Ecotoxicology and Environmental Safety 241 (2022) 113749

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

No adverse dietary effect of a cisgenic fire blight resistant apple line on the non-target arthropods *Drosophila melanogaster* and *Folsomia candida*

Ina Schlathölter [a,b], Michael Meissle [c], Timea Boeriis [a], Dominique Heimo [d], Bruno Studer [b], Giovanni A.L. Broggini [b], Jörg Romeis [c], Andrea Patocchi [a,*]

[a] *Agroscope, Research Division Plant Breeding, Breeding Research Group, Mueller-Thurgau-Strasse 29, 8820 Waedenswil, Switzerland*
[b] *Molecular Plant Breeding, Institute of Agricultural Sciences, ETH Zurich, Universitaetstrasse 2, 8092 Zurich, Switzerland*
[c] *Agroscope, Research Division Agroecology and Environment, Biosafety Research Group, Reckenholzstrasse 191, 8046 Zurich, Switzerland*
[d] *Agroscope, Research Division Methods Development and Analytics, Feed Chemistry Group, Route de la Tioleyre 4, 1725 Posieux, Switzerland*

ARTICLE INFO

Edited by: Professor Bing Yan

Keywords:
Biosafety
Genetic modification
Non-target organisms
Environmental risk assessment
Natural variation
Malus × *domestica*

ABSTRACT

Genetic modification of apple cultivars through cisgenesis can introduce traits, such as disease resistance from wild relatives, quickly and without crossing. This approach was used to generate the cisgenic apple line C44.4.146, a 'Gala Galaxy' carrying the fire blight resistance gene *FB_MR5*. In contrast to traditionally bred apple cultivars, genetically modified (GM) plants need to undergo a regulatory risk assessment considering unintended effects before approval for commercial release. To determine potential unintended effects of C44.4.146, we assessed major leaf components and effects on the fitness of the decomposers *Drosophila melanogaster* (fruit fly) and *Folsomia candida* (collembolan), which were fed a diet amended with powdered apple leaf material. Leaf material of 'Gala Galaxy', several natural 'Gala' mutants, and the unrelated apple cultivar 'Ladina' were used for comparison. The genetic modification did not alter major leaf components and did not adversely affect survival, growth, or fecundity of the two decomposers. Consistent with previous studies with other GM crops, the differences between conventionally bred cultivars were greater than between the GM line and its non-GM wild type. These data provide a baseline for future risk assessments.

1. Introduction

Risk assessments of genetically modified crops include the molecular characterization of the transformed plant, a comparative analysis of compositional, phenotypic and agronomic properties, a safety assessment for humans and animals (toxicology, allergenicity, nutritional value), and a safety assessment for the environment (Craig et al., 2008; OECD, 1986; Devos et al., 2014; European Food Safety Authority, 2010). Environmental risk assessments (ERAs) in the European Union evaluate the effects of intentional and unintentional modifications in the genome and phenotype on non-target organisms (NTOs) and biogeochemical processes (Devos et al., 2014; European Food Safety Authority, 2010). Intentional modifications are caused by the expression of the introduced trait, while unintended modifications can result from pleiotropic interactions of the gene product, from the insertion site, from gene disruption resulting from gene integration and somaclonal mutations introduced via tissue culture and transformation (Ladics et al., 2015). Many ERAs investigating the interaction of GM plants with NTOs have been performed. Most studies focused on transgenic plants producing insecticidal Cry proteins from *Bacillus thuringiensis* (*Bt*) and provided intensive data on the safety for NTOs (Romeis et al., 2019). However, some studies, especially those using plant material as a test substance, did reveal differences between the GM and corresponding non-GM material (Bakonyi et al., 2006; Cascone et al., 2018; Yuan et al., 2011, 2013). In some cases, these differences were likely linked to unintended differences in plant composition rather than to the introduced trait (Yuan et al., 2011; Poerschmann et al., 2005; Saxena and Stotzky, 2001). Such differences, e.g., a higher lignin content and C:N ratio in leaves, were shown to reduce food quality for *Folsomia candida* (Collembola: Isotomidae) (Yuan et al., 2011; Saxena and Stotzky, 2001). If significant

---

Abbreviations: Bt, *Bacillus thuringiensis*; *Dm*, *Drosophila melanogaster*; ERA, environmental risk assessment; *Fc*, *Folsomia candida*; IVG, in vitro cultured 'Gala Galaxy'; GG, 'Gala Galaxy' from a Swiss nursery; GGB, 'Gala Galaxy' from an Italian nursery; RG, 'Royal Gala'; GSR, 'Gala Schniga® SchniCo red'; GO, 'Gala' original; GM, genetically modified.

* Corresponding author.
E-mail address: andrea.patocchi@agroscope.admin.ch (A. Patocchi).

https://doi.org/10.1016/j.ecoenv.2022.113749
Received 28 February 2022; Received in revised form 4 June 2022; Accepted 4 June 2022

differences between GM and their non-GM counterparts are found, they trigger further investigation into potential harmful effects. For assessing potential harm, a proper study design, including comparisons to variations found among conventionally bred varieties or between different plant tissues or locations, are recommended (European Food Safety Authority, 2010; Chen et al., 2021; Garcia-Alonso and Raybould, 2014; Wang et al., 2017). Studies on NTOs exposed to GM plants have shown that variations among conventional varieties were generally greater than the variations caused by the genetic modification (e.g., Herman and Price, 2013; Lazebnik et al., 2017a; Zurbrügg et al., 2010).

Cisgenesis, i.e., the transfer of genes from the same or crossable species by means of genetic modification, has great potential in perennial, outcrossing plant species with long breeding cycles. In apple, cisgenesis could reduce the time to improve a market-established cultivar by adding disease resistance or other favourable traits. The introduction of disease resistance genes into susceptible cultivars will also reduce the use of plant protection products (Haverkort et al., 2016). A well-studied example of a cisgenic plant is A15–31, a potato line derived from the late blight susceptible cultivar 'Desirée'. A15–31 was amended with the resistance gene *Rpi-Vnt1.1*, obtained from *Solanum venturii*, which confers resistance to the causal agent of late blight, *Phytophthora infestans* (Haesaert et al., 2015). Studies on this cisgenic potato line found no effect of the genetic modification on the composition of arthropod (Lazebnik et al., 2017b), nematode (Ortiz et al., 2016), or microbial communities (Krause et al., 2020) in the soil. Two studies, however, reported possible unintended effects of A15–31 compared to its wild type 'Desirée' when leaf material was fed to two aphid species (Cascone et al., 2018; Lazebnik et al., 2017a). Yet, these effects occurred in the 1st but not in the 2nd generation of aphids and were insignificant compared to the variation among conventionally bred potato varieties (Lazebnik et al., 2017a).

Full risk assessments of GM plants commonly include first-tier laboratory tests with a range of non-target species covering different taxonomic groups and ecological functions (European Food Safety Authority, 2010). For the current study, we selected two soil arthropods *F. candida* (*Fc*) and *Drosophila melanogaster* (*Dm*) (Diptera: Drosophilidae) (fruit fly). Many soil arthropods are essential ecosystem components because they increase the decomposition of litter and are foods for higher trophic levels (Fountain and Hopkin, 2005). Such arthropod species may be exposed to GM plant material through ingestion of root material, plant debris, or fruit that falls to the ground. Collembolans occur in many habitats including greenhouses, forest soils, and grasslands (Fountain and Hopkin, 2005). They are widely used in ERAs and toxicology tests because of their asexual mode of reproduction, rapid life cycle, easy maintenance in the laboratory, and high sensitivity to environmental contaminants (Fountain and Hopkin, 2005; OECD, 2016). To date, *Fc* has been used with different transgenic GM plants including cotton, potato, corn, rice, and wheat (Yuan et al., 2011, 2013; Bai et al., 2011; Romeis et al., 2003; Yang et al., 2018; Yu et al., 1997; Zhang et al., 2017). *D. melanogaster* is another well-known laboratory species. While many soil-dwelling dipterans are important decomposers of leaf litter (Savage, 2002), few studies have focused on the effects of GM plants on dipterans (Haller et al., 2016, 2017; Knecht and Nentwig, 2010; Peter et al., 2010).

