

**SZENT ISTVÁN UNIVERSITY**

**DEVELOPMENT OF IMMUNANALYTICAL METHODS FOR  
DETECTION OF *BACILLUS THURINGIENSIS* ENDOTOXIN(S)**

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## PhD School

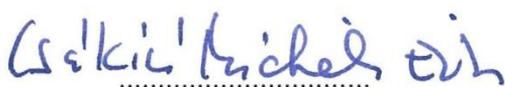
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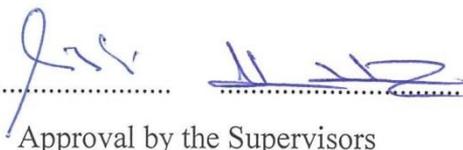
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## 1. SCIENTIFIC BACKGROUND AND AIMS

*Bt* insecticides containing endotoxins of *Bacillus thuringiensis* (*Bt*) represent one of the most frequently used biological pesticide classes, and their efficacy is well documented in Hungary (DARVAS *et al.* 1979). Beside the orally effective *Bt* insecticides genetically modified (GM) plants producing Cry toxins have been developed by agricultural biotechnology. Benefits of *Bt* plants are based on their specific effect; their applicability against different insect orders is facilitated by the immense number of *cry* genes appearing in nature and the diversity of the proteins being expressed. Cry toxins being produced continually in the cells of *Bt* plants provide permanent protection against target pests and are not being exposed to unfavourable weather conditions. However continuous Cry toxin production means constant environmental load, therefore, the *Bt* plant as a plant protection technology doesn't fulfil the basic principle of integrated pest management. Claims that application of *Bt* plants prevents the use of wide spectrum insecticides is mentioned as an advantage, however, this cannot be confirmed in Hungary for GM crops resistant to the European corn borer, because the pest occurs in population densities substantial for damage only once or twice in every decade, thus farmers do not often take protection steps against them (DARVAS *et al.* 2007). For the same reason the 0–5% increase in crop yield is not an internal advantage of the variety comparing to the isogenic control, because yield depends on damage by the European corn borer (BETZ *et al.* 2000, FÜSTI 2007).

Possible gene escape is an important problem in GM plant applications, where the pollen of the modified plant fertilizes flowers of other species (intraspecific hybridization) or congener species (interspecific hybridization) (HESZKY 2007). A further problem is that due to constitutive promoter Cry toxin is produced in plant organs, where it is not required from the plant protection aspect. For example *MON 810* maize produces substantial amounts of Cry1Ab toxin even in its roots, and 1–8% of the toxin content produced in the plant can be determined one year upon sowing in the stubble (SZÉKÁCS and DARVAS 2007).

Non-target organisms may come in contact with toxins of *Bt* plants during their food consumption. Phytophagous organisms may become exposed to effects of Cry toxins due to consumption of *Bt* plants or pollen drifting and polluting their host plant; predators and parasitoids due to prey or host consuming Cry toxin; decomposers due to plant material residues; pollinators due to visiting flowers; symbionts due to their mutual connections (DARVAS and LÖVEI 2006). An outstanding problem is the exposition of non-target species belonging to the same taxonomic group as the target pest. Evolvement of possible Cry toxin resistance and cross-resistance appear also as a problem that may developed for *MON 810* maize as early as the tenth generation in *Plodia interpunctella* (Hübner) as a model organism under laboratory circumstances (DARVAS and LAUBER 2007). For management of cross-resistance 20–50% border rows of isogenic line that maintain the sensitive pest population are recommended by seed companies.

GM plants being present on the market as food and feed have been authorized based on the so-called substantial equivalence principle. Skepsis in food safety of GM plants has been established by the experiments of Árpád Pusztai and co-workers, where lack of growth, disturbance in the immune system and malformations in several organ tissues were observed in rat consuming GM potatoes containing a snowdrop lectin gene (EWEN and PUSZTAI 1999, PUSZTAI *et al.* 2003). In application of every plant protection technology, availability of analytical methods with proper sensitivity for determination of the active ingredients is essential. Cry toxins are important active ingredients both for environmentally friendly plant protection and for agricultural biotechnology. Determination of Cry toxins is a difficulty in residue analysis due to the protein characteristic and degradation features of these analytes. In investigation of plant protection technologies safety assessment of distribution and environmental fate of the active ingredients is essential. For Cry toxin determination enzyme-linked immunosorbent assays (ELISAs) are the widespread method of choice.

The subject topics of my PhD work were investigation of ELISA systems feasible for determination of Cry1Ab and Cry34/35Ab1 toxins

produced by *MON-ØØ81Ø-6* (hereafter *MON 810*) and *DAS-59122-7* (hereafter *DAS-59122*) *Bt* maize varieties, respectively, and of Cry4 toxin applied in larval mosquito control. The aims of my PhD work were the following:

- analytical comparison (calibration, reliability, reproducibility) of commercial EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and QualiPlate, Abraxis Bt-Cry1Ab/Ac ELISA and Agdia Bt-Cry1Ab/1Ac ELISA systems appropriate for Cry1Ab toxin determination,
- quantification in an interlaboratory study of the high standard deviation seen in the scientific literature for Cry1Ab concentration determination originated from the application of ELISA technique as widespread applied analytical method,
- investigation of the applicability of commercial ELISA systems (kits) in Cry1Ab concentration determination in plant and animal tissues by description of matrix effects,
- development of ELISA system for Cry4 toxin determination,
- determination of environmental factors that influence the Cry1Ab production of GM maize carrying *MON 810* genetic event,
- environmental risk assessment of GM maize carrying *DAS-59122* genetic event on larval development, full life cycle and reproductive parameters of the seven spotted ladybird (*Coccinella septempunctata*).

