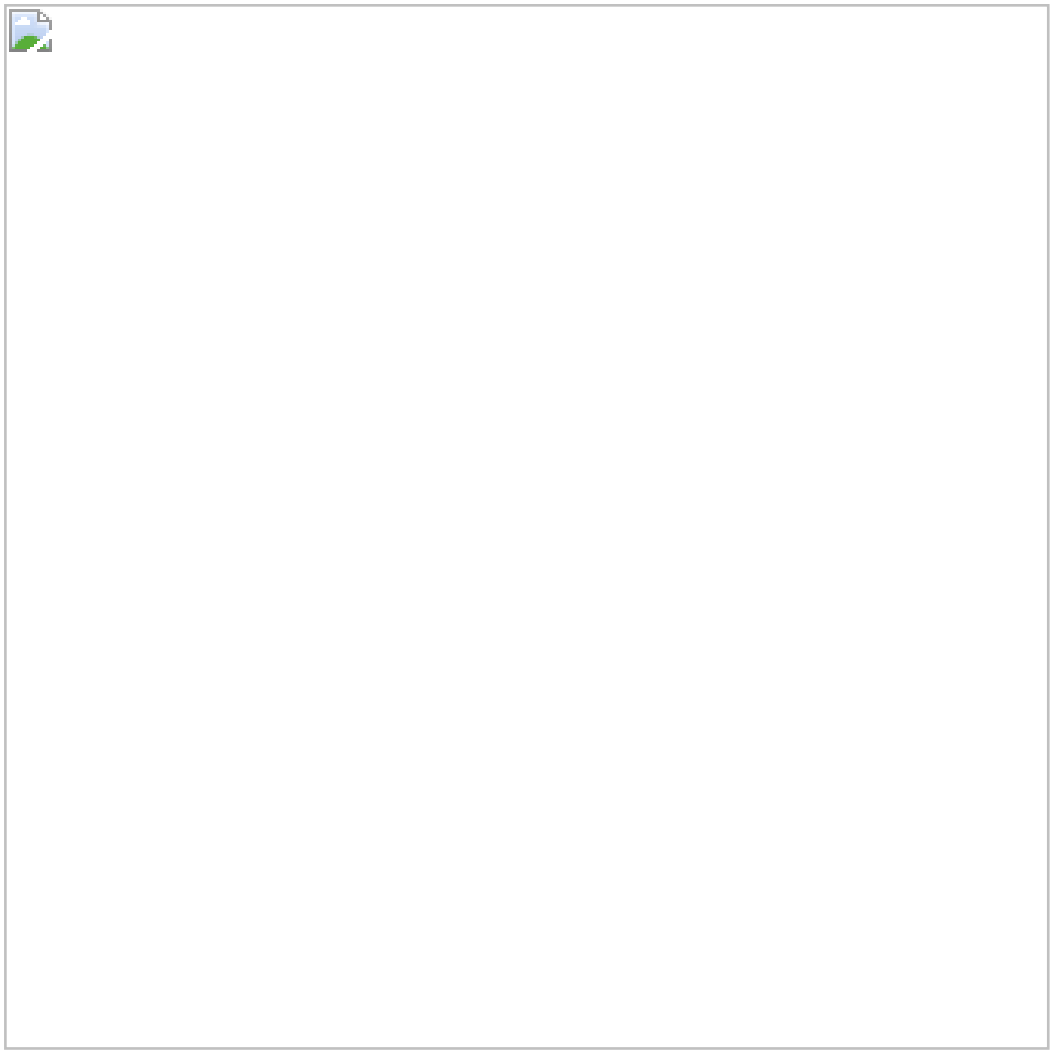

When the Whole is Not Greater than the Sum of the Parts: A Critical Review of Laboratory Bioassay Effects Testing for Insecticidal Protein Interactions

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Review

Transgenic Plants and Insects

When the Whole is *Not* Greater than the Sum of the Parts: A Critical Review of Laboratory Bioassay Effects Testing for Insecticidal Protein Interactions

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Abstract

Many studies have been conducted to investigate synergism among insecticidal proteins; however, a consensus on minimal data requirements and interpretation is lacking. While some have concluded that all additive predictive-type models should be abandoned, we advocate that additivity models can remain useful as assessment tools and that an appropriately designed interaction study will never systematically underestimate the existence of synergism, irrespective of which additivity model (or none at all) may be used. To generate the most meaningful synergy assessment datasets in support of safety assessments, we highlight two beneficial steps to follow: (i) select a testing model which is the most consistent with current knowledge regarding the action of the insecticidal proteins and (ii) avoid using bioassay methods which may result in excess response heterogeneity. We also outline other experimental design elements to consider, which may be crucial for conducting future studies of this type. A contrast of underlying testing assumptions associated with the additivity models is provided, along with a comprehensive review of interaction data for Cry1, Cry2, Cry3, Cry9, and Vip3A insecticidal proteins. Our review captures four recurrent findings: i) experiments reporting synergistic interactions are a minority, ii) the degree of synergism reported is low in magnitude, iii) reported interactions are sometimes equivocal/inconclusive due to unconfirmed model assumptions or other bioassay challenges, and iv) due to biological response variation many of the reported interactions may be artefactual. A brief overview of the positioning of interaction testing data in safety assessments of GM food crops is also provided.

Key words: *Bacillus thuringiensis*, synergism, interaction, Cry, Vip

An increase in transgenic crop insecticidal protein combinations has given rise to an assessment of the potential antagonistic, synergistic, or potentiating toxicological interactions of the multiple proteins being included in a dossier in support of product registration (US EPA 2009a, Raybould et al. 2012a). Synergism or potentiation might be of benefit from a product standpoint, as active principles (e.g., insecticidal trait proteins, active ingredients) might then achieve higher levels of control. With regard to risk assessment, such an interaction might not introduce a new hazard concern per se (in many cases, individual agents will have already undergone a risk assessment), but would indicate that the hazard of the combination of agents to non-target organisms may be greater than the sum of the hazards of the individual agents. Antagonism, on the other hand, might mean that the potency of a combination of agents against target pests is less than that of the sum of the components, which may have adverse consequences for product efficacy and insect resistance management.

With regard to synergistic interactions for any two agents (not specific to insecticidal proteins), such occurrences have been described as rare in low-dose mixtures (i.e., using No Observable Effect Concentration [NOEC] doses in mammalian testing) and seldom has more than a 10-fold effect been observed (Cedergreen 2014). Furthermore, an estimate of the likelihood of such occurrences may even be somewhat inflated due to reporting bias (Boobis et al. 2011, Cedergreen 2014).

