Adviesraad voor Bioveiligheid
Conseil consultatif de Biosécurité

Advice of the Belgian Biosafety Advisory Council on notification B/BE/22/V1 from VIB for deliberate release in the environment of genetically modified plants for research and development

24 March 2022
Ref. SC/1510/BAC/2022_0348

The notification B/BE/22/V1 has been submitted by the VIB to the Belgian Competent Authority (CA) in January 2022 for a request of deliberate release in the environment of genetically modified higher plants for research and development according to Chapter II of the Royal Decree of 21 February 2005.

The title of the notification is: *Scientific field evaluation of maize with increased resistance against DNA damage causing environmental stress.* The purpose of the release is to measure the performance of the modified maize plants under normal field conditions and learn whether also in these conditions they would have an improved growth.

The notification has been officially acknowledged by the CA on 14 January 2022 and forwarded to the Biosafety Advisory Council for advice.
Within the framework of the evaluation procedure, the Biosafety Advisory Council, under the supervision of a coordinator and with the assistance of its Secretariat, contacted experts to evaluate the dossier. Two experts from the common list of experts drawn up by the Biosafety Advisory Council and the Biosafety and Biotechnology Unit (SBB), answered positively to this request.
The experts assessed whether the information provided in the notification was sufficient and accurate in order to state that the deliberate release of the GM maize lines would not raise any problems for the environment, animal or human health in the context of the intended use.

On 2 March 2022, based on a list of questions prepared by the Biosafety Advisory Council, the CA requested the notifier to provide additional information. Answers to the questions were received by the Secretariat on 9 March 2022.

For the purpose of the scientific evaluation, the following legislation has been considered:
- Royal Decree of 21 February 2005 (Belgian Official Journal of 24.02.2005, p. 7129) modified by the Royal Decree of 19 February 2020 (Belgian Official Journal of 02.03.2020, p. 12666).

In parallel to the scientific evaluation, the CA made the dossier available on its website for a one-month public consultation as required in the abovementioned Royal Decree. No questions of the public tackling biosafety issues of the GM maize were received.

Summary of the scientific evaluation

1. Information related to the recipient or parental plants

Zea mays is an allogamous plant that propagates through seed produced predominantly by cross-pollination. Maize pollen can be collected by honeybees and other insects, however these pollinating insects play a minor role in the cross-pollination of maize plants which relies mainly on wind for the dispersal of its pollen (OECD, 2003[1]). Data on pollen dispersal in maize demonstrated that the levels of cross-fertilisation drop rapidly over the initial meters around the pollen source and that most of the released pollen is deposited within about 30 m of the source (Devos *et al*., 2005[2]). At distances farther than 30 - 50 m from the source, pollen dispersal is very low but not zero. However, vertical wind movements can lift up pollen and distribute it over distances up to kilometers under suitable climatic conditions. In Belgium (and in Europe) there are no sexually cross-compatible indigenous wild relatives with which maize can hybridise and form progeny (OECD, 2003; EFSA, 2016[3]). Teosinte, regarded as an invasive weed in Europe since its first occurrence in France (1990) and Spain (2009), has so far not been reported in Belgium. The only recipient plants that can be cross-fertilised by maize in Belgium are therefore other cultivated maize varieties.

Seed dispersal of individual kernels of domesticated plants are mainly the result of field operations of harvesting the crop and transporting the grain from the harvested fields to storage facilities. Spilled maize seeds can overwinter, germinate and appear in the field as volunteers. However, maize is incapable of sustained reproduction outside the domestic cultivation area as it has lost its ability to survive in the wild due to its long process of domestication (OECD, 2003). Volunteers will only occur after a warm winter period (with no temperatures lower than 0°C for more than 6 to 8 hours) and will be characterised by a low probability of cross-pollination (Grüber *et al*., 2008[4]; Palaudelmàs *et al*., 2009[5]). Given the Belgium weather conditions, volunteers are not likely to occur.

2. Information on the design and management conditions in the field trial

The field trial will be conducted during three growing season (from April 2022 until October 2024). The surface of the area for cultivation will not exceed 310 m^2.

Prior to complete formation, tassels from the GM maize will be removed by hand in order to prevent the dispersal of GM pollen. Once the last leaf has been formed, monitoring of upcoming tassels will take place every two days until all tassels have been removed and will be maintained until September 15. Removed tassels will be transported in closed bags and inactivated.