The cisgenic apple line C44.4.146 evaluated in this study was developed by adding the gene *FB_MR5* of the wild apple *Malus* × *robusta* 5 to the 'Gala Galaxy' cultivar through *Agrobacterium tumefaciens*-mediated transformation (Kost et al., 2015). The susceptible 'Gala Galaxy' cultivar thereby acquired resistance to fire blight, which is a devastating bacterial disease of apples. Because *FB_MR5* is expressed at similar transcript levels in C44.4.146 as in conventionally bred accessions (Kost et al., 2015), they should share the same risks posed by the transcription of *FB_MR5*. Our work therefore aimed to identify unintended effects in the cisgenic line C44.4.146. We assessed effects on arthropod fitness using diets supplemented with powdered leaf material in bioassays with *Dm* and *Fc*. Major leaf components were analysed to assess correlations with bioassay results. In order to place the obtained results within the context of natural variation we performed these studies with a large control group consisting of 'Gala Galaxy', 'Gala', several 'Gala' sports, and the 'Gala'-unrelated cultivar 'Ladina'.

2. Materials and methods

2.1. Plant material

We used the cisgenic apple line C44.4.146 (CIS), its non-transformed 'Gala Galaxy', 'Royal Gala' (RG), 'Gala Schniga® SchniCo red' (GSR), the original 'Gala' cultivar (GO), and the cultivar 'Ladina' (LAD). 'Gala Galaxy', RG and GSR belong to the group of 'Gala' sports, i.e., natural mutants of 'Gala' propagated from shoots in which mutation(s) in the meristem generated a new, stable phenotype. Sports are optimal controls for the evaluation of the cisgenic 'Gala' line because they share the genetic origin and show a limited number of trait modifications. 'Gala Galaxy' was obtained from three sources: in vitro cultured 'Gala Galaxy' (IVG), i.e., the line used to generate CIS (wild type of CIS in this study), and 'Gala Galaxy' from commercial nurseries in Switzerland (GG) and in Italy (GGB). These sources of 'Gala Galaxy' were treated as separate genotypes. With the permission of the Swiss Federal Office of Environment (FOEN) for a field trial (reference no. B15001), the apple trees were planted in spring 2016 at a protected field site at Agroscope Reckenholz (Zurich, Switzerland, www.protectedsite.ch) (Brunner et al., 2021; Romeis et al., 2013) as described in Schlathölter et al. (Schlathölter et al., 2021a, 2021b). The field experiment consisted of four blocks (planting rows) with a group of five consecutive trees of every 'Gala' genotype in each block. The genotypes within each block were arranged in a randomized order. At the beginning of each group of five trees, one LAD tree was planted. LAD originated from a cross between 'Topaz' and 'Fuji' (Leumann et al., 2013) and was used as the outgroup. The trees were drip-irrigated, herbicides were applied to maintain the soil around the trees free from grasses, and pest and disease control followed the international guidelines of integrated production (Wijnands et al., 2018; Alaphilippe et al., 2019).

2.2. Leaf sampling for non-target studies

Leaves were collected just before leaf fall on October 10, 2018. Twenty-five leaves were randomly collected from each tree. Leaves from the trees of each genotype within the same block were pooled in a paper bag, resulting in four independent biological samples per genotype. The plant material was dried for 2 days at 32 °C in a drying oven and was milled to a fine powder ($d = 1$ mm) with an ultra-centrifugal mill (ZM200, Retsch GmbH, Haan, Germany) resulting in 26–54 g of powdered leaf material per sample. The samples were stored at room temperature in closed glass bottles in the dark until used in the experiments.

2.3. Analysis of major leaf components

For analysis of total carbon (C) and total nitrogen (N), leaf samples (400 mg per sample) were further dried at 60 °C and analysed with a VarioMax CN elemental analyser (Elementar, Langenselbold, Germany) following a dry combustion method (Bremner and Mulvaney, 1983).

Leaf dry matter, crude fibre, cellulose, hemicellulose, and lignin were analysed according to Association of German Agricultural Analytic and Research Institutes (VDLUFA, 2012) and equivalent International Organization for Standardization (ISO) methods. Samples for the analysis of dry matter were processed on a prepASH® 340 Series (Precisa Gravimetrics AG, Dietikon, Switzerland). Crude fibre was analysed following ISO method 6865:2000. The neutral detergent fibre (αNDF) method (ISO 16472:2006) was used to assess hemicellulose, cellulose, and lignin; the acid detergent fibre (ADF) method was used to assess cellulose and lignin; and the acid detergent lignin (ADL) method was used to determine lignin content (both ISO 13906:2008). Cellulose content was calculated by subtracting ADL from ADF, while hemicellulose was calculated by subtracting ADF from αNDF. Crude fibre, αNDF, ADF, and ADL were analysed with a FIBRETHERM® FT-12 (C. Gerhardt GmbH & Co. KG, Königswinter, Germany). All analyses were done with at least two technical replicates.

2.4. Arthropods

Dm was cultured at Agroscope on cornmeal-sucrose-yeast agar medium containing 4-hydroxybenzoate to prevent fungal growth (Haller et al., 2016, 2017). *Fc* was provided by Innovative Environmental Services Ltd. (Witterswil, Switzerland), cultured at Agroscope in boxes with plaster of Paris and activated charcoal, and fed with baker's yeast as described by Romeis et al. (2003).

2.5. Feeding experiments with Dm

The *Dm* bioassays were based on a "single-fly" protocol (Haller et al., 2016). Adjustments for plant material were adapted from (Haller et al., 2017), who showed that a ratio of one part cotton or maize leaf powder to two parts solid components of the artificial diet allowed normal development of the flies. For the present study, one-third of the solid ingredients of the cornmeal-sucrose-yeast agar diet was replaced by apple leaf powder. To prepare the diet, cornmeal, sucrose, yeast, and agar were added to water and heated, while stirring constantly, until it boiled. When the diet cooled to 55 °C, the leaf powder was added with steady stirring before the diet was poured into plastic boxes for solidification. The standard cornmeal diet was used as a negative control (NC), and cryolite (Na_3AlF_6, sodium hexafluoroaluminate) (purity: ≥ 97.0%) (Sigma-Aldrich, Buchs, Switzerland) at a concentration of 0.4 μg/g standard cornmeal diet served as a positive control (PC). Cylinders of artificial diet were cut with a cork drill (6 mm diameter) and placed individually in 8-tube strips (art. no. 4413, Corning Incorporated, Corning, NY, USA). The 8-tube strips rather than 96-well plates (Haller et al., 2017) were used to avoid heat-sealing and facilitate monitoring. One *Dm* egg (< 4 h old) was placed on top of the diet in each tube. The 8-tube strips were closed with 8-cap strips (art. no. 4418, Corning Incorporated, Corning, NY, USA) containing three 0.45-mm holes for ventilation. The experiment was checked daily until all eggs had developed to adults. The treatments used for the experiment were the leaf-based diets of the eight genotypes from the field as described above plus the negative and positive controls. The experiment was performed four times with four 8-tube strips (32 tubes) per treatment (one strip per block for the leaf-based treatments). It was conducted in a climate chamber at 25 ± 1 °C, 70 ± 10% relative humidity, and a 16-h photoperiod. If the diet in a tube dried out or was heavily contaminated with fungi, the tube was excluded from further analyses, resulting in a total of 89–124 tubes per treatment.

Survival, developmental time (days to the emergence of an adult fly), and adult dry weight were recorded at the end of the experiment. For dry weight measurements, the tubes containing the adult flies were frozen at − 20 °C; the tubes were tilted so that the flies would not freeze to the surface of the medium; after 20 min, the dead flies were transferred to empty 8-tube strips and dried at 35 °C in a drying oven over night. After the sex of the flies was determined by examining the abdominal segments with a stereo microscope, dry weight was measured with a micro balance (MX5, Mettler Toledo, Greifensee, Switzerland).

