Surrogate Species Selection for Assessing Potential Adverse Environmental Impacts of Genetically Engineered Plants on Non-Target Organisms

Conference Proceedings

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1. INTRODUCTION

According to 2011 data, genetically engineered crops employing one or more genes for insect resistance were grown on approximately 60 million hectares globally, and that number is expected to increase (James, 2011). As a part of the larger environmental risk assessment process for genetically engineered insect-resistant crops, most regulatory authorities require that developers evaluate the potential for these crops to have adverse impacts on organisms not intended to be controlled by the trait, referred to as non-target organisms (NTOs). Conducting NTO testing poses both conceptual and logistical challenges for researchers, and the challenges are likely to increase as these crops are considered for commercial planting in a growing number of new geographic locations.

Typically, NTO testing begins in the laboratory with high-dose screening studies, also called early tier testing, which may be followed by semi-field and field testing[1] (Garcia-Alonso *et al*., 2006; Romeis *et al*., 2008), and a fundamental challenge for the researcher is the selection of test species that will be used. The key is to select test species that best represent the valued NTOs in the area in which the GE crop is going to be introduced, and there are many variables to be considered: surrogates must be either field collected and reared, or purchased, as large, uniform populations; they must perform well on an artificial diet and be amenable to manipulation under laboratory conditions; and validated test protocols must be available. The use of surrogates should result in data that consistently meet specific assay performance criteria and are statistically robust. But the most important goal from the standpoint of regulatory decision making is for the data derived from surrogates to accurately predict any adverse impacts likely to be observed from the use of the crop in the agricultural context.

As a result of over a decade of NTO testing of genetically engineered insect resistant crops, numerous surrogate species have been identified that meet these criteria. Test results using these species have effectively assessed the environmental safety of insect-resistant crops and informed regulatory decision making. But as more and more nations, representing a wider variety of agro-ecosystems, consider the adoption of insect-resistant crops, it is timely to re-evaluate the selection criteria for appropriate surrogate species for NTO testing. Specifically, there is reason to address the need, especially for regulators in developing countries, for NTO test data to be transportable across national borders.

These issues were the subject of the conference "Surrogate Species Selection for Assessing Potential Adverse Environmental Impacts of Genetically Engineered Plants on Non-Target Organisms" convened by the Center for Environmental Risk Assessment (CERA), Agriculture & Food Systems Institute, June 26-28, 2012. The objectives of the conference were

1. To identify key criteria for surrogate species selection for laboratory, semi-field, and field testing and
2. To identify best practices for surrogate testing, with a particular focus on facilitating data transportability.

This report summarizes the proceedings of the conference, including the presentations, case studies, breakout and plenary discussions, and the points of consensus agreed to by the participants.

2. METHODOLOGY

The conference agenda is presented in Annex 1. Presentations by the invited speakers provided a common basis of understanding of several key topics: the development of risk hypotheses relevant to impacts on non-target organisms by genetically engineered (GE) crops; the identification of common ecological functions provided by arthropod families across different cropping systems, in different geographic location; the use of surrogate species for pesticide testing and in the evaluation of natural-enemy biocontrol agents and how these evaluations can inform GE crop testing; and the feasibility of collecting ecological toxicity test data that will be transportable to multiple geographic locations.

1 Also called "Tier 1," "Tier 2," and "Tier 3" testing, respectively.

Breakout groups met to discuss (1) the ecological functions to be represented in environmental testing of arthropod-active GE crops and (2) the selection criteria for appropriate surrogate species to be used in laboratory, semi-field, and field tests. In a subsequent breakout exercise, participants discussed two case studies (Annexes 2 and 3), which presented novel combinations of crops and arthropod-active traits. With the case studies, the groups were directed to evaluate the applicability of the ecological functional groups and surrogate species selection criteria that they developed in the first breakout exercise.

Following each breakout exercise, the groups presented their discussions and conclusions back to the plenary session. The plenum was then asked to consider the following issues in light of the deliberations from the two breakout exercises: functional versus phylogenetic approaches to surrogate selection; selection criteria for laboratory, semi-field, and field testing; and best practices for surrogate testing, with a focus on facilitating data transportability. The plenum achieved consensus on a number of points (See Section 6, below).

3. INVITED PRESENTATIONS

3.1 RETROSPECTIVE ON THE EVOLUTION OF SURROGATE SPECIES SELECTION FOR PESTICIDE TESTING

Marco Candolfi, Ph.D., CEO, Innovative Environmental Services, Ltd., Switzerland

The summary was not available at time of publication.

3.2 HOST RANGE TESTING FOR NATURAL ENEMY INTRODUCTIONS

Barbara Barratt, Ph.D., AgResearch Limited, New Zealand

The risks associated with natural enemy introductions for biological control of pests include impacts on non-target species and their populations, host range expansion of the biological control agent post-release, and competition or hybridization with existing natural enemies in the receiving environment. There is also potential for complex and unpredictable indirect impacts at other trophic levels. Risk analysis can be initiated before a biological control agent has been imported, as well as host range testing in quarantine. There has been considerable interest in biosafety of biological control over the last 20 years from researchers and regulatory agencies[2,3,4,5,6].

Pre-entry biosafety considerations include investigation of the natural host range of possible biological control candidates to determine the taxonomic breadth of their natural hosts and the non-target impacts, if any, reported for these organisms in cases where this has been evaluated. The fauna of a new proposed receiving environment can then be examined to determine whether there are organisms related to confirmed hosts in the natural geographical range, or other new areas of introduction, which could be at risk. These data can assist in the preparation of quarantine test species lists.

2 Follett, P.A. and Duan, J.J. (2000). *Nontarget effects of biological control.* Kluwer Academic Publishers, Norwell, Massachusetts, USA.

3 Wajnberg, E., Scott, J.K. and Quimby, P.C. (2001). *Evaluating indirect ecological effects of biological control.* CABI Publishing, Wallingford, Oxon., UK.

4 Bigler, F., Babendreier, D. and Kuhlmann, U. (2006). *Environmental impact of arthropod biological control: methods and risk assessment.* CABI Publishing, Delemont, Switzerland.

5 Food and Agriculture Organisation (1996). *Code of conduct for the import and release of exotic biological control agents.* Food and Agriculture Organisation, Rome, Italy.

6 OECD. (2003). *Guidance for information requirements for regulation of invertebrates as biological control agents (IBCAs).* OECD Environment, Health and Safety Publications Series on Pesticides. Organisation for Economic Co-operation and Development, Paris, France.

Biotype considerations are important to ensure that tests are carried out on natural enemies and their hosts that correspond genetically with those studied in the natural geographical range and other new areas of introduction.

Regulators generally rely most heavily on data collected during host range testing in quarantine when deciding whether to approve the introduction and release of a biological control agent. Critical to the host range testing process is deciding on the test species list, including selection of surrogate species if necessary, the type of tests to conduct, and what variables to measure.

For weed biological control agents the "centrifugal phylogenetic testing sequence"[7] and refinements[8] of this developed in Australia have proved to be largely reliable in developing a taxonomic profile of plants likely to be at risk from phytophagous natural enemies. For invertebrate targets there are the additional challenges of far greater numbers of species to be considered compared with plants, and they are generally less well known taxonomically. In addition, it is necessary to rear test species, the plants they feed on, as well as the natural enemies to conduct tests. Tests lists can be constructed from knowledge of phylogenetic and ecological affinities of the target with species in the receiving environment, with the addition of relevant beneficial or iconic species. An alternative, potentially more objective method of test species selection developed for non-target species selection for biosafety testing of GM plants[9] is currently under evaluation for biological control biosafety. The method depends upon a database and model (PRONTI – Priority Ranking of Non-Target Invertebrates) to assess simultaneously each of the invertebrate species in the receiving environment and prioritize them for biosafety testing with the biological control agent. The database holds biological, ecological, and physiological information on invertebrate species in the receiving ecosystem, as well as information on the likely interactions between each of these species and the proposed biological control agent. The PRONTI model uses this information to prioritize species for biosafety testing using five selection criteria, the potential hazard, degree of exposure, potential ecological impact, the anthropocentric value and testability of each species. Each criterion is assessed individually for each species by applying scores to the species attributes in the database.

The use of surrogate species has not been widely adopted for biological control risk assessment, although it has been acknowledged that in the case of rare species, cautious selection of substitute species can be made[10,11]. Examination of existing laboratory and field host data for the braconid *Microctonus aethiopoides* Loan, however, suggested that in some instances, congeneric species would apparently serve well as surrogates for another, and in some cases they would not[12,13].

The risks identified from pre-entry investigation and quarantine testing with proposed biological control agents are usually assessed individually by regulators for likelihood and magnitude, and this analysis, in combination with expert

7 Wapshere, A.J. (1974). A strategy for evaluating the safety of organisms for biological weed control. *Annals of Applied Biology* 77: 201-211.

8 Wapshere, A.J. (1989). A testing sequence for reducing rejection of potential biological control agents for weeds. *Annals of Applied Biology* 114: 515-526.

9 Todd, J.H., Ramankutty, P., Barraclough, E.I., and Malone, L.A. (2008). A screening method for prioritizing non-target invertebrates for improved biosafety testing of transgenic crops. *Environmental Biosafety Research* 7: 35-56.

10 Messing, R.H., Roitberg, B.D., and Brodeur, J. (2006). Measuring and predicting indirect impacts of biological control, competition, displacement and secondary interactions. Pp. 64-77. In: *Environmental impact of invertebrates for biological control of arthropods – methods and risk assessment.* F. Bigler, D. Babendreier, and U. Kuhlmann, Eds. CABI Publishing, Wallingford, UK.

11 Kuhlmann, U., Mason, P.G., Hinz, H.L., Blossey, B., De Clerck-Floate, R.A., Dosdall, L.M., McCaffrey, J.P., Schwarzlaender, M., Olfert, O., Brodeur, J., Gassmann, A., McClay, A.S., and Wiedenmann, R.N. (2006). Avoiding conflicts between insect and weed biological control: selection of non-target species to assess host specificity of cabbage seedpod weevil parasitoids. *Journal of Applied Entomology* 130: 129-141.

12 Barratt, B.I.P., Evans, A.A., Ferguson, C.M., Barker, G.M., McNeill, M.R., and Phillips, C.B. (1997). Laboratory nontarget host range of the introduced parasitoids *Microctonus aethiopoides* and *Microctonus hyperodae* (Hymenoptera: Braconidae) compared with field parasitism in New Zealand. *Environmental Entomology* 26: 694-702.

13 Barratt, B.I.P. (2004). Microctonus parasitoids and New Zealand weevils: comparing laboratory estimates of host ranges to realized host ranges. Pp. 103-120. In: *Assessing host ranges for parasitoids and predators used for classical biological control: A guide to best practice.* R. G. Van Driesche and R. Reardon, Eds. USDA Forest Service, Morgantown, West Virginia, USA.

opinion, informs the decision-making process. Post-release validation of pre-entry and quarantine test predictions is also critical to improving the process in the future.

3.3 COUNTRY-SPECIFIC APPROACHES TO ASSESSING ADVERSE ENVIRONMENTAL IMPACTS OF GE PLANTS ON NON-TARGET ORGANISMS

3.3.1 Argentina's Approach to Assessing Adverse Environmental Impacts of Genetically Engineered Plants on Non-Target Organisms

Patricia Gadaleta, Ph.D., Ministry of Agriculture, Livestock, and Fisheries, Argentina

Argentina was a pioneer in the early adoption of GM crops. Activities related to genetically modified organisms (GMOs) started to be regulated in 1991, and the first commercial release of a GM crop was approved in 1996.

So far, 27 commercialization permits have been granted (4 for soybean, 20 for maize and 3 for cotton), placing Argentina in one of the international leading positions with 24 million hectares planted with GM crops. Argentina never had any safety-related issues with any of these deregulated materials, and we continue revising our regulatory system in order to address new developments in agro-biotechnology and state-of-the-art approaches in biosafety assessment.

The Constitutional mandate of the Argentine legal system is to protect its inhabitants and its natural resources. Bearing this in mind, the regulatory administration does not favor nor discard any technology; but rather ensures that the use of a said technology shall pose no new or greater risks to its people and natural resources under a scientific-based assessment. Based upon scientific and technical principles, Argentine GMO's regulations included plants, animals, and microorganisms. For GM plants the regulations address the experimental confined releases for field trials, the counter-season seed production (winter nursery) of GM soybean and maize and the risk/safety assessment for the commercial release of GM crops.

