FORMAT FOR THE PRESENTATION OF THE RESULT OF DELIBERATE RELEASE INTO THE ENVIRONMENT OF GENETICALLY MODIFIED HIGHER PLANTS IN ACCORDANCE WITH ANNEX XI OF ROYAL DECREE 178/2004

1 General information

1.1 European notification number: B/ES/21/01

1.2 Member State of notification: Spain

1.3 Date of consent and consent number: 27/11/20, B/ES/21/01

2 Report status

2.1 Please indicate whether, according to Article 3 of the present Decision, the current report is:

- the final report **X**

- a post-release monitoring report

  - final - intermediary

3 Characteristics of the release

3.1 Scientific name of the recipient organism: *N. tabacum* cv K326

3.2 Transformation event(s) (acronym(s) or vectors[1] used (if transformation event identity not available):

The GM plants included in this release are lines derived by self-pollination from the transformation events: FT3-4, FT15-5, SPL2-3, SPL10-5, SPL11-3, SPL15-1, SPL22-2, FT-SPL3-7, FT-SPL4-7, FT-SPL5-1, FT-SPL7-4, FT-SPL9-8, MPO24-1-4-2, MPO24-1-7-1, BBL133 y BBL135.

Transformation vectors: GB2710 (FT lines), GB2713 (FT-SPL lines), GB2714 (SPL lines), GB2484 (MPO1 lines) and pDE-CAS9-BBLs-sgRNA (BBL lines).

T-DNA elements present in the transformation vectors GB2710, GB2713, GB2714 and GB2484: transcriptional units for the expression of DsRed and NptII proteins (selection markers), transcriptional units for the expression of Cas9 protein, transcriptional units for the expression of gRNAs directed to the target genes (FT - GB2710, SPLs - GB2714, FT/SPLs - GB2713, and MPO - GB2484).

[1] In the case of small-scale field trials where several lines may be tested, the vectors used should be mentioned, which gives insight into the introduced traits and/or genetic elements. In the case of large®-scale trials, the number of events notified is limited to only one or a few events.

T-DNA elements present in the transformation vector pDE-CAS9-BBLs-sgRNA: transcriptional unit for the expression of NptII proteins (selection marker), transcriptional units for the expression of Cas9 protein, transcriptional unit for the expression of gRNAs directed to the BBL target genes.

T1 lines FT3-4, FT15-5, SPL2-3, SPL10-5, SPL11-3, SPL15-1, SPL22-2, FT-SPL3-7, FT-SPL4-7, FT-SPL5-1, FT-SPL7-4, FT-SPL9-8, MPO24-1-4-2, MPO24-1-7-1, BBL133 y BBL135 do not contain the T-DNA; the lines released are therefore devoid of any exogenous DNA. The genetic modifications introduced in these plants consist of mutations generated by CRISPR/Cas9 in the target genes.

3.3 Unique identifier, if available: not applicable

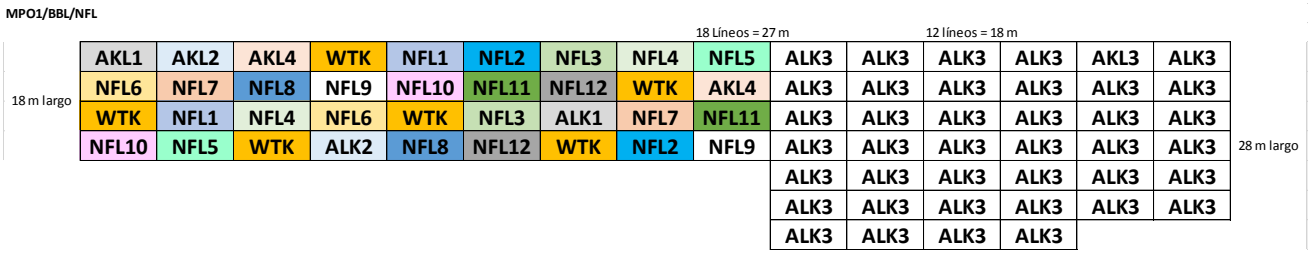
3.4 Please provide the following information as well as the field(s) layout:

