



Comments to the application under Regulation 1829/2003 for authorisation of 59122-maize in the European Union

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Summary

An application for the authorisation of the genetically modified (GM) insect resistant 59122 maize for food and feed uses, import and processing has been submitted by Pioneer Hi-Bred International and Mycogen Seeds to the EC. EFSA recently published a favourable opinion on the application. However, there remain many scientific uncertainties regarding the safety of 59122-maize for the environment, human and animal health.

- The studies undertaken by Pioneer/Dow and submitted to the EC show numerous significant differences between Herculex RW and comparable non-GE maize. This should be a cause for alarm and investigated further. The significant differences found include:
 1. Compositional differences in the content of maize and its kernels.
 2. Liver weights in females in a 42 day poultry study.
 3. Blood parameters in examined following a 90 day feeding trial with rats
- The findings in the parameters of short term feeding trials are of particular importance because these effects are noticed after only a short time. They could give an indication of toxicity in the longer term. Worryingly, the European Food Safety Authority (EFSA) dismiss these differences (as they did with MON863) claiming results are within historical or literature ranges or simply that they are “unlikely to be of any biological significance”.
- The environmental risk assessment is woefully inadequate. The applicant does not deliver any data on the fate of the Cry35Ab1 and Cry35Ab1 proteins fed to animals upon excretion. They could pose a risk to soil organisms and hence soil health.

As long as these uncertainties are not resolved, the precautionary principle should be applied and the import and use for food and feed of 59122-maize in the EU should be refused.

Introduction

A notification for authorisation of the genetically modified (GM) 59122-maize according to Regulation 1829/2003 was submitted by Pioneer Hi-Bred International and Mycogen Seeds (c/o Dow AgroSciences) to the Netherlands Competent Authority (NL-2005-12). On 2.April 2007, EFSA published a favourable opinion on the application. The scope of the application includes all food and feed uses and import and processing. Cultivation of 59122-maize is not included.

The transformation event 59122 contains three transgenes: (I) a 372 bp maize-optimised cry34Ab1 gene from *Bacillus thuringiensis* strain PS149B1; (II) a 1152 bp maize-optimised cry35Ab1 gene from *Bacillus thuringiensis* strain PS149B1; and (III) a 552 bp plant-optimised phosphinothricin acetyl-transferase gene (pat) from *Streptomyces viridochromogenes*. The proteins produced by the cry34Ab1 and cry35Ab1 genes confer together resistance against certain coleopteran insect pests. The protein produced by the pat gene confers resistance to the broad-spectrum herbicide glufosinate.

As discussed below, there remain many scientific uncertainties regarding the safety of 59122-maize for the environment, human and animal health.

Toxicology

Oral toxicity studies with the newly expressed protein

To demonstrate the safety of the newly expressed proteins Cry34Ab1 and Cry35Ab1 an oral toxicity study should be performed by the applicant. In the initial application the applicant provided the results of a two-week acute oral toxicity study in mice (Brooks & DeWildt 2000a, Brooks & DeWildt 2000b, Brooks & DeWildt 2000c). After request of the EFSA the applicant accomplished a repeated dose 28-day oral toxicity study in mice (EFSA 2007). In both studies no indications of adverse effects have been reported.

According to the applicant it was technically infeasible to obtain sufficient quantities of high purity Cry34Ab1 and Cry35Ab1 proteins from 59122-maize to perform an oral toxicity assessment (Gao et al. 2004). Therefore the Cry34Ab1 and Cry35Ab1 proteins used in the above mentioned toxicity studies were produced in recombinant *Pseudomonas fluorescens* strains MR1253 and MR1256 respectively. According to EFSA guidance for risk assessment, it is essential that a microbial-derived protein used for oral toxicity studies is equivalent to the newly expressed protein as it is expressed in the GM plant (EFSA 2006). In the case of Cry35Ab1 protein, this equivalence is questionable because the amino acid sequence of the microbial derived Cry35Ab1 protein differs slightly from the amino acid sequence of the Cry35Ab1 produced in 59122-maize. Due to some PCR-primers chosen in early research stage in the development of 59122-maize four alternate amino acid residues were introduced in the microbial derived Cry35Ab1 protein (Gao et al. 2004). Taking into account these amino acid changes, it remains unclear if equivalence of microbial derived test material to Cry35Ab1 protein from 59122-maize can be established by the data provided by Gao et al. (2000) and Schafer et al. (2003). These data include analysis of glycosylation, molecular weight, appearance of material in SDS-Page analysis, immunoreactivity, and MALDI-TOF fingerprints.