The competent authority for the Argentine regulatory framework is the Secretariat of Agriculture, Livestock and Fisheries, within the Ministry of the same name, who approves the commercial release of GM crops. However, GMOs assessment is conducted by regulatory bodies through their specific advisory commissions: the National Advisory Commission on Agricultural Biotechnology, (CONABIA by its Spanish name) and the Technical Advisory Committee on GMO Use (CTAUOGM by its Spanish name). Both agencies are in charge of the assessment of the applications for the commercial release of new GMOs and determine their risk/safety. In the first case, the biosafety of a new GM crop is assessed by CONABIA with respect to the environment, while the second (CTAUOGM) assesses the safety of the GMO for human and animal consumption. Then, a third regulatory body, the National Directorate of Agrifood Markets, provides advice about the reasonableness of the approval in light of domestic production interests and provides country trade guidelines.

The advisory commissions (CONABIA and CTAUOGM) share several characteristics. These advisory commissions consist of a multidisciplinary group of experts, which are representatives of the public and private sectors (*i.e.*, governmental agencies and ministries, academia, private associations, *etc.*). Each member has a different background, ranging from agronomy to chemistry and molecular biology, thus ensuring that all aspects of the GMOs are thoroughly assessed. The risk/safety assessment is based upon scientific principles and it is done on a case-by-case basis.

In order to obtain the commercial approval of a GM crop, three reports, issued by each of the regulatory bodies, are submitted to the Secretary of Agriculture, Livestock and Fisheries. With these reports, the competent authority decides to grant or to deny the approval of a GM crop. Such approval entails the authorization for the placing of the GM crop on the market (for food, feed and processing) and its unconfined release for planting at a commercial scale.

In 2010, the Secretariat of Agriculture, Livestock, and Fisheries commissioned the Biotechnology Directorate to organize a series of multi-sectorial workshops to review the regulations and propose appropriate amendments to assure the

fulfillment of the constitutional mandate and, at the same time, enable the use of new agro biotech developments with predictable and efficient regulatory policies. After a year working with national experts and stakeholders, new and updated regulations were drafted and enacted for experimental and commercial releases of GM plants and also for counter-season production of GM maize and soybean seeds. Thus, the regulations for the Release of Genetically Modified Plant Organisms into the Environment that was established in the Resolution No. 39/03 has been replaced by the Regulation (SAGYP) No. 701/11. The documents are available at the website of the Ministry of Agriculture, Livestock and Fisheries: http://64.76.123.202/site/agregado_de_valor/biotecnologia/index.php.

In regard to the effects of GM plants on non-target organisms, the previous regulation inquired about the effects of GM plants on the flora, fauna, and microbial populations in two different sections: one in relation with the general description required for risk assessment and the other in relation with the expected impact/effect in the production of the crop in a commercial scale. This was confusing and the information submitted by the applicant was usually unpredictable. The amended regulations reduce redundancy and also try to direct the evidence about the effects on the flora, fauna and microbial population to a more accurate risk assessment and to open the possibility of different approaches for this purpose. So on, the point of the application where the applicant must give information on the effects of the GM plant on the flora, fauna and microbial population of the rhizosphere present in the agro ecosystem is split into sub-items. The sub-item D.11.1. looks into the effects of the GM plant on non-target organisms (or substitute species) that directly or indirectly interact with the crop by assessing the product expressed in the GM plant, the plant material and/or by planting the GM plant. The sub item D.11.2. looks into the effect of the GM plant on species and ecologic interactions relevant for the local agro ecosystem. The first assessment refers to different approaches (laboratory bioassays, semi field trials, or field trials) to assess the risk hypothesis for pest resistant traits and their effect on non-target organisms. The second sub-item comprises all other information specifically related to species and ecological interactions that could be relevant for the local agro ecosystem for a particular trait that have not been addressed in the first item and must be addressed case-by-case. In relation to these points of the application form, last year we conducted a two-day meeting with experts on four relevant crops (soybean, maize, cotton and sugarcane) to analyze which are the valued entities (particular valued species, gilds of species or ecological interactions) in the Argentina's agro ecosystem for each of these crops.

Another new issue in the amended regulations is the introduction of the Previous Consultation Instance (ICP in Spanish). This is an evaluator-applicant exchange mechanism that aims to clarify some of the information to be included in the form and provide details of the criteria to be used in the application. This instance is optional and designed for the benefit of the applicant rather than an instance of debate of regulatory criteria.

The evidence to be provided by the applicant for the non-target organisms risk assessment must entail: the setting of testable risk hypotheses, the definition of the criteria for appropriate selection of test species and ecological functional group, the laboratory and field studies results with an appropriate experimental design and the estimation of risk/safety based on conclusions of these studies. Some specific issues are taken into account for the information submitted and those must be consistent with the hypothesis to be tested. These issues include: the mode and spectrum of action of the expressed proteins and biochemical interactions, the exposure pathway and the level of exposure. In all cases, the evidence provided must be relevant, accurate, complete and reliable. Regarding reliability, those studies that use validated or standardized methods and/or are conducted under good laboratory practices (GLP) as well as peer-reviewed literature and also those studies based on consensus documents of international organizations are considered among reliable sources of information to pursue a risk analysis.

3.3.2 Brazil

Fernando Valicente, Ph.D., EMBRAPA, Brazil

In Brazil, Law Nº 11.105, of 24 March 2005 regulates items II, IV and V of Paragraph 1 of Article 225 of the Federal Constitution, provides for safety norms and inspection mechanisms for activities that involve genetically modified organisms (GMOs) and their by-products, implements the National Biosafety Council (CNBS), restructures the National

Biosafety Technical Commission (CTNBio), provides for the National Biosafety Policy (PNB), revokes Law Nº 8.974, of 5 January 1995, Provisional Measure Nº 2.191-9, of 23 August 2001, and Arts. 5, 6, 7, 8, 9, 10 and 16 of Law Nº 10.814, of 15 December 2003, and provides for other measures. The Brazilian legislation includes one law, two decrees, seven communications, CNBS resolutions, and nine normative resolutions. Normative Resolution No 05, of March 12, 2008, gives provisions on rules for commercial release of Genetically Modified Organisms and their derivatives, and includes the norms on non-target organisms.

General and Preliminary Provisions

In Article Nº 1, the law provides for safety norms and inspection mechanisms for the construction, culture, production, manipulation, transportation, transfer, import, export, storage, research, marketing, environmental release and discharge of GMOs and their by-products, guided by the drive for attaining scientific development in the biosafety and biotechnology area, the protection of life and human beings, of animal and plant health, and the compliance with the principal of environmental precaution. The Annex IV governs the Environment Risk Assessment and gives provisions on rules for commercial release of GMOs and their derivatives. These rules include plants, organisms used for biological control, and invertebrate animals.

The first part of these rules includes the plants.

A) PLANTS: Negative and positive effects to target and non-target organisms, that may take place with the released GMO, listing the species assessed, reason of the selection and techniques used to explain the impacts;

The second part of these rules includes all the organisms used for biological control including information about the non-target organisms.

B) ORGANISMS USED FOR BIOLOGICAL CONTROL: Seven basic pieces of information must be given by the applicant:

1. Target species of biological control and direct effects of GMO on such species compared with the effects on the parental organism;
2. Spectrum of organisms susceptible to the GMO and susceptibility of non-target organisms to the GMO, describing the criteria employed in the choice of organisms assessed;
3. Ways of GMO dispersion from one individual to another and factors that affect such dispersion;
4. Secondary effects that may happen to predators, preys, competitors, and parasites of the target species;
5. Metabolites produced by the GMO that may cause direct or indirect harmful effects on other species through concentration along the food chain;
6. Effects resulting from horizontal transfer to another organism, as the case may be;
7. Possible genetic modifications that may happen in populations of the target organism as a result of the GMO use.

The third part of these rules includes all the invertebrate animals, including information about non-target organisms.

C) INVERTEBRATE ANIMALS: Eight basic pieces if information must be provided by the applicant:

1. GMO effects in the invertebrate's food chain;
2. Possible production of new metabolites or toxins by the GMO that are able to cause harmful effects on the invertebrate's parasites or predators;
3. Possible adverse effects of such GMO releasing in the local ecosystem;

4. Records of likely natural populations of the parental organism within Brazil and, in the affirmative, discuss their effects, either beneficial or harmful, to agriculture, environment, and public health;
5. Likelihood of the transgene to be transmitted to other species through non-conventional reproduction mechanisms and, in the affirmative, specify the transfer mechanism, listing the species;
6. Possible existence of experimental work on the phenotypic expression of the transgene in breeds of specific lineages modified with wild organisms. In the affirmative, describe what these results were;
7. Change in distribution and abundance of natural populations by the possible integration of the transgene to the genic set of such populations, reporting on the possible effect of such change;
8. Mechanisms to be used to check dispersion of the GMO to other environments.

Normative resolution Nº 5 also rules the Post-Commercial Release Monitoring that is required in Brazil. Some basic information is also required regarding the non-target organisms.

1. The monitoring shall be conducted by the applicant with the purpose of oversee the effects resulting from commercial release of a GMO and its derivatives to the environment and human and animal health.
2. The monitoring shall be conducted under strict observance of the principles of precaution, transparency, and scientific independence.
3. The monitoring shall be guided by internationally recognized scientific methodology and experimental designs adequate to the inferences to be made.

Considerations

The expression in plants of foreign genes of agronomic interest using modern transgenic technologies has provided different options to produce important genetically modified (GM) crops. Despite the high rate of adoption of GM crops, there are many concerns about the possible impact of these crops on the environment. The primary ecological concerns to the release of transgenic plants include those related to their possible invasiveness in ecosystems, out-crossing, horizontal gene transfer, development of pest resistance and effects on non-target organisms (Conner *et al*., 2003). One of the primary concerns related to the adoption of insect resistant transgenic plants in the environment is the detrimental effect that these may pose on non-target organisms, including entomophagous arthropods (parasitoids and predators), which have an important function in regulating pests (Dutton *et al*., 2003). Effects of GM plants on non-target entomophagous arthropods (predators and parasitoids) have been a major concern, as these organisms often play an important role in natural pest regulation and are considered to be of economic value. Moreover, this group of organisms may be a good indicator of potential ecological impacts of transgenic plants as they belong to the third trophic level in the food chain (Groot and Dicke, 2002).

In Brazil, CTNBio members (regulators) are identified with their area of expertise. These areas include: Crop Science and Environment, Human, and Animal Science. At least two regulators, depending on the dossier, are chosen to evaluate each GMO to be commercially released. Each dossier is evaluated case by case, and step by step. So, the possible effects of GM crops on non-target organisms follow the same rules and regulations.

According to the *The Economist* (2010) in less than 30 years Brazil has turned itself from a food importer into one of the world's great breadbaskets. It is the first country to have caught up with the traditional "big five" grain exporters (America, Canada, Australia, Argentina and the European Union). It is also the first tropical food-giant; the big five are all temperate producers. Due to a favorable climate, crops are planted throughout the year, and the farmers plant a second crop of corn or cotton called the safrinha. This new scheme of crop rotation is to first plant a rain-fed crop, such as rice, soybean or maize, and then after these crops are harvested, plant a second crop of soybean, sorghum or even maize. Safrinha can also be defined as a farming strategy whereby the farmer takes advantage of a long tropical growing season to produce two crops in a single growing season, thereby maximizing revenue per acre. This new fact also causes concerns because (GM) crops are planted after (GM) crops, and insects are always exposed to crops even during the dry

season. Usually insects may be exposed to the same *Bacillus thuringiensis* (Bt) genes, although different crops are planted. Farmers will have to plan a "gene rotation" instead of (GM) crop rotation.

It also should be considered that a GM crop is attacked by different insect pests; however, these insect pests may attack more than one crop and may be exposed to more than one toxin (in this specific case, Bt toxins), and these insect pests will be the target of parasitoids and predators. Other problem that may evolve is that the non-target organisms may be exposed to different toxins.

In Brazil post-commercial release monitoring is required by law, however, for some researchers it doesn't make any sense, because to be commercially released, a GM crop must be fully studied and a dossier must be fully completed. So, when a GM crop is commercially available it is considered to be safe. On the other side, some researchers state that this monitoring is extremely important because some problems may occur in the future and the studies showed in the dossier are not enough. Some researchers recommend following these GM crops in the field for many years. However, another issue that is not clear is how to address the post-commercial monitoring. Some important issues are still discussed such as:

- If a GM crop is commercially approved and available in the market, there´s no need to do all the research again. Monitoring is different from research.
- Research on non-target organisms only if some questions arises. This research step should be GM crop and non-target organism specific.
- Monitoring all possible effects in the environment, however it should be considered when evaluating and based on actual events. Scientific evidence should be considered.
- It should be considered that sampling and surveying large areas of commercially available GM crops is totally different from sampling field trials.
- Monitoring should also consider the presence of single genes and stacked genes for insect pests. Possible interactions should be considered.

