Bioveiligheidsraad
Conseil de Biosécurité

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Secretariaat
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Title: Advice of the Belgian Biosafety Advisory Council on guidance on risk assessment of Living Modified Organisms under the Cartagena Protocol on Biosafety

Context

On 20 November 2009, the Minister of Climate and Energy, Mr Paul Magnette, asked the Biosafety Advisory Council (BAC) for giving an advice concerning scientific aspects related to some draft decisions submitted to the Parties of the Cartagena Protocol on Biosafety.
This request was addressed in the frame of the forthcoming fifth Meeting of the Parties to the Protocol (MOP-5, Nagoya, Japan, 11-15 October 2010) in particular taking into account that Belgium will hold the Presidency of the European Union at that time.

The Cartagena Protocol on Biosafety is an international Treaty governing the movements of genetically modified organisms (GMOs) (called “living modified organisms – LMOs” in the Protocol) from one country to another. It was adopted on 29 January 2000 and entered into force on 11 September 2003 (Belgium is a Party to the Protocol since 2004). It addresses the transfer of LMOs from one country to another through various procedures for ensuring that countries are provided with the information necessary to make informed decisions before agreeing to the import of such organisms into their territory.

For the purpose of making informed decisions regarding transboundary movements of LMOs, Parties to the Protocol shall ensure that risk assessments are carried out. Annex III of the Protocol describes in general terms the objective, general principles, methodology and points to consider for the risk assessment. Given the general nature of this annex, the Parties to the Protocol decided to facilitate and enhance its effective use by elaborating technical and scientific documents providing further guidance on specific aspects of the risk assessment.

In considering the need for further guidance, the Parties to the Protocol established an open-ended online forum on specific aspects on risk assessment through the Biosafety Clearing-House (BCH) of the Cartagena Protocol and an Ad Hoc Technical Expert Group on Risk Assessment and Risk Management (AHTEG).

The discussions under the Open-ended Online Expert Forum and the AHTEG resulted in the drafting of a "Guidance on Risk Assessment of Living Modified Organisms" (published in document UNEP/CBD/BS/COP-MOP/5/INF/15[1]). This document is divided in four sections :

- A "Roadmap for risk assessment of living modified organisms". This part provides general guidance on how to use Annex III of the Protocol and all other articles related to risk assessment, by elaborating the technical and scientific process of how to apply the steps and points to consider in the process of risk assessment.

[1] Available at http://bch.cbd.int/protocol/meetings/documents.shtml?eventid=3018; Previously circulated as UNEP/CBD/BS/AHTEG-RA&RM/2.

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- A guidance on "Living modified organisms with stacked genes or traits".
- A guidance on "Risk assessment of living modified crops with tolerance to abiotic stress".
- A guidance on "Living modified mosquitoes".

In answer to the request of the Minister, the BAC drafted the current advice, relating to the four sections of the abovementioned "Guidance on Risk Assessment of Living Modified Organisms".

Procedural aspects

At its meeting of 18 June 2010, the BAC mandated the Division of Biosafety and Biotechnology (SBB) to prepare a draft advice on the "Guidance on Risk Assessment of Living Modified Organisms" published in document UNEP/CBD/BS/COP-MOP/5/INF/15, after consultation of external experts when needed.

On this basis, the SBB (acting as technical expert) offered to evaluate the "Roadmap for risk assessment of living modified organisms" and the guidance on "Living modified organisms with stacked genes or traits". In addition, several external experts were contacted to provide a scientific opinion on the guidance on "Risk assessment of living modified crops with tolerance to abiotic stress" or on the guidance on "Living modified mosquitoes". The experts were chosen firstly from the common list of experts drawn up by the Biosafety Advisory Council and the SBB, but also outside of this list in order to benefit from the best expertise in these very specific fields.

When assessing the documents, the SBB and the experts considered in particular whether:
- The roadmap and the guidance documents can be considered useful tools to support the risk assessment of LMOs ;
- Elements presented in these documents are scientifically sound and sufficiently described ;
- These documents are compatible with similar documents developed at EU level (notably by EFSA) ;
- There are important missing elements that should be added.

A compilation of the contributions received from the external experts is provided in Annex I.

Scientific evaluation

1. Roadmap for risk assessment of living modified organisms

Broadly speaking, the roadmap can be considered a useful tool to explain in more details the process and criteria to be applied in the risk assessment of living modified organisms (LMOs). It is indeed complementary to Annex III of the Cartagena Protocol but remains very general and needs therefore to be combined with existing guidance and working tools for risk assessment.
There are no incompatibilities or discrepancies between the roadmap and the EFSA guidance for the risk assessment of LM plants.

As regards the content of the roadmap, the following general comments can be made:
- The roadmap has been developed largely based on information and experience available from risk assessment of LM crops. It is therefore important that this bias is corrected in the near future by further updating and improving the roadmap in light of new experience and information gained as a result of the evaluation of new types of LMOs. For instance,

in case of transgenic animals, mechanisms and metapopulation features allowing suppression of LMOs geographic and demographic spread should be considered.
- The use of terms in the roadmap should be consistent throughout the document.
- The roadmap highlights important characteristics of the risk assessment such as the fact that it is an integrated process, to be conducted in an iterative manner, and performed on a case-by-case basis in comparison with the risks posed by the non-modified recipient organism in the likely receiving environment. Ideally, the non-LM comparator provides a baseline for comparison when it is grown at the same time and location as the LMO. As regards this last point however, defining a solid baseline to do comparative risk assessment may prove difficult in certain cases, such as for the risk assessment of LM mosquitoes or LM plants resistant to abiotic stress. This could be indicated in the roadmap.
- The roadmap should underline the importance that sound statistical tests should be used and completely described in the risk assessment process.
- It is not clearly described in the roadmap what is meant with phenotypic characteristics, is this solely expression levels or does it also include plant characteristics ? Referring to the wording "genotypic, phenotypic and biological changes in the LMO", it is also not clearly described which changes are covered by biological changes which are neither phenotypic nor genomic.
- Under "Overarching issues", the importance of having multiple and contradictory expertise when analyzing data supporting the risk assessment should also be mentioned.
- Under "Overarching issues", the fundamental conceptual difference between uncertainty analysis (UA) and variability analysis (VA) could be mentioned. A fundamental difference is that VA can be characterized by collecting more data and further research. However, as stated in the roadmap, further research is not necessarily a guarantee for reducing UA.

2. Guidance on "Living modified organisms with stacked genes or traits"

Detailed comments on the guidance are provided in Annex I.
Generally speaking, attention should be given to the consistency in the terminology of terms related to genetic, phenotypic, biological changes. In addition, more clear information should be provided on the scope of the risk assessment of stacked events. More specifically, it should be made more explicit (1) if unintentional stacked events fall under the scope of the guidance document and (2) that the focus of the risk assessment of stacked events is on the assessment of combinatorial effects.
No incompatibilities or discrepancies were observed between this guidance and the EFSA Guidance Document for the risk assessment of genetically modified plants containing stacked transformation events.

3. Guidance on "Risk assessment of living modified crops with tolerance to abiotic stress"

Although this guidance is generally considered a useful tool to support the risk assessment of living modified (LM) crops with tolerance to abiotic stress, some major weaknesses, redundancies or missing elements were identified, in particular related to specific issues linked to the environmental risk assessment of new traits conferring abiotic stress tolerance.
It should be underlined that this document is not a full guidance and should therefore be used together with relevant background documents or risk assessment tools.

Detailed comments on the guidance are provided in Annex I. Amongst these, the following one can be highlighted :
- In its current structure, the document may confuse the reader because it does not follow the logical five steps of the risk assessment. Given that the guidance should be seen as

an appendix to the roadmap, it would gain in clarity if it would follow the same basic structure.