In addition, toxicological studies with isolated transgene products are of limited relevance because potential pleiotropic effects in the transgenic plant as well as differences in protein quality remain unexplored.

Subchronic feeding study with grains derived from 59122-maize

The applicant performed a 90-day feeding study in rats with grains from 59122-maize (Malley 2004). The statistical analysis provided by the applicant in the original study report was inadequate. Therefore EFSA requested a new analysis. This analysis revealed several statistically significant differences in haematological parameters between rats fed 59122-maize and rats fed the isogenic control line. The parameters in the 59122-maize group that were statistically significant compared to the isogenic control group were the terminal mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, red cell distribution width and absolute reticocyte values in males and terminal platelet count values in females (Malley et al. 2007).

EFSA do not consider the observed differences as toxicologically relevant (EFSA 2007). EFSA argue that the values were generally comparable with those of other control groups used in the study and/or fell within the ranges for the historical control means for rats of the same strain in other subchronic feeding studies. However, as has been shown in the MON863 maize, methods for comparing a broad set of different diets are usually not adequate for assessments of differences between a GM maize diet and a single comparable non-transgenic maize variety. In addition, it remains unclear whether the used historical control data fulfil all of the following criteria: that the historical control data were obtained with animals of the same species and strain and from the same breeder. The data were obtained in the same laboratory, the study design, experimental methods and assessment criteria were the same, and the studies used for the comparison were carried out within a limited time window.

Comparative assessment

The applicant carried out a comparison of the composition of 59122-maize grain and grain from a near isogenic non-transgenic control maize. The compositional data provided in the original application dossier were obtained from field trials carried out at six locations in Chile (Essner & Coats 2003), from field trials carried out at three locations in the USA (Buffington 2004) and from field trials at two locations in Canada (Buffington 2004). Based on the compositional data from these field trials the

applicant concludes, that 59122-maize grain is comparable to grain from the near-isogenic control maize. After request from EFSA the applicant submitted further compositional data, which were obtained from field trials carried out at three locations in Bulgaria during 2003 and 2004, and from field trials carried out at three locations in Spain during 2004.

EFSA used the data from field trials performed in Europe as the primary source for the comparative assessment of the composition of 59122-maize (EFSA 2007). The reason for focusing on these data remains unexplained. However, as 59122-maize will not be cultivated in the EU, 59122-maize consumed by humans and animals in Europe will be imported. To date, 59122-maize is allowed for commercial cultivation in the USA and in Canada (Agbios 2007). Compositional data from field trials in Bulgaria and Spain can be used as an indication, but they give no information about the composition of 59122-maize in the actual growing regions. Therefore, the focus for the compositional assessment should be on the data derived from field trials in the USA and Canada. It is remarkable, that in its guidance document for risk assessment EFSA argues, that the comparison between GM plants and the most appropriate comparator should cover multiple geographical locations representative of the various environments in which the GM plants is cultivated (ESFA 2006).

An analysis of the compositional data obtained from field trials in the USA and Canada shows, that these data are of limited value for risk assessment, mainly for two reasons. First, the field trials carried out for the production of the analysed material covered only one growing season. According to EFSA the field trials should cover more than one representative growing season (EFSA 2006). Second, not all of the compounds listed in the OECD consensus document on the comparative analysis of maize (OECD 2002) were analysed by the applicant.

