A SUPPORT FRAMEWORK FOR DEPLOYMENT OF GENETICALLY MODIFIED SUGARCANE: IDENTIFYING POTENTIAL RISKS FROM SEXUAL REPRODUCTION OF COMMERCIAL CULTIVARS

By

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Abstract

GLOBAL investments in developing genetically modified (GM) sugarcane (*Saccharum* spp. hybrids) to enhance its agronomic performance generate novel bioproducts, and bio-industries based on the crop's attributes for high biomass production are increasing. As for all GM crops, GM sugarcane will have to go through regulatory systems prior to commercial release, which will encompass environmental safety assessments. However, sexual reproduction for the vegetatively propagated crop has not been studied except to improve breeding. Consequently, knowledge of sexual reproduction in a commercial production setting is lacking and, thus, the baseline to determine possible environmental effects of a GM sugarcane is non-existent. We have initiated research in Australia to close this historical and global knowledge gap for sugarcane to provide baseline information for determining any potential environmental risks and, if necessary, for developing risk management strategies for the deployment of GM sugarcane. A proposed research framework developed and refined for identifying and addressing risks arising from potential sexual reproductive avenues of non-intentional gene escape from a GM sugarcane crop is described. We have engaged Australian regulatory authorities frequently during the development of the framework and the subsequent research to address the important issues for which they consider there is insufficient information. The principles and approaches described in the research framework would be equally applicable for assessing potential issues for the deployment of GM sugarcane in other agro-environments and for future developments of novel GM grass crops.

Introduction

Increasingly, global investments are aimed at the development of GM sugarcane (*Saccharum* spp. hybrid) germplasm. Traits to improve the sugarcane business agronomically and those that will expand the crop's scope of agro-industrial end-uses are being targeted. During the development and prior to the release of GM sugarcane, regulatory systems in many jurisdictions will encompass rigorous, science-based environmental safety assessments. Initially, the potential routes of transgene escape and establishment (within and outside cultivation) for the environment receiving the GM sugarcane require identification. Future evaluations of environmental effects of individual transgenes can then be conducted and compared from such generic baseline.

Relative to the abundance of agronomical and environmental studies on factors affecting growth and development of the vegetatively-propagated crop, sugarcane sexual reproduction has been globally and historically ignored except by breeders. Kock (2007) highlighted the lack of any sugarcane-derived data in a presentation on biosafety regulation of GM crops as no data for cane existed in the scientific literature at that time. Such a situation was noted by Bonnett *et al.* (2007) in the context of a potential introduction of GM sugarcane, which would initially be addressed by conducting studies for an understanding of the crop's sexual reproduction. Sugarcane seed holds no caloric or economic importance in the harvested product. In most other crops, flowering is a pre-requisite to obtaining the harvestable product, seeds. In sugarcane, flowering is not required to obtain the next filial generation nor economic yield. Consequently, there are no studies on the reproduction of sugarcane in the field let alone gene dispersal via pollen or seed. For other grass crops, where the production of seed for planting material needs to be of high genetic purity and quality, the isolation distances of genotypes have been determined. Further, the absence of flowering of sugarcane in some areas has been used to assert sugarcane is a 'secure biofactory' (Wang *et al.,* 2005); indeed, an untested assumption in many sugarcane growing regions. Finally, while sugarcane is unlikely to pose any new risk not evaluated for other transgenic crops, the necessary data are not available for sugarcane. Hence, a comprehensive understanding of the sexual reproductive biology and ecology of sugarcane is required to provide baseline information for the decision-making process of regulatory authorities to manage the safe release of GM sugarcane.

Elements in risk assessments

Within regulatory systems, the assessment of environmental risks for the approval and management of GM crops involves (Craig *et al.,* 2008): a) the identification of potential environmental effects or perceived hazards that would arise from its release; b) an evaluation of the potential consequences; and, c) the determination of the probability (likelihood) of their occurrence.

Identifying potential environmental effects or hazards is generally based on biological features of the modified species/crop, and is dependent on the ecological context in which it is proposed for release. The general focus is on the potential for: a) unintentional escape of the GM organisms that would, if unmanaged, elicit a detrimental effect to the environment; and, b) transfer of the transgene(s), and thus those trait(s), to sexually compatible species (or to non-GM cultivars from GM crops) that would affect their fitness, alter their role in various environmental niches or facilitate the introgression to other non-targeted genetic pools.