## 2. MATERIAL AND METHODS

In the scope of our investigation regarding the calibration of commercial ELISA systems, linear calibration recommended by the manufacturers of the quantitative Abraxis Bt-Cry1Ab/Ac ELISA *kit* (#PN 51001, Warminster, PA, USA)<sup>1</sup> and the EnviroLogix Cry1Ab/Ac QuantiPlate (#AP 003; Portland, ME, USA)<sup>2</sup> were compared to four parametric sigmoid calibration typical for sandwich ELISA systems. In addition, sigmoid calibration — obtained with standard solution from Abraxis Inc. — of Agdia Bt-Cry1Ab/1Ac ELISA *kit* (#PSP 06200, Agdia Inc., Elkhart, IN, USA)<sup>3</sup> allowing qualitative determination was compared to the same parameters of the Abraxis and EnviroLogix ELISA methods. In the case of the Abraxis kit sigmoid calibration curves were obtained in the concentration range of 0–50 ng/ml with Cry1Ab standard solutions from three origins (Abraxis Inc.; Szent István University – Sándor Szoboszlay; National Research Council of Canada – Luke Masson). The linear calibration curve provided by the kit allows analytical determination in a lower, but much more narrow concentration range (0–4 ng/ml) than the sigmoid calibration curve. After normalization of sigmoid and linear calibration curve values, their reproducibilities were investigated and limits of detection (LOD) were determined.

Sigmoid calibration curves in the Agdia Bt-Cry1Ab/1Ac ELISA kit and the EnviroLogix Cry1Ab/Ac QuantiPlate were obtained also in a concentration range of 0–50 ng/ml with Cry1Ab standard from Abraxis Inc. Average sigmoid regression from 3 individual measurements of both kits were compared to the sigmoid curve in the Abraxis Bt-Cry1Ab/Ac ELISA kit obtained with the same toxin standard.

Internal quality control of 0.5, 2.5 and 5 ng/ml standard solutions of the EnviroLogix Cry1Ab/Cry1Ac QuantiPlate withdrawn from the market in 2005 (SZÉKÁCS *et al.* 2010), and of positive and negative controls of the

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<sup>1</sup> <http://www.abraxiskits.com/moreinfo/PN510001USER.pdf>

<sup>2</sup> <https://www.yumpu.com/en/document/view/18625470/quantiplatetm-kit-for-cry1ab-cry1ac-envirologix>

<sup>3</sup> <https://orders.agdia.com/Documents/m172.pdf>

EnviroLogix Cry1Ab/Cry1Ac QualiPlate being on market at present was investigated by Shewhart Control Charts. Reproducibility of the positive and negative controls of the Cry1Ab/Cry1Ac QualiPlate on one plate was assessed by Control Charts of the Range of Duplicates.

Applicability of various ELISA systems suitable for Cry1Ab determination in *MON 810* GM maize was assessed in the frame of an international cooperation. The aim of the round robin study was to assess, to what extent the high variability of Cry1Ab concentrations reported in the scientific literature is explained by differences in the various ELISA methods applied in toxin concentration determination. Cry1Ab content was determined in standard plant samples by the Bt-Cry1Ab-1Ac ELISA kit (#PSP06200, Agdia Inc., Elkhart, IN, USA) in a joint protocol and in own protocols in different laboratories. Results of analytical measurements from were statistically analysed based on the ISO 5725-2 standard. (ISO 1994).

Possible matrix effects in maize leaf were determined in two separate studies. The sigmoid calibration curve with Abraxis Cry1Ab toxin was obtained in leaf matrix of DK 440 isogenic maize line and the corresponding IC<sub>50</sub> value was compared to the same parameter of the sigmoid curve obtained in buffer solution. LOD was also determined in the leaf tissue. Matrix effects were monitored by the standard addition method in the frame of the same study series (KEBEKKUS and MITRA 1998). Effects of *MON 810* (PR34N44) GM maize and its near isogenic maize line (PR34N43) were investigated in feeding studies on swine by the Department of Biology of the Central Environmental and Food Research Institute (currently research institutes of the National Agricultural Research and Innovation Centre) within an EU Research Framework Programme (FP7/2007-2013). Based on the analytical results of the study we determined LODs for all swine organ tissues and assessed analytical goodness of the detection.

For development of a sandwich ELISA system, Cry4 protein and Cry4 specific antiserum were provided by the Laboratory of Microbial Ecology, New York University (New York, NY, USA) and EnviroLogix Inc. (Portland, ME, USA), respectively. Cry4 proteins were a mixture of Cry4A and Cry4B,

purified from *B. thuringiensis* var. *israelensis* cultures isolated from TEKNAR Flowable Formulation by Abbott Laboratories (Chicago, IL, USA). Conjugation of antibodies to horseradish peroxidase was carried out by glutaraldehyde and periodate methods (HARLOW and LANE 1988). The ELISA system was devised based on the principle of a direct, sandwich type immunoassay (TIJSSEN 1985). Analytical standard curves were obtained with formulated VECTOBAC WDG (granulate) and VECTOBAC 12AS (suspension) preparations and were compared to the sigmoid standard curve obtained with pure Cry4 toxin. LODs were determined for both formulations.