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3.3.3 EFSA'S Specific Approach to Assess Adverse Environmental Impacts on Genetically Engineered Plants on Non-Target Organisms

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In the European Union (EU) a scientific opinion (SO) on the assessment of potential impacts of genetically modified (GM) plants on non-target organisms (NTOs), hereafter referred to as NTO SO, was issued in November 2010 (EFSA, 2010a). The drafting of this document was an initiative undertaken by the European Food Safety Authority's (EFSA) Panel on genetically modified organisms (GMOs), with the aim of providing guidance for risk assessors on assessing potential effects of GM plants on NTOs together with a rationale for data requirements. Issues to which special attention was paid were (i) criteria for non-target (NT) species selection and (ii) advice on testing approaches.

Criteria for non-target species selection

Because not all NTOs present in the environment where a GM plant is grown can be tested in an environmental risk assessment (ERA), a representative subset of species (named "focal species" by EFSA) is selected. For the selection of focal species, a 4-step approach combining the strengths of two existing species selection approaches - the ecological and ecotoxicological approach - is proposed.

Starting with problem formulation (step 1), functional groups (*e.g.*, herbivores, pollinators, natural enemies, decomposers) relevant to consider in the ERA are defined. Subsequently (step 2), NT species occurring in the GM plant's receiving environment are categorised within the identified relevant functional groups. The GM plant's receiving environment to be considered is the European agro-ecosystem (EFSA, 2010a). If relevant, endangered species also need to be listed. A first prioritisation of species (step 3) is based on ecological criteria (*e.g.*, species' exposure to the GM plant, abundance, feeding habits, sensitivity to trait) as done in the ecological approach. When selecting the most appropriate species for testing (step 4) - the focal species - practical criteria (*e.g.*, species' availability and testability) considered in the ecotoxicological approach are applied. In the end, this approach results in the selection of testable species belonging to relevant functional groups in the receiving environment. The NTO SO requires that at least one focal species is tested per relevant functional group.

Although the 4-step approach suggests that European species need to be selected, this does not preclude the use of non-EU species as a focal species in lower-tier studies. Indeed, experience with the assessment of impacts on NTOs in the EU reveals that species selected for testing in lower-tier studies can be non-European species that represent species present in the European agro-ecosystems, if this choice is justified.

Issues that remain vague in the NTO SO are whether the species selection approach applies to lower- and/or higher-tier studies and whether the selected focal species should be tested or considered in the ERA (EFSA, 2010b). This latter question is particularly relevant for endangered species, which one may prefer not to test.

Testing approaches

The NTO SO states that potential adverse effects on NTOs due to GM plant cultivation should be considered in the problem formulation phase. These effects, being "unintended" - as not intended by the genetic modification - can be the result of the trait(s). For example, the expression of the Bt-toxin or an intended change in composition of a GM plant, may affect plant-NTO interactions in the field. Besides these anticipated unintended effects, any potential unintended effect of an unpredictable nature - and hereafter referred to as unanticipated unintended effect - needs to be considered in the ERA according to the NTO SO. Such effects are the result of a consistent (non-transient) difference between the GM plant and its comparators that goes beyond the intended trait(s).

For the assessment of potential anticipated unintended effects on NTOs, a tiered testing approach needs to be followed, where one starts looking for negative effects on NTOs in the lab under worst-case conditions (Romeis *et al.*, 2008). Depending on the outcome of the lab studies one then decides if further testing at a higher tier is needed to come to a conclusion on the risks to NTOs. If no adverse effect is detected at a certain tier, it is inferred that the risks to particular NTOs will be negligible.

For the assessment of unanticipated unintended effects a weight-of-evidence approach is recommended that relies on *in planta* (event-specific) data. Sources of such data are the comparative data of the GM plant with its comparator at molecular, compositional, and agronomic/phenotypic level, and data on NTO-plant interactions for at least one focal species per relevant functional group. Comparative data are generally provided in applications for marketing, but could be extended to inform an ERA. For example, one may analyse pollen and nectar for its composition or look into more detail into differences in disease occurrence. To retrieve data on NTO-plant interactions, field trials are considered the most appropriate means by EFSA. However, as an alternative, lower-tier studies with *in planta* material may provide

those data. The outcome of this approach decreases the uncertainty on the occurrence of unintended effects. If differences are identified, their biological relevance will need to be further assessed.

EFSA was criticised because of the undue emphasis put on the assessment of unanticipated unintended effects. It was postulated that the commonly submitted event-specific data on molecular, compositional and phenotypic/agronomic level would provide sufficient evidence to reliably reach a conclusion on unintended effects and that the weight-of-evidence approach could not rule out the occurrence of unanticipated unintended effects. Going more into details, the concept of extended compositional analysis was questioned as it may be challenging due to the lack of standard protocols and the difficulty of interpreting obtained results. The use of plant material was considered not always feasible and may complicate interpretation of results due to increased variability.

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3.3.4 Mexico

Ariel Alvarez, Ph.D., CIBIOGEM, Mexico

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3.3.5 Biosafety in the Philippines: Assessing Adverse Environmental Impacts of Genetically Engineered Plants on Non-Target Organisms

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Biosafety in the Philippines came into focus in 1987 when rice farmers, together with scientists in the University of the Philippines Los Baños (UPLB) and the non-government organization Magsasaka at Siyentipiko Para sa Ikauunlad ng Agham Pang-Agrikultura (MASIPAG), protested the plan of the International Rice Research Institute (IRRI) on the experimentation of different rice blast strains. Earlier there was already a brewing debate at UPLB on the use of radioactive materials (^{14}C) at IRRI rice field. An Ad-Hoc Committee on Biosafety was created to address this concern. It was made up of the UPLB, IRRI, Philippine Council for Agriculture Resources Research and Development (PCARRD), and the Department of Agriculture (DA). The committee recommended the creation of a National Committee on Biosafety and the formulation of national policies and guidelines on biosafety.

Some of the important milestones of biosafety regulations in the Philippines are as follows:

- Executive Order 430, series of 1990, created the National Committee on Biosafety of the Philippines (NCBP).

- The DA Administrative Order No. 8, series of 2002, provided the guidelines for the importation and use (except contained use) and release into the environment of plants and plant products derived from the use of modern biotechnology.
- The Executive Order 514, series of 2006, strengthened the NCBP role.

The responsible agencies of the Department of Agriculture for safety assessment and compliance in the Philippines are (1) the Bureau of Plant Industry (BPI) which is the lead agency and single entry point for applications and responsible for the issuance of permits; (2) the Bureau of Agriculture and Fisheries Products Standards (BAFPS), which takes care of food safety assessment; and (3) the Bureau of Animal Industry (BAI), which is in charge of feed safety assessment. If the regulated article is a pest-protected plant, it has to be duly registered with the Fertilizer and Pesticide Authority (FPA).

The different areas of safety assessment for genetically modified plants (GM) are (1) the importation for contained use in greenhouses and laboratories with the approval based on compliance with safety requirements of the NCBP; (2) for field testing with the approval based on the satisfactory completion of safety testing under contained conditions; (3) for propagation, wherein the approval shall only be given after field trials and a risk assessment show no significant risk to human and animal health and the environment and; and (4) for direct use as food and feed or for processing, where importation of a regulated article shall only be allowed if it has been authorized for commercial distribution as food or feed, in the country of origin and poses no significant risk to human and animal health.

The risk assessment principles are carried out in a scientific and transparent manner. They are based on available scientific and technical information. The lack of scientific knowledge or consensus should not be interpreted as indicating a particular level of risk, absence of risk, or acceptable risk. The identified characteristics of the genetically modified organism (GMO) and its use shall be compared to those of non-GMO from which it is derived and its use under the same conditions. In case new information becomes available, the risk assessment shall be readdressed.

Field testing of Bt corn in the Philippines commenced in August 25, 1999 when the approval of the first limited field testing of Bt corn was accorded to Agroseed Corporation and the Institute of Plant Breeding, UPLB. In June 6, 2001 the approval of the multi-location bioefficacy field trials in major corn growing areas in the Philippines in 13 sites for two growing seasons (2002 – 2003) was given to Pioneer Hi-Bred Philippines.

One of the requirements for the pre-commercialization of Bt corn was the assessment of any possible unwanted effects on non-target organisms (NTO's). Hence monitoring activities were intended to address effects on NTO safety and compliance with the conditions of the field test. The post-commercialization monitoring of the performance of Bt corn required the detection of any development of Asian Corn Borer (ACB), (*Ostrinia furnacalis* Guenee) resistance at the earliest possible time and the possible effect of Bt corn on native biodiversity. Three projects that were funded by the Program on Biosafety System (PBS) were (1) the investigation of secondary ecological effects of Bt corn in the Philippines; (2) the post commercialization monitoring of ACB resistance to Bt corn in the Philippines and the impact of pollen dispersal on non-target Lepidoptera and; (3) the ecosystem-level assessment of the impacts of herbicide tolerant corn on wild biodiversity in corn production systems in Luzon, Philippines. Related studies on Bt corn and NTOs were (1) the influence of Yieldgard (YG) on the effectiveness of *Trichogramma evanescens* (Hymenoptera: Trichogrammatidae), an egg parasitoid of ACB; (2) biodiversity, community structure and population abundance of arthropods in Bt corn agroecosystem; (3) characterization of fungal microflora and evaluation of the level of mycotoxin contamination in YG and non-Bt corn grains in the Philippines; and (4) feeding value for broilers of two YG corn hybrids versus their isogenic counterparts, treated with and without insecticides. Additional studies on NTOs were also done particularly on bees and butterflies.

3.3.6 USA: Approach to Assessing Environmental Impacts of Genetically-Engineered Plants on Nontarget Organisms[14]

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U.S. Federal Oversight of Genetically Engineered Plants

Genetically engineered plants are regulated within the United States by three Federal government agencies under the Coordinated Framework for Regulation of Biotechnology developed by the Office of Science and Technology Policy in 1986 to allow the use of existing Federal statutes for the regulation of Biotechnology (51 FR 23302; June 26, 1986). These Federal agencies include the U.S. Department of Agriculture Animal and Plant Health Inspection Service, the U.S. Environmental Protection Agency (EPA), and the U.S. Food and Drug Administration.

Under the Coordinated Framework, GE products are regulated according to their intended use, with some products being regulated under more than one agency.

- The USDA-APHIS protects agriculture and the environment from pests, diseases, and weeds.
- The EPA protects human health and the environment, as it evaluates PIPs, microbial pesticides, and intergeneric microorganisms.
- The FDA protects the safety of the food and feed supply.

Thereby, each agency will view the product differently based upon their statutory responsibilities.

U.S. Environmental Protection Agency's Role in Regulation of Plant Incorporated Protectants

EPA is responsible for regulating substances produced by genetically modified plants or their produce that are intended to act as pesticides. These are referred to as "Plant Incorporated Protectants" (PIPs), which include the pesticidal substance as well as the genetic material necessary for its production. EPA's Office of Pesticide Programs regulates the sale, distribution, and use of pesticides in order to protect human health and the environment under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). For each pesticide registration, EPA must ensure that the FIFRA regulatory standard is met that use of the pesticide will cause no unreasonable adverse effects on the environment. Within FIFRA, "unreasonable adverse effects on the environment" are defined in part as "any unreasonable risk to man or the environment, taking into account the economic, social, and environmental costs and benefits of the use of any pesticide" (in addition to dietary risks assessed under the Federal Food, Drug, and Cosmetic Act). In making regulatory decisions, EPA evaluates the risks of pesticide use and balances these risks with the benefits derived from pesticide use. Ultimately, if the benefits of the pesticidal product outweigh the risk, then EPA considers the product to be in the interest of the public. While there is no specific monitoring component for environmental effects of PIPs (with the exception of monitoring related to insect resistance management), FIFRA requires reporting of any adverse effects and periodic review for all pesticides to ensure that their registrations continue to meet its regulatory standard.

U.S. EPA's Approach to Accessing the Environmental Risk Assessment of Nontarget Organisms

The environmental risk assessment of PIPs is a formalized, objective, science-based process, and utilizes the same approach as with other pesticides, wherein risk is considered a function of hazard and exposure. The actual data required is determined prior to regulatory submissions during pre-submission meeting(s), in which an applicant has the opportunity to consult with the Agency prior to submitting their data packages to increase the likelihood of supporting the proposed regulatory action. Pre-submission meetings are important because they provide the opportunity for the applicant to present preliminary analysis of the inherent risks, such as existing scientific literature and initial product analysis, to the Agency for comment and discuss what data are appropriate to satisfy data requirements. Additionally, the

14 The contents of this presentation reflect the thoughts and opinions of the speaker and do not represent an official policy statement from the U.S. Environmental Protection Agency or other federal government agencies. Any mention of a product does not constitute an endorsement by the U.S. federal government.

applicability of any existing data to address data requirements through means other than testing (*e.g.*, scientific rationale supported by literature) can also be discussed.

