Preface

In May 2009, the IOBC/WPRS working group ‘GMOs in Integrated Plant Production’ had hold its fourth full working group meeting. The first meeting of the working group had taken place in Prague, Czech Republic, in November 2003 [IOBC/WPRS Bulletin 27(3), 2004], the second meeting in Lleida, Spain, in June 2005 [IOBC/WPRS Bulletin 29(5), 2006] and the third meeting in Warsaw, Poland, in May 2007 [IOBC/WPRS Bulletin 33, 2008].

Similar to the previous meetings, there was a vast interest in this event with 74 participants from 14 countries attending. Besides colleagues from public research institutes, participants were retrieved from private industry and regulatory agencies. This is an indication that the meeting provides a good platform for scientific communication among the different stakeholders dealing with GM crops.

During the meeting, three keynotes, 24 oral contributions and 22 posters were presented. In total, 17 contributions are published in this bulletin.

I would like to thank the members of the scientific organizing committee for their help in putting together an interesting programme and those that had agreed to act as session organizers. On behalf of all participants, I would also like to thank Dr. Thomas Thieme and his team from BTL Bio-Test Labor GmbH for their excellent job in organizing this meeting and their hospitality. I would furthermore like to express my thanks to the University of Rostock for providing us the venue for the meeting.

The next full working group meeting is planned for 2011. The exact dates and location will be announced in time.

Jörg Romeis
Convenor IOBC/WPRS working group
‘GMOs in Integrated Plant Production’
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Managing weeds in herbicide-tolerant GM maize for biological control enhancement

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Abstract: Deployment of transgenic herbicide-tolerant maize that allows post-emergence treatment with broad-spectrum herbicides may lead to changes in the composition and abundance of weed flora. The consequences of these changes on maize arthropods and particularly on insect pest natural enemies are studied in this work. Weeds, insect herbivores and their natural enemies were monitored in maize plots treated twice with glyphosate (V4 and V8) in comparison with plots treated once with conventional pre-emergence herbicides. Plots were sampled by visual observation, pitfall and yellow sticky traps during two consecutive years (2007 and 2008). In spite of the significant differences recorded in weed abundance between the two herbicide treatments, there were very few significant differences in the arthropod groups monitored, in contrast with results of a previous study comparing plots with two glyphosate treatments (as in the present work) and with no herbicide treatment in order to identify the most responsive arthropod to weed abundance alteration. It seems that when maize weed abundance is not drastically altered, populations of arthropod herbivores and natural enemies are not greatly affected. However, more studies are needed to determine the potential impacts of modifying herbicide use on arthropods and particularly on conservation biological pest control.

Key words: GM maize, herbicide-tolerant corn, arthropod, insect, predator.

Introduction

Tolerance to herbicides is the most commonly introduced trait in cultivated transgenic crops worldwide. Herbicide-tolerant (HT) maize, alone or stacked with Bacillus thuringiensis (Bt) toxin-expressing genes, occupied 38 million ha in 2008 (James, 2008). Several direct consequences of the deployment of HT crops have been pointed out, among which are the modification and intensification of herbicide use and the increase of non/low-tillage techniques that may lead to impacts on non-target organisms such as arthropods, birds and other wildlife, non-target plants, plant pathogens and soil biota.

The composition and abundance of weed flora and organisms of higher trophic levels may also change as a consequence of altered weed management practices. It has been reported that maize in the study area hosts a variety of predators that can keep herbivore population densities at tolerable values (Albajes et al., 2003). Therefore, a major concern of impacts of HT maize on arthropods is the preservation, and ideally enhancement, of the natural enemy fauna. In a preliminary work, for two consecutive years we compared the herbivore, predator and parasitoid fauna on plots sown with a maize variety based on the transformation event NK603 treated twice each season with broad spectrum herbicides and on herbicide-untreated plots in order to identify the most responsive species (Albajes et al., 2009). Samples were taken by visual inspection, pitfall traps and yellow sticky traps and it was concluded that herbivore, predator and parasitoid groups were differently affected by the intensive post-emergence treatment. As a general pattern it was concluded that on glyphosate-treated plots there were more herbivores and predators on plants but fewer soil-dwelling predators in
comparison with untreated plots. Among parasitoids, only mymarids showed higher catches in yellow sticky traps on treated plots, whereas the remaining predators were unaffected.

In the present work we aimed to check whether this general pattern is confirmed when the intensive glyphosate treatment is compared with a conventional herbicide regime based on pre-emergence treatments. For this, plant and soil-dwelling predators were recorded by visual plant inspection, pitfall traps and yellow sticky traps catching flying insects in 2007 and 2008. This work was conducted in the framework of a 4-year agreement between the National Institute of Agriculture and Agrofood Technology (INIA) and the University of Lleida (UdL) and is sponsored by the Spanish Ministry of Environment and Agriculture.

Material and methods

Experimental fields and treatments

The study was conducted in Lleida (northeast Iberian Peninsula, 42°N). The experimental field was surrounded by winter cereals and alfalfa with a 1.5 m margin between them, and no other maize field was within a radius of 300 m. In both years the field was tilled one week prior to planting so that weeds were absent at sowing. The field was irrigated using sprinklers and the cultural practices were the common ones in the region. A complete random block design with two treatments and 4 replications was used. The two treatments were randomly assigned to each block in the first year but randomization was not used in the second year as the treatments were repeated on the same plots. The experimental units were plots of about 0.5 ha in size. The two treatments consisted of two applications of glyphosate at V4 and V8 maize growth stages at a rate of 1.08 kg of a.i./ha and one conventional herbicide preemergence treatment (atrazine+alachlor at a dose of 1.75 and 1.0 kg a.i/ha respectively) in 2007 and acetochlor at a dose of 1.26 kg a.i./ha and aclonifen+isoxaflutol at 0.075 and 0.5 kg a.i./h, respectively in 2008). The plots treated with glyphosate in 2007 and 2008 had been already treated with two applications of glyphosate in 2006 whereas the plots treated with conventional pre-emergence herbicides had not been treated in 2006. Seed was dressed with Imidacloprid. The whole experimental field was sown with the same variety (TEB652-E), including the transformation event NK603, which confers tolerance to over-the-top applications with glyphosate herbicide.

Sampling

Results of weed counts were provided by weed scientists at the INIA. Abundance of weeds per m² was estimated by counting the number of individuals within a 0.25 m² ring; on each plot rings were randomly distributed 16 times on each principal diagonal. Weeds were identified to genus level, and when possible to species level. Counts were carried out just before herbicide application and 10/15 days after the last herbicide treatment. Only weed counts after herbicide application are reflected here.

Three techniques were used to estimate arthropod densities or activities: visual counting, pitfall traps and sticky yellow traps. Samples were taken 7 times per season with each of the techniques at the following maize growth stages: V6-7, V8-10, V12-14, V14-15, R1, R3 and R5 [nomenclature of Ritchie et al., 1992].

Abundance of crop-plant dwelling predators and herbivores was estimated by visually counting the number of individuals on 25 plants per plot early in the morning. Three pitfall traps (a glass jar of 8 cm ∅ and 17 cm depth half-filled with water and 20% ethylene-glycol) were arranged in each plot, regularly distributed along the plot length but at least 10 m from the field border, and left active for 5 days. They were protected from irrigation sprinkles by a 25 x 17.5 cm² roof placed at 3 cm height from the ground. Three yellow sticky traps (21 x 31 cm, only one sticky side, Serbios®, Italy) per plot were placed on a stake at canopy height (until V12) or
at ear level (from V15 onwards) and left active for 5 days. Individuals caught in pitfall and yellow sticky traps were taken to the laboratory, kept in the refrigerator until they could be processed and identified in all cases to different taxon levels. Voucher specimens of the main arthropods identified were deposited in the Laboratory of Entomology (University of Lleida, Lleida, Spain).

**Statistical analysis**

In the combined analyses of variance a split-split-plot-like model (Gomez and Gomez, 1984) was used in which year (2006 vs. 2007) was considered the main plot. Subplot was the treatment (glyphosate vs. conventional treatments) and sub-subplots were the sampling dates (seven). All factors except blocks were considered fixed and crossed with each other, except again for blocks that were nested within year. To normalize the original data as much as possible, they were transformed by SQRT (x+0.5) prior to analysis. The level of significance was P<0.05 in all cases. The Statgraphics Plus computer package (Statgraphics, 1997) was used for the analyses.

**Results and discussion**

Table 1 shows the composition and densities of weeds in the two kinds of treatment. These results were provided by weed scientists at the INIA who participate in the INIA-UdL agreement (Garcia-Baudin *et al.*, personal communication). Two thirds of weeds were grasses belonging to two species *Echinochloa crus-galli* and *Setaria verticillata* (recorded together when young plantlets). These two species together with *Amaranthus retroflexus* and *Chenopodium album* represented 84% of the total weeds recorded. Gramineae were considerably (8 times) and significantly (P =0.03) more abundant on plots treated with conventional herbicides. Johnson grass, *Sorghum halepense*, was also significantly more abundant on these plots, with a mean density of 1.4 plants/m², a considerable value that could cause some damage to maize. Only *Veronica persica* numbers were significantly higher on glyphosate-treated plots, although the effects depended on the year.

When the number of plant-dwelling arthropods recorded in visual counting in glyphosate-treated and conventional herbicide-treated plots was compared (Table 2), no significant treatment effects were detected. There were only two significant effects of two-way interactions associated with ‘treatment’ but with very little relevance as the means in both kinds of plot were quite similar. Year had by far the most influential effect on the number of arthropods on plants, being significant in 8 of the 18 arthropod groups examined. A similar pattern was found in pitfall trap catches (Table 3). In the 13 arthropod groups analyzed, no effect of herbicide treatment was found, whereas there was only one case of significant two-way interaction associated with treatment. On the other hand, in 5 arthropod taxons the year significantly influenced the density of individuals per plant. In yellow sticky traps only phytophagous thrips showed significant differences between glyphosate-treated and conventional herbicide-treated plots, although treatment effects interacted with year and sampling dates. The origin of the higher phytophagous thrips catches on conventional treatment was probably the higher density of grass weeds recorded on those plots, as visual on-plant counts did not record any differences between herbicide treatments in phytophagous thrips.

In general, all the arthropod groups monitored with the three sampling techniques during the two years showed very weak responses to the differences in weed composition and abundance. The maize arthropods identified as the most responsive to dramatically altered weed management [intensive treatment with glyphosate in comparison with no herbicide
treatment, Albajes et al. (2009)] did not clearly react to changes in weed abundance when this was not as dramatically altered as it was in the previous work. More research is probably needed to find how weeds interact with herbivorous or natural enemies, what can be expected from modifying use of herbicides and how these modifications may be managed to enhance conservation biological control in maize fields.

Table 1. Number of weeds/m² (±S.E.) on plots treated twice with glyphosate or with a conventional pre-emergence herbicide (in the material and methods section the conventional herbicides applied are detailed) [García-Baudin, personal communication].

<table>
<thead>
<tr>
<th>Weed</th>
<th>% of total</th>
<th>Herbicide treatment</th>
<th>Year (Y)a</th>
<th>Treatment (T)b</th>
<th>Y x T²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glyphosate</td>
<td>Conventional</td>
<td>F, P</td>
<td>F, P</td>
</tr>
<tr>
<td>Abutilon theophrasti</td>
<td>3.3</td>
<td>0.38±0.21</td>
<td>1.95±0.71</td>
<td>0.13, 0.74</td>
<td>2.29, 0.23</td>
</tr>
<tr>
<td>Amaranthus retroflexus</td>
<td>11.0</td>
<td>1.38±0.68</td>
<td>6.47±2.43</td>
<td>0.43, 0.56</td>
<td>1.54, 0.30</td>
</tr>
<tr>
<td>Chenopodium album</td>
<td>6.7</td>
<td>1.27±0.57</td>
<td>3.51±1.37</td>
<td>0.42, 0.56</td>
<td>0.93, 0.41</td>
</tr>
<tr>
<td>Echinochloa crus-galli + Setaria verticillata</td>
<td>66.6</td>
<td>5.25±1.46b</td>
<td>42.10±16.85a</td>
<td>2.33, 0.22</td>
<td>17.33, 0.03</td>
</tr>
<tr>
<td>Lolium multiflorum</td>
<td>1.0</td>
<td>0.02±0.02</td>
<td>0.68±0.34a</td>
<td>6.64, 0.08</td>
<td>7.74, 0.07</td>
</tr>
<tr>
<td>Medicago sativa</td>
<td>1.3</td>
<td>0.83±0.60</td>
<td>0.06±0.04</td>
<td>1.43, 0.32</td>
<td>3.82, 0.15</td>
</tr>
<tr>
<td>Portulaca oleracea</td>
<td>4.6</td>
<td>1.63±0.43</td>
<td>1.63±1.33</td>
<td>0.19, 0.69</td>
<td>0.48, 0.54</td>
</tr>
<tr>
<td>maize volunteers</td>
<td>0.4</td>
<td>0.10±0.04</td>
<td>0.19±0.09</td>
<td>66.06, &lt;0.01</td>
<td>5.80, 0.10</td>
</tr>
<tr>
<td>Sorghum halepense</td>
<td>2.1</td>
<td>0.11±0.06b</td>
<td>1.41±0.50a</td>
<td>0.21, 0.68</td>
<td>9.97, 0.05</td>
</tr>
<tr>
<td>Veronica persica</td>
<td>1.4</td>
<td>0.95±0.38a</td>
<td>0.06±0.04b</td>
<td>53.84, 0.01</td>
<td>23.39, 0.02</td>
</tr>
<tr>
<td>Minor weeds</td>
<td>1.6</td>
<td>0.70±0.25</td>
<td>0.42±0.31</td>
<td>10.35, 0.05</td>
<td>1.61, 0.29</td>
</tr>
<tr>
<td>TOTAL WEEDS</td>
<td>100</td>
<td>12.60±2.03</td>
<td>58.49±20.22</td>
<td>1.65, 0.29</td>
<td>5.62, 0.10</td>
</tr>
</tbody>
</table>

*a df=1,3
Table 2. Number of arthropods/plant (±S.E.) found in visual counts on plots treated twice with glyphosate or with a conventional pre-emergence herbicide.

<table>
<thead>
<tr>
<th>Arthropod group</th>
<th>Herbicide treatment</th>
<th>Year (Y) (^a)</th>
<th>Treatment (T) (^a)</th>
<th>YxT (^a)</th>
<th>Txs (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glyphosate</td>
<td>Conventional</td>
<td>F, P</td>
<td>F, P</td>
<td>F, P</td>
</tr>
<tr>
<td>HERBIVORES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aphids total</td>
<td>7.44±1.43</td>
<td>6.48±1.29</td>
<td>10.39, 0.05</td>
<td>0.76, 0.45</td>
<td>0.65, 0.48</td>
</tr>
<tr>
<td>Leafhoppers</td>
<td>34.7±8.1</td>
<td>33.5±6.1</td>
<td>0.20, 0.68</td>
<td>0.01, 0.92</td>
<td>0.15, 0.72</td>
</tr>
<tr>
<td>Herbivore thrips</td>
<td>7.76±1.73</td>
<td>7.36±1.57</td>
<td>24.54, 0.02</td>
<td>0.35, 0.59</td>
<td>2.00, 0.25</td>
</tr>
<tr>
<td>PREDATORS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orius adults</td>
<td>1.25±0.16</td>
<td>1.17, 0.15</td>
<td>11.13, 0.05</td>
<td>0.36, 0.59</td>
<td>0.05, 0.84</td>
</tr>
<tr>
<td>Orius nymphs</td>
<td>1.34±0.24</td>
<td>1.26±0.24</td>
<td>3.74, 0.15</td>
<td>0.82, 0.43</td>
<td>0.32, 0.61</td>
</tr>
<tr>
<td>Orius total</td>
<td>2.59±0.35</td>
<td>2.43±0.32</td>
<td>3.69, 0.09</td>
<td>0.51, 0.53</td>
<td>0.09, 0.79</td>
</tr>
<tr>
<td>Nabis adults</td>
<td>0.03±0.01</td>
<td>0.03±0.01</td>
<td>0.28, 0.63</td>
<td>0.89, 0.41</td>
<td>0.35, 0.59</td>
</tr>
<tr>
<td>Nabis nymphs</td>
<td>0.01±0.01</td>
<td>0.01±&lt;0.01</td>
<td>0.04, 0.86</td>
<td>1.69, 0.29</td>
<td>0.45, 0.55</td>
</tr>
<tr>
<td>Nabis totals</td>
<td>0.03±0.01</td>
<td>0.04±0.01</td>
<td>0.06, 0.83</td>
<td>4.71, 0.12</td>
<td>0.42, 0.56</td>
</tr>
<tr>
<td>Mirids total</td>
<td>0.07±0.01</td>
<td>0.07±0.01</td>
<td>3.68, 0.01</td>
<td>0.27, 0.64</td>
<td>10.67, 0.05</td>
</tr>
<tr>
<td>Carabids</td>
<td>0.23±0.05</td>
<td>0.23±0.04</td>
<td>98.1, &lt;0.01</td>
<td>0.01, 0.92</td>
<td>6.85, 0.08</td>
</tr>
<tr>
<td>Staphylinids</td>
<td>0.22, 0.04</td>
<td>0.24, 0.04</td>
<td>0.65, 0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total coccinellids</td>
<td>0.15±0.03</td>
<td>0.10±0.02</td>
<td>2.11, 0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chrysopids total</td>
<td>0.08±0.02</td>
<td>0.08±0.02</td>
<td>14.29, 0.03</td>
<td>0.00, 0.98</td>
<td>3.02, 0.18</td>
</tr>
<tr>
<td>Syrphids total</td>
<td>0.06±0.01</td>
<td>0.04±0.01</td>
<td>12.19, 0.04</td>
<td>1.04, 0.38</td>
<td>0.85, 0.42</td>
</tr>
<tr>
<td>Predatory thrips</td>
<td>0.09±0.02</td>
<td>0.10±0.03</td>
<td>3.73, 0.15</td>
<td>0.15, 0.72</td>
<td>0.04, 0.85</td>
</tr>
<tr>
<td>Spiders</td>
<td>1.47±0.20</td>
<td>1.55±0.22</td>
<td>0.13, 0.75</td>
<td>0.05, 0.83</td>
<td>1.21, 0.35</td>
</tr>
<tr>
<td>Trombidids</td>
<td>0.06±0.02</td>
<td>0.05±0.01</td>
<td>22.08, 0.02</td>
<td>0.02, 0.90</td>
<td>0.51, 0.53</td>
</tr>
<tr>
<td>Total predators</td>
<td>6.63±0.86</td>
<td>6.44±0.85</td>
<td>18.81, 0.02</td>
<td>0.10, 0.77</td>
<td>0.44, 0.55</td>
</tr>
</tbody>
</table>

\(^a\) df=1,3; \(^b\) df=6,59
Table 3. Number of arthropods/trap (±S.E.) caught in pitfall traps on plots treated twice with glyphosate or with a conventional pre-emergence herbicide.

<table>
<thead>
<tr>
<th>Arthropod group (%)</th>
<th>Herbicide treatment</th>
<th>Year (Y) (^{a})</th>
<th>Treatment (T) (^{a})</th>
<th>YxT(^{a})</th>
<th>TxS(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glyphosate</td>
<td>Conventional</td>
<td>F, P</td>
<td>F, P</td>
<td>F, P</td>
</tr>
<tr>
<td>Harpalus rufipes</td>
<td>9.27±1.47</td>
<td>11.24±2.02</td>
<td>2.69, 0.20</td>
<td>0.17, 0.71</td>
<td>1.12, 0.37</td>
</tr>
<tr>
<td>Pterostichus sp.</td>
<td>2.23±0.35</td>
<td>2.55±0.44</td>
<td>2.01, 0.25</td>
<td>0.08, 0.79</td>
<td>0.48, 0.54</td>
</tr>
<tr>
<td>Agonum dorsale</td>
<td>0.86±0.31</td>
<td>1.03±0.34</td>
<td>0.02, 0.88</td>
<td>0.06, 0.82</td>
<td>0.03, 0.88</td>
</tr>
<tr>
<td>Other carabids</td>
<td>0.50±0.32a</td>
<td>0.10±0.04b</td>
<td>0.05, 0.83</td>
<td>0.08, 0.79</td>
<td>5.10, 0.11</td>
</tr>
<tr>
<td>Carabid larvae</td>
<td>0.15±0.07</td>
<td>0.07±0.02</td>
<td>0.27, 0.64</td>
<td>0.29, 0.63</td>
<td>1.95, 0.26</td>
</tr>
<tr>
<td>Carabids total</td>
<td>12.35±2.02</td>
<td>14.74±2.06</td>
<td>1.53, 0.30</td>
<td>0.16, 0.72</td>
<td>1.04, 0.38</td>
</tr>
<tr>
<td>Staphylinids adults</td>
<td>3.52±0.66</td>
<td>3.79±0.85</td>
<td>52.91, 0.01</td>
<td>0.0, 0.97</td>
<td>2.17, 0.24</td>
</tr>
<tr>
<td>Staphylinid larvae</td>
<td>0.02±0.01</td>
<td>0.01±0.01</td>
<td>3.00, 0.18</td>
<td>3.00, 0.18</td>
<td>3.00, 0.18</td>
</tr>
<tr>
<td>Staphylinids total</td>
<td>3.76±0.65</td>
<td>3.92±0.84</td>
<td>34.06, 0.01</td>
<td>0.15, 0.73</td>
<td>4.05, 0.14</td>
</tr>
<tr>
<td>Elongate Collembola</td>
<td>12.37±4.77</td>
<td>17.05±7.37</td>
<td>100, &lt;0.01</td>
<td>0.67, 0.47</td>
<td>0.67, 0.47</td>
</tr>
<tr>
<td>Earwigs</td>
<td>0.76±0.17</td>
<td>0.70±0.12</td>
<td>8.89, 0.06</td>
<td>0.00, 0.97</td>
<td>0.14, 0.73</td>
</tr>
<tr>
<td>Spiders</td>
<td>5.70±0.77</td>
<td>5.10±0.57</td>
<td>196, &lt;0.01</td>
<td>0.19, 0.69</td>
<td>24.450, 0.02</td>
</tr>
<tr>
<td>Centipedes Millipedes</td>
<td>28.55±8.50</td>
<td>25.11±5.70</td>
<td>44.51, 0.01</td>
<td>0.11, 0.76</td>
<td>0.03, 0.88</td>
</tr>
<tr>
<td>Total predators</td>
<td>51.59±8.90b</td>
<td>49.94±10.06a</td>
<td>265, &lt;0.01</td>
<td>0.0, 0.95</td>
<td>4.92, 0.11</td>
</tr>
</tbody>
</table>

\(^{a}\) df=1,3; \(^{b}\) df=6,60
Table 4. Number of arthropods /trap (±S.E.) caught in yellow sticky traps placed in plots treated twice with glyphosate or with a conventional pre-emergence herbicide.

<table>
<thead>
<tr>
<th>Arthropod group (%)</th>
<th>Herbicide treatment</th>
<th>Year (Y)*</th>
<th>Treatment (T)*</th>
<th>YxT*</th>
<th>TxB*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glyphosate</td>
<td>Conventional</td>
<td>F, P</td>
<td>F, P</td>
<td>F, P</td>
</tr>
<tr>
<td>Phytophagous thrips</td>
<td>244±39b</td>
<td>424±61a</td>
<td>1932, &lt;0.01</td>
<td>11.99, 0.04</td>
<td>17.55, 0.02</td>
</tr>
<tr>
<td>Leafhoppers</td>
<td>173.3±29.8</td>
<td>183.3±30.6</td>
<td>0.06, 0.81</td>
<td>1.02, 0.39</td>
<td>0.77, 0.44</td>
</tr>
<tr>
<td>Planthoppers</td>
<td>28.03±3.56</td>
<td>26.67±3.23</td>
<td>0.99, 0.39</td>
<td>0.05, 0.83</td>
<td>1.25, 0.35</td>
</tr>
<tr>
<td>Aphids</td>
<td>7.70±1.95</td>
<td>7.60±1.57</td>
<td>67.94, &lt;0.01</td>
<td>0.50, 0.53</td>
<td>0.89, 0.41</td>
</tr>
</tbody>
</table>

**HERBIVORES**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PREDATORS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orius</td>
<td>4.74±0.62</td>
<td>5.57±0.93</td>
<td>57.09, &lt;0.01</td>
<td>0.25, 0.65</td>
<td>2.17, 0.24</td>
</tr>
<tr>
<td>Other Heteroptera</td>
<td>0.24±0.09</td>
<td>0.30±0.11</td>
<td>16.49, 0.03</td>
<td>0.34, 0.60</td>
<td>0.34, 0.60</td>
</tr>
<tr>
<td>Coccinellids</td>
<td>1.05±0.19</td>
<td>1.34±0.23</td>
<td>33.42, 0.01</td>
<td>0.83, 0.43</td>
<td>0.03, 0.87</td>
</tr>
<tr>
<td>Staphylinids</td>
<td>1.30±0.22</td>
<td>1.19±0.21</td>
<td>25.78, 0.01</td>
<td>0.06, 0.83</td>
<td>0.57, 0.50</td>
</tr>
</tbody>
</table>

**PARASITOIDS**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Braconids</td>
<td>4.83±2.18</td>
<td>4.80±2.12</td>
<td>7.22, 0.07</td>
<td>0.58, 0.50</td>
<td>1.66, 0.29</td>
</tr>
<tr>
<td>Ichneumonids</td>
<td>0.19±0.18</td>
<td>1.36±0.24</td>
<td>135, &lt;0.01</td>
<td>5.07, 0.09</td>
<td>7.50, 0.07</td>
</tr>
<tr>
<td>Chalcidoids</td>
<td>15.89±6.53</td>
<td>48.01±22.86</td>
<td>86.58, &lt;0.01</td>
<td>2.13, 0.24</td>
<td>2.63, 0.20</td>
</tr>
<tr>
<td>Mymarids</td>
<td>49.15±9.36</td>
<td>30.34±4.22</td>
<td>25.50, 0.02</td>
<td>4.12, 0.14</td>
<td>7.40, 0.07</td>
</tr>
</tbody>
</table>

**OTHER ARTHROPODS**

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Other Cyclorrhapha</td>
<td>6.54±0.93</td>
<td>8.06±1.04</td>
<td>5.66, 0.10</td>
<td>6.29, 0.09</td>
<td>4.76, 0.12</td>
</tr>
<tr>
<td>Chloropidae</td>
<td>49.66±4.62</td>
<td>45.12±4.92</td>
<td>34.52, 0.01</td>
<td>1.35, 0.33</td>
<td>0.21, 0.68</td>
</tr>
</tbody>
</table>

*df=1,3; bdf=6,60

Acknowledgements

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References

Staphylinids (Coleoptera: Staphylinidae) in genetically modified maize ecosystems: species densities and trophic interactions

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Abstract: In this paper we present results on rove beetles (species, guilds, densities) from a three-year field experiment conducted in Hungary with Bt maize (MON810, Cry1Ab) and its corresponding near isogenic variety. According to our results there were no significant differences in density for species belonging to the non-aphidophagous predator and parasitoid guilds; however the aphidophagous guild showed differences between the two maize varieties in some years. The abundance of aphidophagous staphylinids did not correlate with the total annual and monthly Rhopalosiphum padi density (its prey) in the same year but higher aphid density in one year may have influenced the larval development of rove beetles in that year influencing beetle densities in the following year.

