THESIS OF PhD DISSERTATION

Cry1 toxin content of *MON 810 Bt*-corn and the effect of its pollen on protected butterfly species in Hungary

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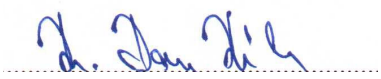

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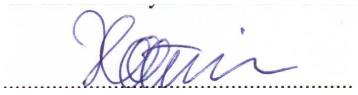

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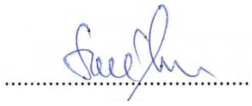

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BACKGROUND OF THE RESEARCH AND AIMS

Introduction

Upon the accession of Hungary and neighbouring countries, the European Union (EU) has been enriched by a region constituting an individual bio-geographical unit, the Pannonian Region. Unique species and habitats are located in this region. The proportion of natural values and habitats near to nature is considerably higher in Hungary than in many others European countries.

At present four genetic events are authorized for cultivation in the EU: the genetic event EH 92-527-1 potatoe (known as Amflora), the genetic event MON 810 (Yieldgard) and ACS-ZM3-2 (T25) maize, as well as Moonshadow 1 carnation. Genomes of the insect resistant varieties of the first generation of GM plants contain the modified/truncated genes of *Bacillus thuringiensis* (*Bt*). By means of these genes, Cry toxins are produced in all plant parts, which have lethal effect on insects. The general accepted assays for non-target effects cannot be disregarded in case of these varieties, as an agent with plant protection effect is emitted with *Bt* plants too, although: in a special formulation.

Bacillus thuringiensis

The *Bacillus thuringiensis* is an insect-pathogen bacterium, generally present in our environment. During sporulation, most *Bt* stocks compose parasporal body, which contains δ-endotoxin(s) and in some case parasporin. One of the large group of δ-endotoxins is the Cry-toxins, which are *per os* type, and have order-level specificity on insects (Cry1 – mostly Lepidoptera, Cry2 – primarily Lepidoptera and Diptera, Cry3 – principally Coleptera, Cry4 – Diptera).

The effect of Cry toxins leads to the lyse of cells of insects midgut in multiple steps, therefore larvae perish because of sepsis. The presence of the Cry toxin is sufficient in itself for the lethal effect, the *Bt* bacterium is not required. The sensitivity of insect species differs against a particular toxin, and the effects of different toxins differ on particular species to a various degree.

Target effects of MON 810 *Bt* maize and advantages of use

The modified *cry1Ab* gene of *Bt* are inserted into the DNA of MON 810 *Bt* maize, whereby the plant produces Lepidopteran specific, modified ~Cry1Ab toxin in all its parts. Compared to spray insecticide, *Bt* plants ensure permanent defense against the target pest and its relative species that damage the plant in a similar way. However, this also means that the Cry toxin is being produced in the plant constantly which means a permanent environmental load. The toxin is shielded by the wall of plant cell in a capsule-like manner. The owner companies mention as an advantage that the cob infections caused by *Fusarium* species decrease with the reduced caterpillar damage. It is refined by national investigations inasmuch as only a minor part of the *Fusarium* infection relates to the larvae of

cotton ballworm and European corn borer. On the whole, increased amount of yield are mentioned as the most important benefit of *Bt* plants, however, this is dependent upon the damage of corn borer larvae in case of Cry1 maize. Since this damage is not remarkable in Hungary, the amount of yield is invariable.

Non-target effects of MON 810 *Bt* maize and disadvantages of use

Our knowledge on natural *Bt* stocks does not apply automatically to the genetically modified *Bt* plants. Critics in several fields of science have been published about authorised GM plants producing Cry toxins.

