Public information concerning the deliberate release of genetically modified sugar beet

Notifier

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Title of the project

Validation of a concept of long-term resistance to the rhizomania virus (beet necrotic yellow vein virus, BNYVV): field experimentation of sugar beet genetically modified to be resistant to rhizomania

Notification number :

B/BE/02/V3

The release of genetically modified organisms (GMOs) in the environment is at the European level regulated by directive 90/220/EC (recently replaced by directive 2001/18/EC of 12 March, 2001) and at the Belgian level by the Royal decision of 18 December 1998 on the "regulations for the deliberate release into the environment or marketing of GMOs or products containing GMOs". To ensure the safe use of GMOs both legislations indicate that the release of GMOs for experimental purposes is prohibited without prior written authorisation of the competent minister. The decision whether or not to grant the consent is based upon a thorough biosafety evaluation of the planned release (risk assessment), conducted by the Biosafety Council.

In order to obtain the necessary authorisation from the competent minister, SES EUROPE - Advanta has submitted an application file to the General Inspectorate of Raw Materials and Processed Products of the competent authority. Following the positive advice (with conditions) of the Biosafety council the competent minster granted a consent for the company SES Advanta to carry out trials with transgenic oilseed rape in the year 2002 in accordance with their application B/BE/02/V3.

The release is to be carried out on a trial location in Flanders on the territory of the municipality of Verrebroek and will follow the normal growing period for sugar beet going from April till October.

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1. Description of the genetically modified plant

Sugar beet is a plant of the Chenopodiaceae family. The plant is grown for its root rich in sugar and is one of the main crops in the European regions. Sugar beet is an essential part of the crop rotation and contributes significantly to the grower's income.

The sugar beets described in this application were transformed to resist to rhizomania, a viral disease caused by a furovirus, the beet necrotic yellow vein virus (BNYVV) (Tamada et Baba, 1973, Kuszala et Putz, 1977), that is transmitted to the beet root by the soilborne virus *Polymyxa betae* (Keskin 1964).

Importance of the rhizomania

The disease affects significantly acreage of the area where sugar beet is grown for industrial use in the world. Observed first in Italy, the disease spread rapidly to different countries in Europe, from which France, Germany, the Eastern countries and more recently the United Kingdom, The Netherlands and Belgium (Asher, 1993). In parallel, the spread of the disease was observed in several countries all over the world, such as Japan (Tamada et al, 1971), the United States (Duffus *et al.*, 1984) and China (Gao *et al*., 1983).

The root yield of 60 to 70 tons per hectare in non-infected areas can drop to 20 tons in BNYVV contaminated fields. In parallel the sugar yield that normally yields up to 16-17% drops to 8%. Moreover, the increase of the Na+ and K+ ions in the roots shows a decrease of the juice purity and consequently a serious reduction of the sugar extraction yield for the sugar industry (Valentin *et al*., 1995).

Biology of BNYVV

The genome of beet necrotic yellow vein virus consists of four ribonucleoprotidic particles including each one single-strain RNA coding for specific virus functions. RNA 1 and RNA 2 encode functions essential for virus replication, movement and transmission. RNA 3 and 4 are implicated in the fungus vector-mediated (*Polymyxa betae)* infection of sugar beet (Richards and Tamada, 1992; Tamada, 1999).

Based on molecular characterisation, the BNYVV strains can be classified in two major groups, the pathotypes A and B. The BNYVV type A is found in most European countries from which Belgium, in the USA, in China and in Japan. The BNYVV Type B is found in Germany and in France. These two pathotypes have a 97% homologous nucleotidic sequence (Kruse *et al*, 1994).

Some BNYVV strains found in Japan and in France have a fifth RNA (RNA 5). The derived protein is involved in the disease symptomatology in sugar beet (Koenig *et al*,

1997). Inoculation tests with BNYVV strains containing the RNA 5 in sugar beet, show more symptoms than strains lacking this particular RNA. In France, this aggressive strain containing the RNA 5 is exclusively found in the area of Pithiviers. It constitutes a third class of BNYVV that is called pathotype P.

