Potatoes, pathogens and pests

Effects of genetic modification for plant resistance on non-target arthropods

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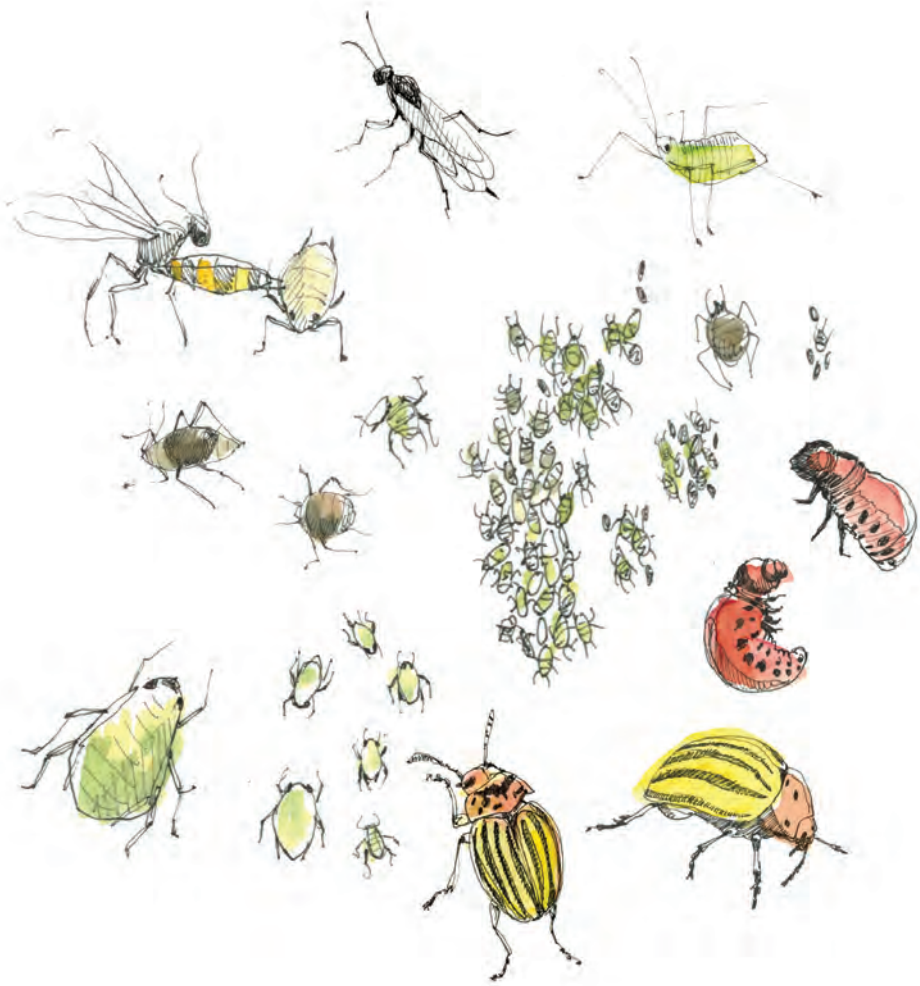
Chapter 1

Introduction & thesis outline



Plants, pathogens and pests have a long history together. Millions of years of evolutionary history have shaped the interactions between plants and their communities of herbivores, pollinators, carnivores, detritivores, and pathogens (Mitter *et al.*, 1991). To understand how plants interact directly and indirectly with these organisms, insights can be gained from the scientific fields of biochemistry, genetics, physiology and ecology (Austin & Ballaré, 2014; Dicke & Hilker, 2003; Strauss & Zangerl, 2002). The more we learn, the more we understand how plant interactions with organisms like pathogens and insects can shape the structure of entire ecosystems (Stam et al., 2014; Tack & Dicke, 2013).

The term "pest" however, anthropocentrically originated when people started shaping their ecosystems through agriculture. Since then, people have been competing with the pests and pathogens that use the same food resource. For centuries the adaptable nature of plants to escape their enemies has been exploited through breeding. In addition, crop protection made use of cultural and biological control methods. Currently, the agricultural industry relies heavily on chemical methods of managing plant pests and pathogens, further altering our environment. For example, the use of fungicides is currently a necessity for growing most potato cultivars in maritime climates such as north-western Europe, and the use of chemical control for the potato industry has a huge environmental impact (Haverkort *et al.*, 2009). The damp and cool climates are ideal for the propagation of one of the world's most damaging potato pathogens: *Phytophthora infestans* (Mont.) de Bary (Fry, 2008). Recent developments in technology have offered a more sustainable solution to chemical pathogen control, and make use of the extensive knowledge gained from breeding for resistance. The introgression of resistance genes through genetic modification is a much more sustainable solution to the environmentally damaging option of chemical pest management (Mundt, 2014).

The regulation of such genetically modified (GM) crops for environmental safety is considered just as relevant as the regulation of crop protection chemicals. In the regulation of GM crops, a major point of focus is the effect of the genetic modification on non-target organisms (NTOs), defined as any organisms that are unintentionally affected by the modification of the crop. This thesis is about the effects of a genetic modification of potato plants (*Solanum tuberosum* L.) on NTOs found in potato agro-ecosystems. The specific modification addressed in this thesis confers resistance to the most aggressive pathogen known on potato, the "plant destroyer" late blight, the oomycete *P. infestans* (Fry, 2008). Although the modification in question is highly specific (see next section), GM plants in Europe are subject to a risk assessment to evaluate possible risks of introducing such plants in the ecosystem including risks to the non-target arthropod community associated with the potato crop. Although there are already European guidelines in place composed by the European Food Safety Authority (EFSA) for their evaluation

(EFSA, 2010), the European Union has called upon the research community to evaluate GM crops including maize and potato. The research consortium AMIGA (Assessing and Monitoring the Impacts of Genetically modified plants on Agro-ecosystems, 2011-2016) was commissioned to carry out the evaluation. AMIGA aimed to systematically test the protocols in the current EFSA-guidance document for environmental risk assessment (ERA) of genetically modified crops, explore new strategies and further develop protocols (Arpaia *et al.*, 2014). The research of AMIGA was conducted by 22 partners in 15 countries and focused on functional ecological components of agro-ecosystems.

This thesis concerns one of eleven work packages of this project: 'trophic structure analysis of agro-ecosystems', and therefore the chapters in this thesis aim to understand the effects of a genetic modification for plant resistance at several levels of interaction between the plants and other trophic levels. Since the nature of the questions and model systems involve the interactions between plants, insects and pathogens, a thorough literature review on the interaction of insects and pathogens was conducted, focused on how feeding strategies and arrival sequences influence these interactions through the plant (Chapter 2). In Chapter 3, we investigate how susceptible and *P. infestans* resistant potato plants respond to single and dual (sequential) herbivory by leaf chewing or phloem-feeding potato pests. I approach this topic by quantifying the expression of genes known to be important in the induced defense responses of plants against the two most important herbivores on potato: the phloem-feeding aphid *Myzus persicae* (Sulzer), and the leaf-chewing Colorado potato beetle *Leptinotarsa decemlineata* (Say).

Figure 1. Third instar *Leptinotarsa decemlineata* larva on a potato leaflet (photo by Jitte Groothuis).

Leptinotarsa decemlineata

The Colorado potato beetle (CPB) (Figure 1) is a gregarious leaf chewer belonging to the family Chrysomelidae (order Coleoptera). Larvae and adults are specialized in feeding on *Solanum* species, and prefer potato plants. They can defoliate a potato plant until there is no remaining aboveground foliage. The Colorado potato beetle is historically and currently one of the most destructive pests of potato crops. This beetle has become resistant to several insecticides used in chemical control strategies (Alyokhin *et al.*, 2008), and if weather permits can have several generation cycles per year (Isely, 1935). Not only are they avid defoliators, but females have impressive fecundity, laying several hundreds of eggs in a lifetime (Harcourt, 1971). The Colorado potato beetle is the number one insect

pest on potato in the EFSA arthropod database (Meissle *et al.*, 2012).
While *L. decemlineata* and *M. persicae* are featured in Chapter 3, and often occur together on potato plants, *M. persicae* is the main herbivore used in several chapters of this thesis as the main non-target herbivore. In Chapter 4, *M. persicae* is thoroughly examined as a non-target insect using several different events of the same target modification in potato plants for resistance against late blight.

Figure 2. *Myzus persicae* adult (in small photo by David Cappaert) and colony on a potato leaf (photo by the author).

Myzus persicae
The green peach-potato aphid (Figure 2) is a generalist phloem feeder, feeding on over 400 different plant hosts. They are viviparous and reproduce by parthenogenesis, and reproducing sexually only once per year for overwintering as eggs, or as adults in mild climates (Van Emden *et al.*, 1969). *Myzus persicae* is a vector of over one hundred plant viruses, and is the most important phloem-feeding pest of potato (Radcliffe, 1982). *Myzus* species can vector twelve different potato viruses (Kennedy *et al.*, 1962; Ng & Perry, 2004; Van Emden *et al.*, 1969), including several leaf-roll viruses. These widespread aphid pests are also prey to many carnivorous arthropod families such as larval Syrphidae (Diptera) (Raj, 1989), ladybird beetles (Coleoptera: Coccinellidae) (Majerus, 1994), lacewings (Neuroptera), spiders (Araneae) and also host to many parasitoids (Müller *et al.*, 1999).

One of the main parasitoids of *M. persicae* is featured in Chapter 5 of this thesis. Studying the third trophic level is not very common for greenhouse studies in a risk assessment context; yet in Chapter 5 we approach this topic with a study on *Aphidius colemani* (Figure 3), a common parasitoid of *M. persicae* . This study also features plants inoculated with the target pathogen *P. infestans*. We investigate how pathogen inoculation of the plants can affect the second (*M. persicae*) and third (*A. colemani*) trophic levels. These solitary generalist parasitoid wasps of the family Braconidae (order Hymenoptera) lay single eggs inside aphid nymphs. One female can parasitize up to 400 aphid nymphs (Torres *et al.*, 2007). The larvae develop inside and consume their host, emerging from it as an adult. Just before pupation, the aphid host integument hardens. In this state the aphid is referred to as a mummy. The adults emerge by

Figure 3. *Aphidius colemani* adult female (photo by Erling Fløistad).

chewing open the mummy. Their efficiency in controlling aphid pests is recognized for crop protection and several *Aphidius* species have become commercially available for biological control.

Figure 4. A potato plot decimated by the late potato blight pathogen *Phytophthora infestans* (photo by the author).

Phytophthora infestans

This near-obligate hemibiotrophic oomycete is a specialist on solanaceous crops and can destroy a whole crop (Figure 4) in a matter of days, reducing leaves, stems and tubers to necrotic waste. Visibly, no symptoms are detectable for a couple of days after infection, after which small necrotic lesions develop. Under high humidity and low temperatures, sporangiophores are produced with hundreds of thousands of sporangia per lesion (Fry, 2008). Its persistence in the soil between seasons, sexual and asexual reproduction as well as a very effective water, air and soil dispersal make this pathogen a serious threat and very prone to overcoming natural plant resistance (Fry, 2008). Currently in The Netherlands, this oomycete pathogen is combatted by spraying fungicide up to 20 times per year (Cooke *et al.*, 2011). It is also historically and currently one of the best-studied plant pathogens, after being the cause of the Great Irish Famine in 1845.

Although we can attempt to recreate interactions between pathogens and plants and their multiple trophic levels in greenhouse experiments, the most realistic biological experiments can only be done in the field. In field tests, we can measure the effect of our introduced genotype with true *P. infestans* pathogenic pressure under a combination of conditions, and also in combination with realistic agricultural management practices. The last experimental chapter presents a community analysis of the potato agro-ecosystems in The Netherlands and Ireland which has been conducted in the field for a period of two years (Chapter 6) taking into consideration the effects of plot management strategy as well as the potato genotype.

Lastly, Chapter 7 discusses the results found in the studies above and comments on the relevance of these in the ecological context of the agro-ecosystem and the variation among currently existing potato varieties. Here I discuss the future of research and policy concerning genetically modified plants with R gene resistance.

How do potato R genes confer resistance?

The resistance of the potato plants discussed in this thesis is ensured via an introgressed major resistance gene (R gene). R genes code for plant protein receptors, which recognize a specific cue from the pathogen. In their interaction with hosts, pathogens secrete various proteins, some of which are effectors. An effector can lead to virulence in susceptible plants. In resistant plants (containing R genes), effectors may be recognized by the plant, leading to successful plant defense against the pathogen. The latter interaction is considered to be an important driver in the co-evolutionary arms race by means of resistance in plants eventually driving pathogen development through new R gene mutations (Jones & Dangl, 2006). The successful recognition of the pathogen protein is followed by encapsulation of the pathogen by callose deposits and death of surrounding cells (Kamoun *et al.*, 1999; Vleeshouwers *et al.*, 2011; Vleeshouwers *et al.*, 2000). Several R genes effective against *P. infestans* are known from wild crossable relatives of potato (Mattheij *et al.*, 1992; Smyda *et al.*, 2013; Van Der Vossen *et al.*, 2003), therefore the GM plants containing these are considered to be cisgenic. Transgenic crops contain a gene from a different non-related species, as is the case for *Bt*-potato, containing a gene from the bacterium *Bacillus thuringiensis* coding for an insecticidal endotoxin. A marker gene is sometimes used in the genetic modification process in order to easily test if the transformation was successful. In some of the GM-plants used in this study, the marker gene *nptII* (Beck *et al.*, 1982) had been inserted along with the R gene from the wild potato species which allows the transformed plant to be grown on a selective medium. The *nptII* gene was originally isolated from the bacterium *Escherichia coli* (Beck et al., 1982) (NPTII, EC 2.7.1.95) and confers resistance to various antibiotics, enabling transgenic cells expressing the inserted gene to successfully propagate on a bacterial medium. In this thesis, we refer to plants containing these bacterial marker genes as transgenic, since they contain a gene from an unrelated organism. In this thesis GM potato plants with and without these marker genes are tested for their effects on NTOs in Chapter 2. The genetic modification for insertion of the R gene is done using *Agrobacterium tumefaciens* which can insert the gene in question into the potato genome semi-randomly, meaning that each separate occasion ('event') of a successful introgression is a genotype with the R genes (or marker genes) inserted in a different genomic position. The influence of the genomic position of the R genes on non-target insects was investigated in Chapters 2, 5 and 6.

Thesis outline

Chapter 2: This chapter summarizes the literature on sequential interactions between insects and pathogens. The focus of the review is on phytohormonal mechanisms, and how they are influenced by pathogen trophic strategy or insect feeding mode. A general working hypothesis is proposed with a summarizing diagram compiled from recent literature. Knowledge gaps in this field are recognized and future research is proposed.

Chapter 3: Herbivore performance and gene expression of potato plants with and without an R gene modification for resistance to *P. infestans* is assessed under herbivory by one or a combination of two non-target herbivores. The isogenic cultivar (Désirée) as well as two cisgenic events of the same R gene modification are compared in order to clarify whether position of the R gene can influence non-target interactions.

Chapter 4 concerns the effects of R gene resistance to *P. infestans* on *M. persicae*. We studied several aspects of the insertion of R genes, i.e. position of the R gene in the genome, insertion of two R genes or co-insertion of a marker gene and tested the effects on *M. persicae* survival and reproduction. The study was reproduced in two labs in Europe and compared in the context of *M. persicae* performance on several existing cultivars varying in their resistance to *P. infestans*.

Chapter 5: Performance of the aphid *M. persicae* and its parasitoid *A. colemani* were investigated with several events of the R gene modification for late blight resistance with and without pathogen inoculation. This study tested whether the inoculation of the pathogen on different events would influence the performance of these NTOs. The study also explored the effect of insertion position or marker-gene presence in the GM potato genotype on interactions with these members of the second and third trophic level.

Chapter 6: This chapter analyses the effects of potato plant genotype and pest management on the arthropod community associated with a potato agro-ecosystem. The experiments were conducted in Ireland and in The Netherlands over the years 2014 and 2015. The factors of year and geographic location are analyzed for their impacts on the arthropod community, and within each year and location we considered the potato genotype as well the effect of fungicide regime on the arthropod biodiversity. We consider several methods for analyzing biodiversity and compare the biological conclusions derived from each, with the aim of providing recommendations for future biodiversity analyses conducted in the context of risk assessment.

Chapter 7: In this General Discussion, I discuss the leap from statistical significance to ecological relevance in risk assessment of GM crops, especially those with R gene modifications. I consider analyses done at the molecular level, at the level of single indicator species, several tropic levels and in the community context. At each level of specificity, the relevance of the conclusions that can be drawn in the context of ecological risk assessments is discussed. We propose ideas for future developments of ERA protocols, in light of expected progress in the field of agricultural sciences.

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Chapter 2

Interactions between herbivores and plant pathogens:

Phytohormonal mediation and the importance of insect feeding mode and pathogen trophic strategy

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Abstract

Induced plant defenses against either pathogens or herbivore attackers are regulated by phytohormones. These phytohormones are increasingly recognized as important mediators of interactions between organisms associated with plants. In this review, we discuss the role of plant defense hormones in sequential tri-partite interactions among plants, pathogenic microbes, and herbivorous insects, based on the most recent literature. We discuss the importance of pathogen trophic strategy in the interaction with herbivores that exhibit different feeding modes. Plant resistance mechanisms also affect plant quality in future interactions with attackers. We discuss exemplary evidence for the hypotheses that (i) biotrophic pathogens can facilitate chewing herbivores, unless plants exhibit effector-triggered immunity, but (ii) facilitate or inhibit phloem feeders. (iii) Necrotrophic pathogens, on the other hand, can inhibit both phloem feeders and chewers. We also propose herbivore feeding mode as predictor of effects on pathogens of different trophic strategies, providing evidence for the hypotheses that (iv) phloem feeders inhibit pathogen attack by increasing SA induction, whereas (v) chewing herbivores tend not to affect necrotrophic pathogens, while they may either inhibit or facilitate biotrophic pathogens. Putting these hypotheses to the test will increase our understanding of phytohormonal regulation of plant defense to sequential attack by plant pathogens and insect herbivores. This will provide valuable insight into plant-mediated ecological interactions among members of the plant-associated community.

Key Words

Tripartite interactions; phytohormones; plant pathogens; herbivorous insects; trophic strategy; feeding mode, plant-mediated indirect interactions

Introduction

Plant growth, reproduction and defense against biotic and abiotic stressors are regulated by phytohormones (Pieterse *et al.*, 2012). The most common and diverse biotic stressors of plants are pathogens and herbivores (Pieterse & Dicke, 2007; Schoonhoven *et al.*, 2005). The populations of these organisms have their intrinsic dynamics, yet they influence one another indirectly through changes in quality of the shared plant, i.e., plant-mediated indirect interactions (Kaplan & Denno, 2007; Ohgushi, 2005; Stam *et al.*, 2014; Utsumi *et al.*, 2010)). Upon insect or pathogen attack, plants are able to mount defensive responses, which underlie plant-mediated indirect interactions. These defenses are regulated mainly by phytohormones that are induced differently depending on the identity, sequence, and intensity of attack of the different stressors (Awmack & Leather, 2002; Howe & Jander, 2008; Stam *et al.*, 2014; Thaler *et al.*, 2012). Plant responses to pathogens and herbivores can affect the whole community through changes in phytohormones and their downstream signaling pathways (Poelman *et al.*, 2008; Tack & Dicke, 2013). Plants are at the core of terrestrial ecosystems and understanding how phytohormones modulate interactions among different members in the community can yield important insight into ecological interactions.

The role of phytohormones in signal-transduction pathways underlying induced defense has been well documented (Gimenez-Ibanez & Solano, 2013; Maffei *et al.*, 2007; Pieterse *et al.*, 2012; Stam *et al.*, 2014; Walling, 2009). Crosstalk between these pathways is one way in which plants can fine-tune their responses by modulating gene expression. Ultimately, each plant-insect or plant-pathogen interaction is a product of its unique evolutionary history, and is the result of an "arms-race" between the two parties in which plant secondary metabolites play a central role (Ehrlich & Raven, 1964). However, phytohormonal pathways induced in plants can be partly predicted based on herbivore feeding mode: it is well documented that insects employing piercing-sucking or biting-chewing feeding modes elicit different responses in plants (Bonaventure, 2012; Broekgaarden *et al.*, 2011). Induction of several defense signaling pathways is known for aphids, piercing-sucking insects that feed on phloem sap (De Vos *et al.*, 2005; Kusnierczyk *et al.*, 2008; Mai *et al.*, 2014). The same applies to pathogens employing different trophic strategies, i.e. necrotrophic and biotrophic (Pieterse *et al.*, 2012). Within the same feeding-mode, whether the attacker is a generalist or specialist also may be an important factor, although this paradigm has recently been challenged in favor of feeding modes as better predictors of phytohormonal responses (Ali & Agrawal, 2012; Bidart-Bouzat & Kliebenstein, 2011; Kliebenstein & Rowe, 2008). In addition to feeding mode, the susceptibility of a plant to a particular attacker also may influence phytohormonal response even within the same insect feeding mode; for example, after aphid attack, salicylic acid-reponsive transcripts accumulated quicker and to higher levels in leaves of resistant plants than in susceptible ones (De Ilarduya *et al.*, 2003). Plant ontogeny

also can affect the defense response of plants to the same attacker; for example, seedlings may allocate more resources to defensive chemicals than mature plants (Barton & Koricheva, 2010; Boege, 2005; Lawrence *et al.*, 2003). Each of these factors results in hormonal responses that influence the subsequent or concurrent attacker. Chronological order of stress initiation on plants as well as duration also come into play, leading to considerable variability in multi-partite interactions (Dicke *et al.*, 2009).

In the case of multiple attacks on plants by organisms of different kingdoms, relatively little is known about the influence of one attack on the next. Much work has been devoted to understand how plant hormones modulate interactions between plants and their associated insect herbivores (Mithöfer & Boland, 2012) or pathogens (Glazebrook, 2005), but plant hormone modulation of three-way interactions among these players is a field that has remained relatively unexplored (Bennett, 2013; Hatcher *et al.*, 2004). In this review, we explore if predictive factors can be identified that influence the biosynthesis of plant defense hormones in tripartite interactions among plants, plant pathogenic microbes, and herbivores. In order to arrive at testable hypotheses, we focus especially on the most studied hormones, jasmonic acid, salicylic acid, and ethylene. Other phytohormones such as auxin, giberellins, cytokinins, and brassinosteroids also are involved in plant defense responses, yet their roles in tripartite interactions have been much less studied (Bari & Jones, 2009; Pieterse *et al.*, 2012). To generate hypotheses about these interactions, it is important to first discuss what is known about regulation by phytohormones of plant responses to microbial stressors like bacteria, fungi, and viruses, and to insect herbivory and how it affects subsequent attacks.

Plant-Pathogen Interactions

Plant Responses to Pathogen Infection

Phytohormonal changes induced by pathogens differ depending on their trophic strategy. Pathogens with a biotrophic strategy usually overcome plant defenses and colonize the plant by producing virulence effectors that manipulate the defense system of the plant, making it susceptible for infection. Resistant plants, however, can recognize the virulence effectors of the pathogen, through resistance gene (R gene) products. This initiates a defense response that arrests the pathogen before it can colonize any further. This often is referred to as effector triggered immunity (ETI) (Fu & Dong, 2013; Glazebrook, 2005), which is the product of closely co-evolved species-specific interactions between plant pathogens and their hosts (Mengiste, 2012; Pieterse *et al.*, 2009). At the site of pathogen infection, ETI leads to a localized response usually related to the production of reactive oxygen species (ROS), or an oxidative burst leading to localized programmed cell death, also known

as hypersensitive response (HR) (Baxter *et al.*, 2013; Kerchev *et al.*, 2012; Kliebenstein *et al.*, 2008; Overmyer *et al.*, 2003; Torres *et al.*, 2006; Van Breusegem & Dat, 2006). This process will ultimately deprive the pathogen of water and nutrients and prevent its growth (Fu *et al.*, 2013; Glazebrook, 2005). Recent evidence also demonstrates that in the absence of HR, ETI can still arrest the pathogen through other cell-wall-breaking defenses (Johansson *et al.*, 2014). Effector triggered immunity against biotrophic pathogens commonly triggers the synthesis of salicylic acid (SA), a phytohormone with a systemic effect in distal parts of the plant. This systemic response commonly upregulates defenses, or allows them to be triggered more quickly (i.e., priming), allowing the whole plant to become resistant to pathogen infection; this process is known as systemic acquired resistance (Pieterse *et al.*, 2012).

With few exceptions, ETI has not been demonstrated in plant responses to necrotrophic pathogens. As necrotrophic pathogens sustain themselves on dead tissue, programmed cell death associated with ETI would benefit them, and be a poor plant defense (Mengiste, 2012; Oliver & Solomon, 2010; Spoel *et al.*, 2007). Necrotrophs can in fact trigger ROS associated with HR to their benefit. This shows that although manipulation of plant defense is essential for biotrophic and necrotrophic pathogens, the mechanisms they use are different. Necrotrophic pathogens actively destroy host tissue throughout the infection with various toxins and cell-wall degrading enzymes (Laluk & Mengiste, 2010; Veronese *et al.*, 2006). Biotrophs, however, manipulate plant defenses, thus maintaining the living cells required for their development (Laluk *et al.*, 2010; Mengiste, 2012; Veronese *et al.*, 2006). Hemibiotrophic pathogens like *Pseudomonas syringae* or *Phytophthora infestans* (potato late blight) have both a biotrophic and necrotrophic phase. This two-phase trophic strategy is reflected in their interactions with plant defenses. In resistant potatoes, the early biotrophic phase of the pathogen is recognized by the plant with effector-induced HR leading to plant resistance. This cuts off resources to the pathogen before it can spread or switch to the necrotrophic phase (Vleeshouwers *et al.*, 2011). In susceptible plants, *P. infestans* can evade the HR triggered by the plant with its own effector protein that down-regulates ligases, enzymes that join large cell-wall molecules. With the pathogen's switch to necrotrophy, the effector protein accumulation is reduced and HR-related proteins increase, leading to the observed necrotic lesions of the necrotrophic phase of *P. infestans* (Bos *et al.*, 2010).

Regardless of the pathogen lifestyle, plant resistance can be triggered by another species-specific route, which does not necessarily involve gene-for-gene recognition, as with ETI. Plants may recognize the pathogen through molecular signals, known as microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs), leading to pattern-triggered immunity (PTI) (Glazebrook, 2005; Lai & Mengiste, 2013; Mengiste, 2012). These patterns emerge from highly evolutionary conserved areas in the pathogen's molecular patterns (Huffaker *et al.*, 2013; Laluk *et al.*, 2010). Pattern

recognition activates specific signaling cascades tightly shaped by plant pathogen co-evolutionary history, which can in some cases also contribute to systemic acquired resistance (Mishina & Zeier, 2007).

The Importance of Pathogen Trophic Strategy for Plant-Mediated Effects on Insect Herbivores

To predict outcomes of plant-mediated interactions between pathogens and insect herbivores, it is especially important to consider the trophic strategy of the pathogen. Since biotrophs and necrotrophs induce plant responses in very different ways (Pieterse *et al.*, 2012; Spoel *et al.*, 2007), their effects on plants will influence the phytochemical environment the insect attacker will encounter. In the case of hemibiotrophs, considering in which trophic phase of the pathogen the interaction is taking place is of importance, since the phase switch also can affect the plant's hormonal profile. Conceivably, a susceptible plant will be sensitive to pathogens manipulating its defensive responses. These plants will have an altered defense system relative to a resistant plant, and thus certain immunity-related hormones and their downstream products may be under expressed. A resistant plant rather, may have a higher concentration of immunity-related hormones in its tissues. Since plants have limited resources to mount defensive responses, and different hormonal routes may antagonize each other, this can have varying effects on insect attackers (Table 1). Plant mediated indirect effects of pathogens on herbivores also will vary based on the feeding mode of the insect (See Figure 1). It previously was believed that phloem feeders were more affected by SA-mediated responses (Goggin, 2007; Li *et al.*, 2006); thus, biotrophic pathogens are likely to negatively impact them, whereas the opposite might be expected for chewing insects as the result of the SA-JA pathway antagonism (Thaler *et al.*, 2012). Recent evidence from studies on plant-aphid (Hogenhout & Bos, 2011), and plant-pathogen compatibility (Gururani *et al.*, 2012), suggests that the highly specific R gene mediated resistance may be more relevant in predicting effects on phloem feeders than phytohormones.

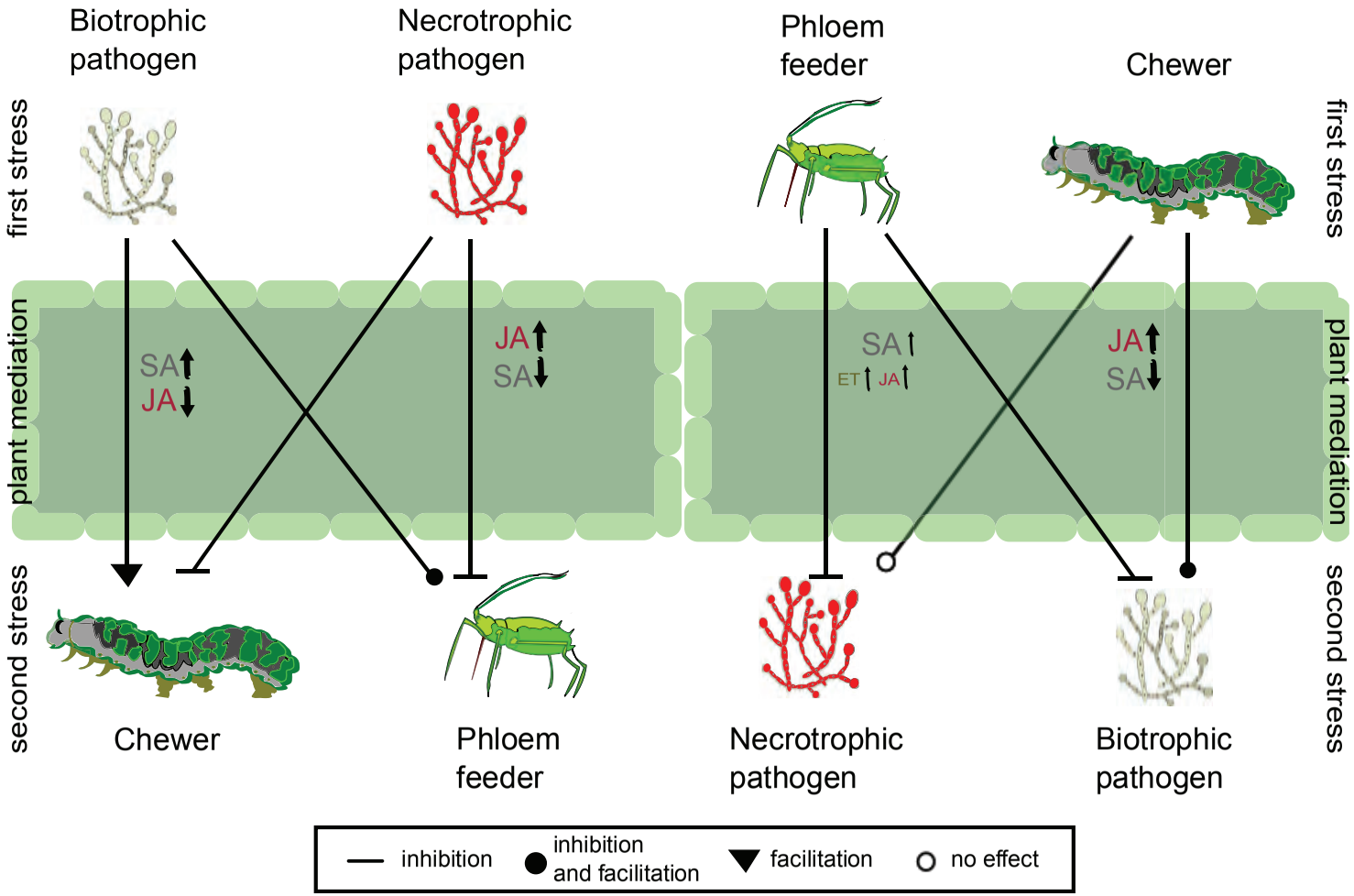

Figure 1. Overview of plant-mediated effects of pathogens on insects and of insects on pathogens of different trophic strategies or feeding modes; including hypothetical phytohormone-mediated mechanisms. Arrow endings represent findings from references discussed in this article, though mechanisms are not necessarily addressed in each reference. Acronyms shown as follows: SA=Salicylic acid; JA=Jasmonic acid; ET=Ethylene.

