Non-target effects of GM potato : an eco-metabolomics approach

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Contents

Introduction and outline

In 2012, genetically modified (GM) plants were planted on an estimated 170 million hectares in 28 countries worldwide (James 2012)[1]. The most common introduced traits in GM crops are herbicide tolerance and insect resistance. Other introduced traits are virus resistance, delayed ripening, metabolic alterations and drought tolerance. Genetic engineering techniques are also used to transfer genes within or between crossable species ("cisgenics"). An increasing number of GM crops carry multiple engineered traits ("stacked events").

A wide range of benefits have been proposed for GM crop cultivation, among which are yield increases, production stability in the face of global warming, extension of geographical ranges and reduced environmental impact through reduced pesticide use. Literature surveys and meta-analyses have concluded that positive effects of GM crops vary across time and space, but seem to confirm an overall positive trend for yield increases and pesticide reductions (Finger *et al.* 2011; Brookes & Barfoot 2012; Mannion & Morse 2012; Morse, Mannion & Evans 2012; Areal, Riesgo & Rodriguez-Cerezo 2013). However, given the relatively short history of GM crop cultivation, it has been suggested that initial benefits may not be sustainable in the long term, given the reports of resistance evolution to herbicides in weeds (Owen & Zelaya 2005; Powles 2008) and resistance to Bt-toxins in insects (Tabashnik *et al.* 2008; Gassmann *et al.* 2011), as well as cases of non-target pest outbreaks as a result of altered pest management in *Bacillus thuringiensis* (Bt) crops (Lu *et al.* 2010).

A thorough analysis of the benefits of GM crops is beyond the scope of this thesis. However, a consideration of both risks and benefits is often lacking in the public debate on GM crops. Qualitative research suggests that public opposition against GM crops may be more related to a lack of perceived benefits than to perceived risks (Gaskell *et al.* 2004). Both risks and benefits have to be considered on a case-by-case basis, since both will depend on the particular modification and geographic region, and both will interact with management practices and socio-economic factors.

Safety concerns

Concerns about the safety of GM crops have been raised since the first generation of genetically engineered plants. Public opposition is particularly strong in Europe, where safety concerns have led several countries to stop the market release of GM crops. In general, the

1 Figures are published annually by the biotechnology-friendly "International Service for the Acquisition of Agri-biotech Applications" (ISAAA).

envisioned problems with GM crops can be grouped into two main categories: 1) harm to humans or livestock through consumption as food or feed, and 2) harm to ecosystems through field cultivation (EFSA 2010a; EFSA 2011)[2]. Food and feed derived from GM plants may pose risks when toxic or allergenic constituents are newly expressed or increased in concentration. GM crops are therefore subjected to extensive compositional analyses. Recently, the use of metabolomics tools has been discussed as a tool in food/feed safety analysis, because of their potential to discover unintended and unexpected metabolic changes in plants (Rischer & Oksman-Caldentey 2006; Chassy 2010; Davies 2010; EFSA 2011). Most studies have found no significant compositional differences between GM crops and their non-GM counterparts (Defernez *et al.* 2004; Catchpole *et al.* 2005; Ioset *et al.* 2007; Kim *et al.* 2009; Barros *et al.* 2010; Misra *et al.* 2012; Kim *et al.* 2013).

Ecological risks can be classified into two main types: 1) transgene escape and 2) effects on non-target organisms. Transgene escape may occur when the crop plant establishes feral populations in the cultivation area or when it outcrosses with wild relatives. Reports of transgene escapes are relatively rare to date, but introgression has been documented e.g. in canola (Hall *et al.* 2000; Warwick *et al.* 2008; Schafer *et al.* 2011) and creeping bentgrass (Zapiola *et al.* 2008; Zapiola & Mallory-Smith 2012). This thesis focuses on the occurrence and detection of GM effects on non-target organisms, which will be discussed in the following paragraphs.

Effects on non-target organisms

A non-target organism (NTO) is an organism that is exposed to a GM plant but is not intended to be affected by the newly expressed trait (EFSA 2010b). This may include animals, plants and microorganisms that occur in the area of cultivation. Non-target organisms can be considered worth protecting for a range of reasons. Some species are beneficial for human agricultural activities, such as predators of pest species, pollen and seed dispersers and decomposers. Some species may also be considered worth protecting due to their charismatic value to humans, independent of their ecological roles and agricultural benefits (Andow & Hilbeck 2004).

While a number of studies have found negative effects of GM crops (mostly insecticidal Bt plants) on NTO in laboratory trials (Hilbeck *et al.* 1998; Birch *et al.* 1999; Losey, Rayor & Carter 1999; Lovei, Andow & Arpaia 2009), their significance for field cultivation is often unclear and widely debated. Marvier *et al.* (2007) performed a meta-analysis of 42 field studies reporting non-target invertebrate abundances on Bt cotton and maize carrying different bacterial *cry* proteins. They found that on average, NTO abundances were significantly higher on Bt fields compared to insecticide-treated non-GM fields, but significantly lower compared to untreated non-GM fields.

Current risk assessment regulations in Europe require that effects on non-target organisms on a given GM crop be tested on a selection of "focal" species (EFSA 2010b). Initially, species are identified on the basis of their ecological functions and their relevance to predefined environmental protection goals (such as preserving an ecological service or biodiversity). The species list is then prioritized according to criteria of ecological relevance, exposure, vulnerability and practicability. For the resulting list of focal species, appropriate measurement endpoints are

2 This thesis focuses on the regulatory framework on GM crops within the European Union, based on the "Directive on the Deliberate Release into the Environment of Genetically Modified Organisms" (2001/18/EC), the "Regulation on Genetically Modified Food and Feed" (1829/2003) and the guidance documents provided by the European Food Safety Agency (EFSA).

to be chosen, and thresholds (limits of concern) be defined for each species/endpoint.

The above approach can be considered the current consensus in trading off ecological knowledge and practical considerations in non-target risk assessment: since not all species in an ecosystem can be measured or tested, the assessment is limited to a choice of relevant and testable species. From a purely ecological point of view, however, this approach has some important limitations. Most importantly, the current approach breaks down complex ecosystems into a number of isolated endpoints and thresholds for individual species, thus ignoring changes at the community level. Ecological systems are complex and interlinked, and an effect in one species might cause system-changes that are difficult to predict. For example, non-target herbivore species are usually not considered of high relevance in terms of ecological services, but may become secondary pests as a result of eliminating their natural enemies (Lu *et al.* 2010; Meissle, Romeis & Bigler 2011). Thus, prioritizing species in risk assessment according to agricultural relevance and testability may not reflect their relevance to ecosystem stability. Furthermore, the focal-species-approach does not account for the possibility that ecological services can be conveyed by multiple species, or that services may be linked to species diversity. In this thesis, patterns of variation in insect communities are therefore partitioned using multivariate analyses, thus accounting for system-level changes.

From a practical point of view, measuring system-changes may often be challenging, as it requires the inclusion of a larger number of species and environments. One way to overcome this limitation may be to examine system-changes at the level of the plant, rather than at the level of the receiving ecosystem. Effects on non-target systems are ultimately the result of unintended changes in plant phenotypes. Metabolomics are a powerful tool in studying the links between genotypes, phenotypes and ecosystems (Fiehn 2002; Dixon *et al.* 2006; Macel, van Dam & Keurentjes 2010; Sardans, Penuelas & Rivas-Ubach 2011). Metabolomic analyses have been used successfully to reveal chemical mechanisms of plant-insect interactions (Widarto *et al.* 2006; Poelman *et al.* 2008; Jansen 2009; Leiss *et al.* 2009a; Leiss *et al.* 2009b; Steinbrenner *et al.* 2011). The broad coverage of compounds and the potential to discover unexpected metabolic alterations make these techniques well-suited to safety assessment problems, because no prior knowledge on the quality of possible changes is needed (Konig et al. 2004; Catchpole et al. 2005; Parr et al. 2005; Rischer & Oksman-Caldentey 2006; Barros et al. 2010). When the goal is to detect and avoid unanticipated effects of genetically engineered plants, it is necessary to view plants as "complex systems embedded in poorly understood, complex, and interacting ecosystems" (Ervin *et al.* 2003, p. 12). However, understanding system-changes in GM plants and non-target communities requires basic knowledge on natural variability in these systems across space and time. Establishing a baseline of variation for evaluating GM effects is a crucial step towards a systems biology approach to NTO risk assessment.

In this thesis, we examine effects of a GM potato on the above-ground, non-target insect community in the Netherlands. The GM line "Modena" (modified in starch composition by means of amylose knock-down; grant No: NRR 30805, AVEBE UA, Foxhol, The Netherlands/BASF Plant Science Company GmbH) serves as a case study and is compared to its non-GM, near-isogenic parent cultivar "Karnico", as well as four commercially available non-GM potato cultivars. We measure insect community variability across the different plant genotypes over time and space and compare these findings with chemical phenotyping data acquired with ^{1}H NMR metabolomics, with the goal of evaluating the relevance of system-changes in plants to insect communities.

In both insect communities and plant metabolomics, we quantify the relative importances of genotypic and environmental factors, as well as genotype-by-environment interactions. These data may help to improve future risk assessment designs and data interpretation on NTO from a systems biology perspective.

This study was funded by the ERGO (Ecology Regarding Genetically Modified Organisms) program of the Dutch Research Organisation (NWO) (project no. 838.06.070). Effects on soil fungal and microbial communities have been studied in parallel studies on the same experimental fields (ERGO project no. 838.06.052 and 838.06.051; Hannula, de Boer & van Veen 2010; Inceoglu *et al.* 2010; Hannula, de Boer & van Veen 2012).

Thesis outline

In chapter 2 of this thesis, an ecological dataset of non-target insect communities from a field experiment with GM potato is presented. The GM potato is compared to its non-modified counterpart and four commercial non-GM potato cultivars and communities are monitored at two experimental locations in two years and at three plant developmental stages within each year. Using multivariate analyses, variation in insect communities is partitioned with respect to genotypic and environmental factors, as well as their interactions.

In chapter 3, a closer look is taken at statistical and practical implications of setting limits of concern for individual species. In lack of biological knowledge, limits of concern are often set arbitrarily to standard percentages, with consequences for statistical power and testability of highly variable non-target species. Using standardized effect sizes to quantify effects in relation to variability is discussed as an alternative approach.

In chapter 4, the application of NMR metabolomics as a tool in ecological safety assessment is introduced. The leaf chemistry of GM and non-GM potato plants is analysed in a laboratory study, with the aim of providing chemical baseline information for the potato system. Metabolic system-changes in response to aphid herbivory and virus infection are assessed as a proxy for the degree of metabolic plasticity that is likely to occur in a field situation.

In chapter 5, an eco-metabolomics approach to NTO risk assessment is applied in the field. Using the same experimental field setup as in chapter 2, a baseline of variation in leaf chemistry is measured in a GM potato, its non-GM counterpart and four commercial varieties across two locations, two years and three developmental stages within years. The biologicalsignificance of metabolomic profile changes in plants towards non-target insects is evaluated.

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Variation in non-target insect communities on GM and non-GM potato across years, locations and developmental stages

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Abstract

Genetically modified (GM) plants may cause environmental harm by affecting non-target insect communities. However, little is known about the patterns of variation in agro-ecosystems. Such baseline information is necessary for evaluating the biological relevance of GM effects. Here, we study non-target insect communities on GM and non-GM potato in the field across two years, three plant developmental stages within years and two locations in The Netherlands. Effects of six plant genotypes (one amylopectine GM line, its non-GM counterpart and four commercial non-GM cultivars) on eight operational taxonomic units of insects were small when compared to the total amount of variation in insect abundances. However, we found evidence for genotype-by-environment and genotype-by-development interactions. Thus, GM effects have to be considered in multiple environments and at various developmental stages in order to minimize uncertainty in risk assessment field trials.

Keywords: transgenic plants, environmental effects, GxE interactions

Introduction

The environmental safety of genetically modified (GM) crops has sparked much debate over the past two decades. In spite of arguments that GM crop farming has improved agricultural productivity and reduced pesticide use (e.g. Raybould & Quemada 2010; Yu, Li & Wu 2011), concerns continue to be voiced that GMP cultivation may have negative impacts on the functioning of ecosystems (Tiedje *et al.* 1989; Wolfenbarger & Phifer 2000; Conner, Glare & Nap 2003). One of the envisioned ecological impacts is that GM crops may affect the composition of insect communities. Many insect species fulfill important ecological functions that are worth conserving from an anthropocentric point of view (Andow & Hilbeck 2004). For example, predatory and parasitoid insects may be important top-down forces in ecosystems (Halaj & Wise 2001), and this function makes them valuable pest control agents in agriculture. Non-pest herbivorous insects may have a beneficial function in sustaining parasitoid and predator populations, but may also become secondary pests as a result of eliminating their natural enemies (Lu *et al.* 2010; Meissle, Romeis & Bigler 2011). Some species are also considered worth protecting due to their charismatic value to humans, independent of their ecological roles and agricultural benefits (Andow & Hilbeck 2004).

Despite the large number of studies (reviewed by e.g. Marvier *et al.* 2007; Sanvido, Romeis & Bigler 2007) that have set out to test the possible effects of GMPs on "non-target organisms" (NTOs), the interpretation of results in the light of environmental harm is often subject of further debate. This may in part be due to a misalignment between the goals of ecological research and the needs of decision-makers (Ervin *et al.* 2003; Raybould 2007). The guidelines for environmental safety assessment have been gradually improved over the years in order to harmonize scientific methods with decision-makers' needs for conclusive results (Marvier 2002; Conner, Glare & Nap 2003; Snow *et al.* 2005; Craig *et al.* 2008; Romeis *et al.* 2008; Perry *et al.* 2009; EFSA 2010b). In Europe, there is now extensive guidance on the selection of non-target species, the choice of measurement endpoints, the design of field trials and their statistical analysis (EFSA 2010a).

However, despite the detailed guidelines, it is unclear how the relevance of effects found in GM/non-GM comparisons should be evaluated. Applicants are required to define thresholds ("limits of concern") for NTO abundances that would indicate environmentally harmful effects. But the necessary fundamental ecological knowledge is not available for many non-target groups. Furthermore, considering GM effects separately per species does not take into account changes at community levels. Given the complexity of agro-ecosystems, more information on baseline variability in insect communities is necessary to inform risk assessments on non-target organisms.

Here, we study insect abundances on GM and non-GM potato in experimental fields in the Netherlands. We compare GM effects to a baseline of variation between five non-GM genotypes and across two years, three developmental stages within years and two locations. Using multivariate data analysis tools, we quantify the effects of different sources of variation and their interactions at community levels.

Methods

Data on insect abundances were collected in two years (2010 and 2011) and on two locations with contrasting soil conditions. Insect sampling was conducted during three months (June – August) using three different methods: (1) whole plant sampling at three developmental stages (vegetative, flowering and senescence), (2) sticky traps (weekly sampling) and non-destructive visual inspection (Colorado beetle counts; upon occurrence). Insects were grouped into 10 operational taxonomic units. Because the genetic modification of the GM variety "Modena" is targeted at metabolic traits, all insects were considered non-target organisms.

Plant Material and field setup

The genetically modified variety "Modena", which is modified in starch composition (amylose knock-down; grant No: NRR 30805, AVEBE UA, Foxhol, The Netherlands/BASF Plant Science Company GmbH, Research Triangle Park, USA) was used for this case study. Modena's parent cultivar "Karnico" was used as a near-isogenic comparator. In addition, four varieties representing a broad phenotypic spectrum of commercial cultivars were used as additional comparators: two consumption varieties ("Premiere" and "Desiree") and two starch varieties ("Aveka" and "Aventra"). Plants were grown from tubers at two field locations in Drenthe, The Netherlands, with a distance of 12 km between locations. The locations were characteristically different in soil conditions: sandy peat (location A) and loamy sand (location B). Fields were shifted in the second year to an area adjacent to the area of the previous year, following the common practice of crop rotation in potato, which prevents the build-up of diseases and pests in soil.

Each field was planted as a randomized 6 x 6 latin square with six replicate plots per variety. Each plot consisted of 28 plants (4 ridges of 7 plants = ca. 10 m2). The fields were planted with tubers at the end of April and were harvested in October. All fields received standard fungicide and fertilizer treatment. In 2010, location B was treated with an insecticide (Calypso, Bayer CropScience, Monheim am Rhein, Germany) after an infestation with Colorado potato beetles. Beetles were counted before spraying, and all other insect sampling activities started at least two week after spraying.

Plant development

The average developmental state of each replicate plot was determined weekly. Developmental stages were scored on a scale from 1 to 14, with 1 = vegetative state, 5-10 = flowering, 7 = flowering peak, 13=senescence, 14 = death. Plants harvested for whole plant insect sampling were weighed immediately after harvesting (fresh weight), and their dry weight was measured after insect sampling was completed. Weather data were kindly provided by Averis Seeds BV (Veendam, Netherlands).

Insect sampling

a) Whole plants

Plants were sampled by cutting the stems of one individual plant per plot closely above the ground and transferring the complete above-ground material into a labeled plastic bag. All plants were weighed in the field, transported back to the laboratory and stored at 4°C. All

sampled plants were visually inspected for the presence of insects on any plant part. Insect counts were summarized in eight operational taxonomic units (OTUs) with taxonomic ranks between ranging from orders to families: Thysanoptera (thrips), Hemiptera\Aphididae (aphids), Hemiptera\Cicadellidae (leafhoppers), Lepidoptera (mostly moth eggs), Hemiptera\Heteroptera (bugs), Neuroptera (lacewings), Diptera\Syrphidae (hover flies) and Hymenoptera (detected as aphid mummies). Whole plant sampling was conducted at three time points during the growing season, corresponding to three phenological stages: early season before flowering (50 days after planting), at flowering (75 days after planting) and senescence (115 days after planting).

b) Sticky traps

Insect sampling by sticky traps was done weekly for 11 times from June to August. One blue and one yellow sticky trap (25 × 10 cm, double-sided, Koppert BV, Berkel en Rodenrijs, The Netherlands) were attached to bamboo sticks closely above the leaf canopy of each plot in the morning (between 10.00 and 11.00 h) and collected ca. 6 hours later. Sticky traps were frozen at -20°C until insects were identified and counted by visual inspection. Two OTUs were counted on the traps: Hemiptera\Cicadellidae (leafhoppers), Diptera\Syrphidae (hover flies).

c) Visual inspection

Colorado potato beetles (CPB) *Leptinotarsa decemlineata* were directly counted on plants in the field. The field on location B was invaded by CPB in both years early in the growing season (May). After insecticide treatment (Calypso, Bayer CropScience, Monheim am Rhein, Germany) in 2010, the field was recolonized by newly hatched beetles, this time in lower numbers, and a population remained in the field until the plants were senescent.

Data analysis

All count data obtained from whole plant samplings, sticky trapping and visual inspections were statistically analyzed for differences between cultivars. These tests were done separately per sampling time point. When data conformed to assumptions about normality, ANOVA was used to test for differences between cultivars, and multiple comparisons between cultivars were analyzed with Tukey's honest significance test. When normality assumptions were not met, Kruskal-Wallis tests were performed and multiple comparisons were analyzed with a non-parametric method described in Siegel, S. and Castellan N.J. (1988).

In addition, count data of whole plant samplings were analyzed with non-parametric permutational MANOVA (as described in Anderson 2001), which allows the partitioning of variation to different factors. This was used to describe the relative importance of environmental versus genetic sources of variation and their interactions. For community level analysis, count data were transformed to Bray-Curtis-dissimilarities. For analyses per species, count data were log-transformed, and dissimilarities were calculated based on Euclidean distances. A significance level of α=0.05 was used for all tests.

Results

Insects sampled on whole plants (harvested above-ground foliage) were assigned to eight operational taxonomic units (OTUs), including herbivores (Aphididae, Thysanoptera, Cicadellidae, Lepidoptera) and higher trophic level insects (Heteroptera, Syrphidae, Neuroptera, Hymenoptera). Most of the aphid individuals belonged to *Myzus persicae*, *Aphis nasturtii*, and *Macrosiphum euphorbiae*. A sample of Thysanoptera individuals were identified as *Thrips fuscipennis* Haliday (Plant Protection Service, sample no. PRISMA 4328863, 30 March 2011) and a sample of lepidopteran eggs was hatched and identified as *Autographa gamma.* Cicadellidae counted on whole plants were mostly nymphs with few adults, and were therefore not identified to species level. However, they were assumed to belong to either *Eupteryx* or *Empoasca*, the two genera of Cicadellidae that adults on sticky traps were attributed to. The majority of Heteroptera individuals belonged to the genus *Orius*.

On sticky traps, abundances of Cicadellidae and Syrphidae adults were quantified. Cicadellidae were identified to belong to either of the two genera *Eupteryx* or *Empoasca*. Of the total 12010 leafhopper individuals, 72% were *Eupteryx* spp. Species from the genus *Empoasca* included *E. vitis*, *E. decipiens* and *E. pteridis* (Domenico Bosco, pers. comm.). Syrphidae adults belonged to 31 species, of which *Episyrphus balteatus*, *Eupeodes corollae*, *Melanostoma mellinum*, and *Sphaerophoria scripta* were most abundant (3659 individuals / 29 species in 2010 and 1192 individuals / 17 species in 2011) (Barendregt 2009; Reemer *et al.* 2009; van Veen 2010).

