction among mCry3A + eCry3.1Ab

Tests for interactions between mCry3A and eCry3.1Ab were conducted in bioassays with Colorado potato beetle (*Leptinosara decemlineata*) (Seastrum 2010). Freshly hatched larvae were exposed to several treatments via a commercially available CPB diet. The treatments were placed into 24-well plates, with 100 µl diet per well. A single, randomly selected larva was placed into each well.

The treatments were various concentrations of mCry3A, eCry3.1Ab, and a mixture of mCry3A + eCry3.1Ab (= Mixture C). The proteins were supplied as a microbially produced test substances. The concentrations of the proteins were chosen to give a range of predicted responses to mCry3A + eCry3.1Ab based on preliminary experiments and historical data (Walters *et al*. 2008, Walters *et al*. 2010). The concentrations used in the bioassay are given in Table 3. The bioassay included controls to assess the effect of the buffer used to dilute the proteins.

Percent mortality of the larvae was recorded for each treatment or control at 96 and 120 hours after the insects were placed on the diet. The CPB bioassays were conducted independently in triplicate on different days using fresh treatments and different batches of freshly hatched insects.

The occurrence of synergism or antagonism between mCry3A and eCry3.1A was assessed by comparing the observed mortality on diets containing protein mixtures with the predicted mortality based on bioassays of the proteins separately. The mortality observed for each treatment was corrected to allow for the mortality observed in the negative control using Abbott's formula (Abbott 1925). Predicted responses were calculated based on an assumption of independent action, using the Colby method (Colby 1967): if mCry3A alone gives x% mortality, and eCry3.1Ab alone gives y% mortality, then under the assumption of independent action, the predicted response to the mixture of mCry3A + eCry3.1Ab is $[x + y – (xy)/100]$. Interpretation of the results uses the same method as the ECB and FAW studies, above.

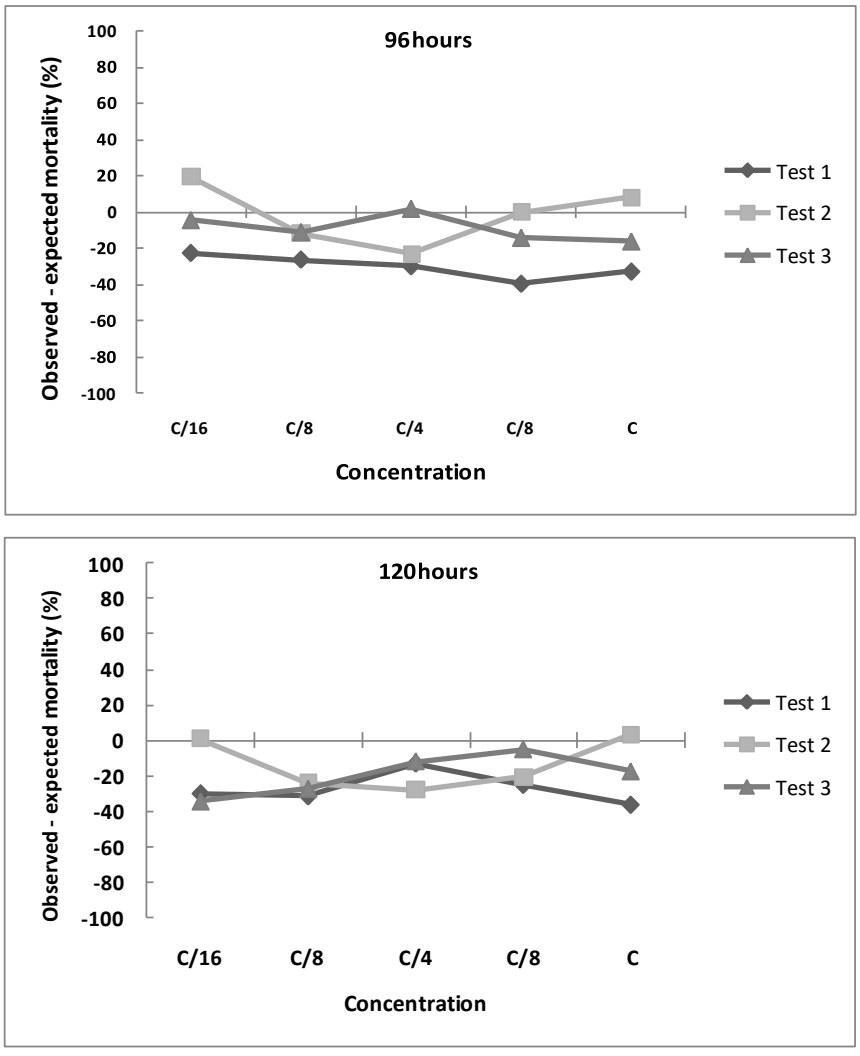
TABLE 3 Treatments in test for interaction between mCry3A and eCry3.1Ab