Table 1. Pathogen first. Overview of the effects of pathogens of different trophic strategies on insects of different feeding guilds, showing known mechanisms causing these effects.

First stress: pathogen	Trophic strategy	Second stress: insect	Feeding guild	Plant	Effect on Insect	Mechanism	Citation
Pseudomonas syringae	hemibiotroph	*Trichoplusia ni*	chewing	*Arabidopsis thaliana*	facilitation	Anti-herbivore defenses are suppressed by SA and ethylene, which can be (partially) countered by COR and ETI	Cui 2002; Cui 2005; Groen, 2013
Erwinia tracheiphila	hemibiotroph	*Acalymma vittatum*	chewing	*Cucurbita pepo*	facilitation	Increased volatile emissions and decreased defensive toxins in leaves from wilted tissues	Shapiro *et al.*, 2013
Puccinia punctiformis	biotroph	*Apion onopordi*	chewing	*Cirsium arvense*	facilitation	?	Bacher *et al.*, 2002
Pseudomonas syringae	hemibiotroph	*Scaptomyza nigrita*	chewing	*Cardamine cordifolia*	facilitation	Not directly tested but SA induction implied	Humphrey *et al.*, 2014
Uromyces viciae-fabae	biotroph	*Aphis fabae*	phloem feeding	*Vicia faba*	facilitation	increased nitrogen content, phytohormones not tested	Al-Naemi and Hatcher, 2013
Marssonina betulae	biotroph	*Euceraphis betulae*	phloem feeding	*Betula pendula*	facilitation	leaves contained higher concentrations of free-amino acids	Johnson *et al.*, 2003
Xanthomonas axonopodis	biotroph	*Myzus persicae*	phloem feeding	*Capsicum annum*	inhibition	?	Lee *et al.*, 2012
Alternaria brassicae	necrotroph	*Phaedon cochleariae*	chewing	*Brassica rapa*	inhibition	?	Rostas *et al.*, 2002
Botrytis cinerea	necrotroph	*Aphis fabae*	phloem feeding	*Vicia faba*	inhibition	decreased nitrogen content, phytohormones not tested	Al-Naemi and Hatcher, 2013
Botrytis cinerea	necrotroph	*Rhodobium porosum*	phloem feeding	*Rosa hybrida*	inhibition	?	Mouttet *et al.*, 2011

Effects of Pathogens on Chewing Insects

Although little information on the effect of necrotrophic pathogens on chewing insects is available, it might be expected that necrotrophic pathogens like *Botrytis cinerea* affect insects at the local level only. This a priori expectation is based on the fact that these pathogens do not commonly cause systemic acquired resistance (Govrin & Levine, 2000). This effect was reported by Rostás and Hilker (2002) who found that the necrotroph *Alternaria brassicae* deterred the leaf beetle *Phaedon cochleariae* at the local level. Although the mechanisms were not fully elucidated, the authors attributed such local effects to toxins released by the fungus. Conversely, we have found five examples of biotrophic or hemibiotrophic pathogens that cause systemic induced susceptibility (SIS) leading to facilitation of chewing insects (Table 1). For example, the stemboring weevil *Apion onopordi* exhibited better survival on *Cirsium arvense* plants affected by the biotrophic rust fungus *Puccinia punctiformis* (Bacher *et al.*, 2002), and *Pseudomonas syringae* infection increased herbivory by the fly *Scaptomyza nigrita* on bittercress (Humphrey *et al.*, 2014). The finding of SIS to insects after pathogen attack also has been observed in several other biological systems (Rostás *et al.*, 2003), though the biochemical mechanisms were not studied in all cases (see Table 1 for recent examples and Rostás et al. 2003 for an extensive list of examples).

One important model species for testing plant responses to pathogenic bacterial attacks is the hemibiotroph *P. syringae*. Plant defense to this pathogen is mediated mainly by the SA- signaling pathway, although other pathways like JA and ethylene also are involved (Fu *et al.*, 2013; Groen *et al.*, 2013; Moran & Thompson, 2001). Early work showed that pathogen attack mainly induced SA in *Arabidopsis thaliana*, which resulted in SIS to chewing insects(Cui *et al.*, 2002). Mutants with elevated levels of SA were more susceptible to the cabbage looper, *Trichoplusia ni* after pathogen attack (Cui *et al.*, 2002). However, plants with higher constitutive levels of SA and known R gene mediated resistance showed resistance to *T. ni* after pathogen treatment (Cui *et al.*, 2005; Cui *et al.*, 2002). Furthermore, plants with and without functional genes involved in SA-signaling were both more susceptible to herbivory by *T. ni* after initial infection by *P. syringae*. This suggests that the SA pathway is not the only factor mediating SIS to the insect. In this system, plant responses against the pathogen importantly are mediated by ethylene signaling. Recent research with *Arabidopsis* mutants has revealed that plants with disrupted ethylene signaling, are more resistant to herbivory (Groen *et al.*, 2013). Interestingly, ethylene also is required in this system to mediate interactions between chewing insects and the SA-inducing phloem feeder *Bemisia tabaci* (Zhang *et al.*, 2013). Since ethylene often acts in concert with JA (Pieterse *et al.*, 2009; Thaler *et al.*, 2012; Xu *et al.*, 1994; Zhang *et al.*, 2013), it is important to take this latter pathway into account. Plant hormones interact through complex pathways, but JA is triggered chiefly in plants attacked by necrotrophic

pathogens, chewing herbivores, and certain phloem feeders. Based on the JA/SA-antagonism (Thaler *et al.*, 2012), , Groen *et al.* (2013) hypothesized that pathogens that can trigger ETI through bacterial effectors, also can induce SIS to herbivory. However, these authors found that with ETI, the ethylene signalling pathway is bypassed, which triggers a cascade independent of the main JA-SA pathways, cancels SIS, and induces resistance to chewing herbivores. A similar situation might apply when plants recognize pathogens through molecular patterns (PTI). In susceptible *Arabidopsis* plants, for instance, SA is up-regulated after the release of bacterial coronatine (COR), which antagonizes the JA response pathway while at the same time up-regulates ethylene biosynthesis (Brooks *et al.*, 2004; Groen *et al.*, 2013). This process antagonizes the JA response but via a separate pathway than through JA-SA crosstalk. In both cases, JA downregulation increases susceptibility to chewing herbivores. If plants are resistant to the pathogen, however, SA is suppressed, and the pathogen-released COR disrupts ethylene signaling, consequently inducing resistance to the herbivore (Groen *et al.*, 2013). These examples are a clear demonstration that plant defenses are triggered in a multi- layered process. This may reflect a plant's trade-offs in the context of a complex community of attackers.

Effects of Pathogens on Phloem Feeding Insects

Based on five published studies, we did not find any clear effect of pathogen trophic strategy on subsequent performance of phloem feeders (Table 1). In three studies with biotrophic pathogens, inhibition and facilitation of aphids were demonstrated (Al-Naemi & Hatcher, 2013; Johnson *et al.*, 2003; Lee *et al.*, 2012). These studies used different pathogens and aphids, hampering generalizations about the effect of biotrophs on subsequent attack by phloem feeders. In one of the aforementioned studies, however, biotrophic and necrotrophic pathogens induced opposing effects on aphid development, survival, and fecundity (Al-Naemi *et al.*, 2013). The biotrophic rust fungus *Uromyces viciae-fabae* enhanced the performance of the aphid *Aphis fabae*, whereas the necrotrophic fungus *B. cinerea* attenuated it. A decrease of aphid fecundity after *Botrytis* infection also has been reported for the aphid *Rhodobium porosum* (Mouttet *et al.*, 2011). These results might seem counterintuitive because biotrophs and aphids usually trigger the SA defense pathway (Goggin, 2007; Guerrieri & Digilio, 2008; Li *et al.*, 2006; Moran *et al.*, 2001). In a similar way, the necrotroph is expected to stimulate the JA pathway at the cost of reduced SA expression, which would in turn benefit the aphid- which is opposite of the finding in the aforementioned paper. In certain cases, the observed aphid fitness did match our a priori expectations. For example, in comparison to uninfested leaves, the aphid *Euceraphis betulae* grew larger, developed faster, and preferred the leaves of the silver birch *Betula pendula* previously infested with the pathogen *Marssonina betulae* (Johnson *et al.*, 2003). These contrasting results could be explained by assuming a role for other plant defensive mechanisms. This downplays the relevance of phytohormone-

mediated mechanisms, or suggests still unknown intricate interactions among phytohormones. The aforementioned results, for instance, were attributed to an increase in phenolic compounds and free amino acids in the infected tissues that occurs downstream of phytohormonal signaling (Johnson *et al.*, 2003). Since aphids are known to be sensitive to changes in nitrogen levels in plants, opposing results also could be explained by a relative increase of nitrogen level when leaves are infected with the biotrophic rust fungus, whereas the opposite might happen as a result of *Botrytis* infection (Al-Naemi *et al.*, 2013). It is clear that different pathogens, which use the same trophic strategy, have different effects on aphids (Table 1).

Since aphids are able to induce both the JA/ethylene and SA pathways(Thaler *et al.*, 2010; Thaler *et al.*, 2012; Thompson & Goggin, 2006), responses may be variable, or not solely determined by the crosstalk in these pathways. Furthermore, phytohormonal interactions are not limited to crosstalk between JA and SA. Aphids also are able to induce ethylene (Thaler *et al.*, 2010; Thompson *et al.*, 2006) or to manipulate cytokinin levels and, therefore, source-sink nutrient flows in plants, ultimately allowing plant colonization (Giron *et al.*, 2013; Mok & Mok, 2001; Sakakibara, 2006). Although detailed information is available on how different plant hormones interact after pathogen infestation (Spoel *et al.*, 2007), their consequences for the community of insects sharing the plant are still little understood.

Thus far, few studies have investigated sequential plant-fungus (or plant-oomycete)-insect interactions. In many cases infections by fungi trigger defense mechanisms similar to bacterial pathogens (Jiang & Tyler, 2012; Latijnhouwers *et al.*, 2003), yet more examples of sequential tri-partite interactions with fungi or oomycetes are needed to better understand the role of plant hormones in mediating indirect interactions with herbivores. Jasmonic acid-deficient tomato plants were more susceptible to the oomycete pathogen *P. infestans*, demonstrated by larger lesions and more spores (Thaler *et al.*, 2004). This suggests that an infection that downregulates JA (from biotrophy, for example) might also induce susceptibility to chewing insects, whereas JA-inducing fungi might induce resistance. The aforementioned study, however, reported that plants may have variable responses to JA-deficiency, which do not necessarily correspond with our predictions based on trophic strategy of the pathogen. That being established, Thaler et al. (2004) concluded that biotrophy and necrotrophy are better viewed as a continuum rather than a dichotomy.

Proposed Hypotheses for Plant-Mediated Effects of Pathogens on Insect Herbivores

The examples reported reveal that although general predictions can be made, the complexity of plant phytohormonal responses hampers our understanding of how plants mediate interactions between insects and pathogens with different trophic strategies. Based on the recent literature (Table 1), we propose the following hypotheses:

1. Biotrophic pathogens facilitate chewing herbivores (through SIS) unless plants exhibit effector-triggered immunity to the pathogen. Biotrophic pathogens can have a facilitating effect on chewing herbivores by upregulating SA- and downregulating JA-mediated defenses.

2. Biotrophic pathogens facilitate or inhibit phloem feeders. The mechanisms are not yet clear but they may be mediated by the plants' recognition of the pathogen or insect attackers, in particular whether ETI or PTI is triggered.

3. Necrotrophic pathogens can inhibit both phloem feeders and chewers through mechanisms that are not yet clear (Mouttet *et al.*, 2011; Rostás *et al.*, 2003). More evidence for the plant-mediated effects of necrotrophic pathogens on insects of the two feeding modes is particularly needed to formulate a more explicit hypothesis.

Plant-Insect Interactions

Plant Responses to Herbivory

The ETI-paradigm which often is associated with pathogen attack on plants also is becoming relevant to plant-herbivore systems, especially with regard to piercing-sucking insects like aphids and whiteflies (Cooper *et al.*, 2004; Erb *et al.*, 2012; Hogenhout *et al.*, 2011). Although still limited to a few model systems, R genes have been discovered that confer resistance against particular clones of the Hessian fly (Grover *et al.*, 1989), aphids, whiteflies, psyllids, and nematodes (Hogenhout *et al.*, 2011). There still is limited information about plant responses after R gene mediated resistance, although more is known about how insect feeding mode affects phytohormone signaling pathways. Several studies have concluded that phloem feeders and biotrophic pathogens can trigger SA-mediated defenses (De Vos *et al.*, 2005; Moran *et al.*, 2001; Walling, 2000), whereas herbivory by chewers and cell content feeders are thought chiefly to induce JA (Bari *et al.*, 2009; De Vos *et al.*, 2005; Howe *et al.*, 2008; Mouttet *et al.*, 2013; Schmelz *et al.*, 2009). Although exceptions to these patterns have been found, we now have strong empirical evidence that hormonal regulation is at least partially predicted by insect feeding mode. Specificity

of the insect-plant interaction also is considered a predictive factor, since specialists are expected to be more resistant to defensive phytochemicals of the specific plant taxon they exploit (Hopkins *et al.*, 2009). However, recent reviews of this paradigm have demonstrated that even specialists suffer from high levels of plant toxins, and that feeding mode has more predictive value for which plant defenses are induced (Ali *et al.*, 2012; Bidart-Bouzat *et al.*, 2011). These responses, however, are dependent on many other variables, including the plant or insect developmental stage (Goggin, 2007; Mouttet *et al.*, 2013; Walling, 2000). For example, one day after oviposition by the leafminer fly Liriomyza sativae, JA-inducible genes were upregulated, whereas after hatching, larval feeding induced SA-regulated genes in tomato plants (Kawazu *et al.*, 2012).

The Importance of Insect Feeding Mode for Plant-Mediated Effects on Pathogens

Based on what we currently know of the effects on insect herbivores using different feeding modes and at certain densities, formulating hypotheses on tripartite interactions between plants, microbial pathogens and insects is difficult, considering the idiosyncrasies of each study system. It is expected that phloem feeders, through induction of the SA pathway, will facilitate colonization by biotrophic pathogens, while inducing resistance against necrotrophs. Thaler et al. (2010) tested this hypothesis in a study of the interactions among *P. syringae*, tomato mosaic virus, caterpillars, and aphids. Caterpillar feeding negatively affected both aphids and the biotrophic pathogen through JA induction; aphid feeding triggered both JA and SA induction, which benefitted caterpillars yet hindered *P. syringae* infection. The predictions and hypotheses tested in this study were limited to two pathogen species, whereas important taxa like fungi and oomycetes were not considered.

We found thirteen examples from ten different references, investigating effects of prior insect feeding on pathogen infection (Table 2). From these, the conclusion that effects on pathogens depend entirely on the insect feeding mode is not warranted.

Effects of Phloem Feeding Insects on Pathogen Infection

Phloem feeders induce resistance in plants against hemibiotrophic or biotrophic bacterial and fungal pathogens. For example, feeding by the green peach aphid *Myzus persicae* on pepper plants reduced infection by the biotrophic bacterium *Xanthomonas axonopodis* (Lee *et al.*, 2012). This was attributed to the aphids triggering the SA-mediated pathway in the same way that biotrophic pathogens do. This effect also was found for another phloem feeder, the silverleaf whitefly, *Bemisia argentifolii*, which reduced the incidence of powdery mildew *Erysiphe cichoracearum* (Mayer *et al.*, 2002). Rice was less likely to contract the rice blast disease *Pyricularia*

grisea, if previously exposed to the phloem-feeding white-backed leafhopper *Sogatella furcifera* (Kanno & Fujita, 2003; Kanno *et al.*, 2005). Another rice pathogen showed a similar trend, as pre-infestation with *S. furcifera* reduced infestation by the rice blast fungus, *Magnaporthe grisea* (Satoh *et al.*, 2009). This evidence suggests that insects with phloem feeding habits will upregulate SA, and through crosstalk will downregulate JA-mediated plant defenses, leading to the inhibition of biotrophic pathogens.

The opposite might be expected in the case of necrotrophic fungi, as shown by Mouttet et al. (2011) who found that pre-infestation with *Rhodobium porosum* aphids on rose plants inhibited *Botrytis cinerea*. In the same study, pre-infestation by cell-content feeding thrips did not reduce the size of lesions caused by *B. cinerea*. Although no specific plant metabolites were measured, this study suggests that piercing-sucking aphids elicit signaling pathways different from cell-content feeding thrips. While thrips and chewing herbivores induce similarly elevated JA levels in *Arabidopsis*, they differ in the particular downstream responsive genes they induce (De Vos *et al.*, 2005). In the study by de Vos et al. (2005), it was concluded that although the phytohormones JA, SA, and ethylene were all important for the defense responses of Arabidopsis against biotrophs, necrotrophs, phloem feeders, cell-content feeders, and chewers, each attacker induced a unique phytohormonal signature and consequently a particular set of genes.

Effects of Chewing Insects on Pathogen Infection

Chewing herbivores can either inhibit or facilitate biotrophic or hemibiotrophic pathogens. In only four studies necrotroph infections were made after insect feeding, and in three of these, no effect on the pathogen was found. Having few examples available makes it difficult to develop a generalized hypothesis about the outcome of a necrotrophic infection following insect feeding. An emerging pattern may be that cell content feeders or chewers (usually associated with JA induction) may not have any effect on necrotrophic pathogens, whereas phloem feeders may inhibit them. Phytochemical mechanisms have not yet been studied, and phytohormone signaling is addressed in only three out of the thirteen examples cited.

De Vos et al. (2006) demonstrated that *Arabidopsis* mutants deficient in either JA, SA or ethylene signaling could still inhibit the growth of the hemibiotroph *P. syringae*, after previous induction by the chewing herbivore *Pieris rapae*. This indicates that caterpillar-induced resistance to *P. syringae* does not depend exclusively on any of these phytohormones alone. Interestingly, *P. rapae* feeding primes *A. thaliana* plants for SA-mediated defense against turnip crinkle virus, and ethylene that was induced by caterpillar feeding acted synergistically on this plant response (De Vos *et al.*, 2006). Induction by chewing herbivores also can systemically increase the growth (surface area of sporangia) of the biotrophic rust fungus *Melampsora allii-fragilis* (Simon & Hilker, 2003, 2005).

Table 2. Insect first. Overview of the effects of insects of differentfeeding guilds on pathogens of different trophic strategies, showing known mechanisms causing these effects.

First stress: insect	Feeding guild	Second stress: pathogen	Trophic strategy	Plant	Effect on pathogen	Mechanism	Citation
Scaptomyza nigrita	chewing	*Pseudomonas syringae*	hemibiotroph	*Cardamine cordifolia*	facilitation	Not directly tested but SA induction implied	Humphrey *et al.*, 2014
Plagiodera versicolora	chewing	*Melampsora allii-fragilis*	biotroph	*Salix cuspidata* (a hybrid of *S. fragilis and S. pentandra*)	facilitation (systemically)	?	Simon and Hilker, 2003
Aphid, *Aphis gossypii*	phloem feeding, piercing sucking	*Colletottichum orbiculare*	hemibiotroph	Watermelon, *Citrullus lunatus*	inhibition	?	Russo *et al.*, 1997
Thrips, *Thrips tabaci*	cell content feeding	*Colletottichum orbiculare*	hemibiotroph	Watermelon, *Citrullus lunatus*	inhibition	?	Russo *et al.*, 1997
Frankliniella occidentalis	cell content feeding	*Botrytis cinerea*	necrotroph	*Rosa hybrida*	no effect	?	Mouttet *et al.*, 2011
Phaedon cochleariae	chewing	*Alternaria brassicae*	necrotroph	*Brassica rapa*	no effect	?	Rostas *et al.*, 2003
Pieris rapae	chewing	*Alternaria brassicicola*	necrotroph	*Arabidopsis thaliana*	no effect	No specific dependence on JA; it was induced, though no inhibition observed	De Vos *et al.*, 2006
Pieris rapae	chewing	*Xanthomonas campestris*	hemibiotroph	*Arabidopsis thaliana*	inhibition	No specific dependence on JA, SA or Ethylene as deficient mutants still exhibited resistance to pathogen	De Vos *et al.*, 2006
Pieris rapae	chewing	*Pseudomonas syringae*	hemibiotroph	*Arabidopsis thaliana*	inhibition	No dependence on JA, SA or Ethylene as deficient mutants still exhibited resistance to pathogen	De Vos *et al.*, 2006
Myzus persicae	phloem feeding	*Xanthomonas axonopodis*	biotroph	*Capsicum annum*	inhibition	SA induction, attracted beneficial bacteria *Bacillus subtilis* to roots	Lee *et al.*, 2012
Bemisia tabaci	phloem feeding	*Xanthomonas axonopodis*	biotroph	*Capsicum annum*	inhibition	SA induction, attracted beneficial bacteria to roots	Yang *et al.*, 2011
Sogatella furcifera	phloem feeding	*Pylicuralia grisea*	hemibiotroph	*Oryza sativa*	inhibition	?	Kanno and Fujita, 2003
Sogatella furcifera	phloem feeding	*Magnaporthe grisea*	hemibiotroph	*Oryza sativa*	inhibition	PR-protein expression, likely SA induction	Kanno *et al.*, 2005; Satoh *et al.*, 2009
Bemisia argentifolii	phloem feeding	*Erysiphe cichoracearum*	biotroph	*Solanum lycopersicum*	inhibition	? Possibly foliar chitinases, peroxidase	Mayer *et al.*, 2002
Rhodobium porosum	phloem feeding	*Botrytis cinerea*	necrotroph	*Rosa hybrida*	inhibition	?	Mouttet *et al.*, 2011

Proposed Hypotheses for Herbivore Effects on Pathogens

To put forward hypotheses about plant-pathogen-insect interactions, more cases are needed in which the effect of different insect feeding modes on pathogens is evaluated in concert with quantification of different phytohormones and the transcription of phytohormone-regulated genes. Additionally, more studies on the effects of chewing herbivores on subsequent interactions with pathogens (particularly fungi) and the mechanisms determining the outcomes of these interactions are needed to provide a stronger basis for proposing testable hypotheses.

Even though few and conflicting findings are published we can hypothesize that:

1. Phloem feeding herbivores inhibit pathogen attack by SA induction; but evidence for negative effects on necrotrophs is still currently scant.

2. Chewing herbivores tend not to affect necrotrophic pathogens and may either inhibit or facilitate biotrophic pathogens.

Future Perspectives

The molecular revolution has allowed ecologists to get a deeper understanding of the dialogue between plants and their associated organisms. During the last few decades, this knowledge has revealed strategies of plants to fine-tune their responses against different attackers, while balancing growth in the face of abiotic stressors (Harrison, 2012; Jones & Dangl, 2006; Pieterse *et al.*, 2009; Pieterse *et al.*, 2012). We now know that this dialog is not limited to pairwise interactions between plants with either insects or pathogens (Biere & Bennett, 2013). A broader approach that takes into account how plants deal with the whole community of organisms colonizing them will provide new breakthroughs in our understanding of plant- based ecosystems.

Although many studies have explored plant-mediated interactions between insect herbivores and plant pathogens (Biere *et al.*, 2013; Pieterse *et al.*, 2007; Ponzio *et al.*, 2013; Stout *et al.*, 2006) the mechanisms, and in particular the roles that phytohormones play in such indirect interactions are still poorly understood. Many studies lack concrete evidence of induced pathways, or they attribute the effects to phytohormonal signalling without having directly measured hormone levels. Knowledge on pairwise interactions can help make general predictions, but available evidence suggests that tripartite interactions are much more complex than we previously thought. The few examples for which phytohormones are measured reveal that the general predictions do not always hold. The best-studied phytohormones act in concert with others, and downstream metabolic pathways also are likely to have a strong impact on the resulting plant phenotype for the insect

or the pathogen of interest. It now has become feasible, thanks to the genomic revolution, to make global transcriptomic analyses, which provide insight into plant physiological responses to different stresses in non-model plants.

Insect herbivores and plant pathogens have a long evolutionary history in common, and coevolutionary forces have shaped their effects on plant phenotype and morphology. In this review, we focussed on how these different attackers introduced sequentially can interact through the plant, although recent evidence shows that insect herbivores and plant pathogens that are found simultaneously on the plant also can establish intimate mutualistic symbiosis mediated by plant physiology (Frago *et al.*, 2012). As reviewed by Casteel and Hansen (2014), insects can exploit bacteria to elicit plant responses that will ultimately benefit the insect, and the same is known for bark beetle-fungi-conifer interactions (Paine *et al.*, 1997). Insects that vector plant pathogens (including viruses, phytoplasmas and fungi) have established mutualistic symbioses that modify plant physiology to the benefit of the insect vector (Casteel *et al.*, 2014; Sugio *et al.*, 2011; Zhang *et al.*, 2012; Ziebell *et al.*, 2011). Although there is ample evidence of herbivores vectoring plant pathogens (Hogenhout *et al.*, 2008; Paine *et al.*, 1997), this type of interaction has not been discussed in detail in this review, as we focused on sequential infestations. Insect vectors, however, also may be influenced by prior infection of plants by pathogens they can transmit. For example, in the wild gourd (*Cucurbita pepo* ssp. *texana*), previous infection by the zucchini yellow mosaic virus induced high levels of SA, which slowed down the spread of the beetle-vectored bacterial wilt *Erwinia racheiphila* (Shapiro *et al.*, 2013). Although this may be selected through a mutualistic interaction between the vector and the bacterium, it also reveals the intricate interactions between hormones underlying interactions among plant-associated community members. How plant pathogens have colonized insects and evolved with them into mutualistic symbionts is an intriguing question that will spark future studies. Insect herbivores are attacked by a diverse community of natural enemies; after herbivore attack, for example, plants can emit volatiles that attract natural enemies of the insect (i.e., indirect defenses) (Turlings *et al.*, 1995; Vet & Dicke, 1992). However, how plant pathogens modulate these interactions is still poorly understood (Ponzio *et al.*, 2013; Ponzio *et al.*, 2014). Understanding how plants can modulate the balance in their secondary metabolite-based responses against pathogens and insect herbivores, and between direct and indirect defenses against herbivores will surely provide a new view on how efficient plant responses operate in multitrophic systems. Ultimately, such studies will require data from field studies to take into account not only different trophic levels, but also highly diverse communities.

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Chapter 3

Genomic position of a resistance gene in potato affects gene-expression and performance of two non-target insects

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Abstract

Breeding for resistance or introgression of resistance genes through genetic modification are considered sustainable solutions to the biggest potato threat: late potato blight *Phytophthora infestans*. In this study, the main research question was whether the *Rpi-vnt1* gene conferring resistance to *P. infestans* in potato plants can influence interactions between the plant and two important non-target potato herbivores: the peach-potato aphid *Myzus persicae* and the Colorado potato beetle *Leptinotarsa decemlineata*; both as single stressors and in combination. We also measured plant responses to these two non-target herbivores by quantifying transcription of the plant-defence-related genes *PR1* and *LOX*. We found that insertion position of the R gene influenced non-target insect performance measured in terms of *L. decemlineata* biomass gain and *M. persicae* offspring numbers. The genomic position and the insect infestation treatment also affected *PR1* and *LOX* gene expression. This study demonstrates that different gene insertion events may differ in their effects on non-target herbivores. Practically, the findings suggest that pre-screening GM events for possible non-target effects on co-occuring species may be relevant before proceeding with further risk assessment trials.

Key Words

Late blight resistance, *Solanum tuberosum*, *Myzus persicae*, *Leptinotarsa decemlineata*, sequential herbivory, non-target insect, genetic modification, genotypic location.

Introduction

Protecting crops against pests and pathogens is not an easy task. Plant feeders are most often present simultaneously on crop plants, which can result in significant losses. Virus transmission by aphid pests such as the peach-potato aphid *Myzus persicae* (Sulzer) and the defoliation caused by the Colorado potato beetle (*Leptinotarsa decemlineata* Say) are an important concern for potato production (Alyokhin, 2009; Radcliffe, 1982). Yet, for potato plants the biggest threat is the late blight pathogen, the "plant destroyer" *Phytophthora infestans* (Mont.) de Bary, which causes losses of billions of dollars worldwide (Fry, 2008). The use of fungicides is basically necessary in damp and cool climates for growing susceptible potatoes. Furthermore, the necessary and often numerous chemical applications for control of *P. infestans* have a huge environmental impact (Haverkort *et al.*, 2009). Breeding for resistance or introgression of resistance genes through genetic modification are considered more sustainable solutions to this problem (Mundt, 2014).

Resistance genes can occur naturally in wild relatives of crops, or can be bred into crops from natural populations for resistance against pests and pathogens. Several resistance genes (R genes) from wild relatives are known to confer resistance to late blight (*P. infestans*) in potato (Mattheij *et al.*, 1992; Smyda *et al.*, 2013; Van Der Vossen *et al.*, 2003). These R genes code for plant proteins which recognize corresponding pathogen effector proteins. This is known as a gene-for-gene interaction, and initiates signal transduction leading to callose deposits, and cell death around the infection site called hypersensitivity response (HR). This prevents the pathogen from spreading through the plant tissue (Kamoun *et al.*, 1999; Vleeshouwers *et al.*, 2011; Vleeshouwers *et al.*, 2000).

A recent study assessed the effects of one major resistance gene (*Rpi-blb1*) in potato against *P. infestans* on a generalist non-target herbivore *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) (Abreha *et al.*, 2015). The performance of the herbivore was similar on the isogenic and transformed plants whether the plants were infested with *P. infestans* or not. However, when the plants were inoculated with *P. infestans*, the moth preferred to oviposit on the isogenic line rather than on the transformed line (Abreha *et al.*, 2015). Although this study highlights the importance of non-target testing under conditions of combined pathogen and insect infestation, *S. littoralis* is not known as a pest insect on potato plants. Furthermore, the performance assays in the study by Abreha *et al.* (2015) were performed on detached leaves, potentially affecting the plant's highly inter-connected defense response system. Although induced responses were suggested as a mechanism for their finding, no phytochemical or molecular experiments were conducted (Abreha *et al.*, 2015). Our recent research revealed unintended effects of certain *Rpi*-gene insertion events on the peach-potato aphid *M. persicae* compared to the un-transformed susceptible plant (Lazebnik *et al.*, 2017). The latter study was not aimed at investigating the

mechanisms, but to advise on risk assessment methodology. Based on our previous findings, we further investigate the role of *Rpi*-gene insertion position in intact potato plants on non-target insect response, this time with two economically relevant non-target insects, i.e. the previously mentioned phloem feeder *M. persicae* and the leaf chewing beetle *L. decemlineata.*

We designed the study bearing in mind the effects of different feeding modes of the non-target insects. Phytohormonal signal-transduction pathways regulate the physiological responses of plants to insects, pathogens and other environmental stimuli (Gimenez-Ibanez & Solano, 2013; Maffei *et al.*, 2007; Pieterse *et al.*, 2012; Stam *et al.*, 2014; Walling, 2009). Although several other factors play an important role, the general consensus remains that hormonal pathway regulation is driven in part by plant recognition of insect feeding mode or phytopathogenic trophic strategy (Lazebnik *et al.*, 2014). It is generally acknowledged that phloem feeders, biotrophic and hemibiotrophic pathogens induce salicylic acid (SA)-mediated responses (Glazebrook, 2005; Pieterse *et al.*, 2012), whereas herbivory by chewers and necrotrophic pathogens induce the jasmonic acid (JA) phytohormonal pathway (Bari & Jones, 2009; De Vos *et al.*, 2005; Howe & Jander, 2008; Kawazu *et al.*, 2012; Mewis *et al.*, 2006; Mouttet *et al.*, 2011; Schmelz *et al.*, 2009). For this reason, we quantified the expression of two key plant defense-related genes: one marker gene in the SA pathway (*PR1*), and one in the JA pathway (*LOX*); both important for plant responses to phloem feeding and leaf chewing herbivores. Under multiple stress conditions, the sequence of attackers can also play an important role for plant-mediated interactions (Stam *et al.*, 2014), and thus for the whole plant-associated community (Erb *et al.*, 2011; Poelman *et al.*, 2008; Soler *et al.*, 2012). Therefore, we performed experiments varying the sequence of arrival on the non-target insects on the plants.

