Preface

In June 2005, the IOBC/WPRS working group ‘GMOs in Integrated Plant Production’ had hold its second full working group meeting. The first meeting had taken place in Prague, Czech Republic, in November 2003 [see IOBC/WPRS Bulletin 27(3), 2004]. Similar to the first meeting in Prague, there was a vast interest in this event with more than 80 participants from 20 countries attending. Besides colleagues from public research institutes, about 20 percent of the 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. I would also like to point to the strong (7 colleagues) participation of colleagues from North America which has added value to the event since they were able to bring in the expertise from commercial growing of GM crops since many years.

During the meeting, three keynotes, 31 oral contributions and 25 posters were presented. According to the talks and posters that were submitted, the meeting had a strong focus on non-target risk assessment and environmental monitoring of GM crops. These are two areas that are receiving a huge interest not only in the WPRS. On the day after the full working group meeting, a special activity workshop was held to discuss future activities on non-target risk assessment and regulation of GM crops. In total 28 contributions and the protocol from the workshop are published in this volume of the bulletin. This includes both full papers and extended abstracts.

I would like to thank all the colleagues that had helped me to set up the scientific programme and those that had agreed to act as session organizers. On behalf of all participants, I would like to thank Ramon Albajes and his team from the Universitat de Lleida for their excellent job in organizing this meeting including an informative meeting website and enjoyable social activities. I think that I can speak for all when saying that we had a great time in Lleida.

The next full working group meeting is planned for the first half of 2007. 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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Key Notes
Recent advances in transgenic insect pest control

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Abstract: Contemporary plant molecular biology has now come of age and this has been in turn translated into a wide range of plant biotechnology applications. Transgenic insect pest resistance is at the forefront of such activities. The first insect resistant crops were commercialised in the mid to late 90s with experimental growing of such crops now dating back to over a decade and a half. Among key issues that occupy all of us working in this area is the sustainability and long-term effectiveness of transgenic crop plants expressing insecticidal genes. This, coupled with the ever increasing stringent legislation regulating transgenic crops, particularly in Europe, make it essential that a lot of effort, time and monetary investment is placed in strategies to prolong the useful life of such products, while at the same time maintaining the exceptional safety standards that have so far been applied selectively to genetically enhanced (GE) crops. We discuss here two different approaches that our own group is currently engaged in to achieve sustainability of insect pest control using plants expressing insecticidal transgenes. One is based on the concept of transgene pyramiding in which multiple transgenes targeting the same insect through different mechanisms are co-expressed in the same plant. The second approach involves the use of insecticidal fusions between conventional, well studied insecticidal genes, for example Bt, with lectin moieties that have additional domains to increase binding interactions between the toxin fusion(s) and receptors in the insect midgut. We exemplify these two strategies with transgenic rice and maize plants that have been specifically engineered to resist attack by lepidopteran and homopteran insects. We conclude by discussing how different drivers and perspectives, often non-scientific in nature, influence the way research in the field of insect pest resistance using transgenic plants is conducted.

Key words: Insect resistant crops, genetic engineering, transgenic plants, food security, Bt genes, sustainable insect pest control

Introduction

Eight hundred and forty million people in the developing world are chronically undernourished, surviving on fewer than 2000 calories per day (Pinstrup-Andersen et al., 1999; FAO, 2001). Many more people, perhaps half of the world’s population in total, suffer from diseases caused by dietary deficiencies and inadequate supplies of vitamins and minerals (Graham et al., 2001). Most of the poor are rural farmers in developing countries, depending entirely on small-scale agriculture for their own subsistence and to make their living. Because of limited purchasing power, the poorest farmers generally cannot irrigate their crops nor buy herbicides and pesticides. This leads to soil exhaustion and falling yields, and leaves the crops susceptible to pests, diseases and natural disasters such as drought. Many farmers are eventually forced to abandon their land and move to cities, adding to the growing problem of urban poverty and hunger (Royal Academy of Sciences, 2000). The world’s population is predicted to double over the next 40 years, with over 95% of people being born in developing countries (Byrnes & Bumb, 1998). Therefore, food production must increase by at least 40% to feed all the extra mouths, while land and water resources dwindle due to increased
urbanization, industrialization and pollution (Byerlee et al., 2000). The only solution is to increase the yields of major food crops, particularly cereal grains, using currently available land and less water. This will need to be achieved with a variety of approaches, including the efficient use of organic and inorganic fertilizers, irrigation strategies, soil and water conservation, pest and disease management and the production of improved plant varieties with higher yields.

It is envisaged that crop varieties with improved agronomic performance will be generated using a number of methods, some based on conventional breeding, others on more recent developments in biotechnology, and perhaps some by combining both conventional and molecular strategies (Huang et al., 2002a). The use of transgenic plants offers the greatest promise for rapid integration of improved varieties into traditional cropping systems because improved plant lines can be generated relatively quickly and with precision once suitable genes for transfer have been identified. It is recognized that biotechnology is not a magic wand that can free the world from poverty, hunger and malnutrition, but we support the view that the use of transgenic plants as one component of a wider strategy including conventional breeding and other forms of agricultural research, can contribute in a substantial manner towards the achievement of food security now and in the future. Significant challenges remain, not only in the achievement of the necessary scientific breakthroughs, but also in ensuring technology transfer to locally adapted crops and the negotiation of intellectual and technological property rights that protect inventors without encumbering the subsistence farmers for whom the improved crops are intended. Different transgenic strategies that can be used to improve agricultural productivity can be utilised, either by reducing extrinsic constraints or increasing the intrinsic yield potential of our crops. In this report we will focus on one facet of the issue, sustainable and durable insect pest control using transgenic plants.

Genetically enhanced crops and insect pest resistance

Many of our crop plants are also food for insect pests, and devastating losses occur throughout the world due to pest infestations either in the field or in stored products such as cereal and legume grains. In the developing world, about half of all crop production is thought to be lost to insects, 15% of these losses occurring due to post-harvest consumption and spoilage (Gatehouse et al., 1993). Farmers spend billions of US$ every year to provide effective control using chemical insecticides. A number of major crops, for example cotton, cannot be cultivated without heavy chemical inputs in the form of insecticides. Insects not only cause direct yield losses by damaging and consuming plants, they also act as vectors for many viral diseases and the damage they inflict encourages microbial infections.

In the industrialized world, pest control is heavily dependent on chemical inputs, which are expensive and damaging to the environment. The chemicals are relative non-selective, killing harmless and beneficial insects as well as pests, and accumulating in water and soil. Another major problem is that insects may evolve resistance to chemical insecticides (Schuler et al., 1998). In the developing world, many farmers are too poor to afford pesticides and are left at the mercy of nature. In any case, chemical control measures are ineffective against some of the worst pests, such as the rice brown planthopper (Nilaparvata lugens) which feeds by sucking sap from the phloem. Biological control has been pursued, however, results are in most cases marginal in row crop situations. We are thus faced with a problem in terms of being able to control damaging insect pests using conventional strategies. The genetic enhancement of plants to express insect-resistance genes offers the potential to overcome all of the shortcomings listed above, since genes that show exquisite specificity towards particular pests have been isolated from bacteria and other sources, thus minimizing the threat
towards non-target organisms. Furthermore, the expression of such proteins within plants allows effective control of insects that feed or shelter within the plant, and the degree of protection is not influenced by the weather, as is the case for topical chemical pesticide applications. Finally, the likelihood of insects becoming resistant to the transgenic plants can be reduced by a number of strategies, such as pyramiding resistance genes affecting different receptors in the target insect, conditional expression and the provision of ‘safe-havens’ or refuges, to reduce selection pressure. Several different types of genes have been exploited to control insect pests, including bacterial toxins, enzyme inhibitors and lectins.

**Bt genes**

Although several microbial species have been used as sources of insecticidal genes, the most popular source by far is *Bacillus thuringiensis*. This is a spore-forming bacterium that produces insecticidal toxins during the sporulation process. They are expressed as inert protoxins that are activated by proteinases in the highly alkaline environment of the insect gut. This provides an important safety barrier since the environment in which the toxins are activated is unique to insects, and thus it is safe for other animals, and humans, to consume plants expressing *Bt* toxins. Once activated, the toxins interact with receptors on the midgut epithelium cells creating pores in the plasma membrane by disrupting osmotic balance. This results in paralysis and the ultimate death of the insect.

Nearly 30% of all the commercially grown genetically enhanced plants in the world contain synthetic insecticidal toxin genes from *Bacillus thuringiensis* (James, 2004). Different subspecies and strains of bacteria produce toxins with different, but always highly specific, host-ranges allowing individual pests to be targeted (van Frankenhuyzen & Nystrom, 2002). Over thirty plant species have been engineered with modified *Bt* genes and many of these have been commercialized or are undergoing field trials (reviewed by Schuler et al. 1998; Llewellyn & Higgins, 2002). Therefore, a large amount of quantitative data has been obtained in terms of improved yields and reduced insecticide use. For example, the NewLeaf® potato variety developed by Monsanto contains a modified cry3A toxin gene. It demonstrated very effective control of the Colorado potato beetle and allowed a 40% reduction in pesticide applications (Carpenter & Gianessi, 2001). In the case of YieldGuard® maize, there has been effective control of the target pest (the European corn borer, *Ostrinia nubilalis*) but only a small reduction in pesticide use (1.5%) reflecting the fact that pesticides are ineffective against this species and are rarely used (Carpenter & Gianessi, 2001). An important knock-on effect of *Bt* maize is the reduction in insect-mediated feeding damage, which limits adventitious access and further spoilage by fungal pathogens and mycotoxin contamination (Llewellyn & Higgins, 2002). Mycotoxin contamination is a very serious health issue that can be minimized or even eliminated through the cultivation of *Bt* maize. This is not often recognised as a benefit of *Bt* crops.

In the developing world, *Bt* cotton varieties are leading the way in the fight against poverty alleviation by showing how biotechnology can increase agricultural yields. *Bt* cotton was adopted by China in 1997 and now accounts for about one third of all cotton grown in that country (Pray et al., 2002). Rural farmers have reported moderate yield gains year on year (5-10%) and a reduction in labor and agrochemical costs. *Bt* cotton varieties are now grown commercially in six developing countries (China, India, Argentina, Indonesia, Mexico and South Africa), with particularly encouraging results in Mexico where these varieties have all but eliminated infestation problems on smallholder farms caused by the pink bollworm (*Pectinophora gossypiella*) (Traxler et al., 2003). The provision of locally adapted *Bt* cereal crops would have a more direct impact on hunger in the developing world, and this is beginning to happen in the case of *Bt* maize, which is grown in both Argentina and South Africa by smallholders for commercial exploitation and their own consumption. *Bt* maize
varieties are nearing approval in China, and are being field-tested in Egypt, the Philippines
and Kenya. While in some countries the technology has been developed by the US company
Monsanto and adapted for local varieties by backcrossing, Bt maize in China and Kenya has
been developed by domestic scientists working in public research institutions (Bohorova et
al., 2001; Huang et al., 2002b).

Several groups have reported enhanced resistance to the striped stem borer and yellow
stem borer (Scirpophaga incertulas) in rice plants transformed with cry1Ab (Wunn et al.,
1996; Datta et al., 1998). Mortality rates of up to 100% have also been reported with rice
plants expressing cry1Ac (Nayak et al., 1997) and cry2A (Bano-Maqbool et al., 1998). Bt rice
is now being field-tested in several developing countries and is reaching the last stages of the
approval process in China (Tu et al., 2000; Ye et al., 2001a). The field evaluations in China
have been very successful. Tu et al. (2000) showed that a Bt commercial hybrid variety
expressing the cry1Ab gene produced a 28% yield increase compared to wild type plants. Ye
et al. (2001a) produced a rice line expressing cry1Ab that was entirely protected against stem
borer attack throughout the growing season, while more than 80% of untransformed plants
were damaged.

Recent developments include the stacking of multiple Bt genes and the use of fusion
genes to provide enhanced resistance against a range of insect pests. For example, Cheng et
al. (1998) produced rice plants expressing both cry1Ab and cry1Ac, which were shown to
eliminate stem borer larvae within 5 days. Ye et al. (2001b) described transgenic plants
expressing a cry1Ab/cry1Ac fusion gene, and showed that damage from four lepidopteran pests
was almost completely eliminated. In an adjacent plot, non-transgenic plants were protected
from attack by lepidopteran insects by pesticide application, but instead succumbed to
planthoppers, which are homopteran (sap-sucking) pests. This direct reduction in pest
damage, plus the secondary reductions in insecticide-induced planthopper infestation and
insect-transmitted viral diseases has great potential to increase yields and contribute
significantly to food security throughout Asia and the rest of the developing world.

Protease and amylase inhibitors
While bacterial toxins have been widely used as insecticides, there is also increasing research
into the use of enzymes that inhibit the digestion of food in the insect gut and therefore act as
anti-feedants. Many plants appear to express such enzymes as a defensive strategy and the
genetic manipulation of crops now allows the corresponding genes to be transferred between
species. Depending on whether the dietary intake of the insect is predominantly protein or
carbohydrate, the expression of protease inhibitors (Lawrence & Koundal, 2002) and alpha-
amylase inhibitors (Chrispeels et al., 1998) should prevent the uptake of nutrients and
therefore starve or delay the development of insect larvae. Insects that predominantly
consume plant proteins produce gut proteases such as trypsin and elastase, which are required
for digestion. Many plants express trypsin inhibitors, particularly in their seeds, and these can
be manipulated to protect susceptible plants from insect pests. In China, a rice variety
resistant to three rice pests is coming to the end of its environmental release trials and is likely
to be commercialized in the near future. This variety contains a Bt gene, the gene for cowpea
trypsin inhibitor and the Xa21 gene for resistance against bacterial blight.

Lectins
The Bt toxins and digestion inhibitors discussed above are active mainly against lepidopteran
and coleopteran species. There are few insecticidal proteins that are active against the
significant threat of homopteran (sap-sucking) pests, such as the rice brown planthopper,
which not only destroy plants directly but act as vectors for a number of very severe viral
diseases (e.g. Rice Tungro Virus, Grassy Stunt Virus and Ragged Stunt Virus). One group of
proteins that does possess such activity is the lectins, with snowdrop lectin (Galanthus nivalis
agglutinin, GNA) the most widely exploited because it has been shown to have no toxic effect on mammals (Gatehouse & Gatehouse, 1998). Importantly, several groups have shown that the gna gene can be stacked with Bt toxin genes to provide simultaneous resistance to a broad range of insect pests. For example, Bano-Maqbool et al. (2001) have produced transgenic rice plants expressing two Bt genes and gna, and these plants show resistance to the three most important rice pests in Asia: rice leaf folder, yellow stemborer and brown planthopper.

**Sustainable and durable insect pest control using genetically enhanced plants**

Transgenic plants expressing Bt toxins have been used successfully to provide resistance against a number of insect pests for several years, with insect resistance being the second most widely used trait in transgenic crops (after herbicide resistance) in world agriculture. One potential issue with Bt based insecticides is that they are very widely used, with up to 90% of microbiological insect control products based on topically applied Bt toxins. For this reason, there is concern that insects might develop resistance to Bt toxins and indeed the diamondback moth (*Plutella xylostella*) has evolved resistance in some open field populations in response to repeated exposure to foliar sprays containing Bt proteins, while laboratory selection experiments with other insect pests have shown that recessive mutant alleles can confer resistance to multiple Bt toxins. It is worth noting, however, that the evolution of resistance in insects against transgenic plants expressing Bt toxins has yet to be seen in the field, defying dire predictions based on computer simulations and various deterministic models. This is despite the fact that insect resistance crops have been cultivated on millions of hectares worldwide for many years. This, of course, does not mean that insects will not evolve resistance under field conditions at some point in the near or in the distant future. It is always prudent to devise strategies to assure the long term usefulness of transgenic crops expressing insecticidal genes. It will indeed be very unfortunate if the effectiveness of such crops that took years to develop at great effort and expense is compromised through inappropriate deployment strategies. Deployment strategies encompass multiple components ranging from the nature of the genetic construct and its expression profile, combining multiple transgenes in the same plant, deployment strategies to discourage the development of homozygous resistant insect populations, etc. One strategy to address potential limitations in conventional transgenic insect pest control involves the stacking or pyramiding of multiple transgenes in the same transgenic plant. We reported perviously the simultaneous introduction of *cry1Ac, cry2A* and *gna* (snowdrop lectin from *Galanthus nivalis*) into rice (Bano Maqbool et al., 2001). The transgenes provided protection against three of the most important insect pests of rice: rice leaf folder (*Cnaphalocrocis medinalis*), yellow stemborer (*Scirpophaga incertulas*) and brown planthopper (*Nilaparvata lugens*). The triple transformants showed synergistically greater resistance to these insects than plants expressing single transgenes and in our experiments we observed that the greatest reduction in insect survival and the greatest reduction in plant damage occurred in plants expressing all three transgenes. Bioassays using the triple transgenic plants showed complete eradication of the rice leaf folder and yellow stemborer, and 25% reduction in the survival of the brown planthopper. We therefore concluded that combinations of genes whose products disrupt different biochemical or physiological processes in the same insect, provide a multi-mechanism defence.

We have now devised an alternative strategy to extend/complement transgene pyramiding, in which a conventional insecticidal molecule such as the Bt toxin *cry1Ac* is fused to the non-toxic ricin B-chain (Mehlo *et al.*, 2005). The recognition of toxin binding sites in the insect midgut is an important factor determining the spectrum of Bt toxin activity. Several groups investigating the mechanism of toxin recognition have identified *N*-acetyl
galactosamine residues as an important component of Bt toxin-binding receptors. We therefore selected the non-toxic ricin B subunit as a fusion partner for the Bt toxin, since it binds such residues with very high affinity. We postulated that a fusion protein comprising an N-terminal Bt toxin and a C-terminal lectin polypeptide would provide a novel binding domain that would allow the hybrid protein to bind to a wider repertoire of receptors than the control toxin, in this case *cry1Ac*. Furthermore, the fact that single alleles in homozygous form can confer resistance to Bt toxins suggests that each toxin interacts with a single receptor, and loss or modification of this receptor leads to resistance in otherwise susceptible insects. Therefore, by increasing the number of binding domains on each toxin, the likelihood of resistance evolving in target populations is reduced, as mutations affecting several different receptors are highly unlikely to occur simultaneously because of the mathematical compounding of mutation frequencies.

The results of insect bioassays in our experiments showed that transgenic rice and maize plants expressing the fusion protein were synergistically more toxic to a range of insect pests than those expressing *cry1Ac* alone. Furthermore, the fusion protein conferred resistance to a broader spectrum of insect pests than those normally susceptible to *cry1Ac*. In our greenhouse experiments, we were able to demonstrate effective control of the striped stemborer (*Chilo suppressalis*), the cotton leaf worm (*Spodoptera littoralis*) and also the leafhopper *Cicadulina mbila*. The aphid *Rhopalosiphum padi* was not affected in any way. Our experiments thus demonstrated that: (a) insects that are normally susceptible to Bt genes are even more susceptible to the fusion protein between Bt and the ricin B chain, suggesting that we may not require a high dose deployment strategy for transgenic crops expressing such molecules, (b) a major lepidopteran pest that is resistant to Bt now becomes susceptible, (c) a homopteran pest that is outside the normal host range of Bt is now highly susceptible to the hybrid molecule and (d) not all insects become susceptible to the fusion protein molecule. The last point is extremely important as it demonstrates a degree of selectivity in the mode of action of the fusion protein. This also provides a starting point for further engineering experiments to construct highly specific toxins targetted against particular insect pests but not others.

**Adoption, deployment and benefits of insect resistant crops**

Standard Bt cotton, maize and potato crops have been released commercially and have been readily adopted by farmers while Bt rice, although yet to be commercialized, has been tested successfully in several field trials. Rice plants expressing Bt genes are expected to be commercialized in China in the very near future, and are already being cultivated illegally by farmers. Experience has shown the benefits of such enhanced crops in terms of increased yields, reduced chemical inputs and, as a knock-on effect, improved farmer and consumer health (Huang et al., 2005). Sustainable resistance against insect pests is the cornerstone of any sensible deployment strategy that utilizes transgenic plants expressing insecticidal proteins, either alone or, preferably, within an integrated pest management system. It is in the context of resistance management that crops expressing fusion proteins such as those described in our work could be the most beneficial. Bt crops in the US must be co-maintained with refuges that decrease the selection pressure on target pests and reduce the theoretical likelihood of resistance becoming established, while in China refuges in Bt cotton crops are provided by alternative host plants that supports the major pest species, *Helicoverpa armigera*. This type of mandatory refuge management system may be difficult to implement for crops such as rice and maize in developing countries with many smallholder farms, and where substitute hosts for insect pests are not available. Fusion proteins such as those we have
developed have the potential of providing strong and sustainable resistance requiring multiple counter-adaptive mutations, but would require only a single toxin transgene.

Lessons learned following the widespread use of chemical pesticides for the control of insect pests over the past several decades call for reason and caution in how we deploy transgenic plants expressing insecticidal genes in the present and in the future and a requirement to learn about the basic mechanisms. This, however, does not translate to a de facto moratorium on research to achieve such sustainability. Bt transgenic plants are remarkably specific in their activity, with little or no effect on non-target organisms. Field tests with Bt rice, maize and other crops have revealed no negative impact on biodiversity and indeed a positive impact resulting from the reduction in pesticide use (James, 2004; Huang et al., 2005). Another strong advantage of conventional Bt crops is that there is no credible evidence for toxicity or allergenicity in humans.

The environmental, economic and social benefits of insect resistant crops are beyond any doubt, in particular benefiting developing countries, despite claims to the contrary by opponents of biotechnology applications. For Bt cotton, farmers in India have seen a 70% reduction in the use of chemical insecticides translating to a very substantial savings in expenditure on insecticides by subsistence farmers who are the individuals the least likely to afford them. Up to 85% increases in cotton yields have been seen by Indian farmers who have adopted the technology. Similarly in China, cotton growers who are cultivating Bt cotton have been able to reduce the number of insecticide applications to one third, compared to farmers that grow conventional cotton. In addition, the number of farmers in China reporting pesticide poisoning in conjunction with cultivation of Bt cotton was 4.7% (less than 2,500) compared to 22% (50,000) amongst farmers cultivating conventional cotton (Huang et al., 2005).

Wider application of novel insect pest control in transgenic crops will necessitate testing a broad range of insects encompassing agriculturally important pests and also beneficial insects and other organisms to ascertain the full efficacy and safety of novel insecticidal proteins. However, exhaustive safety evaluations and environmental impact assessments although prudent and necessary need to be performed in a rational and scientific manner. It is necessary to ascertain effects on non-target organisms, however, we need to keep such evaluations in the appropriate biological context and if for example there are some minor detrimental effects on the growth rate of the green lacewing, this author believes the plight of hundreds of millions of people in the developing world who are on the brink of starvation or those subsistence farmers that are forced to use toxic chemicals to combat insects should also be a component of the assessment exercise!

**Conclusions**

Advances in plant transformation and gene expression technology allow the introduction of novel traits into our crop plants. Genetically enhanced crops have the potential to address some of the causes of hunger, both directly (by increasing the availability of food) and indirectly (by reducing poverty in developing countries). Crop failure, due to pests and diseases, could be averted by the adoption of plants that are resistant to such biotic stresses. An increasing number of resources are being diverted towards the development of ever more sensitive methods to detect so called „unintended effects on non-target organisms“ in insect resistant transgenic crops. So far most, if not all of the results are negative. Is it not time to apply scientific reasoning and come to the logical conclusion that if a battery of tests fails to demonstrate any substantial detrimental effects perhaps the answer might be that these plants do not pose any real threat to the environment, rather than pursuing further research to develop even more sensitive methods in the hope that such detrimental effects might be detected?
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GMO’s in crop production and their effects on the environment: methodologies for monitoring

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Abstract: The Food and Agriculture Organization (FAO) hosted an Expert Consultation on "Genetically Modified Organisms in Crop Production and their Effects on the Environment: Methodologies for Monitoring and the Way Ahead" from 18-20 January 2005, in Rome. The main objective of the consultation was to review the scientific basis for, and procedures to establish, effective post-release monitoring of genetically modified (GM) crops and develop guidelines to strengthen member countries’ capacities to design and carry out monitoring programs. The experts developed a robust design template for monitoring that could work across a reasonable range of resource levels. The design template incorporated a series of core values including a serious commitment to engage and consult with people with a stake in the final outcome throughout the process, a requirement that indicators are selected to address stakeholder concerns, to meet basic requirements for scientific rigor, and which trigger appropriate management or regulatory responses. The experts did not wish the template to be adopted as an inflexible, linear process, but as the basis for a toolbox of concepts and approaches that will support advances towards sustainability.

Some key elements in the template included:

- Development and statement of monitoring program goals in consultation with individuals and groups that have a stake in the final outcome, including particularly growers, and stewards of protected or important natural resources.
- Identification at the outset of potential barriers to achieving the goals of the program, and the development of plans to overcome or minimize these.
- Development of simple, robust, conceptual models for the farming system in question, built from stakeholder and expert knowledge of potential risks and benefits of GM crops, and ways to measure these.
- Identification of limited suites of potential indicators that are connected to the key elements of the conceptual model and which can be used to guide actions and decisions once monitoring has taken place.
- Determination of appropriate trigger values for the selected indicators, and identification of the decisions or management actions that would result if these values are exceeded.
- Development of a transparent and effective process to ensure follow-through with appropriate actions and decisions, including continuing commitment to stakeholder engagement, clarity in analysis and reporting, effective connections to policy development and capacity building where this is required.

The experts demonstrated that full stakeholder engagement throughout the process was essential and should be fostered through formal and informal networks, alliances and initiatives that promote communication and information dissemination. Full stakeholder engagement is vital to build trust and transparency, and the only way to sustain an effective link between monitoring and the resulting actions.

Key words: Monitoring, GM crop, FAO
**Introduction**

The expert panel developed the basis for monitoring program design for countries with substantial knowledge of potential hazards and programs for monitoring environmental effects of GM crops, and countries with limited knowledge of potential hazards, and little experience with GM crop monitoring. This paper provides an outline of the procedure recommended by the experts for the latter case, using herbicide tolerant rice in Asia as a model system. A report of the meeting (FAO, 2005), from which this paper is abstracted, and the background paper that outlines the monitoring program design template and the scientific justification for this approach (Jepson, 2005) should be consulted for further details.

The experts outlined a number of principles that guided monitoring program design, including a definition of monitoring that provides a clear statement of its scope and purpose. These principles were elucidated from the substantial international literature concerning effective program design (e.g. Heywood, 1995; Noon, 2003; Stork & Samways, 1995). They stated that successful monitoring procedures must build upon existing ecological data sources that have established the status of the system under investigation. Where such data are lacking they may need to be collected prior to GM crop introduction. Monitoring should not be confused with general environmental surveillance or ecological inventory: monitoring is goal-oriented, and designed to detect change in comparison to reference sites, and/or pre-treatment condition. When effective, monitoring addresses the priorities of people with a stake in its outcome, and feeds back to inform management and policy development.

In addition, the experts stated that deployment of GM crops must encompass the whole process of technology development from pre-release risk assessment through to post-release monitoring. Monitoring programs should recognize and take into account important sources of variation between farming systems and GM crop types in order to properly address potential interactions between the GM crop and the environment. The positive and negative effects of GM crops will vary with location and context, and monitoring will require a new model of working in order to inform actions at the farming system scale in countries that aim to adopt this technology. The capacity to undertake monitoring varies globally however, and reflects the level of ecological knowledge associated with particular systems, the local capacity to plan, implement and analyze the data, and the integrity of the pathway that leads from the data to decision making, and back to effective management.

**Monitoring program design**

Two separate expert working groups undertook program design exercises, using examples that reflect the range of capacities internationally to develop and undertake monitoring. They proposed processes and mechanisms for developing a monitoring program that meet the needs of countries or regions with substantial knowledge of potential hazards and with existing programs for monitoring environmental effects of GM crops and countries or regions with limited knowledge of potential hazards and little experience in monitoring environmental effects of GM crops. The monitoring program design template for the latter case is presented below in Table 1. Monitoring program development is a greater challenge in cases where possible hazards are not clearly understood, the stakeholder community is not well defined, the level of protection afforded by environmental protection measures is low, and there is a lack of capacity and resources (Jepson, 2005). The experts therefore considered this to be a particularly important case to address.
Table 1. The outline below presents a monitoring design template which includes the basic elements of program design, points to be considered and the challenges of implementing the various elements in the context of herbicide tolerant lowland rice in Asia.

<table>
<thead>
<tr>
<th>Program design where there is limited information and experience</th>
<th>Elements for program formulation</th>
<th>Points to be considered</th>
<th>Hypothetical example: monitoring program for herbicide tolerant (HT) rice in a developing country in Asia.</th>
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</thead>
<tbody>
<tr>
<td>Develop and state program goals in consultation with stakeholders in the final outcome (e.g. farmers, stewards of local protected areas etc.)</td>
<td>Identify and engage stakeholders, recognizing that different skills tend to be found in different sectors. Define the ultimate goals of the monitoring program, expressed in terms that stakeholders value. Develop consensus on precisely stated goals to enable effective monitoring design, and eventual follow up.</td>
<td>Are the goals clear and simple enough to be addressable? If there are broader concerns, should the program be nested within a larger process? Does the program adhere to laws and relevant conventions? Has a fair and equitable selection program been used to identify relevant stakeholders?</td>
<td>Goals: To avoid weedy rice becoming more weedy because of gene flow and selection. To maintain the native gene pool of rice. To maintain the livelihoods of Asian farmers.</td>
</tr>
<tr>
<td>Identify barriers to achieving goals</td>
<td>Identify all the practices, and stressors that may compromise the system. Identify the resource affected by each practice or stressor. This will aid the later identification of indicators. Summarize the characteristics of the above in terms of frequency, extent, magnitude, selectivity and variability.</td>
<td>Competing interests and marketing forces could prevent consensus. Lack of success can result from failure to engage civil society: people with important expertise may be excluded from communication and access to resources. Poor communication between stakeholders limits goals setting, and engagement.</td>
<td>Weedy rice is already widespread in direct seeded areas, less so in transplanted areas. Good management practice is well understood, but not always practiced for various reasons. Marketing forces will influence the adoption of GM rice, and may not acknowledge risks. No obvious technical barrier to effective monitoring.</td>
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<td>Develop a simple, robust, conceptual model for the system based upon stakeholder and expert knowledge</td>
<td>Outline interconnections between system components, the strength and direction of links and the state of the system. Outline the scales at which processes operate and consider how the system ‘works’ with an emphasis on response to practices or.</td>
<td>Engage all sources of knowledge from farmer, public, private and civil society sector. Need to ensure their participation throughout the program.</td>
<td>GM technology, with low adoption of good practice leads to HT gene flow into wild relatives. Herbicide resistance in weedy rice is selected by increased use of herbicides (which can happen with or without gene flow). Weedy rice densities can increase and production consequently decreases.</td>
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<tr>
<td>Program design where there is limited information and experience</td>
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<tr>
<td>stressors. What is acceptable variability and what constitutes a normal pattern?</td>
<td>Identify possible indicators that are connected to key elements of the conceptual model, and to the concerns of stakeholders</td>
<td>Indicators may work, but must be able to be measured cost-effectively. Need provisions for entry and validation of data received from farmers and other stakeholders.</td>
<td>Counts of weedy rice m²² Yield loss Seeding rate, kg / ha Frequency of herbicide use Need to establish sampling regime that may be undertaken by extension services, farmer groups, farm consultants etc.</td>
</tr>
<tr>
<td>Make measurements that reflect agricultural and ecological processes that are sensitive to change across the range of GM crop release and provide information on the status of unmeasured resources. Temporal and spatial scales must be stated.</td>
<td>Estimate the status and trends in the indicator, in comparison with control areas, baseline values before crop release or ideally both</td>
<td>The choice of reference site or condition is complicated where adoption is rapid or widespread. Reference points and baselines may be hard to identify if GM cropping becomes the norm</td>
<td>Reference point – non-GM systems (may want to use sentinel plots / farms) Need to report variation in indicator responses, as well as mean values Important to clearly visualize results and express in terms that have clear meaning to stakeholders</td>
</tr>
<tr>
<td>Determine appropriate magnitude of effect size for a response, based on an understanding of spatial and temporal variation in response relative to baseline or reference condition.</td>
<td>Determine trigger values for the selected indicators that lead to management action</td>
<td>The trigger value must be connected to an adverse effect on resources of concern to stakeholders. Intensively managed systems tend to become depleted and trigger values must take into account broader goals for sustainability, as well as the status and trends in the indicator in the reference site(s) Placing long-term societal goals for sustainability ahead of short-term, possibly unsustainable goals is a challenge and requires confidence building measures among</td>
<td>To be effective in early warning about serious hazards, triggers are needed that result in a change in farmer behavior in time to reverse adverse impacts Need to address balance between long- and short-term costs and benefits May ask farmers to make decisions that are not cost-effective or valued in the short term</td>
</tr>
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</table>
**Program design where there is limited information and experience** | **Elements for program formulation** | **Points to be considered** | **Hypothetical example: monitoring program for herbicide tolerant (HT) rice in a developing country in Asia.**
---|---|---|---
Link monitoring results to decision making through clarity, transparency, effective policy development and capacity building | List and evaluate all possible interpretations of indicator values, the likelihood of each being true and the societal values associated with each interpretation. This engages stakeholders and provides guidance in effective decision making | The experts recognized that there were few effective models for this process in the recent history of adopting new technologies in agriculture. Full stakeholder engagement however, is essential for adaptive and effective technology adoption. | Establish chains of multi-way communication that extend from local government to farmer, to researcher, educator, regulator and policy developer

**Conclusions**

The experts were optimistic that monitoring could work, within reasonable resource levels, and the outline for program design was considered to be a powerful basis for developing a monitoring system. The monitoring system will however work best if nested within other processes that address wider goals, otherwise the process can easily become burdened with multiple tiers of questions and concerns that may go beyond agriculture or the concerns of growers and those that live within the farm community and adjoining areas.

The key insight for all the participants in the workshop was that stakeholder engagement is intrinsic to the system, from the beginning right through to the end, and it is vital to the building of trust, legitimacy and transparency. The experts considered this to be the only way to deliver an effective link between the goals of monitoring on the one hand and triggers and decisions for actions on the other.

Expertise in monitoring and agricultural systems is available in both the formal and informal sectors, but it needs to be identified and engaged if the process is to be successful in each country where monitoring is required. The experts proposed establishment of pilot workshop processes on a small scale in several areas to work the monitoring design process through as a thought experiment, and establish pilot systems that include collection, management and reporting of field data.

The experts also provided detailed recommendations for scientists, policy makers and international organizations that can be used as road map for the effective implementation of GM crop post-release monitoring (FAO, 2005).

**Acknowledgements**

References


Presentations
Parasitization of *Chromatomia horticola* Goreau in experimental fields with genetically modified canola

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**Abstract:** The global surface invested with transgenic crops is rapidly increasing in many areas of the world. Similarly, the awareness about the environmental safety of genetically modified plants has recently increased. Europe is a centre of origin for many cultivated and wild Brassicaceae, therefore the biosafety assessment of such plants is particularly important in view of their possible commercial release. Genetically modified plants resistant to insects may potentially alter the trophic structure, interfering with the regular activity of some functional groups (e.g. predators, parasitoids) both directly and indirectly. With the aim of evaluating the natural parasitization activity on non target herbivores, experimental field with transgenic and control canola were prepared for two growing seasons (2001 and 2003). The genetically modified canola was resistant to Lepidoptera via expression of a synthetic Cry1Ac gene.

Among non target herbivores linked to the crop, the leafminer *Chromatomya horticola* Goreau, was particularly abundant in the later part of the cropping season in both years. The dynamics of natural parasitization on this dipteran, was then comparatively investigated in transgenic and control plots. Twenty infested leaves per each of the three transgenic and three control plots were collected weekly for three weeks when plants were in full blossom. Active parasitization rates on *C. horticola* larvae were calculated by opening all mines under a stereomicroscope. In the second field trial it was also calculated the hatching rate of adult parasitoids that were the identified at least at a genus level.

For field season 2001, the parasitization rate and the percentage of surviving *C. horticola* larvae in the two treatments was comparable with a maximum of 33.87% and 28.57% in control and transgenic plots. The more detailed study run in the second field trial confirmed that the activity of parasitoids was not impaired in transgenic areas. Among the most active parasitoids, *Chrysocharis* sp. furnished the maximum contribution to the natural control of the leafminer. In both treatments this hymenopteran provided around 70% of the total parasitization, *Dygliphus* sp. and *Pediobius* sp. contributed for about 1/5 of total parasitization.

**Key words:** biosafety, natural control, transgenic plants, *Bacillus thuringiensis*

**Introduction**

In 2004, genetically modified canola plants were cultivated on 4.3 millions hectares (6% of the global world area invested with genetically modified crops (James, 2004).

Europe is a centre of origin for many cultivated and wild Brassicaceae. Oilseed rape is thought to have developed in the western Mediterranean from the hybridisation between *B. oleracea* and *B. rapa*. A large number of relatives of oilseed rape exist throughout Europe, some of which are cultivated as crops and others that are known as weeds in farming systems and wild flowers outside cultivated areas (Eastham & Sweet, 2002). There are at least seven species naturally interfertile with *B. napus* (Scheffler & Dale, 1994), but for many more it has
been possible to produce hybrids in controlled conditions. Spontaneous hybridization between a *B. campestris* vegetable cultivar and its wild conspecific has been measured (Manasse, 1992). In some cases the percentage of hybrids with wild brassicas was as high as 93% and hybrids were more fit than their wild parent (Jørgensen et al., 1996).

Canola is also a very attractive plant for pollinators and flower visiting insects as it is rich in nectar (Mc Gregor, 1976). Oilseed rape pollen grains are typical of insect pollination, being fairly heavy and sticky; bees carrying many viable oilseed rape pollen grains can be found emerging from a hive (Ramsay et al., 1999).

For all these reasons, the biosafety assessment of genetically modified canola plants is particularly important in view of their possible commercial release.

Genetically modified plants (GMP) resistant to insects may potentially alter the trophic structure, interfering with the regular activity of some functional groups (e.g., predators, parasitoids) both directly and indirectly (Agrawal, 2000). We setup experimental fields using genetically modified canola plants resistant to Lepidoptera via expression of a synthetic *cry1Ac* gene with the aim of comparing the natural parasitisation activity on a non target herbivores in transgenic and non-transgenic plots.

**Materials and methods**

**Experimental Fields**

The genetically modified canola line GT-2 (Halhill et al., 2001) harbouring the *cry1Ac* gene expressing a δ-endotoxin from *Bacillus turingiensis* Berl., active against lepidoptera, and its untransformed control line were used according to the current EU regulations (Permit n° B/IT/00-022) for two field experiments in the growing seasons 2001 and 2003.

The experimental fields were prepared according to a completely randomised design with 200 m²-plots with three replications for each treatment. Plants were manually transplanted in the experimental plots at a distance of 35 cm between rows and 10 cm along the row, with a final planting density of about 30 plants/m². The total number of plants for both the transgenic and control lines was approximately 18,000.

**Plant characterisation**

The presence of the transgene was assessed with PCR molecular analysis. Two Bt transgene-specific primers (Bt L for - 5’ CAA CAA CTA TCT GTT CTT GAC GGG 3’ and Bt L rev 5’ CAT ACC GTA CAC GAA CTC GAT ATC 3’) were used.

*Cry1Ac* expression in transgenic lines was assessed with ELISA assay performed with commercial kits *Cry1Ab/Cry1Ac* ELISA Plate Kit and *Cry1Ab/Cry1Ac* QuickStix™ (Envirologix Inc.) in 20 plants for each lines.

**Sample collection**

Among the non target herbivores linked to the crop, the leafminer *Chromatomyia horticola* Goreau, was particularly abundant in the later part of the cropping season in both years. The dynamics of natural parasitisation affecting this dipteran was then comparatively investigated in transgenic and control plots.

Twenty infested leaves from each of the three transgenic and three control plots were collected weekly for three weeks when plants were in full blossom. Active parasitisation rates on *C. horticola* larvae was calculated by opening all mines under a stereomicroscope. In the second field trial pupae of *C. orthicola* found in the leaf mines were maintained in an incubator at 26°C ± 1 until eclosion, and the emergence of adult parasitoids was recorded.
Results and discussion

Canola experimental fields were extensively infested by the leafminer Chromatomyia horticola. Some biological features also make it an interesting non-target species to be studied as potential indicator. The larvae are certainly exposed to the toxin through all their development as they feed on the palisade tissue of plant leaves. As immatures develop mining leaves, the species occupies a specific niche in the agro-ecosystem and it represents an important secondary host for parasitoids of economic relevance for the region (e.g. Diglyphus isaea – Walker -, one of the few parasitoids providing natural pest control of the Citrus leafminer in the study area).

Several wasp parasitoids are known to provide natural control of this herbivore in agro-ecosystems (Gencer, 2004). In our samplings during 2001, the active parasitisation rate and consequently the leafminer larval survival were rather similar (Figure 1) with the highest values reached on April 10th when larval parasitisation rate was 33,87% and 28,57% in control and transgenic plots respectively. The difference in active parasitisation values was not statistically significant. A more detailed study repeated during 2003, considering both active parasitisation rate and parasitoids emerging from leafminer pupae, confirmed that parasitisation of C. horticola was not impaired in transgenic fields (Figure 1). The total percentage was even higher in GT2 plots (Table 1) while this difference was, again, not statistically significant (p=0.08).

Three main parasitoids were active in the experimental field (taxonomic identification still needs to be completed), with Chrysocharis sp. providing most of the natural control of the herbivore. In both treatments this chalcidoid was responsible for about 70% of the whole parasitisation while Dygliphus sp. and Pediobius sp. accounted for about 1/5 of total parasitisation (Table 1). Among the other active parasitoids collected, it has to be remarked the finding of a species belonging to the Eurytomidae family, so far never identified as a parasitoid of Chromatomyia horticola.

Table 1. Parasitisation of Chromatomyia horticola in transgenic and control canola field in 2003 (average of three sampling dates).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Larval parasitisation¹</th>
<th>Pupal parasitisation²</th>
<th>Total</th>
<th>Chrysocharis sp. %³</th>
<th>Dygliphus sp. %³</th>
<th>Pediobius sp. %³</th>
<th>Others %³</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT2</td>
<td>30,65</td>
<td>46,92</td>
<td>77,57</td>
<td>65,52</td>
<td>10,35</td>
<td>10,35</td>
<td>13,78</td>
</tr>
<tr>
<td>Control</td>
<td>22,67</td>
<td>29,57</td>
<td>52,24</td>
<td>68</td>
<td>16</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Means followed by the same letter are not significantly different (Mann-Whitney U-test).

¹ Indicates the percentage of larvae of the leafminers that were observed undergoing active parasitisation when mines were open.

² Parasitoid adults emerged from the pupae of the leafminer.

³ Percentage refers only to adults that emerged from pupae.
Table 2. Additive Correspondence Analysis for field data 2003. Absolute contribution of single taxa to the species assemblage along the first axis.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. gossypii</em></td>
<td>0.005</td>
</tr>
<tr>
<td><em>M. persicae</em></td>
<td>0.0024</td>
</tr>
<tr>
<td><em>P. xilostella</em> larvae</td>
<td>0.0198</td>
</tr>
<tr>
<td><em>Pieris</em> larvae</td>
<td>0.0025</td>
</tr>
<tr>
<td>Coccinellidae</td>
<td>0.0167</td>
</tr>
<tr>
<td>Chrysoperla eggs</td>
<td>0.0015</td>
</tr>
<tr>
<td>Thripidae</td>
<td>0.415</td>
</tr>
<tr>
<td>Alticinae</td>
<td>0.135</td>
</tr>
<tr>
<td><em>C. horticola</em> larvae</td>
<td>0.263</td>
</tr>
<tr>
<td>Staphilinidae</td>
<td>0.0677</td>
</tr>
<tr>
<td><em>Meligethes</em> sp.</td>
<td>0.07</td>
</tr>
<tr>
<td><em>Tropinota hirta</em></td>
<td>0.0001</td>
</tr>
<tr>
<td>Miridae</td>
<td>0.0004</td>
</tr>
<tr>
<td><em>Cantharis</em> sp.</td>
<td>0.0001</td>
</tr>
<tr>
<td>Araneae</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

In conclusion, from our field observations it can be affirmed that the activity of larval-pupal parasitoids and overall leafminer larval survival were rather similar between the transgenic GT2 canola line and its near isogenic control line, and therefore no major effects due to the use of the Bt-expressing line were identified. New insights on the complex of natural enemies of *C. horticola* (extent of parasitisation rate, early season activity, most active species) were acquired that indicate this species as an adequate candidate for long-term studies of non target effects. Indeed, in the second field season, *C. horticola* showed the...
highest rank for absolute contribution along the first axis in an Additive Correspondence Analysis, accounting for about 26% of the total variation along the axis (Table 2).

However, the major limitation of this study is due to its lack of temporal continuity imposed by regulatory obligations. In fact, the long term evaluation of these field observations should be the next logical step in order to confirm the validity of these experimental observations.

Acknowledgements

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Monitoring of Roundup Ready soybean in Romania

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Abstract: According to the Romanian Biotech Law regarding the regime of obtaining, testing, using and marketing of genetically modified organisms (GMOs), the aim of post-marketing monitoring is to confirm the conclusions of the environment risk assessment and to identify unanticipated adverse effects of the GMO on human health and on the environment. Based on the conclusions of the environment risk assessment, it has been estimated that a general surveillance activity is sufficient to detect potential modifications of the biology of Roundup Ready® (RR) soybean that may occur in time. Surveillance activity consisted in repeated visits to agricultural fields cultivated with RR soybeans, and where subsequently crop rotation has been implemented according to Good Agricultural Practices. Additionally, a survey has been conducted with farmers who have grown RR soybean for several years, regarding: disease and pest sensitivity as indicator of the maintaining of the specific varietal traits; yield as an indicator of normal reproduction; number of treatments for weed control to confirm the advantage of RR technology as compared to conventional technology (in terms of reduction of herbicide consumption); presence of soybean volunteers as indicator of capacity of survival; presence of soybean plants in the vicinity of the fields where RR soybeans were previously grown as indicator of modification of dissemination potential. Surveillance of the effect of this technology on the annual and perennial weed populations revealed that maintaining of this GM crop would not affect the structure and the size of weed populations. General surveillance with the purpose of identifying potential changes in the biology of RR soybeans confirmed the lack of risk for the RR soybean to become an invasive species in agro-ecosystems or natural habitats. Since the release approval has been granted in Romania there were no significant changes in the RR soybean plants survival capacity. The results of a field study conducted to evaluate the impact of RR versus conventional technology on the arthropod fauna showed no significant differences on epigeal insects (in terms of population size and/or composition). The interaction between RR soybean and invertebrate species is not different from the interaction of conventional soybean. The results of monitoring activities are consistent with the prediction of the earlier ecological risk assessment.