Geographical location(s) (administrative region and, where appropriate, grid reference)	**Size of the release site(s) ([2]) (m2)**	**Identity ([3]) and approximate number of GM higher plants per event actually released (number of seeds/plants per m2)**	Duration of the release(s) (from … (day/month/year… until… (d/m/y)
CTAEX, Villafranco del Guadiana, Badajoz 38º 53' 45.68" N, 6º 51' 17.07" O	950 m^2	NFL1 (SPL11-3): 48 plants; NFL2 (FT15-5): 48 plants; NFL3 (FT-SPL3-7): 48 plants; NFL4 (SPL2-3): 48 plants; NFL5 (FT3-4): 48 plants; NFL6 (FT-SPL4-7): 48 plants; NFL7 (SPL15-1): 48 plants; NFL8 (FT-SPL5-1): 48 plants; NFL9 (SPL10-5): 48 plants; NFL10 (FT-SPL7-4): 48 plants; NFL11 (SPL22-2): 48 plants; NFL12 (FT-SPL9-8): 48 plants; ALK4 (MPO24-1-4-2): 48 plants; ALK3 (MPO24-1-7-1): 854 plants ALK1 (BBL133): 48 plants; ALK2 (BBL135): 48 plants; WT (K326): 144 plants. Approximate plant density: 2 plants /m^2	Seedbed (in greenhouse): From 09/03/21 to 08/06/21 Filed release: From 14/05/21 to 31/10/21

([2]) Specify the size of the GM area and, where appropriate, the size of the non-GM area (e.g. non-GM border)

([3]) Vectors used

Field layout: randomized block design



4 Any kind of product that the notifier intends to notify at later stage

4.1 Does the notifier intend to notify the released transformation event(s) as product(s) for placing on the market under Community legislation(s) at a later stage?

☐ Yes (by another juridical entity of the group) ☐ **No** **X** Unknown to date

If yes, indicate the country (ies) of notification:……..

If yes, specify for which use(s):

- Import.
- Cultivation (e ;g ; seed/planting material production).
- Food.
- Feed.
- Pharmaceutical use (or processing for pharmaceutical use).
- Processing for pour.
    - Food use
    - Feed use
    - Industrial use.
- Others (specify):

5 Type(s) of deliberate release(s)

Please select the main type(s) (in boxes) as well as subtype(s) of the release(s). In the case of multi-sites, multi-events and/or multi-annual release(s), please provide a general overview of the type(s) of deliberate release(s) which has/have been carried out for the full duration of the consent. Please tick the appropriate type(s):

5.1 Deliberate release(s) for research purposes **X**

5.2 Deliberate release(s) for development purposes **X**

- Event screening.

- Proof of concept [2].

- Agronomic performances (e.g. efficiency/selectivity of plant protection product, yield capacity, germination capacity, crop establishment, plant vigour, plant height, susceptibility to climatic factors/diseases, etc.) (specify). **X**

  Analysis of the effect of the introduced mutations on flowering time and duration of the vegetative phase (lines FT, SPL and FT-SPL).

- Altered agronomic properties (e.g. disease/pest/drought/frost-resistance, etc.) (specify).

- Altered qualitative properties (prolonged shelf-life, enhanced nutritional value, modified composition, etc.) (specify). **X**

  Analysis of alkaloid content in BBL and MPO lines.

- Stability of the expression.

- Multiplication of lines.

- Hybrid vigour study.

- Molecular farming[3]. **X**

- Phyto-remediation.

- Others : (specify) .....

5.3 Official testing ☐

- Variety registration on a national variety catalogue

  - DUS (=Distinctness, Uniformity and Stability)
  - VCU (=Value of Cultivation and Use)

- Others: (specify): …….

[2] For example, testing the new trait under environmental conditions.

[3] « Molecular farming » means the production of substances (for instance, proteins, pharmaceuticals) by plants, which have been genetically modified for a particular trait. "Molecular farming" could be defined as well as the production of plant-synthesised pharmaceuticals, plant-made pharceuticals, plant-based proteins production, etc.

5.4 Herbicide authorization ☐

5.5 Deliberate release(s) for demonstration purposes ☐

5.6 Seeds multiplication ☐

5.7 Deliberate release(s) for biosafety/risk assessment research ☐

- Vertical gene transfer studies.
    - Out-crossing with conventional crops
    - Out-crossing with wild relatives
- Horizontal gene transfer studies (gene transfer to micro-organisms).
- Management of volunteers.
- Potential changes in persistence or dispersal.
- Potential invasiveness.
- Potential effects on target organisms.
- Potential effects on non-target organisms.
- Observation of resistant relatives.
- Observation of resistant insects.
- Others: (describe) .....
-

5.8 Other(s) type(s) of deliberate release(s): ☐

(describe) : .....

6 Method(s), result(s) of the release, management and monitoring Measure(s) in respect of any risk to human health or the environment.