Key words: guilds, interactions, prey, rove beetles

Introduction

A number of studies have been conducted on the potential impacts of insect-resistant Cry1Ab-expressing Bt maize varieties on a diversity of non-target organisms. However, potential short- and long-term effects of Bt maize on soil inhabiting Staphylinids (rove beetles) have received little attention. This issue is critical to the soil biodiversity considerations of insect-resistant GM crops (Saxena et al., 2004). Staphylinidae is one of the largest beetle families and is distributed worldwide in almost all types of ecosystems. Within an ecosystem they also occur in a wide range of habitats and are strongly influenced by the structure of cultural landscapes and different intensities of land-use management (Bohac, 1999; Markgraf and Basedow, 2002). However, rove beetles have not frequently been considered in biological pest management largely due to taxonomic constrain and lack of information on species ecology and prey preferences (Balog et al., 2008; Balog and Marko, 2008). Rove beetles are effective predators in agro-ecosystems since they are dominant in terms of abundance, diversity and predatory activity (Shah et al., 2003). Staphylinids feed on small, soft-bodied insects and insect eggs, and they are important mite and aphid predators. One individual of Tachyporus hypnorum (F.) can consume about 10 to 20 spider-mites and aphids per day (Sunderland et al., 1987; Dennis et al., 1991). Some studies were carried out to investigate the functional role of rove beetles in agro-ecosystems, their response to prey spatial heterogeneity and their prey (aphids and mildew) preferences (Bryan and Wratten, 1984; Good and Giller, 1991; Balog et al., 2008), but their role in maize fields has not been fully investigated.

Previous studies have revealed that the variability in activity–density patterns of the rove beetle fauna was mainly influenced by the year, but no detrimental effects could be attributed to Bt maize. In contrast, imidacloprid-treated maize caused a reduction in species richness of rove beetles, even though the abundance of the main species was not reduced (Farinós et al.,
In other studies carried out in Spain the transgenic variety had more rove beetles at one site in Lleida in 2001, whereas the non-transgenic one exhibited a significantly higher abundance at another site in Madrid in 2000 (de la Poza et al., 2005). A recent meta-analysis by Wolfenbarger et al. (2008) revealed no significant effects of Bt crops on detritivores in general, including five collembolan families or their carabid and staphylinid predators, or on the non-collembolan detritivore families Lathridiidae and Japygidae.

During our study we aimed to assess the potential impact of MON810-transgenic maize on staphylinids by assessing their abundances and trophic interactions in experimental GM and isogenic maize stands in Hungary.

**Material and methods**

The three-year (2001, 2002, 2003) experiment was carried out in an experimental field surrounded by large peach and apricot orchards west of Budapest (47° 25’ N, 18° 47’ E), Hungary. Plots (30m x 30m each) with Bt maize (DK 440 BTY; transformation event MON810) and its near isogenic line (DK 440) were established on chernozem soil and arranged alternately, with 6 replications each. An alley distance of 3 m was used between replications. Maize was planted between late April and early May, and harvested between mid-October and early November, depending on the year. There were no insecticide applications during the experiment (except one row application of 0.75 kg/ha diazinon at planting in the first year). A maize hybrid of similar maturity group to the test hybrid was planted in the retention zone (as a pollen capture crop surrounding the entire test field) in accordance with the requirements of the release permit.

**Insect sampling**

Rove beetles were collected using pitfall traps (300 cm³ in size, 8 cm in diameter, half-filled with 4% formaldehyde solution as killing and preservative). Three pitfall traps were placed in the central part (15th row) of each plot at 10m distance from each other and from the left and right borders of the plot. Sampling lasted from May until harvest, and the traps were emptied weekly. Rove beetles were sorted and identified up to species level with a stereo microscope. Larval stages captured in traps in 2008 were counted for each sampling date without identification to species level.

**Statistics**

The most common staphylinid species were classified into three different guilds: (1) parasitoids, (2) aphidophagous species and (3) predators, mainly non-aphidophagous species. Analyses of variance were performed and similarities were compared using O’Brien and Levene test (Pielou, 1984) to determine whether there were any differences in abundance of guilds between GM and isogenic plots. F and p values were computed using the SPSS software and back-transformed means and p ≤ 95% confidence limits were considered as statistically significant differences.

**Results and discussion**

During the three year survey in MON810 maize and its near isogenic stands a total number of 1745 rove beetle individuals were collected belonging to 21 species (Table 1). The most frequently found species were *Aleochara bilineata, A. bipustulata, Platystethus spinosus* and *Tachyporus hypnorum*. These species accounted for more than 82% of the total individuals.
captured during the study. The three year’s total abundance of the species showed no differences between the Bt and isogenic stands.

Table 1. Species of rove beetles, their distribution by year and relative abundance in Bt maize MON810 compared to the isogenic variety (Iso).

<table>
<thead>
<tr>
<th>Species / year</th>
<th>2001 Bt</th>
<th>2001 Iso</th>
<th>2002 Bt</th>
<th>2002 Iso</th>
<th>2003 Bt</th>
<th>2003 Iso</th>
<th>R (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aleochara bilineata</td>
<td>2</td>
<td>2</td>
<td>21</td>
<td>12</td>
<td>25</td>
<td>3</td>
<td>6.934</td>
</tr>
<tr>
<td>Aleochara bipustulata</td>
<td>2</td>
<td>7</td>
<td>14</td>
<td>13</td>
<td>11</td>
<td>4.527</td>
<td></td>
</tr>
<tr>
<td>Aloconota gregaria</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>2.178</td>
<td></td>
</tr>
<tr>
<td>Amisha analis</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>0.115</td>
<td></td>
</tr>
<tr>
<td>Anotilus nitidulus</td>
<td>6</td>
<td>4</td>
<td>7</td>
<td>7</td>
<td>12</td>
<td>8</td>
<td>2.636</td>
</tr>
<tr>
<td>Drusilla canaliculata</td>
<td></td>
<td></td>
<td>7</td>
<td>4</td>
<td>7</td>
<td>7</td>
<td>1.605</td>
</tr>
<tr>
<td>Heterothops dissimilis</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>7</td>
<td>1</td>
<td>7</td>
<td>1.318</td>
</tr>
<tr>
<td>Lordithon trinotatum</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.057</td>
</tr>
<tr>
<td>Ocypus olens</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>0.630</td>
</tr>
<tr>
<td>Omalium caesium</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>6</td>
<td>3</td>
<td>2.350</td>
</tr>
<tr>
<td>Paederus litoralis</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.115</td>
</tr>
<tr>
<td>Philonthus nitidulus</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
<td>0.344</td>
</tr>
<tr>
<td>Platydracus stercorearius</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.115</td>
</tr>
<tr>
<td>Platystethus spinosus</td>
<td>24</td>
<td>12</td>
<td>1</td>
<td>4</td>
<td>456</td>
<td>646</td>
<td>65.673</td>
</tr>
<tr>
<td>Quedius cinctus</td>
<td>2</td>
<td>0.115</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stenus sp.</td>
<td>3</td>
<td>0.229</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tachinus signatus</td>
<td>4</td>
<td>2</td>
<td>7</td>
<td>1</td>
<td></td>
<td>1.261</td>
<td></td>
</tr>
<tr>
<td>Tachyporus chrysomelinus</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td>4</td>
<td>1</td>
<td>0.344</td>
</tr>
<tr>
<td>Tachyporus hypnorum</td>
<td>1</td>
<td>11</td>
<td>17</td>
<td>29</td>
<td>5</td>
<td>5</td>
<td>5.444</td>
</tr>
<tr>
<td>Xantholinus linearis</td>
<td></td>
<td>14</td>
<td>6</td>
<td>13</td>
<td>18</td>
<td></td>
<td>3.152</td>
</tr>
<tr>
<td>Xantholinus longiventris</td>
<td></td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>4</td>
<td>0.860</td>
<td></td>
</tr>
</tbody>
</table>

| No. of individuals       | 51      | 30       | 91      | 103      | 566     | 759      | 1745    |
| No. of species           | 9       | 5        | 15      | 15       | 14      | 18       | 21      |
Comparing the activity-density of the guilds, significant differences \((p = 0.01)\) were only observed in 2001 and marginally significant differences \((p = 0.05)\) in 2003 (Figure 1).

Figure 1. The activity-density with the three year’s total abundance of different rove beetles guilds in Bt maize MON810 and isogenic maize (Iso) stands.

Comparing Bt with non-Bt maize stands, we found significant difference for the aphidophagous guild in 2002 and a marginally significant \((p = 0.05)\) difference in 2003 (Table 2).

Table 2. The effects of treatments on the abundance \((\pm \text{S.D.})\) of the aphidophagous guild (Welch's modified t test).

<table>
<thead>
<tr>
<th>Year</th>
<th>Bt</th>
<th>Isogenic</th>
<th>Welch t ((P) values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001</td>
<td>7.8 ± 5.6</td>
<td>4.0 ± 1.7</td>
<td>1.05 ((0.342))</td>
</tr>
<tr>
<td>2002</td>
<td>3.7 ± 1.6</td>
<td>8.5 ± 3.6</td>
<td>-3.43 ((0.006))</td>
</tr>
<tr>
<td>2003</td>
<td>78.0 ± 23.4</td>
<td>125.5 ± 49.7</td>
<td>-2.012 ((0.052))</td>
</tr>
</tbody>
</table>

The main potential prey for aphidophagous and predatory rove beetles was the aphid \(R.\) \(padi\). The abundance of \(R.\) \(padi\) was significantly affected by the sampling period. During the vegetation period we often found numerically more aphids in Bt stands. However, the \(R.\) \(padi\) abundance was significantly and marginally significantly higher at the end of the vegetation period in the isogenic stands in 2001 and 2002, respectively. Lumbierres et al. (2004) showed
that *R. padi* benefits from developing on Bt maize (Compa CB1, Syngenta Seeds, Basel, Switzerland, containing the Event Bt176 that expresses the Cry1Ab toxin) at the very young maize developmental stages while their abundance decreased and became numerically lower in Bt maize compared to isogenic maize later in the growing season. The adults (alate and apterous) and young nymphs of *R. padi* were significantly more abundant on the same (Compa CB) transgenic plants than on the isogenic plants in the field study by Pons et al. (2005), particularly on young plants. The performance of aphids on Bt maize probably connected to other factors than the expression of the Cry1Ab toxin (Lumbierres et al., 2004; Eizaguirre et al., 2006) and the main factor affecting the predators of *R. padi* is its abundance on Bt maize and not the very low concentration of Cry1Ab protein in their body (Dutton et al., 2003; Eizaguirre et al., 2006). In our study the abundance of the aphidophagous staphylinid guild did not correlate with the total annual and monthly *R. padi* density in the same year. One explanation for this may be that the higher aphid density may have influenced the larval development of the rove beetles; therefore the influence is higher for catches of adults in the next year. A relatively good correlation was observed between the cumulative abundance of the aphidophagous guild and cumulative aphid density (Figure 2).

![Figure 2](image)

Figure 2. Correlation between the total abundance of *Rhopalosiphum padi* and aphidophagous staphylinids in plots with Bt (black squares) and isogenic (white squares) maize (cumulative dates from 2001, 2002 and 2003).

In similar studies in Bt Compa CB (Cry1Ab toxin, Event Bt176), Farinós et al. (2008) found large temporal variation in the activity-density of rove beetles in the years of collection regardless of the treatment. They stated that numerous other factors rather than the use of Bt-transgenic plants could affect the populations. Significant differences in species richness and diversity due to the year, but not to the treatment were found in other abundant arthropod groups, namely spiders and ground beetles. In the case of rove beetles, differences in species
richness were found due to the lower number of species in the corresponding non-transformed hybrid Dracma with imidacloprid insecticide seed-treatment (Farinós et al., 2008). Furthermore, analysis of the abundance of the three dominant species found (A. aterrima, P. varians and P. nitens) showed that they were not significantly affected by Bt maize (Farinós et al., 2008). Thus, the fluctuation in activity density of rove beetle seems to be the result of several other factors and could not be directly attributed to the presence of the Bt toxin. A decrease in the total number of rove beetles was found by Jasinski et al. (2003), in one of six Bt maize plots (MON810) using sticky traps. They suggested that this result might be an artefact of an insecticide application of permethrin (applied against mites) rather than because of some factor related to the transgenic nature of the plants. According to our results, we consider that the effects of Bt toxins on rove beetles can not be directly evaluated studying only the activity-density and prey preferences of the adults. Larval stages can be more influenced by toxins through its prey thus the effect on adults might be indirect. Further studies are thus needed to clarify the factors that influence the abundance of rove beetles in Bt maize fields.

Acknowledgements

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References


Reduction of damage caused by *Ostrinia nubilalis* Hbn. in south-eastern Poland in 2007 through the cultivation of transgenic maize varieties

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**Abstract:** The objective of the study carried out in 2007 was to evaluate the susceptibility of selected varieties of Bt-transgenic maize (DKC3421YG and Bacilla; transformation event MON 810) and their conventional types without the Cry1Ab protein (DKC3420 and Clarica) to damage caused by the European corn borer (*Ostrinia nubilalis* Hbn.) under the conditions of south-eastern Poland. The study confirmed the high resistance of the Bt maize varieties to damage caused by the caterpillars of this moth. The use of Bt varieties allowed the average reduction of damaged plants in location A (Podkarpackie voivodeship) by 95.2% and damaged cobs by 98.1%. In location B (Lubelskie voivodeship) the number of transgenic plants damaged by caterpillars was reduced by 99.4% and that of cobs by 98.9%. In addition, the level of plant damage was lower: for Bt plants, i.e. the plants hosted significantly lower numbers of caterpillars, and a lower number of holes gnawed by them was observed in comparison to the corresponding non-transformed control maize varieties.

**Key words:** Bt maize, Cry1Ab, European corn borer

**Introduction**

The European corn borer (*Ostrinia nubilalis* Hbn.) is currently the most dangerous maize pest in south-eastern Poland (Lisowicz, 2001). The caterpillars of this pest can cause losses in maize grain yield in local cultivations of 20-30%, and even up to 40% (Lisowicz and Tekiela, 2004). Additional damage, mainly in plant quality, is caused because of the invasion of pathogens inside the damaged tissues, especially by fungi such as *Fusarium*, *Trichothecium*, *Trichoderma* and *Penicillium*, which are responsible for the synthesis and accumulation of mycotoxins inside the plants, which are harmful to humans and animals (Tekiela *et al.*, 2005).

Based on estimates, in 2005 the European corn borer caused a loss in over 40% of the maize cultivation area intended for grain in Poland, which translates into 272,000 hectares, and economic losses associated with caterpillars feeding in that period were estimated at PLN 230 millions (Warzecha and Bereś, 2008).

Large losses in maize yield caused by the European corn borer demand urgent control of this pest. Chemical and biological control methods do not fully protect the plants against damage (Bereś, 2006, 2008; Bereś and Lisowicz, 2005; Lisowicz, 2003).

Since 2006, some Polish farmers have been using one of the most effective methods for the control of *O. nubilalis*, which involves the cultivation of Bt-transgenic varieties carrying the transformation event MON 810. These varieties have a bacterial gene transferred from *Bacillus thuringiensis* Berliner var. *kurstaki*, which is responsible for the synthesis of the Cry1Ab protein toxic to the pest over the entire maize vegetation period.
The objective of the study carried out in 2007 was to evaluate the susceptibility of selected varieties of transgenic maize and their conventional (isogenic) types to damage caused by *O. nubilalis*.

**Materials and methods**

The study was carried out in 2007 in south-eastern Poland in two locations of Podkarpackie (A) and Lubelskie (B) voivodeships. In location A the study was carried out with two transgenic varieties expressing Cry1Ab – DKC3421YG and Bacilla, and the two corresponding non-transformed varieties, i.e. DKC3420 and Clarica. In location B the study was carried out with DKC3421YG and DKC3420.

The level of damage to maize plants caused by caterpillars of the European corn borer at both locations was estimated in two periods:

- **20-23 August 2007**, when plants were at the ripening dough stage (BBCH 85). The percentage of plants and cobs damaged by caterpillars was calculated by the evaluation of 100 consecutive plants in four places of cultivation (in total 400 plants of each variety). Additionally, the number of holes bored by caterpillars and the number of caterpillars feeding on plants below and above the cob and inside the cob was counted. The count was obtained by cross-section of 25 plants from four places of cultivation (in total 100 plants for each variety).
- **3-6 October 2007**, when plants were at the stage of full kernel maturity (BBCH 87). The percentage of stalks broken above and below the cob was calculated (including those lying on the ground) and cobs gnawed at the base (including those fallen on the ground). Analysis was carried out taking 100 consecutive plants from each cultivation place (in total 400 plants for each variety).

The statistical significance of differences between treatment means was analysed using Tukey’s HSD test at a significance level *p*<0.05.

**Results and discussion**

In 2007, weather conditions were favourable for the development of maize and the European corn borer. Results from experiments in location A are provided in Tables 1 and 2, and for location B in tables 3 and 4.

In location A, the European corn borer was highly abundant, and the maternal variety DKC3420 was the most susceptible to damage (Table 1). Caterpillars damaged 72.2% of this variety of plants and 37.2% of cobs. The percentage of stalks broken above the cob was also high and amounted to 30.7%. Stalks broken below the cob, and gnawing of the cob base were particularly harmful to yield level. In this studied variety the caterpillars of the European corn borer caused broken stalks below the cob in 18% of plants and gnawed 3.7% of cobs. The analysis carried out for plants at the ripening dough stage demonstrated that on average 1.75 caterpillars of *O. nubilalis* were feeding on a DKC3420 plant, which gnawed 2.4 holes (Table 2).

The transgenic variety DKC3421YG demonstrated a high resistance to *O. nubilalis* (Table 1). Evidence of pest damage was observed in only 3.7% of plants and was most frequently reflected in small size gnawing inside the stalks. From the analysed plants, stalks broken above the cob were observed in 1.5%, and stalks broken below the cob in 0.5% of plants. Caterpillars damaged 0.7% of cobs in the transgenic variety. However, no stalks
gnawed by the pest lying on the ground were found, nor were the cobs gnawed at the base fallen on the ground. In average 0.12 caterpillars were fond per plant that gnawed 0.37 holes (Table 2).

The conventional variety Clarica was damaged to a lesser extent than DKC3420. Caterpillars damaged 64.2\% of Clarica plants and 30.5\% of the cobs (Table 1). Additionally, caterpillars feeding inside the stalks caused broken stalks above the cobs in 24.0\% of plants and in 17.5\% below the cob. On average, on one plant of the Clarica variety there were 1.67 feeding caterpillars which gnawed 2.1 holes (Table 2).

Bacilla, the Bt-transgenic counterpart to Clarica, demonstrated a high resistance to \textit{O. nubilalis} (Table 1). In this variety, caterpillars damaged 2.75\% of plants and 0.5\% of cobs. Additionally, the number of stalks broken above and below the cob was reduced. The cross-section of plants demonstrated no presence of \textit{O. nubilalis} caterpillars in transgenic plants, but on average 0.29 holes gnawed by them were observed (Table 2).

In 2007 in location A a high number of broken stalks was additionally facilitated by strong winds blowing in September which broke away stalks damaged by the pest.

In location B (Lubelskie voivodeships), the European corn borer was less abundant in 2007. In the conventional variety DKC3420, caterpillars damaged 43.5\% of plants and 23.7\% of cobs (Table 3). Additionally, broken stalks above the cobs were observed in 17.5\% of plants and broken stalks below the cob in 6.0\%. On average 1.1 caterpillars and 1.5 holes were recorded per plant (Table 4).

In plants of the transgenic DKC3421YG variety only 0.2\% of plants were damaged (Table 3). A similar low percentage damage to cobs, and stalks broken above the cobs were observed. The cross-section of plants demonstrated no presence of \textit{O. nubilalis} caterpillars, but on average 0.04 holes per plant were found (Table 4).

Table 1. Damage caused by \textit{O. nubilalis} to selected Bt and non-Bt maize varieties in location A (Podkarpackie voivodeship) in 2007.

<table>
<thead>
<tr>
<th>Variety</th>
<th>% damaged plants</th>
<th>% damaged cobs</th>
<th>% of broken stalks below cob</th>
<th>% of stalks fallen on the soil</th>
<th>% of cobs gnawed at the base</th>
<th>% of cobs gnawed at the base fallen on the soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>DKC3420</td>
<td>72.2 a</td>
<td>37.2 a</td>
<td>18.0 a</td>
<td>3.2 a</td>
<td>30.7 a</td>
<td>3.7 a</td>
</tr>
<tr>
<td>DKC3421YG*</td>
<td>3.7 b</td>
<td>0.7 b</td>
<td>0.5 b</td>
<td>0.0 b</td>
<td>1.5 b</td>
<td>0.2 b</td>
</tr>
<tr>
<td>CLARICA</td>
<td>64.2 a</td>
<td>30.5 a</td>
<td>17.5 a</td>
<td>1.5 ab</td>
<td>24.0 a</td>
<td>1.0 b</td>
</tr>
<tr>
<td>BACILLA*</td>
<td>2.7 b</td>
<td>0.5 b</td>
<td>0.5 b</td>
<td>0.0 b</td>
<td>1.2 b</td>
<td>0.0 b</td>
</tr>
</tbody>
</table>

* transgenic variety

Mean values marked with the same letter do not differ significantly (P = 0.05, Tukey's HSD).
Table 2. Average number of holes and caterpillars per plant in location A (Podkarpackie voivodeship) in 2007.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Number of holes in stalk</th>
<th>Total in plant</th>
<th>Number of caterpillars in stalk</th>
<th>Total in plant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>below cob</td>
<td>above cob</td>
<td>below cob</td>
<td>above cob</td>
</tr>
<tr>
<td>DKC3420</td>
<td>1.15</td>
<td>1.0</td>
<td>0.25</td>
<td>2.40 a</td>
</tr>
<tr>
<td>DKC3421YG*</td>
<td>0.25</td>
<td>0.10</td>
<td>0.02</td>
<td>0.37 b</td>
</tr>
<tr>
<td>CLARICA</td>
<td>1.0</td>
<td>0.90</td>
<td>0.20</td>
<td>2.10 a</td>
</tr>
<tr>
<td>BACILLA*</td>
<td>0.09</td>
<td>0.15</td>
<td>0.05</td>
<td>0.29 b</td>
</tr>
</tbody>
</table>

* transgenic variety
Mean values marked with the same letter do not differ significantly (P = 0.05, Tukey's HSD).

Table 3. Damage caused by *O. nubilalis* to selected Bt and non-Bt maize varieties in location B (Lubelskie voivodeship) in 2007.

<table>
<thead>
<tr>
<th>Variety</th>
<th>% damaged plants</th>
<th>% damaged cobs</th>
<th>% of broken stalks below cob</th>
<th>% of stalks below cob fallen on the soil</th>
<th>% of cobs gnawed at the base</th>
<th>% of cobs fallen on the soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>DKC3420</td>
<td>43.5 a</td>
<td>23.7 a</td>
<td>6.0 a</td>
<td>0.2 a</td>
<td>17.5 a</td>
<td>0.2 a</td>
</tr>
<tr>
<td>DKC3421YG*</td>
<td>0.2 b</td>
<td>0.2 b</td>
<td>0.0 b</td>
<td>0.0 a</td>
<td>0.2 b</td>
<td>0.0 a</td>
</tr>
</tbody>
</table>

* transgenic variety
Mean values marked with the same letter do not differ significantly (P = 0.05, Tukey's HSD).

Table 4. Average number of holes and caterpillars per plant in location B (Lubelskie voivodeship) in 2007.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Number of holes in stalk</th>
<th>Total in plant</th>
<th>Number of caterpillars in stalk</th>
<th>Total in plant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>below cob</td>
<td>above cob</td>
<td>below cob</td>
<td>above cob</td>
</tr>
<tr>
<td>DKC3420</td>
<td>0.50</td>
<td>0.90</td>
<td>0.15</td>
<td>1.55 a</td>
</tr>
<tr>
<td>DKC3421YG*</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
<td>0.04 b</td>
</tr>
</tbody>
</table>

* transgenic variety
Mean values marked with the same letter do not differ significantly (P = 0.05, Tukey's HSD).

The results from this study are similar to those obtained by Bereś and Gabarkiewicz (2008) and Twardowski et al. (2008), who studied the resistance of transgenic varieties to feeding *O. nubilalis* caterpillars in Poland.
Conclusions

1. In 2007, the European corn borer was highly abundant in Podkarpackie voivodeship (location A), where the pest damaged, on average 68.2% of plants and 33.8% of cobs in the two conventional maize varieties.

2. The corresponding Bt-transgenic varieties demonstrated high resistance to the pest. The use of varieties with the Bt gene allowed the reduction of the percentage of damaged plants in location A on average by 95.2%, and damaged cobs by 98.1%. In location B the number of transgenic plants damaged by caterpillars was reduced by 99.4% and that of cobs by 98.9%.

3. In the areas with high abundance of *O. nubilalis* (southern Poland), Bt-transgenic maize varieties resistant to this pest are thus a viable alternative to chemical and biological pest control methods.

References


A perspective on problem formulation and exposure assessment of transgenic crops

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Abstract: Risk assessment is a science-based decision making process. When risk assessment follows an established framework, it allows for transparency, predictability, and consistency in the regulatory process. Problem formulation is a critical first stage in the risk assessment process; it involves 1) the characterization of the transgenic plant and receiving environment, 2) definition of harm, 3) identification of potential exposure pathways or potential harm, and 4) establishment of assessment endpoints to evaluate the potential for harm based on the plant’s characteristics. Risk to non-target organisms can be defined as the co-occurrence of hazard (or toxicity) and exposure. Exposure characterization has often been overlooked in the literature regarding the risk assessment of transgenic plants, with many recent publications focusing only on the hazard portion of the risk assessment equation. Exposure assessment informs the risk assessment and assists in the determination of which types of non-target organisms should be tested in hazard characterization. The purpose of risk assessment is to provide a framework for efficient decision-making, rather than to generally increase scientific knowledge; therefore data collection for risk assessment must be directed toward answering specific questions identified in the problem formulation stage.

Key words: ecological risk assessment, genetically modified crops

Problem formulation

Ecological risk assessment (ERA) uses science to answer questions about the safety of a product or action in the environment. For transgenic crops, this process is used to quantify the risk of introducing the genetically modified plant into the environment. One such risk is the potential impact of insecticidal transgenic crops on non-target organisms. Risk is defined as the likelihood of harm to be manifested under environmentally relevant conditions; thus risk is a function of the likelihood of the co-occurrence of hazardous effects caused by the GM plant and exposure of non-target organisms to the transgenic protein contained in the plant tissue.