- The definition of "abiotic stress" should be revised in the light of other definitions proposed by leading authors on the subject (see proposals in the annex). A clear definition of "tolerance" could be also useful, in particular to highlight the distinction between the wordings "stress tolerance" and "stress resistance".
- As for other LM plants, identifying protection goals, assessment end-points (a valued ecological entity and its measurable attributes providing a framework for assessing stress response relationships in a quantitative manner) and risk hypotheses should be an important aspect in setting the context and scope for the risk assessment of LM crops with tolerance to abiotic stress. The implementation of this "problem formulation" in the specific case of LM crops with tolerance to abiotic stress is not addressed sufficiently in the guidance.
- Risk hypothesis could be tested according a "tiered approach", thereby avoiding the gathering of a large set of agronomic data of limited safety relevance.
- As indicated in the guidance, the choice of a good non-LM comparator and the comparative analysis in general could be a challenge. More guidance could be given on how the comparative approach should be performed for this particular type of LMO.
- According to some experts, the proposal to use of the "omics" technologies as a potential tool to contribute to the comparative analysis is not convincing for this particular type of LMO. Indeed, natural variation is expected for many endogenous proteins in fluctuating environments, with no *a priori* relevance for the environmental and health safety.
- A major difficulty in performing the safety assessment of LM crops with tolerance to abiotic stress via comparative analysis is the multiple interactions between the new trait, the genetic background and the receiving environment. The guidance document is of limited use for deciding how to address these interactions in a realistic and informative manner, providing relevant information on safety.
- As there are significant connections between both the signalling pathways and the genes and molecules involved in diverse abiotic stresses, it is likely that some modified traits will cause increased tolerance to more than one abiotic stress. Therefore combinational effects associated with a modification of a specific trait should not necessarily be considered as "unintended effects". They rather fall sometimes under one of the objectives within the field of abiotic stress tolerance, which is to find traits providing crops with increased tolerance against multiple and/or combinations of abiotic stress. The guidance should better address how the risk assessor define the cross talk between different stress responses of the plant and when this information must be considered relevant from a safety point of view.
- When appropriate, the LM crops and their comparator(s) should also be tested in non-stressed conditions (in case the LM crops will not face permanently the stressing conditions). In addition, it would be important to test if the molecule(s) associated with tolerance is (are) expressed constitutively or in answer to stress conditions only and, in the later case, if other elements than the expected stressing conditions can trigger the tolerance properties.
- The guidance should be more explicit on how to consider the impact of the recipient genetic background on the intended and unintended predicted effects. This is a very important aspects for LM crops with tolerance to abiotic stress, because different plant varieties may initially show different stress tolerance capacities, meaning that the added value of the introduced gene - the intended effect of improved stress tolerance - will depend from one variety to the other.
- The guidance should address the difficulties (i) of controlling/measuring the environmental conditions in the field experiments analyzing plant phenotype and (ii) of defining the plant phenotype itself, which results from a complex relationship between external and physiological parameters. It is therefore important to scope which phenotype to assess and to check phenotypic differences based on the mode of action of the introduced gene(s), especially when relationship between transgene(s) and intended differences is not clear.

- Under "Increased persistency in agricultural areas and invasiveness", gene flow between feral and wild relatives should also be considered. The introduction of stress-tolerant traits could indeed result in an increase of crop ferality (due to some factors as variety of pollinators, longer or continuous seed production, high seed output in different environments, increasing seed dispersal, broad germination requirements...).
- It is particularly relevant in the case of LM crops with tolerance to abiotic stress to consider the importance of regional aspects. Regions and locations selected to collect data or conduct field trials should represent the range of agricultural, plant health and environmental conditions the LMO is expected to encounter when commercially cultivated.
- LM plants used in phytoremediation should attract a special attention. In that case, in addition to the LM plant intrinsic characteristics one should also address evolving compositional characteristics of the plant accumulating products from the contaminated environment. The effect of the LMO on the abiotic environment should also be assessed.

4. Guidance on "Living modified mosquitoes"

It is considered that the proposed document is a useful tool to guide the risk assessment of Living modified (LM) mosquitoes. However, taking into account the several comments of the experts, some points could be added and/or could be further elaborated:

- The development of LM mosquitoes, in particular in the frame of a self-propagating strategy, raises specific questions as regards their risk assessment and also the application of the provisions of the Cartagena Protocol. Indeed, it should be underlined that mosquitoes, being LM or not, have very broad dissemination spectra and that it will be very unlikely (if not impossible) to deal with their containment in the importing country only. Releasing LM mosquitoes will therefore intentionally or unintentionally affect several countries. This should be taken into account in the frame of the risk assessment. This should also trigger a broader reflexion on how the Protocol should apply to this type of LMOs, taking also into account the provisions of Article 17 on Unintentional Transboundary Movements, and Article 25 on Illegal Transboundary Movements.
- Given the use of two kind of strategies, i.e. self-limiting and self-propagating systems, and the different biological risks that these strategies may impose, it is suggested to elaborate the guidance for both strategies in a more distinct and explicit manner. Within this respect, the population suppression strategy (mostly relying on the self-limiting system) and aiming at reducing the number of disease-transmitting mosquitoes could be addressed separately from the strategy of permanent replacement (using gene driving systems) which aims at replacing wild mosquito populations by LM mosquitoes, and which could need more constraining risk assessment guidelines.
- Although the focus of the guidance is on LM mosquitoes, it is mentioned that the guidance may be useful for the risk assessment of similar non-LM mosquito strategies. Within this respect, paratransgenesis could be mentioned in the introduction as an alternative approach to introduce effector genes into mosquitoes by utilizing genetically modified insect symbionts to express molecules within the vector that are deleterious to pathogens they transmit. Another reason to mention this approach is that paratransgenesis can be used in both population suppression strategies and population replacement strategies.
- The general approach of using a near isogenic line in the comparative assessment will be a difficult point to meet for mosquitoes. The line used for transformation may serve as control. In addition, defining a species is too vague as for mosquitoes with a worldwide distribution, a lot of subspecies or strains were described with different properties, including ecological niche and capacity of pathogen transfer. Within this respect, one of the first step should consist in the complete taxonomic characterisation of the strain used, including the use of reliable molecular markers and its biogeographic origins.
- While addressing the interaction between LM mosquitoes and any other species, emphasis should be made to evaluation of the fitness of the LM mosquito and particularly its competitive capacity with the native strains and with other species of the same guild

sharing the same kind of environment. This should be done for the aquatic larvae as for the adults. Potential modifications in mosquito fitness and their competitivity should also be considered in light of the consequence of veterinary of health measure management (such as insecticide treatments or medication taken by the host) and their potential consequence on the gene expression in the LM organism.

- Potential adverse effects as a result of the interaction between LM mosquitoes and Wolbachia could be given particular attention because mosquitoes are currently infested by these bacteria, horizontal gene transfer between those species appear to occur, Wolbachia appear to reduce host fitness and to hamper virus transmission, such as for the Dengue viruses.
- The risk of dispersal due to anthropogenic activities, such as transport and trade of potential source of breeding sites such as tyres or lucky bamboos should be considered. The consequences of water management practice, irrigation, sewage water treatment etc. on the introduction of LM strains should also be taken into account.

Overall Conclusion

Broadly speaking, the Biosafety Advisory Council (BAC) is of the opinion that the roadmap and the three guidance published in document UNEP/CBD/BS/COP-MOP/5/INF/15 can be considered useful tools to support the risk assessment of living modified organisms (LMOs). This is particularly true for people having limited practical experience in performing or revising environmental risk assessment of LMOs.
Drawing up guidance on LM stacked events, LM plants with tolerance to abiotic stress and LM mosquitoes is also timely as risk assessors are already or will be soon facing the challenges of evaluating data relating to these products.

The roadmap and the associated guidance documents, in their current form, should be regarded as general guidance aiming at facilitating access and use of more detailed guiding tools to the corresponding topics. These documents are therefore not self-sufficient and should be completed by more detailed tools developed, if appropriate, in the context of the Cartagena Protocol and/or should be considered complementary to guidance developed by other bodies such as EFSA, OECD, IPPC, OIE..., providing these other guidance documents are coherent with each other and consistent with the specific objectives of the Cartagena Protocol as regards risk assessment of LMOs.
In that respect, it should be pointed out that, in their current form, the roadmap and the guidance on LM stacked events do not show incompatibilities or discrepancies with similar guidance published by EFSA.

The BAC is of the opinion that, to make the roadmap and associated guidance documents really useful:
- links should be established quickly to relevant background documents;
- testing should be performed using practical case-studies.

Although the roadmap and the three guidance are useful documents, the Biosafety Advisory Council would like to stress that its experts have identified many points for improvements, which are detailed here above and in the annex. It is therefore important that these documents are viewed as "living document" that can be modified and improved on over time in the light of new experience, information and developments in the field of applications and risk assessment of LMOs.

The BAC advises the Belgian authorities to ensure that a process aiming at amending the current version of the roadmap and the guidance (notably on the basis of comments made by the Belgian experts) is set up rapidly.

p.p. Dr. Ph. Herman

Prof. D. Reheul
President of the Belgian Biosafety Advisory Council

Annex: Compilation of comments of experts in charge of evaluating the guidance on risk assessment of Living Modified Organisms under the Cartagena Protocol on Biosafety (ref: BAC_2010_778)

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Compilation of comments of experts in charge of evaluating the guidance on risk assessment of Living Modified Organisms under the Cartagena Protocol on Biosafety

Mandate for the Group of Experts: Mandate of the Biosafety Advisory Council (BAC) of 18 June 2010
Coordinator: SBB
Experts: Didier Breyer (WIV-ISP, SBB), Adinda De Schrijver (WIV-ISP, SBB), Linda De Vooght (Institute Of Tropical Medicine), Patrick du Jardin (ULg - Gembloux Agro Bio Tech), Michel Edmond Ghanem (UCL), Jean-Claude Grégoire (ULB), Thierry Hance (UCL), Jean-Luc Hofs (CIRAD), Katia Pauwels (WIV-ISP, SBB), Frank Van Breusegem (UGent)
Domains of expertise of experts involved: Risk assessment, GM stacked events, GM mosquitoes, GM plant tolerant to abiotic stress
Secretariat (SBB): Didier Breyer, Adinda De Schrijver, Martine Goossens, Philippe Herman, Katia Pauwels

INTRODUCTION

This compilation relates to the evaluation of a "Guidance on Risk Assessment of Living Modified Organisms" published under the Cartagena Protocol on Biosafety. This document is divided in four sections :

- A "Roadmap for risk assessment of living modified organisms".
- A guidance on "Living modified organisms with stacked genes or traits".
- A guidance on "Risk assessment of living modified crops with tolerance to abiotic stress".
- A guidance on "Living modified mosquitoes".