Risk assessments are structured such that risk is determined first from estimates of hazard under "worst-case" exposure conditions. A lack of adverse effects under these conditions would provide enough confidence that there is no risk and no further data would be needed. Hazard to nontarget organisms is determined through testing according to a tiered scheme. This process and rationale was developed by the U.S. EPA's Biopesticides and Pollution Prevention Division within the Office of Pesticide Programs for evaluating hazard of PIPs to nontarget organisms based on recommendations from several EPA FIFRA Scientific Advisory meetings, ongoing findings from scientific literature, as well as EPA's regulatory experiences since the advent of regulating PIPs in 1996.

EPA's Tiered-Testing Framework

The EPA uses a tiered (Tiers I-IV) testing system to assess the toxicity of a PIP to representative non-target organisms that could be exposed to the toxin in the field environment. Tiered tests are designed to first represent unrealistic worst case scenarios and only progress to real world field scenarios if the earlier tiered tests fail to indicate adequate certainty of acceptable risk. Testing methods which utilize the tiered approach were last published by the EPA as Harmonized OCSPP Testing Guidelines, Series 850 and 885 (EPA 712-C-96-280, February 1996)[15]. These guidelines, as defined in 40 CFR 152.20, apply to microbes and microbial toxins when used as pesticides, including those that are naturally occurring, and those that are strain-improved, either by natural selection or by deliberate genetic manipulation. Therefore, PIPs containing microbial toxins are also covered by these testing guidelines.

Tier I high dose studies reflect a screening approach to testing designed to maximize any toxic effects of the test substance on the test (non-target) organism by representing exposure concentrations several times higher than the highest concentrations expected to occur under realistic field exposure scenarios. The Tier I screening maximum hazard dose (MHD) approach to environmental hazard assessment is based on some factor (whenever possible >10) times the maximum amount of active ingredient expected to be available to terrestrial and aquatic non-target organisms in the environment (EEC)[16]. Tier I tests serve to identify potential hazards and are conducted in the laboratory at high dose levels which increase the statistical power to test the hypotheses. Elevated test doses, therefore, add certainty to the assessment, and such tests can be well standardized. The screening tests evaluate single species in a laboratory setting with mortality as the end point. The trigger for additional higher-tier testing is 50% mortality. Less than 50% mortality under these conditions of extreme exposure suggest that population effects are likely to be negligible given realistic field exposure scenarios. Tiers II–IV generally encompass definitive hazard level determinations, longer term greenhouse or field testing, and are implemented when unacceptable effects are seen at the Tier I screening level.

Selection of Surrogate Testing Species

Tier I testing is the first step in an ecological risk assessment of pesticides. The surrogate concept using indicator organisms is a fundamental part of hazard testing within an ecological risk assessment. Testing of all potentially exposed invertebrates will never be possible and thus the selection of appropriate indicator species for laboratory testing is very important. Logical consideration and defined criteria may be useful when identifying appropriate individual indicator species (*e.g.*, Dutton *et al.* 2003). Non-target invertebrates identified for testing may be representative of those that are important in the crop of interest. The subset of species selected may represent different habitats (*e.g.*, below the soil surface, soil surface, plant canopy), ecological functions (*e.g.*, predator, parasite or decomposer), and taxonomic groups (*e.g.*, relationship to the target pest). The species tested may be chosen based upon their ecological and economic importance, and their consistent performance in Tier I tests.

15 OPPTS Testing Guidelines, Series 850 and 885 website: http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/885Microbial_Pesticide_Test_Guidelines/Series

16 The dose margin can be less than 10x where uncertainty in the system is low or where high concentrations of test material are not possible to achieve due to test organism feeding habits or other factors. High dose testing also may not be necessary where many species are tested or tests are very sensitive, although the test concentration used must exceed 1X EEC.

Test organisms are typically chosen case-by-case according to the potential for exposure to the insecticidal protein (taking into account the crop and the region of introduction), as well as the ability to test the organism in the laboratory (EPA-SAP 2000). In cases where a representative exposed non-target organism cannot be adequately tested in the laboratory, a closely-related indicator that can be easily reared may be substituted (EPA-SAP 2000). Representative indicator species are typically chosen from primary ecological functional groups that may be exposed to an insecticidal protein in the field.

Functional groups may include:

1. beneficial natural enemies (*e.g.*, predators and parasitoids),
2. pollinators (*e.g.*, bees, syrphids, lepidopterans),
3. decomposers (*e.g.*, Collembola, earthworms, nematodes, mites, psocids),
4. non-target herbivores (*e.g.*, monarch butterfly), and
5. herbivores that can serve as alternative prey for key natural enemies (EPA-SAP 2002). Standardized indicator species are chosen based on the reliability, repeatability, and cost effectiveness of suitable laboratory tests (EPA-SAP 2002).

Conclusion

Early tier lab testing helps eliminate unnecessary lines of investigation from further consideration by focusing on the collection of data that are useful for the risk assessment. This approach utilizes the minimum amount of data needed to make scientifically sound regulatory decision. It the Agency's collective experiences that have shown the value and effectiveness of early tier lab testing for the assessing impact on NTOs. There is still a need for validated tests to harmonize the methods used to evaluate effects of GM crops on non-target arthropods. This ensures comparability of data, recognition of data provided by various labs, and acceptability of data by regulatory agencies in addition to continual overall improvement of quality of risk assessment. However, one of the most critical elements to the Agency's environmental risk assessment of PIPs is the applicant-to-regulator consultations held during pre-submission meetings and it is highly recommended that applicants request these meetings prior to their data submissions. In conclusion, the combination of the established tiered-testing approach, pre-submission meetings, seeking outside expert opinion, ongoing scientific findings, and addressing the recommendations included here helps to ensure sound regulatory decision based on transparency and the best available science and EPA's mission to protect the environment from unreasonable adverse effects can be more readily fulfilled.

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3.4 TRANSPORTABILITY OF ECOLOGICAL TOXICITY TEST DATA FOR AN ARTHROPOD-ACTIVE GE EVENT IN THE CONTEXT OF USING THE SURROGATE SPECIES APPROACH FOR NONTARGET ORGANISM TESTING

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Tiered testing with representative surrogate species to assess toxicity to nontarget organisms (NTOs) was originally developed as a relevant and reliable process to assess the effects of conventional pesticides to nontarget organisms. Laboratory NTO testing for conventional pesticides generally follows internationally harmonized test guidelines which enables data transportability and mutual acceptance of data. In contrast, country- or region-specific ecological effects information is typically required for arthropod-active GE events. This diversity of region-specific requirements can be the result of various circumstances such as differences in national laws, regulatory mandates, and regulatory climates. This situation can result in duplicative efforts and, at times, the generation of incongruent conclusions regarding the quality and utility of ecological effects data. International efforts, including this meeting on surrogate species, are working towards harmonization and transportability of Tier 1 ecological effects test methods that use the surrogate species approach. Achieving harmonization of Tier 1 test methods will expedite best practices, minimize differences in core data sets, maximize efficiency of data development, and facilitate the transportability of core data packages across geographies (Romeis *et al.*, 2008; Romeis *et al.*, 2011).

The surrogate species approach evolved because not all NTOs can be tested, and it enables selection of representative taxa and functional guilds for effects testing. Test organisms for laboratory and semi-field studies are typically chosen based on potential for exposure to the plant-produced pesticidal substance (*i.e.*, relevance) as well as the ability to dependably test the organism in the laboratory (*i.e.*, reliability) (Barrett *et al.*, 1994; Rose, 2006; Romeis *et al.*, 2008). The Tier 1 laboratory NTO battery provides: (1) valuable information on taxonomic specificity which can significantly reduce the scope of testing if biological activity is shown to have high taxonomic specificity; and (2) provides a conservative screen to identify potential adverse effects to beneficial organisms in cropping systems (*e.g.*, biological control, pollination, decomposition). Uncertainties associated with the surrogate species approach are addressed by requiring sufficient margins of safety (*i.e.*, safety factors) between conservative estimates of environmental exposure and measurement endpoints (*e.g.*, no observed adverse effect level (NOAEC) or LCx value). Safety factors allow for extrapolation among related species, inter-assay variation, lab to field extrapolations, and uncertainties associated with routes and levels of exposure (Klaassen, 2008). Addressing these uncertainties, with adequate margins of safety, provides a foundation for transportability of laboratory effects data.

Selection of appropriate surrogate species requires an analysis of routes of exposure, identifying appropriate and representative ecological receptors, and the ability to reliably test the organism in the laboratory. A recent scientific review reported that for most arable crops, the taxa identified in the major beneficial insect groupings are comparable across the geographies in the European Union (Meissle *et al.*, 2012). The same conclusion was reached in a similar in-house assessment for maize, where the major beneficial insects in maize were found to be similar across geographies. In other words, for most arable crops, beneficial NTO groupings identified in a given geography are typically similar across geographies. This finding supports the use of a Tier 1 NTO battery that includes appropriate and representative indicator organisms. To evaluate the predictive and protective capability of the Tier 1 NTO battery, Duan *et al.* (2010) performed a retrospective validation of the tiered approach for arthropod-active GE events. This meta-analysis concluded that Tier 1 laboratory studies have been accurate and conservative in evaluating the environmental safety of Bt crops. This conclusion is consistent with findings by Rauschen *et al.* (2010), which recommended that potential effects on key biocontrol agents (*e.g.*, Coccinellids) are most reliably assessed in lower tiered lab studies because of the high level of natural variability observed in the field.

Opponents of biotech crops frequently refer to unintended effects and claim that such effects may be hazardous and unsafe. This position has created barriers to implementing tiered testing and implementation of the surrogate species

approach. Several national and international organizations have carefully evaluated this topic. An EPA FIFRA Scientific Advisory Panel Report (2004) clearly states that unintended effects from the addition of transgenic DNA represents a hypothetical hazard and that there is no evidence to support that significant risks exist, over and above those associated with conventional plant breeding. Similarly, the U.S. National Academy of Sciences (2004) concluded that biotech crops do not pose any more health risks than do crops developed by other techniques and that food safety evaluations should be based on the resulting food product, not the technique used to develop it. A work group within the Organization of Economic Cooperation and Development (OECD) came to a similar conclusion stating "The risks associated with biotechnology-derived foods are not inherently different from the risks associated with conventional ones" (OECD, 2000). While experts acknowledge there is a potential for unintended effects to occur, Kuiper *et al.* (2000) and Haslberger (2003) state that such unintended effects are also possible for conventional plant breeding. The important distinction however, is that biotech crops undergo a thorough safety assessment prior to approval by regulatory authorities, while conventional crops do not undergo assessments to address potential unintended effects. For example, with biotech crops, extensive agronomic, performance, phenotypic and field analyses select against potential unintended effects and eliminate >99% of all transformation events. Additionally, detailed molecular and biochemical characterization of an expressed arthropod-active trait is performed to support the safety assessment. Potential unintended effects are also evaluated by compositional analysis of key nutrients and anti-nutrients as well as with animal nutrition and performance studies to confirm lack of any meaningful unintended effects.

3.4.1 Transportability of Tier 1 NTO Studies to Support Combined Trait Products

For over a decade, arthropod-active GE products, developed by combining previously registered events through conventional breeding, have been on the market. Many regulatory authorities have developed specific data requirements for import and/or cultivation approval that enable bridging to, and transportability of, existing product safety packages for the single trait arthropod-active products. As an example, in 2009 the USEPA codified their requirements to bridge existing data from a previously registered arthropod-active event to a new combined trait product. The EPA requirements permit a science-based approach that supports the use and portability of existing NTO safety data developed with the surrogate species approach (USEPA, 2009). The USEPA approach calls for bridging data from three separate areas. The first condition is confirmation that the presence and structure of the inserted material has been conserved in the combined trait product. The second condition is that expression of the arthropod-active trait is comparable in the single and combined trait product, which confirms no biologically meaningful increase in exposure to NTOs. The third condition is demonstrating the lack of synergism between arthropod-active traits using sensitive insect bioassays. Demonstrating the lack of synergism permits the application of the principle of independent assessment. The principle of independent assessment has a long history of use in toxicology. This principle states that provided each substance in a mixture acts independently, and the substances are below their no observed adverse effect level, their toxicity can be assessed independently (USEPA, 2004; USEPA, 2009). As stated above, satisfying the requirements of this principle enables the use of existing hazard studies performed separately for the individual substances to assess the safety of the combined trait product.

3.4.2 Conclusions

Recent analyses of beneficial insect communities for most arable crops concluded that the major taxa groupings are comparable across the geographies. Therefore, representative surrogates can be selected for Tier 1 testing that provides a conservative screen to assess potential adverse effects and can be used in ecological effects assessments performed across geographies. Additionally, a core set of bridging studies can be performed to evaluate the safety of a combined trait product and enable the use and transportability of the NTO studies performed for individual traits. Provided that general bridging conditions are satisfied, no additional NTO testing with all of the insecticidal proteins in combination should be required (EPA, 2009).