The main research question in this study is whether the *Rpi-vnt1* gene conferring resistance to *P. infestans* in potato plants can influence interactions between the plant and two important non-target potato herbivores: *M. persicae* and *L. decemlineata,* both as single stressors and in combination. We studied two events of the genetic modification to test the hypothesis that genomic position of the R gene affects the plant-insect interaction. In other experiments conducted for this thesis, *M. persicae* differed in the rates of population increase on these two cisgenic events (Chapter 4) and parasitoids differed in parasitism rates of these aphids on these same two events (Chapter 5). Therefore, in this study, we targeted our efforts to understanding the changes in plant defense-related responses. These transcriptional responses were studied for different insect treatments on the plants, in an attempt to understand the mechanisms behind the effects on insect performance.

Materials and Methods

Plant Material

The cisgenic events (A15-31 and A15-45) investigated in this study were developed by the Laboratory of Plant Breeding of Wageningen University and Research (Haesaert *et al.*, 2015; Haverkort *et al.*, 2016). They were created using marker-free transformation methods with the *Rpi-vnt1* gene from *Solanum venturii* (Hawkes and Hjert.), a wild congener of *S. tuberosum* L. The events were selected as "true to type" as they were morphologically indistinguishable from non-transformed Désirée (Haverkort *et al.* 2016).

All genetically modified events and conventional cultivars were maintained *in vitro*, on agar medium (purified agar 0.8% + 2.2 g L^{-1} 0.5 Murashige-Skoog + vitamins; pH = 6) in sterile containers. Containers were kept in a climate room at 16:8 light:dark conditions, 21 ±2°C during light hours and 15 ±2°C when dark, and 60-70% relative humidity. Cuttings were transplanted to potting soil (Lentse potgrond, Lent, The Netherlands) five weeks before the experiments to allow for root growth. Plants of 5-6 weeks old since transplanting were used in the experiments.

Insects

Green peach-potato aphids, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) were collected in 2004 from fields around Wageningen, The Netherlands (51°59'11.5"N 5°39'48.4"E) and reared at the Laboratory of Entomology, Wageningen University. *Myzus persicae* were reared on Désirée plants at 24 ± 2°C, 60-70% RH with a photoperiod of 16:8 L:D. Aphid infestation was done by transferring twenty, second or third instar nymphs and distributing these over different leaves of each plant. Additionally, four seven-day-old *M. persicae* were placed in a clip cage on the same plant (placed at a middle-aged leaf of the plant) for offspring counting just before tissue collection (after four or five days, depending on the experiment). Tissue was collected from leaves with aphids present, flash frozen and stored in a -80 °C freezer until RNA extraction.

Adult Colorado potato beetles, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) were collected in August 2014 from Valthermond, The Netherlands (52°52'25.8"N, 6°56'36.9"E) and mated with a population collected from a field near Wageningen in July 2015. They were reared at the Laboratory of Entomology, Wageningen University on cv. Désirée under the same climate cell conditions as described for aphids. Eggs were kept separate in small containers and emerging larvae were fed on excised leaves until second instar. Plants were infested with four late-first instar *L. decemlineata* larvae. Larvae were weighed before being placed on plants and again just before leaf tissue collection, four or five days later, depending on the experiment. Tissue was collected from leaves with *L. decemlineata* damage, flash frozen and stored in a -80 °C freezer until RNA extraction.

Experimental design

1. *Leptinotarsa decemlineata* pre-treatment

Sixteen plants were used from each genotype: Désirée, A15-31 and A15-45. The following treatments were applied to four plants from each genotype:

a) Three weighed second instar *L. decemlineata* larvae were placed on each of three random potato leaves and one day later infested with *M. persicae* as described above (while leaving the *L. decemlineata* larvae on the plant), tissue samples and insect measurements (counting aphid offspring from clip cages and weighing beetle larvae) were collected four days later (after five days of *L. decemlineata* feeding, and four days of *M. persicae* feeding).
b) Three weighed second instar *L. decemlineata* larvae were placed on each of three random potato leaves and left without a second stress (leaving the *L. decemlineata* larvae on the plant), tissue samples and insect measurements were collected four days later (after five days of *L. decemlineata* feeding).
c) Left untreated for the one day and on the next day plants were infested with *M. persicae* only as described above in the insects section. Aphid offspring were counted from inside the clip cages four days later.
d) Left completely untreated (for use as gene-expression controls), and tissue was collected and frozen for RNA extraction after five days, together with the samples.

2. *Myzus persicae* pre-treatment

Sixteen plants were used from each genotype: Désirée, A15-31 and A15-45. The following treatments were applied to four plants from each genotype:

a) Four seven-day-old *M. persicae* aphids were contained in a clip cage for offspring counting and concurrently twenty aphid nymphs were randomly placed on potato leaves. One day later, the plants were infested with four second instar *L. decemlineata* larvae (while leaving the *M. persicae* on the plant), tissue samples and insect measurements were collected four days later (after five days of *M. persicae* feeding and four days of *L. decemlineata* feeding).
b) Four seven-day-old *M. persicae* aphids were contained in a clip cage for offspring counting and concurrently twenty aphid nymphs were randomly placed on potato leaves and left without a second stress (leaving the *M. persicae* on the plants), tissue samples and offspring were counted from clip cages four days later (after five days of *M. persicae* feeding).
c) Left untreated for one day and on the next day treated only with *L. decemlineata* larvae only as described in above the insects section. Larvae were weighed four days later.
d) Left completely untreated (for use as gene-expression controls), and tissue was collected and frozen for RNA extraction after five days, together with the other samples.

Gene Expression

Extraction of RNA was done using the 'Isolate RNA Plant Kit' (BIOLINE, London, UK). Concentration of purified RNA was tested by Nano-drop spectrophotometer (Nano Drop 2000, Thermo Scientific) and adjusted to 1μg/μL. The extracted RNA was treated with the iScript cDNA Synthesis kit (BIORAD®) for cDNA synthesis. Quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed in a CFX96 Touch™ Real-Time PCR Detection System (BIORAD). Each reaction contained 12.5 μl SYBR Green Supermix (BIORAD), 5 μl cDNA and 10 μM of a gene-specific primer pair (Table 1) in a total volume of 25 μl. For each reaction two technical replicates were performed and average Ct values were used in the analyses. The following PCR program was used for all PCR reactions except for the housekeeping gene *GAPDH*: 3 min 95°C, followed by 40 cycles of 15 s at 95 °C, and 45 s at 60 °C. For *GAPDH*, we used a 62 °C annealing temperature after optimization. A melting curve analysis was performed to check the consistency of each sample product at the end of the cycles for each sample.

Gene expression was calculated by using the geometric mean of threshold cycle (Ct) values (Vandesompele et al., 2002) for the housekeeping genes *GAPDH* and *EF1-α* as internal standards with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Primer sequences used in these reactions are given in Table 1. For analyses of gene expression in untreated control plants only, Désirée plants were used as the biological control, with expression therefore presented relative to the Désirée genotype, while for comparisons between treatments and genotypes, gene expression was in all cases presented relative to the untreated control of the same genotype.

Table 1. Primers used for expression analysis by Quantitative real-time PCR.

Name	Sequence (5'-3')	Pathway/ Type
*GAPDH*_fwd	GGACATTGTCTCCAACGC	Housekeeping
*GAPDH*_rev	ATGAGACCCTCCACAATG	
*EF1a*_fwd	ATTGGAAACGGATATGCTCCA	Housekeeping
*EF1a*_rev	TCCTTACCTGAACGCCTGTCA	
*LOX*_fwd	GAGTTCTCCTCATGGTGTTCGTTTA	Biosynthesis JA
*LOX*_rev	AGTAGTCTGACACCCAACTT	
*PR1*_fwd	GGTGCAGGAGAGAACCTT	Downstream SA
*PR1*_rev	GGTACCATAGTTGTAGTTTGGCT	

Statistical Analysis

Log-transformation was applied on gene-expression data before analysis. The residuals of each model were tested for normality using Shapiro-Wilk tests. For all the experiments, a linear model was used to analyze the response variables: biomass, offspring produced or relative gene expression; with the fixed factors of potato genotype, infestation treatment and their interaction. An ANOVA was used to analyze the effects of the main factors and their interaction on each response variable. If the factors significantly impacted the response variable, the data were subset by factor and main effects were compared within each factor (either genotype or treatment), using an ANOVA to compare levels within a particular factor. The statistical software R (© R Foundation for Statistical Computing, 2014) was used to analyze the data.

Results

Experiment 1: *Leptinotarsa decemlineata* pre-treatment

Biomass gain of *L. decemlineata* larvae

Genotype was a significant factor influencing *L. decemlineata* biomass gain (P = 0.0007) (Figure 1, Table 2A). Whether *L. decemlineata* larve were feeding alone or with the presence of *M. persicae* aphids, that were introduced one day later, did not influence the weight gain of the beetle larvae (P = 0.8768). There was no significant interaction between genotype and infestation treatment on *L. decemlineata* biomass (P = 0.0897). When feeding alone, beetle larvae gained less biomass on the cisgenic A15-31 and marginally less on A15-45 event compared to Désirée (A15-31: P = 0.0354; A15-45: P = 0.0542). Whereas beetles fed on plants and infested one day later with aphids, gained less biomass only on the A15-45 event compared to the isogenic Désirée (P = 0.0027) (Figure 1).

Offspring production by *M. persicae*

The total number of *M. persicae* offspring was affected by the genotype on which the aphids fed (P = 0.0153); and the infestation treatment applied (P = 0.0134) The interaction between the two factors was not significant (P = 0.0822) (Figure 2, Table 2B).

On plants only infested with *M. persicae*, aphids had produced more offspring after 4 days on Désirée plants than on A15-45 plants (P = 0.0315); yet they produced similar offspring numbers on Désirée plants as on A15-31 plants (P = 0.1619) (Figure 2). On plants treated first with *L. decemlineata* larvae and then *M. persicae*, more offspring were produced on A15-31 than on A15-45 plants (P = 0.0127) whereas the number of offspring was similar on A15-31 and Désirée plants (P = 0.0836).

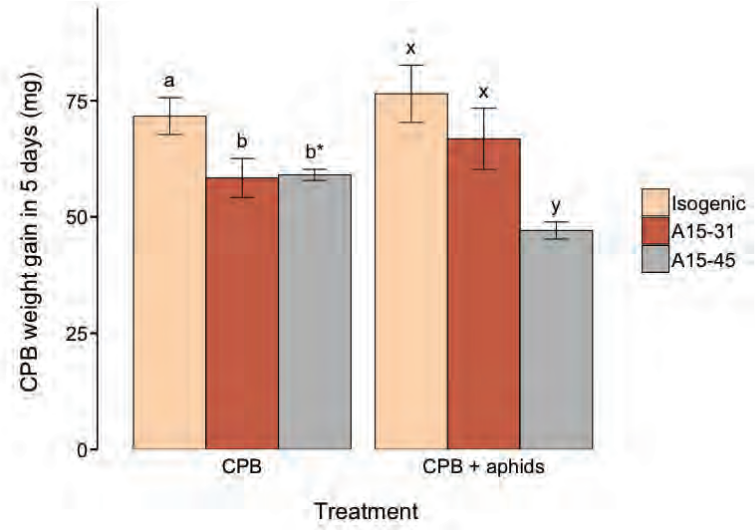

Figure 1: Mean biomass gain (± SE) of *L. decemlineata* larvae (CPB) over a 5-day period on three different potato genotypes: the isogenic cultivar (Désirée), susceptible to *P. infestans,* and two cisgenic *P. infestans*-resistant genotypes A15-13 and A15-45 using two different plant infestation treatments: only CPB or CPB followed by aphids (*M. persicae*). N = 4 plants for each genotype/treatment group. Different letters indicate differences between genotype means ($P < 0.05$) within a treatment type. * indicates $P = 0.0542$ within the CPB treatment group with respect to the isogenic genotype.

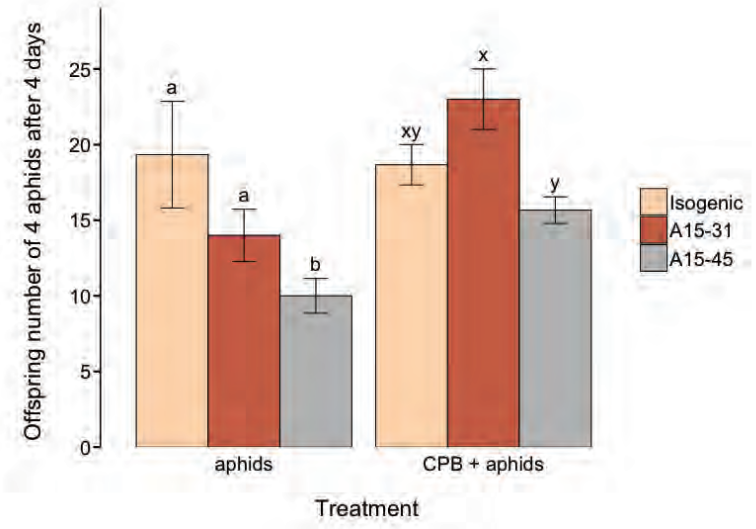

Figure 2: Mean number of offspring (± SE) produced by four *M. persicae* aphids in a four day period on three different genotypes of potato: the isogenic cultivar (Désirée), susceptible to *P. infestans* and two cisgenic resistant genotypes A15-13 and A15-45, and two different plant treatments: aphids alone or *L. decemlineata* (CPB) followed by aphids. N = 4 plants for each genotype/treatment group. Genotype mean values having no lowercase letters in common differ significantly ($P < 0.05$) within an infestation treatment.

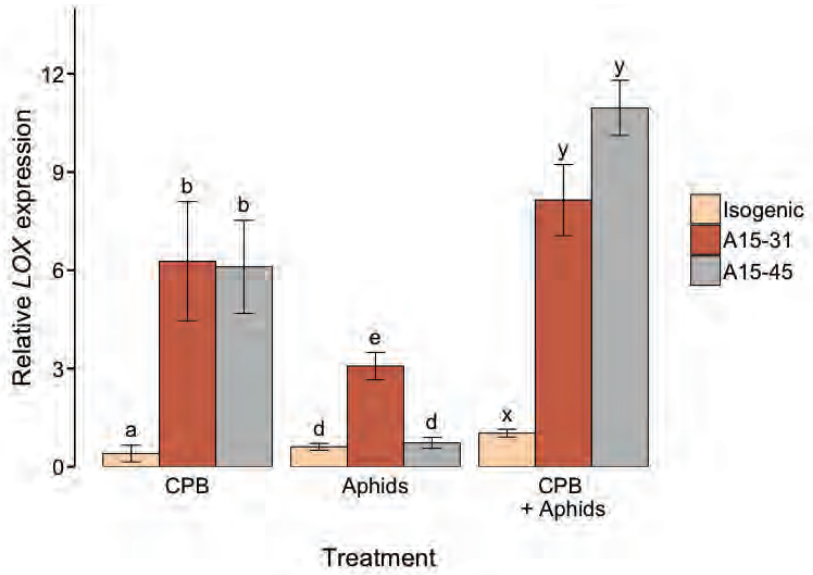

Figure 3: Mean relative *LOX* gene expression in three different genotypes of *S. tuberosum* plants: *P. infestans* susceptible Désirée, and two cisgenic resistant events with *Rpi-vnt1* insertion: A15-31 and A15-45. Plants were treated with either: *L. decemlineata* only (CPB), *M. persicae* only (Aphids) or treated first with *L. decemlineata* and one day later with *M. persicae* (CPB + Aphids). Values were normalized relative to the reference genes *GAPDH* and *EF1*-α and quantified relative to control plants. Different lowercase letters represent differences between genotype means ($P < 0.05$) within the same treatment.

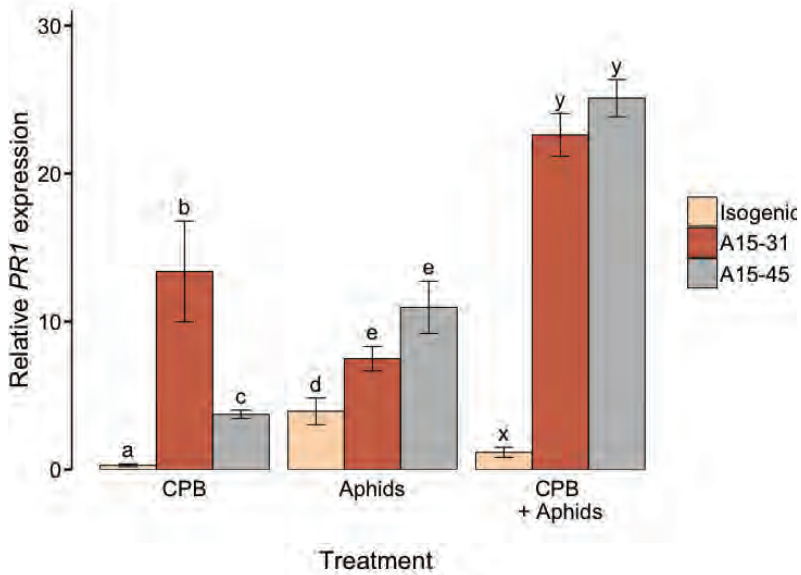

Figure 4: Mean relative *PR1* gene expression in three different genotypes of *S. tuberosum* plants: the isogenic, *P. infestans* susceptible cultivar (Désirée), and two cisgenic resistant events with *Rpi-vnt1* insertion: A15-31 and A15-45. Plants were treated with either: *L. decemlineata* only (CPB), *M. persicae* only (aphids) or treated first with *L. decemlineata* and one day later with *M. persicae*. Values were normalized relative to the reference genes *GAPDH* and *EF1*-α and quantified relative to control plants. Different lowercase letters represent differences between genotype means ($P < 0.05$) within the same treatment.

Gene Expression

LOX expression

LOX expression in potato plants was significantly affected by potato genotype, infestation treatment and their interaction ($P < 0.001$ for both main effects and their interaction) (Figure 3, Table 2C).

On plants treated with *L. decemlineata* larvae, either alone or in combination with *M. persicae* feeding, expression of the *LOX* gene was higher in both cisgenic plants than in the susceptible Désirée genotype (for larvae alone, and combined with aphids : A15-31 & A15-45: $P < 0.0001$; Figure 3). Yet when plants were only infested with *M. persicae, LOX* was expressed more in A15-31 plants than in the other two genotypes (Désirée: $P = 0.0013$; A15-45: $P = 0.0021$).

Table 2: Statistical results of linear model for each tested response variable (A-D) in Experiment 1 for the factors genotype, infestation treatment and their interaction.

	Experiment 1:	***L. decemlineata* pre-treatment**		
A	***L. decemlineata* weight gain (mg)**	**df**	**F**	**P**
	Treatment	1	0.025	0.877
	Genotype	2	12.152	**0.001**
	Treatment x Genotype	2	2.844	0.090
B	**Number of *M. persicae* offspring**			
	Treatment	1	8.4	**0.013**
	Genotype	2	6.033	**0.015**
	Treatment x Genotype	2	3.1	0.082
C	**Relative *LOX* expression**			
	Treatment	2	48.638	**1.76×10^{-5}**
	Genotype	2	140.274	**7.39×10^{-9}**
	Treatment x Genotype	4	21.683	**6.65×10^{-4}**
D	**Relative *PR1* expression**			
	Treatment	2	32.162	**5.63×10^{-7}**
	Genotype	2	125.499	**4.79×10^{-12}**

PR1 expression

Both main factors significantly affected *PR1* gene expression in the potato plants: genotype ($P < 0.0001$), treatment ($P < 0.0001$), and the interaction between genotype and treatment ($P < 0.0001$) (Figure 4, Table 2D).

In all infestation treatments, *PR1* expression of potato plants was higher in the cisgenic lines than in Désirée plants (Figure 4). Plants treated with *L. decemlineata* only had higher levels of *PR1* expression in the A15-45 event than Désirée ($P = 0.0002$); and was higher in the cisgenic A15-31 event than in A15-45 ($P = 0.0214$, Figure 4).

Regarding plants treated with *M. persicae*, *PR1* expression was higher in the cisgenic events than in Désirée (A15-31: P = 0.0404; A15-45: P = 0.0072). Cisgenic plants treated with *L. decemlineata* and then *M. persicae* also had a higher *PR1* expression than similarly treated Désirée (P < 0.0001 for both A15-31 and A15-45). Cisgenic A15-31 and A15-45 plants had similar expression levels of *PR1* when infested only with *M. persicae*. The expression of *PR1* was also similar in the cisgenic events when infested with *L. decemlineata* and *M. persicae* (Figure 4).

Experiment 2: *Myzus persicae* pre-treatment
Biomass gain of *L. decemlineata* larvae
Biomass gain of *L. decemlineata* was neither influenced by plant genotype (P = 0.0709) nor by treatment (P = 0.1432). There was a slight interaction effect between genotype and treatment on *L. decemlineata* biomass (P = 0.0403) (Figure 5, Table 3A).

Offspring production by *M. persicae*
Myzus persicae offspring production in this experiment was affected by genotype (P = 0.0498), treatment (P = 0.0462) and the interaction between these two main factors (P = 0.0292) (Figure 6, Table 3B).

On plants with only *M. persicae* infestation during 5 days, the number of offspring was similar for aphids feeding on all three genotypes (P = 0.7233). On plants that were first infested with *M. persicae* and one day later with *L. decemlineata*, more offspring were produced on A15-31 plants than on the other two genotypes (Désirée: P = 0.0042; and A15-45: P = 0.0111; Figure 6).

Gene Expression
LOX expression
In the aphid pre-treatment experiment *LOX* expression was influenced by plant genotype (P < 0.0001), treatment (P = 0.0006) and their interaction (P < 0.0001) (Figure 7, Table 3C).

Plants treated with a single herbivore species had higher *LOX* expression in cisgenic events than in the isogenic Désirée (for *L. decemlineata* only: P = 0.0082 for A15-31, P = 0.0022 for A15-45; and for *M. persicae* only: P = 0.0311 for A15-31, P = 0.0006 for A15-45). However, Désirée plants treated with both herbivores (*M. persicae* followed by *L. decemlineata*) had a similar *LOX* expression to the cisgenic event A15-31 (P = 0.3561) yet higher *LOX* expression than the event A15-45 (P = 0.0038). For all infestation treatments, the expression of *LOX* between the two cisgenic events differed. On plants treated with *M. persicae*, *LOX* expression was higher in A15-31 than A15-45 (for *M. persicae* alone: P = 0.0311; for combined treatment with *M. persicae* and *L. decemlineata*: P = 0.0009).

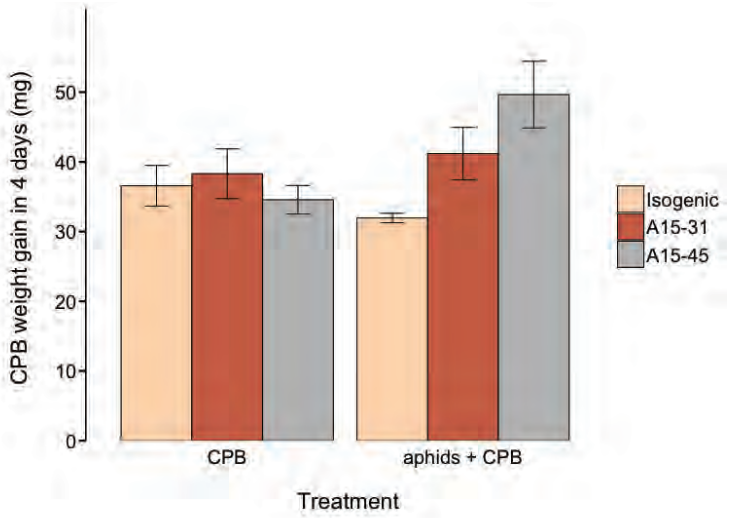

Figure 5: Mean gain in *L. decemlineata* (CPB) biomass per beetle in a four day period on three different genotypes of potato: the susceptible isogenic cultivar (Désirée), and two cisgenic (*P. infestans* resistant) genotypes A15-13 and A15-45 and two different plant treatments: CPB alone or *M. persicae* (aphids) followed by *L. decemlineata* (CPB). N=4 plants per for each genotype/treatment group.

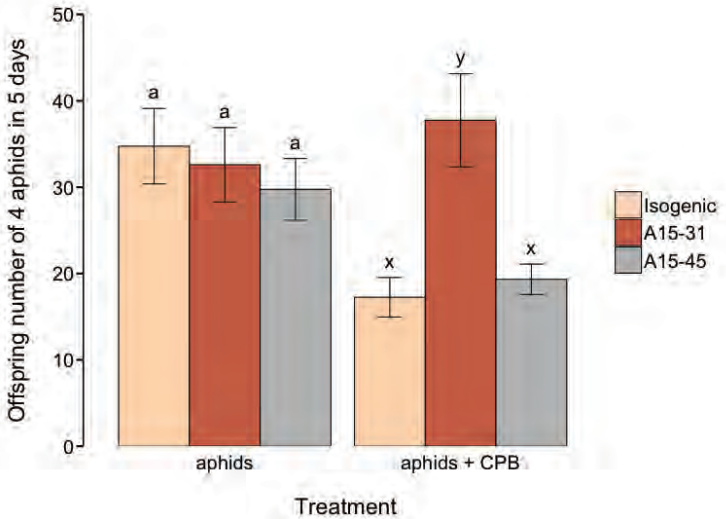

Figure 6: Mean number of offspring produced by four *M. persicae* (aphids) in a five day period on three different genotypes of potato: the isogenic, *P. infestans* susceptible variety (Désirée) and two cisgenic resistant genotypes A15-13 and A15-45 and two infestation treatments: aphids alone or aphids followed by *L. decemlineata* (CPB). N = 4 plants per for each genotype/treatment group. Genotype mean values having no lowercase letters in common differ significantly ($P < 0.05$) within an infestation treatment.

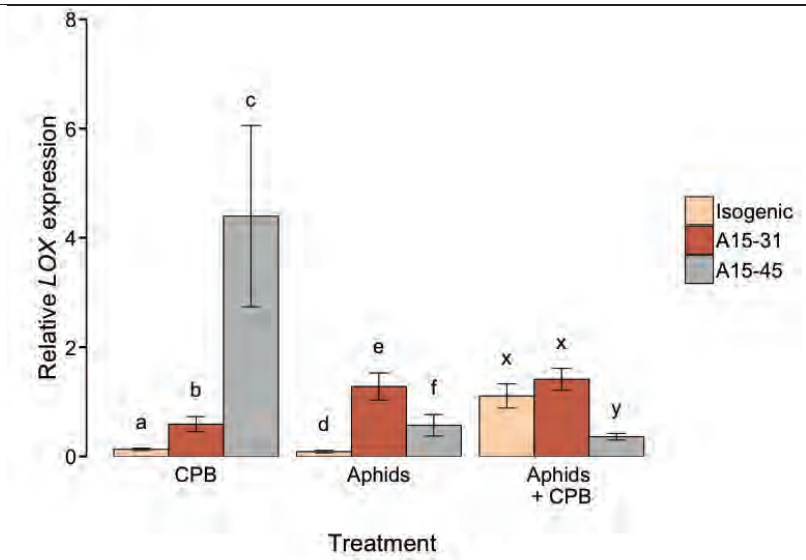

Figure 7: Mean relative *LOX* gene expression in three different genotypes of *S. tuberosum* plants: the isogenic, *P. infestans* susceptible cultivar (Désirée), and two cisgenic resistant events with *Rpi-vnt1* insertion, A15-31 and A15-45. Plants were treated with either *L. decemlineata* only (CPB), *M. persicae* only (Aphids) or treated first with *M. persicae* and one day later with *L. decemlineata*. Values were normalized relative to the reference genes *GAPDH* and *EF1*-α and quantified relative to control plants. Different lowercase letters represent differences between genotype means ($P < 0.05$) within the same treatment.

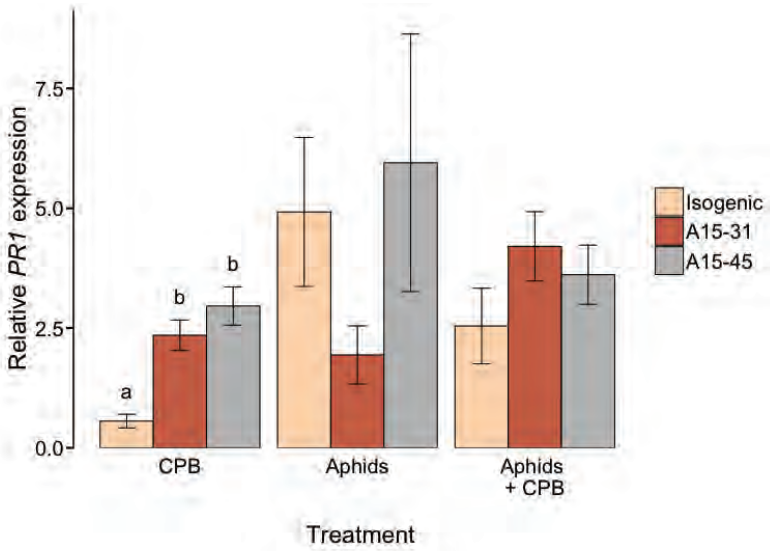

Figure 8: Mean relative *PR1* gene expression in three different genotypes of *S. tuberosum* plants: *P. infestans* susceptible isogenic (Désirée), and two cisgenic resistant events with *Rpi-vnt1* insertion, A15-31 and A15-45. Plants were infested with either *L. decemlineata* larvae only (CPB), *M. persicae* only (aphids) or treated first with *M. persicae* and one day later with *L. decemlineata* larvae. Values were normalized relative to the reference genes *GAPDH* and *EF1*-α and quantified relative to control plants. Different lowercase letters represent differences between genotype means ($P < 0.05$) within the same treatment.

Table 3: Statistical results of the overall linear model for each tested response variable (A-D) in Experiment 2 for the factors genotype, infestation treatment and their interaction.

	Experiment 2:	***M. persicae pre-treatment***		
A	***L. decemlineata* weight gain (mg)**	**df**	**F**	**P**
	Treatment	1	2.343	0.143
	Genotype	2	3.076	0.071
	Treatment x Genotype	2	3.860	**0.040**
B	**Number of *M. persicae* offspring**			
	Treatment	1	4.586	**0.046**
	Genotype	2	3.560	**0.050**
	Treatment x Genotype	2	4.326	**0.029**
C	**Relative *LOX* expression**			
	Treatment	2	10.649	**5.83×10^{-4}**
	Genotype	2	40.277	**4.43×10^{-8}**
	Treatment x Genotype	4	26.872	**3.45×10^{-8}**
D	**Relative *PR1* expression**			
	Treatment	2	3.804	**0.039**
	Genotype	2	2.138	0.143
	Treatment x Genotype	4	2.948	**0.044**

PR1 expression

Plant genotype did not significantly influence *PR1* expression (P = 0.1428). Infestation treatment significantly affected expression levels of *PR1* (P = 0.0389), as did the interaction between genotype and treatment (P = 0.0443) (Figure 8, Table 3D).

The expression of *PR1* was lower in the isogenic Désirée than in both cisgenic genotypes on the plants treated only with *L. decemlineata* (A15-31: P = 0.0014; A15-45: P = 0.0007). For plants treated with *M. persicae*, either alone or combined with *L. decemlineata*, there were no differences in *PR1* gene expression levels between the genotypes (*M. persicae* alone: P = 0.4964; *M. persicae* then *L. decemlineata*: P = 0.3277; Figure 8).

Discussion

In this study, we investigated the effect of a cisgenic *Phytophthora infestans* resistance gene (*Rpi-vnt1*) in potato on the performance of two non-target insects feeding separately or in combination. We also investigated the role of genotypic position of the *Rpi-vnt1* gene on non- target insect performance by comparing two different insertion events. Insect performance experiments were coupled with plant gene expression analyses in order to further understand the effects of *Rpi-vnt1* gene presence and location on the plant responses associated with stress from single or dual insect herbivory. Despite variation observed in the experiments of this

study, our data provides support for effects of genotypic position on non-target insect performance as well as changes in the plant transcriptional responses. These findings are interesting from a theoretical as well as applied perspective, and their implications are discussed below.