Depending on their expertise, the experts were asked to evaluate one or several of these four sections. The experts were asked in particular to considered whether:

- The roadmap and the guidance documents can be considered useful tools to support the risk assessment of GMOs ;
- Elements presented in these documents are scientifically sounded and sufficiently described ;
- These documents are compatible with similar documents developed at EU level (notably by EFSA) ;
- There are important missing elements that should be added.

Comment 1

Broadly speaking, the roadmap can be considered a useful tool to explain in more details the process and criteria to be applied in the risk assessment of living modified organisms (LMOs). It is indeed complementary to Annex III of the Cartagena Protocol but remains very general and needs therefore to be combined with existing guidance and working tools for risk assessment.
There are no incompatibilities or discrepancies between the roadmap and the EFSA guidance for the risk assessment of GM plants.

As regards the content of the roadmap, the following general comments can be made:

- The roadmap has been developed largely based on information and experience available from risk assessment of LM crops. It is therefore important that this bias is corrected in the near future by further updating and improving the roadmap in light of new experience and information gained as a result of the evaluation of new types of LMOs.
- The use of terms in the roadmap should be consistent throughout the document.
- The roadmap highlights important characteristics of the risk assessment such as the fact that it is an integrated process, to be conducted in an iterative manner, and performed on a case-by-case basis in comparison with the risks posed by the non-modified recipient organism in the likely receiving environment. Ideally, the non-LM comparator provides a baseline for comparison when it is grown at the same time and location as the LMO. As regards this last point however, defining a solid baseline to do comparative risk assessment may prove difficult in certain cases, such as for the risk assessment of LM mosquitoes or LM plants resistant to abiotic stress. This could be indicated in the roadmap.
- It is not clearly described in the roadmap what is meant with phenotypic characteristics, is this solely expression levels or does it also include plant characteristics ? Referring to the wording "genotypic, phenotypic and biological changes in the LMO", it is also not clearly described which changes are covered by biological changes which are neither phenotypic nor genomic.
- Under "Overarching issues in the risk assessment process", the importance of having multiple and contradictory expertise when analyzing data supporting the risk assessment could also be mentioned.
- Under "Overarching issues", the fundamental conceptual difference between uncertainty analysis (UA) and variability analysis (VA) could be mentioned. A fundamental difference is that VA can be characterized by collecting more data and further research. However, as stated in the roadmap, further research is not necessarily a guarantee for reducing UA.
- In addition, regarding the establishment of scientifically robust data, emphasis could be made on the quality and transparency of statistical tests.

Comment 2

Concerning the general road map, I have some comments on the overarching issue section (page 14 of the UNEP/CBD/BS/AHTEG-RA&RM/2/5 document, actual page 3). As we had already problems in the past with some dossier concerning plant GMOs, I think that concerning the establishment of scientifically robust criteria it is very important that sound statistical tests should be used and completely described in the risk assessment process. It seems obvious when we are speaking of science, but in practice, in the past the transparency and the quality of statistical tests were not always reached which hampered a correct evaluation by the experts. For instance the statistical guidelines proposed by the EFSA should at least be recommended.

Comment

PART II:

SPECIFIC TYPES OF LMOs AND TRAITS

A. RISK ASSESSMENT OF LIVING MODIFIED **ORGANISMS** WITH STACKED GENES OR TRAITS

Comment : Better to speak of « PLANTS or CROPS » here. Is more specific. Is there a preference to use the term « LM crops » or « LM plants » throughout document?

INTRODUCTION

Worldwide, a growing number of LMOs with stacked **transgenic traits**, particularly LM crops, are being developed for commercial uses. As a result, the number of stacked genes in a single LMO and the number of LMOs with two or more transgenic traits is growing.

Comment : The term « transgenic trait » is only used in this section on RA of stacked events. It would be better to use the same term, i.e. just "trait" throughout the document. Moreover, the trait is not transgenic…it's the plant/LMO that is transgenic.

Stacked transgenic traits can be produced through different approaches. In addition to the cross-hybridising of two LMOs, multiple trait **characters** can be achieved by transformation with a multigene cassette, retransformation of an LMO or simultaneous transformation with different **transgene cassettes** (i.e., cotransformation).

Comment : No stacked traits are produced, but LMOs…Why waiting till second page to introduce the term StaEv?
Comment : "characters" => Not the right wording…I would rather say « … multiple traits »…
Comment : Elsewhere one uses the term « gene cassette, tranformation cassette ». I think it is preferable to use the terminology as defined in the protocol.

This guidance document focuses on stacked transgenic traits that have been produced through cross-breeding of two or more LMOs.

LMOs with multiple transgenic traits resulting from re-transformation, co-transformation or transformation with a multigene cassette should be assessed according to the Roadmap.

This guidance document complements the Roadmap for Risk Assessment developed by the AHTEG on Risk Assessment and Risk Management, and focuses on issues that are of particular relevance to the risk assessment of **LMOs with stacked events** generated through cross breeding of single or multiple event LMO.

Comment : Better to say « LMO with stacked traits » According to definition, a stacked event is an LMO, so the phrasing "LMOs with stacked event (with LMO)" is not correct. Either say "… RA of stacked events…" or "… RA of LMOs with stacked traits…"

This is intended to be a "living document" that will be shaped and improved with time as new information and/or experience becomes available and new developments in the field of applications of LMOs occur, as and when mandated by the Parties to the Protocol.

OBJECTIVE

The objective of this document is to give additional guidance on the risk assessment (RA) of LMOs with stacked events generated through conventional crossing of single or multiple event LMOs. Accordingly, it is meant to complement the Roadmap for Risk Assessment and address special aspects of LMOs with stacked transgenes/traits resulting from the conventional crossing. For the time being it will be restricted to plant LMOs.

USE OF TERMS

Transformation event (TraEv)

For the purpose of this document, a transformation event (TraEv) is an LM plant which results from the use of modern biotechnology applying *in vitro* nucleic acid techniques that may involve, but is not limited to, single or multiple **gene transformation** cassettes. In either case, the result will be one transformation event.

Comment : Better to use the term « gene cassette » throughout document?

Stacked event (StaEv)

For the purpose of this document, a stacked event (StaEv) is an LM plant generated through conventional cross breeding of two or more single parental transformation events (TraEvs) or two already stacked events. Accordingly the transgene cassettes may be physically unlinked (i.e. located separately in the genome) and may segregate independently.

Unintentional stacked event

Unintentional stacked events are the result of outcrossing of stacked events into other LMOs or compatible relatives in the receiving environment. Depending on the segregation pattern of the stacked genes this may result in new and/or different combinations of TraEvs.

SCOPE

Comment : It is not clear if « unintentional stacked events » fall within or out of the scope of this document.

This guidance document focuses on stacked events (StaEv) resulting from conventional crossings between two or more single transformation events (TraEv) as parental lines so that the resulting LMO contains two or more transgenic traits. It is understood that the individual TraEvs making up the StaEv have been assessed previously in accordance with Annex III of the Cartagena Protocol on Biosafety and as described in the Roadmap.

ISSUES TO BE CONSIDERED IN THE RISK ASSESSMENT

Comment : An introductionary paragraph could be included specifying that the RA of StaEvs – as becomes clear from the text below – focuses on the assessment of combinatorial effects.

Assessment of sequence characteristics at the insertion sites and genotypic stability *(see Step 1, Point to consider (c) of the Roadmap for Risk Assessment)*

Rationale:

~~Although recombination, mutation and rearrangements are not limited to LMOs, t~~he combination of transgenic traits via cross breeding may further change the molecular characteristics of the inserted genes/gene fragments at the insertion site and/or influence the regulation of the expression of the transgenes. In addition, changes to the molecular characteristics may influence the ability to detect the LMO, which may be needed in the context of risk management measures (see Step 5 of the Roadmap. **The reappraisal of the molecular sequence at the insertion sites, and the intactness of the transgenes may be confirmative to the molecular characteristics of the parental LMOs, but may also be a basis** for assessing any intended or unintended possibly adverse effects on the conservation and sustainable use of biological diversity in the likely potential receiving environment and of potential adverse effects on human health. The extent of the reexamination may vary case by case and take into account the results of the parental LMO risk assessment.

Comment : Redundant, this does not explain way changes at molecular level may occur due to cross-breeding.
Comment : What is meant with this? Reappraisal?

Assessment of potential interactions between combined events and the resulting phenotypic effects *(see Step 1, Point to consider (d) of the Roadmap for Risk Assessment)*

Comment : I would not speak of « phenotypic effects ». Better « phenotype »?
In addition, it is not clear from the Roadmap what is meant with phenotypic characteristics. This should be clarified in the Roadmap. According to this paragraph here phenotypic characteristics = protein expression. According to the EFSA guidelines, phenotypic characteristics also include characteristics of the GM plant, e.g. plant height. If combinatorial effects have occurred, these could be determined on the basis of phenotypic and agronomic plant and characteristics. The latter is not addressed in the Roadmap and could be included.