In both years the field at location B was invaded by adult Colorado potato beetles in late May when the plants were still very small (average diameter ca. 20 cm). No beetles were encountered on location A. In 2010, beetle numbers reached such a high level on location B that insecticide treatment was necessary to protect plants. The experimental plot was invaded again after two weeks by a second cohort of adult beetles. In both years, no significant differences were found in the numbers of adults, eggs and larvae on the different potato cultivars. Only the number of egg batches in May 2011 showed a significant difference between cultivars (ANOVA: $F=8.25$, $df=5$, $P<0.001$, Tukey HSD: significant differences between Desiree-Premiere, Desiree-Aventra, Desiree-Karnico and Desiree-Modena; Figure 1).

The data obtained from harvested whole plants and sticky traps allow a more detailed analysis of the sources of variation at the community level, which will be presented in the following sections.

Differences between years

Both herbivores and higher trophic level OTUs occurred in much lower numbers in 2011 compared to 2010 (Figures 2-5). Non-parametric permutational MANOVA was used as a statistical tool to partition variation in NTO abundances obtained from whole plant sampling. The community level analysis (using all OTUs to calculate Bray-Curtis dissimilarities between samples) shows that the factor "year" explains a large part of the total variation in insect abundances (Figure 6, Table 1). The importance of year-to-year differences is varying per species (shown for Thysanoptera, Aphididae and Heteroptera in Figure 6). A large fraction of the effect of "year" is also attributed to interaction terms with other factors, indicating that the year effect is not consistent e.g. across locations. Strong differences between years were also found in the abundances of Syrphidae and Cicadellidae sampled on sticky traps (Figures 4+5): all species occured in much lower numbers in 2011 compared to 2010, with some species being absent for most of the year in 2011.

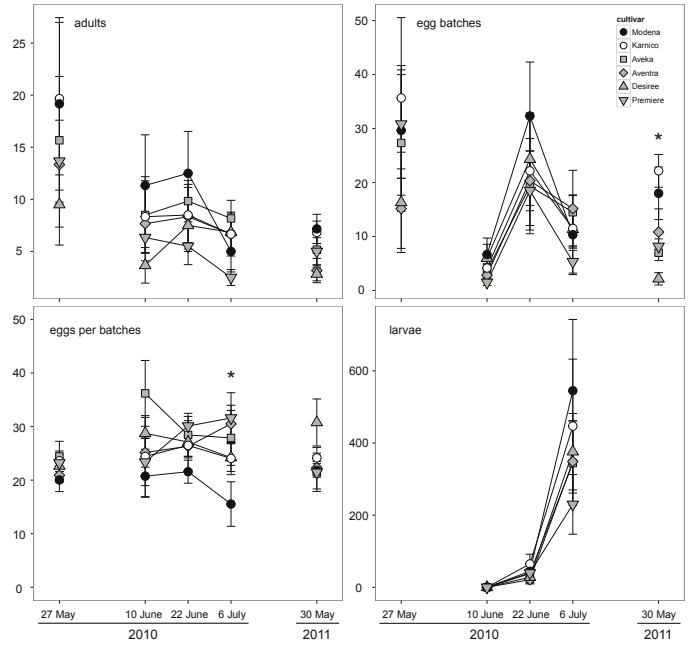

Figure 1: Colorado potato beetle (*Leptinotarsa decemlineata*) counts on different cultivars of potato (mean ± SE, n=6) on five days in 2010 and 2011. The five sampling events correspond to three separate invasions from overwintering adult beetles, two in 2010 and one in 2011.

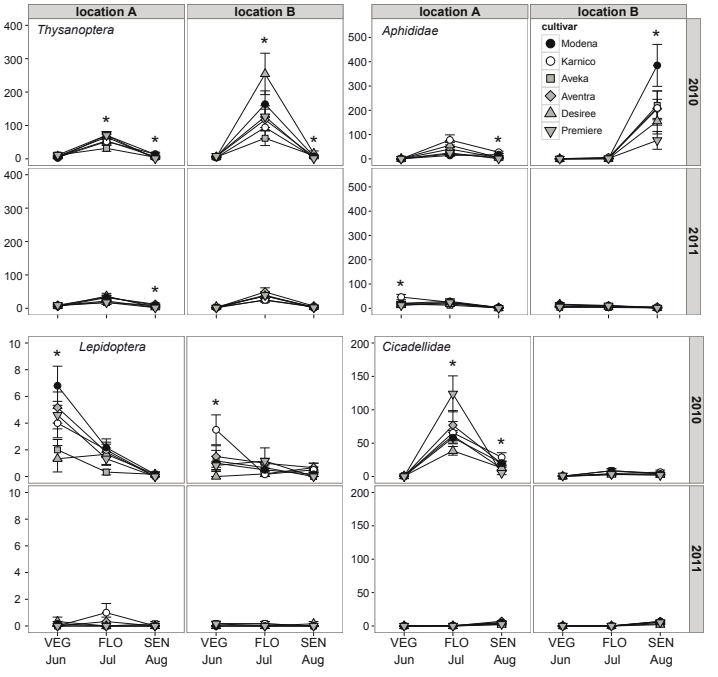

Figure 2: Total counts (mean ± SE, n=6) of four herbivorous insect groups, sampled on whole plants of six potato varieties, on two experimental field locations (A = peaty soil, B = sandy soil) in two years, at three developmental stages of plants (FLO= vegetative; FLO = flowering; SEN = senescent). Asterisks indicate significant differences between cultivars ($P < 0.05$) as derived from ANOVA's and Kruskal-Wallis tests (see Appendix I).

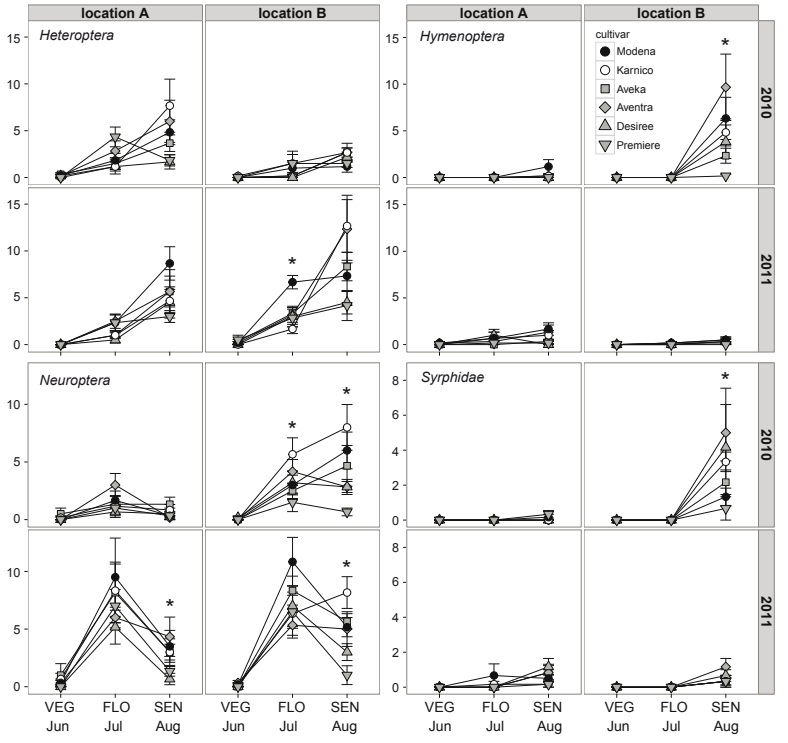

Figure 3: Total counts (mean ± SE, n=6) of four higher trophic level insect groups (predators and parasitoids), sampled on whole plants of six potato varieties, on two experimental field locations (A = peaty soil, B = sandy soil) in two years, at three developmental stages of plants (FLO= vegetative; FLO = flowering; SEN = senescent). Asterisks indicate significant differences between cultivars (P < 0.05) as derived from ANOVA's and Kruskal-Wallis tests (see Appendix I).

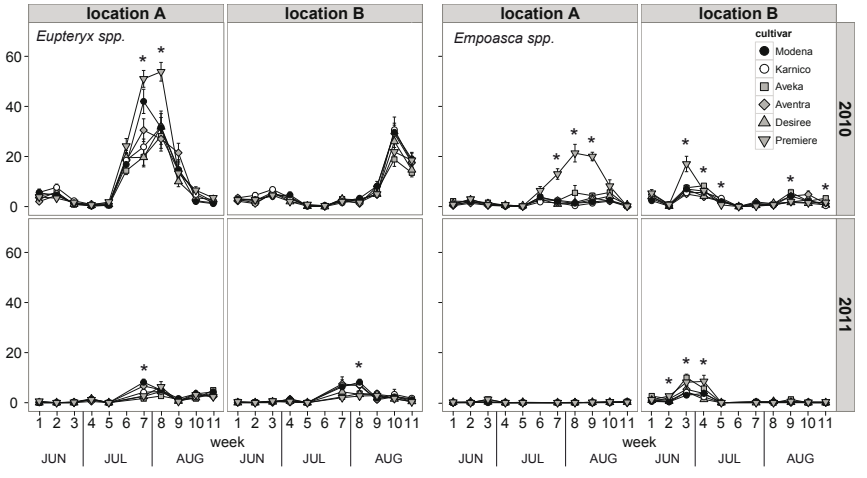

Figure 4: Total counts per sticky trap (mean ± SE, n=6) of two genera of Cicadellidae. Sticky trapping was conducted weekly from beginning of June to end of August on six potato cultivars on two locations (A = peaty soil, B = sandy soil) in two years. Asterisks indicate significant differences between cultivars (P < 0.05) per sampling event, as derived from ANOVAs and Kruskal-Wallis tests (see Appendix II).

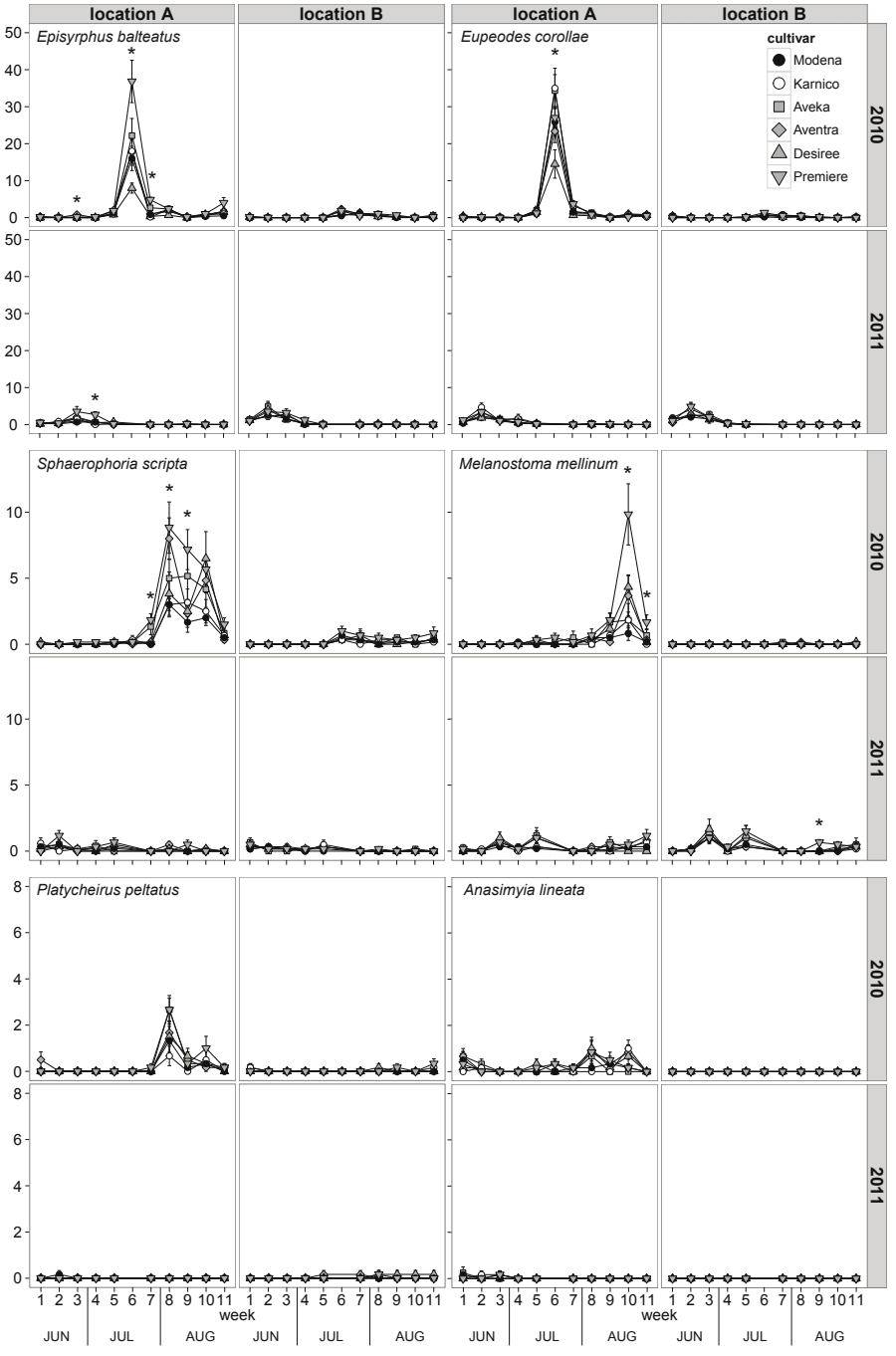

Figure 5: Total counts per sticky trap (mean ± SE, n=6) of four species of Syrphidae. Sticky trapping was conducted weekly from beginning of June to end of August on six potato cultivars on two locations (A = peaty soil, B = sandy soil) in two years. Asterisks indicate significant differences between cultivars ($P < 0.05$) per sampling event, as derived from ANOVAs and Kruskal-Wallis tests (see Appendix II).

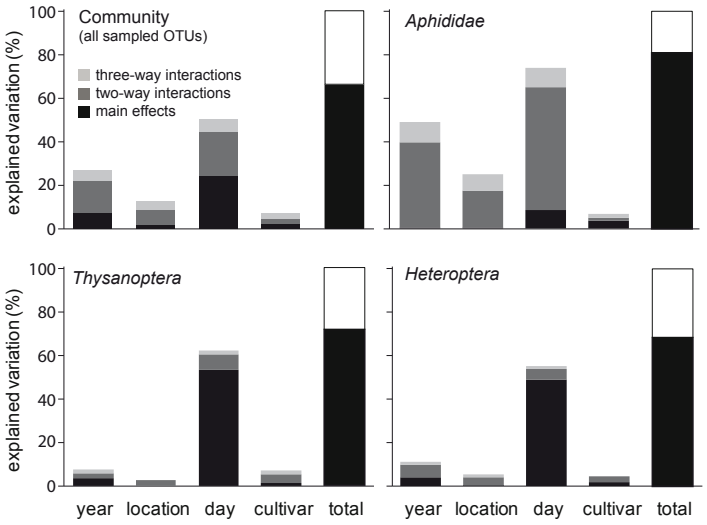

Figure 6: Explained variation (in percent of total variation) in insect abundances on whole plants, partitioned to explanatory variables. Sources of variation are shown for community data (all sampled OTUs) and selected groups (Thysanoptera, Aphididae and Heteroptera); see Appendix III for numerical results from all groups. Community data were analyzed with permutational multivariate analysis of variance based on Bray-Curtis distance matrices. Individual species abundances were analyzed with permutational ANOVA. Only statistically significant (α=0.05) main effects and interactions are shown. Note that percentages may add up to more than 100% because interaction terms are plotted redundantly.

Table 1: Partitioning of variation in insect community data (all sampled OTUs) to different sources of variation and their interactions, using permutational multivariate analysis of variance.

source of variation	DF	SS	%	F	P
year	1	9.02	7.23	76.05	0.001
location	1	2.01	1.61	16.95	0.001
developmental stage	2	30.67	24.57	129.29	0.001
cultivar	5	2.67	2.14	4.50	0.001
year × location	1	2.58	2.06	21.71	0.001
year × developmental stage	2	15.77	12.63	66.47	0.001
location × developmental stage	2	5.84	4.67	24.60	0.001
year × cultivar	5	0.65	0.52	1.09	0.317
location × cultivar	5	0.76	0.61	1.28	0.106
developmental stage × cultivar	10	2.94	2.35	2.48	0.001
year × location × developmental stage	2	3.58	2.87	15.10	0.001
year × location × cultivar	5	0.79	0.63	1.33	0.099
year × developmental stage × cultivar	10	2.19	1.75	1.85	0.001
location × developmental stage × cultivar	10	1.65	1.32	1.39	0.025
year × location × developmental stage × cultivar	10	1.40	1.12	1.18	0.136
Residuals	357	42.35	33.92		
Total	428	124.84			

Differences between locations

Most OTUs showed strong abundance differences between locations. In 2010 more Thysanoptera and Aphididae were found on location B, accompanied by a higher occurrence of Hymenoptera and Syrphidae (larvae) (Figures 2+3). Cicadellidae and Heteroptera occurred in higher abundances on location A (Figure 3). In 2011, the differences between the two locations in insect abundances were less pronounced. This change in the effect of locations between years is also reflected by the statistical interaction between "year" and "location" in permutational MANOVA (whole plant counts, Figure 6). On sticky traps, strong location effects were found in 2010, but not in 2011. Both Syrphidae (adults) and Cicadellidae occurred in higher numbers at location A.

Differences between developmental stages of plants

The most prominent source of variation in insect abundances was the difference between samplings at three different plant developmental stages, which in part reflects seasonal insect population developments. Higher trophic level insects generally occurred later in the season (on senescent plants) than herbivores. Some OTUs showed peak abundances in the middle of the season (e.g. Thysanoptera, Figure 2), others towards the end (e.g. Heteroptera, Figure 3). Syrphidae (adults on sticky traps, Figure 5) showed particularly sharp abundance peaks. The times of peak abundances were species-specific, but changed dramatically across locations and years. The two most abundant species of Syrphidae (*E. balteatu*s and *E. corollae*) showed strong peaks of abundance in mid-July (on flowering plants) on location A, but occurred at much lower abundances on location B. Peaks were very low in 2011 and were shifted towards the early season (vegetative plants, June). Aphididae (on whole plants, Figure 2) showed a sharp increase in abundance on senescent plants at the end of the season in 2010 (location B), while numbers decreased in the course of the season in 2011 (Figure 2). This is reflected by the large amount of variation explained by statistical interactions between the factor "developmental stage" and the factors "year" and "location" in permutational MANOVA (whole plant counts, Figure 6, Table 1).

Genotype effects

The effects of the different cultivars on insect abundances were generally small in comparison to the others factors considered in this study. Insect abundances on all cultivars varied across developmental stages and showed strong differences between years and location (Figures 2-5). Despite this large amount of variation, significant differences between cultivars were observed in many cases. As shown by post-hoc analyses (see Appendices I and II), cultivar differences often involved Premiere, a cultivar known to be "early" in terms of development. A significant difference between the GM line "Modena" and its counterpart "Karnico" was found only on one sampling occasion (out of 48) for Heteroptera (whole plant sampling, location B, flowering plants, see Appendix 1). In general, the presence, quality and size of cultivar differences varied between years, locations and sampling time points, indicating the importance of genotype-by-environment (GxE) interactions. Cultivar differences were often found at peak abundances (e.g. Figures 4+5). For some insect groups, cultivar ranks changed between developmental stages (e.g. Cicadellidae, Figure 2). For data obtained from whole plant sampling, this is indicated by the large fraction of variation that is explained by two-way and three-way interaction terms between the cultivar effect and environmental factors (Figure 7).

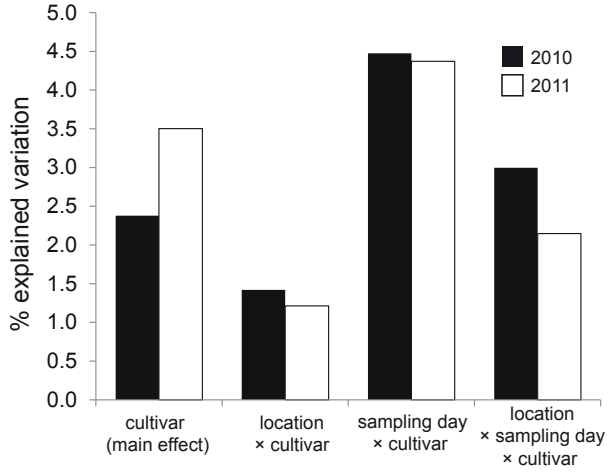

Figure 7: Genotype (=cultivar) effects on insect communities (all sampled OTUs) obtained from whole plant sampling in two years, partitioned to main effects and interactions using non-parametric permutational MANOVA. This Figure is a detail of the cultivar effect depicted in Figure 6 (top-left panel).