During harvest, cobs of the GM maize plants will be collected by hand and transported in closed bags to the lab. Material will be inactivated if no longer needed for research. Stems and leaves, except for a

[1] OECD, 2003. Consensus Document on the biology of *Zea mays* subsp. *Mays* (maize). Series on Harmonisation of Regulatory Oversight in Biotechnology (ENV/JM/MONO(2003)11), No. 27:1-49. http://www.olis.oecd.org/olis/2003doc.nsf/LinkTo/NT00000426E/$FILE/JT00147699.PDF.

[2] Devos *et al*., 2005. The co-existence between transgenic and non-transgenic maize in the European Union: a focus on pollen flow and cross-fertilization. Environmental Biosafety Research 4, 71-87.

[3] EFSA (European Food Safety Authority), 2016. Relevance of new scientific evidence on the occurrence of teosinte in maize fields in Spain and France for previous environmental risk assessment conclusions and risk management recommendations on the cultivation of maize events MON810, Bt11, 1507 and GA21. EFSA supporting publication 2016:EN-1094. 13 pp.

[4] Grüber *et al*., 2008. Post-harvest gene escape and approaches for minimizing it. CAB International 2008 (http://www.cababstractsplus.org/cabreviews).

[5] Palaudelmàs *et al*., 2009. Effect of volunteers on maize gene flow. Transgenic Res. 18, 583-594.

few which will be harvested, will be shredded on the field. Roots and the lowest part of the stem will be left in the ground.

After the field trial, the field will be left fallow and ploughed at the latest during next spring.

3. Information related to the genetic modification

The GM maize lines ZmNAC52-A and ZmNAC52-B obtained by the CRISPR-Cas9 technology (introduced via *Agrobacterium tumefaciens*-mediated transformation), are subject of this field experiment.

The maize lines ZmNAC52-A and ZmNAC52-B contain a knocked out *ZmNAC52* gene which has been achieved by the addition of one basepair, that results in a frameshift mutation. The *ZmNAC52* gene encodes for a transcription factor involved in the regulation of plant growth when DNA damage occurs. Knocking out this gene is expected to result in growth improvement when DNA damage occurs.

The two maize lines were obtained using a vector containing Cas9 and guide RNA (gRNA) genes on a T-DNA construct. The T-DNA construct used for transformation also contains a *blpR* gene that served as a marker for the selection of transformants after *Agrobacterium tumefaciens*-mediated transformation. The *blpR* gene produces the phosphinotricin acetyl transferase (PAT) enzyme, which acetylates phosphinotricin (also known as glufosinate, the active ingredient of several broad-spectrum herbicides), thereby rendering it inactive. The vector backbone contains a spectinomycin resistance marker gene.

Transformed plants were selected on the basis of glufosinate and subsequently backcrossed with B104 and finally selfed to obtain homozygous plants solely containing the mutation (and no T-DNA or vector DNA). The homozygous plants included in the field trial (lines ZmNAC52-A and ZmNAC52-B) were tested for the lack of vector sequences, including the antibiotic resistance marker gene, via their glufosinate sensitivity and via quantitative real-time PCR using several construct-specific primers. These tests confirmed the absence of vector DNA.

4. Potential risks for the environment, animal or human health associated with the release of the GM maize

No increased persistence in the field or invasiveness into natural habitats of the GM maize lines compared to non-GM maize is expected. The intended changed characteristic (increased resistance against DNA stress) may result in a selective advantage to survivability. However, the measures taken (removal of tassels and manual collection of cobs) rule out the development and survival of the GM maize in the year(s) after the field trial.

Vertical gene transfer to cultivated maize in the surroundings through pollen can virtually be ruled out due to the removal of the tassels.

Horizontal gene transfer between plants and micro-organisms is considered as a rare event under natural conditions (Keese, 2008[6]). In case gene transfer from the GM maize to micro-organisms would take place and gene expression would occur (although unlikely as the maize gene is non-functional due to a frameshift mutation), negative effects on the environment and humans are not expected. The *ZmNAC52* gene, expressing a protein involved in the regulation of plant growth, will not confer a selective advantage to bacteria.