Rationale:

The combination of two or more TraEvs resulting in a StaEv may influence the expression level of each of the transgenes and there may be interaction between the genes and the expressed products of the different transgenes. In addition, the stacked transgenes may alter the expression of endogenous genes.

Therefore, in addition to information about the characteristics of the parental single-TraEv LMOs, specific information on potential for interactions between the altered or inserted genes, stacked proteins or modified traits and endogenous genes and their products in the StaEv LMO should be considered and assessed. For example, it should be assessed whether the different transgenes affect the same biochemical pathways or physiological processes, or are expected to or may have any combinatorial effects that may result in potential for new or increased adverse effects relative to the parent LMOs.

Assessment of combinatorial and cumulative effects of stacked event LMOs on the conservation and sustainable use of biological diversity in the likely potential receiving environment, taking also into account potential adverse effects to human health *(see Step 1, Point to consider (c), Step 2, Point to consider (c) and Step 3, Point to consider (b) of the Roadmap for Risk Assessment)*

Rationale:

Assessment of combinatorial and cumulative effects is based on the environmental risk assessment data for the StaEv LMO in comparison to the closely related non-modified recipient species and the parent LMOs in the likely receiving environment, taking into consideration the results of the genotypic and phenotypic assessments outlined above.

If potential new or increased adverse effects on the conservation and sustainable use of biological diversity or on human health are identified in relation to the StaEv through the above analysis of possible interactions, additional supporting data on StaEv may be required, such as:

(i) Phenotypic characteristics, including the levels of expression of any introduced gene products or modified traits, compared to the parent LMOs and to relevant non-modified recipient organisms (plants);
Comment : Is this not a repetition of the former point « Assessment of potential interactions & resulting phenotypic effects ». What is the difference?

(ii) Compositional analysis (e.g. levels of expression in the LMO and persistence and accumulation in the environment, such as in the food chain) of substances with potentially harmful effects newly produced by the StaEv, (e.g. insecticidal proteins, allergens, anti-nutritional factors, etc.) in amounts that differ from those produced by the parental LMOs or non-modified recipient organisms;
Comments : Not levels of expression are determined during compositional analysis, but amount of substances; Insecticidal proteins, do not fall under substances to be tested in compositional analysis according to OECD guidelines; Alternative text: "Compositional analysis to determine the amounts of substances with potentially harmful effects (e.g. allergens, anti-nutritional factors etc.) detected in the parental LMOs or non-modified recipient organisms, in the StaEv."

(iii) Additional information depending on the nature of the combined traits. For example, further toxicological analysis of the StaEv may be required to address any combinatorial effects arising from the stacking of two or more insecticidal traits that result in a broadened target range or increased toxicity.

Also, indirect effects due to changed agricultural management procedures, combined with the use of the transgenic stacked event LMO, should be taken into consideration.

Intentional and unintentional StaEvs may have altered environmental impacts as a result of cumulative and combinatorial effects of the stacked traits prevalent in different LMOs of the same species in the receiving environment. **Unintentional StaEvs may arise from outcrossing with other LMOs of the same species or cross compatible relatives (see "Use of Terms").** If a number of different StaEvs are cultivated in the same environment a number of varying unintentional StaEvs may occur. Changed impacts on non-target organisms or a change in the range of non-target organisms in the likely receiving environment should be taken into account.

Comment : No need to repeat this.

Development of specific methods for distinguishing the combined transgenes in a stacked event from the parental LMOs *(see Step 5, Point to consider (d) of the Roadmap for Risk Assessment)*

Rationale:

Some of the risk management strategies for StaEvs may involve methods for the detection and identification of these LMOs in the context of environmental monitoring. Currently,

many detection methods for LMOs rely on DNA-based techniques, such as polymerase chain reaction (PCR) or protein based ELISA tests targeted to single transformation events. The methods used **to detect the transgene** in the parental lines may not be sensitive or specific enough to differentiate between single parental transformation events and the same event being part of a stacked event. A special problem may arise particularly in the cases where the StaEv contains multiple transgenes with similar DNA sequences. Therefore, the detection of each and all individual transgenes in a StaEv may become a challenge and need special consideration.

Comment : Not the transgene is detected, but the event!

GUIDANCE ON "RISK ASSESSMENT OF LIVING MODIFIED CROPS WITH TOLERANCE TO ABIOTIC STRESS"

Comment 1

This document drafts a guidance for specific risk assessment of GM crops with improved tolerance to abiotic stress (in context of Annex III of Cartagena protocol). In general, this document is a useful tool to support the risk assessment of GM crops with tolerance to abiotic stress. However, I would like to point out a few remarks to be taken into consideration:

a) In view of comparing the LM crop with its non-modified version it is proposed to use transcriptomics and metabolomics technologies to hunt for changes at molecular and metabolite level. To my opinion, there is no sound argument why the use of "omics" technologies should be implemented in the risk assessment for abiotic stress tolerant crops, while this is not the case for other GM traits (e.g. Bt). In addition, it is not more relevant to perform these assays on GM crops than on crops obtained by conventional breeding.
b) Unintended characteristics. As there are significant connections between both the signaling patways and the involved defense genes and molecules of diverse abiotic stresses, it is indeed likely that some modified traits cause increased tolerance to more than one abiotic stress. This is actually one of the objectives within the field of abiotic stress tolerance to find traits that provide crops with increased tolerance against multiple and/or combinations of abiotic stress[1]. Therefore I advise to not consider these additional beneficial traits as 'unintended characteristics'. These type of pleiotropic traits will not cause potential adverse effects concerning the risk assessment.
c) To my opinion, it is not necessary to impose additional control measures for biotech abiotic stress tolerant crops in terms of increased potential for persistency of seeds of plants in agricultural or natural habitats as compared with abiotic stress tolerant crops obtained through conventional breeding.

Minor Comments
In the "Use of terms", waterlogging could be added as another type of abiotic stress.

Reference
[1] Mittler and Blumwald (2010) Annual Review of Plant Biology Vol. 61: 443-462

Comment 2

1. Risk assessment

Progress and new development in ERA lead to re-structure concepts and produce new terms. Steps in developing ERA for stress tolerant GMP (STGMP) are: i) identify assessment endpoints (which are mentioned as "criteria/data to be considered relevant" in the UNEP document), ii) develop a conceptual model that is used to develop risk hypothesis (step 1 of the flowchart), iii) draft an analysis plan (steps 2 and further of the flowchart) based on (i) and (ii). Because it is the key for a valuable ERA, one should define what assessment endpoint is, in the case of stress tolerant plants: it is a valued ecological entity and its measurable attributes providing a framework for assessing stress response relationships (see Nikson, 2008). For example, this would be honey bees and their abundance or volunteers plants and their frequency in the fields.
The conceptual model describes relationships between valued entities, the stressor, the pathway of exposure and potential effects in the environment. In this later point, the problem formulation, based on the STGMP characteristics, must consider whether the trait could expand the range in which the plant could grow. Finally the conceptual model would consider the plant phenotype and the way it could alter the plant interactions within biotic communities inside and outside the field (if the GM plant is capable to colonise a new environment, it can become a new host for new insect species (beneficials or pests).

2. Characterization (page 29)

- second paragraph - One should rather formulate that "the identification of genotypic or phenotypic ***meaningful (or significant)*** changes in the STGMP….is typically done with the non-modified organism."

- In "points to consider", I suggest to replace the existing points with:
    a) Phenotypic characterization of the LMO in the likely potential receiving environment;
    b) Phenotypic characterization of the LMO compared to the counterpart under stressed and non-stressed conditions;
    c) Phenotypic characterization of the LMO under different stress, if necessary;
    d) Likelihood of gene flow to wild or domestic relatives ***in conventional and potential environments (where the wild vegetation might be distinct from original agri-system)***.

3. Unintended characteristics (page 30)

- In "points to consider", I suggest adding:
"d) A change in the crop management directly linked with the genetic modification that could indirectly affect biotic and abiotic factors."

4. Increased persistency in agricultural areas and invasiveness…

Should be considered: the gene flow between ferals and wild relatives. Because of the introduction of the stress-tolerant trait, crop ferality might be increased due to some factors as (Bagavathiannan & Van Acker, 2008):

- variety of pollinators,
- longer or continuous seed production,
- high seed output in different environments
- increasing seed dispersal
- broad germination requirements….

5. The special issue of phytoremediation

Special care should be made regarding GM plants used in phytoremediation. In that case, in addition to the GM plant intrinsic characteristics one should address evolving compositional characteristics of the plant accumulating products from the contaminated environment (Cherian & Oliveira, 2005).
The accumulation or transformation pathway must be carefully taken into account. Impact assessment on the environment must include soil and air properties. For example, in the case of mercuric phytoremediation GM plants evaporate mercury in the atmosphere (Rugh et al, 1996, Bizily et al, 1999).

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Comment 3

1. Structure of the document and semantics :

1.1. Overall structure of the text :
Although the rationale of the RA and the widely accepted flowchart for RA are recalled in the first part of the document (Roadmap), they are not properly used in the part B focusing on Abiotic stress tolerant GM crops ('AST-GM crops'), where the different steps of the RA are mixed up. This makes this part of limited use for the risk assessors.

