Public information

PLANT RESEARCH INTERNATIONAL B.V.

Testing genetically modified apple trees with increased resistance to fungus

European Notification Number
B/BE/02/V1

The release of genetically modified organisms (GMOs) in the environment is strictly regulated at the European level by the European Directive 90/220/EEG (recently replaced by Directive 2001/18/EG of 12 March 2001) and at the national level in Belgium by the Royal Decree (KB) of 18 December 1998 "regulating the deliberate environmental introduction and/or marketing of GMOs or products that contain GMOs". To ensure the safe use of GMOs, both legislations stipulate that the release of GMOs for experimental aims is prohibited without prior permission from the competent Minister. This decision is based on a thorough evaluation of the biosafety of the planned release (risk-assessment), which is to be conducted by the Biosafety Council.

To acquire the necessary permission from the authorised Minister, the research centre Plant Research International submitted an application dossier to the General Inspectorate of Raw Materials and Processed Products of the competent authority. Regardless the positive advice (with conditions) of the Biosafety Council for the first phase of the envisaged trial the competent Minister did not grant the permission to Plant Research International to conduct the experiments with transgenic apple trees from 2002 till 2008 as stipulated in the application B/BE/02/V1. The release was planned at one location in Flanders within the municipality of Melle.

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0. Table of Contents:

1. Description of the genetically modified plants:

The cultivated apple, *Malus pumila* Mill., is an important fruit crop in the Netherlands and Belgium. Apples are usually grown in monoculture and are affected by insects and fungal diseases, including scab. By improving the natural resistance to diseases, the use of pesticides can be reduced. This resistance can be improved by introducing a resistance gene.

In the present study, a piece of genetic material with such a resistance gene was inserted into the apple genome. This gene codes for the production of hordothionin, a protein that occurs naturally in barley and which has been shown to inhibit the growth of the scab fungus in laboratory experiments. In addition, a marker gene and a selection gene have been included to distinguish the transformed tissue from non-transformed tissue.

This genetic modification took place in the laboratory by culturing leaf strips with a special bacteria, *Agrobacterium tumefaciens* strain AGL0, which is capable of transferring pieces of genetic material (DNA) to plant cells. After an initial *in vitro* characterisation, the potentially genetically modified plants were rooted and taken to a greenhouse. In greenhouse tests, a number of these plants demonstrated increased resistance to scab.

2. Aim of the experiment:

This field trial with the varieties Elstar and Gala is intended to determine whether the effects found in the laboratory and the greenhouse also occur in the field. In the greenhouse, only scab resistance was tested. During *in vitro* bioassays, an inhibitory effect on fruit tree canker (*Nectria galligena)* was found, and a literature study on the role of thionin has shown that genetically modified organisms may have increased resistance to multiple pathogens. Therefore, the effects on the most prevalent apple pathogens, scab (*Venturia inaequalis*), mildew (*Podoshaera leucotricha*) and fruit tree canker (*Nectria galligena*), will be studied. The clones that displayed clearly reduced scab infestation in the greenhouse will be tested in the field trial.

The aim of this field trial is:

- to test whether this fungus resistance is also present at the tree level under field conditions,
- testing trees in the field for resistance to other fungal diseases (mildew, canker), and
- evaluating the fruit produced on the genetically modified trees for the occurrence of scab.

The experiment comprises two phases:

1. initial phase (3 years), disease observations to determine whether the intended differences in resistance are present and if these differences are sufficiently large,
2. second phase (3-4 years), evaluating the effects on flowering properties and the fruits.

Note that a market introduction of this transgenic material is emphatically not an aim of this project, nor is it part of the project in any way.

3. Summary of previous and future activities:

The transgenic apple plants were produced in the laboratory in 1997 by inserting the resistance genes. In 1998, the plants were large enough to be transferred from artificial nutrient media to potting soil and were moved into the greenhouse. When the plants reached a size of about 25 cm, they were first tested in the greenhouse for their resistance to the scab fungus. A number of plants turned out to be less susceptible. During the winter, buds were grafted onto rootstocks to create multiple individuals from each transgenic plant (clone); these individuals were also more homogenous in terms of size and vitality. A replicate experiment was conducted with this grafted material. A number of theses clones again displayed reduced susceptibility (up to 50% fewer symptoms). Now we want to know whether, and to what extent, this reduced susceptibility is maintained under practical conditions in the field. To acquire the most complete picture possible, the field trial must continue for 3 to 7 years. If it turns out that the grafted plant material is also less susceptible to scab in the field, nothing more will be done with this material, but we will then know that the inserted gene works! A new series of transgenic apples will then be created with this gene, where only this gene will be added; no other added genes will be present in the final product, such as a gene for antibiotic resistance (for selection purposes) or a GUS gene (as a reporter). These are no longer needed for a market introduction. Obviously, creating this new series will require another 6 to 7 years before we arrive at a point where its resistance will have been tested and confirmed. Other tests, such as taste tests, will then follow before the new trees (and apples) become available to growers and consumers.