2.6. Feeding experiments with Fc

The *Fc* bioassays were conducted with mature 10-day-old individuals, as recommended in OECD guideline 232 (OECD, 2016). Individual collembolans were randomly placed in plastic boxes (diameter 70 mm, height 39 mm) containing a 10-mm layer of a solidified mixture of plaster of Paris (art. no. 51828, LANDI Schweiz AG, Dotzigen, Schweiz), activated charcoal (art. no. 134340010, Acros Organics B.V.B.A., Fair Lawn, NJ, USA), and distilled water at a ratio of 8:1:9 (w/w/w) with scratches in the surface to facilitate egg deposition. Distilled water was added as needed to keep the plaster moist. Leaf powder alone is an unsuitable food for *Fc (*Romeis et al., 2003; Yu et al., 1997). Therefore, the collembolans were fed a 10:1 (w/w) mixture of leaf powder and brewer's yeast (art. no. 368080010, Acros OrganicsTM, NJ, USA). This ratio supported the survival, development, and reproduction of *Fc* in bioassays using leaf powder from wheat or rice (Romeis et al., 2003; Yang et al., 2018). The leaf-based diets were prepared as described by Yang et al. (2018) and stored at − 20 °C until used. The treatments used for the experiment included the leaf-based diets of the eight genotypes from the field as described above plus brewer's yeast (NC). The experiment consisted of 32 boxes per treatment (eight boxes per block for the leaf-based treatments). The collembolans had access to food *ad libitum*. The diets were placed on the plaster surface and were replaced twice per week using a small spatula to reduce fungal growth. The assay was carried out in a climate chamber in total darkness (20 ± 1 °C, 70% relative humidity). Mouldy eggs and injured collembolans were excluded from analysis, resulting in a total of 26–32 boxes per treatment.

The collembolans were checked daily for survival and oviposition. If clusters of eggs were found, the collembolan was transferred to a new box. Observations of the initial collembolans were terminated after 4 weeks. Collembolans were killed with CO_2 and individually weighed (MX5, Mettler Toledo, Switzerland). The boxes containing the eggs were checked for hatching daily from 7 days after oviposition. After hatching, neonates were fed with the same diet as their parent for 1 week before the final number of offspring was determined. For this purpose, the box was flooded with water, and the specimens floating on the surface were counted. The following data were recorded: duration to 1st, 2nd, and 3rd oviposition; number of neonates of the 1st, 2nd, and 3rd oviposition; and weight after 4 weeks.

2.7. Statistical analysis

All statistical analyses were conducted in R 4.1.1 (R Core Team, 2021). All data are presented as means ± SD unless otherwise indicated. Boxplots were generated using the 'ggplot2' package (Wickham, 2016).

Leaf components were analysed using a linear mixed model in the package 'lme4' (Bates et al., 2015) with genotype as a fixed effect and block as a random effect, followed by an ANOVA with type III sums of squares ('stats' package) (R Core Team, 2021).

The effect of dietary treatments on survival in the *Dm* bioassay was assessed using a generalized linear mixed model (glmm) applying a binominal distribution with treatment as a fixed effect, and experiment, experiment × treatment interaction, and block as random effects ('lme4' package), followed by a Chi-square test. Data for developmental time were analysed using a glmm applying a Poisson distribution using the same effects, followed by an F-test. Female and male dry weights were analysed using a linear mixed model (lmm) using the same factors plus sex and sex × treatment interaction as fixed effects, followed by an F-test. If the sex × treatment interaction was significant, the statistical analyses for this endpoint were repeated for each sex separately. Statistical analyses were generally done in two steps: (1) the two control treatments, NC and PC, were compared to each other, and (2) the plant treatments were compared with the NC.

In the *Fc* bioassay, the effects of treatments on survival after 4 weeks were tested with a glmm applying a binominal distribution with treatment as a fixed effect and block as a random effect, followed by a Chi-square test. Data for the duration to 1st, 2nd, and 3rd oviposition were analysed using a glmm applying a Poisson distribution using the same effects, followed by an F-test. Collembolan weight and the number of neonates from the 1st, 2nd, and 3rd oviposition were analysed using a lmm using the same effects, followed by an F-test.

If a treatment effect was significant, means were separated by Tukey's honestly significant difference (HSD) post-hoc tests using the 'multcomp' package (Hothorn et al., 2008). The *p*-values of the Tukey comparisons were converted to letters indicating significantly different groups ('multcompView' package) (Graves et al., 2019).

3. Results

3.1. Analysis of major leaf components

A genotype effect ($p < 0.05$) was identified for all leaf components assessed except cellulose (Fig. 1, Table S1). CIS was not statistically different from its non-GM wild type IVG for any of the measured parameters. CIS had lower concentrations of crude fibre than the 'Gala' genotypes GGB and GO, lower total N concentrations than GSR, and higher C:N ratios than GGB and GSR. Among the non-GM 'Gala' genotypes, total C concentrations in GGB were lower than in GO, total N concentrations in IVG were lower than in GSR, and the C:N ratio in IVG was higher than in GGB and GSR.

Compared to the 'Gala' genotypes, the outgroup LAD had lower lignin concentrations, higher total N concentrations, and lower C:N ratios. LAD also had lower crude fibre concentrations than GGB and GO and lower hemi-cellulose concentrations than GGB.

3.2. Feeding experiments with Dm

The survival rate (percentage emerged flies) in the control (NC) of the third replication was 63.2% (Table S2). Because NC survival rates < 80% raise concern about the reliability of the test (Haller et al., 2016; Rose, 2007), the data of the third replication were excluded from further analyses. Subsequent calculations are thus based on three experimental replications. The survival rates of *Dm* fed either the GM or non-GM leaf treatments were > 78% in the three experimental replications with an average of 88.5% (Table S2).

Survival did not significantly differ between CIS and any of the non-GM treatments including LAD (Fig. 2 A). In addition, differences between the leaf-based diets and the cornmeal diet (NC) were not significant. The only significant difference was a higher survival rate in the GO treatment than in the RG treatment (Fig. 2 A). Developmental time from egg to adult fly ranged from 8.3 ± 0.5 (NC) to 8.9 ± 1.1 days (GSR) without significant differences among the treatments ($p = 0.28$, Fig. 2B). Treatment effects, however, were significant for dry weight. Because the interaction of sex × treatment was significant for dry weight ($p < 0.01$), statistical analyses were separately conducted for males and females.

Both female and male dry weight were significantly affected by the treatments (Fig. 2 C, D, Table S3), but no significant differences were detected among the leaf-based treatments. Female flies were heavier when fed cornmeal diet (NC) compared to all leaf-amended diets (Fig. 2D). A similar difference was observed for male flies, except that RG and GO did not differ from NC (Fig. 2 C). Survival, developmental time, and female/male dry weight significantly differed between NC and the PC, confirming the sensitivity of the assessed traits to the quality of the diet (Table S3).