Strategy developed in the project

As in other furoviruses, cell-to-cell movement of BNYVV is governed by a set of three successive, slightly overlapping viral genes on RNA2. This three gene cluster is known as the triple gene block (TGB) (Gilmer *et al*, 1992) and encode three viral proteins identified respectively as P42, P13 and P15 according to their molecular mass in kilodalton. These three proteins are assumed to participate in the formation of a specific complex necessary to the viral cell-to-cell movement.

The detailed study of this mechanism helped to develop a new BNYVV resistance strategy. This strategy is expected to confer BNYVV resistance to the sugar beet plant by blocking the cell-to-cell movement of the virus. To achieve this goal, the sequence of the 'triple gene block' coding for the P15 protein and necessary to the movement mechanism of the virus, was isolated and mutated to be non-functional in the virus while its expression in the plant still interferes with the virus multiplication and diffusion mechanism into the plant (Lauber *et al*, 2001).

The P15 gene was modified so that the product it encodes is different from the original viral protein and is consequently inactive in the virus.

It is proposed that the expression of the modified P15 sequence in the plant gives a product that competes with the 'wild' viral protein, to form a complex with the other TGB proteins or with certain sites or cell components.

The *pat* gene (Wohlleben *et al*, 1988) was also integrated in the transgenic beets. This gene that confers tolerance to the herbicide glufosinate was used as selection marker during the phases of transformation and of *in vitro* selection.

2. Purpose of the release

The experimental transformants described in this application encode a sequence corresponding to the modified P15 protein of BNYVV.

The experimental protocol that is proposed for the season 2002 is essential to confirm:

1. if the mechanism used blocks rapidly the multiplication and the diffusion of the virus in sugar beet roots in natural infection conditions;
2. if the constitutive expression of the tested sequences induces sugar beet resistance all over the season;

3. if the expression of the tested sequences is sufficient to restore root and sugar yield in the plant.

3. Revue of previous and future activities

Breeding resistance sources from the Beta genus

Because the disease is shown to expand in many countries or areas and as there exists no practical method to effectively control the spread of a soil-borne virus at a large scale by chemical or physical means (Henry *et al,* 1992), the main focus of the breeders has been to identify natural genetic sources of resistance within the sugar beet germplasm. A variety of such tolerance genes have been identified and, some have been successfully used in the breeding of commercial sugar beet varieties.

Since 1986, rhizomania tolerant varieties have been distributed in the French market. Only the use of these tolerant varieties enable farmers to grow sugar beets in areas heavily infected by the virus, where sugar beet is an essential part of the crop rotation and contributes significantly to the grower's income. These varieties represented 23% of the sugar beet seed sales in 1999 and 32% in 2000 (La Technique betteravière, 1999).
In Belgium, the first rhizomania tolerant varieties were commercialised in 1998 and they represented 2% of the sales in 2001.

Different sources of rhizomania tolerance from the *Beta* genus exist. However, there are still few reports which indicate clearly that the tolerance genes, even from differing sources of sugar beet germplasm or wild relatives germplasm (Whitney, 1989), would provide distinct mechanisms of resistance.

The rapid progression of the disease in the sugar beet crop areas and the discovery of very aggressive BNYVV strains, such as the pathotype P, demonstrate that a diversification of the resistance mechanisms, used separately or in combination, would represent a more manageable situation to design long lasting BNYVV resistance strategies.

Genetic modification and virus resistance strategies applied to sugar beet

Since 1986, number of reports and publications have described the use genetic modification to express isolated viral gene sequences in plants, to confer a high level of resistance against the virus or even to confer broad spectrum resistance against a number of related viruses (Powel *et al*, 1986; Fritchen and Beachy, 1993; Wilson, 1993).

One of the most documented viral resistance strategies based on genetic engineering is the use of the viral gene sequences encoding the coat protein (CP) of the target virus under the control of appropriate regulatory sequences.

