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 plants, *Solanum tuberosum* L. (Solanaceae), with the late blight pathogen *Phytophthora infestans* (Mont.) de Bary (Peronosporales: Pythiaceae) on an aphid species commonly infesting potato crops and one of the aphid’s major parasites. We observed the peach-potato aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae), and its natural enemy, the biocontrol agent *Aphidius colemani* Viereck (Hymenoptera: Braconidae), on potato either 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 Désiré and genetically modified (GM) isogenic lines carrying genes conferring resistance to *P. infestans*. Effects of *P. infestans* inoculation on the 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 different positions of 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 various trophic levels.

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 (Birch et al., 2007; Romeis et al., 2011, 2013; Andow 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, *Solanum tuberosum* L. (Solanaceae), 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.
The GM plants were modified through insertion of a major resistance gene (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 GM 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 (Radcliffe, 1982; Meissle et al., 2012). 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 *S. tuberosum* 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, the aphid natural enemy and biocontrol agent *Aphidius colemani* Viereck (Hymenoptera: Braconidae). *Aphidius 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 & 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 compared the population growth of the aphid, 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* (Migula) Castellani & Chalmers – containing either one or two R-genes to answer the question 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 whether 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 (Smith & Townsend) Conn-mediated transfer of the native Rpi-vnt1 gene, from *Solanum venturii* Hawkes & Hjert., using marker-assisted (event A13-17) and marker-free transformation methods (events A15-31, A15-45) to the *S. tuberosum* cultivar Désirée. Also, marker-assisted transformation was employed using a single T-DNA harbouring the native Rpi-vnt1 and Rpi-stol (from *Solanum stoloniferum* Schltdl.) genes (event A16-02). The selectable gene for marker-assisted transformation was the *Escherichia 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).

All GM events and the cultivar Désirée were maintained in vitro, on agar medium (purified agar 0.8% + 2.2 g l\(^{-1}\) Murashige & Skoog + Duchefa 4.4 g l\(^{-1}\) + Sacharose 20 g l\(^{-1}\) + Micro Agar 8 g l\(^{-1}\); pH = 5.8) in sterile containers. Containers were kept in a climate room at L16 (21 ± 3 °C):D8(15 ± 3 °C) and 70 ± 5% r.h. Two independent experiments were performed. For aphid life-history experiments, cuttings were transferred from agar to soil 5 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.
Pathogen infection

*Phytophthora infestans* IPO-C isolate was used for all infected plants (Laboratory of Phytopathology, Wageningen University; Haverkort et al., 2016). The pathogen was maintained on both excised leaves kept in Petri dishes and tuber slices of cultivar *Décériserée* 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⁻¹ by measuring the spore concentration with a Fuchs-Rosenthal haemocytometer with a depth of 0.200 mm and 16 squares of 0.0625 mm² 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; 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% r.h. for 24 h. Two days after the inoculation, aphids were placed on plants. The phenotype of the GM 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, 6 days after inoculation.

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 ca. 20 generations on *S. tuberosum* cultivar *Décériserée* before experiments began under the same climate room conditions as described above.

Each experiment began with 1-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 (2.5 cm diameter, 1 cm high) on the abaxial surface of three leaves of each plant. Ten plant replicates of each event (Table 1) and the non-transformed *Décériserée* 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, total fecundity, and aphid mortality. Intrinsic rate of population increase was calculated as described in Wyatt & White (1977):

\[
rm = 0.74 \times \ln(Md)/d,
\]

where *Md* is the effective fecundity and *d* the length of the pre-reproductive period.

Parasitoid performance and life history

*Aphidius colemani* parasitoids were provided by Koppert Biological Systems (Berkel en Rodenrijs, The Netherlands). Table 1 shows the GM events investigated in this study and their characteristics.

### Table 1 GM events of the cultivar *Décériserée* investigated in this study and their characteristics

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

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 cultivar *Décériserée* and arrows indicate areas of visible hypersensitive response (HR) on genetically modified (GM) events. [Colour figure can be viewed at wileyonlinelibrary.com]
Netherlands). The mummies delivered were placed for 2 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 3-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, a female A. colemani was introduced and given access to hosts for 24 h and removed. The plants were checked for mummies each day; these 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 a freezer for 2 h after which the sex was determined using a dissecting microscope and fresh biomass measured using a Sartorius CP2P model microbalance.

Statistical analysis
Aphid rm, parasitoid biomass, and % parasitism were tested with a mixed linear model with the residual maximum likelihood method ('REML'), or generalized linear mixed model assuming a Gaussian distribution, using R package 'lme4' when data did not meet the assumptions of normality, with 'potato event' and 'P. infestans infection' as fixed factors (R Development Core Team, 2014). 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 with a generalized linear mixed model with binomial distribution, with 'inoculation treatment' and 'genotype' as fixed factors. For analyses of intrinsic rates of aphid population increase and individual parasitoid traits (such as biomass, emergence time, and others) 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 with a Cox proportional hazards regression model (Cox, 1972). This model included the same random effects as above and used the 'survival' package in R (R Development Core Team, 2014). Each genotype was also tested separately 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 rm but genotype and the interaction between genotype and treatment were both predictive factors (Table 2A). Désirée was the only genotype on which aphid rm was negatively affected by P. infestans inoculation (Figure 2). On all GM events, rm of aphids was similar whether inoculated with water or P. infestans (Figure 2).