3.3. Feeding experiments with Fc

The treatments did not significantly affect *Fc* survival or duration to the 1st, 2nd, and 3rd oviposition (Fig. 3A-D; Table S4). Treatment effects were significant for the numbers of hatched neonates from the 1st, 2nd, and 3rd oviposition, but no differences were observed among the 'Gala' treatments. The average number of neonates obtained from collembolans fed on leaf-based diets of the 'Gala' treatments ranged from 5.0 ± 3.4 (GG) to 7.5 ± 3.7 (IVG) in the 1st, from 7.3 ± 4.4 (GG) to 11.1 ± 6.2 (RG) in the 2nd, and from 7.6 ± 5.5 (GG) to 11.9 ± 9.3 (GGB) in the 3rd oviposition (Fig. 3E-G, Table S4). In the first two ovipositions, neonate counts were higher in the outgroup LAD and the yeast-based diet (NC) than in some of the 'Gala' treatments (Fig. 3E, F). The average number of neonates obtained in the first two ovipositions for LAD were 9.3 ± 3.4 and 14.6 ± 7.1, respectively. These numbers are similar to those obtained in the NC treatment (1st oviposition: 10.2 ± 4.1, 2nd oviposition: 13.2 ± 5.2). The number of neonates in the 3rd oviposition was significantly higher in the NC treatment than in all the other treatments, while the number in the LAD treatment was similar to those of the other leaf-based treatments (Fig. 3 G). Significant effects in collembolan weight at the end of the experiment were mainly due to differences between leaf-based diets and NC, which showed a 2.4-fold increased weight with 85.9 ± 20.4 μg compared to a range of 25.6 ± 12.9 μg (IVG) to 44.9 ± 26.3 μg (GO) among the leaf-based diets (Fig. 3H). The only statistical differences for collembolan weight within the 'Gala' treatments was between IVG and the treatments GO and GGB. For this parameter, no difference between LAD and the 'Gala' treatments was observed.

4. Discussion

This study showed that the genetic modification of 'Gala Galaxy' that resulted in the cisgenic apple line C44.4.146 did not alter the levels of the major leaf components assessed in this study in a way that affected the survival, growth, or fecundity of two important detritivorous arthropods fed on processed leaf material.

The differences in lignin, N, and C:N ratio between the conventionally bred 'Gala' cultivars, including natural mutants, and 'Ladina' were greater than the differences between the cisgenic line and its non-GM wild type. Similar results were reported for other GM crops; in Zurbrügg et al. (2010) and Poerschmann et al. (2008), for example, differences in plant composition were greater between conventional maize varieties than between GM-maize lines and their closest non-GM comparators. Studies at transcriptome and metabolome levels obtained similar results for *Bt* rice lines, their non-*Bt* parental rice lines, and conventionally bred rice lines (Liu et al., 2020). In the latter study, genetic modification and crossbreeding resulted in a comparable number of changes, and the largest changes were among conventionally bred non-GM lines.

Leaf composition can influence litter decomposition. That a low C:N ratio and a low lignin:N ratio increase litter decomposition was demonstrated in previous studies (Poerschmann et al., 2005; Taylor et al., 1989). A high rate of N deposition may benefit herbivores (Throop and Lerdau, 2004) and could have positively affected the quality of 'Ladina' leaves for decomposition in our study. Yuan et al. (2011) hypothesised that the lower reproduction of *Fc* fed on the *Bt* rice variety Huahui1 rather than its conventional isoline Minghui63 was either due to a higher C:N ratio in the leaf material or to other unintentional changes in plant metabolism. However, this hypothesis could not be confirmed (Yuan et al., 2013), and the connection to the high C:N ratio did not hold true for a different *Bt* rice line (Yuan et al., 2011). Lignin content was also higher in a *Bt* maize line than in its non-GM near isogenic line (Saxena and Stotzky, 2001). Because of lignin's low decomposability and its protective function in the plant cell, a lower lignin content in 'Ladina' leaves compared to 'Gala' (Fig. 1D) may have increased the ability of *Fc* to digest the leaves, resulting in increased fecundity.

Diets supplemented with GM leaf material (C44.4.146) did not adversely affect survival, growth, or fecundity of *Dm* or *Fc*. Statistical differences between NC and PC in all parameters in the *Dm* bioassay confirmed that this assay could detect the sensitivity of *Dm* to standard toxic substances (Table S3). Assays with both arthropods showed that the diets prepared with powdered apple leaf material supplied sufficient nutrients for survival, growth, and reproduction. Survival rates were similar for the leaf-based diets vs. the standard diets (NC) for *Dm* and *Fc* (Figs. 2A, 3A).

Nevertheless, *Dm* and *Fc* body weights and *Fc* fecundity were significantly lower with leaf-based diets than with the respective standard diet (NC) (Fig. 2 C, 2D, and 3 H). This indicated that the leaf

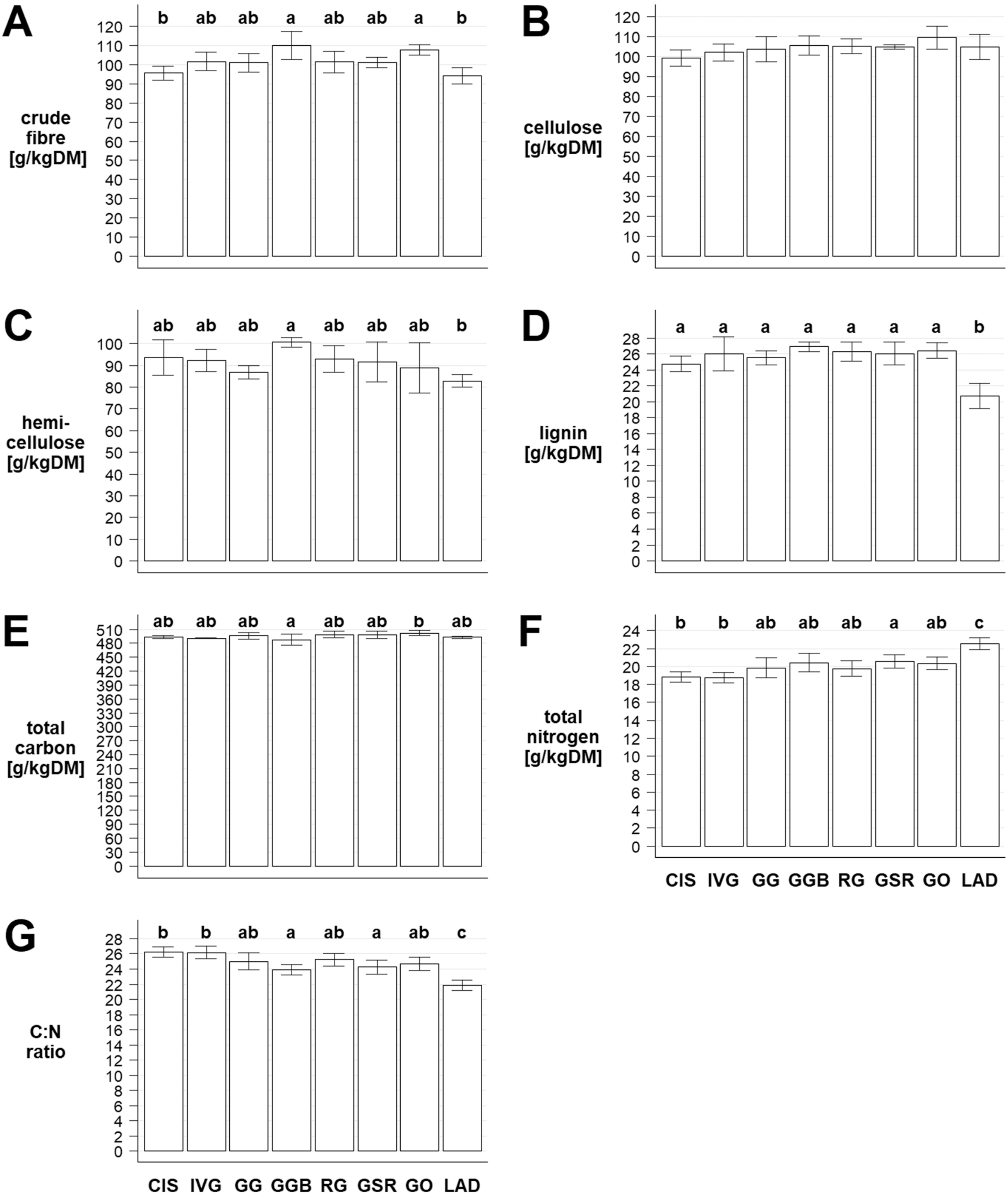

Fig. 1. Comparison of crude fibre (A), cellulose (B), hemicellulose (C), lignin (D), total C (E), total N (F), and C:N ratio (G) in leaves of the seven investigated ‘Gala’ genotypes and ‘Ladina’ (LAD). The sample size per parameter and genotype was four. The components were measured per kg of dry matter (DM). Different letters above the bars indicate significant differences among the genotypes based on Tukey’s HSD post-hoc analyses ($p < 0.05$). No letters are presented in (B) because the genotype effect was not significant. IVG: in vitro cultured ‘Gala Galaxy’, GG: ‘Gala Galaxy’ (budwood purchased from a Swiss nursery), GGB: ‘Gala Galaxy’ (purchased as 1-year-old-plant from an Italian nursery), RG: ‘Royal Gala’, GSR: ‘Gala Schniga® SchniCo red’, GO: ‘Gala’ original.