R gene presence and genotypic position

Under stress from single or dual herbivory, insect performance differed depending on the potato plant genotype and aphids were more sensitive to these genotypic differences than Colorado beetles. From the experiment where plants were pre-treated with *L. decemlineata* larvae, it can be concluded that the effect of plant genotype on *L. decemlineata* biomass can be attributed to the presence of the resistance gene *Rpi-vnt1*. While this is not reproduced in the second experiment, this could be due to experimental factors such as the reduced larval feeding time. Our study also indicates that the insertion of the *Rpi-vnt1*- gene as well as its position, can affect the aphid as well as plant responses.

Considering that the two cisgenic genotypes tested differ only in the position in the genome of the *Rpi-vnt1* resistance gene, two possible mechanisms for these effects can be proposed. First, a possible change in expression levels of the *Rpi-vnt1* gene itself at different genomic positions may influence the physiological responses to stress of the cisgenic events. Second, the random insertion of the R gene within the plant's genome may be related to the response of plants to herbivory or stress. The first option is less likely, as we have observed that the expression of these R genes is at barely detectable levels (unpublished), and that *Rpi* genes in general are not differentially inducible (Gyetvai *et al.*, 2012; Śliwka *et al.*, 2013), and high expression levels are often lethal (Zhang *et al.*, 2016). The second hypothesis was tested in a preliminary experiment wherein the phloem-feeding patterns of *M. persicae* on the two cisgenic genotypes were compared to the isogenic cultivar Désirée using the electrical penetration graph technique (unpublished results). The results of these tests did not provide evidence for any differences in aphid phloem feeding behaviours between the two cisgenic genotypes, or between isogenic and cisgenic genotypes. This indicates that if the location of insertion of the R gene affects the surrounding genes involved in the plant's response to insect feeding, the effect may be due to the nutritional qualities of the phloem rather than mechanical hindrance of phloem feeding. Elucidating the mechanisms behind the observed gene expression and non-target insect effects of the two different cisgenic events observed in this study will require further investigation.

Gene expression and insect performance

Generally, our results do not show a clear correlation between the insect performance results and the expression of two genes in potato plants, *LOX* and *PR1*. In treatments with Colorado beetle larvae, however, we show that higher expression

of the JA-marker gene *LOX* was often correlated with a decrease in *L. decemlineata* biomass gain. The *LOX* gene codes for lipoxygenase, an important enzyme in the biosynthesis of jasmonic acid (Gobel *et al.*, 2001). This gene is key in the response of plants to wounding and pathogen attack. *Leptinotarsa decemlineata* and many other insects are known to be sensitive to JA- induced plant responses (Lortzing & Steppuhn, 2016). Furthermore, both *L. decemlineata* and *Spodoptera exigua* were shown to gain more weight on *LOX*-depleted potato plants (Royo *et al.*, 1999); and *Manduca sexta* caterpillars were also heavier on *LOX*-depleted wild tobacco plants in the lab (Halitschke & Baldwin, 2003) and in the field (Kessler *et al.*, 2004).

Leptinotarsa decemlineata has also been shown to actively suppress JA in plants. Symbiotic bacteria in oral secretion activate the SA-response pathway in plants, which reduces JA accumulation through the JA-SA pathway antagonism known as cross-talk (Chung *et al.*, 2013; Koornneef, 2008). The activation of both plant response pathways by *L. decemlineata* could be an explanation for the lack of reciprocity seen in the JA and SA marker genes expression in our study. Another important factor which influences gene expression is timing. Timing of maximum gene expression peak levels is pathway-specific. Namely, *PR1* is expressed downstream the SA pathway, and exhibits its peak expression later than the *LOX* gene does (since *LOX* is active early in the JA pathway) (Kawazu *et al.*, 2012). Gene expression is also known to be herbivore-density sensitive (Kroes *et al.*, 2015) so finding reciprocal gene expression in these two genes may be difficult to capture with only one time-point for each gene, or one single density tested for each insect. Timing of expression levels in stress-responsive genes have been shown to be ecotype specific in *Arabidopsis thaliana* (Miller *et al.*, 2015). However, genetic variation between ecotypes may differ much more than between Désirée and our cisgenic events. Another possible hypothesis to explain the variability in gene expresssion between the genotypes may be that R gene position can influence surrounding genes through changes in timing or speed of defence gene expression cascades. The particular treatments applied and their durations can also influence the timing and duration of the gene expression cascades. Further research would be needed to determine whether differences lie in the timing or in absolute differences in gene expression among the different cisgenic events. Future work should include time-series analysis of gene expression for several genes in the pathways, as well as phytohormonal quantification coupled with insect feeding.

Single vs. sequential dual herbivory

In our experiments, the effect of infestation treatment affected insect performance as well as plant responses. Infestation treatment affected aphids more than the Colorado beetle larvae; yet affected expression of both defence response genes. From both insect and plant perspectives, stress by single herbivory triggers different responses than dual herbivory (Kroes *et al.*, 2016; Rodriguez-Saona *et al.*, 2010; Soler

et al., 2012). These interactions can depend on the insect species, as well as the plant genotype (Li *et al.*, 2014)2014. Our experiments show that the interaction between the plant genotype and the infestation treatment affects insect performance and plant responses. In essence this interaction means that the variation in measured responses under different treatment conditions varies depending on the plant genotype. It is not common practice to test non-target effects of GM crops under multiple stress conditions in a greenhouse setting in a risk assessment context. Usually, early studies are conducted in the greenhouse with single representative species, while field experiments test effects on whole communities (Charleston & Dicke, 2008). Here we show that testing these interactions in the early experimental stages of comparing genotypes may also provide information about potential effects with commonly interacting non-target herbivores.

Practical implications and next steps

In previous studies, aphids on the cisgenic event A15-31 showed an increased intrinsic population increase (Lazebnik *et al.*, 2017), although an increase in aphid performance was not confirmed in this study. It seems that even small differences in experimental design can influence aphid performance. In this study, we did not assess the rate of intrinsic population increase, since this measure would require much longer observation periods. The infestation times and also numbers of individuals per plant differed between the two studies. In general we have seen that both insect performance and gene expression are dependent on the experimental conditions (Chapter 5).

Nevertheless, this work provides support for an effect of the insertion of an *Rpi*-gene on single and dual herbivory by non-target insects. We also showed that the *Rpi*-gene insertion influenced gene expression in different ways depending on the applied insect treatment. In Chapter 5 we elaborate on the dependency of non-target effects on the interactions of the plant with its target *P. infestans*. In the context of different insertion events, the effect of the sequence in which insect stressors arrive merits further investigation. It is well known that the order in which the insects arrive can influence plant-insect interactions from gene expression to the whole community dynamics (Stam *et al.*, 2014; Voelckel & Baldwin, 2004). Taking these results into account when choosing the most true to type genetically modified crop could be a worthwhile step before proceeding to more elaborate non-target testing in the field. Further experiments are needed to investigate how these effects translate to realistic field growing conditions; and to better understand the mechanisms behind the positional effects.

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Chapter 4

Effects of a genetically modified potato on a non-target aphid are outweighed by cultivar differences

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Abstract

Insect-plant interactions may be unintentionally affected when introducing genetically modified (GM) crops into an agro-ecosystem. Our aim was to test the non-target effects of a late blight resistant GM-potato on *Myzus persicae* in greenhouse and climate room experiments and understand how position and number of R-gene insertions can affect non-targets in GM events. We also aimed to compare results to baseline differences among three conventional potato varieties varying in resistance to late blight. Aphid development and survival was affected by some GM events in the first generation, although effects disappeared in the second generation. Effects were not dependent on the presence of a marker gene or the insertion of a second resistance gene. Positional effects of gene insertion influenced aphid performance on certain GM-events. However, aphid fitness varied considerably more between conventional potato varieties than between Désirée and the GM events. Comparing different GM-events to the non-transformed variety is relevant, since unintended effects of insertion can occur. Our protocols can be recommended for *in planta* risk assessments with aphids. Ecological perspective is gained by selecting several measured endpoints, and by comparing the results with a baseline of conventional cultivars.

Key Words

Genetic modification, non-target testing, greenhouse, environmental risk assessment, *Phytophthora infestans, Solanum tuberosum, Myzus persicae*

Introduction

To be considered for cultivation in agriculture, genetically modified (GM) crops must be subject to environmental risk assessment (ERA). The biodiversity and ecology of organisms in the agro-ecosystem are an important consideration in ERA. Genetically modified plants would be the primary producers supporting the trophic webs of these agro-ecosystems, the direct and indirect consequences of introducing these crops is therefore a relevant concern (Arpaia, 2010; EFSA, 2010). Risk assessments should be done in several stages or tiers, starting with experiments that have a high likelihood of detecting effects on non-targets to more complex and realistic field conditions (Andow & Hilbeck, 2004; Andow & Zwahlen, 2006; Houshyani, 2012; Kos *et al.*, 2009; Romeis *et al.*, 2011). Each consecutive tier in the ERA should use the feedback acquired in previous steps. Trials in confined conditions are important in early tiers of ERA to establish if direct effects occur on the life history of particularly important members of the agro-ecosystem, or representatives of important functional groups (Andow *et al.*, 2013; Birch *et al.*, 2007; Houshyani, 2012; Romeis *et al.*, 2011; Romeis *et al.*, 2013).

Before the introduction of GM plants into the ecosystem, testing for non-target effects of a GM-crop in the greenhouse first requires a thorough and transparent selection of appropriate non-target organisms (NTO)(Carstens *et al.*, 2014; EFSA, 2010). These tests should be more reproducible and reliable compared to field tests, and are an important step in the ERA process. A selection procedure of relevant functional groups and endpoints to test must also be included in the ERA. In this study, we based the selection on the protocol outlined in the EFSA guidance document on ERA of GM plants (2010) as well as on several other sources (Andow *et al.*, 2013; Gillund *et al.*, 2013; Romeis *et al.*, 2014; Romeis *et al.*, 2013; Scholte & Dicke, 2005). We selected the non-target aphid *Myzus persicae* Sulzer to test the non-target effects *in planta* of a late blight resistant genetically modified potato.

Most conventional potato cultivars are susceptible to late blight which is caused by the widespread pathogen *Phytophthora infestans* (Mont.) de Bary, a hemibiotrophic oomycete which colonizes potato leaves, stems and tubers. The modification of the cultivar Désirée confers resistance to *P. infestans* through the insertion of one or two resistance genes (R genes) from crossable potato (*Solanum tuberosum* L.) relatives, *Solanum venturii* Hawkes & Hjert., *(Rpi-vnt1*), and *Solanum stoloniferum* Schltdl & Bouché (*Rpi-sto1*). R genes code for receptor proteins which recognise distinct pathogen effectors (in this case from *P. infestans*). This recognition initiates signal transduction cascades leading to callose deposits and cell death in infected and surrounding cells preventing the pathogen from further spread, which is macroscopically visible as a hypersensitivity response (HR) (Kamoun *et al.*, 1999; Vleeshouwers *et al.*, 2011; Vleeshouwers *et al.*, 2000).

Late blight R genes can be co-inserted with a selectable marker gene from a bacterium coding for resistance to an antibiotic (transgenesis) or using a marker-free transformation protocol. Because the R genes used in this study are derived from crossable species and the transformation events contain no "foreign" DNA the latter protocol is referred to as cisgenesis. We tested two transgenic and two cisgenic events containing the same single R gene (*Rpi-vnt1*). Also we tested two transgenic events harbouring two R genes (*Rpi-vnt1* and *Rpi-sto1*). The location of the R gene insertion in the genome may have an impact on other plant functions, and indirectly on non-target aphids. By testing two transformation events of each construct, position effects could be assessed. We also assessed the reproducibility of the experimental protocol by performing the assays on the same plant clones in two laboratories with different *M. persicae* colonies.

In order to compare the magnitude of the effects of these modifications with the variation among commercially available conventional potato varieties we compared a cisgenic event (also used in concurrent field experiments) with four conventional varieties (including Désirée) varying in their susceptibility to *P. infestans* (Table 1).

Table 1: Cultivars and events used in this study.

Event/Cultivar	Event type	Resistance rating to *Phytophthora* on foliage	R gene insertion, wild relative	Marker-gene
A15-31	Cisgenic	Very High	*Rpi-vnt1, Solanum venturii*	None
A15-84	Cisgenic	Very High	*Rpi-vnt1, Solanum venturii*	None
A15-45**	Cisgenic	Very High	*Rpi-vnt1, Solanum venturii*	None
A13-13	Transgenic	Very High	*Rpi-vnt1, Solanum venturii*	NPTII (kanamycin resistance)
A13-17	Transgenic	Very High	*Rpi-vnt1, Solanum venturii*	NPTII (kanamycin resistance)
A16-02	Stacked transgenic	Very High	*Rpi-vnt1, Solanum venturii* & *Rpi-sto1, Solanum stoloniferum*	NPTII (kanamycin resistance)
A16-24	Stacked transgenic	Very High	*Rpi-vnt1, Solanum venturii* & *Rpi-sto1, Solanum stoloniferum*	NPTII (kanamycin resistance)
Désirée	Isogenic, conventional	Low- Medium*	None	None
Bintje	Conventional	Low*	None	None
Première	Conventional	Low-Medium*	None	None
Sarpo Mira	Conventional	Very High*	None	None

*rating taken from the European Cultivated Potato Database (ECPD, 2015)
**not used for Figure 1 and 3 due to availability restriction at the time of experiment

Selection of non-target species *Myzus persicae* for *in planta* testing

Many species may be exposed to GM plants in any agro-ecosystem. Since not all species can be tested, a representative subset of NTO's should be selected for consideration in the risk assessment of each GM plant. The GMO Panel of the European Food Safety Authority (EFSA) propose a species selection approach (EFSA, 2010). *Myzus persicae* Sulzer (Hemiptera: Aphididae) was chosen based on a final ranking using the aforementioned approach, which includes several important factors. First, the simplicity in rearing this species in many laboratories and aphid reproductive biology, which allows for the measurement of survival and intrinsic rate of increase, which can be used to estimate the population dynamics of this pest; and, it is listed as the most collected phloem feeder in the EFSA arthropod database (Riedel *et al.*, 2016), and second most collected species on potato giving it high relevance as a focal NTO.

Aphids are the most important insect pests of potato (Meissle *et al.*, 2012; Radcliffe, 1982), and the polyphagous *M. persicae* is the most prevalent and studied among those. Aphids can feed on potato and cause direct damage through piercing and sucking from the plant's phloem. More problematic is the fact that *M. persicae* is a vector of over one hundred plant viruses, with about twelve directly affecting potato crops (Kennedy *et al.*, 1962; Ng & Perry, 2004; Van Emden *et al.*, 1969); including several leaf-roll viruses. Although they are widespread pests, aphids they are prevalent in various agro-ecosystems including potatoes. They are a major prey species host many parasitoids (Müller *et al.*, 1999), and are prey to insect families such as larval syrphid flies (Raj, 1989), ladybugs (Majerus, 1994), lacewings, spiders and others (Van Emden *et al.*, 1969). Despite the specificity of an R gene for resistance against *P. infestans*, it is nevertheless important to understand if any modification or insertion can affect an important NTO like the *M. persicae*, and its population dynamics.

Experimental procedures

Plant material

The GM-events tested in this study were developed by the Laboratory of Plant Breeding of Wageningen University and Research Centre (Haesaert *et al.*, 2015; Haverkort *et al.*, 2016). They have been created using *Agrobacterium tumefaciens*-mediated transfer of the native *Rpi-vnt1* gene, from *Solanum venturii*, using marker-assisted (events A13-13, 17) and marker-free transformation methods (events A15-31, 45, 84). Also, two marker-assisted transformation events (A16-02 and A16-24) were used that were generated using a single T-DNA harbouring the native *Rpi-vnt1* and *Rpi-sto1* (from *Solanum stoloniferum*) genes. The tested conventional cultivars and GM events (defined here as clones with gene-insertions conferring resistance to the target *P. infestans*) are described in Table 1. Events were selected as apparently "true

to type" as they were morphologically indistinguishable from non-transformed Désirée under tuber sown field conditions (Haverkort *et al.* 2016).

All GM-events and conventional cultivars were maintained *in vitro*, on agar medium (purified agar 0.8% + 2.2 g /L Murashige&Skoog + Duchefa 4.4 g/L +Sacharose 20 g/L+ Micro Agar 8 g/L; pH = 5.8) in sterile containers. Containers were kept in a climate room at 16:8 light:dark conditions, and 21°C during light hours and 15°C when dark, and 70% relative humidity. Cuttings were transplanted five weeks before the experiments to allow for root growth, seedlings then transplanted to larger pots, and allowed to grow for five weeks before being used in experiments.

Aphid rearing and experimental setup
Wageningen University
Myzus persicae were collected in 2004 from Wageningen, The Netherlands (51°59'11.5"N 5°39'48.4"E) and reared at the Laboratory of Entomology, Wageningen University. They were originally kept on radish but maintained for several generations on *S. tuberosum* cultivar Désirée before experiments began under the same climate room conditions described above.

ENEA
The colony was started from a laboratory strain originally reared at the University of Bologna. The strain was maintained on *S. tuberosum* cultivar Désirée for several generations before experiments began. The *M. persicae* colony was maintained under 16:8 light:dark conditions, and 24°C during light hours and 18°C when dark, and 70% relative humidity

Testing the GM potato events and conventional potato varieties
First tested the intrinsic rate of increase and survival of aphids between the non-transformed Désirée and the following GM (from Désirée) events: A15-31, A15-45 (both cisgenic), A13-13, A13-17 (both transgenic), A16-02, and A16-24 (both transgenic with two R genes) all events are described in Table 1. Then, to test reproducibility, Wageningen University and ENEA performed similar experiments comparing specifically the cisgenic events A15-31 and A15-45 to the non-transformed Désirée. Lastly, we compared several conventional potato cultivars: Désirée, Bintje, Première and Sarpo Mira (described in Table 1) with the same measured endpoints as for the aforementioned experiments.

One-day-old aphid nymphs were used in each experiment. Aphid nymphs were placed singly in clip-cages (25 mm diameter; 10 mm high) on the abaxial surface of two (at ENEA) or three leaves (Wageningen University) on each plant. Ten (at Wageningen University) to fifteen (at ENEA) plant replicates of each event and the non-transformed Désirée cultivar were used, and randomly distributed in the

climate room. Due to space limitations this was split into two or three rounds, each round testing five plants from each event and non-transformed Désirée.

We monitored the fitness of *M. persicae* for two generations. Aphids were checked every day for mortality and for offspring production; neonate nymphs were counted and removed daily. At Wageningen University, once the first generation produced its first nymphs, one of these was caged on another leaf of the same plant; at ENEA second generations were transferred to a new plant. The parameters collected were: pre-reproductive period and total fecundity, for calculation of intrinsic rate of increase (R_m), and aphid mortality of both generations. Intrinsic rate of increase was calculated as described in Wyatt and White (1977): $R_m = 0.74 (\ln Md) / d$, where Md is the effective fecundity and d the length of the pre-reproductive period. The means for all aphid parameters used to calculate survival and intrinsic rate of increase are documented in Appendix A-C.

The same methodology was applied to a second experiment in a greenhouse comparing the first generation of aphid life-history parameters on one cisgenic event (A15-31, highly resistant) and four conventional cultivars varying in their foliar resistance to *P. infestans*. Cultivar Bintje has a resistance rating of low to very low, cultivar Première and Désirée rate low to medium, and Sarpo Mira rates highly resistant to *P. infestans* (ECPD, 2015).

Statistical analysis

Based on a preliminary small scale experiment (15 individuals), we conducted a prospective power analysis. The measurement endpoint selected was the length of the pre-reproductive period. The mean difference deemed to be biologically relevant was set to 1.9 and the common within-group standard deviation was fixed at 2.5 (based on the variability registered in the experiment). This effect was selected as the smallest relevant effect. The criterion for significance (alpha) was set at 0.050 for a 2-tailed test. The analysis was conducted using the Power and precision 2.1 software (Borenstein *et al.*, 2000). The results indicated a sample size of 28 individuals for each group, the study will have power of 81.3% to yield a statistically significant result for the differences indicated.

Intrinsic rate of increase was tested with a mixed linear model, or generalized linear mixed model when data did not meet the assumptions of normality; with fixed factors being 'potato event' and 'aphid generation' and random factors including the 'plant or pot number' (since there were three clip cages per plant), nested within 'round' (experiment was replicated in two rounds). The model was chosen by backwards selection comparing AIC values of simpler models (Burnham *et al.*, 2010). The fixed factor 'aphid generation' (first or second generation) proved to have an influence on aphid intrinsic rates of increase ($P = 0.0034$). For some events,

there was an interaction effect between 'aphid generation' and the 'potato event'. For this reason, we separated the two aphid generations, and used separate models for each using the same random factors as above. Analysis for comparisons to baseline cultivars were done in a similar way as above, though the experiment was conducted in one round, for one aphid generation and the only random effect included in the model was 'plant or pot number'. Analyses for intrinsic rates of increase were conducted using R Statistical Software (R Development Core Team, 2014), with the 'nlme' package.

Survival analyses were conducted using a Cox proportional hazards regression model. This was also separated by generation, which played an important role in aphid survival (P = 0.0005) and interacted with the fixed effect of 'potato event'. This model included the same nested random effects as above, and was performed using R Statistical Software (R Development Core Team, 2014), with the 'survival' package.

Results

Désirée compared to GM events

Comparison of events: In the first generation, aphid intrinsic rate of increase was generally higher on all GM-events than on the non-transformed Désirée plants, though the only events significantly differing from Désirée were the transgenic event A13-17 (P = 0.0122) and the cisgenic event A15-31 (P = 0.0198) (Figure 1A). The trend of higher intrinsic rate of increase was no longer observed in the second generation, the events no longer differed from non-transformed Désirée (Figure 1B; Figure 2).

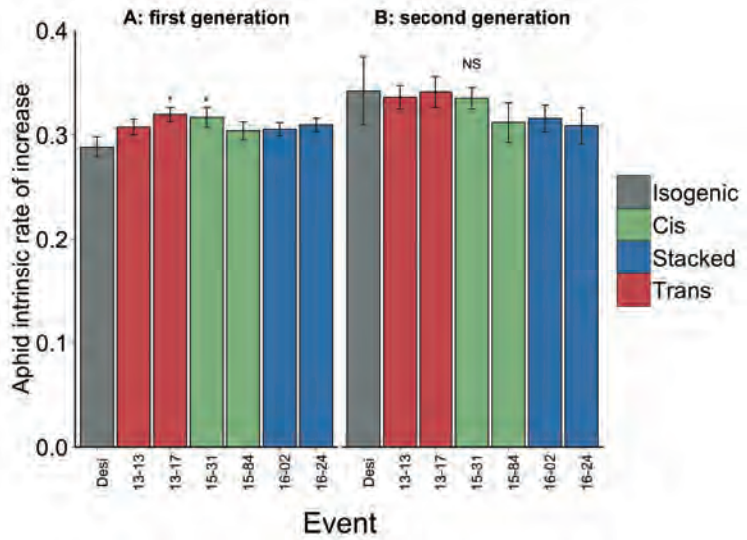

Figure 1: Mean aphid intrinsic rate of increase (±SE) on *Solanum tuberosum* isogenic cultivar Désirée, compared to several genetically modified events for two aphid generations. Two events of cisgenic, transgenic and stacked transgenic potatoes were compared. Asterisk (*) indicates significant differences from the isogenic cultivar within the generation.

Reproducibility between labs:

The higher rate of increase of intrinsic population aphids in the first generation on the cisgenic event A15-31 was observed in the labs at Wageningen University (Figure 2A; P = 0.0138), and at ENEA (Figure 2B; P = 0.0243). However, at Wageningen University, aphids generally had a lower intrinsic rate of increase in the second generation (Figure 2A; P = 0.0223); whereas in ENEA, it was generally higher in the second generation (Figure 2B; P = 0.0177).

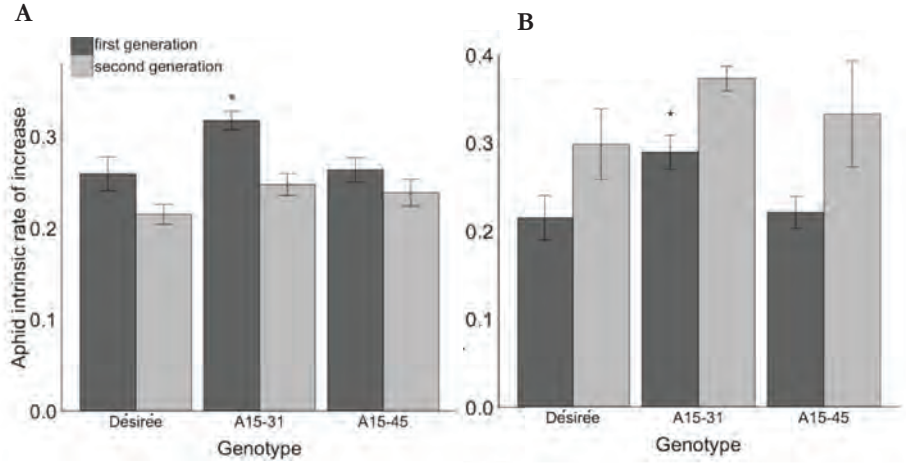

Figure 2: Mean aphid intrinsic rate of increase (±SE) on *Solanum tuberosum* isogenic cultivar Désirée, compared to genetically modified events A15-31 and A15-45, for two aphid generations in A) at Wageningen University Laboratory of Entomology, and B) at ENEA laboratory. Asterisk (*) indicates significant differences from the isogenic cultivar in the first generation.

Aphid survival:

Probability of aphid survival over time also tended to be higher on the GM events as compared to the non-transformed Désirée. However, only in the first generation significant differences were observed in one transgenic event A13-13 (P = 0.028) with a single R gene and one transgenic event event with two R genes, A16-02 (P = 0.039) (Figure 3A). In the second generation, there were no longer differences between the probability of survival of aphids on GM events compared to non-transformed Désirée (Figure 3B).

No differences were found in the survival of aphids on Désirée compared to A15-31 or A15-45 at either Wageningen University or ENEA (Appendix A).

Baseline comparison with commercially available cultivars

In order to put these results into context of the differences found among conventionally bred and commercially available potato varieties, we tested aphids on three different varieties known to differ in level of resistance against *P. infestans*. Compared to Désirée, on the other three conventionally bred varieties, aphids had a lower intrinsic rate of increase (Désirée vs. Bintje: P = 0.002, and Désirée compared to

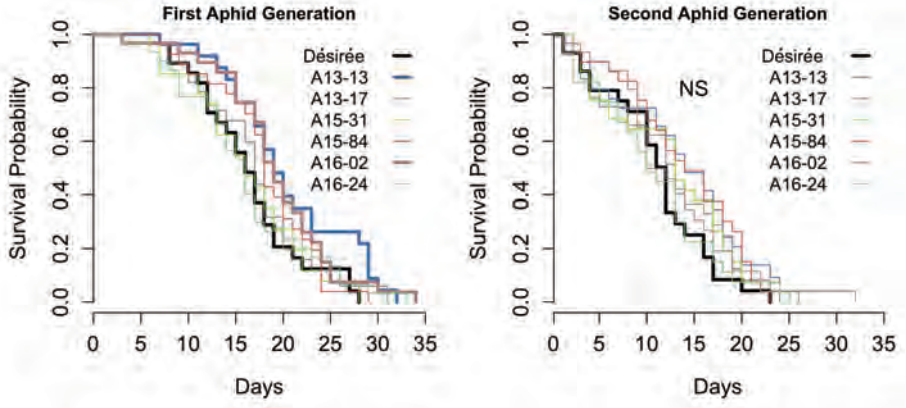

Figure 3: Probability of aphid survival per generation on *Solanum tuberosum* isogenic cultivar Désirée, compared to several genetically modified events. Bold coloured lines indicate significant differences from the isogenic cultivar (Désirée) within the generation.

Première and Sarpo Mira: $P < 0.0001$). When put into context of the conventionally bred varieties there was no longer any difference between aphid rate of increase on the cisgenic event (A15-31) and Désirée ($P = 0.1282$). Although not different from the isogenic *P. infestans*-susceptible Désirée, the highly resistant cisgenic event (A15-31) also did not differ from the highly susceptible conventional variety Bintje ($P = 0.1198$) but aphids had significantly higher intrinsic rate of increase than on the highly *P. infestans*-resistant conventional variety Sarpo Mira ($P < 0.0001$) (Figure 4).

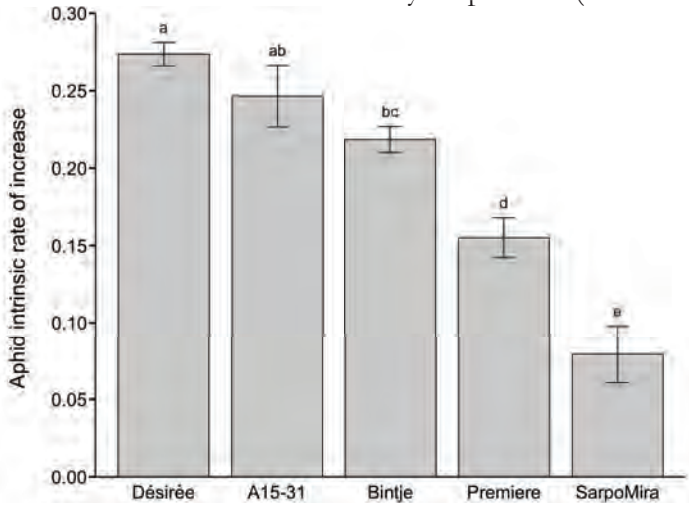

Figure 4: Mean aphid intrinsic rate of increase (±SE) on *Solanum tuberosum* isogenic cultivar Désirée, compared to a cisgenically-modified event (A15-31), and three conventional cultivars Bintje, Première and Sarpo Mira. Different letters indicate significant differences between bars.

Probability of aphid survival did not differ between Désirée, Bintje and the cisgenic resistant event A15-31 (Désirée vs. Bintje, $P = 0.2919$; Désirée vs A15-31, $P = 0.2225$). However, aphid survival was significantly lower on Première ($P = 0.0096$) and Sarpo Mira ($P < 0.0001$) (Figure 5).

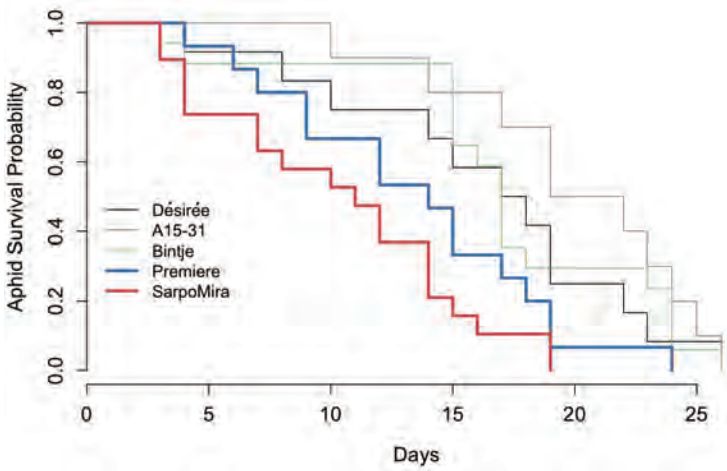

Figure 5: Probability of aphid survival per generation on *Solanum tuberosum* isogenic cultivar Désirée, compared to cisgenically-modified event (A15-31), and three conventional cultivars Bintje, Priemière and Sarpo Mira. Bold red and blue bold lines indicate significant differences from the isogenic cultivar (Désirée).

Discussion

Influence of selection markers, number of R genes, collateral effects and endpoint-choice on detection of non-target effects

The results of our experiments show that genetic modification in potato for resistance to *P. infestans* through R gene insertion may have effects on non-target aphids in the first generation, yet these effects were no longer evident in the second generation of aphids. These effects cannot be attributed to marker-gene use in the modification, since intrinsic rate of increase was higher both in a cisgenic as well as a transgenic event. The differences found between events can not be attributed to the number of R genes either, since survival probability was increased in events both with one and two R genes.