Different experimental approaches have been put forth to consider potential insecticidal protein interactions (Tabashnik 1992, Greenplate et al. 2003, Raybould et al. 2012a, Tabashnik et al. 2013, Levine et al. 2016, Graser et al. 2017), but they commonly rely on one of three models to interpret the additivity of tested components (Table 1). Two of these somewhat related dose–response models, which have been utilized the most, have been termed 'concentration addition' (CA) or 'response addition' (RA), respectively. Implementation of the CA and RA models has been summarized and

Table 1. Three common models used for interaction testing and interpretation of additivity of insecticidal proteins

Model	Assumptions/requirements	Model use/interpretation		Experimental	
		Strengths	Challenges	Strengths	Challenges
CA[a]	Proteins have the same MoA Dose–response has same upper threshold and same slope A single relative potency factor applies for comparison of the dose-response of any two agents	Conservative from an ERA standpoint Can be designed to assess doses in the active range	MoA may not always be a priori clearly defined/established Well-established, full dose–response data of a similar shape are prerequisite	Any uniform ratio of the combined agents could be selected for a given dose–response dataset	Requires full set of dose–responses to generate comparison parameter (e.g., LC_{50})
RA[b]	Proteins have different MoA Dose–response has same upper threshold Doses selected must have a measurable effect when each agent is presented alone	The most conservative from an ERA standpoint By default, the design assesses doses in the active range	MoA may not always be a priori clearly defined/established Have to avoid testing at a sublethal dose	Minimal dose requirements (e.g., can work with as few as 1 or 2 selected doses)	Requires parallel testing of individual agents at same selected dose(s)
Empirical[c]	One (or more) of the agents to be tested has no toxicity to the test organism	No direct relation to MoA interpretations No need to calculate an expected response (e.g., default expectation for one agent is zero activity)	If the agents act against the same target pest, establishing a nominal sublethal dose to use can be difficult	Minimal dose requirements (e.g., can work with as few as 1 or 2 selected doses)	May require a larger dataset for robust statistical analysis

[a]Also known as dose addition, simple similar action, similar joint action, or Loewe additivity.
[b]Also known as independent joint action, independent action, Bliss independence, or effect additivity.
[c]Also known as simple empirical or simple statistical test.

contrasted in numerous reviews (e.g., Berenbaum 1989, Newman 2013, Cedergreen 2014); and they are derived from original works of Loewe and Muischnek (1926) or Bliss (1939) for the CA or RA model approaches, respectively. A variation on these models, which involves consideration of a more dynamic or multidimensional surface where the mode of action only appears to be similar or where the relative potency is not constant at all effect levels has been termed isobologram analysis (or a Combination Index) (Tallarida 2001, Foucquier and Guedj 2015). Although it provides an interesting approach, isobologram analysis quickly becomes extremely complex and has not yet been demonstrated to add much predictive function (Rodea-Palomares et al. 2015). It has not been utilized for insecticidal protein interaction testing and will not be considered further in this present review. The third most common model for insecticidal protein interaction testing is simply termed 'Empirical' as it is based on an empirical test of the response data following an interaction study using a combination of agents.

The CA model (alternatively named dose addition, simple similar action, similar joint action, or Loewe additivity) is based on the assumption that the toxicants have the same mode of action (MoA) at the target site and can be described as dilutions of each other, although perhaps with differing potencies (Table 1). The RA model (alternatively named Independent joint action, independent action, Bliss independence, or effect additivity) assumes the existence of a different molecular target for the toxicants, and that the two components produce independent effects (from a probabilistic standpoint), leading to the actual mortalities (not the doses) being described as additive. Both additivity models have been routinely used to determine if an effect exceeds the expected amount for a combination of agents. Exactly what constitutes an 'expected response,' though, has been and continues to be the subject of much debate (Berenbaum 1989, Berthoud 2013, Geary 2013, Cedergreen 2014, Sucher 2014, Foucquier and Guedj 2015). The Empirical testing model involves no real prerequisite assumptions, but for practical usage is limited to situations where one or more of the tested components are inactive (Table 1).

Some experimental similarities do exist between the RA and CA models. One situation is over a linear range of similar dose–response curves, where the RA model can routinely be considered equivalent to the CA model (Gennings et al. 2005, Price 2010); however, this is not the case in the subthreshold regions of the dose–response curves, where the RA model technically considers these doses as not contributing to an effect. For either model, a steeper slope can be interpreted as reflecting a more homogeneous population for the measured response. Also applicable to either model, there can be a considerable challenge in reproducibly obtaining estimates of a single parameter (e.g., an LC_{50} obtained by probit analysis) to accurately describe the dose–responses. Due to inherent variation, several authors have recommended use of a built-in threshold of at least twofold above the predicted response for even considering a result as being more than additive (Belden et al. 2007, Cedergreen 2014, Rodea-Palomares et al. 2015).

Related to the reproducibility challenge noted earlier, probit analysis (which is routinely used in insecticidal protein bioassay), is itself an estimation technique and admittedly a simplification of reality. In many ways, however, this analysis is well-suited to support the interaction assessment needs for insecticidal proteins. The assumptions for probit analysis are reasonable when used for binary type responses (e.g., living or dead, or meeting a defined response metric), which possess a sigmoidal response curve with probability constrained to be in the interval of 0–100% response (Gennings et al. 2005); furthermore, the log-normal distribution (by dose) assumption is capable of confirmation by testing (e.g., passes a goodness-of-fit or heterogeneity test for the distribution).

If these confirmations are intact, it is also reasonable to use a 'fractional product method' additivity model (such as the RA model) to quantify and compare the median effect responses for a combination of mutually non-exclusive agents, as the method approximates what would be seen for agents obeying the law of mass action (Berenbaum 1985). This method can also be reasonably extended to describe the probabilistic effects from insecticidal proteins (which are actually the manifestation of a series of different steps in the MoA, leading up to a single metric of the binary response) even though these are likely not truly derived from a single or predominant mass action relation. It has been observed that these more complex series of responses as measured for insecticidal proteins may be better described as reflecting the distribution of sensitivities (Berenbaum 1989) to the protein agents. In contrast, responses which are non-binary (e.g., gradual/quantitative changes in growth, fecundity, behavior, degree of binding), may have differing maximal effects and, therefore, not be as suitable for probit analysis and would present an extra challenge to adoption of either a CA or RA additivity approach (Putzrath et al. 1997, Geary 2013).