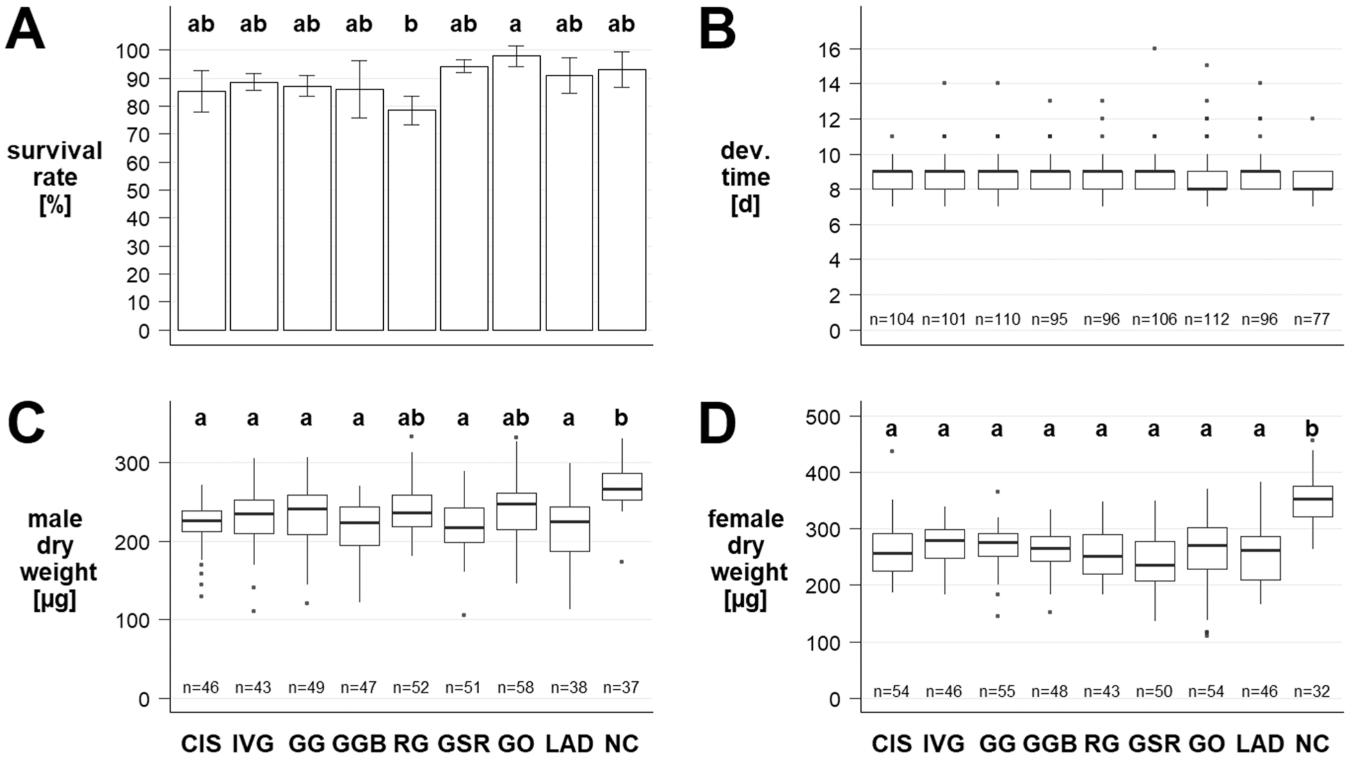

Fig. 2. Effects of dietary treatments containing leaf material of the seven investigated 'Gala' genotypes and 'Ladina' (LAD) on (A) survival rate, (B) developmental time, (C) male dry weight, and (D) female dry weight of *D. melanogaster* (*Dm*). The standard cornmeal diet for *Dm* served as a negative control (NC). Survival rates are means ± SD for the three experimental replications. Boxplots of developmental time and dry weight indicate the distribution of the data pooled from three experimental replications (centre line, median; box limits, first and third quartiles; whiskers, 1.5 × interquartile range; dots, outliers). The total number of considered *Dm* specimens (n) is indicated. In each panel, means with different letters are significantly different ($p < 0.05$) according to Tukey's HSD post-hoc analyses. No letters are presented in (B) because the treatment effect was not significant. CIS: C44.4.146, IVG: in vitro cultured 'Gala Galaxy', GG: 'Gala Galaxy' (budwood purchased from a Swiss nursery), GGB: 'Gala Galaxy' (purchased as 1-year-old-plant from an Italian nursery), RG: 'Royal Gala', GSR: 'Gala Schniga® SchniCo red', GO: 'Gala' original.

material had a reduced nutritional quality for the arthropods. Food of lower quality made with rice, cotton, wheat, or potato leaf material impaired the development and fecundity of *Fc* (Bai et al., 2011; Romeis et al., 2003; Yu et al., 1997; Stam et al., 1996) but not of *Dm* (Haller et al., 2017). The difference between our results and those of Haller et al. (Haller et al., 2017) suggests that as a food for *Dm*, the apple leaves used in the current study might have been inferior to the cotton and maize leaves used in the previous study. The parameters that did not differ among treatments in the current study, i.e., *Dm* developmental time and *Fc* oviposition periods, were similar to the values described in the literature (Tables S3 and S4) (Romeis et al., 2003; Strasburger, 1935).

Fc fecundity was significantly higher with 'Ladina' than with some of the 'Gala' treatments (Fig. 3E, F). Besides the previously mentioned lower lignin and C:N ratios of 'Ladina' (Fig. 1D, G), other undetected compositional differences could have contributed to this effect. Interestingly, *Fc* fecundity was similar when fed 'Ladina' vs. the optimal diet (NC) in the first two ovipositions, but lower in the 3rd oviposition. The final *Fc* weight in the 'Ladina' treatment was comparable to the 'Gala' treatments and lower than in the optimal diet (NC) (Fig. 3G-I). The hypothesis by Stam et al. (Stam et al., 1996), who found that collembolan weight was positively correlated with fecundity, was only supported for the 3rd oviposition, but not for the 1st and 2nd. This indicates that factors in addition to weight may influence fecundity. In another study that compared cisgenic late blight resistant potato lines to their non-GM cultivar 'Desirée' and to two conventionally bred cultivars for their suitability as food for a non-target aphid, aphid fitness varied more among conventional potato varieties than between 'Desirée' and the GM lines (Lazebnik et al., 2017a). In accordance with our compositional analyses and the results of Lazebnik et al. (2017a), the current non-target bioassays also indicate that the variation between conventionally bred varieties is greater than between the cisgenic line and its non-transformed wild type.

In conclusion, the evaluation of potential adverse effects on NTOs is an important part of the ERA of GM crops. This is the first assessment of cisgenic apple leaf material in arthropod bioassays. No adverse effects due to the genetic modification were observed on survival, growth, or fecundity of the two arthropods fed on artificial diets containing powdered leaf material. The lack of effects on these NTOs, as well as the unchanged levels of major leaf components indicates that litter produced by the cisgenic apple genotype C44.4.146 will be decomposed by soil arthropods in a comparable manner to non-GM apple tree litter. Our data support previous reports showing greater differences among conventional cultivars than between GM and non-GM plants. This observation highlights the value of including several conventional cultivars in ERA-studies (Chen et al., 2021; Wang et al., 2017; Sanvido et al., 2012). Our study therefore supports the hypothesis that cisgenic GM plants are as safe as conventionally bred plants (National Academies of Sciences, E. and Medicine NASEM, 2016).