Guidance outlining the general principles for conducting ecological risk assessments has been available for over a decade (U.S. EPA 1992, 1998); the guidance establishes ecological risk assessment as a science-based decision-making process in which the risk of introducing a new product or activity into the environment is quantified using a well-defined, hypothesis-driven process. In this light, ERA is clearly distinguished from exploratory or basic research, i.e. research for its intrinsic value, in that ERA follows a prescribed process that is designed to answer very specific pre-determined questions about risk (Raybould, 2006). Additionally, the framework distinctly outlines the process of risk assessment, which includes three main stages: problem formulation, analysis, and risk characterization (shown in Figure 1). Use of an established framework benefits regulators, applicants, and stakeholders by providing transparency, predictability, and consistency.
The first, and often forgotten, stage in risk assessment is problem formulation. Problem formulation is multi-faceted; risk assessors consider the context of the proposed product or action and the many factors that play into that context, e.g. characteristics of the stressor(s), the ecosystem potentially at risk, and management goals. Throughout the problem formulation process, a series of questions should be asked, with the central focus being identification of potential problems and defining harm in the context of the proposed product and ecosystem.

**Figure 1. Process of ecological risk assessment, and main steps for problem formulation.**

**Stressor characteristics and ecosystem context**
To answer questions about risk, certain components of the system are critically analyzed. What are the characteristics of the stressor? Considerations include identification of the stressor(s), its intensity, frequency, and duration in the environment, and temporal and spatial considerations that may be relevant to biological cycles. In the case of genetically modified plants, the “stressor” would be the transgenic protein in the GM plant, assuming all else is equivalent to the non-transgenic conventional plant. When outlining intensity, duration, and spatial importance, one should consider the trait that is being introduced, the promoter used for expression of the transgene, and subsequently the concentration of expressed protein in various plant tissues, as well as geographic range of crop production. Following these considerations, movement of the transgenic plant tissues and proteins should be considered, so as to predict potential routes of exposure of non-target organisms to the protein.

Existing literature can often provide insight to determine if further studies are needed based on a tiered testing system (Garcia-Alonso et al., 2006). During product development data are often generated that can inform problem formulation; e.g. an insecticidal protein may show activity on lepidopteran pests and not on coleopteran pests during early product screening, therefore it is more efficient to focus the ecological risk assessment on non-target lepidopterans and closely related species. A literature review and examination of available data can provide meaningful information throughout the problem formulation process (Garcia-Alonso et al., 2006).
The context of the ecosystem should be considered when identifying the habitats, species, and/or ecological processes that are potentially at risk. For example, when assessing the risk of a proposed logging activity, alteration of a natural system (forest) and immediate surrounding ecosystems are likely to be considered. In the case of transgenic crops, the context would be the agroecosystem and possibly semi-natural systems adjacent to the agricultural land. Further considerations include stressor-ecosystem interactions and properties of the ecosystem that may impact the stressor; for example, farming practices, soil characteristics including temperature, soil texture and organic matter content, pH, and microflora activity are likely to greatly and quickly reduce concentrations of transgenic proteins in the soil, thereby decreasing potential exposure to soil organisms.

Setting management goals, assessment endpoints, and defining harm

To be successful, ecological risk assessments must be scientifically valid, as well as relevant to public concerns and regulatory needs. Risk managers are charged with protecting societal values, and help to ensure that the ERA will provide relevant information to aid in making decisions on the issue under consideration (e.g. cultivation of transgenic crops). Management goals should be defined in the context of the proposed stressor and ecosystem; this is often best achieved following the development of a conceptual model (see next section). Establishment of management goals and definition of harm in the course of problem formulation leads to identification of assessment endpoints, those explicit expressions of the actual environmental value that is to be protected. Measurement endpoints are measureable responses to a stressor that are related to the assessment endpoint. An example of each can be found in the case of Karner blue butterflies; Karner blues are endangered in the U.S., and concern was expressed that these endangered populations might be exposed, and potentially negatively impacted by transgenic maize expressing lepidopteran-active insecticidal proteins (Peterson et al., 2006). Since Karner blues are protected organisms, they cannot be evaluated in laboratory testing; an alternative to this could be testing of another member of Lycaenidae (surrogate species concept). In this case example, the assessment endpoint would be population levels of Karner blue butterflies that might be exposed to transgenic maize; the measurement endpoint could be increased mortality (e.g. significantly greater than isoline control) of a surrogate species, as measured in a laboratory assay. Likewise for exposure assessment, a measurement endpoint could be determination of the geographic range of the Karner blue, paired against likelihood of temporal overlap with pollination and long-range pollen dispersal from nearby maize fields, as described in Peterson et al. (2006).

Measurement endpoints must align with the definition of harm as outlined in the problem formulation process. Endpoints must be sensitive, timely and relevant to the characteristics of the stressor (transgenic trait), as well as the characteristics of the ecosystem. For example, in the case of non-target Lepidoptera exposed to insecticidal proteins, our management goal is related to population levels of the organisms; an endpoint relevant to the management goal would be increased mortality or large decrease in reproduction following exposure to the protein. Other measurement endpoints such as biochemical change in a surrogate (e.g. decreased cholinesterase activity or protein binding to a receptor) are not necessarily equal to a population change in the assessment species.

An adequate definition of harm must be established to ensure success of the environmental risk assessment. Based on the characteristics of the transgenic crop, what types of effects are of concern? Based on the characteristics of the ecosystem and other confounding stressors already present, what degree of effect is of concern? For example, in the case of an acutely toxic insecticidal protein, mortality as an endpoint may make sense; for a protein inhibiting feeding or absorption in the gut, a non-recoverable 50% decrease in
growth may be a pertinent endpoint. Establishment of a relevant definition of harm allows for a determination of hazard; when this is paired with an exposure assessment, risk can be adequately characterized. Tangible outcomes of the problem formulation process are identification of assessment endpoints and subsequent formulation of a conceptual model and relevant testable hypotheses; these steps are followed by the establishment of the analysis plan, which identifies data needed to inform the risk assessment (Raybould, 2006; Romeis et al., 2008).

**Developing a conceptual model, generating risk hypotheses, and determining an analysis plan**

Assessment endpoints are best determined following careful consideration of trait-crop and ecosystem characteristics; once those characteristics are understood, what are the concerns? A conceptual model considers possible sources of the stressor, including potential transport routes, partitioning and bioavailability of the stressor in the ecosystem, and degradation of the stressor. With these clearly defined potential routes of exposure, the conceptual model also identifies non-target organisms that potentially may be exposed, thus further defining the assessment endpoints. Next, hypotheses are identified based on the factors that are most likely to contribute to risk; the hypotheses then guide the approach for the analysis phase, including the determination of the types of data and analytical tools needed, and experiments to be conducted. This prescribed method of problem formulation is in keeping with the case-by-case approach to ecological risk assessment that is utilized worldwide (e.g. U.S. EPA, 1992; Raybould, 2006; Garcia-Alonso et al., 2006; Romeis et al., 2008). Conceptual models should be developed for each new trait-by-crop combination, thus fitting the case-by-case approach.

An excellent example from Prihoda and Coats (2008; summarized in Figure 2) details a conceptual model for potential exposure of aquatic systems to transgenic maize. Insecticidal proteins from transgenic maize could enter the aquatic system through several routes, including pollen shed, crop dust during harvest, or runoff or leaching from soil or post-harvest crop residue. Subsequently, aquatic organisms could be exposed through several functional pathways, including filter-feeding, ingestion of detritus, or indirectly through prey. Following problem formulation and considering the characteristics of the proposed product and ecosystem, the conceptual model is used to identify which hypotheses are most likely to contribute to risk (shown in highlighted boxes, Figure 2). In the case presented here, the most probable and potentially significant input of insecticidal protein from Bt maize is likely to result from crop residue runoff into a stream. Based on protein characteristics, bioaccumulation in prey is unlikely, therefore the most concerning exposure would be to filter-feeding or detritivorous arthropods. From these deductions, testable hypotheses can be formed.

Example hypotheses would be 1) protein X from crop residue enters the aquatic system and is rapidly degraded (e.g. DT₅₀ (time to 50% dissipation) <3 days) and 2) filter feeding insects and detritivores will not be adversely affected by concentrations of protein X found in plant tissue (e.g. H₀: treatment mortality = control). Subsequently, an analysis plan can be formed to test such hypotheses. For exposure assessment (hypothesis 1), a first step would be to quantify, or provide informed estimates of, crop residue transport out of maize fields; the next important step would be to determine the fate and concentrations of non-degraded protein in aquatic systems (water column versus detritus/sediment) from crop residue. The latter study could be performed using microcosms in the laboratory. For hazard characterization (hypothesis 2), studies could be performed in which a representative filter feeder (e.g. Tier 1 *Daphnia* test or *Chironomus* spp.) and a representative detritivore (e.g. amphipod) could be assayed at worse-case concentrations of protein. Once hypotheses have
been identified and appropriate tests chosen, the second phase of ERA (data generation and analysis) can commence.

Figure 2. Conceptual model of potential transport of insecticidal proteins from transgenic maize to aquatic systems, and possible exposure pathways for aquatic invertebrates (summarized from Prihoda and Coats, 2008). Hypotheses most likely to contribute to risk are outlined in bold.

**Exposure Assessment — A Case Study**

*Introduction*
Exposure assessment, as previously mentioned, is one half of the risk equation. Of late, many published reports have focused on the hazard, or toxicity testing, portion of the risk equation, but few have addressed the significance of exposure. Determination of environmental exposure concentrations allows for a comprehensive assessment of potential risk when coupled with information on hazard. Exposure assessment reveals the relevant sources and pathways of a stressor, as it seeks to measure how much of a stressor can be absorbed by an exposed organism, and how much of the absorbed amount is actually biologically active and available to produce a biological effect (Garcia-Alonso *et al.*, 2006). Exposure assessment can be defined as the process of estimating the intensity, frequency and duration of exposure to a stressor, along with the characteristics of the population exposed.

In the course of registering maize events containing coleopteran-active Cry proteins, applicants have been faced with questions regarding the concern for potential effects of the Cry protein on predatory coccinellids. Following the problem formulation and exposure assessment schema, applicants consider potential sources of the protein, its bioavailability in the environment, potential for bioaccumulation in prey species, and degradation or transformation in the prey species. Further considerations include concentration of protein
within the plant at critical points in the life cycle of prey, and temporal and spatial distribution of prey and predators. In the course of developing a conceptual model (Figure 3), we identified mites and mite-feeding coccinellids as a potential tri-trophic pathway that could contribute to risk to non-target coccinellids. A case study focused on this tri-trophic pathway is described herein.

The product in question is transgenic maize containing Cry34/35Ab1 proteins that result in insecticidal activity against coleopteran pests, specifically corn rootworm (e.g. *Diabrotica virgifera virgifera*). The key concern, as previously described, was whether mite-feeding predators (e.g. lady beetle *Stethorus punctillum*) could potentially be harmed by being exposed to increased levels of Cry34/35Ab1 as a result of bioaccumulation. As part of the problem formulation process, literature and data reviews were conducted. Previous work had shown lack of effects of Cry34/35Ab1-containing pollen on growth, development, and mortality in a known pollen-feeding coccinellid, *Coleomegilla maculata* (data not presented).

What route could potentially result in increased exposure greater than direct feeding on pollen? We hypothesized that exposure could be increased if the proteins bioaccumulated in prey. A survey of bioaccumulation literature showed that chemicals known to bioaccumulate are typically hydrophobic, may be heavily halogenated, or contain cyclic hydrocarbons (e.g. Kenaga and Goring, 1980; Meylan et al., 1999); chemical characteristics indicate proteins are not likely to bioaccumulate. However, some previous studies have reported high concentrations of Cry proteins in mites (Obrist et al., 2006; Álvarez-Alfageme et al., 2008). Based on the literature review, our final exposure hypothesis was Cry34/35Ab1 does not bioaccumulate in mites feeding on 59122 maize; we subsequently designed an experiment to test this hypothesis, with the measurement endpoint being concentration of insecticidal protein in mites fed on transgenic maize.

**Case study methods**

Event 59122 maize and near-isoline maize were grown in the laboratory at room temperature under ambient lighting. At the V3 stage, adult mites (*Tetranychus urticae*) were used to infest event 59122 and near-isoline maize. After 24 and 48 hours of feeding on event 59122 and near-isoline maize, mites (0.2 – 0.5 mg total wet wt) were then manually removed from plants, and weighed into 1.2-ml tubes. *T. urticae* adults were extracted closely following the methods outlined in Álvarez-Alfageme et al. (2008). Mites were extracted in chilled sterile phosphate buffered saline using a GenoGrinder (SPEX CertiPrep, Metuchen, NJ, USA) by shaking for two, 30-second cycles at 1500 strokes per minute. Samples were centrifuged at
4000 rpm for 10 min. at 4°C. The supernatant was transferred into sterile, 1.5-ml centrifuge tubes and centrifuged further at 12,000 x g for 5 min. at 4°C. Extracts were analyzed for concentrations of Cry34Ab 1 and 35Ab1 using a quantitative western blot technique. The upper and lower limit of quantification (ULOQ and LLOQ) for this method were 24 ng and 0.75 ng; therefore, the corresponding LLOQ for Bt Cry34Ab1 and Bt Cry35Ab1 in the mite extract was <0.05 µg/ml.

Case study results and discussion
There was no Bt Cry34Ab1 or Bt Cry35Ab1 detected in extracts of mites feeding on near-isoline control maize or event 59122 maize after 24 and 48 hours. In order to fall below the LLOQ, the concentration of Bt Cry34Ab1 and Bt Cry35Ab1 in mites would have to be less than 74 ng/mg, assuming 0.5 mg mites (wet weight) are extracted. The event 59122 leaf tissue used in the present study had a Bt Cry34Ab1 concentration at the V3 stage of 43 ng/mg (wet weight).

Results from this study indicate that T. urticae feeding on event 59122 maize do not ingest Bt Cry34/35Ab1 proteins in great enough quantities to be detected using an appropriate analytical method. Based on the LLOQ, the concentration of Bt Cry34/35Ab1 was less than 74 ng/mg (wet weight), which results in a maximum bioaccumulation factor of <1.7. In general, bioaccumulation factors are considered meaningful only if they are greater than 10 or even 100. The results from this study indicate that Bt Cry34/35Ab1 exposure to predators of T. urticae in the field would be negligible. Since T. urticae do not bioaccumulate Bt Cry34/35Ab1 proteins after 48 hours of feeding on event 59122 maize, direct feeding on pollen would present a higher exposure scenario than that of predators feeding on T. urticae in the field. The results of this exposure analysis indicate that the initial study conducted with a coccinellid feeding directly on pollen is the most relevant hazard evaluation. Further, based on the results from that assay, it is unlikely that transgenic maize containing Cry34/35Ab1 proteins would result in harm to coccinellid populations in maize fields. This case study outlines the critical steps important for success in problem formulation and exposure assessment: development of a conceptual model, literature and data reviews, and finally development of risk hypothesis and an analysis plan to test that hypothesis.

Conclusions
Properly performed problem formulation identifies areas of concerns and questions about the safety of a proposed product or action. Development of risk hypotheses, as part of the problem formulation step, allows for organized data collection. Following a logical stepwise approach to risk assessment, which includes the important problem formulation phase, helps to ensure consistency and transparency, and is conducive to harmonization of testing methods. As part of the risk assessment process, exposure assessment helps to identify needs for hazard studies, including appropriate test species and route of exposure (e.g. pollen-feeding coccinellid vs. predatory species), and finally exposure concentration, which helps guide planning of appropriate hazard tests.

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Absence of Cry1Ab resistance in a Spanish *Ostrinia nubilalis* population from an infested greenhouse

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**Abstract:** Transgenic corn expressing *Bacillus thuringiensis* (Bt) toxin Cry1Ab has been planted in Spain to control corn borers as *Ostrinia nubilalis* (Hübner), since 1998. Indeed, 79 thousand hectares have been planted to Bt-maize in different Spanish areas in 2008. The high selective pressure may produce the development of resistance in populations of the target pest, decreasing the effectiveness of the transgenic crop. The selection may be even higher in neighbour crops in which *O. nubilalis* is a pest and where it is controlled by conventional Bt-spray products containing Cry1Ab.

A sample of *O. nubilalis* was collected from a greenhouse in south-eastern Spain that was suffering high levels of infestation after repeated treatments with different Bt products. Insects were brought and reared in our laboratory and their susceptibility to activated Cry1Ab toxin, Cry1Ab protoxin and the Bt standard HD-1-S-2005 product was tested. As a susceptible control, insects from France kept in the laboratory for more than 10 years without exposure to Bt were used. The “effective growth inhibition” was recorded seven days after treatment and accounted for both dead larvae and larvae which not passed the first instar. PROBIT analyses of the data revealed no significant different response between the strains to activated Cry1Ab toxin and to HD-1-S-2005. Cry1Ab protoxin showed 7-fold lower activity in the laboratory strain when compared to the field strain. These data suggest an absence of a relevant shift in the resistance to Cry1Ab in the insects from the field strain as compared to the laboratory one, and point to a deficient Bt product application in the greenhouses.

**Key words:** *Bacillus thuringiensis*, resistance

**Introduction**

*Bacillus thuringiensis* (Bt) is a Gram positive bacteria, commonly found in the soil that produces some crystal proteins during the sporulation which are toxic to the larvae of several species of insects (Schnepf *et al*., 1998). These toxins have been widely used in agriculture, as formulations based on a mix of spores, parasporal crystals and additives, or by inserting the genes codifying for the insecticidal Cry proteins in the germplasm of cultivated plants, to make them resistant to the insect pests. Among the Cry toxins, the family Cry1 is highly effective against Lepidopteran pests (van Franzenhuyzen, 2009). Particularly, Cry1Ab is widely used in the control of the European Corn Borer (ECB) *Ostrinia nubilalis* (Hübner) that is the key pest of corn in Europe and North America. While corn is the preferred host plant of ECB, this insect species is highly polyphagous, attacking a wide variety of herbaceous plants (like peppers, beans, weeds, etc.). Control of ECB is problematic with non-systemic pesticides, such as Bt-based formulations. In general, larvae mine tunnels into the stalk few days after hatching and complete their development inside the plants. Consequently, the larvae are vulnerable to Bt formulations only during few days after hatching. This is in contrast to Bt plants expressing Cry1Ab in each tissue constitutively, providing permanent protection from boring larvae (Koziel *et al*., 1993). In case of the Bt-sprays, the susceptible
larva ingests the formulation, the parasporal crystal is released, and the alkaline midgut environment solubilises the crystal. The protoxins are activated by midgut proteases and the activated toxins can pass through the peritrophic membrane. Cry proteins recognize and bind specific receptors located on the brush border membrane of the epithelial cells, insert in the membrane and form pores that lead to the cell death because of osmotic lysis (Bravo et al., 2007). In contrast, the toxins produced by the genetically modified plants are provided directly in the activated form.

Resistance of the target pest to the Bt toxin is the worst threat for the successful use of the Bt technology, either as formulations or transgenic plants. So far, resistant field populations have only been reported for two Lepidopteran species (Tabashnik et al., 1990; Jannmaat and Myers, 2003). Nevertheless, the potential of the lepidopteran larvae to evolve resistance has been well studied and several resistant colonies of a number of species have been selected in laboratory, *O. nubilalis* among them (Huang et al., 1997). Bt resistance can evolve at each of the activation steps, but the most commonly characterized mechanism for higher levels of resistance is the lack of the toxin binding to the receptors located in the epithelial membrane (Ferré and Van Rie, 2002).

Spain is the only biotech mega-country in Europe and Bt corn is commercialized and cultivated since 1998, reaching 80,000 ha in 2008 (James, 2008). Until 2002 only the event Bt176, that expressed the Cry1Ab toxin only in the green tissues and pollen (Koziel et al., 1993), was available. Since 2002 event MON810 is used that expresses the cry1Ab gene under control of a constitutive promoter. Bt corn is grown mainly in the North of Spain: in the regions of Aragon (31,857 ha) and Catalonia (25,298 ha), where Bt varieties are grown on almost 90% of the whole corn area. Monitoring the changes in the susceptibility of ECB individuals from field is thus necessary to preserve the effectiveness of the Bt-based technologies and to design management strategies to delay the appearance of resistance. So far, the screening of field populations from different corn areas of Spain has resulted in no apparent changes in the susceptibility (Farinós et al., 2004). However, no report of *O. nubilalis* populations exposed to Bt on other crops has been published.

The present work reports the baseline susceptibility to the protoxin, to the activated Cry1Ab form and to the standard formulate HD-1-S-2005 of an *O. nubilalis* population from a Spanish greenhouse sprayed with Bt-formulations versus a control laboratory strain.

**Material and methods**

**Insect rearing**

*O. nubilalis* larvae were collected from an high infested greenhouse located in the province of Alicante (S.E. Spain) that were frequently and exclusively treated with Bt-based bioinsecticides. A total of 140 *O. nubilalis* larvae were collected from damaged peppers (from 1 to 3 larvae per plant), and they were taken to the laboratory to establish the colony PH. A control (10 years without Bt exposition) laboratory colony (France) was obtained from INRA (Le Magneraud, France). Pupae were desinfected with bleach solution (1% v/v) during 2 min., rinsed and placed at 19°C. Larvae were reared on an artificial maize diet (Lozzia and Manachini, 2003) until pupation in a controlled environment chamber at 25°C, with a photoperiod of 16:8 (L:D) hours and 60% relative humidity.

**Bt toxins**

Cry1Ab was produced as inclusion body from a recombinant *Escherichia coli* PDB140 strain supplied by R.A. de Maagd (Wageningen, The Netherlands), following the protocol described by Herrero et al. (2004). The protein was solubilised in carbonate buffer with DTT 10mM
(pH 10.5). To activate the protoxin, total proteins of the preparation were measured by Bradford assay (1976) and trypsin (Sigma, St. Louis, MO) was added (1 mg each 10 mg of total protein). The solution was incubated during two hours at 37°C and then centrifuged at 21000 x g at 4°C for 10 min. The toxin and protoxin were quantified by SDS-PAGE/densitometry, using BSA as standard. The formulation Standard Bt HD-1-S-2005 was obtained as a powder from Valent Biosciences and dissolved in carbonate buffer (pH 10.5). This formulation is based on B. thuringiensis var. kurstaki HD1 strain and it is used to standardize the calculation of potency of Bt products.

**Bioassays**

Two ml of diet were placed in each cell of a 128-cell bioassay tray (2 cm²/cell) and 50 µl of the product solution were added to the surface, including carbonate buffer without toxin as control. Cry1Ab toxin and protoxin were supplemented to the surface of the diet at seven different doses with protein concentration ranges of 0.06-45 ng/cm² and 0.003-7 ng/cm², respectively. The formulate Standard Bt concentrations ranged from 0.11-81 ng/cm². One neonate larva (<24 h old) was placed in each well and 16 larvae were used for each dose. The experiments were performed, at least, in duplicate. Bioassay trays were placed in a controlled environment chamber at 25°C, with 60% relative humidity at constant darkness. The effective growth inhibition (GI₅₀) was scored for dead larvae and for the ones which remain in the first stage after seven days, and analyzed with Probit analysis (Finney, 1971) using the POLO-PC program (LeOra Software, Berkeley, CA). Absence of significant differences in the bioassays slopes calculated by Probit analysis allowed the potency calculations to compare the susceptibility between the O. nubilalis populations.

**Results and discussion**

The field-collected (PH) and laboratory (France) O. nubilalis colonies showed a high susceptibility to all tested products (Table 1) as has been described previously (Li et al., 2005; Farínós et al., 2004). Both colonies exhibited a similar sensitivity profile; the protoxin was the most active and activated toxin and HD-1-S-2005 being about 3 to 10-fold less active. However, direct comparisons should be analyzed carefully since part of HD-1-S-2005 and the protoxin are not active components. The rate of response to the increase of the dose reflected by the slope was similar for both O. nubilalis strains in all tested compounds. This fact allowed the calculation of the potencies between the insect colonies for each product. No significant differences in susceptibility were obtained for activated Cry1Ab toxin and HD-1-S-2005, however a 7-fold increase in susceptibility to the Cry1Ab protoxin was found in the PH colony (Table 1).

Increased susceptibility in the O. nubilalis PH colony was not expected. Insects were collected from a Bt-treated greenhouse, in which common pests susceptible to Bt were absent, suggesting the effectiveness of the Bt-sprays and therefore some level of resistance in the O. nubilalis population. The slight intraspecific changes in susceptibility can be attributed to natural variability (González-Nuñez et al., 2000).
Table 1. Susceptibility of *O. nubilalis* larvae from the greenhouse-collected (PH) and susceptible control (France) colonies to Bt Cry1Ab toxin and protoxin, and to a standard formulate product.

<table>
<thead>
<tr>
<th>Colony</th>
<th>Toxin/formulation</th>
<th>n</th>
<th>Slope</th>
<th>GI50*</th>
<th>Potency**</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH</td>
<td>Activated Cry1Ab</td>
<td>245</td>
<td>1.9 ± 0.4</td>
<td>1.4 (0.5 -2.4)</td>
<td>Not significantly different</td>
</tr>
<tr>
<td>France</td>
<td>Activated Cry1Ab</td>
<td>246</td>
<td>1.9 ± 0.3</td>
<td>2.1 (1.2 -3)</td>
<td></td>
</tr>
<tr>
<td>PH</td>
<td>Cry1Ab protoxin</td>
<td>206</td>
<td>2.7 ± 0.6</td>
<td>0.1 (0.05 – 0.13)</td>
<td>0.13 (0.08 – 0.23)</td>
</tr>
<tr>
<td>France</td>
<td>Cry1Ab protoxin</td>
<td>254</td>
<td>2.0 ± 0.3</td>
<td>0.7 (0.5 – 1)</td>
<td></td>
</tr>
<tr>
<td>PH</td>
<td>HD-1-S-2005</td>
<td>256</td>
<td>1.6 ± 0.2</td>
<td>1.6 (0.7 – 3.1)</td>
<td>Not significantly different</td>
</tr>
<tr>
<td>France</td>
<td>HD-1-S-2005</td>
<td>253</td>
<td>1.2 ± 0.2</td>
<td>2.9 (1.7 – 4.6)</td>
<td></td>
</tr>
</tbody>
</table>

*Concentrations expressed in ng cm⁻², with 95% fiducial limits in parentheses.

** Potency ratio between PH and France strains, with 95% fiducial limits in parenthesis.

Even if several Lepidoptera populations have successfully responded to laboratory selection for Bt resistance, the selection pressure exercised by transgenic crops in the field is stronger because of the high concentrations of Bt toxins in the plants during the whole cropping season (Chafaux *et al.*, 2001). On the other hand, when ECB larvae are controlled by Bt formulations the insects could be exposed to sublethal doses (similarly to the laboratory selection), making the appearance of resistance more likely. To date, one case of resistance to Bt that evolved in the greenhouses has been described in the literature (Janmaat and Myers, 2003). There is a potential that Bt tolerant ECB individuals could be selected in the greenhouses sprayed with Bt formulations and then move to the field where corn expressing Cry1Ab is grown. There they might take advantage from the first greenhouse selection to evolve resistance to the high-dose plants. However, so far no resistant ECB individuals selected in laboratory could survive by feeding on Bt corn tissue with high Cry1Ab expression levels but a resistant strain from the US exhibited the ability to survive on transgenic corn by feeding on tissue showing low Cry1Ab expression (Crespo *et al.*, 2009).