The CA Model Requirement for a Parallel Slope if a Single Relative Potency Factor is Assumed

If one agent can truly perform as a mere dilution of a similarly acting agent, then under the CA model, the shape (and slope) of the dose–response curve must be similar (statistically the same or at least parallel) (Berenbaum 1989, Villeneuve et al. 2000, Tallarida 2001, Cedergreen et al. 2008, Gennings 2010). Furthermore, the shape of this curve would not be expected to change in the presence of the other agent (Gennings et al. 2005). For these combinatorial analyses, it is important to compare doses over similar regions of the dose–response relationship that were used to establish the initial slope estimates. This requirement highlights an advantage of using binary response data (which are constrained between 0 and 100% effects), where this condition would normally be met. Having a similar shape of the dose–response is in accord with a classic understanding of the CA model, whereby such agents are mutually exclusive and share the same binding site(s) or molecular target. If the dose–responses of any two agents being compared are not parallel, then a single number does not accurately describe their relative potency. It is readily apparent that when similar slopes are not observed, the deviations can cause large errors in predictions of the relative potency of any two (or more) agents (Villeneuve et al. 2000, Ritz et al. 2006, Wagner et al. 2013).

Regardless of whether the CA or RA additivity model is used for insecticidal protein interaction testing, determination of an expected response must match the underlying assumptions for the model on which it is based. Unfortunately, this point has often been overlooked and our review identified this as a concern for the interpretation of these datasets in the context of environmental risk assessments. In addition, bioassay response variability and other challenges associated with designing and conducting insect bioassays may have led to incorrect conclusions of synergistic interactions among insecticidal proteins. Even for experiments where no additivity model is being used (e.g., testing for potentiation or simple interaction where one component in a mixture has no activity) (Tabashnik 1992, Belden et al. 2007, Raybould et al. 2012a, Graser et al. 2017), the experimental design and methodology play an important part in directing subsequent conclusions and interpretations. For example, by choosing an experimental approach which seeks to reduce design elements that could introduce response variability, one allows more room for the inherent insect response heterogeneity. Such inherent variability always remains a challenge due to working with living organisms (Robertson et al. 1995, Marçon et al. 1999, Da Silva et al. 2016). Overall, the most common concern we highlighted in our review of publications was the use of the CA model in calculations from a single defined effect level without prior confirmation that the combined insecticidal proteins could be considered as mutually exclusive substituents for one another (by virtue of a minimal requirement that the slope for the dose–response is parallel).

Materials and Methods

Experiment Selection Criteria

Prior review papers were examined and database searches were employed to compile the present summary of studies which investigated potential *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) (*Bt*) insecticidal protein interactions. Searches were performed in the following databases: Agricola (National Agricultural Library of the U.S. Department of Agriculture, USA; worldwide coverage of agriculture and related fields since 1970); BIOSIS (Clarivate Analytics (United Kingdom) Limited, covering selected US patent and the non-patent literature from the bioscience area since 1926); CABA (CAB International (United Kingdom), covering the worldwide literature from all areas of agriculture and related Sciences since 1973); HCAPLUS (Chemical Abstracts Service (USA), bibliographic/chemical structure and dictionary databases covering the patent and non-patent literature since 1907); Medline (U.S. National Library of Medicine, covering the worldwide biomedical literature since 1946). Various combinations of truncated keywords and classification codes were used in comprehensive search strategies for retrieval from the aforementioned databases:

The word stems cry, vip, or cyt were searched in combinations of two allowing at least five words between the stems, that is, cry within five words of vip, cry within five words or cyt, vip within five words of cyt. In an alternative approach, the word stems were searched separately.

Both answer sets were further specified for different combinations of concepts applying keywords and synonyms in truncated form: synergy/interaction, in vitro/bioassay, mixture/combination.

Experiments Summarized Included Only Those Which:

- were from peer-reviewed articles and published in English;
- made comparisons using either purified proteins (e.g., studies that used cell lysates/sporulated cultures are not included) or used transgenic event material as a source for the insecticidal protein comparisons, but also included information on the protein expression or quantification;
- used native or engineered versions of Cry1, Cry2, Cry3, Cry9, or Vip3A insecticidal proteins.

In general, for studies where more than one insecticidal protein combination was investigated, all the insecticidal proteins used for a respective species are listed together to conserve table space. Individual experimental combinations within the same reference were only captured on a separate line entry if the level of synergy reported or particular comments warranted (e.g., for future discussion). All studies used a mortality parameter unless otherwise noted.

Additionally, experiments were excluded from our review summary if:

- interactions of binary toxins (with or without other insecticidal proteins) were examined;
- the proteins examined were primarily mosquitocidal toxins (e.g., *Bt* cytolysins, Bin toxins, Mtx toxins, Cry4, Cry10, Cry11);
- resistant strain insects were used as a test organism;
- some other non-insect invertebrate was used as a test organism;

- the study design did not permit the possible detection of synergistic interaction (e.g., mortality was very high for the concentration of individual proteins used, therefore, unreliable for ruling out synergy)

Results

We identified a total of 179 interaction experiment results which met the acceptance criteria to be included in our summary. A large majority of tests (66% of 179) concluded an additive or less than additive result (Table 2, Fig. 1) for the insecticidal protein combinations. Following the guidance of requiring a more than twofold result as a threshold prior to a conclusion of synergistic interaction (Belden et al. 2007, Cedergreen 2014, Rodea-Palomares et al. 2015), 84% of the test results (151 out of 179) can be described as not more than additive. Of the 61 tests where authors originally concluded a greater than additive result (Table 3), 46% were estimated at more than twofold; 34% at more than threefold; and only two tests (3% of 61) were originally described as having an interaction at >10-fold over additivity (Table 3, Fig. 1). Importantly, a number of the studies we examined, including the latter two reports of an interaction at >10-fold over additivity, possessed deviations from the model assumptions or other methodological concerns which render the original interpretations of a synergistic interaction as problematic.

Discussion

What is the Likely True Incidence of Synergism Among Non-Binary Cry and Vip Insecticidal Proteins in Currently Registered Transgenic Crops?

In general, the literature strongly supports that synergistic interactions are rarely found between Cry1, Cry2, Cry3, Cry9, and Vip3A insecticidal proteins, and specifically, that resultant supra-additive interactions are unlikely to occur for cross-class protein mixtures where the activity spectra do not overlap (Raybould et al. 2012a, de Schrijver et al. 2015, Graser et al. 2017). A 2009 U.S. EPA Scientific Advisory Panel observed that 'with respect to Cry1 and Cry3 proteins used in *Bt* crops, given their proven safety record, unless a greater than 10-fold degree of synergism is observed, there would seem to be no need to test for human health or non-target effects' (U.S. EPA 2009b). A review of the data continues to suggest that the hypothesis of no interaction has been tested and corroborated sufficiently with respect to Cry1 and Cry3 proteins for the purposes of risk assessment.