Funding

This work was supported by the Swiss National Science Foundation (SNSF) [grant number 31003A_163386].

CRediT authorship contribution statement

Ina Schlathölter: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft,

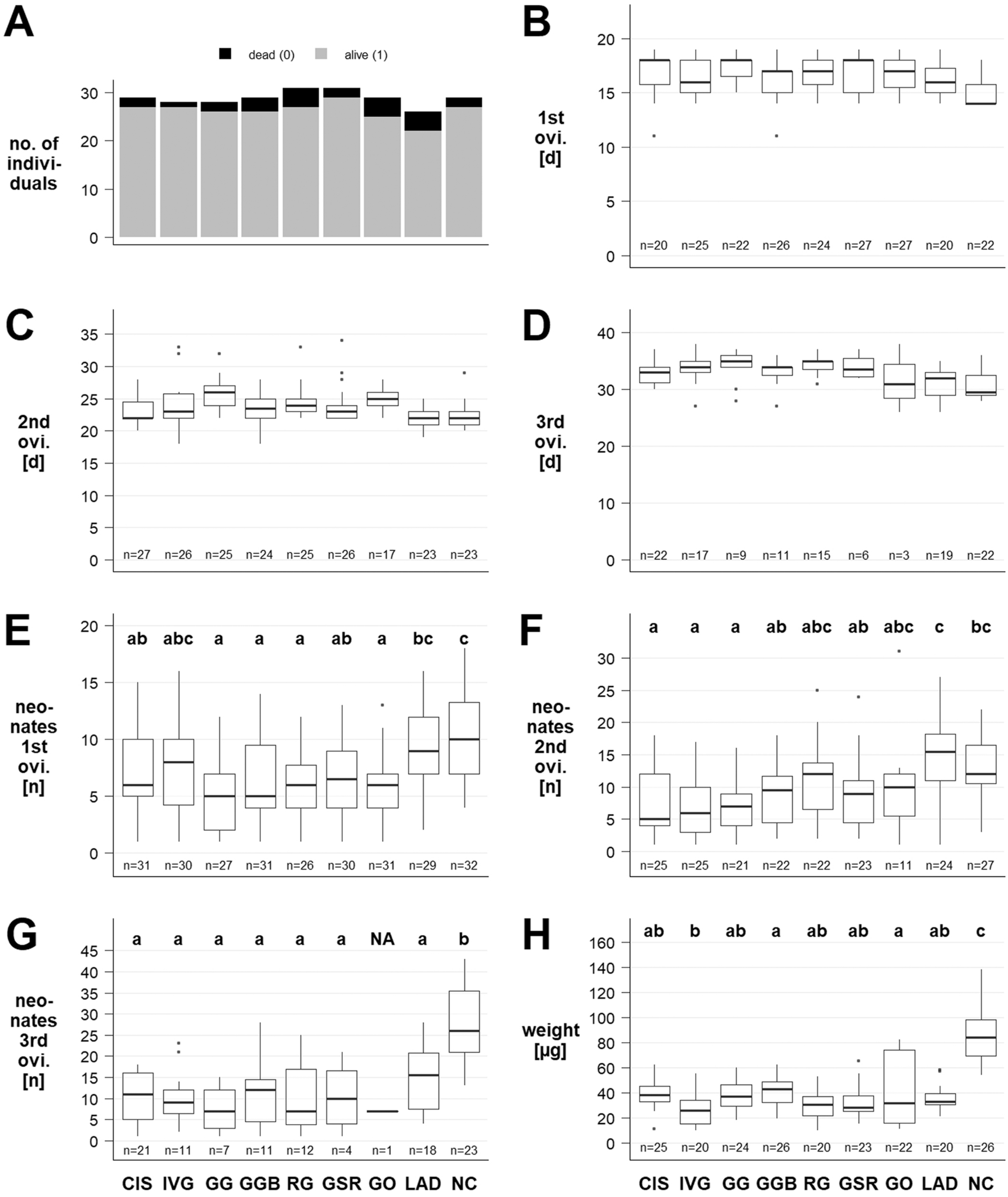

Fig. 3. Effects of dietary treatments containing leaf material of the seven investigated 'Gala' genotypes and 'Ladina' (LAD) on survival (A), time to the 1st, 2nd, and 3rd oviposition (B-D), number of neonates hatched from the 1st, 2nd, and 3rd oviposition (E-G), and final weight (H) of *F. candida* (*Fm*). The standard diet of *Fc* (brewer's yeast) was used as a negative control (NC). Survival is presented as a stacked barplot of the number of survived (grey) and dead (black) collembolans. The boxplots represent the distribution of the data (centre line, median; box limits, first and third quartiles; whiskers, 1.5 × interquartile range; dots, outliers). The total number of considered collembolans (n) is indicated; in panels B–H, means with different letters are significantly different ($p < 0.05$) according to Tukey's HSD post-hoc analyses. "NA" indicates that the corresponding data were not included in the analyses because of a low number of surviving specimens. If the treatment effect was not significant, no letters are presented. CIS: C44.4.146, IVG: in vitro cultured 'Gala Galaxy', GG: 'Gala Galaxy' (budwood purchased from a Swiss nursery), GGB: 'Gala Galaxy' (purchased as a 1-year-old plant from an Italian nursery), RG: 'Royal Gala', GSR: 'Gala Schniga® SchniCo red', GO: 'Gala' original.

Writing – review & editing. **Michael Meissle**: Conceptualization, Methodology, Supervision, Writing – review & editing. **Timea Boeriis**: Investigation, Methodology. **Dominique Heimo**: Investigation, Writing – review & editing. **Bruno Studer**: Supervision, Writing – review & editing. **Giovanni A. L. Broggini**: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Jörg Romeis**: Conceptualization, Methodology, Supervision, Writing – review & editing. **Andrea Patocchi**: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Mario Waldburger for maintaining the collembolans, Sarah Wolf for providing the *D. melanogaster* strain, and Martin Zuber for analysis of total C and total N and for advice on the milling of leaf samples (all Agroscope Reckenholz). We also thank Susanne Vogelgsang and Andreas Kägi (both Agroscope Reckenholz) for giving us access and advice on the use of the centrifuge mill, and Dominik Suter, Emmanuel Kopp, and Anna Dalbosco for their help with milling the leaf samples (Agroscope Reckenholz and Waedenswil). Thanks also to the Extension Horticulture team of Agroscope Waedenswil for the construction and maintenance of the apple orchard and to Susanne Brunner for the management of the protected field site.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113749.

References

Alaphilippe, A., Belien, T., Ioriatti, C., & Malavolta, C., 2019, Crop specific technical guidelines for integrated production of pome fruits. https://www.iobc-wprs.org/ip_practical_guidelines/index.html. Accessed: 30/01/2022.

Bai, Y., Yan, R., Ke, X., Ye, G., Huang, F., Luo, Y., Cheng, J., 2011. Effects of transgenic *Bt* rice on growth, reproduction, and superoxide dismutase activity of *Folsomia candida* (Collembola: Isotomidae) in laboratory studies. J. Econ. Entomol. 104, 1892–1899. https://doi.org/10.1603/ec11095).

Bakonyi, G., Szira, F., Kiss, I., Villányi, I., Seres, A., Székacs, A., 2006. Preference tests with collembolas on isogenic and *Bt*-maize. Eur. J. Soil Biol. 42, 132–S135. https://doi.org/10.1016/j.ejsobi.2006.06.005).

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Soft 67, 1–48. https://doi.org/10.18637/jss.v067.i01).