Aphids had a lower rm on the cisgenic potato event A15-45 than on A15-31, containing the same R-gene insertion at a different genotypic location (Figure 2). Aphid rm did not differ on the transgenic genotypes, whether they contained one or two R-genes (A13-17 vs. A16-02). Aphid rm on the single-R-gene transgenic genotype A13-17 did not differ from that on the marker-free cisgenic counterpart with the same R-gene, A15-31. Aphid rm was lower on the cisgenic clone A15-45 than on the transgenic A13-17 (Figure 2).

Aphid survival
The overall model indicated that effects of inoculation treatment and genotype as well as their interaction significantly affected aphid survival (Table 2B). Survival on susceptible Désirée plants was lower on plants inoculated

Figure 2 Mean (± SE) intrinsic rate of population increase (rm) 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 (linear mixed effects model: P<0.001); the asterisks indicate a significant treatment effect within genotype (P<0.0001).
with *P. infestans* than on water-treated plants (Cox proportional hazards regression model: *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* (Figure 3).

On water-inoculated plants, aphids had a similar survival probability over time on all genotypes (Figure 3). On *P. infestans*-treated plants, aphids had a higher survival probability on A15-31 than on D/C19esire plants (Cox proportional hazards regression model: *P* < 0.0001), and there was no difference in aphid survival between A15-31 and 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 A15-31, the marker-free cisgenic counterpart with the same R-gene (*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: parasitism rate**

The percentage of aphid parasitism by *A. colemani* on *P. infestans*-inoculated susceptible Désirée potato plants tended to be lower than on water-inoculated plants (Figure 4). Parasitism rate was not influenced by inoculation treatments in any of the GM genotypes. The main effect of inoculation treatment was not a predictive factor of % parasitism nor was the interaction between the genotype and

| Table 2 | Results of statistical analyses testing the main factors for aphid (A, B) and parasitoid (C–G) performance traits |
|---------|---------------------------------------------------------------------------------------------------|
| Variable | Factor | d.f. | Test statistic (F or χ²) | P |
| A Ph | Aphid rm<sup>1</sup> | Treatment | 4 | 0.7308 | 0.39 |
| | | Genotype | 1 | 12.7886 | <0.0001 |
| | | Treatment*genotype | 4 | 5.0961 | 0.0005 |
| B Ph | Aphid survival<sup>2</sup> | Treatment | 4 | 7.8651 | 0.005 |
| | | Genotype | 1 | 24.4888 | <0.0001 |
| | | Treatment*genotype | 4 | 51.6980 | 0.0003 |
| C % Parasitism<sup>3</sup> | Treatment | 4 | 1.12584 | 0.29 |
| | Genotype | 1 | 3.56077 | 0.009 |
| | Treatment*genotype | 4 | 0.83753 | 0.50 |
| D Female eclosion probability<sup>4</sup> | Treatment | 4 | 0.0072 | 0.93 |
| | Genotype | 1 | 11.671 | 0.02 |
| | Treatment*genotype | 4 | 3.8662 | 0.42 |
| E Parasitoid | Sex | 1 | 35.432 | <0.0001 |
| | Genotype | 4 | 1.376 | 0.24 |
| | Treatment | 1 | 0.392 | 0.53 |
| | Sex*genotype | 4 | 0.377 | 0.83 |
| | Sex*treatment | 1 | 0.740 | 0.39 |
| | Treatment*genotype | 4 | 1.192 | 0.32 |
| | Sex*treatment*genotype | 4 | 1.106 | 0.35 |
| F Parasitoid time to mummy<sup>2</sup> | Sex | 1 | 9.5560 | 0.002 |
| | Genotype | 4 | 5.4651 | 0.24 |
| | Treatment | 1 | 3.6646 | 0.056 |
| | Sex*genotype | 4 | 6.1864 | 0.19 |
| | Sex*treatment | 1 | 0.0908 | 0.92 |
| | Treatment*genotype | 4 | 3.7612 | 0.44 |
| | Sex*treatment*genotype | 4 | 244.8663 | <0.0001 |
| G Parasitoid time to eclosion<sup>3</sup> | Sex | 1 | 1.4689 | 0.23 |
| | Genotype | 4 | 1.1221 | 0.89 |
| | Treatment | 1 | 0.1450 | 0.70 |
| | Sex*genotype | 4 | 6.4630 | 0.17 |
| | Sex*treatment | 1 | 0.0006 | 0.98 |
| | Treatment*genotype | 4 | 8.0943 | 0.09 |
| | Sex*treatment*genotype | 4 | 32.192 | <0.0001 |

