The ecological relevance of chemical diversity in plants: pyrrolizidine alkaloids in Jacobaea species

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Chapter 6

The effects of phytohormone application on metabolite profile and the feeding of chewing, piercing-sucking and cell-content herbivores: a study in *Jacobaea vulgaris* and *Jacobaea aquatica*

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Abstract

Inducible defences are common strategies in plants. In a previous study methyl jasmonate (MeJA) application on the roots did not increase the total pyrrolizidine alkaloid (PA) concentration while the PA composition was shifted from less toxic senecionine-like PAs to more toxic erucifoline-like PAs. Here, we analyse whether levels of primary and secondary metabolites are influenced by MeJA and salicylic acid (SA) application. Furthermore, we tested whether herbivores with different feeding modes were affected by application of these phytohormones. We used *Jacobaea vulgaris* and *Jacobaea aquatica* as plant species and the chewing caterpillar *Mamestra brassicae*, the cell-content feeder leafminer *Liriomyza trifolii* and the piercing-sucking thrips *Frankliniella occidentalis* as herbivores. PCA analysis showed a clear distinction between the metabolome of MeJA treated, SA treated and control plants. Both phytohormone applications led to significant changes in bins (compounds) of the ^{1}H-NMR spectrum. More bins were up-regulated than down-regulated after MeJA treatment while the reverse was true for the SA treatment. We found significant less feeding damage of *M. brassicae* and *L. trifolii* in plants treated by MeJA while no significant difference was found between control and SA treated plants in both plant species. SA treatment rendered *J. aquatica* more resistant to thrips. Because the concentration of many bins changed, it is difficult to identify the bins that are related to resistance. For thrips resistance we could combine available data for thrips silver damage and NMR analysis for a segregation F2 population of a cross between *J. vulgaris* and *J. aquatica* and identified NMR bins that were positively or negatively correlated with thrips resistance. We then checked whether or not these bins were down-regulated or up-regulated after SA treatment in *J. aquatica*. This analysis led to three candidate compounds related to susceptibility (sucrose, glucose and 3,5-dicaffeoylquinic acid (3,5-DCQA)) and three compounds related to resistance (citric acid, threonine and alanine). The positive effects of sucrose, glucose and 3,5-DCQA and the negative effects of citric acid on thrips survival were confirmed by results of unpublished mortality bioassay done previously in our research group. Literature data showed that β-alanine was positively correlated to thrips mortality.

Key words

Metabolome, ^{1}H-NMR, Methyl jasmonate, Salicylic acid, Jacaranone, Isoleucine, Alanine, Threonine

Introduction

Plants have to cope with various enemies, such as herbivores and pathogens in the natural environment. They therefore have evolved different strategies including chemical defences and mechanical defences, such as trichomes and thorns. Chemical defences are mainly based on secondary metabolites (SMs) of different chemical origins, often characteristic for certain plant taxa and effective at least against generalist herbivores (Schoonhoven et al. 2005). Besides constitutive defences, many metabolites can be induced when the plants are attacked by pathogens or herbivores (Karban and Baldwin 2007).

Induction of chemical defence after attack is mediated by phytohormones like jasmonate (JA), ethylene (ET) and salicylic acid (SA). As a general rule, the JA pathway that frequently acts synergistically with ET is hypothesized to be up-regulated if the plant is attacked by chewing-biting herbivores, cell-content feeders and necrotrophic pathogens (Glazebrook 2005; Walling 2000). While the SA pathway is hypothesized to be activated especially in response to piercing-sucking insects and biotrophic pathogens (Kawazu et al. 2012; Walling 2000). Many studies revealed that the JA and SA pathways are not distinct but interact by a complex network of regulatory interactions (Beckers and Spoel 2006; Pieterse et al. 2001). These interactions include priming (De Vos et al. 2006), complementary additive or synergistic effects (Devadas et al. 2002; Schenk et al. 2000) and mutual antagonism (Diezel et al. 2009; Zhang et al. 2013).

