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FORUM

Problem Formulation in Environmental Risk Assessment of Genetically Modified Crops: A Brazilian Workshop

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Formulação de Problema em Análise de Risco Ambiental de Cultivos Geneticamente Modificados: Workshop no Brasil

RESUMO – O International Life Sciences Institute (ILSI) e a Empresa Brasileira de Agropecuária (EMBRAPA) realizaram um workshop para reunir cientistas do governo, da indústria e da academia, com o intuito de discutir o processo de formulação de problema para o levantamento de risco ambiental (LRA) de culturas geneticamente modificadas (GM). O workshop se concentrou na aplicação do conhecimento científico vigente do LRA para as culturas GM, presentes no meio ambiente brasileiro. Devido às características peculiares e à importância econômica que a cana-de-açúcar e o algodão representam para a economia e o meio ambiente brasileiro, estas culturas foram utilizadas como modelos para estudos de caso, onde os conceitos discutidos foram aplicados à luz da Resolução Normativa Nº05 (RN05) da Comissão Técnica Nacional de Biossegurança (CTNBio). O objetivo deste documento é sumarizar as conclusões obtidas durante este workshop.

PALAVRAS-CHAVE – biossegurança, análise de risco, plantas geneticamente modificadas.

ABSTRACT – A workshop organized by the International Life Sciences Institute (ILSI) Research Foundation, ILSI Brasil, and Brazilian Agricultural Research Corporation (EMBRAPA) brought together scientists from government, industry and academia to explore problem formulation for environmental risk assessment (ERA) of genetically modified crops. The workshop focused on the application of current scientific knowledge related to the ERA of GM crops in the Brazilian context of the National Biosafety Technical Commission (CTNBio) Normative Resolution #05 requirements and the local environment. Due to the importance of cotton and sugarcane to Brazil's economy and the potential environmental impacts associated with introducing genetically modified varieties, these crops were used as

case studies to illustrate and discuss the general concepts in problem formulation. This report is a summary of the discussions from this workshop.

KEYWORDS – biosafety, risk analysis, genetically modified plants.

Transgenic crops have been widely adopted around the world since their initial commercialization in 1996, and in 2008 reached a total of 125 million hectares (James 2008). The cultivation of GM plants is strictly regulated and requires approval from a relevant competent authority prior to commercial use. Most countries have developed a regulatory system that requires the risks of these products to the environment and to human and animal health be assessed and determined to be acceptable before field trials or commercial releases are approved.

Environmental Risk Assessment (ERA) is commonly used by regulatory authorities as part of decision-making for the approval of a genetically modified organism (GMO). Brazil recently implemented Normative Resolution No. 05 to provide guidance for regulators and developers of GM products in the conducting ERAs. Specifically, this resolution "[p]rovides for the rules of planned release in the environment of Genetically Modified Organisms (GMO) of plant origin and derivatives therefrom".

The International Life Sciences Institute (ILSI) Research Foundation, ILSI Brasil, and Brazilian Agricultural Research Corporation (EMBRAPA) brought together 50 scientists from government, industry and academia to explore problem formulation (PF) for ERA of GM crops. The workshop was held in Brasilia over 2 days. In addition, several internationally recognized scientific experts participated in the workshop.

The specific objectives of the workshop included:

- Present and discuss the conceptual basis of PF in ERAs.
- Discuss PF in the context of the Brazilian environment and legislative requirements, in particular to Comissão Técnica Nacional de Biossegurança's (CTNBio) Normative Resolution No. 05 of March 12th, 2008. (http://www.ctnbio.gov.br/index.php/content/view/11444.html).
- Discuss PF for potential risks to desirable organisms – concentrating on non-target arthropods.
- Discuss in detail PF for two case studies, GM cotton and GM sugarcane focusing on gene flow and potential effects on non-target arthropods.

The workshop was designed to meet the needs of Brazilian scientists who must now interpret and implement the recent legislation. Regulators and developers of GM products from both public and private sector needed to discuss these new rules with regard to the established principles of ERA. Achieving the objectives of the workshop could facilitate the adoption of a structured process for initiating ERAs with appropriate PF as a focusing step, which will increase the efficiency and transparency in the decision making process and increase harmonization of approaches to ERA globally. This paper presents the consensus of the group of experts participating in the workshop and is not intended to include a review of the literature. The goal is to produce a pragmatic scheme that assesses risk sufficiently for decision-making without unncesssarily delaying introduction of products that may be beneficial.