Concerning the ecological impacts, geneflow, Cry toxin production and impacts of stubbles on soil organisms as well as development of pest resistance to Cry toxins cause several problems. In addition, the effects of *Bt* plants on non-target animals means a definite problem. Non-target organisms can be exposed to Cry toxin produced by *Bt* plants during their feeding: phytophags by the drifted pollen and plant parts contaminating their foodplants or living area; predators and parasitoids by the prey or host organisms consuming Cry toxin; decomposers by plant stubbles; pollinators by flower attendance; symbiotic organisms by their mutuality associations. The effects on non-target species related to the pest is a prominent problem; in case of MON 810 the Lepidoptera species are involved. The drifting pollen settles down on the foodplant of the caterpillars and they consume it with the pollen. Occuring sublethal effects (lower larval weight, slower development, lower pupal and adult weight) increase the affected individuals' and indirectly the population's mortality. This is especially critical in the case of protected Lepidoptera species. In addition, different aquatic arthropods (*Cladocera*, *Trichoptera*) and molluscs can also be affected, which are not the members of the known Cry1-toxin-sensitive group.

Cry1 toxin content of MON 810 varieties

In the past decades only a few articles were published about the ~Cry1Ab toxin production of MON 810 hybrids, so the background of the variable toxin content is still unclear. One thing is certain, MON 810 cultivars produce ~Cry1Ab toxin in a tissue- and time-specific manner. Soil quality especially its nitrogen fertility also has strong influence on ~Cry1Ab toxin expression; and not only the production site, but the year has also considerable effects. Multi-stack varieties have been reported to have expressed toxin content two fold higher than in single stack MON 810 cultivars. Wide range of the published values occurs as well in a single experiment. Similiarly extreme values have been published about the pollen.

Technical questions arise about the measurement of plant originated ~Cry1Ab toxin. The MON 810 varieties contain the truncated *cry1Ab* gene, which produces a smaller, 91 kDa protein, than

the 131 kDa protoxin produced by the original gene. It is improper in the analytical sense to directly apply ELISA systems directed against Cry1Ab/Cry1Ac protoxins to the measurement of preactivated plant toxin levels by using analytical standards of the protoxin protein.

Goals

Our aims were

- to compare the applicability of two commercial ELISA systems distributed for quantitative determination of Cry1Ab endotoxin on active toxin measurement and on measurable toxin level of the same plant sample;
- to determine the Cry1 toxin level of plant organs and follow their seasonal trends in organs; as well as to compare the toxin expression planting on the same production site in more years;
- to estimate the production and distribution of pollen in case of MON 810 hybrid and its near isogenic line;
- to execute/follow the analysis of the protected lepidopteran species in Hungary emphasizing the potentially affected species by MON 810 maize;
- to determine the Cry1 toxin sensitivity of several protected butterfly species (*Nymphalis io*, *Vanessa atalanta*, *Polygonium c-album*) and to compare them to the Cry1 toxin sensitivity of two maize pests, European corn borer (*Ostrinia nubilalis*) and cotton ballworm (*Helicoverpa armigera*);
- to select a model species (*N. io*) and investigate the effects of pollen concentration characterizing natural pollen drifting on its larvae with a shorter (L1-L3) and a longer (L1-L5) exposition.

MATERIALS AND METHODS

Cultivation and sampling of plants

MON 810 cultivars and their near isogenic lines were sown at the Ecological Experimental Station of the Plant Protection Institute, Hungarian Academy of Sciences (Julianna-major, Nagykovácsi, Hungary) in four years. ~Cry1Ab toxin production in MON 810 maize cultivars was followed for 4 months upon planting. Plant samples were taken from various plant parts including, when appropriate, leaves, stem, root, anther, pollen and grain. Plant samples were immediately processed upon sampling.

Determination of Cry1 toxin

Cry1 toxin levels were determined by two commercial enzyme-linked immunosorbent assays (ELISAs), EnviroLogix Cry1Ab/Cry1Ac QUANTIPLATE ® (#AP 003, Portland, MN, USA) and Abraxis Bt-Cry1Ab/Ac ELISA kit (#PN 51001, Warminster, PA, USA).

Enzymatic activation of Cry1Ab protoxin

Microbial Cry1Ab protoxin was cleaved by trypsin. Enzyme treatments were carried out using carbonate buffer containing dithiotreitol as a solubilising agent. The toxin/trypsin ratios ranged between 0.4 and 55. After incubation, to stop enzymatic activity, phenylmethanesulfonyl fluoride was added. Upon appropriate dilution in PBST buffer, the binding affinity was detected by performing the ELISA protocol.