1.2. Redundancies :
The text suffers from several redundancies : *e.g.* the issue of unintended effects is addressed in the three sections : *Characterization of the LM crop* … (page 29), *Unintended characteristics* (page 30), *Increased persistency in agricultural area and* … (which is just an example of unintended effect, page 30).

1.3. Definition of stress :
The section "Use of terms" (page 28) defines *Abiotic stresses* in a way which disagrees with leading authors on the subject (see Schulze et al 2005 and cited references) and introduces a confusion between "stress" (which refers to a physiological state of an organism deviated from the normal type due to external conditions) and "stress factors" or "stressors" which are the external conditions responsible for the deviation. I would not say that "*Abiotic stresses are environmental conditions caused by non –living factors that are detrimental or suboptimal to the growth, development and/or reproduction of a living organisms*" but I would propose the definition of Schulze et al (2005) (or a similar one), regarding stresses as *"deviations from the physiological normal type as reactions to suboptimal or damaging quantities or intensities of environmental factors"*.

2. Shortcomings :

2.1 Problem formulation, assessment endpoints and protection goals
The roadmap and associated flowchart for RA underline the need of starting the RA by identifying protection goals, assessment endpoints (*i.e.* valued entities amenable to quantitative assessment) and risk hypotheses (describing potential harmful effects resulting from exposure of a valued entity to a hazard). See Raybould (2006, 2007), Raybould and Wilkinson (2005), and Nickson (2008) for an introduction to these concepts. This hypothesis-driven approach is critical for avoiding to consider any change in a biotic community or ecological entity as unacceptable risk. Although this concern is general to RA, it appears specially relevant for GM crops altered in their responses to abiotic factors of their environment. This is due to the fact that changes in plant phenotype is precisely the breeding objective and that the stress factors limiting crop productivity are also expected to contribute to the structure of the biotic communities of the environment. Hence transgene introgression into wild relatives via gene flow from the GM crop must be risk assessed (Tiedje et al. 1989). However, in order to avoid the '*all change is bad*' doctrine as a misinterpretation of the precautionary principle (Raybould and Wilkinson, 2005), protection goals and risk hypotheses must be defined at the start of the RA. Although this "problem formulation stage" is described in the roadmap, its implementation in the proposed guidelines for AST-GM crops is clearly insufficient.

2.2. Assessing agricultural performance is not assessing safety.
Plant performance in the field is analyzed as part of the RA procedure, but the relevance of the intended and unintended effects identified for environmental and food safety remains to be critically considered. For example, increased seed yield under stress conditions can be a consequence of the genetic modification, but no additional risk to the environment can be intrinsically related with this effect. By contrast, changed seed physiology, like increased overwintering capacity and seed dormancy, can have impacts on the frequency of volunteers and feral plants in the environment. Turning back to the initial concern of defining appropriate assessment endpoints and risk hypotheses, any change in plant behaviour does not cause a risk, nor indicates a possible risk. The move from

changed plant phenotypes to hazard potentials is not discussed in the document and no methodological guidelines are proposed.

Some authors have proposed the "tiered approach" coined for risks of pesticides and xenobiotics (Raibould and Wilkinson 2005, Garcia-Alonso et al 2006), but adaptable to the environmental risks associated with the genetic engineering of abiotic stress tolerance (Nickson 2008). Practically, risk hypotheses are evaluated by a progressive approach, the information collected in "lower tiers" directing the extent and nature of the experimentation conducted in higher tiers (see quoted references). Practically, the lower tier test uses the worst-case conditions in laboratory experiments and the outcome of this test is used for deciding whether higher tier data collection, *e.g.* by field testing, is needed. This approach has the advantage to be based on risk hypotheses and to orient the field-collected data toward safety relevant information.

Let us take the example of increased drought tolerance provided by the genetic engineering of hormone (ABA) metabolism. A risk hypothesis may be that altered metabolism aimed at improving drought tolerance also improves frost tolerance of seeds and their overwintering capacity, hence the persistence of volunteers and ferals in the field environment. This risk hypothesis may be evaluated by a tiered approach where "tier 1" laboratory experiments will first identify a possible temperature regime allowing survival of the GM seeds while killing the conventional seeds. Only in the case that such a temperature regime can be identified and providing this regime is regarded as realistic in the crop environment, will higher tier experiments be conducted, using *e.g.* field experiment assessing the overwintering capacity of left-over seeds. This example emphasizes the usefulness of risk hypotheses and of the tiered approach for environmental risk analysis, avoiding the gathering of a large set of agronomic data of limited safety relevance.

2.3. The comparators

This is a key issue for assessing both the intended and unintended phenotypes. The complexity is increased for the AST-GM crops, due to the need to evaluate the expression of the new trait in a range of environmental conditions (with different stressor intensities and durations). Although the document acknowledges the importance of the careful choice of the comparators, it is bit confused on how to achieve this choice and the comparative analysis in general. I am puzzled by the comment on the non-isogenic reference (page 29), claimed to "*make it more difficult to identify statistically meaningful differences*", and very reluctant to propose the “omics” technologies as a way to contribute to the comparative analysis. Indeed, natural variation is expected for many endogenous proteins in fluctuating environments, with no *a priori* relevance for the environmental and health safety.

2.4. How to tackle the "trait x genetic backround x receiving environment" interactions ?

A major difficulty in performing the safety assessment of an AST-GM crop via comparative analysis is the multiple interactions between the new trait, the genetic background and the receiving environment. The present guidance document is of limited use for deciding how to address these interactions is a realistic and informative manner, providing relevant information on safety. I try to summarize this interaction in the figure annexed to this note.

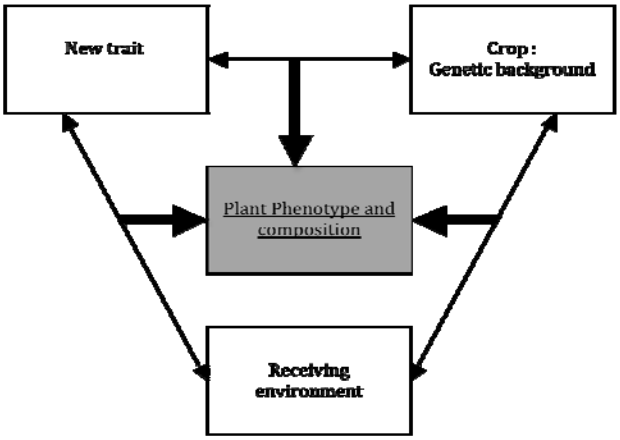

Figure explaining that plant phenotype and composition (including intended and unintended effects) results from the multiple interactions between the new trait, the genetic background of the crop and the receiving environment. Any comparative analysis of a new trait in the framework an environmental risk assessment needs to tackle these interactions.

I elaborate on specific issues related to the new trait, the genetic backgound of the recipient variety and the receiving environment.

New trait : in plants, any gene or gene combinations providing increased tolerance to some abiotic stress is expected to have pleiotropic effects on the stress physiology of the plant : *e.g.* drought, temperature and salt stress are interconnected and plant responses to these stresses share multiple components and genes. This is well accounted for by the guidance document, but, once again, no guidelines are provided for decision making in the RA procedure. Although such pleiotropic effects may be classified as "unintended predicted effects" recognized by the RA roadmap, how does the risk assessor define the cross talk between different stress responses of the plant and when is this information relevant from a safety point of view ?

Genetic background : although the improved stress response driven by the transgene(s) is designated as a "new trait", the recipient plant variety has some capacity to tolerate the stress at start. As different plant varieties may initially show different stress tolerance capacities, the added value of the introduced gene – the intended effect of improved stress tolerance - will depend from one variety to the other. This situation contrasts with the available experience based on the first generation of marketed GM crops, where herbicide tolerance or insect resistance is (close to) zero before adding the transgene, whatever the recipient varieties. As authorization of the GM crop is expected to cover a range of recipient varieties and the risk assessor is expected to conclude on the safety of the future products on the market, RA needs to conclude on the impact of the recipient genetic backgound on the intended and unintended predicted effects. This is challenging and few guidelines are proposed in the reviewed document.

Recipient environment : stress tolerance must be defined with respect to a set of environmental conditions, characterized by different intensities, durations and rates of installation and withdrawal of the stressor (like drought, flood, suboptimal temperatures, salt or other toxic ions, etc.). This poses the two difficulties (i) of controlling/measuring these conditions in the field experiments analyzing plant phenotype and (ii) of defining the plant phenotype itself, which is not a univoqual attribute of the GM crop but a complex relationship between external and physiological parameters. No guidelines are proposed in this context.

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Comment 4

I have read with much interest the guidance document on “Risk assessment of living modified crops with tolerance to abiotic stress” that was developed in order to enhance the effective use of Annex III by elaborating technical and scientific documents providing further guidance on specific aspects of the risk assessment.
I will try to respond to the following questions by addressing some specific and general remarks on the document.

The questions were:
1. Can the guidance document be considered a useful tool to support the risk assessment of GM crops with tolerance to abiotic stress?
2. Are elements presented in the guidance scientifically sounded and sufficiently described ?
3. Are there any important missing elements that should be added ?