Interestingly, on the same event intrinsic rate of increase could be significantly higher whereas survival did not differ. In our findings, significant effects on aphid life history traits were never seen on both events transformed with the same construct. This brings to light the issue that detection of non-target effects depends on the measured endpoint (Charleston & Dicke, 2008; Lövei *et al.*, 2009). For example, in the case of the variety Bintje, it differed from Désirée in terms of aphid intrinsic rate of increase, yet had similar survival probability. Similarly, aphids on Désirée plants transformed to express enhanced chitinolytic activities showed increased population growth, while survival probability did not differ (Saguez *et al.*, 2005). In the GM events, aphids had higher intrinsic rates of increase on A15-31 and A13-17, yet these were not the same events on which survival differed. Therefore, it is important to carefully select biologically relevant endpoints for testing in the greenhouse that can most closely translate to effect differences in the field. Considering several selected measurement endpoints when testing for environmental risk and non-target testing

can be misleading if not all endpoints lead to differences in the same events. This considered, for the events tested at both Wageningen University and ENEA, we came to comparable results with regards to both endpoints. Testing multiple endpoints in several events considerably strengthens the reliability of results of early tier risk assessments, but would require separate testable hypotheses and protection goals specific to each in order to reliably inform the assessment.

The location of the inserted R gene in the genome is the only difference between events transformed with the same construct. Since one event can influence aphid life-history traits, whereas another does not, we conclude that these are unintended effects associated with the location of insertion. These are known as position effects (Miki *et al.*, 2009). These insertions may have occurred in a location that can affect interactions with insects such as defence response pathways. However, insertions usually result in loss of function rather than gain of function (Wang, 2008). Loss of function effects are complemented by the three remaining copies in the tetraploid potato genome. A more likely explanation of the observed position effects could be a difference in expression level of the inserted R gene. Substantial differences in the expression level of the *Rpi-vnt1* gene are observed among different transformation events (J.H. Vossen, unpublished data). In this case, overexpression of a late blight R gene may have a trade-off with resistance to aphids. Generally, these results emphasize the usefulness of a pre-screening for position effects on relevant non-target insects before proceeding with an entire environmental risk assessment on a single modified event. These early tests can help detect possible position effects resulting from genetic modification.

Detection of non-target effects over two insect generations

Our findings show that differences could be detected in the first generation of aphids feeding on GM-events, however, these differences had disappeared in the second generation of aphids. Although transgenic resistance based on the expression of *Bacillus thuringensis* (Bt) proteins has a very different mode of action, *Rhopalosiphum padi* aphids on Bt (transgenic) maize had higher performance in the first generation (Lumbierres *et al.*, 2004). *Aphis gossypii* aphids also had higher intrinsic rates of increase on Bt cotton in the first, but not in the second or third generation (Liu *et al.*, 2005). Since aphids were reared on the untransformed cultivar Désirée, it is possible that the effects seen in the first generation are a consequence of the aphids switching host plants rather than an effect of the transformation itself. This possibility can be tested in future experiments by rearing insects on an alternative host, or on each of the test events separately.

The second generation of aphids was kept on the sample plants at Wageningen University, yet at ENEA second generation aphids were transferred to new plants. Although there were no differences in intrinsic rates of increase between genotypes

detected in the second generation of aphids in either laboratory, the difference in performance of the second generation aphids between experiments conducted at ENEA and Wageningen, may have been caused by induced defence mechanisms since both generations were kept on the same plant in Wageningen. Feeding by conspecifics on the same plant can have negative effects on the life history traits of *M. persicae*, due to systemic defence mechanisms of the plant (Dugravot *et al.*, 2007).

Aphids are considered as good model organisms for understanding epigenetic effects (Srinivasan & Brisson, 2012). The formation of winged offspring is a well-known epigenetic effect in aphids, and can be triggered both pre- or post-natally by appropriate environmental cues (Brisson, 2010; Sutherland, 1969). The formation of sexual aphids is another example of epigenetic responses (Halkett *et al.*, 2004). Although rapid epigenetic responses to changes in plant quality have not yet been studied, this could be an explanation for the changes we observed between rates of increase in two generations.

In aphids it is a natural situation for two generations (or more) to be present on the same plant. In our statistical models, we found in some cases that survival and rate of increase are significantly affected by the interaction of the factors 'generation' and 'event', which may also explain why observed effects are significant in the first, though not in the second generation. Additionally, the present paper allowed the setup of a protocol that proved to be sensitive and reproducible and can be suggested as a standard for *in planta* studies with aphids in ERA.

Significant effects in non-target tests should be compared to variation among conventionally bred varieties

Furthermore, our results point to the importance of comparing the differences found between GM-events and the non-transformed variety to the variation among available conventional varieties in the agro-ecosystem. The concept of baseline variation has been documented before and is considered a necessary part of environmental risk assessment (EFSA, 2010; Houshyani, 2012). We show that when conventional cultivars are included in the comparison of the intrinsic rate of increase, the non-transformed and GM events no longer significantly differ, and rather the variation between conventionally bred varieties is much greater than between a non-transformed cultivar and derived GM events. Though significant effects may be found between the GM potato and its non-transformed progenitor when compared pairwise, this may be insignificant compared to the extent of variation already found between different conventionally bred potato varieties. In the case of our blight resistant events, despite our sensitive assays, no biological relevance was detected for the non-target effect on aphids, since it proved to be in the range of effects present among available commercial varieties.

Acknowledgements

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Appendix (A-C). Aphid fitness parameters used to quantify aphid intrinsic population increase (mean and standard error (SE)) for the experiments at Wageningen University and ENEA on genotypes Désiree A15-31, A15-45 (A). Aphid fitness parameters measured for Rm calculations in Wageningen for experiments on all other GM events (B) and for parameters measured in baseline comparisons (C).

A

Lab	Genotype	Generation	Intrinsic rate of increase (R_m)	SE(R_m)	Effective fecundity (Md)	SE(Md)	Pre-reproductive period in days (d)	SE(d)	Survival time (days)	SE (Survival)
ENEA	Désirée	1	0.22	0.03	11.04	1.89	7	0.43	20.64	1.63
		2	0.3	0.04	11.38	2.85	5.38	0.53	18.7	3.3
	A15-31	1	0.29	0.02	11.79	1.59	6	0.49	19.86	1.53
		2	0.37	0.01	21	2.55	6	0.38	16.27	3.46
	A15-45	1	0.22	0.02	12.38	1.58	7.57	0.33	18	1.54
		2	0.33	0.06	9.75	1.47	6	0.91	20.45	3.22
Wageningen	Désirée	1	0.26	0.02	17.76	2.38	7.86	0.33	21.29	2.4
		2	0.22	0.01	14.75	1.33	9.72	0.46	21.95	2
	A15-31	1	0.32	0.01	25.56	1.63	7.6	0.21	23.32	2.5
		2	0.25	0.01	20.17	1.61	8.84	0.28	23.6	1.75
	A15-45	1	0.26	0.01	22.58	1.94	8.7	0.36	24.04	1.97
		2	0.24	0.01	18.89	2.35	9.32	0.56	25.05	1.78

B

Genotype	Generation	Intrinsic rate of increase (R_m)	SE (R_m)	Effective fecundity (Md)	SE (Md)	Pre-reproductive period in days (d)	SE(d)	Survival time (days)	SE (Survival)
Désirée	1	0.29	0.01	24	1.52	8.14	0.23	20.14	1.14
	2	0.34	0.03	30.6	3.85	7.6	0.81	17.6	1.86
A15-31	1	0.32	0.01	29.27	2.4	7.87	0.19	20.27	1.04
	2	0.34	0.01	35	4.5	7.7	0.33	19.4	0.9
A15-84	1	0.3	0.01	27.83	2.44	8	0.18	20.67	0.82
	2	0.31	0.02	28.45	3.84	8.09	0.31	19.64	0.68
A16-02	1	0.31	0.01	27.19	1.97	7.95	0.15	21.29	1.02
	2	0.32	0.01	26.44	2.44	7.78	0.22	19.11	1.23
A16-24	1	0.31	0.01	29.75	2.24	8.06	0.25	20.94	1.39
	2	0.31	0.02	28.86	4.56	8	0.44	19	1.59
A13-13	1	0.31	0.01	30.37	2.3	8.11	0.15	22.21	1.27
	2	0.34	0.01	34.47	3.4	7.8	0.31	18.47	0.9
A13-17	1	0.32	0.01	29.11	2.17	7.72	0.16	19.67	0.97
	2	0.34	0.01	33.22	4.9	7.44	0.41	19.78	1.79

C

Genotype	Intrinsic rate of increase (R_m)	SE (R_m)	Effective fecundity (Md)	SE (Md)	Pre-reproductive period in days (d)	SE(d)	Survival time (days)	SE (Survival)
Désirée	0.27	0.01	30.5	1.77	9.25	0.21	19.9	1.62
A15-31	0.25	0.02	28.43	3.53	9.5	0.2	16.25	1.86
Bintje	0.22	0.01	17.38	1.71	9.44	0.16	17.06	1.54
Premiere	0.16	0.01	11	1.95	10.82	0.3	13.33	1.45
SarpoMira	0.08	0.02	4.38	1.24	10.75	0.53	10.32	1.22

Chapter 5

Inoculation of susceptible and resistant potato plants with the late blight pathogen *Phytophthora infestans*: effects on an aphid and its parasitoid

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Abstract

Plants are exposed to microbial pathogens as well as herbivorous insects and their natural enemies. Here, we examined the effects of inoculation of potato with the late blight pathogen (*Phytophthora infestans*), on an aphid species commonly infesting potato crops, and one of the aphid's major parasitoids. We observed the peach - potato aphid *Myzus persicae* and its natural enemy, the biocontrol agent, *Aphidius colemani*, on potato plants inoculated with water or *P. infestans*. Population growth of the aphid, parasitism rate of its natural enemy and other insect life-history traits were compared on several potato genotypes, the susceptible cultivar Désirée and genetically modified (GM) isogenic lines carrying genes conferring resistance to *P. infestans*. Effects of *P. infestans* inoculation on intrinsic rate of aphid population increase and the performance of the parasitoid were only found on the susceptible cultivar. Insect traits were similar when comparing inoculated with non-inoculated resistant GM genotypes. We also tested how GM-plant characteristics such as location of gene insertion and number of R genes could influence non-target insects by comparing insect performance among GM events. Different transformation events leading to a different position of the R-gene insertion in the genome influenced aphids either with or without *P. infestans* infection, whereas effects of position of R-gene insertion on the parasitoid *A. colemani* were evident only in the presence of inoculation with *P. infestans*. We conclude that it is important to study different transformation events before continuing with further stages of risk assessment of this GM crop. This provides important information on the effects of plant resistance to a phytopathogen on non-target insects at different trophic levels.

Key Words

Plant-insect interactions, genetic modification, non-target effects, trophic interactions, plant-pathogen-insect interactions, *Phytophthora infestans, Solanum tuberosum, Myzus persicae, Aphidius colemani*

Introduction

Sustainable agro-ecosystems that support natural control of pests and diseases, are characterized by complex and diverse trophic webs (Bukovinszky *et al.*, 2008; Crowder & Jabbour, 2014; Schmidt *et al.*, 2014). Plants constitute the base of agro-ecosystems and phenotypic plasticity of plant traits can shape trophic webs (Poelman *et al.*, 2008). In early tiers of environmental risk assessments (ERA) for genetically modified (GM) crops, trials in confined conditions are important to establish possible effects on the life history of representatives of important functional groups such as parasitoids of plant pests (Andow *et al.*, 2013; Birch *et al.*, 2007; Romeis *et al.*, 2011; Romeis *et al.*, 2013). Testing selected non-target organisms (NTOs) *in planta* in greenhouse or climate room assays are compulsory for ERA of GM crops in Europe (EFSA, 2010), according to the rationale that GM plants and their metabolites represent a potential disturbance to the environment.

In the present study, we examine the effects of GM potato plants resistant to the late blight pathogen *Phytophthora infestans* Mont. De Bary (Peronosporales: Pythiaceae) in the context of ERA. The oomycete *P. infestans* infects leaves, stems and tubers of potato plants, and is aptly named the "plant destroyer", causing over 3 billion US$ of economic damage per year worldwide in lost production and control (Fry, 2008). The GM plants were modified through insertion of a major resistance gene, an R gene coding for a hypersensitive response to infection by *P. infestans.* The R gene was therefore not expected to affect herbivorous insects or their natural enemies which are non-targets of the genetically modified trait; however, such effects cannot be excluded *a priori* (Abreha et al., 2015). We studied two non-target insects, representing organisms at the second and third trophic levels of the potato agro-ecosystem. At the second trophic level, we observed the phloem-feeding peach-potato aphid *Myzus persicae* Sulzer (Hemiptera: Aphididae), a notorious vector of plant viruses and globally one of the most important insect pests of potato (Meissle *et al.*, 2012; Radcliffe, 1982). In our previous work, *M. persicae* was selected according to EFSA (European Food Safety Authority) guidelines as a focal species for potato crops. In a previous study we observed that aphids may have a higher population growth on certain GM events of *P. infestans*-resistant GM-potato and that position of the R gene insertion was a factor affecting the life-history traits of the aphid *M. persicae* (Lazebnik *et al.*, 2017). However, these findings were obtained for healthy plants, *i.e.* not infected by *P. infestans*. The present study builds on those findings by investigating the influence of the plant-pathogen interaction on non-target insects on the susceptible cultivar Désirée and the GM events derived from it. Furthermore, to better understand how these interactions may have indirect trophic effects, we used a representative of the third trophic level in this system: an aphid natural enemy and biocontrol agent, *Aphidius colemani* Viereck (Hymenoptera: Braconidae). *A. colemani* is a solitary generalist, currently reared and used worldwide for biological

control of several aphid pests. Whether in the context of risk assessment or not, the effects of plant-pathogen interactions on members of the third trophic level are rarely studied (but see Ponzio *et al.* (2013), Rostás and Turlings (2008)).

In the present study, we addressed two main questions; first: how does the effect of *P. infestans* inoculation on susceptible and resistant potato plants influence aphids and their parasitoids? To answer this question, we tested population growth of the aphid and parasitism rate of its natural enemy as well as other insect life-history traits on both susceptible plants and several resistant GM isogenic lines stemming from different transformation events. For each genotype, we tested the effect of *P. infestans* inoculation on insect performance traits.

The second question we addressed was: how do GM transformation events, differing in position or number of R genes, affect non-target aphids and their parasitoids? To address the question of R gene location, we tested clones resulting from two events of cisgenic transformation of the same R gene. Cisgenic transformation is defined here as the insertion of a gene from a crossable species in the same family. We also tested two transgenic events (due to the presence of a co-inserted antibiotic resistance marker gene NPTII) from *Escherichia coli*) containing either one or two R genes to shed light on the question of whether a second R gene could affect life-history traits of the non-target insects. Effects of position and number of R genes were tested by comparing two events, with both events either inoculated with water or with *P. infestans*, to determine if there is an interaction between particular events of the GM crop and the *P. infestans* inoculation. Both questions aim to understand the effects of pathogen inoculation on insects at the second and third trophic levels, and potentially provide support for protocol formulation in the context of risk assessments of GM crops.

Materials and Methods

Plant material

The GM events tested in this study were developed by the Laboratory of Plant Breeding of Wageningen University (Haesaert *et al.*, 2015; Haverkort *et al.*, 2016). They have been created using *Agrobacterium tumefaciens*-mediated transfer of the native *Rpi-vnt1* gene, from *Solanum venturii*, using marker-assisted (event A13-17) and marker-free transformation methods (events A15-31, A15-45) to the cultivar Désirée. Also, marker-assisted transformation was employed using a single T-DNA harboring the native *Rpi-vnt1* and *Rpi-sto1* (from *Solanum stoloniferum*) genes (event A16-02). The selectable gene for marker-assisted transformation was the *Escherischia coli* neomycin phosphotransferase type II gene (NPTII) conferring resistance to kanamycin. The tested events are described in Table 1. Events were selected as apparently "true to type" as they were morphologically indistinguishable from non-transformed Désirée under tuber-sown field conditions (Haverkort *et al.*, 2016).

Table 1: GM events of the cultivar Désirée investigated in this study and their characteristics

Name	GM-Type	Marker gene	R gene source	Name of the R gene
A15-31	Cisgenic	N/A	*Solanum venturii*	*Rpi-vnt1*
A15-45	Cisgenic	N/A	*Solanum venturii*	*Rpi-vnt1*
A13-17	Transgenic	NPTII	*Solanum venturii*	*Rpi-vnt1*
A16-02	Transgenic	NPTII	*Solanum venturii and Solanum stoloniferum*	*Rpi-vnt1; Rpi-stol*

All GM events and the cultivar Désirée were maintained *in vitro*, on agar medium (purified agar 0.8% + 2.2 g /L Murashige&Skoog + Duchefa 4.4 g/L +Sacharose 20 g/L + Micro Agar 8 g/L; pH = 5.8) in sterile containers. Containers were kept in a climate room at 16:8 light:dark conditions, and 21 ± 3 °C during light hours and 15 ± 3 °C when dark, and 70 ± 5 % relative humidity. Two independent experiments were performed. For aphid life-history experiments, cuttings were transplanted from agar to soil five weeks before the experiments to allow for root growth; the rooted cuttings were transplanted to soil in the same climate room conditions as above. For parasitoid experiments, cuttings were transplanted from agar to soil three weeks before the experiments on parasitism rate and parasitoid life-history.

Pathogen infection

Phytophthora infestans IPO-C isolate was used (Haverkort *et al.,* 2016) for all infected plants (Laboratory of Phytopathology, Wageningen University). The pathogen was maintained on both excised leaves kept in Petri dishes and tuber slices of cultivar Désirée in a cooled climate cabinet (19°C) before use in experiments. Sporangia were harvested by immersing infected leaves in cold water. Spore concentration was adjusted to 10,000 sporangia/mL by measuring the spore concentration with a Fuchs-Rosenthal haemocytometer with a depth of 0.200 mm and sixteen squares of 0.0625 mm^2 by Labor Optik and adding cold water to adjust to the desired concentration. Two droplets of 15 µL spore solution (or water, for controls) were pipetted on the underside of three leaves per plant, and for parasitoid assays, two droplets were applied on two leaves per plant to accommodate the smaller plant size in parasitoid experiments (see section 'Plant material'). To provide adequate humidity and temperature conditions for fungal growth, all plants (including water inoculated) were covered with black plastic bags and kept in a climate room at 15 ± 3 °C at *ca.* 100% RH for 24 h. Two days after the inoculation, aphids were placed on plants. The phenotype of the genetically modified events was generally similar over time after inoculation, varying slightly in the degree of visible hypersensitive response. An example of each genotype is given in Figure 1, six days after inoculation.

Figure 1. Photos of the potato genotypes used in this study after 6 days of inoculation with either water or *Phytophthora infestans*. Circles indicate necrotic regions on susceptible Désirée and arrows indicate areas of visible HR on GM events.

Aphid life history

Myzus persicae were collected in 2004 in the vicinity of Wageningen, The Netherlands (51°59'11.5"N 5°39'48.4"E) and reared at the Laboratory of Entomology, Wageningen University. They were maintained for several generations on *S. tuberosum* cultivar Désirée before experiments began under the same climate room conditions as described above.

Each experiment began with one-day-old aphid nymphs produced by adults from the rearing that had been isolated on an excised potato leaf in a Petri dish. Aphid nymphs were taken from the Petri dish after 24 h and placed singly in clip cages (25 mm diameter; 10 mm high) on the abaxial surface of three leaves of each plant. Ten plant replicates of each event (Table 1) and the non-transformed Désirée cultivar were tested; all plants were randomly distributed in the climate room. Aphids were checked every day for mortality and for offspring production; neonate nymphs were counted and removed daily. The parameters quantified were: pre-reproductive period and total fecundity, for calculation of intrinsic rate of population increase (r_m), and aphid mortality. Intrinsic rate of population increase was calculated as described in Wyatt and White (1977): $r_m = 0.74 (\ln Md) / d$, where Md is the effective fecundity and d the length of the pre-reproductive period.

Parasitoid performance and life history

The *Aphidius colemani* parasitoids were provided by Koppert Biological Systems (Berkel en Rodenrijs, The Netherlands).

The mummies delivered were placed for two days at 12 °C, then in a Petri dish inside a Bugdorm-42222F Insect Rearing Cage (Megaview Science, Taichung, Taiwan) to emerge. The eclosed adults were left for several days in the cage to mate before use in the experiments. Two bottles of water and honey were provided as a food source for the adults.

Three-week-old plants were infested with 20 three-day-old aphid nymphs and placed in a clear 1 L cylindrical container covered with fine mesh. Ten plants in containers were used per genotype. After 24 h, one female *A. colemani* was introduced and given access to hosts for 24 h and removed. The plants were checked for mummies each day and if found, they were removed from the plant and placed in a Petri dish on moistened filter paper, to record eclosion time. Petri dishes were checked daily to monitor eclosed parasitoids. Each eclosed adult was placed in the freezer for two hours after which the sex was determined using a dissecting microscope and fresh biomass measured using a Sartorius CP2P model microbalance.

Statistical analysis

Aphid intrinsic rate of population increase, parasitoid biomass and proportion parasitism were tested using a mixed linear model, or generalized linear mixed model with R Statistical Software (R Development Core Team, 2014) package 'lme4' when data did not meet the assumptions of normality; with fixed factors being 'potato event' and '*P. infestans* infection'. For *A. colemani* biomass, 'sex' was included as a fixed factor in biomass analyses. The probability that female *A. colemani* eclosed from an aphid mummy was calculated using a generalized linear mixed model with binomial distribution with fixed factors 'inoculation treatment' and 'genotype'. For analyses of aphid intrinsic rates of population increase and individual parasitoid traits (i.e. biomass, emergence time, etc.) we included 'plant number' as random factor since the effect of the individual plant was a large source of variation in aphid and parasitoid performance traits. When inoculation treatment or interaction between inoculation treatment and genotype was significant, we used subsets of the data to make pairwise comparisons between genotypes within each treatment type.

Analyses of survival, time to mummy formation and eclosion were conducted using a Cox proportional hazards regression model (Cox, 1972). This model included the same random effects as above and was performed using R Statistical Software (R Development Core Team, 2014), with the 'survival' package. Each genotype was also tested separately to test for effects of inoculation treatment; then by treatment category, to test for differences between genotypes within a treatment type.

Results

Aphid life history: intrinsic rate of population increase and survival

Overall, inoculation treatment did not influence intrinsic rate of population increase ($P = 0.393$), but genotype ($P < 0.0001$) and the interaction between genotype and treatment ($P = 0.0005$) were both predictive factors. Désirée was the only genotype on which aphid intrinsic rate of population increase was negatively affected by *P. infestans* inoculation ($P < 0.0001$, Figure 2). On GM events, however, rates of population increase of aphids were similar whether inoculated with water or *P. infestans*.

Aphids on the cisgenic A15-45 potato event had a lower intrinsic rate of population increase than on cisgenic event A15-31, containing the same R gene insertion at a different genotypic location (A15-45 vs A15-31 on water inoculated plants: $P < 0.0001$; and A15-45 vs A15-31, *P. infestans* inoculated plants: $P = 0.0008$; Figure 2). Aphid intrinsic rate of population increase did not differ on the transgenic genotypes, whether they contained one or two R genes in (A13-17 vs. A16-02: on water inoculated plants: $P = 0.61$; on *P. infestans* inoculated plants: $P = 0.60$). Intrinsic rate of aphid population increase on the single-R gene transgenic genotype A13-17 did not differ from the marker-free cisgenic counterpart with the same R gene, A15-31 (on water inoculated plants: $P = 0.062$; on *P. infestans* inoculated plants: $P = 0.34$). Aphid r_m was lower on the cisgenic clone A15-45 than on the transgenic A13-17 (on water-inoculated plants: $P < 0.0001$; on *P. infestans*-inoculated plants: $P = 0.0001$) (Figure 2).

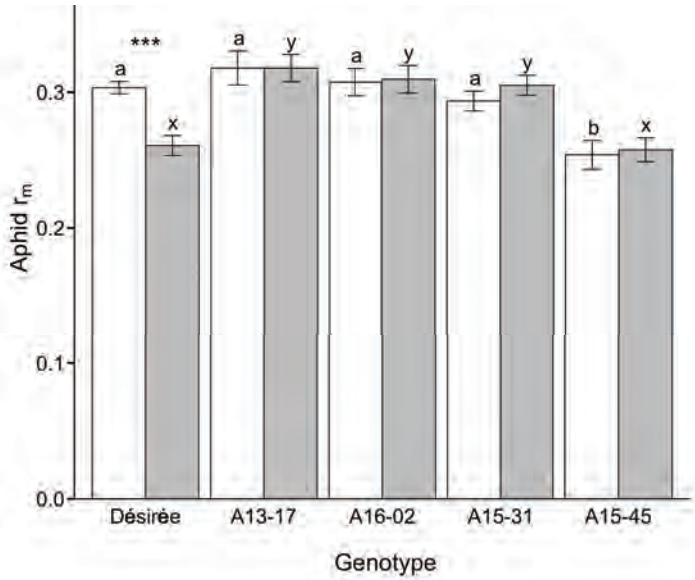

Figure 2: Mean (± SE) intrinsic rate of population increase (r_m) of *Myzus persicae* on water-inoculated (white bars) and *Phytophthora infestans*-inoculated (grey bars) potato plants. Number of plants used was 30 for Désirée, 20 for A15-31, and 10 for all other genotypes; up to three aphids per plant were monitored. Means within a treatment capped with different letters are significantly different between genotypes; the asterisks indicate a significant treatment effect within genotype.

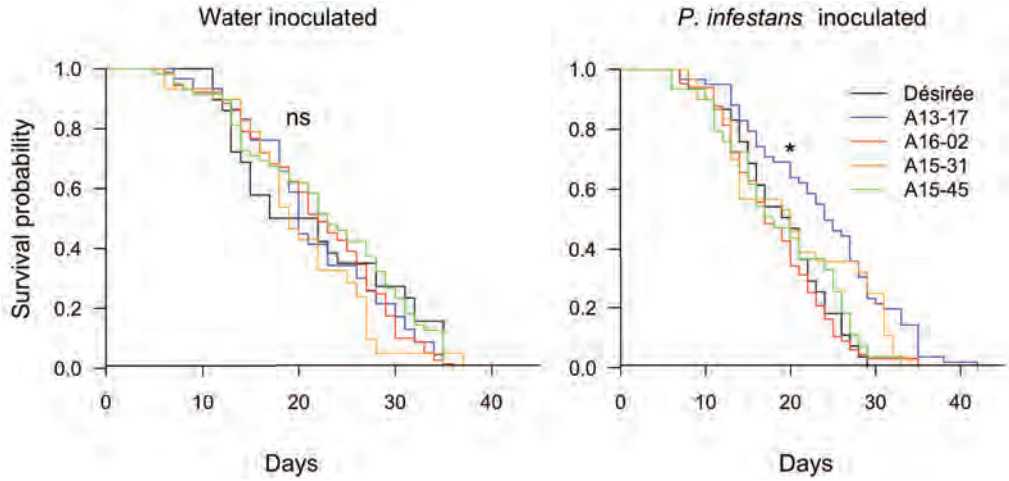

Figure 3: Aphid probability of survival over time on different genotypes of potato plants either inoculated with water or *Phytophthora infestans*. The asterisk indicates significant difference (P = 0.0017) from Désirée.

Aphid survival

The overall model showed that effects of inoculation treatment (P = 0.005) and genotype (P < 0.0001) as well as their interaction (P < 0.001) significantly affected aphid survival. When comparing water and *P. infestans* treated plants, survival on susceptible Désirée plants was lower on plants inoculated with *P. infestans* (P < 0.001). On GM events, however, the effect of inoculation treatment on aphid survival did not differ between plants inoculated with water or *P. infestans* (A13-17: P = 0.083; A16-02: P = 0.33; A15-45: P = 0.23; A15-31: P = 0.56).

On water-inoculated plants, aphids had a similar survival probability over time for all genotypes (Figure 3). However, on *P. infestans*-treated plants, though aphids had a higher survival probability on A15-31 than on Désirée plants (P < 0.0001), there was no difference in survival between aphids on A15-31 compared to the other cisgenic event A15-45 (P = 0.12). No differences in survival were found between aphids on genotypes with one or two R genes (A13-17 vs. A16-02: P = 0.79). Aphid survival probability was lower on the single-R gene transgenic genotype A13-17 than on the marker-free cisgenic counterpart with the same R gene, A15-31 (P = 0.0017), yet aphid survival was similar on the transgenic event A13-17 and the cisgenic event A15-45 (P = 0.15) (Figure 3)

Parasitoid performance: percentage parasitism

On the susceptible Désirée potato plants the percentage of aphid parasitism by *A. colemani* on *P. infestans*-inoculated plants tended to be lower than on water-inoculated plants (P = 0.062). The percentage parasitism was not influenced by inoculation treatments in any of the GM genotypes. The main effect of inoculation treatment was not a predictive factor of percentage parasitism (P = 0.29), nor was

the interaction between the genotype and treatment (P = 0.50); yet, plant genotype did significantly influence percentage parasitism overall (P = 0.009). The factor of plant genotype was only important when plants were inoculated with *P. infestans* since on water-inoculated plants, percentage parasitism of *M. persicae* aphids by *A. colemani* was similar for all genotypes (Figure 4).

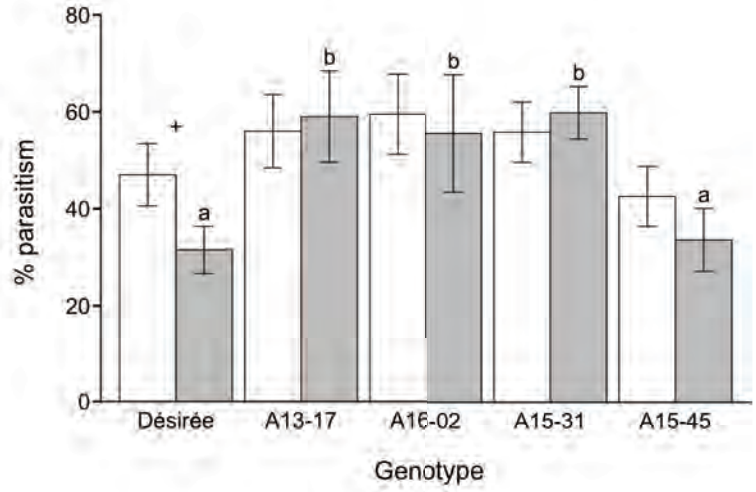

Figure 4: Mean (± SE) parasitism (%) of *Myzus persicae* nymphs by *Aphidius colemani* parasitoids on potato plants of different genotypes either inoculated with water (white bars) or with *Phytophthora infestans* (grey bars; n = 20 plants for Désirée and A15-31, n = 10 for the other genotypes). Means within the *P. infestans* inoculation treatment capped with different letters are significantly different among genotypes. Comparing treatments within the genotype Désirée, '+' indicates P = 0.0617.

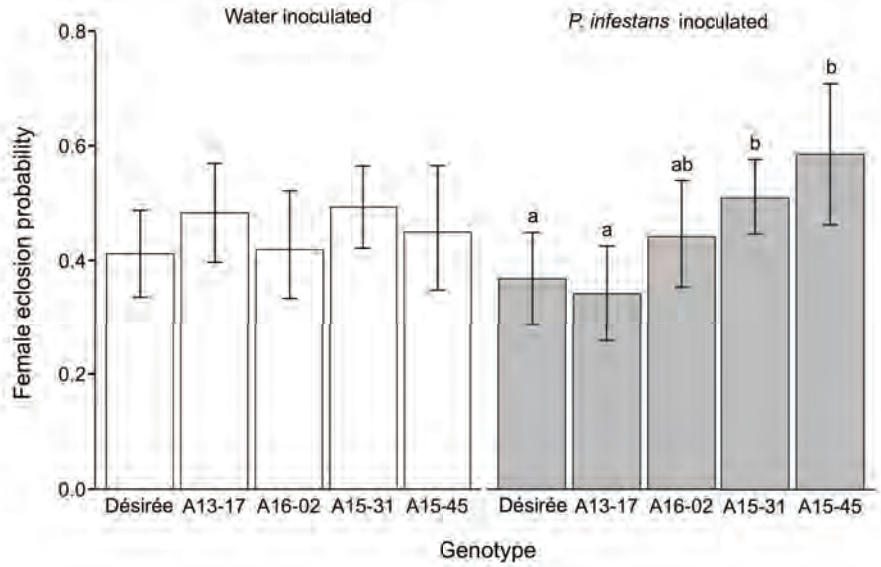

Figure 5: Probability (±95% confidence interval) of female *Aphidius colemani* parasitoids eclosing from *Myzus persicae* nymphs on different genotypes of potato plants either inoculated with water (white bars) or with *Phytophthora infestans* (grey bars). Means within the treatment group capped with different letters are significantly different.