The workshop was opened with an introduction to the work of ILSI presented by Aldo Baccarin, President of ILSI Brasil and included welcomes from Eduardo Romano, Empresa Brasileira de Agropecuária (EMBRAPA), and Julie Fitzpatrick, ILSI Research Foundation. Walter Colli, President of the Comissão Técnica Nacional de Biossegurança (CTNBio), presented an introductory message detailing the importance of bringing together scientists to address issues of common concern. José Geraldo Eugênio de França, Executive Director of EMBRAPA, presented a review of biotechnology crops in Brasil and Julie Fitzpatrick presented the current work of the ILSI Research Foundation's Environmental Risk Assessment for Genetically Modified Crops program on PF.

Background presentations on PF, non-target organisms, ecological risk assessment research, and CTNBio's Normative Resolution No. 05 were presented by Paul Keese from Australia's Office of the Gene Technology Regulator, Alan Raybould from Syngenta, and Jörg Romeis from the Agroscope Reckenholz-Tänikon Research Station. The following is a summary of these presentations and the results of the multi-stakeholder discussions.

The ERA Problem Formulation

Problem formulation is the "formal, structured, opening stage" of a risk assessment that determines its purpose and scope, and so guides the gathering of informative data (Patton 1998). The importance of PF in risk assessments is often overlooked. Poor PF may compromise the entire risk assessment and impair subsequent decision-making. One outcome of this

failure has been the production of data of limited relevance for risk assessment (Craig et al., 2008). Irrelevant data increase rather than allay concerns about the impacts of GMOs (Johnson et al. 2007; Raybould 2006), and may even increase environmental risk because of delays in the introduction of environmentally beneficial products (Cross, 1996; Raybould 2006).

There is a simple question at the heart of PF for regulatory risk assessments of GM crops: "*What could go wrong if this GM crop is cultivated in this country*?" Unfortunately, this question is often subtly but significantly modified to: "What will change if this GM crop is cultivated in this country?" This more open-ended question has led to seeking any form of biological or environmental effect, whereas, risk assessment is concerned with the much smaller subset of effects, namely, those considered harmful. Clearly distinguishing between studies that predict change and those that predict harm is central ensuring regulatory efficiencies and avoiding the generation of irrelevant data (Raybould, 2007). To solve this problem we need to answer another deceptively simple question: "*What is harm*?"

Having an unambiguous (operational) definition of harm resulting from the cultivation of a GM crop is essential so that suitable risk hypotheses can be formulated and tested in the risk assessment. Harm should directly relate to clearly stated objectives or management goals of environmental law or other instruments of policy under which the GM crops are regulated, and should provide the scope and boundaries of the risk assessment. Clear definitions of harm and its seriousness can be difficult to establish because what is harmful cannot be discovered by experiments and objective analysis. Harm is an expression of subjective societal values, which differ between people, and vary over time. Consequently, legislation and management goals are often expressed as high level concepts, such as the protection of biodiversity, ecosystems, natural and physical resources, or the quality of locations, places and areas. The derivation of specific, unambiguous, and therefore scientifically tractable definitions of harm to these concepts is a crucial step in PF.

Scientifically tractable definitions of harm are central to PF and have two elements: the assessment endpoints and unacceptable conditions of those endpoints. The assessment endpoints are the specific environmental components that the risk assessment seeks to protect, and they comprise a component of the environment and a property of that component (e.g., Suter, 1990). They may be comprised of biological, chemical and physical variables: for example, common assessment endpoints are the population sizes of particular species, and the concentration of certain compounds in water bodies. The definition of harm is completed by specifying an unacceptable condition: for example, a population size below a specified threshold, or a concentration above a specified threshold.

Once harm is defined, PF can tackle a potentially open-ended question: "*How may harm arise*?" Answers to this question describe plausible sets of circumstances (scenarios) that could give rise to harm. In other words, this part of PF considers how use of the genetically modified crop may bring about an unacceptable condition of the assessment endpoints. In deriving plausible scenarios for harm, consideration should be given to existing knowledge of the crop's biology, the intended effect of the genetic modification, potential unintended effects of transformation, and the proposed use of the crop, including where and how it will be cultivated, how it will be processed, and the fate of its products and waste materials.

With sufficient imagination, the number of scenarios that leads to harm is almost unlimited, which raises an important question: "*Which, if any, of these scenarios warrant detailed assessment?*" In general, many scenarios are closely related and can be eliminated from further assessment because one or more steps in the pathway to harm can be dismissed as highly unlikely from existing knowledge.

It is useful to consider the philosophical basis for eliminating certain scenarios from further consideration. Scientific risk assessment can be viewed as conforming to the model of the continuous development of scientific knowledge proposed by the Austrian philosopher Karl Popper (1902-1994) (Raybould 2007). Popper proposed that logically science cannot prove theories by finding evidence in their favor, but can test theories by find errors in them. Popper argued that a theory can only be classified as scientific if it is possible to falsify it, and that this logic of scientific discovery can be applied to the development of all objective knowledge. In Popper's view, all objective knowledge is acquired according to a simple scheme: a problem is identified; a trial solution to the problem is proposed; the solution is tested to eliminate errors; and corroboration or falsification of the trial solution provides new knowledge with associated new problems (Popper 1972). The process can be schematically represented as in Figure 1.