Determining the likelihood of an environmental effect or hazard to occur would define the level of exposure to the environment. Once effects or hazards have been identified for a target environment, the competent authority within regulatory systems would assess any consequence of the particular transgenes in relation to any such hazard. While the evaluation of the consequences (for that effect or hazard to occur) is a process largely dependent on the specificity of the genes/traits modified, it is possible to generate a generic research framework to identify the effect or hazard component of risk assessment.

Development of a research framework

A conceptual framework to identify the environmental effect or hazard component of environmental risk is shown in Figure 1. The generic inquiries start with the most basic sexual reproductive process, flowering, and ends with seed germination and establishment of new plants in the target environment(s). Depending on the answers to the questions sequentially asked, the researcher is directed to more research. Should the process under study give rise to no potential effect or hazard, the information gathering can cease. For any particular transgene, a risk analysis would encompass additional analyses to verify if any biological parameter changed as a result of genetic improvement and, thus, if there are potential effects or hazards to be addressed. A risk analysis would then be prepared by the competent authority based on the data, methods, reproducibility, likelihood and relevance of the effect, with a subsequent preparation of a risk management strategy to mitigate or manage any risk.

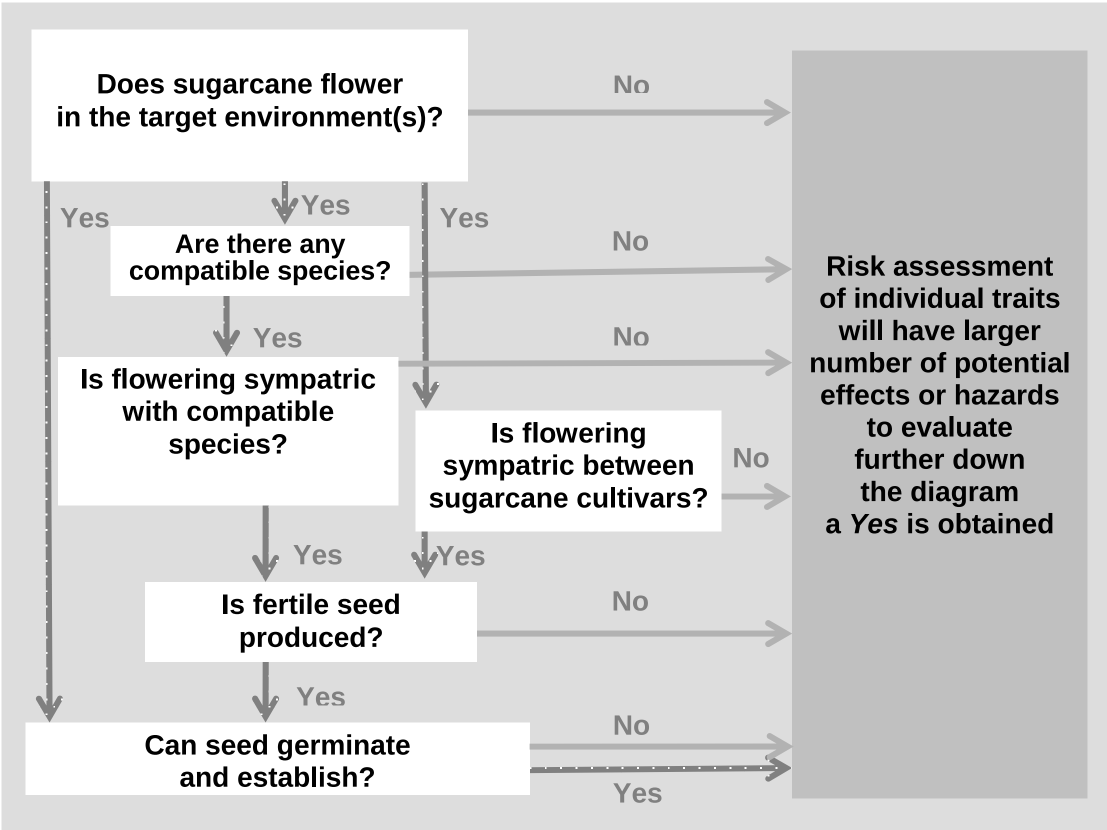

Fig. 1—A generic framework for the identification of potential environmental effects or hazards within a risk assessment, resulting from the sexual reproduction of sugarcane in commercial fields.

