Cultivation of insect resistant maize MIR604

COGEM advice CGM/110404-01

This application concerns the cultivation of the genetically modified maize MIR604. MIR604 expresses the mCry3A *gene conferring resistance to certain coleopteran insects and the* pmi *selectable marker allowing the plant to use mannose as a sole carbon source. Previously, COGEM advised positively on the import of MIR604 as well as import of several stacked maize lines containing MIR604.*

During its long domestication process, maize has lost its ability to survive in the wild. In the Netherlands, the appearance of maize volunteers is rare and establishment of volunteers in the wild has never been reported. There are no reasons to assume that the introduced traits will increase the potential of maize to establish feral populations. The introduced genes cannot spread to closely related species since wild relatives of maize are not present in Europe.

COGEM is of the opinion that the molecular characterization of MIR604 is adequate.

The applicant conducted several laboratory and field studies to assess whether maize MIR604 affects non-target organisms (NTOs) adversely. Instead of using purified recombinant protein in laboratory experiments, the applicant used the entire E. coli *suspension, containing a mixture of 40% expressed mCry3A protein and 60% of a larger mCry3A protein version.*

The laboratory experiments also exhibit other shortcomings: a predatory spider was not tested, the statistical power of the experiments and the obtained P value is not always given, the choice for the statistical test is not always explained and the applicant did not provide an explanation for the high mortality (over 15%) in certain control groups. In addition, in most NTO experiments, population growth, the method of choice for measuring both sublethal effects and mortality, was not studied.

The applicant presents two studies on NTOs in the field, carried out with stacked maize line Bt11xMIR604xGA21. COGEM is of the opinion that a field test with the stacked line cannot replace a field test using the single event for risk analysis. Also, the field trials started quite late in the season, hampering the analysis of relevant life cycle stages of NTOs present in the field.

COGEM is of the opinion that the provided data are insufficient to allow a conclusion that cultivation of MON89034xNK603 exerts negligible effects on NTOs. Therefore, COGEM cannot advise positively on cultivation of maize line MIR604. COGEM points out that a food/feed safety assessment is carried out by other organizations. Therefore, COGEM abstains from advice on the potential risks of incidental consumption.

Introduction

The scope of the present notification (EFSA/GMO/UK/2010/83) by Syngenta Seeds S.A.S. concerns the cultivation, import and processing of genetically modified insect resistant maize MIR604. COGEM has been asked to evaluate the safety of commercial cultivation, import and

processing of this maize line with respect to human health and the environment, within the framework of the European application process. Import and processing of MIR604 maize has been authorised for the European Union in December 2009.[1]

Maize line MIR604 is genetically modified by the introduction and expression of the gene *mCry3A* that confers resistance to the Western corn rootworm (*Diabrotica virgifera virgifera*), the Northern Corn rootworm (*Diabrotica longicornis barberi*) and other related coleopteran species. The insertion and expression of the selectable marker gene *pmi* (*manA*) allows MIR604 to utilize mannose as a sole carbon source.

Previous COGEM advice

COGEM advised positively on import and processing of maize line MIR604 in 2005[2] and on import and processing of stacked maize lines containing MIR604 in 2008, 2009 and 2010.[3,4,5,6,7,8] For import applications Bt11xMIR604 and MIR604xGA21, COGEM questioned some aspects of the molecular characterisation and the general surveillance plan provided. In 2010, the sequence data of maize line MIR604 were revised. COGEM was of the opinion that both the original and the revised sequence data gave no reason to expect any adverse effects on the environment. However, COGEM pointed out that it is of the utmost importance that data provided by the applicant are correct in order to perform a proper risk assessment.

Aspects of the crop

Maize (*Zea mays* L.) is a member of the grass family *Poaceae*. Maize is cultivated as an agricultural crop, originating from Central America. Although insect pollination cannot be completely excluded, maize is predominantly wind pollinated.[9,10] According to literature, pollen viability varies between 30 minutes and 9 days.[10,11,12] In Europe, no wild relatives of maize are present and, therefore, hybridization with other species cannot occur.[13]

Throughout the world the appearance of volunteers is very rare. Seed kernels are the only survival structures of maize.[13] Due to the structure of the corn cob (ear on a stiff central cob enclosed in husks) natural dissemination of the kernels rarely occurs. Maize needs human intervention to disseminate its seed.[13] In addition, kernels exhibit poor dormancy resulting in a short persistence. Besides, maize can only survive within a narrow range of climatic conditions and, as maize is originally a subtropical crop, it is frost-sensitive.[14] Maize is very sensitive to weed competition.[15] During the long process of domestication, maize has lost the ability to survive in the wild.[13] Establishment of maize plants in the wild has never been observed in the Netherlands and COGEM is not aware of any reports of wild maize plants elsewhere in Europe.