Recently, Herman et al. (2007) published a further compositional analysis of 59122-maize based on plant material from field trials carried out in the USA and Canada over two growing seasons. The results of this analysis showed, that the mean carbohydrate level for 59122-maize forage is statistically lower than the level in the non-transgenic control (Herman et al. 2007). In addition, it was found, that location-specific analyte levels were outside of the values reported for conventional maize for 12 of the tested compounds (forage crude fiber, grain cystine, glycine, methionine, threonine, beta-carotene, vitamin B1, delta-tocopherol, gamma-tocopherol, inositol, raffinose and phytic acid). In all cases this variability was found for the non-transgenic control maize and in most cases for the 59122-maize also.

Taken together, based on the data provided by the applicant it cannot be claimed that 59122-maize is substantial equivalent to its non-transgenic parental lines.

Nutritional assessment

To evaluate the nutritional equivalence of 59122-maize grain and non-transgenic controls the applicant performed a 42-day poultry feeding study (Delaney & Smith 2004). The statistical analysis revealed that the liver weight in females fed with 59122-maize was significant higher than the liver weight from female fed the control diet. For Delaney & Smith (2004), the difference in liver weight is not of biological relevance because the observed values are still within the tolerance range calculated for this study. EFSA shares this opinion (EFSA 2007). After consideration of the multiplicity of the tests performed and the variability calculated from data relating to the non-transgenic control varieties, EFSA considers that the difference is unlikely to be of any biological difference. This is a matter of grave concern and demonstrates EFSA's inadequacy to deal with uncertainties in food and feed safety of GM crops.

Allergenicity

For assessing the allergenic potential of Cry34Ab1 and Cry35Ab1 proteins the applicant takes a weight-of-evidence approach, which includes (I) an assessment of the allergenicity potential of the source of the proteins, (II) a search for homology with known protein allergens, (III) in vitro simulated digestibility studies, (IV) an evaluation of glycosylation and (V) an assessment of heat stability. Based on the results obtained by this approach the applicant concludes that the Cry34Ab1 and Cry35Ab1 proteins do not pose any significant allergenic risk to humans. EFSA shares this opinion (EFSA 2007). Based on all information made available, EFSA considers that the newly expressed Cry34Ab1 and

Cry35Ab1 proteins are not likely to be allergenic. However, as will be shown below, there are several shortcomings in the assessment of the potential for allergenicity of Cry34Ab1 and Cry35Ab1 proteins.

Assessment of the allergenicity potential of *Bacillus thuringiensis*:

An important factor to consider in assessing allergenic potential is whether the source of the genes being introduced into plants is known to be allergenic. According to the applicant as well as to EFSA *Bacillus thuringiensis* is not commonly known to cause allergy including occupational allergy in workers using or producing *Bacillus thuringiensis* products. However, neither the applicant nor EFSA discuss recent studies, which indicate at least some allergenic potential of *Bacillus thuringiensis*. One of these studies has been performed by Bernstein et al. (1999). They discovered that migrant health workers developed positive skin tests and elevated specific IgE and IgG antibody levels to *Bacillus thuringiensis* spore extracts containing Cry1Aa and Cry1Ab proteins after respiratory exposure to crop spraying. These results are not proof that *Bacillus thuringiensis* or Cry proteins are allergens, but they are preliminary evidence, that *Bacillus thuringiensis* or Cry proteins may be allergenic. Doekes et al. (2004) also found some evidence for an allergenic potential of *Bacillus thuringiensis*. They performed a longitudinal respiratory health study with more than 300 greenhouse workers to determine the effect of using *Bacillus thuringiensis* products. The presence of IgE antibodies to Bt in the blood sera suggested some workers were being sensitized to the *Bacillus thuringiensis* products. The authors conclude that their results may be a reason for concern that frequent use of *Bacillus thuringiensis* pesticides is a risk factor for occupational IgE-mediated allergic sensitization. In a further study done by Vazquez-Padron et al. (2000) the immune response induced by Cry1Ac protoxin was examined. The data obtained showed that the Cry1AC protoxin is a potent immunogen. Vazquez-Padron et al. (2000) conclude, that the high immunogenicity of Cry1A proteins administered intragastric should be taken into account before releasing Cry-containing products for human use. Furthermore, the US EPA assessed the potential allergenicity of Cry9c protein and determined that Cry9c had a medium likelihood of being an allergen (EPA 2001).