4. Advantages for the environment, the fruit grower and the consumer:

Scab is the most important disease affecting fruit cultivation in Northwest Europe. To control scab, conventional growers, environmentally conscious growers and even organic growers apply chemical agents many times each year (10–15 times). In organic growing, sulphur compounds are used for this purpose; synthetic chemical fungicides are used in the other forms of cultivation.

European policy, which calls for a 90% reduction in the use of chemical agents, increases the risk of greater infection pressure and the spread of this fruit disease (scab). Increasing the level of resistance by means of genetic modification can lead to a reduction in the use of chemical control agents and is compatible with this policy; it can also lead to more efficient apple production by reducing harvest losses. It is expected that infestation of the fruits by storage scab (caused by the same fungus) will also decrease.

5. Biology and lifecycle of the plant under study:

5.1 General biology of the plant under study:

The experiment concerns apple, *Malus pumila* Mill. Besides cultivated (orchard) varieties of apple, there are also wild and ornamental varieties. Vegetatively propagated plants are used in cultivation. For this purpose, a piece of stem is grafted onto a rootstock (in winter) or budded on a rootstock (in summer). The rootstock is not only used for rapid vegetative propagation, but also to limit vegetative growth and bring the tree quickly into production. In most cases, cross fertilisation is required for good fruit set. This cross fertilisation takes place primarily by means of insects and the wind. The generation time is 4 to 7 years in natural ecosystems and under culture. For cultivation purposes, however, only vegetatively propagated material (grafted or budded onto a rootstock) is used. The cultivated apple can cross with wild *Malus* species and ornamental *Malus* varieties. *Malus* species are grown both for consumption (*Malus pumila*) and planted in parks and gardens for their ornamental value. There are several dozen *Malus* species occurring in Belgium and the Netherlands. Some of these are the result of interspecies crosses (spontaneous or intentional crosses). Many *Malus* species can breed with each other and produce fertile offspring. 'Wild' apple seedlings often grow from seeds in discarded apple cores. This can be seen from the size of the fruit and the phenotype of the tree. In practice, such trees are occasionally found in recreation areas. In addition, birds can disseminate seeds from ornamental *Malus* varieties. The seedlings that originate in this way, when they finally grow into fruit bearing trees, usually bear much smaller fruits, and the trees have a ‘wilder’ appearance (smaller leaves, more branching). These seedlings are encountered sporadically in nature areas. For these seedlings, too, the chances of dispersal are quite limited. In addition, night frost during flowering can limit reproduction. The genetically modified apples in this trial do not differ from any of the above in any respect. No orchards or wild apple relatives are located near the trial location.

5.2. Reproductive biology of the plant under study

5.2.1. Generative reproduction

- **pollen and pollination**

In nature, apple reproduces generatively by means of cross-pollination, i.e. other individuals must be present in the vicinity to supply pollen. The spread of apple pollen is quite limited. Studies in orchards with marker genes have indicated that the majority of the pollen remains within a radius of approximately 5 metres. Very little pollen can be found 20 or 30 metres from a tree. This is why multiple varieties are planted together in orchards to ensure good pollination and fruit set. Nonetheless, some pollen can still be found hundreds of meters away, probably due to high wind velocities. In practice, i.e. in the orchard, beehives are used to improve pollination. Although bees can theoretically fly a great distance, they tend to remain within a short distance of an orchard (‘flower fidelity’: i.e. they seek only one type of flower at a time).

- **seeds and fruits**

In Northwest Europe, apples can survive only with difficulty in the wild. Humans are an important cause of apple seedlings growing in nature areas (discarded apple cores). However, the seeds require very specific conditions for germination and rot quickly under damp conditions and moderate temperatures. In addition, the seedlings grow very slowly. The most important additional limitation in the Netherlands is that the plant ultimately requires so much space that only a few seeds can grow into a tree. The chance that a wild seedling will reproduce itself is limited even more by its small radius of pollen distribution.