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3.5 PRACTICAL CONSIDERATIONS FOR SURROGATE SPECIES SELECTION FOR SEMI-FIELD AND FIELD TESTS

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3.5.1 Introduction

In the tier-based system for an environmental risk assessment (ERA) of genetically engineered (GE) crops, semi-field and field tests are conducted when uncertainly in the risk assessment remains after lower tier laboratory tests are conducted (Romeis *et al.* 2008). Higher tier tests are not necessary, accordingly, if effects are not detected in laboratory tests. The reliability of the tier approach is supported by meta analyses that show effects found in the laboratory studies, at least for *Bacillus thuringiensis* (Bt) crops, are consistent with or more conservative than those found in field studies (Duan *et al.* 2009). Even so, field tests often are conducted when they are not scientifically justified because of cultural and legal ramifications. Whether field tests that monitor for unintended effects of GE crops on NTO are necessary is controversial, but most scientists agree there is a need to improve the design of such studies and particularly to identify appropriate survey taxa. For this presentation surrogate species are replaced by representative taxa, which for reasons that follow appear to be a better fit. This presentation focuses on many aspects of using field studies for the ERA of GE crops with emphases

on considering the complexity and challenges of field tests, designing efficient field tests, and developing criteria for selecting representative taxa.

3.5.2 Complexity and Challenges of Field Tests

Field tests are more realistic than laboratory tests but by and large are more complex, have less defined protocols and have lower statistical power. In the case of Bt crops, disturbance by the stressor, Bt protein, is generally small compared with disturbances that commonly occur in the field from farming practices (*e.g.*, previous crop, planting, tillage, harvesting, and use of fertilizers and herbicides), natural disturbances (*e.g.*, local storm flooding, temperature extremes, plant diseases and pests), and topography variation (*e.g.*, soil type, elevation, and vegetative residue). Researchers must consider this signal-to-noise ratio when designing studies. Because of these limitations, studies should be as focused as possible with testable hypotheses generated from problem formulation. Census or fauna-type studies with nebulous objectives are not appropriate, although baseline studies in some cases may be useful.

Understanding how a stressor influences the interactions of species within a complex cropping system is simplified by considering probable exposure to the stressor and degrees of separation from this exposure. In the case of Bt maize this means focusing on arthropods that feed directly on maize tissue: herbivores and pollen and silk feeders—one degree of separation, and then predators or parasitoids—two degrees of separation. Higher degrees of separation generally are not necessary because Bt proteins degrade rapidly and are diluted in the food chain. Certainly from a risk assessor's perspective, the value of considering other interactions quickly reaches a point of diminishing returns.

3.5.3 Efficient Field Tests

Many sampling techniques are available for conducting biotic surveys in agricultural fields (*e.g.*, visual counts and sticky cards to survey above ground arthropods, and litter bags and pitfall traps to survey surface and below ground arthropods). Sampling plans should target those taxa that are considered the most informative to the risk assessment. Field sampling is complicated by seasonal and location fluctuations of many arthropod populations, as found with ground beetle species (Lopez *et al.* 2005), so determining when and where to sample is a challenge. Of course, properly designed experiments must have appropriate controls, including a near-isoline crop and, if possible, the near-isoline crop treated with traditional control measures.

Even the most efficient sampling techniques cannot overcome problems if plot size is not sufficient and representative taxa move among research plots. This is a thorny problem because the appropriate plot size can vary dramatically depending on the mobility of the sampled taxa. For example, adequate plot size for an ERA for ground-dwelling Collembola may be one square meter, but an adequate plot size for some flying insects may be too large to be practical. With Bt proteins, focusing on immature insects may be preferred especially since Bt is most effective against larval stages of specific insects and immature insects generally are less mobile. Pilot studies may be required to determine the most efficient plot size for a representative taxa; a general guideline is plots should not be smaller than 9 m^2 (Prasifka *et al*. 2005). Also, researchers should be aware of possible border effects because many insect populations are higher along field margins, especially if they are undisturbed. In such cases guard rows are often used to buffer such effects.

3.5.4 Representative Taxa

Rather than identifying surrogate species for field tests, a more practical approach may be identifying representative taxa. Important factors to consider when selecting such taxa are probable exposure, likelihood species would be affected, sufficient populations (regional and season-long presence), straightforward sampling, and simple identification (Prasifka *et al.* 2008). Perceived importance of the organism also should be considered. Transportability of data from field studies could be facilitated if representative taxa are selected that commonly occur in other regions of the world. For example, 3-4 families of beetles could be used as representative taxa to test coleopteran-active Bt crops, which could be selected

based on abundance, ecological function or both (see Romeis presentation). Research is needed to determine whether such field data could be transportable among different cropping systems, which arguably could occur if taxa within these systems are similar. From an ecological perspective one could argue that since agricultural systems are disturbed habitats they continually attract succession species. This may suggest that other than the crop species there are no "keystone" species in agricultural habitats, to paraphrase a colleague "there are no grizzly bears in a cornfield".

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3.6 APPLYING PROBLEM FORMULATION TO HELP IDENTIFY RISK HYPOTHESIS RELEVANT TO ENVIRONMENTAL IMPACT OF ARTHROPOD-ACTIVE GE PLANTS ON NON-TARGET ORGANISMS

Alan Raybould, Ph.D., Syngenta, United Kingdom

Presentation summary to be provided

3.7 ARTHROPODS CONTRIBUTING TO ECOSYSTEM SERVICES IN DIFFERENT CROPPING SYSTEMS

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Arthropods form a major part of the biodiversity in agricultural landscapes. Many species are valued because they provide ecosystem services, including biological control, pollination, and decomposition, or because they are of conservation value. A common concern addressed in the ecological risk assessment (ERA) that precedes regulatory approval of genetically engineered (GE) varieties is their potential to adversely affect such valued non-target arthropods (NTAs) present in the receiving environment, *i.e.*, the area in which the GE plant is likely to be grown. Since not all NTAs present in the receiving environment can be tested, surrogate species must be identified to represent the entities to be protected. This, however, requires a description of the arthropod fauna in the receiving environment.

For this purpose, the European Food Safety Authority (EFSA) has commissioned a project to establish a database of bio-ecological information on NTAs found in relevant agro-ecosystems in Europe. To build the database, a large quantity of literature has been screened (>23,000 references) in a systematic manner, and data from >1,100 publications have been entered. At the current state, the database contains more than 13,000 arthropod records for 3,030 species and seven crops (maize, potato, beet, oilseed rape, rice, soybean, and cotton). Information contained in the database includes the taxonomy, geographical distribution, abundance, habitat, ecological function, and feeding guild of each arthropod species as well as collection details (*e.g.*, sampling method, study duration, taxonomic range).

Arthropods contained in the database are distributed over 30 orders and 269 families. Coleoptera, Hemiptera, Araneae, Diptera, Lepidoptera, Hymenoptera, and Collembola represent 92% of all species recorded. Predators and herbivores constitute more than 70% of the species, followed by decomposers, parasitoids, aquatic species (in rice), and pollinators.

The herbivore communities differ substantially among crops. Key pests of each crop are usually the most collected arthropods. In general, the main herbivorous species are butterflies and moths (Lepidoptera), beetles (Coleoptera), bugs, leafhoppers, and aphids (Hemiptera), and midges and flies (Diptera).

Overall, the taxonomic composition of predators, parasitoids, pollinators, and decomposers is relatively similar for all crops (except rice). The predators are largely dominated by beetles (Coleoptera) and spiders (Araneae) followed by hoverflies (Diptera: Syrphidae) and predatory bugs (Hemiptera: Heteroptera). Among beetles, ground beetles (Carabidae), rove beetles (Staphylinidae), and ladybird beetles (Coccinellidae) have been collected most frequently in all crops except rice, where aquatic beetles dominated. Among spiders, Linyphiidae and Lycosidae are the main families collected.

The parasitoids in the database belong to three orders, with parasitic wasps (Hymenoptera) being by far the most important, followed by tachinid flies (Diptera) and rove beetles from the genus *Aleochara*. Pollinators, which have been frequently collected in oilseed rape and less frequently in potato, beet, and maize, are dominated by bees and bumblebees (Hymenoptera: Apidae). Non-predatory aquatic species in rice fields are mostly represented by mosquitoes (Diptera). Decomposers (including soil-inhabiting fungivores) are dominated by springtails (Collembola) and mites (Acarina), followed by flies and midges, and beetles.

If evidence indicates that phylogeny could be a reasonable predictor of arthropod susceptibility to an arthropod-active protein newly expressed by a GE plant, then the taxonomic information in the database could be used to identify species in the receiving environment most likely at risk. We currently see three ways in which the database could be used to support the ERA for GE crops:

(i) Identification of test species for laboratory studies that are representative of NTAs potentially at risk because they are likely to be exposed to the arthropod-active protein.

(ii) Identification of species for higher-tier studies in cases where non-target studies need to be conducted under more realistic semi-field or field conditions.

(iii) Identification of appropriate species for case-specific monitoring. This includes those species or groups of arthropods that are abundant, widely distributed, and most relevant to meeting protection or management goals in the receiving environment.

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4. DISCUSSION

A requirement of all regulatory programs governing the development and use of GE crops is the determination of whether the presence of the crop in the environment is likely to have unexpected or inadvertent effects. Central to this determination is an evaluation of the likelihood of adverse impacts on non-target organisms (NTOs).

Because it is not possible to test every organism in the environment that may come in contact with the GE crop, researchers typically use a select group of species that act as surrogates for species that are present in the environment in question but, for various reasons, cannot be tested in the laboratory (Garcia-Alonso *et al.*, 2006; Romeis *et al.*, 2008). Many regulatory authorities readily accept such surrogate testing data in their safety assessment processes, and the data generated to date has been predictive of impacts from GE crops on natural NTO populations. The question arises, however, as to whether the global adoption of GE crops in diverse geographic locations, with NTO populations different from the location where the crop was first developed and tested, necessitates a re-examination of how surrogate species are selected for NTO testing.

For the purposes of this conference, participants were asked to focus their discussions on two fundamental questions regarding surrogate species testing:

1. Which ecological functional groups should be represented in the testing of arthropod-active GE crops and why?
2. What are appropriate criteria for the selection of surrogate species for laboratory, semi-field, and field tests?

The findings of this discussion were then applied to the analysis of two case studies involving novel GE crops with insect-resistance traits to determine if the findings from the earlier discussion provided useful, robust guidance in selecting appropriate surrogate species.

4.1 FUNCTIONAL GROUPS TO BE REPRESENTED IN ECOLOGICAL TESTING OF ARTHROPOD-ACTIVE GE CROPS

There was agreement among the participants as to the primary ecological functions that should be considered when assessing arthropod-active GE crops: herbivores, pollinators, predators and parasitoids, and decomposers. It was noted that herbivores are generally not valued species within an agroecosystem, but they may have charismatic value, such as the monarch butterfly, or other valued functions, and their abundance may affect food webs. It was determined that the particular functional groups to be represented in the assessment of a specific GE crop should be chosen on a case-by-case basis. Because ecological function largely determines the path of exposure, functional groups with likely exposure to a particular GE crop should be the focus of the assessment, while the testing of functional groups for which exposure to the crop would be unlikely or remote, temporally or geographically, would be unnecessary.

The participants identified and discussed several nuances in the selection of appropriate functional groups for GE crop assessment, as well as the selection of species within those groups. For example, some species, such may have multiple functional roles, such as syrphid flies, which function as predators when larvae and as pollinators when adults. Conversely, a single ecological functional group may comprise multiple specialized functions. For example, the herbivore group contains many specialized feeding sub-types or guilds—leaf versus root consumers, as well as phloem feeders—and these sub-types may be represented by diverse species. The participants determined that functions (and functional sub-types) associated with exposure to the GE crop are the crucial ones for inclusion in the assessment. Once a particular route of exposure is identified, a surrogate could be selected that adequately represented the organism that is exposed to the GE crop. The basis of representation is therefore not shared function, but shared susceptibility to the toxin.

In light of these nuances, the participants considered whether reliance on ecological function was as useful as phylogenetic relatedness, especially for Tier 1 testing. The rationale was that, in laboratory tests, the species used are physiological surrogates, not functional ones, because function is unlikely to correlate with toxicological sensitivity. Although this assumption is not always borne out, it was held generally true that phylogeny predicts physiology. Therefore in the context of Tier 1 testing, it made more toxicological sense to select surrogates based on their relatedness to the species of concern. For example, in conducting a Tier 1 test of the hypothesis that the GE crop will not negatively affect a key pollinator, the test should be conducted on a species closely related to the pollinator, rather than on a species that merely functions as a pollinator. Similarly, if impacts on an endangered species are in question, the appropriate surrogate would be a species that is phylogenetically related, rather than functionally related.