A close inspection of the literature which does assert that synergistic interactions exist (for Cry1, Cry2, Cry3, Cry9, and Vip3A insecticidal proteins) led to three recurrent findings, regardless of the insect species tested: i) the degree of synergistic interaction reported is low in magnitude (97% of tests reported less than a 10-fold increase in activity, ~66% reported less than a threefold increase); ii) the reported interactions are sometimes equivocal/inconclusive due to underlying model assumptions not being confirmed or other bioassay limitations/challenges (e.g., protein not purified or status undetermined, potential for saprophagy, non-binary response metric used) (Notably, this is true for the only four reported examples that asserted a fivefold or more degree of synergy.); iii) given the demonstrated variation in biological response across individuals within a given study (e.g., the majority of probit estimates had well over a 1.5-fold 95% CI spread), many of the reported interactions may be artifactual (54% of tests reported less than a twofold increase over the predicted value).

What is a Suitable Model for Additivity Testing of Insecticidal Proteins?

Both the CA and the RA additivity models have been applied across different fields of biology and likewise, within entomology, both have been used to examine the potential for interaction of insecticidal proteins. Our review shows, however, that for testing of insecticidal protein interactions many labs have adopted the CA model by virtue of following a methodology outlined by a single publication where the expected LD_{50} response is calculated as the harmonic mean of the component LD_{50}s (Tabashnik 1992). While this work gave sound and much-needed guidance, the calculations therein were built on a stated premise that the CA model was 'most appropriate for testing synergism of chemically similar poisons such as *B. thuringiensis* toxins.' An understanding of insecticidal protein MoA is far from complete, but it has progressed substantially since 1992, and although similar in the sense of being membrane-active, clearly a number of insecticidal proteins have very different molecular targets and steps in their MoA. This is particularly relevant for certain insecticidal proteins (e.g., Vip3A, Lee et al. 2003) that were not even identified at the time of the Tabashnik (1992) reference; in addition, a number of other Cry proteins had been identified but not yet cloned and purified for in vitro studies at that early date. In other fields of biology, restricting the use of the CA model to agents which have been demonstrated to be mutually exclusive in their action is a bit more consistent, but not perfect (Wagner et al. 2013, Geary 2013); likely the complexity of *Bt* insecticidal protein MoA has hindered applying a similar restrictive principle for use of the CA model within entomology.

As described by Groten et al. (2001), an empirical approach to assessing compound interactions would be outcome-based, where only information regarding the doses/concentration and corresponding effects are available. In contrast, a mechanistic approach can take into account more information about known reaction steps which have established quantitative relationships (e.g., following Michaelis–Menten kinetics) (Groten et al. 2001). We advocate the use of a hybrid approach between a strictly empirical versus strictly mechanistic view, in that if the insecticidal proteins have demonstrated non-competitive binding for the receptor(s) (equivalent to molecular target sites), this is interpreted in line with the proteins having discrete modes of action. This judgment about the existence of a discrete MoA (or not) is then taken into consideration for model selection and guides how the potential interactions will be examined in the ensuing experiments.

It is interesting that both the CA and RA models can often yield similar results (Liao et al. 2002, Gennings et al. 2005, Price 2010), and furthermore, a comparison of the two models concluded that neither one can be selected/preferred just based on inherent accuracy (Cedergreen et al. 2008). It should be acknowledged that in actual practice, both additivity models are imperfect and that there are identifiable flaws in each model. For example, with the CA model, differential metabolic activity affecting two agents (otherwise identified as having the same MoA) and/or differential access to a presumed identical binding site could affect the actual shape/sigmoidicity of the dose–response curve. These steps can be difficult to parse out, resulting in uncertainty regarding the validity of underlying identical binding and mechanism of toxicity assumptions (Berenbaum 1989, Ritz et al. 2006). Accurately estimating the dose–response curve and demonstrating that any two curves should be considered equivalent is, therefore, a practical limitation for use of the CA model (Ritz et al. 2006, Foucquier and Guedj 2015), and can warrant a large prerequisite dataset. On the other hand, conceptually for the RA model, one wonders whether two toxicological agents can really be

Table 2. Published examples reporting an additive or less than additive result for insecticidal protein combinations

Number of examples	Insecticidal proteins	Insect	Additivity model or empirical test	Assessment method used	Model assumption concerns	Other comments	Reference
5	Vip3Aa, Vip3Ae, Vip3Af, Cry1Aa, Cry1Ac, Cry1Ca	Tobacco budworm (*Heliothis virescens*)	CA	Tabashnik eq. 5[a]	CA model not justified by dose–response slopes (used slopes which varied by ~2–4-fold)	upper end of dose–response not well-represented; precision very poor for LC90 values	Lemes et al. (2014)
1	Vip3Aa, Cry1Ca	Fall armyworm (*Spodoptera frugiperda*)	CA	Tabashnik eq. 5	—[b]	—	Lemes et al. (2014)
2	Cry1Ac, Cry1Fa	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	CA model not justified by dose–response slopes (dose–response could not be determined for 1 of the 2 proteins)	—	Ibargutxi et al. (2008)
1	Cry1Ac, Cry1Fa	*Earias insulana*	CA	Tabashnik eq. 5	—	—	Ibargutxi et al. (2008)
3	Cry1Ac, Cry2Ab	*Earias insulana*	CA	Tabashnik eq. 5	CA model not justified by dose–response slopes (used slopes which varied by 1.8-fold)	—	Ibargutxi et al. (2008)
1	Cry1Ac, Cry2Ab	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	Measured growth inhibition	Ibargutxi et al. (2008)
1	Cry1Ac, Cry2Ab	*Earias insulana*	CA	Tabashnik eq. 5	—	Measured growth inhibition; high heterogeneity across protein responses	Ibargutxi et al. (2008)
2	Cry1Ac, Cry1Fa	*Earias insulana*	CA	Tabashnik eq. 5	—	Measured growth inhibition	Ibargutxi et al. (2008)
1	Cry1Aa, Cry1Ab	Gypsy moth (*L. dispar*)	CA	Tabashnik eq. 5	—	Force-feeding bioassay; measured weight in 4th instar larvae	Lee et al. (1996)
6	Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca	Pink stem borer (*Sesamia inferens*)	CA	Tabashnik eq. 5	—	multiple insects (10) per test arena; purity of proteins not shown; some details of diet incorporation not shown/referenced	Gao et al. (2010)
3	Cry1Aa, Cry1Ab, Cry1Ac	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	used droplet-feeding method	Li and Bouwer (2014)
4	Cry1Aa, Cry1Ab, Cry2Aa, Cry9Aa	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	—	Li and Bouwer (2014)
5	Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca, Cry1Da	Bean shoot borer (*Epinotia aporema*)	CA	Tabashnik eq. 5	—	—	Sauka et al. (2007)
9	Cry1AbMod, Cry1Ac, Cry2Ab	Pink bollworm (*Pectinophora gossypiella*)	RA	Colby method[c] with Fisher's exact test	—	9 out of 11 combinations statistically insignificant.; 7 out of 11 qualitatively greater, but only 3 at plus 13% or greater	Tabashnik et al. (2013)
34	Cry1Ac, Cry2Ab	Cotton bollworm (*Helicoverpa armigera*) susceptible strain	RA	Colby method with *t*-test	—	only 2 out of 36 combinations statistically significant.; 7 out of 36 qualitatively greater	Wei et al. (2015)
1	Cry1Ab, Cry9A	Diamondback moth (*Plutella xylostella*)	RA	Colby method	—	Only 1 combination used; purity of substances not shown; used leaf-dip method	Mittal et al. (2007)