Susceptibility data indicate that no ECB individuals tolerant to Bt were selected in the greenhouses of Alicante. Absence of *Helicoverpa* and *Spodoptera* pests indicated the effectiveness of the Bt formulations employed. Differently from these defoliator pests, ECB is a borer, meaning that neonate larvae are tunnelling the stalk of the herbaceous host plants, to complete larval development inside the plants. To be useful against boring pests, Bt formulations have to be sprayed soon after egg hatching to reach the larvae before they bore into the plant. Desynchronization of the Bt spray applications and the hatching of the larvae may explain the observed high ECB infestation in the greenhouse.

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EFSA’s activities on the environmental risk assessment of GM plants

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Abstract: The European Food Safety Authority (EFSA) plays a central role in the risk assessment of genetically modified (GM) plants in the European Union by providing (1) independent science-based advice on the safety of GM plants and derived food and feed products, and (2) risk assessment guidance to assist applicants in the preparation and presentation of their GM plant market authorisation applications. The EFSA’s scientific panel on genetically modified organisms (GMO Panel) has taken several initiatives to consider the latest experience gained, as well as technological progress and scientific developments made in the field of the risk assessment of GM plants and derived food and feed products. In this respect, the EFSA GMO Panel is currently in the process of revising the environmental sections of its guidance document for the risk assessment of GM plants and derived food and feed products.

Key words: EFSA, environmental risk assessment, GM plants, guidance document

Introduction

Genetically modified (GM) plants and their derived food and feed products are subjected to a risk analysis before they can be commercialised in the European Union (EU). This analysis consists of 3 components: risk assessment, risk management and risk communication. In risk assessment, potential adverse impacts associated with a specific activity are scientifically characterised on a case-by-case basis, whilst in risk management, policy alternatives to accept, minimise or reduce the characterised risks are weighed and, if needed, appropriate prevention and control options are selected. Because risk managers and regulators rely on risk assessments to make an informed decision on whether or not to approve a certain use of a GM plant, it should explain clearly what assumptions have been made during the risk assessment, and what is the nature and magnitude of uncertainties associated with the characterised risks. The decision on whether a certain risk is acceptable and/or tolerable under a particular set of conditions is not part of the risk assessment itself, as this choice is not only based on scientific criteria, but also on socio-ethical and economic considerations. Risk communication is the third component of the risk analysis and is defined as an interactive exchange of information and opinions on risk throughout risk analysis, running between risk assessors, risk managers and other interested parties. It includes the explanation of risk assessment findings and of the basis on which risk management decisions are made.

The role of the European Food Safety Authority (EFSA) is to provide independent, objective and transparent science-based advice on the safety of GM plants and derived food and feed products in the European Union (EU), whilst market authorisation decisions are taken by Member States and the European Commission. The assessment of GM plant market authorisation applications is done by the EFSA’s scientific panel on genetically modified organisms (GMO Panel) that is composed of 21 independent European scientific experts. The EFSA GMO Panel is supported by a number of specialised working groups drawing upon a
pool of more than 40 ad hoc experts in agronomy, biology, biotechnology, ecology, environmental sciences, genetics, immunology, microbiology, modelling, molecular biology, nutrition, plant physiology and toxicology.

In its assessment, the EFSA GMO Panel considers data generated by applicants during product development as part of their GM plant market authorisation applications, as well as any other relevant scientific information published in the scientific literature.

**EFSA GMO Panel guidance document for the risk assessment of GM plants and derived food and feed products**

To guide and assist applicants for the preparation and presentation of GM plant market authorisation applications, the EFSA GMO Panel has developed a guidance document for the risk assessment of GM plants and derived food and feed products (EFSA, 2006). The guidance document describes principles, concepts, data requirements and issues to be considered when performing the risk assessment of GM plants and derived food and feed products.

**Risk assessment principles and concepts**

Risk assessment generally comprises several sequential steps: (1) problem formulation as critical first step; (2) hazard assessment that examines potential hazards and their magnitude; (3) exposure assessment that covers levels and likelihood of exposure; and (4) integrative risk characterisation in which the magnitude of consequences and the likelihood of occurrence are integrated. Risk is recognised as a function of the probability and severity of an adverse effect occurring to human and animal health or the environment following exposure to a hazard, under defined conditions. In the EU, the consideration of mitigation options such as post-market (environmental) monitoring is not included as a fifth step in the risk assessment framework, as risk assessment is kept separated from risk management (Hill, 2005).

In order to identify the areas of greatest concern or uncertainty related to risks, each risk assessment begins with the identification and formulation of the problem (Hill and Sendashonga, 2003; Raybould, 2006; Wolt et al., 2010). In this respect, the most important questions to be solved and meriting detailed risk characterisation are identified. On the one hand, problem formulation involves defining assessment endpoints that are targets for protection, extracted from public policy goals. On the other hand, it involves the development of a methodology that will help to direct the risk characterisation and to produce information that will be relevant for regulatory decision-making. This is achieved on the basis of a conceptual model and an analysis plan. The conceptual model describes consequential exposure scenarios of how harm to the assessment endpoint (valued entity) may arise from GM plant deployment. Detailing the components of the system enables the identification and formulation of relevant risk hypotheses. These risk hypotheses are necessary to make assumptions and predictions about how a potential stressor could affect an assessment endpoint. In the analysis plan, decisions are made about the most appropriate way to measure the response of each assessment endpoint to GM plant deployment. Data needed and the approach to be taken for data acquisition and synthesis are delineated in order to test relevant risk hypotheses formulated in the conceptual model (Hill and Sendashonga, 2003; Raybould, 2006, 2007; Nickson, 2008; Romeis et al., 2008; Storkey et al., 2008; Wolt et al., 2010).

Risk assessment of GM plants should (1) be science-based where quantitative information is available and use qualitative information in the form of expert judgment; (2) be case-specific; (3) use a comparative approach; (4) be iterative and examine conclusions already made based on new information; and (5) follow a tiered approach.
According to the case-by-case principle, biological and agro-ecological characteristics of a GM plant; the nature of the introduced trait(s); the receiving environment in which the plant will be introduced; the scale and frequency of deliberate release; and the interactions among these elements should be considered when performing an environmental risk assessment (Garcia-Alonso et al., 2006).

With the comparative approach, a two-step logic is followed that consists of identifying possible differences between the GM and non-GM plant, and subsequently assessing the potential environmental and food/feed consequences, as well as the nutritional impact of the identified differences.

An environmental risk assessment is generally conducted in a tiered manner, where information collected in lower tiers directs the extent and nature of the experimentation conducted in higher tiers (Wilkinson et al., 2003; Garcia-Alonso et al., 2006; Romeis et al., 2008). Thereby, both hazards and exposure are evaluated within different tiers that progress from worst-case scenario conditions framed in highly controlled laboratory environments to more realistic conditions in the field, if necessary. The conclusion regarding potential risks drawn at each tier will lead to a regulatory decision after the residual uncertainty of the assessment has been defined or to additional investigations. If a risk is identified, decision-making can consider whether risk management should be implemented to reduce the identified risk. It is important that throughout the assessment, the problem being addressed remains appropriate, and is revised, if necessary.

Data requirements
Data requirements for GM plant market authorisation applications include the molecular characterisation of the genetic modification; the assessment of the modification with respect to biological and agronomic characteristics of the GM plant; the assessment of food/feed safety aspects of the GM plant and derived food and feed products; and the evaluation of environmental issues related to the GM plant and its associated farm management practices. Data on composition, toxicity, allergenicity, nutritional value and on the environmental impact provide, on a case-by-case basis, cornerstones of the risk assessment process (Craig et al., 2008).

Environmental issues
In line with Directive 2001/18/EC, the following issues should be considered in the environmental risk assessment of each GM plant: (1) changes in the persistence and invasiveness of the GM plant; (2) potential unintended effects on plant fitness due the genetic modification; (3) the potential for gene transfer; (4) interactions between the GM plant and target organisms; (5) interactions between the GM plant and non-target organisms (NTO); (6) potential effects on human and animal health due to accidental exposure; (7) potential effects on biogeochemical processes; (8) impacts of specific cultivation, management and harvesting techniques associated to the cultivation of the GM plant; (9) potential interactions with abiotic environment; and (10) the scientific quality of the proposed post-market environmental monitoring plan (Craig et al., 2008). Moreover, an assessment of direct and indirect, as well as immediate and delayed effects is required.

Further prospective in the field of environmental risk assessment

Since the EFSA GMO Panel is continuously considering any new scientific information, it has taken several initiatives to further develop and improve scientific approaches on risk assessment of GMOs and to address specific scientific issues (Paoletti et al., 2008). In the
context of the environmental risk assessment of GM plants, some initiatives have been taken to consider the latest experience gained, as well as technological progress and scientific developments made.

**EFSA scientific colloquium on the environmental risk assessment of GM plants**
In June 2007, EFSA organised a scientific colloquium to discuss approaches and challenges related to the environmental risk assessment of GM plants (EFSA, 2008). This colloquium aimed to stimulate discussions on (1) approaches for NTO testing; (2) the use of models for predicting potential risk outcomes of moving to a higher scale of GM plant deployment based on outcomes of risk assessment studies performed at a lower scale of GM plant deployment; (3) the use of models for predicting long-term effects; and (4) broadening the scope of the environmental risk assessment to enable the integration of a much wider range of influences and drivers of agricultural systems. While it was agreed that the environmental risk assessment as outlined in the current EFSA GMO Panel guidance document for the risk assessment of GM plants and derived food and feed products is at the forefront of recent developments in this area, further guidance was found useful in some fields. In this respect, participants from academia, public research sector, national advisory bodies, non-governmental organisation, private sector and competent authorities from Member States made a list of recommendations to EFSA.

**Self-tasking working group on NTO testing**
Following the discussions held and recommendations made on NTO testing at the EFSA scientific colloquium and acknowledging the different NTO testing approaches debated in the scientific literature, EFSA has established a working group on NTO testing. This working group is responsible for harmonising different NTO testing approaches and for the development of more detailed guidance in this area. The focus of the working group is on the development of criteria for the selection of species and ecological functional groups; arthropod diversity; experimental design of NTO studies; statistical power of NTO tests; receiving environment; and post-market environmental monitoring.

**Update of environmental sections of the EFSA GMO Panel guidance document for the risk assessment of GM plants and derived food and feed products**
Since March 2008, the EFSA GMO Panel is working to further update the environmental sections of its risk assessment guidance document on GM plants and derived food and feed products. Following some of the recommendations made at the EFSA scientific colloquium and following a request of the European Commission, EFSA will not only establish more detailed guidance on NTO testing, but also on (1) the design of field studies to assess potential ecological effects of the GM plant in its receiving environment; (2) the identification of EU geographical regions where GM plants may be released; (3) the selection of appropriate techniques for assessing potential long-term effects of GM plants; (4) the assessment of environmental fitness of GM plants and their progeny; (5) the impact of specific farm management practices; and (6) specific requirements for the assessment of GM stacked events.

**Working group on statistics**
Following the outcome of the Biosafenet seminar on the statistical design and analysis of field trials for assessing the risk associated with GM plants – held on 12-15 January 2009 and organized by the International Centre for Genetic Engineering and Biotechnology (ICGEB) – and considering the discussions of the working group on NTO testing, the working group on
statistics is supporting the previously mentioned working groups on statistical issues. It will provide recommendations on field trial design and experimentation; statistical power of environmental risk assessment studies; the use of prospective power analysis; size effects on environmental risk assessment; number of replications for field trials; sample sizes; and on specific statistical methodologies for data analysis of environmental risk assessment studies.

Consultations

Interested parties will be consulted during the revision of the environmental sections of the EFSA GMO Panel guidance document for the risk assessment of GM plants and derived food and feed products. Mid-June 2009, a 3-day stakeholders’ consultation was organized by EFSA. Applicants, Member States and non-governmental environmental organisations were invited to express their views on the current guidance document. In addition of these stakeholders’ consultations, EFSA will organise a public consultation before adopting the revised guidance document. In this respect, interested parties will be invited to submit comments on the revised guidance document by responding to the consultation. Comments will have to be submitted by means of an electronic form available on the EFSA website.

References


Environmental impact of herbicide regimes used with genetically modified herbicide-resistant maize

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Abstract: With the potential advent of genetically modified herbicide-resistant (GMHR) crops in the EU, changes in patterns of herbicide use are predicted. Broad-spectrum, non-selective herbicides used with GMHR crops are expected to substitute for a set of currently used herbicides, which might alter the agro-environmental footprint from crop production. To test this hypothesis, the environmental impact of various herbicide regimes currently used with non-GMHR maize in Belgium was calculated and compared with that of possible herbicide regimes applied in GMHR maize. Impacts on human health and the environment were calculated via the pesticide occupational and environmental risk (POCER) indicator. Results showed that the environmental impact of herbicide regimes solely relying on the active ingredients glyphosate (GLY) or glufosinate-ammonium (GLU) is lower than that of herbicide regimes used in non-GMHR maize. This beneficial environmental impact is reduced or counterbalanced depending upon the use of other herbicides in association with GLY or GLU in GMHR maize.

Key words: biotechnology-based weed management strategy, environment, herbicide regimes, risk assessment

Introduction

Through the insertion of genes from biologically unrelated species, modern biotechnology has enabled the production of genetically modified (GM) crops that are resistant to non-selective herbicides containing the active ingredient glyphosate (GLY) or glufosinate-ammonium (GLU). It became possible to use post-emergence herbicides for controlling many weeds at high efficacy over a wider range of crop growth stages without concern for injury to the crop. Since the first commercial introduction of GM herbicide-resistant (HR) crops in 1995, the area planted with these crops has increased rapidly. This rapid adoption is generally attributed to low cost, simplified, more flexible and selective weed management options provided by the use of broad-spectrum, intrinsically non-selective herbicides (Cerdeira and Duke, 2006).

Switching from conventional crops to GMHR crops has altered crop production with important changes in patterns of herbicide use and production practices (Beckie et al., 2006; Cerdeira and Duke, 2006; Kleter et al., 2007; Gianessi, 2008; Givens et al., 2009b). Herbicide regimes used with GMHR crops have substituted for a set of other regimes. Not only have farmers reduced the rates of soil-applied pre-emergence treatments and used GLY or GLU to effectively control emerging weeds, they also have substituted GLY or GLU for their previously used post-emergence applications. Moreover, GMHR crops have contributed to the increase in adoption of conservation tillage practices (Givens et al., 2009a).
Focussing on changes in patterns of herbicide use, this paper investigates the potential changes in the agro-environmental footprint of switching from conventional crops to GMHR crops by comparing the environmental impact of various herbicide regimes used with GMHR and non-GMHR crops. Maize is taken as a case study as it is a major crop in Belgium and as current herbicide regimes in non-GMHR maize may not always provide consistent season-long weed control. The pesticide occupational and environmental risk (POCER) indicator (Vercruysse and Steurbaut, 2002) is calculated for various herbicide regimes applied in GMHR and non-GMHR maize.

Material and methods

**POCER indicator**

The aim of the POCER indicator is to evaluate the impact and risk of pesticide treatments to human health and the environment. This impact is reflected on 10 environmental modules with 3 modules for human occupational, non-diетary exposure (1-3) and 7 modules for the environment (4-10): (1) risk to pesticide operator; (2) risk to worker; (3) risk to bystander; (4) persistence in the soil; (5) risk of ground water contamination; (6) acute risk to aquatic organisms; (7) acute risk to birds; (8) acute risk to bees; (9) acute risk to earthworms; and (10) risk to beneficial arthropods. The 10 modules are based on criteria formulated in Annex VI of the Directive 91/414/EEC concerning the placing of plant protection products on the market.

For each module, the risk is estimated by the use of risk indices. A risk index (RI) is the quotient of the estimated human exposure or the predicted environmental concentration (exposure assessment) and a toxicological reference value (effect assessment). To calculate the total risk, each RI is converted in an exceedence factor (EF). Hereby, a lower and upper limit is defined per module. Based on the upper and lower limit and the RI of each module, the EF is calculated. Calculated EF values $\leq 0$ are scored as zero and reflect a low (negligible) risk, whilst calculated EF values $\geq 1$ are scored as one and reflect a high (unacceptable) risk.

To estimate the risk per herbicide regime, which includes different active substances, the risks calculated separately for each active substance are summed. Subsequently, the total risk is converted in an EF. The total risk is calculated by summing the values of the 10 modules assuming that all components are equally important. Vercruysse and Steurbaut (2002) provide more details on POCER indicator calculations.

**Herbicide management regimes in non-GMHR maize**

In principle, 3 different weed management approaches are possible in non-GMHR maize: grass and broadleaf herbicides applied (a) pre-emergence, (b) early post-emergence, ideally in the 2-4 leaf stage of maize, or (c) sequentially, where a combination of (grass) herbicides with soil activity is applied pre-emergence followed by a mixture of post-emergence (broadleaf) herbicides. Thirteen typical herbicide regimes were selected for the analysis, representing different combinations of activity substances, weed spectrum and doses, as well as timing of application (see Devos et al., 2008 for more details).

**Herbicide management regimes in GMHR maize**

Acting almost exclusively through contact with foliage, GLY and GLU are applied post-emergence when and where they are actually needed with a low crop injury potential. Eighteen herbicide regimes containing the active substances GLY or GLU were established, representing different numbers of applications (single vs. sequential), doses and the presence of residual herbicide (see Devos et al., 2008 for more details).
Results and discussion

**POCER index**

1. **Risk to pesticide operator**, 2. **Risk to worker**, and 3. **Risk to bystander**: No differences in risk to human health were observed between herbicide regimes applied in maize cropping systems, except for the regimes that only contain the active substance GLY (Figure 1). Due to its low human toxicity, GLY has a lower impact on the pesticide operator: it has a higher acceptable operator exposure level (AOEL), which is the maximum amount of active substance to which the operator can be exposed without adverse health effects. The risk to worker was usually small, except for herbicide regimes that contained the active substance terbuthylazine. The risk to bystander of all the regimes studied was very small and transient, ending in zero EF values. Accordingly, the total risk on human health was lower for herbicide regimes that only relied on the active substance GLY than for the other analysed regimes.

4. **Persistence in the soil**: The studied herbicide regimes had EF values ranging between 0.00 and 0.03, reflecting a low persistence of the corresponding active substances in the soil. This is because half-lives of active substances shorter than or equal to 90 days soil persistence are generally considered to be low. GLY is readily degraded by soil microbes, having a moderate half-life ranging from 7 to 60 days with an average value of approximately 47 days, but reaching 174 days in some soils under some conditions (reviewed by Cerdeira and Duke, 2006). This range of half-life values can be explained by different experimental conditions of temperature, moisture and soil (reviewed by Arias-Estévez et al., 2008).

5. **Risk of ground water contamination**: Because GLY and GLU are rapidly adsorbed and tightly complexed by most soils, they show a low potential for transport (Giesy et al., 2000; Cerdeira and Duke, 2006). This phenomenon is reflected in the calculated EF values: compared with the other analysed herbicide regimes, the risk of ground water contamination of GLY and GLU when used alone was slightly lower than for the other analysed herbicide regimes (Figure 1).

6. **Acute risk to aquatic organisms**: The low acute toxicity of GLY and GLU to fish, Daphnia and algae was confirmed by the calculated EF values. When used alone, the EF values of GLY and GLU were lower (= 0.00) than those of a wide range of active substances such as dimethenamide-P (0.38-0.46 dependent upon the dose), S-metolachlor (1.00) and terbuthylazine (0.77-0.92 dependent upon the dose), confirming findings of other studies (Giesy et al., 2000). Once in surface water, GLY and GLU dissipate more rapidly than most other herbicides. GLY does not bioaccumulate, biomagnify, or persist in an available form in the environment (Cerdeira and Duke, 2006).

7. **Acute risk to birds**, 8. **Acute risk to bees**, 9. **Acute risk to earthworms**, and 10. **Risk to beneficial arthropods**: Although GLY and GLU have been shown to be less toxic to invertebrates than were other herbicides (Giesy et al., 2000; Cerdeira and Duke, 2006), this trend was not observed in this study. This is due to the low toxicity to invertebrates and vertebrates of currently used herbicide regimes, reflecting a move towards active ingredients with better (eco)toxicological profiles since the nineties.
Discussion

The agro-environmental footprint from crop production will be altered when switching from conventional crops to GMHR crops. Herbicide regimes used with GMHR crops will substitute for currently used herbicide regimes. By comparing the environmental impact of various herbicide regimes used in maize cropping systems, our results demonstrate that most herbicide regimes used with GMHR maize have a better environmental impact than those currently used in non-GMHR maize: the POCER exceedence factor values for the environmental modules were reduced approximately by a sixth when GLY or GLU is used alone. The beneficial environmental effect of herbicide regimes solely relying on GLY or GLU is attributed to their lower potential to leach into groundwater and lower toxicity to aquatic organisms. Similar observations have been made in previous studies on this matter (e.g., Brimner et al., 2005 for GMHR oilseed rape; Bonny, 2008 for GMHR soybean; Kleter et al., 2008 for GMHR sugar and fodder beet). In an experimental study conducted in Canada (Québec), herbicide regimes solely containing the active ingredients GLY or GLU had a reduced environmental impact per hectare (EI; calculated using the environmental impact quotient) compared with herbicide regimes used with non-GMHR maize (Leroux et al., 2006). Relying on data from US pesticide-use surveys, Kleter et al. (2007) concluded that the EI value calculated per hectare decreased from 87.9 to 53.6 – representing a proportional
reduction of 39% – when herbicide regimes applied in non-GMHR maize were replaced by those used in GMHR maize. On a global scale, a cumulative reduction of 4.6% in EI values was calculated due to changes in herbicide regimes from growing GMHR maize since 1997 (Brookes and Barfoot, 2008). Likewise, the adoption of GMHR maize was predicted to reduce herbicide concentrations in surface water of vulnerable US watersheds (Wauchope et al., 2002). Because GLY and GLU have better (eco)toxicological or environmental profiles than the herbicides they replace, the adoption of GMHR maize in the EU might positively contribute to the quality of the receiving environment. However, this beneficial environmental impact can be reduced or counterbalanced depending upon the use of other herbicides in association with GLY or GLU in GMHR maize (Figure 1).

It is important to bear in mind that the POCER indicator focuses on a specific set of assessment endpoints and that the results obtained should be considered in a perspective of a dynamic and continuously evolving agricultural crop management. To enable assessing the agro-environmental footprint of crop production, additional issues will have to be considered such as (1) the rate and scale of adoption of GMHR maize (including stacked maize events) and its associated farm management practices; (2) shifts in weed abundance, diversity and composition; (3) the evolution of resistance in weeds; (4) reductions in botanical diversity that might lead to reductions in invertebrates and possible effects for animals higher in the food chain; (5) the impact of metabolites resulting from the degradation of active substances; (6) the rate and scale of adoption of conservation tillage practices; and (7) weed management regimes and mitigation measures implemented.

References


Effects of Bt maize on non-target lepidopteran pests

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Abstract: Genetically modified (GM) maize with the insecticidal capacity of Bacillus thuringiensis, (Bt maize, expressing Cry1Ab) was first authorized in Spain in 1998. Since then its cultivated area has increased year by year to reach 78’000 ha in 2008, representing 21% of the total maize-growing area in Spain. In the study area (Lleida, Catalonia, NE Iberian Peninsula) it represents almost 80% of the total. Bt maize provides an effective control of two key lepidopteran pests, Sesamia nonagrioides (Lefèbvre) and Ostrinia nubilalis (Hübner). However, in addition to the two corn borers, two other non-target Lepidoptera, Mythimna unipuncta (Haworth) and Helicoverpa armigera (Hübner), cause occasional but severe damage to maize. Effects of Bt maize on these two Lepidoptera were studied in laboratory and field trials. Some larvae of both species can survive and complete development when feeding on Bt maize. Field evaluations carried out from 2005 to 2008 showed no differences in the number of H. armigera larvae per plant between Bt and isogenic varieties in most of the trials. In the laboratory, M. unipuncta showed a larval survival of 15%, which is significantly lower than that recorded in isogenic varieties. Additionally, larval development in survivors was significantly longer when they were fed Bt maize. Adults resulting from larvae developed on transgenic maize laid 13% fewer eggs than those resulting from larvae developed on isogenic maize. When they had the choice, neonate M. unipuncta larvae preferred first Sorghum bicolor, then isogenic maize plants and finally Bt plants for feeding. Recorded differential mortality caused by Bt maize on non-target Lepidoptera in comparison with targeted corn borers may affect the composition and abundance of the Lepidoptera community in maize as a consequence of Bt maize deployment.

Key words: Helicoverpa armigera, Mythimna unipuncta, transgenic maize

Introduction

Bt maize has been sown in Spain since 1998. During the period 1998-2000, the area sown with Bt plants represented 5% of the total maize-growing area. This area has increased year by year and in 2007-2009 it represented 21% of the total (MARM, 2009). From 1998 to 2005 the only Bt maize sown was event Bt176. In 2005 this event was prohibited, and from 2003 to the present the varieties sown have been based on event MON810. Bt maize provides very effective control of the two key pests in our region: the Mediterranean corn borer, Sesamia nonagrioides (Lep., Noctuidae) and the European corn borer, Ostrinia nubilalis (Lep., Crambidae), but the effect on other lepidopteran pests that occur in maize fields is not so well known. In the study area maize is attacked by two other Lepidopteran species belonging to the Noctuidae family, Mythimna unipuncta and Helicoverpa armigera, but we have few data about the effectiveness of Bt maize against them. If these two species could survive on Bt maize they would benefit from the absence of corn borers. M. unipuncta feeds on leaves of several non-agricultural and cultivated gramineous plants such as maize, millet and weeds. When its populations are high it can consume all the leaves of a maize field. In our region for several years we have found maize fields totally devastated by M. unipuncta larvae close to other undamaged field. H. armigera is a species that feeds on crops belonging to very different botanical families. Little is known on the damage caused by this species on maize.
but it is always present in low number on the ear silks. It has also been observed in the study area on sunflower and, increasingly, on alfalfa.

The aims of this work were to determine whether Bt maize was as efficient against *M. unipuncta* and *H. armigera* as it is against corn borers, whether the larvae of the two species – which feed at least partially on Bt maize – could survive, and how feeding on Bt maize affects the development of *M. unipuncta*.

**Material and methods**

To achieve these objectives, the effect of Bt maize on *M. unipuncta* was studied by feeding the larvae with leaves of some Bt (P67 and DKC) and their corresponding isogenic varieties (P66 and TTR, respectively) and the effect on *H. armigera* was evaluated by recording in the field larval occurrence on some Bt and non-Bt varieties over several years.

To study the effect on *M. unipuncta* larvae, a laboratory rearing was established. For this, adults were caught with light traps installed in maize fields and then taken to the laboratory. The adults were caged with 4-5 leaf-maize plants and provided with a solution of 10% sucrose as the water and food source. Neonate larvae were individually placed in transparent cylindrical containers (3 cm ø and 5.5 cm height), in which they were fed with a piece of leaf of the non-transformed commercial varieties assayed. The pieces of leaf were renewed every day. The larvae developed in the same container until pupation and mortality and the duration of the development of each instar were recorded.

Pupae resulting from these larvae were sexed and kept at 8 ºC until experimental use.

The viability of adults coming from these pupae whose larvae developed on Bt maize and non-Bt maize was studied by caging the adults (10 couples) in separate cages with non-Bt maize plants, counting the number of eggs laid by females and recording egg hatching.