6.1 Risk management measure(s)

Please report the risk-management measures, which have been used to avoid or minimise the spread of the GMO(s) outside the site(s) of release, and in particular those measures:

- Which were not originally notified in the application,

- Which were applied in addition to the conditions in the consent,

- Which the consent required only under certain conditions (e.g. dry periods, flooding),

- For which the consent allowed the notifier a choice among different measures.

Tick the examples where appropriate:

The risk-management measures implemented to avoid the spread of the genetically modified plants are those notified in the application; no additional measure has been necessary.

6.1.1 Before the sowing/planting:

- Clear labelling of the GM seeds (distinct from other seeds/tubers/etc.) (describe).

  For the shipment of the seeds from IBMCP to CTAEX, they were placed in screw-top tubes sealed with parafilm, in a heat-sealed bag inside a padded envelope. Both the envelope and the heat-sealed bag with the seeds had a datasheet attached to it with information on (i) the identification of the content as genetically modified seeds, and (ii) the name and contact details of the sender and recipient.

  Upon arrival at CTAEX, seeds were sown as indicated in the section below.

- Segregation during the processing and transport of the seed/planting material (describe the method involved; provide example(s) of containment to prevent spillage during the processing and transport).

  Seeds were transported from IBMCP to CTAEX by private car. Upon arrival to CTAEX, seeds were manually sown in 384-wells polystyrene trays that were adequately label for the correct identification of the plants. An independent tray was used for each of the lines to avoid possible cross contamination between them. The area occupied by the genetically modified seedling trays was isolated from the rest of the greenhouse by means of a mesh.

- Destruction of superfluous seeds/planting material (describe the method involved).

  The tubes were the seeds were transported together with any unused seeds were destroyed by autoclave.

- Temporal isolation (specify).

  Not applicable

- Rotation (specify the previous crop).

  In the previous growing season, the plot used for the cultivation of the genetically modified plants had been cultivated with tomato for industry.

- Other(s): (specify) .....

6.1.2 During the sowing/planting activities:

- Method of sowing/planting.

  The seedlings grown in the greenhouse were taken to the release site as described below and transplanted manually.

- Emptying and cleaning of the sowing machinery on the field of release.

  Not applicable. Sowing and planting were done manually.

- Segregation during the sowing (provide example of containment to prevent spillage during the sowing/planting).

  The polystyrene trays containing the genetically modified seedlings were transported to the release plot by foot. Trays were cover with plastic to avoid the risk of accidental dispersal. Planting was done manually and each experimental block was identified by means of a planting label placed on the ground. Once the transplanting was completed, the polystyrene trays were returned to the greenhouse where they were kept for a few days until the survival of the transplanted seedlings was verified. After this time, any plants and substrate that were left over after transplanting were destroyed by autoclave. Germination trays were cleaned with water and hypochlorite.

- Other(s): (specify)

6.1.3 During the period of release:

- Isolation distance (x meters)

  - From sexually compatible commercial plant species.

    The distance between the release area and the closest commercial tobacco cultivated areas was greater than 100 km.

  - From sexually compatible wild relatives.

    Not applicable. There are no wild relatives in Europe that are sexually compatible with tobacco.

- Border rows (with the same crop or a different one, with a non-transgenic crop, x meters, etc).

  The distance between the modified plants and the closest crops (tobacco plants from deliberate release trial B/ES/21/02) was 126 m.

- Cage/net/fence/signpost (specify).

  The plot where the release site was located is surrounded by a fence. Additionally, the deliberate release area was surrounded with a second wire fence for restricted access to authorized personnel. The area was signposted as a GM area. The different blocks of the experimental design were identified with planting labels.

- Pollen trap (specify).

Not applicable. The tobacco plants were topped before the opening of the first flower bud; therefore, no pollen was produced.

- Removal of GM inflorescences before flowering (indicate the frequency of removal).
  Plants were inspected at least twice a week and those with flower buds were topped. After the topping plants were treated with pendimethalin 33% at a dose of 0.8 L / 100 L and 4% fatty alcohol to inhibit the growth of axillary buds. Any axillary bud that escaped chemical control was manually eliminated on a weekly basis and the chemical treatment repeated.

- Removal of bolters/relatives/hybrid partners (indicate the frequency of the removal, x meters around the GM field, etc).

  Not applicable

- Other(s): (specify)…….

6.1.4 At the end of the release:

- Harvest/destruction methods (of crop or part of it) / other means (e.g.: sampling) (describe).

  The aerial part of the plant (leaves and stems) was manually harvested. Part of the harvested material was air-dry within the release site. This material was covered with a mesh to avoid the accidental dispersal of the leaves. The remaining part was dried in an oven. Once dried, this **non viable** plant material was stored in boxes until shipment to a processing company for analysis.

