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Commentary

Relevance of *Bt* toxin interaction studies for environmental risk assessment of genetically modified crops

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Summary

In recent years, different *Bacillus thuringiensis* (*Bt*) toxin-encoding genes have been combined or 'stacked' in genetically modified (GM) crops. Synergism between *Bt* proteins may occur and thereby increase the impact of the stacked GM event on nontarget invertebrates compared to plants expressing a single *Bt* gene. On the basis of bioassay data available for *Bt* toxins alone or in combination, we argue that the current knowledge of *Bt* protein interactions is of limited relevance in environmental risk assessment (ERA).

Introduction

In the first generation of commercialized GM crops, single genes encoding *Bt* toxins were incorporated to make them resistant to certain insect pests. These were genes encoding crystal (Cry) proteins or the vegetative insecticidal protein Vip3Aa. Other types of *Bt* proteins, such as the cytolytic (Cyt) proteins, have not been used in GM crops so far. Over the past decade, transgenes from different GM events have been combined through traditional breeding, resulting in commercially available plant lines expressing multiple insecticidal *Bt* protein genes. For example, *Bt* maize MON89034 x TC1507 x MON88017 x 59122 produces the Cry34Ab/Cry35Ab binary toxin and the Cry3Bb toxin to control *Diabrotica* spp., and Cry1A.105, Cry2Ab and Cry1Fa for the control of lepidopteran maize pests (http://www.isaaa.org/gmaprovaldatabase/default.asp).

Before cultivation of a GM crop is permitted, it needs to be demonstrated that its cultivation poses negligible ecological risk to invertebrates not targeted by the GM crop, hereafter referred to as nontarget invertebrates, and the ecosystem services (e.g. pest control, pollination, soil nutrient cycling) they provide. Bioassays with pest invertebrate species have demonstrated that antagonistic or synergistic interactions between two or more *Bt* toxins can occur (e.g. Lee *et al.*, 1996; Sharma *et al.*, 2010). In the case of synergism, the effect on nontarget invertebrates may be greater than that of the GM crop expressing just a single *Bt* gene. In many jurisdictions, it is therefore common practice in the ERA of GM crops expressing multiple *Bt* toxin genes to evaluate whether interactions between the *Bt* proteins occur and whether such interactions could impact nontarget invertebrates (EFSA, 2010; OGTR, 2003; Raybould *et al.*, 2010; US EPA, 2007, 2010, 2011). Data from *in vivo* bioassays testing for synergistic interactions of the combined *Bt* proteins on a sensitive species, often the target pest, are usually provided in regulatory dossiers preceding approval of commercial cultivation of stacked GM crops. If synergism were detected, this would trigger further assessment of the impact on nontarget invertebrates. In this context, the relevance of laboratory toxicity studies for assessing the impact of combined *Bt* toxins on nontarget invertebrates has been questioned. We examined currently available published evidence of *Bt* protein specificity and of interactions occurring among *Bt* proteins to explore to what extent existing knowledge of *Bt* protein interactions can aid in assessing impacts on nontarget invertebrates. Further, we investigated the utility of novel studies of *Bt* protein interactions in both target and nontarget invertebrates for ERA.

Specificity of *Bt* proteins

The past few decades have revealed an astounding diversity of *Bt* genes encoding proteins that together are toxic to a broad array of taxa (van Frankenhuyzen, 2013). Available data undoubtedly underestimate that diversity because characterization of biological activity lags far behind gene discovery. The specificity picture

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is fragmentary at best, considering that (i) more than half (171) of the 329 holotype proteins that had been documented by the end of 2013 have not been tested in any bioassay, (ii) the majority of the 158 tested proteins were used in bioassays with a limited number of species (10 or less), and (iii) species and toxins tested are not equally distributed across protein families and taxa.