Key words: RR soybean, post-marketing monitoring, arthropod fauna, earthworms

Introduction

According to the Romanian biotech law regarding the regime of obtaining, testing, use and marketing of genetically modified organisms (GMOs), the aim of post-marketing monitoring is to confirm the conclusions of the environmental risk assessment and to identify unanticipated adverse effects on the environment and on human health. In Romania, GM soybean is commercially grown in pre-existing agro-ecological environments, and the direct and indirect ecological effects of the Roundup Ready® (RR) technology are likely to be broadly similar to those resulting from conventional chemical spraying. The results of many research projects to determine the effects of growing GM herbicide tolerant varieties for
agriculture and the environment, revealed no significant difference between GM and non-GM crops in terms of weed diversity (Buckelew et al., 2000; Watkinson et al., 2000; Elmegaard & Pedersen, 2001; Jasinski et al., 2001; Strandberg & Pedersen, 2002; Dewar et al., 2003; Witmer et al., 2003; Bennett et al., 2004; Yoshimura et al., 2004). Furthermore, any effects identified were due to the herbicide technology applied, not whether the crops were GM or not. Based on the conclusion of many studies undertaken in different locations in the European Union and on the conclusions of the environmental risk assessment of Roundup Ready soybean carried out by the applicant, it has been estimated that a general surveillance activity is sufficient to detect potential modification of the RR soybean biology that may occur in time.

Surveillance activities consisted of repeated visits to agricultural fields cultivated with RR soybean and where subsequent crop rotation has been implemented according to Good Agricultural Practices.

In order to confirm some conclusions of the environmental risk assessment conducted by the applicant, field experiments (case-specific monitoring) were undertaken for evaluating the impact of RR versus conventional technology on the arthropod fauna and weed population. Results of these monitoring activities of RR soybean crops in Romania are presented.

**Material and methods**

The objectives of the surveillance activities are presented in Table 1.

<table>
<thead>
<tr>
<th>Surveillance target</th>
<th>Purpose</th>
<th>Period and frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 RR soybean plants</td>
<td>Evaluation of growth and development</td>
<td>Monthly</td>
</tr>
<tr>
<td>2 Weed in the plantlet stage</td>
<td>Estimation of weeding level and impact of first herbicide treatment</td>
<td>Before first treatment</td>
</tr>
<tr>
<td>3 Weeds after each treatment</td>
<td>Estimation of impact of RR technology on weed populations</td>
<td>After each treatment</td>
</tr>
<tr>
<td>4 Natural vegetation from ruderal areas surrounding the crop</td>
<td>Estimation of habitat and nutritive resources for other species in the trophic chain</td>
<td>Monthly, from planting to harvest</td>
</tr>
<tr>
<td>5 Health state of the crop</td>
<td>Estimation of effects of GM crops on other biodiversity elements</td>
<td>Monthly</td>
</tr>
</tbody>
</table>

**General surveillance**

A general surveillance was conducted during the period 2002-2004. Farmers cultivating RR soybean were asked questions regarding plant behaviour in the new agro-ecosystem (Table 2). Responses were considered indicators for soybean behaviour related to invasiveness, persistence, rate and/or mode of reproduction, dissemination, survival, etc., and were used by state institutions involved in policy regarding GMOs.
Table 2. General surveillance (RR soybean biology) undertaken during 2002-2004.

<table>
<thead>
<tr>
<th>Traits/characteristics</th>
<th>Indicators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pest sensitivity</td>
<td>Maintenance of line-specific characteristics</td>
</tr>
<tr>
<td>Yield</td>
<td>Normal reproduction, inter-species relations (symbionts, etc.)</td>
</tr>
<tr>
<td>Presence of soybean volunteers in rotation</td>
<td>Changes in survival capacity</td>
</tr>
<tr>
<td>Presence of soybean plants in the vicinity of fields</td>
<td>Changes in dissemination potential</td>
</tr>
</tbody>
</table>

Case-specific monitoring

Case-specific monitoring has been carried out in 2004, in a monoculture (2002-2004) soybean experimental field located at the Moara Domnească Didactic Experimental Station and focused on aspects listed in Table 3. The experimental protocol included several variants of conventional cropping systems: FRONTIER 900 EC 2l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha; TREFLAN 48% EC 2 l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha; and one of Roundup Ready technology (ROUNDUP READY 3 l/ha at 6 leaves stage + 3 l/ha at 12 leaves stage), applied on two RR soybean cultivars, SP9191RR and S2454RR.

Table 3. Case-specific monitoring (ecological impact) undertaken during 2002-2004.

<table>
<thead>
<tr>
<th>General trends in biodiversity as recorded in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure and composition of the weed population. In 2004, the number and weight of weeds species was determined by counting and gravimetric measures (standard methods as used in herbicide experiments).</td>
</tr>
</tbody>
</table>
| Structure and composition of invertebrate fauna. At an interval of 2 weeks, Barber traps (2 replications/variant) were placed in the field. Traps were filled with 4% formaldehyde and opened for a 24 hour period. During the interval of two openings of the Barber traps, adhesive Pherocone traps (1 yellow sticky traps/variant; 72 cm²/trap) were used additionally being analysed. Before each opening of the Barber traps, Pherocon traps were replaced, recorded and the data was interpreted. The epigeal fauna was collected and counted two times a week from May until September, from 12 soil traps. Also, samples of the beneficial fauna from 10 plants grown in variant 1 (FRONTIER 900 EC 2l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha, variant 2 (TREFLAN 48% EC 2 l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha) and from 10 RR soybean plants, in 4 replications, was counted and recorded. Furthermore, acariens (*Tetranychus urticae*) from 10 plants, in 4 replications/variants were counted and recorded. Insects collected from both traps and plants were preserved in 70% alcohol and taxonomically determined in the laboratory. Where the species level could not be determined, systematic position for genus, family, order or class were established. Invertebrate community structure from the variants of conventional and RR technology was compared by calculating Sörensen Similarity Index according to the formula: 
| Is = (2c/a+b)*100, where: ‘Is’ is the Sörensen Index, ‘c’ is the number of common species, ‘a’ is the number of species from one of the fields, and ‘b’ is the number of species from the other field. 100% indicates that there is no difference between the two faunas compared and 1% - that they are completely different. |
Results and discussion

Results from the general surveillance programme confirmed the lack of risk for the RR soybean to become persistent in agro-ecosystems or to invade natural habitats. There have been no significant changes in the RR soybean plants survival capacity since the release approval was granted in Romania in 2000. Soybean itself never appeared as a volunteer crop. Within the naturally occurring plant populations located in the immediate vicinity of the soybean crop fields or in the ruderal areas, feral soybean plants were never observed, a logical situation for Romanian climate which does not allow *Glycine* species to overwinter. The results of the surveillance activities demonstrated that RR soybean has not been altered in growth and development characteristics when compared to conventional soybean cultivars.

Normal yields registered with RR soybean confirmed that the complex relationships at the biocoenosis level between RR soybean plants on one side and the soil micro-organisms, invertebrate and vertebrate populations on the other side, have not been altered by the genetic modification. With other words, RR soybean cultivars had a similar behaviour than the conventional varieties grown in the same ecosystem, with regards to relationships mentioned above.

**Results of case specific monitoring**

*Structure and composition of the weed population*

Weed species were drastically eliminated from soybean crops by herbicide applications. The Roundup cropping system produced almost the same results as conventional cropping systems (Table 4).

After the treatments, some annual species germinated from remaining seeds, developed, flowered and recovered the seed bank in the soil. All weed species proliferated in the field edges, and along the access pathway and produced a large quantity of seeds. Their existence is not jeopardised.
Table 4. Frequency, mass and species structure of the weeds, determined in several cropping systems, in a three year monoculture of RR soybean.

<table>
<thead>
<tr>
<th>Period of assessment in 2004</th>
<th>Variant of cropping system – weed control – applied previously (2002-2003)</th>
<th>From which:</th>
<th>Species structure (no./g/m² from total weeds)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total weeds (no./g/m²)</td>
<td>Grasses</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Broad</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>leaves</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Setaria</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>glauca</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Echinochloa crus-galli</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sinapis arvense</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amaranthus hybridus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anthracnema arvensis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xanthium arvense</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chenopodium album</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Solanum nigrum</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Convolvulus arvensis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Convolvulus arvensis</td>
<td></td>
</tr>
<tr>
<td>Early spring, before any tillage</td>
<td>V₁</td>
<td>33/208</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>V₂</td>
<td>14/71</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>V₃</td>
<td>15.8/104</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>V₄</td>
<td>19.3/137</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>V₅</td>
<td>19.5/134</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>V₆</td>
<td>23/167</td>
<td>-</td>
</tr>
<tr>
<td>20 days after first tillage</td>
<td>V₁</td>
<td>97/277</td>
<td>74/160</td>
</tr>
<tr>
<td></td>
<td>V₂</td>
<td>49/181</td>
<td>33/73</td>
</tr>
<tr>
<td></td>
<td>V₃</td>
<td>33.2/162</td>
<td>25/60</td>
</tr>
<tr>
<td></td>
<td>V₄</td>
<td>39.5/149</td>
<td>23/47</td>
</tr>
<tr>
<td></td>
<td>V₅</td>
<td>42.2/166</td>
<td>26/58</td>
</tr>
<tr>
<td></td>
<td>V₆</td>
<td>43/182</td>
<td>23/57</td>
</tr>
<tr>
<td>20 days after second tillage</td>
<td>V₁</td>
<td>28.6/112</td>
<td>12/31</td>
</tr>
<tr>
<td></td>
<td>V₂</td>
<td>25/105</td>
<td>12/31</td>
</tr>
<tr>
<td></td>
<td>V₃</td>
<td>22.2/100</td>
<td>13/31</td>
</tr>
<tr>
<td></td>
<td>V₄</td>
<td>26.5/107</td>
<td>13/39</td>
</tr>
<tr>
<td></td>
<td>V₅</td>
<td>27/90</td>
<td>15/33</td>
</tr>
<tr>
<td></td>
<td>V₆</td>
<td>29.2/112</td>
<td>14/32</td>
</tr>
<tr>
<td>30 days after ppi herbicide application</td>
<td>V₁</td>
<td>55/238</td>
<td>19/38</td>
</tr>
<tr>
<td></td>
<td>V₂</td>
<td>55/238</td>
<td>19/38</td>
</tr>
<tr>
<td></td>
<td>V₃</td>
<td>12/51</td>
<td>7/22</td>
</tr>
<tr>
<td></td>
<td>V₄</td>
<td>9/70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>V₅</td>
<td>9/70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>V₆</td>
<td>7.6/72</td>
<td>-</td>
</tr>
</tbody>
</table>

to be continued ....
<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 days after ppi herbicide application</td>
<td>V₁</td>
<td>95/759</td>
<td>54/244</td>
<td>41/515</td>
<td>38/102</td>
<td>16/42</td>
<td>1/20</td>
<td>1/69</td>
<td>1/27</td>
<td>31/211</td>
<td>1/30</td>
<td>1/15</td>
<td>3/102</td>
<td>2/41</td>
</tr>
<tr>
<td>V₂</td>
<td>8/133</td>
<td>1/3</td>
<td>7/130</td>
<td>1/3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3/70</td>
<td>-</td>
<td>-</td>
<td>2/31</td>
<td>2/29</td>
<td></td>
</tr>
<tr>
<td>V₄</td>
<td>11/237</td>
<td>-</td>
<td>11/207</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9/129</td>
<td>-</td>
<td>-</td>
<td>1/79</td>
<td>1/29</td>
<td></td>
</tr>
<tr>
<td>V₅</td>
<td>11/237</td>
<td>-</td>
<td>11/207</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9/129</td>
<td>-</td>
<td>-</td>
<td>1/79</td>
<td>1/29</td>
<td></td>
</tr>
<tr>
<td>V₆</td>
<td>18/263</td>
<td>2/22</td>
<td>16/241</td>
<td>1/10</td>
<td>1/12</td>
<td>-</td>
<td>1/80</td>
<td>1/12</td>
<td>12/121</td>
<td>1/5</td>
<td>-</td>
<td>1/20</td>
<td>1/15</td>
<td></td>
</tr>
<tr>
<td>70 days after ppi (15 days after Roundup app.)</td>
<td>V₁</td>
<td>157/1296</td>
<td>115/286</td>
<td>42/1110</td>
<td>77/195</td>
<td>38/91</td>
<td>1/50</td>
<td>1/99</td>
<td>1/51</td>
<td>32/446</td>
<td>1/42</td>
<td>1/30</td>
<td>3/214</td>
<td>2/78</td>
</tr>
<tr>
<td>V₂</td>
<td>157/1296</td>
<td>115/286</td>
<td>42/1110</td>
<td>77/195</td>
<td>38/91</td>
<td>1/50</td>
<td>1/99</td>
<td>1/51</td>
<td>32/446</td>
<td>1/42</td>
<td>1/30</td>
<td>3/214</td>
<td>2/78</td>
<td></td>
</tr>
<tr>
<td>V₃</td>
<td>25.5/153</td>
<td>21/37</td>
<td>4.5/116</td>
<td>15/25</td>
<td>6/12</td>
<td>-</td>
<td>0.5/10</td>
<td>-</td>
<td>2/35</td>
<td>-</td>
<td>-</td>
<td>1/41</td>
<td>1/30</td>
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</tr>
<tr>
<td>V₄</td>
<td>36.1/417</td>
<td>22/34</td>
<td>14.1/383</td>
<td>15/20</td>
<td>7/14</td>
<td>-</td>
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<td>12/201</td>
<td>0.1/10</td>
<td>-</td>
<td>1/109</td>
<td>1/63</td>
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</tr>
<tr>
<td>V₅</td>
<td>36/517</td>
<td>22/37</td>
<td>14/480</td>
<td>17/21</td>
<td>5/16</td>
<td>-</td>
<td>1/101</td>
<td>-</td>
<td>11/199</td>
<td>-</td>
<td>-</td>
<td>1/140</td>
<td>1/70</td>
<td></td>
</tr>
<tr>
<td>V₆</td>
<td>36/517</td>
<td>22/37</td>
<td>14/480</td>
<td>17/21</td>
<td>5/16</td>
<td>-</td>
<td>1/101</td>
<td>-</td>
<td>11/199</td>
<td>-</td>
<td>-</td>
<td>1/140</td>
<td>1/70</td>
<td></td>
</tr>
<tr>
<td>90 days after ppi (35 days after Roundup app.)</td>
<td>V₁</td>
<td>229/2335</td>
<td>186/499</td>
<td>43/1836</td>
<td>117/317</td>
<td>69/182</td>
<td>1/50</td>
<td>2/211</td>
<td>1/71</td>
<td>32/715</td>
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<td>3/511</td>
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<tr>
<td>V₂</td>
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<td>5/40</td>
<td>2/84</td>
<td>2/15</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/45</td>
<td>1/39</td>
<td></td>
</tr>
<tr>
<td>V₃</td>
<td>7/124</td>
<td>5/40</td>
<td>2/84</td>
<td>2/15</td>
<td>3/25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/45</td>
<td>1/39</td>
<td></td>
</tr>
<tr>
<td>V₄</td>
<td>19.2/568</td>
<td>4/18</td>
<td>15.2/550</td>
<td>2/8</td>
<td>10/6</td>
<td>-</td>
<td>0.2/4</td>
<td>-</td>
<td>12/261</td>
<td>0.1/20</td>
<td>-</td>
<td>1/170</td>
<td>1/95</td>
<td></td>
</tr>
<tr>
<td>V₅</td>
<td>22/584</td>
<td>6/32</td>
<td>16/552</td>
<td>3/14</td>
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<td>-</td>
<td>14/261</td>
<td>-</td>
<td>-</td>
<td>1/190</td>
<td>1/101</td>
<td></td>
</tr>
<tr>
<td>V₆</td>
<td>22/584</td>
<td>6/32</td>
<td>16/552</td>
<td>3/14</td>
<td>3/18</td>
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<td>14/261</td>
<td>-</td>
<td>-</td>
<td>1/190</td>
<td>1/101</td>
<td></td>
</tr>
<tr>
<td>Harvest</td>
<td>V₁</td>
<td>22/584</td>
<td>6/32</td>
<td>16/552</td>
<td>3/14</td>
<td>3/18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14/261</td>
<td>-</td>
<td>-</td>
<td>1/190</td>
<td>1/101</td>
</tr>
<tr>
<td>V₂</td>
<td>22/584</td>
<td>6/32</td>
<td>16/552</td>
<td>3/14</td>
<td>3/18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14/261</td>
<td>-</td>
<td>-</td>
<td>1/190</td>
<td>1/101</td>
<td></td>
</tr>
<tr>
<td>V₃</td>
<td>11.4/139</td>
<td>8/46</td>
<td>3.4/93</td>
<td>4/28</td>
<td>4/18</td>
<td>-</td>
<td>0.4/15</td>
<td>-</td>
<td>1/37</td>
<td>-</td>
<td>1/20</td>
<td>0.3/5</td>
<td>1/16</td>
<td></td>
</tr>
<tr>
<td>V₄</td>
<td>25.2/705</td>
<td>10/66</td>
<td>15.2/639</td>
<td>6/49</td>
<td>1/17</td>
<td>-</td>
<td>0.2/10</td>
<td>-</td>
<td>12/301</td>
<td>1/40</td>
<td>-</td>
<td>1/207</td>
<td>1/101</td>
<td></td>
</tr>
<tr>
<td>V₅</td>
<td>26.1/789</td>
<td>10.1/77</td>
<td>16/712</td>
<td>7/53</td>
<td>3.1/24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14/369</td>
<td>-</td>
<td>-</td>
<td>1/224</td>
<td>1/119</td>
<td></td>
</tr>
<tr>
<td>V₆</td>
<td>54/1099</td>
<td>31/94</td>
<td>23/1005</td>
<td>17/72</td>
<td>14/22</td>
<td>-</td>
<td>1/71</td>
<td>-</td>
<td>17/515</td>
<td>2/71</td>
<td>1/70</td>
<td>1/161</td>
<td>1/117</td>
<td></td>
</tr>
</tbody>
</table>

Experimental variants:
V₁ – Check 1, without weed control
V₂ – Check 2, manual total weed control
V₃ – ROUNDPUP READY 3 l/ha postrising (6 leaves) + 3 l/ha postrising (12 leaves)
V₄ – GUARDIAN 860 EC 2.5 l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha
V₅ – FRONTIER 900 EC 2 l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha
V₆ – TREFLAN 48% EC 2 l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha
Structure and composition of invertebrate fauna

In all three experimental variants, the dominant groups were epigeal Coleoptera and Arachnida (Table 5).

Table 5. Structure of the invertebrate fauna captured in soil traps.

<table>
<thead>
<tr>
<th>Order</th>
<th>VARIANT 1*</th>
<th>VARIANT 2*</th>
<th>VARIANT 3*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crustacea</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Miriapoda</td>
<td>35</td>
<td>36</td>
<td>37</td>
<td>108</td>
</tr>
<tr>
<td>Arachnida</td>
<td>101</td>
<td>91</td>
<td>83</td>
<td>275</td>
</tr>
<tr>
<td>Insecta</td>
<td>911</td>
<td>788</td>
<td>920</td>
<td>2619</td>
</tr>
<tr>
<td>TOTAL</td>
<td>1049</td>
<td>918</td>
<td>1043</td>
<td>3010</td>
</tr>
</tbody>
</table>

* Experimental variants:
1) FRONTIER 900 EC 2l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha
2) TREFLAN 48% EC 2 l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha
3) ROUNDUP READY 3 l/ha at 6 leaves stage + 3 l/ha at 12 leaves stage

The most common species of insects collected belonged to the orders Coleoptera, Hymenoptera and Orthoptera (Table 6). Coleoptera captured in soil traps mainly belonged to the species *Pterostichus vulgaris* L., *Harpalus pubescens* Mull., *H. griseus* Panz. and *Carabus coriaceus* L. (Table 7).

Cropping systems, conventional variants and RR technology, did not produced any qualitative or quantitative significant differences with regards to structure of coleopteran populations. Together with potentially harmful species from the group *Harpalus*, predator species from genera *Pterostichus*, *Carabus*, *Cicindella* and *Amara* were the most abundant. On the soybean plants, the highest abundance showed species belonging to the orders Heteroptera, Coleoptera-Coccinellidae, Neuroptera and spiders (Arachnida) (Table 8) without significant differences in quality and quantity between the conventional cropping systems and RR technology.

Table 6. Structure of insect fauna captured in soil traps.

<table>
<thead>
<tr>
<th>Order</th>
<th>VARIANT 1*</th>
<th>VARIANT 2*</th>
<th>VARIANT 3*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthoptera</td>
<td>152</td>
<td>128</td>
<td>173</td>
<td>453</td>
</tr>
<tr>
<td>Dermaptera</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Hemiptera</td>
<td>58</td>
<td>72</td>
<td>65</td>
<td>195</td>
</tr>
<tr>
<td>Homoptera</td>
<td>10</td>
<td>14</td>
<td>21</td>
<td>45</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>173</td>
<td>129</td>
<td>195</td>
<td>497</td>
</tr>
<tr>
<td>Coleoptera</td>
<td>511</td>
<td>432</td>
<td>458</td>
<td>1401</td>
</tr>
<tr>
<td>Lepidoptera</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Diptera</td>
<td>5</td>
<td>8</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>TOTAL</td>
<td>911</td>
<td>788</td>
<td>920</td>
<td>2619</td>
</tr>
</tbody>
</table>

* Experimental variants: see footnote of Table 5.
Table 7. Main Coleopterans species captured in soil traps.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>VARIANT 1*</th>
<th>VARIANT 2*</th>
<th>VARIANT 3*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harpalus pubescens Mull.</td>
<td>123</td>
<td>99</td>
<td>109</td>
<td>331</td>
</tr>
<tr>
<td>H. griseus Panz.</td>
<td>81</td>
<td>85</td>
<td>69</td>
<td>235</td>
</tr>
<tr>
<td>H. zabroides Dej.</td>
<td>39</td>
<td>25</td>
<td>41</td>
<td>105</td>
</tr>
<tr>
<td>H. aeneus F.</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>H. distinguendus Duft.</td>
<td>3</td>
<td>5</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>Pterostichus vulgaris L.</td>
<td>117</td>
<td>109</td>
<td>110</td>
<td>336</td>
</tr>
<tr>
<td>P. cupreus L.</td>
<td>9</td>
<td>6</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>P. melas Creutz.</td>
<td>4</td>
<td>7</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>Cicindella soluta Dej.</td>
<td>5</td>
<td>5</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>Carabus coriaceus L.</td>
<td>67</td>
<td>54</td>
<td>50</td>
<td>171</td>
</tr>
<tr>
<td>C. cancelatus Illig.</td>
<td>44</td>
<td>25</td>
<td>32</td>
<td>101</td>
</tr>
<tr>
<td>Drasterius bimaculatus Ross.</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Amara aenea Deg.</td>
<td>7</td>
<td>5</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>Tanymecus dilaticollis Gyll.</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Bothynoderes punctiventris Germ.</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>TOTAL</td>
<td>511</td>
<td>432</td>
<td>458</td>
<td>1401</td>
</tr>
</tbody>
</table>

* Experimental variants: see footnote of Table 5.

Table 8. Mean number of predatory arthropods found per plant.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>VARIANT 1*</th>
<th>VARIANT 2*</th>
<th>VARIANT 3*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SP 9191 RR</td>
<td>S 2454 RR</td>
<td>SP 9191 RR</td>
</tr>
<tr>
<td>Nabis pseudoferus</td>
<td>1,0</td>
<td>1,25</td>
<td>0,75</td>
</tr>
<tr>
<td>Nabis feroides</td>
<td>0,1</td>
<td>0,1</td>
<td>0,02</td>
</tr>
<tr>
<td>Nabis ferus</td>
<td>0,5</td>
<td>0,75</td>
<td>1,0</td>
</tr>
<tr>
<td>Nabis rugosus</td>
<td>0,1</td>
<td>0,1</td>
<td>0,25</td>
</tr>
<tr>
<td>Anthocoris sp.</td>
<td>0,1</td>
<td>0,1</td>
<td>0,1</td>
</tr>
<tr>
<td>Orius sp.</td>
<td>0,02</td>
<td>0,1</td>
<td>0,2</td>
</tr>
<tr>
<td>Coccinella 7-punctata</td>
<td>0,1</td>
<td>0,02</td>
<td>0,02</td>
</tr>
<tr>
<td>Chrysopa sp.</td>
<td>0,07</td>
<td>0,05</td>
<td>0,05</td>
</tr>
<tr>
<td>Arachnide</td>
<td>1,0</td>
<td>1,0</td>
<td>1,25</td>
</tr>
</tbody>
</table>

* Experimental variants: see footnote of Table 5.

The acarian *Tetranychus urticae*, one of the main pest of soybean, did not show significant differences among the technology variants investigated (Table 9). Within the fauna captured on adhesive Pherocon traps, aphids, thrips and cicadellids where predominant. There were no significant differences among the weed control technologies applied (Table 10).

High similarity of the fauna from soybean grown in different weed control cropping systems, proved by only small and non-significant variations of the Sörensen Index, shows that RR technology did not seem to influence the fauna on the ground and on the soybean vegetation (Table 11).
Table 9. Mean number of acariens (Tetranychus urticae)/leaf.

<table>
<thead>
<tr>
<th>DATE</th>
<th>VARIANT 1*</th>
<th>VARIANT 2*</th>
<th>VARIANT 3*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SP 9191 RR</td>
<td>S 2454 RR</td>
<td>SP 9191 RR</td>
</tr>
<tr>
<td>30 V</td>
<td>0,75</td>
<td>0,5</td>
<td>0,25</td>
</tr>
<tr>
<td>22 VI</td>
<td>1,5</td>
<td>1,25</td>
<td>1,75</td>
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<tr>
<td>11 VII</td>
<td>1,75</td>
<td>1,75</td>
<td>1,5</td>
</tr>
<tr>
<td>1 VIII</td>
<td>1,75</td>
<td>2</td>
<td>2,25</td>
</tr>
<tr>
<td>22 VIII</td>
<td>3,5</td>
<td>4</td>
<td>4,25</td>
</tr>
<tr>
<td>12 IX</td>
<td>1,75</td>
<td>2</td>
<td>1,5</td>
</tr>
<tr>
<td>TOTAL</td>
<td>11</td>
<td>11,5</td>
<td>11,5</td>
</tr>
</tbody>
</table>

* Experimental variants: see footnote of Table 5.

Table 10. Mean number of insects captured in Pherocon traps/72 cm².

<table>
<thead>
<tr>
<th>Species and Insect Group</th>
<th>VARIANT 1*</th>
<th>VARIANT 2*</th>
<th>VARIANT 3*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SP 9191 RR</td>
<td>S 2454 RR</td>
<td>SP 9191 RR</td>
</tr>
<tr>
<td>Aphids</td>
<td>217</td>
<td>325</td>
<td>297</td>
</tr>
<tr>
<td>Thrips</td>
<td>35</td>
<td>37</td>
<td>21</td>
</tr>
<tr>
<td>Cicadellids</td>
<td>57</td>
<td>43</td>
<td>61</td>
</tr>
<tr>
<td>Syrphus sp.</td>
<td>12</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Chrysopa sp.</td>
<td>5</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Coccinella 7-punctata</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Adalia sp</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Subcoccinella 24-punctata</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Psyllobora 22-punctata</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Poppylea 14-punctata</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Experimental variants: see footnote of Table 5.

We summarize that RR soybean is not persistent in agricultural habitats and its invasiveness into natural habitats is not altered as compared to conventional soybean. Annual and perennial weed species are drastically eliminated from soybean crops by herbicide applications. After the treatment, some annual species germinate from remaining seeds, develop, flower and recover the seed bank in the soil. All weed species proliferate in the field edges and along the access pathway and produce a large quantity of seeds. Their existence is not jeopardised. The results of a field study conducted in order to evaluate the impact of RR versus conventional technology on the arthropod fauna showed no significant differences in epigeal and beneficial insects from the soybean plants (in terms of population size and/or composition). No obvious adverse effects on biodiversity were identified upon cultivation of
RR soybean. The environmental risk of utilisation of RR soybean technology can therefore be regarded as negligible.

Table 11. Similarity of fauna from the fields cultivated with two RR soybean cultivars treated with 3 formulas of herbicides (Sörensen Index).

<table>
<thead>
<tr>
<th>VARIANT</th>
<th>VARIANCE 1</th>
<th>VARIANCE 2</th>
<th>VARIANCE 3</th>
<th>VARIANCE 4</th>
<th>VARIANCE 5</th>
<th>VARIANCE 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>VARIANT 1</td>
<td>81 -100 %</td>
<td>81 -100 %</td>
<td>81 -100 %</td>
<td>81 -100 %</td>
<td>81 -100 %</td>
<td></td>
</tr>
<tr>
<td>VARIANT 2</td>
<td>39;35;38 90.9</td>
<td>81 -100 %</td>
<td>81 -100 %</td>
<td>81 -100 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VARIANT 3</td>
<td>41;35;38 88.6</td>
<td>41;33;39 82.5</td>
<td>81 -100 %</td>
<td>81 -100 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VARIANT 4</td>
<td>35;31;38 84.93</td>
<td>35;36;39 97.3</td>
<td>35;35;41 81 -100 %</td>
<td>81 -100 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VARIANT 5</td>
<td>43;40;38 98.8</td>
<td>43;37;39 90.24</td>
<td>43;37;41 88.09</td>
<td>43;37;35 94.87</td>
<td>81 -100 %</td>
<td></td>
</tr>
<tr>
<td>VARIANT 6</td>
<td>39;32;38 83.11</td>
<td>39;34;39 94.44</td>
<td>39;34;41 97.5</td>
<td>39;32;35 86.48</td>
<td>39;38;43 92.68</td>
<td></td>
</tr>
</tbody>
</table>

Experimental variants:
1. Cultivar (SP 9191 RR) FRONTIER 900 EC 2l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha
2. Cultivar (S 2454 RR) FRONTIER 900 EC 2l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha
3. Cultivar (SP 9191 RR) TREFLAN 48% EC 2 l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha
4. Cultivar (S 2454 RR) TREFLAN 48% EC 2 l/ha ppi + BASAGRAN 48 LC 3 l/ha + FURORE 100 EC 1 l/ha
5. Cultivar (SP 9191 RR) ROUNDUP READY 3 l/ha post + 3 l/ha post
6. Cultivar (S 2454 RR) ROUNDUP READY 3 l/ha post + 3 l/ha post

References


First experiments on unintended effects of Bt maize feed on non-target organisms in Poland

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Abstract: Due to strict regulatory restrictions the first experiments of the Department of Applied Entomology, Warsaw Agricultural University on unintended effects of transgenic crops and their products could not start before 2003. Two species of stored product pests were selected for testing: (a) the grain mite (Acarus siro) for studies on unexpected unintended effects and (b) the Mediterranean flour moth (Ephestia kuehniella) for expected and desired effects. The effects of Bt maize diets made from kernels of the maize line DKc307Bt (MON 810) and bio-pesticide Biobit 3,2 WP mixed with the isogenic non-Bt cultivar Monumental on A. siro development, survival and female fecundity are herein reported.

Key words: unintended effects, Bt maize, Biobit 3,2 WP, Acarus siro L.

Introduction

The Polish society and scientists are deeply divided in their opinion on cultivating transgenic crop cultivars and safety of their products. Both groups supporting and rejecting the use of transgenic crops demand more studies on risk assessment and monitoring of unintended effects of transgenic crops and other GMOs.

In spite of the fact that between 1998-2000 more than 50 field experiments were carried out on transgenic maize, sugar beet and oilseed rape on the request of AgrEvo (now Bayer CropScience) and Monsanto throughout the country (Twardowski & Michalska, 1998; Sip, 2003), presently there are no field experiments on transgenic crops, neither herbicide tolerant nor pest resistant.

Due to strict regulatory restrictions, the first experiments of the Department of Applied Entomology, Warsaw Agricultural University on unintended effects of transgenic crops and their products did not start until 2003. Two species of stored product pests were selected for testing: (a) the grain or flour mite (Acarus siro L.; Acari: Acaridae) for studies on unexpected unintended effects and (b) the Mediterranean flour moth (Ephestia kuehniella Z. Lepidoptera: Pyralidae) for expected and desired effects of ground kernels of the Bt maize line MON 810. In 2005, permission was granted by the Ministry of Environment to start greenhouse experiments with MON 810.

Material and methods

Flour mites (Acarus siro L.) used in the experiment were obtained from a large colony maintained in our laboratory. Three diet treatments were prepared from ground kernels of two maize cultivars:

A) DKc307 Bt - F1 line (MON 810) with full expression of the Bt gene cry1Ab,
B) Isogenic line (Monumental) with 2.8% of the bio-insecticide Biobit 3.2 WP added (3.2% crystals of *Bacillus thuringensis* var. *kurstaki*), and
C) Isogenic line (Monumental) as the control.

Ten eggs of the flour mite were placed in small glass rearing cages containing one of the 3 diets and kept at a constant temperature of 25° C and a relative humidity of 85%. Each treatment consisted of 10 replicates resulting in a total of 100 eggs. Data were recorded in 2-3 day intervals to determine the time of development from egg to adult and the percentage of larval and nymphal mortality. Emerging adults were checked for the sex ratio and removed from the rearing cages.

In a second experimental series, one-day-old females and males were paired in separate cages and provided with one of the three diets. Each treatment consisted of 25 replications. Laid eggs and dead individuals were counted and removed every 3-4 days to calculate female fecundity and adult longevity.

**Results and discussion**

The type of diet affected the developmental period of larval and nymphal stages of the flour mite. The diets made of Bt maize and the mixture of conventional maize with Biobit significantly extended the developmental period of mites in comparison to 13.5 days on the non-Bt cultivar (Table 1) Even larger differences were observed for larval and nymphal survival on the tested diets. The highest mortality of up to 86.4% was recorded on the diet with Biobit in comparison to 45.5% on Bt maize and 21.6% on non-Bt maize flour (Table 1).

Table 1. Selected life-table parameters of *Acarus siro* on diets containing Cry1Ab toxin derived from the transgenic maize cultivar DKc 307Bt or the bio-pesticide Biobit 3.2 WP in comparison to non-Bt (cv. Monumental) diet. Means followed by different letters within the same column are significantly different (Tukey HSD test).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Development (days ±SD)</th>
<th>Mortality (in %)</th>
<th>Sex ratio (Females:Males)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Females</td>
<td>Males</td>
<td>Population</td>
</tr>
<tr>
<td>Non-Bt maize with 2.8% Biobit 3.2 WP</td>
<td>17.5±0.5</td>
<td>17.8±0.1</td>
<td>17.6±0.1</td>
</tr>
<tr>
<td>DKc 307 Bt (MON 810)</td>
<td>19.2±0.3</td>
<td>19.7±0.3</td>
<td>19.5±0.1</td>
</tr>
<tr>
<td>Non-Bt cv. Monumental</td>
<td>13.5±2.8</td>
<td>14.5±0.3</td>
<td>13.9±0.1</td>
</tr>
<tr>
<td>P</td>
<td>0.0046</td>
<td>0.0005</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

The fecundity of a female in a given time (mₓ) was significantly affected by the type of diet (Table 2, Figure 1a).

Conventional flour with Biobit reduced the average fecundity to 12.1 eggs/female compared to 22.5 on Bt maize and 28.5 eggs/female on the control flour without insecticide. Differences were also noted for the female average life span: females on conventional maize with Biobit and on Bt maize flour lived in average 39.7 and 41.5 days, respectively, whereas females on untreated control flour lived longer (47.3 days) (Table 2, Figure 1b).
Table 2. Life duration and average fecundity of *Acarus siro* females on diets with Cry1Ab toxin in comparison to non-Bt cultivar. Different letters indicate significant differences among treatments (Tukey HSD test).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Life duration (min.-max.)</th>
<th>Av. fecundity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Bt maize with 2.8% Biobit 3.2 WP</td>
<td>39.7 (26-47)</td>
<td>12.1±0.6</td>
</tr>
<tr>
<td>DKc 307 Bt (MON 810)</td>
<td>41.5 (31-52)</td>
<td>22.5±0.5</td>
</tr>
<tr>
<td>Non-Bt cv. Monumental</td>
<td>47.3 (42-56)</td>
<td>28.5±2.9</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0,0001</td>
<td>0,0001</td>
</tr>
</tbody>
</table>

Figure 1. Effect of bio-pesticide Biobit 3.2 WP (A), Bt maize line DKc 307Bt (B), and non-Bt cv. Monumental (C) on (a) fecundity and (b) longevity of *Acarus siro* females.

Results on the duration of development of *A. siro* from egg to adult, mortality of different developmental stages and fecundity and longevity of females will be used to construct a life table, which can be compared to other treatments. We will continue our experiments with additional diets made from kernels of two other maize cultivars and kernels of F2 Bt-maize populations. Furthermore, we study expected and desired effects on the Mediterranean flour moth *Ephestia kuehniella*. The Monsanto laboratory in France is assisting us in estimating the Cry1Ab toxin levels in the tested flours. Those additional data will allow us to interpret the preliminary results presented here.

Acknowledgements

We are grateful to Ms. Francesca Tencalla of Monsanto Company for providing samples of feed made from transgenic maize kernels and recent shipment of maize seeds allowing us to start first experiments on unintended effects of GMOs in Poland.
References


RISE - a tool for the management of large data sets collected during field studies

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Abstract: Field studies of non-target arthropods, which last for one or more seasons, generate a large amount of data. Processing this data using a table calculation program is very demanding on time spent rearranging and controlling the data. BTL has greatly improved a common method of evaluating large-scale field studies by developing a tool to evaluate all the data collected during such studies. The method is called RISE – Reliable Impact Study Evaluation. It is supported by a database and suitable procedures for purposes of interconnection, data storage and calculation hence BTL currently employs it for handling large data sets.

Key words: field studies, evaluation, database, statistical analyzes

Introduction

Field studies of non-target arthropods, which last for several seasons, generate a large amount of data. During a large-scale field study, consisting of four plots of each of four treatments, the arthropods in 1600 pitfall trap samples were determined. There can be up to 2500 samples when operating three types of traps. Thus, more than a million specimens can be caught over a period of four months.

If the dataset has to be presented according to GLP (good laboratory practice) then it becomes very difficult to monitor the data processing, starting with the transfer of the hand written raw data onto a computer and finishing with a report. An important element that must be included in such studies is a QAU (Quality Assessment Unit). This is an external institution, which checks the study plan, the data creation, collection, consolidation and evaluation.

Validating 1500 pages of information requires time, accuracy and a suitable method. The work of the QAU is done in parallel with the evaluation process in accordance with the guidelines of GLP. These guidelines insist on precise planning and documentation of all activities. But above all the method used for evaluating the huge amount of data from such studies needs to be reliable.

Clear guidelines and the use of a computerized system for data handling resulted in a method called RISE – Reliable Impact Study Evaluation, which facilitates and reduces the amount of time needed to complete a study for registration purposes.

Material and methods

The identification process
The arthropods in the samples were assigned to particular groups prior to identification by taxonomists. All data must be written down and each error (e.g. wrong number, number written in a wrong line or column etc.) must be indicated, dated and signed to meet the requirements of GLP.
The initial sorting of the samples can result in animals being placed in the wrong group and sent to the wrong specialist. This may happen if the animals stick together or are covered with mud. In most cases the specialist identifies the animal and inserts the data into his written report.

If the specialist uses a different identification key (including different revisions) then the information for a particular species may be entered under a synonym or differently spelt. If this is not corrected before evaluation by the QAU, all calculations relevant to this taxon and all higher determination levels are erroneous, as the assumption is there are two taxa.

![Diagram](image)

Figure 1. Integration of RISE into the different processes of a study.

Based only on human mistakes several problems (e.g. different layouts of tables created during the transfer of data from written into electronic form) arise from using table calculation tools. Taxa are entered incorrectly or without reference to higher taxonomic levels, sometimes columns are inserted for additional attributes, and last but not least errors are propagated by using methods like “copy and paste”, which are used to transfer data between different sheets.
As a result control and correction of electronic data is extremely difficult, but it is precisely this step that is very important for reliable evaluation.

**The tool RISE**

After several years of working with Excel as a table calculation program a tool for optimizing data processing and control was designed by BTL. In combination with clear guidelines this tool is now used by BTL for data handling and analysis when studying the impact of products or plants on organisms in the field. It is based on the MS-SQL Server and interfaces supported by MS-Access in the VBA (Visual Basic for Applications) programming environment.

The use of queries (i.e. definitions in SQL for data to be read out of the database) allows the transfer of data into files as well as into statistical and graphical software. An extensive trial of RISE revealed it is an effective and reliable method for evaluating complex studies on the impact of agrochemicals or of GMOs on non-target organisms. Studies that have a similar structure regarding treatments, plots and replicates can be evaluated using RISE. Beginning with the study plan and ending with the final report the whole evaluation procedure of a study is now more reliable (Figure 1).

**The input process**

The logical structure of large field studies was virtualized inside a database managed by MS-SQL Server. A study with its detailed information is divided into treatments and plots, and all the data inserted into the database. The different sampling methods used (e.g. pitfall traps, soil samples or sticky traps) are definable and sampling dates can be allocated to each. Every sample is recorded within the database and the abundance of each taxon within it is entered with a reference to that particular sample. Furthermore, the identification of the different kinds of arthropods is based on the same taxonomic literature. These arthropods are identified to order, family, subfamily, genus and species. Ecological functions can be entered along with different life stages and gender.

Once the master data is entered it is possible to create templates for a selection of taxa and samples using a consistent layout, similar to a pivot table in Excel (Figure 2). Taxa belonging to a group that is not listed in the templates (e.g. due to partition mistakes, synonyms or country-specific systematic placements, missing in selection or in database) can be added and verified later. The study code, location, sampling date and single samples are included. This is useful for minimizing the errors generated when producing and entering written data. The templates can be printed and appended by the taxonomists. Additionally there are spaces for the signatures that are required by GLP.

![Figure 2. Excerpt of a printed template including header, footer and sample data.](image)
Redundancies and thus the high probability of generating errors when using Excel can be avoided by the use of a database. Every record is unique and allocations made by referencing existing records. By using constraints the input of duplicate records is avoided. Thus, a record is either an error that must be corrected or an additional value that has to be added to an existing one; otherwise it cannot be inserted. All written raw data relevant to that sample must be reviewed to solve this problem, as it is the basic information of the study. This ensures the correct collection and entry of data.

To cater for changes in taxonomy, functions are implemented to add, change and reallocate taxa. The input of data is done sample by sample for each taxon, no matter what the level of determination (i.e., the five levels named above). The first control is done by comparing the paperwork with the electronic raw data that is read out of the database. The electronic data is presented in a pivot table of the same structure as the templates mentioned above (Figure 3). Afterwards the hand written records and a copy of the electronic raw data (different formats like tab/comma separated values or Excel sheet) are sent to QAU for additional checking.

The evaluation process
After the input and final checking of the raw data by QAU evaluation is done by queries and statistical software. All the data and calculated results are transferred to QAU for immediate checking. In cases where the calculated values cannot be validated the written and electronic data are compared again.

Suitable queries allow calculations for particular taxonomic levels and special routines determine the most abundant or dominant taxa, either based on the whole catch or the catch of particular orders or families. The level of dominance can be expressed in terms of 0 to 100, in steps of 0.1. This is useful for analyzing taxa that are not so abundant but ecologically important.

During the study preliminary results can be produced at any time by the use of suitable queries, statistical tools and graphics software like SAS, Systat and SigmaPlot (Figure 4). All results can be produced in file format, as stated above. RISE allows the calculation of several biodiversity indices, mean values and information like the number of taxa as well as lists of all the taxa collected during the study. RISE determines the numbers of predators and parasitoids and analyses the results of different sampling methods or the whole study. Even comparisons between different locations are possible using the records in the database.

Study reports (i.e. draft and final report) are created after the confirmation of the statistical results by QAU. The whole evaluation procedure requires further explanation of the

<table>
<thead>
<tr>
<th>S5am</th>
<th>Taxon genus</th>
<th>Taxon name</th>
<th>Taxon level</th>
<th>Taxon gender</th>
<th>Excel function</th>
<th>C</th>
<th>T1</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>Etna dentipalpis</td>
<td>Adult</td>
<td>Female</td>
<td>Predator</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>Laphyphantes</td>
<td>Adult</td>
<td>Female</td>
<td>Predator</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Melaneta</td>
<td>Male</td>
<td></td>
<td>Predator</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>Cadaverecridae</td>
<td>Adult</td>
<td>Female</td>
<td>Predator</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Electronic copy of raw data; missing columns contain further taxonomic and study information (Excel).
ecology and biology of the taxa, which is included manually into the report. All other results are inserted as calculated and formatted by the statistical software. The study ends with the confirmation (i.e. comparison of results and figures included with a separate output produced by the statistical software; checking of texts and formats) of the final report by QAU.

Figure 4. Graphical presentation: a filled symbol indicates it is significantly different from the control (Sigma Plot).

**Results and discussion**

Initially the input of raw data into this database is more labour intensive than when using an Excel spreadsheet, but the evaluation of the latter requires manual rearrangement of the data. This can be avoided by using databases and data mining tools, which perform these tasks automatically on command. As a result the amount of work necessary to verify the data is much reduced and speed of analysis of the data greatly increased. The ability to search within Excel is very limited compared to the techniques for finding, filtering, aggregation and grouping provided by queries.