In the experimental orchard of Plant Research International at Wageningen, spontaneous apple seedlings (volunteers) have never occurred; this is caused by the absence of the required germination conditions and because weed control is applied between the trees. During the trial being discussed here, conventional weed control will also be used.

5.2.2. Vegetative propagation

No forms of vegetative dissemination by roots or other plant parts are known for apple. Moreover, it is important to note that the transgenic plants are grafted onto non-transgenic rootstocks.

6. Possible environmental effects or risks:

6.1. Out-crossing and dissemination in natural ecosystems:

- <u>Spread of transgenic pollen:</u>

The plants are grafted onto the conventional M9 root stock (a non-genetically modified rootstock); the two-year-old saplings will be planted by hand in the spring. During the first phase (3 years), disease observations will be conducted (infestation by scab, mildew and canker). From the second year on, the trees would normally come into flower. During this first phase, flowering will be prevented. For this purpose the saplings will be sprayed with a natural plant hormone gibberellin during the first and following growing seasons (during which the flower buds for the following year are formed). This is a method that is frequently used in fruit growing research. As a result, significantly less flowering occurs. During the spring (April/May), any flowers that form despite the spraying will be removed manually.

If the results during the first phase regarding the intended differences in resistance are positive, the experiment will proceed to the second phase (approximately 3-4 years). During this phase, flowering is essential to study possible effects on the flowering properties and to evaluate the fruits. Therefore a portion of the trees will no longer be treated with gibberellin. On these trees, the flowers will be encased in a timely fashion and pollinated by hand, thereby preventing any spread of the pollen. The flowers on the other group of trees will still be sprayed or removed if necessary.

These measures ensure that the effects remain limited to the trial parcel.

· <u>Spread of transgenic seeds:</u>

As indicated above, flowering and seed formation are to be prevented until the final phase of the experiment, thereby making it impossible for transgenic seeds to spread into the environment. During the final phase, the fruits will be marked in August and harvested manually at the end of September. Every week, any windfall fruit will be collected so that no seeds can be released. Volunteers (spontaneous seedlings) will also be monitored during the experiment and for two years afterwards. As stated previously, volunteers have never been observed in the orchards, and this is not expected to occur in this trial.

· <u>Selective advantage:</u>

The spread of pollen and seeds will therefore be prevented. Leaf waste as such does not contribute to the spread of the newly introduced properties. The hordothionin present in these leaves will have as little effect on the soil, flora and fauna as the protein that is naturally present in barley and that enters the environment during the cultivation of this crop. In principle the seeds and the *hth* gene that is expressed in the seeds may be more resistant to fungi and bacteria due to the activity of the hordothionin. This could increase the duration of survival. For the plants themselves, it should be noted that the GMOs, at least in a young stage in the greenhouse, are more resistant to scab. This field test will study whether this also applies in a field situation. The trees may also be more resistant to other pathogens. This will also be investigated in the field test. Theoretically, this could give the GMOs a selective advantage. However, infection by crop-specific pathogens plays a role primarily in monocultures, where infection pressure can be high. Nonetheless, a selective advantage in nature is possible.

6.2. Interactions with target organisms:

It is expected that these interactions will be negligible because the genetic modification only concerns a reduced susceptibility to the scab fungus. The fungus will therefore not be entirely eliminated and will be able to survive in the environment (although at a decreased level), especially on the susceptible, non-transgenic control plants included in the experiment. The disease pressure during the crop period will be reduced and will not disappear entirely. Following completion of the experiment, the population can recover. After all, the cultivation of barley has not resulted in the eradication of this disease.

6.3. Interactions with non-target organisms:

The transformed plants produce hordothionin. Thionins occur in many cultivated grain crops and are therefore already consumed in large quantities. Animals consume large quantities of unprocessed grains on a daily basis. People consume the economic portions of the crop in processed and unprocessed form. Thionin is an arginine/lysine-rich protein and is therefore very sensitive to proteases and trypsins in saliva and in the gastro-intestinal tract. These proteins are therefore digested immediately upon consumption. Because significant interactions are not expected, the possible environmental effects of these interactions are expected to be absent or extremely small.

6.4. Impact of large scale and long term use:

The exclusive aim of this experiment is to study whether potentially increased resistance to pathogens also occurs under field conditions, resulting in a possible decrease in the use of crop protection agents. The genetically modified apple trees to be tested in this experiment are not intended for market introduction. Data about possible large-scale use are therefore not important to this experiment.