Direct exogenous application of jasmonate has been commonly used to simulate attack by herbivores and to analyse the subsequent plant responses (Howe and Jander 2008). For instance, glucosinolate and nicotine concentrations rise after JA treatment in *Brassicae* and *Nicotiana attenuata* (Baldwin 1996; van Dam et al. 2004). The growth of both *Mamestra brassicae* and *Pieris rapae* was retarded when feeding on the leaves of JA -induced plants of *Brassicae oleracea* (van Dam and Oomen 2008). The role of SA in plant responses to pathogens has been well documented, but its effects on plant responses to insects are not so well understood (Penninckx et al. 1996; Tateda et al. 2014).

These studies showed the effects of JA application on the production of SMs or the performance of herbivores. However, there are not many studies that couple the induction of SMs to the changes in herbivore resistance, to identify the candidate compounds involved (but see Schweiger et al. 2014; Sutter and Müller 2011).

A plant's resistance to herbivore attack is thought to be principally determined by its SMs, which can be remarkably plastic and responsive to different grades and types of herbivory (Schwachtje and Baldwin 2008). The role of primary metabolites (PMs) is often neglected in plant defence. For instance, isoleucine (Ile) produced at the attack site is rapidly conjugated to jasmonate (JA), forming JA-Ile, a key activator of defence signalling (Chini et al. 2007; Thines et al. 2007). The

hundreds of genes down- or up-regulated during the plant-herbivore or -pathogen interaction have been analysed with microarray studies, and almost all aspects of metabolism are represented, with a substantial fraction coming from primary metabolism (Both et al. 2005; Hui et al. 2003). Therefore, the role of primary metabolism in plant defence may be underestimated.

In this study, we investigated how the plant metabolite profile, and the amount of damage inflicted by herbivores with different feeding modes, were affected by MeJA and SA application. *Jacobaea vulgaris* and *Jacobaea aquatica* both notorious for their pyrrolizidine alkaloids (PAs) were used to address these questions. A previous study (chapter 5 of the thesis) showed that the total PA concentration was not affected by root application of MeJA while a significant shift in PA composition occurred from senecionine-like PAs to erucifoline-like PAs. However, it is unclear in as much other plant chemical components are affected and therefore we used nuclear magnetic resonance (NMR) spectroscopy to profile the major organic molecules present in leaf tissue. A chewing herbivore *Mamestra brassicae*, a cell-content feeder *Liriomyza trifolii* and a piercing-sucking herbivore *Frankliniella occidentalis* were used in this study. We compared our results with a previous experiment (Cheng et al. 2011b) on thrips resistance off a population of 102 segregating F2 genotypes from a cross between the two species from the present study and combined these results with the results from an ^{1}H-NMR analysis of the same genotypes (Kirk et al. 2012). The combination of the two experiments and the present experiment reduces the chance on false positives. Our experiments were designed to answer the following questions: (1) Do plant metabolite profiles change after MeJA and SA application? (2) Are the chewing herbivore and the cell-content feeder negatively affected by MeJA application while the piercing-sucking herbivore is negatively affected by SA application? (3) Are the changes in the metabolome related to the changes in herbivore feeding? (4) Do the effective compounds also confer resistance to thrips attack in an independent study using F2 hybrids between *J. vulgaris* and *J. aquatica* in this study? (5) Are the identified metabolites involved in resistance to thrips also effective if tested separately?

Materials and Methods

Plant species

The *Jacobaea vulgaris* and *Jacobaea aquatica* individuals used in this study are the parental plants of a well-studied hybrid cross (Cheng et al. 2011a). *J. vulgaris* was derived from the seeds collected at the Meijendel Nature Reserve (52°7′54″N, 4°19′46″E, The Netherlands), and *J. aquatica* was derived from the seeds collected at the Zwanenwater Reserve (52°48′38″N, 4°41′7″E, The Netherlands). *J. vulgaris* is attacked by over 60 species of herbivores and grows in dry and sandy soils while *J. aquatica* is fed on by relatively few herbivores and grows in marshy environment.

Genotypes of both species are maintained in tissue culture and can be easily be cloned to produce replicates. Each plant was grown in 5 ml of MS medium to induce a fully developed root system in a climate room (humidity 50%, light 16h /dark 8h, 20°C/20°C).