- Harvest / destruction before the ripeness of the seeds.

  Not applicable. Tobacco plants were topped before the opening of the first flower bud; therefore, no seeds were produced.

- Effective removal of plant parts.

  The stems and roots left after the harvest were crushed with a disc harrow and buried in the ground.

- Segregated storage and transport of crop/waste (provide examples of containment to prevent spillage of collected seeds/crops/wastes).

  As mentioned above, the stems and roots left after the harvest were crushed with a disc harrow and buried in the ground. The harvested material, once dried, was stored in cardboard boxes in a room next to the CTAEX seed chamber. The stored material is non-viable material. Part of this material was sent to a company for its compositional analysis. Shipment was done in hermetically sealed cardboard boxes with a plastic packaging. The remaining material was destroyed by incineration.

- Clean up of machinery on the release site.

  Not applicable. The harvest was manually performed.

- Destination of the waste, treatment of waste/ surplus yield/plant residues (describe).

Plant material left after harvest was crushed with a disc harrow and buried in the ground. The remains of the dried harvested material were destroyed by incineration

- Post-harvest treatment and cultivation measures on the release site (describe the method for preparing and managing the release site at the end of the release, including cultivation practices).
  During the autumn and winter the soil will be harrowed. It is intended (if approved) to perform a new deliberate release in this site in the period between May and December 2022. This new release would include tobacco of the same variety, T-DNA free, and with editions in the very same genes as those analysed in the present deliberate release. For this purpose, the site will be managed according to common agricultural practices (application after the winter of herbicides, fertilizers, etc.).

- Other(s): (describe):

6.1.5 Post-harvest measures:

Please indicate which measures were taken on the release site after harvest:

Frequency of visits (average): weekly by CTAEX personnel

- Subsequent crop (specify). **X** Repetition of deliberate release (if approved). This new release would include tobacco of the same variety, T-DNA free, and with editions in the very same genes as those analysed in the present deliberate release.
- Crop rotation (specify).

- Fallow/no crop (specify).

- Superficial soil work / no deep ploughing. **X**

- False-sowing beds.

- Control of volunteers (specify intervals and duration). **X**

  After the harvest, the plot will be weekly inspected for a period of one year to detect and remove any potential volunteer plants.

- Appropriate chemical treatment(s) (specify).

- Appropriate soil treatment(s) (specify).

- Other(s) (specify)

6.1.6 Other(s) measure(s): (describe)

6.1.7 Emergency plan(s).

Indicate:

a) If the release proceeded as planned:

Yes **X**

The deliberate release was carried out without any incident being recorded. Disc harrow passes after the last harvest, initially scheduled for November 3, had to be delayed until November 10 because the rain fallen in the previous days prevented it from being carried out.

- No (describe for which reason, e.g. vandalism, climatic conditions, etc.)

b) if measures according to the emergency plan(s) (Article 6(2)(a)(vi) and Annex III.B of Directive 2001/18/EC) had to be taken:

- No **X**
- Yes (describe)

6.2 Post-release monitoring measures

Due to the fact that the current report format can be used for the final and post-release monitoring report(s), the notifier is asked to clearly make the difference between both types of report through this section 2 of Chapter 6. Please indicate whether

- **The post-release monitoring plan will start** (in the case of a final report, after the last harvest of the GM higher plants).**X**

  The week after the end of the trial

- **The post-release monitoring plan is ongoing** (in the case of an intermediary post-release monitoring report).

- **The post-release monitoring plan has been completed** (in the case of the final post-release monitoring report).

- **No post-release monitoring plan has to be fulfilled.**

The results of this monitoring are meant to confirm or invalidate earlier assumptions in the risk assessment.

According to the aforementioned cases, please indicate which monitoring measure(s) will be/are/were taken and where (on the release site/near the site (e.g. on fields edges)). Please be aware that all post-release monitoring measures taken during the whole post-release period shall be indicated here.

Specify:

- Monitoring measures within site

Duration: from November 2021 to October 2022

Frequency of visits (average): weekly by CTAEX personnel

- Observation of resistant relatives.
- Observation of resistant insects.
- Control of volunteers (specify intervals and duration). **X**
  The plot will be weekly inspected to detect and remove any potential volunteer plants.
- Monitoring of gene flow (specify).
- Appropriate chemical treatment(s) and/or soil treatment(s).
- Others (specify).

- Monitoring measures of adjacent areas:

  Not applicable. There is no risk of dissemination to adjacent areas as plants were topped before the opening of the first flower bud (avoiding any risk of dissemination of pollen or seeds) and the plant material remaining after the harvest was destroyed at the place of release.