Molecular characterization

An overview of the construction and inserted genetic elements of both parental lines as well as the properties of the introduced genes is given below.

Origin and function of the introduced genes

Maize line MIR604 is genetically modified via *Agrobacterium*-mediated transformation. A modified version of the gene c*ry3A* (*mcry3A*) has been introduced and confers resistance to the Western corn rootworm, Northern corn rootworm and other related species. MIR604 expresses the *pmi* selectable marker gene and is therefore capable of using mannose as a sole carbon source during the plant transformation procedure.

An overview of the introduced sequences is given below:
Active ingredient cassette

- MTL promotor, derived from the *Zea mays* metallothionein-like gene; provides root-preferential expression
- *mcry3A* gene, a modified version of the *cry3A* gene from *Bacillus thuringiensis* subsp. *tenebrionis*; confers resistance to coleopteran insects
- NOS, terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens*

Selectable marker cassette:

- ZmUbiInt promotor, derived from the *Z. mays* polyubiquitin gene; provides constitutive expression in monocots
- pmi gene, from *Escherichia coli*; catalyzes the isomerization of mannose-6-phosphate to fructose-6-phosphate
- NOS, terminator sequence from the nopaline synthase gene of *A. tumefaciens*

Properties of the introduced genes
Insect resistance
Maize line MIR604 expresses a modified version of the *cry3A* gene. The *cry3A* gene is derived from *B. thuringiensis* (subsp. *tenebrionis*). Two alterations were made to the gene *cry3A* to enhance the activity of the expressed protein against its target pests. The N-terminus is shortened by 47 amino acids and a protease cleavage site has been introduced. The *mcry3A g*ene encodes a δ-endotoxin specific for certain insects of the order Coleoptera. The δ-endotoxins are solubilized in the midgut of susceptible insects and are activated by midgut proteases to release a toxin fragment. The toxin fragment binds to specific receptors on the epithelial surface of the midgut. Subsequently, pores are formed in the membranes of the gut cells of the insect, enabling midgut bacteria to enter the body cavity, which leads to septicemia and death.[16]

Selectable marker
Maize line MIR604 was genetically modified with the gene *pmi* (*manA*) encoding for the enzyme phosphomannose isomerase (PMI). As a result of the expression of *pmi*, plants are capable of using mannose as a sole carbon source. Mannose is phosphorylated to mannose-6-phosphate (M6P) which can be converted to fructose-6-phosphate with the help of PMI. In non-GM plants lacking PMI, conversion of M6P will not occur. M6P will accumulate, block glycolysis, and inhibit plant growth. Consequently, the insertion of *pmi* has led to the introduction of a selection system in MIR604. Mannose is used as the selective agent and is applied to cell cultures to select transformed cells. It is not used as a selective agent in mature plants.

Molecular analysis

The molecular characterization of maize MIR604 was previously evaluated by COGEM for several applications concerning import and processing.[2,3,4,5,6,7,8] COGEM concluded that the molecular characterization of MIR604 was adequate, though a comment was made on the analysis of ORFs spanning the junction of the insert and the maize genomic DNA. The applicant only analysed ORFs between a start and a stop codon that were longer than 50 amino acids.

For the current application, the applicant performed an analysis of the ORFs at the junction between maize genomic DNA and the insert using updated databases. The applicant analysed all ORFs from stop codon to stop codon with a minimum length of 7 amino acids. This meets the criteria laid down by the COGEM for analysis of these ORFs.

The applicant analysed whether the ORFs showed any homology to the amino acid sequences of known allergens or toxins. Based on the results of the analysis, the putative proteins from these ORFs are not expected to be toxic or allergenic.

The applicant submitted other new data concerning the molecular characterisation of MIR604, in addition to the old data present in application EFSA/GMO/UK/2005/11 concerning import and processing of maize MIR604. The applicant performed homology searches in updated databases using the inserted sequences and the genomic sequences flanking the insert. Also, expression of the insert and differences in reproduction, dissemination and survivability of MIR604 were investigated in several recent European field trials. The updated data on the (molecular) characterisation of MIR604 maize does not give rise to any new concerns.