An important feature of the process of developing and refining this research framework has been regular engagement with Australian regulatory agencies during the planning and conduct of the research. This has ensured that their most important questions are being addressed, allowing us to modify both the strategy and experimental approaches as a consequence of feedback. With the publication of results as a final objective, this would allow: (*i*) the work to be available to a wider audience including regulators and technology developers; and, (*ii*) other researchers to apply it to their agro-environments of interest, and to identify any omission for their particular situation in order for them to design experiments that would fill those knowledge gaps. The subsequent sections summarise the results we have found when developing and applying the framework to sugarcane growing regions in Australia.

Does sugarcane flower in the target environment(s)?

Flowering varies greatly between years and locations (Berding *et al.*, 2004). In some instances, such observations have led to the location of breeding facilities in areas of highest flowering. They have also led, in areas of no or low flowering, to the notion that sugarcane is an ideal GM crop because it is vegetatively propagated and does not flower. However, prior to the release of GM crops, a determination of whether flowering occurs is crucial and, if it does, if any viable seed is produced. As sugarcane is grown over a range of latitudes and altitudes (even in one country), multiple environments should be monitored. In Australia, sugarcane is grown from latitude 16ºS in the north to 29ºS in the south, with flowering varying across seasons and cropping regions (Cox *et al.*, 2000). Our approach has been to monitor the flowering of sugarcane in commercial fields in several regions in north Queensland over several years; multiple sites within each region were monitored at monthly intervals. Flowering occurred to some degree in all regions in all years studied, with the peaks of flowering for cropping seasons 2007 and 2008 in the Mulgrave and Herbert River regions shown in Table 1. Additionally, we also have quantified the extent of flowering in other sugarcane growing regions at higher latitudes. Flowering, as expected, was less frequent and less intense.

Table 1—Time of highest flowering of sugarcane cultivars (*Saccharum* spp. hybrids) and of *Saccharum spontaneum* L. in the Mulgrave and Herbert River regions of north Queensland, Australia, seasons 2007 and 2008.

		2007		2008	
Region	Month	*Saccharum spontaneum*	Sugarcane hybrids	*Saccharum spontaneum*	Sugarcane hybrids
Mulgrave	March			7.34 (4.90)[C]	
	April	39.2 (23.4)[A]			
	June		48.6 (25.1)[B]		
	July				29.5 (36.4)[D]
Herbert	June	44.9 (34.5)[E]		45.7 (34.6)[G]	
	July		64.8 (38.8)[F]		61.5 (40.1)[H]

Note: The data represent the mean proportion of flowering stalks of replicates assessed in different locations across the season (S.D., standard deviation). The data shown is the maximum value observed across the season for the wild relative and the crop.

Number of assessed thickets/paddocks= [A], 30;[B], 46; [C], 32; [D], 46; [E], 23; [F], 26;[G], 25; [H], 17.

Are there any sexually compatible species to sugarcane?

Sugarcane and its closest relatives are not native in many sugarcane growing countries. A good source of information for identifying species and genera present in such different countries and regions are botanical flora and herbarium specimens. Internationally published flora compendia and taxonomical atlases are other valuable sources. In some instances, details of the occurrence and locations of plants related to sugarcane can be found in work describing the collection of germplasm for breeding purposes either indigenous (*e.g.*, Nagatomi and Degi, 2007) or endo-exogenous (*viz.*, regional or world germplasm repositories: Sugarcane Breeding Institute Collection, Coimbatore, India; Sugarcane Development Program, Canal Point, USA).