  Duration:

  Frequency of visits (average):

  Area monitored:

  - Observation of resistant relatives.
  - Observation of resistant insects.
  - Control of volunteers and/or monitoring of feral populations (specify intervals and duration).
  - Monitoring of gene flow (specify).
  - Appropriate chemical treatment(s) and/or soil treatment(s).
  - Others (specify).

6.3 Plan for observation(s)/methods(s) involved

In this section the observation plan and the methods used to collect the effects which have to be reported under the next section (section 6.4) need to be specified. Any amendments or modifications to the plan as proposed in the application and the SNIF[4] part B need to be specified in detail.

During the time between the notification and the final report submission, new scientific insights or methods may be developed which cause a change in the methods used. In particular these modifications need to be specified under this section.

[4] Summary notification information format (=SNIF)

The observation plan followed during the release corresponds to that provided for in the notification, and no additional measure has been necessary. CTAEX staff has carried out a continuous inspection of the plants and the release area in order to detect any anomalies that might occur. No effect of the genetically modified plants on human health or the environment has been observed.

6.4 Observed effect(s)

6.4.1 Explanatory note.

All results of the deliberate release(s) in respect of any risk for human health or the environment shall be stated, without prejudice to whether the results indicate that any risk is increased, reduced or remains unchanged.

The main objectives of the information given in this section are:

- to confirm or invalidate any assumption regarding the occurrence and impact of potential effect(s) of the GMO(s) which was/were identified in the environmental risk assessment,

- to identify effect(s) of the GMO(s) which was/were not anticipated in the environmental risk assessment.

The observed **effect(s)/interaction(s)** of the GMO(s)

- with respect to any risk to human health,
- with respect to any risk to the environment

shall be reported under this section.

Particular attention shall be drawn to unexpected and unintended effect(s).

Indications as regards the effects, that the notifier may have to report, are provided hereunder. The effects have obviously to be considered in the light of the crop, the new trait, the receiving environment as well as the conclusions of the environmental risk assessment, which is carried out on a case-by-case basis.

In order to structure the information and to facilitate and efficient search within the given information, the notifier shall use, as far as possible, specific keywords to fill in the text fields under Chapter 6, especially sections 6.4.2, 6.4.3 and 6.4.4. A most updated list of those specific keywords is available on the Internet at: http://gmoinfo.jrc.it.

6.4.2 Expected effect(s)

This section concerns « expected effects », that is to say, potential effects which were already identified in the environmental risk assessment of the notification and could therefore be anticipated.

Notifiers should supply data from the deliberate release(s) which validate the assumptions made in the environmental risk assessment.

As stated in the authorization application, the environmental risk assessment did not identify any potential effects of the genetically modified plants on human health or the environment other than that of the unmodified tobacco plant.

6.4.3 Unexpected effect(s) [5]

"Unexpected effects" refer to effects on human health or the environment which were not foreseen or identified in the environmental risk assessment of the notification. This part of the report should contain any information with regard to unexpected effects or observations relevant for the initial environmental risk assessment. In case of any observed unexpected effects or observations, this section should be as detailed as possible to allow a proper interpretation of the data.

No unforeseen effects on human health or the environment were observed during the release.

6.4.4 Other information

Notifiers are encouraged to supply information, which is outside the scope of the notification but which might be relevant to the field trials in question. This may also include observations of beneficial effects.

The purpose of this release was (i) the evaluation of the effect of the mutations introduced in the FT, SPL and FT-SPL lines (coded in the field as NFL) on the duration of the vegetative phase by determining the flowering time and (ii) the evaluation of the alkaloid content in the MPO and BBL lines (coded in the field as ALK). The FT and FT-SPL lines showed absence of flowering. No other difference was observed between the behaviour of these lines and conventional tobacco. Likewise, no difference was observed between the behaviour of the BBL and MPO lines and conventional tobacco. The material harvested from these lines will be analyzed to determine its alkaloid content.

7 Conclusion

In this chapter, the notifier should specify the conclusions drawn and the measures taken or to be taken on the basis of the results of the release with regard to further release(s) and where appropriate, make reference to any kind of product the notifier intends to notify at a later stage.

The field trial has been carried out according to plan, and no incident has been recorded during its development. As expected from the risk evaluation carried out before the release, the edited plants have not shown any effect on human health or the environment different from that of conventional tobacco. Based on the results of the current release, it can be

[5] Without prejudice to Article 8 OF Directive 2001/18/EC as regards handling of modifications or new information.

concluded that the risk evaluation carried out and the risk control procedures applied are adequate, and no modifications for future releases are necessary.

DATE:

December 20, 2021