Plant treatments

After two weeks of growth in Murashige and Skoog (MS-0) medium, the shoots were randomly assigned to one of the following treatments: (1) Control (CON) in which the shoot was dipped in 0.1 % Triton water solution for 30 seconds; (2) Methyl jasmonate (MeJA) in which the shoot was dipped in 5 mM methyl jasmonate (dissolved in 0.1% Triton solution) for 30 seconds; (3) Salicylic acid (SA) in which the shoot was dipped in 5 mM salicylic acid (dissolved in 0.1% Triton solution) for 30 seconds. Each treatment was replicated 10 times for both species. After dipping, the plants were put on a paper tissue to absorb solution left on the leaves. The treated plants were propagated in a new tube with MS medium for another two weeks. After that, three leaf discs (1.2 cm diameter) per plant were punched for herbivore tests. The remaining leaf material were wrapped in aluminium foil, put in liquid nitrogen and then kept in a -80 ºC freezer until freeze drying.

Herbivore source and rearing

Eggs of *Mamestra brassicae* were obtained from Entomology Lab, WUR (Wageningen, The Netherlands) and caterpillars were reared on cabbage (*Brassica rapa*) in a climate room (humidity 50%, light 16h /dark 8h, 20°C/20°C).

Leafminer *Liriomyza trifolii* with a cell-content feeding mode and thrips *Frankliniella occidentalis* with a piercing-sucking feeding mode were both from cultures maintained in our lab. Both species were reared on chrysanthemum (*Dendranthema grandiflora*) plants in a climate room (humidity 50%, light 16h /dark 8h, 20°C/20°C).

Herbivore performance tests

The non-choice feeding bioassays were conducted in petri dishes (60mm * 15mm) lined with a layer of agar to maintain a consistent humidity. Leaf-discs were gently pressed on the agar with the abaxial side up. Each trial had 10 replicates per treatment per species totalling 10 replicates * 3 treatments * 2 plant species = 60 assays.

In tests with *M. brassicae*, each trial included one leaf-disc and one 2nd instar caterpillar. The caterpillars fed for four hours and the damaged leaf-discs were scanned. The leaf area eaten was estimated to the nearest mm^2 by subtracting the damaged leaf area from the whole leaf-disc.

For the bioassay with *L. trifolii* we used two female leafminers and one leaf-disc in each trial. After 24 hours, the number of feeding punctures were counted under microscope.

For *F. occidentalis* we used four female thrips and one leaf-disc for each trial.

After 7 days, for each leaf the amount of silver damage was visually scored in mm^2 using a microscope.

Nuclear magnetic resonance measurements

Freeze-dried plant material was extracted and analysed by NMR spectroscopy according to Kim et al. (2010). Briefly, 20 mg of lyophilized sample was taken into a 2.0 ml microtube and 0.5 ml methanol-d_4 and 0.5 ml D_2O (KH_2PO_4 buffer, pH= 6.0) containing 0.005% TMSP-d_4 (trimethyl silyl propionic acid sodium salt-d_4, w/v, Sigma-Aldrich, Zwijndrecht, the Netherlands) were added. The mixture was extracted at room temperature for 20 min with ultrasonication (Branson 5510E-MT, Branson Ultrasonics, Danbury, CT, USA) and subjected to centrifugation at 17,000 ×g at room temperature for 5 min. 300 ml of the supernatant was transferred to a 3 mm NMR tube and analysed.

The ^{1}H-NMR spectra were recorded using a Bruker DMX 600 spectrometer (Bruker, Karlsruhe, Germany). For each sample, 64 scans were recorded with the following parameters: 0.167 Hz/point, pulse width (PW) = 4.0 µs, acquisition time (AQ) = 3.17 s, dummy scans (DS) = 4 and relaxation delay (RD) = 1.5 s. Free induction decays (FIDs) were Fourier transformed with line broadening (LB) = 0.3 Hz. Phase adjustment and baseline correction were applied manually.