Taken together, these studies are not proof that *Bacillus thuringiensis* or Cry proteins are allergens, but the results suggest that they could be. Therefore the assessment of the allergenic potential of 59122-maize should be done with great accuracy and not only should include indirect tests (as applied by the applicant) but also more direct tests.

Homology with known protein allergens:

One step in the allergenicity assessment is the use of bioinformatics to determine whether the sequence of amino acid residues of the newly introduced proteins is similar to known allergenic proteins. The applicant conducted a search for eight amino acid strings occurring in the CRY34Ab1 and Cry35Ab1 amino acid sequences that match strings in allergen databases (Song 2003). The selection of eight contiguous amino acids to identify matches in the databases is questionable. There are allergenic protein known, which have IgE epitopes shorter than eight amino acids (Becker 2001, Banerjee et al. 1999, Beezhold et al. 1999). Therefore FAO and WHO recommends the use of six contiguous amino acids to do the database search (FAO/WHO 2001). The applicant is aware of this recommendation, but emphasizes that the use of six amino acids would increase the chances for false positives (Song 2003). However, from a consumer safety point of view care should be taken not only to reduce false positives, but also to reduce false negatives. Moreover, a combination of methods have been proposed to reduce the false positive rate resulting from the use of six amino acids instead of eight (e.g. Kleter & Peijnenburg 2002). The six-amino-acids threshold reflects a precautionary approach, which should be taken by the applicant in his risk assessment.

Generally, it has to be said, that the searches for homology have their limitation, because they are limited to sequence analysis of known allergens that are available in the database (Kuiper & Kleter 2003).

Heat stability:

To test the heat stability Cry34Ab1 and Cry35Ab1 were incubated at 60, 75 and 90 for 30 minutes. After exposure to heat treatment the biological activity of the two proteins was measured in a bioassay with southern corn rootworm. The bioassay indicates that Cry34Ab1 and Cry35Ab1 are deactivated after exposure to heat, as shown by loss of biological activity. The heat stability tests were performed with the Cry34Ab1 and Cry35Ab1 proteins derived from recombinant *P. fluorescens*.

The data submitted by the applicant refer to loss of insecticidal activity as measured in a bioassay. The use of insecticidal activity as the parameter of heat stability is questionable, as it is implicitly assumed that insecticidal mode of action is relevant to allergenic potential, and that loss of insecticidal activity somehow correlates with loss to allergenic potential. This assumption is not consistent, because it is the size of the breakdown fragment not loss of insecticidal activity, which is of interest for potential allergenicity (Freese 2005). Loss of insecticidal activity could involve nothing more than (partial) denaturation, with little or no breakdown of the protein's primary structure (Freese 2005). WHO and FAO recommend methods to directly measure the size of fragments resulting from the heating process; they do not mention bioassays (FAO/WHO 2001).

In vitro simulated digestibility studies:

As part of the allergenicity assessment the digestibility of Cry34Ab1 and Cry35Ab1 proteins in simulated mammalian gastric fluid was investigated (Herman et al. 2003). The *in vitro* digestibility studies were performed with the Cry34Ab1 and Cry35Ab1 proteins derived from recombinant *P. fluorescens*. The two recombinant Cry-Proteins were rapidly degraded *in vitro*. However, it has to be stated, that the relevance of the gastric fluid assay to both *in vivo* digestion and allergenic potential remains uncertain and that the true predictive value of this assay is not understood (Herman et al. 2006). Therefore, there is no evidence available that the Cry34Ab1 and Cry35Ab1 proteins are degraded *in vivo*.