However, the functional role of an affected species becomes increasingly relevant as the testing moves to higher tiers. For example, if Tier 1 testing indicates an adverse effect on coleopterans, then the assessment should include higher-tier testing to evaluate the impact on the functions performed by coleopteran insects in the environment where the crop will be grown.

4.2 CRITERIA FOR SURROGATE SPECIES SELECTION

As a result of the discussion, several criteria were identified for the selection of relevant and reliable surrogate species for laboratory and semi-field testing of arthropod-active GE crops. Of primary concern was that the test organisms have a substantial history of use in the laboratory and/or semi-field context and that test protocols developed for these organisms be validated and have the power to detect potential adverse effects. Each organism chosen should be easily reared under controlled, standardized conditions so as to provide large numbers of consistent individuals with a high level of fitness, and the organisms should be amenable to the kinds of manipulations necessary to administer the tests. (See, generally, Carstens *et al.*, 2012.) Assuming validated protocols have been established for them, indicator species, that is, species known to be particularly sensitive to ecological change, may be appropriately included as candidate surrogate species. The desired outcome, following the application of the selection criteria, would be a large number of candidate surrogate organisms, representative of relevant species in many diverse geographic locations, which together would become a "toolbox" for researchers from which appropriate species could be drawn for laboratory or semi-field testing.

Participants concurred that, subject to acceptability by regulatory agencies, data species other than those typically used in nontarget testing may be informative to risk assessment: even a known pest species could be an appropriate surrogate. For example, pest Lepidoptera can be surrogates for phylogenetically related protected butterfly species. Data generated while determining the activity spectrum of a pesticide on various pest species can provide valuable information to inform NTO selection and testing.

The question arose as to the selection of appropriate species when the GE trait does not result in the production of a pesticidal substance or if the pesticidal substance produced has an unknown spectrum of activity. The participants determined that such circumstances underscored the need to include as many species in the toolbox as met the selection criteria, to enable the imposition of additional criteria, on a case-by-case basis, to identify appropriate organisms, depending on the specific trait and crop.

Additional discussion elucidated how the choice between a functional approach and a phylogenetic approach to surrogate species selection could affect selection criteria. For example, when using a functional approach, researchers should select surrogates performing functions present in the locality where the crop will be planted. On the other hand, when using a phylogenetic approach, the ubiquity of an organism may be correlated with the significance of its role in the environment, and selecting a surrogate for an abundant species may result in more powerful statistical data. The participants did agree that an approach including some test species selected on a functional basis and others on a phylogenetic basis may be appropriate. For example, the participants observed that a function, such as herbivory, is unlikely to correlate well with susceptibility to a particular toxin, due to the variety of organisms that feed on leaves. Similarly, phylogeny is not consistently correlated with toxicological sensitivity, as it is known that not all lepidopteran species are susceptible to known Lepidoptera-active Bt toxins.

In other cases the test results themselves may direct surrogate selection for additional laboratory and semi-field testing. Tier 1 tests may inform the surrogate selection process for semi-field tests. The logistics of performing semi-field tests may also drive the selection of appropriate surrogates. For example, large populations of organisms are desirable to obtain more statistical power. If there is no representative species present in sufficiently large numbers, it may be reasonable to select a genus- or family-level surrogate. Participants agreed that the question of surrogate selection was not relevant to conducting field tests, as the researcher is directly observing the species of concern.

One of the most important conclusions drawn from the discussion was that, as these surrogate choices are made, the researcher must be explicit as to whether a particular test organism is being used as a phylogenetic surrogate or a functional one, or both, and state any assumptions implicit in the choice of a particular surrogate species.

Lastly, discussion acknowledged that non-scientific reasoning may underlie the selection of some test species . Charismatic species such as the monarch butterfly or beneficial species, such as the honeybee, may be included in non-target organisms testing even when a scientific basis for the route of exposure or toxicological sensitivity is lacking. Such a species may be tested due to its economic value (*e.g.*, honeybees), because it has become customary in a particular regulatory jurisdiction, or because the general public demands such testing.

5. CASE STUDIES

Two case studies provided an opportunity to apply the concepts of surrogate selection and non-target organism impact testing to examples of novel crop/trait combinations. The first case study involved a rice variety, developed for use in India, producing two different insecticidal proteins from *Bacillus thuringiensis*—Cry1Ab and Cry1Ac. The second case study involved a soybean variety, developed for Brazil, producing (E)-ß-farnesene, an aphid alarm pheromone. The soybean variety also contained a herbicide tolerance gene.

5.1 APHID-RESISTANT SOYBEAN

The case study involved a trait that has been used only in experimental contexts, so little is known about the mode of action or activity spectrum of the active ingredient. In addition, as opposed to toxic proteins derived from *B. thuringiensis*, the gene in this case study encodes a protein that is not a toxin but an enzyme in the biosynthetic pathway of a volatile secondary metabolite not ordinarily made by soybean. This metabolite, (E)-ß-farnesene, is the active ingredient (a.i.). It is known that the gene comes from peppermint—a plant that is edible and successfully pollinated by bees. In addition, peppermint has been grown as a crop on large acreages for many years without evidence of adverse impacts on the agroecosystem. Information regarding insect pests attacking peppermint may help in assay design, but in the absence of a full toxicological profile for the a.i., participants concluded that testing the a.i. on surrogates spanning a broad taxonomic range, including pest and non-pest species, is advisable. There was little expectation that the enzyme would be toxic or affect insect behavior, but participants agreed that a minimal amount of testing on a few species would be necessary.

Since the a.i. works largely as an aphid repellent and only secondarily as an aphid toxin, the starting hypothesis was that neither the a.i. nor the gene product ((E)-ß-farnesene synthase) would repel non-aphid insects. The hypothesis would be tested using all standard surrogate species phylogenetic groups (Hymenoptera, Diptera, Coleoptera, Neuroptera) and known soybean insect pests. Participants noted that laboratory studies on insect behavior are known to result in a high rate of false positives. If repellent effects are noted, higher-tier testing would be done to ascertain whether the crop will affect insect behavior either on the crop itself or on neighboring plants in ways that might impact biocontrol, herbivory, or pollination functions.

It was agreed that semi-field and field tests could not be conducted simply by spraying the a.i. on plants. The primary challenges with higher-tier testing would be determining the number of plants necessary to produce an environmentally relevant atmospheric concentration of the a.i. and, subsequently, determining whether insect behaviors have measurably changed. Participants assumed that effects from the a.i. are likely concentration dependent, and because the a.i. is volatile and short-lived in the environment, detecting off-field effects would be difficult. In fact, it was noted that small, semi-field tests may not be feasible, since a small number of soybean plants may not generate sufficient a.i. to have a measurable effect. Various trapping and scouting methods were proposed to detect changes in insect movement and distribution, but the participants questioned whether the tests being proposed had been validated.

5.2 LEPIDOPTERAN-RESISTANT RICE

The rice case study involved two well-studied and understood genes used extensively in other crop species, but less so in rice. To determine an appropriate approach to testing the effects of this rice variety on non-target organisms, the discussion focused on ways in which a new use of existing genes could result in environmental harm. A primary question was whether the two genes present in a single plant could cause additive or synergistic effects that could be tested using from one to a few known sensitive species. If no protein interactions were noted, there is a large, pre-existing body of evidence available to establish the environmental safety of a rice variety containing these two Bt genes. The discussion identified the production of rice in an aquatic environment as a significant difference in exposure and noted that Trichoptera (caddisfly) and Odonata (Zygoptera) damselfly might be species meriting further study for non-target impacts. However, there are currently no good surrogates for the damselfly.

The discussion acknowledged that, in spite of existing data documenting the environmental safety of Cry1Ab and Cry1Ac, non-target testing may still be required under various regulatory regimes and suggested that typical assays of non-target effects could be done, including a non-susceptible lepidopteran; a lepidopteran species known to feed on wild rice species; larval honey bees as a surrogate for larval hymenopteran parasitoids; ladybird beetle as a surrogate for coleopterans and predators; and adult honey bees as a surrogate for adult hymenopterans.

6. CONCLUSIONS

Following extensive discussion in the breakout groups and in the final plenary session, the conference participants advanced the following points of consensus:

1. Provided adequate margins of safety, hazard testing that is used to inform the in-field assessment informs the off-field assessment.
2. Surrogate species are the appropriate test organisms for laboratory and semi-field studies.
3. Representative taxonomic groups are the appropriate level of resolution (test unit) for census field studies.
4. Measures of "surrogate" processes representative of ecological function in the field can be a valuable tool.
5. Identifying faunistic similarities across geographies supports data transportability.
6. Field studies should focus on the taxa that are one or two trophic levels away from the crop.
7. Sufficient information for robust/rigorous risk characterization can be developed through problem formulation, the tiered testing process, and the use of surrogate species.
8. Pests can be used as surrogates.
9. There was consensus on surrogate selection criteria for early tier tests.

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ANNEX 1 — CONFERENCE AGENDA

TUESDAY, JUNE 26, 2012

Time	Title	Presenter
0830	Welcome and Introductions Meeting Objectives and Format	Morven McLean *CERA, USA*
0850	Retrospective on the evolution of surrogate species selection for pesticide testing Q&A	Marco Candolfi *IES, Switzerland*
0930	Host range testing for natural enemy introductions Q&A	Barbara Barratt *AgResearch Limited, NZ*
1000	**Break**	
1030	Country-specific approaches to assessing adverse environmental impacts of GE plants on non-target organisms (20 minutes each)	
	• Argentina	Patricia Gadaleta *MAGyP, Argentina*
	• Brazil	Fernando Valicente *Embrapa, Brazil*
	• European Union	Adinda De Schrijver *GMO Biosafety and Biotechnology Unit, Belgium*
	• Mexico	Ariel Alvarez *CIBIOGEM, Mexico*
	• Philippines	Bonifacio Cayabyab *Los Baños, Philippines*
	• USA	Annabel Waggoner *USEPA, USA*
1230	**Lunch**	
1330	Transportability of ecological toxicity test data for an arthropod-active GE event Q&A	Steven Levine *Monsanto Company, USA*
1410	Practical considerations for surrogate species selection for semi-field and field tests	Rick Hellmich *USDA ARS, USA*
1450	Applying problem formulation to help identify risk hypotheses relevant to environmental impact of arthropod-active GE plants on non-target organisms Q&A	Alan Raybould *Syngenta, UK*
1530	**Break**	
1600	Grouping of arthropod families by valued functions in different cropping systems Q&A	Joerg Romeis *ART, Switzerland*
1640	Introduction for Breakout Group Activity	Morven McLean *CERA, USA*
1730	**Close of Day 1**	

	WEDNESDAY, JUNE 27, 2012
0830	Breakout Session I Discuss: 1. The functional groups that should be represented in ecological testing of arthropod-active GE crops (and why); and 2. Criteria for surrogate species selection for lab, semi-field and field tests. Group 1: West Conference Room — Group 2: Board Room Group 3: Malaspina Room — Group 4: 4th Floor Meeting Room (in CropLife America's offices)
1030	**Break**
1030	Report back from Breakout Session I (20 minutes each; West Conference Room) • Group 1 • Group 2 • Group 3 • Group 4 Q&A
1230	**Lunch**
1330	Breakout Session II Based on the findings from the morning session, discuss the case studies and evaluate if the functional groups and selection criteria apply. Group 1: Case Study I (West Conference Room) — Group 2: Case Study II (Board Room) Group 3: Case Study I (Malaspina Room) — Group 4: Case Study II (4th Floor Meeting Room)
1500	**Breaks as needed**
1630	Preparation for reports for Breakout Session II readout
1730	**Close of Day 2**
	THURSDAY, JUNE 28, 2012
0830	Reports from Breakout Session II (20 min per group) • Group 1 • Group 2 • Group 3 • Group 4 Q&A
1000	**Break**
1030	Group Discussion on Consensus Points from Breakout Group Sessions — Convener: Andrew Robers
1230	**Close of meeting**

ANNEX 2 — APHIS-RESISTANT SOYBEAN IN BRAZIL (CASE STUDY 1)

A. INTRODUCTION

One of the most harmful insect pests on soybeans is the soybean aphid, *Aphis glycines*, which can reduce yields by as much as 40%. Current aphid management practices incorporate spray pesticides for severe infestations and reliance on parasitoids and other natural enemies of aphids when insect loads are lower. While there is increasing grower interest in reducing pesticide use, the efficacy of aphid control using natural predators is inconsistent, both geographically and temporally. Because aphid populations can double every 72 hours, inconsistent control results in serious crop losses. A few soybean genes have been identified conferring partial aphid resistance or tolerance, and these genes have been crossed into commercial varieties, but aphid biotypes have already begun to develop resistance to these traits.