Discussion

Insect abundances on potato plants were found to strongly differ between years, locations and plant developmental stages, and to some extent between plant genotypes. The differences between years are probably related to the contrasting weather conditions in the growing seasons (Figure 8): In 2010, low precipitation and warm temperatures resulted in very dry conditions for plants in the early season (June –July). In 2011, precipitation and temperature were more constant throughout the growing season with more optimal growing conditions for plants. This is reflected by higher average weights of plants during mid-season in 2011 compared to 2010 (Figure 9). The lower insect abundances in 2011 are probably the result of a combination of healthy plants on the experimental field and less population growth in general in the region. Lower insects abundances in 2011 were also reported elsewhere, e.g. from aphid monitoring stations in the region (www.nak.nl/aardappelen/bladluisinformatie).

The contrasting soil conditions on locations A and B affected both abiotic (e.g. moisture, organic matter) as well as biotic (e.g. microbial and fungal soil communities) conditions (Hannula, de Boer & van Veen 2010; Inceoglu *et al.* 2010; Hannula, de Boer & van Veen 2012). The low precipitation and high water permeability of the loamy sand on location B led to very dry conditions in 2010 on this field. With sufficient precipitation on both locations in 2011, the differences between locations in insect abundances were less pronounced. Cultivars differences were larger in 2010, probably as a result of the fact that cultivars reacted differently to the dry conditions. This is reflected in the phenology of plants (Figure 10), which shows how plants were caused to terminate flowering quickly during the dry period and started flowering again when precipitation increased again. Premiere suffered most from the lack of rain and did not go back to flowering. In the more constant conditions in 2011, there were more consistent differences between cultivars.

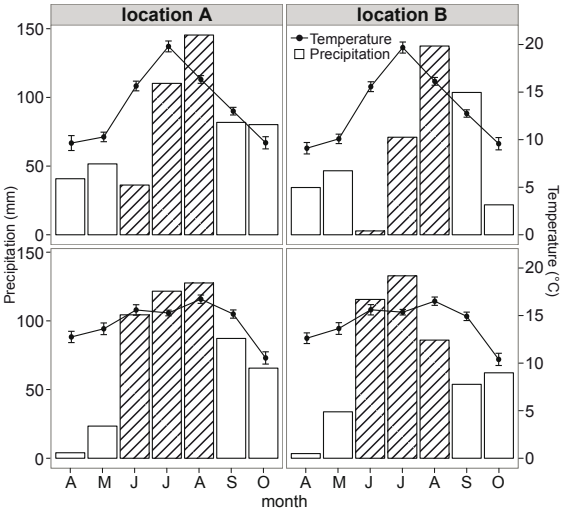

Figure 8: Temperature and precipitation on both experimental field locations and in both years from planting (April) to harvest (October). Striped bars mark the periods of regular insect sampling (June-August).

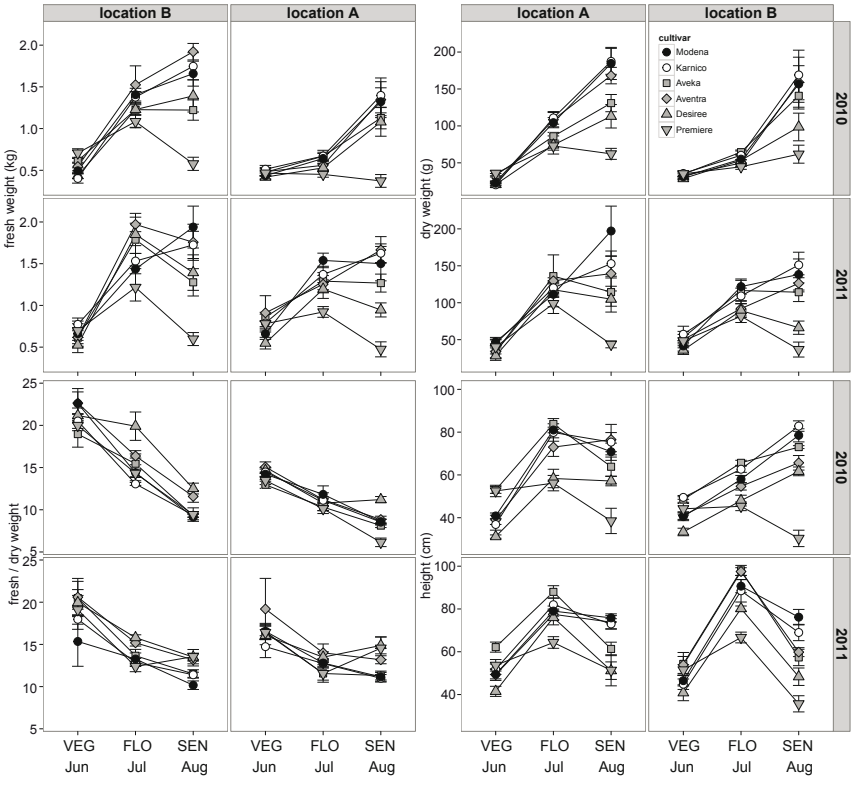

Figure 9: Fresh weight, dry weight, dry matter content and height (mean ± SE, n=6) of potato plants sampled for whole plant insect counting.

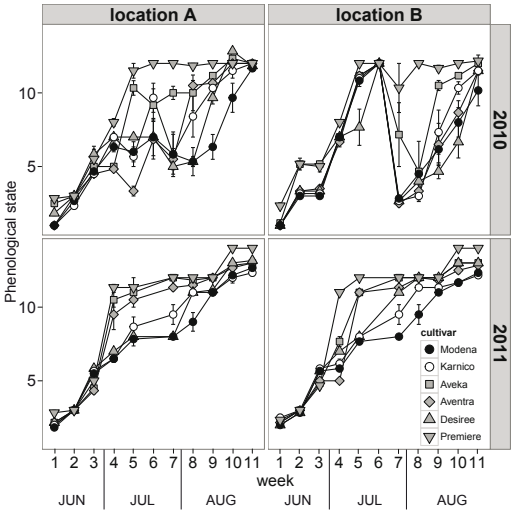

Figure 10: Phenological states (mean ± SE, n=6) of plants on experimental plots throughout the growing season (week 1 = beginning of June = 50 days after planting). Phenological state was scored on a scale from 1 to 14, with 1 = vegetative state, 5-10 = flowering, 7 = flowering peak, 13=senescence, 14 = death.

Differences in insect abundances between plant developmental stages can be caused by a wide range of factors, including changes in abiotic conditions (e.g. temperature, day length), changes in insect populations (intrinsic population growth, multitrophic interactions) and changes in plant phenotypes (morphology and metabolism). Abundance differences between developmental stages ranged from two-fold changes (e.g. Heteroptera), up to 400-fold changes in parthenogenetically reproducing aphids. In general, herbivorous insect species tended to be present on plants earlier in the season than higher trophic level species. The latter were low or absent on vegetative plants in whole plant sampling and in the early weeks of sticky trapping. The large fluctuations in insect communities within a growing season show that conclusions about genotype differences depend heavily on the time of sampling.

Conclusions for risk assessment

In this study, we show that insect communities on potato vary extensively across year, locations and plant developmental stages. Effects of plant genotypes (including GM effects) were small, when compared to the total amount of variation. This emphasizes the importance viewing GM effects in the context of baseline variation in agro-ecosystems. Other studies have found that crop management practices can have large effects non-target insect communities (Di Giulio, Edwards & Meister 2001; Boutin, Martin & Baril 2009). Such comparisons are important in order to put effects of GM crops into a wider perspective of environmental impacts of agriculture in general.

In this study, we found strong evidence for genotype-by-environment as well as genotype-by-development interactions in the effect of plants on insect abundances. These findings suggest that 1) conclusions on non-target safety are of limited validity outside the experimental region and that 2) conclusions depend strongly on the choice of sampling time points within a growing season. Limiting the number of sampling events within a growing season may produce uninformative or biased results. On the other hand, expanding sampling schemes to follow insect populations over

the course of a season will drastically increase the resources necessary to conduct risk assessment field trials. Genotype-by-environment and genotype-by-development interactions add to the level of uncertainty in risk assessment (c.f. Aslaksen, Natvig & Nordal 2006). A thorough discussion of decision-making under uncertainty, however, is not a purely scientific discourse. Under high levels of uncertainty, the ultimate goal of minimizing negative environmental impacts of GM crop cultivation may be better achieved by focusing efforts on post-market monitoring, rather than on imperfect predictions from pre-market risk assessments.

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Appendix I.

Univariate statistical analyses of cultivar differences in insect abundances from whole plant counts (6 cultivars: Modena (=GM), Karnico (=non-GM counterpart), Aveka, Aventra, Desiree, Premiere).

A) Thrips

Year	Location	Developmental stage	Test	F	Df	P	Post-hoc tests
2010	A	FLO	ANOVA	2.75	5	0.037	Aveka-Modena
2010	A	SEN	ANOVA	4.82	5	0.002	Premiere-Desiree Premiere-Karnico Premiere-Modena
2010	B	FLO	ANOVA	3.00	5	0.027	Aveka-Aventra Aveka-Desiree Aveka-Modena Aveka-Premiere
2010	B	SEN	ANOVA	3.79	5	0.009	Premiere-Desiree Premiere-Karnico Premiere-Modena
2011	A	SEN	ANOVA	3.13	5	0.026	-

B) Aphids

Year	Location	Developmental stage	Test	F	Df	P	Post-hoc tests
2010	A	SEN	ANOVA	16.62	5	<0.001	Aveka-Karnico Aveka-Modena Aveka-Premiere Aventra-Karnico Aventra-Modena Aventra-Premiere Desiree-Karnico Desiree-Modena Desiree-Premiere Premiere-Karnico Premiere-Modena
2010	B	SEN	ANOVA	3.41	5	0.015	Premiere-Modena
2011	A	VEG	ANOVA	2.68	5	0.040	Premiere-Karnico

C) Moth eggs

Year	Location	Developmental stage	Test	F	Df	P	Post-hoc tests
2010	A	VEG	ANOVA	3.79	5	0.010	Desiree-Aventra Desiree-Modena
2010	B	VEG	ANOVA	2.75	5	0.037	Karnico-Desiree

D) Leafhoppers

Year	Location	Developmental stage	Test	F	Df	P	Post-hoc tests
2010	A	FLO	ANOVA	3.03	5	0.025	Premiere-Desiree
2010	A	SEN	ANOVA	5.06	5	0.002	Premiere-Aventra Premiere-Karnico Premiere-Modena

E) Predatory bugs

Year	Location	Developmental stage	Test	F	Df	P	Post-hoc tests
2011	B	FLO	ANOVA	4.42	5	0.004	Modena-Aventra Modena-Desiree Modena-Karnico Modena-Premiere
2011	B	SEN	ANOVA	3.00	3	0.026	-

F) Aphid mummies

Year	Location	Developmental stage	Test	F	Df	P	Post-hoc tests
2010	B	SEN	ANOVA	3.31	5	0.017	Premiere-Aventra Premiere-Modena

G) Lacewing eggs

Year	Location	Developmental stage	Test	F	Df	P	Post-hoc tests
2010	B	FLO	ANOVA	2.85	5	0.032	Premiere-Karnico
2010	B	SEN	ANOVA	6.26	5	<0.001	Premiere-Aveka Premiere-Aventra Premiere-Karnico Premiere-Modena
2011	A	SEN	ANOVA	2.87	5	0.031	Desiree-Aventra
2011	B	SEN	ANOVA	7.57	5	<0.001	Premiere-Aveka Premiere-Aventra Premiere-Karnico Premiere-Modena Karnico-Desiree

H) Syrphid larvae

Year	Location	Developmental stage	Test	χ2	Df	P	Post-hoc tests
2010	B	SEN	Kruskal-Wallis	14.65	5	0.012	Aventra-Premiere

Appendix II.

Univariate statistical analyses of cultivar differences in insect abundances from sticky trap sampling (6 cultivars: Modena (=GM), Karnico (=non-GM counterpart), Aveka, Aventra, Desiree, Premiere). Only significant test (α=0.05) are shown.

A) Hemiptera\Cicadellidae

Empoasca spp.

Year	Location	Week	Test	F / χ2	Df	P	Post-hoc tests
2010	A	7	ANOVA	8.72	5	<0.001	Premiere-Aveka Premiere-Aventra Premiere-Desiree Premiere-Karnico Premiere-Modena
2010	A	8	ANOVA	10.65	5	<0.001	Premiere-Aveka Premiere-Aventra Premiere-Desiree Premiere-Karnico Premiere-Modena
2010	A	9	ANOVA	24.66	5	<0.001	Premiere-Aveka Premiere-Aventra Premiere-Desiree Premiere-Karnico Premiere-Modena
2010	B	3	ANOVA	4.82	5	0.002	Premiere-Aveka Premiere-Aventra Premiere-Desiree Premiere-Karnico Premiere-Modena
2010	B	4	ANOVA	2.61	5	0.045	-
2010	B	5	ANOVA	3.26	5	0.018	Premiere-Karnico
2010	B	9	Kruskal-Wallis	14.67	5	0.012	-
2010	B	11	ANOVA	3.50	5	0.013	Aveka-Desiree Aveka-Karnico
2011	A	3	Kruskal-Wallis	13.60	5	0.018	-
2011	B	2	ANOVA	3.34	5	0.016	Premiere-Karnico Premiere-Modena
2011	B	3	ANOVA	3.92	5	0.007	Aveka-Karnico Aveka-Modena Premiere-Modena
2011	B	4	ANOVA	5.05	5	0.002	Aveka-Desiree Premiere-Desiree

Eupteryx spp.

Year	Location	Week	Test	F / χ2	Df	P	Post-hoc tests
2010	A	7	ANOVA	7.60	5	<0.001	Aveka-Modena Aveka-Premiere Desiree-Modena Desiree-Premiere Premiere-Karnico
2010	A	8	ANOVA	3.07	5	0.023	Premiere-Aveka Premiere-Aventra
2010	B	2	ANOVA	3.30	5	0.017	Karnico-Aventra
2011	A	7	ANOVA	4.72	5	0.003	Aveka-Aventra Aveka-Modena Desiree-Modena
2011	B	8	ANOVA	3.21	5	0.019	Premiere-Modena

B. Diptera\Syrphidae

Episyrphus balteatus

Year	Location	week	Test	F / χ2	Df	P	Post-hoc tests
2010	A	3	Kruskal-Wallis	12.36	5	0.030	-
2010	A	6	ANOVA	5.32	5	0.001	Premiere-Desiree Premiere-Modena
2010	A	7	Kruskal-Wallis	17.88	5	0.003	Premiere-Desiree Premiere-Karnico
2011	A	4	Kruskal-Wallis	11.97	5	0.035	-

Eupeodes corrollae

Year	Location	week	Test	F / χ2	Df	P	Post-hoc tests
2010	A	6	ANOVA	4.02	5	0.007	Desiree-Aveka Desiree-Karnico

Sphaerophoria scripta

Year	Location	week	Test	F / χ2	Df	P	Post-hoc tests
2010	A	7	Kruskal-Wallis	22.57	5	>0.001	Premiere-Karnico Premiere-Modena
2010	A	8	ANOVA	3.37	5	0.016	-
2010	A	9	ANOVA	3.00	5	0.026	Premiere-Modena

Melanostoma mellinum

Year	Location	week	Test	F / χ2	Df	P	Post-hoc tests
2010	A	10	ANOVA	5.85	5	<0.001	Premiere-Aveka Premiere-Karnico Premiere-Modena
2010	A	11	Kruskal-Wallis	12.92	5	0.024	-
2011	B	9	Kruskal-Wallis	21.88	5	<0.001	-

Appendix III.

Partitioning of variation in insect abundances to different sources of variation and their interactions, using permutational ANOVA.

A) Thysanoptera

Sources of variation	DF	SS	F	%	P
year	1	37.64	46.70	3.73	0.001
location	1	1.07	1.33	0.11	0.273
developmental stage	2	539.38	334.63	53.47	0.001
cultivar	5	11.38	2.83	1.13	0.013
year × location	1	9.59	11.90	0.95	0.001
year × developmental stage	2	9.34	5.80	0.93	0.008
location × developmental stage	2	18.49	11.47	1.83	0.001
year × cultivar	5	2.10	0.52	0.21	0.743
location × cultivar	5	1.84	0.46	0.18	0.803
developmental stage × cultivar	10	40.34	5.00	3.00	0.001
year × location × developmental stage	2	2.20	1.37	0.22	0.252
year × location × cultivar	5	7.53	1.87	0.75	0.096
year × developmental stage × cultivar	10	20.86	2.59	2.07	0.001
location × developmental stage × cultivar	10	5.21	0.65	0.52	0.774
year × location × developmental stage × cultivar	10	14.02	1.74	1.39	0.075
Residuals	357	287.72		28.52	
Total	428	1008.72		100	

B) Lepidoptera (only 2010)

Sources of variation	DF	SS	F	%	P
location	1	10.26	21.57	5.51	0.001
developmental stage	2	42.79	44.99	22.98	0.001
cultivar	5	9.49	3.99	5.10	0.003
location × developmental stage	2	13.79	14.50	7.41	0.001
location × cultivar	5	4.04	1.70	2.17	0.146
developmental stage × cultivar	10	13.05	2.75	7.01	0.004
location × developmental stage × cultivar	10	8.60	1.81	4.62	0.059
Residuals	177	84.19		45.21	
Total	212	186.22		100	

C) Hemiptera\Aphididae

Sources of variation	DF	SS	F	%	P
year	1	7.15	9.08	0.49	0.003
location	1	5.99	7.59	0.41	0.016
developmental stage	2	122.66	77.82	8.47	0.001
cultivar	5	48.16	12.22	3.33	0.001
year × location	1	9.36	11.88	0.65	0.001
year × developmental stage	2	554.83	351.99	38.31	0.001
location × developmental stage	2	233.38	148.06	16.12	0.001
year × cultivar	5	4.61	1.17	0.32	0.335
location × cultivar	5	2.24	0.57	0.15	0.745
developmental stage × cultivar	10	24.08	3.06	1.66	0.001
year × location × developmental stage	2	107.77	68.37	7.44	0.001
year × location × cultivar	5	5.14	1.31	0.36	0.278
year × developmental stage × cultivar	10	23.89	3.03	1.65	0.002
location × developmental stage × cultivar	10	7.08	0.90	0.49	0.545
year × location × developmental stage × cultivar	10	10.39	1.32	0.72	0.216
Residuals	357	281.36		19.43	
Total	428	1448.09		100	

D)Hemiptera\Cicadellidae

Sources of variation	DF	SS	F	%	P
year	1	219.71	465.26	19.80	0.001
location	1	51.39	108.83	4.63	0.001
developmental stage	2	315.12	333.65	28.40	0.001
cultivar	5	10.36	4.39	0.93	0.001
year × location	1	53.60	113.50	4.83	0.001
year × developmental stage	2	189.67	200.82	17.09	0.001
location × developmental stage	2	33.80	35.79	3.05	0.001
year × cultivar	5	2.16	0.92	0.20	0.456
location × cultivar	5	1.58	0.67	0.14	0.654
developmental stage × cultivar	10	16.82	3.56	1.52	0.002
year × location × developmental stage	2	28.15	29.80	2.54	0.001
year × location × cultivar	5	1.31	0.56	0.12	0.731
year × developmental stage × cultivar	10	7.52	1.59	0.68	0.116
location × developmental stage × cultivar	10	2.11	0.45	0.19	0.925
year × location × developmental stage × cultivar	10	7.66	1.62	0.69	0.092
Residuals	357	168.59		15.19	
Total	428	1109.55		100	

E) Hemiptera\Heteroptera

Sources of variation	DF	SS	F	%	P
year	1	20.53	41.93	3.74	0.001
location	1	0.11	0.23	0.02	0.652
developmental stage	2	266.92	272.55	48.65	0.001
cultivar	5	8.64	3.53	1.57	0.006
year × location	1	21.40	43.70	3.90	0.001
year × developmental stage	2	11.51	11.75	2.10	0.001
location × developmental stage	2	0.16	0.17	0.03	0.856
year × cultivar	5	2.59	1.06	0.47	0.373
location × cultivar	5	1.22	0.50	0.22	0.79
developmental stage × cultivar	10	16.49	3.37	3.01	0.001
year × location × developmental stage	2	6.86	7.01	1.25	0.002
year × location × cultivar	5	1.83	0.75	0.33	0.585
year × developmental stage × cultivar	10	2.74	0.56	0.50	0.834
location × developmental stage × cultivar	10	7.18	1.47	1.31	0.148
year × location × developmental stage × cultivar	10	5.68	1.16	1.04	0.344
Residuals	357	174.81		31.86	
Total	428	548.68		100	

F) Hymenoptera

Sources of variation	DF	SS	F	%	P
year	1	0.83	3.28	0.39	0.054
location	1	2.61	10.34	1.24	0.001
developmental stage	2	37.04	73.42	17.66	0.001
cultivar	5	7.51	5.95	3.58	0.001
year × location	1	12.42	49.24	5.92	0.001
year × developmental stage	2	8.96	17.75	4.27	0.001
location × developmental stage	2	10.92	21.64	5.20	0.001
year × cultivar	5	0.44	0.35	0.21	0.887
location × cultivar	5	1.03	0.82	0.49	0.538
developmental stage × cultivar	10	10.12	4.01	4.82	0.001
year × location × developmental stage	2	15.56	30.83	7.42	0.001
year × location × cultivar	5	2.26	1.79	1.08	0.113
year × developmental stage × cultivar	10	2.37	0.94	1.13	0.491
location × developmental stage × cultivar	10	3.08	1.22	1.47	0.262
year × location × developmental stage × cultivar	10	4.59	1.82	2.19	0.062
Residuals	357	90.06		42.93	
Total	428	209.79		100	