Taken together, based on the information given in the application dossier it cannot be excluded, that there is a potential allergenic risk of 59122-maize.

Environmental risk assessment

The scope of the application includes import and processing of 59122-maize as well as its use for food and feed. Cultivation is excluded. Therefore environmental exposure to 59122-maize will be mainly restricted to the following three routes: (1) accidental spillage of grain during loading/unloading vessels, trains or truck, during transport or during processing for food and feed uses; (2) sowing seed of conventional maize or of transgenic maize other than 59122 accidentally contaminated with the 59122-event during production; (3) manure and faeces from the gastrointestinal tract of animals fed on 59122-maize. Regarding potential effects on non-target organisms the third route deserves special attention.

Exposure through manure and faeces from animals fed on 59122-maize:

The potential distribution of Cry protein and fragments of Cry proteins on fields through the manure of animal fed on GM Bt-plants has been documented in the scientific literature (Lutz et al. 2005, Einspanier et al. 2004). Whereas the applicant does not address this exposure route at all, EFSA takes it into account in the risk assessment (EFSA 2007). However, the conclusions of the EFSA are rather an expert guess than a scientific assessment.

Citing data supplied by the applicant (Herman et al. 2003) and literature on other Cry proteins (Ahmad et al. 2005, Lutz et al. 2005), EFSA concludes that most Cry proteins are degraded in the gastrointestinal tract so that very low amounts of Cry proteins remain intact to pass out in faeces. This conclusion has to be contested, because it cannot be drawn from the work cited by EFSA. The cited work done by Herman et al. (2003) is studying the stability of microbial derived Cry34Ab1 and Cry35Ab1 in a gastric fluid assay and therefore has limited significance for two reasons. First, the study was done with microbial derived Cry34Ab1 and Cry35Ab1 proteins and not with Cry34Ab1 and Cry35Ab1 proteins embedded in plant tissue. Second, the relevance of the gastric fluid assay to *in vivo* digestion is uncertain (Herman et al. 2006). The work of Ahmad et al. (2005) cited by EFSA does not deal with the stability of Cry proteins in the gastrointestinal tract at all. In fact, Ahmad et al. (2005) addressed the stability of Cry3Bb1 protein in soil. In turn, Lutz et al. (2005) investigated the degradation of Cry1Ab in the bovine gastrointestinal tract and showed, that fragmented Cry1Ab protein can be found in faeces. The authors conclude, that a potential effect of fragmented Cry1Ab protein to organisms is unlikely but cannot be excluded (Lutz et al. 2005).

Scientific data on the stability of Cry proteins in the gastrointestinal tract of animals are scarce. The available data show that fragmented Cry proteins can be excreted by animals. Therefore, a potential distribution of Cry protein fragments on fields may be feasible considering the routine spreading of

manure in e.g. dairy farms. Whether this distribution will cause any effects on non-target organisms cannot be answered, due to the fact that data on potential biological effect of fragmented Cry proteins are virtually absent. However, it is possible that this poses risks to soil organisms and hence soil health, which have not yet been investigated.

Taken together, as the applicant does not deliver any data on the fate of the Cry35Ab1 and Cry35Ab1 proteins fed to animals upon excretion, any conclusion about the risk for non-target organisms remains highly speculative.

Monitoring and general surveillance

As it will be discussed below the plan for monitoring and general surveillance delivered by the applicant and accepted by EFSA has several shortcomings.