Pangenetics Seeds (Pangenetics) has developed a new experimental soybean variety that is both herbicide tolerant and aphid resistant. Glyphosate tolerance is conferred by the well-characterized CP4 EPSPS gene. Aphid resistance is conferred by a novel, proprietary technology involving the expression of the (E)-ß-farnesene synthase (EßfS) gene from *Mentha piperita* (peppermint). EßfS catalyzes the conversion of farnesyl diphosphate into (E)-ß-farnesene (Eßf). Eßf is a sesquiterpene olefin that occurs in a wide range of plant and animal species, where it frequently functions as a chemical signal. In aphids, it is an alarm pheromone. Aphids exposed to Eßf become agitated, stop feeding, and disperse from their host plants, either by flying away or by dropping off the plant. In addition, Eßf is acutely toxic to aphids at a dosage of 100 ng/aphid. Eßf also functions as a kairomone, attracting aphid parasitoids and enhancing their foraging behavior.

Aphid control via direct application of Eßf has been ineffective due to the volatility of Eßf and its rapid deactivation through oxidation. However, Pangentics has demonstrated that transgenic soybean leaves can deliver Eßf to above-ground plant surfaces consistently and at biologically effective concentrations. Small-scale confined field trials with Eßf-producing soybean plants have demonstrated efficacy against aphid predation, and Pangenetics is seeking guidance on the testing of impacts of Eßf and EßfS on non-target organisms.

B. PART I: THE NON-TRANSGENIC PLANT

For more information about the biology of soybean, see OECD (2000).

B.1. General Description of the Plant

Cultivated soybean, *Glycine max*, is a diploidized tetraploid (2n=40), in the family Leguminosae, the subfamily Papilionoideae, the tribe Phaseoleae, the genus *Glycine*, and the subgenus *Soja*. It is an erect, bushy herbaceous annual that can reach a height of 1.5 metres. Three types of growth habit can be found among soybean cultivars: determinate, semi-determinate and indeterminate. Soybean is not frost tolerant, and it does not survive freezing winter conditions.

The primary leaves are unifoliate, opposite and ovate, the secondary leaves are generally trifoliolate and alternate. The nodulated root system consists of a taproot from which emerges a lateral root system. The plants of most cultivars are covered with fine trichomes. The papilionaceous flower consists of a tubular calyx of five sepals, a corolla of five petals, one pistil and nine fused stamens with a single separate posterior stamen. A soybean plant can produce as many as 400 pods, with two to twenty pods at a single node. Each pod contains one to five seeds. The pod is straight or slightly curved and consists of two halves of a single carpel which are joined by a dorsal and ventral suture. The shape of the seed, usually oval, can vary among cultivars from almost spherical to elongate and flattened.

Soybean is commonly considered one of the oldest cultivated crops, native to North and Central China. Historical and geographical evidence suggests that soybeans were first domesticated in the eastern half of China between the 17th and 11th century BCE.

B.2. Reproductive Biology of the Species

Soybean is considered a self-pollinated species, propagated commercially by seed. The soybean flower stigma is receptive to pollen approximately 24 hours before anthesis and remains receptive 48 hours after anthesis. The anthers mature in the bud and directly pollinate the stigma of the same flower. As a result, soybeans exhibit a high percentage of self-fertilisation, and cross pollination is usually less than one percent. Artificial hybridisation is used for cultivar breeding.

B.3. Center of Origin and Center of Genetic Diversity

Glycine max belongs to the subgenus *Soja*, which also contains *G. soja* and *G. gracilis*. *Glycine soja* (2n=40) is a wild viny annual with small and narrow trifoliate leaves, purple flowers and small, round, brown-black seeds. It grows wild in Korea, Taiwan, Japan, N.E. China, and areas around the border of the former USSR. *Glycine gracilis*, intermediate in form between *G. soja* and *G. max*, has been observed in Northeast China. Cytological, morphological, and molecular evidence suggest that *G. soja* is the ancestor of *G. max*. *Glycine gracilis* is considered to be a weedy or semi-wild form of *G. max*, with some phenotypic characteristics intermediate to those of *G. max* and *G. soja*, and may be an intermediate in the speciation of *G. max* from *G. soja* or a hybrid between *G. soja* and *G. max*.

In addition to the subgenus *Soja*, the genus *Glycine* contains the subgenus *Glycine*. The subgenus *Glycine* consists of twelve wild perennial species, including *G. clandestina*, *G. falcata*, *G. latifolia*, *G. latrobeana*, *G. canescens*, *G. tabacina*, and *G. tomentella*. These species are indigenous to Australia, South Pacific Islands, China, Papua New Guinea, Philippines, and Taiwan.

B.4. Means of Dispersal and Establishment

Soybean is propagated only by seed: there are no means for vegetative propagation. Neither the seedpod nor the seed has morphological characteristics that would encourage animal transportation.

Soybean seed rarely displays any dormancy characteristics and, only grows as a volunteer in the year following cultivation under certain environmental conditions. Volunteers do not compete well with the succeeding crop, and can easily be controlled mechanically or chemically. The soybean plant is not weedy in character. *Glycine max* is not found outside of cultivation in Brazil.

B.5. Intra-Specific, Inter-Specific and Inter-Generic Hybridization

Hybrids between *G. max* and *G. soja* and between *G. max* and *G. gracilis* have been easily obtained via hand pollination. Hybrids between diploid perennial *Glycine* species show normal meiosis and are fertile. Early attempts to hybridise annual (subgenus *Soja*) and perennial (subgenus *Glycine*) species were unsuccessful. Although pod development was initiated, these eventually aborted and abscised. Intersubgeneric hybrids were later obtained *in vitro* through embryo rescue, between *G. max* and *G. clandestina*; *G. max* and *G. tomentella*; and *G. max* and *G. canescens*, using transplanted endosperm as a nurse layer.

The progeny of intrasubgeneric hybrids (between *G. max* and *G. soja* and between *G. max* and *G. gracilis*) are fertile. The progeny of intersubgeneric hybrids were sterile and obtained with great difficulty.

C. PART II: THE RECEIVING ENVIRONMENT

C.1. Cultivation of the Host Plant in Brazil

Soybean production in Brazil is concentrated in the South (Parana, Santa Catarina and Rio Grande do Sul) and the Center-West (Mato Grosso, Mato Grosso do Sul, Goias, and the Federal District). Land in the South is naturally produc-

tive, while some of the Center-West lands must be supplemented with nitrogen, phosphorous, and lime. To minimize erosion and preserve soil organic matter, no-till management is frequently used. Rotations with corn are common.

C.2. Sexually Compatible Relatives in the Receiving Environment

There is no evidence that populations of any of the wild relatives of *G. max* exist in South America.

C.3. Ecological Interactions in the Receiving Environment

Asian soybean rust, *Phakopsora pachyrhizi* and white mold, *Sclerotinia sclerotiorum*, have recently become severe disease problems in Brazil.

Several insect species limit soybean production in Brazil. The most important insect pests and any known enemies, parasitoids, and biocontrol measures are listed in the table below.

Insect	**Natural Enemy or Biocontrol Measure**
Soybean aphid (*Aphis glycines*)	Various species of syrphid flies are aphid parasitoids
Bean shoot borer (*Epinotia aporema*)	
Velvetbean caterpillar (*Anticarsia gemmatalis*)	*Baculovirus anticarsia* is used as a biocontrol agent
Soybean looper (*Pseudoplusia includens*)	
Black cutworm (*Agrotis ipsilon*)	
Lesser cornstalk borer (*Elasmopalpus lignosellus*)	
Various species of stink bug (mainly *Nezara viridula*, *Piezodorus guildinii*, and *Euschistus heros*)	Several egg parasitoids (Hymenoptera: Scelionidae) are the principal natural enemies of stink bugs, and *Trissolcus basalis* and *T. podisi* are used in Brazil for biological control.
Various species of mealybug	Several species of *Aenasius* are parasitoids of mealybug

Surveys in soybean fields in Brazil have identified a wide variety of beneficial species, including syrphid flies, ladybird beetles, lacewings, ground-dwelling bees, such as the green metallic bee, and honeybees, although honeybees are fairly rare. Honeybees in soybean field have been found carrying soybean pollen.

D. PART III: THE TRANSGENIC PLANT

D.1. Method Used to Introduce the Novel Traits

Agrobacterium-mediated transformation

D.2. Purpose of the Transformation

Insect resistance and herbicide tolerance

D.3. Anticipated Cultivation Region

The variety would be intended for use throughout Brazil's soybean growing regions.

D.4. Summary of the Introduced Genetic Elements

The (E)-ß-farnesene synthase gene from *Mentha piperita*, is under the direction of a constitutive, leaf-specific promoter, RBCS3CP, the Rubisco small subunit promoter from tomato.

The EPSPS gene from *Agrobacterium tumefasciens* strain CP4 is under the direction of a constitutive promoter, 35S, from the cauliflower mosaic virus.

D.5. Inheritance and Stability of Each Introduced Trait

The segregation and stability data were consistent with insertion at a single locus, behaving in a Mendelian hereditary fashion.

D.6. Differences in Genetic and Phenotypic Variability from Non-Transgenic Crop

Genetic and phenotypic variability is within the range of the isogenic comparator.

D.7. Differences in Modes and/or Rate or Reproduction from Non-Transgenic Crop

No differences in reproductive ability have been noted, when compared to the isogenic line.

D.8. Expression Levels of Novel Proteins in Different Tissues over Time

Expression of EßfS in leaves was 19 µg EßfS/g fresh weight. EßfS expression in roots, pollen, and grain was below levels of detection. Transgenic leaf tissue releases 4.4 µg Eßf/g fresh wt•hour as a volatile. EPSPS protein levels in grain averaged 140 µg/g fresh weight and 62 µg/g fresh weight in leaves.

D.9. Differences in Agronomic Characteristics from Non-Transgenic Crop

The agronomic performance of transformed plants is within the range exhibited by the isogenic comparator.

D.10. Differences in Disease and/or Pest Susceptibility from Non-Transgenic Crop

Aside from reduced feeding by aphids, the transformed plants have similar disease and pest susceptibility to the isogenic comparator.

D.11. Potential Impact on Non-Target Organisms in the Receiving Environment

There are many examples in the literature of Eßf used as a chemical signal, such as the following:

- Stimulation of pollination behavior in bumblebees
- Use as a trail pheromone by ants
- Oviposition stimulant to the European corn borer
- Food attractant to the codling moth

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ANNEX 3 — LEPIDOPTERAN-RESISTANT RICE IN INDIA (CASE STUDY 2)

A. INTRODUCTION

Rice production in India is affected by a multiplicity of insect pests, leading to average annual yield losses of approximately 30%. Three of the most significant rice pests are yellow stem borer (*Scirpophaga incertulas*), rice leaf folder (*Cnapholocrocis medinalis*) and rice case worm (*Nymphula depunctalis*).

Yellow stem borer occurs in all of the rice-growing areas of India during both the kharif and rabi seasons. The pest affects the crop in the nursery, soon after transplanting, and also in the pre-earhead stage. Yellow stem borer caterpillars bore into the stem and feed internally causing death of the central shoot during the vegetative stage and "white earhead" during the milky stage, respectively resulting in chaffy grains. The larvae feed on green tissue of the leaf sheath and stem after initially mining the midrib of leaves.

Infestations of rice leaf folder usually occur during the late growth stages of the crop. Nymphs and adults suck the sap from leaves. The leaf folder larvae, which hatch from eggs laid on leaf blades, fold the leaves longitudinally and feed within resulting in a linear pale white stripe. Rice leaf folder is also a vector of yellow dwarf and tungro viruses in rice.

Rice case worm is an important pest of irrigated and rain fed wet land. The pest attacks the rice crop in the early transplanted stage. The larvae cut the leaf tips and roll by spinning both margins to make tubular case. They live inside the tube, feed on leaves, float over the water to move from plant to plant, and defoliate rice plant before maximum tillering. Heavy damage can lead to patches of severe defoliation, stunted growth, skeletonization of leaves and death of plants.

Delhi Rice Inc. (DRI) has developed the transgenic rice event DRI-2012 with resistance to yellow stem borer, rice leaf folder and rice case worm.

B. PART I: THE NON-TRANSGENIC PLANT

For more information, see MOEF/DBT (2011).

B.1. General Description of the Plant

Rice belongs to the genus *Oryza* and the tribe *Oryzeae* of the family Gramineae (Poaceae). The genus *Oryza* contains 25 recognized species, of which 23 are wild species and two, *O. sativa* and *O. glaberrima*, are cultivated. *O. sativa* is the more widely grown of the two cultivated species. It is grown worldwide including in Asia, North and South America, the European Union, Middle East and Africa. *O. glaberrima* is grown in West Africa.

