

Cultivation of glyphosate tolerant soybean 40-3-2

COGEM advice CGM/061128-01

The present application by Monsanto Europe S.A. of file EFSA/GMO/NL/2005/24, 'Glyphosate-tolerant Soybean Transformation Event 40-3-2' concerns the cultivation of a genetically modified soybean.

The recombinant soybean line is genetically modified by insertion of the cp4 epsps gene. As a result, 40-3-2 soybean is tolerant to herbicides containing the active ingredient glyphosate. According to the applicant, the soybean line is already released in the United States of America, Brazil, Canada, Argentina, Uruguay, Romania, the Republic of South Africa, Paraguay and Bolivia.

In Europe, no wild relatives of soybean are present and modern soybean cultivars do not possess any of the attributes commonly associated with problematic weeds. Moreover, there is no reason to assume that the inserted genes would increase the potential of the soybean to run wild. Furthermore, establishment of feral populations in soybean producing countries has never been observed. In addition, soybean can not survive the climatological conditions in North-Western Europe. Survival and establishment of soybean volunteers in the wild has never been reported. The molecular analysis of the soybean line gives no reason to assume that toxic or allergic proteins are being formed as a result of the modification. Therefore, in COGEM's view, cultivation of soybean 40-3-2 will not pose a risk to the environment in the Netherlands.

In addition, COGEM is of the opinion that some parts of the general surveillance plan as well as the performed tests on non-target organisms, can be improved.

Introduction

The scope of the present notification by Monsanto Europe S.A. concerns the cultivation of soybean line 40-3-2. The line contains and expresses the gene *cp4 epsps* conferring tolerance to glyphosate based herbicides.

40-3-2 soybean has been authorized for import and processing in the European Union according to directive 90/220/EEC in 1996. According to the applicant, the soybean line is already released in the United States of America, Canada, Argentina, Uruguay, Brazil, Romania, Republic of South Africa, Paraguay and Bolivia. There is a history of safe use e.g. no adverse health effects concerning handling and consuming of products and derivatives of this line have been reported.

Previous COGEM advices

In 1995 COGEM has advised positively on the import and processing of this soybean line (1). In 2000, COGEM obtained additional information concerning the authorized 40-3-2 soybean line. Additional research had pointed out that besides the primary insert, two additional fragments were inserted in the soybean line. COGEM was of the opinion that the presence of the two additional fragments did not pose a risk to the environment. Furthermore, there were no indications that the presence of the fragments leads to the formation of toxic or allergic proteins (2).

Last April, COGEM advised negatively on the commercial import and processing of A2704-12 soybean (3). The molecular analysis was considered incomplete because the applicant did not provide the required information concerning the 5' flanking region of the insert.

Aspects of the crop

Soybean is a member of the genus *Glycine* and belongs to the *Fabaceae* (*Leguminosae*) family (4). Soybeans are adapted to agricultural regions from equatorial to temperate zones. Depending on cultivar and climate, the growth period can range from 65 to 150 days. The crop starts flowering 25 to 150 days after sowing, depending on the day length, temperature and cultivar. Flowering can take 1-15 days; pod formation 7-15 days; seed filling 11-20 days and ripening to harvest 7-15 days (4). Soybean is a short-day plant and hence, flowers more quickly under short days (4). The optimum temperature for the growth of soybean is between 25°C and 30°C. Temperatures below 21°C and above 32°C can reduce floral initiation and pod set (4). Soybean is very susceptible to frost damage and somewhat susceptible to excessive drought and extended flooding. For the development of a good crop, 7.6 mm of water a day is needed resulting in a minimal requirement of 500 mm water per season (4).

Cultivated soybean is a self-pollinating annual species. Seeds are the only structure of survival (5). The absence of dormancy causes a limited survival rate of seeds (6). Moreover, seeds are susceptible to climatic conditions (6). Due to a relatively large seed size, the dispersal of seeds is limited. Animal transportation is not encouraged by the morphological characteristics of the seedpod or seeds (5). The dispersal of seeds may occur by humans during transport, sowing or harvest (5). As soybean is a self-pollinating species, the dispersal of pollen is limited because the anthers mature in the bud and directly pollinate the stigma of the same flower (5). Insect-borne exportation of pollen is thus very limited. Soybean volunteers are rare and the cultivated soybean plant has never been found outside of cultivation (6). There are no wild relatives of soybean in Europe.