7. Confinement, control and monitoring measures:

7.1. Controlling pollen spread:

During the first phase of the experiment, flowering – and therefore pollen production and dispersal – will be prevented by spraying with gibberellins. Any flowers that form despite this measure will be manually removed and destroyed in the spring.
During the second phase of the experiment, when fruit formation and, therefore, flowering are essential, pollen dispersal will be prevented by encasing the flowers in a timely fashion and pollinating them manually.

7.2. Controlling the spread of transgenic seeds:

The spread of transgenic seeds is in fact impossible, because any apples (or windfall apples) that may form will first be marked and collected periodically at the end of the season, tested and ultimately destroyed.

7.3. Postharvest treatment, succession and monitoring:

Following completion of the experiment, the trees will be lifted and the soil cultivated with a rotary cultivator (the rootstocks of the trees are not genetically modified). The trees will then be chipped, and the waste will be collected and incinerated under the supervision of the responsible scientist. After the trees are removed and destroyed, the parcel will be included in a normal arable crop rotation scheme (maize, wheat, potatoes). During the two years following the completion of the experiment, the plot will be monitored for volunteers. Apple seedlings that survive soil tillage and cultivation, including weed control measures (see the above expectations regarding this point), will be collected and destroyed in an autoclave.

8. Destruction of transgenic material:

Following the completion of the experiment, all plant material will be collected and destroyed by supervised incineration. Waste from pruning and apple testing will also be destroyed by incineration or autoclaving, depending on the quantity of material.

9. Emergency situations:

In cases of emergency, the experiment will be immediately terminated and the plant material will be collected and destroyed by means of controlled incineration, to be carried out at a suitable waste incineration facility.

10. Inspection:

In Belgium, the General Inspectorate of Raw Materials and Processed Products of the Ministry of Small Enterprises, Traders and Agriculture is charged with inspecting field trials using transgenic plants. In order to assist in planning these inspections, the licensee (the legal entity who requests and receives a permit to work with GMOs) is required to inform the authorised service about the sowing and harvest date. On the experimental site, inspectors are to make sure that the sowing and harvest activities are in accordance with the ministerial permit and the various protocols. In addition, the inspectors take samples of the plant material; this material is analysed in official laboratories.

11. Activity report:

At the end of the growing season, an activity report must be drawn up by the licensee and submitted no later than 31/12/2002 to the authorised service, in this case the General Inspectorate of Raw Materials and Processed Products. This activity report must contain at least the following information:

- a copy of the logbook,
- the location and period of release,
- the exact nature of the genetically modified organisms that were released,
- the effective area of the experimental plot,
- the aim or aims of the experiments,
- the frequency with which observations were taken on the experimental plot and the nature of these observations,
- the measures that were taken to prevent any unintentional dispersal of transgenic material outside the experimental plot,
- the method used to destroy the harvest and its effectiveness,
- the results of the experiment,
- a summary of the supervision of the experimental plot.

12. Social-economic aspects:

Crop protection agents are used on a large scale in apple growing to control pests and diseases; this is true in not only conventional cultivation, but also in integrated and organic growing. In all forms of apple growing, the use of crop protection agents has turned out to be essential.

Improving the resistance to disease (especially fungal disease) will not only reduce the quantity of chemical agents being used, it will also reduce the dependence on such agents. This is in complete accordance with the aims of current governmental policy in Europe. Breeding apple by means of conventional crossing techniques is a difficult and long-term process because seed formation and germination are poor. In addition, the time between generations is 4 to 7 years. A programme to provide resistance (from a wild relative or a less commercially attractive variety, for example) to a good commercial variety by means of back-crossing requires at least 4 to 5 back-crosses. The development of a resistant variety in this way therefore requires at least 16 years. In *Malus*, few resistances to the various diseases are available. This also limits the possibilities of attaining results in the short term while maintaining quality. Genetic modification offers the possibility of adding resistance to an existing, commercially valuable variety in a single crop cycle and significantly expands the number of available genes. In our case, these are barley genes that may protect apple against fungal diseases. If the desired effect is indeed shown in this field experiment, the environmental advantages of the GMOs for reducing the use of crop protection agents will certainly be publicised in the press.

13. List of references:

- Florack D.E.A. et al: Thionins: properties, possible biological roles and mechanisms of action. Plant. Mol. Biol. 26 (1994): 25-37
- Wertheim S.J.: Malus cv. Baskatong as an indicator of pollen spread in intensive apple orchards. J. Hort. Sci. 66(1991): 635-642.