COGEM is of the opinion that the molecular characterisation of MIR604 maize has been adequately performed and meets the criteria laid down by COGEM.[17]

Environmental risk assessment

During the long process of domestication, maize has lost the ability to survive in the wild.[13] Maize needs human intervention to disseminate its seed.[13] Maize kernels exhibit poor dormancy resulting in a short persistence. Maize is very sensitive to weed competition and cannot persist as a weed.[13,15] Furthermore, maize is naturally frost sensitive and can only survive within a narrow range of climatic conditions. In the Netherlands, volunteers are rarely found and establishment of maize plants in the wild has never been observed. In Europe, no wild relatives of maize are present and, therefore, hybridization with other species cannot occur.[13]

In the opinion of COGEM, there is no reason to assume that the traits expressed in maize MIR604 will increase the potential of maize to establish feral populations. With regard to potential adverse effects of MIR604 on non-target organisms (NTOs), the applicant refers to laboratory, greenhouse and field studies. These studies will be discussed below.

Laboratory and greenhouse studies

The applicant performed several experiments to study whether MIR604 has an adverse effect on NTOs. None of the studies were performed with plant material of MIR604.

COGEM is of the opinion that plant material from MIR604 is the test substance of first choice for NTO studies, since this reflects the situation in the field. However, not all organisms consume plant material (like predatory insects) and the production and purification of large amounts of non-native protein from plants is inefficient. Therefore, the use of purified recombinant protein, expressed in a bacterial production system, is an acceptable alternative.

To study the effect of MIR604 maize on NTOs, mCry3A protein was produced using an *E. coli* expression system. The applicant did not purify the mCry3A protein from *E.coli*, but used the microbial suspension containing the mCry3A protein in the NTO experiments. Since a bacterial expression system might influence the folding and processing of a plant protein, the applicant provides a study to prove the biological similarity of mCry3A expressed in maize and in *E.coli*. The applicant however does not provide a rationale for the use of the *E.coli* test substance instead of purified protein from *E.coli* for NTO studies.

Moreover, not all the mCry3A protein expressed by *E. coli* is identical. 60% of the produced protein contains the mCry3A sequence as well as an extra N-terminal sequence of 16 amino acids. This larger protein showed a fourfold higher bioactivity in studies with Western Corn Rootworm. COGEM is of the opinion that the presence of the larger protein in the test suspension may change the effect of the test suspension on NTOs.

Previously, COGEM formulated guidelines for the selection of relevant NTO species for GM crops in the North-Western European situation.[18,19] In general, COGEM is of the opinion that all NTOs tested should be relevant to the European crop system. For GM plants expressing Cry3 protein, testing should be done using a predatory ground beetle, a predatory ladybird beetle, a wolf spider and a Hymenopteran pollen feeder.[19]

The applicant used several NTOs, namely carabid beetle (*Poecilus cupreus*), rove beetle (*Aleochara bilineata*), earthworm (*Eisenia fetida*), seven-spotted ladybird beetle (*Coccinella septempunctata),* insidious flower bug *(Orius insidiosus)*, honey bee *(Apis mellifera)*, bobwhite quail (*Colinus virginianus*) and rainbow trout (*Oncorhynchus mykiss*) in laboratory experiments or semi-field studies.

The organisms tested by the applicant include three of the above mentioned arthropods required by COGEM. A wolf spider (*Lycosidae*) was not tested. Wolf spiders are surface-active predatory arachnids. The applicant did test two European ground beetles (*Poecilus cupreus* and *Aleochara bilineata*), representing surface-active predatory insects. Results from these tests may give an indication of effects of MIR604 on predatory arachnids, but COGEM questions the suitability of this alternative.

Three of the NTOs tested by the applicant, i.e. *O. insidiosus, C. virginianus and O. mykiss*, do not occur in the European crop system. However, testing of the insidious flower bug (*O.*

insidiosus) gives supportive evidence for the absence of an effect on NTOs. Though bobwhite quail (*Colinus virginianus*) and rainbow trout (*Oncorhynchus mykiss*) are not native European species, tests of the effects of MIR604 on these organisms gives insight into effects on vertebrates. COGEM notes that the effects of ingestion of MIR604 plant materials by vertebrates are evaluated in a food/feed assessment. This assessment is carried out by other organisations than COGEM.