Pollen production of maize and distribution of the settled pollen

To determine the pollen production, male flowers were isolated with special paper sacks. Spilled pollen was carefully sifted, its weight was measured by analytical scale. With similar methods, further notable amounts of pollen was collected to carry out the sensitivity experiments on caterpillars.

To get the vertical distribution of maize pollen, the number of settled pollens on each leaf-floor of corn was counted under stereo microscope. The measurement was repeated with silicon oil coated traps.

To get the horizontal distribution, silicon oil coated traps were fixed radially on maize leaves in the row before the last row with tassel, as well as in the first and third detasseled rows. In the section of the dominant direction of wind, silicon oil coated traps were placed in a distance of up to 200 metres.

To investigate the amount of settled maize pollen on weeds, we made surveys near Zsámbék and Kömlőd. Horizontal distribution of pollen was investigated on potted European dewberry and blackthorn.

Weed survey in aspects of protected lepidoptera species

To investigate the most typical weed species of Hungarian corn fields, weed surveys were carried out in a dozen randomly chosen maize fields and later in a field near Zsámbék during shedding periods of maize. Weed list was surveyed in the field and at the edges, and coverage of weed species (%) was estimated.

Analysis of the way of life of protected lepidoptera species in Hungary

Analysis of the way of life of protected lepidoptera species were carried out by using collection data of the Lepidoptera Collection at Hungarian National History Museum, data of related literature as well as knowledge and observations of the experts of the Collection. Knowing their food plants, protected species which larvae can feed in weed associations of corn or in shelter belts were selected. To determine the period of larval development, collection time (swarming time in Hungary) of specimens of the Lepidoptera Collection was combined with the behaviour of larvae for each species. Species whose food plants occur in or near cornfields and larvae develop during corn pollen shedding were identified.

Tests with maize pollens

Experiments with *Bt* pollen were carried out between 2002 and 2009. The ~Cry1Ab toxin content of the pollen was determined by ELISA, the density of pollen was counted with stereo microscope. Two types of treatment was used.

At dusting, potted nettle was pretreated with adhesion improver and microsieve was used to disperse the pollen. Distributions of pollens were checked by counting them on leaves and on silicon oil coated traps. In case of peacock butterfly, batches of eggs were placed on the leaves after treatments. Larvae consumed treated nettle leaves during 1-3 stadia, then they were put on untreated plants. Their developments were followed and their weights were measured many times. Weights of pupae were measured by sexes, as well as adult emergence was recorded.

At spraying, suspensions were made by pollen, water and additives. Inefficiency of additives were verified by control treatment containing only these agents. After drying, distributions of pollens were counting on nettle's leaves. Freshly hatched neonates were put on treated leaves. Experiments were checked by the methods mentioned above.

Tests with DIPEL WP

In case of protected lepidoptera, DIPEL WP was sprayed with a microsprayer at suitable concentrations on potted and cut nettles. The control was sprayed with pure water. For these experiments batches of eggs, freshly hatched neonates and larvae at stage before moulting (not eating) in various stadia were used.

To evaluate sublethal effects of DIPEL WP, treated potted nettles were used, that larvae consumed during 1-3 stadia. Experiments were checked by the methods of dusting.

In case of pests, freshly hatched neonates were put on maize leaves treated by various concentrations of DIPEL WP.

Statistical analysis

Data were analysed with STATISTICA (StatSoft Inc., USA) and R 2.10.1 softwares (R Development Core Team). To analyse the data of chemical determinations, linear and sigmoid regression were used and the homogeneous groups were separated by Fisher's least significance test. To analyse the data of biological investigations, one-way *ANOVA*, Tukey- or LSD-tests, χ^2 test with Monte Carlo simulated p-value and probit analysis were used as required.

NEW SCIENTIFIC RESULTS

Comparing our results with the related literature, we put a different complexion on Cry1 toxin determination and reason about the environmental effects of *MON 810* maize on protected lepidoptera species, which can be useful for the national authorisation.