Changes or corrections are instantly available for all calculations of affected data because every record is only stored once and the statistics are compiled using the data in the database. This guarantees the quality of the evaluation process and provides a timesaving of several weeks for a whole study (Figure 5). Query results can be passed immediately through statistics software and the flexibility of the analysis allows a much more detailed evaluation.
Figure 5. Amounts of time spent on different stages of a study when using Excel and RISE.

Acknowledgements

The authors warmly thank the team at BTL, especially Udo Hoffmann who always had time for providing the necessary background information on GLP, taxonomy and field trials, which were essential for the realization of this project.

References


All product names mentioned here are the trademarks of their respective owners.
Transgenic Bt maize: main results of a six-year study on non-target effects

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Abstract: From its introduction in 1998 to 2004, Spain increased the area of Bt maize cultivation to 58,000 ha. Only one Bt maize variety (event 176) was commercially grown until 2002; in 2003 the event MON810 was also authorised for growing in Spain and this event became predominant in 2004. In 1999 we initiated a long-term research project sponsored by the Spanish Ministry of Education and Science aiming to determine non-target effects of Bt maize (event 176). We summarise the main published and unpublished results of the research. (i) Sublethal effects on the corn borer Sesamia nonagrioides. In the laboratory, sublethal concentrations of Bt in the diet caused a significant increase in the critical day length for diapause induction, whereas diapause and post diapause development was prolonged in diapausing larvae collected in Bt fields at the end of the season in comparison with larvae developed in a non-Bt field. (ii) With regard to the effects on non-target pests and natural enemies: consistently higher aphid and leafhopper densities were observed in Bt plots in comparison with non-Bt plots; when Orius majusculus Reuter —which we used as a model predator— was fed with Bt plant leaf and pollen, its development was shorter than when it was fed with non-Bt plant leaf and pollen. In the field, no negative impact of Bt maize (Compa CB, event 176) on either predators or aphid parasitoids was found. (iii) With regard to S. nonagrioides larval dispersal, it was concluded that only mature larvae dispersed in noticeable numbers and they did so at least to adjacent rows to that containing the plant on which the eggs hatched. Adult movement was monitored by catching rubidium-marked males at different distances from the source; a distance of 400 m from the source did not display a significant decrease in male catches.

Key words: Bt maize, Sesamia nonagrioides, predators, GMO

Introduction

Transgenic maize that has incorporated the insecticidal capacity of Bacillus thuringiensis Berliner (Bt maize) has been deployed in Spain and reached 58,000 ha in 2004. In the last two years MON810 has been the prevalent event sown, though Bt176, the only event allowed in Spain until 2002, is still sown in a few areas.

Maize has two main corn borers in Spain: Sesamia nonagrioides Lef. (Lep.: Noctuidae) and Ostrinia nubilalis (Hbn.) (Lep.: Crambidae). The two Bt maize events authorised in Spain are effective in greatly reducing damage caused by both of them (author’s unpublished results).

In order to assess the risks of non-target effects that the deployment of Bt maize might cause, we have carried out two research and development projects since 1999 sponsored by the Spanish Ministry of Education and Science, covering the following areas: (i) sub-lethal effects on target herbivores, (ii) effects on non-target pests, predators and parasitoids, and (iii) dispersal capacity of the corn borer S. nonagrioides with a view to designing a refuge strategy.
to delay development of resistance to Bt. Most of the work carried out within these two projects has been already published. Here we merely review the most significant results to give a comprehensive overview of the risk assessment work.

Materials and methods

Details of the materials and methods used in each item may be found in the original publications that are indicated in each section. Here we merely summarise the main features of the methodology.

(i) Sublethal effects on target herbivores (S. nonagrioides) (Eizaguirre et al., 2005). Two types of experiments were conducted to assess the influence of sublethal concentrations of Bt toxins on larval diapause induction and development and on post-diapause development. To this end, larvae of *S. nonagrioides* were submitted in the laboratory to sublethal concentrations of the commercial Bt product Dipel®, which contains, among others, the same toxin Cry 1Ab as expressed by the Bt maize event 176 and the critical day length for diapause induction was measured. In the field, larvae of the corn borer were collected in October and February, when Bt toxin in the plant is below the lethal concentration, and diapause and post-diapause developments under 16:8 (L:D) day length were measured by recording days to pupation.

(ii) Effects on non-target pests (Pons et al., 2005), on predators in the laboratory (unpublished results) and in the field (Poza et al., 2005), and on parasitoids (Pons & Starý, 2003). During a period of three years (2000-2002) a randomised block design (0.4-0.6 ha) with four replications was used to study at farm-scale the influence of Bt maize on these non-target insects. Samplings of aphids and epigeal predators were performed by visually counting the individuals on 15-25 plants per plot 5-6 times every year. Ground dwelling predators caught in 3 pitfall traps placed in the central rows of each plot were recorded. Leafhopper density was estimated by counting twice per season the number of insects on three leaves of 10 plants per plot. Damage caused by leafhoppers was assessed with a SPAD chlorophyll meter and by measuring leaf area. Most insects were identified to species level, whereas all spiders were considered together as a whole group of Araneae. Statistical analyses were performed with the main predatory groups (mostly families), with the prevalent leafhopper species [*Zyginidia scutellaris* (Herrich-Schäffer)], and the four prevalent aphid species. To determine the effects of Bt maize on parasitoid aphid survival, in 2002—a year with an extraordinary number of aphids on maize—a number of aphid mummies were collected in Bt and non Bt plots and parasitoid emergence was compared by a χ² test. To test the effect of Bt maize on predators in the laboratory we used *Orius majusculus* Reuter as a model species because it is known to feed on the plant, pollen and on a variety of prey species and it is found during most of the season, its potential exposure to Bt toxins in transgenic Bt maize is quite high.

(iii) Dispersal capacity of *S. nonagrioides*. Dispersal was studied in both larvae and adult males. In the first case, a non-Bt field was used for three years. Maize was sown with a distance of 0.70 m between rows and 0.175 between plants within a row. Thirty sites were selected each year. The sites consisted of three rows with 11 plants each, which were inspected to ensure that they were not naturally infested by corn borers. The central plant on the central row was infested with an egg mass of 70-100 eggs each of *S. nonagrioides*, and the number and age of larvae on the rest of the plants at the site were recorded 7, 14 and 32 days after infestation. To estimate the dispersal capacity of adult males, these were marked with Rb by spraying a source field with RbCl; males developed on the RbCl-treated field and caught
in pheromone traps located 0, 100, 200, 300 and 400 m from the source were identified by analysing Rb in captured males by flame atomic emission spectrometry.

Results and discussion

Only the most significant results are shown. More details on results of the experiments can be found in the references included in materials and methods section.

(i) Sublethal effects on target pests \( S. \text{nonagrioides} \). Critical day length in larvae treated with sub lethal concentrations of Dipel increased by about 41 min in comparison with untreated larvae (Figure 1). If this 41 min increase is translated into the field diapause onset, it means that larvae submitted to sublethal concentrations of Bt in transgenic maize may enter into diapause 20 days earlier than those fed on non-Bt maize and give a lower third adult flight taking into account that in the region and in late August each day reduces 2 min the day length.

![Figure 1. Percentage (mean±s.e.) of larvae that entered diapause after being reared on a diet containing a sublethal concentration of Bt toxin or on a control diet. Within each day length, percentages followed by no or the same letter are not significantly different.](image)

Larvae collected in Bt fields with a sublethal concentration of the Bt toxin showed significantly \((P<0.05)\) longer diapause and postdiapause development (Figure 2). It can thus be concluded that larvae of \( S. \text{nonagrioides} \) that survive in Bt fields where they are subjected to sublethal concentrations of the toxin may alter their phenology and this affects their fitness; additionally, population phenology changes may decrease the probability of mating at random between adults emerged from a Bt field and a non-Bt refuge field, thus accelerating development of Bt-resistance in corn borer populations.
Figure 2. Duration (mean + s.e.) of diapause and postdiapause development in *S. nonagrioides* larvae collected in a non-Bt vs Bt field (when Bt toxin was expressed at sublethal concentrations) and submitted in the lab at 16:8 (L:D) and 15°C. To measure diapause and postdiapause developments, larvae were collected in October and February respectively. Durations were significantly (P<0.05) different within each development.

(ii-1) Effects on non-target pests: leafhoppers and aphids. Though there were more leafhoppers—and in particular significantly more mature nymphs—on Bt maize (Figure 3a), the damage caused was not significantly different in Bt vs. non-Bt maize. Aphids were in general more abundant on Bt maize for the three prevalent species (*Rhopalosiphum padi* L., *Sitobion avenae* F., and *Metopolophium dirhodum* Walker) and most developmental stages. The results are only shown for the first species and selected stages (Figure 3b).

(ii-2) Effects on non-targets: predators in the laboratory. *Orius majusculus* nymphs developed in a significantly shorter time when fed on Bt pollen and plant leaves, whereas survival was not significantly affected. This contrasts with what was observed in a previous work, where *O. majusculus* was provided with *Ephestia kuehniella* eggs and fed on Bt vs. non-Bt pollen and plants but there were no differences in developmental time and survival (Pons et al., 2004).

(ii-3) Effects on non-targets: predators in the field. Densities of Anthocoridae, Coccinellidae and Araneae (together they represented more than 80% of the predators recorded in the visual sampling) were not significantly different in Bt vs. non-Bt maize—a similar conclusion to that of the three prevalent groups recorded in pitfall traps: Carabidae, Araneae and Dermaptera (>95% of predators caught) (Figure 4). It is concluded that Bt maize is compatible with naturally occurring predators.

(ii-4) Effects on non-targets: parasitoids. There were no significant differences in the number of parasitoids emerged from mummies collected in Bt and non-Bt maize plots. It can thus be concluded that Bt maize does not affect survival of immatures of aphid parasitoids.

(iii-1) Dispersal capacity of *S. nonagrioides* larvae. Only mature larvae dispersed in significant numbers (Figure 5a). An average of 67% of the larvae that reached last instars moved from the infested to surrounding plants, compared to 1.0 and 8.3% for young and mid-aged larvae. Among larvae that reached last instars, the 12% moved to rows adjacent to those containing the infested plant, and down the row five plants. This might limit the effectiveness of refuges based on the seed mixture or strip planting of Bt and non-Bt corn.
Figure 3. Mean (+ s.e.) densities of different morphs of (A) leafhoppers (*Zyginidia scutellaris*) and (B) the aphid *Rhopalosiphum padi* on Bt and non-Bt maize. Bars with no or the same letter are not significantly different (*P*<0.05) when the two types of maize are compared.

Figure 4. Densities (mean + s.e.) of the prevalent predatory groups recorded in visual samplings (A, left column) and pitfall traps (B, right column) on Bt and non-Bt maize. There were no significant differences between Bt and non-Bt maize for any predator group.

(iii-2) Dispersal capacity of *S. nonagrioides* males. At the end of the season, no significantly different numbers of males flew to distances of 100, 200, 300 and 400 m from
the source plot (Figure 5b). This means that S. nonagrioides males can disperse at least 400 m from where they emerge. Under such a perspective, refuges of non-Bt fields can be placed at a distance of 400 m from Bt fields to delay resistance to B. thuringiensis toxins in S. nonagrioides.

Figure 5. Dispersal capacity of S. nonagrioides. (a, left) Percentage (mean + s.e.) of larvae dispersed of each age. (b, right) Rubidium marked males (mean + s.e) caught at different distances from the source. There were no significant differences among distances.

Conclusions

1. When S. nonagrioides larvae are exposed to sublethal concentrations of Bt toxins, diapause induction and development and post-diapause development may be modified, leading to fitness decrease due to altered population phenology. This can also cause a certain reproductive isolation between adults that emerge from Bt and non-Bt fields, leading to accelerated development of Bt-resistance in the corn borer populations.
2. The mechanisms of enhancement of population densities of sucking insects like leafhoppers and aphids observed in Bt maize should be further investigated.
3. When the predator O. majusculus is fed with Bt pollen and Bt plant leaves, development is faster in comparison with a non-Bt pollen and plant diet. In contrast, survival is not affected by the type of diet.
4. Naturally occurring predators are not affected by the Bt transgene in maize when it is tested in the field.
5. About two thirds of the larvae of S. nonagrioides that reach last instars move away from the initially oviposited plant to colonise neighbouring plants. Of these, 12% migrate to adjacent rows. These findings show that mixing seeds or strips of Bt and non-Bt maize may be ineffective for delaying Bt-resistance in the corn borer populations.
6. Males of S. nonagrioides may fly away from the field where they emerged at least to a distance of 400 m, so non-Bt refuge fields may be located at a distance of 400 m from transgenic Bt fields to delay the development of Bt-resistance in the corn borer populations.

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References


Environmental impact of Bt maize – three years of experience

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Abstract: The aim of our study was to assess the environmental impact of Bt maize MON 810 in comparison with the parental non-transgenic cultivar. Enzyme-linked immunosorbent assay (ELISA) was used to quantify the amount of Bt toxin Cry1Ab in the plants. Selected components of the ecosystem were followed during the vegetation season in three successive years, with focus on the plant-dwelling insects and on the communities of epigeic insects and spiders. The examined species included target and non-target herbivores and predators. The results were evaluated for each sample date and finally for the entire experimental period. The study revealed no significant negative effect of Bt maize on the plant dwelling non-target insects and on the epigeic beetles and spiders.

Key words: GM crops, European corn borer, thrips, aphids, Orius, Carabidae, Staphylinidae, spiders

Introduction

Maize is one of the most important cereal crops in the world; its derivatives and by-products are extensively used as fodder as well as in the food industries ranging from the production of starch and breakfast cereals to oil and distillates. The European Corn Borer (ECB) Ostrinia nubilalis (Lepidoptera: Crambidae) is the major pest decreasing maize yield in the US and Europe. ECB larvae burrowing into the maize cause stem breakage and often collapse of the whole plant. The damage is avoided in genetically modified Bt maize that expresses a Bacillus thuringiensis toxin poisonous to ECB. However, the deployment of Bt maize is hindered by fears about its possible environmental side effects. This concern is addressed by our comparison of entomofauna on the plants and the soil surface in the stands of the Bt and control maize.

Material and methods

Field trials were performed in Southern Bohemia (300 m a.s.l.) in the vicinity of České Budějovice in 2002 – 2004. The Bt maize cultivar MON810 and the parental non-transgenic cultivar were each planted on five 0.5 ha plots that were distributed checker-wise in the fields of 7.6 ha (2002) and 14 ha (2003 and 2004). Stripes of bare land 10 m wide separated the plots. Field margins were seeded with the near isogenic cultivar. At the end of 2003, the plants were shred to small pieces and ploughed into the soil. In 2004, each plot was planted with the same type of maize (Bt or non-Bt) as in 2003.

The amounts of Cry1Ab were measured in fresh plant tissues every 2-3 weeks with enzyme-linked immunosorbent assay (ELISA). Commercial kit (Agdia, purchased from Linaris, Germany) specific for Cry1Ab was used in 2002 and a kit detecting Cry1Ab and
Cry1Ab/1Ac in 2003 and 2004. Sensitivity threshold of the assay was 0.025 µg Cry1Ab in one gram of fresh plant tissue.

The occurrence of insects on plants was recorded visually in the field every week. Every 2-3 weeks, 10 plants from each plot were taken to the laboratory for a thorough quantitative inspection. The epigeic insects were collected once before the maize sowing, four times during the growing season, and once after the harvest. Four pitfall traps (10 cm in diameter) per plot were exposed for 21 day-intervals in 2002 (total exposure time 93 days) and 5 traps per plot for 14 day-intervals in 2003 and 2004 (exposure time 43 days). Entrapped insects were stored in 3-4% formaldehyde and later classified to the species level. The results were evaluated with Canoco statistics for each collection time and for the entire experimental period.

**Results**

### Toxin expression and the target pest

The toxin expression in maize leaves was relatively stable throughout the season. In 2003-2004, the leaves contained 1 to 2.5 ppm Cry1Ab, while only 0.06 ppm was detected in the stems, 0.075 in the roots, 0.05 in the flowers, and 0.08 in the pollen. Cry1Ab content in the grain varied between 0.025 (milk-stage ripening) and 0.088 ppm (ripe grain stored for six months). These values were several times lower than 6.4 to 7.5 ppm Cry1Ab measured in the leaves in 2002. The discrepancy could be due to the use of different ELISA kits and Cry1Ab standards.

Toxin presence prevented infestation by the ECB. Adults and eggs of ECB were found on the Bt and non-Bt plants in similar numbers but boring caterpillars occurred only on the non-Bt plants. Larvae hatching on the Bt-plants apparently died before they could cause visible boring tunnels.

### Non target insects on the maize plants

No significant differences in insect communities on the Bt and non-Bt maize plants were detected. The most abundant insect groups were aphids and thrips and their natural enemies, e.g. Orius sp. (2003-2004) and the predatory thrips Aeolothrips fasciatus (2004).

Two aphid species, Rhopalosiphum padi and Metopolophium dirhodum, were found on maize in all three years from June to July. The aphids were considerably more abundant in 2002 than in 2003 and 2004 (Table 1), probably due to higher annual rainfall in 2002. At the beginning of July 2004, one more aphid species, Sitobione avenae occurred on both Bt and non-Bt plants; 83, respectively 190 specimens per plant were found in course of the season.

Table 1. Numbers of aphids Rhopalosiphum padi and Metopolophium dirhodum found on ten randomly chosen plants per plot in 2002-2004. Average values ± SD are presented.

<table>
<thead>
<tr>
<th>Bt</th>
<th>Rhopalosiphum padi</th>
<th>Metopolophium dirhodum</th>
<th>Control</th>
<th>Rhopalosiphum padi</th>
<th>Metopolophium dirhodum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhopalosiphum padi</td>
<td>490±12</td>
<td>286±11</td>
<td>262±12</td>
<td>449±44</td>
<td>229±229</td>
</tr>
<tr>
<td>Metopolophium dirhodum</td>
<td>248</td>
<td>166</td>
<td>219,6</td>
<td>66</td>
<td>128±128</td>
</tr>
<tr>
<td>2002</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
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<td>2004</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

58
The thrips *Franklinella occidentalis* usually appeared on the plants approximately at the 4-leaf stage and the numbers of nymphs reached a maximum at the flowering time at the end of June and the beginning of July (Figure 1). The peak of nymph abundance preceded that of adults by nearly three weeks. In 2004, the highest abundance was observed at the beginning of August. Thrips persisted on the plants until the last sampling. Thrips infestation of the Bt and control plants was statistically indistinguishable.

![Figure 1. Average numbers of the thrips *Franklinella occidentalis* per maize plant in 2002-2004.](image)

**Epigeic beetles and spiders**

Adult ground beetles (Carabidae), rove beetles (Staphylinidae), and spiders (Araneae) dominated in pitfall catches. The species numbers and abundance of the carabids and spiders were higher in 2004 than in 2002-2003, in the case of staphylinids, the catches of 2002 and 2004 were similar and higher than in 2003 (Table 2).

**Carabidae**

Two basic environmental variables were defined for the Canoco analysis of carabid abundance: the sampling date (exact number of days after the first trap placing in the spring) and the presence or absence of Bt-toxin (Figure 2). Separate analyses with Monte Carlo permutation test confirmed that the date provides for most of the recorded variability (p=0.001; F=5.695), while the presence and absence of Bt toxin is insignificant (p=0.72; F=0.795). When both variables were used together, their influence on the structure of communities of carabid beetles was significant (p=0.039; F=1.245). It must be mentioned that *Pterostichus melanarius* and *Poecilus cupreus* made up about three quarters of all carabid species caught in our fields in all study years.
Table 2. Total numbers of collected species and specimens of the epigeic beetles and spiders.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Non-Bt</td>
<td>Bt</td>
<td>Total</td>
<td>Non-Bt</td>
<td>Bt</td>
<td>Total</td>
<td>Non-Bt</td>
<td>Bt</td>
<td>Total</td>
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<tr>
<td>Carabidae</td>
<td>Species</td>
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<td>31</td>
<td>40</td>
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<td>48</td>
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<tr>
<td></td>
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<td>6149</td>
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<td>1694</td>
<td>3481</td>
<td>6462</td>
<td>5766</td>
<td>12228</td>
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<td>Staphyliniidae</td>
<td>Species</td>
<td>44</td>
<td>42</td>
<td>55</td>
<td>12</td>
<td>11</td>
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<tr>
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<td>Specimens</td>
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<td>880</td>
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<td>23</td>
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</table>

In contrast to the ground beetles and spiders, no clearly dominating species were identified. For example, in 2002 a rare *Atheta* species was only about three times less abundant than the most frequent species *Aleochara bipustulata*. In 2004, *Aleocharinae* species occurred more frequently in the non-Bt than the Bt plots. Other common species included *Philonthus atratus*, *Lesteva longelytrata* and *Xantholinus linearis*. Canoco analysis revealed that Bt had no significant influence on the structure of rove beetle community (p=0.884, F=0.749). No preference for Bt or non-Bt plants was detected in any of the species. The presence or absence of Bt toxin accounted only for 1.2% of ecological variability, while 9.7% variability was attributable to the sampling date (Figure 3).

![Figure 2. Canoco analysis of the distribution of common carabid species (triangles) and their sums per plot (P1, 3, 5, 7, 9 represent the Bt, and P2, 4, 6, 8, 10 the non-Bt plots) in the experimental area in 2004. Horizontal line indicates sampling dates and vertical line indicates partial, but not significant, separation of the Bt and non-Bt plots.](image-url)
Figure 3. Canoco analysis of the distribution of Staphylinidae in the experimental area in 2004. See Fig. 2 for details.

**Spiders**

Enormous differences between the dominating and the rare species (Table 3) were detected in all years of study. *Oedothorax apicatus* represented 90% of all spider species in 2002, 94% in 2003 and 92% in 2004. Other common spiders, *Erigone dentipalpis*, *Trochosa ruricola* and *Pardosa agrestis*, reached only 2.6% (2002), 2% (2003) and 4.3% (2004) of the total spider catches.

<table>
<thead>
<tr>
<th>Species</th>
<th>2002</th>
<th></th>
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<th>2004</th>
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<tbody>
<tr>
<td></td>
<td>Non-Bt</td>
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<td>Non-Bt</td>
<td>Bt</td>
<td>Non-Bt</td>
<td>Bt</td>
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<tr>
<td><em>Oedothorax apicatus</em></td>
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<td>2290</td>
<td>1982</td>
<td>1987</td>
<td>5167</td>
<td>5317</td>
</tr>
<tr>
<td><em>Erigone dentipalpis</em></td>
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<td>89</td>
<td>1</td>
<td>0</td>
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<td>9</td>
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<tr>
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<td>52</td>
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<tr>
<td><em>Pardosa agrestis</em></td>
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<td>14</td>
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<tr>
<td><em>Trochosa ruricola</em></td>
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<tr>
<td><em>Meioneta rarestris</em></td>
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<td>12</td>
<td>3</td>
<td>2</td>
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<td>18</td>
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<tr>
<td><em>Pardosa palustris</em></td>
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<td>12</td>
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<td>0</td>
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<td>20</td>
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<tr>
<td><em>Walckenaeria vigilax</em></td>
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<td>6</td>
<td>3</td>
<td>1</td>
<td>66</td>
<td>70</td>
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<tr>
<td><em>Robertus arundineti</em></td>
<td>8</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>10</td>
<td>7</td>
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<tr>
<td><em>Diplostyla concolor</em></td>
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<td>3</td>
<td>2</td>
<td>4</td>
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<td>5</td>
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<tr>
<td><em>Pardosa paludicola</em></td>
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<td>0</td>
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Discussion

Our data confirm that Bt maize is fully resistant to the target pest, *O. nubilalis* (Clerk et al., 2000). ELISA measurements showed that the expression of Cry1Ab toxin in the MON810 maize cultivar begins long before the ECB begins to fly and deposit the eggs. Field observations confirmed that the egg laying ECB adults do not distinguish between the Bt and non-Bt plants (Orr & Landis, 1997). In both 2002 and 2003, the toxin apparently killed all hatched ECB larvae.

No organism other than ECB seems to be affected by toxin expression. The lack of effect on aphids is not surprising in view of toxin absence in the phloem sap of Bt maize (Raps et al. 2001). While some laboratory studies have revealed prey-mediated effects of Bt maize on larvae of the green lacewing *Chryoperla carnea* (Hilbeck et al., 1998; Dutton et al., 2002), this predator was not found to be affected in a number of field studies (Bourguet et al., 2002; Candolfi et al., 2004).

According to our results, the numbers of lacewing eggs on the Bt and the non-Bt plants were virtually identical in our plots but we did not follow up the development of larvae.

Epigeic predators are an important component of agricultural ecosystems because they keep many potential pests below the threshold of economic damage. Certain predators are occasional herbivores and all of them probably consume some prey contaminated with the Bt toxin, but we found no differences between the Bt and the non-Bt plots either in beetle or spider communities. This is in sharp contrast to the use of insecticides that often kill indiscriminately and reduce populations of the beneficial insects such as carabid beetles (Lee et al., 2001).

Conclusion

Our data show that the presence of Bt toxin in the maize plants and their residues ploughed into the soil had no significant effect on the plant-dwelling insect communities and on epigeic ground beetles, rove beetles, and spiders.

Acknowledgements

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Predicting fitness changes in transgenic plants: testing a novel approach with pathogen resistant *Brassicas*

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Abstract: Research into risks from genetically modified (GM) crops has produced an extensive literature on the probability of hybridisation between crops and their wild relatives e.g. (Ellstrand, 2003). Transgene escape, however, will involve two components, gene flow and establishment. This may be through establishment of transgenic crop plants in the wild, or through hybridisation between crops and wild relatives followed by establishment of the resulting transgenic hybrids. One conjectural risk from genetic engineering is that transgenic plants may show increased fitness compared to the nontransgenic crop variety or wild relatives. This may allow them to become more dominant in semi-natural systems and have negative impacts on other plant species and other ecosystem components (Hails, 2002). This possibility is one component of the assessment of environmental risk from the use of GM crops, but there are serious problems with attempts to predict the potential for changed fitness.

At the moment, there is no proposed middle way for predicting the effects of genetic modification on fitness between the easy, but extremely imprecise, use of lists of traits, and the accurate, but large-scale and expensive, measurement of demographic parameters in a wide range of habitats and geographic regions (Crawley *et al*., 1993; Crawley *et al*., 2001). Bullock (1999) proposed that population modelling could be used to highlight those demographic parameters which, if changed by modification, will have strong effects on fitness. With a model system comprising of oilseed rape, *Brassica napus* ssp *oleifera*, and wild turnip, *B. rapa*, we are developing a risk assessment methodology, centred around population modelling, that may serve to highlight potential, ecologically significant, changes in fitness in transgenic plants, prior to large scale field release.

Key words: *Brassica rapa*, invasions, finite rate of increase, matrix models, environmental stochasticity, genetically modified plants

Introduction

One of the concerns raised over the introduction of genetically modified crops is that transgenes will invade populations of wild relatives, causing ecologically significant changes in fitness. In recent years, this has given rise to several studies estimating hybridisation rates and the fitness of crop-wild relative hybrids (Hails *et al*., 2005). These studies have established that transgenes are likely to move to F\(_1\) hybrids, albeit at low frequency. Hybridisation, however, is not synonymous with introgression, and questions remain as to whether particular transgenes will cause ecologically significant changes in recipient plant populations.

The most direct approach to investigate the invasive potential of transgenes is to release transgenic plants into natural habitats and monitor their fitness. This approach has been taken...
with *Brassica napus*, simulating invasions of the feral crop (transgenic and wild type) in a range of natural habitats across the UK (Crawley *et al*., 1993; Hails *et al*., 1997; Crawley, 2001). However, at the time these experiments were conducted, the only transgenic constructs available were for herbicide tolerance, or marker genes. Nevertheless, these experiments did generate a large demographic dataset for *B. napus* and demonstrate that genetic modification *per se* did not enhance ecological fitness [although seed survival was reduced (Hails *et al*., 1997)]. The disadvantages of such an empirical approach is that it is very labour intensive, would need to be conducted for each crop/transgenic construct combination, and does not provide insight into the mechanisms behind any changes in fitness.

A second approach has been to monitor the performance of transgenic hybrids between crops and wild relatives, comparing fitness components with the unmodified hybrid. This represents the first ecological step between the crop and introgression into the wild relative. These empirical studies have focussed on fitness consequences under experimental conditions, in which they have manipulated the densities of the natural enemies which are transgene targets. For example *Brassica napus* (Stewart *et al*., 1997; Mason, 2003), and F1 hybrids with *B. rapa* containing *Bt* transgenes have been found to have a fecundity advantage under high insect herbivore pressure (Vacher *et al*., 2004). Similarly, coat protein transgenes were found to confer a selective advantage to squash, *Cucurbita pepo*, and three generations of hybrids with its wild relative *C. texana* (F1, BC1 and BC2) under conditions of high disease pressure (Fuchs, 2004). However, what this tells us, principally, is that the transgene functions as expected in the hybrid progeny.

A number of questions remain to be answered before it can be concluded that enhanced fitness under these experimental conditions can be translated to enhanced fitness in natural communities. How frequently do the transgene targets (herbivores or pathogens) impact on plant fitness? Are there any costs to carrying the transgene in the absence of herbivores or pathogens? How will genotype by environment interactions modulate the impact of herbivores or pathogens? To answer these questions requires a combination of census data from natural plant communities, manipulative field experiments, and a theoretical framework to place any fitness changes in context. We draw on all three approaches here in our study of oilseed rape, *Brassica napus* and the wild relative with which it is most likely to hybridise, *B. rapa*. We question whether the introduction of transgenic fungal resistance into *B. rapa* populations would significantly alter their fitness in semi-natural habitats.

**Materials and methods**

**Field sites**

Three field sites were chosen in southern England where *B. rapa* currently thrives: Claverton, Somerset; Radley, Oxfordshire and Wytham, Oxfordshire. All three sites are riverside habitats. Data were collected in two ways: in experimentally manipulated plots and from transects through the established populations.

Over three years a number of manipulative experiments were conducted at these three sites, but the basic design of the plots consisted of 5 parallel lines 2.5 metres in length and 50cm apart (see Figure 1). Along one line, chosen at random, the vegetation was removed for a width of 50cm, and the soil surface lightly dug over – this was the ‘disturbed’ treatment. Along each undisturbed line, 100 seeds were sown, and for each disturbed line, 50 seeds. Within one block, three plots were constructed in this manner – one for *B. napus*, one for *B. rapa*, and a control line to monitor germination from the seed bank. A total of 5 blocks were established in this way at each site. Consequently, eight times as many seeds were sown in the undisturbed treatment as the disturbed treatment, as germination is so much poorer in the
former. Individual plants were recorded in weeks 2 and 4 after sowing, and monthly thereafter, to record germination, growth, survival, flowering and seed production.

Figure 1. Basic design of experimental plots 2001 – 2004.

Fifty seeds of B. napus and B. rapa were buried, in nylon mesh bags (gauze width 0.1mm), at a depth of 10cm at the end of each disturbed and one undisturbed row. These were later retrieved and seeds dissected to determine the survival of buried seed.

Using this basic design as a template, additional treatments were applied in some years in a split plot design. In 2001, additional treatments included the application of fungicide (or water as a control) in a criss-cross design across the disturbed/undisturbed treatment.

Transects were also set-up to monitor the growth of established B. rapa plants at each site, each with at least 80 tagged plants (Claverton = 85, Radley = 100, Wytham = 80). Plants were tagged after they had become established, but subsequent monitoring provided additional information on fecundity. All were monitored each month for fungal infection.

**Combining matrix models with field data**

Matrix models, parameterised by the field data, provide a framework to explore population growth rates and potential persistence of plant populations over many generations. The first step is to construct a transition matrix, which converts a population vector at time t to the population at time t+1. The transition probabilities from one stage to the next are combinations of demographic parameters, and are represented as matrix elements (see Table 1). The dominant eigenvalue of the transition matrix is equal to the finite rate of increase (denoted λ).

The life cycle of B. rapa is relatively simple. It is an annual, with a proportion of seeds remaining dormant for one or two years. One point in the life cycle is chosen (here, this point is July/August, when the adult plants are carrying mature pods). The matrix is then constructed to describe the transition from one year to the next as illustrated in Table 1.
Table 1. A simple transition matrix for *B. rapa*.

<table>
<thead>
<tr>
<th>Seed in year t</th>
<th>Adults in year t</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed in year t+1</td>
<td>Seed survival over one year  Pod per plant * seeds per pod * seed survival over one year</td>
</tr>
<tr>
<td>Adults in year t+1</td>
<td>Seed survival over winter * Pods per plant * seeds per pod * seed survival over winter * probability of germination * probability of survival to adult</td>
</tr>
<tr>
<td></td>
<td>Seed survival over winter * Pods per plant * seeds per pod * seed survival over winter * probability of germination * probability of survival to adult.</td>
</tr>
</tbody>
</table>

The transition matrix converts the population vector at time *t* to the population at time *t+1* as follows:

\[ N_{(t+1)} = A_t N_{(t)} \]

The transition matrix described in Table 1 assumes that one set of conditions prevails. However, in reality, the environment changes from year to year. We explored two forms of environmental stochasticity; the presence and absence of pathogens and the occurrence (or not) of disturbance. Data gathered from our field experiments and transects allowed us to parameterise the four transition matrices: \( A_DI, A_{DH}, A_{UI}, A_{UH} \) corresponding to disturbance (\(+ = D, - = U\)) and pathogen infection (\(+ = I, - = H\)). We can then simulate what will happen to a population in a variable environment by picking one of the four matrices with probability of disturbance \( p_D \), and probability of infection \( p_p \).

In a deterministic matrix model, the population growth rate is the logarithm of the dominant eigenvalue of the transition matrix, \( \log \lambda \). In a stochastic matrix model, the long term population growth rate can be estimated as

\[ \log \hat{\lambda} = \frac{1}{T} \sum \log R_{(i)} \text{ where } R_{(i)} = \frac{\left\| N_{(t+1)} \right\|}{\left\| N_{(t)} \right\|} \text{ and } \left\| N_{(i)} \right\| = \sum n_i \text{ with } n_i \text{ being the individual matrix elements.} \]

This represents the ability of the population to increase when rare, and is often used as a measure of the ability of a genotype to expand. In density independent models, (as described here) individual fitness is equivalent to the population growth rate.

**Results**

**Impact of disturbance and fungicide on the population growth rate of *B. rapa***

*B. rapa* behaves as a typical annual species, with its population growth rate being enhanced by disturbance (Figure 2 illustrates this for one habitat). In undisturbed habitats, \( \log(\lambda) \) is insignificantly different from zero, so \( \lambda = 1 \), and the population is replacing itself. *B. napus* has a similar response to disturbance, but in undisturbed habitats has a \( \lambda \) very much less than 1 (Crawley et al., 1993; Hails, 2002). The application of fungicide had no significant impact, suggesting that in this year (2001) at least fungal pathogens were either not prevalent or not virulent at this particular site.
Figure 2. Log(λ) ± SE for B. rapa at Claverton in 2001-2002; illustrating the impact of disturbance (only apparent in some sites) and lack of impact of fungicide. Note that populations are replacing themselves in undisturbed habitats

**Presence and impact of pathogens in natural populations of B. rapa**

Over 2001-2002, four pathogens were found at our three field sites: Leptosphaeria maculans, L. biglobosa, Alternaria brassicae and A. brassicicola. In 2003, a new isolate of A. brassicicola swept through the Claverton site, and provided us with the opportunity to observe and monitor a natural experiment. A comparison of infected and uninfected pods illustrated that this pathogen had a significant impact on fecundity – reducing the numbers of seeds per pod ($t_{218} = 11.0, p < 0.001$) and reducing the viability of those seeds which did form ($t_{105} = 14.9, p < 0.001$, Figure 3).

![Figure 3. Mean number of seeds per pod ± SE (Figure 3a) and the proportion of viable seed per pod ± SE (Figure 3b) in healthy and Alternaria infected pods of B. rapa at Claverton](image)

**Relative impact of pathogens and disturbance on the long term population growth rate**

Two sets of simulations were run over 10,000 generations; the first focussed on the impact of disturbance on log(λ). Each generation, the transition matrix $A_{DH}$ was picked with probability $p_d$, and $A_{UH}$ with probability $(1-p_d)$. Population growth over that generation is calculated, and the population vector re-scaled before the next iteration. As expected, disturbance enhances
the long term population growth rate with the slope of \( \log(\lambda_s) \) against \( p_d \) rising sharply (see solid line in Figure 4). The second set of simulations fixed the probability of disturbance (\( p_d \)) at 0.1, and picked the transition matrix \( A_{D1} \) or \( A_{U1} \) as appropriate with probability \( p_p \), or matrix \( A_{Dh} \) or \( A_{Uh} \) as appropriate with probability \( (1-p_p) \). Again as expected the presence of pathogens reduces the population growth rate, but the slope of \( \log(\lambda_s) \) responds very weakly to changes in \( p_p \).

Figure 4. The impact of the frequency of disturbance (\( p_d \)) or pathogen attack (\( p_p \)) on the long term population growth rate (\( \log(\lambda_s) \)) of \( B. \) rapa.

Discussion

Enhanced fitness has been demonstrated for certain transgenic plants under specific ecological conditions, principally when the herbivores or pathogens that are transgene targets are manipulated to be present at high density (Hails et al., 2005). Few studies have addressed these same questions under field conditions where natural enemy pressure is unmanipulated. One exception to this involved the \( Bt \) transgene backcrossed into wild sunflower populations and grown in conditions where they were exposed to natural levels of herbivory. The BC1 transgenic line experienced reduced herbivory and enhanced fecundity compared to the BC1 controls, illustrating a selective advantage under realistic field conditions (Snow et al., 2003). Similarly, we illustrate here that when a pathogen sweeps through a resident population of \( B. \) rapa, it has a significant impact on fecundity. Thus plants resistant to such pathogens, either naturally or through the presence of a transgene would have a selective advantage. It would be tempting to conclude from this that ‘modified wild populations could prosper and spread’ (Snow et al., 2003).

This is in contrast with years in which exclusion of fungal pathogens using fungicide has no significant impact on \( \lambda_s \), presumably because pathogens are only important in some years and some places. This suggests that the impact of fungal pathogens, and therefore the consequences of transgenic pathogen resistance, needs to be evaluated in the context of stochastic models. Our preliminary results here suggest that in a stochastic environment, the population growth rate responds weakly to the presence of pathogens, relative to the response to disturbance. One possible explanation for this is that in undisturbed years, germination is limited by the number of microsites available (Hails et al., unpublished data). Thus strong density dependence operates between seed production and the establishment of seedlings in
the following generation, reducing the fecundity advantage enjoyed by individuals which escaped pathogen attack in the previous generation.

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References

A framework for evaluating possible non-target effects of transgenic corn in the United States: Standardizing laboratory tests

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Abstract: Collaborators in the United States have been working on various aspects of evaluating possible effects of transgenic crops on non-target organisms. These efforts include developing a comprehensive approach for evaluating effects of Bacillus thuringiensis (Bt)-corn pollen on monarch butterflies and developing guidelines for monitoring transgenic crops in the field. This report focuses on efforts to standardize Tier I and Tier II laboratory tests to assess non-target effects of genetically engineered (GE) plants. An overview of U.S. Environmental Protection Agency testing guidelines is provided; and suggestions are offered for ways to standardize these tests. A core group of harmonized tests to assess non-target effects of GE plants would foster communication among scientists and regulators and ultimately would contribute to science-based decisions related to the regulation of GE crops.

Key words: Bt crops, maize, tiered tests, harmonization

Introduction

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) mandates that the United States Environmental Protection Agency (USEPA) regulate the use and sale of pesticides to protect human health and preserve the environment while at the same time taking into account the benefits provided by those pesticides. Under this Act the USEPA regulates plants that are genetically engineered to express insecticidal proteins. Before a genetically-engineered (GE) plant is registered, an ecological risk assessment is conducted to determine whether there are potentially any unreasonable adverse effects from the use of these plants. The corn and cotton plants that express proteins derived from the bacterium Bacillus thuringiensis have been evaluated with tests adapted from guidelines for microbial pesticides. USEPA used these guidelines to minimize variations among tests that were being conducted. They are readily available on the USEPA Office of Prevention, Pesticides and Toxic Substances (OPPTS) (e.g., USEPA, 1996a).

The current USEPA guidelines provide a starting point for developing protocols for testing GE plants; but critics have suggested that they could be improved. A USEPA Scientific Advisory Panel that assessed non-target organism data requirements for GE plants recommended that the USEPA should provide applicants with detailed recommendations regarding experimental design and data analysis (USEPA, 2000). At present there are several different efforts among entomologists and risk assessment scientists to standardize Tier I and
Tier II tests so that test protocols are unambiguous, results are easily interpreted, and there is consistent logic in the approaches to testing and their relationship to monitoring. In this paper, an overview of existing USEPA–OPPTS test guidelines is provided, and suggestions are offered for ways to develop standardized tests to assess possible effects of GE plants on non-target arthropods.

**Testing guidelines**

The tests that are more easily standardized and are the present focus of this paper are lab-based Tier I and Tier II tests. Tier I tests simulate worst-case scenarios and often have exposure levels that exceed (>10×) the expected environmental concentration (EEC). Diets are usually artificial with incorporated proteins administered in maximum limit dose, short duration studies. If warranted on the basis of Tier I results or the nature of concern, Tier II tests and higher level tests may be conducted. Tier II tests are a step closer to reality because plant tissues are used, usually at the expected environmental concentration (i.e., 1× EEC) and with exposure routes and duration that better represent the field environment. If Tier II tests indicate hazard (or toxicity) is sufficient to have an effect then Tier III testing is required. The advantage of Tier I and Tier II tests are that they allow for increased replication and control over testing conditions. Tier III tests are long-term laboratory or semi-field tests, which sometimes are followed by Tier IV simulated or actual field testing.

The USEPA has numerous harmonized test guidelines for tiered tests in categories including Spray Drift, Residue Chemistry, Ecological Effects, Microbial Pesticides, Biochemicals, among others. Microbial Pesticide test guidelines, which are most relevant to GE crops, are divided into the following groups: A, Product Analysis; B, Residues; C, Toxicology; D, Non-target Organism; and E, Environmental Expression. The following tests are within the Non-target Organism group:

<table>
<thead>
<tr>
<th>USEPA #</th>
<th>Test Guidelines Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>885.4050</td>
<td>Avian oral, Tier I</td>
</tr>
<tr>
<td>885.4100</td>
<td>Avian inhalation test, Tier I</td>
</tr>
<tr>
<td>885.4150</td>
<td>Wild mammal testing, Tier I</td>
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<td>885.4200</td>
<td>Freshwater fish testing, Tier I</td>
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<td>885.4240</td>
<td>Freshwater aquatic invertebrate testing, Tier I</td>
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<td>885.4280</td>
<td>Estuarine and marine animal testing, Tier I</td>
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<tr>
<td>885.4300</td>
<td>Nontarget plant studies, Tier I</td>
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<td>885.4340</td>
<td>Nontarget insect testing, Tier I</td>
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<td>885.4380</td>
<td>Honey bee testing, Tier I</td>
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<tr>
<td>885.4600</td>
<td>Avian chronic pathogenicity and reproduction test, Tier III</td>
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<td>Aquatic invertebrate range testing, Tier III</td>
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<tr>
<td>885.4700</td>
<td>Fish life cycle studies, Tier III</td>
</tr>
<tr>
<td>885.4750</td>
<td>Aquatic ecosystem test</td>
</tr>
</tbody>
</table>

The other set of test guidelines that might prove useful for developing standardized tests for arthropods are in the category Ecological Effects. These include two honey bee tests (850.3020 and 850.3030), pollinator test (850.3040), earthworm test (850.6200) and two daphnia tests (850.1010 and 850.1300).

Many of these guidelines provide generalized protocols that sometimes lack detail. For example, the Microbial Pesticide Test Guidelines for Tier I testing of honey bees entails only
These harmonized protocols provide flexibility for adapting tests to new products. One challenge in developing tests is establishing the level of detail. There are tradeoffs between too little detail, which may preclude repeatability of studies among laboratories, and too much detail, which might stifle experimental flexibility. During the registration process, the development of testing protocols involves consultation among USEPA and applicant representatives. The applicant starts with the tests guidelines and associated publications and develops detailed protocols suitable for testing their product. The modified protocols in some case are then reviewed by the USEPA and recommended changes are made. This process may be repeated until the USEPA determines the tests are appropriate. This iterative approach has some advantages because it allows the applicant and the USEPA to make necessary adjustments to the tests. For example, in the case of honey bee tests, the appropriate food source (pollen or honey) or age of bees (larvae or adults) may depend on the route of exposure or type of stressor. These types of adjustments are logical and can be made after consultation. This approach, however, also has disadvantages. There is the possibility that each applicant or associated testing laboratories could develop unique protocols. Under these conditions, tests from different laboratories and sometimes tests within the same laboratory are fundamentally different and cannot be compared. These limitations open the door for outside scrutiny and call into question whether some of these tests should be standardized.

**Framework for standardizing tests**

The first step toward standardizing tests would involve forming a steering committee to oversee the standardization process. Members of the committee could include representatives from regulatory agencies, academics, government laboratories, gene suppliers, and testing laboratories. This committee would then select candidate arthropod(s), which could include traditionally tested taxa, such as honey bees, earthworms, daphnia, collembola, lady beetles, parasitic wasps, and green lacewings; and other taxa, such as ground beetles (carabids), monarch butterflies, and minute pirate bugs (anthocorids). In some cases separate tests for adults and immatures might be necessary. Individuals appointed by the steering committee then would oversee a harmonization process that would combine protocols from taxon-specific tests derived from various publications (e.g. Candolfi *et al.*, 2000) and testing laboratories. In many cases protocols would be modified for testing GE plant products. The harmonization process would be followed by inter-laboratory testing or ring tests for validation.