In 2004, soybean was grown commercially in 93 countries (4), with a total production of 206 million metric tonne of soybeans (7). The major producers of soybean are the

United States of America, Brazil, Argentina and China. Together, these countries are responsible for almost 90% of the total soybean production (7). About 60% of the total soybean production is genetically modified (8). Within the European Union, approximately 64% of all soybeans are grown in Italy. It should be noted that only non-gm soybean is grown in Europe.

Molecular aspects of the GM-plant

Soybean line 40-3-2 is genetically modified by means of particle acceleration. Plasmid vector PV-GMGT04 contains, amongst others, two copies of the *cp4 epsps* gene and one copy of the *uidA* gene. The *uidA* gene encodes for a β -D-glucuronidase (GUS) protein. The CP4 EPSPS protein confers tolerance to herbicides containing the active ingredient glyphosate.

An overview of the components of the vector PV-GMGT04 is given below:

- E35S, promoter derived from *Cauliflower mosaic virus* (CaMV);
- CTP4, The N-terminal chloroplast transit peptide sequence from the *Petunia hybrida epsps* gene (2 copies);
- *cp4 epsps*, gene derived from *Agrobacterium tumefaciens CP4*; encoding 5-enolpyruvylshikimate-3-phosphatesynthase (CP4 EPSPS) (2 copies);
- Nos 3', terminator from *A. tumefaciens* (2 copies);
- *nptII*, gene derived from *Escherichia coli* Tn5, encoding neomycin phosphotransferase;
- *ori-pUC*, bacterial origin of replication derived for *E. coli* pUC 119;
- P-MAS, promoter derived from *A. tumefaciens*;
- *uidA*, GUS coding sequence from *E. coli*;
- P-FMV, constitutive promoter derived from *Figwort mosaic virus* (FMV).

Properties of the introduced genes conferring herbicide tolerance

A functional *cp4 epsps* gene is present in soybean line 40-3-2. This gene encodes for a CP4 EPSPS protein possessing a high tolerance to glyphosate. EPSPS is a naturally occurring enzyme involved in the biosynthesis of aromatic amino acids. In non-transgenic soybean lines, glyphosate acts by binding to and inhibiting the function of naturally occurring EPSPS. Consequently, aromatic amino acids are no longer formed, leading to plant death. In contrast, CP4 EPSPS is not affected by glyphosate because of a reduced binding affinity. Because 40-3-2 expresses *cp4 epsps*, it has acquired a high tolerance to glyphosate (9).

EPSPS proteins are active in the chloroplasts of a plant cell. The sequence encoding the chloroplast transit peptide is fused to the *epsps* gene, resulting in the transport of the transgenic CP4 EPSPS protein to the chloroplast (10).

Molecular analysis

It was shown by hybridization analysis that soybean 40-3-2 contains a single functional DNA insert comprised of one single copy of the *cp4 epsps* gene cassette under control of a E35S promoter.

PCR analysis of the flanking regions of the insert showed that 354 base pairs (bp) of the promoter sequence are missing. These base pairs comprise a duplicated portion of the ES35 enhancer region. Odell *et al.* showed that this missing enhancer region is not likely to have a significant effect on the functionality of the promoter, since the region necessary for transcriptional initiation of the gene remains intact (11). COGEM shares this opinion. Immediately adjacent to the 3' region of the functional insert, a 250 bp segment of the *cp4 epsps* gene is located. Furthermore, a second insert comprising a 72 bp *cp4 epsps* DNA segment, was identified in an other part of the plant genome. Analysis of the 5' and 3' flanking regions of the 40-3-2 insert including the 250 bp segment, demonstrates that the flanking regions consist of (rearranged) soybean genomic DNA.

Southern blot and PCR analyses were performed in order to check the absence of PV-GMGT04 vector backbone sequences. PCR analysis showed that no complete origin of replication (ori-pUC) or *nptII* gene is present. However, presence of shorter ori-pUC or *nptII* fragments can not be completely ruled out. Southern blot analysis shows that no intact *uidA* gene or P-FMW promoter are present. In view of the above, COGEM is of the opinion that no functional vector backbone elements are present in the soybean line.