1. Difference in the measurement of active Cry1Ab toxin by two commercial ELISA systems (EnviroLogix Cry1Ab/Cry1Ac QUANTIPLATE and Abraxis Bt-Cry1Ab/Ac ELISA kit) approved to quantitative measurement of Cry1Ab toxin was proven. The difference of immunoreactivity between the two systems was 3.2-fold corrected by the protoxin/active toxin cross-reactivity. So the measurements of different ELISA systems are similar in order of magnitude, but they are not directly comparable.

2. Trends of ~Cry1Ab toxin content of MON 810 maize by plant organs and phenological stages were determined. The highest toxin level was measured in leaves (~17 μg/g at fith-leaf stage).

3. A difference in pollen capture ability of ruderal weeds of maize fields was verified. Stinging nettle (*Urtica dioica*; 328 ± 200 pollen/cm^2), European dewberry (*Rubus caesius*, 431 ± 334 pollen/cm^2) and Jimsonweed (*Datura stramonium*, 339 ± 266 pollen/cm^2) are proven to be frequent species and good at pollen capture.

4. Protected lepidoptera species in the Pannonian Biogeographical Region, which are exposed to the pollen of MON 810 maize comparing the pollen shedding period with the food plant and habitat preferences of the species were determined. During pollination, larvae of comma butterfly (*Polygonia c-album*), peacock butterfly (*Inachis io*), red admiral (*Vanessa atalanta*) and small tortoiseshell (*Aglais urticae*) feed on stinging nettle; larvae of cardinal (*Pandoriana pandora*), lesser marbled fritillary (*Brenthis ino*), niobe fritillary (*Argynnis niobe*) and red underwing skipper (*Spialia sertorius*) feed on European dewberry, while caterpillars of death's-head hawkmoth (*Acherontia atropos*) feed on Jimsonweed.

5. Cry1 toxin sensitivity of larvae of the investigated protected lepidoptera species is (LC_{50} values): *Nymphalis io* L2 (1.93 ppm), *N. io* L3 (2.99 ppm), *N. io* L1 (4.39 ppm), *N. io* L4 (5.74 ppm), *N. io* L5 (6.17 ppm), *Nymphalis c-album* L1 (7.24 ppm) and *Vanessa atalanta* L1 (15.14 ppm).

6. The effects of L1-L3 (20-40 % mortality, lower larval and pupal weight, slower development) and L1-L5 (>80% mortality, lower larval and pupal weight, slower development) exposure of MON 810 pollen at concentration characterizing natural pollen drifting on larvae of peacock butterfly were investigated. When the toxin content of MON 810 pollen was lower than 30 ng/g, effects were observed only at high pollen density (~1000 pollen/cm^2).

7. According to our investigations, we suggest the peacock butterfly as a model species to assess the environmental impacts of corn borer resistant MON 810 and similar Bt maize hybrids in the Pannonian Biogeographical Region.

PUBLICATIONS IN THEME OF THE DISSERTATION

Book chapters

Darvas B. és **Lauber É.** (2006): Genetikailag módosított élő szervezetek a növénytermesztésben. *In* Darvas B. és Székács A. (szerk.): *Mezőgazdasági ökotoxikológia*. L'Harmattan Kiadó, Budapest. 315-319. old.

Lauber É., Polgár A. L. és Darvas B. (2007): A *MON 810*-es kukorica pollenje és a védett lepkék. 39-42. old. *In* Darvas B. (szerk.): *Mezőgazdasági géntechnológia – Elsőgenerációs GM-növények*. Országgyűlés Mezőgazdasági Bizottsága, Budapest.

Darvas B. és **Lauber É**. (2007): A Cry1-toxinrezisztenciáról. 64-66. old. *In* Darvas B. (szerk.): *Mezőgazdasági géntechnológia – Elsőgenerációs* GM-*növények*. Országgyűlés Mezőgazdasági Bizottsága, Budapest.