Specificity of *Bt* strains was initially thought to be limited to specific insect orders, in particular Lepidoptera, Coleoptera and Diptera (and most of them still are), and often to a limited number of species within those orders. The first classification of crystal proteins (Höfte and Whiteley, 1989) reflected that order specificity and included only one rank family (Cry2) with dual specificity. Since then, testing has revealed that toxicities outside those orders of primary specificity (cross-order activity) are common (Figure 1), occurring in at least 21 proteins that are distributed over 16 primary rank families across all three protein classes (Cry, Cyt and Vip). Cross-toxicities that are supported by reasonable evidence are so far limited to the class of Insecta. Indeed, cross-activities need to be viewed with caution until they are confirmed through additional testing, because validation by independent studies is limited to coleopteran toxicity of Cry1Ba, Cry1Ia and Cyt1Ba and hemipteran toxicity of Cry3Aa and Cry11Aa.

Interactions among *Bt* proteins

Interactions between *Bt* proteins have been studied in the context of resistance management and broadening of the activity spectrum of *Bt*-based pesticides, and more recently also in the context of risk assessment of GM crops. Studies of *Bt* protein interactions, gathered from approximately 50 publications covering 24 proteins (Cry, Cyt and Vip) are, with a few exceptions, limited to target pest species. The majority of studies examined interactions between *Bt* proteins active against mosquitoes (Diptera), being important vectors of human diseases, and between *Bt* proteins active against Lepidoptera, which include major agricultural pests. A few interaction studies among *Bt* proteins with nematode activity have also been conducted.

In general, mosquitocidal *Bt* proteins that are co-expressed in *Bt* subsp. *israelensis* act synergistically, but there is insufficient evidence to state that overall synergism among *Bt* proteins is a common phenomenon in other *Bt* subspecies expressing multiple proteins. Studies with Lepidoptera-active proteins show that the nature of the interaction (synergistic, additive or antagonistic) depends on the insect species tested. For example, Cry1Ab-Cry1Ac acted antagonistically against *Lymantria dispar* (Lee *et al.*, 1996), additively against *Bombyx mori* (Lee *et al.*, 1996) and synergistically against *Chilo partellus* (Sharma *et al.*, 2010). The limited information at hand indicates that insect species-dependent interaction may also occur for Cyt and Vip proteins. However, more research is needed to substantiate this assumption.

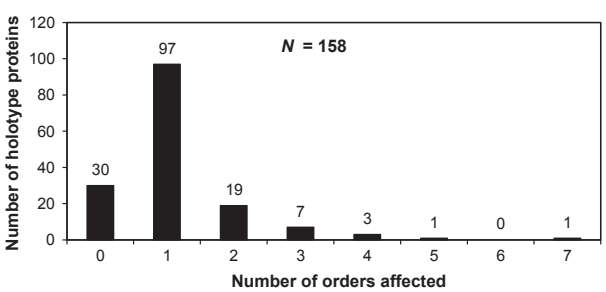

Figure 1 Distribution of the number of Bt pesticidal proteins (Cry, Cyt and Vip) as a function of the number of orders that is affected. The number of proteins reported (suspected) to be active across more than one order (31) includes three proteins with dual specificity and seven proteins for which cross-toxicity is unsubstantiated. The number of cases substantiated to date is thus 21.

Genetically modified crops combine not only *Bt* proteins that are active within one insect order, but also *Bt* proteins targeting species from different insect orders. Available studies with Cry proteins having different primary order activities show that synergism among these proteins is not likely to occur. For example, no change in activity towards lepidopteran or coleopteran target pest species was found when Cry34/35Ab was combined with Cry1Fa, Cry3Bb with Cry1A.105 and Cry2Ab, Cry3A with Cry1Ab, or Cry3Bb and Cry34/35Ab with Cry1A.105, Cry1F and Cry2Ab (Raybould *et al.*, 2010; US EPA, 2007, 2010, 2011). Additional evidence that these interactions are not likely to occur comes from specificity data of the individual Cry proteins. The Cry1 and Cry2 families act against Lepidoptera, while the Cry3 family affects Coleoptera. The reason for this distinction in activity most likely lies within the molecular structure of the *Bt* proteins and their mode of action: for example, the type of receptors they bind to or the fact that Lepidoptera- and Coleoptera-active *Bt* proteins need different pHs to be activated in the insect midgut.

