Environmental Risk Assessment for Breeding Stack Products: Methods and Regulatory Implications

CropLife International Environmental Risk Assessment Project Team

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Position

CropLife International has concluded that where crops containing individual single events have been determined to be as safe as their conventional counterparts, it can generally be concluded, based on the knowledge and experience of conventional breeding, that the breeding stack of the single events is also as safe as the stacked product of non-biotechnology-derived traits in conventional varieties (CropLife International 2015). We further conclude that in most cases breeding stack products also do not create new environmental risks. Unless there is a clear indication of an interaction leading to increased hazard or exposure for the stacked product, the environmental risk of the stacked product can be considered to be equivalent to that of the parental events and, if those parental events have already been assessed, no further regulatory review should be needed.

Purpose

While in most cases risk assessment for breeding stack products is unnecessary, there may be some cases where a need for additional evaluation might be considered to assess the potential environmental effects. The purpose of this document is to describe the methods that can be used to conduct environmental risk assessments for stacked agricultural biotechnology products that, through conventional breeding, contain multiple transgenic events (stacked products) that have already undergone significant regulatory scrutiny and discuss the regulatory implications of assessing the risk of stacked products under different risk scenarios such as imported grain versus cultivation.

Rationale for the Position - Overview

The environmental risk of stacked agricultural biotechnology products can be evaluated using the standard risk assessment process: problem formulation and risk characterization. During problem formulation for the stacked products that combine single events through conventional breeding, data for the single parent events or if available the stacked product are evaluated. For the single parent events, these data may include an environmental risk assessment and regulatory review. Exposure scenarios for the stacked product are also evaluated as part of problem formulation. As occurs with all types of crop breeding, stacked products undergo extensive agronomic and quality testing prior to commercialization.

Risk characterization studies for stacked products, whether imported or cultivated, are only needed in cases where plausible hypotheses can be developed during problem formulation indicating that a

combination of traits would interact in some way that creates a hazard or exposure scenario that would result in a significantly increased probability of unacceptable adverse environmental effects as compared to the single event parent products. Unless there is a clear indication of an interaction leading to increased hazard or exposure for the stacked product, additional data or risk assessment characterization should not be required. In this case the environmental risk of the stacked product can be considered to be equivalent to that of the singles which have already been assessed and no further regulatory review would be needed. For the purposes of this paper, "interaction" is defined as a biologically significant non-additive change in the hazard or exposure profile of the stacked product compared to the parental events. Any such interaction would need further assessment to determine whether the result may cause unacceptable environmental harm.

Regulation of breeding stacks of previously assessed events can be accomplished for agencies or countries that currently regulate breeding stacks through notification to the regulatory agency that evaluates environmental risk of the intention to commercialize a specific stacked event product or set of products. The notification can include a description of the application of the principles described in this paper to the stacked event product(s), a summary of the risk assessments for the parental events, and any additional data on the stack(s) that are deemed to be relevant to the environmental risk assessment. For imported grain containing previously assessed single insect control or herbicide tolerance traits, additional data are not likely needed to assess environmental risk of stacks. For example, for imported grain a conclusion of negligible risk can be made based on (a) the environmental risk of the single parent events has been shown to be acceptable, (b) interactions that have resulted in adverse environmental effects have not been seen , (c) there is no scientific basis to support the notion that the use of conventional breeding to combine GM lines is any more risky than breeding of non-GM lines or combining GM and non-GM lines, and (d) there is a very low probability of exposure of non-target organisms to the grain which would result in significant adverse environmental effects. For cultivation, in most cases of breeding stacks of insect protection traits with herbicide tolerance traits, or breeding of two or more herbicide tolerance traits, it is difficult to envision a plausible scenario which would result in some unacceptable environmental harm and it is not anticipated that any new data would be warranted. For stacks of two or more insect protection traits where plausible hypotheses might be developed that indicate that a combination of the traits could result in unacceptable adverse environmental effects, data on interaction of the insecticidal ingredients may be warranted for commercial cultivation. As new events are developed that act in new ways, problem formulation might reveal plausible scenarios where the combination of traits could possibly cause some type of unacceptable environmental harm and additional risk characterization or mitigation might be required. In most cases, since no or limited new data are generally warranted, review of such notifications should be completed in a short defined period prior to commercial launch.