On *P. infestans*-inoculated plants, however, percentage parasitism on the cisgenic A15-45 event was lower than on A15-31, containing the same R gene insertion at a different genomic position (P =0.012; Figure 4). Percentage parasitism on transgenic genotypes with one or two R genes did not differ (A13-17 vs. A16-02: P = 0.77). Percentage parasitism on the single-R gene transgenic genotype A13-17 did not differ from the marker-free cisgenic counterpart with the same R gene, A15-31 (P = 0.94), but percentage parasitism on genotype A13-17 was higher than on the cisgenic event A15-45 (P = 0.034) (Figure 4).

Female eclosion probability

Overall, the genotype influenced the probability of female parasitoids eclosing (P = 0.020). Neither inoculation treatment (P = 0.93) nor interaction between genotype and inoculation treatment (P = 0.42) affected female eclosion probability. When potato plants were inoculated with *P. infestans* more females eclosed from both cisgenic genotypes A15-31 (P = 0.013) and A15-45 (P = 0.006) than from the transgenic counterpart A13-17 (Figure 5). No differences in female emergence probability were found between A15-31 and A15-45 nor between A13-17 vs. A16-02 (Figure 5).

Parasitoid life history: biomass, time until mummy formation and eclosion

Generally parasitoid sex was the only factor significantly influencing biomass. Biomass of eclosed parasitoids in both treatments was overall higher for females than males (P < 0.0001; Figure 6). For both sexes, biomass was unaffected by plant genotype, inoculation treatment or the interaction between the two.

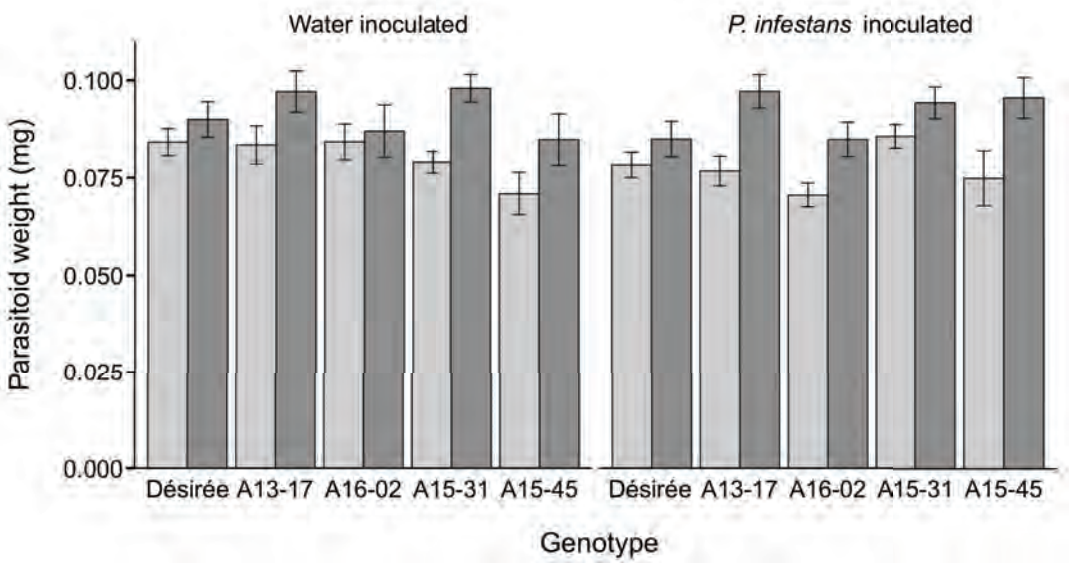

Figure 5: Mean (± SE) biomass (mg) of male (light grey bars) and female (dark grey bars) *Aphidius colemani* parasitoids eclosed from *Myzus persicae* aphids on potato plants of different genotypes.

Time until mummy formation was significantly affected by the sex of the parasitoid ($P = 0.002$) as well as the interaction between the factors: sex, inoculation treatment and genotype of the plant. Sex of the eclosed parasitoid influenced mummy formation only in Désirée and in A15-31, with males having a shorter time until mummification than females (Désirée: $P = 0.011$; A15-31: $P = 0.014$). For male and female parasitoids, time until mummy formation was influenced by the interaction of genotype and treatment (males: $P < 0.0001$; females: $P < 0.0001$). Yet, neither genotype nor treatment alone were good predictors of mummy formation in either sex.

Time until adult emergence was not influenced by parasitoid sex, yet the interaction between genotype, treatment and parasitoid sex did influence the emergence time of the parasitoids ($P < 0.0001$). In both sexes emergence time was influenced by the interaction between genotype and treatment (for females: $P = 0.012$; for males; $P = 0.0001$). Neither male nor female parasitoid emergence times could be predicted by the factors genotype or treatment alone.

Discussion

We studied how inoculation of potato plants with the late blight pathogen affects an aphid and an its parasitoid, non-target organisms of GM potatoes modified for resistance to the late blight pathogen *P. infestans*. We investigated whether different events of a genetic modification for the same trait could affect the interactions with the non-target insects.

Aphid survival and growth were affected by the interaction between potato plants and *P. infestans* on the susceptible cultivar Désirée , yet not in any of the resistant GM events. There are very few studies investigating the plant-mediated effects of oomycete plant pathogens on aphids. *Phytophthora* root rot was shown to negatively impact pea aphids on arrowleaf clover (Ellsbury *et al.*, 1985); however, to this day limited generalizations can be made concerning tripartite interactions among plants, plant pathogens and phloem feeders. The current evidence shows that biotrophic (or hemibiotrophic) pathogens can either facilitate or inhibit phloem feeders (Lazebnik *et al.*, 2014). For higher trophic levels, it is becoming clear that pathogen infection can alter the entire associated food web (Tack & Dicke, 2013), though more studies on tripartite interactions with multiple biotic stressors considering the third trophic level are needed to draw conclusions on consistent patterns. Incompatible interactions such as between the resistant GM-events and the *P. infestans* pathogen did not affect performance of the non-target pest *M. persicae*.

Reduction in aphid parasitism by *A. colemani* was seen on susceptible late-blight-infected Désirée plants. We conclude from this finding that infection by *P. infestans* reduced plant quality, and in turn reduced aphid performance (r_m) and subsequently

parasitoid performance (percentage parasitism). Reduction in plant quality has been shown to influence multiple trophic levels in several systems, for example root herbivores influencing aphids and their parasitoids (Soler *et al.*, 2005) or mildew-infected *Plantago lanceolata* plants reducing the parasitoid quality and slowing down the development time of its larval host *Melitaea cinxia* (Van Nouhuys & Laine, 2008). Parasitoid performance is known to be highly dependent on aphid host quality (Jarošík *et al.*, 2003; Schädler *et al.*, 2010). In these experiments we have shown that on the genotypes on which aphids developed poorly, the parasitoids performed poorly as well, parasitizing lower proportions of aphids and producing offspring of lower biomass. Interestingly, the reduction in aphid performance on infected Désirée plants did not lead to negative effects on parasitoid life-history traits such as biomass, development time or sex ratio. This confirms that effects of host plants can become attenuated higher up the trophic chain (Schädler *et al.*, 2010), or that some parasitoid traits are less susceptible to host quality.

It was clear from the phenotype of the plants used in this study that Désireé plants were severely affected by the inoculation of the pathogen whereas resistant genotypes exhibited a hypersensitivity response that halted the spread of infection. Since the only reductions in insect performance traits were seen on susceptible plants with notable pathogen infection this indicates that these reductions in performance may be caused by the infection. This suggests that on any GM-genotype in a mono-culture (or mono-genotype) field situation, the presence of the target pathogen on the events we tested should not affect the non-target insects on the resistant plants. In terms of risk assessment, this finding suggests that testing for non-target effects of *P. infestans*-resistant GM potatoes inoculation of the pathogen would not provide further useful information. In a recent study by Abreha et al. (2015) however, oviposition preference of S*podoptera littoralis* was affected by the plant-pathogen interaction between a resistant GM-potato and *P. infestans,* indicating that gene-for-gene interactions between the R gene avirulence protein and the pathogen effector can have consequences for organisms not involved in this interaction. There is a lack of research testing gene-for-gene interactions on non-target stress inducers. Yet, recently, Ponzio *et al.* (2016) , showed that compatible and incompatible interactions with the bacterial pathogen *Xanthomonas campestris* result in differential emission of volatile blends by *Brassica nigra* plants and that both are highly attractive to *Cotesia glomerata* parasitoids. Further investigation is warranted to better understand the effects of gene-for-gene interactions on non-target organisms.

Whether position or number of R genes in GM crops is a factor in plant responses to non-target insects is a novel discussion. There are several generalizations that can be made based on our findings. Differences in *M. persicae* r_m and % parasitism by *A. colemani* found between genotypes A15-31 and A15-45, were only found in the presence of the *P. infestans*-inoculation treatment. Therefore, effects of GM

characteristics may be dependent on the interaction with the target, and in the absence of this interaction, few differences in terms of NTO performance could be attributed to the particular GM event. In our study the effects of position of the R gene insertion and presence of the antibiotic marker cannot be separated from each other. To test the effects of the NPTII marker gene directly, a genotype containing only the NPTII marker and no R genes would have to be included in the comparisons. With our comparison, there is no conclusive effect of marker gene insertion on the non-target organisms. The effect of R gene position, however, was noted for aphids in both *P. infestans*-inoculated and water-inoculated plants. This could indicate that not all insertion events of the same gene, achieved through *Agrobacterium tumefaciens*-mediated transfer, have the same effects on non-target insects. Genetic modification of the Desirée cultivar has previously been shown to have unexpected effects on *M. persicae*, and pleiotropic effects can influence aphid developmental traits both positively and negatively (Alla *et al.*, 2003). We can also conclude that *A. colemani* female weight and eclosion time are traits which are not sensitive to changes in the GM event, and thus might be inconclusive measurable traits for potential risk assessments of this particular GM trait. Lastly, and in congruence with our previous study (Lazebnik *et al.,* 2016), results show that for the *P. infestans*-resistant potato lines, there was no influence of the inclusion of the additional R gene *Rpi-stol*, on any of the non-target insect traits measured. With respect to sustainability of GM potatoes, this additional R gene in the cultivar is predicted to create more durable resistance to *P. infestans* (Haesaert *et al.*, 2015; Haverkort *et al.*, 2016).

In previous experiments, we found a higher intrinsic rate of population increase of *M. persicae* on the cisgenic event A15-31 and no differences for the event A15-45 compared to Désirée. The differences between the experiments may be attributed to the water inoculation and cold treatment (15°C, 100% humidity and dark conditions for 24 h), a necessary control in the current experiments to adequately compare plants treated with *P. infestans*. We tested this hypothesis by comparing water-inoculated and cold-treated plants and found that water inoculation treatment does in fact influence aphid intrinsic rate of population increase (unpublished). Another possible factor is location, either climate room or greenhouse, which affects plant growth and therefore conditions for aphid development. Abiotic interactions can influence plant-insect interactions (Atkinson & Urwin, 2012), and growing conditions of the plants must be tightly controlled to obtain reproducible results.

This research contributes to our understanding of how plant resistance traits can impact non-target insects at different trophic levels. Studying this in the context of GM or naturally resistant cultivars is a relatively underexplored area of research. We exemplify the need for testing several GM events for possible effects on non-target organisms, and show in general that inoculation treatment by the target pathogen does not in itself affect responses of a non-target aphid and its parasitoid. Our

study indicates that a pre-screening of several GM-events for non-target effects in the presence of the target is advisable before proceeding with a complete risk assessment of a GM candidate.

Acknowledgements
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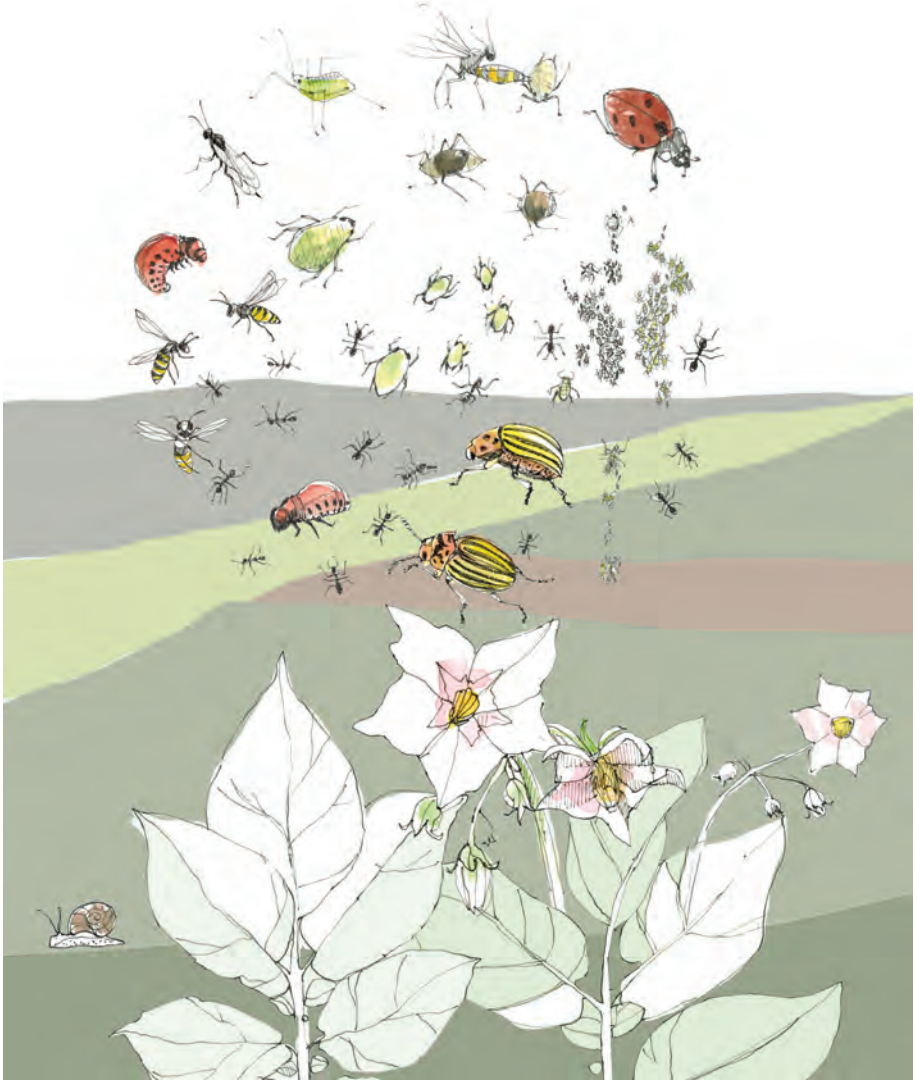
Chapter 6

Biodiversity analyses for risk assessment of genetically modified potato

Jenny Lazebnik, Marcel Dicke, Cajo J. F. ter Braak & Joop J. A. van Loon



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Abstract

An environmental risk assessment for the introduction of genetically modified crops includes assessing the consequences for biodiversity. In this study arthropod biodiversity was measured using pitfall traps in potato agro-ecosystems in Ireland and The Netherlands over two years. We tested the impact of site, year, potato genotype, and fungicide management regime on arthropod community composition. Three potato genotypes were compared: the cultivar Désirée, susceptible to the late blight pathogen *Phytophthora infestans*, a genetically modified cisgenic clone of Désirée resistant to *P. infestans* and the cultivar Sarpo Mira, also resistant to late blight. We aimed to test several ways to measure biodiversity in the context of risk assessment by using both univariate biodiversity indices and multivariate ordination methods, categorizing the pitfall trap catch by taxonomic or functional category. The Shannon-Wiener and Simpson biodiversity indices both showed strong differences between sites, years and potato genotypes, but showed no effects of the fungicide management regime. The effect of genotype was due to cultivar differences between Désirée and Sarpo Mira rather than between the GM-event (A15-31) and its isogenic comparator Désirée. Multivariate permutation analyses and RDA ordination confirmed these findings and also showed interactions between year, site and either genotype or treatment. The added value of the multivariate analysis was that it provided information on the specific arthropod groups or taxa that contributed to community structure. Multivariate analyses are recommended for use as a sensitive method to compare functionally important arthropod groups driving community structure within the framework of environmental risk assessments, or for the process of indicator species selection.

Key Words

Biodiversity, environmental risk assessment, genetically modified crops, biodiversity index, multivariate biodiversity analysis, functional groups

Introduction

The value of biodiversity for ecosystem functioning has been demonstrated in many environments (Hooper *et al.*, 2005; Reiss *et al.*, 2009; Tilman *et al.*, 2014). Greater biodiversity is commonly associated with higher agricultural yields, better biological control (Aquilino *et al.*, 2005), more efficient land use, and many other ecosystem services (Benayas *et al.*, 2009; Swift & Anderson, 1994; Tilman *et al.*, 2014). Therefore, when it has been decided that environmental risks need to be assessed under field conditions (EFSA, 2010), monitoring of changes in biodiversity is essential.

Methods for assessing environmental risks of genetically modified (GM) crops are still being developed and debated (Devos *et al.*, 2016; Johnson *et al.*, 2007; Stirling, 2007; Todt & Luján, 2014). Although the guidance documents of the European Food Safety Authority (EFSA) emphasize the importance of conservation and protection of biodiversity in the European Union (EFSA, 2007, 2010), there are no uniform guidelines for assessing biodiversity. Quantifying biodiversity is a prerequisite for being able to reach set targets. The European biodiversity targets for 2020 aim to "halt biodiversity loss and the degradation of ecosystem services… [by] protecting and restoring biodiversity and associated ecosystem services, enhancing the positive contribution of agriculture and forestry and reducing key pressures on EU biodiversity, [thereby] stepping up the EU's contribution to global biodiversity" (European Commission, 2011) . While setting biodiversity targets is essential to make progress towards sustainability of agriculture, we are still hindered by the lack of consensus about how to measure biodiversity for environmental risk assessment (ERA). There are many ways to measure biodiversity (Balvanera *et al.*, 2006; Magurran, 2004), all of which may provide different information about the species assemblages in a given community. We aim to compare several ways of measuring biodiversity, and to evaluate advantages and disadvantages of using these different measures in the context of risk assessment.

There are several guidance articles about choosing focal, indicator or surrogate species for ERA (Arpaia, 2010; EFSA, 2010; Hilbeck *et al.*, 2006; Hilbeck *et al.*, 2008; Hilbeck *et al.*, 2014). There are also very concrete directives for setting limits of concern for endpoints, which are often total counts of the individuals belonging to focal or indicator species. Single-species counts are unsuitable for representing community structure, nor do they take into account any trophic links between species or groups present. Single-variable biodiversity indices, however, were also deemed insufficient for accurately measuring effects on biodiversity (EFSA, 2010; Perry *et al.*, 2009). For this reason, multivariate analyses are considered useful in the guidance documents (EFSA, 2010; Perry *et al.*, 2009). In both cases, there is no concrete advice given to the manner in which biodiversity in the receiving environments of GM crops should be quantified.

In 2012, the European Union commissioned the four-year project "Assessing and Monitoring the Impacts of Genetically modified plants on Agro-ecosystems" (AMIGA) (Arpaia *et al.*, 2014). This project made use of EFSA guidance documents for testing environmental impacts of GM crops (EFSA, 2007, 2010), and developed methodology and executed proof-of-concept studies for testing non-target effects of GM potato and maize. Non-target organisms (NTOs) are defined as "all those species directly and/or indirectly exposed to the GM plant, and which are not the targets of the newly expressed metabolite(s) in these plants" (Arpaia, 2009). For testing of GM potato modified for resistance to late blight (*Phytophthora infestans*), experimental fields were set up from which arthropod biodiversity data were collected. We have conducted field experiments with GM potatoes in Ireland and The Netherlands, two countries in the Atlantic biogeographic zone as defined by the European Commission (Pinborg, 2016).

This study has two main aims: first, to investigate the importance of collection site and year in predicting arthropod biodiversity; second, to assess within each site how fungicide spraying regime (none, weekly or IPM 2.0 (Kessel *et al.*, 2016)) and genotype (the susceptible potato cultivar Désirée, resistant GM Désirée (transformed with a gene conferring resistance to *P. infestans*), and the resistant non-GM cultivar Sarpo Mira) influence the assemblage of arthropods.

We analyzed biodiversity at the two sites using both univariate (linear mixed effects model) and multivariate analyses: Nonmetric Multidimentional Scaling (NMDS) and redundancy analysis (RDA) with permutation tests; either using taxonomic (family level) or functional grouping. We used relative diversity (total richness divided by total abundance); and two well-known biodiversity indices: Shannon-Wiener (H') and Simpson (1-D). The Shannon-Wiener index takes into account species richness and diversity such that unique species and higher evenness increase the value. The Simpson index indicates the chance that two random draws from a population represent individuals of the same type (in this case taxon or functional group), and subtraction from 1 ensures that the index increases with diversity. These different statistical approaches were evaluated for their sensitivity to reveal differences by comparing the significance of explanatory factors and comparing them for consistency of biological conclusions made from the results of each approach. We then discuss the feasibility of these approaches and usefulness of each grouping method in ecological risk assessment. We aim to provide advice for monitoring biodiversity as part of the risk assessment of genetically modified crops, and more generally for cases where biodiversity is deemed an important trait to be assessed.

Methods

Plant material and experimental design

Two potato cultivars and one GM event were used in the field trials: the highly susceptible cultivar Désirée, the highly resistant cultivar Sarpo Mira and the highly resistant Désirée-derived, cisgenically modified event A15-31 (detailed description in Haesaert *et al.* (2015); Haverkort *et al.* (2016)). Jacobsen and Schouten (2007) generated A15-31 through cisgenic modification of the Désirée cultivar through the transfer of an R gene coding for resistance to *P. infestans: Rpi-Vnt1.1* (Pel *et al.*, 2009), originally obtained from *Solanum venturii*. The GM event in this study was created at the Laboratory of Plant Breeding of Wageningen University and Research.

Field trials were carried out in 2013 and 2014 in The Netherlands (Valthermond; GPS coordinates 52.871829846N and 6.942662896E) and in Ireland (Oak Park, Carlow; GPS coordinates 52.8560667N and 6.9121167W). These trials were carried out under permit IM10-006 for The Netherlands and in Ireland were licensed by the Environmental Protection Agency as per Notification No. B/IE/12/01. Valthermond has predominantly reclaimed peat soil (90.1% sand, 9.9% organic matter, pH = 5.1). The Oak Park campus in Carlow has a mix of light textured gravelly and heavy textured soils derived from limestone till, commonly known as boulder clay. The experimental design at both sites is described in Table 1. Plots (6 x 6 m in The Netherlands and 3 x 3 m in Ireland) were 6 m apart at both sites with grass in between. The nine plots per replicate (block) were randomly assigned to one of the nine combinations of genotype and management regime (no fungicide, weekly fungicide spraying or management using IPM2.0). Plot management regimes and specific qualities of each site are described in detail by Kessel *et al.* (2016).

Table 1: Experimental field designs in The Netherlands and in Ireland

The Netherlands		
Potato genotypes	3	Désirée, A15-31 and non-GM resistant cultivar: Sarpo Mira
Fungicide regimes	3	None, Weekly, IPM 2.0
Blocks	7	Total of 3 * 3 * 7 = 63 plots
Plot size	6 x 6 m	
Ireland		
Potato genotypes	3	Désirée, A15-31 and non-GM resistant cultivar: Sarpo Mira
Fungicide regimes	3	None, Weekly, IPM 2.0
Blocks	6	Total of 3 * 3 * 6 = 54 plots
Plot size	3 x 3 m	

At both sites, two pitfall traps were placed in the center of each plot, 1 m apart and connected by 10 cm high plastic edging pressed into the soil, to facilitate insect edging behaviour. Pitfall traps were 1 L plastic containers with an opening of 10 cm diameter, each containing 100 mL 70% ethylene glycol. Both traps were covered with an aluminum cover about 2 cm off the ground to protect the trap from rain, leaving room for ground dwellers to enter. Traps were left in the plots for one week, three times throughout the field season, with about four weeks between two trapping sessions.

Identification of species to family and to functional groups

The identification of arthropods, mollusks or oligochaetes from pitfall trap samples was done using appropriate dichotomous taxonomic keys (Goulet *et al.*, 1993; Triplehorn *et al.*, 2005). Family level was used by default for taxonomic grouping, though when identification to family was not feasible, the order, sub-order or super-family was used, for example the super-family Aphidoidea (aphids) or Entomobryomorpha (sub-order of Collembola). Each family grouping was assigned to one or two of the following ecological functional groups: predators, detritivores, parasitoids, fungivores, herbivores, hyperparasitoids or unknown (see Appendix A). This means that in some cases, functional groups may contain the same family, exclusivity per functional group could not be achieved with family level identification. This was determined using several resources on the biology of each insect taxon ("BugGuide," 2016; Goulet *et al.*, 1993; Oosterbroek, 2006; Triplehorn *et al.*, 2005).

Statistical analysis and sampling data

For each site and plot, we summed the arthropod abundances per plot and (collected in all one-week trapping sessions over the whole season) and divided by the number of trapping sessions (usually 3, but sometimes trap data were unavailable due to flooding, or other plot specific incidents) to obtain mean abundance per plot. For multivariate analyses at family level, data was discarded from the analyses if the mean sum in all traps over all weeks for a given family was less than 1. The total numbers of arthropods collected and identified to family is given in Table 2. All statistical analyses were performed using R version 3.0.2 (R Development Core Team 2013).

Univariate analyses

The mean abundances were used to compute Shannon-Wiener and Simpson indices, familial richness or functional group diversity, defined as a ratio of the number of taxa (i.e. richness) and the number of arthropods collected. Each index was calculated using the R package 'vegan' with the function 'diversity'. A mixed effects linear model was performed using the R package 'lme4' to calculate the effects of year, site, genotype, treatment (fungicide regime) and interaction effects of those factors on biodiversity (for both functional and taxonomic groupings). Since treatments in

Table 2. Numbers of arthropods collected and identified to family at each year and site.

Site	Year	Number identified
Valthermond (NL)	2013	25080
Valthermond (NL)	2014	20600
Carlow (IR)	2013	10439
Carlow (IR)	2014	23458
	Total:	79577

both field sites were arranged into blocks the random factor 'block' was accounted for in the mixed effects linear model. After determining the significance of each of the factors, a post-hoc test was performed using the R package 'multcomp' in order to better understand the cause of variability between the genotypes. Since there were several interaction effects between genotype and site, and genotype and year, the post-hoc pairwise comparisons between genotypes were split by site and year.

Multivariate ordination

To graphically visualize the effects of site and year on the arthropod communities at each site, we used Non-Metric Multidimensional Scaling (NMDS; ecodist package in R), an ordination method well-suited to community data with many zeroes (McCune and Grace, 2002). We used NMDS analysis for two types of data: arthropod families and functional groups (predators, detritivores, parasitoids, fungivores, herbivores, hyperparasitoids and unknown). We performed a separate NMDS analysis for families and functional group data, considering factors year and site. Bray–Curtis dissimilarity matrices were used for distance calculations. Distances between samples were ranked and represented in a two-dimensional configuration as in all cases stress values did not exceed 0.3. The most similar points, each point representing a plot, in terms of taxon diversity and abundances were displayed closer together. Vectors represent functional groups or orders and families that had an $R^2 > 0.3$ as well as a significant effect of $P < 0.001$ as calculated by the 'envfit' function in R with 999 permutations. This was done to ensure that only the most relevant groups for determining community structure would be displayed, and for legibility of the ordination. The 'envfit' function calculates the correlation between the point distribution and a given taxonomic or functional group, and gives coordinates to plot a vector with the strength and direction of the correlation.

Multivariate constrained ordination (RDA) and permutation tests

In order to test the effect of the four factors (site, year, genotype and treatment) and their interactions on the multivariate community (both for functional and taxonomic groupings) using the 'vegan' package in R, we performed a redundancy analysis (RDA). The abundance data were first log transformed to make the model multiplicative on the abundance scale - which is more ecologically meaningful than the additive scale - and to avoid undue influence of extremely high values. The significance of each term of the model (comprised of the four main factors and their interactions) and axes were tested sequentially against the results of 999 permutations (Legendre & Anderson, 1999). The tests on genotype and treatment used permutations restricted within each block.

Results

Data of the mean number of arthropods caught per plot at each site, year, and for functional groups, have been graphically summarized for each genotype–treatment combination (Appendix B). For taxonomic categories, means with standard error are summarized in four tables, each representing one year and one site (Appendix C, which can be accessed at this publically accessible link: https://goo.gl/KlmwUF

Effects of potato genotype and fungicide treatment on biodiversity also depend on the site and year of sampling

The results of the univariate analyses showed that the three biodiversity indices measured were affected by the site, year and potato genotype. The interactions between any two of those three factors as well as the interaction between all three were also significant for the models conducted using taxonomic groups (Table 3). The tests conducted with the Shannon-Wiener and the Simpson indices resulted in the same conclusions with regards to significant factors as when the index was calculated using taxonomic groups. When calculating the indices with functional groups, however, the indices did not always result in the same relevant factors (Table 3A, 3B). For example, when testing the indices using functional groups the Simpson index did detect year as a relevant factor, whereas the Shannon-Wiener index did not, and the interaction between site year and genotype was seen as significant when calculated with the Shannon-Wiener index, but not with the Simpson index. Relative diversity results were mostly in agreement with the other two indices for the main effects of site, year and genotype, yet differed in the significance of the interactions between the factors year and genotype, even when calculating the relative diversity using the taxonomic groups (Table 3C).

In order to determine which genotype was the source of the variation in biodiversity measured by these indices, a post-hoc test was conducted by separating the groups by

Table 3. Results of univariate biodiversity analyses of arthropods collected in Valthermond NL in 2013 and 2014 and in Carlow IR in 2013 and 2014 from pitfall traps identified to functional group or to family level. Three different measures of biodiversity were considered: Shannon-Wiener index (A), Simpson index (B), and relative diversity (C) (diversity/ abundance: number of individuals per trap). Overall effects are shown by F and P values, and significant factors or interactions are given in bold.