Introduction
Recent advances in functional genomics have led to the discovery of genes associated with tolerance to abiotic stresses such as cold, heat, water, and salt (Vij and Tyagi, 2007). Some of these genes show promise in major crops like maize (Nelson *et al*., 2007) and rice (*Oryza sativa*; Hu *et al*., 2006). As such, a discussion on planning an environmental risk assessment of GM abiotic stress-tolerant crops is timely. Regulators are now confronting the challenges involved in evaluating data from these new and potentially beneficial products. Products expressing stress tolerance phenotypes are now being widely tested in field trials around the world. Technology providers will submit data and information on GM crops with stress-tolerant phenotypes to regulatory authorities for review that will include an environmental risk assessment as part of a request for commercial release. For this, it is very helpful to restrict this guidance to abiotic stress tolerance in plants.

First, and as I will detail later, I think that this document should focus on emphasizing on identifying differences on the risk assessment and risk management of these particular group of LMOs (resistant to abiotic stresses); in relation to others LMOs that Annex III and the Roadmap already deal with. This topic is already quite vast considering all possible types of abiotic stress. The document on abiotic stress may certainly include general considerations on resistance to different types of abiotic stress in crops. Nevertheless, it would be useful to go into more depth on those types of abiotic stress for which more scientific information is already available (drought and salt stress for instance).
Having said that, the draft is generally well written keeping plants in mind, as what applies to those specific LMOs may not be relevant to other LMOs. I think the questions that were posed are generally relevant for undertaking an assessment work on living modified plants that have been developed to tolerate abiotic stresses. However, I do think this document needs some conceptual and practical improvement.

The use of terms: some missing definitions
Due to their sessile environment, plants have developed myriad of strategies to ensure their metabolism and efficient ways to reproduce while anchored in one place and submitted all the time to "stressful" conditions. Plants cannot simply rely on one strategy or one family of compounds to cope with their changing environment. I think the definition of “stress” developed in this document needs to be changed. I prefer the use of the definition used by Levitt (1980) which defines stress as the action of an environmental factor (here a non-living organism), or the combination of different factors that

limits the realisation of the genetically determined potentialities of growth, development and reproduction of plants (Levitt, 1980).

Additionally, in the "Use of terms" a clear definition of "Tolerance" is somehow unclear and needs specification. Abiotic stresses are responsible for more than 50% yield loss worldwide. As the world population is rising exponentially, this problem needs to be dealt with, especially taking into account the deleterious effects of global warming. Ultimately, developing new "stress-tolerant" crop varieties will require further understanding and modulation of the physiological and molecular processes underlying plant stress responses. Thus far, most studies have concentrated on transgenic modifications that create plants that "survive" extreme stress conditions -hence the use of the "tolerance" term-, usually at the expense of biomass accumulation. Thus, definition of agronomic parameters -that candidate genes or trait are most likely to impact on (e.g. growth rate, biomass, yield, quality etc.) are of paramount importance for this purpose.
Unfortunately, most scientists nowadays use without any distinction the terms of "stress tolerance" and "stress resistance". For me the two terms are not equivalent and I would rather use "resistance" than tolerance especially for crop species as it involves a more active defense mechanism than simply "tolerating" the stress –which sounds more like a survival mechanism. If the document is focusing on crops, then the aim would be the development of “resistant” plants. In any case, it would be interesting in the document to define the used "tolerant" terminology especially when we are talking about crops. The goal of using manipulations is to reduce the degree of plant growth inhibition (and thus yield reduction) under stress conditions and not simply make the plant survive.

Emphasizing the use of "realistic" conditions for LMO evaluation
The use of controlled conditions makes easier -to a certain extent- the laboratory studies and most scientists work under those specific conditions. This is not the real situation since plants are constantly exposed to stress in natural environment. The analysis of any results must thus take into consideration the differences that exists between the kinetics of stress imposition.

Therefore, the use of "realistic" conditions for assessing "tolerance" and risk in phenotyping studies should be mentioned as most studies use abnormally high stress conditions for a short time which is completely unrealistic. Those conditions do not correspond to what plants normally face in field conditions. Therefore, basing risk assessment on unrealistic conditions would be completely irrelevant.

I’d like to add to these basic points to consider, the importance of scoping which phenotypes to be checked because there are too many phenotypes to be test all. To put it concretely, I think it is important to scope which phenotype to be assessed and to check any other phenotypical difference except for intended differences, based on the mode of introduced genes action, especially when relationship between introduced genes and intended differences is not clear.

Testing the LMOs in representative environments
Since LMOs are intended for use and developed to be cultivated under abiotic stress conditions, it is essential to consider the importance of regional aspects for the evaluation of specific characteristics and the environmental behavior of the LMO as well as of interactions of the GMP with the environment. We need to outline clearly the requirement of an assessment of potential adverse effects of the GMP on the ‘potential receiving environments’. In the Annex of the Cartagena Protocol, the case-by-case principle is clearly recommended for the ERA because ‘of the broad range of individual characteristics of different organisms (LMO-by-LMO) and different environments (region-by-region). This requirement has not been fulfilled so far and that guidance for its implementation is urgently needed.

Hence, regionally differing factors that may influence the characteristics and the behavior of the LMO as well as the interactions of the LMO with the environment must be taken into account during the risk assessment procedure. Regions and locations selected to collect data or conduct field trials should thus represent the range of agricultural, plant health and environmental conditions the LMO is expected to encounter when commercially cultivated.

Different environments may be defined e.g. by the differences in occurrence or in the number of generations of target organisms different agricultural practices and agronomic structures (e.g. nitrogen

input), different cultivation systems (e.g. low-tillage farming), different crop rotation practices, different climatic conditions, different occurrence of other organisms in non-target environments as well as other abiotic and biotic conditions.
Such relevant factors of a specific region or location should be determined at the start of the ERA which calls, again, for a broad and integrative ERA concept. This is important as these factors may lead to differences in potential adverse environmental effects which only become evident if assessed on a regional level.

Develop criteria for the assessment of effects of the LMO on the abiotic environment
Risk assessment practice in this draft does not further consider effects of the LMO on the abiotic environment. It focuses mainly on the effect of the LMO on the environment. Changes of the abiotic environment by the use of LMOs will depend largely on the introduced trait, and may be relevant for LMOs with altered tolerance of certain environmental conditions, such as climate, abiotic soil fractions or gases (EFSA 2006a).
Although the level of assessment will have to be decided on a case-by-case basis, criteria are still needed for the decision for which GMPs such an assessment will be relevant.

Assessment of effects related to land use and cultivation techniques
From an environmental point of view changes in land use or cultivation techniques when cultivating LMO resistant to abiotic stresses are of high relevance when the cultivation of the novel crop involves changes in other cultivation techniques and/or land use that might in many cases affect biodiversity agricultural landscapes. Thus the description of the current baseline and an evaluation of identifiable changes when growing a GMP can give a first insight into future developments of agriculturally used areas.

Adding specific information that could not be covered by the Annex III
I believe the risks associated with abiotic stress tolerance can be -up to a certain extent- assessed in the same way as other types of genetically engineered crop plants, following the steps for risk assessment in Annex III in the Cartagena protocol. The first step is to identify adverse effects that may be associated with any novel genotypic and phenotypic changes associated with the abiotic stress tolerant LMO. By comparing the LMO to its traditional counterpart, any novel changes associated with the abiotic stress tolerance can be identified, including any changes to the biology of the crop plant (e.g., if the genes may alter multiple characteristics of the plant) or to the potential receiving environment (e.g., if the plant can grow where it has not been grown before). After the adverse effects associated with these changes have been identified, then the likelihood and consequences can be considered together to determine the risk and the need for any additional risk management.

However, I am not sure that there is ENOUGH additional information in the document for considering abiotic stress tolerance so far that would not be covered by the steps currently described in Annex III and in the roadmap. However, I am aware that the Annex III is a well-crafted and generally applicable document. What is needed to support risk assessments, then, is not a set of specific instructions for reviewing a particular abiotic stress trait (which would seem contrary to the general principle of case-by-case assessments from paragraph 6 of Annex III), but rather a useful collection of available information that can help risk assessors obtain what information they need and guidance on how to use that information when performing an assessment.

It would be helpful if the document could use an abiotic stress tolerant plant to demonstrate how the risk assessment process can be applied to this type of LMO (see later example of drought-maize for example).

Furthermore, there are many variations on the definition of abiotic stress, but in general they include the idea that it is a non-living stress on a living organism in a specific environment. I think a further specification on which types of abiotic stresses to include in this work is needed. At first, it is needed to look at what information is available from “conventional” (non-Genetically Engineered (GE)) research into abiotic stress resistance and then what research is available for GE stress tolerance. The primary focus should be on developing useful information to help risk assessors examine the abiotic stress traits they are likely to see first. For example, water stress has long been an area of research by plant scientists (e.g., osmotic regulation in the face of both too much and too little water). Tolerance to heat, cold, and salinity have also been the subject of considerable research. Thirty years ago, a great deal of research focus was placed on air pollution stressors such as ozone and nitrous oxides that are common components of smog. In more recent years, scientists have pressed forward to develop crops that can better withstand these biotic stress factors. It is thus needed to make some of the most important literature resources more readily accessible to people who will do risk assessments of plants engineered to tolerate abiotic stresses. Then we can look at the research into GE abiotic stress tolerance and the products under development to see how it relates to this earlier work. In this way we can develop a foundation addressing the most pressing risk assessment needs. This procedure is not clearly stated in the document.