Articles in English

Lang, A., **Lauber, É.** and Darvas, B. (2007): Early tier tests insufficient for GMO risk assessment. *Nature Biotechnology,* **25**: 35-36. (IF: 22,850)

Lauber, É. and Darvas, B. (2009): Increased mortality of isolated first instar larvae of *Inachis io* (Lepidoptera). *Acta Phytopath*. *Entomol. Hung*., **44**: 111-117.

Székács, A., **Lauber, É.**, Juracsek, J. and Darvas, B. (2010): Cry1Ab toxin production of MON 810 transgenic maize. *Environ. Toxicol. Chem*., **29**: 182-190. (IF: 2,310)

Székács, A., **Lauber, É.**, Takács, E. and Darvas, B. (2010): Detection of Cry1Ab toxin in the leaves of MON 810 transgenic maize. *Anal. Bioanal. Chem*., **396**: 2203-2211. (IF: 3,328)

Darvas, B., Bánáti, H., Takács, E., **Lauber, É.**, Szécsi, Á. and Székács, A. (2011): Relationships of *Helicoverpa armigera*, *Ostrinia nubilalis* and *Fusarium verticillioides* on MON 810 maize. *Insects*, **2**: 1-11.

Articles in Hungarian

Darvas B., Csóti A., Gharib, A., Peregovits L., Ronkay L., **Lauber É.** és Polgár A. L. (2004): [Some data to the risk analysis of Bt-corn pollen and protected lepidoptera species in Hungary]. *Növényvédelem*, **40**: 441-449.

Darvas B., Székács A., Bakonyi G., Kiss I., Biró B., Villányi I., Ronkay L., Peregovits L., **Lauber É.** és Polgár A. L. (2006): [Authors' response to the statement of the European Food Safety Authority GMO Panel concerning environmental analytical and ecotoxicological experiments carried out in Hungary]. *Növényvédelem,* **42**: 313-325.

Darvas B., **Lauber É.**, Bakonyi G., Békési L., Székács A. és Papp L. (2007): A *MON 810*-es GM-kukoricák környezettudományi megítélése. *Magyar Tudomány*, **167** (8): 1047-1056.

Takács E., **Lauber É.**, Bánáti H., Székács A. és Darvas B. (2009): [*Bt* plants in plant protection]. *Növényvédelem*, **45**: 549-558.

Darvas B., **Lauber É.**, Takács E. és Székács A. (2009): GM-növények mérlege a növény- és környezetvédelemben I-II. *Környezetvédelem*, **17** (1): 24-25; (2): 26-27.

Conference Proceedings

Darvas, B., **Lauber, É.**, Polgár, L. A., Peregovits, L., Ronkay, L., Juracsek, J. and Székács, A. (2004): Non-target effects of DK-440-BTY (Yieldgard) *Bt*-corn. *Abs. First Hungarian-Taiwanese Entomological Symposium*, Page 5.

Darvas B., **Lauber É.**, Kincses J., Vajdics Gy., Juracsek J. és Székács A. (2005): *Bt*-kukorica eredetű Cry1Ab toxinra rezisztens *Plodia interpunctella. Abs. 51. Növényvédelmi Tudományos Napok,* 9. old.

Darvas, B., **Lauber, É.**, Csóti. A., Peregovits, L., Ronkay, L., Polgár, A. L. and Székács, A. (2006): The impact of *Bt*-maize (MON 810) pollen on protected butterfly species in Hungary. *Abs. First European Congress of Conservation Biology*, Page 21.

Székács A., Juracsek J., Maloschik E., **Lauber É.**, Polgár A. L. és Darvas B. (2006): A Cry1Ab toxin eloszlása a DK-440 BTY növényben, tarlómaradványa és annak lebomlása. *Abs. 52. Növényvédelmi Tudományos Napok,* 32. old.

Lauber É., Kincses J., Polgár A. L., Juracsek J., Székács A. és Darvas B. (2006): Az *Inachis io* és *Polygonia c-album* első stádiumú lárváinak érzékenysége Dipel-re és Cry1Ab-toxint tartalmazó GM-pollenre. *Abs. 52. Növényvédelmi Tudományos Napok,* 36. old.