A. **Shannon-Wiener index**		*By taxonomic groups*		*By functional groups*	
	DF	**F**	**P**	**F**	**P**
Site	1	68.359	**<0.0001**	66.875	**<0.0001**
Year	1	102.217	**<0.0001**	3.025	0.084
Genotype	2	4.242	**0.016**	5.774	**0.004**
Treatment	2	0.208	0.812	0.467	0.628
Site x Year	1	94.719	**<0.0001**	104.676	**<0.0001**
Site x Genotype	2	9.289	**<0.001**	8.000	**<0.001**
Year x Genotype	2	4.389	**0.014**	5.338	**0.006**
Site x Treatment	2	0.067	0.935	0.122	0.885
Year x Treatment	2	0.259	0.772	0.526	0.592
Genotype x Treatment	4	0.64	0.634	0.806	0.523
Site x Year x Genotype	2	4.127	**0.018**	7.629	**<0.001**
Site x Year x Treatment	2	0.797	0.452	0.202	0.817
Site x Genotype x Treatment	4	0.25	0.909	0.562	0.690
Year x Genotype x Treatment	4	0.733	0.570	0.495	0.740
Site x Year x Genotype x Treatment	4	0.753	0.557	0.577	0.680

B. **Simpson index**		*By taxonomic groups*		*By functional groups*	
	DF	**F**	**P**	**F**	**P**
Site	1	122.958	**<0.0001**	34.939	**<0.0001**
Year	1	143.217	**<0.0001**	29.099	**<0.0001**
Genotype	2	5.123	**0.007**	5.157	**0.007**
Treatment	2	0.225	0.799	0.067	0.935
Site x Year	1	127.737	**<0.0001**	148.258	**<0.0001**
Site x Genotype	2	7.864	**<0.001**	4.447	**0.013**
Year x Genotype	2	5.326	**0.006**	3.817	**0.024**
Site x Treatment	2	0.042	0.958	1.085	0.340
Year x Treatment	2	0.187	0.829	2.690	0.070
Genotype x Treatment	4	0.553	0.697	1.700	0.152
Site x Year x Genotype	2	4.707	**0.01**	1.737	0.179
Site x Year x Treatment	2	0.582	0.560	0.378	0.686
Site x Genotype x Treatment	4	0.248	0.911	0.507	0.731
Year x Genotype x Treatment	4	0.532	0.713	2.317	0.059
Site x Year x Genotype x Treatment	4	0.816	0.516	2.039	0.091

C. **Relative diversity**	*By taxonomic groups*			*By functional groups*	
	DF	**F**	**P**	**F**	**P**
Site	1	13.540	**<0.001**	34.939	**<0.0001**
Year	1	20.136	**<0.0001**	29.099	**<0.0001**
Genotype	2	3.572	**0.030**	5.157	**0.007**
Treatment	2	0.314	0.731	0.067	0.935
Site x Year	1	130.108	**<0.0001**	148.258	**<0.0001**
Site x Genotype	2	8.827	**<0.0001**	4.447	**0.013**
Year x Genotype	2	4.136	0.174	3.817	**0.024**
Site x Treatment	2	0.414	0.662	1.085	0.340
Year x Treatment	2	0.879	0.417	2.690	0.070
Genotype x Treatment	4	1.055	0.380	1.700	0.152
Site x Year x Genotype	2	3.434	**0.034**	1.737	0.179
Site x Year x Treatment	2	1.521	0.221	0.378	0.686
Site x Genotype x Treatment	4	0.766	0.548	0.507	0.731
Year x Genotype x Treatment	4	1.944	0.105	2.317	0.059
Site x Year x Genotype x Treatment	4	1.881	0.115	2.039	0.091

Table 4: Results of post-hoc tests comparing genotypes sampled in The Netherlands and Ireland in 2013 and 2014 for three different biodiversity indices (Shannon-Wiener, Simpson or Relative diversity) for arthropods identified to either functional group or family level. Due to the relevant interactions between site and genotype and year and genotype, the post-hoc tests were constrained by site (A) or year (B) in order to make pairwise comparisons.

A. By SITE	**Shannon-Wiener**		**Simpson**		**Relative diversity**	
	NL	IR	**NL**	IR	**NL**	IR
Désirée - A15-31	0.618	0.241	0.803	0.598	0.078	0.295
Désirée - Sarpo Mira	**0.000**	0.98	**0.000**	0.992	**0.020**	0.935
A15-31 - Sarpo Mira	**< 0.0001**	0.33	**< 0.0001**	0.676	**<0.0001**	0.487

B. By YEAR	**Shannon-Wiener**		**Simpson**		**Relative diversity**	
	2013	2014	**2013**	2014	**2013**	2014
Désirée - A15-31	0.870	0.408	0.929	0.726	0.668	0.946
Désirée - Sarpo Mira	**0.005**	0.734	**0.001**	0.917	**0.012**	0.995
A15-31 - Sarpo Mira	**0.001**	0.856	**0.000**	0.929	**<0.001**	0.972

year and site, since these were relevant interacting factors. This post-hoc showed no differences between the Désiree and the GM comparator A15-31. When differences were detected, these were only in comparison with Sarpo Mira (Table 4).

Multivariate analyses can detect important components of community structure

The effects of site and year are also clear from the NMDS ordination graphs. Notably, for each year and site combination the point clusters clearly separate when classified based on functional group (Figure 1C), but the separation is much more distinct when data are classified based on taxonomy (Figure 2C). The ordinations show which functional groups or taxonomic groups play a significant role in the separation between the plots in terms of community composition.

In 2013 herbivores, predators and detritivores were the most important functional groups underlying the separation between arthropod communities found in Irish and Dutch pitfall traps (Figure 1A). In Figure 1A a positive correlation is shown (acute angle between vectors) between parasitoids and detritivores, and a weaker yet positive correlation between herbivores and predators and between parasitoids and hyperparasitoids. Vectors with angles at 90 or 270 degrees, such as between herbivores and detritivores are not correlated. Figure 2A shows that the taxa driving the separation between sites in 2013 in Ireland are the positively correlated Aphidoidea (aphids), Thysanoptera (thrips), Carabidae and Poduromorpha. In The Netherlands, the community was shaped by positively correlated detritivores Sciaridae, Mesostigmata and Entomobryomorpha as well as predatory Coleoptera larvae (unknown families) (Figure 2A).

Vector length is indicative of the strength of the relationship to the ordination points, so not all vectors will have correspondence in the family level ordination. For example the 'parasitoids' vector in Figure 1A is not as long compared to the other vectors, and thus no parasitoid families are shown in Figure 2A, since only the most significant families were displayed, for which $P < 0.001$ and $R^2 > 0.3$.

In Ireland in 2014 the arthropod community was mostly characterized by the detritivores and the positively correlated predator and parasitoid functional groups (Figure 1B). Hyperparasitoids and fungivores were also highly correlated, though with shorter vectors, playing a smaller role in community structure (Figure 1B). In The Netherlands, herbivores were an important functional group, though they played a smaller role than the other functional groups in determining the overall community structure.

The important arthropods in the 2014 Dutch and Irish pitfall traps have been specified to family or order level in Figure 2B. In Ireland detritivores were comprised of Mesostigmata, Opiliones, Poduromorpha and Symphypleona and the most important

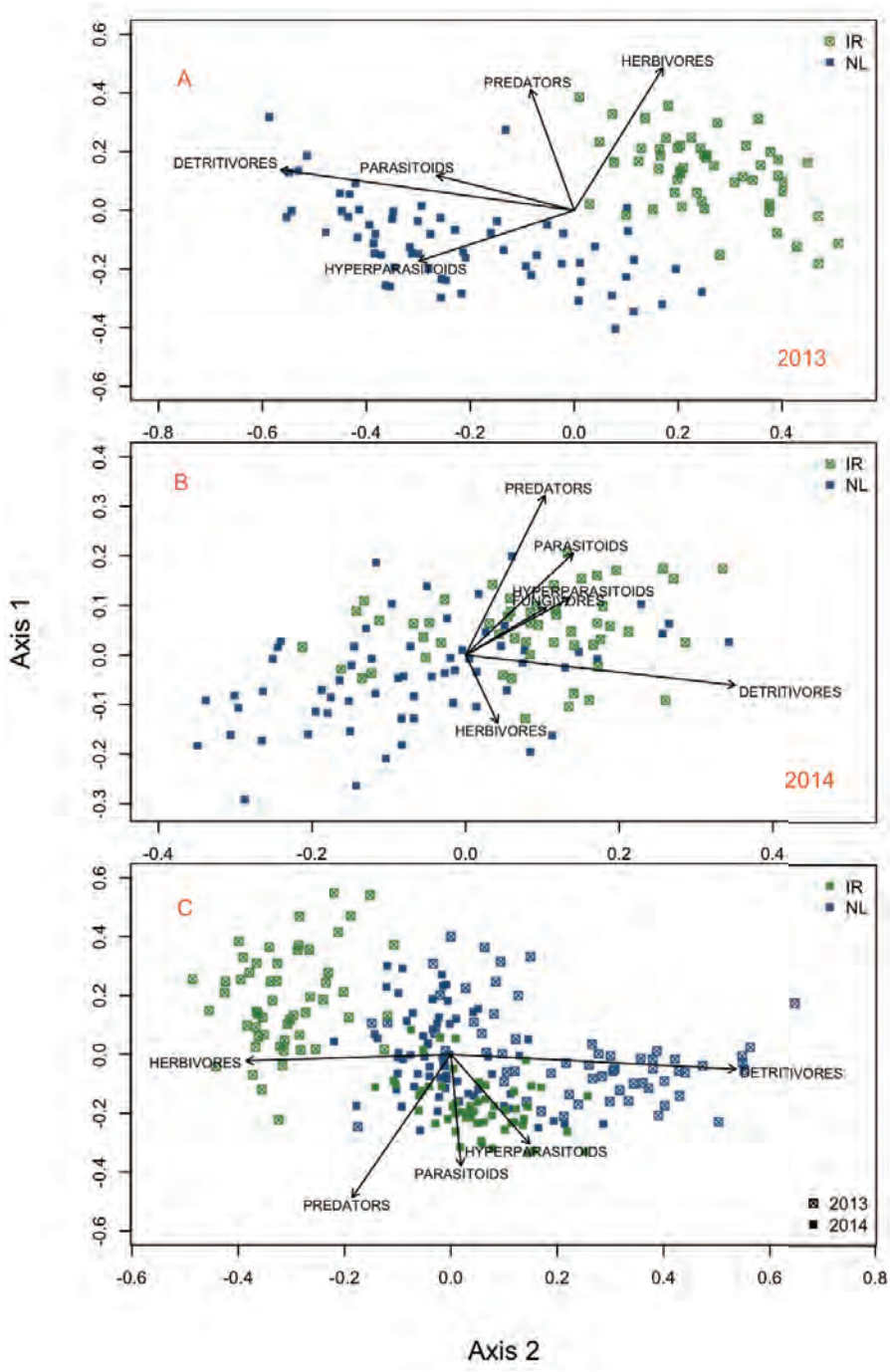

Figure 1: Graphical representation of the effect of site in (A) year 2013 (IR = Ireland and NL = The Netherlands); (B) year 2014 (IR and NL) and (C) 2013 & 2014 (IR and NL) on the arthropod community as defined by functional groups using Nonmetric Multidimensional Scaling (NMDS).

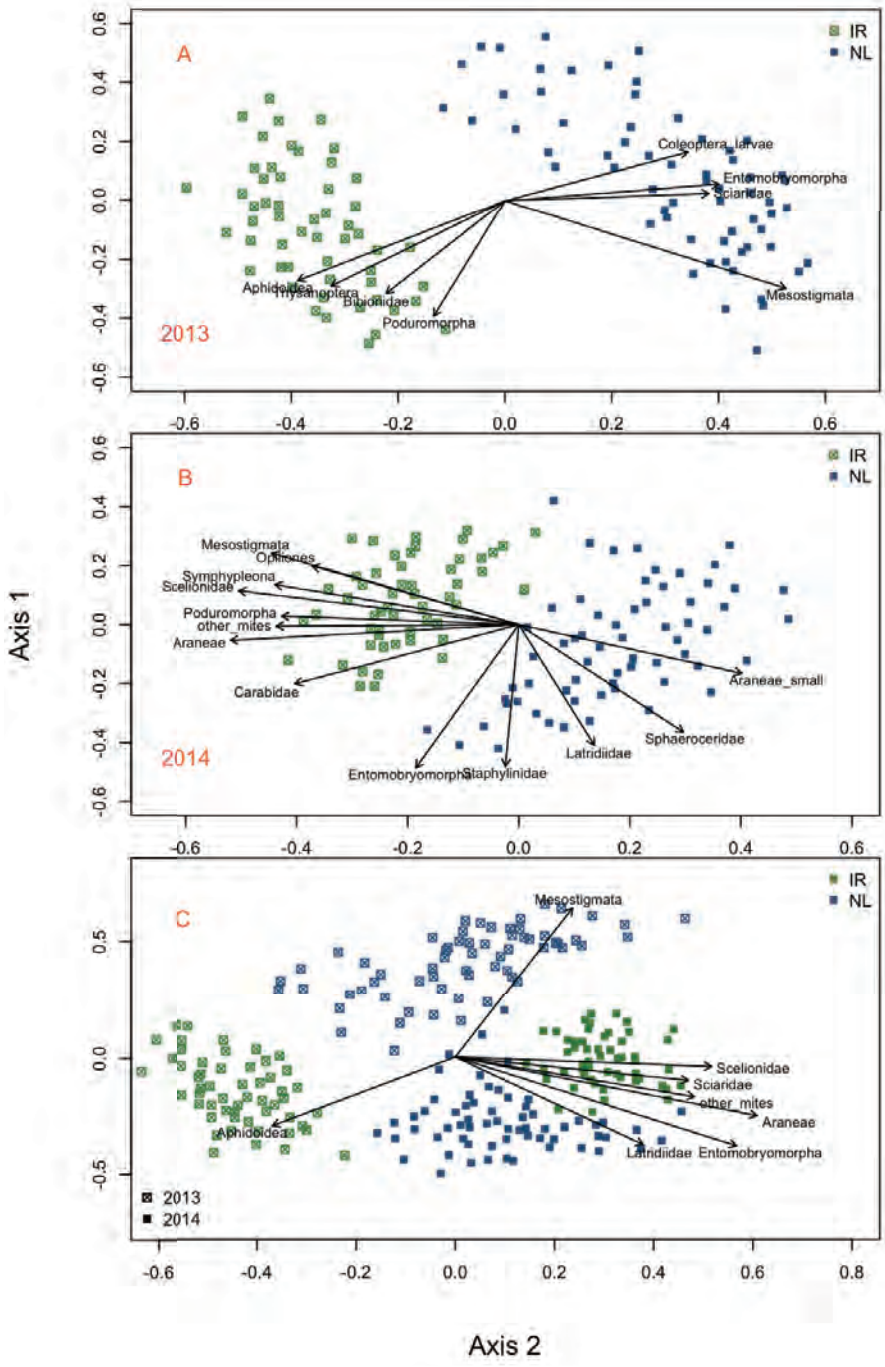

Figure 2: Graphical representation of the effect of site in (A) year 2013 (IR = Ireland and NL= The Netherlands); (B) year 2014 (IR and NL) and (C) 2013 & 2014 (IR and NL) on the arthropod community as defined by family or order groupings using Nonmetric Multidimensional Scaling (NMDS).

predator taxa were Carabidae and Araneae. Scelionidae were important parasitoids in Ireland. In The Netherlands, detritivore and predator families also significantly affeced arthropod community separation. Entomobryomorpha, Latridiidae and Sphaeroceridae were the detritivores and small Araneae and Staphilinidae were common predators in The Netherlands, but their higher numbers in Ireland drove the vector direction in Figure 1B. Herbivores were less important in predicting the community structure, nevertheless aphids (Aphidoidea) were common herbivores found in Dutch pitfall traps compared to Irish ones: having a high correlation with plot clustering $P < 0.001$, yet a relatively low fit: $R^2 = 0.133$, which was below our threshold for displaying the vector representing the taxa correlation with pitfall trap catch in Figure 2B.

Overall, for all years and locations, the main functional groups driving the arthropod communities were the negatively correlated detritivores and herbivores (Figure 1C). Parasitoids, hyperparasitoids and predators were the most positively correlated and also important drivers of the arthropod communities in both countries (Figure 2C). The arthropod communities were more distinguished from each other when identified to the family or order level than when grouped by functional category. Overall Aphidoidea in Ireland, and Mesostigmata in The Netherlands were the most important arthropods for the 2013 communities, while the 2014 communities were generally associated with the Scelionidae parasitoids, Sciaridae, Entomobryomorpha, mites and Latridiidae (detritivores) and the Araneae (predators), which were all positively correlated groups (Figure 2C).

Multivariate constrained ordination (RDA) and permutation tests

The multivariate permutation tests (Table 5) confirmed effects of site and year and their interaction as significant for the collected arthropod community. The genotype factor was found to be significant for functional groups, but not for taxonomic groups. On the other hand, treatment was detected as a significant factor for taxonomic groups but not for functional groups. Both models (using taxonomic and functional groups) were able to explain much of the variation in the measured biodiversity (adjusted R^2=0.44-0.45, both models, Table 5).

The effects of genotype and treatment are small compared to the site and year effects (Table 5) and, moreover, their interactions with site or year are also significant (Table 5). The ordination plots in Figure 3 show the significant interactions: the site by genotype mean plot scores conditional on year and treatment (Figure 3A) and year by treatment mean plot scores conditional on site and genotype (Figure 3B). In Figure 3A the differences among sites are much bigger than those among the genotypes, and the configuration of the genotypes within sites differs among sites. The genotype Sarpo Mira in the Dutch plots is most distinctively characterized by its detritivore population (Figure 3A). Similarly, in Figure 3B, the differences

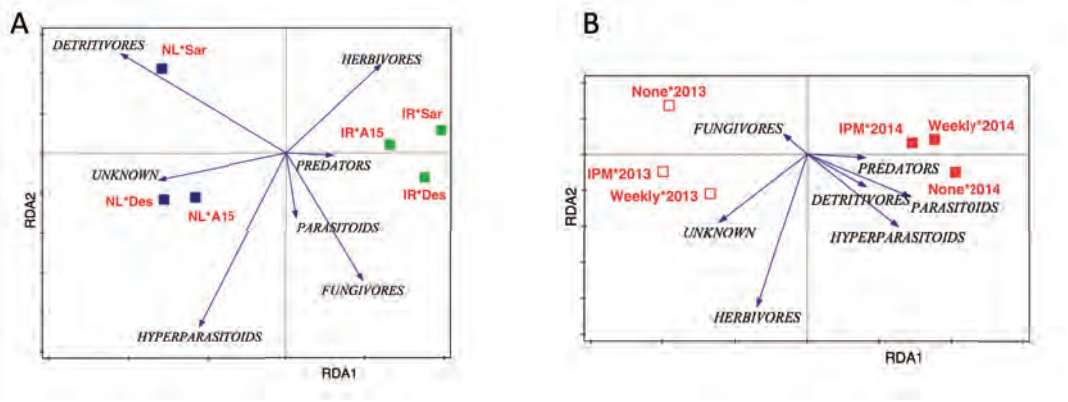

Figure 3: An RDA ordination made from functional group data showing A) the site by genotype mean plot scores conditional on year and treatment. The sites are noted as: NL=The Netherlands, IR= Ireland, and the genotypes: Des= Désirée, A15=P. infestans resistant event A15-31, Sar= Sarpo Mira. B) the year by treatment mean plot scores conditional on site and genotype. The treatments are noted as: None= untreated plots, IPM=treated conditionally with fungicide, see methods, Weekly= sprayed weekly with fungicide.

among years are much bigger than those among fungicide regimes. The pitfall trap collections from the two different years also separate clearly, with the untreated plots in 2013 having the most distinctive separation (Figure 3B).

Discussion

This study aimed to monitor biodiversity of arthropods using pitfall traps in potato agro-ecosystems in Ireland and The Netherlands over two years and determine the importance of site, year, genotype and fungicide treatment on the community structure. Finally, we aimed to compare both univariate and multivariate methods to quantify biodiversity in the context of risk assessments by exploring the effects of these factors using two different ways to group individuals trapped in the pitfalls: by taxonomic group or by functional category.

Insight from biodiversity indices

Relating the taxonomic or functional group richness with abundance of organisms per trap by calculating the 'relative diversity' index was able to detect most of the same factors affecting biodiversity as identified by the Shannon-Wiener or Simpson indices, even with few functional group categories. Relative diversity is one of the simplest univariate ways to measure the biodiversity response, yet is generally considered to be flawed (Buckland *et al.*, 2005; Gotelli & Colwell, 2001). This variable assumes that richness increases linearly with abundance, which is generally not the case for most environments; however, it is sometimes used as a comparator to other biodiversity indices (Bishop *et al.*, 2009; Sapkota *et al.*, 2010) as we have done here. In comparison, the Shannon-Wiener and Simpson indices both consider the distribution of the taxa. Both indices satisfy the most important criteria for a

Table 5. The results of RDA permutation test for effects of year, site genotype and treatment (and their interactions) on biodiversity in The Netherlands (NL) and Ireland (IR) in 2013 and 2014 as determined by separating arthropods by taxonomic groups or by functional groups.

RDA model permutation test	*df*	***By taxonomic groups*** *(adj. R^2= 0.4463)*			***By functional groups*** *(adj. R^2=0.4426)*		
		variance	**F**	**P**	**variance**	**F**	**P**
Site	1	11.757	66.421	**0.03**	0.534	39.865	**0.012**
Year	1	13.917	78.627	**0.03**	0.692	51.657	**0.012**
Genotype	2	0.401	1.132	0.11	0.067	2.498	**0.004**
Treatment	2	0.483	1.365	**0.004**	0.027	1.012	0.374
Block	6	1.833	1.726	**0.03**	0.191	2.382	**0.012**
Site x Year	1	7.164	40.472	**0.03**	1.072	80.062	**0.012**
Site x Genotype	2	0.339	0.959	0.345	0.051	1.896	**0.036**
Year x Genotype	2	0.338	0.955	0.48	0.042	1.559	0.097
Site x Treatment	2	0.389	1.100	0.075	0.034	1.287	0.194
Year x Treatment	2	0.522	1.475	**0.003**	0.052	1.953	**0.029**
Genotype x Treatment	4	0.674	0.952	0.515	0.066	1.238	0.18
Site x Year x Genotype	2	0.337	0.951	0.378	0.035	1.312	0.169
Site x Year x Treatment	2	0.348	0.982	0.309	0.022	0.805	0.603
Site x Genotype x Treatment	4	0.735	1.037	0.112	0.068	1.261	0.14
Year x Genotype x Treatment	4	0.602	0.850	0.868	0.025	0.474	0.971
Site x Year x Genotype x Treatment	4	0.673	0.951	0.332	0.049	0.907	0.513
Residual	192	33.984	*NA*	*NA*	2.571	*NA*	*NA*

biodiversity index including the aspects of overall abundance, evenness and number of taxa or groups (Buckland *et al.*, 2005). Of these three criteria Shannon-Wiener and Simpson indices differ in how they represent evenness (Smith & Wilson, 1996), and the main focus here was on how these differences influenced the conclusions drawn from their use in a risk assessment context.
In the context of our research questions, the use of Shannon-Wiener or Simpson indices based on taxon level identifications generally resulted in the same biological conclusions. The discrepancies that were found between the two indices resulted in a loss of detected significance in the analyses conducted using functional grouping compared to that using taxonomic groups. This could be a result of the decrease in resolution of the results: grouping the count data into fewer categories, can give the index less variability between plots. In general, the biodiversity indices were comparable in their outputs and the results from these are more reliable (or more congruent with each other) when calculated based on higher resolution grouping (taxonomic) rather than on pooling into fewer groups (functional).

The conclusions drawn from the multivariate analyses were only partly in agreement with the univariate analyses. The main factors of site and year again prevailed as significant factors for pitfall trap biodiversity in potato plots. This was confirmed by univariate and multivariate analyses as well as the NMDS ordination plots. The univariate post-hoc tests showing the distinction of the Sarpo Mira genotype in The Netherlands, were supported by the RDA plots with the added advantage of showing which functional groups contributed to the distinctions. This supports the idea that a multivariate ordination can provide relevant nuance to the univariate biodiversity analysis.

In the multivariate permutation analyses however, the resolution of the data affected whether the single factors genotype or treatment were found to be significant, as well as which two-way interactions were important for explaining variation between the community composition. This could have resulted from an increase in power of the calculations made with higher count values (functional groups). Whether genotype or treatment was found to be significant, the interactions between the year and site as well as sites and genotype were also congruent with the univariate analyses. A new interaction which was not detected by the univariate analysis was that between year and treatment. Therefore, it is possible that taking a different approach to understanding biodiversity could lead to slightly different biological insights about the data collected.

The EFSA guidelines for assessments of potential impacts of GM plants on non-target organisms disregarded indices "because it is most unlikely that studies will yield sufficient samples of individuals to characterize indices adequately or that a sufficient degree of ecological background information will exist to give confidence

that biodiversity can be represented adequately as a single number" (EFSA, 2010). In an analysis of several diversity indices it was concluded that diversity indices should only be used in the context of other indices since they may at times generate misleading findings and that validation is necessary through other statistics-based means (Boyle *et al.*, 1990). The Shannon-Wiener index is generally advised by several publications comparing and describing indices for relatively small sample sizes (<1000) (Buckland *et al.*, 2005; Magurran, 2004). The Simpson index is considered less sensitive to underlying species abundance distributions (Buckland *et al.*, 2005), but also expected to yield similar biological conclusions as the Shannon-Wiener index, which is supported by our study. Poelman et al. (2009) arrived at similar biological conclusions when comparing the results of the Shannon-Wiener and Simpson indices to test the effects of cultivar and time (and their interaction) on herbivore diversity in cabbage plots. Considering our results, we could argue that for questions regarding the importance of effects of one or two categorical factors on arthropod biodiversity, the use of a biodiversity index can result in similar general biological conclusions as a multivariate permutation test for determining the effects of those factors on the community structure and the multivariate approach can give further insights which may not be detected with an index approach. However, discounting the Shannon-Wiener or Simpson diversity indices for failure to come to the adequate biological conclusions seems unwarranted.

Most of the analyses concluded that there is an effect of genotype on pitfall trap biodiversity. Through further post-hoc investigations, these effects were mostly noted in a single year and single location, and attributed to the differences in biodiversity associated with Sarpo Mira containing potato plots compared to Désirée or A15-31, while these latter two were in all cases found to have equivalent arthropod assemblages. This is not a surprising result, since the only difference between A15-31 and its isogenic comparator is a single resistance gene. When comparing aphid intrinsic rates of increase in a baseline comparison with these same genotypes in a greenhouse setting, large differences between cultivars were noted while Désiree and A15-31 remained the most similar (Lazebnik *et al.*, 2017). Baseline comparisons to conventional crops both in field and greenhouse are therefore crucial for maintaining the ecological perspective when it comes to assessing the risk of GM crops in the agro-ecosystem.

Our analyses indicated that biodiversity is significantly influenced by the year and the site at which sampling was done, indicating that the conditions under which biodiversity is tested matter a great deal; thus, conducting risk analyses for just one year in one location is unlikely to provide a broad enough support for important regulatory decisions. Agricultural fields in particular may require multi-year experiments since crop rotation and field size and the frequent changes in vegetation create unique ecosystems every year (Fahrig *et al.*, 2015; Rusch *et al.*, 2013).

Since site was also a significant predictor of biodiversity, even though Ireland and The Netherlands were grouped within the same biogeographic zone, our findings indicate that collecting data from only one site would not be representative for the biodiversity in that zone as a whole.

Insights from the multivariate approach

The community structure can be much better interpreted through multivariatae ordinations with vectors representing the groups that most shape the separation between arthropod communities. This can be useful for data exploration (using NMDS) without setting any constraints on the tested factors. An RDA ordination can complement a permutation test and serve as a post-hoc analysis explaining in greater detail which factors are responsible for differences detected.

In the context of risk assessment of GM crops, the identification of arthropods to species level would give the most accurate representation of biodiversity. However, this level of identification requires highly specialized knowledge of taxonomy and a considerable time investment often not feasible in early stages of environmental risk assessment. In our study, arthropods were categorized by family or order, in many if not most cases harboring several ecological functions. Therefore, with some families categorized in two functional groups, the functional categories provide less specificity in quantifying biodiversity, and thus will result in a less detailed description of the arthropod community.

Failure of the univariate or multivariate analyses to detect any differences in biodiversity within a single year and site was likely due to the decreased statistical power from reduced plot replication in the genotype-treatment combinations compared to the number of plots in the analysis per year-site combination. However, arthropods in the families shown as particularly significant in the ordination would be logical to select for the indicator species approach. This could be another use of the ordination plots in cases where the effect sizes are low.

The bias of pitfall trapping

Although pitfall trapping is the most common way to trap ground-dwelling invertebrates, it does have its limitations as a method to catch rare or charismatic species (Duelli *et al.*, 1999) and may also incur sex-ratio biases within taxonomic groups based on mate finding or sex-biased foraging behaviors (Enge, 2001; Topping & Sunderland, 1992). In this case, pitfall trapping allowed for standardization between plots and provided a high enough catch count for standard statistical analyses. This choice, however, does limit our understanding of the arthropod community to just ground dwellers and may overestimate the importance of certain groups in terms of the broader arthropod community present in flight or on the crops themselves. That being said, pitfall traps are highly practical and widely used for indicating habitat

quality and measuring nature conservation values (Eyre & Rushton, 1989). It is also remarkable that groups such as aphids and parasitoids were still commonly found in the traps despite not necessarily being ground-dwelling species. Luff and colleagues were able to correctly predict seven out of ten habitat types with an ordination analysis of ground beetle fauna alone, caught by pitfall trapping (Luff *et al.*, 1992). For these reasons, the use of this trap type can be justified and even recommended for general feasibility in risk assessments situations and we recommend it for future use with the aforementioned caveats.

Pitfall trapping was also one of the most popular methods used in the recently updated database on arthropods commissioned by the European Food and Safety Authority (Riedel *et al.*, 2016), and the only one used in the Irish entries on potato crops. Although the entries were qualitative rather than quantitative, we found a congruence in the taxa that were significant in discriminating community compositions in our study: carabids in Ireland, for example, and aphids in The Netherlands were the entries shown as 'highly' abundant in the database and important factors in our NMDS ordinations for those locations.

Future directions and conclusions

New methods and technologies for detecting biodiversity in, on and above ground will be instrumental in future environmental risk assessments. Analyzing environmental DNA (eDNA) for example is an emerging field of technology whereby genetic material can be obtained directly from environmental abiotic samples like water or soil. The eDNA method is predicted to be used for monitoring biodiversity of entire ecosystems at multiple spatial and temporal scales (Thomsen & Willerslev, 2015). Tracking certain biodiversity elements from space is another recent proposition (Maron *et al.*, 2015). In the future, biodiversity e-infrastructure is predicted to enable a faster, easier integration of ecological information from across the globe (La Salle *et al.*, 2016). This would also open doors for better citizen science in documenting ecological data. An important initiative which will be essential for setting biodiversity targets, practically measuring and finally analyzing biodiversity data is the proposition on 'Essential biodiversity variables' by the Group on Earth Observations Biodiversity Observation Network (Pereira *et al.*, 2013). This tool is being developed specifically for its use in biodiversity monitoring on a global scale by GLOBIS-B (Global Infrastructures for Supporting Biodiversity research), which is a European Union commissioned program that aims to develop scalable workflows for analyzing, visualizing and sharing biodiversity information (Kissling *et al.*, 2015).

Until the promising new technologies proposed above have been tested, approved for use in risk assessment and become more readily available, we have to work with the limitations of currently used and rather basic trapping methods. A recent article exemplified that with current monitoring schemes developed for detecting

effects of GM crops, which can detect adverse effects on biodiversity of 1 - 5% annually, requires considerable resources, roughly 320 ± 170 person days per year per monitoring scheme depending on taxonomic group and number of sites (Schmeller & Henle, 2008). With this in mind it is understandable why species indicator selection methods like ranking matrices are currently advised to choose specific species for testing non-target effects of GM crops (Hilbeck *et al.*, 2014). While these methods are established for choosing appropriate targets for assessments, the overall assessment of arthropod community structure or biodiversity remains untested. The use of multivariate ordinations could therefore be useful, either within the framework of the selection process mentioned, or as a more sensitive method to compare functionally important arthropod groups driving the community structure as a whole.