Mixture	Protein concentration (µg/ml diet)	
	mCry3A	eCry3.1Ab
C	4	4
C/2	2	2
C/4	1	1
C/8	0.5	0.5
C/16	0.25	0.25

3.1.3.1 Results of the CPB bioassay

Plots of observed – expected mortality are displayed in Figure 3. There is a tendency for lower than expected mortality in all tests; therefore the results corroborate the hypothesis that there is no synergism between mCry3A and eCry3.1Ab, but do not corroborate the hypothesis of no antagonism between mCry3A and eCry3.1Ab.

FIGURE 3 Comparison of observed and expected mortality of CPB on diet containing mixtures of mCry3A + eCry3.1Ab 96 and 120 hours after treatment



3.1.3.2 Conclusions for interaction of mCry3A + eCry3.1Ab

Bioassays of first-instar CPB corroborated the hypothesis of no synergism between mCry3A and eCry3.1Ab. These results suggest that the effects on NTOs of mixtures of mCry3A + eCry3.1Ab will be no greater than those predicted from the effects of these proteins when tested separately.

3.1.4 Bioassay for interactions between Cry1Ab + Vip3Aa20 + Cry1F and mCry3A + eCry3.1Ab

The final interaction study compared the effects on ECB of different concentrations of a mixture of Cry1Ab + Vip3Aa20 + Cry1F in the presence and absence of a mixture of mCry3A + eCry3.1Ab; the study also compared the effects on CPB of different concentrations of a mixture of mCry3A + eCry3.1Ab in the presence and absence of a mixture of Cry1Ab + Vip3Aa20 + Cry1F (Seastrum *et al*. 2010).

Each test species was exposed to two concentrations of the protein mixture to which they are sensitive (the "active mixture"): "dose 1", intended to produce about 30% mortality after 120 hours; and "dose 2", intended to produce about 70% mortality after 120 hours (Table 4). Each dose was presented with and without dose 2 of the protein mixture to which the species is insensitive (the "inactive mixture"), giving 4 treatments for each bioassay. Buffer controls and inactive mixture only treatments were also conducted. For both species, mortality was assessed 96 and 120 hours after the beginning of the bioassay.

TABLE 4 Concentrations of insecticidal proteins used in the bioassays to test for synergism or antagonism between Cry1Ab + Vip3Aa20 + Cry1F and mCry3A + eCry3.1Ab

Treatment	Protein concentration
ECB dose 1	25 ng Cry1Ab + 12.5 ng Vip3Aa20 + 50 ng Cry1F / ml diet
ECB dose 2	50 ng Cry1Ab + 25 ng Vip3Aa20 + 100 ng Cry1F / ml diet
CPB dose 1	1 µg mCry3A + 1 µg eCry3.1Ab / ml diet
CPB dose 2	4 µg mCry3A + 4 µg eCry3.1Ab / ml diet

Data from bioassays were subjected to analysis of variance using the model

$$Yijk = U + Di + Ij + Tk + DIij + eijk$$

where *Yijk* = observed % mortality
U = overall mean
Di = dose effect of the active ingredient
Ij = effect inactive ingredient
Tk = effect of the test
*DI*ij = dose x inactive ingredient interaction
eijk = residual error

For each bioassay, *F* tests were used to assess the statistical significance of the effects of dose, inactive ingredient and their interaction.