Protocols will vary depending on the taxon, but a general outline could include the following:

- **Organism** – common and scientific names
- **Rearing methodology** (deliver stage specific organisms)
- **Bioassay**
  - Life stage(s) tested (egg, larvae, adult, multiple, etc.)
  - Length of test
  - Endpoint(s) (LC50, size/weight, etc.)
  - Test system and conditions
  - Test material (origin, form)
  - Dose calculation (based on 10X highest expressing tissue, dry weight)
  - Treatment application (protein in artificial diet, plant material, etc.)
- **Replicate number**
- **Positive control (compound used)**
Negative control
Validity criteria (e.g., control mortality limits)
Statistical methods (power tests, t-test, ANOVA, etc.)

As mentioned previously, one challenge will be to develop tests with appropriate amount of detail to avoid problems with test repeatability and at the same time allow for experimental flexibility. For some tests, variances could be incorporated into the standardized tests to allow for at least some flexibility. These tests could be made available to the public through peer-reviewed journal articles, handbooks, websites or a combination of these outlets.

Discussion

Most regulatory agencies rely on a science-based iterative process to improve testing protocols. The USEPA periodically updates their guidelines to incorporate new information and improve testing details. Testing of GE products is relatively new, so it is likely the tests used today will improve over time as the science progresses. The high-profile nature of GE plants has encouraged some scientists to consider expediting this process. Yet, even if standardized tests were developed it would be necessary to re-evaluate these periodically and update when needed. Although this paper has a U.S. bias, there is merit in considering global harmonization of tests. This may be possible because several scientists at this conference have expressed interests in pursuing standardized tests. A stepwise approach to standardizing the first test(s) might include: formation of steering committee, develop basic criteria for tests and publications, identify leaders for individual test development, submit tests and manuscript to steering committee for peer review, and pursue publishing results.

The bottom line, a core group of harmonized tests to assess non-target effects of GE plants would foster communication among scientists and regulators and ultimately would contribute to science-based decisions related to the regulation of these plants. This would be a major step forward.

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References


Pair-wise combination of toxin genes in transgenic crops: the risk of cross-resistance development

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The evolution of insect resistance is one of the main threats of the wide adoption of transgenic crops expressing Bacillus thuringiensis (Bt) insecticidal protein genes (Ferré & Van Rie, 2002). The use of more than one Cry toxin in the same plant is a strategy to delay or minimize the appearance of resistance (Ferré, 2003). The toxins to be considered should have different modes of action to avoid development of cross-resistance. The best characterized mechanism to confer high levels of resistance to Bt toxins and cross-resistance among Cry1A toxins is binding site modification (Ferré & Van Rie, 2002). Toxins using the same binding sites exert their toxicity cannot be considered as alternatives or complements of each other. In the present study, we have tested the hypothesis that Cry1A, Cry1Fa, and Cry1Ja share the same receptor in Helicoverpa armigera, H. zea, and Spodoptera exigua.

Using $^{125}$I-Cry1Ac toxin and unlabeled Cry1Ac, Cry1Fa, and Cry1Ja toxins as competitors, we performed binding competition experiments. Cry1Fa and Cry1Ja toxins competed for the Cry1Ac binding site in the three species tested (Figure 1). The three toxins have higher $K_d$ values for Cry1Fa and Cry1Ja as compared to Cry1Ac in the three insect species (Table 1). Although toxicity and binding affinity not always correlate (Wolfersberger, 1990), the low affinity of Cry1Fa in H. armigera and H. zea agrees with its lack of toxicity against these species (Liao et al., 2002), and the toxicity of Cry1Fa to S. exigua (Chambers et al., 1991) agrees with its higher affinity for the binding sites in this species.

Complete competition of the $^{125}$I-Cry1Ac was only observed in S. exigua, in the range of concentrations tested, indicating that Cry1Ac does not have binding sites other than those shared with the Cry1Fa and Cry1Ja. In the Helicoverpa species, complete competition with Cry1Fa and Cry1Ja was not reached, but neither was it a plateau indicating the occurrence of single Cry1Ac binding sites.

To test for the reciprocal competition, Cry1Fa was biotinylated and used in competition experiments with the other toxins. The results showed that both Cry1Ac and Cry1Ja competed for the Cry1Fa binding site in the three species (Figure 2). Furthermore, there is no evidence of unshared sites for Cry1Fa, since competition by unlabeled Cry1Fa was not more effective than that by the heterologous toxins.
Figure 1. Binding competition between $^{125}$I-Cry1Ac and increasing concentrations of unlabeled Cry1Ac (●), Cry1Fa (○), or Cry1Ja (△) to BBMV from *H. armigera* (A), *H. zea* (B), and *S. exigua* (C). Each data point is the mean of two independent replicates.
Table 1. Dissociation constant ($K_d$) and concentration of binding sites ($R_t$) for Cry1 toxins binding to BBMV from *H. armigera*, *H. zea* and *S. exigua*, determined using Cry1Ac as the labeled ligand. $K_d$ values of heterologous ligands are estimated assuming the same $R_t$ as the labeled ligand.

<table>
<thead>
<tr>
<th>Toxin</th>
<th>$K_d$ (nM) ± SD</th>
<th>$R_t$ (pmol/mg protein) ± SD</th>
<th>$K_d$ (nM) ± SD</th>
<th>$R_t$ (pmol/mg protein) ± SD</th>
<th>$K_d$ (nM) ± SD</th>
<th>$R_t$ (pmol/mg protein) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Ac</td>
<td>1.6 ± 0.2</td>
<td>16.4 ± 0.2</td>
<td>0.34 ± 0.04</td>
<td>29.6 ± 0.5</td>
<td>0.7 ± 0.5</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>Cry1Fa</td>
<td>150 ± 40</td>
<td>220 ± 10</td>
<td>29 ± 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry1Ja</td>
<td>250 ± 20</td>
<td>640 ± 2</td>
<td>22 ± 2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Binding of biotinylated-Cry1Fa to BBMV from *H. armigera*, *H. zea*, and *S. exigua*, in the absence of competitor (lanes labeled as —) or in the presence of a 120-fold excess of competitor (1Fa, 1Ac, and 1Ja lanes).

In addition to this study, all available information on binding site competition reported so far suggests that Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja share a common binding site in most, if not all, susceptible Lepidoptera: (i) cross-resistance to Cry1Fa and Cry1Ja has been detected in populations selected with Cry1A toxins (Gould et al., 1995; Lee et al., 1995; Siqueira et al., 2004; Tabashnik et al., 1997b); (ii) in *Plutella xylostella* and in *Heliothis virescens* this type of cross-resistance has been proposed to be due to the alteration of a common receptor for Cry1A toxins, Cry1Fa, and Cry1Ja (Granero et al., 1996; Jurat-Fuentes & Adang, 2001; Tabashnik et al., 1997a; Tabashnik et al., 2000); (iii) in six lepidopteran species, Cry1Ac and Cry1Ja competed for the same binding sites (Herrero et al., 2001); (iv) Cry1A, Cry1Fa and Cry1Ja show more amino acid sequence similarity in domain II, involved in specificity and receptor binding, than the rest of Cry toxins (Bravo, 1997; Tabashnik et al., 1996; Thompson et al., 1995).

Species such as *H. armigera* and *H. zea*, non-susceptible to Cry1Fa, can be controlled with the Cry1Ac toxin in Bt-cotton expressing both Cry1Ac and Cry1Fa in the same plant. The Cry1Fa toxin expressed in this Bt-cotton is effective against *Spodoptera* spp., which are little affected by Cry1Ac. However, from the perspective of resistance management, pair-wise combinations of Cry1Ac, Cry1Fa, and Cry1Ja expressed in transgenic plants will not offer a
good strategy for those insects susceptible to more than one of these toxins, neither will do rotations of Bt-crops containing single genes of these three toxins.

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Wolfersberger, M.G. 1990: The toxicity of two Bacillus thuringiensis delta-endotoxins to gypsy moth larvae is inversely related to the affinity of binding sites on midgut brush border membranes for the toxins. Experientia 46: 475-477.
Ecological investigations on the effect of Bulgarian GM plants on the arthropod fauna

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Abstract: In 2004 we studied the effect of genetically modified (GM) cultivars from three agriculturally important species (tomato, potato and alfalfa) in Bulgaria on the key groups of animal species, typical representatives of these agroecosystems. **Tomato:** The effect of GM tomato cultivar resistant to tomato spotted wild virus (TSWV) on the arthropod fauna was studied. The main pest insects were aphids and thrips. Among predatory insects the bugs *Macrolophus pygmaeus,* and *Orius* spp. were most numerous. There was no statistically significant difference between the insect communities in plots with GM and non-GM tomatoes. In laboratory experiments, the effect of GM and non-GM tomatoes on larval development of Colorado potato beetle (CPB) was studied. Tomato was not a suitable food for CPB and both GM and non-GM tomato leaves caused more than 80 % larval mortality. **Potato:** Under laboratory conditions, the effect of three Bulgarian Bt potato cultivars (Bor®, Koral® and Kalina®) on the aphid *Myzus persicae* and adults of the grasshopper *Tettigonia viridissima* were studied. Bt potatoes had no negative effect on aphid development and reproduction. Potatoes are not a suitable food for the grasshopper and adults reared on Bt or non-Bt potato leaves died after a mean of 12–14 days. In the field *M. persicae,* *T. viridissima,* aphidophagous ladybirds *Coccinella septempunctata,* *Propylea quatuordecimpunctata,* *Hippodamia variegata* and spiders were observed. Their abundance during the season was at a very low level and there were no statistically differences between Bt and non-Bt plots. **Alfalfa:** In field and laboratory experiments, the effect of GM alfalfa with reduced lignin content on arthropod fauna was evaluated. No differences between arthropod communities on GM and non-GM alfalfa were found in the field. Laboratory experiments indicated that GM alfalfa had no effect on larval development and larval mortality of the leaf beetle *Phytodecta fornicata.*

Key words: Bt potatoes, GM alfalfa, GM tomato, pest insects, beneficial arthropods

Introduction

Throughout the history of plant breeding, ’new technologies’ have regularly been utilized to develop new gene combinations for improving crop cultivars (Simmonds et al., 1999). These included: the artificial manipulation of chromosome number, the development of addition and substitution lines for specific chromosomes, chemical and radiation treatments to induce mutations and chromosome rearrangements, as well as cell and tissue culture approaches such as embryo rescue, in vitro fertilization and protoplast fusion to allow the recovery of interspecific and intergeneric hybrids. The genetic gains from the integration of these technologies into mainstream plant breeding have substantially improved the performance of resulting cultivars. They continue to make a major contribution to genetic improvements in yield, environmental adaptation, resistance to specific diseases and pests, and specific quality
attributes that are constantly demanded by farmers, the food industry and consumers (Conner et al., 2003).

Despite numerous advantages, there is a multitude of concerns about the impact of GM crops on the environment. Key issues in the environmental assessment of GM crops are putative invasiveness, vertical or horizontal gene flow, other ecological impacts like the effect on biodiversity and non-target arthropods, the impact of presence of GM material in other products.

The main aim of our investigations is to evaluate the efficacy and potential impacts of the Bulgarian transgenic cultivars (tomato, potato and alfalfa) on key groups of animal species, typical representatives of these agroecosystems.

**Material and methods**

**Tomato**

Tomato spotted wild virus (TSWV) is among the most important viral diseases affecting tomato and causing significant economic losses worldwide. TSWV is a thrips transmitted plant virus, which infects more than 650 plant species of different botanical families (Peters et al., 1990; Goldbach & Peters, 1994). During the first year of experiment in open field conditions we used 300 transgenic plants (TSWV resistant transgenic tomato carrying the nucleoprotein N gene of the Bulgarian L3 isolate of the virus) and 300 control plants. The plants were grown in one field using a randomized block design (Cochran & Cox, 1950). Thirty plots were used – 15 with the transgenic line and 15 with the non transgenic control. Plots consisted of 20 plants each. Every week from the middle of June till the end of September, we observed 10 whole plants from each plot. At the end of the trial period the numbers of fruits with bite damages were counted in order to evaluate the possible interaction with herbivores (birds and mice). In addition we reared Colorado potato beetle larvae in the laboratory on transgenic and non transgenic tomatoes. Newly hatched larvae were randomly divided in groups of 10 and reared in 18 cm Petri dishes on GM or non-GM tomato leaves. Sixty larvae were tested on each diet.

**Potato**

Three Bulgarian Bt potato cultivars expressing Cry3A protein against CPB (Bor®, Kalina®, Koral®) were studied. There were three Bt plots (4 x 30 m) and three conventional plots 4 x 10 m separated from Bt plots by other conventional potato cultivars. Potatoes were planted at the beginning of June. Every week from the end of June until the end of August we observed 20 randomly selected plants from each Bt and non-Bt plot. Immediately thereafter arthropods were sampled by beating these plants over an entomological net.

Under laboratory conditions, the effect of Bulgarian Bt potato (Bor®) on the aphid *Myzus persicae* and the grasshopper *Tettigonia viridissima* were studied. Aphids were reared either on Bt or non-Bt potato plants. Grasshoppers were collected as larvae and reared in the laboratory on alfalfa till imago. Newly emerged adults were fed with Bt, non-Bt potato leaves and alfalfa as a control. Potatoes were cultivated in big pots inside nylon isolators at a temperature of 20-25°C, relative humidity 53-75 % and 16L:8D photoperiod. The experiments were done at a temperature of 25±2°C, a relative humidity 64-78 %, and 16L:8D photoperiod. The developmental time, longevity and fecundity of aphids and the longevity of adult grasshoppers were recorded.

**Alfalfa**

There were two experimental plots (100 m² each) with transgenic (CCoAOMT-down-regulated alfalfa plants, possessing up to 20 % reduced lignin content) and control plants. Between both plots, 200 m² of maize were planted. Arthropods were collected with a standard
entomological net (5 x 5 sweeps). Samples were taken weekly from the end of June till the middle of November. In laboratory experiments we reared larvae of the leaf beetle *Phytodecta fornicata* on GM and control alfalfa plants. Each experiment was conducted with 10 pairs.

Experiments with tomatoes, potatoes and alfalfa were carried out in 2004 near Sofia. No insecticides were applied to both GM and control plots. Arthropods were identified in the field. In case where the taxonomic identity of the arthropod species could not be confirmed in the field, voucher specimens were collected for later identification by expert taxonomists. Data were analyzed by using ANOVA and LSD test (least significant difference test, $\alpha=0.05$).

**Results and discussion**

**Tomato**

Among pest insects only aphids, thrips, single specimen of white fly and the bug *Dicyphus stachidis* were found on GM and non-GM tomatoes (Table 1). Although the number of aphids was very low (5 – 10 per plant) this group was the most prominent one on tomato plants from June till September.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Mean number*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Transgenic plants</td>
<td>Control plants</td>
</tr>
<tr>
<td>Aphids</td>
<td>5.287</td>
<td>5.007</td>
</tr>
<tr>
<td>White flies</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Thrips</td>
<td>0.435</td>
<td>0.390</td>
</tr>
<tr>
<td><em>Dicyphus stachidis</em></td>
<td>8.47</td>
<td>10.60</td>
</tr>
<tr>
<td>Lacewing eggs</td>
<td>4</td>
<td>2.64</td>
</tr>
<tr>
<td><em>Macrolophus pigmaeus</em></td>
<td>68.0</td>
<td>41.9</td>
</tr>
<tr>
<td>Spiders</td>
<td>6.50</td>
<td>4.29</td>
</tr>
<tr>
<td>Damaged fruits</td>
<td>4.52</td>
<td>4.05</td>
</tr>
</tbody>
</table>

* The number of aphids and white flies per plant, of thrips per flower and of other arthropods per ten plants.

Thrips were observed in August and beginning of September at a number 0.2 – 0.5 per flower (Figure 1). The larvae and adults of bugs, spiders and lacewing eggs dominated among predatory arthropods. Most numerous was the bug *Macrolophus pygmaeus* (Table 1). There was no statistically significant difference between the number of observed arthropods on transgenic and non transgenic plants ($p>0.05$, Table 1). There was a correlation in changes of abundance between thrips and their predator *M. pygmaeus*. Increasing abundance of *M. pygmaeus* in August was followed by decreasing abundance of thrips.

Our laboratory experiments showed that tomatoes were not a good food for Colorado potato beetle larvae. When fed on transgenic or non transgenic plants more than 80% of the larvae died. It was surprising because experts consider Colorado potato beetle among tomato pests.
Figure 1. Abundance of thrips (left) and the bug *Macrolophus pigmaeus* (right) on tomato plants during the 2004 season.

**Potato**

In the field only aphid *M. persicae*, grasshopper *Tettigonia viridissima*, three species of aphidophagous ladybirds (*Coccinella septempunctata, Hippodamia variegata, Propylea quatuordecimpunctata*) and spiders were observed.

The abundance of aphids (1 – 5 per plant), grasshoppers (0.04 - 0.06 per plant), ladybirds (0.1 - 0.2 per plant) and spiders (0.4 – 0.5 per plant) during the season was very low and there were no statistically differences between Bt and non-Bt potato plots (ANOVA, p>0.05).

Under laboratory conditions, Bt potatoes had no negative effect on recorded life-table parameters of the aphid *M. persicae* (Table 2). Although the grasshopper *T. viridissima* was present in potato fields, potatoes were not suitable as food and adults reared on Bt or non-Bt potato leaves died after a mean of 12 - 14 days (Table 3).

Table 2. Effect of Bt and non-Bt potatoes on some life history parameters of *Myzus persicae*. Figures in a column, followed by the same letter, are not significantly different from one another (LSD test).
Table 3. Effect of Bt and non-Bt potatoes on longevity of adults of *Tettigonia viridissima*. Figures followed by the same letter are not significantly different from one another (LSD test).

<table>
<thead>
<tr>
<th>Potatoes</th>
<th>Tettigonia viridissima</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Bor Bt</td>
<td>12</td>
</tr>
<tr>
<td>Bor non-Bt</td>
<td>12</td>
</tr>
<tr>
<td>Control (alfalfa)</td>
<td>12</td>
</tr>
</tbody>
</table>

**Alfalfa**

Table 4. Dominant arthropods in GM and non-GM (control) alfalfa fields.

<table>
<thead>
<tr>
<th>Species</th>
<th>Percentage</th>
<th>Percentage</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>control</td>
<td>GM</td>
<td></td>
</tr>
<tr>
<td>Pest insects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf beetles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phytodecta fornicata</em></td>
<td>3.6</td>
<td>5.1</td>
<td>0.090</td>
</tr>
<tr>
<td>Phytophagous ladybird beetles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Subcoccinella 24-punctata</em></td>
<td>11.7</td>
<td>14.9</td>
<td>0.382</td>
</tr>
<tr>
<td>Weevils</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phytonomus variabilis</em></td>
<td>10.5</td>
<td>9.3</td>
<td>0.544</td>
</tr>
<tr>
<td><em>Apion apricans</em></td>
<td>2.5</td>
<td>2.6</td>
<td>0.680</td>
</tr>
<tr>
<td><em>Sitona humeralis</em></td>
<td>22.5</td>
<td>14.0</td>
<td>0.114</td>
</tr>
<tr>
<td>Phytophagous bugs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Adelphocoris lineolatus</em></td>
<td>30.2</td>
<td>33.1</td>
<td>0.322</td>
</tr>
<tr>
<td><em>Lygus pratensis</em></td>
<td>16.7</td>
<td>19.1</td>
<td>0.185</td>
</tr>
<tr>
<td>Other phytophagous insects</td>
<td>2.3</td>
<td>1.9</td>
<td>0.448</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Predatory arthropods</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bugs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Nabis pseudogerus</em></td>
<td>9.2</td>
<td>10.2</td>
<td>0.377</td>
</tr>
<tr>
<td>Ladybird beetles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Coccinella septempunctata</em></td>
<td>9.2</td>
<td>8.0</td>
<td>0.264</td>
</tr>
<tr>
<td><em>Propylea quatuordecimpunctata</em></td>
<td>13.0</td>
<td>13.3</td>
<td>0.734</td>
</tr>
<tr>
<td><em>Hippodamia variegata</em></td>
<td>6.3</td>
<td>6.9</td>
<td>0.647</td>
</tr>
<tr>
<td>Lacewings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chrysoperla carnea eggs</em></td>
<td>4.8</td>
<td>5.8</td>
<td>0.118</td>
</tr>
<tr>
<td>Spiders</td>
<td>57.5</td>
<td>55.8</td>
<td>0.597</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Dominating pest and predatory arthropods were very similar in GM and non-GM alfalfa plots (Table 4). Among pest species the bugs *Adelphocoris lineolatus* and *Lygus pratensis*, the
weevils Sitona humeralis and Phytonomus variabilis, the phytophagous ladybird Subcoccinella 24-punctata and the leaf beetle Phytodecta fornicata dominated. Spiders were most numerous among predators, followed by aphidophagous ladybirds (Coccinella septempunctata, Propylea quatuordecimpunctata, Hippodamia variegata), the bug Nabis pseudogerus and lacewing Chrysoperla spp. eggs. There was no statistically significant difference between the number of observed pest and predatory arthropods on transgenic and non transgenic plants (p>0.05, Table 4).

In laboratory experiments GM alfalfa had no negative effect on larval development and larval mortality of leaf beetle Phytodecta fornicata (Table 5). When fed on GM or non-GM alfalfa, larval development lasted 23 - 24 days and mortality was 38 and 42 %, respectively.

Table 5. The effect of GM and non-GM alfalfa on larval development of Phytodecta fornicata in the laboratory. Figures followed by the same letter are not significantly different from one another (LSD test).

<table>
<thead>
<tr>
<th>Plant</th>
<th>Mean larval development</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>GM alfalfa</td>
<td>12</td>
</tr>
<tr>
<td>Control alfalfa</td>
<td>12</td>
</tr>
</tbody>
</table>

Our initial results showed that TSWV resistant transgenic tomato, Bt potatoes, and GM alfalfa with low lignin content had no specific effect on arthropod fauna both in field surveys and laboratory experiments. Presently there is no published data on similar investigations with GM tomato and GM alfalfa. Some authors only suppose that any alterations in lignin biosynthesis might affect feeding and population growth rates of defoliators (James et al., 1998). On this point of view more laboratory experiments are needed.

Our previous investigations demonstrated no measurable effect of Bt potato Newleaf® on epigeic arthropods (Spitzer et al., 2001, and unpublished results). Newleaf® potatoes were highly effective against CPB larvae and adults, and more effective than weekly sprays of Bt-based microbial insecticides, bi-weekly applications of permethrin, or early and mid-season applications of systemic insecticides phorate and disulfoton (Reed et al., 2001). Newleaf® potatoes appear to have an advantage over broad-spectrum foliar applied insecticides in promoting the role of natural enemies (Duan et al., 2004; Kalushkov & Nedved, 2005). Aphid Myzus persicae cultured on transgenic Bt and conventional potatoes were suitable food for aphidophagous ladybird Propylea quatuordecimpunctata according to the rate of larval development, larval mortality and adult fresh weight. Females of P. quatuordecimpunctata fed with M. persicae cultured on Bt potato, M. persicae cultured on non-Bt potato and mixture of M. persicae from Bt potatoes and aphid Aphis craccivora, laid a little more eggs than those fed only with A. craccivora (Kalushkov & Hodek, 2005).

The findings we reported are not surprising because the Cry3A protein is known to be highly selective in its activity, affecting only Coleoptera in the family Chrysomelidae (MacIntosh et al., 1990; Eckberg & Cranshaw, 1994).
Acknowledgements

These investigations were funded by UNEP-GEF project. The experiments with Bt potatoes were partially supported by grant B-1105 from the Bulgarian National Science Fund.

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Barley cystatin variants against phytopathogenic fungi, pests and their impact on natural enemies

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Abstract: The goal of this study has been to know the effects of the barley cystatin Hv-CPI and seven derived variants generated by direct-mutagenesis, on the growth and digestive physiology of the Colorado potato beetle (CPB), Leptinotarsa decemlineata and to assess the potential impact of these proteins on the spiner soldier bug (SSB), Podisus maculiventris, a generalist hemipteran predator. Among the different cystatins tested, the variant C⁶₈→G, in which the only cysteine residue was changed to glycine, showed the highest inhibitory activity when tested in vitro against commercial cysteine proteases and CPB digestive enzymes. Feeding trials conducted with CPB larvae reared on transgenic potato plants expressing this variant, resulted in significant lower weight gains compared to those fed on non-transformed plants. No effects on survival, development, and weight, were observed when SSB nymphs fed on prey CPB reared with transgenic potato plants. To investigate the physiological background, biochemical analysis were carried out on guts of insects dissected at the end of the feeding assays. The effects of the barley inhibitor Hv-CPI and its variants on the growth of the the necrotrophic fungus Fusarium oxysporum have been also analysed. The cystatin Hv-CPI inhibited fungal spore germination by 25%, the five point mutations inhibited spore germination by 18 to 40%, while the two truncated forms had no antifungal effect.

Key words: cystatin, insect pest, natural enemy, protein defence, transgenic potatoes

Introduction

Plant cystatins are a group of specific inhibitors of cysteine proteases that have been involved in the regulation of protein turnover and in plant defence. The protective role is supported by in vitro inhibition data and bioassays against different pests and by the enhanced resistance towards insects, nematodes, slugs and potyviruses in transgenic plants expressing plant cystatins (for a review see Haq et al., 2004). As far as we know, only few cystatin genes from rice, maize and Arabidopsis have been used as transgenes in crop protection assays. Fungicidal and antimicrobial activities have been also described for several cystatins (Pernas et al., 2000; Martinez et al., 2003, 2005). However, the mechanism responsible of their antifungal properties is still unknown, although we have shown that is not associated with its role as cysteine protease inhibitor (Martinez et al., 2003).

We have reported previously the characterization of a barley cystatin Hv-CPI (Gaddour et al., 2001) and several variants derived from it with different Ki (inhibition constant) against cysteine proteases and antifungal properties against Botrytis cinerea (Martinez et al., 2003). Our aim is to search for improved cystatin genes to be used as transgenes and to investigate their direct and indirect impacts on insect pests, their natural predators and on fungal pathogens.
The necrotrophic fungus *Fusarium oxysporum* causes vascular wilt diseases in many plant species worldwide. Their clamydospores persist in dead tissues or are released into the soil where they remain dormant until suitable conditions allow them to germinate and enter the vascular tissues producing the death of the plants (Jimenez-Casco *et al.*, 2004).

The Colorado potato beetle (CPB) *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) is one of the most serious pest of potato worldwide. Protein digestion in CPB larvae and adults is carried out by a complex proteolytic system. Purification and characterization of their digestive protease activities have shown that they are mainly of the cysteine protease type (Novillo *et al.*, 1997). The spined soldier bug (SSB), *Podisus maculiventris* (Hemiptera: Pentatomidae) is a generalist predator used as a biological control agent of lepidopteran and coleopteran pests. Protease activity in SSB midguts is mainly based on cysteine proteases (Bell *et al.*, 2005). The presence of digestive cysteine-proteases both in prey (CPB) and in the predator (SSB) suggested their potential for studying a possible interference mediated by cystatins.

This study has been focused on two main goals, i) to determine the *in vitro* inhibitory activity of the Hv-CPI cystatin and their derived variants against commercial cathepsin B and the phytopathogenic fungus *F. oxysporum* and ii) to analyse the effects of transgenic potato plants expressing a selected variant of the barley cystatin on the *L. decemlineata* and on its natural enemy, *P. maculiventris*.

**Materials and methods**

*Inhibitory activity of recombinant barley cystatins in vitro*

The wild type Hv-CPI, five mutant variants and two truncated forms derived from it were purified from recombinant *E. coli* cultures as described previously (Martinez *et al.*, 2003). Their inhibitory properties *in vitro* against commercial cathepsin B and digestive cathepsin B-like activity from guts extracts of CPB larvae and SSB nymphs were assayed, using ZAA$_2$MNA (N-carbobenzoxy-alanine-arginine-arginine-4-methoxy-β-naphtylamide) as substrate and the conditions described by Novillo *et al.* (1997). Fungal growth inhibitory assays were performed as described Martinez *et al.* (2005), by incubating $10^4$ spores of *F. oxysporum* in potato dextrose broth (PDB) liquid medium in the absence and presence of 6 µM of each cystatin variant. Inhibition constant ($K_i$) values against commercial cathepsin B were determined from Dixon plots ($1/V$ versus $[I]$). The effect on insects and fungi was monitored by measuring the percentage of enzyme inhibition and of spore germination, respectively.

*Insect bioassays with potato transgenic plants*

Different transgenic potato lines expressing the C$^{68}$→G barley cystatin variant were produced (Alvarez-Alfageme *et al.*, submitted) and used to feed second instar *L. decemlineata* for 4 days. In addition, third instar nymphs of *P. maculiventris* were reared with CPB fed on potato lines until they reach the fifth instar. To determine the effect of transgenic cystatin-potato plants on both insects, CPB larvae and SSB nymphs were weighed and dissected for digestive protease assays (Ortego *et al.*, 1999).
Results and discussion

Inhibition of fungal growth and determination of Ki values against cathepsin B

In vitro bioassays of the necrotrophic fungus *F. oxysporum* were carried out adding at the PDB medium the seven recombinant cystatin variants and the wild type Hv-CPI purified from *E. coli* cultures at the 6 \( \mu M \) concentration. After incubation of 48 h, the wild-type Hv-CPI produced an inhibition of fungal spore germination of 35\% and the five point variants inhibited between 18 (R\(^{58}\) → G) to 40\% (Q\(^{63}\) → L), as compared to the 100\% of germination control obtained in the medium without cystatin (Table 1). The two truncated forms did not affect spore germination, even at concentrations of 12 \( \mu M \) (data not shown), as well as the protein purified from *E. coli* transformed with the expression vector alone.

The biochemical characterization of these cystatin variants against commercial cathepsin B showed that the variant Q\(^{63}\) → P was unable to inhibit cathepsin B, as well as other cysteine proteases (Martinez et al., 2003), but maintained the antifungal properties against *F. oxysporum*, indicating that the cystatin role as fungicide is not associated with its function as inhibitor of cysteine proteases. We have previously reported this effect mediated by Hv-CPI cystatin and their variants after studying the in vitro inhibition growth of another important phytopathogenic fungus such is *B. cinerea* (Martinez et al., 2003).

Table 1. Inhibition of spore germination of *F. oxysporum* and of digestive cathepsin B-like activity (CTB) from *L. decemlineata* midgut extracts and determination of Ki values against commercial cathepsin B. \(^{a}\)6 \( \mu M \) of each cystatin was used for fungal assays, and \(^{b}\)1 \( \mu M \) was used for insect digestive extracts. ni: no inhibitory activity detected.

<table>
<thead>
<tr>
<th>Cystatin variants</th>
<th>(^{a})Spore germination of <em>F. oxysporum</em></th>
<th>(^{b})Inhibition of CTB digestive activity of CPB (%)</th>
<th>Ki (M) against cathepsin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>No protein</td>
<td>100 ± 2</td>
<td>ni</td>
<td>ni</td>
</tr>
<tr>
<td>HvCPI</td>
<td>75 ± 6</td>
<td>22 ± 1</td>
<td>2.5 x 10(^{-6})</td>
</tr>
<tr>
<td>Q(^{63}) → L</td>
<td>60 ± 4</td>
<td>26 ± 3</td>
<td>4.5 x 10(^{-5})</td>
</tr>
<tr>
<td>Q(^{63}) → P</td>
<td>75 ± 6</td>
<td>ni</td>
<td>ni</td>
</tr>
<tr>
<td>R(^{58}) → G</td>
<td>82 ± 8</td>
<td>10 ± 1</td>
<td>5.9 x 10(^{-5})</td>
</tr>
<tr>
<td>C(^{68}) → G</td>
<td>67 ± 7</td>
<td>43 ± 2</td>
<td>2.7 x 10(^{-8})</td>
</tr>
<tr>
<td>K(^{92}) → P</td>
<td>88 ± 6</td>
<td>8 ± 1</td>
<td>3.1 x 10(^{-4})</td>
</tr>
<tr>
<td>N-term(\Delta Q^{62})</td>
<td>100 ± 3</td>
<td>ni</td>
<td>ni</td>
</tr>
<tr>
<td>(\Delta Q^{63})C-term</td>
<td>100 ± 3</td>
<td>ni</td>
<td>ni</td>
</tr>
</tbody>
</table>

Inhibition of CPB and SSB digestive proteases

Barley cystatin variants were tested in vitro against the main midgut protease activity belonging to the cathepsin B-like from CPB larvae. The wild-type protein produced an inhibition of 22\% of enzyme activity. However, the variant C\(^{68}\) → G was a much better inhibitor than the wild-type because this substitution reduced the digestive activity to almost 45\%. This data indicated that the G\(^{68}\) substitution was an important amino acid for the inhibitory properties of the Hv-CPI cystatin. The other point variants seemed to be less effective as inhibitors, being the Q\(^{63}\) → P protein, in which the target Q of the reactive site was...
substituted by the cyclic amino acid proline, devoid of cysteine protease inhibitory capacity (Table 1). No effect was observed when the two truncated forms were used. These results are according with the $K_i$ values determined against cathepsin B.

Similar in vitro assays were performed with the wild-type and the $C^{68}\rightarrow G$ proteins against extracts from midgut of SSB nymphs and no inhibitory activity of cathepsin B-like was observed (data not shown). However, it is important to mention that the digestive activity in SSB midguts is mainly based on cysteine proteases (Bell et al., 2005).

Taking in mind all these data, we decided to use the $C^{68}\rightarrow G$ cystatin variant as transgene to fight against the main potato insect pest, the Colorado potato beetle and to study their impact on its natural enemy, the spined soldier bug.

**Effect of transgenic potatoes expressing the $C^{68}\rightarrow G$ cystatin on CPB larvae and SSB nymphs**

Larvae of CPB were reared on the transgenic potato lines expressing the $C^{68}\rightarrow G$ variant as well as on non-transgenic plants (NT), to assess the cystatin effect on larval growth (Alvarez-Alfageme et al., submitted). While insect survival was not affected, larvae that had fed on some transgenic potato lines showed presented significantly lower weight gains, particularly those fed on line G16 (Table 2). Protein extracts from this line showed the highest inhibitory activity against cysteine proteases (Alvarez-Alfageme et al., submitted). Moreover, biochemical analysis carried out on guts of CPB insects dissected at the end of the feeding assay, showed higher cathepsin B-like activity levels than those fed on non-transformed isogenic plants, probably produced as an adaptation response to the presence of the cystatin in the tranegenic lines (Alvarez-Alfageme et al., submitted).

<table>
<thead>
<tr>
<th>Potato plants</th>
<th>NT</th>
<th>G2</th>
<th>G5</th>
<th>G10</th>
<th>G11</th>
<th>G14</th>
<th>G16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larval growth</td>
<td>(mg fw)</td>
<td>59 ± 3</td>
<td>57 ± 3</td>
<td>62 ± 3</td>
<td>58 ± 3</td>
<td>56 ± 4</td>
<td>52 ± 3</td>
</tr>
</tbody>
</table>

Table 2. Growth of *L. decemlineata* larvae feeding on different lines of transgenic potato plants expressing the $C^{68}\rightarrow G$ cystatin (G) or on its corresponding non-transformed isogenic plants (NT). *Feeding assays were performed for 4 days from second to fourth instar. Larval growth is expressed in mg of fresh weight. Data are the mean ± SE (n = 48-64). *Significantly different from NT (Dunnet two-tailed test P ≤ 0.05).

To study the prey-mediated effects at the third trophic level, SSB nymphs were fed on CPB larvae reared with G16 and NT potato lines. No differences in development or weight of this natural enemy were observed, whereas a slight reduction on midgut cathepsin B-like activity of SSB nymphs fed on CPB larvae reared on G16 plants was detected (Alvarez-Alfageme et al., submitted). These results indicate that the cystatin interactions that take place in the prey produce an insecticide effect and trigger an over-expression of the target digestive proteases of the Colorado potato beetle, while the possible impact on the spined soldier bug seems to be no harmful. The absence of detrimental effects on other insect predators via their herbivorous prey feeding on different species of transgenic plants such are potato and oilseed rape expressing the oryzacystatin-I from rice or the chicken egg white cystatin, has been also reported (Bouchard et al., 2003; Ferry et al., 2003; Cowgill et al., 2004). More work has to be done for clarifying the importance of direct and indirect effects of transgenic plants in multitrophic interactions.
Acknowledgements

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References


Bt formulated products: should there be more concern about resistance development with the introduction of Bt transgenic plants?

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Abstract: Because both Bt transgenic plants and Bt formulated products contain crystal (Cry) proteins, and the introduction of Bt transgenic plants has increased fears regarding Bt resistance development, concerns have been raised that the utility of using Bt formulated products is now more at risk. Here I will try to present various reasons as to why this concern may be premature. Several points to consider: (i) Resistance to Bt transgenic plants has yet to occur in any part of the world after up to 9 years of extensive use. The only cases of documented Bt resistance in the field occurred with Bt formulations, but only under intense selection pressure either in the subtropics-tropics, or in enclosed areas such as grain silos or glasshouses. (ii) Bt plants such as maize and cotton, are targeting insect pests not usually treated with Bt formulated products. (iii) The Cry proteins expressed in transgenic plants are not in the same form as those found in Bt formulated products. (iv) All Bt Cry proteins are not the same, and some Bt Cry proteins can overcome resistance to other Bt Cry proteins such as in the case of controlling diamondback moth, *Plutella xylostella* in *Brassica* crops. (v) Essentially all Bt formulated materials may contain various other insecticidal components associated with the Bt strain such as spore, zwittermycin, VIP proteins, chitinases, phospholipases, etc. Some of these compounds have even been shown to synergize the insecticidal activity of some Cry proteins. An example can be made using the beet armyworm, *Spodoptera exigua*, that was selected for resistance to a single Cry protein, similar to what would be found in a Bt transgenic plant. This highly resistant population (>100 fold) was susceptible to the Bt Cry protein + spore, and was only 5-fold more tolerant to a Bt formulated product (compared to a susceptible population) that contained the protein that *S. exigua* was selected against. However, it is important to keep in mind that every insect species may react differently to these various Bt proteins and compounds, and therefore, resistance concerns should be treated on a species by species basis.

Key words: GMO’s, transgenic crops, insecticide resistance

Introduction

Bt formulated products have been successfully used for over 40 years primarily to control Lepidoptera and Coleoptera in vegetable and fruit crops, and mosquitoes and blackflies that vector human pathogens. Bt formulated products are extremely safe to non-target organisms including humans with few exceptions. As a result, Bt formulated products are certified for use in most organic production systems. One concern of widespread use of insecticides, including Bt formulated products is the development of insecticide resistance. To date, the only documentation of Bt resistance in the field (including grain silos and glasshouses) is with the overuse of Bt formulations to primarily control diamondback moth, *Plutella xylostella*, Indian meal moth, *Plodia interpunctella*, and cabbage looper, *Trichoplusia ni* (Jannaat & Myers, 2003; Tabashnik, 1994). However, with the recent introduction of Bt transgenic crops that express relatively high levels of Bt protein in most tissues essentially season long the concern for Bt resistance development has increased. One question that arises from these
resistance concerns is: What will be the impact of insects developing resistance to Bt transgenic crops have on the utility of using Bt formulated products?

To help understand the potential for insects developing resistance to Bt transgenic crops having a negative impact on the of use of Bt formulated products, we first must understand the conditions that promote resistance. From the insecticide aspect, the primary issue is selection pressure, i.e. exposure of the insect population to a particular mode of action. Selection pressure is primarily attributed to three different means: 1) Increased rate (concentration) of insecticide, 2) Increased number of applications of one mode of action, 3) Increased persistence (residual) of the insecticide. So for Bt formulations, the major selection pressure could come from increased rate of the Bt formulation and the increased number of applications. For Bt crops, the main concern would be persistence, followed by rate the concentration expressed in the plant and also the continuing production of the Bt protein. There are also factors associated with the insect population itself that can have an impact on resistance development. Some of those factors would be: 1) Reproductive potential of the insect, 2) Number of generations per year, 3) Number of alternative hosts, 4) Propensity of insect species to develop resistance. Perhaps the best way to determine whether a particular situation is amenable for Bt resistance development is to look at the specific attributes of the cropping and pest system in question. Specific attributes to evaluate might include: 1) The specific pest(s) targeted for control with a specific Bt formulation, 2) The specific Bt crop expressing a specific Bt Cry protein(s), 3) The pest(s) targeted for control with the specific Bt crop. The adoption of Bt crops in the United States has been quite substantial since the introduction of Bt cotton in 1996 and Bt corn in 1997. Even though adoption of these two crops currently represent at least 20-30% of the overall acreage of cotton and corn grown in the United States, there still is no detectable Bt resistance in any target pest of Bt crops, and furthermore, no detectable Bt resistance change in target pests of Bt formulations.

With the increase in the use of Bt in the field since the introduction of Bt crops, what could be some of the primary reasons as to why there is no detectable change in resistance to Bt formulations and why the use of Bt formulations would most likely pose little increased risk to resistance development on insects targeted for control by Bt crops? Some of the possible reasons might be: 1) Bt formulations do not target same pests as targeted by Bt crops, 2) Current target pests of Bt crops feed internally, therefore Bt formulations ineffective, 3) Bt formulations not as efficacious as Bt crops, 4) Bt formulations have limited field persistence.

As an example, if we look at the current European scenario with the use of Bt maize for controlling primarily *Ostrinia nubilalis*, *Sesamia* spp., *Helicoverpa armigera*, the author is unaware of any Bt formulations used for controlling these pests in any crops. (Note that this would not be surprising as all three of these pests are primarily internal feeders.). Therefore, there currently should be little concern about Bt crops affecting the use of Bt formulations to control the above pest species.

Besides the pest and crop aspect, there are various Bt Cry proteins and other insecticidal compounds that can be found in either Bt formulations or Bt crops. These various insecticidal compounds can affect different insect species in different ways, and some can still be active against insects that have developed resistance to a particular Bt formulation or compound. In other words, all Bt formulations are not alike, all Bt’s in Bt crops are not alike, and different insect species respond differently to Bt compounds. Several examples are: A population of diamondback moth, *Plutella xylostella*, was resistant to Dipel and was also resistant to Cry1Ab and Cry1Ac (found in Dipel), but not Cry2Aa (found in Dipel but at low levels) and not to Cry1C (found in other Bt formulations such as Xentari) and not to the Bt formulation Xentari (Tabashnik, 1994). Although the beet armyworm *Spodoptera exigua* was selected for
high levels (>300-fold) of resistance to Cry1C (Moar et al., 1995) this same population was only 2.9 fold resistant to the Bt formulation Xentari (Table 1). 

Table 1. Toxicity of susceptible and Cry1C-resistant Spodoptera exigua to Xentari.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Susceptiblea LC50 (95%FL)</th>
<th>Susceptible slope (SE)</th>
<th>Resistantb LC50 (95%FL)</th>
<th>Resistant slope (SE)</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1C</td>
<td>0.97 (0.7-1.19)</td>
<td>2.1 (0.2)</td>
<td>61% mortality at 320 g/g</td>
<td>N/A</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Xentarid</td>
<td>17.2 (14.8-19.8)</td>
<td>2.0 (0.1)</td>
<td>49.2 (30.8-88.9)</td>
<td>1.9 (0.3)</td>
<td>2.9</td>
</tr>
</tbody>
</table>

a Mortality in µg/g diet  
b After 34 generations of selection using Cry1C toxin  
c Resistance ratio  
d Primary Spodoptera exigua-active protein is Cry1C

Although the current discussion surrounding Bt cross resistance centers on the expression of different Bt Cry proteins and other insecticidal compounds (e.g. Vip 3A), this same phenomena has been occurring with Bt formulations since their initial use. Specifically, most Bt strains found in Bt formulations contain: 1) Various Cry proteins with potentially different modes of action or binding (Cry1Ac, Cry2Ab, Cry1C), 2) Spores that have been documented to synergize Cry proteins against many insect pests including Spodoptera spp. and overcome Bt resistance (Moar et al., 1995), 3) Chitinases, 4) Phospholipases, 5) VIP’s (vegetative insecticidal proteins), 6) Zwittermycin, and undoubtedly other as yet unknown compounds.

So in conclusion, Bt formulations contain numerous insecticidal components, most with unique modes of action and/or binding characteristics. Perhaps the best example of a Bt formulation widely used with no detectable field resistance is with the use of Bti against mosquitoes and blackflies. There is still no resistance to Bti for mosquitoes and blackflies even though there are lab-selected strains for each Bti insecticidal component; the Cry and Cyt proteins work in synergy (Wirth et al., 1997). Therefore, resistance development should proceed much slower for Bt formulations than for Bt crops. However, the primary reason(s) why we already have documented field cases of Bt formulation resistance is essentially the same reasons when using traditional chemical insecticides; overuse. Because Bt crops only contain 1-2 insecticidal compounds, even if resistance did occur, this does not necessarily imply that the resistant insect would be resistant also to a particular Bt formulation as per the reasons discussed above.

The current and future state of Bt crop adoption in the US looks positive. This is partly due to the fact that the technology used to develop Bt crops is still speeding ahead. In fact, within the next 2-3 years, there will be at least 3 new cotton cultivars, each “pyramided” with Cry1Ac and another Bt compound. This strategy will most likely hold true also for Bt maize. Additionally, there are various other insecticidal genes (e.g. non-Bt) on the horizon for expression in Bt crops.

The potential for Bt resistance development in the US in the near term most likely will be dependant on the rate of adoption of the new pyramided Bt crops; if Bt resistance does not
occur before the “pyramided” Bt crops are widely adopted, there will most likely be negligible potential for widespread Bt resistance to occur in the near future. Therefore, the threat for Bt resistant insects to move over to non-Bt crops that are treated with Bt formulated products and be cross resistant will be extremely low. In fact, if there was a concern for Bt resistance to be transferred between insects feeding on either Bt crops or on crops treated with Bt formulations, the current scenario would most likely be for Bt formulation-resistant insects moving over to Bt transgenic crops (the Bt crops would only contain Cry proteins found in Bt formulations).

In conclusion, there is no data available to suggest that there is an inherent threat to the use of Bt formulations to control the targeted pest(s) by the use of Bt crops, partly because there are no reports of an insect species resistant to a single Bt Cry toxin (e.g. Bt maize) being cross resistant to a Bt strain formulation (Akhurst et al., 2003). However, each insect species is different, and each Bt formulation contains various insecticidal components at different levels. Therefore, this concern will ultimately need to be addressed on a case by case basis.