Three laboratory experiments, on earthworms, rove beetles and honey bees, were carried out with only four independent replicates, with each replicate respectively containing ten, twenty or hundred organisms. The number of replicates in combination with the variability within the experiment determines the ability to detect effects accurately. COGEM is of the opinion that an effect that is present should be detected in at least 80% of the cases, therefore experiments should have a statistical power of 0.8 or more.[20] However, information on the statistical power of the experiments is not given and consequently it is unclear how well the experiments are able to detect an effect. If the statistical power of the experiments is below 0.8, the number of replicates should be increased to ensure an accurate detection of any effect that might be present.

Different statistical tests have been used without explanation for the chosen method. COGEM is of the opinion that the applicant should clarify why a certain statistical test was chosen. In addition, in most experiments the obtained P value is not given. The applicant should give information about the obtained P-values.

In the laboratory experiments on ground beetles and flower bugs, mortality in the control groups exceeded 15% slightly. The applicant did not provide an explanation for the mortality percentages in these species. Mortality was 28,5% in the controls for the honey bee experiment. For honey bee, the applicant argues that the level of mortality was similar to other honey bee experiments conducted by the laboratory that performed the tests. The mortality of the control population in the rove beetle experiment was 34%, but mortality was not considered an important aspect of the experiment by the applicant. COGEM points out that a high mortality in control groups could indicate problems with the experimental setup[21] and could mask an effect that is present. Preferably, mortality in control groups should not exceed 15%.

The laboratory experiments on ladybird beetle, ground beetle, rove beetle and honey bee investigated sublethal effects (development time, adult emergence, reproduction and brood development), mostly in addition to mortality. A significant deviation was found for the development time from larvae to adult in ladybird beetles. Larvae in the control group developed slower into adult beetles than larvae ingesting mCry3A protein. The applicant indicates that it is unclear why this difference has occurred and concludes that at least no adverse effects have been found of mCry3A protein. As a possible explanation, the applicant states that the extra dietary protein supplied by mCry3A protein might have been the cause of faster development time.

COGEM is of the opinion that any significant effect on development should be taken seriously and investigated thoroughly. A faster development could lead to less reproduction, thus influencing population size and eventually overall fitness of the species. These consequences

cannot be excluded on forehand. A study on population growth could have confirmed or negated the importance of the observed effects for the population.

Moreover, in most of the NTO experiments population growth was not studied. COGEM is of the opinion that it is important to study whether maize MIR604 has sublethal effects on non-target organisms because sublethal effects can affect population size significantly. In a previous advice COGEM stated that she considers measurement of population growth the method of choice when studying whether a genetically modified crop has an adverse effect on non-target organisms, because both mortality and sublethal effects are reflected in this parameter.[20]

Field trials

The applicant provides field trials executed in Spain and Hungary for NTO analysis. The field studies have been performed with a stacked maize line, Bt11xMIR604xGA21. In addition to mCry3A, this maize line contains another Cry protein, Cry1Ab, and a herbicide tolerance protein, mEPSPS. COGEM is of the opinion that a field test with the stacked line may give supportive evidence for effects of the genetic modification on NTOs, but cannot replace a field test using the single event.

Also, the maize field trial in Spain was planted quite late in the season, on June 10th 2008. Moreover, on the first sampling date, June 28th, only visual counting of non-target arthropods was performed. The yellow sticky traps and the pitfall traps were not installed. Therefore, results from this first sampling date are not statistically robust.

The maize field trial in Hungary was also planted more than a month later than planned, on June 11th 2009. Because of the late start of both experiments, the development of maize will be asynchronic with the course of development of NTOs. It is possible that relevant NTOs are no longer present by this time, while other insects are attracted to this field because of the unusual presence of maize pollen. Secondly, cultivation at a later time, results in lower yields, which also can affect the presence of certain herbivorous insects.

Conclusion on NTOs

Overall, COGEM is of the opinion that the studies that have been carried out do not provide enough information to endorse the conclusion of the applicant that MIR604 does not affect NTOs adversely.

General surveillance

General surveillance has been introduced to be able to observe unexpected adverse effects of genetically modified crops on the environment. The setting or population in which these effects might occur is either not, or hardly predictable. The central tool for general surveillance in case of cultivation of MIR604 maize is an annual farmer's questionnaire which is addressed to a subset of farmers that cultivate MIR604 maize.