Scientific Rationale for the Position

Introduction

Stacked products combine multiple traits in a single plant line or variety. Breeding stack products can be composed of single events that have already undergone significant regulatory scrutiny to evaluate potential environmental risk. Breeding stacks are developed using conventional breeding where each parent plant containing the parental event or events contributes one or more traits to the next generation of plants. As an alternative, multiple traits can be added to plant lines or varieties by transformation using multi-gene constructs, retransformation (a plant containing a transgene is transformed with another transgene), or using co-transformation (simultaneous transformation with multiple independent transgenes) (ISAA 2014). These types of stacks are outside of the scope of this document since they constitute new GM products developed by means other than conventional breeding. Examples of commercial lines created using stacking include multiple traits that confer insect protection, herbicide tolerance, output traits, etc. Some of the advantages of stacking traits include increasing the spectrum of pest protection and providing multiple modes of action to support insect and/or weed resistance management plans.

Environmental risk assessment includes two basic phases: problem formulation and risk characterization. During problem formulation (a) protection goals are identified, (b) existing data, including information from the single event products, are gathered and reviewed for utility, (c) a conceptual model is developed to identify exposure pathways and organisms or biological systems where unacceptable adverse effects might occur, and (d) significant areas of uncertainty are identified and discussed. If at the end of the problem formulation phase it is determined that additional data are needed to make a regulatory decision, then hypotheses-driven research plans are developed to reduce uncertainties needed to assess potential environmental risks and help make regulatory decisions. This is the risk characterization phase. However, in many cases, data gathered and analyzed during the problem formulation phase are sufficient to support risk management decisions and additional data are not needed to characterize risk (Raybould 2006, Wolt et al. 2010).

In most cases for stacked products a considerable amount of data already exists for the traits contained within the single parental events (e.g. mode of action, binding specificity, spectrum of activity, etc.) and an environmental risk assessment has already been conducted for each of the parental events. The data from the single events can be used to investigate whether a plausible scenario(s) exists where the combination of traits created by stacking would cause an unacceptable environmental harm. A plausible scenario is one that is likely to occur or is feasible, while an implausible scenario would be one that would not appear to be reasonable based on all available information. If no plausible scenarios exist for the combination of traits which would result in unacceptable environmental harm, then the risk of harm for the stacked product is equivalent to that of the single event products and no further data or

regulatory scrutiny are needed. For example, there have been no indications in laboratory or field tests that indicate interaction between insect resistant traits and herbicide tolerance traits. Also, laboratory and field data support the assertion that current insect resistant traits have not been shown to interact in a way to cause environmental harm. If a plausible scenario is found that might result in unacceptable environmental harm, then hypotheses can be developed and studies designed to test whether the scenario might actually occur and to understand the potential environmental significance of these effects within the context of the agricultural system.

How does conventional breeding history inform the ERA?

Plants were domesticated by selecting varieties with specific qualities or traits. Modern crops represent a long history of selection and breeding. Crop varieties are commercialized after being tested in the field over many years and in many environments – crop lines that have unacceptable characteristics are eliminated prior to commercial release. Breeding stack products are part of this agricultural context and are the product of modern breeding techniques. They have been shown to be compositionally equivalent to crops produced using traditional breeding methods that have a long history of safe use (Harrigan et al. 2010). For centuries, breeding for host-plant resistance to insects and disease has been a common theme when breeding new crop varieties. One classic and highly successful example is Hessian fly resistance in wheat varieties, where multiple modes of insect resistance were developed in different wheat varieties and rotated in fields to stave off multiple pest biotypes. Imidazolinone and sulfonylurea herbicide-tolerant crops are examples of herbicide-tolerance traits that were developed using conventional breeding methods. In addition to insect and herbicide tolerance, other examples of traits include disease tolerance, yield enhancement, improved nutrition profiles, cosmetic changes (e.g. purple cabbage and red grapefruit), and plant architecture changes (e.g. stout wheat), etc. For centuries, plant breeders have routinely crossed plant lines to combine beneficial traits into a single commercial variety. The ERA for stacked products can be built upon this foundation of “history of safe use” of conventional breeding for the development of new plant varieties.