G) Neuroptera

Sources of variation	DF	SS	F	%	P
year	1	40.91	103.47	7.03	0.001
location	1	18.90	47.80	3.25	0.001
developmental stage	2	266.35	336.83	45.77	0.001
cultivar	5	23.91	12.09	4.11	0.001
year × location	1	8.23	20.83	1.42	0.001
year × developmental stage	2	18.61	23.53	3.20	0.001
location × developmental stage	2	20.90	26.43	3.59	0.001
year × cultivar	5	1.76	0.89	0.30	0.477
location × cultivar	5	5.73	2.90	0.98	0.012
developmental stage × cultivar	10	12.91	3.27	2.22	0.001
year × location × developmental stage	2	2.96	3.74	0.51	0.023
year × location × cultivar	5	0.51	0.26	0.09	0.941
year × developmental stage × cultivar	10	8.14	2.06	1.40	0.02
location × developmental stage × cultivar	10	7.61	1.93	1.31	0.042
year × location × developmental stage × cultivar	10	3.34	0.85	0.58	0.558
Residuals	357	141.15		24.26	
Total	428	581.92		100	

H) Diptera\Syrphidae

Sources of variation	DF	SS	F	%	P
year	1	0.45	2.83	0.31	0.106
location	1	3.88	24.17	2.65	0.001
developmental stage	2	36.33	113.16	24.76	0.001
cultivar	5	1.87	2.33	1.28	0.04
year × location	1	6.59	41.03	4.49	0.001
year × developmental stage	2	1.59	4.94	1.08	0.007
location × developmental stage	2	9.70	30.20	6.61	0.001
year × cultivar	5	0.75	0.93	0.51	0.456
location × cultivar	5	2.14	2.66	1.46	0.023
developmental stage × cultivar	10	4.13	2.57	2.82	0.007
year × location × developmental stage	2	10.61	33.05	7.23	0.001
year × location × cultivar	5	1.78	2.21	1.21	0.042
year × developmental stage × cultivar	10	1.97	1.23	1.34	0.278
location × developmental stage × cultivar	10	4.16	2.59	2.83	0.005
year × location × developmental stage × cultivar	10	3.47	2.16	2.36	0.019
Residuals	357	57.31		39.06	
Total	428	146.7		100	

Effect measurement and limits of concern in non-target risk assessment of GM crops

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Abstract

A central requirement in assessing the risks of genetically modified (GM) plants towards non-target organisms (NTO) is the definition of threshold values for GM/non-GM differences, the so-called "limits of concern". Due to a lack of ecological information, these limits of concern are often chosen arbitrarily and set to the same value for all tested species. Here, we show some of the consequences of this current practice for experimental field design as guided by power analysis. Arbitrary limits of concern result in large differences in statistical power between species, although the biological relevance of the assumed thresholds is unclear. Observed variation in field trials may indicate biological baseline variation that could guide the definition of thresholds. Here, we discuss standardized effect sizes as a way of incorporating information about variability in the measurement of effects. Furthermore, we suggest the use of multivariate analyses in non-target risk assessment, because the current approach of measuring effects separately per non-target group lacks a systems biology perspective.

Keywords: insect communities, transgenic plants, power analysis, environmental effects

The safety of genetically modified (GM) crops towards non-target organisms (NTO) is usually assessed by comparing a GM line to its near-isogenic, non-modified counterpart. A central requirement in NTO risk assessment is the definition of threshold values for the difference between the GM and its counterpart in any given measurement endpoint, e.g. the abundance of non-target insect species. These so-called 'limits of concern' are defined as the minimum differences of sufficient magnitude to cause ecological harm (EFSA 2010). However, little guidance is currently provided on how limits of concern for non-target organisms should be determined. Ideally, limits of concern should be based on species-specific information about population dynamics and ecological interactions. This approach of establishing ecologically justified limits of concern has been followed for individual species using existing knowledge and mathematical modeling (e.g. O'Callaghan, Soboleva & Barratt 2010). However, such detailed ecological information is rarely available for the wide range of non-target organisms that often is considered in field trials. Therefore, it has become common practice to work with fixed, arbitrary limits of concern for all of the tested species. Typical limits of concern across species are between 30-50% abundance difference between the GM plant and its counterpart (e.g. Prasifka *et al.* 2008; EFSA 2010; Albajes *et al.* 2012).

In this study, we examine some of the problems and consequences of the current practice. First, we show that arbitrary limits of concern have implicit consequences for experimental field designs as guided by power analysis. Second, we discuss standardized effect sizes as an alternative way of quantifying effects in relation to biological variation. Finally, we point out the limitation of the current per-endpoint-analysis when effects at the community level may be ecologically as relevant as changes in individual species.

Arbitrary limits of concern and power analysis

Power analysis in non-target risk assessments ensures that experiments generate data that can inform the decision-making process, rather than being interesting from a purely scientific point of view (Romeis, Lawo & Raybould 2009). Statistical power is the probability of correctly rejecting a false null hypothesis of no difference in a statistical test. Statistical power is defined as 1-β, where β is the Type II error rate, or the chance of accepting the null hypothesis while in fact it was false. Calculating the power of a statistical test requires an estimate of the expected variation and the definition of a desired effect size that one wishes to detect. Estimating statistical power before the start of an experiment (prospective power analysis) aids the design of experiments that are capable of detecting differences when they are present (Marvier 2002; Perry *et al.* 2003). Power analysis deserves particular attention in risk assessment, where avoiding Type II errors (concluding no difference when there actually is a difference) is more important than avoiding Type I errors (concluding that there is a difference when there actually is no difference), because the former may suggest a false sense of safety to regulators and decision makers with potentially hazardous consequences to the environment (Perry *et al.* 2009). While the Type I error rate (α) is typically set to 5% in scientific practice, β is usually uncontrolled.

In NTO risk assessment, the desired effect size that is defined in power analysis will equal the limits of concern. For example, one may want to be able to detect a difference of 30% difference between GM and counterpart, because this effect size constitutes a biologically relevant effect. When an effect size is defined in power analysis, it is assumed that variation around

means (i.e. variation between replicate plots in a field experiment) indicates sampling quality. Consequently, power analysis will indicate low statistical power for species with high abundance variation between replicates. When limits of concern are set arbitrarily at the same value for all non-target species, this results in large differences between species in statistical power and required sample sizes, even though it is unclear whether the originally assumed limit of concern constitutes a biologically relevant effect in all species.

We performed a power analysis using variability estimates from a field trial conducted in the Netherlands where a number of non-target insect groups were counted on the GM potato "Modena" (grant no. NRR 30805, AVEBE UA, Foxhol, The Netherlands/BASF Plant Science Co. GmbH) and its near-isogenic comparator "Karnico" on six replicate plots (chapter 2, this thesis). Insect sampling was done by harvesting the complete above-ground material of one plant per plot and counting all insects on it. For this example, we used insect data obtained by counting insects on flowering plants at one of two locations (location A) in one of two years (2010). Assuming a two-sample t-test for the difference between GM and non-GM, we performed a power analysis to calculate the number of replicates that would be required to a range of desired effect sizes with a power of 0.8 and two-sided $\alpha = 0.05$. Figure 1 shows the resulting power curves for different taxa, indicating the amount of replicate plots that would be necessary in order to detect effect sizes between 0% and 100% (percent difference on GM plant compared to counterpart). This analysis shows that when limits of concern are set to 30%, a minimum of 50 replicates would be necessary to detect such an effect with a power of 0.8 in the least variable species, and a multiple of 50 for more variable species. Already for the rather small plot size in this experiment (10 m^2 per replicate plot), the minimum required surface area would be 500 m^2 per cultivar, assuming that only the species with the highest power are tested. Although larger replicate plot sizes are likely to reduce variabilities, and thus the number of replicates needed, it is clear that experimental fields in non-target risk assessment require large surface areas, and that large differences in variability between species will make many species practically untestable. Some authors have therefore suggested using statistical power as a criterion for selecting focal species for field trials, thus omitting taxa with low statistical power from further analysis (Prasifka *et al.* 2008; Albajes *et al.* 2012). However, when the causes for the differences in variation are unclear, selecting taxa by power can create an undesirable bias in the selection.

The above-mentioned problems of large differences in power and sample size requirements between species are a result of the assumptions that 1) the size of biologically relevant effects is the same across species and 2) that variability between replicate plots indicates sampling precision. However, when differences in variability between species have biological reasons, it may be useful to incorporate a measure of variability in the definition of effects. This is the idea behind standardized effect sizes, which will be discussed in the following paragraphs.

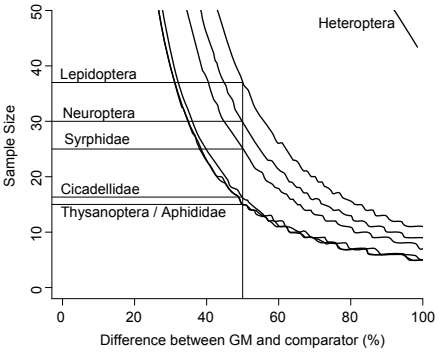

Figure 1: Power curves showing the sample size that is required to test for a given difference (%) between a GM potato plant and its comparator with a power of 0.8 in a two sample t-test. Data were taken from a field field experiment conducted in the Netherlands (chapter 2, this thesis: insect abundances on flowering plants at location A, 2010). Due to the large differences between species in variability, the required sample sizes to test for commonly used limits of concern of 30-50 % strongly differ between species.

Standardized effect sizes

Standardized effect sizes quantify the size of a difference in relation to the variation in the measured variable. Since the parameter of interest in risk assessment is the difference between a GM plant and its non-modified counterpart, we focus here on Cohen's (1988) standardized difference (*d*). Cohen's *d* is calculated by dividing the difference between two means ($m_1 - m_2$) by a standard deviation (*s*) derived from the data. Cohen (1988) suggested using either of the standard deviations of the two compared groups for the calculation of *d.* Other authors have suggested using pooled standard deviations (s_{pooled}) for the calculation, where the standard deviations of the two groups (s_1 and s_2) are weighted by their sample sizes (n_1 and n_2) (Hedges 1981):

$$s_{pooled} = \sqrt{\frac{(n_2 - 1)s_2^2 + (n_1 - 1)s_1^2}{n_1 + n_2 - 2}}$$

For a more detailed description of SES calculations in different situations the reader is referred to Nakagawa & Cuthill (2007).

Standardized effect sizes are increasingly reported in many disciplines as a supplement to statistical hypothesis tests indicating the magnitude and relevance of an effect, and they are routinely used for comparing effects across studies in meta-analyses. For example, Marvier *et al.* (2007) performed a meta-analysis of 42 field studies reporting non-target invertebrate abundances on Bt cotton and maize carrying different bacterial *cry* proteins, using Hedges *g* (=Cohen's *d* with pooled standard deviations).

Standardized effect sizes quantify the size of an effect in relation to the variability in the measured parameter. In contrast, percentages quantify the size of an effect in relation to the size of the mean of the measured parameter. Thus, SES represent a different measurement scale for

effects. The choice of a measurement scale is also a decision on how risks are characterized (see also Andow 2003). In risk assessment, the relevance of a change in the abundance of a non-target organism may depend more on whether or not that change exceeds a certain level of biological baseline variation rather than on how large the change is compared to the mean abundance. When differences are quantified (and limits of concern are set) as SES, highly variable species are allowed to exhibit larger absolute differences in means in a comparative field trial than less variable species. High variation between replicate plots may be caused by high local reproduction rates or spatial aggregation behavior. In such cases, larger standard deviations would indicate higher biological baseline variation. It would therefore be reasonable to assume higher thresholds for biologically relevant effects (i.e. higher limits of concern). Thus, SES may be a way to account for biological baseline variation in the definition of effects on non-target organisms.

There has also been some criticism of SES, especially in toxicological literature, because "combined" (difference + standard deviation) effect size metrics may obscure absolute changes in variables (e.g. Lenth 2001; EFSA 2011). In toxicology, SES may indeed be less useful, because there is no reason to assume that high variability in the concentration of a toxic compound would affect the relevance of a concentration change for human health. The usefulness of SES for NTO risk assessment should also be confirmed by more ecological research on the relationship between abundance variability and population resilience on a larger spatial scale.

Standardized effect sizes can be used to perform power analyses in a similar way as other effect metrics. As shown in Figure 2, the power analysis becomes uniform across species with a single power curve for all taxa as a consequence of using Cohen's *d* to quantify effects. The number of replicates that is necessary to detect a given effect has now become independent of the particular species, because differences in variability between species are incorporated in the *d* statistic. From this analysis more generic conclusions can be drawn with respect to experimental design. For example, detecting an effect of size *d* = 0.8 with a power of 0.8 requires 25 replicates.

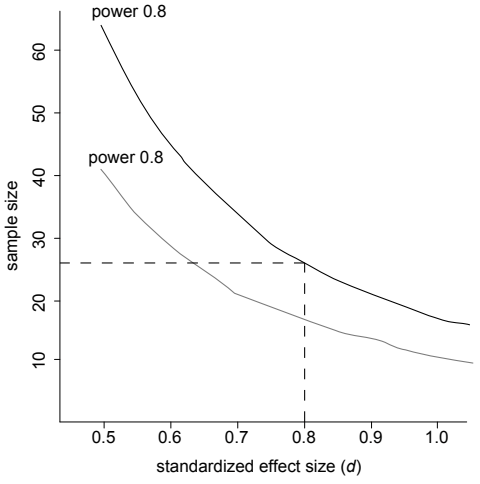

Figure 2: Power analysis of a two-sample t-test using standardized effect size *d* as a measure of difference instead of percentages, which results in uniform power curves across species. For example, in order to detect an effect size of *d* = 0.8 with a power of 0.8, 25 replicates would be necessary.

Standardized effects sizes can also be used to perform so-called equivalence tests. The idea behind equivalence testing is that the "onus is placed back on to those who wish to demonstrate the safety of GMOs to do high quality, well-replicated experiments" (Perry *et al.* 2009). In equivalence testing, a null hypothesis of 'difference' is assumed instead of the traditional null hypothesis of 'no difference'. Equivalence is concluded when the difference between the GM plant and its comparator is shown to be significantly smaller than a given threshold value, which is defined *a priori* by the limits of concern. Equivalence tests can be illustrated by presenting the size of a difference and its confidence interval (CI), along with the zero line of no difference and the limits of concern (Cohen 1994; Nakagawa & Cuthill 2007). The outcomes of both difference tests and equivalence tests can be conveniently depicted in a single graph using CIs. Equivalence may only be concluded when the CI of the difference falls entirely within the specified limits of concern (for examples see Perry *et al.* 2009; EFSA 2010b). Confidence intervals can be calculated for standardized effect sizes by using the MBESS package (Kelley & Lai 2011) of the statistical software R (R Development Core Team 2010). Figure 3 shows, for different values of Cohen's *d*, how the width of the CI for *d* depends on the sample size. Confidence intervals for *d* become narrower with larger sample sizes, thus increasing the chance that they fall within the limits of concern. On the other hand, the chance of concluding equivalence decreases with increased *d*.

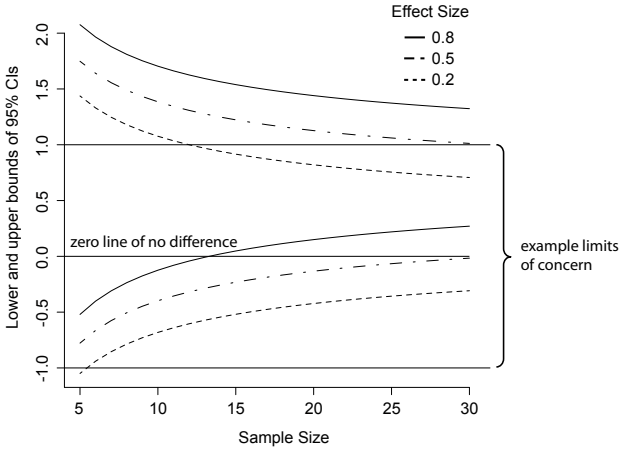

Figure 3: Upper and lower bounds of 95% confidence intervals of standard effect size *d*, as a function of sample size for three values of *d* (0.2, 0.5 and 0.8). For example, an effect size of *d* = 0.8 can only be concluded to be significantly different from *d* = 0 (i.e. confidence interval does not include 0) with a minimum sample size of n = 15 in a test of difference. In equivalence testing, when the limits of concern are set to *d* = 1.0, a same sample size of n = 15 only allows the conclusion of equivalence for effect sizes smaller than *d* = 0.2 (confidence limits fall within *d* = ±1.0).

Univariate versus multivariate analysis

Measures of effect sizes and limits of concern should reflect relevant changes in ecosystems. However, system-level changes are rarely considered in NTO risk assessment studies. GM effects are usually quantified separately per measurement endpoint, and limits of concern are set for each endpoint independently. Considering GM effects independently per species in univariate analyses lacks an ecological community perspective and could potentially miss important

changes at the system level. Consider the hypothetical example in Figure 4: abundances of three species (1, 2 and 3) are estimated for each of three genotypes (A, B and C). The abundances of all three species are above the limit of concern (LC) in all three genotypes, and would thus not be considered at risk. However, when all three species are considered simultaneously, it becomes clear that the three genotypes have (quantitatively) distinct NTO communities. These distinct communities may potentially exhibit distinct functionalities in terms of ecological services. Such system-level changes cannot be detected with univariate analyses, but instead require multivariate approaches. Some studies have used diversity metrics (e.g.Whitehouse, Wilson & Constable 2007; Farinos *et al.* 2008) or quantitative food webs (von Burg *et al.* 2011) for studying non-target effects of GM plants. However, community changes have to be linked to ecological service functionality, and standards for defining multivariate limits of concern need to be defined. These are important future challenges in NTO risk assessment.

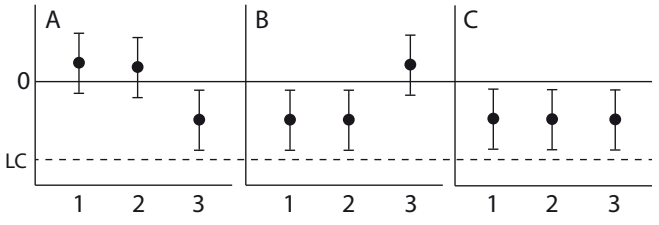

Figure 4: Hypothetical example of three species (1, 2 and 3) sampled on three plant genotypes (A, B and C). All three species are above the limit of concern (LC) in all three genotypes. However, from a multivariate perspective, all three genotypes host three (quantitatively) distinct communities. Bars represent 95 % confidence intervals.

Concluding remarks

The current practice of setting limits of concern in NTO risk assessment often lacks an ecological justification. Arbitrary limits of concern may be a starting point for detecting effects, but have important consequences for statistical power and experimental design. Using standardized effect sizes as an alternative way of quantifying effects may offer a biologically more meaningful way of quantifying effects in NTO risk assessments. However, the main challenge is the lack of knowledge about the biological causes of variability and whether or not this variability is related to the resilience of a species at larger spatial scales.

Multivariate analysis techniques offer a systems biology perspective to non-target risk assessment that is lacking in the current way of consideration endpoints separately. However, defining standards for multivariate analyses and for the setting of multivariate limits of concern are important future challenges.

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Metabolomic plasticity in GM and non–GM potato leaves in response to aphid herbivory and virus infection

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Abstract

An important aspect of ecological safety of genetically modified (GM) plants is the evaluation of unintended effects on plant–insect interactions. These interactions are to a large extent influenced by the chemical composition of plants. This study uses NMR–based metabolomics to establish a baseline of chemical variation to which differences between a GM potato line and its parent cultivar are compared. The effects of leaf age, virus infection and aphid herbivory on plant metabolomes were studied. The metabolome of the GM line differed from its parent only in young leaves of non–infected plants. This effect was small when compared to the baseline. Consistently, aphid performance on excised leaves was influenced by leaf age while no difference in performance was found between GM and non–GM plants. The metabolomic baseline approach is concluded to be a useful tool in ecological safety assessment.

Keywords: Solanum tuberosum, genetic modification, risk assessment, potato virus Y, *Myzus persicae*, NMR, metabolomics

Introduction

One of the concerns regarding the cultivation of genetically modified (GM) plants is their possible impact on insect ecology and biodiversity in agricultural fields (Conner, Glare and Nap 2003). Measuring such effects, however, is not straightforward because ecological impacts are neither easily defined nor is their chance of occurrence easily predicted. Fundamental knowledge of complex ecological interactions would often be required and this knowledge is in most cases not readily available. Comparative risk assessment is an alternative that provides clear criteria for safety without directly predicting ecological processes (Perry *et al.* 2009; EFSA 2010). In comparative risk assessment the changes introduced by genetic modification are compared to a baseline of variation present in the system under study. For example, a change in insect performance on a plant due to genetic modification would be considered safe when that change does not exceed the baseline of variation in insect performance on this plant. Baselines should capture the variability in the agricultural system under study and consist of a selection of relevant factors, e.g. variation among different cultivars of the same plant species, different environmental conditions, locations, etc.