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Spiders in Bt and non-Bt potato fields in Bulgaria

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Abstract: The present study was conducted to investigate the influence of potatoes genetically transformed to express an insecticidal toxin derived from Bacillus thuringiensis on the spider coenosis under conventional farming conditions in western Bulgaria. Samples from pitfall traps showed that Bt-potatoes [Superior Newleaf® expressing Cry3A protein against Colorado potato beetle (CPB) Leptinotarsa decemlineata Say] preserved spiders in comparison to conventional potatoes sprayed with chemical insecticides twice in the season. Several species seemed to be sensitive to insecticides, but not to Bt plants.

We collected 3056 individuals of 57 species of spiders in the season 2000. There were 42 species of spiders collected in the Bt field, while only 26 species were found in the conventional field (cultivar Santana®) sprayed with a pyrethroid insecticide. Abundance of spiders in the conventional field was low already in the beginning of season, medium during summer, and low in autumn. In the Bt field, there was medium initial abundance, which temporally decreased, then peaked in late summer and dropped in autumn. In 2001, we collected 2478 individuals of 62 species of spiders; 41 species in the Bt field, and 39 species in the conventional field (cultivar Arinda®) sprayed with fipronil. The abundance of spiders in both fields increased more or less over the entire season.

The canonical correspondence analysis revealed that the date of sampling (seasonality) was the only statistically significant (p=0.001) variable, explaining 15–19 % of species data variability, while field type (Bt) and insecticide spraying were weak predictors of the spider community composition. The most common species was Pardosa agrestis representing 53–79 % of all individuals, showing no preference for the field type.

Key words: Colorado Potato Beetle; Bacillus thuringiensis; natural enemies; GMO; Aranea;

Introduction

Genetically modified plants have come under attack for potentially posing a threat to non-target organisms (Poppy, 2000). When assessing side effects of this new technology, it is important to remember that any human intervention to protect crops from pests will have some negative impact on those arthropods that depend on pests as prey. So any negative impact of genetically modified plants on populations of non-target insects has to be considered in comparison to other pest control measures (Schuler, 2000).

The major arthropod pest of potatoes in Bulgaria includes the Colorado potato beetle (CPB), Leptinotarsa decemlineata (Say). In some regions, in addition to CPB, there are other groups of pests such as aphids and click beetles (Elateridae). Bt-transformed potato plants express a Bt-toxin (δ-endotoxin or Cry3Aa protein specific against beetles originating from the entomopathogenic bacterium Bacillus thuringiensis Berliner), throughout the plant.

In this article, we report the results of a field study conducted near Sofia, Bulgaria, to evaluate the efficacy and potential non-target impacts of a transgenic Bt potato cultivar and
insecticides against spiders (Aranea), mostly epigeic, caught in pitfall traps. Parallel results dealing with effect on predatory ladybirds (Coleoptera: Coccinellidae) and ground beetles (Coleoptera: Carabidae) are presented elsewhere (Kalushkov & Nedvéd, 2005; Spitzer et al., unpublished). In those studies, seasonality (date of sampling) was the strongest predictor of community composition. Insecticide spraying had usually a strong short-lasting effect on beetle abundance, while the presence of Bt-toxin had a marginal impact on both abundance and species diversity.

Duan et al. (2004) studied ground dwelling arthropods in Bt and non-Bt potato fields, treated with diverse insecticides (sprays of a microbial Bt-based formulation, permethrin, and systemic insecticides) or untreated, in Oregon for two years. The abundance and species composition of diverse arthropods did not differ significantly among treatments except for the permethrin treated plots with decreased abundance of Aranea and increased numbers of springtails.

The effect of transgenic potatoes resistant to CPB on the abundance of generalist predators was compared to nontransgenic fields in the USA during the entire season by Riddick et al. (2000). Low doses of insecticides were used even in the transgenic fields to suppress nontarget pests. Heteropteran predators and ladybirds were sampled by sweeping, ground-foraging carnivorous carabids, ants, and spiders were sampled by pitfall traps. None of the coccinellids, carnivorous carabids and ants were affected, but spiders were more abundant in the transgenic fields.

Spiders constitute an important element of invertebrates in agro-ecosystems as generalist predators (Nyffeler & Sunderland, 2003). Since Bt potatoes expresses the Bt toxin continuously during the season, spiders are potentially exposed to this toxin by feeding on Bt toxin contaminated prey. The present study was conducted to investigate the influence of Bt potatoes and of insecticide sprays in the conventional cultivar on the epigeic spider coenosis under conventional farming conditions in Bulgaria.

Materials and methods

Experimental plots
The investigated fields were situated in western Bulgaria: near Samokov at 900 m a.s.l. in 2000 and near Ihtiman at 600 m a.s.l. in 2001. The sampling design was limited by the real farming situation, as the investigated fields were production fields, not scientific experimental plots.

In 2000, transgenic potatoes containing Bt-toxin (Superior Newleaf®) were planted in a 1.6 ha monoculture. One hundred meters from this field separated by bare land was a conventional field with a standard cultivar (Santana®, 4 ha), which was sprayed twice in the season (8th and 26th July) with the pyrethroid alfa-cypermethrin (Vaztac® - 10 EC, 100 ml/ha). Both fields were almost free of weeds. In 2001, experiments were carried out in two similar plots – 1.5 ha Bt potatoes Superior Newleaf® on one side and 1.5 ha conventional cultivar (Arinda®) on the other side of a 5 m wide road. Around both plots were other fields with standard cultivars (Santana®, Arinda®). The conventional field and surrounding standard cultivars were sprayed twice in the season (23th June and 9th July) by fipronil (Regent® - 800 WG, 0.02 kg/ha). These fields were strongly infested by weeds.

Sampling
Spiders were sampled in ten pairs of pitfall traps (Greenslade, 1964) in each field, six times in the season 2000: June 15 and 28, July 11 and 21, August 10 and 28. Each pitfall trap pair consisted of two 0.5 l plastic vessels of bright white colour containing a solution of
ethyleneglycol : water (1:1) (Work, 2002). The trap pairs were 15 m apart in three rows, each also 15 m apart, situated in the center of each field. In 2001, samples were taken eight times (May 15, June 1st, 16th, 29th, July 14th and 28th and August 9th and 23rd). Pairs of traps were 15 m apart in a single row in the center of each field, and formaldehyde : water (1:8) (Pekar, 2002) was used as the preserving solution.

**Data processing**

All collected spiders were indentified to species or genus level. Numbers of males, females, and juveniles were pooled. The samples were analysed by canonic correspondence analysis using Canoco 4.5 (Ter Braak & Šmilauer, 1998). Three environmental variables were introduced to the analyses: the type of field / cultivar (Bt), the insecticide residue (spray) and seasonality (date). The field type variable (Bt) was coded either 1 (yes – Bt) or 0 (no – conventional). The amount of insecticide decreasing with time from the last spray application (variable spray) was calculated as the reciprocal value of the number of days from the spray. Variable date was simply the number of days from the day when the first sample was taken + 1. These environmental variables were analysed as a stay-alone factor or together with the others as covariables. We also considered a possible interaction of the variables. Samples from individual pairs of pitfall traps were pooled for each date and field type for the analysis or used as pseudoreplications in the monte Carlo permutation test.

**Results**

**Species abundance and dominance**

In the season 2000, there were 42 species of spiders collected in the Bt field and only 26 species in the conventional field (Santana®) sprayed with the pyrethroid. Altogether, we collected 3056 individuals of 57 species of spiders. The most common species was the wolf spider *Pardosa agrestis*, representing 79 % of all individuals and showing no preference for the field type. Abundance of spiders in the conventional field was low already in the beginning of season, medium during summer, and low in autumn. In the Bt-field, there was medium initial abundance, which temporally decreased, then peaked in late summer and dropped in autumn. *Pardosa pratensis* and *P. palustris* were typical species in the Bt-field, while *P. roscai*, and the linyphiids *Erigone dentipalpis* and *Oedothorax apicatus* were typical in the conventional field.

In 2001, we collected 41 species of spiders in the Bt field, and 39 species in the conventional field (Arinda®) sprayed with fipronil. The total annual catch was 2478 individuals of 62 species of spiders. *P. agrestis* represented 53 % of all individuals, the second dominant linyphiid *Meioneta rurestris* 19%. The abundance of spiders in both fields more or less increased over season. The linyphiid spider *Trichoncoides piscator* was more abundant in the Bt field, while the crab spider *Xysticus kochi* was typical in the conventional field.

The pooled or mean abundance of spiders did not differ between Bt and conventional fields in both years (paired t-test based on samples: 2000: p=0.48, 2001: p=0.35). The insecticidal treatment in standard cultivars had no direct effect on spider abundance [Figures 1 and 2; paired t-test based on individual traps in subsequent samples: 2000: p=0.58, 0.48; 2001: 0.14 (increase), 0.16 (decrease)].
Figures 1 and 2. Seasonal changes in abundance of spiders (all species pooled) in the potato fields in 2000 (Fig. 1 –left), and 2001 (Fig. 2 – right). Squares and dashed line - Bt, circles and solid line - conventional. Arrows show the spraying dates (alfa-cypermethrin used in 2000, fipronil in 2001). Sampling started on June 15, 2000 and May 15, 2001.

**Community structure**

Canonical correspondence analysis was run for the evaluation of the role of three environmental variables for species composition in 2000 and 2001. There was no significant difference between Bt and conventional plots (p=0.24 and 0.22). Variable spray, evaluated as the reciprocal time from the date of spray to the date of sampling had no effect (p=0.41 and 0.50). Seasonality had a significant impact on different species abundances in each year (p=0.001) (Figures 3 and 4).
Figure 4. Canonical correspondence analysis of the species composition of spiders in Bt and non-Bt (control) potato fields in the seasons 2000 (Fig. 3) and 2001 (Fig. 4), and the environmental variables: dny=date—the seasonal component; spray—the influence of insecticides on species composition; Bt and C—the global effect of plant cultivar (transgenic or control) indicated by black triangles. Individual samples indicated by numbers Bt1-8 and C1-8. Position of selected species are also plotted – Arhu: Araneoncus humilis, Aulalb: Aulonia albimana, Biaaur: Bianor aurocinctus, Drla: Drassodes lapidosus, Erde, Eriden: Erigone dentipalpis, Hada: Haplodrassus dalmatensis, Hapaen: Haplodrassus aeneus, Hasi: Haplodrassus signifer, Hora: Hogna radiata, Meirur: Meioneta rurestris, Mifia: Micaria fulgens, Oeap, Oedapi: Oedothorax apicatus, Oedret: Oedothorax retusus, OxySP: Oxyptilla sp., Pabi, Parbif: Pardosa bifasciata, PacSP.: Pachygnatha sp., Paho: Pardosa hortensis, Parpal: Pardosa palustris, Parpr: Pardosa pratensis, Pavi: Pardosa vittata, Phfe: Phrurolithus festivus, Prva: Prinerigone vagans, Sidi: Sitticus distinguendus, Stealb: Steatoda albomaculata, Trpi: Trichoncoides piscator, Waca: Walckenaeria capito, Walwig: Walckenaeria vigilax, Xyko, Xyskoc: Xysticus kochi, Zehes: Zelotes hermani, ZelSP: Zelotes sp.

Discussion

Spiders represented in our pitfall traps were mainly adult hunters, and the families Lycosidae, Linyphiidae and Thomisidae. For comparison, the foliage-dwelling spider fauna collected in maize fields and adjacent nettles in South Germany by drop cloth sampling and suction sampling was dominated by juvenile spiders, web-building spiders, and spiders of the families Theridiidae, Linyphiidae, Tetragnathidae and Araneidae (Ludy & Lang, 2004).

There was a relatively high number of species in both the Bt-transgenic and the non-transgenic potato fields. Despite the number of species, the fields were dominated by just a few of them. A similar species dominance structure was found for ground beetles (Carabidae; Spitzer et al., unpublished) in the same plots in Bulgaria. Our catches of 3056
individuals of 57 species and 2478 individuals of 62 species have a higher species richness than the above mentioned foliage-dwelling spider fauna (647 individuals of 40 species; Ludy & Lang, 2004), and less equitability. The abundance peaked similarly to our study mid August. A total catch of 2357 foliage-dwelling spiders was received in another study in maize fields (Meissle & Lang, 2005), but only 29 species were identified to specific level. However, juvenile spiders, which are difficult to identify, dominated the catch. The abundance peak was shifted to September.

Bt cultivar or weekly sprays of a microbial Bt-based formulation did not significantly impact the beneficial predators in potato in Oregon (Reed et al., 2001). However, bi-weekly applications of permethrin significantly reduced the abundance of several major generalist predators. In the case of our potato fields, the differences in species composition were likely due to natural variations in seasonal dynamics.

Populations of predators including spiders were consistently as high or higher in 11 transgenic cotton cultivars compared with non-Bt cotton (Hagerty et al., 2005). Duan et al., (2004) evaluated nontarget impacts of transgenic Bt potato (Newleaf®) and conventional insecticides on ground-dwelling spiders (Aranea). Weekly sprays of permethrin significantly reduced the trap capture of spiders, but there were no significant differences in the capture of spiders between Bt and non-Bt-potato fields treated with Bt sprays, systemic insecticides, or no insecticides.

In a field study in South Germany, plant-dwelling spider assemblages were recorded in Bt maize fields and conventional maize fields, with and without pyrethroid application. Bt maize had no substantial effects on species richness and abundance of spiders, whereas insecticide application reduced spider densities (Meissle & Lang, 2005).

Only repeated and large-scale insecticide treatments destroy the populations of both prey and predators to such an extent that a restoration of the equilibrium situation is slow, if it appears at all (Hilbeck, 1996). The repeated spraying in our plots in 2000 seemed to have destroyed the carabid community so much that it did not stabilize until the end of the season (Spitzer et al., unpublished). On the other hand, the effect of spraying in 2001 did not persist and the community of carabid beetles of various food specializations was restored. In contrast to the carabids, a recovery of coccinellid populations attributable to the flying adults was observed in the sprayed fields in both 2000 and 2001 (Kalushkov & Nedvěd, 2005). Spiders were almost not affected by the spraying. In the study of foliage-dwelling spider fauna (Ludy and Lang, 2004), more species occurred in nettle margin strips than in maize fields. We avoided the margin effect in our study by placing the traps only in center of the plots.

An effect of either spraying or Bt plants undetectable with available methods may cummulate over several insect generations. French et al. (2004) performed a canonical correspondence analysis of their data pooled over three consecutive years. The effect of individual years on the ground beetle assemblage was very strong, determining the first two canonical axes. The crop type – either corn or soybean – was less important. However, a study of possible long-term effect could not be performed in our study in the region of western Bulgaria where transgenic Bt potatoes were planted in a different location every year.

The cultivars used (Newleaf Superior®, and either Santana® or Arinda®) are similar but not isogenic. If any differences in insect community had occurred, they could be attributed not only to the Bt-toxin but also to other possible differences between the plants. Only one plot with Bt and one plot with classical cultivar were observed, as they were real production plantations and not a scientifically designed experiment. No real replications were possible and individual traps were used as pseudoreplications. The two plots each year might differ also in other parameters than the cultivar planted, and such a difference might occur in the ordination analysis. However, no such differences were found. Although Bt transgenic crops
have been reported frequently to have no adverse effects on predatory arthropods in field experiments, our study confirms such conclusion in a farm-situation agricultural system in Europe.

Acknowledgements

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Do environmental impacts differ for Bt, Ht and conventional corn with respect to pesticide use in Europe? An empirical assessment using the Environmental Impact Quotient

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Abstract: In this paper we assess and compare the environmental impact of pesticide programs for Bt, Ht (glufosinate) and conventional corn using the Environmental Impact Quotient. Results show that Bt management will always have a lower environmental impact than any of the conventional insecticide programs. Results are less straightforward for the different, country-specific herbicide programs. Conventional herbicide treatment in Germany has a much lower environmental impact than that of the Ht variety, which is in contrast with any of the other herbicide programs that have been considered. Using three other assessment tools including the oral LD50 dose for rats, million acre treatment method, and volume active ingredient applied generates the same results.

Key words: EIQ, pesticide use, Bt, Ht corn

Introduction

Growing transgenic crops can provide important social and private benefits. Some of this social and private benefits are irreversible in nature. Examples of irreversible private benefits includes an increase in farmer’s health due to a change in pesticide use. Irreversible social benefits may include a reduced impact on the environment. In this paper we assess and compare the environmental impact of pesticide use on both non-transgenic and transgenic crops to investigate whether these social benefits indeed may be gained. The outcomes are used to be included in an economic decision making framework (see Demont et al., 2005; Scatasta et al., 2005).

There are many tools specifically designed to assess the environmental impact of pesticide use. Levitan (1997) provides an excellent overview and discussion of these methods and their applicability. In this paper we confine ourselves to those methods that have been applied empirically to compare pesticide use for transgenic and non-transgenic crops. The main focus will be on one such methods the so-called Environmental Impact Quotient (EIQ) developed by Kovach et al. (1992), which will be applied to data for corn. The robustness of our results is tested by comparing them with the outcomes of three other assessment tools (rat oral LD50 dose, million acre treatment, and volume active ingredient applied).

The remaining of the paper is set out as follows: section 2 provides an introduction to the environmental impact quotient; section 3 discusses the transgenic corn varieties we use in our application; section 4 describes the data and presents the results, and section 5 concludes.
The Environmental Impact Quotient (EIQ)

The EIQ was initially designed by IPM specialists to help farmers in their choice for pest-control options. The underlying premise of the EIQ is that environmental and health impacts result from the interaction of toxicity and exposure. The EIQ incorporates the impacts of active ingredients of formulated products on farm workers, (application and harvest worker) consumers, and ecology (non-target organisms: fish, birds, honeybees, and other beneficial insects) (Kovach et al., 1992). Separate impacts are calculated based on inherent properties of certain pesticides, for example toxicity towards certain organisms and exposure of these organisms to these pesticides. The inherent properties are assigned ratings that range from 1-3, or 1-5 where 1 denotes the lowest toxicity or harmfulness, and 3 or 5 the highest, based on predefined boundary values (Kleter & Kuiper, 2005). Summing the separate impacts results in a single number, the EIQ for one specific active ingredient. Annex 1 provides the mathematical presentation of the EIQ. For those pesticides that contain multiple active ingredients, the EIQ’s are summed. However, the EIQ alone does not provide any information about the dosage and application rate yet. Therefore the EIQ is multiplied by the active ingredient, the rate per hectare used and the number of applications, resulting in the field rate EIQ.

Before continuing on the EIQ though we should also realize that there are, as with any other methods, some shortcomings of the EIQ that should be kept in mind. We will mainly replicate concerns reported earlier by Dushoff et al. (1994) and Levitan et al. (1995) which are discussed below. An important limitation is that there are only three scores that can be assigned, either, 1, 3 or 5, which limits the range of scores. For example, a pesticide that is 1000 times more toxic than another will receive a toxicity rating that is at most only five times as high. Related to this problem is the rating score of 1 instead of 0 for neutral effects, thereby decreasing the ‘distance’ between relatively benign substances and extremely hazardous ones. In addition, the relative importance of factors depends largely on what other factors they are multiplied by. For example, in the farmworker component, the value for chronic toxicity is always multiplied by the value of dermal toxicity. Thus, a substance with known long-term health effects on humans, but which showed no acute dermal effects (i.e, dermal toxicity is 0) would not be considered a risk to farmworkers. Another problem is the weighing of persistence versus toxicity. The EIQ measures toxicity by exposure with the possible result that a non-toxic but persistent pesticide, may receive a higher EIQ than a more toxic but less persistent pesticide. Furthermore, a single number ignores the fact that the environmental effect of a pesticide depends on the conditions on which it is used (including soil type, hydrology, local ecology and type of crop) which are not taken into account when using the EIQ. By combining various components with different weights implicit value judgements are made that would require a more explicit examination. Also, the effect of pesticides may accumulate over time, but dynamic effects are not taken into account. Lastly, the use of a single number hides information gaps and gives an illusion of firm knowledge.

All in all, there are some weakness that should be kept in mind when using the EIQ. However, provided that results are indeed treated with some caution we believe the EIQ can still be of use (missing explanation: other methods only consider toxicity but not exposure or make no difference at all among active ingredients). Besides, EIQ values have been generated for a large number of pesticide active ingredients by the New York State Integrated Pest Management Program (NYSIPM), which makes it a ready-to use and easy applicable method. In the next section we will describe the corn varieties to which the EIQ will be applied in our empirical setting.
Transgenic corn varieties

The environmental impact assessment of pesticide use is applied, in this study, to non-transgenic and transgenic corn. The transgenic varieties include Bt and Ht corn, which are described below.

Bt corn
Insect resistant plants produced through biotechnology express traits derived from the Bacillus thuringiensis (Bt), a species of soil-borne bacteria. When these spores are ingested by an insect the protein crystal gets dissolved, thereby releasing protoxins, which are in turn activated by specific enzymes. When a susceptible insect tries to feed on a transgenic crop expressing the Bt protein, it stops feeding and will die of a result of the binding of the Bt toxin to its gut wall (Gianessi et al., 2002). There are several varieties of Bt, each with a specific insecticidal activity against certain groups of insects, the specificity depending on the characteristics of the Bt toxin itself. In 1981, a Bt gene was cloned and successfully transferred to and expressed into another organism. Bt corn and potato plants were developed soon thereafter. The Bt technology in corn is particularly used to control the European Corn Borer (ECB) a moth larva, which is considered to be the most damaging pest to corn.\(^1\)\(^,\)\(^2\)

Ht corn
Transgenic crops have been developed to express tolerance to one of three post emergence herbicides (glufosinate, glyphosate and bromoxynil) to control a wide range of weeds with a minimal damage to crops. Because of genetic transformations these herbicides can be sprayed on transgenic crops without damage, while nearby weeds are being killed. The herbicides are toxic to untransformed conventional crop cultivars. In our application we focus on crops that are glufosinate-tolerant. Glufosinate is a broad-spectrum herbicide that inhibits glutamine synthetase, a plant enzyme essential to the processing of accumulated ammonia into a form of nitrogen usable by plants. Interference with the activity glutamine synthetase leads to toxic cellular accumulation of ammonia. The inhibition of glutamine synthetase also indirectly inhibits carbon fixation with cascading destructive effects that quickly kill the plant (Gianessi et al., 2002). Glufosinate is a modified, synthetic version of a naturally occurring compound, bialaphos, which is produced by the soil bacterium Streptomyces. To avoid being poisoned by their own bialaphos production, Streptomyces species also produce an enzyme that detoxifies bialaphos and glufosinate. The responsible gene has been isolated and used to produce glufosinate–tolerant crops (Gianessi et al., 2002).

In the next section we will turn to the empirical part of the study.

Data analysis

Both primary and secondary data have been used. To assess the differences in environmental impact of Bt and conventional corn we used field trial data from Narbonne, France. For Ht corn we had no field trial data available. Therefore, we used data from an earlier empirical application of Gianessi et al. (2003) who estimated the economic impacts of glufosinate corn for several countries in Europe. Additional information on the German herbicide program was

\(^1\) Reported damages due to ECB attacks include: interference with nutrient flows in the host plant; entranced infection by stalk diseases and stalk dbrakage and ear drop prior to harvest which may all result in considerable yield losses (Brookes, 2002).
\(^2\) Alternatives to the use of GM technology to deal with ECB are treatment with insecticides or, as in 80% of all cases, no treatment at all (Benbrook, 2003; Brookes, 2002).
being provided by K. Hurle (University of Hohenheim, Germany; pers. comm. 2005). This section starts by analysing the results for Bt corn management.

Table 1. Field Rate EIQ for Bt and conventional corn production.

<table>
<thead>
<tr>
<th>Management Program</th>
<th>Treatment</th>
<th>Active Ingredient (g/l)</th>
<th>Dosage (l/ha)</th>
<th>Application rate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bt crop management of Bt-variety A-Bt</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetochlor</td>
<td>Herbicide</td>
<td>400</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Dichlorphim</td>
<td>Herbicide</td>
<td>66</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Isoxaflutole</td>
<td>Herbicide</td>
<td>75</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Aclonifen</td>
<td>Herbicide</td>
<td>500</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total Environmental Impact</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Iso non Bt – crop Management of Bt</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Acetochlor</td>
<td>Herbicide</td>
<td>400</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Dichlorphim</td>
<td>Herbicide</td>
<td>66</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Isoxaflutole</td>
<td>Herbicide</td>
<td>75</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Aclonifen</td>
<td>Herbicide</td>
<td>500</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>Insecticide (against wireworms, seed corn and other pests)</td>
<td>n.a.</td>
<td>78.4</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total environmental impact</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Iso non-Bt crop management-variety A</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetochlor</td>
<td>Herbicide</td>
<td>400</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Dichlorphim</td>
<td>Herbicide</td>
<td>66</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Isoxaflutole</td>
<td>Herbicide</td>
<td>75</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Aclonifen</td>
<td>Herbicide</td>
<td>500</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>Insecticide (against wireworms, seeds corn maggots and other pests)</td>
<td>n.a.</td>
<td>78.4</td>
<td>1</td>
</tr>
<tr>
<td>Lambda-cyhalothrine</td>
<td>Insecticide against ECB</td>
<td>100</td>
<td>0.15</td>
<td>1</td>
</tr>
<tr>
<td>Deltamethrine</td>
<td>Insecticide against ECB</td>
<td>15</td>
<td>1.33</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total environmental impact</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Other non Bt variety crop management</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Acetochlor</td>
<td>Herbicide</td>
<td>400</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Dichlorphim</td>
<td>Herbicide</td>
<td>66</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Isoxaflutole</td>
<td>Herbicide</td>
<td>75</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>Aclonifen</td>
<td>Herbicide</td>
<td>500</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
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<td>Insecticide (against wireworms, seed corn maggots and other pests)</td>
<td>n.a.</td>
<td>78.4</td>
<td>1</td>
</tr>
<tr>
<td>Lambda-cyhalothrine</td>
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<td>0.15</td>
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</tr>
<tr>
<td>Deltamethrine</td>
<td>Insecticide (against ECB)</td>
<td>15</td>
<td>1.33</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total environmental impact</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*n.a. is not applicable*
The field rate EIQ’s of the crop management program used in the French field trials for Bt and conventional corn are presented in Table 1. The results show that the Bt management program has the lowest environmental impact. This result is consistent with the fact that Bt reduces the usage of ECB specific insecticides to zero. If farmers do not protect against the ECB with conventional methods, non-Bt and Bt crop management have the same EIQ.

Next we will see whether these results are replicated when using alternative assessment tools. We successively consider the rat oral LD50 method, the million acre treatment and the volume active ingredient.

The rat oral LD50 method is used by Nelson & Bullock (2003), to quantitatively assess environmental impacts of GM soybeans compared to conventional soybeans. The oral LD 50 dose for rats is an acute mammalian toxicity measure, taking into account the milligrams of formulated ingredient per kilogram of bodyweight that kills 50% of the rat population (Nelson & Bullock, 2003). For our Bt varieties this is however an inappropriate indicator as Bt is known to be harmless to mammals, but might be toxic to butterflies, certain beetles, flies, freshwater fish and invertebrate animals (U.S. Department of Agriculture, 2005).

The million acre treatment method is applied by Fernandez-Cornejo & McBride (2002) and Carpenter & Gianessi (2001) to assess environmental impacts of reduced pesticide use. Pesticide impacts are calculated by multiplying the number of active ingredients applied per acre with the number of repeated applications. This is then finally multiplied by the total number of acres per region or country to get an overall estimate. In our experimental field trial data there is only one small area (plot) to which this specific management strategy is being applied. Hence we cannot multiply by the total number of acres, so comparisons can only be made on a per acre basis.

Volumes of active ingredient are used by Benbrook (2003) to assess environmental impacts of genetically engineered crops in the United States. As Bt substitutes for the use of any insecticide against the ECB applying this method, like the EIQ, always favours the Bt technology over conventional practices.

In summary, because all the methods described above consider only pesticide use, assessing and comparing environmental impacts of Bt and non-Bt crop management always leads to obvious results.

Next we assess and compare specific herbicide programs.

The Field Rate EIQ’s for Ht and conventional corn management programs are presented in Table 2. In addition to the herbicides that were used for the field trial program, we used information with respect to Ht corn from Gianessi et al. (2003), and compared some well-known conventional herbicide programs for the EU, Germany, France, and Italy respectively.

Comparing the results for Ht corn and several conventional herbicide programs we see a surprising result. In nearly all cases Ht corn would have a lower environmental impact than the conventional programs, except for the German program (6.45 vs 25.43). Thus, when applying the specific German tank mix of Nicosulfuron, Flufenacet and Metsulam, the environmental impact of the conventional herbicide program is lower than that of Ht corn. In other words conventional corn would in this case be preferred to the transgenic variety. By contrast, the conventional programs in France, Italy and the typical EU program do worse than the Ht variety, and here growing Ht would thus be the preferable choice. One point is worth noting though. For the French as well as for the Italian program only the aggregate amount applied was known, and not the amount per active ingredient. We thus divided the total load by the number of active ingredients to get the amount per active ingredient. Figures for these programs may thus have been over-or understated. However, this measurement error
is expected to be small as there is not much variance in the EIQ values across active ingredients applied in those programs, with the exception of Dimethanamid.

Next we apply the LD50 method, the million acre treatment and the volume active ingredient methods successively. Results are presented in Table 3.

Table 2. Field Rate EIQs for different herbicide programs.

<table>
<thead>
<tr>
<th>Management Program</th>
<th>Tank mix</th>
<th>Volume active ingredient (g/ha)</th>
<th>Application rate</th>
<th>EIQ</th>
<th>Field Rate EIQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ht corn</td>
<td>Glufosinate</td>
<td>450</td>
<td>2</td>
<td>28.25</td>
<td><strong>25.43</strong></td>
</tr>
<tr>
<td>Field trial data</td>
<td>Acetochlor</td>
<td>400</td>
<td>1</td>
<td>22.98</td>
<td><strong>36.77</strong></td>
</tr>
<tr>
<td>(Narbonne)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dichlormid</td>
<td>66</td>
<td>1</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>Isoxaflutole</td>
<td>75</td>
<td>1</td>
<td>22.67</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>Aclonifen</td>
<td>500</td>
<td>1</td>
<td>22.98</td>
<td>5.75</td>
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<tr>
<td><strong>Total environmental impact</strong></td>
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<td></td>
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<td></td>
<td><strong>43.37</strong></td>
</tr>
<tr>
<td>EU</td>
<td>Flufenacet</td>
<td>600</td>
<td>1</td>
<td>11.33</td>
<td>6.80</td>
</tr>
<tr>
<td></td>
<td>Therbuthylazine</td>
<td>800</td>
<td>1</td>
<td>22.98</td>
<td>18.38</td>
</tr>
<tr>
<td></td>
<td>Nicosulfuron</td>
<td>40</td>
<td>1</td>
<td>18.9</td>
<td>0.76</td>
</tr>
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<td></td>
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\(^3\) The information on the German program was provided by K. Hurle, University of Hohenheim through personal communication.
Table 3. LD50 dose, million acre treatment and volume active ingredient for different herbicide programs.

<table>
<thead>
<tr>
<th>Management program</th>
<th>Oral LD50 dose for rats</th>
<th>Volume active ingredient</th>
<th>Million acre treatments conventional corn</th>
<th>Million acre treatment Ht corn</th>
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<td>1.04</td>
<td>n.a.</td>
<td>n.a.</td>
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<tr>
<td>Ht glufosinate</td>
<td>443.35</td>
<td>0.9</td>
<td>n.a.</td>
<td>n.a.</td>
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<td>900.27</td>
<td>1.74</td>
<td>18108000</td>
<td>9054000</td>
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<tr>
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<td><strong>250.40</strong></td>
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<td><strong>794000</strong></td>
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<td>1.8</td>
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<td>Italy</td>
<td>899.48</td>
<td>2.5</td>
<td>4436000</td>
<td>2218000</td>
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</table>

Table 3 shows that for the LD50 method, results are the same as when using the EIQ. The German herbicide program for conventional crops here also does better than the Ht management practice. This result is replicated when using Benbrook’s volume active ingredient method. The volume applied in the German program is only about half of that applied in the Ht program. We also observe that the German conventional program not only is better than the Ht program, but also highly preferable over any of the other country-specific programs.

The only method that shows a somewhat different result is the million acre treatment. The number of million acre treatments for Ht corn is calculated per country as the number is dependent on the number of acres that are planted with corn in a specific country. In order to compare the possible decline in treatments we calculated the number of treatments for Ht for each country (column 3), if Ht would have been grown there, and compared this to country-specific treatments for conventional corn (column 4). Here, the Ht variety would actually need less treatments than the conventional German program.

Conclusions

In this paper we calculated the environmental impact of pesticide use for conventional and transgenic corn varieties to investigate whether possible irreversible social benefits may be gained by growing Bt and Ht corn. Preliminary results suggest that indeed irreversible benefits may be gained by growing Bt corn through reduced insecticide use. However, some caution is warranted, with respect to the interpretation of these results. Perceived environmental gains may be overstated if the area has not been sprayed in the past. Moreover, if insecticides are not only sprayed to control ECB but also other target pests the reduction in pesticide use may be much smaller than assumed.

Results are more ambiguous for the herbicide programs. Here we have seen that a specific tank mix used in Germany shows a much lower environmental impact than the Ht variety would have. In this case the use of a conventional corn management program would be preferred to the use of Ht corn. However, this only applies to the specific tank mix that is being used in Germany and not to herbicide programs used in France and Italy. Future studies should look into further (non-transgenic) crop management programs to provide solid information about the expected environmental impact of changes in pesticide use from planting transgenic crops.
References


Annex 1. Mathematical presentation of the EIQ

The EIQ consists of three components, the farm worker, the consumer and the ecology component, with an equal weight for each component.

Farm worker component:

\[ C(DT * 5) + (DT * P) \]

Consumer component:

\[ (C * (S + P) / 2 * SY) + (L) \]

Ecology (fish, birds, honeybees, other beneficial insects) component:

\[ (F * R) + (D * S + P) / 2 * 3) + (Z * P * 3) + (B * P * 5) \]

Total (Farm worker + Consumer + Ecology)/3

\[ EIQ = \frac{[C[(DT * 5) + (DT * P)] + [C*((S + P) / 2) * SY] + (L)] + [(F * R)] + (D * (S + P) / 2) * 3) + (Z * P * 3) + (B * P * 5)]/3}{3} \]

Where DT= dermal toxicity, C= chronic toxicity, SY= systemicity, F= fish toxicity, L = leaching potential, R= surface loss potential, D=bird toxicity, S= soil half-life, Z=bee toxicity, B= beneficial arthropod toxicity, and P= plant surface half-life.

The field rate EIQ is then calculated as:

Field rate EIQ = EIQ* % a.i.* rate used
Genetic structure of *Sesamia nonagrioides* populations: Implications for Bt-maize resistance management

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The Mediterranean corn borer, *Sesamia nonagrioides* (Lepidoptera: Noctuidae), is one of the main pests of maize in the Mediterranean area. The average yield loss caused by both *S. nonagrioides* and the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae), is estimated between 5 and 7%. Bt maize expressing Cry1Ab toxin, which is commercially grown in Spain since 1998, can effectively control *O. nubilalis* as well as *S. nonagrioides*, reducing at the same time environmental costs associated with the use of conventional insecticides. The development of resistance in target pests to Bt plants is considered one of the main risks for the success of this powerful control tool. Resistance can be affected by a variety of interacting influences, such as genetic, environmental and management factors. The main resistance management strategy (high dose/refuge) implies, on the one hand, the use of Bt crops expressing high doses of toxin during all the season and, on the other hand, the conservation of non Bt crop refuges. In Spain, the use of refuges is recommended to be a 20% of the total area of the crop, only for Bt maize fields bigger than 5 ha. The high dose/refuge strategy stands that in order to maintain the frequencies of the resistance alleles under a threshold it is important that susceptible and resistant individuals mate at random. Thus, the characterization of genetically differentiated populations is of critical importance, since the potential to develop Bt resistance is strongly related to the migration within and between populations. Genetic studies about *O. nubilalis* have been carried out (Pornkulwat *et al.*, 1998; Bourguet *et al.*, 2000; Coates & Hellmich, 2003), but little is known about the genetic structure of *S. nonagrioides* populations.

A resistance monitoring programme funded by the Spanish Ministry of Environment has been operating for the last seven years to monitor target pest resistance to Bt-maize in Spain (Farinós *et al.*, 2004a,b). In addition, a European-wide programme “ProBenBt” (Protecting the benefits of Bt-toxins from insect resistance development by monitoring and management) has been funded by the EU to accomplish targeted investigations into various aspects of ECB/CMC genetics and Bt resistance. As part of these ongoing projects, we have studied the genetic structure of *S. nonagrioides* populations in Spain and other European countries (France, Italy and Greece) to understand how gene flow can influence the Bt resistance management of this important pest. The analysis was performed by the RAPD-PCR technique, a very useful method to study genetic variability and applied to the study of many insect population genetics (Taberner *et al.*, 1997; Zhou *et al.*, 2000).

The RAPD markers were obtained using seven primers (10 bp oligonucleotides of random sequence) showing good efficiency and reproducibility. Present and absent bands were scored and Nei and Li similarity indexes (Nei & Li, 1979) between individuals were calculated. The within-population values were all greater than 0.7, which is consistent with the fact that most individuals within a population share similarities in their genetic makeup. The between-population values were greater than 0.6, indicating that these populations are
genetically very similar to each other. Nei genetic distances (Nei, 1972) between populations were also calculated; the mean distance obtained was 0.07, which indicates a low genetic differentiation between the populations studied. The maximum values of genetic distances were obtained when comparing the Italian and Greek populations vs. the Spanish and French ones. The dendrograms constructed based on the between-population similarity values and the genetic distances show that the populations from the Italian and Greek populations tend to cluster together separated from the Spanish and French ones.

The effective rate of migration ($N_m$) was calculated by three methods (Wright, 1951; Weir & Cockerham, 1984; Lynch & Milligan, 1994) as an estimate of gene flow among all the populations studied, being $N_m$ higher than 1 in the three cases. A slightly higher estimated gene flow among $S. \text{nonagrioides}$ populations from Spain, France and Morocco was obtained by Buès et al. (1996) using allozime markers. These values are considered high enough to make gene flow overcome genetic drift, and thus prevent the genetic differentiation between populations. In addition, behavioral studies carried out with $S. \text{nonagrioides}$ suggest that males and females from adjacent maize fields could mate at random (Eizaguirre et al., 2004). From these studies, we can conclude that gene flow exists within and between $S. \text{nonagrioides}$ populations, meeting one of the requirements for the deployment of non Bt refuges for resistance management. Up to now, no increase in tolerance to Cry1Ab expressed in Bt maize has been detected in Spain (González-Núñez et al., 2000; Farinós et al., 2004b), however, resistance management practices and regular resistance monitoring must be continued in order to detect possible changes in susceptibility and to maintain the effectiveness of Bt plants.

References


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A microscopic approach to determine the impact of *Bacillus thuringiensis* Cry proteins on non-target organisms: Lack of Cry1Ac binding to *Chrysoperla carnea* (Stephens) midgut epithelial cells

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**Abstract:** The green lacewing *Chrysoperla carnea* (Stephens) is an important natural enemy in maize and cotton, and for this reason it has been chosen for the analysis of non-target side-effects of these crops expressing insecticidal proteins from *Bacillus thuringiensis* (Bt crops). Besides appropriately designed bioassays, microscopic studies can provide decisive information about the effect and mode of action of Cry proteins on this beneficial insect. An indispensable step for their toxicity is the binding to the epithelial cells of the midgut. *In vivo* binding experiments have been performed by feeding *C. carnea* and *Helicoverpa armigera* (Hübner) with concentrated purified Cry1Ac. Specific binding and cell damage was found in epithelial midgut cells of the Cry1Ac susceptible control, *H. armigera*. However, neither any histopathological effect of Cry1Ac nor immunodetection of this protein in *C. carnea* was found. Our results show that in the case that Cry1Ac was ingested by *C. carnea* through its prey, no binding of this protein to the midgut of the predator would occur and, therefore, no toxicity is expected. In conclusion, Bt crops expressing *cry1Ac*, and thus other *cry1A* genes, do not seem to pose any risk for *C. carnea*.

**Key words:** Bt crops, GMO environmental impact, *in vivo* binding, immunodetection

**Introduction**

*Bacillus thuringiensis* (Bt) is a gram-positive bacterium with its main characteristic of producing parasporal crystal proteins (Cry proteins) highly specific against several orders of insects, nematodes, mites and protozoa. Bt based biopesticides conform the 90 % of the global biopesticides market and are considered to be the best alternative to chemical insecticides because of their innocuousness to non-target organisms. Many crops have been engineered during the last decade to make them resistant to insects by expressing Bt toxin-encoding genes. The most widely planted insect-resistant crops worldwide in 2004 were Bt maize and Bt cotton, which respectively occupied the 14% and 6% of the global area of transgenic plants (James, 2004). The genes expressed in varieties of Bt-maize are *cry1Ab* or *cry1Fa*, whereas varieties of Bt-cotton express either *cry1Ac* (Bollgard I), both *cry1Ac* and *cry2Ab* (Bollgard II) (http://www.monsanto.com), or both *cry1F* and *cry1Ac* (WideStrike) (http://www.dowagro.com).

Briefly, the mode of action in insects of Cry proteins begins after ingestion, when the protoxin is processed by the gut proteases. Following binding of the activated protein to specific receptors in the brush border membrane of midgut epithelial cells, insertion and further pore formation lead to an osmotic imbalance, cell lysis and insect death. In this study we present a novel approach to determine the putative impact of Cry proteins in non-target organisms. We hypothesized that binding of Cry proteins to epithelial cells followed by cell damage should be found in case they were toxic to *C. carnea*.
Materials and methods

Insect rearing
Eggs and larvae of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and adults of *C. carnea* were collected in cotton fields in the province of Sevilla (Spain). Both colonies were maintained in laboratory under controlled conditions of 16 h light: 8 h dark photoperiod, 75-80% r. h. and 26 ± 2 ºC. Larvae of *H. armigera* were reared on corn flour based artificial diet while adults were maintained with 10 % honey solution. The diet of *C. carnea* consisted of *Ephestia kuehniella* eggs (Koppert Biological Systems, The Netherlands) for larvae and specific artificial diet for adults (Vogt *et al*., 1998).

Toxin preparation
Cry 1Ac and was obtained from the recombinant *B. thuringiensis* strain EG 1170 (Ecogen, Inc., Langhorne, Pa.).Toxin purification was performed as described previously (Estela *et al*., 2004). Cry1Ac was activated by trypsin treatment, dialyzed overnight and purified by ion-exchange chromatography in order to obtain highly purified activated toxin. Toxicity of this protein was confirmed against *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). The purity of samples was checked by SDS-PAGE and the protein concentration was determined by densitometric analysis using bovine serum albumin as a standard.

**C. carnea Cry1Ac feeding, tissue preparation and sectioning**
Fifty green lacewing larvae (L3 stage) were provided with a drop of fluorella blue-stained water containing either 0 or 4 µg of Cry1Ac purified toxin, after 4 h starvation. Similarly, 40 larvae of *H. armigera* (L4 stage) were starved for 6 h and then provided with a drop containing either 0 or 7 µg of Cry1Ac. The susceptibility of *H. armigera* to Cry1Ac is well documented (Liao *et al*., 2002), (Avilla *et al*., 2005) and for this reason, it was included as a control insect. Midguts were dissected at 4ºC 15 min after larva completely consumed the drop. Whole midguts were fixed in Bouin-PFA (70% picric acid, 25% paraformaldehyde, 5% acetic acid) fixative solution overnight at 4ºC, washed 1 h in distilled water, then transferred to 70% ethanol and stored at 4ºC until used. Seven min incubation in toluene was performed after the dehydration of the tissue. Then, the midguts were embedded overnight in paraffin. Longitudinal sections (5µm) were prepared with a microtome and placed on mounting glasses coated with poly-L-lysine (Menzel GmbH, Braunschweig, Germany). Tissue sections were dried by incubating for 2 h at 40ºC. After rehydration, the tissue sections were transferred to TS-t buffer (10 mM Tris, 150 mM NaCl, 100 µM thimerosal, 0.1% Triton X-100, pH 7.6).

**Bright field microscopy**
Rehydrated sections were incubated for 20 min in 0.1% azokarmin in 1.5% acetic acid at 56ºC and rinsed in distilled water. Further incubations, 1 min each, in anilin (1% anilin in 70% ethanol), acid ethanol (1% acetic acid in 96% ethanol) and distilled water were performed. A final incubation in 5% phosphotungstic acid and water rinsing prepared the sections ready for further dehydration. The sections were covered with DPX mounting medium (Fluka, Switzerland) and observed under a Nikon E800 microscope. Images were captured with a high performance CCD camera.

**Fluorescent microscopy**
Sections from *H. armigera* and *C. carnea* were incubated with anti-Cry1Ac rabbit polyclonal antibody (5µg/ml) for 16 h at room temperature after rehydration. After equilibration with TS-t buffer, a fluorescent anti-rabbit antibody diluted to 1:500 (Alexa Fluor 488, Molecular Probes, Madrid, Spain) was added and incubated for 2 h. Then, the sections were rinsed with TS-t buffer and Fluorescent Mounting Medium (Dako Cytomation, CA, USA) was added before further observation with a Nikon microscope E800 equipped with epifluorescence and
the appropriate filters. Image capturing and processing was performed as described above. As expected, no staining was found in any of the controls in which the primary and/or secondary antibodies were omitted or in larvae that had not ingested the toxin.

Results and discussion

Midgut sections from green lacewing and *H. armigera* larvae that had ingested Cry1Ac, as well as their controls not exposed to the toxin, were observed under the microscope to determine any histopathological effects that could be associated to the ingested toxin. Azokarmine staining showed in *H. armigera* control (Figure 1A) a continuous midgut epithelium where no cell damage was observed. After 20 min Cry1Ac ingestion (Figure 1B), brush border membrane became disrupted, and some cells sloughed into the lumen. The above described effects were not observed in *C. carnea*, where no differences between midgut sections of control and Cry1Ac-fed larvae were found (Figures 1C and 1D).