The AMIX software (Bruker) was used to reduce the ^{1}H-NMR spectra to an ASCII file. The spectra, which were measured in chemical shift (ppm), were binned (bucketed) into bins of equal width (0.04 ppm). Spectral intensity was scaled to internal standard (TMSP) and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of d10.00-0.40. The region of δ 4.88-4.72 was excluded from the analysis because of the residual signal of H_2O and CH_3OH-d_4, respectively. The regions of δ 10.00−7.68 and δ 0.8−0.4 were excluded from the analysis because these contained no significant peaks.

Correlation with datasets of previous studies

We used the NMR measurements (Kirk et al. 2012) and silver damage data of thrips (Cheng et al. 2011b), obtained for a segregating population of 102 F2 hybrids from a cross between the two species, and used these in the present study to correlate the NMR bins from the NMR spectra with silver damage. This analysis for the F2 genotypes allows for an independent assessment of the metabolites involved in the resistance against thrips. The NMR bins that were positively or negatively correlated with thrips resistance were then checked whether or not these bins were down-regulated or up-regulated after SA treatment in *J. aquatica*. From the significant bins in both tests, the candidate compounds involved in thrips resistance were identified. These compounds were among the compounds tested previously in thrips mortality bioassay in our research group.

Statistical analysis

Principal component analysis (PCA) was performed with the SIMCA-P (ver. 13.0, Umetrics, Umeå, Sweden). The effects of the phytohormone treatments were evaluated by analysis of 170 NMR data bins. For each bin it was tested if the metabolite profile changed after phytohormone treatment. The number of up- and down-regulated bins were tested by χ^2 tests.

The NMR bins were compared between each phytohormone treatment and the control group with Mann-Whitney U-tests. *P*-values of the bins in a specific pairwise comparison were adjusted according to Benjamini & Hochberg method (Benjamini and Hochberg 1995) to correct for multiple testing. Volcano plots including all the bins of a given treatment-control comparison with fold changes (peak intensity in treatment bin divided by peak intensity in control bin) were plotted in R 3.0.2.

Different herbivore performances between control, MeJA treatment and SA treatment were evaluated using Kruskal-Wallis one-way analysis of variance, followed by post hoc Dunn's tests in R 3.0.2.

In the available datasets of the F2 hybrids for thrips damage and NMR, spearman rank correlations between peak intensity of the NMR bins and thrips silver damage were performed. Significant bins were then compared with the NMR bins of the SA application in *J. aquatica.* We selected the NMR bins that were increased after SA application and negatively correlated with silver damage in the F2 hybrids and the NMR bins that were reduced after SA application and positively correlated with silver damage in the F2 hybrids. For the selected bins we identified the metabolites represented by these bins.

Results

Metabolome profiles of leaf tissue

For the dataset obtained from the ^{1}H- NMR analysis, a 13-component model explained 97.5% of the variance, with the first two components explaining 63.0 % (the third one explaining 9.2%) in *J. vulgaris* (Fig. 1a). In *J. aquatica*, a 13-component model explained 98.4% of the variance, with the first two components explaining 64.8% (the third one explaining 10.0%) (Fig. 1b). PCA plots of the first two components showed that the plants treated by MeJA were clearly separated from the control and SA treated plants for both species. The separation between the control plants and the SA treated plants was less clear.

Compared to the control group, significant up- or down-regulated NMR bins were found for both phytohormone treatments. MeJA application caused significant changes in 51 bins and 122 bins in *J. vulgaris* and *J. aquatica*, respectively (Table 1, Fig. 2). SA application led to significant changes in 7 bins and 78 bins in *J. vulgaris* and *J. aquatica*, respectively (Table 1, Fig. 2). Among the significant NMR bins, MeJA treatment resulted in more up-regulated (44) than down-regulated (7) bins in *J. vulgaris* (χ^2=565.14, *df*=1, *P*<0.001) and in *J. aquatica* (96 and 26 bins, respective-

ly) (χ^2=4557.5, *df*=1, *P*<0.001) (Table 1, Fig. 2). For the SA treatments 1 NMR bin was up-regulated and 6 bins were down-regulated in *J. vulgaris* (χ^2=20.513, *df*=1, *P*<0.001) while in *J. aquatica* 22 were up-regulated and 56 bins were down-regulated (χ^2=110.831, *df*=1, *P*<0.001) (Table 1, Fig. 2).