3.1.4.1 Results of the ECB bioassay – Cry1Ab + Vip3Aa20 + Cry1F and mCry3A + eCry3.1Ab

As expected, mortality in the buffer control and mCry3A + eCry3.1Ab treatments was low, with all but one replicate containing 0, 1 or 2 dead larvae (= 0, 4.2 or 8.3% mortality). One replicate exposed to mCry3A + eCry3.1Ab contained 4 dead larvae (= 16.7% mortality). The results from the remaining insecticidal protein treatments are summarised in Table 5. Analysis of these data revealed no statistically significant effect of mCry3A + eCry3.1Ab on the potency of Cry1Ab + Vip3Aa20 + Cry1F: for the data at 96 hours, the probability of no effect of mCry3A + eCry3.1Ab was 0.639, and for the data at 120 hours, the probability of no effect was 0.792.

TABLE 5 Results of the ECB bioassay to test the effect of mCry3A + eCry3.1Ab on the potency of Cry1Ab + Vip3Aa20 + Cry1F

Dose	x Inactive ingredient	Mean % Mortality 96 hours	Mean % Mortality 120 hours
1	Absent	16.8	26.8
1	CPB dose 2	18.0	29.1
2	Absent	57.5	76.0
2	CPB dose 2	50.5	70.5
Mean of dose across inactive ingredient [a]			
1		17.4	28.0
2		54.0	73.2
Mean of inactive ingredient across dose [b]			
Absent		37.2	51.4
CPB dose 2		34.3	49.8

***F*-test probabilities**	**96hours**	**120 hours**
Dose	0.001	<0.001
Inactive ingredient	0.639	0.792
Dose x Inactive ingredient	0.512	0.514
Standard deviation	10.2	9.8

[a]The percent mortality for each dose was averaged across the absence or presence of the inactive ingredient.
[b]The percent mortality for either the absence or presence of the inactive ingredient was averaged across the dose.
The highlighted row indicates the statistical significance of the test of the hypothesis that mCry3A + eCry3.1Ab has no effect on the potency of Cry1Ab + Vip3Aa20 + Cry1F.

3.1.4.2 Results of the CPB bioassay – Cry1Ab + Vip3Aa20 + Cry1F and mCry3A + eCry3.1Ab

As expected, mortality in the buffer control and Cry1Ab + Vip3Aa20 + Cry1F treatments was low, with all replicates containing 0, 1 or 2 dead larvae (= 0, 4.2 or 8.3% mortality). The results from the insecticidal protein treatments are summarized in Table 6. Analysis of these data revealed no statistically significant increase in the potency of mCry3A + eCry3.1Ab in the presence of Cry1Ab + Vip3Aa20 + Cry1F: for the data at 96 hours, the probability of no effect of Cry1Ab + Vip3Aa20 + Cry1F was 0.064, and for the data at 120 hours, the probability of no effect was 0.147.

TABLE 6 Results of the CPB bioassay to test the effect of Cry1Ab + Vip3Aa20 + Cry1F on the potency of mCry3A + eCry3.1Ab

Dose	x Inactive Ingredient	Mean % Mortality 96 hours	Mean % Mortality 120 hours
1	Absent	36.1	51.4
1	ECB dose 2	36.1	48.6
2	Absent	45.0	61.8
2	ECB dose 2	66.7	81.9
Mean of dose across inactive ingredient [a]			
1		36.1	50.0
2		55.8	71.9
Mean of inactive ingredient across dose [b]			
Absent		40.5	56.6
ECB dose 2		51.4	65.3

***F*-test probabilities**	**96 hours**	**120 hours**
Dose	0.006	0.006
Inactive ingredient	0.064	0.147
Dose x Inactive ingredient	0.064	0.070
Standard deviation	8.3	9.0

[a]The percent mortality for each dose was averaged across the absence or presence of the inactive ingredient.
[b]The percent mortality for either the absence or presence of the inactive ingredient was averaged across the dose.
The highlighted row indicates the statistical significance of the test of the hypothesis that Cry1Ab + Vip3Aa20 + Cry1F has no effect on the potency of mCry3A + eCry3.1Ab.