The present study applies the comparative approach to the study of risks regarding ecological interactions between a plant, an insect and a virus species, using a GM potato cultivar and its non–GM counterpart as a case study. Because it is practically impossible to measure all ecological interactions between a plant and its associated insect species in all possible environmental conditions, leaf chemistry of plants is used here as an indicator of possible changes to plant–insect interactions. The strong influence of plant chemical traits on ecological relationships with insects has been shown repeatedly: both primary and secondary plant metabolites have been found to affect food webs over several trophic levels above and below ground (Van der Putten *et al.* 2001; Inbar and Gerling 2008; Poelman, van Loon and Dicke 2008; Schwachtje and Baldwin 2008). Thus, demonstrating chemical equivalence between a GM plant and its comparator(s) with a broad, non–targeted method may be a global indication for its safety with respect to insect ecology.

Plant chemistry, however, is a plastic trait that varies over space and time and this plasticity has been shown to play an important role in ecological interactions (Turlings, Tumlinson and Lewis 1990; Gols *et al.* 2007; Poelman *et al.* 2008). Therefore a baseline of variation in plant chemistry needs to be established. In this study, plants were grown in climate chambers and subjected to a set of internal and external factors that are assumed to influence plant chemistry in the field: virus infection (potato virus Y), aphid herbivory (*Myzus persicae*) and leaf age. In order to test to what extent the measured chemical variation can indeed serve as an indicator for changes in plant–insect interactions, we measured the performance of *Myzus persicae* in a bioassay on leaves.

Using chemical information in ecological risk assessment requires broad, non–targeted metabolomic profiling techniques since no prior knowledge on the nature of possible specific changes is available (Jansen 2009; Leiss *et al.* 2009). In the present study, nuclear magnetic resonance (NMR) spectroscopy was chosen due to its broad coverage of compounds. In a risk assessment framework, NMR is of particular value due to the simple sample preparation and its good reproducibility across machines (McArdle and Anderson 2001; Widarto *et al.* 2006; Barros *et al.* 2010). NMR is non–destructive and can therefore be easily combined with other methods

that are less broad in terms of compound range but more sensitive to low concentrations. NMR has been previously applied to food classification studies (e.g. Kim *et al.* 2010), risk assessment in GM plants (e.g. Barros *et al.* 2010) and studies of plant–insect interactions (e.g. Widarto *et al.* 2006; Leiss *et al.* 2009).

In summary, we ask: a) what is the baseline of variation in potato leaf chemistry in response to internal and external factors such as leaf age, virus infection and aphid herbivory, b) how do chemical changes introduced by genetic modification compare quantitatively to this baseline and c) how does the measured chemical variation in plants relate to aphid performance on these plants?

Materials and methods

Plants

In this study the GM potato cultivar "Modena" (grant No: NRR 30805, AVEBE UA, Foxhol, The Netherlands/BASF Plant Science Company GmbH) and its non–GM counterpart "Karnico" were used. The genetic modification of "Modena" results in higher relative amylopectin yields in tubers, which is achieved by blocking amylose production with an antisense knock–down of the granule–bound starch synthase. All plants were grown from tubers in a growth chamber (16:8 hours light:dark photoperiod, light intensity 112.3 ± 18.5 µmol m^{-2} s^{-1}, 23°C, 70% relative humidity) in 5 liter pots, covered in insect–proof gauze sleeves. For testing the effect of potato virus Y (PVY) infection on metabolomic profiles, six–week–old, PVY–infected plants were compared to healthy control plants of the same age. The effect of aphid–herbivory on plant chemistry was tested by infesting six–week–old healthy plants with aphids for three weeks and taking leaf samples from these nine–week–old infested plants as well as from nine–week–old healthy control plants.

Potato virus Y infection treatment

Potato virus Y (PVY) infection occurred naturally in ca. 25 % of both GM and non–GM plants grown from tubers in the laboratory. Infection was presumably acquired during the growing season in the field before tuber harvest. The infection status of all plants in the experiment was determined by both visual inspection for symptoms during plant growth and by ELISA antibody tests performed on freeze–dried leaf samples by the Dutch General Inspection Service for agricultural seeds and seed potatoes (NAK). After six weeks of growth, leaf samples were taken from eight PVY infected and eight healthy plants of each cultivar (GM and non–GM). From each plant, one young leaf (first fully grown leaf from top) and one old leaf (third leaf from bottom) was sampled.

Aphid herbivory treatment

Several individuals of the peach–potato aphid (*Myzus persicae*) were taken from a clonal laboratory population and reared for at least one generation on whole plants of the potato cultivar "Nicola", in order to avoid adaptation to either of the experimental cultivars. Sixteen GM and sixteen non–GM plants that were grown in a climate chamber (see above) were used in the experiment. All of these plants were virus–free. Half of the plants of each cultivar were infested

with 20 adult aphids, plants were covered with insect–proof gauze sleeves and populations were allowed to build up for three weeks. The other half of the plants of each cultivar was kept aphid–free. After this period, young (first fully developed) and old leaves (third leaf from bottom) were sampled from both aphid–infested and aphid–free plants.

Extraction of plant material

All sampled leaves were frozen in liquid nitrogen immediately after sampling and stored at -20°C until analysis. Leaf material was extracted and prepared for NMR analysis according to the protocol of Kim et al. (2010). Leaf samples were freeze–dried and ground to fine powder (3 min at 30 Hz) in a mixer mill (MM200, Retsch, Germany). Equal amounts of ground material (30 mg) were transferred into 2 ml centrifuge tubes, and 600 µl KH_2PO_4 buffer (90mM, pH 6.0) in D_2O and 600 µl methanol-*d4* (1:1) were added for extraction. As an internal standard, 0.05% trimethyl silyl propionic acid sodium salt (TMSP; w/w) was used. The mixtures were vortexed, ultrasonicated for 10 min, and centrifuged at 13,000 rpm for 10 min. The supernatants were transferred to a 1.5 ml tube and centrifuged again for 1 min at 13.000 rpm, before 700 µl of each extract was transferred to an NMR–tube.

NMR analysis

Spectra of ^{1}H NMR measurements, as well as J–resolved, COSY and HMBC spectra were recorded at 25 °C on a Bruker 600 MHz AVANCE II NMR spectrometer (600.13 MHz proton frequency) equipped with TCI cryoprobe and Z–gradient system. CD_3OD was used as an internal lock. For a detailed description of the measurement parameters see Kim et al. (2010). The resulting spectra were manually phased and baseline corrected, and calibrated to the internal standard TMSP at 0.0 ppm using XWIN NMR (version 3.5, Bruker). ^{1}H NMR spectra were automatically reduced to ASCII files using AMIX (v. 3.7, Bruker Biospin). Intensities of spectra were scaled to the intensity of the internal standard (TMSP, 0.05% w/v) and reduced to integrated regions (“buckets”) of equal width (0.04) corresponding to the region of δ 0.4–δ 10.0. Residual signals of water and MeOH were excluded from the analysis by deleting the respective spectral regions of δ 4.8–δ 4.9 and δ 3.28–δ 3.34. Structure elucidation of compounds was facilitated by J–resolved, COSY and HMBC spectra and an in–house reference library of isolated compound spectra. Quantification of specific compounds (α-chaconine and α-solanine) was done by measuring peak heights of the signals corresponding to H-6 protons of the aglycone in MestReNova software (version 6.0.2–5475, Mestrelab Reseach S.L.).

Aphid performance bioassay

Variation in chemical profiles of healthy plants caused by genetic modification and leaf age was related to the performance of the peach–potato aphid (*Myzus persicae*): First, the population growth of aphids during the aphid–induction experiment was measured by counting the number of aphids on the plant after three weeks. Instantaneous rates of population increase (r_i) were compared between GM and non–GM plants and between young and old leaves. In a second bioassay the scale of the experiment was reduced from whole plants to excised leaves that were placed on a layer of sterile agar in petri–dishes. Gauze was embedded into petri–dish lids to allow for air–flow and dishes were sealed with parafilm. Two young leaves (two first fully grown leaves) and two old leaves (third and fourth leaf from the bottom) were excised from

six–week–old plants grown under insect–free conditions in a climate chamber. Sixteen replicate plants were used and five adult *Myzus persicae* individuals were placed on each leaf. The number of offspring per leaf after five days was recorded.

Data analysis

The bucketed metabolomics data were mean–centered and standardized (variance = 1) prior to all multivariate analyses. The metabolomic distances between samples and groups of samples were determined by non–parametric MANOVA based on permutation of Euclidean distance–matrices (McArdle and Anderson 2001). This method is similar to the metabolomic distance method introduced by Houshyani et al. (2012), except that no data reduction is performed prior to the calculation of distances. The analysis was performed in R version 2.12.1 (R Development Core Team 2010) with package "vegan" version 1.17–6 (Oksanen *et al.* 2011), using 999 permutations.

Principal component analysis (PCA) was used as an unsupervised method to visualize variability and clustering in the data set. Partial least squares–discriminant analyses (PLS–DA) is a supervised multivariate analysis technique, which maximizes the covariance between the X–matrix (^{1}H NMR spectral intensities) and the Y–matrix (group information). Although qualitatively the same grouping patterns were found in PLS–DA and PCA, the separation of groups was stronger in PLS–DA. The latter was therefore used to identify the variables (and the corresponding compounds) that were most influential to the group separation. Both PCA and PLS–DA were performed with *SIMCA–P* software (v. 11.0, Umetrics, Umeå, Sweden). Components were added only when significant according to the cross–validation function of the software. For ^{1}H NMR data from the PVY infection experiment, a PLS–DA with four significant components explained 60.9 % of the total variation. In the aphid herbivory experiment, a PLS–DA model with three components explained 73.8 % of the total variation in metabolomic data. Relative levels of α-chaconine and α-solanine were compared between treatments by performing ANOVAs on data after square–root transformation. Data obtained from the whole–plant bioassay were tested for a difference in means of instantaneous rates of aphid population increases using Student's t–test. Data obtained from bioassays with aphids on excised leaves in petri–dishes were analyzed by fitting a generalized linear model (GLM) with poisson–distribution and log–link function to the data in R version 2.12.1. The model was compared with reduced models in a stepwise manner in order to determine significance of factors.

Results and discussion

The presence of a number of common primary metabolites was confirmed by NMR, such as glucose: α-glc at δ 5.18 (d, *J* = 3.5 Hz) and β-glc at δ 4.58 (d, *J* = 7.9 Hz), sucrose at δ 5.40 (d, *J* = 3.8 Hz) and δ 4.16 (d, *J* = 8.7 Hz), alanine at δ 1.48 (d, *J* = 7.2 Hz), glutamate at δ 2.40 (m), threonine at δ 1.33 (d, *J* = 6.5 Hz), acetic acid at δ 1.93 (s), fumaric acid at δ 6.56 (s), choline at δ 3.24 (s), cytosine/uracil at δ 5.90 (d, *J* = 8.0 Hz) and δ 7.47 (d, *J* = 8.0 Hz). Among the group of secondary metabolites, which are often species–specific in plants, a complex pattern of glycoalkaloid (GA) signals in the methyl region δ 0.8–1.3 was found, corresponding to H-18, H-19 and H-21 of the aglycone (Lawson *et al.* 1997). Glycoalkaloids occur in plants of the Solanaceae family and have long been known for their bioactivity (reviewed by Maga (1994) and Friedman (2006). The two main glycoalkaloids α-chaconine and α-solanine (Friedman 2006) were identified by alignment with NMR spectra obtained from isolated compounds. NMR peak assignments of glycoalkaloids have also been previously reported by (Abouzid *et al.* 2008). In particular, signals corresponding to H-6 of the aglycone part proved characteristic for the distinction between the two alkaloids in the mixture: the respective signal of α-chaconine was shifted down–field at δ 5.16 (s) compared to the signal of α-solanine at δ 5.12 (s). Characteristic compounds detected in the phenolic region (δ 6.0–8.0) were 5-caffeoylquinic acid (chlorogenic acid) at δ 6.36 (d, *J* = 16.0 Hz) and its analogues 3- and 4-caffeoylquinic acid at δ 6.40 (d, *J* = 16.0 Hz and δ 6.44 (d, *J* = 16.0 Hz) respectively, as well as the alkaloid trigonelline at δ 9.16 (s), δ 8.86 (m) and δ 8.12 (m).

Table 1. Sources of variation in leaf metabolomic profiles in a potato virus Y (PVY) infection experiment: non–parametric MANOVA based on Euclidean distances between samples.

source of variation	***df***	***SS***	***explained variation [%]***	***F***	***P***
GM	1	558.8	5.38	5.49	0.001
PVY infection	1	1738.3	16.73	17.07	0.001
leaf age	1	2241.9	21.58	22.01	0.001
GM : PVY infection	1	491	4.73	4.82	0.001
GM : leaf age	1	247.9	2.39	2.43	0.020
PVY infection : leaf age	1	424.8	4.09	4.17	0.001
residuals	46	4684.9	45.10		
Total	52	10387.7			

Table 2. Sources of variation in leaf metabolomic profiles in an aphid herbivory (*Myzus persicae*) induction experiment: non–parametric MANOVA based on Euclidean distances between samples.

source of variation	***df***	***SS***	***explained variation [%]***	***F***	***P***
aphid herbivory	1	465.9	4.00	4.53	0.012
leaf age	1	5501	47.25	53.49	0.001
aphid herbivory : leaf age	1	636.7	5.47	6.19	0.003
residuals	49	5039.4	43.28		
total	52	11642.9			

Table 3. Sources of variation in relative α-solanine and α-chaconine contents in leaves in a potato virus Y (PVY) infection experiment

a) α-Solanine content

source of variation	***df***	***SS***	***F***	***P***
leaf age	1	228.2	10.635	0.002
PVY infection	1	862.76	40.208	< 0.001
Residuals	49	1051.42		
b) α-chaconine content				
leaf age	1	84.37	4.2854	0.044
PVY infection	1	639.52	32.4848	< 0.001
leaf age: virus infection	1	102.47	5.205	0.027
Residuals	48	944.96		

Table 4. Sources of variation in relative α-solanine and α-chaconine contents in leaves in an aphid herbivory (*Myzus persicae*) induction experiment

a) α-Solanine content

source of variation	***df***	***SS***	***F***	***P***
leaf age	1	983.35	34.91	< 0.001
Residuals	50	1408.36		
b) α-chaconine content				
leaf age	1	4826.00	106.01	< 0.001
Residuals	50	2276.1		

Chemical baseline variation: leaf age, virus infection and aphid herbivory

In both experiments (PVY infection and aphid herbivory) leaf age had the biggest effect on chemical profiles. This becomes evident by the amounts of explained variation in metabolomic profiles (Table 1 and Table 2) and by the clear separation of young and old leaves in PLS–DA score plots along the first component (Figure 1). The most influential variables in PLS–DA causing this age effect in both experiments were spectral peaks assigned to the alkaloid trigonelline and phenolic compounds which were present in higher amounts in young leaves. Furthermore, sugars (glucose, sucrose) and choline were increased in young leaves. Levels of secondary metabolites are generally expected to be higher in, with respect to fitness, more valuable plant parts such as young leaves, as part of an 'optimal defense' strategy (VanDam *et al.* 1996; McCall and Fordyce 2010). Trigonelline is generally associated with biosynthesis regulation in response to abiotic stressors and with the accumulation of secondary metabolites (Minorsky 2002). The within–plant distribution of glycoalkaloids has previously been reported to show lower levels in the top leaves, to increase with leaf maturity, and to decrease again in older leaves (Brown, McDonald and Friedman 1999). A similar pattern was found in the plants of the aphid herbivory experiment: old leaves had lower glycoalkaloid contents than young leaves (Figure 3B, Table 4). Curiously, this relationship was reversed in the plants of the PVY infection experiment (Figure 3A, Table 3). Since these plants were three weeks younger, 'young and old' leaves in these plants may have been 'developing and mature' rather than 'mature and senescent' leaves, respectively.

The second largest effects on chemical profiles, following the effect of leaf age, were the effects of PVY infection and aphid herbivory (Table 1 and Table 2). As apparent from the PLS–DA score plot of the PVY infection experiment (Figure 1A), control and PVY infected plants are mostly separated along the second component. Potato virus Y infection coincided with a general increase in phenolic compounds in the spectral region 6.0–8.0 ppm (Figure 2) including chlorogenic acid and its isomers. Sucrose and choline were reduced in infected plants. However, this shift in metabolomic profiles after PVY infection was not observed in young leaves of the non–GM cultivar where samples from healthy plants grouped together with samples from infected plants (Figure 1A). Both α-chaconine and α-solanine levels increased in response to PVY infection in both young and old leaves (Figure 3A, Table 3). Aphid herbivory had a weak effect on metabolomic profiles of old leaves, but a stronger one in young leaves (Figure 1B). Leaves of aphid induced plants had lower levels of sucrose and showed an increase of phenolics and malic acid. Glycoalkaloid levels were not affected by herbivory (Figure 3, Table 4).

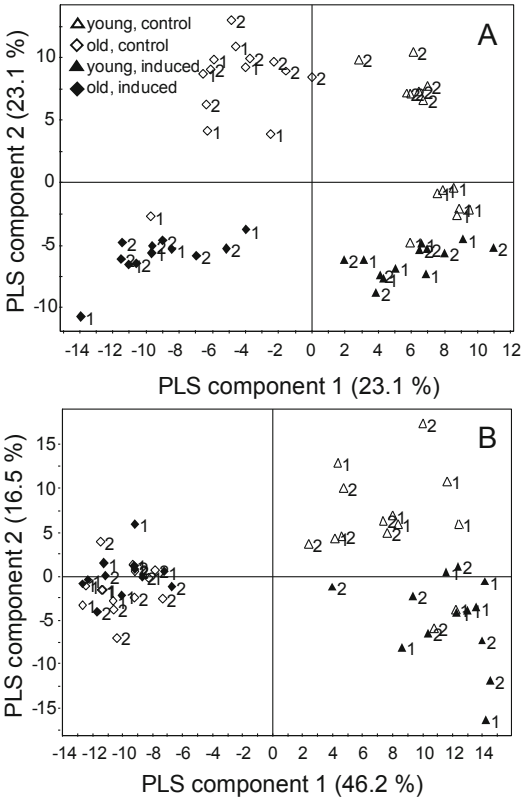

Figure 1. PLS–DA score plots showing groupings in ^{1}H NMR metabolomic profiles of non–GM ('1') and GM plants ('2'). Groupings occur between young and old leaves along component 1, and between healthy and (A) potato virus Y infected or (B) aphid infested leaves along component 2.

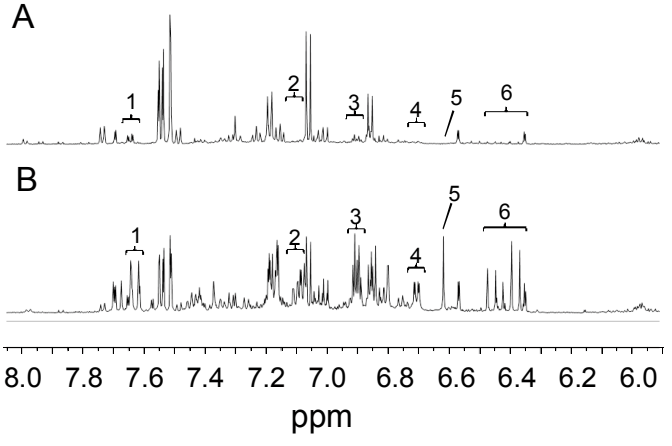

Figure 2. Differences in phenolic compounds in old leaves between (a) healthy and (b) potato virus Y infected GM potato plants in the spectral region of 6.0–8.0 ppm. Some phenolics were increased in virus infected plants (1 = unknown, 3 = unknown, 6 = 3-, 4- and 5-caffeoylquinic acid), while others were synthesized de novo (2 = unknown, 5 = unknown).

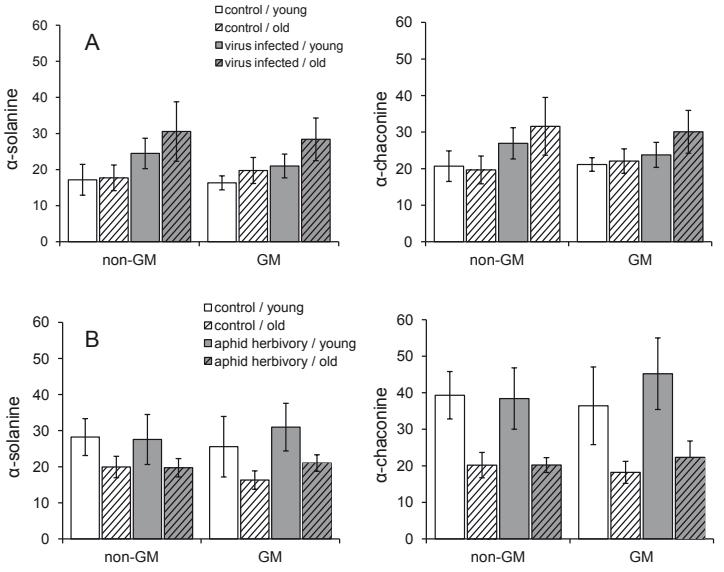

Figure 3. Relative amounts of the two main glycoalkaloids α-solanine and α-chaconine in a potato virus Y infection experiment (A) and an aphid herbivory experiment (B). Values are relative peak heights (square–root transformed) of ^{1}H NMR signals corresponding to H-6 of the aglycone. Error bars represent standard deviations.