In general, multiple traits from traditionally bred varieties are combined in commercially grown crops without conducting environmental risk assessments or regulatory oversight. Conventional breeding of GM lines with non-GM lines does not trigger any novel risk concerns (Pilacinski et al. 2011, Weber et al. 2012, Steiner et al. 2013) and so no additional data should be needed to make a regulatory decision. Crops with stacked traits are composed of single events that have already undergone significant regulatory scrutiny to evaluate potential environmental risk. The certainty of the ERA is increased because, in addition to the traditional field testing conducted for traditionally bred crops, new traits introduced through techniques such as transgenesis have already been extensively evaluated for both human safety and environmental risk (Cellini et al. 2004).

Incorporating information about combined traits into the ERA

Risk is a function of the probability and seriousness of harm resulting from an activity. Risk is high if a severe harmful effect is likely, whereas risk is negligible if the most serious possible effect is small and unlikely. Approved single GM events and traits have been determined to pose negligible environmental risk. The increased prevalence of crops containing various combinations of traits raises questions of how to effectively assess the risks posed by their use. In particular, how can data generated for the single events (that have already been found to be acceptable in terms of environmental risk) inform the risk assessment of crops containing stacked traits or events?

Interaction was defined in regards to evaluating the potential for adverse effects with respect to food and feed uses of genetically engineered (GE) breeding stacks as "an effect, such as a new or modified metabolic activity, resulting from a combination of transgene-derived products in a GE stack that is not occurring in any of the parental single events" (Steiner et al. 2013). Interaction can be defined similarly for the purposes of environmental risk assessment for stacked products – interaction is a biologically significant non-additive change in the hazard or exposure profile of the stack product compared to the single parental events that is caused by the combination of transgenes.

If the risk of an adverse effect posed by the use of event A is R_A, and the risk of an adverse effect posed by use of event B is R_B, the risk of an adverse effect posed by similar use of a product comprising events A and B is R_{AB} and given by a simple formula:

$$R_{AB} = R_A + R_B \quad (1)$$

So in the case of event A and B that have already been determined to have acceptable risk R_{AB} is also likely to be acceptable unless there is some type of plausible hypothesis that an interaction or a case where the sum of the individual effects exceeds some acceptability threshold due to the combination of events A and B that would result in an unacceptable adverse effect

If a plausible hypothesis can be developed for some type of potential interaction, formula (1) can be modified - where $IR_{A.B}$ is the risk of an adverse effect posed by the interaction between the combined use of events A and B. The formula would be expanded if more than two events or traits were stacked.

$$R_{AB} = R_A + R_B + IR_{A.B} \quad (2)$$

Interaction in this case does not denote mere contact between the traits or events, but rather that the risks of adverse effects posed by event A or event B alone are different from the risks they pose in the presence of the other event. This could include a new or modified metabolic activity or some type of synergism. If there is no plausible interaction between R_A and R_B then the interaction term $IR_{A.B}$ is negligible (or can be considered to be zero) and the data from the individual events is sufficient to assess the risk of the crop containing the stacked traits or events (equation 1). However, if after review of available information there is reason to believe that the combination of traits might result in

unacceptable harm, then the data from the individual events may not be sufficient and additional risk characterization may be required. When assessing the risks from stacked traits, the risk assessor tests the hypothesis that $IR_{A.B}$ is not greater than zero. The testing of the hypothesis may not require development of any new data; for example, the risk assessor might use existing knowledge to conclude that the stack of unrelated traits (i.e. herbicide tolerance and insect resistance) is unlikely to produce any harmful effects because there is no plausible hypothesis for interaction.

Significant non-harmful interactions may occur, for example, if there is synergism between the insecticidal proteins which could result in an enhanced ability to control insect pests but at the same time not affect non-target organisms. An example of synergism would be an increase in the efficacy of one insecticidal protein in the presence of a second insecticidal protein. Another example of a positive additive interaction is when the traits provide complementary functions, such as the breeding of a crop engineered with an increased water-use efficiency trait with a crop engineered to have increased heat tolerance. Having both traits might possibly allow the crop to spread to a habitat that is unavailable to one of the parental traits, and this may require a new evaluation of potential risk.