3.1.4.3 Conclusions from the ECB and CPB bioassays – Cry1Ab + Vip3Aa20 + Cry1F and mCry3A + eCry3.1Ab

The presence of mCry3A + eCry3.1Ab had no statistically significant effect on the potency of Cry1Ab + Vip3Aa20 + Cry1F to ECB; and Cry1Ab + Vip3Aa20 + Cry1F had no significant effect on the potency of mCry3A + eCry3.1Ab to CPB. When combined with results from previous studies that show no synergism among the components within each mixture, these results corroborate the hypothesis that the effects on NTOs of mixtures of Cry1Ab + Vip3Aa20 + mCry3A + Cry1F + eCry3.1Ab will be no greater than those predicted from the effects of these proteins when tested separately.

3.2 Concentrations of Cry1Ab, Vip3Aa20, Cry1F, mCry3A, and eCry3.1Ab in 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize

Protein expression levels exhibit a certain level of variability depending on genetics and environment. However, due to the conservative manner for setting NTO effects test concentrations this variability is unlikely to erode margins of exposure for NTOs to levels that are unacceptable (McDonald *et al.* 2020).

Nevertheless, the protein expression levels in 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize were examined. Bt11, MIR162, MIR604, TC1507, 5307, 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize, and a nontransgenic, near-isogenic maize were grown concurrently in field trials in Germansville PA, Stewardson IL, and Delavan WI (Bednarcik 2021). Samples of leaves, roots, and whole plants from developmental stage V6; leaves, roots, pollen, and whole plants from developmental stage R1; forage from R4; and kernels from developmental stages R6 and senescence were collected from each genotype. The samples were processed and analyzed by enzyme-linked immunosorbent assay to measure the dry-weight concentrations of the transgenic proteins produced in these tissues.

In most tissues, the mean concentrations of the insecticidal proteins were not statistically greater in 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize when compared with the respective single component events (Table 7). The exceptions were the mean concentration of Cry1Ab in V6 and R1 leaves, Vip3Aa20 in V6 whole plants, Cry1F in V6 and R1 whole plants and R6 kernels, and eCry3.1Ab in V6 roots.

TABLE 7 Summary of the study comparing the dry-weight concentrations of transgenic insecticidal proteins in 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize (stack) with those in Bt11, MIR162, MIR604, TC1507, and 5307 maize

Tissue	Protein				
	Cry1Ab	**Vip3Aa20**	**Cry1F**	**mCry3A**	**eCry3.1Ab**
V6 leaves	Stack by 8%	NS	NS	NS	NS
V6 roots	NS	NS	NS	NS	Stack by 15%
V6 whole plants	NS	Stack by 13%	Stack by 10%	NS	NS
R1 leaves	Stack by 19%	NS	NS	NS	NS
R1 roots	NS	NS	NS	NS	NS
R1 pollen	NR	NS	NS	NS	NR
R1 whole plants	NS	NS	Stack by 14%	NS	5307
R4 forage	NS	NS	NS	NS	NS
R6 kernels	NS	NS	Stack by 10%	MIR604	NS
Senescent kernels	NS	NS	NS	MIR604	NS

NS – difference is not statistically significant.
NR – not reported; data did not allow for statistical comparison due to some of the data points falling below the limit of quantitation.
Genotype given where the difference is statistically significant; genotype with the higher concentration is listed. If the stack is higher the percentage increase was calculated [((concentration in stack / concentration single) × 100%) – 100%] and presented as "by %".

3.3 Nontarget organism risk assessment for 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize

The hypotheses that cultivation of Events Bt11, MIR162, MIR604, TC1507, and 5307 maize in the United States will not harm NTOs were corroborated by weight of evidence (US EPA 2001, 2009, 2010a, 2010b, and 2012). Corroboration is based principally on laboratory data that show the absence of adverse effects of these proteins to representative surrogates for valued taxonomic or functional groups of NTOs at concentrations in excess of those to which these organisms are likely to be exposed via cultivation of transgenic maize. 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize is also unlikely to harm NTOs provided that the potential for these proteins to cause adverse effects is not increased when they are combined, or that the concentration of the proteins is not significantly higher in 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize than in the respective single events.