Cry1Ab toxin production of *MON 810* GM maize was investigated at various leaf levels and within a given leaf level. Effects of necrosis, of nitrogen-phosphor-potassium mixed fertilizer, of cultural circumstances and of soil type (FLORIMO general potting soil and brown forest soil with clay) on Cry1Ab production were also assessed.

Environmental risk assessment of *DAS-59122* was implemented by sponsorship from Pioneer Hi-Bred International Inc. (Ankeny, IA USA). Development time of the seven-spotted ladybird (*Coccinella septempunctata*) preying the bird cherry-oat aphid (*Rhopalosiphum padi*) or pea aphid (*Acyrtosiphon pisum*) at three different temperatures and prey consumption of certain larval stages were determined in preliminary experiments. A subsequent open field study was performed to investigate the effects of *DAS-59122* GM maize on L1 and L2 larval stages of the seven-spotted ladybird and on the full life cycle of the non-target organism, where mortality, development time, imago weight, fertility and fecundity parameters were compared to control treatments.

### 3. RESULTS

The objective of my PhD work was to investigate the analytical reliability of different commercial ELISA systems feasible for Cry1Ab toxin determination. On the basis of ELISA method assessment, it has been pointed out that manufacturer supplied linear calibration of the EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and QualiPlate, and the Abraxis Bt-Cry1Ab/Ac ELISA is located on the lower curvature of the sigmoid calibration curve typical for ELISA systems, where reliability and reproducibility of the determination is not optimal. Linear calibration is well-reproducible during determinations on the basis of the regression coefficients, but relative standard deviations are higher near the low LOD than at the IC<sub>50</sub> level, which affects – with the high dilution rate considered – the precision of the toxin content determination in the plant samples as well. Internal quality control of the EnviroLogix Cry1Ab/Cry1Ac QuantiPlate and QualiPlate has confirmed the uncertainty of the measurement on the lower curvature of the sigmoid calibration. For the 0.5 ng/ml standard solution the calculated concentration values were  $0.572 \pm 0.106$  ng/ml, corresponding to an 18.5% relative standard deviation. Relative standard deviations in individual measurements ranged between 0.0% and 141.4% and between -1327.0% and 595.4% at the level of optical density (OD) values detected and concentrations calculated, respectively. On the Shewhart Control Chart, 3 points have fallen out of the warning limits and 1 point out of the control limits, reflecting that the analytical determination in the lower Cry1Ab concentration is not under statistical control. The same phenomenon occurred for the negative control of the EnviroLogix QualiPlate that may lead to false negative results in Cry1Ab determination.

In the scope of an international inter-laboratory ring trial test it was established that the same ELISA method (joint protocol) led to 15.5–31.6% relative standard deviation, making the comparability of the results from different laboratories questionable. Average Cry1Ab toxin content determined in the maize samples in the laboratories by their own protocols (differing from the joint protocol in sample preparation and the setup of the ELISA system)

were in the -66.5%–160.1% range compared to the average values determined in the same laboratory with the joint protocol.

Matrix effects were determined in plant (maize) and animal (porcine) tissues in the evaluation of the analytical applicability of commercial ELISA kits. No matrix effect occurred in Cry1Ab determination in maize leaf, however, among porcine tissues, the limit of detection in muscle tissue, determined in three independent measurements, was quantified to be  $7.95 \pm 7.99$  ng/ml. This extremely high standard deviation indicates that this tissue is a complex matrix in Cry1Ab determination, which may cause difficulties in a food safety context.

My PhD work also included the development of an ELISA for quantitative determination Cry4 toxin applied in larval mosquito control. In a direct, sandwich type immunoassay; a conjugate of the analyte-specific antibody to a reporter enzyme is a component of the immunocomplex of major importance. Cry4-specific antibodies were conjugated to horseradish peroxidase (HRP) by two methods: a condensation reaction using glutaraldehyde and an oxidative process using sodium periodate. The enzymatic activity of HRP was retained during the conjugation process in both cases. Conjugation by periodate coupling was found to be stable, thus, it was successfully applied in the sandwich ELISA system. Parameters of the optimal method were: coating Cry4-specific antibody coating dilution 1:500, antibody–HRP conjugate dilution 1:200. Standard curves were obtained with analytical standard of Cry4 toxin, granulated toxin formulation (VECTOBAC WDG) and suspension toxin formulation (VECTOBAC 12 AS) in water. The LOD was found to be 2 ng/ml for pure Cry4 toxin, practical LODs were 170 ng/ml and 900 ng/ml for *Bti* preparations VECTOBAC WDG granulate and VECTOBAC 12 AS suspension, respectively.

Investigating Cry1Ab production in *MON 810* maize we determined toxin distribution among leaf levels and within a single leaf. Average Cry1Ab toxin content in the leaves of *MON 810* maize cultivar DK-440 BTY ranged between 4821 and 10054 ng toxin/g fresh leaf weight. The lowest Cry1Ab toxin content ( $4821 \pm 1042$  ng Cry1Ab toxin/g) determined at the lowest leaf level

differed significantly from all other leaf levels. As for longitudinal toxin distribution within a single leaf, the highest toxin concentration ( $8924 \pm 1507$  ng Cry1Ab toxin/g fresh leaf weight) was measured in the middle section of the leaf, significantly decreasing towards the leaf tip ( $4579 \pm 1864$  ng/g) and the sheath ( $1892 \pm 223$  ng Cry1Ab toxin/g fresh leaf weight). As for diagonal toxin distribution within a single leaf, the highest toxin concentration ( $9885 \pm 877$  ng/g) was determined near the leaf vein and the lowest ( $8194 \pm 480$  ng/g) at the leaf edge. Actual Cry1Ab content in leaf tissue is strongly affected by necrotisation: 68% and 28% of fresh green leaf Cry1Ab content was determined in yellow (half-necrotized) and brown (necrotized) leaf tissues, respectively.