To determine whether *M. unipuncta* neonates show any preference for feeding among different plants, 5 couples of adults were placed in cages with 6 different potted plants: 2 pots with two Bt maize (P67), 2 pots with two non-Bt maize (P66) and 2 pots with two *Sorghum bicolor* plants. This design was repeated 6 times. After the eggs hatched, the plants on which larvae started to feed were annotated every day for two weeks.

To determine whether *H. armigera* larvae can survive on Bt maize plants, the number of larvae on several non-commercial varieties of Bt and non-Bt maize were counted (Table 1). Experimental units were plots of 27 x 38 m² in size. A complete randomized block design with 3 blocks (2005) and 4 blocks (2006-2008) was used. The plants observed were chosen at random on rows 10 to 14 and at a minimum of 7 m from the edge of each plot in order to minimize plot edge effects as much as possible. Fifteen plants per plot (3 points x 5 plants/point) were inspected carefully five times in the season and the number of *H. armigera* larvae was recorded. As a significant number of *H. armigera* larvae were usually found only on the 4th and 5th sampling dates, we used only these data for statistical analysis.

**Statistical analysis**

A one-way (Bt vs. non Bt) ANOVA was used to analyze the effects of Bt maize on the variables measured. Percentages were transformed by ASIN(SQRT(%/100)) to normalize data as much as possible.
Table 1. Number of Bt and non-Bt plots and data of the visual sampling performed to evaluate the effect of Bt maize on *H. armigera* larvae. Each year 2 fields with a complete randomized block design with 3 blocks (2005) and 4 blocks (2006-2008) was used.

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<th>2005</th>
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<td>16</td>
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<tr>
<td>Number of Non-Bt Plots</td>
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<td>09/26/05</td>
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**Results**

Figure 1 compares the percentage of mortality of *M. unipuncta* larvae fed with Bt maize vs. the corresponding isogenic variety: when the larvae were fed with leaves of the two transgenic varieties (DKC and P67) the resulting mortality was significantly higher than in the larvae fed with leaves of the corresponding isogenic varieties (Tietar and P66). Although the mortality of the larvae fed with leaves of transgenic plants was high, a percentage of these larvae (14% of larvae fed on DKC and 17% on P67 varieties) survived to pupation.

![Figure 1](image)

Figure 1. Percentage of *M. unipuncta* larval mortality when fed with two Bt maize varieties and the corresponding isogenic varieties. Within each treatment different letters above the columns indicate significantly different mortality.

Survival of larvae during development can be observed in Figure 2. The survival percentage of larvae fed with leaves of transgenic maize was similar in the two transgenic varieties (DKC and P67) and lower than in larvae fed with the two isogenic varieties (Tietar
and P66). The highest mortality occurred in the first instar (L1) and in the last instar (the last one before pupation).

![Graph showing survival rates of M. unipuncta larvae fed with two Bt maize varieties and the corresponding isogenic varieties during the different larval instars.](image)

Figure 2. Survival of *M. unipuncta* larvae fed with two Bt maize varieties and the corresponding isogenic varieties during the different larval instars (Li indicates the last instar before pupation in all cases).

There were significant differences in the development duration of larvae fed with the two transgenic varieties in comparison with the larvae fed with the corresponding isogenic varieties (p<0.05): larvae on the two transgenic varieties showed longer development times than the isogenic ones (Figure 3).

![Graph showing duration of larval development.](image)

Figure 3. Duration in days of the larval development of *M. unipuncta* larvae fed with two Bt maize varieties and the corresponding isogenic varieties. Different letters above the columns indicate significantly different mortalities.
The majority of larvae fed with leaves of the two non-Bt varieties (P66 and Tietar) pupated after the 5th larval instar (Figure 4); none of them reached the 8th instar. None of the larvae fed with leaves of the Bt varieties (P67 and DKC) pupated after the 5th instar; the majority of them pupated after the 7th instar, and some after the 8th instar.

![Figure 4](image)

Figure 4. Percentage of *M. unipuncta* larvae that pupate after the 5th, 6th, 7th or 8th instar when fed on different Bt (Bt) or non-Bt (No) varieties.

The number of eggs per female coming from larvae fed with non-Bt maize vs. Bt maize can be seen in Figure 5. Females coming from larvae fed on Bt maize mated and laid viable eggs, indicating that larvae that survive on Bt maize can reproduce.

![Figure 5](image)

Figure 5. Number of eggs per female laid by adults of *M. unipuncta* that developed from larvae fed on the Bt and non-Bt maize varieties.
Host plant preference: Neonate larvae coming from *M. unipuncta* adults placed in cages with pots with *Sorghum bicolor* plants, Bt (P67) maize plants, and non-Bt (P66) maize plants started to feed on *S. bicolor* plants; when they finished with the leaves of this host, they moved to non-Bt maize and only when all leaves of this host plant were finished did they move to the Bt maize, whose leaves they also finished. These results are similar to the behavior of *M. unipuncta* larvae observed in the field. When one field is sown with a Bt (MON810) variety and an adjoining one with a non-Bt variety, the first suffers extremely high damage by *M. unipuncta*. When most of this field has been exhausted, the larvae start to feed on the leaves of the second field.

Figure 6. Number of larvae of *H. armigera* per plant in plots sown with Bt and non-Bt varieties that were monitored in 2005, 2006, 2007, and 2008.

Figure 6 shows the differences in the number of *H. armigera* larvae between Bt and non-Bt maize at the fourth sampling date in 2005, 2006, 2007, and 2008. The highest number of larvae per plant was recorded in 2005, when 50% of the plants had one larva, whereas in 2006 there were almost no plants with larvae. Only in one year, 2007, were significantly fewer larvae on non-Bt plants. These results – particularly those of 2005 – show that *H. armigera* larvae can feed and survive on some Bt maize varieties.

Discussion

The efficacy of Bt maize plants against corn borers has been widely studied, and among the most recent works are those of Huang *et al.* (2006) and Van den Berg and Van Wyk (2007). However, there are fewer works concerning the effect of Bt maize on other lepidopteran maize pests such as leaf and silk feeders. Pilcher *et al.* (1997) evaluated the efficacy of Bt maize with event 176 against *M. unipuncta* and against *H. zea* and obtained in the field similar results to those of this work. Although mortality was higher in *M. unipuncta* larvae fed on Bt maize than in those fed on the isogenic maize, 15% of the larvae reached pupation, but with longer developmental times. An absence of differences in the number or larvae per plant on Bt and isogenic varieties was also reported by Pilcher *et al.* (1997) for *H. zea*. More recently, Schaafsma *et al.* (2007) evaluated the effectiveness of three Bt corn events (176 and
Mon 810 with the Cry 1Ab toxin and TC1507 with the Cry 1F toxin) against *M. unipuncta*; they observed considerable plant damage on the three varieties assayed but did not record the larval survival rate. Our study shows that larval mortality occurred mainly just after the first and the last larval instars, being low in the intermediate instars. Larvae fed on the Bt maize took longer to complete development and needed more instars to pupate. These features are a common response to low quality, as stated by Esperk *et al.* (2007). To plan strategies for Bt-resistance prevention in insect pests, it is important to know the performance of the larvae surviving Bt maize. In the case of *M. unipuncta*, adults resulting from larvae fed on transgenic plants laid viable eggs though in lower numbers than those fed isogenic maize. Several authors (e.g. Van der Berg and Van Wyk, 2007) have studied females oviposition choice between Bt and non-Bt maize with *Sesamia calamistis*, but there are very few works that have studied the behavior of neonate larvae. In our work we demonstrate that when they have the choice of host plant, neonate *M. unipuncta* larvae choose first *Sorghum bicolor*, then isogenic maize plants, and finally Bt maize plants. It seems that neonate larvae can distinguish Bt plants and try to avoid them. Similarly, Yang *et al.* (2008) found indications that *H. armigera* larvae can distinguish parts of the plant with fewer Bt toxins to feed on and are more likely to survive on Bt cotton.

In summary, this work indicates that larvae of *M. unipuncta* and *H. armigera* can survive, at least partially, when feeding on Bt maize. As mortality of corn borers on Bt maize is very high, changes in the status of lepidopteran pests on maize must be considered. It may also be concluded from this work that when strategies to prevent Bt-resistance in corn borers are planned, potential consequences for *H. armigera* and *M. unipuncta* must be considered.

**Acknowledgements**

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Current challenges in environmental risk assessment: The assessment of unintended effects of GM crops on non-target organisms

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Abstract: As part of the regulatory approval process for commercial cultivation of genetically modified crops in the EU applicants have to assess the potential adverse effects that GM crops may have on human and animal health and the environment. This includes an assessment of potential adverse effects on non-target organisms arising from intended and unintended results of the genetic modification. The methodology to be used for the environmental risk assessment of GM crops to non-target organisms has been a major subject of debate for many years. However, after much research, a conceptual framework based on a tiered approach is now widely accepted by risk assessors, regulators and the scientific community. This methodology works well when the assessment is aimed at establishing the risk associated with intended effects of the genetic modification or unintended effects that have been identified during the comparative assessment. There the transformed plant is grown alongside its conventional counterpart and a number of plant characteristics are measured and compared. However, the regulatory process in the EU now also considers the requirement to assess the risk of potential unintended effects that have not been identified during the comparative assessment. This represents a major challenge for risk assessors in that there is no clear basis in which to set testable hypothesis. This paper discusses some of the problems encountered by risk assessors when trying to fulfill this regulatory requirement and explores ways forward.

Key words: risk assessment, unintended effects, genetically modified crops, regulation

Introduction

As part of the regulatory approval process for commercial cultivation of genetically modified (GM) crops in the European Union (EU) applicants have to assess the potential adverse effects that GM crops may pose to human and animal health and the environment. These potential adverse effects are evaluated following risk assessment principles and methodologies. This paper focuses on the environmental risk assessment and in particular on the potential adverse on non-target organisms (NTO).

The methodology to be used for the environmental risk assessment of GM crops to non-target organisms has been a major subject of debate for many years. However, after much research, a conceptual framework based on a tiered approach is now widely accepted by risk assessors, regulators and the scientific community (Garcia-Alonso et al., 2006; Romeis et al., 2008). The first step within this framework is “Problem formulation” which consists on collection and consideration of all the data available on the GM crop to allow the formulation of testable hypotheses and the design of a plan to test them (Raybould, 2007). There is often a misconception that the data used in the problem formulation phase of the environmental risk assessment is data generated exclusively for this purpose. In reality, the data used in the problem formulation step is data previously available and includes all the data that applicants need to generate for their cultivation applications, as well as data available in the literature.
The information generated for any cultivation application in the EU usually includes data on the receiving crop, on the genes intended for insertion, their sources, the proteins they express, molecular characterization data on the resulting GM crop, expression data, compositional analysis, agronomic characterization, toxic and allergenic potential, etc. Guidance on the studies that all cultivation applications must contain is provided in EFSA documents (EFSA, 2006, 2008). Failure to provide these data results in applications failing the thorough EFSA completeness check, leading to delays while the missing data is generated and the application can be validated and enter the regulatory process. Thus, there is a large amount of data available to the risk assessor at the problem formulation step that allows the focused planning of the risk assessment. The problem formulation helps the risk assessor in the formulation of testable hypothesis and in the design of any studies that may need to be conducted for the risk characterization (Raybould, 2007). These studies are then organized and evaluated in a tiered manner, as previously described by Garcia-Alonso et al. (2006) and Romeis et al. (2008).

This risk assessment methodology works well for the evaluation of potential adverse effects of the GM crop on NTO due to characteristics of the GM plant that have been identified as potentially harmful. However, the EU regulatory process currently requests that applicants conduct additional studies to further assess potential adverse effects of the GM crop due to unintended changes introduced in the GM plant even when these have not been identified during the risk assessment. This paper discusses the challenges that this request represents for risk assessors and suggests ways forward.

Identification of intended and unintended differences: the comparative assessment

It is widely accepted that traditionally cultivated crops have a history of safe use and familiarity for consumers or animals and the environment (EFSA, 2008); this is the basis of the concept of familiarity. The risk assessment for a new GM crop therefore starts with a comparison of the GM crop with its non-GM conventional counterpart. The outcome of this comparative assessment allows the risk assessor to establish what is different in the GM plant under assessment. The comparative assessment is based mainly on data from three different sources: molecular characterization, compositional analysis and agronomic comparison (see Figure 1).

The molecular characterization includes an analysis of the transgene flanking regions to establish whether the gene insertion has resulted in any disruption on the function of key endogenous genes. It also includes an open reading frame analysis (ORF) to determine whether proteins other than the intended protein could be produced by the GM plant. If any endogenous genes were disrupted or new proteins (other than the intended protein(s)) were produced, these would be considered as unintended effects of the genetic modification. A risk assessment to determine whether these differences could result in potential adverse effects on human or animal health and the environment would then have to be conducted.

The compositional analysis comprises data generated in several field trials in a range of locations representative of different environments where the GM crop may be grown. In these field trials the GM plant is grown side by side its comparator. Plant samples are taken for analysis to determine whether any differences in key components have occurred as a result of the genetic modification. The components that are analyzed are chosen following the OECD guidance documents published for each crop to provide some harmonization and allow the construction of databases that compile the information gathered for conventional crops and allow establishing safe ranges for these crops. Given the large number of components
analyzed, it is not uncommon to detect some statistically significant differences by chance when in reality no difference exists. Any differences identified are then assessed for their biological relevance by comparing the values obtained for a particular analyte in the GM plant with the range of values known for the conventional crop and which are known to be safe. If no biologically significant differences are detected, other than those intended by the genetic modification, the risk assessor can concentrate the risk assessment on the intended differences. If biologically significant differences that can constitute potential harm to humans and animals or the environment are identified, these constitute “unintended identified” differences that need to be evaluated in the risk assessment.

Figure 1. Process used for assessing whether the genetic modification has resulted in any unintended changes in the GM plant or not.

A similar approach is followed for agronomic comparisons. The GM plant is grown in several field trials conducted at a range of locations representative of different environments where the GM crop may be grown. Agronomic characteristics are recorded and compared to establish any differences between the GM and non-GM crop. The parameters that are considered for comparison include, for example, characteristics that may indicate changes in weediness potential to the GM plant that could lead to potential adverse environmental effects. Again, any differences identified are then assessed for their biological relevance by comparing the values obtained in the GM plant with the range of values known for the
conventional crop. Any differences that are biologically significant are evaluated in the environmental risk assessment.

Differences between the GM plant and its non-GM counterpart or comparator do sometimes occur, often at random, given the large number of comparisons made. However, these differences do not necessarily indicate adverse effects. The risk assessor’s first task after identifying a difference is to establish whether this difference is biologically relevant or not and the probability that this difference will lead to an adverse effect in the field. If this is the case, then the risk assessment is focused on these identified biologically relevant differences. For most GM crops the only differences identified are those expected due to the intended effect of the genetic modification (intended differences). In other cases, other differences may be identified, like for example differences in composition or in agronomic characteristics (unintended differences). For the purpose of the risk assessment, any differences considered biologically relevant, are assessed in the same way, regardless of whether these differences were intended or unintended. The methodology used to conduct this environmental risk assessment for GM crops on NTO has been thoroughly described in previous publications (Garcia-Alonso et al., 2006; Romeis et al., 2008).

In summary, there are many sources of data in the standard application package for cultivation applications in the EU that provide information to assess whether the genetic modification has resulted in unanticipated differences that may result in adverse effects to the environment and to NTO. The potential adverse effects of these biologically relevant differences to NTO can then be assessed using the tiered risk assessment approach.

The assessment of potential adverse effects on NTO due to unidentified differences

When it comes to assessing potential adverse effects of the GM crop on NTO due to unidentified differences, the methodology to follow is not so clear. In the EU, according to Directive 2001/18/EC, the environmental risk assessment should focus on the “identified characteristics of the GM crop and its use which have the potential to cause adverse effect”. This directive also establishes the need to submit a monitoring plan for all cultivation applications. One of the objectives of the monitoring plan is “to identify the occurrence of adverse effects of the GMO or its use on human health or the environment which were not anticipated in the environmental risk assessment”. Therefore, all applications for cultivation of GM crops in the EU have to contain a monitoring plan which aims to detect any unanticipated adverse effects as a result of the cultivation of the GM crop, once the product is commercialized. However, it appears that the regulatory process in the EU is now going a step further and applicants are now requested to conduct additional studies to detect potential adverse effects on NTO due to “unintended unidentified” characteristics of the GM plant pre-commercialization.

The current proposal from EFSA is to ask applicants to conduct NTO field studies in the EU to assess the potential unintended effects of the GM crop on NTO. The rationale behind this proposal is that field evaluations provide a measure of the potential effects under realistic field conditions. While this request may appear logical and simple at first, any risk assessor trying to implement it is faced by many challenges.

First, to design a sound field trial to detect potential adverse effects on NTO the risk assessor needs a hypothesis to test. If these trials are designed as part of the tiered approach (Garcia-Alonso et al., 2006; Romeis et al., 2008), the risk assessor has enough information to decide which NTO are at risk and design a field trial that will maximize the chances to detect
differences with confidence. The risk assessor will have done a comparative assessment, identified all differences found between the GM plant and the non-GM (whether intended or unintended), established whether they are biologically relevant or not and if they are, they would have identified which NTO groups may be at risk. The right methods can then be designed to test the hypothesis of no harm to those particular NTO. However, when no unintended differences have been identified after the comparative assessment, what is then the hypothesis to test? If the hypothesis, as some suggest, is that the GM plant does not have an adverse effect on any NTO, a field trial designed to collect data on all NTO would be highly unlikely to provide any clear answers. This type of field trial is very difficult to design as it is unclear which organisms are to be tested. The plot size, trapping methodology and assessment would have to fit all NTO. The term NTO normally describes organisms that are very diverse taxonomically, with different feeding habits and therefore different exposure pathways. NTO are also unpredictable and it is not always possible to anticipate their abundance, very often many are not present in large enough numbers to make appropriate statistical comparisons. This results in very large, very complex data sets that are difficult to interpret.

Another challenge is the interpretation and use of the data obtained. During the risk assessment, the risk assessor first aims to detect differences between the GM crop and the conventional crop and then establishes the biological relevance of these differences. As discussed above, the more comparisons are made in a study, the higher the chance of observing a statistical significant difference by chance, due to random variation, when in reality no differences exist. Thus, faunistic NTO studies, even when comparing two conventional varieties of the same crop, are likely to detect statistically significant differences of unknown biological relevance. Therefore, for any differences detected in faunistic NTO studies, the assessor would face a real challenge when trying to establish their biological relevance, since no baselines are available and no data exists on what is “normal” in agricultural ecosystems. The usefulness of these data is therefore questionable and their contribution to adding information to the risk assessment for decision making unclear.

Another important challenge in the EU arises from practical constraints. Currently many EU countries have a negative position against GM crops and do not allow research field trials in their territory. Other countries are more open to the evaluation of this technology and while they allow research field trials they do so with caution and impose strict compliance measures in terms of isolation, containment and crop destruction. For NTO field trials this means limitations on the size of the plots and degree of replication. Isolation measures often mean that the trials have to be surrounded by several rows of non-GM crop and further isolated by at least 200m from other crops of the same type. A relatively small NTO trial therefore often results in the management of several hectares of land for at least two years, the first while the trial takes place, the second (or more, depending on the country) while monitoring for volunteers takes place. This makes finding suitable locations very difficult and not many farmers are amenable to let their lands, especially considering that they need to negotiate with their neighbours the location of their crops to protect the isolation distance. Furthermore, plant material from these trials can not enter the food and feed chain and has to be destroyed at the end of the trial, for an NTO trial this is often the disposal of several tons of plant material to destroy and bury with the evident environmental disruption that this brings. All this makes faunistic NTO field trials very difficult to perform in practice, especially considering that they are expected to be conducted in representative environments.

Where a clear need to perform NTO trials is identified through the tiered risk assessment approach, applicants have to overcome these difficulties and conduct the trials as there is a clear need to generate these data to allow a proper risk characterization that helps in the decision making process. However, when the comparative assessment has been conducted
and all biologically relevant differences identified assessed through the tiered assessment and the conclusion is that there are no risks to NTO, the need to conduct these faunistic NTO field studies is questionable.

**Ways forward**

As discussed above, the current data requirements for cultivation applications of GM crops and the comparative assessment allow the detection of unintended differences between the GM crop and the conventional crop. The mandatory requirement in the EU to monitor after the crop is commercialized provides further assurance that there are mechanisms in place to ensure that any unanticipated adverse effects that were not detected during the pre-market risk assessment could be detected. However, it appears that one of the concerns is that the comparative assessment is more suitable to detect unintended differences between the GM plant and the non-GM that are relevant to the food and feed safety assessment, but not so much to the risk assessment for NTO and the environment.

A potential way forward could be the gathering of additional data in the field trials that applicants have to conduct for the completeness of their cultivation applications. One possibility would be the gathering of more “ecologically relevant” data in agronomic studies. These studies tend to be performed in small plots and therefore would not be suitable for trapping and counting NTO, but the studies are conducted at several locations and the plots are replicated allowing statistical analysis. Additional parameters, like interaction of the plant with some pests or with stages of NTO that are not very mobile, could provide further indication of whether the GM plant is interacting with the agroecosystem fauna in a similar way to the conventional plant. Any unanticipated events like sudden pest resurgence could serve as indicators of potential system disruption. The inclusion of these additional measurements in the comparative analysis would strengthen the problem formulation phase and provide more confidence that unanticipated effects of the GM crop on NTO are unlikely to occur. This could be further complemented with the monitoring that all products have to go through once they are commercialized in the EU.

**Conclusions**

Under the regulatory process in the EU all applications for cultivation of GM crops have to include an assessment of potential adverse effects of the crop on NTO, this includes not only an assessment of the potential adverse effects due to the intended modification but also an assessment of potential harm resulting from unintended effects. Current data requirements that all cultivation applications need to comply with in the EU provide a large amount of data, generated both in the laboratory and in the field, which is used to perform a comparative assessment. This comparative assessment allows the identification of any differences between the GM crop and the conventional crop whether they are intended or unintended effects of the genetic modification. Despite this extensive characterization, additional field data are being requested to confirm the assessment of potential unintended adverse effects on NTO. Such field data are problematic because they have limited power to detect biologically significant differences, while detecting many statistically significant differences of unknown relevance. An alternative approach to provide more confidence in the assessment of unintended effects on NTO is to collect additional data from the agronomic field studies that applicants already conduct. This would provide risk assessors with additional information to assess the occurrence of unintended effects and regulators with extra confidence that the potential occurrence of unintended affects has been assessed pre commercialization.
References


A faunistic database as a tool for identification and selection of potential non-target arthropod species for regulatory risk assessment of GM maize

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Abstract: In order to assess the similarities in arthropod species composition and abundance in maize fields in different geographic areas of Europe, we are compiling a database of species found and their ecological functions in five selected European maize producing countries (Czech Republic, France, Germany, Hungary and Spain). Countries were selected to represent both the extent of maize production as well as relevant ecological zones in which maize is grown. The database contains information on the taxonomy, distribution, abundance and ecological function(s) of each species. By comparing the species representing particular ecological functions and/or taxonomic groups in each country, we will determine the extent of faunal similarities or differences among the countries studied and identify those species that could supply the most widely applicable data for non-target arthropod risk assessment of GM maize. This database will have broad utility in the EU and is designed to allow for inclusion of other world geographies in future.

Key words: corn, environmental monitoring, non-target arthropods, receiving environment, tiered testing, Zea mays

Background

In the European Union and elsewhere, the legislation governing genetically modified (GM) plants requires a pre-commercial environmental risk assessment as part of the regulatory approval process (SCBD, 2000; EC, 2001). Arthropods are among the most species-rich groups of non-target macro-organisms exposed to GM crops in agricultural landscapes and they provide important ecological functions such as pollination, pest regulation and decomposition of organic materials. For practical reasons, only a small fraction of all arthropod species living in a crop can be considered when assessing potential effects of a novel GM plant. It is therefore frequently necessary to select appropriate species to serve as surrogates that can represent taxonomic groups as well as the ecologically and economically important functions in the crop as the receiving environment (Romeis et al., 2008). In addition, regulatory risk assessment of non-target arthropods may consider species with
special cultural value or species classified as threatened that cannot be used in testing, but for which risk assessment needs eventually to be performed.

The general principle proposed to assess risks of GM plants to non-target arthropods is a tiered approach in which the surrogate concept for non-target organisms is accepted (Dutton et al., 2003; Garcia-Alonso et al., 2006; Rose, 2007; Romeis et al., 2008). Early in the process, the problem formulation stage defines the scope of non-target risk assessment and formulates testable hypotheses that are subsequently addressed. Information considered during the problem formulation relates to the specific properties of the stressor (e.g., a plant-expressed Bt toxin), the likely sensitivity of non-target arthropod taxa or guilds to the stressor, the likely exposure of species and specific characteristics of the receiving environment, i.e., the maize field in our case.

Despite the fact that maize cultivation has many common features in Europe (Meissle et al., 2010), maize crops and their arthropod fauna may vary according to climate, geographic location, general features of the landscape, soil properties and cultivation techniques. Arthropod species with known ecological features and functions that are common in maize and widespread over Europe could be potential candidates to serve as surrogates for regulatory testing. In particular, such species would be appropriate representatives of the receiving environment if higher tier testing (i.e., laboratory tests with plant material and/or tests under semi-field and field conditions) is triggered by early tier results (Dutton et al., 2003; Garcia-Alonso et al., 2006; Rose, 2007; Romeis et al., 2008). Currently, there is no compilation of information on arthropod species composition and abundance in maize in different geographic areas of Europe that would allow the selection of the most appropriate species (based on a number of criteria) for regulatory non-target arthropod testing. This kind of information is particularly useful since the development of test systems is complex, requires considerable resources and expertise and long development times for validation. There is thus a critical need for a database documenting the relevant taxonomy, distribution, abundance and ecological function(s) of arthropod species found in European maize fields.

**Objective**

The objective of the project is to create a searchable database of information on arthropod species within maize crops in European countries. Once developed, comparisons of the species representing particular functions and/or taxonomic groups in defined geographic regions, can be used to determine the extent of faunal similarities or differences and identify those species that would eventually best satisfy the criteria of appropriate test species for higher tier testing of GM maize and supply the most widely applicable data for maize as the receiving environment. The data generated in this project could provide the scientific base to support the selection of test species and the harmonization of regulatory non-target arthropod risk assessment of GM maize. While this first phase of the project necessarily was limited to select EU countries the database is designed for expansion to include other world areas.

**Materials and methods**

The database is based on a format developed by Todd et al. (2008). We used a simplified version, which nevertheless retains the ability to hold additional information on the species’ ecology and their potential interactions with GM maize expressing specific traits, in case this is required for environmental risk assessments in the future. References used in this study were recorded and stored in an electronic Endnote library. The type of reference (e.g., peer-reviewed research article, web-published list, research report, etc.) was also recorded so that
different types of information can be filtered if desired. The database contains information on each species’ taxonomy, ecological function, habitat and abundance. Most of the information about the European maize fauna was gathered from published literature (peer-reviewed and others) and through direct contacts with affiliated research groups. Additionally, the Web-of-Science (online academic database) was used to collect further publications containing data on maize arthropods.