Risk assessment of GM crops fits Popper's scheme because the safety of a GM crop cannot be proved, but acceptable risk can be established by testing hypotheses (Raybould 2006): the development of scenarios describing how the GM crop may cause harm is the initial problem (P_1); trial solutions (TS) are risk hypotheses that propose the scenarios will not be fulfilled; error elimination (EE) are tests of those hypotheses; the tests lead to increased knowledge of risk and new problems, which could be decision about whether to require further testing or to complete the risk assessment. When rejecting a scenario for further

consideration, one has, in effect, decided that a hypothesis that a scenario will not occur has been tested and corroborated with sufficient rigor. It may appear "obvious" to the assessor that certain scenarios cannot lead to harm; however, "obvious" is really shorthand for a highly corroborated hypothesis of no harm via that scenario. As no explicit hypothesis testing may occur, it is important for the purposes of transparency to document which scenarios were considered and judged to be implausible.

Figure 1. Popper's scheme for the development of objective knowledge.

Scenarios that require detailed assessment are those for which there is considered to be insufficient corroboration of the risk hypotheses: in other words, the risk hypotheses require further testing. Testing the risk hypotheses tackles the final question that must be answered in PF: "*How will risk be characterized?*" Risk hypotheses postulate the absence of phenomena necessary for harm to occur (Raybould 2006); for example, if harm to the environment could arise because of toxicity of a transgenic protein to a particular species, a suitable risk hypothesis is that the species will not be exposed to concentrations of the protein in excess of the lowest concentration that could have an adverse effect. This hypothesis can be tested by comparing the concentration of the protein that produces adverse effects with predictions of the environmental concentrations of the protein as a result of cultivation of the GM crop (i.e., expected environmental concentrations). Strictly speaking the corroboration or falsification of the hypothesis falls outside PF. Importantly, the risk hypotheses formulated during PF must be testable, and therefore possible methods for testing hypotheses are an important consideration.

Risk hypotheses should be rigorously tested with data acquired from the literature, expert judgment or from new studies. It is important to emphasize that new studies should be required only if existing data or other relevant information are not available to test the risk hypotheses with sufficient rigor to adequately characterize the risk. For example, regulatory risk assessments of a new GM soybean cultivar in Brazil would most likely not require new data on horizontal gene flow because sufficient data already exist to satisfactorily corroborate the hypothesis that harm will not arise by this route from cultivation of soybeans.

CTNBio's Normative Resolution #05

CTNBio's Normative Resolution #05 provides the goals, scope and boundaries for PF. It also serves as the basis for defining harms (linked to assessment endpoints) necessary for identifying potential meaningful risks.

Normative Resolution #05 decrees that case-by-case risk assessment is required for all commercial releases of GMOs into the Brazilian environment for the purpose of protecting human, animal, and plant health, and the environment. The scope includes biosafety issues arising from the "construction, cultivation, production, manipulation, transport, transfer, import, export, storage, research, marketing, consumption, disposal to the environment and discarding of genetically modified organisms, GMO and GMO derivatives."

In addition, Annex IV (part A) of Normative Resolution #05 sets out specific requirements for ERA of plants. The requirements include consideration of the GMO parental organism, its ancestors and wild relatives; spread and persistence of the GMO and its progeny in the same or different environments; gene transfer to the same species or other sexually compatible species; possible effects on environmentally important indicator species; negative and positive effects on target and non-target organisms; horizontal gene transfer; and, biotic and abiotic interactions with the environment, including soil and water.

Brazil is signatory to the Cartagena Protocol on Biosafety, and as such Normative Resolution #05 was written in a manner for to Brazil maintain compliance with this international agreement. The principles, methods and general considerations outlined in Annex III of the protocol are consistent with the information in Normative Resolution #05. Examples of how the Normative Resolution #05 is applied to PF can be found in the case studies described below.