Figure 1. Lack of histopathological effects induced by Cry1Ac in the midgut of *C. carnea* and *H. armigera*. (A, C) larvae not exposed to Cry1Ac; (B, D) larvae exposed to Cry1Ac for 15 min. BM: basal membrane; L: lumen; BBM: brush border membrane. Scale bar = 50 µm. Cell damage was observed in *H. armigera* treated midguts (arrows).
Similar histopathological effects were found after observation by fluorescent microscopy. The well organized global structure observed in H. armigera control was not found in Cry1Ac-fed insects where typical cell damage signs were found. Immunodetection of ingested Cry1Ac toxin was performed using a fluorescent green antibody that bound along the brush border membrane and also to the peritrophic membrane of H. armigera. However, no binding was detected in C. carnea (data not shown). Results from in vivo binding strongly suggest that the lacewing larval midgut lacks specific receptors for Cry1Ac.

The green lacewing cannot ingest in the field the amounts of Cry1Ac used in our experiments. Bt-crops express Cry proteins in nanograms per milligram of fresh weight. Thus, for the lacewing to ingest microgram amounts of toxins, its prey would need to feed on amounts of plant tissues far exceeding their consumption capacity. In addition, no binding of Cry1Ac or histopathological effects in the midgut of the predator have been detected and therefore no toxicity is expected. In conclusion, Bt-crops expressing cry1Ac are a negligible risk to this beneficial predator.

Acknowledgements

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References


Assessing the performance and non-target effects of wheat engineered with the *kp4* gene to mediate smut resistance under semi-field conditions

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Abstract: An open glasshouse system was established at Agroscope FAL Reckenholz in Zurich to conduct experiments with annual transgenic plants under environmental conditions that are close to the field situation and simultaneously maintaining biological containment. Two KP4-transgenic Swiss spring wheat varieties, previously shown to mediate quantitative stinking smut (*Tilletia caries*) resistance in a climate chamber study, were tested in this system. The objectives of the study were to assess plant performance and potential non-target effects on insects. While the study revealed some differences in plant performance between the two wheat varieties, only one parameter (days till seedling emergence) was affected by the plant transformation status. No effect of the *kp4* transgene on wheat infesting insects was revealed.

Key words: environmental risk assessment, *Folsomia candida*, *Oulema melanopus*, *Rhopalosiphum padi*, transgenic plants

Introduction

Fungal crop infection can cause serious reduction in yield and genetic engineering is considered a promising approach for conferring disease resistance to agronomically important crops (Punja, 2001; Campbell *et al*., 2002). Spring wheat varieties were engineered with the *kp4* gene derived from a double-stranded RNA virus infecting maize smut, *Ustilago maydis*, which is an important fungal disease of maize (Clausen *et al*., 2000). The gene codes for a so-called ‘killer protein’ (KP) that is expressed by the infected *U. maydis* and inhibits the growth of competing *U. maydis* strains that lack virus infection (Hankin & Puhalla, 1971; Koltin, 1986). The biological activity of these KP proteins is known to be highly specific. While a number of bacteria and fungi have been tested for their sensitivity towards *U. maydis* KP toxins, including KP4, they have been found to affect only a small group of grass-infecting species in the order Ustilaginales which cause smut and bunt diseases in cereals (Koltin & Day, 1975; Schlaich *et al*., 2006). Thus, KP proteins may confer resistance to specific fungal diseases if used in transgenic plant protection strategies. Climate chamber tests of the KP4-transgenic spring wheat plants infected with the model fungal disease, stinking smut (*Tilletia caries*; synonym: *T. tritici*), revealed a more than 30% reduction of the infection rate as compared to non-transgenic parental plants (Clausen *et al*., 2000; Sautter *et al*., 2000). A recent study has also revealed an increased fungal resistance under field conditions (Schlaich *et al*., 2006).

The aim of the present study was to evaluate the performance of KP4-transgenic and non-transgenic wheat plants under environmental conditions that are close to the field situation and to assess non-target effects on three insect species feeding on the plants, i.e. the bird-cherry aphid, *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae), the cereal leaf beetle,
Oulema melanopus (L.) (Coleoptera: Chrysomelidae), and the springtail, Folsomia candida Willem (Collembola: Isotomidae).

Materials and methods

KP4-transgenic wheat plants
The two Swiss spring wheat varieties Greina and Golin were genetically engineered with the kp4 gene from Ustilago maydis virus P4 (Clausen et al., 2000). Plants were transformed with three genes, i.e. the kp4 gene controlled by the corn ubiquitin promoter, the selectable BASTA resistance marker gene (bar) controlled by the rice actin promoter, and the ampicillin resistance gene from the bacterial cloning plasmid pUC19. Transgenic and non-transgenic wheat plants of the varieties Golin (transgenic line 5) and Greina (transgenic line 16) were used in this experiment (Clausen et al., 2000; Sautter et al., 2000). Transgenic plants were grown from T5 seeds (5th generation after transformation).

The presence of the kp4 gene in our experimental plants was confirmed by Southern blot analysis and specific RT-PCR. The KP4 protein itself could not be directly detected since antibodies for this protein were not available. However, earlier in vitro and glasshouse studies, using the same transgenic lines, have confirmed anti-fungal activity (Clausen et al., 2000), suggesting expression of the active KP4 protein.

The semi-field system
An open glasshouse system has been established at FAL Reckenholz near Zurich, Switzerland, which allows to perform experiments with transgenic plants under environmental conditions that are close to the field situation, but ensuring biological containment comparable to a closed glasshouse. The glasshouse is kept open during daytime and only under sub-optimal weather conditions (rain, strong wind) and at night the roof and side walls are automatically closed. Plants were grown in 20 thermally insulated plastic containers (80 cm wide, 120 cm long, and 80 cm high) aligned in two rows. Each container was separated in two plots using a plastic wall (40 plots in total), each plot consisting of a separated central cylinder (26 cm diameter) for the transgenic plants and a surrounding area for non-transgenic buffer plants. Insulation ensured that soil temperatures (measured at a depth of 10 cm) were similar to those in the field, not exceeding 26°C during the course of this experiment. The plants were grown in a loamy field soil with an organic matter content of 2-5% and pH 7-8.

To ensure biological containment, access to the open glasshouse was restricted and a net and fences protected the experimental plants from larger animals like mice and birds. Small paper bags were placed over individual flowering ears and were sealed with clips in order to prevent wheat pollen from escaping the system. Plants were manually watered as required and excess water was collected and recycled in the system. All soil and plant material was heat treated prior to discharge.

Experimental setup
The experiment involved eight treatments established with five replications. Each replication was assembled in a randomized block with the eight treatments in four adjacent containers. The two varieties Greina and Golin were used as KP4-transgenic and non-transgenic (NT) genotypes. Each genotype was either not inoculated or inoculated with Tilletia caries (stinking smut) (see Table 1 for treatments). Ten experimental plants were grown in each central cylinder and were surrounded by corresponding non-transgenic, uninoculated plants as a buffer. The wheat plants were grown during 2001 for a period of about five months from March 12 (sowing) until August 11 (harvest).
**Plant parameters**

Date of seedling emergence was recorded for each grain (20 seeds per cylinder). Observations were taken daily. A few days after emergence, 10 plants per cylinder were left and surplus plants were removed. A number of plant parameters were recorded for each plant: (i) Plant height - measured at maturity from the soil surface to the top of the ear excluding the awns; (ii) Number of ears per plant - counted at harvest; (iii) Total grain yield per cylinder; (iv) 1000-kernel weight. After harvest, ears were kept separate per plant in paper bags at room temperature for a few days. They were then stored at 4°C till threshing.

Data recorded per plant were pooled per cylinder to avoid pseudo-replications resulting in five replications per treatment. Mean data were analysed using a 3-way ANOVA with wheat variety, transformation status and *T. caries* infection as factors for date of seedling emergence, plant height and the number of ears per plant. A 2-way ANOVA with wheat variety and transformation status as factors was conducted for grain yield and 1000-kernel weight. Means were subsequently separated using Fisher’s LSD.

**Entomological analyses**

*Rhopalosiphum padi* - Aphids were reared on wheat plants in the glasshouse at 24 ± 4 °C and 50 ± 10% r.h. Experiments were conducted in the open glasshouse on KP4-transgenic and non-transgenic Greina and Golin plants that had not been inoculated with *T. caries*. Two reproductive adults from the colony were caged on the flag leaf of the plants in a clip cage (3.4 cm diameter). Only one cage was attached per plant. On the next morning, all but one newly laid nymphs were removed from the cage. The development of the nymphs was checked daily between 10 and 12 am until the start of reproduction. Subsequently, the number of offspring produced by the individual aphids was counted daily and all nymphs were removed. Aphid performance on the different wheat genotypes was assessed by calculating the intrinsic rate of natural increase \[ r_m = 0.74 \ln \left( \frac{F_D}{D} \right) \], where \( F_D \) = number of aphids produced over a period of time equal to that of the nymphal development period (D) (Wyatt & White, 1977). The mean development time and \( r_m \) values were compared between transgenic and non-transgenic plants using Student’s *t*-test. Aphid performance on the two wheat varieties could not be compared due to variety-dependent differences in plant growth stages at the sampling dates. In total, between 19 and 24 aphids were studied for each of the four wheat genotypes.

*Oulema melanopus* - Adult cereal leaf beetles migrated into the open glasshouse and oviposited on the experimental plants. After the larvae had completed development and disappeared into the soil for pupation in early July, the typical feeding damage caused by the beetle larvae on the wheat leaves was scored using a scheme developed for scoring infection by yellow rust (*Puccinia striiformis*) (Figure 1). Leaf damage was determined using five scores based on the percentage of the leaf surface damaged: 0 (no damage), 1 (1-5%), 2 (5-15%), 3 (15-25%), and 4 (25-50%). Only plants that had not been inoculated with *T. caries* were evaluated. For each plant, two leaves (flag leaf and the subsequent leaf) from the three strongest shoots were scored and average damage score was calculated per plant. These two leaves were selected based on results from previous studies showing that they are preferably infested by *O. melanopus* larvae (Jossi & Bigler, 1985). To avoid pseudo-replication, a cylinder mean was calculated resulting in five replications per wheat genotype. Leaf damage scores were analysed by 2-way ANOVA with wheat variety and transformation status as independent factors.
Folsomia candida - A laboratory culture of *F. candida* was maintained as described by Romeis et al. (2003). Since soil samples taken from the central cylinders in March and April only contained few collembola, each cylinder was infested with a mixed-age population of 200 *F. candida* individuals from the laboratory colony two month after planting (May 15). Soil samples were subsequently taken from each central cylinder on three sample days (June 12, July 5, and August 2). Uninoculated plants and plants that had been inoculated with *T. caries* were not differentiated as an effect of inoculation on soil arthropods was not expected, resulting in a total of ten samples for each wheat genotype for each sampling date. Soil samples were collected with a 1.4 cm diameter soil coring device to a depth of 10 cm. Sampling holes were subsequently filled with surrounding soil. Six soil cores were taken from each central cylinder on each sampling date and pooled into one clean plastic bag resulting in sample sizes of about 40 g fresh soil. Approximately 10 g fresh soil was used to gravimetrically determine its water content (12h, 105°C). The rest of each sample was suspended in water. Collembola floating on the water surface were collected, microscopically identified as collembola, and counted. Numbers were standardized per 100 g soil (dry weight equivalent). To evaluate the population development of *F. candida*, repeated-measures ANOVA was used. In order to homogenize variances, data was transformed by square-root (x+1) prior to analysis.

Percentage damaged leaf area

Figure 1. Scheme to score infection by yellow rust (*Puccinia striiformis*) or leaf damage caused by cereal leaf beetles (EPPO, 1997).
Results and discussion

Plant parameters

Plant performance is summarized in Table 1. Days till seedling emergence differed significantly between KP4-transgenic plants and non-transgenic plants (ANOVA, F = 11.6, df = 1, 32, P = 0.0018) and was affected by inoculation with *T. caries* (F = 4.14, df = 1, 32, P = 0.0501). In contrast, wheat variety had no impact on the days till seedling emergence (F = 3.15, df = 1, 32, P = 0.085). For plant height a significant difference was detected between the two wheat varieties (F = 219.8, df = 1, 32, P < 0.0001) and for fungal infection status (F = 8.52, df = 1, 32, P = 0.006) but not for the transformation status (F = 0.61, df = 1, 32, P = 0.44). Similarly, the number of ears per plant was affected by wheat variety (F = 134.2, df = 1, 32, P < 0.0001) but not by fungal infection (F = 0.12, df = 1, 32, P = 0.73) or transformation status (F = 2.93, df = 1, 32, P = 0.097). Grain yield was affected by wheat variety (F = 30.6, df = 1, 16, P < 0.0001) but not by the transformation status (F = 1.26, df = 1, 16, P = 0.28). Similarly, the 1000-kernel weight was affected by the wheat variety (F = 52.3, df = 1, 16, P < 0.0001) but not by the transformation status (F = 2.71, df = 1, 16, P = 0.12). There was no significant two-way interaction (P > 0.05) for any of the parameters assessed.

Entomological analyses

*Rhopalosiphum padi* - No significant difference was detected in the pre-reproductive development and in the intrinsic rate of natural increase of *R. padi* reared on KP4-transgenic or non-transgenic plants of either of the two wheat varieties (Table 2). These results suggest that the phloem-sap of the transgenic plants was of the same nutritional quality as compared to the non-transgenic parental plants.

Table 1. Performance of KP4-transgenic and non-transgenic (NT) plants of two spring wheat varieties grown in an open glasshouse under close to field environment (n =5). [inf. - plants infected with *T. caries*; non-inf. - non-infected plants.]

<table>
<thead>
<tr>
<th>Variety/Genotype</th>
<th>Seedling emergence (days)</th>
<th>Plant height (cm)</th>
<th>Number of ears per plant</th>
<th>Grain yield (g)</th>
<th>1000-kernel weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golin NT inf.</td>
<td>14.2 ± 0.25 <em>ab</em></td>
<td>89.9 ± 0.89 <em>b</em></td>
<td>4.9 ± 0.19 <em>a</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Golin KP4 inf.</td>
<td>13.3 ± 0.55 <em>cd</em></td>
<td>88.6 ± 1.32 <em>b</em></td>
<td>5.2 ± 0.12 <em>a</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Golin NT non-inf.</td>
<td>14.7 ± 0.0.42 <em>a</em></td>
<td>90.9 ± 2.38 <em>ab</em></td>
<td>5.0 ± 0.24 <em>a</em></td>
<td>4.7 ± 0.62 <em>a</em></td>
<td>35.3 ± 0.97 <em>a</em></td>
</tr>
<tr>
<td>Golin KP4 non-inf.</td>
<td>13.4 ± 0.26 <em>bcd</em></td>
<td>93.5 ± 1.27 <em>a</em></td>
<td>5.1 ± 0.07 <em>a</em></td>
<td>5.5 ± 0.25 <em>a</em></td>
<td>37.3 ± 0.55 <em>a</em></td>
</tr>
<tr>
<td>Greina NT inf.</td>
<td>13.6 ± 0.26 <em>bcd</em></td>
<td>76.4 ± 0.84 <em>c</em></td>
<td>3.6 ± 0.11 <em>b</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Greina KP4 inf.</td>
<td>12.7 ± 0.25 <em>d</em></td>
<td>77.0 ± 0.60 <em>c</em></td>
<td>4.0 ± 0.11 <em>b</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Greina NT non-inf.</td>
<td>13.8 ± 0.25 <em>abc</em></td>
<td>78.5 ± 1.00 <em>c</em></td>
<td>3.9 ± 0.10 <em>b</em></td>
<td>2.9 ± 0.27 <em>b</em></td>
<td>27.3 ± 1.32 <em>b</em></td>
</tr>
<tr>
<td>Greina KP4 non-inf.</td>
<td>13.8 ± 0.23 <em>abc</em></td>
<td>79.3 ± 0.61 <em>c</em></td>
<td>3.9 ± 0.15 <em>b</em></td>
<td>3.0 ± 0.33 <em>b</em></td>
<td>29.1 ± 1.42 <em>b</em></td>
</tr>
</tbody>
</table>

Means within a column followed by similar letters were not significantly different (P>0.05, Fisher’s LSD test).
Table 2. Pre-reproductive development and intrinsic rate of natural increase ($r_m$) of *Rhopalosiphum padi* reared on KP4-transgenic or non-transgenic (NT) plants of two spring wheat varieties grown in an open glasshouse.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Genotype</th>
<th>n</th>
<th>Development (days ± SE)</th>
<th>$r_m$ ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Golin</td>
<td>NT</td>
<td>23</td>
<td>8.87 ± 0.130</td>
<td>0.33 ± 0.008</td>
</tr>
<tr>
<td>Golin</td>
<td>KP4</td>
<td>19</td>
<td>9.11 ± 0.105</td>
<td>0.32 ± 0.008</td>
</tr>
<tr>
<td>Greina</td>
<td>NT</td>
<td>24</td>
<td>9.79 ± 0.170</td>
<td>0.28 ± 0.006</td>
</tr>
<tr>
<td>Greina</td>
<td>KP4</td>
<td>23</td>
<td>9.96 ± 0.172</td>
<td>0.28 ± 0.006</td>
</tr>
</tbody>
</table>

Differences between KP4-transgenic and non-transgenic plants were not significant (P > 0.05; Student’s t-test).

*Oulema melanopus* - The mean (± SE) scores of the leaf surface damage caused by cereal leaf beetles was 1.20 ± 0.114 and 1.14 ± 0.054 for non-transgenic and KP4-transgenic Golin plants, respectively, and 1.08 ± 0.097 and 1.08 ± 0.066 for non-transgenic and KP4-transgenic Greina plants. There was no significant effect by wheat variety (2-way ANOVA, $F = 1.02$, df = 1, 16, P = 0.33) or transformation status (F = 0.10, df = 1, 16, P = 0.76). These damage scores translate to leaf surface damage levels of 1-5%, indicating that infestation by the beetles in our study was very low. Earlier studies revealed that a single cereal leaf beetle larva causes damages of about 2.5 to 4 cm² leaf surface during its development, corresponding to roughly 10-30% of the surface of a flag leaf (Bigler, 1985). Also, for winter wheat varieties in Switzerland, much higher damage levels have previously been reported (Jossi & Bigler, 1985).

*Folsomia candida* - The introduced population of *F. candida* increased significantly over time (repeated-measures ANOVA, $F = 3.68$, df = 2, 72, P = 0.030) (Figure 2). However, population sizes remained low with an average ranging from 16 to 31 collembola per 100 g soil (dry weight equivalent) found on the last sample date, ten weeks after infestation. Collembola populations did neither differ between soil planted with the two wheat varieties ($F = 0.16$, df = 1, 36, P = 0.70) nor between soil with transgenic and non-transgenic plants ($F = 0.07$, df = 1, 36, P = 0.79) (Figure 2). This is in accordance with earlier glasshouse studies that did also not detect any effect of the KP4 transformation on *F. candida* populations (Romeis et al., 2003). However, in the glasshouse, a significant variety effect was observed. Laboratory studies in which individual *F. candida* were fed a mixture of baker’s yeast and dried root material from the experimental plant genotypes did not reveal any negative effects of either wheat variety or plant transformation on different life-history parameters of this soil microarthropod (Romeis et al., 2003).
Figure 2. Population development of *Folsomia candida* in soil planted with KP4-transgenic and non-transgenic (NT) plants of the spring wheat varieties Golin and Greina.

### Conclusions

Plant performance in this semi-field system was good and allowed to assess a number of different plant parameters. With exception of the days till seedling emergence, the two spring wheat varieties differed in all plant parameters evaluated. A significant transformation related effect was only recorded for the days till seedling emergence. Inoculation with the fungal pathogen *T. caries* had a significant negative effect on plant height at harvest. However, this effect of the fungal infection was not influenced by expression of the kp4 gene. Studies on the different insect species did not reveal any significant effects related to the KP4-transgenic genotype.

The open glasshouse system established at FAL Reckenholz in Zurich was found to be suitable to conduct experiments with annual transgenic plants under environmental conditions that are close to the field situation and simultaneously maintaining biological containment comparable to a closed glasshouse. The system could thus provide an opportunity to study transgenic plants prior to field release for which much more stringent regulations apply.

### Acknowledgements

We thank Christof Sautter and Thomas Schlaich (ETH Zurich) for conducting the Southern blot and RT-PCR analyses of the KP4-transgenic plants and Sabine Keil and Martina Battini for technical support during the experiment.
References


Impact of genetically modified corn on arthropod communities

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Abstract: Agricultural research is engaged increasingly to manipulate genes, among other biological methods. The evaluation of hazards connected with the creation and release of GMOs should consider, among other things, their environmental impact. The new technology of corn cultivation, based on the use of genetically modified corn hybrids [insect resistant (Bt) corn or Roundup Ready® (RR) corn] have offered the possibility to observe if this new technology has an impact on the surface fauna captured in pitfall (Barber) traps or on existing fauna on corn plants. To assess possible environmental effects, we examined the epigeic fauna in corn fields, knowing that the use of chemical pesticides affects both the composition and the abundance of agrocoenoses. Our objective was to evaluate the potential impact of Roundup corn and Bt corn on the epigeic fauna and on plant-dwelling predators. The epigeic fauna was collected biweekly in pitfall traps from May till September in 2000 and 2004. The traps were opened for 24 hours. At the dates of trap setting, visual count technique was used to register the predator fauna on corn plants. The “Sörensen” index of similarity was used to evaluate differences between GM and non-transgenic corn fields. Data obtained regarding resemblance of the whole insect fauna from the corn varieties investigated, represented by value of Sörensen index, confirm the hypothesis that Bt corn and Roundup Ready corn do not influence the insect fauna.

Key words: Roundup Ready® corn, Bt corn, epigeic fauna, predators

Introduction

Agricultural research is engaged increasingly to manipulate genes, among other biological methods. The evaluation of hazards connected with the creation and release of GMOs should consider, among other things, their environmental impact. The new technology of corn cultivation, based on the use of genetically modified corn hybrids [insect resistant (Bt) corn and Roundup Ready® (RR) corn] have offered the possibility to observe if this new technology has an impact on the surface fauna captured in pitfall (Barber) traps or on the existing fauna on corn plants (Rosca & Sabau, 2001; Rosca, 2002, 2004). In Romania, the European corn borer is considered the most important pest of corn after panicle apparition, being spread throughout all cropping zones in the country. Grain damages caused by this pest can sometimes reach up to 40%. Damages caused by weeds can sometimes reach up to 30%.

Material and methods

Experiments were conducted at the Experimental Didactic Station “Moara Domneasca” near Bucharest, in 2000 and USAMVB, Timisoara, in 2004. The experiments had two objectives: to observe the potential impact of Bt corn and Roundup Ready corn on the epigeal fauna and on plant-dwelling predators under conventional farm practice.

Field experiments were conducted from May–September in 2000 with the following variants: 512 Dekalb Bt, AW 641 RR and check (AW 641 and F 376), and in 2004 involving the following treatments: NK 603 RR, check (NK 603).
Plot sizes were over 1200 m² for each variant in one replication (field). The herbicide Roundup Ready was applied post emergent in variants AW 641 RR and NK 603 RR (2 l/ha, corn stage 1-3 leaves and 3 l/ha, corn stage 6-8 leaves in 150 liters of water per hectare). There was no insecticide treatment in any of the field plots.

Evaluation of the epigeal fauna was done biweekly by capturing in pitfall (Barber) traps, opened for 24 hours. Ten traps were placed per field plot. The distance between traps was 10-20 m and traps were filled with formalin (3-4%). Visual count technique was used, at the same sample date to register the plant-dwelling predators on 10 corn plant in 4 replicates. Animals collected in pitfall traps or on corn plants were preserved in 70% alcohol for later identification in the laboratory. In 2004, three Pherocone traps (Yellow sticky traps) were placed per plot for 48 hours. Arthropods caught were counted on an area of 72 cm².

An useful tool to assess whether the structure of animal communities differs among corn fields is comparison by the “Sörensen” index of similarity (100%, means no difference between faunas and 1% signify two complete different faunas). In addition, data were analysed by two-way ANOVA with species or animal groups and cultivation variant as factors.

Results and discussion

Insects captured in pitfall traps
In 2000, a total of 1053 insect specimens were identified: 359 in 512 Dekalb Bt corn fields, 373 in AW 641 (check) fields and 321 in F 376 (check) fields. The most common species belonged to the Coleoptera (784 specimens): 261 in 512 Dekalb Bt corn field, 289 in AW 641 fields and 234 in F 376 fields. The second most abundant group of species belonged to the Hymenoptera, with most common ant species: Formica fusca (15/4/20), F. rufa (8/6/12), Lasius flavus (16/9/17) and L niger (12/5/1). The third most abundant group of species belonged to the Heteroptera, most common being Pyrchocoris apterus (32/22/27). There were 329 identified specimens of main predators insects belonging to Carabidae family: 117 in 512 Dekalb Bt corn field, 105 in AW 641 corn field and 107 in F 376 corn field.

Table 1. Structure of the fauna captured in soil traps in Roundup Ready corn and control plots in 2004.

<table>
<thead>
<tr>
<th></th>
<th>RR corn (NK603RR)</th>
<th>Check NK603</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthoptera</td>
<td>62</td>
<td>73</td>
<td>135</td>
</tr>
<tr>
<td>Dermaptera</td>
<td>1</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Heteroptera</td>
<td>29</td>
<td>43</td>
<td>72</td>
</tr>
<tr>
<td>Homoptera</td>
<td>27</td>
<td>32</td>
<td>59</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>43</td>
<td>35</td>
<td>78</td>
</tr>
<tr>
<td>Coleoptera</td>
<td>615</td>
<td>643</td>
<td>1258</td>
</tr>
<tr>
<td>Lepidoptera</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Diptera</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>TOTAL</td>
<td>829</td>
<td>783</td>
<td>1612</td>
</tr>
</tbody>
</table>

In 2004 there were 1612 insects specimens identified: 829 in RR corn field and 783 in the control fields. The most common species belonged to the Coleoptera (1258 specimens): 615 in RR corn field and 643 in the control fields (Table 1).
Ground beetles were the most abundant predators in pitfall traps. The most abundant species in 2000 were *Pterostichus vulgaris* L., *Pterostichus melas* Creutz. and *Harpalus distinguendus* Duft. (Table 2). The most abundant species in 2004 were *Harpalus pubescens* Mull., *Pterostichus vulgaris* L. and *Carabus coriaceus* L. (Table 3). There were no differences between fields (P>0.05; two-way ANOVA).

Table 2. Main Coleopteran species captured in pitfall traps in Bt and Roundup Ready corn and two control varieties in 2000.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>512 Dekalb Bt</th>
<th>AW 641 RR</th>
<th>AW641 (Check)</th>
<th>F 376 (Check)</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Harpalus distinguendus</em> Duft.</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td><em>Pterostichus cupreus</em> I.</td>
<td>11</td>
<td>8</td>
<td>10</td>
<td>2</td>
<td>31</td>
</tr>
<tr>
<td><em>Pterostichus vulgaris</em> L.</td>
<td>85</td>
<td>75</td>
<td>78</td>
<td>69</td>
<td>307</td>
</tr>
<tr>
<td><em>Pterostichus melas</em> Creutz.</td>
<td>17</td>
<td>15</td>
<td>11</td>
<td>21</td>
<td>64</td>
</tr>
<tr>
<td>TOTAL</td>
<td>117</td>
<td>103</td>
<td>105</td>
<td>107</td>
<td>432</td>
</tr>
</tbody>
</table>

Table 3. Main Coleopteran species captured in pitfall traps in Roundup Ready corn and the control variety in 2004.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>RR corn</th>
<th>Check</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Harpalus pubescens</em> Mull.</td>
<td>163</td>
<td>162</td>
<td>325</td>
</tr>
<tr>
<td><em>H. griseus</em> Panz.</td>
<td>90</td>
<td>93</td>
<td>183</td>
</tr>
<tr>
<td><em>H. zabroides</em> Dej.</td>
<td>17</td>
<td>21</td>
<td>38</td>
</tr>
<tr>
<td><em>H. aeneus</em> F.</td>
<td>4</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td><em>H. distinguendus</em> Duft.</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td><em>Pterostichus vulgaris</em> L.</td>
<td>119</td>
<td>126</td>
<td>245</td>
</tr>
<tr>
<td><em>P. cupreus</em> L.</td>
<td>9</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td><em>P. melas</em> Creutz.</td>
<td>4</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td><em>Cicindella soluta</em> Dej.</td>
<td>4</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Cereals fleas (<em>Phyllotreta</em>, <em>Chetocnema</em>, <em>Crepidotera</em>)</td>
<td>38</td>
<td>42</td>
<td>80</td>
</tr>
<tr>
<td><em>Carabus coriaceus</em> L.</td>
<td>98</td>
<td>93</td>
<td>191</td>
</tr>
<tr>
<td><em>C. cancelatus</em> Illig.</td>
<td>51</td>
<td>60</td>
<td>111</td>
</tr>
<tr>
<td><em>Drasterius bimaculatus</em> Ross.</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td><em>Amara aenea</em> Deg.</td>
<td>6</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td><em>Tanytmescus dilaticollis</em> Gyll.</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td><em>Bothynoderes punctiventris</em> Germ.</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

**Main species of natural enemies living on corn plants**

The most abundant group of species which were found on corn plants belonged to the Heteroptera (*Nabis* sp.), Coleoptera (*Coccinellidae*), Neuroptera (*Chrysopa* sp.) and spiders.

For the evaluation of changes in the corn biocenoses it is suggested to study the Heteroptera fauna since these predator species sit at the top of the trophic chain and are easy to be studied through visual inspections or through pherocone traps (Table 4).
Table 4. Structure of the Heteroptera fauna on RR corn in 2004.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>Plant</th>
<th>Pherocone trap (72 cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anthocoris nemorum</em> L.</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td><em>Anthocoris nemoalis</em> F.</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td><em>Orius niger</em> W.</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td><em>Aptus</em> (Himacerus) <em>myrmecoides</em> C.</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Nabis feroxoides</em> Rm.</td>
<td>25</td>
<td>6</td>
</tr>
<tr>
<td><em>Nabis ferus</em> L.</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td><em>Nabis pseudoferus</em> Rm.</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td><em>Nabis rugosus</em> L.</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>82</strong></td>
<td><strong>38</strong></td>
</tr>
</tbody>
</table>

**Similarity of faunas from corn fields**

Data obtained regarding resemblance of whole insect fauna from the corn variants investigated represented by value of Sörensen index (71.43, 79.25, 86.66 in 2000 and 91.3 in 2004), confirmed the hypothesis that genetically modified corn (Bt or RR), has no impact on the insect fauna in corn field (Tables 5 and 6).

Table 5. Resemblances (Sörensen Index) of whole insect fauna in Bt corn and control plots in 2000.

<table>
<thead>
<tr>
<th>YEAR 2000</th>
<th>512 Dekalb <em>Bt</em></th>
<th>AW 641 (check)</th>
<th>F 376 (check)</th>
</tr>
</thead>
<tbody>
<tr>
<td>512 Dekalb <em>Bt</em></td>
<td></td>
<td>61 – 80 %</td>
<td>61 – 80 %</td>
</tr>
<tr>
<td>AW 641 (check)</td>
<td>21; 15; 21</td>
<td>71.43</td>
<td>61 – 80 %</td>
</tr>
<tr>
<td>F 376 (check)</td>
<td>29; 26; 31</td>
<td>86.66</td>
<td>31; 21; 22</td>
</tr>
<tr>
<td></td>
<td>86.66</td>
<td>79.25</td>
<td>79.25</td>
</tr>
</tbody>
</table>

Table 6. Resemblance (Sörensen Index) of whole insect fauna in Roundup Ready corn and control plots in 2000.

<table>
<thead>
<tr>
<th>YEAR 2004</th>
<th>RR corn</th>
<th>Check</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR corn</td>
<td></td>
<td>81 -100 %</td>
</tr>
<tr>
<td>Check</td>
<td>48; 42; 44</td>
<td>91.3</td>
</tr>
</tbody>
</table>

In conclusion it is possible to affirm that the cultivation of genetically modified corn (Bt or RR) does not appear to influence the epigal fauna or plant-dwelling predators. Pherocone traps could be an useful tool for studying biodiversity in corn field. The structure of the predatory Heteroptera species complex seems to be an indicator for biodiversity in corn fields. Because
being predaceous, their number and species composition could reflect changes in whole biocoenoses.

References

Tier-based testing for effects of proteinaceous insecticidal plant-incorporated protectants on non-target arthropods in the context of regulatory risk assessments

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Abstract: A White Paper was written to synthesize available scientific and regulatory information, thereby providing guidance to the regulated community regarding the conduct of non-target invertebrate testing for evaluation of new products expressing insecticidal proteinaceous plant-incorporated protectants (PIPs). The information discussed in this document forms the basis for conducting non-target invertebrate ecological risk assessments for proteinaceous PIPs intended to control insect pests. Ecological risk assessment is the process by which regulatory authorities such as the USDA Animal and Plant Health Inspection Service (APHIS) and EPA Office of Pesticide Programs (OPP) use scientific data on potential hazards and exposure to assess the likelihood of unintended impacts on organisms in the environment. The hazard potentially associated with transgenic crops is the potential toxicity of the PIP trait to representative non-target organisms. The exposure assessment is based on the likelihood that a non-target organism will come in contact with the expressed PIP. The ecological risk is determined by the interaction of these two factors on a case-by-case basis. The focus of this document is the PIP ecological risk assessment scheme for non-target invertebrates, including detailed analysis of exposure and risk characterization as described in the EPA's Ecological Risk Assessment Framework document (EPA, 1998). Both laboratory testing of select, representative non-target species and appropriate field testing are addressed. This testing is conducted to determine if there is a hazard associated with the PIP trait that could pose a risk to non-target invertebrates. This type of testing is covered by the tiered testing scheme, with early tier studies, such as Tiers I and II, representing worst-case exposure scenarios with a built-in safety factor and use relatively simple testing procedures. Higher tier studies encompass more complex laboratory, greenhouse or field studies that enable a more realistic assessment of field exposure should lower tier, worst case studies reveal unacceptable toxicity.

Key words: plant incorporated protectants, ecological risk assessment

Introduction

The United States Department of Agriculture (USDA) Animal Plant Health Inspection Service (APHIS) Biotechnology Regulatory Services (BRS) and Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) Biopesticides and Pollution Prevention Division (BPPD) have developed a tier-based approach to conducting non-target arthropod ecological risk assessment for genetically engineered crops intended to control insect pests. These crops are considered plant incorporated protectants (PIPs). The term PIP was designated by the EPA to describe substances that are incorporated into plants to protect them from damage caused by insect pests and diseases. A PIP is defined as the pesticidal substance that is produced in a plant and the genetic material necessary for the production of that substance (Federal Register, Volume 66, No. 139, July 19, 2001).
EPA and USDA authority to require data for environmental effects assessment

**USDA-APHIS.**
The Plant Protection Act (June 20, 2000) gives APHIS authority to regulate any organism altered or produced through genetic engineering using genetic material derived from plant pathogens or plant pests as vectors or where there is a potential to present a plant pest risk. The term “plant pest” means any living stage of any of the following that can directly or indirectly injure, cause damage to, or cause disease in any plant or plant product: a protozoan, a nonhuman animal, a parasitic plant, a bacterium, a fungus, a virus or viroid, an infectious agent or other pathogen, and any article similar to or allied with any of these.

APHIS considers genetically engineered plants to be “regulated articles” and oversees their introduction (importation, interstate movement, and release into the environment) under 7 Code of Federal Regulations (CFR) 340. Based on the potential for effects on non-target organisms beneficial to plants, APHIS currently considers all plants engineered to express proteins that are PIPs as regulated articles. Introduction of a regulated article requires an APHIS permit (7 CFR 340.4) or certain regulated articles may be introduced under the notification process, provided they meet specified eligibility requirements and performance standards for confinement. In addition, 7 CFR 340.6 provides for a petition process for determination of non-regulated status. If APHIS grants a petition for non-regulated status for a genetically engineered plant, that plant is no longer a regulated article and is not subject to oversight by USDA and permits or notifications are no longer required for field testing, importation, or interstate movement of the article or its progeny.

**EPA.**
EPA is responsible for regulating the sale, distribution, and use of pesticides to protect human health and the environment. If a genetically engineered plant produces a substance that is intended to be used for “preventing, repelling or mitigating any pest”, the substance and the genetic material necessary to produce the substance are pesticides under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA).

FIFRA Section 3(a) requires, with some exceptions, that a pesticide be registered under the Act prior to distribution or sale in the U.S. To register a pesticide, EPA evaluates the proposed pesticide to ensure that its use will not pose an unreasonable risk to human health or the environment. Under FIFRA Section 5, EPA issues Experimental Use Permits (EUPs) for field tests greater than or equal to ten acres to allow prospective registrants to generate information or data necessary to register a pesticide. In addition, the Federal Food, Drug, and Cosmetic Act (FFDCA) authorizes EPA to establish tolerances (maximum limits) or exemptions from the requirement of a tolerance for residues of pesticides in food. Regulatory requirements, criteria, and procedures applicable to PIPs are outlined in 40 CFR 174 and 40 CFR 152 (66 FR 37772: 40 CFR Parts 152 and 174 Plant-Incorporated Protectants; Final Rules and Proposed Rule; July 19, 2001).

In the United States, the EPA has taken the lead in developing ecological risk assessment processes for safety determinations in the application of biotechnology to plant protection. An EPA risk assessment is based on a risk versus benefit analysis and considers the potential for unreasonable adverse effects to occur. Regulatory guidance for ecological risk assessment of products containing PIPs is governed principally by the 1982 Subdivision M guidelines for microbial pesticides as amended in 1989. Additional regulation of PIPs was implemented

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4 Field testing, use or movement of a regulated article outside a laboratory or greenhouse is considered release into the environment.
Ecological risk assessment

Ecological risk assessment is the process by which regulatory authorities such as BRS and BPPD use scientific data on potential hazards and exposure to assess the likelihood of adverse impacts on populations of organisms in the environment. A risk exists if the PIP has hazardous effects on non-target organisms and these organisms are exposed to the PIP. Estimates of hazard and exposure allow the regulatory agencies to determine the likelihood that a PIP may cause a problem and the scale of that problem, and therefore acquisition of relevant hazard and exposure data is the objective of any testing program.

The hazard potentially associated with transgenic crops is the potential toxicity of the PIP trait to non-target beneficial organisms. The exposure assessment predicts the likelihood that non-target organisms will have a dietary exposure to the expressed PIP at or above the hazard threshold level. The ecological risk is determined by the interaction of these two factors on a case-by-case basis. Both laboratory testing of select, representative non-target species and, when warranted, appropriate field testing are addressed. This testing is conducted to determine if there is a hazard associated with the PIP trait that could pose an unacceptable risk to non-target arthropods.

For PIPs, there are several components of the preliminary assessment that are particularly important including: 1. Overall management goals and assessment endpoints; 2. Hazard identification; 3. Exposure identification; 4. Test endpoints (e.g., mortality or developmental rate); 5. Iterative or tiered approach; 6. Scientific and public concerns.

Iterative or tiered approach

Tiered testing is commonly used by regulatory agencies such as the US EPA to efficiently make decisions on low-risk scenarios. For example, a well-defined tiered testing scheme is used within EPA/OPP for the regulation of conventional and biological crop protection chemicals in the areas of ecological and human health risk assessments. In each case, lower tiers represent worst-case exposure scenarios, while scenarios in higher tiers are more refined and realistic. Lower tier tests conducted in the laboratory are designed to be conservative in nature. Passing lower tiers may indicate to a reviewer that there is little or no risk of acute toxicity to the tested and related organisms. By extension, and because of the high dosing levels used, the lower-tier results may also indicate long-term exposures (and thus, chronic testing) are not necessary to further characterize risk. Failing lower tiers does not necessarily indicate the presence of an unacceptable risk in the field, but it may trigger the need for additional exposure and/or effects information to be collected before deciding if the risk is acceptable or unacceptable. Higher-tier tests better represent environmental realism in terms of exposure scenarios and lower safety factors are generally used when evaluating results from such tests because the test endpoint and assessment endpoint are more closely linked than with lower-tier studies.

Higher tier studies encompass more complex laboratory, greenhouse or field studies, and enable a more realistic assessment of field exposure should lower-tier, worst-case studies...
reveal unacceptable toxicity. Movement from lower to higher tiers is driven by the need and the ability to test hypotheses resulting from the problem formulation and by the need for additional data to satisfy regulatory requirements. Some hypotheses can be tested quickly by conducting relatively simple assays, while other hypotheses can probably never be tested because of the lack of resources or technical limitations. The goal in designing an appropriate testing framework is to satisfy the needs within the bounds of current capability and without unnecessary regulatory burden.

For PIPs, the following tiered study framework for non-target arthropod testing has been proposed:

**Tier I** Laboratory tests using exposure levels representing at least 10x the highest Expected Environmental Concentration (EEC). The test compound consists of protein mixed with artificial diet. That protein should be similar to what is expressed *in planta*. The protein is often produced microbially and should be in an activated form where *in planta* data indicate that it is appropriate.

**Tier II** Laboratory tests using plant material alone or mixed with artificial diet. For example, arthropods may be exposed to the protein using pollen or leaf discs from the genetically modified plant. Because plant materials are used, exposure levels generally reflect 1x the EEC.

**Tier III** Long-term laboratory and/or semi-field tests. Examples of long-term laboratory tests include full life-cycle tests and tri-trophic tests. Extended laboratory or semi-field tests may be conducted under greenhouse conditions. Controlled semi-field tests employ cages or other techniques to provide some measure of experimental control under simulated field conditions.

**Tier IV** Field tests. These tests use larger plots that may be distributed across an area or region. Studies may involve looking at specific groups of sentinel organisms or census studies.

**Tiers I and II**

**Selection of indicator organisms**

The surrogate concept in ecological risk assessment involves the selection of indicator organisms to represent a group of taxonomically or functionally related organisms. The concept of selecting indicator organisms evolved because not all components of an ecosystem can be tested. Test organisms should be chosen on a case-by-case basis according to the potential for exposure to the pesticidal protein (taking into account the PIP, the crop and the region of introduction) as well as the ability to test the organism in the laboratory (EPA-SAP 2000). The surrogate should also be chosen based on its ability to represent the non-target organisms associated with the crop plant or target pest. Because Bt proteins are specific to certain insect taxa, insects that are related to the target pest should receive particular consideration. In cases where a representative exposed non-target organism cannot be adequately tested in the laboratory, a closely related indicator that can be easily reared may be substituted (EPA-SAP 2000).
Non-target arthropods should be identified for testing that are representative of those that are important in the crop of interest. As it is not possible to test all species that are potentially present, the subset selected should represent different habitats (e.g., below the soil surface, soil surface, plant canopy), levels of mobility, and function (e.g., predator, parasite or decomposer). The species tested should be chosen based upon their ecological and economic importance, relationship to the target pest and their consistent performance in such tests. For each proposed test organism, the following criteria should be considered: 1. The mode of action and specificity of the insecticidal protein in non-target species closely related to the target pest should be given particular consideration; 2. Exposure based on habitat and field abundance; 3 Functional groups (e.g. predators, decomposers) that are ecologically important; 4. Taxonomic and ecological breadth and relationship to target pests; 5. Ability to conservatively estimate field exposure; 6. Whether a suitable test system exists for laboratory analysis.

Dose selection
An uncertainty factor is often used in extrapolating from the indicator organism response to the level used for environmental regulation because the indicator may not be the most sensitive member of the group. This process ensures that the indicator organism test is indeed representative of the group of non-target organisms. Studies are conducted at a conservative maximum hazard dose (MHD) to allow extrapolation from the surrogate species tested to potentially more sensitive species within the same taxonomic and/or functional group, and to address both direct (i.e., consumption of plant material) and indirect (i.e., multi-trophic exposure) exposure routes. For PIPs, it would be appropriate to define the MHD as the PIP expression levels in the plant via the expected route of exposure.

Duration of exposure and endpoints
Test endpoints must be compatible with the duration of exposure. For example, if the endpoint is based on mortality, short-duration (e.g., 7-14 days) exposures will be adequate for most test organisms. This duration is appropriate for Bt Cry proteins mode of action that targets the digestive system of susceptible insects and generally take no more than 3 to 5 days to cause visible symptoms in exposed insects. If growth or development time is the endpoint, longer exposures (e.g., 14 to 30 days) may be needed to adequately detect any adverse effect. Test duration should reflect the biology of the surrogate (e.g., susceptible stage, generation time), the life cycle of the plant species, the nature of the PIP and the dose selected. For example, a higher dose may allow for a shorter duration and the length of time the PIP is expressed by the plant or within a specific plant tissue must be considered. Test diet should be periodically changed and concentrations of active toxin should be verified.

Controls
Controls are typically included in experiments as indicators of the suitability of the test system and for comparison to the data generated for the treatment(s) of interest. For non-target organism (NTO) testing, several specific attributes of the test system are of interest. Negative controls are included to assess the suitability (health) of the test organism and the test conditions (e.g., temperature and diet) and/or to evaluate potential effects of the matrix or formulation in which the test protein is delivered. Positive controls are included to confirm that the test organism is exposed to the test protein and, in some cases, to assess the sensitivity of the test organism to a standard toxicant.
Triggers for testing beyond Tiers I and II

Higher tier tests may be triggered when results of lower-tier laboratory studies indicate a potentially unacceptable risk. Limitations to laboratory-based testing may also result in the need for field or semi-field testing. However, further lower-tier studies may be more suitable depending on the hypothesis to be tested.

Field testing may be needed for a risk assessment, particularly for new or novel proteins that lack existing research or history of safe use. Tier III and IV testing may be needed because levels and routes of test material exposure used in the laboratory may not be realistic; effects assessments are made after short-term exposure of organisms, not lifetime exposure as might occur in the field; organisms in the field are subject to supplementary stresses that have additive effects that may amplify impacts that occur under the optimal physical and biological conditions of laboratory tests; and laboratory tests cannot practically evaluate all species that are actually exposed to test substances in the field. However, these perceived limitations can be addressed by an appropriate tier-based testing approach which combines hazard data generated in the laboratory with field-based exposure data to place laboratory results within a field context.