Table 2. *Continued*

Number of examples	Insecticidal proteins	Insect	Additivity model or empirical test	Assessment method used	Model assumption concerns	Other comments	Reference
3	Cry1Ac, Cry2Aa	Cotton bollworm (*Helicoverpa armigera*)	CA, RA	Tabashnik eq. 5 or Colby method	—	—	Liao et al. (2002)
1	Cry1Ab, Cry2Aa	Mediterranean flour moth (*Ephestia kuehniella*)	CA	Tabashnik eq. 5	—	purity information not given; lyophilized proteins adsorbed to crushed peanuts; multiple insects (10) per test arena	Azizoglu et al. (2016)
1	Cry1Ab, Cry2Aa	Indian meal moth (*Plodia interpunctella*)	CA	Tabashnik eq. 5	—	—	Azizoglu et al. (2016)
4	Cry1Aa, Cry1Ab, Cry9Ca	Spruce budworm *(Choristoneura fumiferana)*	CA	Tabashnik eq. 5	Slopes of dose-responses not described	used droplet-feeding method vs. 6th instars and frass-failure response	Pang et al. (2002)
1	Cry1Ab, mCry3A	European corn borer (*Ostrinia nubilalis*)	Empirical	Low dose/high dose combinations with ANOVA	—	—	Raybould (et al. 2012a)
1	Cry1Ab, mCry3A	Colorado potato beetle (*Leptinotarsa decemlineata*)	Empirical	Low dose/high dose combinations with ANOVA	—	—	Raybould et al. (2012a)
1	Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C, Cry1D, Cry1F, Cry1I, Cry2Ab, Cry9B, Cry9E	Beet armyworm (*Spodoptera exigua*)	CA	Tabashnik eq. 5	—	Used mixtures of washed crystals from 2 different *Bt* isolates; expression levels of isolate genes not determined	Konecka et al. (2012)
1	Cry1Ab, Cry1F, Vip3A	European corn borer (*Ostrinia nubilalis*)	RA	Colby method	—	—	Graser et al. (2017)
1	Cry1Ab, Cry1F, Vip3A	Fall armyworm (*Spodoptera frugiperda*)	RA	Colby method	—	—	Graser et al. (2017)
1	mCry3A, eCry3.1Ab	Colorado potato beetle (*Leptinotarsa decemlineata*)	RA	Colby method	—	—	Graser et al. (2017)
1	Cry1Ab, Cry1F, Vip3A, mCry3A, eCry3.1Ab	Fall armyworm (*Spodoptera frugiperda*)	Empirical	Low dose/high dose combinations with ANOVA	—	—	Graser et al. (2017)
1	Cry1Ab, Cry1F, Vip3A, mCry3A, eCry3.1Ab	Colorado potato beetle (*Leptinotarsa decemlineata*)	Empirical	Low dose/high dose combinations with ANOVA	—	—	Graser et al. (2017)
1	Cry1Ab, Cry2Ab	Corn earworm (*Helicoverpa zea*)	RA	Colby-like plus Chi-square test	—	used event material plus ELISA; multiple insects (4) per test arena	Greenplate et al. (2003)
1	Cry1Ab, Cry2Ab	Fall armyworm (*Spodoptera frugiperda*)	RA	Colby-like plus Chi-square test	—	used event material plus ELISA; multiple insects (4) per test arena	Greenplate et al. (2003)
1	Cry1Ac, Cry1F	Beet armyworm (*Spodoptera exigua*)	Empirical	ANOVA comparisons	No apparent interactions, but experimental design cannot support the conclusions	used event material but no ELISA	Adamczyk and Gore (2004)

Table 2. *Continued*

Number of examples	Insecticidal proteins	Insect	Additivity model or empirical test	Assessment method used	Model assumption concerns	Other comments	Reference
1	Cry1Ac, Cry1F	Fall armyworm (*Spodoptera frugiperda*)	Empirical	ANOVA comparisons	No apparent interactions, but experimental design cannot support the conclusions	used event material but no ELISA	Adamczyk and Gore (2004)
1	Cry1Ac, Cry1B	Coffee leaf miner (*Perileucoptera coffeella*)	CA	Tabashnik eq. 5	—	used infused leaf material	Guerreiro Filho et al. (1998)
1	Cry1Ac, Cry2Ab2	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	Pooled data from populations which varied by 37-fold; slopes varied over six-fold; large ranges for 95% CI	used formulated MVP and lyophilized maize leaf as source material	Brévault et al. (2009)
2	Cry1Ac, Cry2Ab2, Vip3Aa19	Cotton bollworm (*Helicoverpa zea*)	CA	Tabashnik eq. 5	—	Measured growth inhibition; used event material plus ELISA; used a shared(?) slope parameter	Levine et al. (2016)
1	Cry2A, Cry9C	Cotton leafworm (*Spodoptera litura*)	Empirical	ANOVA comparisons	—	used event material plus ELISA; low expression of Cry2A; multiple insects (10) per test arena	Li et al. (2014)
1	Cry1Ab, Vip3Aa19	Tobacco budworm (*Heliothis virescens*)	Empirical	ANOVA plus LSMEANS	—	used event material but no ELISA; multiple insects (3) per test arena	Adamczyk and Mahaffey (2008)
1	Cry1Ab, Vip3Aa19	Corn earworm (*Helicoverpa zea*)	Empirical	ANOVA plus LSMEANS	—	used event material but no ELISA; multiple insects (3) per test arena	Adamczyk and Mahaffey (2008)
2	Cry1Ba, Cry1Ca, Cry1Da	Fall armyworm (*Spodoptera frugiperda*)	Empirical	GLM and Kruskal-Wallis test	—	—	Costa et al. (2014)
3	Cry1Aa, Cry1Ac, Cry1Ca, Vip3Aa	Oriental fruit moth (*Grapholita molesta*)	RA	Colby-like plus Chi-square test and Fischer's test	—	—	Scaramal Ricietto et al. (2016)
6	Cry1Aa, Cry1Ca, Vip3Aa	Oriental fruit moth (*Grapholita molesta*)	CA	Tabashnik eq. 5	CA model not justified by dose-response slopes (used slopes which varied by 1.9–2.3-fold); Broad LC50 estimate for 2 components (9–11-fold); 2 components with base of 32-fold differing potency	—	Scaramal Ricietto et al. (2016)