Further, it is not expected that the GM maize would have significant effects on organisms (invertebrates, vertebrates and soil micro-organisms) and humans, as no trait that could affect the behaviour or

[6] Keese, P. 2008. Risks from GMOs due to horizontal gene transfer. Environ. Biosafety Res. 7: 123-149.

development of organisms via contact or feeding has been integrated. Given the restricted scale of the field trial, any potential effect to organisms and biogeochemical processes - if these would occur - will be of a local and temporal nature. As the release of GM pollen in the environment is prevented, a possible altered allergenicity potential of the transgenic pollen (allergy from maize pollen may occur in case of occupational exposure to high amounts of pollen grains, see *e.g.* Oldenburg *et al.*, 2011[7]) does not form a concern for human health.

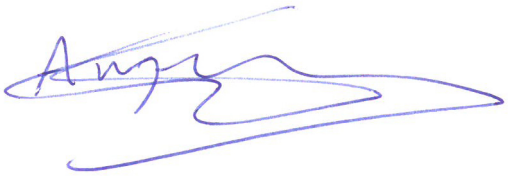
5. Information related to the control, monitoring, post-release and waste treatment

The management measures proposed are considered as sufficient to prevent potential adverse effects to the environment, animal and human health during and after the field trial. The monitoring and removal of any appearing tassel in the transformed lines will prevent gene flow by pollen spread. Careful manual harvesting of the cobs and storing them in closed bags will prevent seed dispersal. The seeds and the few collected plants will be destroyed after analysis.

Years of experience have shown that no volunteer plants appear in the year following the field trial. Therefore, the field will be left fallow (without monitoring of volunteers) and ploughed at the latest during next spring.

Conclusion

Provided that the trials are conducted as described in the dossier, the Biosafety Advisory Council concludes that it is very unlikely that this proposed small scale field trials with GM maize will harm human health, animals or the environment.



Prof. Dr. ir. Geert Angenon
President of the Biosafety Advisory Council

Annex I: Compilation of comments of experts in charge of assessing the dossier B/BE/22/V1 (ref: BAC_2022_0287)

[7] Oldenburg 2011. Maize pollen is an important allergen in occupationally exposed workers. Journal of Occupational Medicine and Toxicology 6: 32.

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Compilation of comments of the experts in charge of evaluating notification B/BE/22/V1

Ref. SC/1510/BAC/22_0287

Coordinator: Geert Angenon
Experts: Patrick du Jardin (ULiege), Nina Papazova (Sciensano, GMOLAB), Jan Van Doorsselaere (VIVES)
SBB: Adinda De Schrijver

INTRODUCTION

Dossier **B/BE/22/V1** concerns a notification of the VIB, for deliberate release in the environment of genetically modified higher plants (GMHP) according to Chapter II of the Royal Decree of 21 February 2005.

The notification has been officially acknowledged on 14 January 2022 and concerns a field trial with maize modified with increased resistance against DNA damage caused by environmental stress

Experts were invited to evaluate the GMHP considered in the notification as regards their potential impacts on the environment, including human and animal health, and information relating to pre- and post-release treatment of the site.
The comments of the experts are roughly structured as in
- Annex II (principles for the risk assessment) of the Royal Decree of 21 February 2005
- Annex III (information required in notifications) of the Royal Decree of 21 February 2005

The comments below served as basis for a list of questions that the competent authority forwarded to the notifier with a request to provide additional information. The comments highlighted in grey correspond to the questions/comments selected and sent to the notifier.

B. INFORMATION RELATED TO THE RECIPIENT OR (WHERE APPROPRIATE) PARENTAL PLANTS

Have evaluated this section and had no comments/questions: 1 expert

Comment:

1) On page 2 of the technical dossier, the possibility of pollen spread from feral maize individuals in the Belgian context is acknowledged, however there is a only vague quotation of expert judgement ("*Maïs experts van ILVO zijn van mening dat ook in België niet kan worden uitgesloten dat opslagplanten van maïs het stadium van mannelijke vruchtbaarheid bereiken en stuifmeel gaan verspreiden.*"). Could the dossier be substantiated on this point by giving the sources of the data, kinds of observation and any useful information for a quantitative assessment?

2) the report quotes a report on maize pollen dispersal which seems to be a non-refereed document: Emberlin et al. 1999. I think non peer-reviewed documents should be avoided. Still, this review quotes peer-reviewed journals to which it can be referred.
Following Emberlin, the technical dossier mentions that the life span of pollen varies between 30 minutes to 9 days (*De levensduur van maïspollen is afhankelijk van luchtvochtigheid en temperatuur en kan variëren van 30 minuten tot 9 dagen (Emberlin et al, 1999*)). I think that the maximum of 9 days indicated by this sentence is a misleading interpretation of Emberlin, which says. "*Firstly, they refrigerated the pollen in the tassel and found that the pollen would set seed with decreasing success over 9 days.*" "*Decreasing success over 9 days*" does not mean that viability is zero beyond 9 days and that 9 days should be regarded as a maximum.