Bioinformatics analyses revealed that the flanking regions of the 250 bp *cp4 epsps* segment and the 72 bp segment do not contain known promoter elements, nor could a complete set of polyadenylation signals be observed. Because these elements are not found COGEM is of the opinion that transcription is unlikely. In addition, all putative open reading frames consisting of the DNA sequences containing and flanking the 72 and 250 bp segments, were examined on biological similarity with know toxins, allergens or other proteins. No similarity was found. Concluding, COGEM is of the opinion that the presence of the additional 250 bp and 75 bp fragments poses no risks to the environment.

Northern blot analyses disclosed the production of secondary transcripts which comprise a sequence flanking the 3' end of the primary functional insert. The largest transcript encompassed 7.4 kilo base pairs. COGEM endorses the applicants view of the 7.4 kb fragment being a read-through transcript of the *cp4 epsps* gene. Translation of this transcript produces probably only the CP4 EPSPS protein. Therefore, these findings do

not alter the aforementioned conclusions that 40-3-2 contains a single functional gene cassette.

In view of the above, COGEM is of the opinion that the molecular analysis of soybean 40-3-2, does not indicate that the cultivation of the soybean line poses a risk to the environment.

Advice

The present application concerns the cultivation of soybean 40-3-2. As stated above, soybean growth is very sensitive to temperature. A reasonably high temperature is required in all stages of development. The Dutch climate is not optimal for growing soybean. During the warmest months (May to September), the average temperature in the period of 2000-2006 is around 16 °C (12), while the optimum temperature for the growth of soybean is between 25 °C and 30 °C. Furthermore, because soybean is a short-day plant, development to maturity usually will take longer in countries like The Netherlands where the days are long during summer. In addition, in the growing season, the average rainfall in The Netherlands measures 150 mm while 500 mm is required (12). Moreover, the fact that periods with frost are common in The Netherlands, in combination with the absence of seed dormancy, makes it impossible for soybean to survive and to establish itself here.

Modern soybean cultivars do not possess any of the attributes commonly associated with problematic weeds and there are no reasons to assume that the genes inserted will increase the potential of the soybean to run wild. Furthermore, establishment of feral populations in soybean producing European countries has never been observed. In addition, no wild relatives of soybean are present in Europe and therefore outcrossing with wild relatives is not possible.

The molecular analysis of the soybean line gives no reason to assume that toxic or allergic proteins are being formed as a result of the modification. Furthermore the transgenic variety is cultivated in several countries in large acreages and no adverse health effects concerning handling and consuming of products and derivatives of this line have been reported.

Conclusion

In view of all aspects considered, COGEM is of the opinion that the cultivation of soybean line 40-3-2 poses negligible risks for the environment.

Additional remarks

As the application of soybean 40-3-2 regards cultivation of this plant, the possible effects on non-target organisms are also taken into consideration. Given the data provided by the

applicant, COGEM is of the opinion that the CP4 EPSPS protein is likely to be not toxic to non-target organisms. However, the experiments performed do not include dose-response curves. Consequently, it can not be stated with certainty that high doses of CP4 EPSPS protein are not toxic to non-target organisms. COGEM has pointed out this omission before (13). Recently, COGEM published a research report in which a method for the selection of non-target arthropods is described (14). Within her research program, COGEM has recently funded a follow up desk study in order to be able to propose laboratory experiments for the effects of GM-crops on non-target arthropods.

Assessing the agronomic effects occurring as a result of the application of the herbicide is not within the scope of COGEM. However, it is noteworthy that the effect of the application of the herbicide on non-target plants will be limited to field margins and verges since the herbicide will only be sprayed on the field. Moreover, due to the rapid degradation of glyphosate in the soil, it is not to be expected that any effects will persist for a long time.

The general surveillance plan is supplied by the applicant. General surveillance will be performed by selected networks or organizations and/or specific company monitoring programs. However, it is unclear how the results of the monitoring will be reported back to the applicant. Furthermore, the applicant makes a distinction between reporting direct and indirect effects in the monitoring plan. According to the applicant, direct effects will be reported annually and indirect effects only at the re-evaluation stage or at the end of a given consent. COGEM is of the opinion that the applicant should report both direct and indirect effects annually.

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