B.2. Reproductive Biology of the Species

B.3. Pollination and Fertilization

O. sativa is generally self-pollinated, with limited degree of outcrossing (< 0.5%). The factors limiting the receptivity of rice flowers to outcrossing include a short style and stigma, short anthers, limited pollen viability and brief period between opening of florets and release of pollen (between 30 seconds and 9 minutes). Immediately after the spikelet opens at flowering, pollen is shed on the protruded stigma of the same spikelet or neighboring spikelets of the same plant. The maturation of pollen in an anther is synchronized with the maturation of the ovule within the same spikelet.

All wild and cultivated rice are wind-pollinated, with a few varieties having scented flowers that attract bees. It has been reported greater out-crossing is observed when honeybees are present. Although wind assisted pollen dispersal distances have been estimated up to 110 meters, pollen is short-lived with most pollen grains loosing viability after approximately five minutes under typical environmental conditions. The morphology of the pollen grain also changes dramatically

after shedding from the anther. Initially grains are spherical but within minutes they begin to collapse, coinciding with a measured loss of viability.

Although, the germinability of pollen lasts only for few minutes after being shed from anther under favorable temperature and moisture conditions, ovules keep their viability to receive pollens for several days after maturation. Fertilization is completed within six hours and occurs in the spikelet. Only one pollen tube reaches an ovule to initiate double fertilization. During fertilization rice is most sensitive to cold temperature.

B.4. Seed Dispersal

The probability of seed dispersal from rice plants varies widely within the *O. sativa* species. Most cultivars have limited dispersal ability, whereas in wild rices and some cultivars, mature rice seeds can be shed from the plant through seed shatter. Shattered seed can either be buried in the soil for subsequent germination or be eaten/dispersed by animals. Another cultivar specific trait is the presence or absence of awns at the tip of the lemma. When present, awns can vary in their rate of development, length, diameter and bristle length. The presence or absence of awns influences the potential for seed dispersal through attachment to passing animals.

B.5. Seed Dormancy

Seed dormancy is generally weaker in cultivated rice than in wild or weedy rice. The longevity of rice seeds has not been well studied however wild rice seeds are believed to be long lived and may be dormant for several years. It has been reported that of the three *O. sativa* ecotypes, Indica cultivars display the greatest degree of dormancy, followed by Javanica and then Japonica cultivars. Although dormancy is a heritable trait, environmental conditions during seed maturation also appear to influence the degree of dormancy present in the seeds. For example, Indica cultivars have stronger dormancy after maturation in rainy weather, but drying the seeds at high temperature (40ºC to 50ºC for up to two weeks) after harvest removes dormancy from all rice seed.

B.6. Mating Systems

O. sativa is largely an autogamous plant (self-fertilizing) propagating through seeds produced by self-pollination. Cross pollination between wild species and *O. sativa* cultivars has been reported to occur in natural habitats. The degree of outcrossing has been reported to be generally higher in Indica cultivars and wild species than in Japonica cultivars.

B.7. Asexual Reproduction

Although *O. sativa* is cultivated annually, the rice plants can grow vegetatively and continuously under favorable water and temperature conditions, even after they have borne seeds. This perennial character in O. sativa is considered to have been inherited from the ancestral species *O. rufipogon*. Under natural conditions, tiller buds on the basal nodes of rice plants start to re-grow after rice grains have been harvested. These new tillers, called ratoons, grow best under long day conditions and are used in some countries to obtain a second harvest. Cell/tissue culture techniques can be used to propagate calli and reproduce tissues or plants asexually under the appropriate cultural conditions. Haploid plants can be easily obtained through anther culture and they become diploid spontaneously or when artificially treated with chemicals.

B.8. Center of Origin and Center of Genetic Diversity

The center of origin and centers of diversity of the two cultivated species *O. sativa* and *O. glaberrima* have been identified using genetic diversity, historical and archaeological evidence and geographical distribution. It is generally agreed that valleys of the Yangtze and Mekon rivers could be the primary centers of origin of *O. sativa*, and the Niger River delta may be the primary center of origin of *O. glaberrima*. The foothills of the Himalayas, Chhattisgarh, Jeypore Tract of Orissa, northeastern India, northern parts of Myanmar and Thailand, and the Yunnan Province of China are some of

the centers of diversity for Asian cultigens. The inner delta of the Niger River and some areas around Guinean coast of the Africa are considered to be centers of diversity for *O. glaberrima*.

India has abundant resources of wild rice particularly *O. nivara*, *O.rufipogon*, *O.officinalis*, and *O. granulata*. The wild species of rice can be found in many different natural habitats, from shade to full sunlight, and can be either annual or perennial in nature. The habitats of *O. nivara* are ditches, water holes, and edges of ponds, whereas *O. rufipogon* is usually found in deepwater swamps. Some wild species occur as weeds in and around rice fields and even hybridize naturally with the cultivated forms. This complex association between cultivated and wild forms has also enhanced the diversity of rice crop in traditional agricultural systems. Northeastern hills, Koraput region of Orissa, Raipur region of Chhattisgarh and peninsular region of India are considered important centres of diversity based on germplasm collections.

B.9. Means of Dispersal and Establishment

Gene flow through conventional sexual hybridization is limited to *O. sativa* varieties and to the AA type genome species within this genus. Gene flow between more distantly related species, particularly those outside of the *Oryza* genus, is restricted to artificial breeding methods such as embryo rescue and somatic hybridisation (the regeneration of plants following the fusion of two protoplasts).

C. PART II: THE RECEIVING ENVIRONMENT

C.1. Cultivation of the Host Plant in India

Rice is grown under widely varying conditions of altitude and climate in the country and so the rice growing seasons also vary, depending upon temperature, rainfall, soil types, water availability and other climatic conditions. In the eastern and southern regions of the country, where the mean temperature is found favorable for rice cultivation throughout the year, two or three crops of rice are grown annually. In the northern and western parts of the country, where rainfall is high and winter temperature is fairly low, only one crop of rice is grown during the months of May to November.

C.2. Sexually Compatible Relatives in the Receiving Environment

Rice plants (*O. sativa* or other *Oryza* species) that are grown unintentionally in and around rice growing areas are regarded as weeds. Rice has a tendency to become weedy in areas where wild and cultivated rice plants grow sympatrically. In these areas, wild and cultivated rice plants can hybridize, producing plants that compete with the cultivars and produce inferior seed, thus decreasing the yield from the rice crop. However, weedy rice can also develop in areas without native wild rice populations.

In the case of *O. sativa*, weedy rice (*Oryza* spp.) is viewed as a major economic problem when it occurs in rice fields as it causes losses in yield through competition with the cultivars as wells as decreasing the value of the harvested grain through its color. Characteristics of weedy rice contributing to its potential weediness include similar growth attributes with cultivars due to common progenitors, high seed shedding rate, dormancy, and persistence, adaptation to different habitats and relatively higher outcrossing ability. In view of the above, populations of weedy rice tend to be genetically diverse and highly heterogeneous, and often have intermediate characteristics between wild and cultivated varieties.

C.3. Ecological Interactions in the Receiving Environment

The pest and disease species of major significance in India's rice producing regions are listed in the table below:

Major Insect Pests	Major Nematode Pests	Major Diseases
Yellow stem borer *Scirpophaga incertulas*	Root knot nematodes *Meloidogyne graminicola*	Rice blast *Magnaporthe grisea*
Rice gall midge *Orseolia oryzae*	White tip nematode *Aphelenchoides besseyi*	Sheath blight *Rhizoctonia solani*
Green leafhoppers *Nephotettix* spp.	Stem or ufra nematode *Ditylenchus angustus*	False Smut *Ustilaginoidea virens*
Brown plant hopper *Nilaparvata lugens*	Root nematode *Hirschmanniella* spp.	Brown spot *Bipolaris oryzae*
Leaf folder *Cnapholocrocis medinalis*	Root lesion nematode *Pratylenchus* spp.	Black sheath rot *Gaeumannomyces graminis*
Gundhi bug *Leptocorisa oratorius*		Bakanae disease *Gibberella fujikuroi*
Rice hispa *Dicladispa armigera*		Bacterial leaf blight *Xanthomonas campestris* pv. *Oryzae*
Rice case worm *Nymphula depunctalis*		Bacterial leaf streak *Xanthomonas campestris* f.sp. *translucens*
Rice thrips *Stenchaetothrips biformis*		Rice tungro virus
Climbing cutworm *Mythimna separata*		Grassy stunt virus
Rice root aphid *Tetrineura nigriabdominalis*		Ragged stunt virus

The predominant, naturally occurring predators that offer control of pests in rice crop are as follows:

Spiders: wolf, lynx, and orb spiders are known to consume a large number of prey and play an important role in reducing the densities of plant hoppers and leafhoppers in rice fields.

Beetles: The adults and nymphs of species such as *Ophionea nigrofasciata* (ground beetle) and *Paederus fuscipes* (rove beetles) prey upon aphids, leafhoppers, and planthoppers. Lady beetles such as *Micraspis crocea* are also important insect predators in rice.

Bugs: *Microvelia douglasi atrolineata* is a water bug that can survive for long periods even without food provided the field is saturated or flooded as in rice fields. Both the adults and nymphs live on the water surface and attack insects that fall onto the surface.

Damselfies: *Agriocnemis femina femina* and *Agriocnemis pygmaea* are common in rice and feed on lepidopterans and hoppers.

Crickets: Two species of crickets, *Anaxipha longipennis*, and *Metioche vittaticollis*, are common predators of rice insect pests.

Parasitoids of common rice insect pests are very important components of the natural enemy complex of insect pests and have been the most common type of natural enemy introduced for biological control of insects. The different parasitoids attacking the various growth stages of rice pests are provided in the table below:

Host	Parasitoid Species (listed by host growth stage)		
	Egg	Larvae	Pupae
Yellow stemborer	*Tetrastichus schoenobii* *Trichogramma japonicum* *Telenomus rowani*	*Stenobracon nicevil*lei *Bracon chinensis*	*Tetrastichus ayyari*
Leaf folders	*Copidosomopsis nacoleiae* *Trichogramma chilonis*	*Cotesia augustibasis*	*Xanthopimpla flavolineata* *Tetrastichus ayyari*
Brown planthopper	*Oligosita yasumatsui* *Anagrus* spp.	*Pseudogonatopus* spp.	*Pseudogonatopus* spp.
Gall midge		*Platygaster oryzae*	

D. PART III: THE TRANSGENIC PLANT

D.1. Method Used to Introduce the Novel Trait(s)

Event CRI-2012 was produced using a direct DNA transfer system. The elite Indica rice cultivar Pusa Basmati-1 was co-bombarded with two gene expression cassettes: a translational fusion of two *Bacillus thuringiensis* genes, *cry1Ac* and *cry1Ab*; and, the hygromycin resistance gene, *hpt*, used as a selectable marker.

PCR and segregation analyses of T1 and T2 progeny resulted in the recovery of 4% marker-free T2 transformation events

D.2. Purpose of the Transformation

Resistance to lepidopteran pests.

D.3. Anticipated Cultivation Region

Varieties derived from DRI-2012 are expected to be deployed wherever rice is grown in India.

D.4. Summary of the Introduced Genetic Elements

Pusa Basmati-1 was co-bombarded with two gene expression cassettes:

Cassette 1: a codon optimized, synthetic translational fusion *cry1Ac-1Ab* gene under direction of the light-inducible, tissue specific promoter phosphoenol pyruvate carboxylase (PEPC).

Cassette 2: *hpt* under direction of a double CaMV35S promoter.

D.5. Inheritance and Stability of Each Introduced Trait

Progeny analysis confirmed stable inheritance of the *cry1Ac-1Ab* gene in event DRI-2012.

D.6. Differences in Genetic and Phenotypic Variability from Non-Transgenic Crop

None were observed except for the desired trait of resistance to the target pests, as confirmed in bioassays.

D.7. Differences in Modes and/or Rate of Reproduction from Non-Transgenic Crop

None were observed.

D.8. Expression Levels of Novel Proteins in Different Tissues over Time

Cry1Ac and Cry1Ab expression in young and mature leaves ranged from 0.6 – 5.4 and 1.2 – 7.8 µg/g fresh weight, respectively. Neither protein was detected in root tissue, pollen, or grain.

D.9. Differences in Agronomic Characteristics from Non-Transgenic Crop

None were observed except for the desired trait of resistance to the target pests.

D.10. Differences in Disease and/or Pest Susceptibility from Non-Transgenic Crop

None were observed except for the desired trait of resistance to the target pests.

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