Effects of cultivation conditions and soil type were determined on Cry1Ab production of two *MON 810* varieties. Cry1Ab production in plants grown in the greenhouse was lower than that under plants cultivated under open field conditions. Moreover, further differences were detected in the toxin production of the two *MON 810* maize varieties as well. Despite of the same genetic event integrated in their genome, Cry1Ab production levels determined were not equal in the tissue samples. Application of nitrogen-phosphor-potassium fertilizer did not cause higher Cry1Ab concentration in the *MON 810* maize leaf tissue, however a nearly 1.5-fold increase in the biomass of the GM and near isogenic lines was detected, thus, Cry1Ab production per hectare was also higher in case of fertilizer application.

Environmental risk assessment of *DAS-59122* GM maize was performed on the L1 and L2 larval development and entire life cycle of the seven-spotted ladybird (*Coccinella septempunctata*) with the bird cherry-oat aphid (*Rhopalosiphum padi*) as the herbivore species in a tritrophic system and the pea aphid (*Acyrtosiphon pisum*) in colony maintaining. Larval development time was determined at three different temperatures on the two aphid prey species (*R. padi* and *A. pisum*). Development was the most intensive at 30°C,  $9.17 \pm 0.41$  days until adult emergence for consumption of *A. pisum* and  $11.00 \pm 0.63$  days for *R. padi*. There were no significant differences in adult body weights when the predator was raised on these two aphid species. Prey

consumption at larval stages (L1-L4) was also determined for both aphid species; results for *R. padi* were applied in environmental risk assessment.

No significant differences in survival and development of L1 and L2 *C. septempunctata* at different leaf levels of the isogenic line (PR36D79) and *DAS-59122* GM maize. Three further negative control hybrids (PR36V52, PR37N01, PR37M34) were also used in the full life cycle study. Body weights of ladybird imagos from larvae reared on previously infected maize plants under whole plant isolator were determined. The weight of adult females occurred to be the lowest in the *DAS-59122* and PR36V52 treatment ( $45.28 \pm 6.18$  mg and  $47.07 \pm 6.34$  mg, respectively), while the highest values were determined for the isogenic line (PR36D79) and PR37N01 treatment groups ( $52.61 \pm 3.75$  mg and  $51.90 \pm 4.38$  mg, respectively). The weights of adult males were the lowest also in the *DAS-59122* maize treatment group ( $36.74 \pm 5.42$  mg), representing a significant decrease relative to the near isogenic ( $43.76 \pm 4.50$  mg), PR37M34 ( $40.54 \pm 4.85$  mg), PR36V52 ( $42.94 \pm 3.14$ ) and PR37N01 ( $41.98 \pm 5.20$  mg) hybrid maize lines. Investigations of the reproduction parameters (fertility and fecundity) showed that egg batch sizes ranged between 5 and 80, average size of eggs batches were 25 eggs for *DAS-59122*, 33 for the near isogenic line, and 25–33 for the three conventional hybrid maize lines. At an average, 17–30 L1 larvae hatched from the eggs. Average fertility rates among treatments on maize lines differed from 58–77% with no statistically significant differences.

#### 4. NEW SCIENTIFIC RESULTS

1. Thesis: Linear calibration of the Abraxis Bt-Cry1Ab/Ac ELISA kit is situated on the lower curvature of four parametric sigmoid curve characteristic to sandwich ELISA systems, where reliability, accuracy and reproducibility of the analytical measurement is worse compared to the neighbourhood of the inflexion point. Linear calibration among independent measurements are well reproducible, however the applied 0.125 ng/ml limit of detection instead of 5 ng/ml decrease the precision and increase the accuracy of measurement.
2. Thesis: Performing internal quality control for positive and negative control of EnviroLogix Cry1Ab/Cry1Ac QualiPlate calculated value for positive control was  $1.45 \pm 0.22$  ng/ml, among individual measurements relative standard deviations were 0.0–28.8% at the level of calculated concentrations. The positive control was proved as reliable and precise reference point based on the Shewhart Control Chart. The calculated value of the negative control (nominal value: 0.00 ng/ml) was  $0.0009 \pm 0.0785$  ng/ml. Based on the Control Chart, the negative control is not considered as an appropriate reference point.
3. Thesis: In a round robin study 15.5–31.6% relative standard deviations were determined due to application of a standardized ELISA system in Cry1Ab content determination of *MON 810* leaf samples. Results of own protocols in certain laboratories were -66.5%–160.1% of the average value determined by the joint protocol.
4. Thesis: Limit of detection values were determined in heart, lymph node, brain, muscle, liver, spleen and placenta porcine tissues, as well as in serum and colostrum by the EnviroLogix Cry1Ab/Cry1Ac QualiPlate ELISA system validated with Cry1Ab standard for quantitative determination.
5. Thesis: A direct sandwich ELISA system was developed for determination of Cry4 toxin. Parameters of the optimized system were 1:500 dilution rate of coating Cry4 specific antibody and 1:100 dilution

rate of antibody-HRP conjugate. The limits of detection for the toxin were 2 ng/ml, 170 ng/ml and 900 ng/ml for pure Cry4, for VECTOBAC WDG granulate and for VECTOBAC 12 AS suspension, respectively.