Problem Formulation applied to protecting non-target (valued) arthropods

The majority of commercially grown GM crops are modified for enhanced insect resistance or herbicide tolerance (James 2008). One of the potential harms associated with growing insect-resistant GM plants is adverse effects on beneficial (valued) non-target

arthropods (NTAs, also referred to as non-target organisms or NTOs). To assess the potential risks, regulatory authorities need to construct reasonable risk hypotheses based on the characteristics of the crop, the introduced trait, the likely receiving environment and the interactions among these. Because it is not reasonable or necessary to test every arthropod species individually for potential harmful effects, appropriate ERA methods must be developed based on well-formulated hypotheses

Choice of Species

For practical reasons, only a small fraction of the terrestrial arthropods potentially exposed to the insecticidal proteins can be considered for regulatory testing. As generally adopted in many parts of the world, it is necessary to initially select appropriate species that can be tested under worst-case conditions in the laboratory; these species serve as surrogates for the broader diversity of ecologically and economically desirable organisms. Selection of species should be evident from the exposure scenarios, and should be chosen based reasonable risk hypotheses developed considering the crop and introduced trait. Furthermore, they can represent different ecological functions within the agroecosystem (herbivors, pollinators, decomposers, predators, etc.). To reflect biogeographical variation, it is crucial to determine which taxa are likely to occur in the cropping systems where the transgenic plant is expected to be grown. Another important source of information that serves as a basis for selecting relevant surrogate species is the information on the insecticidal protein. This includes the known specificity, mode of action and the temporal and spatial exposure profile. This information is accumulated at the time of PF (plant characterization). The information collected in these previous steps will direct the selection of representative NTAs from a proposed set of species that capture key ecological functions, that are amenable to testing and for which standardized testing protocols exists (Romeis *et al*. 2008).

Generally, species selected for testing should be those that provide the most rigorous test of the risk hypotheses for a particular insect-resistant genetically modified plant in a specific agricultural and environmental setting. The application of the surrogate species concept enhances the transferability of data from lower tier tests to a wide range of regions and crops (e.g., Romeis et al., 2009).

Choice of Test Methods

A typical risk hypothesis resulting from the PF phase may be that the insecticidal protein does not cause an adverse effect to NTAs at the concentration expressed in the field. Both seriousness and likelihood of harm can be evaluated within different levels or "tiers" that progress from worst-case hazard and exposure to more realistic scenarios as shown in Figure 2 (Garcia-Alonso et al. 2006; Rose 2007; Romeis et al. 2008). Lower tier tests are generally conducted in the laboratory to provide high levels of replication and study control. Lower tier tests add conservatism such as high doses to account for uncertainty. When harm is detected in low tier tests, additional information may be required. In these cases, higher tier tests can serve to confirm if an adverse effect might still be detected at more realistic rates and routes of exposure. Higher tier studies including semi-field or field-based tests offer greater environmental realism, however these tests only make sense when early tier studies in the laboratory indicate potential harm at environmentally relevant levels of exposure. In cases where acceptable risk cannot be concluded in lower tiers (typically laboratory studies with purified toxins or plant material) with sufficient certainty, higher tier studies would be conducted. The aim is to evaluate whether the adverse effect detected is present under more realistic conditions. Higher tier studies might be conducted at the initial stage when early tier tests are not possible; for example, when the species to be tested is not available or amenable for laboratory testing.

Movement between tiers is based on the sufficiency of information that is available as shown in Figure 2. If sufficient data and experience from toxicological testing and exposure analyses are available to characterize the potential risk as being acceptable, then there is no need to undertake additional testing. The process is thus designed to balance the expense related to time and resources needed to identify and define sources of potential risk with the need for more information.

Case Studies

GM cotton (*Gossypium hirsutum*) and GM sugarcane (*Saccharum* X *officinarum*) were presented as case studies for discussing the development of effective PF for ERAs. For each case study two scientists presented background documents after which the participants divided into three breakout groups that addressed gene flow and NTAs. In the course of these conversations other issues were raised including horizontal gene transfer, physical and chemical alterations in the soil and adjacent water bodies, and capacity to survive in different environments. Workshop participants recognized that a complete PF for a GM plant would be much more extensive, but all aspects could not be covered in the course of this workshop. The Workshop focused on these three areas (gene flow, NTAs and "other issues) to be discussed in the workshop. Eduardo Romano and Fátima Grossi-de Sa from EMBRAPA presented background material on genetically modified cotton (BR Cotton) resistant to coleopteran Boll Weevil (*Anthonomus grandis*) and the main Lepdopteran insect-pests in Brazil. Adriana Gianotto and Jesus Ferro from Allelyx presented background material on sugarcane genetically modified to herbicide and insect resistance. Following the background presentations the workshop participants split

into three groups to discuss the various aspects of PF. The highlights of the breakout group discussions were presented to all workshop participants in a plenary session for further discussion.