Adding environmental remediation, agricultural production under extreme conditions
Additionally, I think that the specific subjects like environmental remediation, agricultural production under extreme condition could be covered in this draft dealing with the definitions and description of various abiotic stress tolerance traits. Abiotic stress includes not only draught and heat, but also contaminated soil with heavy metal and POPs (Persistent Organic Pollutants).

Comparing with the appropriate controls
The following items must also be considered and clarified concerning the characteristics of LMO in abiotic stress. Therefore the comparative studies with Null crop are much more important in abiotic stress than the other LMO. This should be further emphasized on in the document. The use of appropriate controls is essential.

1. Phenotypic trait of LMO
2. Difference in such phenotypic trait between stressed and non-stressed (good) condition
3. Difference in internal change between stressed and non-stressed (good) condition
4. How do LMO adapt in stressed condition? Is there any difference due to the intensity of the stress? Intensity of the stress means not only optimal and suboptimal condition, but also the range of stress.

General remarks on the document
Crops with tolerance to abiotic stress are now being developed using the tools of modern biotechnology (Vij and Tyagi, 2007). Because these crops are developed using genetic modification techniques they will be subject to a detailed environmental risk assessment prior to commercial use. As technology providers develop these crops, they will need to understand how to approach the risk assessment starting with a proper problem formulation. Over the years, much counsel has been given on the importance of understanding ecological processes and principles in the context of protecting the environment from the risks posed by GM crops (Snow et al., 2005). Some of these scientists call for an ecological approach to environmental risk assessment on the basis that it is more rigorous and even protective. Unfortunately this approach is predominant in the analysed document.

However, the later approach has been shown to be inappropriate when compared to the ecotoxicological model described by Raybould (2007). A recent article by Nickson (2008) describes an approach beginning with problem formulation that is based on established risk assessment principles that have been applied successfully to both chemical products and GM crops (Raybould, 2007). Problem formulation (PF), Nickson proposes integrates knowledge in a systematic and organized manner to help risk assessors develop conceptual models and analysis plans that will provide information relevant to protecting valued environmental entities. Importantly, a well-developed problem formulation increases the efficiency of the environmental risk assessment and the certainty of its conclusions. Nickson's approach is based on a proven conceptual standard that can be applied on a case-by-case basis to GM crops with improved tolerance to abiotic stress.
The following points for effect of LMO on biodiversity under abiotic stress should also be considered:
1. Whether the transgenic gene act only under stressed condition or not? It means 1) Switch ON/OFF by stress or 2) constantly expressed.
2. Mode of action should be clarified in order to consider unexpected condition other than target stressed condition.
3. From the viewpoint of crop import, import countries basically assess the LMO under their own environment (condition). They had better know how the LMO performs under abiotic stress conditions, however, they do not need to conduct the trial under such conditions, when they do not have such conditions.

Carefully constructing hypotheses: some learning from the field
There is a particular point that should be made in Points to Consider. My experience with conducting a risk assessment for drought-resistant maize and salt-resistant tomato highlighted the importance of carefully constructing hypotheses that account for the intended differences. This is a very different situation than the one occurring with *Bacillus thuringiensis* (Bt)-crops. For insect-protected products based on proteins from Bt, knowledge of the mechanism is relevant to building the conceptual model and analysis plan. In this case, Bt proteins are specifically toxic to certain pests and pose minimal risk to other organisms based on their mode of action (OECD, 2007) and levels of expression in plants. Conversely, if the gene conferring drought tolerance (in the case of Maize for instance) has no reasonable mechanism for conferring toxicity to organisms, it is unlikely that detailed knowledge of the mechanism by which a gene confers drought tolerance will be necessary for the risk assessment. Knowledge that a maize plant is tolerant to water stress is sufficient to guide the development of the conceptual model and analysis plan. For example, for Monsanto's drought maize it was needed to test the hypothesis that the GM maize would be phenotypically unchanged compared to the non-GM maize when water was limited but also when water was optimal. In my opinion and my experience this is a key element that should be better reflected in the draft. Testing these hypotheses is not trivial, and a risk assessor will see this as a challenge.

I strongly recommend that the document employs a specific example such as drought tolerance on a crop like maize (that were already performed), to examine whether any additional consideration on LMO itself and the associated environments. General points have been already addressed and concepts and precaution ARE already in the Annex of the Cartagena Protocol, and it is cardinal to have a guidance document with practicality.

Furthermore, I caution the document to keep concepts like "fitness" in perspective for risk assessment. There is much coming out in the literature about how stress traits enhance fitness. However, the question for the risk assessment is not whether a plant is more fit, but whether it is a

weed/pest, unacceptably invasive, etc. We do not want to give guidance that is interpreted as studying fitness is the same as harm.

Assessment of the socio-economic impacts

Whether or not the adverse effects identified in Step 1 of the risk assessment should include socio-economic impacts is not a question unique to abiotic stress tolerance. Personally I agree, that socio-economic considerations should be included as part of the decision process, and not as part of the risk assessment process as described in Annex III. Indeed, abiotic stress is of particular importance at this stage because of the launch of new initiatives for a green revolution to Africa, for example, where attempts to introduce varieties resistant to drought and other traits related to abiotic stress. The purpose of these initiatives is to include these lands that have traditionally been used in a sustainable manner, which now are called "marginal lands", to a large-scale agricultural mode In this sense, it is not possible to conceive a system of risk assessment without taking into account socio - economic elements, in line with Article 26 of the Cartagena Protocol. Given the limited understanding we have of how these “marginal ecosystems” function in a context of industrial agriculture, the core driver of the risk assessment should be the precautionary principle. Although risk assessment should be made using the best available scientific information, which is low, the precautionary principle should be the lead element for both, the risk assessment process and the decision making process prior the introduction of new LMO crops in these “marginal lands”. It cannot be “a science driven decision. We must consider that there is very little scientific research on all aspects that would be required before the introduction of industrial crops on land that by definition are extremely fragile, and where local populations depend on, and that they have been able to survive due to the delicate balance that they have developed with their environment. Therefore, the effectiveness of risk assessment is limited because of these gaps, which creates great uncertainty and weaken the predictive power of science. The Cartagena Protocol itself acknowledges the existence of these gaps, and also acknowledges that new scientific evidence might arise related with the dangers that this technology entails (See Art.12 of the Protocol).

Unfortunately, in the risk assessment process arbitrary and pre-established assumptions are sometimes made, and extrapolation to not similar conditions are being used. In several national laws and regulations risk assessments use the principle of "familiarity", which is not recognized by the Protocol, and is based on procedures which has no scientific support, since it extrapolates and draws conclusions and decisions based on studies made in different environmental, socioeconomic and cultural contexts, and in the case of stress tolerant LMOs, with different traits, which has not being subject to large scale industrial production. In the case of abiotic tolerant LMO, stress risk assessments should take into account all the variables, especially when dealing with complex problems (both environmental and social ones). RA should consider cumulative and synergistic and long term effects of these new LMOs, they should go beyond conventional risk assessment.

Additionally, risk assessment in land with abiotic stress should include an assessment of the technological package to be implemented, not only of new traits, because the introduction of new crops and agricultural practices totally different from what have been conducted there before, and which would not be possible if it had not been genetically transformed, must be evaluated carefully in order to avoid irreversible damage to the sustainable use of biodiversity, as determined by the Article 1 of Annex 3 of the Protocol

Comment 1

With regards to the roadmap, that general road map is, of course, based on agricultural plant risk assessment. However some points are difficult to meet for mosquitoes such as the use of near isogenic strains as control for GM strains (first §, page 17 of the document, actual page 6 and page 19, actual page 8, step 3). On the other way, species is to vague as for mosquitoes with a worldwide distribution, a lot of subspecies or strains were described with different properties, including capacity of pathogen transfer. So, the line used for the transformation may serve as control.

Page 17, (actual page 6) Point to consider regarding the receiving environment: the risk of dispersal due to transport and trade of potential source of breeding sites such as tyres or lucky bamboos should be also considered particularly for mosquitoes. Consequence of water management practice, irrigation, sewage water treatment etc. on the introduce GM strains and on possible effect on genotype and phenotype of the LM mosquito introduced should also be taken into account.

Page 18 or actual page 7, Points to consider regarding the potential adverse effects resulting from the interaction between the LMO and the receiving environment: a point should be add concerning the consequence of veterinary of health measure management and their potential consequence on the gene expression in the LM organism, such as insecticide treatments or medication taken by the mosquito host. Particularly should these treatments modify the mosquito fitness and their competitivity for other mosquito species sharing the same environment.

Concerning the Guidance of risk assessment of living modified mosquitos, the proposed guidance document is of good quality and is a useful tool for the risk assessment of LM mosquitoes. I have however several comments.