Bremner, J.M., Mulvaney, C.S., 1983. Nitrogen - Total. Methods Soil Anal. 595–624. https://doi.org/10.2134/agronmonogr9.2.2ed.c31).

Brunner, S., Romeis, J., Patocchi, A., Peter, R., 2021. Die Protected Site - sieben Jahre Freilandforschung mit gentechnisch veränderten Pflanzen. Agrar. Schweiz 12, 9–15. https://doi.org/10.34776/afs12–9 g).

Cascone, P., Radkova, M., Arpaia, S., Errico, S., Lotz, L., Magarelli, R., Djilianov, D., Guerrieri, E., 2018. Unintended effects of a *Phytophtora*-resistant cisgenic potato clone on the potato aphid *Macrosiphum euphorbiae* and its parasitoid *Aphidius ervi*. J. Pest Sci. 91, 565–574. https://doi.org/10.1007/s10340-017-0941-1).

Chen, Y., Romeis, J., Meissle, M., 2021. Addressing the challenges of non-target feeding studies with genetically engineered plant material–stacked *Bt* maize and *Daphnia magna*. Ecotoxicol. Environ. Saf. 225, 112721 https://doi.org/10.1016/j.ecoenv.2021.112721).

Craig, W., Tepfer, M., Degrassi, G., Ripandelli, D., 2008. An overview of general features of risk assessments of genetically modified crops. Euphytica 164, 853–880. https://doi.org/10.1007/s10681-007-9643-8).

Devos, Y., Aguilera, J., Diveki, Z., Gomes, A., Liu, Y., Paoletti, C., du Jardin, P., Herman, L., Perry, J.N., Waigmann, E., 2014. EFSA's scientific activities and achievements on the risk assessment of genetically modified organisms (GMOs) during its first decade of existence: looking back and ahead. Transgenic Res 23, 1–25. https://doi.org/10.1007/s11248-013-9741-4).

European Food Safety Authority, 2010. Guidance on the environmental risk assessment of genetically modified plants. EFSA J. 8, 1879. https://doi.org/10.2903/j.efsa.2010.1879.

Fountain, M.T., Hopkin, S.P., 2005. *Folsomia candida* (Collembola): a "standard" soil arthropod. Annu. Rev. Entomol. 50, 201–222. https://doi.org/10.1146/annurev.ento.50.071803.130331).

Garcia-Alonso, M., Raybould, A., 2014. Protection goals in environmental risk assessment: a practical approach. Transgenic Res 23, 945–956. https://doi.org/10.1007/s11248-013-9760-1).

Graves, S., Piepho, H.-P., Selzer, L., & Dorai-Raj, S., 2019, multcompView: Visualizations of paired comparisons. https://CRAN.R-project.org/package=multcompView.

Haesaert, G., Vossen, J.H., Custers, R., De Loose, M., Haverkort, A., Heremans, B., Hutten, R., Kessel, G., Landschoot, S., Van Droogenbroeck, B., Visser, R.G.F., Gheysen, G., 2015. Transformation of the potato variety Desiree with single or multiple resistance genes increases resistance to late blight under field conditions. Crop Prot. 77, 163–175. https://doi.org/10.1016/j.cropro.2015.07.018).

Haller, S., Meissle, M., Romeis, J., 2016. Establishing a system with *Drosophila melanogaster* (Diptera: Drosophilidae) to assess the non-target effects of gut-active insecticidal compounds. J. Ecotoxicol. 25, 1794–1804. https://doi.org/10.1007/s10646-016-1722-y).

Haller, S., Romeis, J., Meissle, M., 2017. Effects of purified or plant-produced Cry proteins on *Drosophila melanogaster* (Diptera: Drosophilidae) larvae. Sci. Rep. 7, 11172. https://doi.org/10.1038/s41598-017-10801-4).

Haverkort, A.J., Boonekamp, P.M., Hutten, R., Jacobsen, E., Lotz, L.A.P., Kessel, G.J.T., Vossen, J.H., Visser, R.G.F., 2016. Durable late blight resistance in potato through dynamic varieties obtained by cisgenesis: Scientific and societal advances in the DuRPh project. Potato Res 59, 35–66. https://doi.org/10.1007/s11540-015-9312-6).

Herman, R.A., Price, W.D., 2013. Unintended compositional changes in genetically modified (GM) crops: 20 years of research. J. Agric. Food Chem. 61, 11695–11701. https://doi.org/10.1021/jf400135r).

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. 50, 346–363. https://doi.org/10.1002/bimj.200810425).

Knecht, S., Nentwig, W., 2010. Effect of *Bt* maize on the reproduction and development of saprophagous Diptera over multiple generations. Basic Appl. Ecol. 11, 346–353. https://doi.org/10.1016/j.baae.2010.02.010).

Kost, T.D., Gessler, C., Jänsch, M., Flachowsky, H., Patocchi, A., Broggini, G.A., 2015. Development of the first cisgenic apple with increased resistance to fire blight. PLoS One 10, e0143980. https://doi.org/10.1371/journal.pone.0143980).

Krause, S.M.B., Nather, A., Ortiz Cortes, V., Mullins, E., Kessel, G.J.T., Lotz, L.A.P., Tebbe, C.C., 2020. No tangible effects of field-grown cisgenic potatoes on soil microbial communities. Front. Bioeng. Biotechnol. 8, 603145 https://doi.org/10.3389/fbioe.2020.603145).

Ladics, G.S., Bartholomaeus, A., Bregitzer, P., Doerrer, N.G., Gray, A., Holzhauser, T., Jordan, M., Keese, P., Kok, E., Macdonald, P., Parrott, W., Privalle, L., Raybould, A., Rhee, S.Y., Rice, E., Romeis, J., Vaugh, J., Wal, J.-M., Glenn, K., 2015. Genetic basis and detection of unintended effects in genetically modified crop plants. Transgenic Res 24, 587–603. https://doi.org/10.1007/s11248-015-9867-7).

Lazebnik, J., Dicke, M., ter Braak, C.J.F., van Loon, J.J.A., 2017b. Biodiversity analyses for risk assessment of genetically modified potato. Agric. Ecosyst. Environ. 249, 196–205. https://doi.org/10.1016/j.agee.2017.08.017).

Lazebnik, J., Arpaia, S., Baldacchino, F., Banzato, P., Moliterni, S., Vossen, J.H., van de Zande, E.M., van Loon, J.J.A., 2017a. Effects of a genetically modified potato on a non-target aphid are outweighed by cultivar differences. J. Pest Sci. 90, 855–864. https://doi.org/10.1007/s10340-017-0831-6).

Leumann, L., Baumgartner, I., Lussi, L., Frey, L., Nölly, M., Kellerhals, M., Weber, M., 2013. Ladina, die neue feuerbrandrobuste Apfelsorte. Schweiz. Z. für Obst- und Weinbau 1, 10–13.

Liu, Q., Yang, X., Tzin, V., Peng, Y., Romeis, J., Li, Y., 2020. Plant breeding involving genetic engineering does not result in unacceptable unintended effects in rice relative to conventional cross-breeding. Plant J. 103, 2236–2249. https://doi.org/10.1111/tpj.14895).

National Academies of Sciences, E. & Medicine (NASEM), 2016. Genetically engineered crops: experiences and prospects. National Academies of Sciences, Engineering, and Medicine. The National Academies Press,, Washington, DC. https://doi.org/10.17226/23395).

OECD, 1986. Recombinant DNA safety considerations: safety considerations for industrial, agricultural and environmental applications of organisms derived by recombinant DNA techniques. Organization for Economic Cooperation and Development. OECD,, Paris.

OECD, 2016. Test No. 232: Collembolan Reproduction Test in Soil. In *OECD Guidelines for the Testing of Chemicals*. Sect. 2. OECD Publ. https://doi.org/10.1787/9789264264601-en).

Ortiz, V., Phelan, S., Mullins, E., 2016. A temporal assessment of nematode community structure and diversity in the rhizosphere of cisgenic Phytophthora infestans-resistant potatoes. BMC Ecol. 16, 55. https://doi.org/10.1186/s12898-016-0109-5).