[a]Equation 5 as described in Tabashnik (1992). $LC_{50}(m)=\dfrac{1}{\dfrac{r_a}{LC_{50(a)}}+\dfrac{r_b}{LC_{50(b)}}}$ with $LC_{50(m)}$ being the expected LC_{50} of the mixture, where $LC_{50(a)}$ and $LC_{50(b)}$ are the expected median lethal concentrations for the individual components, *a* and *b*, respectively. The relative proportions of *a* and *b* components are described as r_a and r_b, respectively.

[b]— = none noted.

[c]As described in Colby (1967). If Component A alone gives *x*% effect and Component B alone gives *y*% effect, then under the assumption of independent action, the predicted percent response to A + B is: $x + y - (xy/100)$.

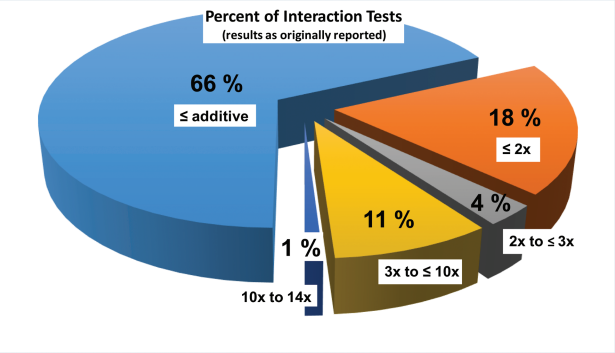

Fig. 1. Reported interaction testing results (original description of ≤ additivity, or degree of supra-additivity) for Cry1, Cry2, Cry3, Cry9, and Vip3A insecticidal proteins.

considered as having no bearing on the action of each other, given the complexities of in vivo physiology which exist. To put it another way, after an initial agent has already acted on a membrane, cell, or tissue, can that site really be considered as unaltered for a second agent that approaches and acts 'independently' (Cedergreen et al. 2008)? Perhaps related to this, when evaluating interactions for *Bt* insecticidal proteins that are being combined for resistance management purposes against the same target pest, an effect that is more than additive might be best interpreted as an additional interaction or downstream effect at the susceptible pest membrane or tissue level rather than a protein–protein interaction at a common receptor (agonist-like). Even with these conceptual challenges, the RA model is capable of providing convincing evidence for more than additive effects, resulting from a combination of agents (Foucquier and Guedj 2015). Also, the existence of 'imperfections' for these models due to underlying biological complexities is not unique to applications for insecticidal protein interaction testing (Berthoud 2013).

Some have concluded that all additive predictive-type models should be abandoned when evaluating combinations of agents, in deference to simpler, direct comparisons of statistically significant increases over the effect of either agent. It is noted that this might help to focus attention on the largest interactions (which would be most useful from a discovery or research standpoint), and has been advocated for registering drug combinations as well as for understanding endocrinological interactions (Berthoud 2013, Geary 2013). In the context of environmental risk assessment, such a shift could simplify the testing requirements but may not actually be helpful toward understanding any biological significance to the organism(s) in question. More importantly, demonstration of a statistically significant increase in an effect (for a combination of agents) would not help determine if an acceptable margin of exposure is in place, assuming an actual route of exposure and likelihood of exposure to the combination of proteins could be first estimated. The idea that 'all models are wrong, but some are useful' (attributed to Box 1979) definitely seems appropriate here, and we advocate that the additivity models remain useful as assessment tools when applied to robust datasets and when the underlying model assumptions are properly taken into account and used in the testing strategy.

Recommendations for Improving the Accuracy of Synergism Testing

Somewhat related to the concerns mentioned in the earlier section for the CA and RA additivity models, there has been criticism regarding inaccuracy of both models as they do not follow a 'sham combination' test. This is illustrated nicely in Foucquier and Guedj (2015), where it can be seen that both RA and CA models conclude synergism more often than a sham control experiment would support. But in actual usage (from the standpoint of risk assessment) this provides a 'built-in' conservative element, with the RA model being even a bit more conservative in this way. Also when working within the linear range of a dose–response (e.g., between 20 and 80% mortality for datasets with a full dose–response that achieve above 90% corrected mortality), a sham experiment would not generate a much higher than expected response, so this blanket criticism is overstated. The guidance to avoid use of the RA additivity model when working in the region of threshold doses (Berthoud 2013), however, remains warranted. Importantly, an appropriately designed interaction study bioassay will never systematically underestimate the existence of a synergistic interaction, irrespective of which additivity model (or none at all) may be used in the experimental design.

The CA model has a basic assumption that one insecticidal protein can substitute for another in terms of describing and quantitating a biological response (Berenbaum 1989, Villeneuve et al. 2000, Tallarida 2001, Cedergreen et al. 2008, Gennings 2010). The RA model has a basic assumption that the agents are tested at a dose which exhibits toxicity, and that the agents can be expected to work through different molecular targets. While the appropriate dose for testing can be reliably determined, establishing a sufficient understanding about the molecular targets can sometimes prove challenging (Vachon et al. 2012, Pardo-Lopez et al. 2013, Adang et al. 2014). If the CA model is used, however, when in fact an understanding of the MoA has progressed enough to demonstrate that the proteins differ in an aspect of the MoA which is known to be critical to toxicity (e.g., binding to different target receptors), there is a risk of weakening the experimental interpretations. If an additional fundamental mathematical relation assumption for the CA model is violated (failing to demonstrate that the dose–response slopes are equivalent), then the 'predicted' effects are clearly not accurate, rendering the downstream interpretations of interaction unsound and unreliable. Avoiding these prediction errors is directly relevant to determination of an expected response using the Tabashnik (1992) equation for the CA model (i.e., the harmonic mean calculation, which is based on a parallel slope assumption), even for a ray-type design (Gennings et al. 2005), where several of the dose combinations would not be near the median lethal point. This type of prediction error was the most common data concern we identified in our summary of interaction tests which concluded a synergistic interaction for insecticidal proteins.