Predictability of interactions between *Bt* proteins

Different types of information of a biochemical and toxicological nature can be used to predict interactions among *Bt* proteins. The biochemical mechanisms behind the interactions among *Bt* proteins are, however, still not clearly understood, and various hypotheses have been proposed (Sharma *et al.*, 2010). Hence, for the time being toxicological activity data are the only tools for making predictions on interactions.

Two tentative conclusions emerge from our literature review on interactions and specificities of *Bt* proteins. Firstly, if the activity spectra of the stacked *Bt* toxins do not overlap, interactions are not likely to occur. Confirmatory data are needed to substantiate whether this holds true for all types of *Bt* toxin combinations in GM crops, especially those including Vip proteins. Secondly, interactions among stacked *Bt* proteins that target pests within the same order cannot easily be predicted. Observations that interactions among *Bt* protein combinations with overlapping specificity can be synergistic to one insect species but neutral or antagonistic to another species of the same order complicates the predictability of interactive effects.

Relevance of interaction studies for ERA

In the ERA of GM crops containing several *Bt* proteins, the potential impact of the combined presence of *Bt* proteins on nontarget invertebrates is evaluated. A logic first step in the ERA would thus be to determine whether interactions, and in particular synergism, between the *Bt* proteins actually occur *in vivo*. While in some jurisdictions, such as the EU (EFSA, 2010), the procurement of *in vivo* laboratory studies with sensitive species, often the target pest, is a standard requirement to assess

whether synergism occurs, this is not the case in other jurisdictions, where such studies are requested on a case-by-case basis (e.g. Japan, http://www.bch.biodic.go.jp/).

The utility of *in vivo* laboratory studies assessing *Bt* protein interactions in target pest species for ERA, as a source of information to assess potential impacts on nontarget invertebrates, seems to be limited. Tests with *Bt* proteins that have the same order specificity may reveal synergistic interactions in a target pest, but will not indicate if this is also the case for nontarget invertebrates of the same order as the target pest. As interactions appear to be species dependent, such an extrapolation cannot be reliably made. Bioassays testing interactions between *Bt* proteins with different specificity can, however, add to the weight of evidence that interactions are not likely to occur in nontarget invertebrates from the same order as the target pest tested.

Direct testing of the potential impact of *Bt* protein interactions on nontarget invertebrates in the ERA of stacked GM events has been the subject of debate. We argue that bioassays with nontarget invertebrates should only be carried out with species that fall within the same order(s) that are affected by the *Bt* proteins present in the GM crop. There is no evidence to date that *Bt* protein interactions can extend activity to species outside the orders affected by the separate proteins. Depending on the protection goals specified in the problem formulation phase of the ERA, one may consider testing nontarget invertebrates with high value for the agro-ecosystem falling within the *Bt* protein order specificity. An example of such nontargets is coccinellid beetles, valued for their role as biological control agents, if the target pest is a coleopteran as well. However, *in vivo* testing will likely only be relevant when one of the *Bt* proteins present in the stacked GM event showed activity against the selected species. If the individual proteins are not toxic to the selected species, observing an unacceptable adverse effect will be unlikely, particularly when there are large margins of exposure, a factor reducing the probability of underestimating risk (see Raybould *et al.*, 2010), for the individual *Bt* proteins.

Concluding remarks

Due to a current knowledge gap, it is difficult to predict synergism between *Bt* proteins in a particular invertebrate species. Moreover, extrapolation of interactions observed in one species to another is not recommendable. In the ERA of a GM crop, the assessment of the combined *Bt* protein effects on nontarget invertebrate species therefore seems to be useless, unless one would test all relevant nontarget invertebrate species, which are part of the protection goals. We postulate that for the nontarget invertebrate species that fall outside the specificity of the *Bt* proteins present in the GM crop, the *in vivo* laboratory studies carried out with the single events can also apply for the ERA of the stacked GM event. For the nontarget invertebrates that fall within the specificity of the *Bt* proteins, testing valued species or applying a theoretical worst-case scenario test (i.e. synergism occurs) would be the preferable approaches to assess the impact of stacked *Bt* gene events on nontarget invertebrates, until our knowledge on *Bt* toxin interactions is more advanced.

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