Statistical model: <sup>1</sup>linear mixed effects model (test statistic: F), <sup>2</sup>Cox proportional hazards regression model (χ²), <sup>3</sup>linear model (F), <sup>4</sup>binomial linear mixed model (χ²).
treatment; yet, plant genotype significantly influenced % parasitism overall (Table 2C). The factor of plant genotype was only important when plants were inoculated with *P. infestans*, as on water-inoculated plants, % parasitism of *M. persicae* aphids by *A. colemani* was similar for all genotypes (Figure 4). On *P. infestans*-inoculated plants, % parasitism on the cisgenic A15-45 event was lower than on A15-31, containing the same R-gene insertion at a different genomic position (Figure 4). Parasitism rate did not differ on transgenic genotypes with one vs. two R-genes (A13-17 vs. A16-02; Figure 4), nor on the single-R-gene transgenic genotype A13-17 vs. the marker-free cisgenic counterpart with the same R-gene, A15-31, but it was higher on genotype A13-17 than on the cisgenic event A15-45 (Figure 4).

**Female eclosion probability**

Overall, the genotype influenced the probability of female parasitoids eclosing, but not the inoculation treatment nor the interaction between genotype and inoculation treatment (Table 2D). When potato plants were inoculated with *P. infestans*, more females eclosed from both cisgenic genotypes A15-31 and A15-45 than from the transgenic counterpart A13-17 (Figure 5). No differences in female emergence probability were found between A15-31 vs. 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 (Table 2E). Biomass of eclosed parasitoids in both treatments was overall higher for females than for males (Figure 6). For both sexes, biomass was unaffected by plant genotype, inoculation treatment, or their interaction.

Time until mummy formation was affected by the sex of the parasitoid as well as by the three-way interaction between sex, inoculation treatment, and genotype of the plant (Table 2F). Sex of the eclosed parasitoid influenced mummy formation only on Désirée and on A15-31, with males having a shorter time until mummification than females (Cox proportional hazards regression model, 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 and females: both *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 (Table 2G). In both sexes emergence time was influenced by the interaction between genotype and treatment (Cox proportional hazards regression model, females: *P* = 0.012; 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 its parasitoid, NTOs 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 hemi-biotrophic) 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 (*rm*) and subsequently parasitoid performance (% 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* L. plants reducing the parasitoid quality and slowing down the development time of its larval host *Melita cinxia* (L.) (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 Désirée plants were severely affected by the inoculation of the pathogen whereas resistant genotypes exhibited a hypersensitivity response that halted the spread of infection. As 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 plant 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 \( P. \) \textit{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 \( Spodoptera \) \textit{littoralis} Boisduval was affected by the plant–pathogen interaction between a resistant GM-potato and \( P. \) \textit{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, Ponzo et al. (2016) demonstrated that compatible and incompatible interactions with the bacterial pathogen \( Xanthomonas \) \textit{campestris} (Pammel) Dowson result in differential emission of volatile blends by \( Brassica \) \textit{nigra} (L.) WDJ Koch plants and that both are highly attractive to \( Cotesia \) \textit{glomerata} (L.) parasitoids. Further investigation is warranted to better understand the effects of gene-for-gene interactions on NTOs.

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 \( M. \) \textit{persicae} \( r_m \) and \% parasitism by \( A. \) \textit{colemani} found between genotypes A15-31 and A15-45, were only found in the presence of the \( P. \) \textit{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 NTOs. The effect of R-gene position, however, was noted for aphids in both \( P. \) \textit{infestans}-inoculated and water-inoculated plants. This could indicate that not all insertion events of the same gene, achieved through \( A. \) \textit{tumefaciens}-mediated transfer, have the same effects on non-target insects. Genetic modification of the Désirée cultivar has previously been shown to have unexpected effects on \( M. \) \textit{persicae}, and pleiotropic effects can influence aphid developmental traits both positively and negatively (Alla et al., 2003). We can also conclude that \( A. \) \textit{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., 2017), results show that for the \( P. \) \textit{infestans}-resistant potato lines, there was no influence of the inclusion of the additional R gene \textit{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 \( P. \) \textit{infestans} (Haesaert et al., 2015; Haverkort et al., 2016).

In previous experiments, we found a higher \( r_m \) of \( M. \) \textit{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 constant darkness), a necessary control in the current experiments to adequately compare plants treated with \( P. \) \textit{infestans}. We tested this hypothesis by comparing water-inoculated and cold-treated plants and found that water-inoculation treatment does in fact influence aphid \( r_m \) (J. Lazebnik, M. Tibboel, M. Dicke, J.J.A. van Loon, unpubl.). 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 NTOs, 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.

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