As indicated in the introduction of this part, one should distinguish between self-limiting systems and self-propagating ones. These two kinds of strategies correspond to different level of risk. Self limiting systems aimed at mosquito population reduction, for example by the use of sterile insect techniques. In that case, inundative release of probably billions of sterile males obtained by genetical tranformation or an other means are made until the possible eradication of the population. In that case, once the release program is stopped, the introduced mosquitoes will rapidly be eliminated from the target population (Beech et al., 2009a). Indeed, only sterile insects are released and with very little risk of gene transfer. Population reduction using strains homozygous for a dominant lethal genetic systems (RIDL) is another way of possible mosquito control (Wilk *et al*.,2009), for instance like it was proposed for 'temperature-sensitive lethal' (tsl) strains of Medfly (Franz 2005). In that case again, once the program is stopped, LM mosquitoes should be eliminated from the population by natural selection.

Self-propagating or self-sustaining systems concern "population replacement" i.e. changing the native strain into a less harmful form. Self-sustaining systems are intended to persist indefinitely, and indeed to increase in prevalence, e.g. allele frequency, in the target area and beyond where a population of mosquitoes is replaced by a LMO one, which for example is unable to transmit a virus or a plasmodium. In this case, only a few individuals may allow a gene transfer through an entire population. In that case, if problems are encounter, even if the program is stopped it becomes very difficult to avoid the propagation of the modify genotype in the native population. This point must be underline in the document and probably should lead to two kind of guidelines, one for self limiting systems and the other one probably more constraining on self propagating systems.

Concerning step one, the systematic of mosquitoes is rather complex, some genus containing a lot of subspecies or even cryptic species, or even complex of species. Now, the ecological niche and the capacity to transmit some pathogens to human being or animals is different from one subspecies or even strain to the other. So the first point of step one should be a complete taxonomic characterisation of the strain used, including the use of reliable molecular markers and its biogeographic origins.

Mosquitoes are currently infested by the Wolbachia endosymbionte/pathogen. Wolbachia are ricketsies like bacteria than can be transmitted maternally and provokes several alterations of the reproductive systems of its host, depending on the host species and Wolbachia strains (cytoplasmic

incompatibility, male killing, feminization, Walker *et al.,* 2009). A risk assessment should thus take into account the possible interactions between the genetic transformation and Wolbachia in the host strain considered. Moreover, the risk of possible horizontal gene transfert through Wolbachia is also to analyse as horizontal genetic transfert have been proven between species (Raychoudhury *et al.,* 2009).
On the other hand some strains of Wolbachia appear to reduce their host fitness or to hamper Dengue transmission for instance and may be use as control method for vector-born diseases (Beech *et al.,* 2009;Bian *et al.,* 2010). Even if mosquito strains containing pathogenetic mutants of Wolbachia are not true LMO, the use of such strains to reduce mosquito populations or pathogen transmission should be include in the risk assessment process.

Mosquito species are currently able to transmit several pathogens from viruses to filaria to human being and animals. A genetic event that modifies the capacity of transmission of one of these pathogens may have a positive effect on the transmission of another pathogens. This point should also be taken into consideration.

Concerning the ecological risk, the replacement for example of *A. aegypti* by *Ae. albopictus* could not only be the consequence of one release but should be monitored through time and at an appropriate geographical scale.

The risk of LM mosquitoes long-range dispersal due to anthropogenic activities, such as tyre trade, should also be evaluated and monitoring plan should be proposed. The susceptibility of the modify strain to the current insecticide use for mosquitoes control should also be evaluated and compare to the native strain.

Before release, the fitness of the LM mosquito is to be evaluated and particularly its competitive capacity (not only interaction which is quite vague as indicated in point (e) page 34, actual page 15) with the native strains and with other species of the same guild sharing the same kind of environment. This should be done for the aquatic larvae as for the adults.

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Comment 2

Three questions regarding the guidance document were posed:

1. Can the guidance document be considered a useful tool to support the risk assessment of genetically modified mosquitoes?
2. Are the elements presented in the guidance scientifically sounded and sufficiently described
3. Are there important missing elements that should be added

1. It is our opinion that this document will be a useful tool to guide the researcher in the process of a risk assessment when planning to use LMO's.

However, we do feel that some LMO-related issues should be defined more into detail since they are important elements in the risk assessment:
- A clear definition should be provided on what living modified mosquito's are. Here, the different modes by which the modified mosquitoes are obtained should be clearly defined since they impose different risks: genetically engineered mosquitoes by transgenesis, modification of mosquitoes by introduction of genetically modified bacterial symbionts (paratransgenesis), introduction of different bacterial symbiont strains (eg. Wolbachia) in non-natural host mosquitoes, mosquitoes that have been made sterile by irradiation.
- In step 2 of the guidance document a clear difference should be made between 2 distinct strategies that involve the release of LM mosquitoes and that impose different biological risks for the environment.

- The first strategy involves population suppression in which mosquitoes are manipulated with the aim to reduce the number of disease- transmitting mosquitoes without affecting the transmission capability of the wild insects (sterile insect technique, GM mosquitoes carrying a lethal gene which is passed on to the offspring causing them to die). Most suppression strategies are self-limiting, designed to remove themselves from the environment after release and so preventing persistence of GM mosquitoes in the wild.
- The second strategy involves a permanent replacement of the wild mosquito populations with GM mosquitoes that are unable to transmit the pathogen. This approach makes use of a gene driving system in which the gene of interest is spread throughout the population.

2. To our opinion elements presented in the guidance are scientifically sounded and sufficiently described

3. There are important missing elements that should be added
- The document does not mention paratransgenesis, this is an alternative approach to introduce effector genes into mosquitoes. Paratransgenesis focuses on utilizing genetically modified insect symbionts to express molecules within the vector that are deleterious to pathogens they transmit. So rather than genetically modifying mosquitoes, the focus is on the genetic modification of bacteria that inhabit the mosquito midgut. Paratransgenesis can be used in both population suppression strategies and population replacement strategies. Most symbionts have developed such a close relationship with their host that over time genome erosion has occurred to a state where the symbiont can no longer survive outside its host and therefore decreasing the risk of spread throughout the environment, however the risk of horizontal gene transfer increases with this approach and should be assessed on a case-by-case basis.
- In the risk assessment, a risk comparison should be included between the use of the proposed LMO and conventional mosquito-targeting methodologies to control the pathogen transmission that are applicable to the target mosquito population.

Comment 3

1. Can the guidance document can be considered a useful tool to support the risk assessment of genetically modified mosquitoes ?

As a general guidance document, **only partly** because, if most of the points that apply to living modified crop plants can be used for living modified mosquitoes, some aspects are more specific to living modified mosquitoes and would have to be added.

Two example:

- Step 1, "Points to consider regarding the characterization of the LMO". A 5th paragraph should be added, along the following lines: "(e) When relevant, mechanisms allowing suppression of the LMO's geographic and demographic spread within a certain time frame (e.g. a gene-drive mechanism that can be self-limiting or reversible over time)" (see e.g. Sinkins & Gould 2006; Fua et al. 2010; Gould et al. 2010);
- Step 2, " Points to consider": change item (c) into: " Available information on the location of the release and the receiving environment (such as geographic and biogeographic information, including, as appropriate, coordinates, information regarding metapopulation features that may hamper dispersal and reduce impact of the LMO in the case of trangenic animals designed to reduce or eradicate a natural population of harmful conspecifics, information on the sexually compatible species and whether they are co-localized with the LMO and whether flowering occurs at the same time, or in general, interbreeding can occur)."

The whole document should be carefully reshaped to take into account the particular features of genetically modified mosquitoes and their intended use. This is out of the scope of my brief review. Note that ERAs have already been developed for living modified mosquitoes (see e.g. Andow D.A. *Ecological risk assessment (era) for lm mosquitoes*. Unpublished).

As a tool connected to the Cartagena Protocol, **no**, the document cannot be considered as useful because it completely misses the Protocol's major point. The Protocol "… shall apply to the transboundary movement, transit, handling and use of all living modified organisms that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health." (article 4 – Scope.)

The Cartagena Protocol restrictively addresses organisms that can be contained at every moment (in the exporting country, in transit, in the importing country). Organisms designed for mass release (sterile insect technique (SIT) approaches) or massive reproduction once released (e.g. mosquitoes designed to propagate a reduced ability to transmit malaria or dengue virus) do not belong to these categories of containable organisms because their efficiency is dependent upon their longevity, mobility and fecundity, all traits which favour wide spreading and efficient establishment.

The Protocol already excludes pharmaceuticals (article 5). Perhaps it should concentrate on genetically engineered plants and another Protocol should be built up regarding genetically modified organisms for pest (or possibly weed) control, which will have to be highly mobile to achieve the goals they have been assigned to.

2. Are the elements presented in the guidance scientifically sound and sufficiently described ?

What is present in the guidance is sound, but both partly incomplete and partly irrelevant (see above).

3. Are there important missing elements that should be added ?

IF mosquitoes must be considered within the frame of the Cartagena Protocol (which I doubt), then the Protocol itself must be reconsidered and, parallely, important components (such as those outlined above) must be added to the guidance document.

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