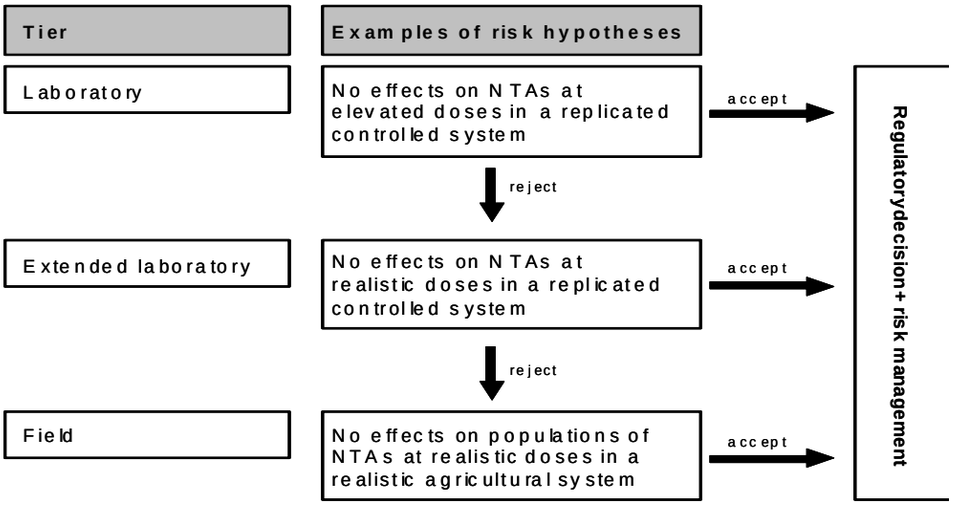

Figure 2. Tiered scheme for ecological risk assessment (adapted from Romeis et al., 2008).

Case Study: Cotton

Background

A crucial and basic component for a proper risk assessment is the definition of an appropriate baseline for comparison. Of different possible baselines (Andow et al., 2006) the members of the workshop were unanimous in defining that for GM crops the appropriate reference point is the environmental impacts associated with traditional crops including practices commonly used in cultivating plants developed by conventional breeding. Therefore participants of the case study agreed that the impact of GM cotton should be compared to the impact of the cultivation of conventional cotton and its associated agronomic practices. Cotton in Brazil is severely damaged by boll weevil (*Anthonomus grandis*) insect-pest and, on average, sixteen pesticide sprays are applied during the season for its control. The transgenic insecticidal trait discussed here would thus likely result in significant reductions in insecticide use. The Normative Resolution #05, which regulates the commercial release of GMOs in Brazil, and recommends that the potential environmental benefits of the transgenic crop cultivation should be taken into account in its risk assessment.

The hypothetical transgenic event was derived from Brazil, called BR Cedro, a commercial variety of *G. hirsutum*, and would be cultivated in the same way as non transgenic varieties in Brazil. The transgenic event named BR Cotton 351 would be used mainly for production of textile fibers and in a smaller scale, feed and oil for human consumption. The BR Cotton 351 contains the *cry8Ka5* gene from *Bacillus thuringiensis* that confers resistance to boll weevil, and the *npt*II gene from *Escherichia coli*, which confers resistance to the antibiotic kanamycin.

Cotton gene flow

The group agreed that gene flow from cotton in Brazil posed some interesting scientific considerations that should be addressed in the PF. Three species of the genus *Gossypium* occur in Brazil, all of them are allotetraploids and sexually compatible among themselves: *G. hirsutum*, *G. barbadense*, and *G. mustelinum.* Additionally, *G. mustelinum* is judged to be a rare species considered to be at risk of extinction. Therefore, genetic compatibility between the transgenic *G. hirsutum* and the remaining *Gossypium* genotypes raises concerns about the maintenance of the genetic variability of the native cotton in Brazil. It is interesting to note that in this case risk assessment considers only the potential adverse outcomes. A thorough PF could also pose an alternative hypothesis that the *Bt* gene in *G. mustilinum* is beneficial for the population of this species by protecting it from extinction.

Gene flow is a natural phenomenon that has many elements which must be considered in a rigorous ERA (Stewart et al., 2003). Firstly, gene flow and hybridization are not the same as introgression. This distinction is important because potential negative ecological effects can be associated at various steps along the process to introgression. PF for this Bt cotton should consider the potential harms associated with both intermediate hybrids and introgressed species. Hybridization is the initial cross between parent plants

of different varieties, subspecies, species or genera while introgression is defined as the permanent incorporation of genes from one set of differentiated populations (species, subspecies, races and so on) into another. The process of introgression is more involved than hybridization. In the case of a hypothetical GM cotton resistant to boll weevil, it would be important to clarify whether the population of wild cotton would benefit from being protected due to the presence of the transgene and no longer likely to control by coleopteran attack. A transgene that confers a selective advantage to the wild relative greater than the sum of the selective disadvantages of loci that are genetically linked with the crop transgene locus is likely to introgress if there are no mitigating factors. If the transgene confers a selective disadvantage, special circumstances are required to fix the gene (Haygood et al 2004). Even if a transgene confers a meaningful advantage, it needs to overcome further barriers to be introgressed into the recipient genome. Several basic conditions must be met for successful introgression: the transgenic crop and sexually compatible wild plant must have overlapping flowering times; the hybrids must persist for at least one generation and be fertile to produce backcross hybrids; and finally, backcross generations to the wild relative must progress to the point at which the transgene is incorporated into the genome of the wild relative (Stewart et al., 2003).