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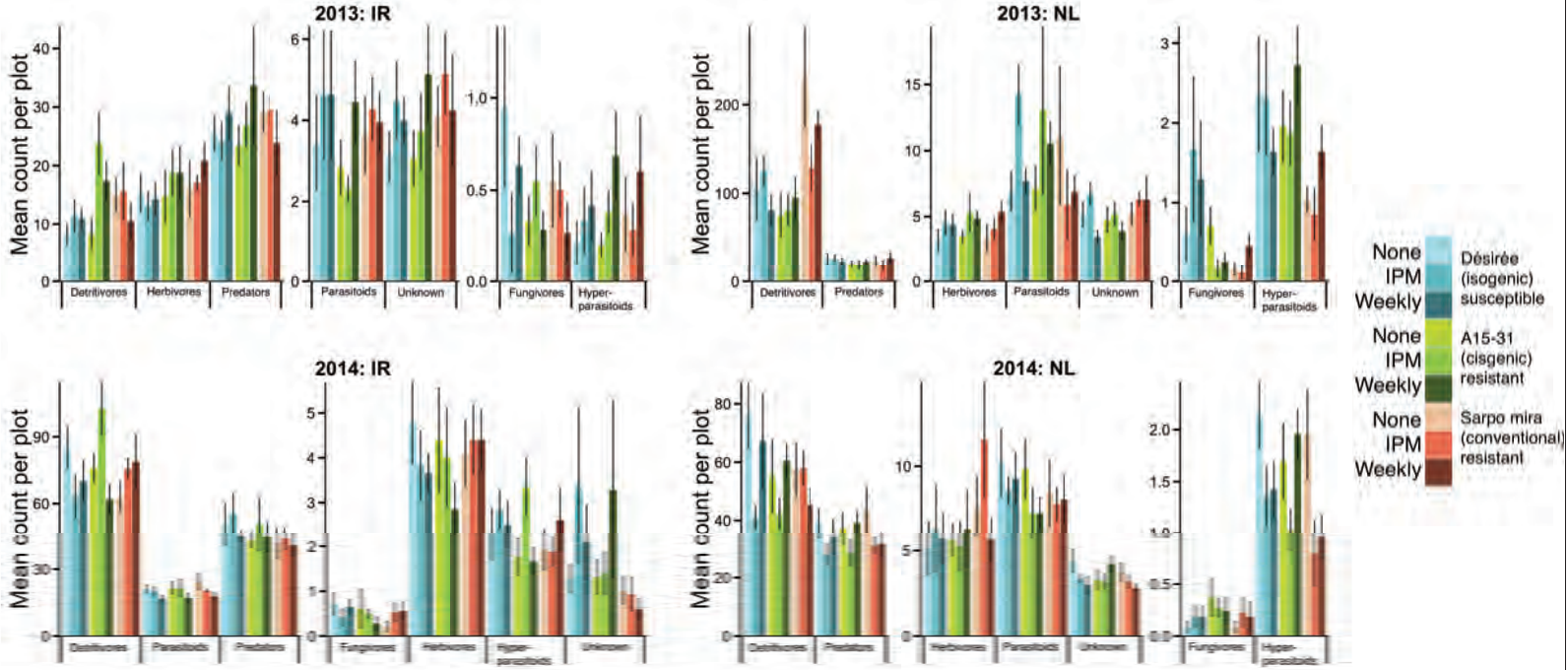
Appendix A: Alphabetic lists of all taxa found in pitfall traps in potato plots in The Netherlands and Ireland between 2013 and 2014 grouped by functional categories.

PREDATORS	DETRITIVORES	PARASITOIDS	FUNGIVORES	HERBIVORES	HYPER-PARASITOIDS	UNKNOWN
Araneae	Armadillidae	Aphelinidae	Drosophilidae	Anthomyiidae	Aphelinidae	Coleoptera larvae
Assilidae	Calliphoridae	Aphid mummies	Mollusca	Aphidoidea	Ceraphronidae	Diptera larvae
Carabidae	Cecidomyiidae	Braconidae	Ptilidae	Apidae	Diapriidae	Diptera pupa
Carabidae larvae	Chironomidae	Ceraphronidae	Scatopsidae	Chrysomelidae	Eucoilidae	Hemerobiidae
Chilopoda	Chloropidae	Chalcidoidea		Chrysomelidae pupae	Figitidae	Heteroptera unknown
Chrysopidae larvae	Diplopoda	Diapriidae		Cicadellidae	Megaspilidae	Psocoptera
Coccinellidae	Drosophilidae	Eucoilidae		Cicadellidae larvae	Mymariidae	Unknown
Coccinellidae larvae	Entomobryomorpha	Figitidae		Curculionidae	Pteromalidae	Coleoptera
Dolichopodidae	Ephydridae	Ichneumonidae		Elateridae	Trichogrammatidae	Unknown Diptera
	Heteroptera larvae					Unknown egg
Empididae	Histeridae	Megaspilidae		Hydrophilidae		Unknown insect
Formicidae	Latridiidae	Mymariidae		Lepidopteran larvae		Unknown larvae
Haematopotus	Megadrilaceae	Platygastridae		Miridae		Unknown insect
Histeridae	Mesostigmata	Proctotrupidae		Mollusca		
Linyphiidae	Mollusca	Pteromalidae		Noctuidae		
Lycosidae	Muscidae/Fanniidae	Scelionidae		Scathophagidae		
Mallophaga	Opiliones			Symphyta		
Miridae	Phoridae			Symphyta larva		
Nabidae	Poduromorpha			Tenthretinidae		
Opiliones	Psychodidae			Tephritidae		
Pachygnatha	Scarabaeidae			Thysanoptera		
Panorpinae	Scathophagidae			Thysanoptera larvae		
Rhagionidae	Sciaridae					
Reduviidae	Sepsidae					
Reduviidae larvae	Silphidae					
Siphonaptera	Sphaeroceridae					
Staphylinidae	Symphypleona					
Staphylinidae	Throscidae					
Syrphidae	Tipulidae					
Tetragnathidae	Unknown mites					
Thomisidae						
Vespidae						

Appendix B. Mean (± SE) number of arthropods in each functional category found in pitfall traps placed in potato plots in Carlow, Ireland (IR) (N = 6 plots per colored bar) and Valthermond, Netherlands (NL) (N = 7 plots per colored bar) in 2013 (top two panels) and 2014 (bottom two panels). All blue bars represent plots of Desiree, green bars represent the resistant cisgenic A15-31 GM event, and the red bars represent a resistant conventional cultivar Sarpo Mira. For each genotype three different fungicide management regimes were tested: no spraying (None), weekly fungicide spraying (Weekly) and an IPM 2.0 regime, where spraying was done only under high pathogen pressures depending on environmental conditions (IPM).



Chapter 7

General discussion: Nothing in environmental risk assessment makes sense, except in light of the baseline

Protecting crops against pests or pathogens is not a simple task. In the course of agricultural history, people have invented many measures to protect their crops against harmful organisms. These measures include manual removal, application of chemicals, biological control, and various cultural tactics like intercropping or crop rotation. These methods can also be combined as part of an integrated pest management system (IPM) (Barzman *et al.*, 2015) that is characterized by coordinated use of complementary methods to develop a systems approach to sustainable crop protection (Lewis *et al.*, 1997). Using cultivated breeds with enhanced resistance to pests and pathogens is yet another crop protection strategy, and an important part of an IPM approach. Selection for desirable crop traits has been applied and refined since the beginning of agricultural history (Bennett, 2010), and yet it is also considered most akin to the very recent innovation of genetic modification (GM) technology (Arber, 2010; Gepts, 2002; Schouten *et al.*, 2006). As opposed to the other known methods of crop protection, both conventional breeding and GM technology bring about crop improvement through the manipulation of the plant genotype.

An environmental risk assessment is required in Europe for the registration of GM crops, and although breeding for resistance is considered the closest to the GM approach to plant protection (Hartung & Schiemann, 2014), environmental concerns are not the main focus of regulation directives for registration of crop cultivars. The main concerns in the development of new cultivars are mainly directed towards distinctness, uniformity, stability (which only under special cases includes environmental testing) and value for cultivation (European Commission, 2016). Thus development of new varieties does not require testing for unintended effects on non-target organisms on a regular basis, since the safety of cultivars is considered a given based on historical use (Kok *et al.*, 2008). To ensure the prevention of unintended harmful effects of crop protection on the environment, an environmental risk assessment (ERA) is required prior to approval of the use of chemical, biological or GM crop cultivation. This includes testing for unintended effects on the organisms occurring in or around the crop, referred to as non-target organisms (NTOs). Sustaining biodiversity (the overall assemblage of organisms at the community level) is therefore an important protection goal.

Plant breeding has resulted in a vast variety of cultivars which are marketed for their unique traits. This leads to many different cultivars with higher resistance levels to a given pest, pathogen or higher tolerance to unfavourable environmental conditions. In the context of this discussion, we refer to the 'baseline' as variation among conventionally bred cultivars in terms of any (or all) associated traits. Each cultivar is associated with a community of organisms and communities may vary with cultivars. For example, in *Brassica oleracea* cultivars, herbivore communities differ widely in species richness and composition (Poelman *et al.*, 2009). Most ERAs compare the

non-target effects between the GM crop and its non-GM isogenic line (from now on referred to as 'comparator') (Paoletti *et al.*, 2008). The assumption is that if any differences (such as variation in effects on the non-target community) between a GM crop and its comparator are within the range of the baseline variation - there should be no higher risk to introducing the GM crop in question than a conventionally bred cultivar with similar resistance properties. Considering that the ERA methods for chemical crop protection products are similar that of the GM crop (Neale, 2000), it may also be valuable to compare effects of GM crop cultivation to the alternative methods for crop protection. Therefore, it is justified to compare results (for any ERA trait in question) from other forms of crop protection programs (manual, chemical, biological, IPM etc.) with those from the cultivation of GM crops (and associated IPM strategies). In Chapter 6, this concept was addressed by comparing different forms of crop management strategies including an IPM regime and weekly fungicide spraying on all genotypes tested, including the susceptible unmodified comparator. Finding statistical differences in a particular trait of interest between a GM crop and its comparator does not imply that those differences fall outside the normal variation between cultivars. Similarly, effects outside the effect range among cultivars do not imply that the environmental risk is greater than that of alternative crop protection methods.

Using examples from this thesis and other research on GM crops, my aim in the following paragraphs is to exemplify the importance of comparing results from the GM crop and its closest non-GM comparator with an ecologically relevant baseline in every aspect of assessing risk to non-target organisms. We begin with the early tier greenhouse tests, with studies at the molecular level, to single or several focal species and end with field experiments testing risks for NTOs at the community level. Any significance found between the GM and its closest non-GM comparator can only be relevant for risk assessments in the context of a current baseline of ecological variation: be it in response to manual, chemical, biological, IPM control, or responses to conventionally crop cultivars with similar traits. I summarize by providing recommendations for the future of ERA.

Risk assessment in the 'omics' era

Apart from molecular characterization of the inserted DNA sequences, neither targeted nor untargeted effects on plant gene expression are required for ERA under the EFSA guidance for GM crops. Untargeted metabolomics are generally regarded as a useful tool in the ERA process (Ladics *et al.*, 2015; Rischer & Oksman-Caldentey, 2006), yet the protocols for interpreting such data need further development (Francke, 2012; Hall & de Maagd, 2014; Nicolia *et al.*, 2014). In Chapter 3 of this thesis, I used the targeted gene expression approach using two genes in the phytohormonal pathways well known to be triggered by two non-target insect herbivores differing in feeding modes (reviewed in Chapter 2). Although this

approach could detect differences between two different events of *Phytophthora infestans*-resistant potato genotypes, the study was not designed to test the ecological relevance of the effects found. A comparison to the gene expression levels in several baseline cultivars with similar phenotypic traits would put these findings into a practical agronomic perspective. The study of Chapter 3 was useful to compare differences in defence responses in two events differing only in the genotypic location of the R gene, demonstrating that non-target testing may be useful even before choosing an event for further testing in an ERA context. Evidence from studies in GM potato (Catchpole *et al.*, 2005; Lehesranta *et al.*, 2005; Plischke *et al.*, 2012; Van Dijk *et al.*, 2010), wheat (Baker *et al.*, 2006), barley (Kogel *et al.*, 2010) and maize (Coll *et al.*, 2010; Van Dijk *et al.*, 2010) at either transcriptomic, metabolomic or proteomic levels demonstrate in nearly all cases that any differences found between the profiles of the GM crop and its comparator fall within the range of the '~omic' profiles of several conventional cultivars of that crop. Approaches and recommendations for comparing large molecular data sets to baselines have been proposed (Houshyani *et al.*, 2014), which may lead to consensus on the use of these new technologies and methods for use in ERA in the near future. Following up on the results of Chapter 3 would require the study of the same non-target insects and same genes of interest on a baseline of conventional potato cultivars differing in resistance to late blight to shed light on the ecological relevance of those findings for use in ERA.

Baselines for the lab and greenhouse

In the European Union guidelines for ERA of GM crops, a set of levels or tiers of experiments are followed, starting with controlled laboratory and greenhouse experiments using the focal species chosen for testing (EFSA, 2010). There is ample literature on the best ways to approach the selection of these focal or indicator species (Andow *et al.*, 2013; Hilbeck *et al.*, 2014; Romeis *et al.*, 2013; Scholte & Dicke, 2005). In practice, however, there are often limits to the range of species from which a selection can be made. Rearing or propagation is not always possible or feasible for all NTOs. For insects, numbers of generations per season/year may set limits to the number of experimental replicates feasible within a year. In general, the early tiers are designed to test the GM crop with its comparator under worst case scenario conditions or exclusive exposure to the GM crop in question. For example, in the case of Bt-crops, insects could be exposed to the purified toxic protein, and in no-choice scenarios (Romeis *et al.*, 2008). If no differences are detected, these are considered to be even more unlikely in a field scenario (Garcia-Alonso *et al.,* 2006). Pre-selected endpoints are used as proxies for fitness of the focal species, and tested under exposure to the GM crop and its comparator. These endpoint variables can be connected with the mode of action of the target. For example, testing effects caused by a particular gene resulting in an introduced metabolite in the plant, or connected with a protection goal that is set by policy makers (EFSA, 2010). If no effects are detected, further tests are deemed unnecessary and the tier system will

have prevented costly and unwarranted field tests. If significant effects of the GM crop on the NTOs are found, or evidence is not sufficient to claim equivalence between the two genotypes, then 'higher' tier semi-field or field experiments are conducted. In Chapter 4, lower tier experiments were conducted with the aphid *Myzus persicae,* and while some differences could be detected in certain events of the GM transformation, all detected differences fell within the range of variation among the baseline cultivars which were tested applying the same greenhouse bioassays. Greenhouse experiments under controlled conditions can never truly replace natural field conditions. However, the greenhouse tests did provide evidence that the differences found between the GM crop and its comparator fell within the baseline variation for the chosen NTO trait. Relating any NTO traits with baseline variation can be a useful tool in the ERA process, also in the lowest tiers of testing.

Although indoor experiments are limited in many ways, trophic processes can be emulated in the greenhouse by choosing focal species that are connected by trophic function. This can provide evidence for any cascading effects in the ecosystem. In Chapter 5, we studied the effect of different genotypic events of GM potatoes on *Aphidius colemani*, a parasitoid of *Myzus persicae*. Choosing appropriate performance traits is important for the evaluation of NTO fitness variables and appropriate estimators should be chosen based on the relevant biology of each focal NTO (Charleston & Dicke, 2008). Intrinsic population increase is deemed appropriate for aphids (Stearns, 1992) while biomass and development times are judged appropriate for parasitoids (Roitberg *et al.*, 2001). Roitberg *et al.* (2001) caution against the use of just one fitness proxy, as this practice may lead to false conclusions. For this reason, we also opted for several developmental endpoints in Chapter 5. These tests indicated that the cascading effects were present only in susceptible plants, but could also affect the parasitoid if the cisgenic potato plants were inoculated with the target organism, *Phytophthora infestans*. These findings support the practice of early tier ERA studies involving the target of the modification in combination with focal NTOs. By including the baseline comparisons as well as the target organisms of the modification, early tier testing can provide information and ecological context to the ERA before taking it to the field.

The complexity of the field and measuring biodiversity

When it comes to field experiments, the testing becomes less targeted at specific endpoints (or non-target focal species) and more concerned with general unintended effects on crops and crop-associated biodiversity. However, untargeted analyses of plant-associated organisms pose a familiar problem. Akin to the ~omics-issues stated above, the debate about how to interpret results from very large datasets resurfaces, this time including data acquired by measuring biodiversity, as well as many other environmental variables. While it seems easy to prescribe the measure of species richness as a solid proxy for biodiversity, this measure has numerous

downsides (Gotelli & Colwell, 2001). Generally, taking abundance measures into account is necessary for an accurate portrayal of diversity, which can also be taken into account by employing biodiversity indices, each with their own sensitivities to particular qualities of species assemblages (Boyle *et al.*, 1990; Buckland *et al.*, 2005; Duelli & Obrist, 2003; Gotelli & Chao, 2013). In Chapter 6 we show that, in general, the biological conclusion derived from relative richness measures is equivalent to two common biodiversity indices, yet getting the additional qualitative information about differences between community structure is only feasible with a multivariate ordination method (Dray *et al.*, 2012). This is a similar approach to that used for molecular or –omics data sets. The two types of data are separated by orders of magnitude in terms of size and physical properties, yet their biological interpretation depends on a solid understanding of how their function relates to their individual classification. Understanding the function of certain genes for example, is necessary to draw meaningful conclusions from their levels of expression. In a similar sense, understanding the ecological function of the taxa in a community is needed to understand how the effects of a change in their relative distribution can affect an agro-ecosystem.

Another option is available, which is also often categorized using the term "biodiversity". This option is the use of indicator species commonly advocated in risk assessment as well as conservation efforts (Feld *et al.*, 2010), alleviating the issues surrounding the feasibility of quantifying all organisms associated with an ecosystem. There is, however, debate about the reliability of this approach (Dray *et al.*, 2012; Siddig *et al.*, 2016). Quantitative testing for the ability of an indicator to predict the health of the ecosystem is also another matter of concern (Halme *et al.*, 2009; Rossberg *et al.*, 2016). Because of these issues with indicators it may be equally useful to collect data on specific species of concern (such as endangered species or species at risk, if any), rather than a surrogate which may not be sufficiently indicative of the state of the ecosystem. Although there are published methods for selecting surrogates for environmental science (Lindenmayer *et al.*, 2015), the methods of measuring biodiversity within an ERA context should still be refined to properly and realistically meet protection goals.

In Chapter 6, biodiversity was assessed in field experiments executed in Ireland and in The Netherlands over two years. Plots were set up to compare biodiversity in the GM crop to its comparator along with one conventionally resistant cultivar. These were also subjected to three crop management regimes. Interestingly, the analyses showed that the factors year and location were most important in determining the arthropod communities. Just as biodiversity is highly dependent on environmental factors, this is also found to be true using –omics techniques: when comparing GM maize with its comparators, environmental factors were more predictive of transcript, protein and metabolite profiles than differences in genotype

(Barros *et al.*, 2010; Röhlig *et al.*, 2009). Variable environmental conditions have been making biological field experiments difficult to reproduce since the beginning of experimental ecology, and risk assessment testing is no exception. The examples mentioned above all used multivariate ordination techniques to interpret the large data sets, and came to similar conclusions on the importance of environmental and temporal factors. Similar conclusions were derived from field tests on GM potato modified for starch composition: variability was due largely to location and timing, and differences between GM crop and comparator were minimal in comparison to the cultivar baseline tested (Plischke, 2013).

The baseline and scaling ERA to future uses

In tiered experiments performed in this thesis as well as the many published experiments on other GM crops, we conclude that making statements about the environmental effects of GM crops is not meaningful for risk assessment without a baseline. This baseline is needed throughout the tier system (from -omics testing to field experiments) for attaching relevance to any significant differences between the GM crop and its comparator. In this thesis I also show that differences can be due to the genomic position of an inserted resistance gene and that these differences may be impacted by the co-inoculation of the target of the modification. In terms of the findings and insights gained from the experimental work of thesis and the discussion above I propose the following ideas for research directions and future practice of ERA for GM crops:

- Early trials involving non-target organisms should be used for selection of the most appropriate plant genotype for further ERA testing.
- A stronger focus on the development of standardized methods for quantification of biodiversity for untargeted endpoints as well as for specific NTOs.
- The development of standardized methods is also necessary for comparing large data sets of transcripts, proteins and metabolites.
- Understanding how to interpret broader biological context from different types of large -omics data sets is necessary for the eventual inclusion of -omics data in ERA protocols.
- Considering the rate at which technology is advancing in the agricultural field, a scalable ERA process should be geared towards the effects of the new traits as such rather than the methods or technology used to develop the end product (Hartung et al., 2014; Miller, 2010).

Basing the ERA process on novel introduced traits rather than the techniques used in product development would make ERA more universally scalable to future developments or new technologies, and safer for the environment (Hartung *et al.*, 2014; Miller, 2010; Morris & Spillane, 2008; Ricroch *et al.*, 2016). This rationale has already been put to the test for more than 10 years in Canadian legislation, for testing

plants with novel traits (Smyth & McHughen, 2008). As technological advances and higher production demands continue, GM crops will become part of our toolkit for integrated pest management (Kos *et al.*, 2009), and our vision and methods used in ERA will mature to benefit sustainable agricultural practices.

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Summary

One of the world's most damaging potato pathogens is late blight, caused by the oomycete *Phytophthora infestans*. Fungicides are currently necessary to protect potato crops against late blight infection in northern maritime climates. The use of chemical control in potato cultivation has a huge environmental impact. Recent developments in technology have offered a more sustainable solution to chemical pathogen control. The introgression of resistance genes through genetic modification may alleviate the environmental effects from the overuse of chemical control. Although the modification for resistance to late blight addressed in this thesis is highly specific to *P. infestans*, introduction of GM plants in Europe is subject to an environmental risk assessment. This includes testing for risks to the non-target arthropod community associated with the potato crop. This thesis investigates possible non-target effects, and how to assess these in greenhouse and field conditions.

A review of the available knowledge on interactions between pathogens, insects and plants is presented in Chapter 2. In this review the role of plant defense-related hormones is discussed for implications in sequential tri-partite interactions among plants, pathogenic microbes and herbivorous insects. The importance of pathogen trophic strategy and herbivore feeding mode as predictors of effects on the insects or pathogens in these interactions are discussed. The chapter also addresses how plant resistance mechanisms may affect their interactions with herbivores or pathogens.

After reviewing the possible effects of plant resistance on herbivores, the effects of the *Rpi-vnt1* gene conferring resistance to *P. infestans* in potato plants were tested on two important non-target potato herbivores: the peach-potato aphid *Myzus persicae* and the Colorado potato beetle *Leptinotarsa decemlineata*, both as single stressors and in combination. Two events of the same R gene modification were considered, in order to test the hypothesis that genomic position of the R gene affects these plant-insect interactions. The insertion position of the R gene influenced non-target insect performance, which was measured in terms of *L. decemlineata* biomass gain and *M. persicae* offspring numbers. The insertion position and the insect infestation treatment also resulted in changes in the plant transcriptional responses measured for *PR1* and *LOX* gene expression. This study demonstrated that testing several events on non-target insects before selecting an event for further testing in a risk assessment can give insights into potential unintended effects of a modification.

In Chapter 4, the insertion position of the resistance gene was found to affect the performance of the aphid herbivore *M. persicae*. In this experiment, the effect of the number of R genes on this non-target herbivore was also tested. Aphid development was not dependent on the insertion of a second resistance gene; yet, positional effects of gene insertion were again shown to influence aphid performance on

certain GM-events. The results were compared to a baseline of three conventional potato varieties, representing a range in potato plant resistance to late blight. Aphid fitness varied considerably more between conventional potato varieties than between Désirée and the GM events. The protocols used in this experiment were recommended for *in planta* risk assessments with aphids.

While testing effects on a single herbivore species is useful, interactions in the field can be much more complex. In Chapter 5, complexity was added to the plant-insect interactions by exposing plants to microbial pathogens as well as herbivorous insects and their natural enemies. Here, the aphid *M. persicae* and its natural enemy, the parasitoid wasp *Aphidius colemani* were exposed to potato plants inoculated with *P. infestans*. Population growth of the aphid, rate of parasitism by its natural enemy and other insect life-history traits were compared between several potato genotypes, the susceptible cultivar Désirée and several GM events tested in the previous chapters. Differences in intrinsic rate of aphid population increase and the performance of the parasitoid were only found on the susceptible cultivar when inoculated with *P. infestans*. Genomic position of the *Rpi-vnt1* gene insertion in the genome was once again observed to affect aphids either with or without *P. infestans* infection. However, effects of genomic position of *Rpi-vnt1* gene insertion on the parasitoid *A. colemani* were only evident in the presence of inoculation with *P. infestans*. The research from this chapter contributed to our understanding of how inserted plant resistance genes can influence the second and third trophic levels.

While investigating multiple trophic levels in the greenhouse is possible, it is still limited in scope. To get a full picture of the effects these GM crops can have on the whole spectrum of non-targets is best done in the field. Assessment of risks to biodiversity is one of the elements in an environmental risk assessment associated with the introduction of GM crops. In Chapter 6, the arthropod biodiversity is observed using pitfall traps in potato agro-ecosystems in Ireland and The Netherlands over two years. The impact of site, year, potato genotype, and fungicide management regime on arthropod community composition was tested. Several methods of quantifying biodiversity were used in the context of risk assessment. Analyses were conducted using univariate and multivariate methods. Categorization of the pitfall trap catch was done by taxonomic as well as functional category. Both the indices and the multivariate permutational RDA analyses, concluded that the arthropod community is generally impacted most by the site and year and their interactions with the genotype and treatment factors. Where genotype was significant in community composition, this was due to differences between the two conventional cultivars, rather between the GM-crop and its isogenic comparator. The multivariate permutation test could provided more nuanced insights than the univariate analyses about the arthropod community. The outcomes of a multivariate

ordination provide great value to a biodiversity analysis, and are recommended for use within the framework of environmental risk assessments, or in the process of indicator species selection.

Drawing on the lessons learned in the experimental chapters, as well as insights gained from the ample literature about GM environmental risk assessments, I concluded the thesis with a discussion about the essential role of the baseline comparison at all levels of the risk assessment. All analyses done at the molecular level, at the level of single indicator species, several trophic levels and in the community context should include a comparison to a relevant baseline in order to have ecological and environmental relevance. Lastly, I propose ideas for the development of protocols for environmental risk assessments in the light of expected scientific progress in agricultural biotechnology.

Curriculum vitae
and
list of publications

Jenny Lazebnik grew up in Ottawa, Canada where she completed her bachelor of science with honours in biology at the University of Ottawa. During her time at university, her summers were spent working at the Canadian National Collection of Insects, Arachnids and Nematodes at Agriculture Canada in the department of Hymenoptera. It was here that her curiosity for entomology and biological control began. After her bachelor she spent some years doing insect related jobs: a field season at CABI Biosciences in Delémont, Switzerland; an internship in insect ecology at the University of Antwerp, Belgium and she identified insects for PhD students at Carleton University in Ottawa. Finally, deciding that a Master's degree was the next step, she found a place at the Forest Entomology lab at University of Alberta. There she studied volatile signaling between pine trees under attack by the Jack pine budworm and how this may affect the invasion of the mountain pine beetle. With insect-plant interactions still on the forefront of her academic interests, she joined the Laboratory of Entomology at Wageningen University in order to pursue the work of this PhD thesis. Her work on environmental risk assessment with the European project AMIGA (Assessing and Monitoring the Impacts of Genetically Modified plants in Agro-ecosystems) was a very appropriate stepping stone to her current position as scientific reviewer at the Board for the Authorisation of Plant Protection Products and Biocides of The Netherlands (College voor toelating van gewasbeschermingsmiddelen en biociden: Ctgb), in Ede.

List of Publications

Lazebnik, J., Tibboel, M, Dicke, M. & Van Loon, J. J. A. (2017). Inoculation of susceptible and resistant potato plants with the late blight pathogen *Phytophthora infestans*: effects on an aphid and its parasitoid. *Entomologia Experimentalis et Applicata* (in press).

Lazebnik, J., Arpaia , S., Baldacchino, F., Banzato, P., Moliterni, S., Vossen, J. H., Van de Zande, E. M., & Van Loon, J. J. A. (2017). Effects of a genetically modified potato on a non-target aphid are outweighed by cultivar differences. *Journal of Pest Science,* published online January 18, 2017; DOI: 2010.1007/s10340-10017-10831-10346.

Lazebnik, J., Frago, E., Dicke, M., & Van Loon, J. J. A. (2014). Phytohormone mediation of interactions between herbivores and plant pathogens. *Journal of Chemical Ecology*, 40, 730-741.

Miller, S. S., E. M. Watson, **J. Lazebnik**, S. Gulden, M. Balcerzak, G. Fedak, and T. Ouellet. (2011). Characterization of an alien source of resistance to Fusarium head blight transferred to Chinese Spring wheat. *Botany* 89, 301-311.

Accepted for publication

Lazebnik, J., Dicke, M., Ter Braak, C. J. F. & Van Loon, J. J. A. (2017). Biodiversity analyses for risk assessment of genetically modified potato**.** *Agriculture, Ecosystems and Environment.*

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Interactions between herbivores and plant pathogens: phytohormonal mediation and the importance of insect feeding mode and pathogen trophic strategy

Writing of project proposal (4.5 ECTS)

- Trophic structure analysis in genetically modified potato crops for environmental risk assessment

Post-graduate courses (7.5 ECTS)

- WIAS Design of experiments; PE&RC (2012)
- Meta-analysis; PE&RC (2012)
- Survival analysis; PE&RC (2013)
- An introduction to mass spectrometry-based plant metabolomics; EPS (2013)
- Zero inflated models and GLMM using R; PE&RC (2013)
- Mixed linear models; PE&RC (2013)

Laboratory training and working visits (4.5 ECTS)

- AMIGA Meetings in INIA, Madrid; ENEA: Rotondella and Brussels(2x); summer school: TEAGASC, Ireland (2012-2016)
- Field trials with GM potato; TEAGASC, Ireland (2013, 2014)
- Visits to Universities: Neuchatel, Lausanne and Basel (2014)

Invited review of (unpublished) journal manuscript (2 ECTS)

- Plant Biology: phytohormonal pathways for defense against Blackleg disease, a novel method for screening in vitro grown potato (2015)
- PLoS One: plant-insect interactions decrease performance of *Myzus persicae* (Sulzer) via increased salicylate signalling (2015)

Competence strengthening / skills courses (3.6 ECTS)

- Techniques for writing and presenting a scientific paper; WGS (2013)
- PhD Competence assessment; WGS (2013)
- Reviewing a scientific paper; WGS (2015)
- Writing grant proposals; Wageningen in'to Languages (2016)

PE&RC Annual meetings, seminars and the PE&RC weekend (25.5 ECTS)

- PE&RC Day: extreme life (2012)
- PE&RC Symposium: traits as a link between systematics and ecology (2012)
- Entomologendag (2012)
- Midterm PE&RC weekend (2012)

- PE&RC Day (2014)
- Last years PE&RC weekend (2015)

Discussion groups / local seminars / other scientific meetings (8.3 ECTS)

- 2nd Wageningen University PhD Symposium (2015)
- Insect–plant interactions (2012-2015)
- R User meeting (2012-2016)

International symposia, workshops and conferences (8.1 ECTS)

- Non-target organisms and GM crops: assessing the effects of Bt proteins (2012)
- 8th Insect Plant Interactions Workshop; Wageningen (2013)
- 15th Symposium on Insect Plant Interactions; Neuchatel (2014)
- 6th European Plant Science Retreat Ecology meeting; Stockholm (2015)
- PR-Proteins and induced resistance against pathogens and insects; Aachen, (2015)

Lecturing / supervision of practicals / tutorials (7.5 ECTS)

- Supervision practical EABI (2012-2014)
- Supervision of practical IPI (2013)
- Supervision of practical MEE (2014)

Supervision of MSc students

- Assessing performance of the aphid (*Myzus persicae*) and its natural enemy (*Aphidius colemani*) on *Phytophthora*-resistant GM potato plants
- The effects of two herbivores and a hemibiotroph on potato defence response
- Multipartite interactions on potato plants: exploring effects of potato plant attackers on secondary attackers
- Plant-mediated interactions between potato (*Solanum tuberosum*), *Phytophthora infestans*, *Leptinotarsa decemlineata* and *Myzus persicae*
- Does insect infestation affect *Phytophthora infestans* infection in resistant potato plants?
- The effect of *Rpi-vnt1* gene position on multiple interactions
- Performance of a non-target aphid and it's parasitoid on *Phytophthora infestans*-resistant GM potato plants

Acknowledgements

If you got this book and went straight to the acknowledgements, this part is probably for you! For me, it is an opportunity to describe how grateful I am to have had the privilege to work at Wageningen on this PhD adventure, with so many amazing people by my side. So thank you. I know there are very few who will bother to read any part of it, but for those who do- I am very honoured.

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Hugs,
Jenny

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