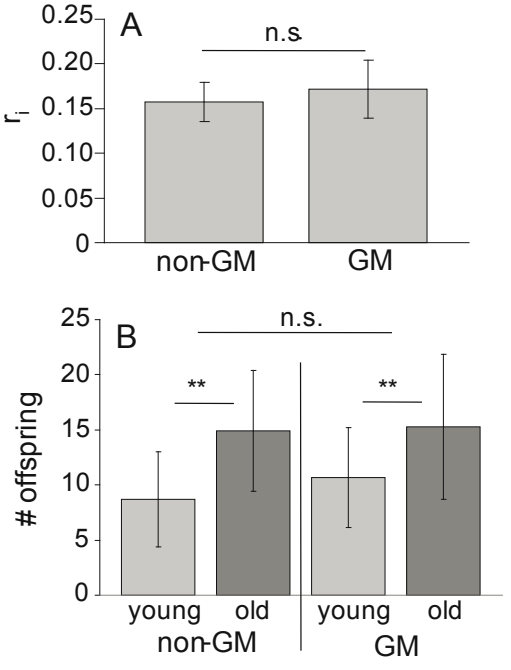

Figure 4. (A) Instantaneous rates of population increase (r_i) of aphids *Myzus persicae* on six–week–old GM and non–GM potato plants; (B) number of offspring per individual aphid on excised leaves in petri–dishes. Error bars represent standard deviations.

Comparative risk assessment: genetic modification vs. chemical baseline

In general, effects of genetic modification on chemical profiles were absent across infection treatments or leaf ages with one exception. A difference between GM and non–GM samples was only observed in young leaves of healthy plants. These young, healthy leaves of GM plants had lower levels of sugars and phenolic compounds compared to their non GM counterparts. Glycoalkaloid levels were similar in both plant types across treatments (Figure 3, Table 3 and Table 4). The observed difference was absent in older leaves of the same plants. It was also not found in PVY infected plants or in any of the treatments in the aphid herbivory experiment. Thus, genetic modification affected metabolomic profiles only in a restricted developmental period (young leaves of six–week–old plants) and under specific environmental conditions (healthy plants). Consequently the genetic modification explained the least amount of variation in non–parametric MANOVA (Table 1) compared to the other treatments. In other words, when compared to the baseline of chemical variation, which in this study consisted of a combination of internal and external factors, the chemical changes caused by this genetic modification should be considered not biologically significant to plant–insect interactions.

An indication that the conclusion drawn from plant chemistry is indeed valid for plant–insect interactions may be the equal rate of population increase of aphids (*Myzus persicae*) on non–GM and GM plants during the aphid herbivory treatment (Figure 4A). We tested this more rigorously in a bioassay with parthenogenetic female aphids (*Myzus persicae*) on excised leaves of the two plant types, using plants of the same age as the ones that were chemically profiled in the aphid herbivory induction experiment. The effect of leaf age was included in the bioassay as part of the baseline that had also been used to capture variation in chemical profiles. Again, aphid performance was not affected by genetic modification, but was significantly lower on young leaves compared to old leaves (Figure 4B). Thus, while the effect with the largest influence on plant metabolomes did affect aphid performance, the minor effect of genetic modification did not. This suggests that the chemical baseline approach is valid at least for this specific plant–insect interaction. The pattern of aphid performance coincides with the relative amounts of glycoalkaloids that were found in plants of the same age in the herbivory induction experiment: we found higher amounts of glycoalkaloids in young leaves compared to old leaves and aphids performed less well on young leaves. While a causal relationship is not tested directly here, the bioactivity of α-solanine and α-chaconine against aphids has been previously shown by (Güntner *et al.* 1997; Fragoyiannis, McKinlay and D'Mello 1998).

We conclude that metabolomic studies can add important information to the assessment of ecological safety of genetically modified plants by revealing natural variation in plant chemistry as a relevant factor in plant–insect interactions. The selection of treatments that are included in a baseline is eventually a decision that has to be made by regulatory authorities. Once a set of criteria for a baseline is established, the comparative approach provides a workable framework for risk assessors.

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Eco-metabolomic variation in GM and non-GM potato is weakly correlated to non-target insect communities

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Abstract

Plant chemistry can influence insect communities across several trophic levels. Therefore, unintended metabolic alterations in genetically modified crop plants are a possible cause for effects on non-target organisms. Here, we use ^{1}H NMR to study eco-metabolomic variation in GM and non-GM potato plants over the course of two years, three developmental stages within years and at two field locations in the Netherlands. Effects of GM potato were small when compared to the amount of variation among non-GM genotypes. However, the presence of genotype-by-environment interactions suggests that conclusions cannot easily be extrapolated to other geographical regions. Few links were found between metabolomics data and variation in insect communities on the same experimental fields. In senescent plants, the abundances of Aphididae and Neuroptera were correlated to malic acid concentrations in leaves. This suggests that effects of plant genotypes on non-target insects are caused to a large extent by factors not detected with ^{1}H NMR metabolomics, such as volatile compounds or morphological characters. More functional metabolomics studies are needed to elucidate the biological relevance of eco-metabolomic variation.

Keywords: metabolomics, GMO, potato, NMR, GxE interactions, non-target, insects

Introduction

The introduction of genes into crop plants by means of biotechnology has raised concerns about unintended, pleiotropic effects on plant metabolism. Pleiotropic effects may result from altered gene regulation or interference of the novel gene product with biosynthetic pathways (Fiehn 2002; Ricroch, Berge & Kuntz 2011). Unintended metabolic changes in genetically modified (GM) plants have been discussed in the context of food and feed safety, because toxic or allergenic compounds may be affected (OECD 1993; FAO/WHO 2000; Konig *et al.* 2004; Chassy 2010; Davies 2010; Doerrer *et al.* 2010; EFSA 2011; Ricroch, Berge & Kuntz 2011). However, pleiotropic effects could also cause effects on non-target organisms (NTO). For example, unintended changes in secondary defense chemicals could affect interactions with secondary pests or beneficial insects (Alvarez-Alfageme, von Burg & Romeis 2011; Axelsson *et al.* 2011). Effects of chemical plant traits on insect communities have been shown repeatedly, and can span multiple trophic levels from herbivores to predators and parasitoids (Wardle *et al.* 2004; Inbar & Gerling 2008; Poelman, van Loon & Dicke 2008; Schwachtje & Baldwin 2008). Changes in leaf or root plant chemistry can also affect soil microbial and fungal communities (van der Heijden *et al.* 1998; Broughton & Gross 2000; Peter, Lindfeld & Nentwig 2010), as well as interactions between above- and below-ground communities (Van der Putten *et al.* 2001; Erb *et al.* 2008). Revealing metabolic changes in GM plants is therefore highly relevant to the assessment of their environmental safety towards non-target organisms.

A recent development in chemical measurement techniques is the use of metabolomics approaches. The non-targeted nature of metabolomics and their broad coverage of compounds make these techniques well-suited to safety assessment problems, because no prior knowledge on the quality of possible changes is needed (Konig *et al.* 2004; Catchpole *et al.* 2005; Parr *et al.* 2005; Rischer & Oksman-Caldentey 2006; Barros *et al.* 2010). Furthermore, the ability to measure large numbers of compounds simultaneously has greatly enhanced the ability to reveal patterns and mechanisms at a systems biology level (Fiehn 2002; Jansen 2009; Davies 2010; Macel, van Dam & Keurentjes 2010). Detecting system-level changes is particularly important in risk assessment, where effects (e.g. human health or non-target organisms) may be the result of combined effects across multiple compounds. Detecting such system-effects requires multivariate analysis approaches, which are currently not considered in risk assessment procedures.

The major challenge in GM risk assessments is to evaluate the biological relevance of metabolic changes. One approach is to compare the magnitude of effects to a baseline of variability, such as variation between commercial cultivars and variation across growing environments (Chassy 2010; Davies *et al.* 2010; Doerrer *et al.* 2010). However, the actual functional relevance of metabolic variation still remains unknown in many cases. Linking metabolic variation to biological effects will therefore greatly improve the ability to avoid risks of GM crops.

In this study, we analyze leaf samples of a GM potato, its near-isogenic parent, and four commercial cultivars in a 2-year field study using ^{1}H NMR metabolomics. In order to evaluate the biological relevance of patterns in leaf metabolomics in terms of environmental safety, we use two approaches. First we compare the differences between the GM plant and its counterpart to a baseline of variation in the field, which includes variation among a set of four commercial cultivars as well as variation between two years, two locations and three developmental stages

within a growing season (vegetative growth, flowering and senescence). Second, we use data on insect abundances obtained from the same experimental fields (and the same plant individuals; see chapter 2, this thesis) to study possible functional relationships between metabolomics and insect abundances.

The study was conducted in synchrony with two other projects studying different aspects of the above- and belowground ecology on the same experimental fields: soil fungal communities (ERGO project nr. 838.06.052; Hannula, de Boer & van Veen 2010; Hannula, de Boer & van Veen 2012) and soil microbial communities (ERGO project nr. 838.06.051; Inceoglu *et al.* 2010).

Methods

Field setup

Six potato cultivars were compared in this field trial. The genetically modified variety "Modena" (modified in starch composition by means of amylose knock-down; grant No: NRR 30805, AVEBE UA, Foxhol, The Netherlands/BASF Plant Science Company GmbH, Research Triangle Park, USA) was grown alongside its near-isogenic parent cultivar "Karnico" and four conventional cultivars representing a broad phenotypic spectrum of commercial cultivars: two consumption varieties ("Premiere" and "Desiree") and two starch varieties ("Aveka" and "Aventra"). Plants were grown in two consecutive years (2010 and 2011) at two field locations in Drenthe, The Netherlands. The locations were 12 km apart and characteristically different in soil conditions: sandy peat (location A) and loamy sand (location B). Fields were shifted in the second year to an area adjacent to the area of the previous year, following the common practice of crop rotation in potato. Each field was planted as a randomized 6 x 6 Latin square with six replicate plots per cultivar. Each plot consisted of 28 plants (4 ridges of 7 plants = ca. 10 m^2). The fields were planted with tubers at the end of April and were harvested in October. All fields received standard fungicide and fertilizer treatment. In 2010, location B was treated with an insecticide (Calypso, Bayer CropScience, Mijdrecht, The Netherlands) after an infestation with Colorado potato beetles.

Leaf sampling

One leaf sample per plot was taken on each of three sampling days within a year at both locations, corresponding to three developmental stages: vegetative growth (June), flowering (July) and senescence (August), respectively. Samples were never taken from plants at the margins of plots, in order to avoid edge-effects. At each developmental stage, one plant per plot was sampled. The first fully grown leaf from the top of each sampled plant was cut with a razor blade. Leaf samples were put into aluminum foil and frozen in dry ice immediately after sampling. In total, 432 leaf samples were taken. All samples were transported to the laboratory within max. 8 hours and freeze-dried thereafter. All leaf samples were taken between 12.00 and 16.00 h on each sampling day. Diurnal effects can therefore not be entirely excluded but are expected to spread randomly across genotypes.

NMR analysis

Leaf material was extracted and prepared for NMR analysis according to the protocol of Kim et al. (2010). Freeze–dried samples were ground to fine powder in a mixer mill (MM200, Retsch, Haan, Germany). Equal amounts of ground material (30 mg) were transferred into 2 ml centrifuge tubes, and 600 µl KH_2PO_4 buffer (90mM, pH 6.0) in D_2O and 600 µl methanol-*d4* (1:1) were added for extraction. Trimethylsilyl propionic acid sodium salt (TMSP; 0.005% w/w) was used as an internal standard. The mixtures were vortexed, ultrasonicated for 10 min, and centrifuged at 13,000 rpm for 10 min. The supernatants were transferred to a 1.5 ml tube and centrifuged again for 1 min at 13.000 rpm, before 700 µl of each extract was transferred to an NMR–tube. ^{1}H NMR spectra, as well as J–resolved, COSY and HMBC spectra were recorded at 25 °C on a Bruker 600 MHz AVANCE II NMR spectrometer (600.13 MHz proton frequency) (Bruker, Billerica, USA) equipped with TCI cryoprobe and Z–gradient system. CD_3OD was used as an internal lock. Spectra were manually phased and baseline corrected, and calibrated to the internal standard TMSP at 0.0 ppm using XWIN NMR (version 3.5, Bruker). Signal intensities were scaled to the total spectral intensity and reduced to integrated regions ("buckets") of equal width (0.04) corresponding to the region of δ 0.4–δ 10.0. Residual signals of water and MeOH were excluded from the analysis by deleting the respective spectral regions of δ 4.8–δ 4.9 and δ 3.28–δ 3.34. In total, the spectra of 426 samples (out of 432) were of suitable quality for further analysis. Peak assignment was facilitated by J–resolved, COSY and HMBC spectra and an in–house reference library of isolated compound spectra.

Data analysis

The bucketed metabolomics data were mean–centered and Pareto scaled (divided by the square root of the standard deviation) prior to all multivariate analyses. Metabolomic distances between samples and groups of samples were determined by non–parametric MANOVA based on permutation of Euclidean distance–matrices (Anderson 2001). The analysis was performed in R version 2.12.1 (R Development Core Team 2010) with package "vegan" version 1.17–6 (Oksanen *et al.* 2011), using 999 permutations. PLS-DA (Partial least squares–discriminant analyses) was used to visualize the effects of environmental variables on metabolomics profiles and to identify key metabolites contributing to the discrimination. PLS–DA maximizes the covariance between the X–matrix (^{1}H NMR spectral intensities) and the Y–matrix (group information). PLS-DA models were validated by comparing the observed ratios of sums-of-squares between and within groups (B/W ratios) to a distribution of B/W ratios obtained by permuting class labels (1000 permutations) (Bijlsma *et al.* 2006). PLS-DAs and permutation tests were performed with Metaboanalyst 2.0 (www.metaboanalyst.ca) (Xia *et al.* 2012). The relationship between metabolomics data and insect count data obtained from the same individual plants was investigated with PLS models and cross-validated analysis of variance (CV-ANOVA) using SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden). Bivariate regression models were analyzed using R software (R Development Core Team 2010).

Results

A total of 22 compounds were identified from ^{1}H NMR spectra of crude leaf extracts, including sugars, amino acids, organic acids and phenolics and glycoalkaloids (Table 1). Citric acid, malic acid, glutamine and GABA (Figure 1) are part of or closely linked to the tricarboxylic acid (TCA) cycle. Two glycoalkaloids (GAs), a group of secondary compounds specific to Solanaceae, were detected in the samples: α-solanine and α-chaconine, with slightly higher concentrations of the latter. A signal assigned to H-6 of the aglycone was used to distinguish between these two GAs (Figure 2), which usually constitute 95% of all GAs in potato (Maga 1994; Friedman 2006). In the phenolic region of the spectra (δ 6.0–8.0), 5-caffeoylquinic acid (chlorogenic acid) and its analogues 3- and 4-caffeoylquinic acid were detected. The alkaloid trigonelline produced characteristic peaks at δ 9.16 (s), δ 8.86 (m), and δ 8.12 (m) (Figure 2). The peaks of malic acid have been reported earlier to be sensitive to pH differences, showing strong chemical shift variation when pH differences between samples exceed the buffer capacity (Kim, Choi & Verpoorte 2006; Mirnezhad *et al.* 2009). This may particularly cause problems when fixed bucket sizes are used in the spectral analysis. In this study, only slight chemical shift variations were found for malic acid peaks (Figure 1). We used the sum of two adjacent buckets to separate the influence of pH peak shifts from actual concentration changes in malic acid. This showed that malic acid concentrations indeed varies across years, locations, developmental stages and cultivars (Figure 3).

Table 1: List of compounds and corresponding signals found in H-NMR spectra of potato leaves.

compound	chemical shift and coupling constants
Acetic acid	1.92 (s)
Alanine	1.48 (H-3, d, J = 7.2 Hz)
Ascorbic acid	4.54 (H-4, d, J = 1.7 Hz)
α-chaconine	5.16 (s, anomeric proton of sugar moiety)
Chlorogenic acid (overlap with peaks of isomers 4- and 5-caffeoylquinic acid)	6.40 (H-8', d, J = 15.7 Hz), 6.92 (H-5', d,J = 8.4 Hz), 7.18 (H-2', d, J = 2.1 Hz), 7.64 (H-7', d, J = 15.9 Hz)
Choline	3.20 (s)
Citric acid	2.76 (H-β, d, J = 15.7 Hz), 2.56 (H- β, d, J = 17.0 Hz)
Fatty acids	0.90 (H-ω, t, J = 7.5 Hz)
GABA (γ-amino butyric acid)	1.88 (H-3, m), 2.3 (H-2, t, J=7.5 Hz), 3.0 (H-4, t, J=7.5)
α-Glucose	5.20 (H-1, d, J = 3.8 Hz)
β-Glucose	4.61 (H-1, d, J = 7.9 Hz)
Glutamine	2.47 (H-4, m), 2.15 (H-3, m)
Glycine	3.52 (s)
Glycoalkaloids (aglycone)	0.8–1.3 (complex pattern, H-18, H-19, and H-21)
Malic acid	4.29 (H-α, dd, J = 9.8, 3.2 Hz), 2.68 (H-β', dd, J = 15.4, 3.1 Hz)
Ouinic acid in ester (multiple isomer signals)	2.20 - 2.00
α-Solanine	5.12 (s)
Sucrose	5.41 (H-1, d, J = 3.9 Hz), 4.19 (H-1, d, J = 8.7 Hz)
Threonine	1.33 (H-5, d, J = 6.6 Hz)
Trigonelline	9.16 (H-1, s), 8.86 (H-5 and H-3, m), 8.12 (H-4, m)
Valine	1.08 (H-3β, d, J = 6.6 Hz), 1.02 (H-3α, d, J = 7.3 Hz)

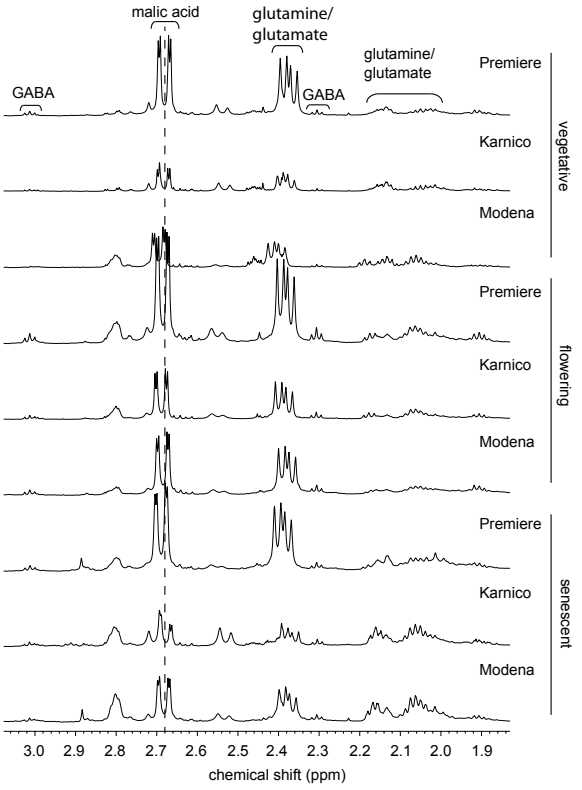

Figure 1: Peak assignments in the region 2.0-3.0 ppm of ^{1}H NMR spectra obtained from leaf samples of field-grown potato plants. Examples of three cultivars (GM + counterpart + one commercial cultivar) at three developmental stages are shown, including the cultivar "Premiere", the GMO "Modena" and its near-isogenic cultivar "Karnico". Samples were taken at location A in 2010. Slight chemical shift variations were found in malic acid peaks across developmental stages and cultivars.

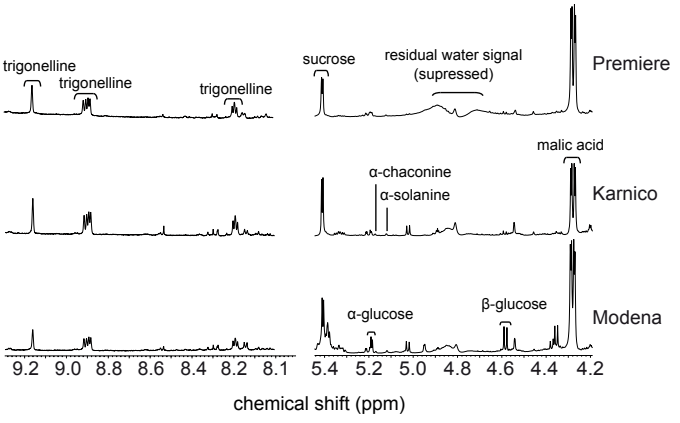

Figure 2: Peak assignments in two regions of ^{1}H NMR spectra obtained from leaf samples of field-grown potato plants. Examples of three cultivars in are shown, including the cultivar "Premiere", the GMO "Modena" and its near-isogenic cultivar "Karnico". Samples were taken from senescent plants at location A in 2010.