There is a low probability of introgression of genes from cotton to other *Gossypium* species including *G. mustelinum*. Despite several centuries of sympatric cultivation of *G. barbadense* and *G. hirsutum*, there is little evidence of interspecific introgression of alleles from cultivated cotton into *G. mustelinum*. Isoenzymatic studies of *G. mustelinum* showed that only 6 out of the 50 loci sampled were polymorphic, without any heterozygous plant being verified (Wendel and Rowley 1994). These data show that the populations are highly monomorphic and indicate that self-pollination may be more common than cross pollination. In addition to the natural barriers to introgression of transgenes from GM plants to wild relatives, CTNBio created large exclusion zones for transgenic cotton in Brazil in 2005 as shown in Figure 3. The participants of the cotton case study considered that due to the natural barriers in introgression along with the adoption of exclusion zones in Brazil it is expected that gene flow from transgenic plants will pose negligible risk to genetic variability of *Gossypium* species compared to the cultivation of conventional cotton.

Cotton non-target Arthropods

The group supported the use of the tiered approach (Romeis et al., 2008) to assess the risk that GM cotton poses to NTAs. The selection of the species should be based on their abundance and ecological role in the context of cultivating cotton. The knowledge on arthropods in Brazilian cotton fields is considerable and allows the participants to identify the most important species (Hilbeck et al. 2006).

In general, the assessment starts with laboratory tests to determine whether the insecticidal proteins could harm the selected insects. The risk assessment may conclude negligible risk in this early tier if no effects are observed under these worst case conditions. However, if unacceptable effects have been identified or cannot be ruled out with sufficient certainty, higher tier tests will be performed where NTAs will be exposed to the toxin under more realistic conditions. In the cotton case study, two scenarios were discussed:

1) laboratory feeding studies conducted under worst-case exposure conditions revealed no detectable adverse effect of the toxin on the selected insects and;

2) the toxin caused detectable adverse effects on the representative insects with LD50 much higher (at least one order of magnitude) than the level present in the GM crop.

In both cases and based on evidence presented, the group considered that the risks to NTAs are lower than the risk of the commonly used alternative technology – the use of chemical pesticides. While it was evident that in the first case (no effects observed in lab tests at high doses) no additional studies would be necessary, the second case may require additional higher tier studies when unacceptable risks cannot be ruled out with sufficient certainty. Moreover, the group discussed the value of LD50 estimates for supporting ERA studies. In general, LD50 values are estimates from dose-response curves and their use is linked to the concept that toxicity is a function of dose and exposure. Thus, by increasing the tested dose it is possible to estimate LD50s to a large number of substances, but how these lethal dose values relate to exposure and therefore ERA is often times not made clear. In other words, an LD50 may be detected in a lab study, but if the expected environmental exposure is much lower (>10x) than the concentration tested the risk can be characterized as negligible. This is standard practice in pesticide risk assessment. Experience with testing proteins with a very narrow host spectrum such as Bt Cry proteins, LD50 values are frequently not calculated because of the lack of effects observed at very high concentrations (scenario 1 above). Products that have been through regulatory systems using this method have not been shown to be associated with environmental harm (e.g. Romeis et al. 2008). For risk assessment pedants, 10x is not a safety factor. Testing at lower than 10x can be used to demonstrate acceptable risk, we do not have to demonstrate no effect at 10x . 10x is desirable because it provides more power to extrapolate than does testing at 1x.

Another very important point that came out of this discussion was the need for appropriate test systems. The group discussed the fact that trophic transfer is an interesting ecological element of any systems. However, recent attempts to test for direct toxic effects of the protein through the trophic system using

susceptible prey are fundamentally flawed. Spurious results can be obtained using moribund larvae as diet for specialist predators (Romeis et al., 2008).

Cotton Additional Issues

The likelihood of horizontal transference of a transgene to soil microorganisms is very small, certainly much lower than the chances of a similar transfer among bacteria (Keese 2008). Bt proteins and Bt DNA, as well as other recombinant proteins and their DNA, can be shed into the soil after the crop is harvested and plant tissues are incorporated in the soil. The potential registrants should be aware of the pertinent literature on environmental fate and discuss it in the context of their products, as to provide sufficient background data to dismiss (or not) a potential impact of these proteins on the soil or water collections.