6. Thesis: Distribution of Cry1Ab toxin produced by *MON 810* GM maize among leaf levels and in within a given leaf level. Effects of necrosis, of potting soil and brown forest soil with clay, of cultivation circumstances on Cry1Ab production were also assessed. No significant differences were observed in Cry1Ab concentration after application of nitrogen-phosphor-potassium mixed fertilizer, however a 1.5-fold increase in biomass of the GM and near isogenic maize lines was observed, thus toxin dosage per hectare appeared to be significantly higher with fertilizer application.
7. Thesis No significant differences were noticed in survival of L1 and L2 larva of seven-spotted ladybird (*Coccinella septempunctata*) in a tritrophic system compared to the near isogenic line (PR36D79). Lowest body weights were observed in *DAS-59122* treatment for male adults ( $36.74 \pm 5.42$  mg), which difference was significant compared to values determined on near isogenic, PR37M34, PR36V52 and PR37N01 hybrids. No significant differences were detected for reproduction parameters.

## 5. CONCLUSIONS AND SUGGESTIONS

Concentration determination is the most reproducible, the most reliable and the most accurate on the quasi-linear part, thus, at the inflexion point ( $IC_{50}$ ) of the four-parametric sigmoid calibration curve typical for sandwich ELISA systems. Linear calibration of the commercial ELISA systems investigated provided quantitative determination in the concentration range of 0–4 ng/ml and 0–5 ng/ml. Regarding this analytical consideration, examination of reproducibility and precision of linear calibration is essential. Linear regression is well reproducible among independent measurements; however, it is situated on the lower curvature of the sigmoid curve. This analytical consideration has been confirmed in case of the EnviroLogix Cry1Ab/Cry1Ac QualiPlate by internal quality control of positive and negative controls and calibration points of 0.5, 2.5 and 5 ng/ml concentrations of a standard purchased from EnviroLogix Inc. For the negative control (nominated concentration is 0 ng/ml) and for the 0.5 ng/ml standard solution the ELISA system was found not to be under statistical control. For higher concentrations no outlier points were detected in quality control as they are located on the lower part of quasi linear section of sigmoid curve. Extremely high relative standard deviations of lower concentrations may modify the slope of linear regression among independent measurements, and therefore, the results of determination as well. The negative control of the commercial EnviroLogix ELISA kit for qualitative determination also has not been proven to be an appropriate reference point. This can occur as a problem in environmental and food safety issues, where a plant or food product found to be GMO-free may still contain Cry toxin. Referring to food safety, a further difficulty is that the LOD for porcine muscle tissue was  $7.95 \pm 7.99$  ng/ml determined by three independent measurements. The 100% relative standard deviation indicates that the muscle tissue is a complex matrix in Cry1Ab determination. Occurring matrix effects can be reduced by sufficient dilution of samples, however, in case of feed containing Cry toxin, the expected Cry toxin concentration in muscle tissue is low, thus, sample dilution does not

appear feasible as a solution. As for plant samples, no matrix effect is expected in examination of leaf tissue with a 1:10 dilution rate.

Results of the round robin experiment highlight the importance of standardized ELISA protocols resulting in comparability of data of environmental studies in various laboratories. The results also show that even in case of a standardized protocol significant differences may occur in determination of Cry1Ab concentration, however, variance in data is far below than that in comparisons of results obtained with different ELISA systems. Relative standard deviations of 15.5–31.6% causing significant differences observed in this study are lower than in another systematic round robin experiment (NGUYEN *et al.* 2008). Plant-to-plant variation in Cry1Ab concentration of *MON 810* GM plant was of similar level, indicating that natural diversity also occurs in toxin production in GM plants (THEN and LORCH 2008). In our study, the same ELISA kit, the same protocol and the same analytical standard solutions were applied in the measurements. Observed relative standard deviations can be further reduced by standardization of remaining differences (sample preparation, microplate reader, software). Automatization of the sample preparation and the measurement processes (automatic extraction apparatus, ELISA robot) would evidently decrease standard deviations determined in our study, since human error source can be excluded, like uneven sample preparation by mortar and pestle as well. Concentrations determined in our study by our own ELISA protocols, occasionally significantly differing from those obtained in the joint protocol, draw attention to limitations of the commercial ELISA kit application (calibration, toxin-prototoxin cross-reactivity) and to accurate and deliberate presentation of results determined by these systems.

Investigation of *MON 810* GM maize toxin production resulted in significantly different Cry1Ab concentrations in the same organ of different *MON 810* species, in a given leaf of a given maize species, further on different soil types, under different cultivar circumstances (greenhouse and open field) and by fertilizer application. Enhanced differences in Cry1Ab determination may occur by parallel presence of influent factors. Differences among studies

carried out by various laboratories — beside errors originated from the analytical determinations — appear due to these factors, thus, comparison of results becomes impossible. Several factors, for example soil type or climatic conditions cannot be eliminated; however, results will be appropriate for comparison by a systematic and well-designed sampling procedure. All these considerations justify the importance that case studies in the course of authorization processes of GM plants have to include related varieties, designation of non-target species and the given biogeographical region. At present EU member states may ban cultivation of certain GM plants in their territory, thus, member states are entitled to make decisions at national level regarding restriction or ban of cultivation of GMOs authorized at EU level (EUROPEAN PARLIAMENT AND COUNCIL 2015).