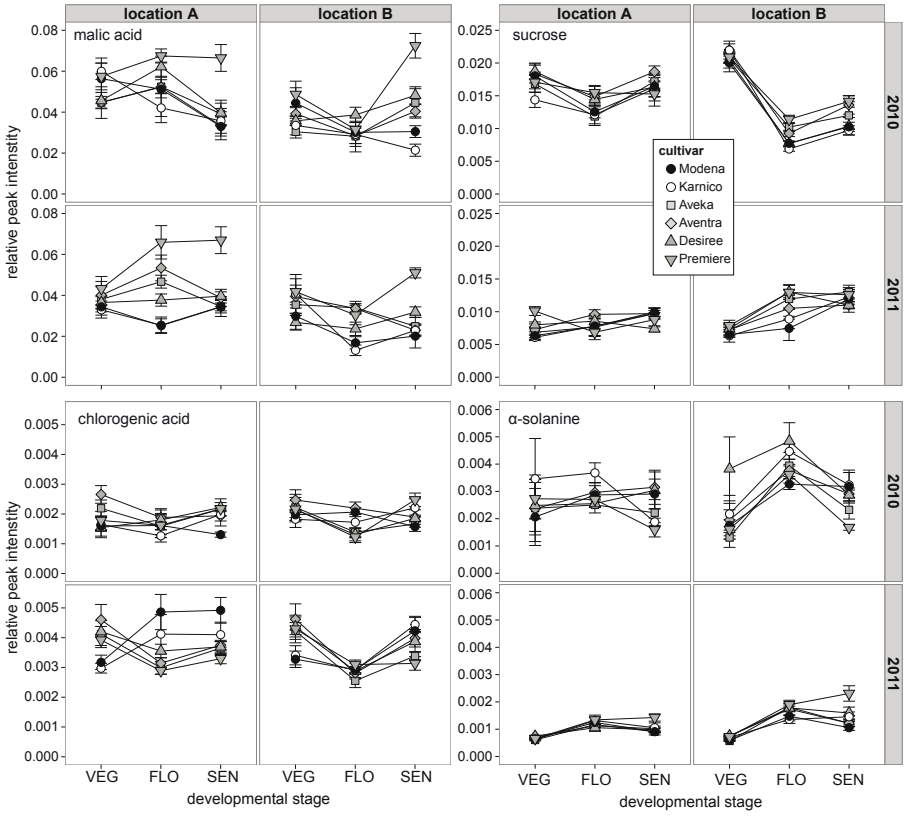

Figure 3: Variation across years, locations and developmental stages in relative peak intensities (arbitrary values) of signals assigned to four compounds in ^{1}H-NMR spectra of field-grown potato plants. Data from six cultivars are shown, including the genetically modified "Modena" and its near-isogenic counterpart "Karnico", as well as four commercial, non-modified cultivars

Variation between years

The largest differences in ^{1}H NMR profiles were found between samples taken in the different years (Table 2). Many two-way and higher order interactions between the factor year and other factors were found, indicating that these other effects varied across years. For further quantitative analysis, the two years were therefore considered separately (Table 3). These separate analyses show that for each factor, the amounts of explained variances differ slightly between years, with less total explained variance in 2010. However, the relative ranking of factors is similar across years.

The differences in metabolomic profiles between the two years are also evident from the significant separation of samples in partial least squares discriminant analysis (PLS-DA) (Figure 4). The discrimination of samples was largely determined by the first PLS component. The loadings of this component indicate a strong influence of the signals assigned to sucrose, malic acid and the glycoalkaloid α-chaconine. These compounds were present in higher amounts in 2010. Another influential group of signals was assigned to the phenolic chlorogenic acid and its isomers, which were higher in samples from 2011.

Variation between locations

The two experimental locations had significant effects on metabolomic profiles in both years (Table 3). This effect was slightly stronger in 2011 (7.93 % of total variation) compared to 2010 (5.00 % of total variation). Some qualitative differences were found for the location effect between years. In 2011, the signals assigned to chlorogenic acid and the glycoalkaloid α-chaconine were stronger in samples from location A and contributed to the discrimination in PLS-DA (Figure 5). In both years, the discrimination was influenced by the signals of malic acid and glutamine, which were higher in samples from location A, as well as citric acid, which was higher in location B.

Table 2: Partitioning of variances in metabolomic profiles of field-grown potato leaves with permutational MANOVA (1000 permutations), performed on bucketed, mean-centered and pareto-scaled NMR spectral data. P-values indicate the fraction of permuted F values larger than the observed F.

source of variation	DF	SS	F	%	P(>F)
year	1	20.23	125.88	14.63	*<0.001*
location	1	5.94	36.95	4.30	*<0.001*
developmental stage	2	11.71	36.44	8.47	*<0.001*
cultivar	5	11.48	14.28	8.30	*<0.001*
year × location	1	1.51	9.40	1.09	*<0.001*
year × developmental stage	2	9.54	29.68	6.90	*<0.001*
location × developmental stage	2	2.88	8.95	2.08	*<0.001*
year × cultivar	5	3.00	3.74	2.17	*<0.001*
location × cultivar	5	1.62	2.01	1.17	*<0.001*
developmental stage × cultivar	10	4.75	2.95	3.43	*<0.001*
year × location × developmental stage	2	3.72	11.59	2.69	*<0.001*
year × location × cultivar	5	0.74	0.92	0.54	0.619
year × developmental stage × cultivar	10	3.61	2.25	2.61	*<0.001*
location × developmental stage × cultivar	10	2.27	1.41	1.64	*0.008*
year × location × developmental stage × cultivar	10	1.90	1.18	1.38	0.109
Residuals	332	53.36		38.60	
Total	403	138.25			

Table 3: Partitioning of variances in metabolomics profiles with permutational MANOVA (1000 permutations), performed on bucketed, mean-centered and pareto-scaled NMR spectral data. Separate analyses for 2010 and 2011 are shown. P-values indicate the fraction of permuted F values larger than the observed F.

Source of variation	DF	SS	F	%	P (>F)
A) 2010					
location	1	3.25	16.25	5.00	*<0.001*
developmental stage	2	10.66	26.67	16.40	*<0.001*
cultivar	5	7.00	7.01	10.78	*<0.001*
location × developmental stage	2	4.37	10.94	6.72	*<0.001*
location × cultivar	5	1.30	1.30	2.00	0.083
developmental stage × cultivar	10	4.26	2.13	6.56	*<0.001*
location × developmental stage × cultivar	10	2.38	1.1932	3.67	0.116
Residuals	159	31.76		48.88	
Total	194	64.98		100.00	
B) 2011					
location	1	4.60	34.11	7.926	*<0.001*
developmental stage	2	11.15	41.34	19.212	*<0.001*
cultivar	5	8.63	12.80	14.873	*<0.001*
location × developmental stage	2	2.43	9.02	4.192	*<0.001*
location × cultivar	5	1.26	1.86	2.165	*0.002*
developmental stage × cultivar	10	4.64	3.44	8.002	*<0.001*
location × developmental stage × cultivar	10	1.99	1.48	3.434	*0.004*
Residuals	173	23.32		40.196	
Total	208	58.02		100	

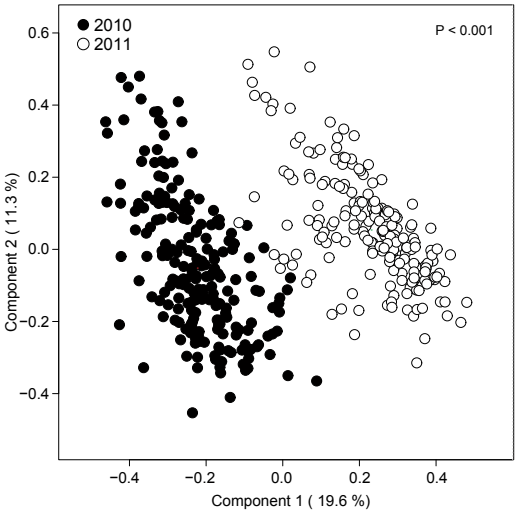

Figure 4: PLS-DA score plot showing the separation of ^{1}H NMR profiles between leaf samples of field-grown potato plants collected in two years (2010 and 2011).

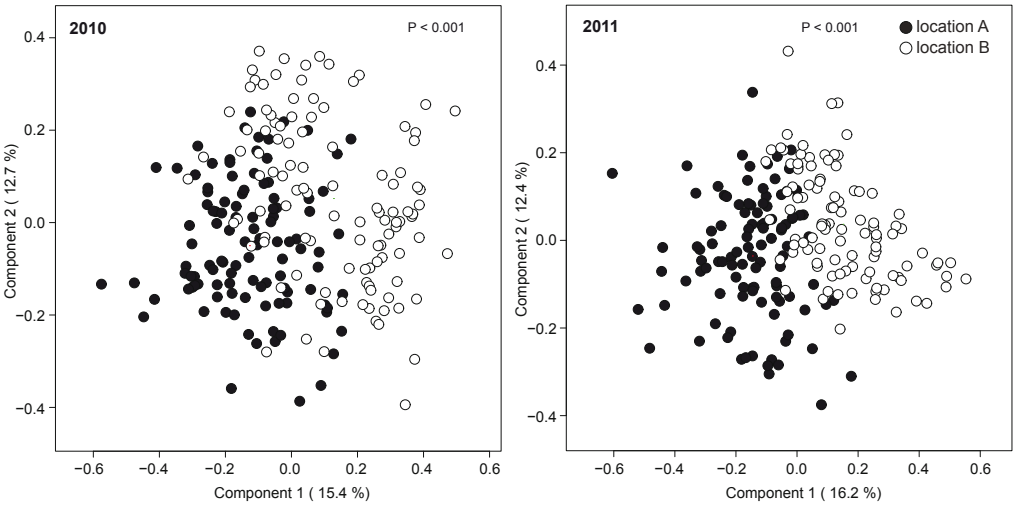

Figure 5: PLS-DA score plots showing the separation in ^{1}H NMR profiles between leaf samples of field grown potato plants taken on two locations in 2010 and 2011. The significance of PLS-DA models was tested with CV-ANOVA.

Variation between developmental stages

The second largest factor influencing metabolomic profiles was plant development. Samples were taken at three different developmental stages: vegetative growth, flowering and senescence. The differences in profiles between the developmental stages accounted for 16 and 19 % of the total variation in 2010 and 2011, respectively (Table 3). The statistical interaction between the effect of developmental stages and the effect of locations indicates that the developmental patterns of cultivars were different on the two locations. This interaction was less strong in 2011. The PLS-DA score plots illustrate the differences between samples taken at the different stages (Figure 6). In both years, the separation was strongest along the first PLS component, which separated the vegetative stage from the flowering and senescence stages. However, the compounds that influenced this separation were different between the two years. In 2010, the first component was strongly influenced by signals assigned to compounds of primary plant metabolism, such as sucrose, α-glucose and malic acid which were higher in vegetative plants. In 2011, sugar signals also influenced the separation between vegetative and flowering/senescent plants, but were lower in the vegetative stage. In addition, the separation of developmental stages in 2011 was influenced by signals of choline, alanine and threonine, which were higher in vegetative plants.

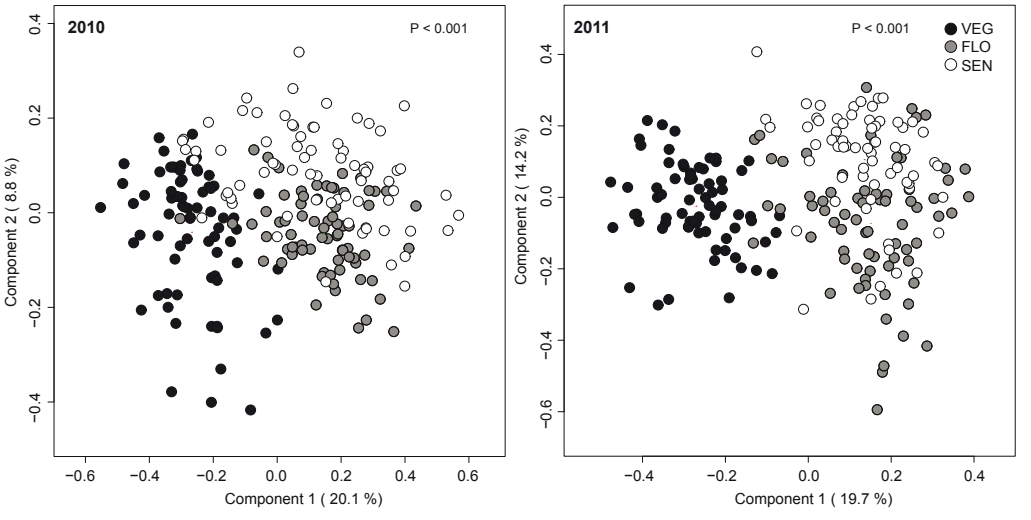

Figure 6: PLS-DA score plots showing the separation in ^{1}H NMR profiles between leaf samples of field-grown potato plants taken at three developmental stages in the growing season: vegetative (VEG), flowering (FLO) and senescence (SEN). Sampling was conducted in June, July and August, respectively. Significance of the PLS-DA model was tested with CV-ANOVA.

Variation between cultivars

Cultivars differed significantly in metabolomic profiles, as indicated by the significant main effect in permutational MANOVA (Table 3). After the effects of years and developmental stages, the differences between cultivars ranked third in their influence on metabolomics profiles. The cultivar effect showed significant interactions with the effect of developmental stages. There was also evidence for genotype-by-environment (GxE) interactions (i.e. cultivar-by- location interactions) in 2011, but not in 2010.

In order to separate genotypic from environmental effects, separate PLS-DAs were performed for each sampling time point. Using permutation tests to validate PLS-DA models, we found significant discriminations between cultivars on all sampling time points except one (2010, location A, senescent plants) (Figure 7). Cultivars clustered to varying degrees across sampling time points, but were often found to cluster in three groups: Karnico/Modena, Premiere and Desiree/Aveka/Aventra. The GM Modena and its counterpart Karnico showed strong overlap on all time points, and differences between the two were always smaller than differences among the set of commercial cultivars. When cultivar effects were detected, this difference was caused by signals of the organic acids malic acid and citric acid, sucrose and glucose, the glycoalkaloid α-chaconine as well as chlorogenic acid isomers and other phenolics (quinic acid in ester isomer signals around δ 2.0). Although these compounds were important determinants of the observed cultivar differences in all cases, their relative concentrations were not consistently higher or lower in any of the cultivars across seasons and locations. For example, in both years malic acid concentrations at flowering were higher in Premiere samples compared to other cultivars on location A, but not on location B.

Relationship between metabolomics data and insect abundances

Plant metabolomes and insect abundances can be expected to change across time and space without any direct causal relationship between the two. Therefore, we analyzed the relationship between metabolomics data and insect abundances separately for each sampling time point. Insect abundances could be predicted from metabolomics data on two sampling time points in 2011: in senescent plants on location A and in senescent plants at location B. This is despite the fact that insect numbers were the lowest on these two sampling time points, and cultivar differences were found in both insect abundances and metabolomics on most other sampling time points (Table 4). As indicated by cross-validated ANOVA of PLS models, these relationships were significant for Aphididae (location A) and Neuroptera (location B). In both cases, malic acid contributed strongly to the PLS model. In bivariate linear regression models, Aphididae numbers ($F_{1,32}$=5.16, P=0.030) and Neuroptera numbers ($F_{1,30}$=20.85, P<0.001) were found to decrease with malic acid concentrations (Figure 8). However, these relationships disappeared after correcting for cultivar differences in malic acid concentrations for both Aphididae (ANCOVA, Aphididae: $F_{1,27}$=0.73, P=0.399; cultivars: $F_{5,27}$=7.07, P<0.001) and Neuroptera (ANCOVA, Neuroptera: $F_{1,25}$=0.26, P=0.631; cultivars: $F_{5,25}$=3.78, P=0.011).

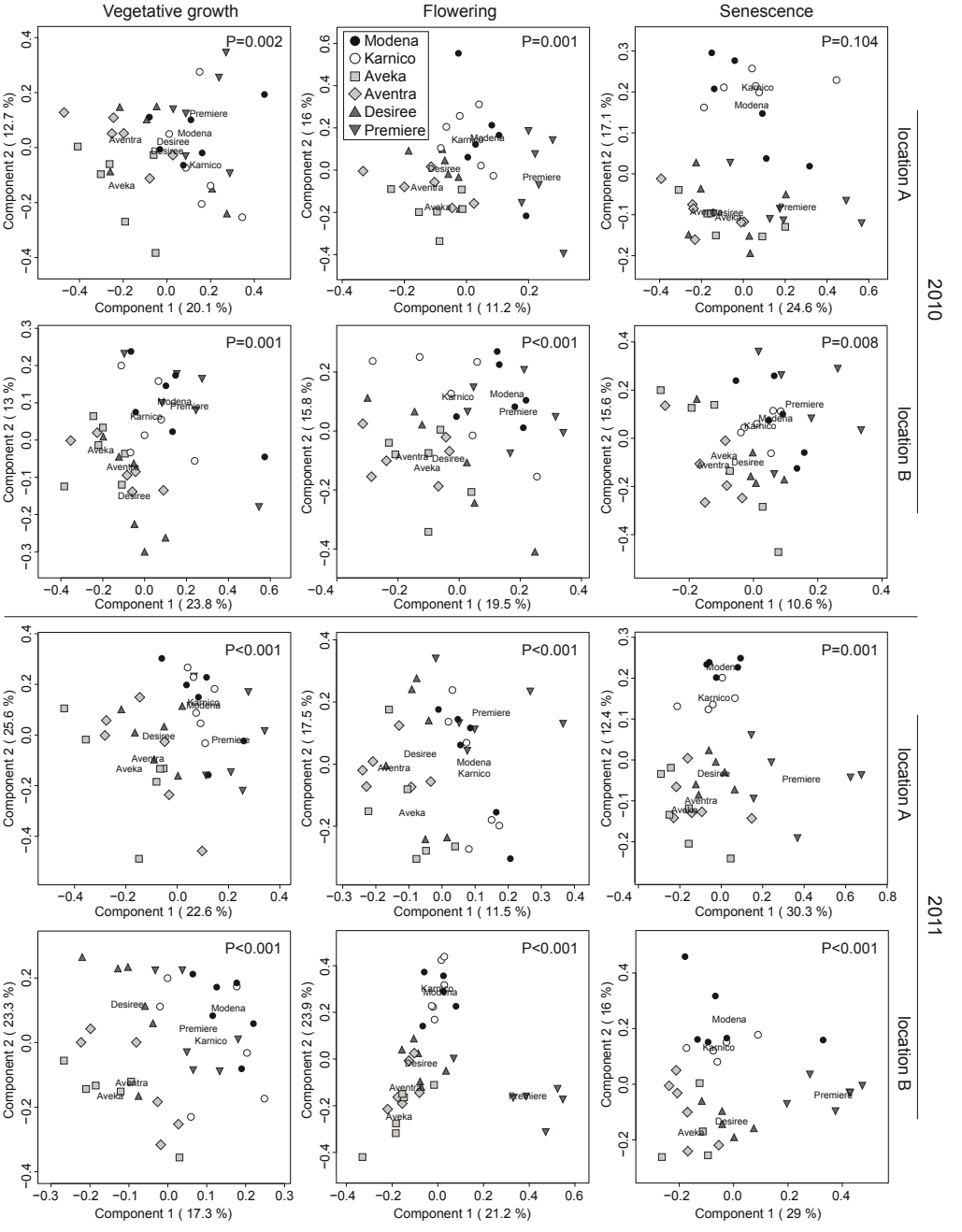

Figure 7: PLS-DA score plots showing the separation between ^{1}H NMR profiles of samples taken from the GM plant "Modena", its non-modified counterpart "Karnico" and four commercial cultivars in two years, at two locations and at three different developmental stages. Cultivar labels are located at the centroids of the respective point clouds and P-values indicate the significance of the discrimination.

Table 4: Overview of A) cultivar differences in insect count data (P-values derived from ANOVA), B) cultivar differences in metabolomics data and C) PLS regression models of metabolomics data and insect count data obtained from the same individual plants.