Other points were raised in the discussion such as extensive analysis of transgene-locus structure including sequencing of the flanking genomic regions of the transgenes could be useful for traceability and inspection issues, but will have little or no environmental biosafety importance. Some authors advise to include nucleotide sequencing of transgenic locus in risk assessment because the integration of exogenous DNA into the plant genome can result in the disruption of host genes resulting in non intended effects. However, this group is aware the phenotype is much more important for ERA than the genotype, and that possible pleiotropic or non intended effects caused by several different reasons including insertional mutagenesis would be better analyzed by substantial equivalence and field tests, both required before the process of release for commercialization of GM plants.

Case Study: Sugarcane

Background

GM sugarcane was derived from a sugarcane commercial hybrid (*Saccharum* X *officinarum*) and information was presented to show that it would be cultivated in the same way as 'traditional' varieties in Brazil. As such, this GM sugarcane would be used mainly to produce sucrose (sugar) and ethanol with the bagasse most likely being burned at the mills to produce energy. Alternatively, the variety could also be used to produce cachaça and other food products such as rapadura, sugarcane syrup and brown sugar. In Brazil, it is also common to use sugarcane for cattle feeding and for *in natura* human consumption.

The main production of sugarcane in Brazil occurs on the Southeast, Mid-East, South, and Northeast regions of the country. The production of sugarcane in the North region is irrelevant. Sugarcane is very important to the Brazilian economy. Brazil is the major exporter of sugar and ethanol to the world market and these two sugarcane products are commodities that occupies the fourth place among the Brazilian agribusiness exporter after soybean and its subproducts, meat and pulp and paper products (CONAB, 2007).

There is no commercial release of GM sugarcane anywhere and this is supposed to be the first one. This variety would include the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS)* gene from *Agrobacterium tumefaciens* sp. strain CP4, which confers tolerance to glyphosate herbicide and the *cry2A* gene from *Bacillus thuringiensis*, which confers resistance to insects belonging to the order Lepidoptera.

Sugarcane Gene Flow

Sugarcane pollen has low viability in normal environmental conditions: its half life is only 12 minutes and it shows no viability after 35 minutes at 26.5°C and 67% RH (Moore 1976; Venkatraman 1922). As a consequence, it is not expected that viable pollen is carried long distances in the field. Furthermore, sugarcane seeds do not have high viability, losing 90% of their viability after 80 days at 28°C, if not properly desiccated (Rao, 1980). They also require high humidity to germinate.

The center of origin of sugarcane is Asia and it is considered an exotic crop in Brazil. It was introduced in the early times of colonization, and has been cultivated for approximately 500 years. It is known that in areas where sugarcane is cultivated, very few plants can grow outside the cultivated area (OGTR, 2008). It is also known that sugarcane requires very specific conditions to flower, and therefore sexual reproduction is only likely to happen near the Equator, in the Northeast Region of the country (Brett, 1951; Moore & Nuss, 1987). Due to this difficulty in flowering, breeders have to manipulate environmental conditions in order to make effective crosses (Matsuoka et al. 1999).

There are no known wild relatives of sugarcane in Brazil, since all the species belonging to the "*Saccharum* Complex", an intercrossing group of species which have given origin to the *Saccharum* species, have their origin center in Southeast Asia (Roach & Daniels (1987) . However, in recent studies, some botanists have classified some Brazilian native plants from the *Erianthus* genus as belonging to the *Saccharum* genus: *S. villosum*, *S. asperum* and *S.* cf. *baldiwinii* (Flora Farenogâmica, 2008). Since very little is known about the biology of these plants, studies are required to check the likelihood of gene flow among these species.

Overall, the breakout group agreed that inter and intraspecific gene flow associated with sugarcane poses no significant concerns for the environmental risk assessment. In this case, the PF would lead to a conclusion that no more data need to be collected for an ERA for this crop.

The insertion of glyphosate tolerance in sugarcane may lead to the appearance of sugarcane volunteer

plants in the field if inadequate management is performed. This can happen due to the current use of glyphosate by growers to eradicate crop ratoons after planting ends, and as such poses a significant stewardship consideration for herbicide tolerant sugarcane. This does not mean that the GM sugarcane with tolerance to glyphosate will become weedier, since it will not have more ability to spread than the conventional variety (for example: more seeds, more dormancy, presence of rhizomes, etc). However, farmers should be aware that it will not be possible to use glyphosate to eliminate transgenic plants with tolerance to this herbicide and would need to adapt their cultural practices to the GM variety.