**Preliminary results**

A preliminary database analysis was carried out using Microsoft MS Access. It was conducted with a total of 587 recorded species from Germany (239 species), the Czech Republic (252 species) and Hungary (287 species). When data from France and Spain were included, the number of recorded species increased to 655. In total, we entered species from 13 orders and 68 families covering a wide spectrum of the existing European arthropod fauna, which makes this database a very useful tool. For the preliminary analyses, three countries (Germany, Czech Republic and Hungary) and two common arthropod groups (Coleoptera: Carabidae; Hemiptera: Cicadellidae) were selected to exemplify the kind of information that can be extracted from the database.

The database currently contains records for a total of 125 different species of carabids found in the three countries. Sixty seven of these species have been recorded from Germany, 68 species from the Czech Republic and 60 species from Hungary. Twenty species were found simultaneously in each of the three countries, representing approximately 30% of each country’s total number of carabid beetle species, indicating a considerable taxonomic overlap. However, only *Harpalus (Pseudoophonus) rufipes* was found in medium to high abundance in all three countries. *Calathus fuscipes* and *Pterostichus melanarius* were highly abundant in two countries and had low abundance in the third one.

Comparing our data with the CABI Crop Protection Compendium distribution maps (available on www.cabi.org/compendia/cpc) we found that the ground beetles *H. (P.) rufipes*, *P. melanarius* and *Poecilus cupreus* are especially widespread and common throughout Europe. In our database, *P. melanarius* was ranked as highly abundant in Germany, the Czech Republic and France, but of low abundance in Hungary. *P. cupreus* was recorded as highly abundant in Spain and the Czech Republic, moderately so in Germany and France, and of low abundance in Hungary. The use of an additional database such as the CABI Crop Protection Compendium might thus be a practicable approach to get a more complete picture of the geographic distribution of species of interest. This combination of wide geographical distribution, abundance, the known function as soil dwelling predators, the prey spectrum, the availability of testing methods (e.g., Heimbach et al., 2000; Stacey et al., 2006), the commercial breeding and the availability of the species shows the potential of such species as appropriate candidates for regulatory testing of insecticidal proteins for which effects on valued non-target beetles cannot be ruled out based on their known spectrum of activity (e.g., *Bt* Cry toxins targeting corn rootworms; Raybould et al., 2007).

The lack of otherwise very common European arthropod species in certain countries does not necessarily mean that they do not inhabit maize crops there. These species may not have been recorded in field studies because of the collecting methods used or because they were not or could not be identified to the species level, due for example to a lack of resources and/or taxonomic knowledge. One obvious example are the Cicadellidae where there were no data recorded for the Czech Republic whereas our database contains information on 69 species recorded in Hungary, and 27 species in Germany. In spite of this current lack of recorded data, we would plausibly argue that a high proportion of the Hungarian and German
Cicadellidae species must also occur in the Czech Republic since it is a neighbouring country and hence in the same climatic region with maize cultivation under similar conditions and agricultural practices. The CABI Crop Protection Compendium provides information only for one of the 76 European species of Cicadellidae in our database (*Empoasca vitis*). While the CABI Crop Protection Compendium is a useful tool to provide information on the geographic distribution of arthropod species, it contains insufficient information on the other parameters needed for species selection. The combination of data from a number of countries, complemented with data from the CABI Crop Protection Compendium and from other sources, is thus a suitable and practicable approach to complete missing data in other regions.

**Conclusions**

The 655 recorded species in the current database probably represent a reasonable proportion of the existing fauna of soil- and plant-dwelling arthropods in European maize fields. The database enables us to describe the arthropod species in maize fields as the receiving environment and to assess the available data on their abundance. It also informs the user of ecological functions such as predation and parasitism (biological control) and hence can facilitate the selection of ecologically and agronomically relevant species. The database thus provides information for the identification of potential surrogate species for early tier regulatory testing of non-target arthropods or later stage ecological interactions evaluations to assist the risk assessment(s) for GM maize. Overall, this database tool and approach should allow for the streamlining of resources in areas as diverse as test system development and in field ecological assessment. We also recognize the utility of the approach as generally applicable not just to the EU but also other world geographies and could help harmonize data needs, transfer and uses in a wide range of countries.

**Acknowledgements**

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**References**


Involving public sector research in regulations and international negotiations on biotechnology

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Abstract: Thousands of research projects in modern biotechnology have been and are carried out in public sector research institutes worldwide to help strengthening sustainable production of food, feed, fiber and fuel. As activities involving biotechnology expand, harmonization of domestic regulations with international agreements, such as the Cartagena Protocol on Biosafety, becomes increasingly important. International regulations and agreements define which role public sector research institutes can play in addressing agricultural challenges. For a long time the public sector has not been represented during international negotiations, which resulted in the confirmation of the misperception that biotechnology is only the domain of big handful multinational companies. The Public Research and Regulation Initiative (PRRI) was established in 2004 with the objective to offer a forum for public sector scientists to be informed about and involved in international discussion about biosafety.

Key words: International regulations on biotechnology, Involvement of public sector, Public Research and Regulation Initiative, Cartagena Protocol on Biosafety

Introduction

The potential of agricultural biotechnology has been widely acknowledged since the 1980’s and considerable financial resources have been invested in biotechnology research. Fast advancement in biotechnology applications, however, led to the recognition that the use of this new technology needs to be regulated. In 1986, the Organization of Economic Cooperation and Development (OECD) published a key document addressing biosafety issues – the “rDNA Safety Recommendations” or so called “Blue Book”. In that same year, the US published its coordinated framework of regulation of modern biotechnology. Four years later the first EU Directives and Regulations followed with the objective to harmonize the regulations of modern biotechnology in the European Union. In 1992, the Convention on Biological Diversity was adopted which includes provisions for the increase of biotechnology transfer (Article 19) as well as the establishment of national biosafety systems.

This was followed in 2000 by the introduction of the Cartagena Protocol on Biosafety, which became an international document regulating the transboundary movement of genetically modified organisms. These and other international negotiations have played a considerable role in defining the role of public sector research in biotechnology. Although their work is directly impacted by regulations, public sector research has for a long time not been represented in international negotiations and, consequently, a voice of interest groups was predominantly divided between industry and environmental NGOs. The voice of science was not heard.

This imbalance was recognized in 2004 by a group of scientists who acknowledged that there was a need for public sector researchers to be present during the negotiations to represent the interest of public sector research institutes worldwide. The idea was further elaborated during several international meetings and received very positive feedback from the
scientific community. As a result, Public Research and Regulation Initiative (PRRI) was established in 2004 with the objective to offer a forum for public sector scientists to be informed about and involved in international discussion about biosafety.

In 2005, public sector scientists participated for the first time in two Meetings of the Parties (MOPs) to the Cartagena Protocol on Biosafety (CPB). Since then PRRI has started to grow in number of members, branch in a number of topics and strengthen its recognition also in other international fora. More than 300 researchers are members of PRRI today and 12 working groups have been established to address a broad range of topics that are relevant to public sector research in modern biotechnology.

Involvement of public sector in international negotiations

Involvement in the meetings of the Cartagena Protocol on Biosafety

The Cartagena Protocol on Biosafety (CPB) was adopted by the Conference of the Parties (COP) in 2000 as a protocol to the Convention of Biological Diversity (CBD). It has currently over 150 Parties. The objective of the CPB is to contribute to the safe transfer, handling and use of living modified organisms that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risk to human health, and specifically focusing on transboundary movement. The Meeting of the Parties (MOP) is the governing body of the Protocol. The MOP takes place every two or three years. In addition to the parties to the protocol, stakeholders granted with an observer status are invited to participate in the negotiations to express their views on and concerns with development of the negotiations. Stakeholders represent non-parties, United Nations and specialized agencies such as FAO, intergovernmental organizations such as the European Parliament, non-governmental organizations such as Greenpeace and industry representatives. Since 2005, PRRI participates in the CPB meetings.

Prior to participation in MOPs, PRRI organizes regional preparatory meetings to inform public sector scientists about the background of the CPB and to explain the relevance of the issues on the agenda of the MOP. The articles of the CPB that are relevant to public sector research in modern biotechnology, such as Article 15 on Risk Assessment, are discussed and written statements addressing potential concerns are prepared. The statements are then read by the PRRI members during the negotiations and recorded by the CBD Secretariat for further considerations. In this context, PRRI brings a balance into stakeholders’ contributions by providing negotiators with independent and scientifically sound information. Since 2005, PRRI has facilitated participation of over 100 public sector scientists in 3 MOPs.

Beyond the Protocol

Next to the participation in MOPs, PRRI has – for example – also been actively involved in the discussions on the EU Directives and Regulations. PRRI is supported, among others, by the Sixth European Framework Program with the project acronym ‘Science4BioReg’. One of the objectives of the project is to stimulate the interaction between public sector researchers and the EU policy makers during the preparation and implementation of the EU Directives and Regulations on GMOs. Political decisions, and consequently, public opinion about agricultural biotechnology in Europe have been for a long time influenced by many myths. Controversial research papers warning about potential negative impact of GM crops have been often quoted in press. With reference to this kind of research, several European countries imposed a ban on the cultivation of GM crops in their territories. The stand of the European countries, however, does not impact only European farmers but also public research. The European policy regarding GMOs also affects the potential for the production of cash crops in
developing countries for export to the EU. To balance the discussion, PRRI has established a blog on its website called “The Ask Force” where it addresses articles about biosafety and biotechnology that have gained much public attention but which are not supported by peer reviewed scientific research.

Another often quoted claim has been that modern biotechnology is a sole domain of big multinational companies. In this context, PRRI launched a pilot project called the “ABC Database”. The ABC Database contains information about the objectives and results of public research in agricultural biotechnology, with the aim to inform all interested stakeholders and with the aim to facilitate collaboration in public research. The database is a collaborative initiative of the International Food Policy Research Institute (IFPRI) and the Public Research and Regulation Initiative (PRRI), in concert with public research institutes and other organisations worldwide. The ABC Database is a first step towards creating an enabling environment that fosters research, cooperation and technology transfer and comprehensively reviews and updates information on the status of public sector biotechnology R&D pipelines.

The way forward

In the years to come, PRRI will continue to support the public sector research in modern biotechnology by promoting scientifically sound, transparent and predictable implementation of regulatory frameworks. Information about PRRI activities can be found at www.pubresreg.org.
Modelling of minimal distances from GM oilseed rape in Lithuania

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Abstract: The study aimed to evaluate distances required for oilseed rape to prevent outcrosses between different cultivars and also between neighboring weeds of Brassicaceae. Evaluation of possible outcrosses of traditional cultivars growing in close proximity is a good model to estimate the probability of gene flow from GM plants. Modelling was made by field experiments, combining them with molecular analysis and pollen study. Five cultivars (‘Maskot’, ‘Sw Savan’, ‘Heros’, ‘Ural’, ‘Landmark’) of spring oilseed rape (Brassica napus L.) were grown in experimental plots in Lithuania. The number of weeds was registered in plots established parallel to the different cultivars. Plastid SSR primers MF1, MF2, MF3, MF4 were used for the molecular experiments. Amplification was successful with all used primers; two SSR loci used in our study were polymorphic after amplification with primers MF2 and MF7. Results of oilseed rape genetic diversity showed a significant genetic variation among individuals and different cultivars. Estimations were done concerning dispersion of pollen of oilseed rape by bees. The amount (as mass proportion, estimated in %) of oilseed rape pollens was 66 and 92 % in two bee hives placed near B. napus cultivars at the beginning of the study period (July 12) and decreased to 24 and 49 % until July 28 in the two hives, respectively. Examined cultivars significantly differed in respect to the concentration of N, Ca, Mg, and S on the pollen surface. Our preliminary data suggest the possibility to use pollen surface element analyses for the identification of specific cultivars.

Key words: Brassica napus, Brassicaceae, weeds, pollen, plastid SSR primers.

Introduction

Oilseed rape (Brassica napus L.) is predominantly self-pollinated (approx. 70 %). In addition to it, pollen dissemination occurs by wind and mainly bees (Thompson, 2003). Variation may also arise from out-crossing species during maintenance of a cultivar (Dulson et al., 1998).

Cultivars of B. napus have been produced by traditional and non-traditional means, so cultivars of a range of genetic complexities are available (Dulson et al., 1998). GM varieties of oilseed rape have been created carrying different traits including herbicide or heavy metal tolerance or a modified protein composition (Misra and Gedamu, 1989; Miki et al., 1990; Altenbach et al., 1992; Bond et al., 2004). Transgenes could spread from the crops to wild species, with unknown consequences for weed control, the biology of the recipient species and the ecosystems (Raybould and Gray, 1993, 1994; Ellstrand, 2003; Johannessen et al., 2006). This makes cultivation of transgenic oilseed rape an extraordinary challenge, creating uncertainties about their environmental risks. Gene flow from GM plants depends on various circumstances including edaphic and climatic peculiarities of the geographical areas where plants are grown.

Edaphic and climatic conditions of Lithuania are favourable for oilseed rape growth, and the area planted with this crop is increasing from year to year. In Lithuania no GM oilseed
rape were cultivated. In 2006 a notification was presented for field trials with GM spring oilseed rape (B. napus) by BASF Plant Science GmbH, which was not approved by the Ministry of Environment.

Microsatellite markers are routinely used to investigate the genetic structuring of natural populations (Balloux and Lugon-Moulin, 2002). Microsatellites or simple sequence repeats (SSRs) have been recognized as useful molecular markers in marker-assisted selection, the analysis of genetic diversity, population analysis and other purposes in various species (Gupta and Varshney, 2000). SSR markers are capable of detecting genetic differences between closely related plants (Lowe et al., 2002, 2004). Several research groups have isolated a number of microsatellites for molecular markers in Brassica species, mainly in B. napus (Kresovich et al., 1995; Truco et al., 1996; Uzunova and Ecke, 1999; Lowe et al., 2004). The wide utility of these markers is demonstrated for haplotype identification and detection of polymorphism in B. napus, B. nigra, B. oleracea, B. rapa (Flannery et al., 2006). Chloroplast DNA generally exhibits lower mutation rates than nuclear DNA and therefore interspecific variation is low in comparison to the nuclear genome (Provan et al., 2001; Panda et al., 2003).

The main task of our field experiment was to model gene flow between cultivars of oilseed rape traditionally grown in Lithuania and also to evaluate their coexistence with neighboring wild species of Brassicaceae and other weeds suggesting some ways for isolating cultivars of oilseed crops from the neighbouring plants.

**Material and methods**

**Study area**

Five cultivars (‘Maskot’, ‘Sw Savan’, ‘Heros’, ‘Ural’, ‘Landmark’) of spring oilseed rape (B. napus) were grown in one experimental field divided to 6 plots in the western part of Lithuania (Vėžaičiai, 55°43′N, 21°27′E). Four selected cultivars (‘Maskot’, ‘Sw Savan’, ‘Heros’, ‘Ural’) were grown on 60 m² each in North-South rank and the distance between plots with different cultivars was 100 m. In addition, for the analysis of pollen dissemination by wind the cultivar ‘Landmark’ was grown in a plot of 200 × 500 m together with weeds, surrounding the other four cultivars in a distance of 10 m on the eastern and western side. For the weed records 14 squares (0.25 m² size) were set parallely to the oilseed rape cultivars ‘Maskot’, ‘Sw Savan’, ‘Heros’, ‘Ural’ in two ‘Landmark’ plots. Distances between two nearest squares were 10 m, between all next squares 20 m. Parallel to the northern part of cultivar ‘Maskot’ two hives of the bees were allocated in a distance of 6 m. The nearest next oilseed rape fields were situated in 232 m distance to the North (cultivars Ukininkas 1) and in 520 m distance to the South (cultivars Ukininkas 2) from our experimental field. All cultivars were sown at the same time.

**Plant material**

The agrogenose of spring oilseed rape was examined. The weeds were eliminated from the plots where cultivars ‘Maskot’, ‘Sw Savan’, ‘Heros’ and ‘Ural’ were grown, while the weeds were left in the plots with ‘Landmark’. All weeds were eliminated from plots between cultivars. The number of the weeds was calculated during their bloom. At that time flowering of oilseed rape was partway done. Species abundance was recorded in squares of 0.25 m².
Pollen preparation and analysis
Pollen was collected for analysis by taking samples of the pollen brought into the bee hives. From the hives pollens were taken at different days in July starting from the beginning of oilseed rape flowering, till the end of the flowering period: 12th, 14th, 16th, 18th, 21st, 23rd, 25th, and 28th July. The bee collected pollen was prepared for analysis and checked with a microscope to determine the proportion of pollen grains from oilseed rape.

Compositional element analyses of the pollen surface by EDXS microscope
From each oilseed rape cultivar pollen samples were taken during the most intensive period of flowering. Pollen samples were dried and stored at 25 °C until further analyses. Pollens were aluminium coated and observed under an ESEM Philips XL 30 microscope as described by Tomasevic et al. (2005). The EDXS spectra for the compositional analyses were performed by point analysis with 1500-2000 cps (count per second) and a collection time of 2 min (three replicates per analysis). The findings of the elemental analysis were expressed as percentages of atoms. The voltage was 20 kV. SEM images were obtained with back-scattered electron or secondary electron imaging.

DNA extraction
DNA was extracted from spring oilseed rape leaves (100 mg) using a genomic DNA extraction kit (MBI Fermentas, Vilnius, Lithuania) with some modifications. The concentration of DNA was estimated with a spectrophotometer (Biophotometer, Eppendorf, Hamburg). DNA was diluted up to 100 ng/µl for use in PCR.

PCR conditions
DNA amplification was carried out in PCR tubes with a total reaction volume of 25 µl. The reaction mixture contained 12.5 µl PCR MasterMix (2×), 0.5 µl primer (10 pmol/µl), 10.5 µl deionised water and 1 µl DNA (100 ng). DNA amplification was performed using a thermocycler (Master cycler gradient, Eppendorf, Hamburg) programmed to 1 cycle at 94 °C for 3 min, following 29 cycles of denaturation at 94 °C for 40 s, annealing at 47 °C or 50 °C for 40 s, extension at 72 °C for 40 s min, final extension at 72 °C for 3 min, and hold at 4 °C. Primers used were synthesised by MBI Fermentas.

Electrophoresis and analysis of amplification products
After amplification, PCR products were separated by electrophoresis in 3.5 % agarose gel with 1× Tris- Borate-EDTA. Agarose gel was stained with ethidium bromide and photographed under UV light (EASY Win32, Herolab, Germany). Gene Ruler™ 50 bp DNA Ladder Plus (MBI Fermentas) was used as a marker.

Results and discussion
Composition of the spring oilseed rape agrocenose
Since the experiment was carried out in the field, it was affected by the local weather conditions. Because of lack of moisture during the period of germination, the oilseed rape crop was rare and the weeds occupied the most part in the field planted with ‘Landmark’ (this field was left to grow naturally, without applying any chemicals, or weeding out). So it was a natural competition in this agrocenose – and the spring oilseed rape ‘Landmark’ constituted only 13 % of the plant community. The largest part in this plant community (86%) consisted of Dicotyledones (Magnoliopsida). 29 species of weeds were identified, depending to 15 families: Brassicaceae, Polygonaceae, Caryophyllaceae, Asteraceae, Poaceae, Fabaceae,
Plantaginaceae, Violaceae, Ranunculaceae, Lamiaceae, Equisetaceae, Chenopodiaceae, Boraginaceae, Euphorbiaceae, Rosaceae. Most of the weeds were crucial, knotgrass and pink grasses. Dominant weed species were white goosefoot (*Chenopodium album* L.) from Chenopodiaceae family (43.5 %) and by curltop lady-thumb (*Persicaria lapathifolia* L.) from Polygonaceae family (22.1 %). Weeds from the Brassicaceae family were treacle mustard (*Erysimum cheiranthoides* L.), shepherd’s purse (*Capsella bursa-pastoris* L.), wild radish (*Raphanus raphanistrum* L.) and field pennycress (*Thlaspi arvense* L.); they composed 6.0 % of all species, 2.7 %, 1.9 %, 0.5 % and 0.9 %, respectively.

**Analysis of weeds of the Brassicaceae family in the agrocenose**

The analysis of the Crucifereae species in the different spring oilseed rape cultivars showed that in the plots with the cultivar ‘Maskot’, situated only 6 m from the bee hives, the weeds species belonging to this family constituted 14.7 % of all plants, i.e. 1.9 to 10.6 times more than in the other cultivars (Figure 1).

Figure 1. Amount of weeds belonging to the Crucifereae in the agrocenose of spring oilseed rape (Lithuania, Vėžaičiai, 2008).

Therefore the potential to outcross was most likely in the cultivar ‘Maskot’. As can be seen in Figure 1 – in the other cultivars the weeds constituted only a very small part of the agrocenose: 4 % in ‘Sw Savan’, 3.4 % in ‘Heros’, 1.4 % in ‘Ural’ and 7.9 % in ‘Landmark’.

Out-crossing of different *Brassica* sp. is possible when compatible hybridisation partners are found nearby, including crops and wild relatives (Anonymous, 2006). Wild radish (*R. raphanistrum*) hybridizes spontaneously with *B. napus* and produces viable hybrids (Baranger et al., 1995). Wild radish seems to cross more readily with *B. napus* than does cultivated radish (*R. sativum*) (Anonymous, 2002). The frequency of gene flow from *B. napus* to four wild relatives, *B. rapa*, *R. raphanistrum*, *Sinapis arvensis* L. and *Erucastrum gallicum* (Willd.) O.E. Schulz, was assessed in greenhouses and/or field experiments, and actual rates
measured in commercial fields in Canada (Warwick et al., 2003). In our experiment a very small part (0.47 %) in the agrocenose of spring oilseed rape constituted of wild radish.

It was estimated that under field conditions, oilseed rape can outcross with turnip (*Brassica campestris* L.) and brown mustard (*Brassica juncea* (L.) Czern.) (Jørgensen et al., 1996). Oilseed rape (*B. napus*) and weedy *B. rapa* were able to hybridize and backcross spontaneously in both experimental plots and cultivated fields (Jørgensen and Andersen, 1994; Jørgensen et al., 1996; Wilkinson et al., 2000). Gene flow from *B. napus* to *B. oleracea* was also reported. In our experimental fields these species did, however, not occur.

**Dispersion of oilseed rape pollen**

The present study aimed to compare the element composition on the surface of the oilseed rape pollen from different cultivars and to assess the dispersal of pollen over time. Among the various species of pollen brought by bees into their hives, oilseed rape pollen comprised 23-92 % (Table 1). The amount of oilseed rape pollen in the hives decreased within the period from 12th to 28th July. There were obvious differences between the two hives that were studied. At the start of the analysis (12th July) the first hive contained 3 times more oilseed rape pollen compared to the second hive. In subsequent samples, this portion decrease by 68% in hive 1 and by only 17% in hive 2.

Table 1. Dynamics of proportion (estimated as mass %) of oilseed rape pollen brought by bees into the hives (July 2008).

<table>
<thead>
<tr>
<th>Pollen sampling day in July</th>
<th>Pollens from the 1st hive</th>
<th>Pollens from the 2nd hive</th>
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<tbody>
<tr>
<td></td>
<td>Oilseed rape</td>
<td>Other species</td>
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<tr>
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</tr>
<tr>
<td>25</td>
<td>40.8</td>
<td>59.2</td>
</tr>
<tr>
<td>28</td>
<td>23.6</td>
<td>76.4</td>
</tr>
</tbody>
</table>

**Compositional element analysis of the pollen surface**

According to the EDXS analyses, the concentrations of carbon and oxygen on the pollen surface did not differ among the oilseed rape cultivars (Table 2). Carbon and oxygen were the prevailing elements on the surface of the oilseed rape pollen with concentrations ranging from 48-63 % and 33-42 %, respectively (Table 2). For both elements, detected differences among oilseed rape cultivars were not significant.
Among cultivars the pollen surface nitrogen concentration ranged between 0.11-8.75 %. The most contrasting cultivars were ‘Maskot’ and ‘Ukininkas 2’, their N concentration differed significantly. Similarly, the sulphur concentration on the pollen surface varied considerable among cultivars, from 0.01 to 11.51 %. The most contrasting and significantly different cultivars were ‘Ural’ and ‘Landmark’. Among cultivars the magnesium concentration on the pollen surface ranged from 0 to 1.8 %. The most contrasting cultivars were ‘SW Savann’ and ‘Ukininkas 2’, showing significant different concentrations. The calcium concentration ranged from 0.48 to 1.06 %, with significant differences between ‘Ural’ and ‘Ukininkas 2’. Taking the pollen surface concentrations of N, S, Mg and Ca into account, the most similar cultivars were ‘Ukininkas 1’ and ‘Ukininkas 2’ and at the same time they were the most distinct from the other cultivars. Different concentrations of the nutritional elements on the pollen surface might reflect the genetical and physiological peculiarities of individual oilseed rape cultivars. The data presented suggest the possibility to use pollen surface element analyses for identification of specific oilseed rape cultivars.

Table 2. Estimated concentration of EDXS elements on the oilseed rape pollen surface (% of pollen dry mass: mean value ± standard deviation).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>C</th>
<th>O</th>
<th>N</th>
<th>S</th>
<th>Mg</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘SW Savann’</td>
<td>60.82±19.74</td>
<td>38.36±13.89</td>
<td>0.12±0.30</td>
<td>0.08±0.01</td>
<td>0±0</td>
<td>0.62±0.15</td>
</tr>
<tr>
<td>‘Landmark’</td>
<td>57.58±18.52</td>
<td>41.16±14.35</td>
<td>0.56±1.18</td>
<td>0.01±0.01</td>
<td>0±0</td>
<td>0.69±0.12</td>
</tr>
<tr>
<td>‘Ukininkas 2’</td>
<td>48.83±19.78</td>
<td>39.31±12.70</td>
<td>8.75±4.03</td>
<td>0.83±0.14</td>
<td>1.80±0.10</td>
<td>0.48±0.10</td>
</tr>
<tr>
<td>‘Ukininkas 1’</td>
<td>53.36±18.52</td>
<td>42.20±13.92</td>
<td>1.58±2.41</td>
<td>0.64±0.17</td>
<td>1.73±0.24</td>
<td>0.48±0.13</td>
</tr>
<tr>
<td>‘Heros’</td>
<td>63.09±20.11</td>
<td>33.83±12.12</td>
<td>1.76±3.22</td>
<td>0.28±0.02</td>
<td>0.01±0.01</td>
<td>1.03±0.17</td>
</tr>
<tr>
<td>‘Ural’</td>
<td>48.09±15.41</td>
<td>38.20±12.64</td>
<td>0.23±0.94</td>
<td>11.51±0.43</td>
<td>0.92±0.03</td>
<td>1.06±0.10</td>
</tr>
<tr>
<td>‘Maskot’</td>
<td>61.77±20.33</td>
<td>36.88±13.89</td>
<td>0.11±0.30</td>
<td>0.22±0.01</td>
<td>0.01±0.01</td>
<td>1.01±0.14</td>
</tr>
</tbody>
</table>

*Brassica napus* cultivars haplotypes identification by SSRs plastids markers

Four plastid SSR primers MF1, MF2, MF3, MF4 created by Flannery *et al.* (2006) were used for the molecular experiments with spring oilseed rape cultivars (Table 3).