In sugar beet, the expression of the BNYVV coat protein sequence was reported by Kallerhof *et al,* 1990, Ehlers, 1991 Kraus *et al,* 1994 and in the patent WO91/13159.
The present notification describes a new strategy of rhizomania resistance by the insertion into the sugar beet of BNYVV derived sequences. The used sequence is slightly modified so that it is not functional in the virus but still confers a high level of virus resistance to the plant.

Strategy developed in the project

The genome of the beet necrotic yellow vein virus (BNYVV) consists of four (or five) plus-sense RNAs, from which RNA 1 and RNA 2 encode functions essential for virus replication, movement and transmission. RNA 3, RNA 4 (and RNA 5) are implicated in the vector-mediated infection of sugar beet (*Beta vulgaris*) (Richards and Tamada, 1992; Tamada, 1999).

As in other furoviruses, cell-to-cell movement of BNYVV is governed by a set of three successive, slightly overlapping viral genes on RNA2. This three gene cluster is known as the triple gene block (TGB) (Gilmer *et al*, 1992) and encode three viral proteins identified respectively as P42, P13 and P15 according to their molecular mass in kilodalton. These three proteins are assumed to participate in the formation of a specific complex necessary to the viral cell-to-cell movement.

The new resistance strategy explored in this project is expected to confer BNYVV resistance to the sugar beet plant by blocking the cell-to-cell movement of the virus. To achieve this goal, the sequence of the 'triple gene block' coding for the P15 protein and necessary to the movement mechanism of the virus, was isolated and mutated to be non-functional in the virus, while its expression in the plant still interfere with the virus multiplication and diffusion mechanism into the plant.

The P15 gene was modified so that the product it encodes is different from the original viral protein and is consequently inactive in the virus.

Several modifications were obtained and compared. The modified P15 sequences were inserted in the sugar beet genome by direct transformation of guard-cell protoplasts.
The primary transformants obtained were rooted in a growth room and grown in rhizomania infected soil. At the end of the experiment, the BNYVV infection level was measured in the roots. A number of primary transformants showed a high level of BNYVV resistance in this experiment performed under controlled conditions (Lauber *et al,* 2001). One modification of the P15 sequence in particular was selected.

It is proposed that the expression of the mutated P15 sequence in the plant gives a product that competes with the 'wild' viral protein, to form a complex with the other TGB proteins or to interfere with certain sites or cell components.

The concept was described in the patents WO 98/07875 and WO 00/03025.

In 2001, first generation hybrids were tested under field conditions in France (release permit B/FR/01/02/02).

The experiment will be continued to confirm under different agronomical conditions that the selected transformation events confer to the derived lines and hybrids resistance to BNYVV.

Two experiments are foreseen in 2002: one trial in France under release permit B/FR/01/02/02 and one trial in Belgium which is described in the application B/BE/02/V3.

4. Advantages for the environment, the farmer and the consumer

The disease affects significantly acreage of the area where sugar beet is grown for industrial use in the world.
The root yield of 60 to 70 tons per hectare in non-infected areas can drop to 20 tons in BNYVV contaminated fields. In parallel the sugar yield that normally yields up to 16-17% drops to 8%.
Moreover, the increase of the Na+ and K+ ions in the roots shows a decrease of the juice purity and consequently a serious reduction of the sugar extraction yield for the sugar industry (Valentin *et al*., 1995).

The disease is shown to expand in many countries or areas, at a speed depending upon the combination of environmental and agricultural factors, but there exists no practical method to effectively control the spread the virus at a large scale by chemical or physical means (Henry *et al*, 1992).
The main focus of the breeders has been to identify natural genetic sources of resistance within the sugar beet germplasm. A variety of such tolerance genes have been identified and some have been successfully used in the breeding of commercial sugar beet varieties.

Since 1986, rhizomania tolerant varieties have been distributed in the European market. Only the use of these tolerant varieties enable farmers to grow sugar beets in areas heavily infected by the virus, where sugar beet is an essential part of the crop rotation and contributes significantly to the grower's income.