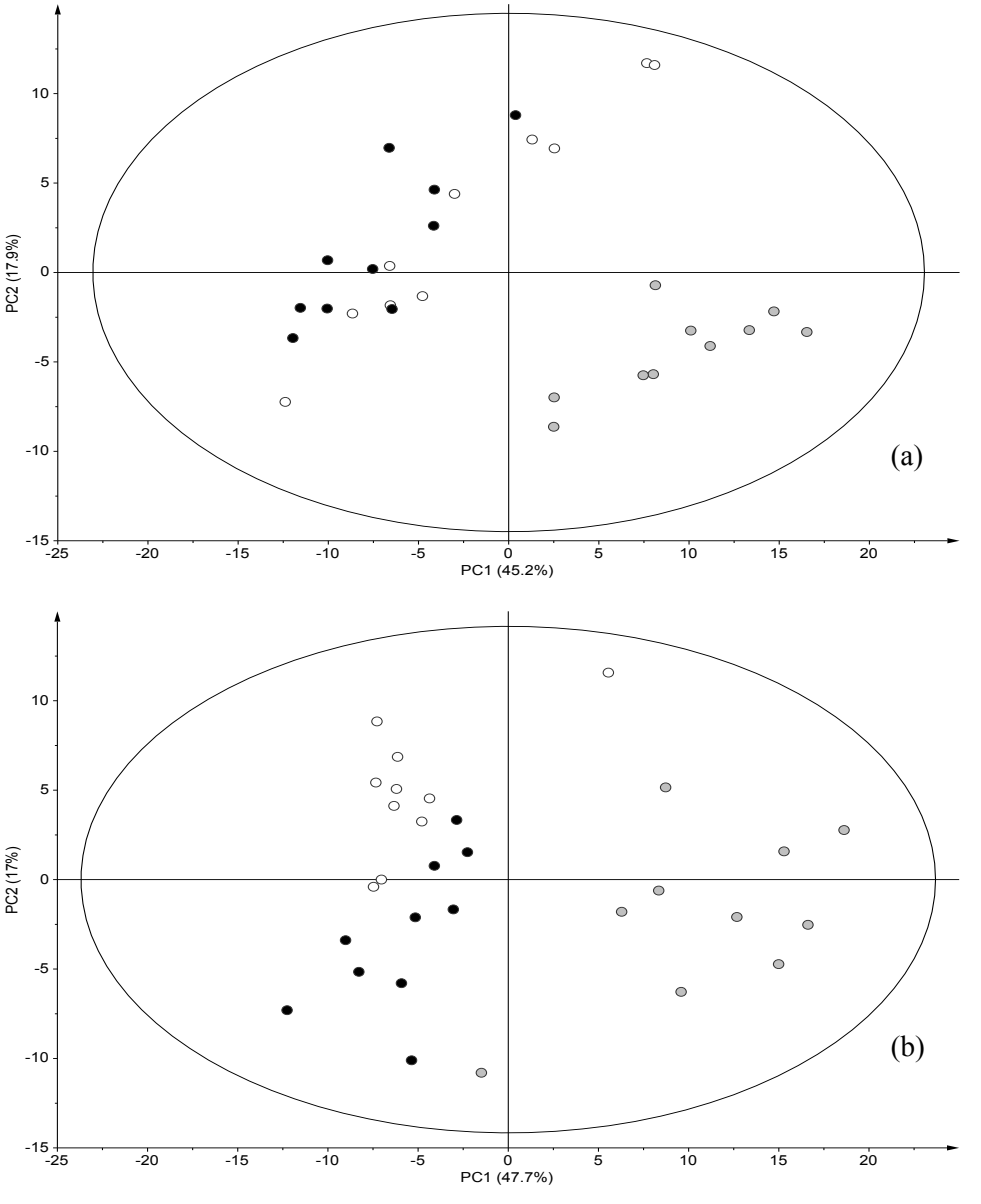

Fig. 1 Plot of principal components 1 and 2 based on PCA of the signal intensity of 170 1H-NMR bins of *Jacobaea vulgaris* (a) and *Jacobaea aquatica* (b). White dots represent control plants, grey dots represent plants treated with methyl jasmonate (MeJA) and black dots represent plants treated with salicylic acid (SA), n=10 for each treatment.