In COGEM's view the questionnaire should not only contain questions about the performance of MIR604 maize on the field, but should also contain questions about unexpected effects of the MIR604 maize on the whole of the farmer's premises. COGEM is also of the opinion that the part

of the farm questionnaire dealing with animals is too general. Birds, deer and insects are assigned to one category 'wildlife'. Information about the occurrence of wildlife should be obtained by different questions for specific groups of organisms (e.g. mammals, (predatory) birds, and insects). In addition, the farmer should be asked whether unusual quantities of other animals were observed and whether dead animals were found. The questions in the farm questionnaire refer to the usual situation, but the usual situation is not well defined. It would be better to rephrase the questions to acquire data that can be used to detect negative or positive trends in populations of organisms relevant to the monitoring scheme.

In addition, the applicant mentions that existing monitoring networks which collect environmental data will be used in the general surveillance of maize MIR604. However, it is not clear which networks the applicant aims to use and whether these networks will agree to cooperate in the general surveillance. COGEM is of the opinion that the applicant should ascertain that information on potential adverse effects is obtained.

Advice

The present application concerns the cultivation of the genetically modified maize line MIR604. This maize line expresses the *mCry3A* gene conferring resistance to certain coleopteran insects and the *pmi* selectable marker allowing the plant to use mannose as a sole carbon source. In the past, COGEM advised positively on the import of this particular maize line.

There are no wild relatives of maize in Europe and the appearance of volunteers is rare. Furthermore, there are no reasons to assume that the inserted traits will increase the now absent potential of the maize line to establish feral populations. Also, COGEM is of the opinion that the molecular characterization is adequate.

The applicant conducted several laboratory and field studies to analyse potential adverse effects of MIR604 on non-target organisms (NTOs). Instead of using purified recombinant protein in laboratory experiments, the entire *E. coli* suspension was used, containing a mixture of 40% expressed mCry3A protein and 60% of a larger mCry3A protein version. COGEM is of the opinion that the presence of the larger protein in the test suspension may change the effect of the test suspension on NTOs.

The applicant did not test a predatory wolf spider for effects of MIR604 on NTOs. The laboratory experiments also exhibit other shortcomings: the statistical power of the experiments and the obtained P value are not always given, the choice for a certain statistical test is not always explained and the applicant did not provide an explanation for the high mortality (over 15%) in certain control groups.

In addition, in most NTO experiments, population growth was not studied. COGEM is of the opinion that it is important to study whether maize MIR604 has sublethal effects on non-target organisms because sublethal effects can affect population size significantly.

The applicant presents two studies on NTOs in the field, carried out with stacked maize line Bt11xMIR604xGA21. COGEM is of the opinion that a field test with the stacked line may give supportive evidence for effects of the genetic modification on NTOs, but cannot replace a field

test using the single event. Also, the field trials started quite late in the season, hampering the analysis of relevant life cycle stages of NTOs present in the field.

COGEM is of the opinion that the data provided are not sufficient to conclude that cultivation of MIR604 exerts negligible adverse effects on NTOs.

Furthermore, the General Surveillance plan could be improved on several points.

Conclusion

COGEM is of the opinion that she cannot perform an adequate risk analysis with regard to the cultivation of MIR604. As a result of the concerns mentioned, COGEM cannot currently issue a positive advice on the cultivation of maize line MIR604.

A food/feed safety assessment is carried out by other organisations. Therefore, COGEM abstains from advice on the potential risks of incidental consumption.

Additional remark

The applicant states that the application for cultivation of maize MIR604 is a formality. The applicant intends to cultivate the stacked maize line Bt11xMIR604xGA21. Before the application of this stacked line will be accepted by EFSA, EFSA requires an approval of the single parental lines MIR604, GA21 and Bt11. Therefore, the authorisation of cultivation of MIR604 only serves the purpose of an application for the cultivation of stacked maize line Bt11xMIR604xGA21.

EFSA apparently wishes to evaluate single parental lines to be better able to assess possible unexpected environmental effects. In her guidance documents, this argument is however not mentioned. [22,23]

COGEM sees no added value of the approval of a single parental maize event before approval may be given for a stacked line, as stated before in comments on the EFSA guidance for the ERA of GM plants. [24] Studies using single parental events may give supportive evidence for the assessment of a stacked line. However, only studies using the stacked maize line itself will give conclusive evidence on the environmental safety of the maize line. Moreover, since maize cannot survive in the wild in Europe, segregation of events from a stacked maize line in the field will not lead to the presence of a single event in a maize plant in the field. Lastly, a formal application is an expensive and time consuming execution for both risk assessors and applicants involved.

Since the current application could nevertheless result in a legal permit for cultivation of a genetically modified maize line in Europe, COGEM has performed a detailed environmental risk analysis for MIR604.

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