However, there are still few reports which indicate clearly that the tolerance genes, even from differing sources of sugar beet germplasm or wild relatives germplasm

(Whitney, 1989), would provide distinct mechanisms of resistance. Such a diversification would represent a more manageable situation to design long lasting BNYVV resistance strategies.

The new resistance strategy explored in this project is expected to confer BNYVV resistance to the sugar beet plant by blocking the cell-to-cell movement of the virus.

5. Biology and life cycle of the plant

Cultivated sugar beet is biennial and requires vernalisation (a period of cold) followed by long days to flower.

During the first year of growth, sugar beet plants form a large root rich in sucrose and during the winter, in appropriate climatic conditions, the plants undergo a period of vernalization. Flowering and seed set is promoted during the spring with the increase in day length and temperatures.

The biennial character is likely to be governed by two major loci, one for the vernalisation phase and the other for the post-vernalisation photoperiod sensitivity (Abe *et al*., 1994).

In normal culture conditions, the sugar beet root crops do not flower.

In the field experimentation of 2002, the transgenic sugar beets will remain vegetative. The plants will be harvested in September 2002, before vernalisation and flowering induction.

6. Potential effects or risks for the environment

6.1 Outcrossing and spread in the ecosystems

6.1.1 Spread of transgenic pollen

As in any sugar beet crop, the plants in trial will not be allowed to flower. There will be no pollen spread.

The risk genetic material spread through pollen towards other compatible species is considered negligible.

6.1.2 Spread of transgenic seed

The trial plants will remain vegetative during the field release. A monitoring plan is prepared to ensure that any plant showing sign of bolting is detected and destroyed before flowering.

The risk of transgene dissemination through seeds is considered to be zero.

6.1.3 Selective advantage

Sugar beet is not recorded as an invasive species, nor is sugar beet or any species of the genus *Beta* a weedy species in any other environment nor in any other crop, except in the sugar beet crop.

It is not expected that the genetic modification will change the survival potential of the sugar beet in the environment, excepted the resistance to rhizomania and to glufosinate.

6.1.4 Post-release treatment of the site.

At the end of the experiment, the beets will be mechanically harvested. The leaves will be cut off, the roots will be harvested, washed and weighted. They will be sliced and pulp samples will be taken and frozen in closed plastic vials. All these operation will be done in the trial area.

The frozen pulp samples will be sent to SES-Europe, in Tienen, for the usual analyses of the sugar content.

At harvest, the leaves and the pieces of roots will be left in the trial site. They will be destroyed by rotary cultivation prior to incorporation into the soil.
The site will not be used for sugar beet cultivation for the following two years, during which all volunteer beets that may appear will be destroyed.

The two years following the release, the only authorized crops will be those using herbicides lethal to the beets (for examples cereals).

6.2 Interaction with target organisms

The strategy explored in this project is to confer BNYVV resistance to the plant by blocking the cell-to-cell movement of the virus. To achieve this goal, a gene of the ‘triple gene block’ necessary to the movement mechanism of the virus, the

P15 gene, was isolated and mutated so that its expression in the plant blocks virus multiplication and diffusion mechanism into the plant (Lauber *et al.*, 2001).

The P15 gene was modified so that the product it encodes is different from the original viral protein and is consequently inactive in the virus.

It is not expected that the genetic modification has an ecological incidence on the BNYVV virus.

6.3 Interaction with non-target organisms

The culture conditions will be similar to those applied in any sugar beet yield trial.

The environmental impact of the interactions between the genetically modified sugar beet plants and non-target organisms is not expected to be different from that arising from a trial of non genetically modified sugar beet.

6.4 Impact of large-scale and long-term use

The transgenic material described in this application is strictly experimental.
The transformation events were developed to evaluate the efficacy of the modified BNYVV P15 sequence to confer to the beet a high level of resistance to this virus.

The data produced in the field releases will contribute to evaluate the opportunity of developing this new technology. The study of the impact of large-scale and long-term use is not part of the proposed experiment.