Finally, two important points about $IR_{A.B}$ should be considered. First, convincingly corroborating the hypothesis that $IR_{A.B} = 0$ does not confirm that R_{AB} is acceptable, even if R_A and R_B are acceptable independently: R_A and R_B may both be below an acceptability threshold, but their sum may not be. For example both R_A and R_B individually might cause some minor and acceptable effect, but when added together the sum of the individual effects might be sufficient for the risk assessor to review whether a re-evaluation is needed. Second, it is not necessary to evaluate $IR_{A.B.}$ (e.g. define the exact mode of interaction) if the risk assessor instead chooses to evaluate R_{AB} directly (e.g. simply measure the end result of the combination). This is most likely to occur if measuring $IR_{A.B}$ is difficult or impossible. For example, testing for synergism may be an effective strategy for determining the risks posed by use of a crop containing two insecticidal proteins for which extensive effects data already exist. However, it should be remembered that the goal is to estimate the potential of interactions that would cause unacceptable adverse effects ($IR_{A.B}$) and not to perform a detailed analysis of the underlying mechanisms leading to the interaction. For example, for insecticidal traits, the simplest approach to estimating $IR_{A.B}$ is likely to utilize a susceptible organism that is exposed either to plant material (or artificial diet) containing a mixture of A and B similar to what they might be exposed to in the field.

Determining plausible interactions for stacked products

The existing knowledge about the single events can be used to determine if there are potential interactions and if this potential exists, to help develop plausible hypotheses that can be tested to determine if these interactions would result in unacceptable adverse effects. Knowledge of how the genes function and potentially interact can be investigated at several levels – at the molecular level, the cellular level, and the organism level or phenotype. Traits with known mechanisms of action, or simple mechanisms of action, have less uncertainty and it is relatively simple to determine whether or not

interactions are plausible. For example, the story of how Bt derived insecticidal proteins are produced and accumulate in plant cells is relatively simple and there have been no indications of harmful interactions between Bt traits when they were stacked. This historical evidence provides risk assessors with greater certainty that future stacks with Bt insecticidal proteins are unlikely to result in harmful effects. If at some point there were traits with unknown mechanisms of action, then they might have greater uncertainty and therefore the risk assessment would need to account for a greater number of plausible interactions. The number of plausible interactions could increase as multiple pathways are affected within the cell. The ERA takes this into account as hypotheses are developed during problem formulation and tested during risk characterization.

Assessing the exposure for traits in stacked products

The potential exposure of non-target organisms to environmental stressors such as insecticidal proteins is evaluated as part of the environmental risk assessment for single event products. This evaluation includes both the temporal and spatial exposure based on measured and modeled scenarios. The question for breeding stacks is whether the exposure profile for the product containing the stacked traits will be significantly different than the exposure profiles for the products containing single traits. During the ERA problem formulation phase, the risk assessor seeks to develop plausible hypotheses of risk. In the case of exposure, the question is whether plausible hypotheses exist for changes in temporal or spatial exposure due to changes in projected use or other factors related to the stacked trait compared to the ERA conducted for the single trait products.

For example, a single maize event with a Bt trait active on corn rootworm stacked with another single maize event with a Bt trait that is active on corn borers is unlikely to significantly change in response to when and where corn is grown. Thus the expected temporal and spatial exposure profile for the single traits and the stacked traits are likely to be the same because the projected use pattern will not change and these data corroborate the hypothesis of implausibility. However, there may be cases where plausible hypotheses can be developed because changes in projected use may occur. For example traits conferring drought tolerance, increased nitrogen use efficiency, or tolerance to high or low temperatures could possibly be stacked in ways that might present a new spatial or temporal exposure profile. For example, the ability to plant corn earlier in the season in a potentially drier area might result in planting in areas previously considered to be economically unsuitable for this crop. When combined with an insecticidal trait, the exposure to non-target organisms might be different than it was for the product containing a single event conferring insect resistance. This change in exposure does not mean that risk is necessarily increased, but rather that the risk assessor should develop and test a hypothesis that the exposure is indeed going to be different and then seek to understand what would be the potential harmful effects of this difference in the context of previously established protection goals.