Darvas B., **Lauber É.**, Vajdics Gy., Pap L., Juracsek J. és Székács A. (2006): *Bt*-kukorica eredetű Cry1Ab toxinra rezisztens *Plodia interpunctella* keresztérzékenysége Dipel-re, és reakciója citokróm P-450 gátlóra. *Abs. 52. Növényvédelmi Tudományos Napok,* 37. old.

Darvas B., **Lauber É.**, Polgár A. L. és Székács A. (2007): Környezettudományi eredmények a DK-440 BTY genetikailag módosított (*MON 810*) kukoricával. *Abs. 53. Növényvédelmi Tudományos Napok,* D old.

Darvas B., **Lauber É.** és Székács A. (2007): Az Európai Unióban engedélyezés alatt álló, géntechnológiai úton módosított növények környezettudományi megítélése. *Abs. 12. Tiszántúli Növényvédelmi Fórum,* 15-27. old.

Székács A., Darvas B., **Lauber É.** és Takács E. (2008): Géntechnológiai úton módosított haszonnövények környezetvédelmi aspektusai. *Tudomány és innováció a jövő szolgálatában*, Budapest, 2008. november 7., 23-27. old.

Lauber É., Bánáti H., Takács E. és Darvas B. (2009): Természetes eredetű proteinázgátlók hatása Cry1-fogékony és Cry1-rezisztens aszalványmolyon. *Abs. 55. Növényvédelmi Tudományos Napok,* 20. old.

Takács E., Bánáti H., **Lauber É.** és Darvas B. (2009): Ovipozitánsok vizsgálata hatása Cry1-fogékony és Cry1-rezisztens aszalványmolyon. *Abs. 55. Növényvédelmi Tudományos Napok,* 21. old.

Székács, A., Takács, E., Juracsek, J., **Lauber, É.** and Darvas, B. (2010): Determination of Cry1Ab toxin content of MON 810 maize pollen by enzyme-immunoassay. *Abs. 9th European Congress of Entomology*, Budapest, Page. 200.

Darvas, B., Bánáti, H., Szécsi, Á., **Lauber, É.** and Székács, A. (2010): Relationships of *Helicoverpa armigera*, *Ostrinia nubilalis* and *Fusarium verticillioides* on MON 810 maize. *Abs. 9th European Congress of Entomology*, Budapest, Page 200-201.

Lauber, É., Peregovits, L., Ronkay, L., Csoti, A., Szekacs, A. and Darvas, B. (2010): Protected lepidopteran larvae and Cry1Ab toxin exposure by Bt maize pollen in the Pannonian Region. *Abs. 9th European Congress of Entomology*, Budapest, Page 205-206.

Fónagy A., Krishnan, M., Bánáti H., **Lauber É.**, Takács E., Székács A., Nyiri A., Herman G., Kugler N. és Darvas B. (2010): Kukoricafajták virágzása, különös tekintettel az intraspecifikus hibridizációra (MON 810 x egyéb fajták) [No 1.] *Abs. 56. Növényvédelmi Tudományos Napok,* 53. old.

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

Bánáti H., **Lauber É.**, Szécsi Á., Székács A. és Darvas B. (2010): A gyapottok-bagolylepke és a kukoricamoly szerepe a csőfuzariózis terjesztésében. *Abs. 56. Növényvédelmi Tudományos Napok,* 56. old.

Darvas B., Bánáti H., Szécsi Á., **Lauber É.** és Székács A. (2010): A gyapottok-bagolylepke, a kukoricamoly és a fuzariózis együttes előfordulása szabadföldi hagyományos és Cry1-toxintermelő kukoricacsövekben. *Abs. 56. Növényvédelmi Tudományos Napok,* 57. old.

Lauber É., Székács A. és Darvas B. (2010): Cry1-toxint termelő (*MON 810*) kukorica pollenjének hatása nappali pávaszemre (*Inachis io*) – eredményrevízió. *Abs. 56. Növényvédelmi Tudományos Napok,* 60. old.