LOD values of a direct sandwich ELISA system developed for determination of Cry4 toxin were found to be 2 ng/ml, 170 ng/ml and 900 ng/ml for pure Cry4 toxin, VECTOBAC WDG granulate and VECTOBAC 12 AS suspension formulations containing Cry4 toxin, respectively. The importance of the ELISA system developed is that there are currently no suitable commercial ELISA methods to detect Cry4 toxin. The LOD values of our developed method for formulations are considerably high; therefore, monitoring of degradation rates at applied dosages under the LOD may be performed by mosquito larval tests (FEJES 2015).

Effects of *DAS-59122* GM maize were investigated on L1 and L2 larval stages and on the full life cycle of the seven-spotted ladybird (*Coccinella septempunctata*) in a tritrophic system. The phytophagous organism in our study was the bird cherry-oat aphid (*Rhopalosiphum padi*) frequently infecting maize. As *Bt* insecticides exert their effects on early larval stages of the target pests, assessment of early larval stages of non-target species is of primary importance. No mortality of L1 and L2 larvae during development was determined on GM and near isogenic maize lines. There occurred no acute effect of maize carrying the *DAS-59122* genetic event on larvae of non-target species. In the full life cycle study larvae from their early life stages through imago stage to eggs laying consumed aphids infecting the given maize species.

Beside the near isogenic line, 3 further conventional maize hybrids were also applied as controls. No differences were observed in the number and sex ratio of imagos developed on the 5 maize species investigated, however, adult weights of males were significantly lower compared to the 4 control maize lines. For reproduction parameters there were no differences among maize lines. The aim of environmental risk assessment is to compare GM and non-GM species, where a basic tool is the comparison of the GM variety to its near isogenic line. The principle of substantial equivalence — considered as a basic assumption in such studies — supposes that such a biotechnological process cannot cause alteration in the genome that would cardinaly change the quality of the species. Studies on GM and isogenic maize lines have quickly denied this supposition, as significant differences in composition or biological effects were confirmed. EFSA has introduced the concept of „comparative risk assessment” into the explanation of environmental risk assessment that involves other species „proven safe as food product” beside the near isogenic line. During the evaluation, differences among the isogenic line and commercial hybrids are built in the negative control, thus, the deviation of the background increases that narrows the circle of significant differences between the GMO and the background (SZÉKÁCS and DARVAS 2012). During investigating effects of *DAS-59122* GM maize on the full life cycle of the seven-spotted ladybird definitely decreased body weights were observed for male imagos, and the difference appeared significant even compared to the four negative controls, indicating that the difference is not a statistically random effect. The results pertain to the seven-spotted ladybird, their generalization and expansion to other ladybug species is possible only on the basis of further studies.

The results of my PhD work draw attention to the limitations of the currently available ELISA systems for Cry1Ab determination in *MON 810* GM maize. Analytical, biological and other factors also play a role in the high differences in Cry1Ab concentration published in the scientific literature. Comparability of the results by different research groups can be greatly improved by considering these factors, reduction of which to minimal levels is being advised.

## 6. REFERENCES

- DARVAS, B., LAUBER, É. (2007): A Cry1-toxinrezisztenciáról. 64-66. p. In DARVAS B. (Szerk.): *Mezőgazdasági géntechnológia - elsőgenerációs GM-növények*. Budapest: Magyar Országgyűlés Mezőgazdasági Bizottsága, 167 p.
- DARVAS, B., LAUBER, É., BAKONYI, G., BÉKÉSI, L., SZÉKÁCS, A., PAPP, L. (2007): A MON 810-es GM-kukoricák környezettudományi megítélése. *Magyar Tudomány*, 168, 1047-1056. p.
- DARVAS, B., LÖVEI, G. (2006): A genetikailag módosított szervezetek környezeti hatásai. 320-326. p. In: DARVAS B., SZÉKÁCS A. (Szerk.): *Mezőgazdasági ökotoxikológia*. Budapest: L'Harmattan Kiadó, 382 p.
- DARVAS, B., SEPRŐS, I., SZÁNTÓ, J. (1979): Környezetkímélő növényvédelmi eljárások rovarok és atkák ellen. I. Biológiai védekezés: entomopatogén baktériumok, entomofág állatok. Agroiinform Kiadó, Budapest. 53 p.
- BETZ, F.S., HAMMOND, B.G., FUCHS, R.L. (2000): Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. *Regulatory Toxicology and Pharmacology*, 32, 156-173. p.
- EUROPEAN PARLIAMENT AND COUNCIL (2015): Directive (EU) 2015/412 of the European Parliament and of the Council of 11 March 2015 amending Directive 2001/18/EC as regards the possibility for the Member States to restrict or prohibit the cultivation of genetically modified organisms (GMOs) in their territory.  
[http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=OJ:JOL\\_2015\\_068\\_R\\_0001](http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=OJ:JOL_2015_068_R_0001).
- EWEN, J.W.B., PUSZTAI, Á. (1999): Effect of diets containing genetically modified potatoes expressing *Galanthus nivalis* lectin on rat small intestine. *The Lancet*, 354, 1353-1354. p.
- FEJES Á. (2015): Perzisztens növényvédő szerek hatástartam- és lebomlásvizsgálatai. *PhD Disszertáció*. Pannon Egyetem Georgikon Kar, Állat- és Agrárkörnyezettudományi Doktori Iskola. 1-124. p.
- FÜSTI MOLNÁR G. (2007): Az állami elismerés előtt lévő géntechnológiai úton módosított fajtákkal végzett hazai fajtavizsgálatok eredményei. 17-19. p. In: DARVAS B. (Szerk.): *Mezőgazdasági géntechnológia - elsőgenerációs GM-növények*. Országgyűlés Mezőgazdasági Bizottsága, Budapest. 167 p.
- HARLOW E., LANE D. (1988): *Antibodies: A Laboratory Manual*. Cold Spring Harbor, New York: Cold Springs Harbor Laboratory, 344-348. p.
- HESZKY L. (2007): Génáramlás, génmegszökés várható következményei. 20-23. p. In: DARVAS B. (Szerk.): *Mezőgazdasági géntechnológia - elsőgenerációs GM-növények*. Budapest: Országgyűlés Mezőgazdasági Bizottsága, 167 p.
- ISO (1994): ISO 5725-4:1994 Accuracy (trueness and precision) of measurement methods and results. Part 4: Basic methods for the determination of the trueness of a standard measurement method.  
<http://www.iso.org/iso/home.html>.
- KEBEKKUS, B.B., MITRA, S. (1998): *Environmental chemical analysis*, Boca Raton, FL, USA: CRC Press, 344 p.