The results of the interaction study indicate no synergism between Cry1Ab + Vip3Aa20 + Cry1F + mCry3A + eCry3.1Ab, and therefore the effects on NTOs of a mixture of Cry1Ab, Vip3Aa20, Cry1F, mCry3A, and eCry3.1Ab that results from cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize will be no greater than those predicted from the effects of these proteins when tested separately.

The results of the comparative expression study indicate that many NTOs will be exposed to similar concentrations of Cry1Ab, Vip3Aa20, Cry1F, mCry3A, and eCry3.1Ab from the cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize as they are from the cultivation of the individual events Bt11, MIR162, MIR604, TC1507, and 5307 maize; therefore, the margins of exposure in NTO effects studies for the single events are applicable to the events in combination. The exceptions may be those species potentially exposed to a tissue type/insecticidal protein combination for which a trend for an increase over multiple developmental stages is observed. For 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize this includes Cry1Ab in leaves. While the concentrations of Cry1F in whole plants (V6 and R1) were higher in the stack compared to the single, this tissue type is not used to calculate worst-case EEC for any NTOs and therefore has no implications on the risk assessment.

The mean concentrations of Cry1Ab in V6 and R1 leaves were greater in 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize when compared to the respective single event. Nontarget arthropods rarely eat leaves of maize. The more likely route of exposure to transgenic proteins is consumption of prey that have fed on maize (Harwood *et al.* 2005). Nevertheless the concentration of Cry1Ab in leaves can be regarded as the worst-case EEC for above-ground nontarget arthropods.

The maximum increase in exposure to Cry1Ab from leaves of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize is predicted to be approximately 1.19X the EECs from Bt11 maize leaves, which is well within the previously determined margins of exposure for above ground arthropods. Margins of exposure of 6 (lady beetles and parasitic hymenoptera) and 5 (green lacewing) were reported (Mendelsohn *et al.* 2003); therefore, the worst-case increase in EEC for Cry1Ab in 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize gives a minimum margin of exposure of 4.25 for above ground

nontarget arthropods. Cry1Ab has no adverse effects on above ground nontarget arthropods at concentrations greater than worst-case predictions of EECs for cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize; therefore, the increase in concentration of Cry1Ab in leaves is not considered biologically significant, and no adverse effects on above ground nontarget arthropods are expected to result from cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize.

4.0 WEEDINESS ASSESSMENT

Maize has lost the ability to survive without cultivation (OECD 2003). It can over-winter and germinate in a subsequent crop as a volunteer weed; for example, maize is a common volunteer in soybean fields. Nevertheless, several features of maize make it unlikely to form self-sustaining weedy populations in agricultural systems: it is easily controlled in subsequent crops by selective herbicides; seed dispersal is limited because seeds are held inside the husks of the cob; and the seeds lack dormancy so that young plants are exposed to harsh winter conditions. Maize does not persist in habitats outside agriculture because, in addition to the features listed above, it requires disturbed ground to germinate and it is very uncompetitive against perennial vegetation. Expression of AMY797E, Cry1Ab, Vip3Aa20, mCry3A, Cry1F, eCry3.1Ab, PAT, mEPSPS, and PMI is highly unlikely to alter the dispersal or competitive ability of maize.

This hypothesis was corroborated in a study comparing agronomic and phenotypic characteristics of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize with those of the nontransgenic, near-isogenic maize (Ward 2021), as described below.

4.1 Agronomic Characteristics of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 Maize

A way to test whether a transgenic crop variety is likely to be weedier than its corresponding nontransgenic variety is to compare their performance in agronomic trials (White 2002, Raybould 2005). If their agronomic characteristics are similar, then it is likely that the potential to form weedy populations is no greater for the transgenic variety than for the nontransgenic variety. If the risks to endpoints potentially affected by weediness are acceptable for the nontransgenic crop, it follows that the risks should be acceptable for the transgenic crop (Raybould 2005). Agronomic characteristics typically used by breeders and agronomists to evaluate maize were compared between 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize and nontransgenic, near-isogenic maize (Ward 2021).