Future studies which examine the potential for interaction among insecticidal proteins should benefit from the following considerations to allow for the most meaningful data interpretations:

- judge if discrete modes of action exist for the insecticidal proteins to be tested;
  - o consider what is known about the MoA and harmonize this understanding with how the combination may be implemented for resistance management purposes;
- use purified protein for the simplest and most robust study designs;
- avoid using multiple test organisms per well or container as the potential for saprophagy arises, which may allow some organisms to escape the treatment;
- avoid methods which may introduce heterogeneity across the testing surface or dosing step (e.g., leaf dip or force-feeding/droplet-feeding) when other alternative methods are available;

Table 3. Published examples reporting a greater than additive result for insecticidal protein combinations

Number of examples	Level of synergy reported (*x*-fold)	Insecticidal proteins	Insect	Additivity model	Assessment method used	Model assumption concerns (type)	Other comments	References
9	1.24 to 5.24	Cry1Aa, Cry1Ab, Cry1Ac	Maize stem borer (*Chilo partellus*)	CA	Tabashnik eq. 5	CA model not justified by dose–response slopes (used slopes which varied by 4–10-fold or more)	—	Sharma et al. (2010)
1	14.3	Vip3Aa, Cry1Ca	Sugarcane borer (*Diatraea saccharalis*)	CA	Tabashnik eq. 5	CA model not justified by dose-response slopes (used slopes which varied by 2.9-fold)	upper end of dose–response not well-represented; precision very poor for LC90 values	Lemes et al. (2014)
1	1.03	Cry1Ac, Cry1Fa	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	CA model not justified by dose–response slopes (dose–response could not be determined for 1 of the 2 proteins)	—	Ibargutxi et al. (2008)
2	1.06 to 1.10	Cry1Ac, Cry1Fa	*Earias insulana*	CA	Tabashnik eq. 5	—[a]	—	Ibargutxi et al. (2008)
3	1.58 to 3.12	Cry1Ac, Cry2Ab	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	—	Ibargutxi et al. (2008)
3	1.38 to 1.75	Cry1Ac, Cry1Fa	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	Measured growth inhibition	Ibargutxi et al. (2008)
1	1.11	Cry1Ac, Cry1Fa	*Earias insulana*	CA	Tabashnik eq. 5	—	Measured growth inhibition	Ibargutxi et al. (2008)
2	1.07 to 1.40	Cry1Ac, Cry2Ab	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	Measured growth inhibition; high heterogeneity across protein responses	Ibargutxi et al. (2008)
2	1.13 to 1.23	Cry1Ac, Cry2Ab	*Earias insulana*	CA	Tabashnik eq. 5	—	Measured growth inhibition; high heterogeneity across protein responses	Ibargutxi et al. (2008)
5	1.6 to 4.92	Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ca	Maize stem borer (*Chilo partellus*)	CA	Tabashnik eq. 5	—	multiple insects (10) per test arena; purity of proteins not shown; some details of diet incorporation not shown/ referenced	Gao et al. (2010)
1	1.6	Cry1Aa, Cry1Ab	Pink stem borer (*Sesamia inferens*)	CA	Tabashnik eq. 5	—	—	Gao et al. (2010)
1	11.0	Cry1Ab, Cry1Ba	Maize stem borer (*Chilo partellus*)	CA	Tabashnik eq. 5	CA model not justified by dose–response slopes (used slopes which varied by 2.4-fold)	—	Gao et al. (2010)
1	4.46	Cry1Ac, Cry1Ba	Maize stem borer (*Chilo partellus*)	CA	Tabashnik eq. 5	CA model not justified by dose–response slopes (used slopes which varied by 1.8-fold)	—	Gao et al. (2010)
1	1.60	Cry1Ac-Cry1Ca	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	used droplet-feeding method	Li and Bouwer (2014)

Table 3. *Continued*

Number of examples	Level of synergy reported (*x*-fold)	Insecticidal proteins	Insect	Additivity model	Assessment method used	Model assumption concerns (type)	Other comments	References
1	4.13	Cry1Ab-Cry1Ca	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	—	Li and Bouwer (2014)
1	1.72	Cry1Aa-Cry1Ca	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	—	Li and Bouwer (2014)
2	1.02 to 1.14	Cry1Ac-Cry2Aa or Cry9Aa	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	—	Li and Bouwer (2014)
1	1.37	Cry2Aa-Cry9Aa	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	—	Li and Bouwer (2014)
1	1.18	Cry1Ca-Cry2Aa	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	—	Li and Bouwer (2014)
1	2.29	Cry1Ca-Cry9Aa	Cotton bollworm (*Helicoverpa armigera*)	CA	Tabashnik eq. 5	—	—	Li and Bouwer (2014)
3	1.01 to 1.43	Cry1Ba, Cry1Ca, Cry1Da	Bean shoot borer (*Epinotia aporema*)	CA	Tabashnik eq. 5	CA model not justified by dose–response slopes (dose–response could not be determined for 1 of the 3 proteins which was used in 2 comparisons)	—	Sauka et al. (2007)
2	≤ 25% increased mortality	Cry1AbMod, Cry1Ac, Cry2Ab	Pink bollworm (*Pectinophora gossypiella*)	RA	Colby method	—	only 2 out of 11 combinations statistically significant.; 7 out of 11 qualitatively greater, but only 3 at plus 13% or greater	Tabashnik et al. (2013)
2	≤ 10% increased mortality	Cry1Ac, Cry2Ab	Cotton bollworm (*Helicoverpa armigera*)	RA	Colby method with *t*-test	—	only 2 out of 36 combinations statistically significant.; 7 out of 36 qualitatively greater	Wei et al. (2015)
1	21% increased mortality	Cry1Ab, Cry1C	Diamondback moth (*Plutella xylostella*)	RA	Colby method	—	Only 1 combination used; purity of substances not shown; used leaf-dip method	Mittal et al. 2007
1	1.5	Cry1Ab, Cry1Ac	Gypsy moth (*L. dispar*)	CA	Tabashnik eq. 5	—	Force-feeding bioassay; measured weight in 4th instar larvae	Lee et al. (1996)
5	2.7 to 4.9	Cry1Aa, Cry1Ac	Gypsy moth (*L. dispar*)	CA	Tabashnik eq. 5	—	Force-feeding bioassay; measured weight in 4th instar larvae	Lee et al. (1996)
1	7.3	Cry1Aa, Cry1Ac	Gypsy moth (*L. dispar*)	CA	Tabashnik eq. 5	—	Force-feeding bioassay; measured weight in 4th instar larvae; wide estimate for the ID_{50} (9.2-fold), but even at its max, it would still suggest a 4.6-fold effect	Lee et al. (1996)