- NGUYEN, H.T., HUNFELD, H., MEISSLE, M., MIETHLING-GRAFF, R., PAGEL-WIEDER, S., RAUSCHEN, S., ZURBRUEGG, C., EBER, S., GESSLER, F., ROMEIS, J., TEBBE, C.C., NENTWIG, W., JEHLE, J.A. (2008): Round robin quantitation of Cry3Bb1 using the qualitative PathoScreen ELISA. *IOBC/WPRS Bulletin*, 33, 59-66. p.
- PUSZTAI, Á., BARDÓCZ, ZS., EWEN, S.W.B. (2003): Genetically modified foods: Potential human health effects. 342-372. p. In: D'MELLO, J.P.F. (Eds.) *Food Safety: Contaminants and Toxins*. Wallingford, Oxon, UK: CAB International. 452 p.
- ROH, J.Y., CHOI, J.Y., LI, M.S., JIN, B.R., JE, Y.H. (2007): *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. *Journal of Microbiology and Biotechnology*, 17, 547-559. p.
- SZÉKÁCS A., DARVAS B. (2007): A *MON 810*-es kukorica Cry1-toxintermelése és annak tarlómaradványokban való bomlása. 162. p. In: DARVAS B. (Szerk): *Mezőgazdasági géntechnológia - elsőgenerációs GM-növények*. Budapest: Magyar Országgyűlés Mezőgazdasági Bizottsága, Budapest, 167 p.
- SZÉKÁCS, A., DARVAS, B. (2012): Álláspontok a *GMO* környezeti kockázatelemzésében alkalmazott statisztikai módszerekről. *II. Ökotoxikológiai Konferencia*, p. 24.
- SZÉKÁCS A., LAUBER, É., JURACSEK, J., DARVAS, B. (2010): Cry1Ab production of *MON 810* transgenic maize. *Environmental Toxicology and Chemistry*, 29, 182-190. p.
- THEN, C., LORCH, A. (2008): A simple question in a complex environment: How much Bt toxin do genetically engineered *MON 810* maize plants actually produce? 17-21. p. In: BRECKLING, B., REUTER, H., VERHOEVEN, S. (Eds.): *Implications of GM-crop cultivation at large spatial scales. Theorie in der Ökologie*: Frankfurt: Peter Lang. 180 p.
- TIJSEN, P. (1985): Practice and theory of enzyme immunoassays. *Laboratory techniques in biochemistry and molecular biology*, vol 15, Amsterdam, Hollandia: Elsevier, Biomedical Press, 549 p.

## 7. SCIENTIFIC PUBLICATIONS

### 1. Scientific papers

#### a) Articles in scientific journals with IF

Székács A., Lauber É., **Takács E.**, Darvas B. (2010): Detection of Cry1Ab toxin in the leaves of *MON 810* transgenic maize. *Anal. Bioanal. Chem.* 396: 2203-2211. *IF: 3.841*

Székács A., Weiss G., Quist D., **Takács E.**, Darvas B., Meier M., Swain T., Hilbeck A. (2011): Inter-laboratory comparison of Cry1Ab toxin quantification in *MON 810* maize by enzyme-immunoassay. *Food. Agr. Immunol.* 23: 99-121. *IF: 0.633*

Fejes Á., **Takács E.**, Fekete G., Darvas B., Ferguson B.S., Saxena D., Székács A. (2012): Aquatic effect duration and degradation study of Cry4 toxin with immunoassay and *Aedes aegypti* larval biotest. *Aquatic Insects*, 34 (Suppl. 1): 211–226. *IF: 0.325*

**Takács E.**, Nagy A, Gelencsér É., Székács A. (2015): Internal quality control of an enzyme-linked immunoassay for Cry1Ab toxin detection applied in animal tissues. *Acta Alimentaria*, 44: 593-600, in press. *IF: 0.40<sup>4</sup>*

#### b) Articles in peer-reviewed scientific journals without IF

**Takács E.**, Darvas B., Székács A. (2012): Analytical difficulties and certain biological aspects of Cry1Ab toxin determination in *MON 810* genetically modified maize. *Acta Phytopathol. Entomol. Hung.* 47: 293-306.