Considering the overall assessment and the knowledge of maize biology, I do not see biosafety concerns here, but I think that the dossiers should comply with the best academic standards.

Note SBB & coordinator: This comment is also valid for B/BE/22/V2 & B/BE/22/V3

C. INFORMATION RELATED TO THE GENETIC MODIFICATION

Have evaluated this section and had no comments/questions: 1expert

Comment:

The name of the vector is pBUN-ZmUBIL-ZmCas9-NosT-OsU3-attB1-ccdB-attB2, however the map and table of genetic elements on the technical dossier do not show any attB1-ccdB-attB2 sequences, which are popular genetic elements used in the Gateway cloning technology. Can the applicant clarify this and amend the map and table if necessary? If these sequences are present somewhere, this raises the issue of their later characterization in the transformants and segregants intended to be released, in particular the demonstration of absence of these sequences in the field-released segregants (see section D.2) and the likelihood of horizontal gene transfer (see section D.6).

D. INFORMATION RELATED TO THE GENETICALLY MODIFIED PLANT

D.1. Information related to the traits and characteristics, which have been introduced or modified

Have evaluated this section and had no comments/questions: 1 expert

Comment:

The dossier does not discuss whether the introduced mutation impacts plant growth in conditions of stress-induced DNA damage only, or potentially the DNA repair mechanisms themselves. If these latter are potentially affected, then the authors should discuss the possible impact of the mutation directed by CRISPR/Cas9 on the rate of other, non-directed mutations and how this might impact the phenotype of the GM plant and the conclusions of the field assessment. Briefly said: what do we assess when we field trial the plants, the site-directed mutation only or also associated 'random' mutations?

Note SBB & coordinator: The purpose of this field trial is to see whether the phenotype observed in the greenhouse also persists in the environment. One can presume that on the basis of this field trial the notifier will decide whether it will be worthwhile (or not) to continue R&D on this modified maize. We are of the opinion that at this stage of R&D, potential impacts of unintended mutations are not worthwhile to consider given the containment measures that will be applied. In addition, we don't see a reason for suspecting a mutator phenotype due to the mutation in the ZmNAC52 gene.

D.2. Information on the molecular characteristics of the final GMO

Have evaluated this section and had no comments/questions: 1 expert

Comment:

The absence of vector (T-DNA and backbone) sequences in the recipient plant is checked by PCR amplification. However, the primers seem not to cover all genetic elements. Indeed, the map in figure 3 of the annex 2 on the molecular characterization and the table on page 3 of the same annex do not indicate primers allowing the detection of the pVS1 StaA gene coding for the stability protein from the Pseudomonas plasmid pVS1. Can the authors clarify and, if no check was done for this sequence, can they justify why? If there is no argument for the absence of this sequence in the maize genome, then a risk assessment of its possible presence should be performed (based at first on the scientific literature).

Note coordinator & SBB: Absence of T-DNA and backbone has been checked with 10 primer pairs, including 3 primer pairs for the backbone. This appears largely adequate for a field trial notification and is - what concerns the demonstration of the vector backbone – in line with what has been accepted as sufficient for field trial notifications so far.

D.3. Information on the expression (of the insert)

Have evaluated this section and had no comments/questions: 2 experts

D.4. Information on how the GM plant differs from the recipient plant

Have evaluated this section and had no comments/questions: 1 expert

Comment:

As acknowledged, the intended change in the maize plant is likely to impact the fitness in stressful environments. However, this aspect is worth considering for large scale cultivation in different field environments and may be regarded as non-relevant for small-scale field trials with the proposed gene-flow control measures.

D.5. Genetic stability of the insert and phenotypic stability of the GMHP

Have evaluated this section and had no comments/questions: 1 expert

Comment:

The authors indicate : "*De fenotypische stabiliteit van de ZmNAC52 mutant planten is hoog aangezien het fenotype behouden blijft in meerdere generaties in de serre*". This is a quite vague statement. The EU regulation wants to verify the stability of the new, intended phenotype, but there is no indication in the dossier about which phenotype is scored in the greenhouse and about the number of generations. If significant changes in any phenotypic traits of the greenhouse-grown plants were observed, I presume they would have been noticed (and likely counter-selected in the R&D pipeline!), but more precision may be expected here.