Analysis of variance was used to test for entry effects both across locations and within each location. Statistical comparisons were made between the test maize and the nontransgenic control maize, and between the test + trait-specific herbicide (TSH) maize and the control maize. The agronomic characteristics with data suitable for analysis of variance were early stand count, days to 50% pollen shed, days to 50% silking, ear height, plant height, stalk-lodged plants, final stand count, days to maturity, grain moisture, grain yield, and hundred-kernel weight. The data for the other characteristics evaluated during the study (root-lodged plants and dropped ears) were not suitable for formal statistical comparison but were assessed based on comparisons of descriptive statistics. In addition, for each agronomic

characteristic assessed, the means for the test maize and for the test + TSH maize were compared with the range of variation for the six nontransgenic maize reference hybrids.

The results of the statistical analyses are as follows: Plant height was significantly lower for both the test and the test + TSH maize when compared to the control; however, the differences were small (7 cm and 8 cm, respectively). Days to 50% pollen shed was significantly higher in the test + TSH maize than in the control maize, but the difference was only 0.8 days. Days to maturity was significantly higher for the test maize compared to the control, but the difference was only 1 day. Hundred-kernel weight for the test + TSH maize was significantly higher than for the control maize by 1.5 grams (5.1%). All of these differences were small and would likely have no effect on cultivation or harvest quality of the crop. No statistically significant differences were observed for early stand count, days to 50% silking, ear height, stalk-lodged plants, final stand count, grain moisture, or grain yield when compared to the control.

Although statistical comparisons could not be performed for the remaining characteristics (root-lodged plants and dropped ears), the means were comparable between both the test and the control maize, and between the test + TSH and the control maize.

The mean values for all agronomic characteristics measured in the untreated and TSH-treated 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize, including those for which analysis of variance was not appropriate, were within the observed ranges for the nontransgenic, near-isogenic control maize and for the six nontransgenic reference hybrids.

The results of this study support the conclusion that 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize (whether test or test + TSH) possesses agronomic characteristics similar to that of the nontransgenic, near-isogenic control maize, and by extension is agronomically similar to conventional, commercial maize.

Therefore, 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize is highly unlikely to be associated with an increase in the abundance of maize volunteers or be more difficult to control than conventional maize volunteers. Similarly, agronomic data provide no evidence that 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize will form persistent feral populations. The probability of spread of the transgenic proteins outside maize cultivation through volunteers and self-sustaining feral populations of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize is therefore low.

5.0 CONCLUSIONS

The risk to NTOs from the cultivation of Bt11, MIR162, MIR604, TC1507 and 5307 maize is regarded as negligible due to the absence of adverse effects associated with the respective insecticidal proteins at concentrations in excess of those to which NTOs will be exposed in the field. Data from protein interaction studies corroborated the hypothesis of no interaction among Cry1Ab, Vip3Aa20, mCry3A, Cry1F, and eCry3.1Ab that increases their potency, and therefore the NOAECs for NTOs are unlikely to be lower when exposed to the proteins in combination. A comparative protein expression study corroborated the hypothesis of no increases in protein concentrations in 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 ×

GA21 maize compared with cultivation of the single component events that erode margins of exposure to an extent that prediction of lack of harm to NTOs is compromised. Therefore, cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize poses no greater risk to NTOs than does the separate cultivation of the single component events. Consequently, the risk to NTOs from the cultivation of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize is negligible.

Conventional maize is not found outside of cultivation. Data from an agronomic performance study corroborated the hypothesis that the expression of AMY797E, Cry1Ab, PAT, Vip3Aa20, PMI, mCry3A, Cry1F, eCry3.1Ab, and mEPSPS is highly unlikely to alter the dispersal or competitive ability of maize. Therefore, 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize is highly unlikely to be weedier than conventional maize or form persistent feral populations. The probability of spread of the transgenic proteins outside maize cultivation through volunteers and self-sustaining feral populations of 3272 × Bt11 × MIR162 × MIR604 × TC1507 × 5307 × GA21 maize is therefore low.

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