These primers have been used for the evaluation of *B. napus*, *B. oleracea*, *B. rapa* haplotype polymorphism. Amplification was successful with all primers used; two SSR loci used in our study were polymorphic after amplification with primers MF2 and MF7. Genetic polymorphism was detected among different individuals of spring oilseed rape and different alleles were determined in both loci (MF2 and MF7) (Figures 2, 3).
Table 3. Plastid SSR primers.

<table>
<thead>
<tr>
<th>SSR locus</th>
<th>Gene region</th>
<th>Repeat</th>
<th>Primer sequences (5’-3’), forward (F) and reverse (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF-1</td>
<td>trnL-F</td>
<td>(A)7–10</td>
<td>F-TCAATTGCACATTCTAGAATTCTAAG R-CAATTCAATATGGTTATATATTAGAG</td>
</tr>
<tr>
<td>MF-2</td>
<td>rpl16</td>
<td>(T)8–13</td>
<td>F- GGTTCGTCGTTCCCATC GC R -CATAATAATTAGATAAATCTGTTCC</td>
</tr>
<tr>
<td>MF-3</td>
<td>trnE-trnT</td>
<td>(T)7–10</td>
<td>F- AATGGTATGACTAGTTATAAAGG R- CTTAACAATGAGATGAGGCAATC</td>
</tr>
<tr>
<td>MF-7</td>
<td>TrnM-atpE</td>
<td>(T)7–16</td>
<td>F- CGGCAGGAGTCATTGTTCAAA R- GATTTTGTAACTAGCTGACG</td>
</tr>
</tbody>
</table>


Loci MF1 and MF3 did not show any differences between different B. napus individuals. Locus MF-7 (found within trnM-atpE intergenic spacer) was the most variable in our experiments and the same results were reported by Flannery et al. (2006). Results of oilseed rape genetic diversity showed a significant genetic variation among individuals and different cultivars.

We believe that the analysis of pollen surface elements in combination with plastid and nucleus SSR primers can be applied to study gene flow initiated by oilseed rape pollen and seeds and for the identification of separate oilseed rape cultivars. It might thus be a useful for the environmental risk assessment of GM oilseedrapes.
Figure 3. Plastids DNR TrnM-atpE spacer amplification applying primer MF7 (M - 50 bp molecular marker. M – ‘Maskot’; S – ‘Sw Savan’; H – ‘Heros’; U – ‘Ural’; L – ‘Landmark’; Uk1, Uk2 – oilseed rape cultivars grown by farmers.

Acknowledgements

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References


Occurrence and field densities of Coccinellidae in the maize herb layer: Implications for environmental risk assessment

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Abstract: One aspect of the Environmental Risk Assessment (ERA) of genetically modified plants is the potential ecological impact on the receiving environment. Plants with genes from *Bacillus thuringiensis* (*Bt*) that produce proteins with entomotoxic properties need to be assessed for their potential effects on non-target organisms (NTO), especially beneficials.

One important group of NTO are the ladybird beetles (Coleoptera: Coccinellidae), as they serve important biological control functions. Their exposure to the Cry proteins from *Bt*-plants depends on their consumption of exposed prey and plant materials such as pollen. *Bt*-plants expressing Cry3 proteins directed against the Western corn rootworm *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) especially pose a potential hazard to beneficial Coleoptera.

To assess the suitability of higher tier test systems (i.e. semi-field and field experiments) within the ERA, we analysed data from two 3-year, field-scale experiments with *Bt*-maize varieties containing the transformation events MON810 and MON88017. We present data on the densities of Coccinellidae in maize and calculated confidence intervals (CI) suitable for a test of equivalence between the *Bt*-maize varieties and their respective near-isogenic lines. We also report the results of a Monte Carlo simulation study assessing the probability that 90% CI are included in pre-specified margins of irrelevant change of mean abundances. The results show the limits of addressing questions regarding the non-target impact of *Bt*-maize on Coccinellidae in field experiments, given their low densities and the large natural variability.

Key words: *Bt*-maize, non-target organisms

Introduction

Genetically modified (GM) plants with genes from *Bacillus thuringiensis* (*Bt*) coding for proteins with entomotoxic properties are used worldwide to control important insect pests. Before they can be placed on the market, GM plants need to undergo an Environmental Risk Assessment (ERA) under Regulation 2001/18/EC. Amongst others, they need to be assessed for their potential effects on non-target organisms, in both crop areas and in surrounding habitats.

One important group of non-target organisms with respect to *Bt*-plants are ladybird beetles (Coleoptera: Coccinellidae), as they serve important biological control functions, especially as aphid predators (Obrycki and Kring, 1998). While they do not get into contact with *Bt*-proteins via their aphid prey on maize (Raps et al., 2001; Lundgren and Wiedenmann, 2005), Coccinellidae may consume pollen when it is available (Lundgren et al., 2005). Other potential prey items may also contain *Bt*-proteins. Coccinellidae are therefore exposed to the Cry proteins and thus the potential impact on ladybirds needs to be assessed. This is
especially true with Bt-plants expressing Cry3 proteins to combat the Western corn rootworm *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae).

ERA does most reasonably follow a tiered approach, starting with simple laboratory experiments under controlled conditions and gaining in complexity and realism when higher tier (i.e. semi-field, field experiments) tests are warranted, based on remaining uncertainties (Romeis *et al*., 2008). However, the suitability of higher tier test systems within the ERA depends on a number of factors, including the occurrence and the densities of studied non-target organisms in the field. We therefore wanted to analyse the suitability of field experiments for assessing the potential impact of the cultivation of Bt-maize on ladybirds. In a first step, we present data on the densities of Coccinellidae in maize from two 3-year, field-scale experiments with Bt-maize varieties based on the transformation events MON810 (expressing the Lepidoptera-specific Cry1Ab) and MON88017 (expressing the Chrysomelidae-specific Cry3Bb1) and calculate confidence intervals (CI) suitable for a test of equivalence between the Bt-maize and the near-isogenic lines. In a second step, we perform a Monte Carlo simulation study to assess the probability that 90% confidence intervals are included in pre-specified margins of irrelevant change of mean abundances. We discuss the relevance of the findings with respect to future ERA approaches focussing on Coccinellidae.

**Material and methods**

**Field experiments and sampling**

Data were gathered during two field experiments. The first was on the potential impact of the cultivation of Bt cultivar Novelis (MON810, details in Eckert *et al*., 2006), the other was on the *Diabrotica*-resistant variety DKc5143Bt (MON88017, see Rauschen *et al*., 2009). The experiments were performed during the periods from 2001 to 2003, and from 2005 to 2007, respectively. The MON810 experiment had a total size of 6 hectares (2 ha with Novelis), the MON88017 was 4 hectares (1.04 hectares with DKc5143Bt).

Different sampling methods were used to assess the diversity of Coccinellidae as a group and the densities of individual ladybird species, including: transect-wise sweep net catches, yellow Moericke traps, panicle samples during anthesis, destructive cob sampling, and visual assessments during the growing season.

**Statistical analyses**

For the analysis of insect abundance data we used generalized linear models assuming a negative binomial distribution (McCullagh and Nelder, 1989; Venables and Ripley, 2002). Based on the mean and variance parameters estimated in these models, confidence intervals for the ratio of mean abundance can be calculated (McCulloch and Searle, 2001). These intervals allow, in contrast to usual significance tests, to discuss the relevance of observed differences, including the uncertainty due to the limited sample sizes possible in field trials.

In the Monte Carlo simulation study we assumed that the Bt plant and its near-isogenic control are in fact equivalent, drew random data from the negative binomial distribution to simulate insect count data, and then calculated confidence intervals for each of the simulated data sets. Mean abundances of 0.5, 1, 5, 10 and 50 individuals per observational unit and situations with high, low and no extra-Poisson variability were modelled. Statistical analysis and the simulation study were performed in R 2.6.2 (R Development Core Team, 2008)
Results and discussion

Abundances of ladybird species in maize

In the first (2001-2003) field experiment, Coccinellidae were generally not determined on species level. For illustration, data are given only for the second (2005-2007) experiment. The overall numbers of ladybirds in the maize field were rather low (Table 1). Most species were caught only as single specimen during individual samplings, only three species occurred regularly. Five other species were identified (not listed in Table 1), but all of them were caught on single occasions only.

Table 1. Total numbers of different ladybird species caught with two sampling methods in the experimental maize field during the 2005 – 2007 project, pooled over all treatments and sampling dates.

<table>
<thead>
<tr>
<th>Species</th>
<th>sweep nets</th>
<th>panicle samples</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propylea 14-punctata</td>
<td>24</td>
<td>53</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>66</td>
<td>51</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>Coccinella 7-punctata</td>
<td>3</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Harmonia axyridis</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>4</td>
<td>15</td>
</tr>
</tbody>
</table>

An analysis of all Coccinellidae data of the two experiments revealed that single species usually occur with such low mean abundances that a meaningful statistical analysis is not possible. For single species with higher abundances or when considered on the family level, the data may exhibit an increased, i.e. extra-Poisson, variability.

Confidence intervals for Coccinellidae field data

Due to the low abundance of the single ladybird species, data were pooled on family level and also over the three experimental years to assure a reasonable medium density.

As an example, the result of the analysis of sweep net data from the MON88017 project is shown: the 90% confidence interval calculated for the ratio of mean abundance comparing DKc5143Bt (MON88017) and its near-isogenic line DKc5143 (Figure 1), indicates equivalence between the two maize lines, given the a priori definition of equivalent abundance ratios of [0.5; 2.0] (i.e. a reduction of abundance in Bt to 50% of that in the near-isogenic line, or an increase of 200%).
Results of the simulation study

Figure 2 summarizes the results of the simulation study. Consider first the experimental setup used in the two projects – with 8 replications per treatment, performed over three years (upper left figure) – and the equivalence margins [0.5; 2.0] (dotted vertical lines). In this situation it is only possible to show equivalence in at least 80%, if the mean abundances are larger than 1 and there is no extra variation in the data. For species with strong extra-variation in abundance counts, one cannot expect to establish equivalence in at least 80% of the cases even with mean abundances as high as 50 individuals per unit.

If equivalence was in fact to be defined by the margins [0.5; 2.0], a greater effort in terms of number of replications and number of consecutive growing seasons would be needed to be able to establish equivalence in at least 80% of the cases. If a stricter definition of equivalence, i.e. margins of [0.8; 1.25] (dashed vertical lines), was to be used, equivalence could not be shown for species that exhibit large variabilities in their densities and/or have low densities below a mean of 5. In these cases, an even larger effort would be needed to establish equivalence in at least 80% of the cases.

Conclusions

The results of the simulation study show that it is extremely difficult to show equivalence between a Bt- and its near-isogenic variety in terms of the densities of Coccinellidae, if the mean densities of the individual species or Coccinellidae as a group are low and they show some extra variability. For DKc5143Bt (MON88017), the mean abundance and the variability of the combined Coccinellidae were just right to show equivalence within the pre-specified margin of [0.5;2.0]. For smaller margins, equivalence could not be shown. This would have needed a much larger effort in terms of replicates and sampling years.

Testing risk hypothesis for Coccinellidae in the field and using their mean densities as an assessment endpoint requires a large number of replicates and long experimental times, if mean densities are low. This would require greater efforts than currently undertaken in field-release experiments. This means that only species occurring in large numbers can be reasonably assessed in the field and should therefore be the focus of research if field tests are warranted. Otherwise, additional laboratory or glasshouse experiments should be conducted if remaining uncertainties about risk have to be addressed.
In addition, given the large natural variability, other conventional varieties should by default be used in field experiments, as they can be used as additional criteria in assessing the equivalence between a genetically modified maize variety and its near-isogenic line.

Figure 2. Ranges in which 90% confidence intervals were included with 80% probability for simulated count data. Shown are results for four experimental setups differing in the number of experimental years and the number of replications within each year.

Acknowledgements

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References


Interplay of arbuscular mycorrhizal fungi with transgenic and non-transgenic wheat

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Abstract: This study aims to assess whether genes transferred to wheat (Triticum aestivum L.), directed against specific pathogenic fungi, may also influence the beneficial arbuscular mycorrhizal fungi (AMF). The results of our field experiment show: first, the level of fertilization used according to current farming practices leads to a significant reduction of mycorrhizal colonization, both in non-transgenic and transgenic wheat lines; second, the destruction of plants in certain experimental field plots by vandals also significantly affected mycorrhiza formation of the remaining healthy plants. Third, although these results must still be confirmed using more samples from other plots, the differences of root colonization between transgenic and non-transgenic plants do not seem to be significant.

Key words: arbuscular mycorrhizal fungi (AMF), wheat, transgenic

Introduction

Arbuscular mycorrhizal fungi (AMF) form mutualistic symbioses with 80% of terrestrial plant families including many agriculturally and horticulturally important crop species (Smith and Read, 1997). Wheat (Triticum aestivum L.), as the major land plant, always lives in symbiosis with AMF. Arbuscular mycorrhizal fungi transfer minerals such as phosphorus and nitrogen and water to the plant and receive carbohydrates in exchange. They can increase the plant to resistant to pathogens and heavy metals as well (Galli et al., 1994; Newsham et al., 1995).

The transgenic wheat lines used in this study were developed by Swiss research groups with the aim to improve their resistance to powdery mildew (Blumeria graminis). One group of transgenic wheat lines carries natural antifungal genes from barley (chitinase and β-1,3-glucanase), while the other one carries Pm3b, a resistance gene directed against powdery mildew from wheat. These genetically transformed wheat lines are expected to be more resistant to fungal plant pathogens compared to non-modified wheat. However, the possible effect of these transgenes on beneficial AMF has never been studied previously.

This report results from a field trial conducted in 2008, which is the second field trial with transgenic wheat in Switzerland. The results of this field trial will contribute to the ongoing discussion on transgenic wheat and its possible use in Swiss agriculture.

Material and methods

Wheat varieties and out group
The transgenic wheat lines used in this field trial are: A13 – Frisal transformed with a barley chitinase gene and a barley glucanase gene; A9 – Frisal transformed with a barley chitinase gene and a silent barley glucanase gene; Pm3b#1–Pm3b#4 – 4 independent lines of Bobwhite...
S26 transformed with the wheat resistance gene Pm3b. The four Pm3b#sl lines are the non transgenic sister lines of Pm3b#1~4. Other non transgenic wild type spring wheat lines are: Frisal, Bobwhite S26, Toronit, Fiorina, Casana and Rubli. Spring triticale and spring barley and two weed mixtures were chosen as outgroups.

**Field design**
The field experiment was carried out in an agricultural research station in Zurich-Reckenholz, Switzerland from March to August 2008. The 6 transgenic wheat lines, 10 non transgenic wheat lines and 4 outgroups were planted in Macro-plots, with 4 replicate blocks each containing 2 nitrogen levels. The subplots were 1.32 x 1.00 meter; they were randomly arranged in each block. All were planted on arable farm land and half of the plots received fertilizer twice (marked N2 in this paper, in total 6 gN/m² in the form of NH₄NO₃ (Lonza, Visp, Switzerland).

**Sampling and laboratory work**
The harvests were conducted twice, first when the plants were 6 weeks old (tillering stage) and second when the plants were 3 month old (watery to early milk-ripe stage). Some plots had been subject to vandalism two weeks before the second harvest, the remaining healthy plants in those plots were sampled and 4 levels of damage in the plots were assigned as an additional, unforeseen parameter: damage < 25%; 25%~50%; 50%~75% and > 75%.

Roots were collected and washed with a pressure hose in the laboratory. Then they were dried with tissues and cut into small pieces. The roots kept in 50% ethanol were stained according to a protocol based on Philips and Hayman (1970) and Brundrett et al. (1984). Briefly, roots were digested in 10% KOH at 75 °C for 4 min in a water bath, rinsed with water, incubated in 2% HCl at room temperature for 2 h minimum, stained with 0.05% Trypan blue in lactic acid : glycerol : water (1:1:1) at 75 °C for 4 min and finally incubated in 50% glycerol at room temperature for a few days. The stained roots were cut into 1−1.5 cm pieces, mounted on slides and observed through a microscope (x 200 magnification). A minimum of 100 crosses were counted per slide. The presence of hyphae, vesicles and arbuscules were recorded to evaluate the mycorrhizal colonization of the wheat roots.

**Results and discussion**
**Fertilizer and vandalism have significant effects on mycorrhization of wheat roots**
Plants from the first harvest displayed very little mycorrhization, which is probably due to the short period of colonization. Plants from the second harvest showed a good level of mycorrhizal colonization. A very clear and expected result was the difference in mycorrhizal colonization between plants grown at two fertilizer levels (Figure 1). An ANOVA analysis of all data showed that the difference in mycorrhiza formation between plants grown under low and high nitrogen levels was significant with a P value of < 0.00001 (Figure 2, ANOVA table not shown).

In addition to nitrogen fertilization, ANOVA analysis showed the plot damage had a significant effect on the root colonization (P=0.0005). Figure 3 shows that a relatively high colonization occurs in the subplots with higher damage levels. This could be explained by the fact that plants in a “stress situation” need to acquire more nutrition thereby relying more heavily on AMF. This situation leads to a higher level of mycorrhizal colonization in the neighbouring healthy plants of the same plots.
Figure 1. Root AMF colonization in the samples of all 20 lines with 2 nitrogen levels from the second harvest. The colonization is in most cases clearly higher at the low nitrogen level (N1) compared to the high nitrogen level (N2).

Figure 2. Root AMF colonization in the samples with two nitrogen levels (combined data).

Figure 3. Distribution of root AMF colonization according to the 4 damage levels.
The transgenic wheat did not exhibit significant effects on the degree of root mycorrhization. The difference in mycorrhization between non-transgenic and transgenic Bobwhite lines carrying powdery mildew resistance genes was not significant. However, when only the low nitrogen level was considered, there was a trend towards a lower colonization in the transgenic Bobwhite lines (P=0.074). When only considering the low nitrogen level, and only the pairs of sister lines of transgenic and non-transgenic Bobwhite, the reduction in colonization was statistically significant (P=0.04, Figure 4) but the residual distribution was not normal since it did not follow an F distribution (not shown). The reason might be that too few samples were considered. These 8 Bobwhite lines were recounted using a double-blind analysis and 4 slides were observed instead of 1 slide for each sample. The staining method was slightly improved and this showed a higher colonization. In the double blind assay, ANOVA analysis showed no significant difference between the transgenic Bobwhite and non-transgenic Bobwhite (P=0.154, Figure 5).

Figure 4. Colonization of transgenic Bobwhite and non-transgenic Bobwhite plants at low nitrogen level.

Figure 5. Colonization of transgenic Bobwhite and non-transgenic Bobwhite plants at low nitrogen level counted in a double blind procedure.
For the wheat cultivar Frisal, transgenic Frisal expressing antifungal enzymes appeared to have a higher colonization than non-transgenic Frisal at the low nitrogen level, but the opposite trend occurred at the high nitrogen level; in both cases, the differences were not significant.

The main conclusion of this field trial is that higher fertilization levels in the soil can lead to a significant reduction of root mycorrhization. Secondly, the destruction of plants significantly affected mycorrhiza formation of the remaining healthy plants in the same plots. Finally, the differences of root colonization between transgenic and non-transgenic do not seem to be significant, although this result needs to be confirmed by studying more samples from other plots in the field trial. The possibilities of quantitative PCR will be explored to determine the colonization of AMF of wheat roots from the field, with the aim of applying this technique to field samples.

Acknowledgements

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References

Ground beetles (Col., Carabidae) in Bt-maize – preliminary results from the first large scale field experiment in Poland

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Abstract: The ground beetle fauna was studied at two experimental field sites in Poland. The aim of this study was to determine the long term impact of Bt maize on non-target organisms in comparison to conventional maize. For this purpose, Bt maize (DKC 3421 YG) expressing the Cry1Ab toxin and the respective isogenic non-Bt variety (DKC 3420) were cultivated under identical conditions. For comparison, two non-Bt cultivars sprayed with a lambda-cyhalotrine were also included. Population density of surface-active invertebrates was monitored using pitfall traps (4 per plot). In the first year of the study, no significant differences between Bt maize and the conventional treatments were detected.

Key words: Cry1Ab, environmental risk assessment, non-target organisms

Introduction

Ground beetles are one of the key organisms in the maize ecosystem and act as generalist predators of many crop pests (Holland, 2002; Lopez et al., 2005). In case of transgenic maize with a gene expressing the δ-endotoxin Cry1Ab from Bacillus thuringiensis Berliner var. kurstaki they are exposed to Bt proteins both directly through ingesting maize litter and maize pollen and indirectly through eating prey which had fed on the maize plants. Till now, no field study on the impact of Bt maize on this important group of non-target organisms has been conducted in Poland.

Material and methods

Studies of similar design were conducted in two maize fields in Poland in 2008, i.e., near Wrocław, in Lower Silesia and near Rzeszów, in the South-East part of the country (Figure 1). The experiments were located in areas where infestation by the target pest Ostrinia nubilalis Hübner (Lepidoptera: Crambidae) is a substantial problem. For this purpose Bt maize (DKC 3421 Yield Gard) (Monsanto Company) and the respective isogenic non-Bt variety (DKC 3420) were cultivated. The control variety was tested both untreated and treated with lambda-cyhalotrine. For comparative analysis two non-Bt cultivars, sprayed with lambda-cyhalotrine were also included as reference control (Ref. 1 – cultivar Bosman, and Ref. 2 – cultivar Wigo). The statistical design consisted of randomized complete blocks with five treatments and four replications. Each experimental plot was 160 m² (40 m x 40 m).

In total, 80 plastic pitfall traps were used to collect the epigeal arthropods (four in each plot). The diameters of the plastic cups were 6 cm and they were sunk into the top level of the soil surface. Traps were filled with 50:50 water and ethylene glycol as preservative. The traps were emptied weekly through the whole vegetation season.
Data on ground beetle catches were analyzed using analysis of variance (ANOVA), and means were separated using Tukey’s HSD (honestly significant difference) test. Differences among means were compared at the 5% level of significance. To analyze species diversity of the beetle fauna ecological parameters of Shannon-Weaver (H’), Pielou (J’) and Simpson were used.

<table>
<thead>
<tr>
<th>Block 1</th>
<th>Block 2</th>
<th>Block 3</th>
<th>Block 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>DKC 3420 Prot.</td>
<td>DKC 3421 YG</td>
<td>DKC 3420 Prot.</td>
<td>Ref. 1 Prot.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40 m</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.5 m</td>
</tr>
<tr>
<td>Ref. 1 Prot.</td>
<td>Ref. 2 Prot.</td>
<td>DKC 3420 Non-Prot.</td>
<td>DKC 3421 YG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40 m</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.5 m</td>
</tr>
<tr>
<td>DKC 3421 YG</td>
<td>DKC 3420 Prot.</td>
<td>Ref. 1 Prot.</td>
<td>Ref. 2 Prot.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40 m</td>
</tr>
<tr>
<td></td>
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<td>4.5 m</td>
</tr>
<tr>
<td>DKC 3420 Non-Prot.</td>
<td>Ref. 1 Prot.</td>
<td>Ref. 2 Prot.</td>
<td>DKC 3420 Prot.</td>
</tr>
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<td></td>
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<tr>
<td>40 m</td>
<td>40 m</td>
<td>40 m</td>
<td>4.5 m</td>
</tr>
<tr>
<td>Ref. 2 Prot.</td>
<td>DKC 3420 Prot.</td>
<td>DKC 3421 YG</td>
<td>DKC 3420 Non-Prot.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40 m</td>
</tr>
</tbody>
</table>

178 m

Figure 1. Design of two field experiments located in Southwestern and Southeastern Poland. DKC 3421 YG: Bt-transgenic variety, no insecticide treatment; DKC 3420: corresponding isogenic variety, no insecticide treatment; DKC 3420 Prot.: isogenic variety, treated with lambda-cyhalothrine; Ref. 1 + 2 Prot.: Two conventional control varieties, treated with lambda-cyhalothrine.

**Results and discussion**

A total number of 72,985 individuals belonging to 47 ground beetles species were recorded in the experiment conducted in Wrocław, Lower Silesia (Table 1).
Table 1. The most numerous ground beetle species collected in Wroclaw, Lower Silesia, in 2008.

<table>
<thead>
<tr>
<th>Species</th>
<th>Ref. 1 Prot</th>
<th>Ref. 2 Prot.</th>
<th>DKC 3420 Prot.</th>
<th>DKC 3420 Non-Prot.</th>
<th>DKC 3421 YG</th>
<th>Total</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudoophonus rufipes (De Geer)</td>
<td>15274 82.1</td>
<td>13881 79.6</td>
<td>10968 80.1</td>
<td>8769 81.3</td>
<td>10103 81.0</td>
<td>58995</td>
<td>80.8</td>
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<tr>
<td>Calathus erratus (Sahlberg)</td>
<td>858 4.6</td>
<td>1147 6.6</td>
<td>752 5.5</td>
<td>492 4.6</td>
<td>544 4.4</td>
<td>3793</td>
<td>5.2</td>
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<tr>
<td>Pterostichus melanarius (Illiger)</td>
<td>643 3.5</td>
<td>784 4.5</td>
<td>729 5.3</td>
<td>593 5.5</td>
<td>766 6.1</td>
<td>3515</td>
<td>4.8</td>
</tr>
<tr>
<td>Calathus fuscipes (Goeze)</td>
<td>839 4.5</td>
<td>787 4.5</td>
<td>738 5.4</td>
<td>502 4.7</td>
<td>606 4.9</td>
<td>3472</td>
<td>4.7</td>
</tr>
<tr>
<td>Dolichus halensis (Schaller)</td>
<td>669 3.6</td>
<td>469 2.7</td>
<td>200 1.5</td>
<td>181 1.7</td>
<td>202 1.6</td>
<td>1721</td>
<td>2.4</td>
</tr>
<tr>
<td>Harpalus affinis (Schrank)</td>
<td>48 0.3</td>
<td>75 0.4</td>
<td>55 0.4</td>
<td>30 0.3</td>
<td>37 0.3</td>
<td>245</td>
<td>0.3</td>
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<tr>
<td>Trechus quadristriatus (Schrank)</td>
<td>38 0.2</td>
<td>51 0.3</td>
<td>33 0.2</td>
<td>58 0.5</td>
<td>24 0.2</td>
<td>204</td>
<td>0.3</td>
</tr>
<tr>
<td>Bembidion lampros (Herbst)</td>
<td>45 0.2</td>
<td>31 0.2</td>
<td>33 0.2</td>
<td>35 0.3</td>
<td>40 0.3</td>
<td>184</td>
<td>0.2</td>
</tr>
<tr>
<td>Zabus tenebriones (Goeze)</td>
<td>40 0.2</td>
<td>32 0.2</td>
<td>51 0.4</td>
<td>24 0.2</td>
<td>37 0.3</td>
<td>184</td>
<td>0.2</td>
</tr>
<tr>
<td>Microlestes minutulus (Goeze)</td>
<td>13 0.1</td>
<td>13 0.1</td>
<td>25 0.2</td>
<td>24 0.2</td>
<td>15 0.1</td>
<td>90</td>
<td>0.1</td>
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<tr>
<td>Carabus cancellatus Illiger</td>
<td>25 0.1</td>
<td>23 0.1</td>
<td>15 0.1</td>
<td>7 0.1</td>
<td>12 0.1</td>
<td>82</td>
<td>0.1</td>
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<tr>
<td>Harpalus tardus (Panzer)</td>
<td>12 0.1</td>
<td>16 0.1</td>
<td>21 0.2</td>
<td>12 0.1</td>
<td>15 0.1</td>
<td>76</td>
<td>0.1</td>
</tr>
<tr>
<td>35 other species</td>
<td>93 123</td>
<td>77 60</td>
<td>69 424</td>
<td>47 72985</td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total beetles</td>
<td>18597 17432</td>
<td>13697 10787</td>
<td>12470 72985</td>
<td>100.0</td>
<td></td>
<td></td>
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<tr>
<td>No. species</td>
<td>36 36</td>
<td>31 31</td>
<td>36 47</td>
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</tr>
<tr>
<td>Shannon-Weaver index H'</td>
<td>0.79 0.88</td>
<td>0.86 0.83</td>
<td>0.82</td>
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<td></td>
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<tr>
<td>Pielou index J'</td>
<td>0.22 0.24</td>
<td>0.25 0.24</td>
<td>0.23</td>
<td></td>
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<td></td>
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<tr>
<td>Simpson index</td>
<td>0.32 0.36</td>
<td>0.35 0.33</td>
<td>0.34</td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

*dominance (%) 

During the first year of the study the most abundant species in each treatment was *Pseudoophonus rufipes* (contributing more than 80% of the beetles caught). The calculated diversity quality indexes (Shannon-Weaver, Simpson) revealed similar values between the treatments. Also the equality Pielou formula was similar for beetle assemblages in the different treatments. In case of seasonal activity-density of captured ground beetles in Wroclaw, significant differences occurred mostly between Ref. 1 and DKC 3420 (sprayed and unsprayed), and in one case between Ref. 1 and Ref. 2, and in one case between Ref. 2 and DKC 3420 (sprayed) (Table 2). Overall, significant differences among the different treatments were only found at three time points (from a total of 15 sampling dates). There were no significant differences between Bt maize DKC 3421 YG and the conventional cultivars.