**Takács E.**, Lauber É., Bánáti H., Székács A., Darvas B. (2009): *Bt*-növények a növényvédelemben. *Növényvédelem*, 45: 549-558.

#### c) Other articles in non-peer-reviewed scientific journals

Darvas B., Lauber É., **Takács E.**, Székács A. (2009): A GM-növények mérlege a növény- és környezetvédelemben I. *Környezetvédelem*, 17: (1): 24-25.

Darvas B., Lauber É., **Takács E.**, Székács A. (2009): A GM-növények mérlege a növény- és környezetvédelemben II. *Környezetvédelem*, 17 (2): 26-27.

### 2. Conference proceedings

#### a) Hungarian( full-length)

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<sup>4</sup> average impact factor of the last 5 years

Székács A., Darvas B., Lauber É. és **Takács E.** (2008): Géntechnológiai úton módosított haszonnövények környezetvédelmi aspektusai. In: Tudomány és innováció a jövő szolgálatában. Budapesti Műszaki Főiskola, Budapest, pp. 23-27.

**Takács E.**, Fejes Á., Fekete G., Darvas B. és Székács A. (2010): Cry4 toxin vízi hatástartam- és lebomlásvizsgálata immunoassay és *Aedes aegypti* lárvateszt segítségével. *Acta Biol. Debr. Oecol. Hung.* 21: 223-232.

*b) Hungarian (abstract)*

Székács A., **Takács E.**, Lauber É., Juracsek J., Darvas B. (2010): A Cry1-toxin eloszlásának mérése *MON 810* kukoricában - ELISA módszerek alkalmazhatósága. *Abs. 56. Növényvédelmi Tudományos Napok* (Kömíves T., Haltrich A., Molnár J., szerk.), 54. old.

**Takács E.**, Bánáti H., Fónagy A., Székács A. (2010): A Herculex RW (*DAS-59122-7*) kukorica Cry3-toxinjainak hatása az azon táplálkozó zselnicemeggy-levéltetűn (*Rhopalosiphum padi*) keresztül a hétpettyes katicabogár (*Coccinella septempunctata*) fiatal lárváira. *Abs. 56. Növényvédelmi Tudományos Napok* (Kömíves T., Haltrich A., Molnár J., szerk.) 59. old.

**Takács E.**, Juracsek J., Gabriele W., David Q., Angelika H., Darvas B., Székács A. (2011): Összehasonlító vizsgálat a *MON 810*-es kukorica Cry1Ab-toxintartalmának mennyiségi meghatározására immunanalitikai módszerrel. In. *Abs. 56. Növényvédelmi Tudományos Napok* (Kömíves T., Haltrich A., Molnár J., szerk.), 28. old.

**Takács E.**, Murányi A., Darvas B., Ködöböcz L., Juracsek J., Székács A. (2011): A műtrágyázás hatása a *MON 810*-es kukorica Cry1Ab-toxintermelésére. *Abs. I. Magyar Ökotoxikológiai Konferencia* (Darvas B., szerk) 29. old.

**Takács E.**, Darvas B., Bánáti H., Lauber É., Juracsek J., Székács A. (2012): A *MON 810*-es GM-kukorica Cry1Ab-tartalmának változása a tenyészedőszak során szabadföldi és üvegházi körülmények között. *Abs. II. Magyar Ökotoxikológiai Konferencia* (Darvas B., szerk) 25. old.

*c) Foreign language (full length)*

**Takács E.**, Bánáti H., Fónagy A., Darvas B. (2010): Short term effect of *DAS-59122-7* maize on L1 and L2 of *Coccinella septempunctata* feeding on *Rhopalosiphum padi*. *Növénytermelés* 59 (Suppl.): 625-628.

**Takács E.**, Fónagy A., Juracsek J., Kugler N., Székács A. (2012): Characterisation of tritrophic effects of *DAS-59122-7* maize on seven-spotted ladybird (*Coccinella septempunctata*) feeding on the bird cherry-oat aphid (*Rhopalosiphum padi*). *IOBC Bull.* 73: 121-134.

*d) foreing language (abstract)*

- Székács A., Fejes Á., Fekete G., **Takács E.**, Nádasy M., Darvas B., Anton A. (2010): Aquatic arthropod biotests for environmental surveys. *Abs. IX. European Congress of Entomology*, p. 93.
- Székács A., **Takács E.**, Juracsek J., Lauber É., Darvas B. (2010): Determination of Cry1Ab toxin content of *MON 810* maize pollen by enzyme-immunoassay. *Abs. IX. European Congress of Entomology*, p. 200.
- Székács A., Weiss G., Quist D., **Takács E.**, Darvas B., Hilbeck A. (2010): A Round Robin interlaboratory comparison of Cry1Ab toxin determination in *MON 810* maize and biological samples by enzyme-immunoassay. *Abs. IX. European Congress of Entomology*, p. 231.
- Takács E.**, Székács A., Weiss G., Quist D., Darvas B., Hilbeck A. (2011): Inter-laboratory comparison of Cry1Ab toxin quantification in *MON 810* maize by enzyme-immunoassay. *Abs. International Conference on Agri-Environmental Chemistry and Toxicology*, p. 53.

**Cumulative impact factor: 5.20**