Table 1 Summary of up- or down-regulated NMR bins after methyl jasmonate (MeJA) and salicylic acid (SA) treatments in *Jacobaea vulgaris* and *Jacobaea aquatica*

Plant species	*J. vulgaris*		*J. aquatica*	
Treatments	MeJA	SA	MeJA	SA
Total bins	170	170	170	170
Down-regulated bins	65	117	42	116
Up-regulated bins	105	53	128	54
Significant bins [a]	51	7	122	78
Significant down-regulated bins	7	6	26	56
Significant up-regulated bins	44	1	96	22

[a] The number of NMR bins that are significant after adjusting for multiple comparisons by the Benjamini & Hochberg method

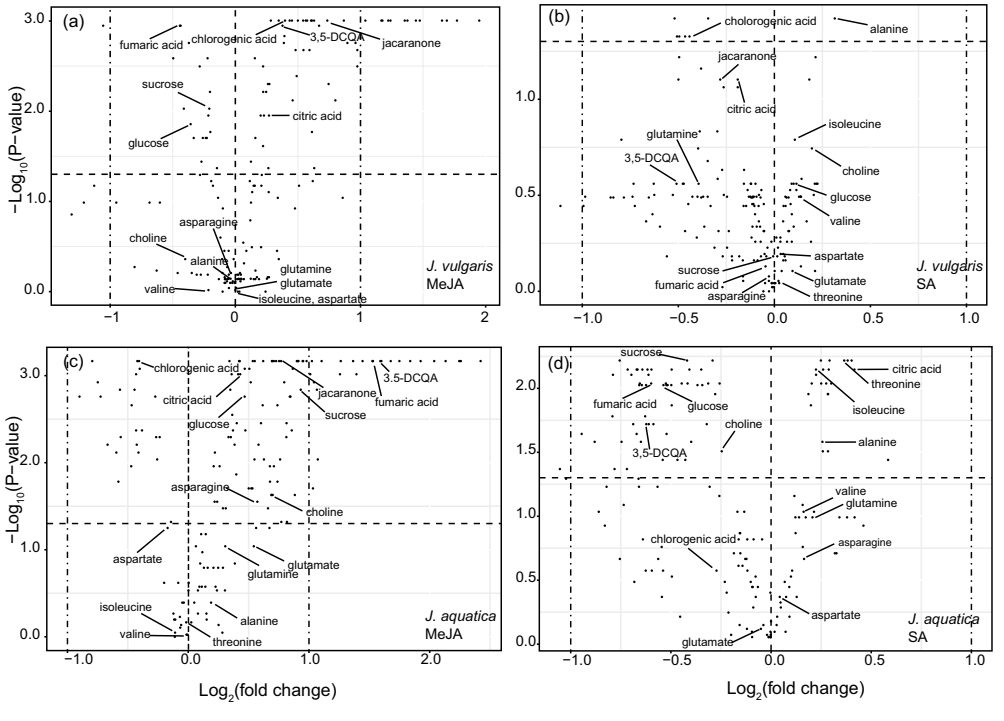

Fig. 2 Volcano plots of 246 bins of NMR analysis of shoots of *Jacobaea vulgaris* and *Jacobaea aquatica*, 2 weeks after phytohormone treatment with methyl jasmonate (MeJA) or salicylic acid (SA). For each bin, the negative log10 of the unadjusted P value (Mann-Whitney U-test, comparison with the control) is plotted against the $\log_2$ of the mean fold change. The horizontal dashed line is P =0.05 and the significant bins (P<0.05) are above this line. The identified metabolites with significant P value of Mann-Whitney U-test were labeled.