Table 2. Significant differences (p values) between the number of ground beetles caught in different treatments in Wroclaw, Lower Silesia (ANOVA, Tukey HSD test). Comparisons from other sampling dates were non-significant (p>0.05).

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref. 2. Prot.</td>
<td>0.003049 (13 August)</td>
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</tr>
<tr>
<td>DKC 3420 Prot.</td>
<td>0.000034 (13 August)</td>
<td>0.026178 (13 August)</td>
</tr>
<tr>
<td>DKC 3420 Non-Prot.</td>
<td></td>
<td>0.04447 (4 August)</td>
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<tr>
<td></td>
<td></td>
<td>0.000034 (13 August)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.001303 (25 August)</td>
</tr>
</tbody>
</table>

In the second experiment conducted in the South-Eastern part of Poland, near Rzeszów, a total of 14,198 specimens of ground beetles were collected (data from 3 out of 15 sampling dates) (Table 3). The predatory *Pterostichus melanarius* (55.1%) and the granivorous *Pseudoophonus rufipes* (34%) were clearly the dominating species. No significant differences in the number of beetles collected were detected among treatments.

Ground beetles are a polyphagous group of epigeal insects highly abundant in maize crops. Thus they can be exposed to toxins produced by transgenic plants through different ways. These arthropods can directly feed on plants or consume plant-produced insecticidal compounds indirectly when preying on non-target phytophagous organisms. In addition, the beetles may come into contact with the toxin that is present within the soil. Till now no clear differences in ground beetle abundance in Bt maize compared to conventional cultivars were detected in our two field experiments. Our preliminary results from 2008 are in accordance with previous studies from other European countries that also failed to detect detrimental effects of Bt maize on species richness and diversity indices for ground beetles (Farinos *et al.*, 2008; Lopez *et al.*, 2005; Manachini, 2000; de la Poza *et al.*, 2005; Sehnal *et al.*, 2004; Toschki *et al.*, 2007). Our preliminary date will be supported with data from two more years (2009-2010) on both experimental field sites.
Table 3. The most numerous ground beetle species collected in Rzeszów, Southeastern Poland in 2008 (results from 3 on 15 dates).

<table>
<thead>
<tr>
<th>Species</th>
<th>Ref. 1 Prot</th>
<th>Ref. 2 Prot</th>
<th>DKC 3420 Prot</th>
<th>DKC 3420 Non-Prot</th>
<th>DKC 3421 YG</th>
<th>Total</th>
<th>D*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pterostichus melanarius</em> (Illiger)</td>
<td>1370</td>
<td>1424</td>
<td>1364</td>
<td>1655</td>
<td>2021</td>
<td>7834</td>
<td>55.1</td>
</tr>
<tr>
<td><em>Pseudophonus rufipes</em> (De Geer)</td>
<td>896</td>
<td>962</td>
<td>1042</td>
<td>961</td>
<td>970</td>
<td>4831</td>
<td>34.0</td>
</tr>
<tr>
<td><em>Dolichus halensis</em> (Schaller)</td>
<td>157</td>
<td>156</td>
<td>116</td>
<td>115</td>
<td>210</td>
<td>754</td>
<td>5.3</td>
</tr>
<tr>
<td><em>Bembidion quadrimaculatum</em> (L.)</td>
<td>49</td>
<td>50</td>
<td>70</td>
<td>49</td>
<td>47</td>
<td>265</td>
<td>1.9</td>
</tr>
<tr>
<td><em>Bembidion femoratum</em> Sturm</td>
<td>46</td>
<td>60</td>
<td>58</td>
<td>35</td>
<td>22</td>
<td>221</td>
<td>1.6</td>
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<tr>
<td>42 other species</td>
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<td>69</td>
<td>58</td>
<td>46</td>
<td>57</td>
<td>293</td>
<td>2.1</td>
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<tr>
<td>Total beetles</td>
<td>2878</td>
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<td>2564</td>
<td>2709</td>
<td>14198</td>
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<td>23</td>
<td>24</td>
<td>31</td>
<td>25</td>
<td>23</td>
<td>47</td>
<td></td>
</tr>
</tbody>
</table>

*dominance %)


Acknowledgements

This subproject called „Impact of MON 810 maize on non-target arthropod species and trophic interactions under the Polish environmental conditions” is realize within the framework project „Environmental and economic aspects of permitting cultivation of GM crops in Poland” funded by Polish Ministry of Science and Higher Education.

References

Assessment of Bt maize effects on non-target arthropods in field studies using the evaluation approach of “good ecological state”

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¹Julius Kuehn-Institute, Federal Research Centre for Cultivated Plants, Institute for Strategies and Technology Assessment in Plant Protection, 14523 Kleinmachnow, Germany; ²Martin-Luther-Universität Halle-Wittenberg, Faculty of Natural Sciences III, Institute for Agricultural- and Nutritional Science, Halle (Saale), Germany
E-mail: claudia.wendt@jki.bund.de

Abstract: In the context of GMO safety research, the occurrence of non-target arthropods were monitored in maize fields and evaluated in order to detect possible effects of Bt maize using the “good ecological state” approach. These studies were performed in half-fields of Bt maize (MON 810) (BT) and conventionally cultivated maize (CV) planted in the Oderbruch region (Brandenburg, Germany), an infestation area of the European corn borer (Ostrinia nubilalis), in the years 2000-2007. Non-target arthropod taxa and densities were determined by counting insects on plants (5 sampling points/half-field) and pitfall traps (6 sampling points/half-field). In most cases, taxa had to be pooled to higher taxonomic units (indicator groups), e. g. Thysanoptera and aphid predators (predator units), due to low density or difficulty in identifying species. Carabids and spiders in pitfall traps were determined to the species level but also pooled for statistical analysis. Using five (visual counting) or six (pitfall trapping) sampling points per CV and BT half-field, we tested for density variation within and between the respective half-field within and between the respective years. The approach utilises baseline values to calculate “corridors of good ecological state”, defined as a range delimited by the 10% and 90% quantiles of densities in the CV half-fields during the last 5 years (2003-2007). Corridors were defined for each indicator group, e. g., for Thysanoptera and carabids, 2.9 to 12.6 individuals per stem and 23.2 to 60.4 individuals per trap and week, respectively. If significant differences between CV and BT half-fields are found in connection with CV or BT values outside the corridor limits, these cases should be subjected to particularly thorough evaluation. This approach proved to be inappropriate for arthropods with extreme abundance variation, e.g. aphids. Its utilisation as a tool for a „case-specific monitoring“ of effects of Bt maize on non-target arthropods is being discussed.

Key words: European corn borer, Ostrinia nubilalis, MON 810, non-target organisms

Introduction

According to Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms, especially Annex VII, as well as Regulation (EC) No 1829/2003 of the European Parliament and of the Council on genetically modified food and feed, monitoring of cultivated GM crops is mandatory. All monitoring programmes need to be appropriate to determine direct and indirect, immediate and cumulative long-term effects of the crops on non-target arthropods. In addition to Annex VII of Directive 2001/18/EC, guidelines (2002/811/EG) have been published describing aims, basic principles and structures of monitoring programmes in more detail. However, many details necessary to realise the implementation of such monitoring programmes remain
unexplained. Take, for example, remaining uncertainties regarding effects on non-target arthropods as well as appropriate parameters for risk assessment. Furthermore, there are insufficient long-term field study data for many non-target arthropods. Thus, representative monitoring methods need to be developed for implementation of long-term monitoring programmes. Council Decision 2002/811/EC explicitly suggests that general surveillance should include long-term monitoring to allow for unexpected effects that may occur after longer periods of environmental exposure.

In the years 2000 to 2007, we performed a field study to elaborate, test and evaluate a case-specific approach to monitoring non-target arthropods in Bt maize as well as to develop a guideline for a monitoring programme which can be implemented with a reasonable effort. The study was designed as a half-field comparison of Bt maize (MON 810) (BT) and conventionally cultivated maize (CV). Half-field comparisons are simple yet generally suitable for ecological effect studies (Perry et al., 2003).

The objective of this investigation were a) to test for variation of density, or density activity, of selected taxa within and between the respective CV and BT half-fields within and between the respective years and b) to calculate and use “corridors of good ecological state” based on variation of densities in CV half-fields during the last 5 years of the study.

The idea of using “corridors of good ecological state” as part of the “good ecological state” approach follows the European Enforcement Project’s “Monitoring the environmental effects of genetically modified plants” Final Project Report Part 1 (Anonymous, 2002). This model was conceived as a tool to support the identification and evaluation of significant differences in occurrence and diversity of arthropods in order to detect possible ecological side effects of Bt maize.

**Material and methods**

Our field studies were carried out in the Oderbruch region, a landscape in the German state Brandenburg. There, maize is grown on about 12% of the overall agricultural area, and the European corn borer (Ostrinia nubilalis) regularly occurs in high densities. The study started in 2000. Monitoring data were collected in field studies from 2003 to 2004 (two field-field comparisons each year) and 2005 to 2007 (five half-field comparisons per year).

Each half-field comparison comprised Bt maize (BT) and conventional maize (CV). Half-fields were planted with Bt variety MEB 307 (Event MON 810) (Cry 1Ab) and the near-isogenic conventional maize variety Monumental. The adjacent half-fields received the same agricultural management and all fields had a similar cropping and management history.

Two different sampling methods were used for data collection: visual counting of arthropods on maize plants and pitfall trapping. Visual counts were performed at the time of flowering of maize (BBCH 65). All arthropods found on the plants were determined to species level if possible. In each half-field, visual counts were performed at 5 sampling points containing three maize plants each. For pitfall trapping, six traps (diameter 10 cm, trapping agent: 2% formaldehyde solution) per half-field were installed. The catches were collected at weekly intervals during a period of four weeks at BBCH 61-69. All epigeic spiders and carabids collected were determined to species level. Sampling points were arranged along the midline of each half-field to exclude effects from the adjacent half-field and from surrounding habitats. In both methods, the distance between the sampling points was 20m.

**Statistical analysis**

Due to the small number of species densities per sampling point, the species were pooled into higher taxonomic units, referred to as indicator groups. Four pooled indicator groups were
assessed: Thysanoptera, aphid predators (predator units, calculation according to Freier et al., 1998), spiders and carabids. Using five (visual counting) or six (pitfall trapping) sampling points per CV and BT half-field, we tested for variation within and between half-fields within a given year and between the different years, respectively. Subsequently, we defined “corridors of good ecological state” as a range delimited by the 10% and 90% quantiles of densities in CV half-fields during the last 5 years. If significant differences between CV and BT half-fields were detected in connection with CV or BT values outside the respective corridor, these cases were subjected to particularly thorough evaluation (Figure 1).

The three baselines used are as follows.

Baseline 1: Mean and standard deviation of density in the conventional maize half-field, also used for significance testing to BT half-fields.

Baseline 2: Variation between CV half-fields (one randomly selected field per year) within the five last years (2003 to 2007). This calculated variation corridor is assumed to describe a relatively stable ecological state. In certain cases, the standard deviation may fall below the x axis and thus describes a value smaller than zero. Because it obviously is not possible to find arthropod species numbers less than zero we used 10% and 90% quantiles instead of standard deviation. The corridor between the 10% and 90% quantiles is defined as the “corridor of good ecological state“. As it changed annually, this corridor must be recalculated every year.

Baseline 3: Variation of mean between different CV fields within a given year and frequency and tendency (CV<BT or CV>BT) of significant differences between CV and BT.
Results and discussion

Thysanoptera, Aphid predators

Figure 2 shows the results for the indicator groups Thysanoptera (above) and aphid predators, measured in predator units (below).

![Graph showing variation within the indicator groups Thysanoptera and aphid predators in conventional (CV) and Bt maize (BT) half-fields in the years 2003-2007 and the respective “corridors of good ecological state”, defined as a range between the 10% (P10) and 90% (P90) quantiles, *=significant, S.D.= standard deviation, C.V.=coefficient of variance.]

In the indicator group “Thysanoptera”, variation was high between the years and between the half-fields in one year. Over all years, however, variation was not unusually high (C.V. \(CV_{cv}\)= 0.4; C.V. \(CV_{BT}\)= 0.3, Figure 2 above). In the case of aphid predators measured in predator units, variation was moderate between the years except in 2007, which showed an overall higher abundance. Regarding the fields of one year the variation was moderate (C.V. \(CV_{cv}\)= 1.2; C.V. \(CV_{BT}\)= 0.7).
BT= 0.7, Figure 2 below). The “corridor of good ecological state” for Thysanoptera was defined as 2.9 to 12.6 individuals per sampling point (3 plants). The corridor for aphid predators was 0.05 to 0.56 predator units per sampling point, representing rather low predator densities. Means of about five predators per m² were observed in wheat fields during 10 years (Freier et al., 2007). As this range is usually found in maize fields in the Oderbruch region, it is assumed that the situation in all fields within the corridor can be characterised as ecologically stable.

When the observed density exceeds or deceeds the corridor limits and the density of one variant differs significantly, as in Figure 2 (above) field 14, p=0.014 (Welch test), this suggests a possible adverse change in the ecological situation and should prompt more detailed analyses of site-specific differences between half-fields combined with additional investigations in the following year, if necessary.

**Spiders, carabids**

Representing the results of pitfall trapping, data for spiders and carabids are presented in the following Figure 3.

In the spider group activity density varied moderately between the years as well as between the half-fields of one year. The overall variation was not unusually high (C.V. cv= 0.45; C.V. BT= 0.44, Figure 3 above). In the carabid group, the overall variation of activity density was high, especially in BT half-fields (C.V. cv= 0.52; C.V. BT= 0.78, Figure 3 below). The “corridor of good ecological state” was defined as a density of 13.0 to 46.6 individuals per trap and week for spiders 23.2 to 68.4 for carabids.

In fields 7, p=0.003 (Welch test) and 14, p=0.027 (Welch test) (Figure 3 above), a significant spider activity density exceeding the corridor limits was found. The same applied to carabids in field 17, p=0.0007 (Welch test) (Figure 3 below) and field 5, p=0.048 (Welch test), where the activity density fell below the corridor limits. These cases should be investigated more thoroughly, looking for reasons such as those given for Thysanoptera and aphid predators. Overall, 15 significant differences between the variants were found in all investigated fields. Our results suggest a trend towards higher densities of aphids, spiders and carabids in Bt maize, but no distinct trend could be identified.

**Criteria for risk assessment**

The approach utilizing P10 and P90 quantiles to define the “corridors of good ecological state” was found to be appropriate for most investigated indicator groups, e. g. Thysanoptera, aphid predators, spiders and carabids. This approach could not be used for aphids due to excessive density variance. In that case, the calculated corridor ranged from P10=0.0 to P90=114.5 individuals per sampling point. There are different reasons for the enormous density variation of aphids in maize: population size in cereal ecosystems before migration to maize, weather conditions during the migration and population settlement of aphids, and predator occurrence in maize (Freier, 2001).

Our experience showed that the number of plants per sampling point should be increased to five to reduce the variation in sampling points in arthropod counts and increase the statistical power.
Figure 3. Variation within the indicator groups Spiders (above) and Carabids (below) in conventional (CV) and Bt (BT) maize half-fields in the years 2003-2007 and the “corridor of good ecological state” delimited by 10% (P10) and 90% (P90) quantiles, *=significant, S.D.=standard deviation, C.V.=coefficient of variance.

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Consideration of the case-specific monitoring of genetically modified potato and appropriate monitoring endpoints

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Abstract: A study on the feasibility and the choice of criteria for developing a general outline for a case-specific monitoring regime for transgenic potato has been performed. Possible impacts of genetic modifications on non-target organisms are assessed, considering the direct or indirect effects of metabolic alterations of the GM plants. Criteria for an appropriate risk assessment and recommendations for case-specific monitoring are suggested based on a review of the scientific literature on crop-organism interactions. First, we identify relevant pathways of plant-organism interactions through nutrients, toxins, or mechanisms of attraction and defence. Second, we consider the need for case-specific monitoring, taking into account variability of existing potato varieties and environmental conditions.

Key words: GMO monitoring, environmental risk assessment, integrated pest management, metabolism, non-target organisms, potato

Introduction

In genetically modified plants (GMP) with novel traits like improved nutritional properties or resistance against abiotic stresses, the modifications could be complex and metabolic networks could be unintentionally affected causing effects on other organisms and resulting in fitness costs to the plants themselves. Metabolic pathways are connective, crosstalk between them occurs and they are governed by complex regulatory mechanisms. Therefore, some of the effects might be difficult to predict on the basis of current knowledge (Rischer et al., 2006).

Case-specific monitoring is supposed to overcome uncertainties in the assessment of a specified risk by investigation of a clearly defined cause-effect hypothesis during environmental release of the GMP. In contrast, general surveillance aims to detect non-anticipated risks as well as long-term adverse effects (Sanvido et al., 2008). There is an ongoing debate as to the appropriate choice of targets of a case-specific monitoring program. Our scope is to clarify whether current knowledge of mechanisms of interactions between potatoes and other organisms generally allows the identification of adverse effects of GMP, the underlying causal chains and a possible need for case-specific monitoring.

Secondary plant metabolites are involved in communication, defence against pests and pathogens, and attraction of natural enemies. Due to the promotion of integrated plant protection in the regulations of European Agriculture (see European Parliament 13.1.09, revision of the directive 91/414/EEC) these compounds and mechanisms are of increasing interest to risk assessment of GMP. Natural variation has been and is being used as a breeding source, and resulting changes in metabolism have not been of much concern. However, this has changed with the need for safety assessments of GMP (Rischer et al., 2006).
Metabolic compounds have diverse biological functions, show a high degree of variability and are influenced by a multitude of biotic and abiotic factors. This raises the question whether unintended adverse effects can be fully assessed prior to an environmental release (within the risk assessment process) or whether and how they may be addressed within the monitoring regime after market release of the GMP.

We undertake a review of the natural variation of metabolic compounds observed in potato with the aim to define parameters (baselines) useful for risk assessment, monitoring purposes and decision making.

Results and discussion

A review of the literature on potato physiology depicts the diversity and variability of metabolic compounds within and between potato species and cultivars. Hence, interactions of potato cultivars with other organisms will/may vary considerably.

A prominent group of metabolites in potatoes are glycoalkaloids (GA) and their derivatives. The accepted safe limit for human consumption for the amount of GA in potato tubers is 20 mg/100 g fresh weight (FW), which is considered a reference value for risk assessment (see black arrow in Figure 1). In commercially grown varieties the amount of GAs found in tubers can sometimes exceed this limit, irrespective of farming system (Hajslova et al., 2005). Numerous GM potato lines with altered carbohydrate metabolism are available, but rarely were examined for changes in secondary metabolism (Richter et al., 2007). Esposito et al. (2002) suggest a routine GA determination for new genotypes. A breeding goal for potato for the future is to increase foliar GA levels to achieve resistance to pests and pathogens whilst at the same time keeping the level of total GA (TGA) in tubers low (Ginzberg et al., 2009; Grafius and Douches, 2008).

Data from 11 different studies have been combined in Figure 1 to illustrate the range of steroidal GA content in wild and cultivated potato species and cultivars. Wild potato species are an important source for breeding material; they usually have higher levels of TGA. Toxic secondary metabolites serve as a type of direct defence against herbivores. The presence of glycoalkaloids and leptines in foliage is associated with resistance, for example, to the Colorado potato beetle *Leptinotarsa decemlineata* and nematodes, or reduced feeding by *Agriotes obscurus* (wireworm). Resistance to *L. decemlineata* is only achieved with high levels of GA in leaves. Leptin (foliar GA) levels of >300mg/100g FW (see grey arrow in Figure 1) resulted in a 50% suppression of feeding. However, even varieties with lower foliar GA levels may be protected against damage because of oviposition preferences displayed by the beetle (Lytytinen et al., 2007). Jonsson and Olsson (1994) have identified TGA as a key factor in predicting wireworm larval feeding. Johnsson et al. (2008) showed only a weak relationship between TGA level and susceptibility to wireworm but the potato variety with the highest TGA (30.9mg/100 g FW) showed the least damage. Lowering the level of TGA could therefore lead to a weakening herbivore resistance.

A second group of alkaloids, calystegines (Tepfer et al., 1988), have also been found in potato. They are strong glycosidase inhibitors. As with GAs, their amount varies among potato cultivars (Griffiths et al., 2008). The ratio of GA and calystegines also varies, implying separate genetic control of the two metabolite classes (Friedman et al., 2003). Calystegines have been shown to have allelopathic properties and are likely to be a source of carbon and nitrogen for certain soil bacteria. Inhibition of plant invertase by calystegine B2 was demonstrated in glasshouse experiments, and plant invertases are involved in the response to biotic and abiotic stress (Roitsch et al., 2004). Calystegines in potato tubers increase with increasing sucrose levels, as observed in sucrose synthase antisense tubers.
This suggests a connection between calystegine biosynthesis, sugar metabolism and activation of defence against pathogens (Berger et al., 2007). Therefore, changes in response, for example, to sucrose availability may result in reduced plant fitness and have an impact on the interaction with non-target organisms. Potential effects have not been quantified yet.

Figure 1. Range of total glycoalkaloid content in potato accessions. Arrows indicate (black) maximum level for human consumption and (grey) level for 50% feeding suppression in Colorado potato beetles.

References: Study (1) Sadowska et al., 2008: 1a cultivar Irga, 1b GM potatoes (PVY resistance); (2) Sotelo et al., 2000: Mexican commercial varieties; (3) Friedman et al., 2003: Commercial varieties; (4) Ramsay et al., 2004: 4a Cultivated taxa; 4b Wild species; (5) Papathanasiou et al., 1999: Commercial varieties; (6) Abreu et al., 2007: Influence of different cropping systems; (7) Shimoi et al., 2007: Different cultivars; (8) Kozukue et al., 2008: 8a S. andigena; 8b S. canasense; 8c S. stenotomum; 8d S. tuberosum (all in tuber cortex); (9) Friedman, 2004: Commercial varieties incl. cv. Lenape; (10) Ronning et al., 2000: Foliar GA S. chacoense; (11) Deahl et al., 1993: Foliar GA;

Volatile plant compounds have probably evolved to repel herbivores, but now perform a remarkable range of functions. Most of the animals that interact with plants are insects, which detect volatiles through their antennae. Plant volatiles attract pollinators, and they act as
indirect defences by attracting parasites and predators that prey upon herbivores. They are also involved in plant-plant or within-plant communication, thereby playing a physiological role in the systemic response of plants to local damage (Dudareva et al., 2006). The composition of the volatile “blend” is highly variable depending on genotype, plant development stage, environmental conditions and biotic factors such as attack by insect herbivores, and the differences can be discriminated by predators and parasitoids (Degen et al., 2004). The effects of genetic modifications on volatile emission can include changes in the attraction of predators and parasitoids (tritrophic interactions), and even have wider consequences for community composition (Dicke, 2009). Volatile emissions fluctuate, however, and the composition of a volatile blend and the change in ratio between concentrations of individual compounds may provide important information (Jansen et al., 2009). Therefore, it may be more useful to pay attention to subtle changes in volatile patterns than to measure absolute concentrations of volatiles emitted by plants. An interesting trend in the analysis of plant volatiles is the application of so-called electronic noses (Laothawornkitkul et al., 2008) to the evaluation of transgenic plants (Zawirska-Wojtasiak et al., 2009).

Proteomics, transcriptomics and metabolomics are profiling techniques that are being used as tools to determine substantial equivalence and to investigate the extent of natural variation between conventional cultivars. With the growing number of conventional cultivars being investigated, the range of natural variation that is considered acceptable will become broader (Cheng et al., 2008). When performing risk assessments, GMP should not only be compared with their near isogenic line, but also with a number of commercial varieties (conventional range). This will provide additional data and ultimately lead to the development of crop composition databases (systems biology approach) that detail key crop parameters/profiles and their natural variation. An example is the Golm Metabolome Database (GMD@CSB.DB; Kopka et al., 2005), which provides public access to custom mass spectra libraries and metabolic profiling experiments. Such databases will provide further background information for risk assessment and management, will contribute to the prevention of unnecessary follow-up analyses and, ultimately, to the development of predictive genetic engineering (Rischer et al., 2006).

There is no single method enabling the analysis of the complete metabolome, and a hierarchical approach is favoured (Catchpole et al., 2005; Rischer et al., 2006). However, the feasibility and usefulness of measuring absolute levels of single metabolites in the field as part of the case-specific monitoring process is doubtful. Gas chromatography methods are slow, require pre-concentration and preparation procedures, and slight changes in settings induce shifts in the data. Metabolic profiling may be useful in experimental settings, in order to perform environmental risk assessments, but it will be challenging to correlate the experimentally gained profiles with reaction modes after environmental release. Multiple factors drive variations and fluctuations in plant metabolites, thereby interfering with a case-specific monitoring. Effects of genetic modifications mediated by secondary metabolites are likely to manifest themselves in shifts of reaction modes or patterns of the plants in response to environmental factors or in the dynamics of non-target organisms. These could be assessed in field experiments at larger sites or during general surveillance over longer time periods with emphasis on local environment, seasonal variation and crop management practice (Birch et al., 2007), using farmer questionnaires (Böttinger and Schiemann, 2007; Schmidt et al., 2008) for tracking. Insights gained and potential effects revealed can then be fed back into risk assessment and when appropriate, into the set-up of case-specific monitoring or experimental investigation.
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