A) Significant cultivar differences in insect groups (ANOVA)

		Vegetative	Flowering	Senescent
2010	**location A**	Lepidoptera, P=0.010	Thysanoptera, P=0.037 Cicadellidae, P=0.025	Thysanoptera, P=0.002 Aphididae, P<0.001 Cicadellidae, P=0.002
	location B	Lepidoptera, P=0.037	Thysanoptera, P=0.027 Neuroptera, P=0.032	Thysanoptera, P=0.001 Aphididae, P=0.015 Hymenoptera, P= 0.017 Neuroptera, P<0.001 Syrphidae (larvae), P=0.012
2011	**location A**	Aphididae, P=0.04	(none)	Thysanoptera, P=0.022 Neuroptera, P=0.031
	location B	(none)	Heteroptera, P=0.004	Neuroptera, P<0.001

B) Cultivar differences in metabolomics (PLS-DA / permutation test)

		Vegetative	Flowering	Senescent
2010	**location A**	P=0.002	P=0.001	P=0.104
	location B	P=0.001	P<0.001	P=0.008
2011	**location A**	P<0.001	P<0.001	P=0.001
	location B	P<0.001	P<0.001	P<0.001

C) PLS regression metabolomics/insects (PLS / CV-ANOVA)

		Vegetative	Flowering	Senescent
2010	**location A**	n.s.	n.s.	n.s.
	location B	n.s.	n.s.	n.s.
2011	**location A**	n.s.	n.s.	Aphididae, P<0.001
	location B	n.s.	n.s.	Neuroptera, P<0.001

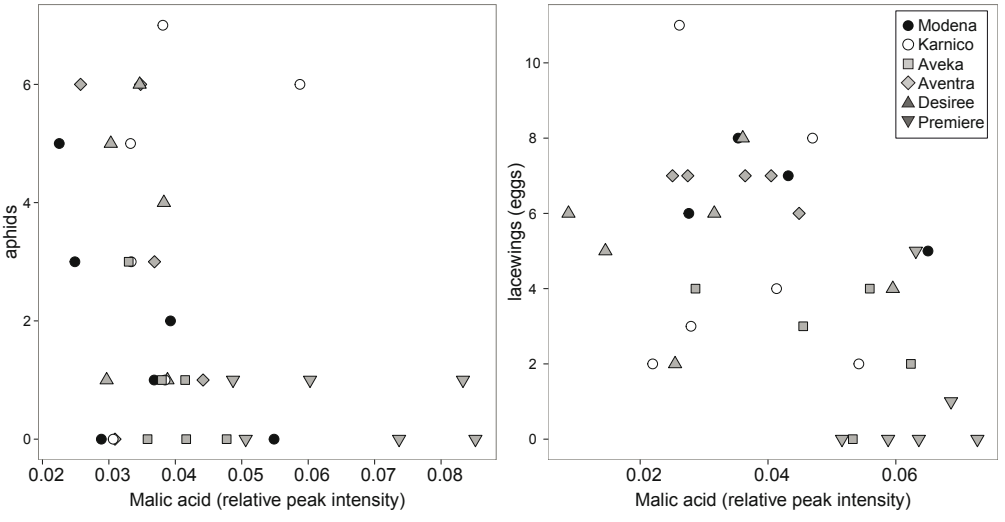

Figure 8: Correlations between relative peak intensities (arbitrary values) of ^{1}H NMR signals assigned to malic acid and Aphididae (2011, location A, senescent plants) and Neuroptera (2011, location B, senescent plants). NMR spectra of leaf samples and insect count data were obtained from field-grown potato plants.

Discussion

Differences in plant metabolism between years can have multiple causes. Plant growth, development and the production of secondary defense compounds are likely to be affected by abiotic and biotic conditions. In 2010, growing conditions were very dry and warm during early growth, causing a delay in flowering. In 2011, weather conditions were more constant, resulting in more constant plant growth and development (Figures 8+9, chapter 2, this thesis). Parallel studies on the same experimental fields have found differences between years in insect communities (see chapter 2, this thesis), soil fungal communities (Hannula, de Boer & van Veen 2010; Hannula, de Boer & van Veen 2012) and soil microbial communities (Inceoglu *et al.* 2010).

Differences between the two locations may be a result of the contrasting soil conditions between the two locations (A = sandy peat, B= loamy sand), which resulted in both abiotic and biotic differences between the two soils (Hannula, de Boer & van Veen 2010; Inceoglu *et al.* 2010; Hannula, de Boer & van Veen 2012). In 2010, there was also a difference in local weather, with less precipitation on location B (Figure 8, chapter 2, this thesis). These differences between the two locations are likely to interact with the development of plants as well as their resource allocation to chemical defenses. The experimental fields at the two locations were also found to differ in the associated above-ground insect communities (chapter 2, this thesis).

Plant metabolomes showed great changes over the course of a growing season, probably reflecting the large morphological and functional alterations caused by plant growth, flowering, tuber onset and senescence. However, also external conditions such as temperature, humidity and light also change in the course of a growing season. Thus, seasonal variation in metabolomics is caused by a combination of external and internal factors that cannot be easily disentangled in the field. While noting the presence of such changes in plants may seem trivial, it is important to quantify their relative importance and the strengths of interactions with other factors, because this information can serve as a reference for evaluating the biological relevance of GM effects.

The effects of developmental stages and cultivars involved similar compounds, and showed statistical interactions within both years, which probably reflects the known phenological differences between cultivars. For example, the cultivar “Premiere” is known as an “early” cultivar in terms of sprouting, early growth, flowering and senescence. Samples from all cultivars were taken on the same days for each sampling time point, in order to avoid effects of weather and temperature or differences in insect communities. Therefore, cultivars differed slightly in their developmental stages at the times of sampling (see also Figure 10, chapter 2, this thesis). This could explain the strong contribution of TCA cycle metabolites and sugars in the effects of cultivars and developmental stages. The GM line Modena and its parent cultivar Karnico showed strong overlap in metabolomic profiles at all sampling times, and differences between the two were always smaller than the differences among the set of commercial cultivars. Similar findings on the relative size of GM effects on metabolomic profiles in comparison to differences between commercial cultivars have been reported for potato tubers before (Defernez *et al.* 2004; Catchpole *et al.* 2005). Here, we show that this pattern also holds for plant chemistry in leaves. Furthermore, we show that in the field-cultivated plants in this study, GM effects remain relatively small over the whole course of a growing season.

The fact that relationships between insect abundances and malic acid were only found

in senescent plants and only across (not within) cultivars, suggests that these relationships were a result of senescence, with lower insect numbers and higher malic acid concentrations in cultivars that senesce earlier. While malic acid may have a direct effect on insects, it is likely that malic acid is an indicator of senescence, and that senescence involves a number of physiological changes that affect insects. The fact that no other relationships were found between ^{1}H NMR peaks and insect abundances could suggest that ^{1}H NMR metabolomics in the present form do not pick up relevant information that drives plant-insect interactions. Such undetected agents may be chemical compounds that are not extracted efficiently (e.g. volatiles or polymers), have too low concentrations or show strong peak overlaps in crude extract spectra. Another explanation for a weak association between chemical plant traits and insects could be that differences in insect abundances between cultivars are largely driven by morphological traits, such as leaf thickness or hairiness (Flanders *et al.* 1992).

Conclusions

This study shows that ^{1}H NMR metabolomics can successfully pick up profile changes in potato leaves across time, space and between genotypes. The difference between the GM line Modena and its counterpart Karnico never exceeded the baseline variation of commercial cultivars, suggesting that few unintended physiological changes were caused by genetic modification. Similar results have been reported for other crops as well (reviewed by Davies *et al.* 2010).

An important question in the application of metabolomics data in GM risk assessment is the question of how relevant of profile changes are to the particular risk studied. Natural patterns of variation in the measured parameters can provide some reference for the evaluation of effect sizes. With respect to plant-insect interactions, however, this study shows that despite the large amount of information contained in ^{1}H NMR profiles, the amount of information relevant to NTO safety assessment is limited. Low sensitivity or extraction efficiency in relevant compounds may be overcome technically in the future. No single metabolomics technique that can cover complete plant metabolomes (Chassy 2010), suggesting that a combination of techniques is necessary. However, for metabolomics to inform GMO safety there is an urgent need for more functional metabolomics approaches.

The statistical analysis of metabolomics data represents another challenge in the context of safety assessment. Profile differences are often found to be dominated by few “usual suspects”, i.e. compounds that respond to many different experimental treatments (Robertson 2005). This includes regulatory or signaling compounds or common pathway intermediates. Univariate analyses may be less sensitive to the influence of usual suspects than multivariate profiling methods, but lack the ability to detect “system-changes”. Statistical analyses of –omics data differ widely between disciplines, and need to be standardized and aligned with the principles of substantial equivalence (see also Davies 2010; Ricroch, Berge & Kuntz 2011 and chapter 3, this thesis).

An important observation is that genotypic effects, including GM effects, can vary in space and time. The possibility for GxE interactions, i.e. the possibility that GM effects occur

in one environment, but not the other, raises questions about the design and interpretation of pre-market safety assessments: How many different environments have to be included? Which sampling time is the most relevant? If effects occur in one developmental stage, but not in others, what does that mean in terms of safety? These questions also require a public debate about the level of acceptable uncertainty in pre-market safety assessments. Ever-increasing costs for field trials in multiple environments have to be weighed against the decrease in uncertainty that they provide. The uncertainty of pre-market assessments also has to be compared to alternative strategies, such as improved post-market monitoring.

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Summary and conclusions

In this thesis, patterns of variation in plant metabolomes and insect communities were described in GM and non-GM potato plants in both laboratory and field experiments. In the following paragraphs, the main findings of the individual chapters of this thesis will be summarized. After discussing some aspects in further detail, conclusions are drawn on the implications of this study for non-target risk assessment procedures.

In chapter 2 of this thesis, patterns of variation in non-target insect communities on experimental potato fields were studied. We found that differences between plant genotypes (including the GM variety and its counterpart) were small when compared to other sources of variation, such as year-to-year differences, location effects and differences between developmental stages of plants. Similar findings – small genotype effects compared to natural baseline variation – have been reported for other crops. Viewing effects of GM plants in the light of natural baseline variation in agricultural systems is an important step in closing the knowledge gap between statistical significance and biological significance. Genotype-by-environment (GxE) interactions accounted for a considerable part of the total variation in insect abundances. GxE interactions imply that effects can be small or absent in some environments, but large in others. Therefore, conclusions from field trials are not valid outside the range of tested environments. This emphasizes the need for a careful choice of representative geographical zones for risk assessment experiments. However, it also shows that a residual level of uncertainty in NTO safety is unavoidable.

In chapter 3, we examined some aspects of the current practice of effect size measurement in NTO risk assessment. Due to a lack of ecological knowledge for most species, thresholds (limits of concern) for GM / non-GM differences are often set arbitrarily without biological justification. Arbitrary fixed thresholds across species have the disadvantage of resulting in large differences in statistical power between species and thus large differences in experimental design requirements for field trials. Standardized effect sizes were discussed as an alternative scale for measuring effects. Standardized effects sizes measure effects in relation to the observed variability, thus making fixed thresholds more biologically appropriate. On the other hand, such combined effect size metrics have the disadvantage of obscuring absolute changes in the data. Furthermore, the biological reasoning of correcting for variability may not always reflect correctly the vulnerability of populations at the landscape level. However, it is important to realize that biological justifications of thresholds are not always given, and that the use of fixed thresholds has important consequences that may contradict biological reason.

In chapter 4, metabolomics were introduced as a method for detecting system-changes at the plant level with possible relevance to non-target organisms. Pleiotropic effects of genetic modification can result in constitutive metabolic changes. However, when inducible pathways are

affected, metabolic changes will only occur under specific conditions. In this laboratory study we took into account the plasticity of plant metabolism in response to internal and external stimuli. We showed that leaf age, aphid infestation and virus infection cause significant alterations in leaf metabolism, but that these changes were similar in quantity and quality in both GM and non-GM plants. In general, GM/non-GM differences were never greater than the metabolic alterations due to internal and external stimuli.

In chapter 5, ^{1}H NMR metabolomic profiles of potato plants were measured in a 2-year field study. Metabolic plasticity across two locations and three developmental stages was taken into account as a baseline of variation. This study showed that large changes in metabolomic profiles occurred across years and locations, and throughout the growth period of plants within years. We found evidence for genotype-by-environment interactions as well as genotype-by-development interactions. Only weak relationships were found between metabolomics data and insect abundances on the same experimental fields, despite the fact that differences between genotypes were found in both insect abundances and ^{1}H NMR metabolomics. This suggests that 1) variation in insect abundances was largely independent of plant chemistry and instead affected by e.g. morphological traits, or 2) that ^{1}H NMR metabolomics did not pick up relevant information in plant metabolism to explain patterns in insect abundances. The latter may be caused by ineffective extraction of some classes of compounds (e.g. volatiles, polymers) or by a lack of sensitivity of the detection method. Overall, effects of GM potato on metabolomics as measured by NMR were small when compared to eco-metabolomic variation. However, the understanding of the functional relevance of eco-metabolomic variation is still limited.

Towards a systems biology approach to NTO risk assessment

Establishing baselines of variability in semi-natural agricultural environments at both plant and ecosystem level is crucial for understanding plants as "complex systems embedded in poorly understood, complex, and interacting ecosystems"[1]. Fundamental knowledge on ecosystem variability and stability is necessary in order to set biologically justified threshold limits for non-target species in GM/non-GM comparisons. A systems biology approach to NTO risk assessment also requires a rethinking of the way that effects in risk assessments are measured. The current practice of considering GM effects independently per endpoint in univariate analyses lacks a systems perspective and could potentially miss important changes at the metabolic network level in plants or at the community level in ecosystems.

Metabolomics is a powerful tool in understanding the complexity and plasticity of plant metabolism. One of the challenges for "eco-metabolomics" is to understand the functional relevance of plant metabolic changes for ecological interactions. Describing natural variation across time and space and in response to stressors will help to understand functional relationships between plant metabolomics and ecological processes. Such baseline information is also crucial for the application of metabolomics in NTO risk assessment, because natural variation in crop metabolomes can serve as a reference to which GM/non-GM comparisons can be compared. In this study, few functional relationships between potato leaf metabolomes and insect communities were found.

1 Ervin, D. E., Welsh, R., Batie, S. S. & Carpentier, C. L. (2003), Agriculture Ecosystems & Environment, 99, p. 12.

Future metabolomics approaches need to aware of limitations in extraction efficiency and sensitivity in relevant compounds. Combining different metabolomics techniques and inserting automated extraction and chromatography steps before the actual detection may significantly improve the power of eco-metabolomics approaches. Ultimately, the functional relevance of metabolomic variation in plants will not be fully understood unless metabolomics are integrated with information at different levels of organization, from genomes to ecology and evolution.

Conclusions for non-target risk assessment

This thesis has shown that non-target risk assessment field trials are dealing with a tremendous amount of natural variation. Sampling insect abundances in the field with sufficient precision therefore requires large surface areas and a large amount of man-power and time. Nevertheless, a considerable degree of residual uncertainty will remain, because genotype-by-environment and genotype-by-development interactions will restrict conclusions to the experimental situations of the conducted field trials. Furthermore, it is unknown how effects at the level of (relatively) small pre-market field trials will translate to larger spatial scales after commercialization. It can be expected that the stability of NTO populations strongly reacts to patterns of habitat fragmentation at the landscape level, and these effects may interact with the management practices that are adopted along with GM crop cultivation. These are uncertainties that lie beyond the immediate statistical uncertainties of an experiment (i.e. Type I and Type II errors). Non-target field trials may remain an important step in the marketing process of GM crops, but the uncertainties associated with them should be communicated clearly and publicly. An effective environmental protection strategy will inevitably need to monitor changes systematically over the course of years after commercialization.

In dit proefschrift worden variatiepatronen in plantenmetabolomen en daarop voorkomende insectengemeenschappen beschreven in zowel GG (genetisch gemodificeerde) en niet-GG aardappelplanten in laboratorium- en veldexperimenten.

In hoofdstuk 2 van dit proefschrift worden variatiepatronen in gemeenschappen van niet-doel-insecten op experimentele aardappelvelden onderzocht. Het effect van verschillende plantengenotypes (waaronder de GG-plant en haar moederplant) op insectengemeenschappen is klein in vergelijking met andere variabelen zoals het jaar, de locatie en het ontwikkelingsstadium van de planten. De vergelijking van effecten van GG planten met ecologische basis variatie is belangrijk om een verband tussen statistische significantie en biologische significantie te kunnen leggen. Een groot deel van de totale variatie wordt veroorzaakt door interacties tussen genotypes en omgeving. Dit toont aan dat effecten van GG planten in bepaalde omgevingen klein, maar in andere omgevingen groot kunnen zijn. Conclusies voor ecologische risico's van GG planten kunnen dus alleen voor de experimenteel geteste omgevingen getrokken worden. Dit benadrukt dat een zorgvuldige keuze van representatieve geografische zones zeer belangrijk is voor risicoanalyses. Het maakt echter ook duidelijk dat restonzekerheden niet uit te sluiten zijn.

In hoofdstuk 3 worden een aantal aspecten van de hedendaagse praktijk onderzocht, waarmee de grootte van effecten op niet-doel-organismen wordt gemeten. Voor veel insectensoorten is er onvoldoende ecologische informatie beschikbaar. Daarom worden grenswaardes voor verschillen tussen GG en niet-GG planten vaak willekeurig voor alle soorten vastgelegd zonder biologische verantwoording. Willekeurige grenswaardes leiden tot grote verschillen in statistische power tussen soorten en dus tot grote verschillen in eisen voor experimentele opzetten. Standardized effect sizes (SES) worden bediscussieerd als alternatieve schaal voor het meten van effecten. Standardized effect sizes meten effecten in relatie tot de gemeten variatie, en houden dus ook bij het gebruik van vaste grenswaardes rekening met biologische verschillen tussen soorten. Een nadeel van SES is dat deze maat geen indicatie meer geeft van de grootte van absolute verschillen tussen metingen. Het is ook onduidelijk of er een verband is tussen de gemeten variatie in een soort en de kwetsbaarheid van deze soort op landschappelijk niveau.

In hoofdstuk 4 worden metabolomics als methode geïntroduceerd om systeemveranderingen in een plant te meten die mogelijke gevolgen kunnen hebben voor niet-doel organismen. Pleiotropische effecten van genetische

modificatie kunnen constitutieve metabolische veranderingen veroorzaken, maar ook induceerbare pathways kunnen beïnvloed worden. Het laatste zou dan alleen onder bepaalde condities tot meetbare veranderingen leiden. In dit hoofdstuk wordt met behulp van NMR metabolomics aangetoond dat de metabolomen van GG en niet-GG aardappelplanten op een vergelijkbare manier reageren op infestatie door bladluizen, virus infectie en het verouderen van bladeren.

In hoofdstuk 5 wordt eco-metabolomische variatie in ^{1}H NMR metaboloom profielen van aardappelplanten in een tweejarige veldstudie beschreven. Er zijn grote verschillen in metaboloom profielen gevonden tussen jaren en locaties, en tussen verschillende ontwikkelingsfases van de planten binnen de jaren. Verder zijn interacties tussen genotypes (waaronder de GG plant en haar moedersoort) en omgeving gevonden, maar ook tussen genotypes en ontwikkelingsstadia. Ondanks het feit dat er verschillen tussen genotypes in zowel ^{1}H NMR metaboloom data en insectengemeenschappen zijn gevonden, was de relatie tussen deze twee metingen zwak. Dit duidt erop dat 1) variatie in insectengemeenschappen voor een groot deel onafhankelijk is van de chemische samenstelling van planten (en in plaats hiervan meer beïnvloed wordt door bij voorbeeld morfologische eigenschappen van de planten), of 2) dat door middel van ^{1}H NMR metabolomics weinig relevante chemische stoffen gemeten kunnen worden, die verschillen op insectengemeenschapsniveau veroorzaken. Redenen hiervoor kunnen onvoldoende extractie van bepaalde groepen chemische stoffen zijn, zoals vluchtige componenten of polymeren, of een te lage sensitiviteit voor belangrijke stoffen. In conclusie zijn de effecten van genetische modificatie op plantenmetabolomen, zoals door NMR gemeten, klein in vergelijking met de eco-metabolomische variatie in het veld. De kennis over de functionele relevantie van eco-metabolomische variatie is echter nog steeds beperkt.

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I, Andreas Plischke, was born on 20 July 1982 in Wesel, Germany. I finished my secondary school program (Abitur) at the Kondrad-Duden-Gymnasium in Wesel, Germany in 2001 and started to study biology at the Westphalian Wilhelms-University in Münster, Germany later that year. After two research projects on topics as diverse as physiological plant-pathogen interactions and animal evolutionary ecology, I took the opportunity to conduct eco-physiological field work on a population of burrowing parrots in El Condor, Argentina under the supervision of Juan Masello from the Max-Planck-Institute for Ornithology at Radolfzell, Germany. I wrote my dissertation (Diplomarbeit) using the data collected in Argentina, and received my Diplom (MSc equivalent) in 2007, with zoology and botany as major subjects and biochemistry as a minor subject.

In the following year I gathered experience in various aspects of life working as a field-assistant in ecological risk assessment studies in Poland for Rifcon GmbH, Germany; travelling by train from Moscow to Bangkok; working as a teaching assistant at the Westphalian Wilhelms-University of Münster, Germany; working as an intern at the Evolutionary Entomology research group at the University of Sheffield, UK and working as an intern at the research-funding Volkswagen foundation in Hannover, Germany.

In order to further explore the world of science and research, I started my PhD at the Ecology and Phytochemistry group at Leiden University, The Netherlands in 2008 under the supervision of Peter Klinkhamer and Paul Brakefield. During this time I conducted laboratory experiments and field work on plant-insect interactions, gained experience in NMR metabolomics measurements and data analysis and presented my work at several national and international scientific meetings. I also enjoyed organizing a weekly seminar series and an annual symposium as a member of the scientific meetings committee of the institute. In 2012, I took the opportunity to do a short internship at the Marketing and Communication department of UNESCO-IHE Water Education Institute in Delft, The Netherlands, marking my return to the world outside academia. Since 2013, I am working as a scientific officer at the Federal Office of Consumer Protection and Food Safety (BVL) in Berlin, Germany.