Table 2 ^{1}H chemical shift (d) of metabolites present in leaves of *Jacobaea vulgaris* and *Jacobaea aquatica* identified by 1D NMR spectra in MeOH-d_4

No	Compounds	Chemical shift (ppm)
1	Alanine	δ 1.48 (d)
2	Asparagine	δ 2.94 (d), 2.96 (d)
3	Aspartate	δ 2.71 (d), 2.68 (d)
4	Chlorogenic acid	δ 7.61 (d)
5	Choline	δ 3.22 (s)
6	Citric acid	δ 2.55 (s)
7	3,5-dicaffeoylquinic acid	δ 6.90 (d), 6.88 (d), 6.49 (d)
8	Fumaric acid	δ 6.53 (s)
9	Glucose	δ 5.18 (d)-α, δ 4.59 (d)-β
10	Glutamate	δ 2.40 (m)
11	Glutamine	δ 2.46 (m), 2.13 (m)
12	Isoleucine	δ 0.96 (t)
13	Jacaranone	δ 6.24 (d), 7.14 (d)
14	Sucrose	δ 5.41 (d)
15	Threonine	δ 1.34 (d)
16	Valine	δ 1.06 (d), 1.00 (d)

In total, 16 up- or down-regulated compounds were identified in the leaf tissue (Table 2), including 3 secondary metabolites (jacaranone, chlorogenic acid and 3, 5-dicaffeoylquinic acid) and 13 primary metabolites. Of these primary metabolites, there are 2 sugars (sucrose and glucose), 8 amino acids (asparagine, aspartate, glutamine, glutamate, alanine, threonine, valine and isoleucine), fumaric acid, citric acid and choline. Jacaranone significantly increased compared to the control after MeJA application in both species, while chlorogenic acid decreased significantly by both phytohormone applications in *J. vulgaris* but not in *J. aquatica* (Fig. 3). In *J. aquatica* 3,5-dicaffeoylquinic acid (3,5-DCQA) increased significantly after MeJA application, while it decreased after SA treatment, but in *J. vulgaris* there was no significant differences among the treatments (Fig. 3b). Both MeJA and SA treatment induced a significant decrease of sucrose and glucose concentrations in *J. aquatica*. In contrast, only glucose decreased after MeJA application in *J. vulgaris*. Fumaric acid decreased after MeJA treatment in *J. vulgaris* while in *J. aquatica* it caused a decrease (Fig. 3). Citric acid increased significantly after MeJA and SA treatment in *J. aquatica*. However, in *J. vulgaris* it increased after MeJA treatment while it decreased after SA treatment. Choline increased in *J. vulgaris* while it decreased in *J. aquatica* after SA application. MeJA application induced a decrease of choline in

both species (Fig. 3). All the amino acid showed similar responses after plant phytohormone treatments in both species, except glutamine. Glutamine decreased in *J. vulgaris* after SA application while it increased in *J. aquatica* (Fig. 3).