Tiers III and IV field testing

Overall, field and semi-field studies are the most direct way to assess potential impacts of Bt crops on non-target organisms at a community-level. Their need should be considered on a case-by-case basis. In addition, field studies have their own limitations. Large plot field studies may result in significant within-plot variation leading to the need for additional sampling for precise estimation (EPA-SAP 2004). Therefore, intermediate testing such as extended laboratory tests with realistic substrates and exposure scenarios and semi-field tests, rather than census studies are recommended. Semi-field tests may test individual organisms or multiple organisms in microcosms (small systems similar to larger systems in constitution, configuration or development), mesocosms (surrogate for real ecosystem that are sufficient in size to meet all of the components of interest), field cages or contained arenas. Semi-field tests provide a bridge between laboratory and field and can be used for gathering acute and chronic data on individuals and populations.

The need for field monitoring studies should be considered case-by-case, based on the level of potential hazard and exposure, and goals should be adjusted as information and experience accumulate (EPA-SAP 2001, 2004). When field studies are deemed necessary, the field studies should be conducted on a large enough scale and with an appropriate design and sampling regime to account for the specificity and season-long expression of the PIP being evaluated. The nature of sampling including the method, scale and timing should be relevant to the species being evaluated. A statistically valid test with enough power to determine treatment effects will require an appropriate study design with respect to plot size, replication, sampling method and sample size (EPA-SAP 2004). Appropriate positive and negative controls, as well as specified endpoints, need to be considered (EPA-SAP 2004). Because of test plot size and power of test considerations (in terms of experimental materials and locations), the conduct of Tier III-IV field studies is frequently not practical prior to commercial registration.
Stacked (Combined) trait and pyramided products

Combined or stacked trait products are crops with two or more genes introduced with different modes of action and/or spectra of activity. For example, crops modified to contain two insect-resistance genes, or an insect-resistant gene and a herbicide tolerant gene, are considered stacked or combined trait products. The term "pyramid" is used to describe the special case where multiple resistance genes encoding PIPs are present that target the same pests with possible overlap in the mode of action. For example, a corn or cotton plant containing a Cry1A protein and a Cry2A protein active against the same lepidopteran pest such as the European corn borer or tobacco budworm is considered to have two "pyramided" PIPs.

It is possible that inserting two genes into a plant may result in different effects on non-target organisms than a single insertion. However, studies conducted thus far with Bt microbial formulations and PIPs have not demonstrated synergistic or antagonistic interactions between different proteins known to be independently active. It is unlikely that a plant containing two different genes as a result of combining two single traits by breeding will result in an increased hazard to non-target arthropods compared to the single-trait parental lines. Therefore, non-target data may not be necessary provided the protein expression levels are the same in single gene plants and susceptibility of target pests to the combined PIPs is comparable to their susceptibility to the individual traits. If there is no difference in susceptibility to the combined versus the individual expressed proteins among susceptible target insects, then it is unlikely that there will be a difference in susceptibility among non-target organisms.

References


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Farm questionnaires for monitoring the cultivation of genetically modified maize

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In cooperation with: Bayer CropScience, KWS SAAT AG, Monsanto, Pioneer Hi-Bred International, Syngenta Seeds GmbH

Abstract: The Federal Biological Research Centre for Agriculture and Forestry (BBA), maize breeders and statisticians have worked out a questionnaire for farmers to report on their observations of effects linked to the cultivation of genetically modified crops forming a part of a mandatory GMO monitoring regime. Questionnaires provide a low-cost opportunity for data acquisition - the farmers involved supply data on the actual cultivation of a crop. In a first step, the questionnaire on genetically modified (GM) maize was designed. The questions focus on potential effects related to the GM maize grown, as well as on background information describing the cultivation methods and the site-specific situations. In further steps the questionnaire will be adapted for additional crops and optimised as a tool and basis for statistical analysis.

Key words: general surveillance, case specific monitoring

General concept

The objective of the GMO monitoring is to confirm or identify the occurrence of adverse effects caused by cultivation of a GMO or its use on animal/human health or the environment. Therefore, effective monitoring regimes have to be established by using different tools and data sources that are appropriate for the specific task and can be combined in an overall analysis.

There are several reasons why farmers should be addressed in a GMO monitoring program:
• many effects of GM crops will firstly occur in agricultural areas
• the farmer “monitors” the fields for his own planning
• the farmer knows his (long-term) farming practice best
• the farmer knows the (long-term) local situation

Farm questionnaires are an efficiently and easily handled tool for GMO monitoring that specifically exploits the farmers’ knowledge and experience. The advantages of such an approach are:
• low organisational efforts
• site specific data
• easy access to detailed agricultural data
• incorporation of farmers’ knowledge.
Nevertheless, there might be limits to the extent and the quality of data:

- farmers’ limited readiness to participate
- limited monitoring objectives that are relevant for farmers
- contrast of statistical and scientific proofs vs. practical efforts.

Thus data acquisition by questionnaires needs to balance the demand on detailed information and achievable answers. Moreover, the outcome needs to be accessible for a scientifically sound analysis and further interpretation.

In Germany, a farm questionnaire for monitoring the cultivation of GM maize has been developed and tested. The questionnaire asks for observations made and additional background information. There are several questions addressing the effects of GM maize (Table 1) as well as the agricultural regime (Table 2).

Table 1. Questions concerning effects of GM maize.

<table>
<thead>
<tr>
<th>Class of questions...</th>
<th>...in relation to GM crop effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant development</td>
<td>Traits of the GM variety</td>
</tr>
<tr>
<td>Diseases and pests of maize</td>
<td>Effects on plant protection regimes</td>
</tr>
<tr>
<td>Abundance of <em>Ostrinia</em> (BT-Maize)</td>
<td>Effects on plant protection regimes (resistance management)</td>
</tr>
<tr>
<td>Abundance of beneficial insects</td>
<td>Effects on biodiversity / sustainability</td>
</tr>
<tr>
<td>Weed populations / abundance (HT-Maize)</td>
<td>Effects on plant protection regimes (Resistance management)</td>
</tr>
<tr>
<td>Comments on cropping and application</td>
<td>Evaluation of agricultural practice</td>
</tr>
<tr>
<td></td>
<td>Indirect effects of cultivation methods</td>
</tr>
</tbody>
</table>

Table 2. Questions concerning the agricultural regime.

<table>
<thead>
<tr>
<th>Class of questions...</th>
<th>...in relation to the GMO monitoring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil quality</td>
<td>Impact of geology</td>
</tr>
<tr>
<td>Weather</td>
<td>Impact of climate</td>
</tr>
<tr>
<td>Tillage</td>
<td></td>
</tr>
<tr>
<td>Dates of sowings and harvest</td>
<td>Impact of agricultural management</td>
</tr>
<tr>
<td>Pesticide management</td>
<td></td>
</tr>
<tr>
<td>Fertilizer management</td>
<td></td>
</tr>
<tr>
<td>Rotation of crops</td>
<td></td>
</tr>
<tr>
<td>Use of products</td>
<td>Traceability</td>
</tr>
</tbody>
</table>

Most questions are supplied with different possible categories of answers (e.g. occurrence of diseases with values: none/less – normal – increased, including the category “uncertain”) to ensure the comparability of answers. Additionally free text entries are possible to include unforced opinions and comments.
All data (answers) are stored in a computerised data base, which enables to check the plausibility and quality of answers. After such a step of pre-processing, the data (tables) are transferred to a statistical software system for analysis. The data are analysed for any differences to normal biological variability, for differences between GM and non-GM maize cultivation (e.g. abundance of diseases in maize fields) or exceeding of given thresholds. Such thresholds have to be defined in advance (e.g. the expected abundance of corn borer in GM maize fields can be defined by the accuracy in seed production: there may be 2 % non-BT plants possibly bearing corn borers). Significant differences are analysed for their causal connection with GM maize cultivation or other impact factors like climate or agricultural practice. Temporal or spatial trends can be followed as well as correlations with such factors or special events (e.g. time lines of abundances of weeds and spatial distributions).

A print version of the questionnaire is available at http://www.bba.de/ (menu “Gentechnik”). In the years 2000 - 2004, 183 questionnaires covering 175 GMO and 173 non-GMO fields have been returned and analysed. No adverse effects of GM maize have been found so far.

**Necessary number of questionnaires and statistical analysis**

The data recorded for GMO monitoring are of different value for analysis: since each character is subjected to complex dynamics in agriculture and nature, it is of limited value to survey only those characters that might be influenced by GMOs and not to analyse the difference between GM crop and conventional cultivation. In fact, all factors that may have a considerable influence on the observed character should be collected. Each character can be assigned to one of the possible types – the intrinsic monitoring characters (e.g. development, diseases, and weed presence) and the influencing factors (e.g. agricultural practices, soil parameters). The intrinsic monitoring characters are of paramount interest whereas the values of the influencing factors are recorded to analyse the causes of any effects on the monitoring characters (Figure 1).

**Statistical procedures**

Using statistical procedures the monitored characters can be analysed for differences or significant deviations from “normality” and correlations for these deviations can be detected in the whole complex system. With this approach the “GM plant cultivation” is also a factor (factor under examination) and any effects (differences) in the monitoring characters can be analysed for their relation to this cultivation. Therefore, the whole statistical model can be described as in Figure 2.

It is recommended that planning and analysing of GMO monitoring data should be performed by sequential procedures. These procedures work without fixing a sample size. A decision about continuing or stopping data sampling can be made at any time on the basis of an interim (meantime) analysis of the data acquired so far; e.g. every year an interim assessment of possible adverse effects can be done. The progression of a sequential procedure can be displayed graphically as a sequential path in a coordinate system, where the sample size is plotted at the x-coordinate and the value for a decision statistic is plotted at the y-coordinate. Within the coordinate system a continuation area will be defined. As long as the path is within this area, the data acquisition will be continued; when the path reaches the border of this area, the test decision will be met (Figure 3).
Figure 1. An intrinsic monitoring character (central box) and its influencing factors.

Figure 2. The statistical model for the monitoring characters.
The main objective of General Surveillance is to detect unforeseen effects. The statistical procedure to answer this question is a statistical test with the null hypothesis $H_0$ that there is no effect (no statistically significant difference between the values of a character in GM and non-GM maize cultivation) and the alternative hypothesis $H_1$ that there is an effect (difference).

The data acquisition in GMO monitoring is to be done over the period of ten years (or more) set by the regulatory framework. Sequential procedures offer a flexible strategy to split the total sample number up to these ten years. It is useful to analyse the accumulated data each year to see any trends or effects at an early stage. On the other hand these procedures can come quickly to a test decision if there is probably no effect at all, allowing the monitoring to be as cost effective as possible. This approach will be especially useful for the Case Specific Monitoring. Whether it will be applicable for General Surveillance – in the existing regulatory framework in Europe – needs further consideration.

Since this kind of analysis considers only one influencing factor (GM plant cultivation) and a range of other factors is also likely to have some influence, it is important to ensure that the GMO influence can be clearly separated from other factors. In order to do this, the compounding impact of the other influences has to be calculated (for instance with propensity scores) and allowances have to be made for these factors.
Sample size determination

GMO monitoring includes data sampling for several characters. Data are generated by existing networks and farm questionnaires. The sample size for existing networks can’t be influenced, but the number of questionnaires to be analyzed has to be fixed in accordance with statistical demands.

Statistical sample size determination – like statistical analysis – can be done only for each character to be monitored separately, since each character has its own distribution. To get an adequate sample size for all characters of the questionnaire either the most important character has to be selected for size determination or the maximum sample size of all characters has to be chosen.

Data that are surveyed to see whether GM crop cultivation results in any differences to “normality” may be regarded as the most important characters. They may be recorded within the questionnaires as mainly ordinally distributed characters with three possible values (e.g. earlier / normal / later).

Transferred to “statistical language” a deviation from normality could be formulated as demonstrated by the following example:

*Character:* germination
*Possible values:* earlier – normal – later
*Normality:* the plants germinate “normal at at least 90% of the fields (sample sites)”, at most 10% germinate earlier or later – with that a certain distribution pattern will be defined

*Difference to normality:* the plants germinate earlier or later at more than 10% of the fields → a different pattern (Figure 4).

![Figure 4. Distribution patterns of an ordinally distributed character with three possible values (left: pattern between GMO and non-GMO differ, right: patterns are similar).](image)

The experimental design for the sequential procedure for the test whether the patterns of an ordinally distributed character differs between GM and non-GM maize cultivation with \( \alpha = 0.05 \) and \( \beta = 0.01 \) (high power of 99% for the test since it is important to find existing effects) leads to an expected total sample size of 668 – 896, a fixed sample size of 1252 and a maximum sample size of 2130 (Figure 5).
In a sequential procedure, the sample size is not fixed. The fixed sample size is for comparison only. The expected sample sizes provide the range how many questionnaires have to be surveyed until a decision will be possible. The maximum describes the number of questionnaires that has to be surveyed in worst case.

The survey will be done by starting with approx. 1/10 of 2130 questionnaires in the first year (if possible; otherwise as many as possible allowing for the phased introduction of the GM crop and scaling up in later years). An interim analysis of all characters of interest will result in the first section of the paths within the continuation areas. The survey will be continued for those characters whose paths did not yet leave the area. At the end, decisions about all characters and of the further monitoring strategy should be possible.

**Conclusions**

In a GMO monitoring regime farm questionnaires offer a low cost option to acquire monitoring relevant data at farm level. Statistical analysis provides a scientifically sound decision process. Sequential statistical procedures allow a clear decision on continuation for Case Specific Monitoring as well as on strategic options for General Surveillance.
References


Toxigenic micromycetes and their mycotoxins in grains of transgenic Bt-maize hybrid and non-transgenic hybrids

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Abstract: In 2002-2004 we have studied the efficacy of Bt -maize to control the European Corn Borer (ECB) and grain infection by toxigenic micromycetes in comparison with biological control by the introduction of Trichogramma wasp and untreated control hybrid in two localities in the Czech Republic (Praha-Ruzyně and Ivanovice na Hané). Injury of plants caused by ECB differed according to locality. At locality Ivanovice na Hané, higher occurrence of pest and toxigenic micromycetes species was recorded. Bt-maize showed a high level of resistance to ECB, there were no injured plants during our survey. In grain samples, a total of 15 taxa of the genus Fusarium and 9 taxa of the genus Penicillium were identified. A similar complex of micromycetes was recorded on Bt maize and the non-transgenic hybrids. But the frequency of Fusarium species was significantly reduced in Bt-maize when compared to the mean of all non-transgenic hybrids. Reduction in frequency in Bt-maize was 35.3% for Fusarium oxysporum, by 100% for F. proliferatum, by 61.6% for F. sporotrichioides, by 32.4% for F. subglutinans and by 77.6% for F. verticillioides. Bt-maize was found to be infected by toxigenic micromycetes at a lower level compared to non-transgenic hybrids and grain was found to contain lower amounts of three selected mycotoxins (FUM, DON and ZEA).

Key words: maize, Bt-maize, Bacillus thuringiensis, toxigenic micromycetes, Fusarium, Penicillium, Ostrinia nubilalis, ECB, biological protection, Trichogramma, mycotoxins

Introduction

The European corn borer (ECB) is a major pest of maize in Central Europe suspected to promote the infection of maize with Fusarium species (Magg et al., 2003). The Fusarium species infect maize at emergence or after plants have been damaged by insect or birds (Reid, 1999). Injuries of plants caused by ECB larvae are often the initial infection sites for Fusarium species (Lew et al., 1991; Munkvold et al., 1997, 1999). The most effective protection of maize against ECB is the use of transgenic Bt maize that contents cry genes derived from the soil bacterium Bacillus thuringiensis. The injuries of plants by ECB and the occurrence of Fusarium species are reduced in Bt-maize and the content of mycotoxins in grains of Bt-maize is significantly lower compared to non-transgenic hybrids (Munkvold et al., 1999; Clements et al., 2003; Magg et al., 2002, 2003). The occurrence of mycotoxins in grains decreases product quality and has unfavourable influence on human and animal health (Magg et al., 2003).

The aim of this study was to determine the spectrum of toxigenic species of micromycetes in grains of Bt-maize after mechanical injury and in non-transgenic maize after injury by ECB larvae. In addition, the content of mycotoxins in grains was determined.
Material and methods

Field experiments were conducted at two localities, Praha-Ruzyně (middle Bohemia) and Ivanovice na Hané (south Moravia), in 2002-2004. The maize treatments used for the evaluation of ECB resistance, occurrence of micromycetes and mycotoxins concentration were: 1) transgenic Bt-maize (MON 810; DKC3421YG), 2) non-transgenic isolinie (DKC3420) 3) local hybrid Raissa, 4) isolinie (DKC3420) with biological protection by Trichogramma wasps, 5) Raissa with biological protection by Trichogramma wasps.

The ears of non-transgenic hybrids injured by ECB and with visible mycelium on the ear surface were collected. In Bt-maize, ears with mechanical injury (caused by birds, environmental conditions) or with visible mycelium on the surface were collected.

Two methods of isolation of micromycetes were used - direct isolation of mycelium from the surface of ears and surface sterilization of kernels with sterile water. The basic media used for isolation were: Malt extract agar (MEA), Cornmeal agar (CMA) and Soil agar with glucose and bengal rose (SEGA). The Fusarium species were identified on: Synthetic nutrient-poor agar (SNA), Oatmeal agar (OA) and Potato-dextrose agar (PDA), the Penicillium species were identified on: MEA and Czapek yeast agar (CYA). The micromycetes were determined using the following keys: Booth (1971), Brayford (1993), Burgess (1988), Pitt (1979, 1991), Ramirez (1982), Samson et al. (1996) and Seifert (1996).

The content of mycotoxins - deoxynivalenol (DON), fumonisins (FUM) and zearalenol (ZEA) was assessed by employing an immunoassay method ELISA. A random sample from each treatment from both localities were taken. A subsample of about 100g was ground and meal was taken for the subsequent analyses of mycotoxin concentrations. The limit of DON detection (LOD) was 0.1 mg/kg (ppm) and the limit of DON quantification (LOQ) was 1.25-12.5 mg/kg (ppm). LOD of ZEA was 50 µg/kg (ppb) and LOQ of ZEA was 250-3000 µg/kg (ppb). LOD of FUM was 0.2 mg/kg (ppm) and LOQ of FUM was 10-60 mg/kg (ppm).

The frequency of species on maize ears were recorded and the effect of environmental factors and treatment strategy was statistically analysed using the multivariate analysis Canoco. Dependence of occurrence of species on years and treatments were tested. Statistical analysis included only species, that were isolated more than once.

Results

In 2002-2004, a total of 180 ears in total (36 ears from each of the five treatments) were obtained (Table 1). In total, 24 taxons of micromycetes were isolated (15 Fusarium species and 9 Penicillium species). The total frequency of all species isolated from preharvest maize from both localities during three years and from the different treatments are given in Table 1. The most frequent species were Fusarium subglutinans (33.8%), F. proliferatum (11.1%), F. verticillioides (10.5%), F. oxysporum (8.0%), F. sporotrichioides (6.4%) and Penicillium hordei (1.9%). The species F. proliferatum was not isolated from Bt-maize. Occurrence of F. subglutinans and F. verticillioides, which are mentioned in literature as species occuring after injury caused by ECB, were reduced on Bt-maize when compared to the non-transgenic hybrids.

The frequency of Fusarium species was significantly lower in Bt-maize with mechanical injury when compared with the mean of all non-transgenic hybrids by 35.3% for F. oxysporum, by 100% for F. proliferatum, by 61.6% for F. sporotrichioides, by 32.4% for F. subglutinans and by 77.6% for F. verticillioides.
Table 1. Frequency of all species of micromycetes isolated from preharvest maize in two localities (Praha-Ruzyně and Ivanovice na Hané) in 2002-2004 (total of 180 ears). Annotation: 1 - Bt-maize, 2 - Isolínie, 3 - Raissa, 4 - Isolinie + Trichogramma, 5 - Raissa + Trichogramma.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species of fungi (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusarium subglutinans</td>
<td>24.4</td>
<td>40.3</td>
<td>34.7</td>
<td>40.3</td>
<td>29.1</td>
<td>33.8</td>
</tr>
<tr>
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<td>12.5</td>
<td>16.7</td>
<td>16.7</td>
<td>11.1</td>
</tr>
<tr>
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<td>2.8</td>
<td>8.3</td>
<td>16.7</td>
<td>2.8</td>
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<td>10.5</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>5.6</td>
<td>6.9</td>
<td>11.1</td>
<td>11.1</td>
<td>5.5</td>
<td>8.0</td>
</tr>
<tr>
<td>Fusarium sp. div.</td>
<td>2.8</td>
<td>11.1</td>
<td>5.6</td>
<td>5.6</td>
<td>6.9</td>
<td>6.4</td>
</tr>
<tr>
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<td>1.4</td>
<td>7.0</td>
<td>9.7</td>
<td>6.4</td>
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<td>0.0</td>
<td>2.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Fusarium culmorum</td>
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<td>4.2</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Fusarium avenaceum</td>
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<td>0.0</td>
<td>1.4</td>
<td>2.8</td>
<td>0.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Fusarium acuminatum</td>
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<td>0.0</td>
<td>1.4</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Fusarium cf. avenaceum</td>
<td>1.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Fusarium cf. equiseti</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Fusarium graminearum</td>
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<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Fusarium incarnatum</td>
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<td>1.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Fusarium solani</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Fusarium tricinctum</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Penicillium hordei</td>
<td>0.0</td>
<td>4.2</td>
<td>1.4</td>
<td>0.0</td>
<td>4.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Penicillium purpurogenum var. rubrisclerotium</td>
<td>1.4</td>
<td>2.8</td>
<td>0.0</td>
<td>4.2</td>
<td>1.4</td>
<td>1.9</td>
</tr>
<tr>
<td>Penicillium sp. div.</td>
<td>0.0</td>
<td>2.8</td>
<td>0.0</td>
<td>2.8</td>
<td>0.0</td>
<td>1.1</td>
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<td>Penicillium islandicum</td>
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<td>0.0</td>
<td>1.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Penicillium cf. islandicum</td>
<td>0.0</td>
<td>0.0</td>
<td>2.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Penicillium pulvillorum</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>1.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Penicillium brevicompactum</td>
<td>1.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Penicillium canescens</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Penicillium cf. spinulosum</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Penicillium thomii</td>
<td>1.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Penicillium viridicatum</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Total no. of species</strong></td>
<td>13</td>
<td>11</td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>27</td>
</tr>
</tbody>
</table>

The spectrum of toxigenic species in maize grains differed among years. In 2002, the most frequent species was *Fusarium oxysporum*, in 2003, the most frequent species were *F. verticillioides* and *F. proliferatum* and in 2004, the most frequent species were *F. subglutinans* and *F. sp. div.* (Figure 1).

The spectrum of toxigenic species in maize grains did not differ among treatments. In Bt-maize, no micromycete species prevailed when compared to the non-transgenic hybrids. The most frequent species on the all treatments was *F. subglutinans*. The frequent species on the non-transgenic isoline were *Fusarium sp. div.*, *F. sporotrichioides* and *P. hordei*, on hybrid Raissa the frequent species were *F. verticillioides* and *P. cf. islandicum*. The frequent species on the non-transgenic isoline with *Trichogramma* wasps applied were *F. proliferatum* and *P. purpurogenum* var. *rubrisclerotium*, on hybrid Raissa the frequent species were *F. verticillioides* and *P. hordei* (Figure 2).
Content of mycotoxins

In 2002, the content of mycotoxins in Bt-maize was below the limit of detection (Table 2). In 2003, the content of mycotoxins in all the samples was below the limit of detection. In 2004, mycotoxin DON was detected in higher content in all samples, including Bt-maize (Table 3). In 2002-2004, Bt-maize samples contained a much lower levels of mycotoxins when compared to the non-transgenic hybrids.

Table 2. Content of mycotoxins above limit of detection from corn in localities Praha-Ruzyně (L1) and Ivanovice na Hané (L2) in 2002.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Bt-maize</th>
<th>Isolinie</th>
<th>Isolinie+ Trichogramma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Locality</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L1</td>
<td>L2</td>
<td>L1</td>
</tr>
<tr>
<td>DON (ppb)</td>
<td>0.0</td>
<td>0.0</td>
<td>22.8</td>
</tr>
<tr>
<td>ZEA (ppb)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>FUM (ppm)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Table 3. Content of mycotoxins above limit of detection from corn in localities Praha-Ruzyně (L1) and Ivanovice na Hané (L2) in 2004.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Bt-maize</th>
<th>Isolinie</th>
<th>Isolinie+ Trichogramma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locality</td>
<td>L1</td>
<td>L2</td>
<td>L1</td>
</tr>
<tr>
<td>DON (ppb)</td>
<td>106.9</td>
<td>40.5</td>
<td>116.4</td>
</tr>
<tr>
<td>ZEA (ppb)</td>
<td>0.0</td>
<td>0.0</td>
<td>50.1</td>
</tr>
<tr>
<td>FUM (ppm)</td>
<td>0.8</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Discussion

Our study revealed that transgenic Bt-maize expressing cry genes from the bacteria Bacillus thuringiensis showed resistance to ECB. The occurrence of cry genes causes the production of δ-endotoxin in green plant tissues. The expression of cry genes in leaves and stalks causes mortality of ECB larvae after feeding (Magg *et al.*, 2001) and consequently, we have not detected any ECB damaged Bt-maize plant during our survey. In correspondence with Clements *et al.* (2003), Bt-maize had significantly lower Fusarium infection of kernels and lower concentration of mycotoxins compared to the non-transgenic hybrids. Reduced Fusarium species in Bt-maize resulted in better grain quality and decreased potential for the development of mycotoxins (Munkvold *et al.*, 1997). The occurrence of *F. proliferatum*, *F. subglutinans* and *F. verticillioides*, which are mentioned in literature as species occurring after injury caused by ECB (Gatch & Munkvold, 2002), was reduced on Bt-maize compared to the non-transgenic hybrids.

It has been reported before that the composition of fungal species differs between Bt-maize and non-transgenic hybrids (Gatch & Munkvold, 2002). In contrast to this, the composition of toxigenic micromycetes was similar between Bt and non-transgenic hybrids in our study. The main difference among hybrids was in the frequency of the occurrence of single species. *F. subglutinans* and *F. verticillioides* were among the three most frequently isolated species from all treatments. Logrieco *et al.* (2002) reported that the occurrence of these species was the most frequent from isolation in Europe in the last ten years.

The results from the mycotoxin analysis show that the occurrence of micromycetes differs among years. However, the occurrence of toxigenic species does not need to be in correspondence with the occurrence of mycotoxins in maize grain since the production of mycotoxins is influenced by many factors, including moisture, temperature, present oxygen and oxide carbonic, and the occurrence of insect herbivores.

Acknowledgements

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Testing the impact on non-target organisms of insecticidal proteins expressed in transgenic crops

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Abstract: The potential effects on non-target organisms (NTOs) of insecticidal proteins expressed by transgenic crops have to be evaluated in ways similar to the testing of conventional plant protection products. The exposure patterns of insecticidal proteins expressed in transgenic crops will be different to conventional insecticidal compounds. Therefore current methods have to be modified to allow successful and reliable testing of these compounds. Syngenta has developed transgenic maize expressing a modified version of a native cry3A gene, which is a member of a class of cry genes found in Bacillus thuringiensis (Bt) subsp. tenebrionis. The modified Cry3A protein (mCry3A) has enhanced activity against the Western corn rootworm (Diabrotica virgifera virgifera) and Northern corn rootworm (Diabrotica longicornis barberi). As part of the environmental safety assessment of this maize, the hazard (toxicity) of mCry3A to various non-target arthropods was tested. The test species were chosen to represent some of the main functional groups present in maize crops, for their taxonomic relatedness to the target pest and also for their suitability for laboratory testing. Species tested included Poecilus cupreus, Aleochara bilineata, Coccinella septempunctata, and Orius insidiosus. Laboratory tests were conducted using microbially-produced protein introduced in the test species’ diets at concentrations at least ten times the expected environmental concentration. This allowed the organisms to be exposed to the protein orally, with continuous exposure to a high ‘worst-case’ dose. An orally-active insect growth regulator was used as a toxic standard and water as a control. Brief descriptions of the methods are given as examples of how protocols developed for studies of crop protection chemicals can be adapted for studies of insecticidal proteins. We also describe diet analysis studies to investigate whether the protein remained active within the diets during the toxicity tests. No adverse effects due to the test protein were observed on any of the NTO species tested and exposure to intact, bioactive mCry3A was confirmed.

Key words: transgenic crops, non-target organisms, insecticidal protein, maize, Cry toxin, Western corn rootworm

Introduction

Some plant protection products (PPPs) can, by their nature, be potentially harmful to non-target organisms (NTOs) such as the predators and parasitoids of crop pests, and other beneficial organisms such as honeybees and earthworms. These compounds must be tested according to government regulations before they can be registered for use (for example, Barrett et al., 1994; Candolfi et al., 2000; OECD, 1984; OECD, 1994). Testing involves measuring the toxicity of the PPP to representative indicator organisms. Initially, several organisms were tested, but experience has shown that two species (Aphidius rhopalosiphi and Typhlodromus pyri) are the most sensitive to most PPPs, and usually only these species are tested in the first instance. In the case of insecticidal proteins expressed by transgenic crops,
the potential effects on NTOs also have to be evaluated. However, existing regulations for transgenic crops are not as specific about the species to test, mainly because the proteins have different and very specific ranges of target pests (in most cases they are limited to a few species in a single family) and because relatively few proteins have been tested against a range of NTOs. Therefore it is not yet possible to recommend a standard set of sensitive indicator organisms. While it is expected that a PPP may have a harmful effect on NTOs, proteins expressed in transgenic crop plants are often more specific so are likely to have limited or no effects on NTOs. The exposure patterns will also be different between proteins in transgenic crops plants and sprayed compounds. Exposure is likely to be oral and long-term with a transgenic crop protein, as opposed to the contact and relatively shorter exposure of sprayed compounds. Therefore current methods have to be adapted to allow successful and reliable testing of proteins expressed in transgenic crops. Syngenta has developed a transgenic maize, Event MIR604, expressing a modified version of a native cry3A gene, which is a member of a class of cry genes found in Bacillus thuringiensis (Bt) subsp. tenebrionis (Sekar et al., 1987), to provide resistance to several beetle pests. The mCry3A protein has activity against the western corn rootworm Diabrotica virgifera virgifera LeConte and Northern Corn rootworm (Diabrotica longicornis barberi) (Coleoptera: Chrysomelidae), two serious pests of maize in the USA, and other related coleopteran maize pests. This insecticidal protein is expected to be expressed throughout the transgenic maize plant. However, only trace levels have been found in the pollen.

As part of the environmental safety assessment of Event MIR604, the hazard (toxicity) of mCry3A to various non-target arthropods was tested. The NTO test species were chosen to represent some of the main functional groups relevant to maize crops, for their taxonomic relatedness to the target pest and for their suitability for laboratory testing. The aim was to keep the test conditions, control mortality and validity criteria at similar levels to those in the established guidelines so that these tests were comparable to conventional tests for sprayed compounds. This paper presents the test methods used for four example insect species.

Materials and methods

Laboratory tests were conducted using preparations of microbially-expressed mCry3A protein introduced in the test species’ diets. The protein was purified from cells of recombinant Escherichia coli expressing the coding gene for mCry3A. The conventional test diets of the various test species were adapted, or alternative ones developed, to allow the protein to be introduced to the organisms orally. This allowed for a continuous exposure of the organism to the protein. The organisms were exposed to a high ‘worst-case’ dose of the protein, based on ten times the expected environmental concentration of mCry3A to which similar organisms would be exposed in a field of Event MIR604. An orally-active insect growth regulator (150 g/L teflubenzuron) was used as a toxic standard (or positive control) to demonstrate the effectiveness of the test system. Water-treated diet was used as a negative control. Treatment concentrations were measured in µg protein per g of diet (µg/mL for the treatment solution used to prepare the diet for C. septempunctata).

Poecilus cupreus
Larvae of the ground beetle Poecilus cupreus (L.) (Coleoptera: Carabidae) are free-living, soil-dwelling predators. In conventional testing they are normally fed blowfly pupae (Heimbach et al., 2000). In this case, the blowfly pupae were individually injected with 1 µL of the treatment solution (either the protein dissolved in water, the toxic standard, or just water) using a fine-tipped pipette. These treated pupae were then fed to the beetle larvae. All the pupae required for the test were prepared together prior to test initiation and stored in a
freeze (-20°C) until needed. Treated pupae (defrosted) were offered to the beetle larvae daily and any uneaten pupae were removed. Larvae were reared for up to 28 days in tubes of standard soil according to Heimbach et al. (2000), until they had developed to adulthood. There were forty replicate arenas per treatment, each holding one beetle larva. Pre-imaginal mortality and the initial weight of emergent adults were recorded.

*Aleochara bilineata*

The rove beetle *Aleochara bilineata* (Gyll.) (Coleoptera: Staphylinidae) is also a soil-dwelling predator. Larvae of *A. bilineata* parasitise dipteran pupae in the soil, therefore they would be difficult to expose directly to the protein. It was decided that the conventional test method would be followed, where first-generation adults are exposed to sprayed deposits of the test compound (Grimm et al., 2000). These beetles then get to parasitise fly pupae, and the second-generation emergence is assessed as the toxic endpoint. In this case, adults were fed a meat-based diet with the treatment solution added, and effects on their reproduction rates and survival of their offspring were assessed. The diet was minced beef, cooked beforehand to denature any enzymes present that could break down the protein, and blended with the treatment solution into a paste. Aliquots of 0.2 g fresh diet were presented to the beetles daily in small containers. The beetles were also supplied with damp cotton wool as a water source. Treated diets were kept frozen (-20°C) until needed.

For the first week of exposure, 20 adult beetles (10 males, 10 females) were kept in 9-cm-diameter plastic pots lined with filter paper. Keeping the beetles in these dishes encouraged regular contact between the beetles and the treated diet. After this time, the beetles were transferred to boxes containing damp quartz sand (as per the usual test method). The beetles were kept in these boxes for a further four weeks and fed the treated diets daily. Treatments were replicated four times. To assess beetle fecundity, batches of 500 pupae of *Delia antiqua* (Meigen) (Diptera: Anthomyiidae) were placed in trenches within the sand on three weekly occasions. After this period, the adult beetles were removed from the sand and discarded, and the sand and pupae left together for a further week. The fly pupae were then removed from the sand and the number of second-generation rove beetles emerging from them was assessed over the next six weeks (until fewer than two beetles per day emerged from the control pupae).

*Coccinella septempunctata*

The ladybird *Coccinella septempunctata* (L.) (Coleoptera: Coccinellidae) and other ladybird species are unlikely to be exposed directly to the protein because their main diet of aphids are themselves unlikely to be exposed to the protein; expression via the maize phloem is predicted to be relatively low. Some ladybird species (e.g. *Coleomegilla maculata*) eat pollen, but no mCry3A has been detected in pollen of Event MIR604. However, because of the taxonomic relatedness of ladybirds to the target pest beetle, and their importance as predators within the crop, it was decided to include them in the study program. The main problem with testing this species is that ladybird larvae only survive successfully on live aphids. Trials using dead, mashed or injected aphids proved unsuccessful. Therefore, procedures that allowed the protein to be introduced to the larvae via live aphids needed to be established. A method was developed whereby live pea aphids (*Acyrthosiphon pisum* Harris) were dipped into the treatment solution so that it coated the aphids’ cuticle. Live aphids were submerged in a solution containing a wetting agent (0.004% Agral 90) and the protein (or toxic standard) for approximately 30 seconds. The aphids were then removed and left to dry for about 30 minutes on filter paper. Most of the aphids survived this process and were actively moving again once the solution had dried.

Second-instar ladybird larvae were reared individually in Petri dishes until they pupated. They were supplied daily with an excess of freshly treated aphids, old aphids being removed.
before new ones added. There were forty replicate dishes per treatment, each holding one ladybird larva. After emerging from the pupae, the adult ladybirds were kept together in treatment groups for a further two weeks, and again supplied with treated live aphids. The negative control in this test was aphids treated with just 0.004% Agral 90. Development time and survival for the ladybird larvae and adults was assessed throughout the test period.

**Orius insidiosus**

*Orius insidiosus* (Say) (Heteroptera: Anthocoridae) is a leaf-foraging predatory bug, common in maize crops. The females lay eggs within plant tissues so may be exposed to plant-expressed compounds in this way. The bugs feed using sucking mouthparts, so exposing them to treated diets is more complicated than for insects with chewing mouthparts. The diet used in conventional tests is moth eggs (Bakker *et al.*, 2000), but these proved too small to inject with a treatment solution. Coating them would prove ineffective because the bugs primarily consume the egg contents, not the shell. A feeding method was therefore developed using an artificial meat-based diet. This diet consisted of minced beef and lambs liver, cooked to denature any enzymes and then blended together in an electric food mixer with yeast and honey. After this, egg, sucrose, water and a preservative (methyl p-hydroxybenzoate) were added and the mixture blended until a smooth paste had formed. The treatments were then incorporated into the diet, and the treated diet stored in a freezer until needed. Approximately 0.2 g of diet was placed into the excised lid of a 1.9-mL-capacity micro-centrifuge tube. A short length of Parafilm® was stretched thinly over the diet to seal it inside the lid and prevent it from drying out. The bugs were capable of piercing the Parafilm® with their mouthparts and suck out the contents. Two- to three-day-old *O. laevigatus* nymphs were reared individually in ventilated plastic containers until they reached adulthood (up to 21 days). Treatments were replicated 40 times. Bugs were provided with a fresh, sealed lid of diet each day, and damp cotton wool as a water source. The survival and development rate of the bugs was assessed.

**Diet analyses**

To show that active protein remained present in the treated diets throughout the studies, samples of the different insect diets (taken during and after the completion of the exposure studies) were quantitatively analysed for mCry3A protein by ELISA (enzyme-linked immunosorbent assay) and its integrity (intactness) was investigated by Western blot analysis. For the ELISA and Western blot analyses, the protein was extracted from the samples of treated diets. Additionally, the bioactivity of the protein in the *A. bilineata* and *O. insidiosus* diets was investigated in bioassays using larvae of the Colorado potato beetle (*CPB*; *Leptinotarsa decemlineata*), which is known to be sensitive to mCry3A, feeding on standard artificial CPB diet supplemented with the test diets. Samples of the water-treated diets used in the studies were used as negative controls. Untreated CPB diet was used as an additional negative control for the CPB bioassay. CPB diets containing mCry3A protein in the same concentrations as calculated for the exposure test diets were used as positive controls. CPB bioassays were not done on the *P. cupreus* and *C. septempunctata* diets because of the impracticalities of feeding those test diets to the CPB larvae.

**Results and discussion**

Control, treatment and toxic standard mortality observed in the four example studies are shown in Table 1, along with the validity criteria from the standard guidelines for these tests. No treatment (mCry3A) data were statistically significantly different from the negative controls in that test (*P* > 0.05). All toxic standard data were statistically significantly different from the controls in that test (*P* < 0.05). Where applicable, all control and toxic standard data
agreed with the values set in the guideline validity criteria for each species. All four tests were completed successfully, first time.

Table 1. Summary of data gathered in the exposure tests on the four example species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Assessment a</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control (untreated)</td>
</tr>
<tr>
<td>P. cupreus</td>
<td>Observed % pre-imaginal mortality</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Corrected % pre-imaginal mortality</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Validity criteria (%)</td>
<td>≤ 20</td>
</tr>
<tr>
<td></td>
<td>Mean adult weight (mg)</td>
<td>81.5</td>
</tr>
<tr>
<td>A. bilineata</td>
<td>Mean number of second generation progeny</td>
<td>647</td>
</tr>
<tr>
<td></td>
<td>% effect on reproduction</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Validity criteria (%)</td>
<td>&gt; 400 progeny</td>
</tr>
<tr>
<td>C. septempunctata</td>
<td>Observed % pre-imaginal mortality</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Corrected % pre-imaginal mortality</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Validity criteria (%) (pre-imaginal)</td>
<td>≤ 30</td>
</tr>
<tr>
<td></td>
<td>Observed % adult mortality</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>Corrected % adult mortality</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Validity criteria (%) (adult)</td>
<td>none published</td>
</tr>
<tr>
<td>O. insidiosus</td>
<td>Observed % mortality</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Corrected % mortality</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Validity criteria (%)</td>
<td>≤ 25</td>
</tr>
</tbody>
</table>

a All validity criteria are based on those stated in the conventional test guidelines for that species. Mortalities corrected with respect to the control according to Abbot (1925).
b No treatment (mCry3A) data were statistically significantly different from controls in that test ($P > 0.05$).
c All toxic standard data were statistically significantly different from controls in that test ($P < 0.05$).
Extraction of mCry3A from the test diets followed by ELISA showed a recovery of the following percent of the nominal diet concentration used in each species test: 95.6% for *O. insidiosus*, 91.7% for *A. bilineata* and 24% for *P. cupreus*; the concentration of mCry3A delivered to *C. septempunctata* via the aphid diet (w/w) was 18% of that in the treatment solution (w/v). No mCry3A was detected in any of the negative control diets.

Western blot analysis for each of the four test diets revealed a single immunoreactive band corresponding to the predicted molecular weight of mCry3A (ca. 67,700 Da). No immunoreactive material was detected in any of the negative control diets.

The results of the CPB bioassay (96 h exposure) for the *A. bilineata* and *O. insidiosus* diets are summarized in Figure 1. The test diets showed high levels of bioactivity, resulting in high, statistically significant larval mortality after 96 h for the CPB diets prepared with 10 and 20% w/w protein-treated test diets (data sets 3 and 4, Figure 1). The positive controls for the bioassays (data sets 5 and 6, Figure 1) gave similar statistically significant responses. The water-treated control diets (data sets 1 and 2, Figure 1) showed only low mortality, similar to the control CPB diets (data sets 7 and 8, Figure 1).

The data from all four exposure tests confirm that the methods adopted for each species were effective ways of orally exposing the test organisms to the test item. The negative (untreated diet) control data show that the organisms were able to develop successfully on the diets, without compromising study validity. The toxic standard data demonstrate that the route of exposure was effective. No adverse effects were observed in any of the mCry3A treatments, demonstrating that this protein is not harmful to *P. cupreus*, *A. bilineata*, *C. septempunctata* or *O. insidiosus* during their development when orally applied at a concentration based on up to ten times the expected environmental concentration within the transgenic maize crop, under laboratory test conditions.

The results of the diet analysis studies confirm that intact, bioactive mCry3A was present in all the mCry3A treated test diets. The high proportion of mCry3A recovered by ELISA supports the conclusion that mCry3A was present in the diets at sufficient concentrations. The Western blot analysis indicates that mCry3A did not degrade under the conditions of test diet preparation. Correspondingly, the bioactivity of the *A. bilineata* and *O. insidiosus* test diets against Colorado potato beetle larvae confirms that these species were exposed to active mCry3A. These results also indicate that mCry3A was stable under the conditions of preparation of the test diets and subsequent storage of the treated diet samples at ca. -20°C or below.

Whilst large parts of the four exposure tests were similar to the standard guidelines, the process of diet preparation and the daily addition of the diets to the arenas made these tests more time-consuming than the standard tests. Injecting the fly pupae, dipping the pea aphids and making up the *O. insidiosus* diet caps was particularly complex. None of the exposure tests were set up to provide a quantitative exposure of the protein to the organisms. A dose-response bioassay could be run using a protein with a known toxicity to the species tested here, to confirm the route of exposure by quantifying the amount of consumed protein. However, the analysis of the diets confirmed that all of the tested organisms were exposed to the protein at sufficient concentrations to confidently predict any effects of the protein on the non-targeted organisms. Although the protein recovery in the *P. cupreus* and *C. septempunctata* diets was lower than in the *A. bilineata* and *O. insidiosus* diets, protein concentrations in all four species’ diets were well above the expected environmental concentration for this transgenic maize crop, indicating the importance of dosing the test organisms with much higher concentrations than calculated from the levels of the risk of exposure.
Figure 1. Insecticidal activity of mCry3A protein in the *A. bilineata* and *O. insidiosus* test diets to Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) larvae, 96 h after exposure.

**Key to data sets:**
1. Control diet: CPB diet containing 10% (w/w) water-treated control diets (without mCry3A)
2. Control diet: CPB diet containing 20% water-treated control diets (without mCry3A)
3. Test diet: CPB diet containing 10% protein-treated test diets (mCry3A)
4. Test diet: CPB diet containing 20% protein-treated test diets (mCry3A)
5. Positive control diet: CPB diet treated with a solution of MCRY3A-0102 (1:1, v/v) to give a final concentration of 12.5 µg mCry3A/g
6. Positive control diet: CPB diet treated with a solution of MCRY3A-0102 (1:1, v/v) to give a final concentration of 50 µg mCry3A/g
7. Untreated control diet: CPB stock diet without any treatment
8. Control for positive control diet: CPB stock diet treated with water (1:1, v/v) to simulate the volume added to controls 5 and 6

Furthermore, the exposure methods described in this report represent a powerful tool for the testing of other orally active compounds, either agrochemicals or crop-expressed proteins.

**Acknowledgements**

The authors would like to thank the following people for help with this work: Demi Vlachos, Robert Joseph, Monica Garcia-Alonso and Stephen Vinall.
References


General surveillance for unanticipated effects of GM crops: the use of existing monitoring and surveillance networks

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(On behalf of the EFSA Working Group on Post Market Environmental Monitoring)

General surveillance of newly marketed GM crops should be largely based on routine observations and should be conducted over a range of sites and environments which are exposed to the GM crop or likely to be affected by it. A wide range of parameters should be observed and if unusual observations are reported, more focussed in-depth studies can be carried out in order to determine cause and relationship with GM crops. Existing surveillance systems should be used where practical e.g. routine farm recording systems, and any "abnormal" effects not usually occurring in similar situations with conventional cropping should be recorded. However, direct comparison with non-GM crop reference areas is not always practical or necessary. Reference can be made to the historical knowledge and experiences of the "observer" (e.g. farmers, inspectors, botanical surveyors) in relation to the situation prior to the introduction of the GM plant.