7. Precautions taken for containment, control and monitoring

A detailed protocol will be produced prior to sowing, and will be communicated to the technicians in charge of the trial.
The protocol will describe all the operations to conduct in the release site, including observations, notations and sampling and in particular the specific measures taken in a release of genetically modified sugar beets.

The technicians in charge of the trial will use the trial logbook to record all the operations carried on in the trial. The logbook will be validated by the responsible scientist.

As described in the trial protocol, regular visits will be conducted by experienced technicians of Advanta.

7.1 Control of pollen spread

The beets in the trial and in the surrounding field will not be allowed to flower. In normal weather conditions, all the beets in a yield trial remain vegetative.

Regular visits of the trial by experienced staff, will allow detecting any bolting plant. Procedures are then followed to destroy the bolting plant long before flowering.

There will be no possibility of transfer of genetic material to the surrounding beet crop.

7.2 Control of seed spread

See 7.1. The plants will not be allowed to flower. There will be no seed release in the trial site.

7.3 Control, monitoring, post release and waste treatment plans

At harvest of the yield and selectivity trials, the beets will be 'topped' to remove the leaves and the roots will be weighed on a mobile tare-house. All the sugar beet root pieces will be chopped up on the trial site and will be left on the field.

Pulp samples will have been collected from the roots, on the mobile tare-house, and transferred to SES laboratories in Tienen, for analysis.

Wastewater, the leaf and the root pieces will be spread over the trial surface and will be incorporated in the soil by rotavation.

The site will not be used for sugar beet cultivation for the following two years, during which all volunteer beets that may appear will be destroyed.

The two years following the release, the only authorized crops will be those using herbicides lethal to the beets (for examples cereals).

If necessary, genetically modified material will be identified using two methods:

- Genomic Southerns or PCR analyses on plant material will demonstrate the presence of the inserted DNA.

- When sprayed with glufosinate-ammonium, modified plants will survive; unmodified plants will die.

- Use of an Elisa test based on an antibody targeted at the *pat* protein (art. N° 24016E07, FWD, Steffens Biotechnische analysen GmbH, Germany) will reveal the presence of the protein in any tissue of the plant.

- When cultivated in BNYVV infected soil, the genetically modified plants will develop normally, when non-transgenic BNYVV susceptible plants will exhibit typical rhizomania symptoms.

- A specific Elisa test (Torrance *et al,* 1996), on lateral roots for examples, will assess the amount of virus present in the beet roots. This test is used to identify rhizomania susceptible and resistant plants. The genetically modified plants derived from the transformation event MOX 63 and MOA 20, cultivated in BNYVV infected soil will show less virus than non-transgenic non-resistant controls grown in the same conditions.

Treatment of the site, other than already described, will be based on previous experience of sugar beet cultivation.

8. Destruction of genetically modified material

Seed will be sown directly in the trial plots. The drill machine will be cleaned in the trial site and all the remaining seeds will be brought back to Tienen to be destroyed.

After sowing, the trial plots will be thinned to leave 90-100 beets per 10m_ plot. This is the usual practice in the yield trials of Advanta to ensure a homogenous plant density in the micro-plots. The plants in excess will be left in the trial site.

At harvest of the yield and selectivity trials, the beets will be 'topped' to remove the leaves and the roots will be weighed on a mobile tare-house. All the sugar beet roots will be chopped up on the trial site and will be left on the field.

Pulp samples will have been collected from the roots, on the mobile tare-house, and transferred to SES laboratories in Tienen, for analysis.

Wastewater, the leaf and the root pieces will be spread over the trial surface and will be incorporated in the soil by rotavation.

The site will not be used for sugar beet cultivation for the following two years, during which all volunteer beets that may appear will be destroyed.

The two years following the release, the only authorized crops will be those using herbicides lethal to the beets (for examples cereals).

9. Emergency plan

Regular visits to the release site during the trial period will ensure that any unexpected events will be identified at an early stage.

The trial will be a trial of vegetative beets. All the trial plants that will start producing a flower stalk will be identified at an early stage and removed from the trial before flowering.

If the need arose, the trial plants can be effectively destroyed by the application of a suitable herbicide, for example glyphosate or metsulfuron methyl.