Post-market monitoring of food and feed

According to Art. 5 83) k) of EU-Regulation 1829/2003 a post-market monitoring plan regarding the use for human consumption should be added to the dossier. Based on its risk assessment, the applicant concludes that a post-market monitoring of food and feed products containing, consisting of or derived from 59122-maize is not necessary (Technical Dossier, p. 59). This opinion is shared by EFSA (ESFA 2007). However, as potential allergenicity of 59122-maize cannot be ruled out completely, it is essential that commercialized 59122-maize is monitored for the unintended occurrence of allergenic reactions. The applicant should deliver an adequate plan allowing an appropriate post-market monitoring.

Case-specific monitoring

Having identified no environmental risks the applicant concludes in the Technical Part of the application dossier, that case-specific monitoring is not applicable for the use of 5922-maize for all food and feed purposes and the import and processing of 59122-maize. This opinion is shared by EFSA (ESFA 2007).

In its risk assessment, EFSA considered the potential impact on non-target organisms resulting from indirect exposure to 59122-maize through manure and faeces (see above). Based on a small data set EFSA concluded that there is no risk for non-target organisms. As the case-specific monitoring has been installed for the validation of case-specific hypotheses of the risk assessment, the distribution and potential effects of Cry34Ab1 and Cry35Ab1 protein fragments on farmland should be addressed in a case-specific monitoring. The applicant should deliver an appropriate monitoring plan.

General surveillance

In the general surveillance plan provided by the applicant, it is stated that a monitoring system will be used by including all the operators involved in the handling and use of viable 59122-maize. In addition substantial accidental release of viable 59122-maize will be monitored for any potential adverse effects. Furthermore, the operators will be required to report to the applicant any unanticipated effects due to environmental exposure to 59122-maize. However, the general surveillance plan of the applicant has to be considered insufficient and should be supplemented with more details:

- Procedures should be specified for detection of 59122-maize unintentionally released in the environment;
- It has to be specified who will be involved in the general surveillance (which institutions are involved; who is collecting the information etc);
- The observatory measures should be specified;
- "Hot spots" for potential dispersion of viable 59122-maize should be determined, i.e. those points along the food and feed chain where viable 59122-maize is handled, stored or transported.

Conclusions

There remain many scientific uncertainties regarding the safety of GM insect resistant Bt 59122-maize for the environment, human and animal health including:

- In a 90 day rat study, many significant differences compared to the isogenic control group were noted including the terminal mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, red cell distribution width and absolute reticulocyte values in males and terminal platelet count values in females. Disturbingly, EFSA does not consider these observed differences as relevant
- In a 42-day poultry feeding study, statistical analyses revealed that the liver weight in females fed with 59122-maize was significant higher than the liver weight from female fed the control diet. Again, EFSA considers that the difference is unlikely to be of any biological difference.
- The data submitted on the potential allergenicity of Bt proteins ignores recent studies that suggest these proteins can be allergenic. From the data in the dossier, it cannot be excluded that there is a potential allergenic risk of 59122-maize.
- The environmental risk assessment is woefully inadequate. The applicant does not deliver any data on the fate of the Cry35Ab1 and Cry35Ab1 proteins fed to animals upon excretion. Therefore, any conclusion about the risk for non-target organisms remains highly speculative.
- The plan for monitoring and general surveillance delivered by the applicant and accepted by EFSA has several shortcomings. It should include monitoring for unintended allergenicity and impacts on non target organism resulting from indirect exposure from the Bt proteins in faeces. General surveillance is too vague.

Importantly, the significant differences found in the feeding trails were only notices after a short time. This raises concerns over the long term exposure of animals to 59122. Worryingly, EFSA simply dismisses these differences as “unlikely to be of any biological significance”. This is no basis upon which to assess food and feed safety and demonstrates the inadequacy of EFSA to handle warning signs and uncertainties over food and feed safety of GM crops.

As long as these uncertainties are not resolved, the precautionary principle should be applied and the import and use for food and feed of 59122-maize in the EU should be refused. Additionally, there should be an immediate suspension of the current authorisation procedure for GMOs, pending a serious review of the risk assessment and risk management measures performed by EFSA and the European Commission.

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