Assessing hazard for breeding stack products

The potential hazard to non-target organisms from environmental stressors such as insecticidal proteins is evaluated as part of the environmental risk assessment for single event products. This is generally accomplished using laboratory tests conducted under controlled conditions with higher than expected exposures. The primary endpoint in these tests is mortality, however other factors such as developmental effects or weight gain are also often observed as part of the tests. During the ERA problem formulation phase for breeding stacks, the risk assessor takes all available data into account to understand if there is a plausible mechanism where interaction might occur between multiple stressors. For example, Bt Cry proteins have been shown to have a very narrow spectrum of activity, generally restricted to a single order or family. The prior knowledge that Cry1 protein activity is restricted to Lepidoptera and Cry3 activity is restricted to Coleoptera, and the results obtained from previous combinations of similar proteins can provide evidence to a risk assessor that a significant interaction is unlikely to occur.

Where significant uncertainties remain, the potential for interaction can be tested in the laboratory (Raybould et al. 2012). For insecticidal proteins this is most logically accomplished by testing the proteins alone and together against target pests known to be sensitive to each one – as these are known to be sensitive to the stressor and any synergistic or antagonistic effects that occur are likely to be detected. These tests can be conducted using later instar larvae and plant material or using purified protein incorporated into artificial diets. For tests utilizing plant material, later instars are commonly used as both the single traits and the stacked traits will cause 100% mortality in early instars obscuring any potential interactions. Use of later instars allows the researcher to use not only mortality, but also sub-lethal end points (e.g. weight gain) to detect any interactions. In the absence of unexpected interactions such as synergy or antagonism in a sensitive organism, the effects of the individual components are presumed to be additive and approaches for determining departure from additivity are described in the scientific literature. For other potential stressors where significant uncertainty remains and plausible harm might occur, hypothesis driven studies can be developed to determine if there are significant levels of interaction between the stacked traits.

Interpretation of hazard data requires care. Because of variability within the test system, the level of detection must be taken into account. An observed difference in results for single traits versus the product containing multiple traits may simply be caused by variability within the test system and organisms rather than actual synergism or other type of interaction. For this reason, risk assessors may choose to use a threshold such as five or ten fold greater effect in the stacked trait product than the singles to indicate whether or not there is a significant likelihood that synergism may be present (USEPA 2009). A second factor to consider in the interpretation is whether any statistically significant differences observed under laboratory conditions would necessarily be predictive of biologically significant effects in insect populations under real world conditions.

It should be remembered that the primary goal in an ERA is not to understand the exact molecular function and interaction, but rather if and how the products of the potential interaction might affect the protected entity. One method to understand the potential interaction is to do a detailed molecular analysis of the mechanism of action of the single events (Ra, Rb, Rc… etc.) and then also conduct a detailed analysis of all potential harmful interactions that might occur (IRa,b,c…. etc.). The information gained in this approach can be used to reduce the level of uncertainty in the risk assessment. Another method is to simply evaluate the environmental risk of the stacked product. For example, if no biologically significant effects on non-target organisms are observed using plant materials or the transgenic proteins from the stacked product using sensitive replicated studies conducted under controlled laboratory conditions, then this should provide sufficient certainty for the risk assessment that exposure to plant materials in the field will also have no effects even if the exact mechanism of action or interaction data may not be available.

Risk assessment for stack trait products

In summary, the basic methods used for environmental risk assessment of crops containing single traits or events are used for crops containing multiple traits or events: problem formulation and risk characterization. The critical additional step during problem formulation is the evaluation of whether combining traits into a single crop would cause an unacceptable harm. If, based on available data, no plausible hypotheses for adverse effects due to the combination of traits can be developed, then the environmental risk assessments for the single events provide sufficient information to perform the risk assessment of the stacked trait product with sufficient certainty to make a regulatory decision. If plausible hypotheses can be developed for interaction to occur, then exposure and hazard of the traits in the stacked trait product can be either modeled or tested experimentally so that the risk assessment can incorporate information on the potential interaction.

Regulatory authorities may require notification of the commercialization of breeding stacks of previously approved events, and may consider the environmental risks of such stacks following the principles presented here. Regulators should generally not require additional data on stacks of insect protection with herbicide tolerance traits, or of two or more herbicide tolerance traits. For stacks of two or more insect protection traits where plausible hypotheses might be developed that indicate that a combination of the traits could result in unacceptable adverse environmental effects, data on interaction of the insecticidal ingredients may be warranted for commercial cultivation. Since little or no new additional data are generally warranted, review of such notifications should be completed in a short defined time period prior to commercial launch.

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