10. Inspection

The General Inspection of the Raw Materials and the Finished Products is in charge of the control of field trials with genetically modified plants. To plan the controls, the notifier is requested to inform beforehand the relevant department of the dates of sowing and harvest. Inspectors will check that the operations of sowing and harvest are conducted in accordance with the ministerial authorizations and the official protocols. Moreover, the Inspectors will sample plant material that will be analysed in the official laboratories.

11. Activity report

At the end of the cropping season, the notifier will provide an activity report to the controlling department, the General Inspection of the Raw Materials and the Finished Products, before 31 December 2002. The activity report will at least contain the following information:

- Copy of the logbook
- Place and period of release
- Precise nature of the released transformation events
- Actual surface of the trial
- Objectives of the experimentation
- Frequency and natures of the observations taken in the trial site
- Measures taken to avoid spread of transgenic material out of the trial site
- Methods used to destroy the harvest and efficacy of the destruction
- Results of the experimentation
- Monitoring plan

12. Socio-economic aspects

The disease progresses significantly in the European different countries. In France, the percentage of the surface used to grow sugar beet that is infected by the BNYVV increased from 39% in 1999 to 46% in 2000. In the Netherlands, the presence of rhizomania has been reported in several areas by Heijbroek (Heijbroek,1984 and Heijbroek, 1989).

As in the rest of Europe, the Rhizomania also spreads in Belgium. In the past, the disease was only found in the area of Antwerp (Wauters, 1996), but since 1995, infection sources have been found in various areas (Wauters *et al*, 1996). In this country, rhizomania tolerant varieties represented 2% of the sugar beet seed sales in 2001.

As no practical method exists to effectively control the spread of a soil-borne virus at a large scale by chemical or physical means (Henry *et al*, 1992), the main focus of the breeders has been to identify natural genetic sources of resistance within the sugar beet germplasm. Several tolerance genes have been identified and, some have been successfully used in the breeding of commercial sugar beet varieties.

Since 1986, rhizomania tolerant varieties have been distributed in the European market. Only the use of these tolerant varieties enable farmers to grow sugar beets in areas heavily infected by the virus, where sugar beet is an essential part of the crop rotation and contributes significantly to the grower's income.

However, there are still few reports which indicate clearly that the tolerance genes, even from differing sources of sugar beet germplasm or wild relatives germplasm (Whitney, 1989), would provide distinct mechanisms of resistance. Such a diversification would represent a more manageable situation to design long lasting BNYVV resistance strategies.

The rapid progression of the disease in the sugar beet crop areas and the recent emergence of particularly virulent BNYVV sources highlight the need for the diversification of the resistance sources and mechanisms, to establish a long-term BNYVV resistance strategy.

The resistance strategy explored in this project is expected to confer BNYVV resistance to the sugar beet plant by blocking the movement of the virus.
The preliminary data obtained in bio-assays conducted with the primary transformants and first generation hybrids, indicate that the modified viral sequence indeed confers a high level of resistance to BNYVV, all over the growing season.

The field experiments are needed to confirm under various agronomic conditions that the mechanism involved blocks the multiplication of the virus and restores root and sugar yield to the beets.

13. References

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14. Glossary

ARN :	ribo-nucleic acid
BNYVV:	beet necrotic yellow vein virus', virus that causes the sugar beet rhizomania disease. This virus is transmitted by the soilborne fungus *Plymyxa betae* to the sugar beet.
CP:	coat protein of a virus
Thinning:	operation that consists of adjusting the population of the beet plants in breeding plots after the germination. The young plants are pulled out and left onto the trial site.
Furovirus:	*fungal rod shape virus.* Group of plant viruses that comprise several RNA particles.
Na+:	natrium ion
K+:	potassium ion
P13, P42, P15:	proteins implicated in the formation of a specific complex required to the cell to cell movement of BNYVV.
TGB:	*triple gene block* Cluster of three genes of the BNYVV RNA2, involved in the cell to cell movement of the virus.