From breeding literature, we identified a list of species that were demonstrated to produce fertile hybrids with commercial sugarcane (Bonnett *et al.*, 2008). When comparing these with related species and genera present in the likely target environments of Australia for the future introduction of GM sugarcane, we identified that *Saccharum spontaneum* L. is the most likely species which could spontaneously hybridise with commercial sugarcane. A wild relative (and one of the progenitors) of modern sugarcane cultivars, *S. spontaneum* has established as naturalised populations in close proximity to commercial sugarcane in five recently recorded locations in north Queensland (Bonnett *et al.*, 2008), in addition to one remote location on the Daly River in the Northern Territory, where no sugarcane is commercially grown. While the north Queensland populations were identified from herbarium samples and local knowledge, we determined their extent by conducting land and river surveys. For the Northern Territory location, there were entries of *S. spontaneum* at the Queensland Herbarium. In countries where the progenitors and relatives of sugarcane occur sympatrically with sugarcane, the most important question is whether their flowering is synchronous with commercial sugarcane. A risk assessment, thus, would focus more on the consequence of the gene being transferred rather than the likelihood of transfer *per se*.

Is flowering sympatric with sexually compatible species?

Flowering times and seed production of commercial crops and of the naturalised populations of *S. spontaneum* were assessed as components of the regional monitoring previously described. The monthly observations indicated that there was synchronous flowering at some locations (Olivares-Villegas *et al.,* 2008). From an analysis of seed collected from the crop and the non-domesticated species (Table 2), we demonstrated that in some instances of synchronous flowering (Table 1), both were sexually fertile and produced viable seed. Thus, in those locations there appears to be an opportunity for hybridisation between commercial sugarcane cultivars and *S. spontaneum*. Consequently, managed experiments have been conducted to determine the ability of some of the naturalised populations of *S. spontaneum* to accept pollen from commercial sugarcane. Hybrid incidence is being assessed via non-radioactive molecular markers.

Table 2—Seed viability peaks of sugarcane cultivars (*Saccharum* spp. hybrids) and of *Saccharum spontaneum* L. in the Mulgrave and Herbert River regions of north Queensland, Australia, during seasons 2007 and 2008.

		2007		2008	
Region	Month	*Saccharum spontaneum*	Sugarcane hybrids	*Saccharum spontaneum*	Sugarcane hybrids
Mulgrave	March			7.11 (4.27)[C]	
	June	7.68 (13.3)[A]			
	July		53.0 (61.6)[B]		103.8 (91.8)[D]
Herbert	May			86.9 (122.1)[G]	
	June	79.4 (115.9)[E]			
	July		14.5 (31.7)[F]		

Note: Data are highest mean (from collections across the season) of (triplicated) seedlings germinating per gram at the constant, optimum germination temperature of 36°C, in controlled chambers. Data shown is the maximum value observed across the season (standard deviation, S.D.). No viable seed was recorded in Herbert River region for sugarcane hybrids in 2008.
Number of assessed thickets/paddocks= [A], 3; [B], 14; [C], 22; [D], 18; [E], 27; [F], 16; [G], 23.

Is flowering sympatric between sugarcane cultivars?

As sympatric, synchronous sexual fertility is mostly observed in commercial cultivars across the monitored regions (Olivares-Villegas *et al.*, 2008, Table 1), we have been evaluating the potential hybridisation among sugarcane cultivars under field conditions. Distance of pollen dispersal and studies on non-intentional, inter-cultivar hybridisation have also been conducted to determine the limits of gene transfer via the microgametophyte to other *Saccharum* spp. hybrids. Such information would establish a comparative baseline for gene escape during testing phases of GM cane crops, which may be dependent on the transformed germplasm and transgene involved.

Is fertile seed produced?

Transgene dispersal and establishment outside of cultivation via seed is one of the main environmental risk issues within the regulatory assessment for the prospective release of GM crops. While sugarcane is a vegetatively-propagated crop, under certain conditions it can produce seed — the basis of sugarcane breeding programs. As previously noted, our monitoring of north Queensland regions included seed collection not only of *S. spontaneum*, but also of sugarcane cultivars. As flowering is not indicative of the plant's ability to produce fertile seed, we tested the seed viability. Initial observations of variable seed fertility in opportunistic collections (Bonnett *et al.*, 2007) led to systematic assessments, where sub-samples of collected seed were subjected to *in vitro* germination tests. Viable seed was produced to some extent in some regions in each year when tested under the optimum germination temperature of 36°C (constant). The highest germinations of seed for 2007 and 2008 in the Mulgrave and Herbert regions are shown in Table 2. While the data is mostly lower than the average seed germination from breeders' crosses for sugarcane (*viz.*, 39 seedlings per gram of fuzz), seed germinates; thus, a risk assessment would have to consider its impact on the target environment. However, flowering may not always lead to viable seed set, either due to mega- or microgametophyte infertility resulting from genetic aberration and thermosensitivity.