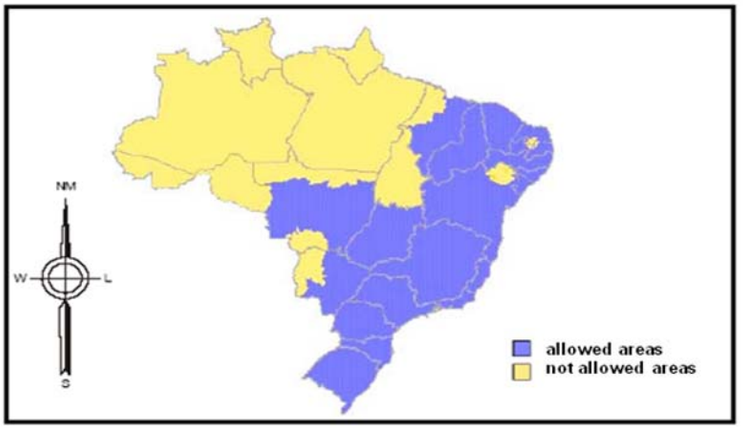

Figure 3. Exclusion zones for transgenic cotton in Brazil (Barroso et al., 2005).

Sugarcane Non-target Arthropods

Although there is no commercial release of a transgenic sugarcane variety, there is extensive experience and knowledge of non-GM sugarcane cultivation, which provides the essential biological and agronomic baseline information for PF. In addition, the data available from other GM crops already in the market with the same integrated traits can be used, allowing regulators to focus on the species that are unique to sugarcane and have not been studied yet. In this manner, it is known that Bt genes have effect on a limited range of insects and that Cry2A affects only lepidopteran insects, making unnecessary to study a broad range of NTAs.

It was suggested at the workshop that the impact of the transgenic variety on the population of ants should be studied. Ants belong to the order Hymenoptera, and some species are considered to be important to Brazilian sugarcane plantations because they act as predators for herbivorous pests. Although there was data on the safety of Cry2A for hymenoptera (honeybees, Duan et al, 2008) no direct data on ants were available in the scientific literature. However, the group recognized that, based on available information, it is expected that Cry2A should have no activity on hymenoptera at expected environmental concentrations Thus, it seemed reasonable to expect negligible risk. So, the conclusion was that the impact of GM sugarcane expressing Cry2 protein on ant populations should be discussed further, taking into consideration the insect biology and all the possible exposition pathways. An appropriate testable risk hypothesis that is linked to a credible causal pathway leading to harm should be drawn. Without this plausible hypothesis, the specificity of Cry2A may limit the relevance and need for further studies.

Sugarcane Additional Issues

Another important issue when dealing with an asexually propagated crop such as sugarcane is that the introduced trait is not easily passed to other varieties by the breeding programs. For sugarcane, it is even more difficult because of the complexity of the genome (D'hont 2005; Piperidis and D'hont 2001). On the other hand, there is a need for different varieties of sugarcane in order to satisfy seasonal operation needs from the mills and the different environmental conditions that the culture is cultivated. All this combined creates a challenge to regulators worldwide: how to evaluate new events of the same crop with the same construction? CTNBio's Normative Resolution #5, states that "GMO that contains the same genetic construction used in a GMO of the same species, with a favorable technical approval for commercial release in Brazil, shall pass through a simplified analysis for its release, under CTNBio's judgment". Without precedents, it is uncertain what kind of data this simplified analysis will require. The conclusions at the meeting were that all the data from risk assessment of the first variety approved should be used with the inclusion of the following data:

- Agronomic characterization of the parental from which the variety derives from;

• Molecular characterization of the transgenic variety;

• Determination of the expression level(s) of the transgene(s);

• Detection methodology for the transgenic variety.

These GM varieties are intended to be planted in Brazil, and their main products (ethanol and sugar) are commodities that are intended to be used for internal consumption and for exportation. Thus, the question of how the commercial release of different events of the same construction in sugarcane is going to happen is not only a concern for Brazilian regulators but also for regulators from countries importing Brazilian GM sugarcane products.

Although the exported Brazilian ethanol is almost completely intended to be used as biofuel, sugar is a product for human consumption. So, this can also raise questions about the food safety of this product and it is a problem that should be formulated and tested. On the other hand, the presence of DNA or Bt proteins at sugar (and also at ethanol) is expected to be negligible due to their high processing.

There is a lot of knowledge on sugarcane biology that has not been published and remains with the professionals that advise sugarcane growers. It is necessary to mine this knowledge base in order to have a complete package of information for the risk assessment prior to the commercial release of a GM sugarcane variety.

Conclusions

The conclusions of the workshop include:

- PF is a critical step in ERAs to ensure that testable risk hypotheses are developed in a structured, transparent manner;
- Effective PF can be used to identify relevant data that is both necessary and sufficient for the ERA, using existing knowledge whenever possible;
- RN#05 provides the essential legislative context for PF of GMO ERAs in Brazil, but requires appropriate interpretation;
- The hypothetical case studies of GM cotton and GM sugarcane with insecticidal traits provided practical and useful examples for applying PF;
- The diversity of participants provided a platform for useful discussions and networking.

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