Table 3. *Continued*

Number of examples	Level of synergy reported (*x*-fold)	Insecticidal proteins	Insect	Additivity model	Assessment method used	Model assumption concerns (type)	Other comments	References
1	1.46	Cry9Ca, Vip3Aa7	Diamondback moth (*Plutella xylostella*)	CA	Tabashnik eq. 5	CA model not justified by dose–response slopes (used slopes which varied by 2.1-fold)	multiple insects (10) per test arena; used leaf-dip/ application method	Dong et al. (2012)
5	2.2 to 5.3	Cry1Ac:Cry1Ie	Asian corn borer (*Ostrinia furnacalis*)	CA	Tabashnik eq. 5	Slopes of dose–responses not described	purity information not given; inconsistent trend as ratios change	Jiang et al. (2016)

[a]— = none noted.

- if using the CA model to apply the defined LD_{50} effect level in the Tabashnik (1992) harmonic mean equation, demonstrate that the parallel slope assumption is valid for the components of the testing mixture;
- generate dose–response curves or targeted level positive control responses for the individual components concurrently with that of the testing mixture;
- avoid using either additivity model in the range of sublethal doses (for a mortality metric) or threshold responses (for any biological response metric);
  - o if using a sublethal dose of any component (essentially testing for potentiation of an active component), include a statistical measure of the difference between the observed versus the expected response;
- avoid using either additivity model with two components of widely differing potency (somewhat related to the above point);
- work from datasets which pass heterogeneity tests for the underlying biological response metric;
- for probit analyses, work from datasets which demonstrate a full range of responses and with reduced variability

Implications of our Findings for Ecological Risk Assessment of GM Insecticidal Protein Stacks

An understanding of the potential for a synergistic interaction is helpful during risk assessment to guide inferences from existing ecotoxicological data (already generated for the trait proteins being assessed in the new combination). In actuality, if an interaction study result suggests that synergism exists, it is not necessarily indicative of a new hazard to NTOs (i.e., it is very unlikely that a new MoA will be created from the new combination), it simply indicates that the assumption of additivity of the risk (at constant exposure) may be unreliable. This would be especially relevant if working with crop events that produce substances with concentrations near the NOECs.

Overall, the findings of this present review support the previously stated conclusion that there is no a priori concern that a new highly active toxin could be created through the combination of these well-investigated insecticidal proteins (U.S. EPA 2005). This is reinforced by the risk assessment assumption that any interaction which may be observed in the sensitive test organism can be applied to the assessment of potential impact on NTOs which have already been demonstrated to be insensitive to the insecticidal proteins being used in the newly tested combination. That is, in most cases, an actual NOEC cannot even be established for the NTOs, or the concentration which exists in the crop is << the NOEC. If a requirement to assess the ecological risk of a protein combination has been pre-determined (e.g., in some cases it may be deemed unnecessary), then the interaction testing strategy fits into a tiered testing scheme for that subsequent risk assessment (Raybould et al. 2012b). It is important to note that an alternative strategy to inform the risk assessment might involve first tier ecotoxicological testing of the actual protein mixture (Raybould et al. 2012b). Following the interaction testing strategy, if data are available to corroborate the hypothesis that the toxicity of the insecticidal proteins in the transgenic crop is no greater when the traits are combined (than when the traits are separate) (Fig. 2), the risk can be assessed based on the ecotoxicological data available for the single proteins and it follows that previously established margins of exposure for the traits alone are applicable to the traits in the new combination (reviewed in Raybould et al. 2012a). Furthermore, a quantitative consideration of any potential impact of a given interaction must also be kept in the proper context of other risk assessment factors such as the potential for a

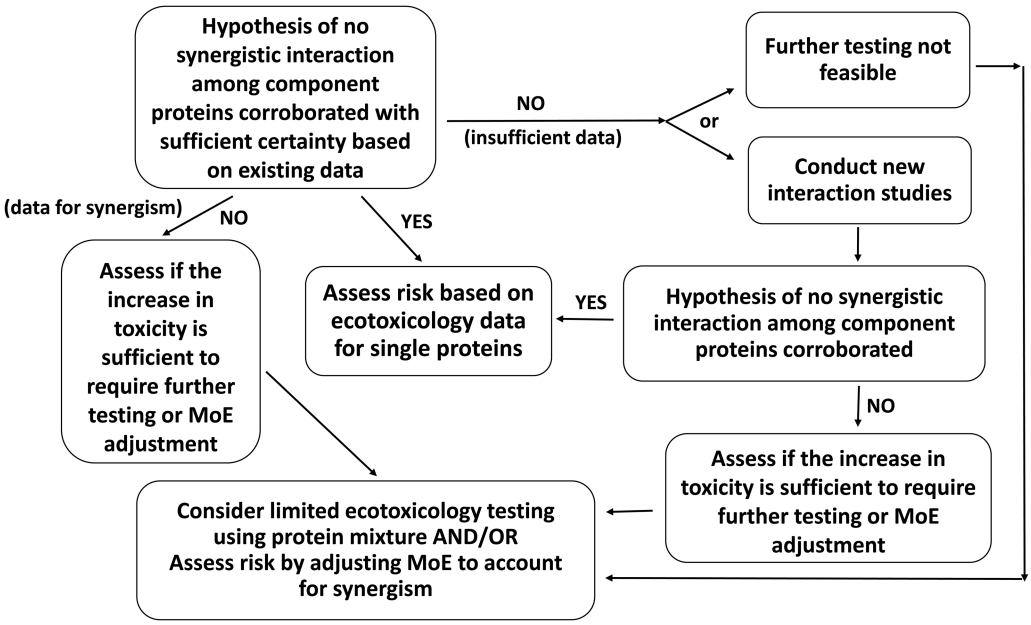

Fig. 2. Interaction testing outcomes in relation to risk assessment decisions.

route of exposure or the actual margins of exposure (Raybould et al. 2012a). And if the above hypothesis (that the toxicity of the insecticidal proteins in the transgenic crop is no greater when the traits are combined than when the traits are separate) is not corroborated, one must still assess whether the increase in toxicity is sufficient to require further information on non-target effects to adequately assess risk (Fig. 2). If more information is deemed as being required, then the assessment of the effects of the mixture of proteins directly on one or more non-target organisms could ensue, using taxonomic relatedness to the species in which synergism was detected as a useful NTO selection criterion (Raybould et al. 2012a).

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