Can seed germinate and establish in the target environment(s)?

Seed germination under *in vitro* ideal conditions is not indicative of seed's ability to germinate *in situ* (*viz.*, under the environmental conditions and niches to which the seed would be exposed). Although there is an information void on sugarcane seed longevity under field conditions and its response to environmental variables, there are some instances where there have been observations of in-field seedling establishment from germinated sugarcane seed, by sugarcane industry personnel. Albeit infrequent, there is potential for these plants to survive until sexual maturity, a fact yet to be verified as those observations were not systematically continued (to see if seedlings progressed to mature sugarcane plants). Currently, we are conducting studies on the potential of seeds and seedlings to germinate and establish, respectively, in various target environments where germination has been observed in the past, and their development (through time) is being followed.

We have also determined the response of sugarcane seed to temperature upon testing both seed produced by breeders and that collected from commercial fields under conditions of non-limiting moisture. We found that the optimum germination occurred between 30–36^{o}C, with a 60% reduction at 24°C and a further reduction of 50% at 18°C, while there was some seed germinating at 15°C and, none was observed at 10°C (Powell *et al.,* in preparation). Thus, temperature *per se* might not prevent germination of viable seed in sugarcane growing regions of Australia. As temperature is not the sole factor that determines germination in the environment, experiments are underway to understand the effects of various moisture stresses on sugarcane seed germination.

Further research to support decision making on environmental issues by regulators

Evolution of increased weediness is a potential, significant environmental consequence of non-intentional gene flow from cultivated plants to wild relatives (Ellstrand *et al.,* 1999). Plant ecologists and population geneticists have studied such gene flow in the context of conventionally-improved crops to anticipate possible risks of transgenic crops. The most discussed resulting effects from crop-to-wild hybridisation is increased fitness through introgression, the evolution of increased weediness or invasiveness in wild relatives, the evolution of pests that are resistant to new strategies for their control, and the impacts on non-target species in associated ecosystems (Dale *et al.,* 2002).

In sugarcane, the centuries-old agricultural practice has been to select against weedy traits in the vegetatively propagated crop, but there are wild relatives of sugarcane that are weeds (Anon., 2008). In particular, some genotypes/ accessions of *S. spontaneum* are aggressive weeds in regions of southeast Asia, Indonesia, the Philippines and, recently, in Panama (Holm *et al.,* 1997; Hammond, 1999); whether environmentally influenced or not, those accessions have particular allele combinations that could explain their resistance to environmental stresses, seed dispersal, vigorous tillering and fast developing rhizomatous root system. In Australia, the naturalised populations of *S. spontaneum* have not become as invasive, but such potential could be latent. Current studies are being conducted to understand those biological and ecological features that could explain the invasiveness of the weedy accession in Panama. A non-intentional elicitation of weediness through the modification of certain traits, such as those conferring abiotic stress tolerance, might potentially become an important issue to monitor in future developments of GM cane.

Conclusions

We have described a research framework for identifying and addressing risks related to potential avenues of non-intentional gene escape from a GM sugarcane crop, providing information that would aid decisions for GM cane management. While the framework was conceived for the cropping context of sugarcane growing in Australia and for assisting decision-making within its regulatory system, it is applicable to other cane-growing industries across the world, particularly where those jurisdictions have legislation based on science-based evaluations (*e.g.*, following legislation adherent to international standards). In addition to its application to other sugarcane agro-environments, the approaches also could be applied to other grass crops potentially useful for biofuel or bioproducts applications where sexual reproduction is not a pre-requisite for economic yield and so may be relatively unknown. The intent of this research was not to address the specific effects of particular genes, but to provide information about generic elements when potentially introducing a GM sugarcane into the environment. The research should, however, address decisions for the development and managed deployment of GM sugarcane.