Note SBB & coordinator: This comment is also valid for B/BE/22/V2 & B/BE/22/V3

D.6. Any change to the ability of the GMHP to transfer genetic material to other organisms

Have evaluated this section and had no comments/questions: 1 expert

Comment:

The possible presence of attB1-ccdB-attB2 sequences in the transformation vector (see section C) should be addressed and risk assessed if present.

D.7. Information on any toxic, allergenic or other harmful effects on human health arising from the genetic modification

Have evaluated this section and had no comments/questions: 2 experts

D.8. Information on the safety of the GMHP to animal health, particularly regarding any toxic, allergenic or other harmful effects from the genetic modification, where the GMHP is intended to be used in animal feedstuffs

Have evaluated this section and had no comments/questions: 2 experts

D.9. Mechanism of interaction between the genetically modified plant and target organisms (if applicable)

Have evaluated this section and had no comments/questions: 2 experts

D.10. Potential changes in the interactions of the GMHP with non-target organisms resulting from the genetic modification

Have evaluated this section and had no comments/questions: 2 experts

D.11. Potential interactions with the abiotic environment

Have evaluated this section and had no comments/questions: 2 experts

D.12. Description of detection and identification techniques for the GM plant

Have evaluated this section and had no comments/questions: 2 experts

Comment:

- Information on the protocols used for DNA extraction is missing. In particular, if a kit is used for DNA extraction from plant material, a reference of the kit should be provided;
- Concerning the sequencing step: The amplicon sequence should be provided to be able to analyse the sequence, and the sequencing strategy should be better described in terms of replicates (how many?), strands to sequence (one or two?), and alignment to the reference sequence. In particular, can it be clarified why another primer (only one) is proposed for sequencing and if the primers for amplification can be used for sequencing?

D.13. Information about previous releases of the GM plant, if applicable

Have evaluated this section and had no comments/questions: 2 experts

E. INFORMATION RELATING TO THE SITE OF RELEASE

Have evaluated this section and had no comments/questions: 2 experts

F. INFORMATION RELATING TO THE RELEASE

Have evaluated this section and had no comments/questions: 2 experts

G. INFORMATION RELATED TO CONTROL, MONITORING, POST-RELEASE AND WASTE TREATMENT

G.1. Any measures taken

Have evaluated this section and had no comments/questions: 2 experts

G.2. Information on methods for post-release treatment of site

Have evaluated this section and had no comments/questions: 2 experts

G.3. Information on post-release treatment methods for the GM plant material, including wastes

Have evaluated this section and had no comments/questions: 2 experts

G.4 Information on monitoring plans and techniques

Have evaluated this section and had no comments/questions: 2 experts

G.5. Information on any emergency plans

Have evaluated this section and had no comments/questions: 2 experts

G.6. Information on methods and procedures to protect the site

Have evaluated this section and had no comments/questions: 2 experts

ANNEX 1. INFORMATION RELATED TO THE RISKS FOR THE ENVIRONMENT

1. Persistence and invasiveness of the GM plant, including of gene flow from plant to plant

Have evaluated this section and had no comments/questions: 2 experts

2. Gene transfer from GM plants to micro-organisms

Have evaluated this section and had no comments/questions: 1 expert

Comment:

See comment in section D.6

3. Interactions of the GM plant with target organisms

Have evaluated this section and had no comments/questions: 2 experts

4. Interactions of the GM plant with non-target organisms

Have evaluated this section and had no comments/questions: 2 experts

5. Effects of the specific cultivation, management and harvest techniques

Have evaluated this section and had no comments/questions: 2 experts

6. Effects on biogeochemical processes

Have evaluated this section and had no comments/questions: 2 experts

7. Effects on human and animal health

Have evaluated this section and had no comments/questions: 2 experts

OTHER INFORMATION

Do you have any other questions/comments concerning this notification that are not covered under the previous items?

It should be acknowledged that the implementing rules for the RA of GMPs released into the environment, as followed here, are not 'fit for purpose' for the assessment of field-tested gene-edited plants.

Note SBB: This comment is also valid for B/BE/22/V2 & B/BE/22/V3