General surveillance (Gen Sur) should complement general environmental monitoring conducted by Member States. The higher the ecological integration and scale (from the individual to a population, from single farms to regions) the more difficult it is to distinguish potential effects of the GM plants from other factors. Initially, general surveillance should focus on each event individually. Ultimately, when several GM plants have been commercialised, the interactions between these GM plants and their management regimes should be examined where appropriate.

Surveillance of ecological interactions between different GMOs at a regional or national level may be considered primarily to be a governmental task and additional to the monitoring requirements for a single application following placing on the market. In the Directive 2001/18/EC, the possibility of additional surveillance by government authorities is described in Item 44 of the Conciliation Committee. Applicants for marketing of GM crops should be aware of all relevant surveys and monitoring in areas where the GM plants will be grown and should refer to the results of this monitoring in reports to the Competent Authority and the Commission, since the approach as stated in paragraph 1.3 of Council Decision 2002/811/EC (EC, 2002b) foresees monitoring in many cases as an evolving process.

Applicants should define the infrastructures that will be established and exploited in order to conduct general surveillance of regions where the GM plant is grown. Applicants should describe how to evaluate and select existing surveillance systems which are already monitoring one or more of the relevant parameters/elements. Applicants should describe how arrangements for collecting, collating and analysing data will be made.

Applicants should also identify which additional surveys will be asked to contribute to the general surveillance (for example, public institutions, farmer associations) in selected Member States. Although detailed arrangements may not have been agreed at the time of the application, applicants should describe how formal agreements and procedures will be
established with the Commission and Member States or other third parties before commercial market introduction.

According to Council Decision 2002/811/EC the responsibility for each step in the monitoring plan should be clearly assigned in the notification. Where third parties are employed or contracted to conduct monitoring studies, the structure of their involvement should be detailed.

The EFSA Working Group on General Surveillance has conducted stakeholder consultations in consideration of the use of existing monitoring networks in different Member States. Presentations have been received on existing agronomic networks in Spain, existing monitoring programs in Germany, Austria, the Netherlands and also existing European biodiversity monitoring networks. The following general conclusions have been reached:

- It is apparent that many of the existing surveys and networks collecting environmental data are unlikely to produce data of relevance or use in monitoring impacts of GM crops. This is for various reasons but often associated with the design of the survey, the time and scale of data collection, the update of the information. Existing European biodiversity monitoring programs are mainly focused on protected habitats or species (specially birds, increasing programs related to plants), have little common language (scope, location, methodology), systems are site-specific, there is no monitoring of unprotected parts of landscapes, and very few long-term (>10 years) monitoring programs. In addition methods and data in surveys are not harmonized within and between Member States. Existing national monitoring programs are often based on the use of volunteers. This may not be adequate for consistent long-term data collection.

- 80% of the biodiversity is in the unprotected part of the landscape and therefore it is often under the responsibility of Member States. In addition many non-agricultural systems are not monitored at all in Europe. Thus in addition to the need to amend the monitoring objectives of existing monitoring schemes and there is also a requirement for additional environmental surveys.

- In order to select appropriate networks it is important to decide which parameters need to be monitored through networks and then seek appropriate networks to do this. There may be a tendency to use a survey just because it exists (e.g. some bird surveys). Therefore it is also important in the Monitoring Plan to describe the types of surveys that could be used and selected. Methods and parameters for Gen Sur should be described in comparison with an established base-line. Potential cumulative long term effects (CLE) of the GMO could either be addressed within case-specific monitoring or general surveillance. The need to target CLE within either one of the two post-market approaches may depend on the level of uncertainty left after the risk assessment step.

The EFSA Working Group are also of the view that national and European systems should to be established to receive monitoring reports, collate and analyse data, compare data across states that are growing the GM crop and investigate adverse reports or incidences to determine cause and effect. All member states will need to establish Central Registers of GM crops to hold information on the geographical location of GM crops each season and to comply with EU regulations. Thus there is the feasibility of establishing Geographic Information Systems which can relate the growing of GM crops to crop production in regions over seasons and to a wide range of other geographic features including proximity of environmentally sensitive areas. This Central Register would be the logical place to also receive Monitoring reports and analyse data submitted. Thus a Central Register also becomes
a Data Base and allows study of many of the interacting factors which may be affecting areas
where GM crops are grown.
A new insecticidal preparation on the basis of *Bacillus thuringiensis* with insecto-acaridical activity

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Abstract: *Bacillus thuringiensis* based microbiological insecticides are widely used to protect crops and forests against insect pests. These insecticides vary in the spectrum of insecticidal activity, e.g. *B. thuringiensis* ssp. *kurstaki* DIPEL (USA) and Lepidocid (Russia) based insecticides are toxic only to Lepidoptera while *B. thuringiensis* ssp. *tenebrionis* NOVODOR (USA) and Colorado (Russia) preparations affect only Coleoptera. We have constructed a novel strain based on a production strain of *Bacillus thuringiensis* ssp. *kurstaki*, which has expanded insecticidal activity. This *Bacillus thuringiensis* strain represents a new generation of bioinsecticides with δ-endotoxins that are not only highly effective against Lepidoptera and Coleoptera pests, but also possess additional insecticidal activity lacking in current industrial insecticides, i.e. against Homoptera, Thysanoptera, Acariformes and other Arthropoda. Most of these pests are globally distributed and represent serious threat to many economically important cultures such as grains, legumes, vegetables, fruits, berries, cotton, forests, ornamental and medicinal plants and herbs. Presently, these pests are mainly controlled by chemical insecticides. Thus the insecticide derived from this strain not only combines effects of two industrial insecticides such as DIPEL and NOVODOR, but has a wider range of insecticidal activity. The new *Bacillus thuringiensis* B-8715 (Tyurin et al., 2004) is genetically stable and harmless to warm blooded animals and beneficial insects. The mechanism and basis of the insecticidal activity against sucking pests is currently unknown.

Key words: *Bacillus thuringiensis*, δ-endotoxins, microbiological insecticides, sucking arthropods, Homoptera, Thysanoptera, Acariformes

Introduction

The development of resistance to synthetic chemical insecto-acaricides has contributed to the increased use of microbials. Crop protection against arthropod pests, including highly resistant populations on transgenic plants, requires new bioinsecticides that combine the advantages of different industrial bioinsecticides, such as DIPEL and NOVODOR, with an expanded range of insecticidal activity.

We have developed a production strain based on δ-endotoxins of *B. thuringiensis* ssp. *kurstaki* with an increased range of insecticidal activity. In addition to high effectiveness against Lepidoptera and Coleoptera pests, the new *B. thuringiensis* strain possesses insecticidal activity that current industrial insecticides lack, i.e. against aphids (Homoptera, Aphidoidea), thrips (Thysanoptera, Thripidae) and spider mites (Acariformes, Tetranychidae).
Laboratory tests have indicated that the new *B. thuringiensis* ssp. *kurstaki* strain is genetically stable, and harmless to warm-blooded animals and beneficial insects. We believe this strain to be a good basis for the development of an industrial production of a new generation of plant protection against a wide range of pests.

**Material and methods**

**Investigated strains**

Bacterial strains used in this study include recombinant strain *B. thuringiensis* ssp. *kurstaki* RCIM B-8715 (Tyurin et al., 2004) and its parent variants, *B. thuringiensis* ssp. *tenebrionis* RCIM B-5081 and *B. thuringiensis* ssp. *kurstaki* HD-1 RCIM B-1226.

**Conditions of cultivation**

All 3 strains were cultivated in 750ml flasks until complete sporulation. The strains were cultured on a circular shaker at 28°C in 200 ml of medium containing 1% hydrolysate of casein, 0.2% yeast extract, 0.005% MnSO₄, 0.03% MgSO₄, 0.1% KH₂PO₄ and 0.6% glucose, pH 7.4-7.5, or 1% peptone. Definition of a credit viable dispute was carried out on agarose in Petri plates containing: 1% peptone 0.2% yeast extract, 0.005% MnSO₄, 0.03% MgSO₄ and 0.1% KH₂PO₄, pH 7.4-7.5.

**Study of δ-endotoxin production**

The culture was centrifuged, washed with 1M NaCl in the presence of 0.001M EDTA, and extracted with 0.1M carbonate buffer (pH 11.0, with 0.01M dithiothreitol) on a magnetic shaker for 1 h at room temperature. Protein concentration in the extract was determined by the Bradford method using bovine serum albumine (BSA) as standard (Bradford, 1976). The degree of dilution was controlled by repeated extraction in the same buffer. After repeated extraction, no more than 7% protein was dissolved. Proteins were separated in 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemli, 1970).

**Insecticidal activity against Lymantria dispar**

A comparative assessment of the insecticidal activity of recombinant strains was done with 2nd instar gypsy moth, *Lymantria dispar* L., larvae. Eggs were collected from oviposition cages and stored at +4°C on a medium containing: 200 g swollen kidney bean seeds, 15 g dry barm, 15 g agar, 6 g small pieces filter paper, 12 g sucrose, 4 g di-substituted potassium phosphate, 4 g ascorbic acid, 1 mg linseed oil, 3 g benzoic acid in 5 ml ethanol, 40% formalin, 4 ml KOH, and 1 g dry oak leaf (crushed).

For bioassays, 3 g of medium were mixed with 1 ml of the test suspension of the spore-crystal mixture (twice washed with physiological saline). The mixture was ground in a porcelain mortar with a pestle. For control, a mixture of 3 g diet and 1 ml water was used. The mixtures were transferred by a spatula to a Petri plate cover and placed in the form of a ring with a diameter of about 3 cm. Ten caterpillars were introduced inside the ring. On the Petri plate bottom, a disk of filter paper was placed. Each BT sample was tested in 3 repetitions in parallel. The experiment was conducted at room temperature and natural light. The number of dead insects was recorded on day 6.

**Insecticidal activity against Galleria mellonella**

Seventh instar wax moth, *Galleria mellonella* L., larvae were placed on Petri plates with 9 g diet (honeycomb + wheat bran). To each plate, 3 ml of test suspension or water was added. After the diet was dried somewhat, 10 *Galleria mellonella* caterpillars weighing 75±5 mg were placed in the center. Each BT suspension was tested in 3 replications. Bioassays were conducted on laboratory tables at a temperature of 25±20°C and a relative humidity of 60-80%. Mortality was documented on day 6.
**Insecticidal activity against sucking arthropods**

When using arthropods with sucking mouthparts (aphids, thrips, mites) as test objects, the optimal technique for treatment under laboratory conditions is the immersion of host plants into solutions of cultural liquid with spores and crystals of BT test strains.

In the case of the web-spinning mite, *Tetranychus urticae* Koch, 2-leaf haricot plants were cut and infested with females a day before the experiment. The plants were then immersed in solutions of test specimens for 6 seconds and were then put in glasses with water at the bottom.

For the melon aphid, *Aphis gossypii* Glov., cucumber leaves were taken, put into solutions of test specimens for 6 seconds, then placed onto wetted filter paper in Petri plates and infested with aphid females.

A different laboratory technique was used for Western flower thrips, *Frankliniella occidentalis* Pergande. Haricot plants in the 2-leaf-phase were taken, on which 7 days before the experiment thrips females had laid eggs. After larvae appeared, the leaves were cut, immersed into solutions of test specimens for 6 seconds and then placed into glasses with water supporting leafstalks with plastic jars smeared with Vaseline along the edges.

For all species, mortality was recorded after 5 days.

**Insecticidal activity against Leptinotarsa decemlineata**

Colorado potato beetle, *Leptinotarsa decemlineata* Say, larvae of younger (2nd and early 3rd) instars were used as test insects. Leaves of a potato plant were immersed in tested solutions of the preparations and placed in Petri dishes (ten larvae in each). Each experiment was run in triplicate. Plates were provided with vented covers and incubated at 27°C. Dead larvae were counted on the fifth day.

**Insecticidal activity against Tenebrio molitor larvae**

3 ml of aqueous suspension of the test material or water (in control) were mixed with 3 g of the diet. The mixtures were divided in 3 equal parts, which were placed in 40 mm plastic Petri dishes, 16 1st to 2nd instar larvae, which had starved for 24 hours, were placed in each Petri dish, plates were provided with vented covers and incubated at 27°C. Each experiment was carried out in triplicate. Total larvae mortality was observed on 15 – 22 days of the experiment.

**Estimation of toxicity of recombinant strain under field conditions.**

In order to test the laboratory specimen of the biopreparation derived from recombinant strain *B. thuringiensis* ssp. *kurstaki* RCIM B-8715 under field conditions, 20g of lyophilized preparation were produced with the following characteristics:

1. Spore titers $22 \times 10^9$ cells/g,
2. Content of $\delta$-endotoxins – 27 mg/g.

In each variant of the experiment, 100 plants (cotton, lemon and rose plants, respectively) were chosen. The dose of preparation in all experiments was $4.4 \times 10^8$ spores/ml. Plants were sprayed with an aerosol solution of the preparation or water. Before treatment, the quantities of aphids and thrips on each plant were determined. To avoid natural locomotion of aphids and thrips, sprayed plants were covered with gauze bags. After 3, 5, 7 and 10 d, the numbers of dead and alive insects were registered. Each variant was repeated 4 times. The numbers of aphids and thrips were calculated under 10-fold magnification.

**Results and discussion**

**Insecticidal effect against Lepidoptera and Coleoptera pests.**

Our novel strain *Bacillus thuringiensis* ssp. *kurstaki* B-8715 synthesises $\delta$-endotoxins that are effective against both Lepidoptera and Coleoptera pests (Table 1).
Table 1. Insecticidal effect against both Lepidoptera and Coleoptera pests.

<table>
<thead>
<tr>
<th>Insects</th>
<th>Lepidoptera</th>
<th>Coleoptera</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacillus thuringiensis</strong></td>
<td>Lymantria dispar</td>
<td>Tenebrio molitor L</td>
</tr>
<tr>
<td>strains</td>
<td>mortality (%)</td>
<td>mortality (%)</td>
</tr>
<tr>
<td>B-5081 (Bt tenebrionis)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>B-8715 (novel Bt kurstaki)</td>
<td>58</td>
<td>100</td>
</tr>
<tr>
<td>B-1226 (Bt kurstaki)</td>
<td>100</td>
<td>16,7</td>
</tr>
</tbody>
</table>

The recombinant strains differed in efficacy against coleopteran and lepidopteran insects. The highest relative coleopteran activity was achieved by the novel strain *Bacillus thuringiensis* ssp. kurstaki B-8715, and the highest lepidopteran activity with Bt ssp. kurstaki strain B-1226. The reason for the different activity was apparently due to the δ-endotoxin composition of the recombinant strains (Figure 1). The protein profile of *Bacillus thuringiensis* B-8715 more closely resembles that of *Bacillus thuringiensis* ssp. tenebrionis. We propose that the gene responsible for synthesis of δ-endotoxin Cry 3Aa is activated prior to the bacteria sporulation stage and earlier than the genes encoding δ-endotoxins of *Bacillus thuringiensis* ssp. kurstaki HD-1, which are activated only during sporulation (Agaisse & Lereclus, 1995).

**Insecticidal effect on sucking arthropods**

The strain *Bacillus thuringiensis* B-8715 demonstrated enhanced activity against representatives of Homoptera, Thysanoptera and Acariformes (Table 2). Mortality reached...
Table 2. Insecto-acaricidal activity against sucking arthropods in investigated strains. Values represent mortality after 5d.

<table>
<thead>
<tr>
<th>Test strain</th>
<th>tenebrionis</th>
<th>Recombinant</th>
<th>kurstaki HD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melon aphid Mortality (%)</td>
<td>12.8</td>
<td>59.2</td>
<td>17.4</td>
</tr>
<tr>
<td>Web-spinning mite Mortality (%)</td>
<td>21.7</td>
<td>91.45</td>
<td>23.6</td>
</tr>
<tr>
<td>Western flower thrips Mortality (%)</td>
<td>12.4</td>
<td>53.3</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Table 3. Toxicity of the recombinant strain under field conditions

<table>
<thead>
<tr>
<th>#</th>
<th>Treatment</th>
<th>Individuals per 10 plants before treatment</th>
<th>Average quantity after treatment (on days following treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>Control-water</td>
<td>173</td>
<td>146</td>
</tr>
<tr>
<td>2</td>
<td>Carbophos- 50%, concentration of emulsion 0.7 l/ha</td>
<td>165</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>Biopreparation</td>
<td>194</td>
<td>24.0</td>
</tr>
<tr>
<td></td>
<td>Cotton aphid (Aphis gossypii Glov.) on cotton</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tobacco thrips (Thrips tabaci Lind.) on cotton</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Melon aphid (Aphis gossypii Glov.) on lemon</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Green peach aphid (Myzus persicae Sulzer) on rose</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
values of more than 4 times above that of the initial parental strains. The encouraging results of laboratory experiments promoted experiments under field conditions.

**Estimation of toxicity of the recombinant strain under field conditions**

In Table 3, the results of field testing with the recombinant Bt strain on sucking pests are presented. Initial data obtained demonstrated that the biopreparation efficiency against aphids on day 10 was the same as carbophos. It was little lower against tobacco thrips. The efficiency of the biopreparation against aphids on day 3 was 70%.

The results have important implications for pest control. The pests examined are globally distributed and represent a serious threat to many economically important cultures, such as grains, legumes, vegetables, fruits, berries, cotton, forests, ornamental and medicinal plants and herbs. The industrial preparation on a basis of the strain *Bacillus thuringiensis* B-8715 has the potential to protect plants, including transgenic plants, from sucking pests. We are currently conducting experiments to determine the aphidicidal component of this preparation in more detail.

**Acknowledgements**

The authors express profound gratitude to the United States Department of Agriculture, Agricultural Research Service, for support of our scientific work.

**References**


Key factors for Bt cotton sustainability in smallholder farming: a modelling approach

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Abstract: Bt cotton is now being grown worldwide and adopted by many smallholders to overcome their bollworm problems. The management of the whole cotton cropping system is a key factor to ensure the sustainability of pest control by Bt cotton. Practices used in large-scale farming conditions to prevent insect resistance are no longer valid and there is controversy about the strategies to be adopted by smallholders. Modelling is a useful tool for simulating resistance patterns and developing strategies for sustainable bollworm management because many factors have to be considered when investigating the evolution of insect resistance in Bt crops. On the basis of preliminary laboratory results and field observations, we used a model to assess the significance of several of these factors in the Bt resistance process. The model highlighted some key factors: the initial level of resistance in wild populations, the heritability of resistance and the outmigration of resistant individuals from cotton fields at the end of the growing season. Some of these points could now be addressed on the basis of our current state of knowledge, but further research is needed to be able to gain insight into others.

Key words: Bt cotton, Helicoverpa armigera, sustainability, modelling

Introduction

Transgenic cotton, engineered to express toxins from \textit{Bacillus thuringiensis} Berliner, was initially designed to control \textit{Heliothis virescens} (F.) populations in USA. It was subsequently adopted by many farmers in the Old World to control an increasingly pesticide-resistant bollworm, \textit{Helicoverpa armigera} (Hübner).

When Bt cotton was released, many scientists were concerned about the sustainability of this approach. Principles for its management, based on high Bt-toxin expression and the use of refuges (i.e. cotton plots planted with conventional cultivars), have been adopted in some countries to prevent the development of insect resistance (Alstad & Andow, 1995; Tabashnik \textit{et al.}, 2004), but cannot be recommended for smallholder farming.

Resistance development is a complex process involving genetic, environmental and management factors. Modelling is an effective way to take this complexity into account when exploring resistance management options on a country- or region-wide scale (Tabashnik & Croft, 1982; Gould, 1998). In a previous paper (Nibouche \textit{et al.}, 2003), we outlined the framework for the prevention of resistance after the introduction of Bt cotton in West Africa. The purpose of the present study was to use the model to rank different factors involved in resistance management.
Bollworm life history and selection for resistance

We ran the model using, as input variables, some elements of the bollworm's life history in West Africa, as well as the results of a laboratory study on Bt-toxin resistance.
In West Africa, cropland in cotton producing areas is organized in a patchwork of small plots (0.5 to 5 ha) in which farmers grow 2/3 cereals (corn and sorghum) and 1/3 cotton during the rainy season. Cotton is the only crop grown from the middle of the rainy season onwards. At the end of the rainy season and during the dry season, some farmers grow vegetables in small irrigated schemes.

_H. armigera_ appears after the onset of the rains (April and May) and the first bollworms colonise wild host plants (especially _Cleome_ sp.). After a few generations, bollworm populations can invade maize and sorghum crops when cotton is in the vegetative growth stage. Bollworms are then observed on cotton as soon as flowers appear. Three successive generations grow on cotton during the rainy season, with the third being the most important in terms of numbers and damage. When the cotton plant matures, a large part of the bollworm population migrates (onto vegetables?), while a smaller portion enters diapause in the soil, emerging before the onset of the next rains. During the dry season, _H. armigera_ is found on vegetables grown in small plots around towns and villages, but the importance of long distance flight (migration) in the process of adaptation to the dry season remains unknown.

The bollworm has recently developed resistance to pyrethroids in West Africa (Martin _et al._, 2000), but its field populations are susceptible to Bt toxins. The susceptibility of _H. armigera_ to Cry1Ac is, however, fivefold less than that of _H. virescens_. A laboratory strain kept under toxin pressure developed after 18 generations a sudden resistance to Cry1Ac, with a resistance ratio of 164 afterwards (Uraichuen, 2002). There was no cross-resistance with Cry2A toxins. The heritability of this mutation-induced resistance was demonstrated as dominant, but its biological cost has yet to be determined.

Modelling

These operational and genetic factors were used to simulate the evolution of resistance (which we considered was achieved when 50% of the genotypes were RR), under various hypotheses, with the aim of developing an effective management strategy. The cropping ratio between cotton and maize was set at 1/3; the biological cost was estimated as 0.15 for RR genotypes, and none for the RS genotype. The initial resistance gene frequency was estimated as 10^{-3}. The LC_{50} of susceptible individuals was 0.24 μg/cm² (Uraichuen, 2002). As suggested by Forrester (pers. comm.), we set the toxin efficacy—as expressed by the cotton plant—at 0.95 for the first generation, 0.80 for the second generation, and half this level, i.e. 0.40, for the last generation.

The variables not considered in the simulation were the ratio of non-Bt to Bt cotton plants (set at 0.05, equivalent to a refuge ratio of 0.25 associated with an efficacy of pesticides used on non-Bt cotton estimated as 70%). We put forward various hypotheses on the proportion of bollworms emerging from the cotton crops at the end of the season to develop a new population on vegetables (0.5 to 0.9), and on the functional dominance of resistance. The presence of two genes (Cry1Ac + Cry2Ab) in some Bt cotton cultivars is represented in the simulation by a decrease in the initial frequency of resistant individuals (from 10^{-3} to 10^{-6}). The results of simulations are presented in Table 1.
Table 1. Key factors involved in the sustainability of Bt cotton.

<table>
<thead>
<tr>
<th>Refuge area (%)</th>
<th>Mortality in the refuge (%)</th>
<th>Migration from cotton</th>
<th>Initial resistance freq.</th>
<th>Dominance of resistance</th>
<th>Years before resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>70</td>
<td>0.5</td>
<td>$1 \times 10^{-6}$</td>
<td>0.1</td>
<td>21</td>
</tr>
<tr>
<td>25</td>
<td>70</td>
<td>0.9</td>
<td>$1 \times 10^{-6}$</td>
<td>0.1</td>
<td>14</td>
</tr>
<tr>
<td>25</td>
<td>70</td>
<td>0.5</td>
<td>$1 \times 10^{-3}$</td>
<td>0.1</td>
<td>11</td>
</tr>
<tr>
<td>25</td>
<td>70</td>
<td>0.9</td>
<td>$1 \times 10^{-3}$</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td>25</td>
<td>70</td>
<td>0.5</td>
<td>$1 \times 10^{-6}$</td>
<td>0.5</td>
<td>6</td>
</tr>
<tr>
<td>25</td>
<td>70</td>
<td>0.5</td>
<td>$1 \times 10^{-6}$</td>
<td>0.9</td>
<td>6</td>
</tr>
<tr>
<td>25</td>
<td>70</td>
<td>0.9</td>
<td>$1 \times 10^{-6}$</td>
<td>0.9</td>
<td>1</td>
</tr>
</tbody>
</table>

Discussion

On the basis of our simulation hypotheses, we considered three key-factors concerning the development of resistance to Bt toxin. The first one is the heritability of resistance, i.e. dominant, semi-dominant, recessive. It depends on the mutation that generates resistance, and seems to differ from country to country. The genetics of resistance can be managed if the level of toxin expression in cotton allows a switch from dominant to functionally recessive. However, this is difficult because: 1) the level of toxin expression is affected by water stress in rainfed conditions; and 2) bollworm infestation mainly occurs late in the cycle, when the plant does not express the toxins as well as during its vegetative growth stage. The second factor considered is the initial resistant allele frequency, which can be reduced by gene pyramiding. The third factor is the gene flow, represented by the bollworms leaving Bt cotton at the end of the season, and considered as Bt-toxin resistant. The emergence of resistant adults from cotton fields can be reduced by manual topping or by spraying the last pest generation.

Further studies are needed to assess other factors. The question on the role of wild plants as refuges in small-scale farming systems has yet to be addressed. This would require determining the proportion of bollworms living on wild or cultivated plants other than cotton during the rainy season, and the number of adults leaving these refuges to mate with bollworms emerging from cotton.

References


Global regulatory perspectives regarding transgenic crop risks to non-target insects: The case of Cry1F maize and butterflies

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Abstract: Regional and national assessments of Bt maize effects on non-target organisms demonstrate considerable convergence in terms of identifying the potential risks posed and the means whereby the risks are evaluated. This is well-exemplified for transgenic maize expressing the Cry1F protein from Bacillus thuringiensis var. aizawai. Cry1F maize (TC1507) has been commercially deployed since 2001 and offers resistance to lepidopteran pests; therefore, potential adverse effects on non-target Lepidoptera are a focus of regulatory assessments. Cry1F maize is currently approved for production in the United States and for export to several markets, including Japan, Mexico, Taiwan and South Korea; cultivation approvals are under consideration in Europe. All risk assessments for current and pending approvals of Cry1F maize include focused evaluation of potential risks to non-target lepidopteran species that may occur in and around maize (in Cry1F production fields, experimental trials, or as inadvertent volunteers from imported grain). While the specific ecological entities considered vary by country or region, there is considerable global consistency in the types of data used and methodologies employed for the assessment of non-target risks to endangered and charismatic butterflies. Common elements of these assessments are: (1) use of a core set of short-term, high-dose laboratory studies to broadly establish non-target effects, augmented as warranted by refined laboratory studies or monitoring; (2) determination of logical ecological entities of concern through evaluation of species most likely to be exposed on the basis of biology and distribution; (3) focused consideration of butterflies that are endangered, threatened, or charismatic; and, (4) exposure analysis to determine probable risk under environmentally relevant exposure scenarios. Recognition of these commonalities, as well as the reasons for differences in approach when they occur, provides insight as to globally-harmonized testing and assessment approaches for evaluating Bt crop risks to non-target insects.

Key words: Herculex I, GMO, genetically engineered

Introduction

Crops transgenically modified for insect resistance were grown on 22.4 million hectares in 2004 (James, 2004). Despite their increased use in global agriculture, there remains unevenness in adoption of insect resistant transgenic plants in some regions of the world. This may be due in part to regulatory uncertainties as to how their ecological safety should be addressed.

Formal frameworks for ecological risk assessment have been advanced as a means to logically address the testing of transgenic plants (Dutton et al., 2003; Wilkinson et al., 2003). These frameworks emphasize the use of a tiered testing strategy to focus scientific resources on those ecological components most likely to be affected by the environmental stressor. In
the case of a plant-expressed Cry toxin, the ecological entities of concern are sensitive species and lifestages of non-target arthropods and the stressor is the protein toxin.

Ecological risk assessment for transgenic plants seeks to understand the likelihood of harm to be manifested under environmentally relevant conditions. This entails detailed consideration of exposure encompassing the route, source, frequency, intensity, and duration of exposure. Exposure characterization – along with knowledge of the bioactivity of the stressor – allows for a rational stepwise design of toxicity tests to characterize effects. It also serves as a means for interpreting the results of toxicity tests in terms of the probability for harm to be manifested under conditions of environmental deployment of the transgenic plant. Thus, exposure and effects characterizations jointly describe risk.

The use of ecological risk assessment principles has been demonstrated for the case of Bt maize expressing Cry1Ab. Monarch butterfly (*Danaus plexippus* L.) has proven a sensitive species for which non-target exposure is a relevant consideration. In this case, ecological risk assessment principles have been applied at several levels to better understand the degree of likely impact. Screening level risk assessment for monarch using the results of tier I laboratory studies, showed that potential adverse effects of Cry1 protein exposure via maize pollen were limited to the Bt maize field and near field edge (Wolt *et al.*, 2003). A higher tier ecological risk assessment evaluating results from tier II laboratory and tier III semi-fields studies (Sears *et al.*, 2001) showed minimal impact from short-duration exposure on monarch populations. Both assessments highlighted the importance of environmentally relevant exposure estimates in arriving at a risk conclusion. A further regional assessment of risks from long-term exposure of Bt maize pollen to monarch larvae, showed that while the chronic effect to monarch was significant there remained minimal impact at the population level (Dively *et al.*, 2004). An additional example for Cry1Ab maize is the analysis of indirect effects on species not susceptible to plant-incorporated Cry1 toxins. While adverse effects have been suggested for green lacewing (*Chrysoperla carnea* Stephens) consuming prey intoxicated from feeding on Bt maize (Hilbeck *et al.*, 1998; Dutton *et al.*, 2002), there is a lack of sensitivity to Cry1Ab (Romeis *et al.*, 2004) and ecological risk assessment principles demonstrate the rationale and evidence for negligible secondary exposure to entomophagous arthropods (Dutton *et al.*, 2003).

These examples for Cry1Ab maize point out the utility of a conservatively cast tiered scheme of for non-target risk assessment. In the case of monarch butterfly, problem formulation led to the assumption of larval sensitivity to the protein toxin and opportunity for exposure via incidental pollen ingestion. When subsequent tier I studies indicated the degree of sensitivity was high, more detailed testing and assessment was warranted. Conversely in the case of green lacewing, problem formulation augmented with tier I testing established that both the probability for exposure and effect was remote; risk was, therefore, negligible, and there was no basis for further tiers of testing and assessment.

The recent regulatory clearances for Bt maize expressing Cry1F provide evidence for the increasing recognition and use of ecological risk assessment principles by regulatory authorities. The ecological risk assessments conducted by these various authorities necessarily address questions of specific national or regional concern; however, as shown for the case of Cry1F maize, they rely on a common core of data on which to base decisions. We review here the types of core studies that have been conducted for the purposes of ecological risk assessment and regulatory clearance for Cry1F maize. These studies provide a relevant starting point for harmonized approaches to testing of non-target insect effects of insect resistant plants.
Bt maize expressing Cry1F

Status of regulatory approvals
Insect resistant transgenic maize expressing Cry1F (TC1507) gained full regulatory clearance in the USA in 2001 (USDA, 2000; USEPA, 2001a). An extension of approval for a maize-optimized version of Cry1F maize (TC6275) was subsequently granted in the USA (USDA, 2003). Canadian approval was obtained in 2002 (CFIA, 2002). Full environmental approval was obtained in Japan as a condition of import (J-BCH, 2005). Approval for cultivation has been recently obtained in Argentina (MECON, 2005). Approval for cultivation is currently under consideration in the EU (EFSA, 2005). Various other approvals for import and feed/food use are in place as well (http://www.dowagro.com/herculex/steward/export.htm).

Nature of non-target insect risk assessments
A commonly recognized component in regulatory evaluations for products of modern plant biotechnology is the need for a case-by-case approach that accommodates the unique features of a given combination of donor crop, transgenic element, and environmental deployment. Because of this, the specific data needed for inclusion in regulatory submissions are not elaborated by most regulatory authorities. There is, however, common understanding of information that needs to be considered in regulatory submissions (see for instance, Nap et al., 2003). Various decision documents and environmental assessments provide insight as to the specific data used by regulatory authorities for assessments of non-target effects of Cry1F maize (USDA, 2000, 2003; USEPA, 2000, 2001a, 2001b; CFIA, 2002; J-BCH, 2005). These documents indicate common elements of product characterization, ecotoxicity testing, and subsequent risk assessment.

Product characterization. Product characterization information serves as the foundation for the risk assessment problem formulation and analysis plan. For Cry1F information on host and donor familiarity was used to establish the nature of product and its history of safe use and environmental exposure. It was also used to determine prior scientific and regulatory experience with related products. Cry1 proteins are known to be specifically active on Lepidoptera and have a history of use in Bt sprayable pesticides and Bt crops, including maize. On this basis, the focus for the Cry1F analysis plan becomes non-target Lepidoptera occurring in and around maize fields.

A precursor to initiation of the analysis plan is the confirmation of activity and specificity. Insecticidal activity spectrum studies with purified bacterially produced Cry1F protein assayed lethality on a variety of insect species (punitive targets for control) and established specificity for Lepidoptera and confirmed lack of activity on representative species for other insect orders (Hymenoptera, Neuroptera, and Coleoptera). Associated studies of the bacterially-produced Cry1F protein compared to plant-expressed Cry1F were conducted to establish that the plant-produced and bacterially-produced proteins were biologically, biochemically, and immunologically equivalent. This is because both the spectrum studies and subsequent ecotoxicity studies use bacterial Cry1F protein. This information also established the relevance of prior knowledge concerning the activity and use of bacterial Cry1F protein to the risk assessment for maize-expressed Cry1F protein.

Expression studies described Cry1F concentration and variance over time as well as among plant parts. This information is a critical aspect of the exposure characterization and aids in determination of the exposure potential to various non-target species.

Ecotoxicological testing. Single species laboratory ecotoxicity tests were used to confirm the anticipated spectrum of activity and hazard to non-target organisms. Selection of appropriate surrogate species for testing should consider activity profile, host crop, environment where deployed, and the amenability to laboratory rearing and testing (Dutton et al., 2003). These
rationaloes for designing the ecotoxicological testing plan are reflected in those studies used for the non-target ecological risk assessment of Cry1F maize (Table 1).

Table 1. Non-target invertebrate studies used for Cry1F maize non-target risk assessment (Hellmich et al., 2001; Pleasants et al., 2001; USEPA, 2001a,b).

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>Protein source</th>
<th>Dose</th>
<th>Effect endpoint</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apis mellifera</td>
<td>Honey bee (larvae)</td>
<td>bacterial derived</td>
<td>640 ug Cry1F per larva</td>
<td>mean survival to emergence</td>
<td>no effect of limit dose at &gt; 200× corn pollen expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>corn pollen (TC1507)</td>
<td>2 mg pollen per larva</td>
<td>adult survival and reproduction, 28 d</td>
<td>no effect of limit dose at &gt; 79× field exposure</td>
</tr>
<tr>
<td></td>
<td></td>
<td>bacterial derived</td>
<td>0.63, 3.1, and 12.5 ug Cry1F per g diet</td>
<td>mean survival to pupation, 13 d</td>
<td>no effect of limit dose at &gt; 15× corn pollen expression</td>
</tr>
<tr>
<td>Folsomia candida</td>
<td>Springtail</td>
<td>bacterial derived</td>
<td>320 ug Cry1F per g diet</td>
<td>mortality at 12 d</td>
<td>no effect of limit dose at &gt; 10× corn pollen expression</td>
</tr>
<tr>
<td>Chrysoperla carnea</td>
<td>Green lacewing (larvae)</td>
<td>bacterial derived</td>
<td>480 ug Cry1F per g diet</td>
<td>mortality at 29 d</td>
<td>no effect of limit dose at &gt; 15× corn pollen expression</td>
</tr>
<tr>
<td>Brachymeria intermedia</td>
<td>Parasitic hymenoptera</td>
<td>bacterial derived</td>
<td>dose-response to Tc1507 pollen on milkweed leaves</td>
<td>growth reduction after 4 d</td>
<td>no effect of limit dose at &gt; 15× corn pollen expression</td>
</tr>
<tr>
<td>Hippodamia convergens</td>
<td>Ladybird beetle</td>
<td>bacterial derived</td>
<td>480 ug Cry1F per g diet</td>
<td>mortality at 14 d</td>
<td>no effect of limit dose at &gt; 104× aquatic exposure</td>
</tr>
<tr>
<td>Danaus plexippus</td>
<td>Monarch (larvae)</td>
<td>bacterial derived</td>
<td>2.26 mg Cry1F per g dry soil</td>
<td>immobilization after 2 d</td>
<td>no effect of limit dose at &gt; 10^4× aquatic exposure</td>
</tr>
</tbody>
</table>

Studies conducted with honey bee, green lacewing, ladybird beetle, and parasitic hymenoptera represent systematic and functional diversity as well as widely differing routes of potential exposure to Cry1F toxin. These tier I acute limit dose studies confirm the adequacy of the problem formulation in terms of how product characterization defines potential risk to non-target insects. These core studies are augmented with further confirmatory studies on arthropods which serve as general indicators of ecosystem health (springtails, earthworm, and daphnia). Finally, a tier II study of a potentially sensitive non-target species (monarch butterfly) with a plausible route of exposure (incidental consumption of maize pollen) supplements the core data. This study regime was augmented by small-scale ecological monitoring studies in some instances (USEPA, 2001b; EFSA, 2005) but these data
were used mainly to confirm assessment results and should be viewed as supplemental information.

It is noteworthy, that while monarch butterfly has proven adequate to assess Cry1F effects to non-target Lepidoptera by most regulatory authorities, the Japanese environmental assessment used instead data for a species of local interest (pale grass blue butterfly, *Zizeeria maha* ssp. *argia*) (J-BCH, 2005).

**Non-target risk findings.** Data specific to Cry1F as well as a broader understanding of the ecological risks from Bt maize deployment lead to a conclusion of negligible concern (see for instance, EFSA, 2005). There is no rationale for further testing for species other then Lepidoptera, since tier I tests confirm the lack of activity at high doses to systematically and functionally diverse non-target organisms.

For non-target butterflies, the degree of concern and requirement for further testing is determined on the basis of (1) the magnitude of effect, (2) the degree to which dose regimes used in testing are conservative, and (3) consideration of biology of butterflies of interest relative to Cry1F maize deployment scenarios. In the case of monarch butterfly there is no need for testing beyond tier II, since the tier II study using a relevant exposure scenario (pollen on milkweed leaves) confirms the lack of toxicity seen in high-dose no-choice studies with bacterial protein in artificial diet (Hellmich *et al*., 2001). The adequacy of this conclusion is confirmed through ecological risk assessment that considers monarch population biology in relation to Bt maize deployment (Sears *et al*., 2001). Similar findings arise for the ecological safety assessment of pale grass blue butterfly (J-BCH, 2005). It is interesting to note that risk findings are similar for monarch butterfly and pale grass blue butterfly even thought these species show very different sensitivities to Cry1F maize pollen. The LC$_{50}$ for monarch is >1,000 grains per cm$^2$ food source (Hellmich *et al*., 2001) and that of pale grass blue butterfly is 100 grains per cm$^2$ (J-BCH, 2005). In both cases, lack of exposure is the controlling factor leading to a conclusion of negligible risk.

Further considerations of Cry1F maize risks to non-target butterflies focus on threatened and endangered species within various national and regional regulatory jurisdictions. These assessments consider species reproductive biology, feeding habits, host plant characteristics, and proximity to maize fields in arriving at a risk determination under the assumption that the species of concern will be highly sensitive to the Cry1F toxin. The endangered Karner blue butterfly was the focus of regulatory assessment within the USA (USEPA, 2001a). In Japan (J-BCH, 2005) and the EU (SNIF, 2001), a broad-based consideration of butterflies occurring on national Red Lists was the focus for assessments. While in the case of US and Japanese assessments exposure probabilities were sufficiently low (primarily because of habitat and reproductive biology) to minimize concern for Cry1F risk to endangered and threatened butterfly species, the adequacy of such an assessment approach for the EU has been questioned (EFSA, 2005).

**Regulatory approaches to transgenic crop ecological safety assessment**

Based on an understanding of the data common to regualtory packages, a philosophy emerges for the way these data may be evaluated within the context of ecological risk. The analysis is for the most part weight-of-evidence based on comprehensive evaluation of the data. This analysis proceeds from a general understanding of the product (nature of the host plant, the donated transgenic element, and characteristics of the event). Those characteristics of the event most critical to the ecological risk assessment are host and donor familiarity, activity and specificity of the protein, protein equivalency, and expression within the host plant. The analysis then considers data specific to ecological entities of concern in terms of ecotoxicity
and likelihood of exposure. The risk-based findings should focus on harm that may be manifested at environmentally relevant exposures and seek to determine whether there are unreasonable adverse effects to the environment.

For a specific protein toxin, the assessment will typically consider the relationship of the findings of limit dose ecotoxicity studies to high end estimates of the environmental concentration as determined through characterization of the route, source, frequency, intensity, and duration of exposure for ecological entities of concern. Confirmation of the adequacy of risk characterization is obtained through correspondence of product specific results with general understanding for the donor, host, and product class being considered; the internal consistency/directional correctness of overall product and risk characterization; the ability to characterize hazard at or above environmentally relevant exposure concentrations; confirmatory data (if available) from field studies and published reports; and consideration whether the conservatism of the risk conclusion is in keeping with the degree of uncertainty found in the weight-of-evidence analysis. The appropriate stopping point for data development (that is, the appropriate tier of testing) is determined from the adequacy of the comprehensive risk assessment as has been described here for the examples of Cry1F maize regulatory considerations.

Conclusions

Risk is the likelihood of harm to be manifested in the environment. Ecological risk analysis is a formal approach to parsing risk from the joint probabilities of expose and effect as discerned from synthesis of broad-based information. As revealed from considerations of regulatory decision documents for Cry1F maize, common elements of these assessments are: (1) use of a core set of short-term, high-dose laboratory studies to broadly establish non-target effects, augmented as warranted by refined laboratory studies or monitoring; (2) determination of logical ecological entities of concern through evaluation of species most likely to be exposed on the basis of biology and distribution; (3) focused consideration of butterflies that are endangered, threatened, or charismatic; and, (4) exposure analysis to determine probable risk under environmentally relevant exposure scenarios.

References


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Workshop report
Non-target risk assessment of GM crops and regulation

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Protocol from a satellite workshop organized by the IOBC/WPRS Working Group ‘GMOs in Integrated Plant Production’ in Lleida (Catalonia), Spain, on 4 June 2005.

Background

The potential risks of genetically modified (GM) crops on non-target (NT) organisms have to be assessed as part of the regulatory risk assessment. While regulatory documents (e.g. EC 2002; CBD Secretariat, 2000) give some guidance on how this assessment should be conducted, there is still a need for a detailed description of the risk assessment process, solid criteria for NT species selection and standard test methods.

Objective

To establish generic environmental risk assessment (ERA) guidelines for GM crops with particular emphasis on non-target organisms.

Stakeholders

Regulatory authorities, biotech industry and public research institutes. Representatives of all three stakeholders will be involved in the activity. The IOBC/WPRS working group will provide the platform to bring the three stakeholders together.

The workshop resulted in the establishment of three activities:

Activity 1. Development of a generic risk assessment process for non-target organisms

The use of a step-by-step or ‘tiered’ approach for NT risk assessment for GM plants has been recommended both by the EU (EC, 2002) and EFSA (2004). Considerable effort has been invested into designing such a tiered risk assessment scheme both in the USA and in Europe. Currently, USDA/APHIS and EPA are preparing a position paper that describes the basis for conducting non-target invertebrate ecological risk assessment for proteinaceous plant-incorporated protectants in the USA (Rose, 2006). Similarly, the Technical Advisory Group (TAG) of EuropaBio’s Plant Biotechnology Unit has developed a guidance document on how to assess the safety of GM plants for NTs (EuropaBio, 2004). Based on the NT risk assessment approaches that are published or under development, a generic NT risk assessment procedure will be developed that could be adopted by different countries while still taking into account their specific regulatory needs.
The NT risk assessment will be divided in two main phases, a problem formulation phase followed by consecutive tiers of assessment. During the problem formulation phase, the hazards and level of concern associated with the deployment of a specific GM plant are defined. All available information on the GM plant, the event and the transgenic compound expressed is collected. Taking this information into account, the problem is formulated in an iterative process. At the end of this phase it will be clear what has to be assessed further and which potential risks can be ruled out with a sufficient level of certainty. This background is not provided in the current risk assessment regulation guidance documents. Once the problem formulation phase has defined the potential risks that have to be addressed, tiered assessment/testing will be conducted following a scientific approach. Complexity and realism of the conducted tests increase with higher tiers. Early tier studies will be conducted in the laboratory followed by extended laboratory or semi-field experiments and large scale field trials. Movement from one tier to the next is triggered by specific assessment endpoints that need to be defined.

**Activity 2. Definition of criteria for the selection of non-target organisms to be assessed**

The group agreed on the following definition of NT species: ‘Wildlife associated with the crop that does not cause economically significant levels of damage. It refers to those organisms which are not the intended targets of a particular use of a GM plant.’

It became clear that, while NT risk assessment has to be done case-by-case, NT testing should be done by drawing from a set of appropriate indicator species. The problem formulation phase is a very important step also for the selection of NT test species. Criteria for species selection could include:
- importance in the crop system (ecological functions)
- importance in the region of GM crop introduction
- potential sensitivity to the insecticidal protein
- cultural importance
- species of conservation importance
- species exposed to the transgenic compound in the field
- existence of a robust test system

**Activity 3. Development of standard test methods for selected non-target species**

The final goal is to develop standardized and validated test protocols for a set of test NT species. These could include a set of surrogates that have to be tested for each transgenic compound and/or species that might have to be assessed on a case-by-case basis depending on the crop, the trait, and the area of introduction.

A list will be compiled with NT species for which standardized and validated protocols already exist. In a second step, the extent to which these protocols can be adopted for the assessment of insecticidal proteins will be evaluated. Once species have been identified for which protocols should be developed, appropriate experts from research institutes, industry and other interested stakeholders will discuss in detail the test protocols for a specific species with the aim of proposing a standard protocol in each case.
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