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UN CADRE POUR L'EXPLOITATION DE LA CANNE À SUCRE GÉNÉTIQUEMENT MODIFIÉE: IDENTIFICATION DES RISQUES POTENTIELS DE LA RÉRPODUCTION SEXUÉE DES CULTIVARS INDUSTRIELS

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**MOTS-CLÉS: Canne à Sucre, Modification Génétique,
Biotechnologie, Réglementation,
Déploiement, Reproduction Sexuée.**

Résumé

LES INVESTISSEMENTS à travers le monde sont en progression pour le développement de la canne à sucre (hybrides de *Saccharum* spp.) génétiquement modifiée (GM) dans le but d'améliorer sa performance agronomique pour générer de nouveaux bioproduits et pour l'utiliser comme bio-industrie grâce à sa production de biomasse élevée. Comme pour toutes les cultures GM, la canne GM aura à se conformer à des systèmes réglementaires, comprenant des évaluations de biosécurité à l'environnement avant son exploitation industrielle. Cependant, la reproduction sexuée de cette culture qui est propagée de manière végétative, n'a pas encore été étudiée, sauf pour les besoins d'amélioration génétique. Conséquemment, une connaissance dans un milieu de production industrielle n'est pas disponible et cela justifie l'obtention des données de base pour évaluer des éventuels effets environnementaux de la canne GM. Nous avons initié une recherche en Australie pour combler cette lacune historique et mondiale pour la canne à sucre. Cette étude consistait à obtenir des informations de base pour déterminer les éventuels risques à l'environnement, et si nécessaire, développer des stratégies de gestion appropriées pour l'exploitation de la canne à sucre GM. Un cadre de recherche développé et raffiné pour identifier et adresser les risques découlant des avenues potentielles de la reproduction sexuée liée à des fuites non-intentionnée des gènes de la canne GM est rapporté. Nous avons engagé les autorités régulatrices australiennes pendant le développement de ce cadre et pour la recherche subséquente pour adresser les aspects importants pour lesquels elles considéraient ne pas détenir suffisamment d'information. Les principes et les approches décrits dans ce cadre de recherche s'appliqueraient aussi pour évaluer les questions potentielles pour l'exploitation de la canne GM dans d'autres agro-environnements et pour le développement futur des nouvelles cultures herbacées génétiquement modifiées.

UNA INFRAESTRUCTURA PARA APOYAR LA LIBERACIÓN DE LA CAÑA DE AZÚCAR GENÉTICAMENTE MODIFICADA: IDENTIFICANDO RIESGOS POTENCIALES DERIVADOS DE LA REPRODUCCIÓN SEXUAL DE CULTIVARES COMERCIALES

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PALABRAS CLAVE: Caña de Azúcar, Modificación Genética, Biotecnología, Regulación, Liberación, Reproducción Sexual.

Resumen

INVERSIONES mundiales están en incremento para desarrollar caña de azúcar (híbridos de *Saccharum* spp.) genéticamente modificada (GM) a fin de aumentar su desempeño agronómico, generar novedosos bioproductos y explotar bioindustrias sustentadas en los atributos del cultivo para producir gran biomasa. Al igual que para todos los cultivos GM y previo a su liberación comercial, la caña de azúcar GM tendrá que cursar sistemas de regulación entre cuyas evaluaciones a conducir se encuentra la seguridad ambiental. Sin embargo, para el cultivo propagado vegetativamente, su reproducción sexual no ha sido estudiada excepto para progresar el fitomejoramiento. En consecuencia, falta la comprensión de la reproducción sexual en un contexto de producción comercial y, por tanto, resulta inexistente el referente para determinar posibles efectos ambientales de una caña de azúcar GM. Iniciamos investigaciones en Australia para cerrar esta brecha histórica y mundial a fin de proveer información referencial para determinar cualesquier riesgos potenciales al ambiente y, de ser necesario, para desarrollar estrategias de manejo de riesgo en la liberación de caña de azúcar GM. Proponemos y describimos una infraestructura de investigación desarrollada y afinada para identificar y atender riesgos derivados de las potenciales avenidas reproductivas sexuales de escape génico no intencional desde un cultivo de caña de azúcar GM. Hemos involucrado frecuentemente a las autoridades regulatorias Australianas durante el desarrollo de la infraestructura e investigación subsecuente a fin de atender las cuestiones más importantes para los cuales consideraron que no existe información suficiente. Los principios y enfoques descritos en la infraestructura de investigación son aplicables, igualmente, a la evaluación de asuntos potenciales en la liberación de caña de azúcar GM en otros agro-ambientes y para desarrollos futuros de cultivos de pastos GM.