Peter, M., Lindfeld, A., Nentwig, W., 2010. Does GM wheat affect saprophagous Diptera species (Drosophilidae, Phoridae)? Pedobiologia 53, 271–279. https://doi.org/10.1016/j.pedobi.2009.12.006).

Poerschmann, J., Gathmann, A., Augustin, J., Langer, U., Gorecki, T., 2005. Molecular composition of leaves and stems of genetically modified *Bt* and near-isogenic non-*Bt* maize - Characterization of lignin patterns. J. Environ. Qual. 34, 1508–1518. https://doi.org/10.2134/jeq2005.0070).

Poerschmann, J., Rauschen, S., Langer, U., Augustin, J., Górecki, T., 2008. Molecular level lignin patterns of genetically modified *Bt*-maize MON88017 and three conventional varieties using tetramethylammonium hydroxide (TMAH)-induced thermochemolysis. J. Agric. Food Chem. 56, 11906–11913. https://doi.org/10.1021/jf8023694).

R Core Team, 2021, R: A language and environment for statistical computing. ⟨https://www.R-project.org/⟩.

Romeis, J., Battini, M., Bigler, F., 2003. Transgenic wheat with enhanced fungal resistance causes no effects on *Folsomia candida* (Collembola: Isotomidae). Pedobiologia 47, 141–147. https://doi.org/10.1078/0031-4056-00177).

Romeis, J., Naranjo, S.E., Meissle, M., Shelton, A.M., 2019. Genetically engineered crops help support conservation biological control. Biol. Control 130, 136–154. https://doi.org/10.1016/j.biocontrol.2018.10.001).

Romeis, J., Meissle, M., Brunner, S., Tschamper, D., Winzeler, M., 2013. Plant biotechnology: research behind fences. Trends Biotechnol. 31, 222–224. https://doi.org/10.1016/j.tibtech.2013.01.020).

Rose, R., 2007. White paper on tier-based testing for the effects of proteinaceous insecticidal plant-incorporated protectants on nontarget invertebrates for regulatory risk assessment. USDA-APHIS US Environ. Prot. Agency, Wash., DC. ⟨https://www.epa.gov/sites/production/files/2015-09/documents/tier-based-testing.pdf⟩. Accessed 30/01/2022.

Sanvido, O., Romeis, J., Gathmann, A., Gielkens, M., Raybould, A., Bigler, F., 2012. Evaluating environmental risks of genetically modified crops: ecological harm criteria for regulatory decision-making. Environ. Sci. Policy 15, 82–91. https://doi.org/10.1016/j.envsci.2011.08.006).

Savage, J., 2002. Exploring the diversity of flies (Diptera). *Cleaning up the world: Dipteran decomposers*. Biodiversity 3, 12–15. https://doi.org/10.1080/14888386.2002.9712613).

Saxena, D., Stotzky, G., 2001. *Bt* corn has a higher lignin content than non-*Bt* corn. Am. J. Bot. 88, 1704–1706. https://doi.org/10.2307/3558416).

Schlathölter, I., Broggini, G.A., Meissle, M., Romeis, J., Studer, B., Patocchi, A., 2021a. Multi-level assessment of field-grown cisgenic apple trees. Acta Hortic. 1307, 239–246. https://doi.org/10.17660/ActaHortic.2021.1307.37).

Schlathölter, I., Dalbosco, A., Meissle, M., Knauf, A., Dallemulle, A., Keller, B., Romeis, J., Broggini, G.A., Patocchi, A., 2021b. Low outcrossing from an apple field trial protected with nets. Agronomy 11, 1754. https://doi.org/10.3390/agronomy11091754).

Stam, E., Leemkule, M., Ernsting, G., 1996. Trade-offs in the life history and energy budget of the parthenogenetic Collembolan *Folsomia candida* (Willem). Oecologia 107, 283–292. https://doi.org/10.1007/BF00328444).

Strasburger, E., 1935. Drosophila melanogaster Meig: eine Einführung in den Bau und die Entwicklung, 1 ed..,. Springer-Verlag,, Berlin Heidelberg. https://doi.org/10.1007/978-3-642-92298-5).

Taylor, B.R., Parkinson, D., William, F.J.P., 1989. Nitrogen and lignin content as predictors of litter decay rates: A microcosm test. Ecology 70, 97–104. https://doi.org/10.2307/1938416).

Throop, H.L., Lerdau, M.T., 2004. Effects of nitrogen deposition on insect herbivory: Implications for community and ecosystem processes. Ecosystems 7, 109–133. https://doi.org/10.1007/s10021-003-0225-x).

VDLUFA, 2012, Handbuch der Landwirtschaftlichen Versuchs-und Untersuchungsmethodik (VDLUFA-Methodenbuch), Bd. III. Die chemische Untersuchung von Futtermitteln. In: VDLUFA-Verlag Darmstadt, Germany. https://www.vdlufa.de/Methodenbuch/index.php?option=com_content&view=article&id=4&Itemid=111&lang=de.

Wang, F., Dang, C., Chang, X., Tian, J., Lu, Z., Chen, Y., Ye, G., 2017. Variation among conventional cultivars could be used as a criterion for environmental safety assessment of *Bt* rice on nontarget arthropods. Sci. Rep. 7, 41918. https://doi.org/10.1038/srep41918).

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag,, New York. ⟨https://ggplot2.tidyverse.org⟩.

Wijnands, F., Malavolta, C., Alaphilippe, A., Gerowitt, B., & Baur, R. 2018 General technical guidelines for integrated production of annual and perennial crops, IOBC-WPRS Commission IP Guidelines, 4th edition. ⟨https://www.iobc-wprs.org/ip_practical_guidelines/tablecode.php?culture=general⟩. Accessed: 30/01/2022.

Yang, Y., Zhang, B., Zhou, X., Romeis, J., Peng, Y., Li, Y., 2018. Toxicological and biochemical analyses demonstrate the absence of lethal or sublethal effects of *cry1C*- or *cry2A*-expressing *Bt* rice on the collembolan *Folsomia candida*. Front. Plant Sci. 9. https://doi.org/10.3389/fpls.2018.00131).

Yu, L., Berry, R., Croft, B., 1997. Effects of *Bacillus thuringiensis* toxins in transgenic cotton and potato on *Folsomia candida* (Collembola: Isotomidae) and *Oppia nitens* (Acari: Orbatidae). J. Econ. Entomol. 90, 113–118. https://doi.org/10.1093/jee/90.1.113).

Yuan, Y., Ke, X., Chen, F., Krogh, P.H., Ge, F., 2011. Decrease in catalase activity of *Folsomia candida* fed a *Bt* rice diet. Environ. Pollut. 159, 3714–3720. https://doi.org/10.1016/j.envpol.2011.07.015).

Yuan, Y., Xiao, N., Krogh, P.H., Chen, F., Ge, F., 2013. Laboratory assessment of the impacts of transgenic *Bt* rice on the ecological fitness of the soil non-target arthropod, *Folsomia candida* (Collembola: Isotomidae). Transgenic Res 22, 791–803. https://doi.org/10.1007/s11248-013-9687-6).

Zhang, B., Yang, Y., Zhou, X., Shen, P., Peng, Y., Li, Y., 2017. A laboratory assessment of the potential effect of Cry1Ab/Cry2Aj-containing *Bt* maize pollen on *Folsomia candida* by toxicological and biochemical analyses. Environ. Pollut. 222, 94–100. https://doi.org/10.1016/j.envpol.2016.12.079).

Zurbrügg, C., Hönemann, L., Meissle, M., Romeis, J., Nentwig, W., 2010. Decomposition dynamics and structural plant components of genetically modified *Bt* maize leaves do not differ from leaves of conventional hybrids. Transgenic Res 19, 257–267. https://doi.org/10.1007/s11248-009-9304-x).