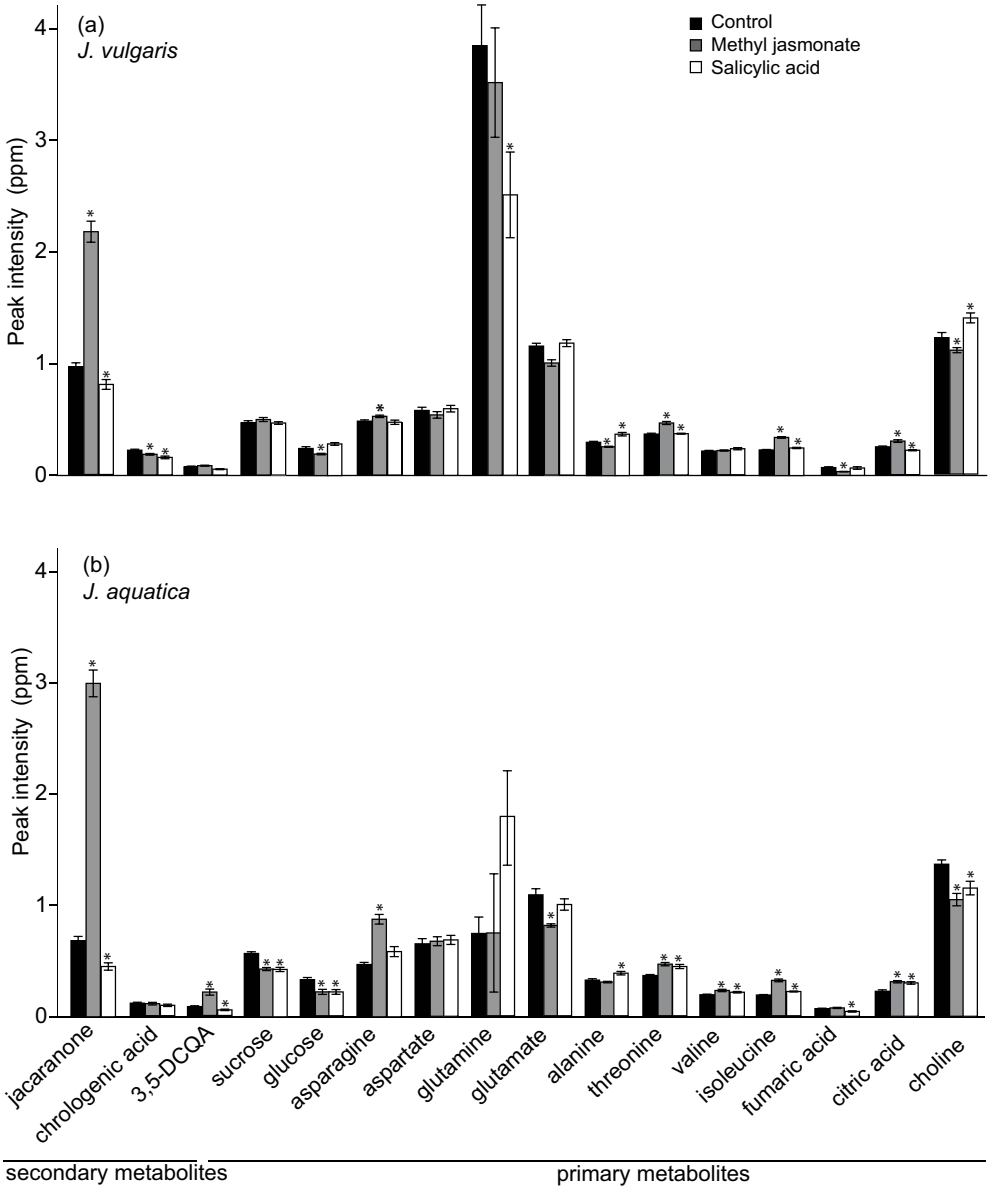

Fig. 3 Semi-quantitative expression of secondary and primary metabolites identified in *Jacobaea vulgaris* and *Jacobae aquatica* leaves after phytohormone application. The Y-axis reprensents peak intensity relative to the internal standard TMSP. Asterisks indicate metabolites that differed significantly from the control after correction for multiple testing with the Benjamini Hochberg method.

Herbivore performance

Damage by the chewing caterpillar M. brassicae

The leaf area eaten by caterpillars significantly differed between phytohormone treatments for both plant species (KW test, *J. vulgaris*: χ^2=14.551, *df*=2, *P*<0.001; *J. aquatica*: χ^2=14.837, *df*=2, *P*<0.001). The leaf area eaten was significantly lower on plants treated with MeJA compared to plants treated with SA and control plants (Fig. 4a).

Damage by the cell-content feeding leafminer L. trifolii

Similar to *M. brassicae*, the number of leaf punctures by leafminers significantly differed between the phytohormone treatments for both plant species (KW test, *J. vulgaris*: χ^2=8.240, *df*=2, *P*=0.016; *J. aquatica*: χ^2=7.747, *df*=2, *P*=0.021). The number of leaf punctures was significantly reduced in the MeJA treatment group compared to the SA treatment group and the control group (Fig. 4b).

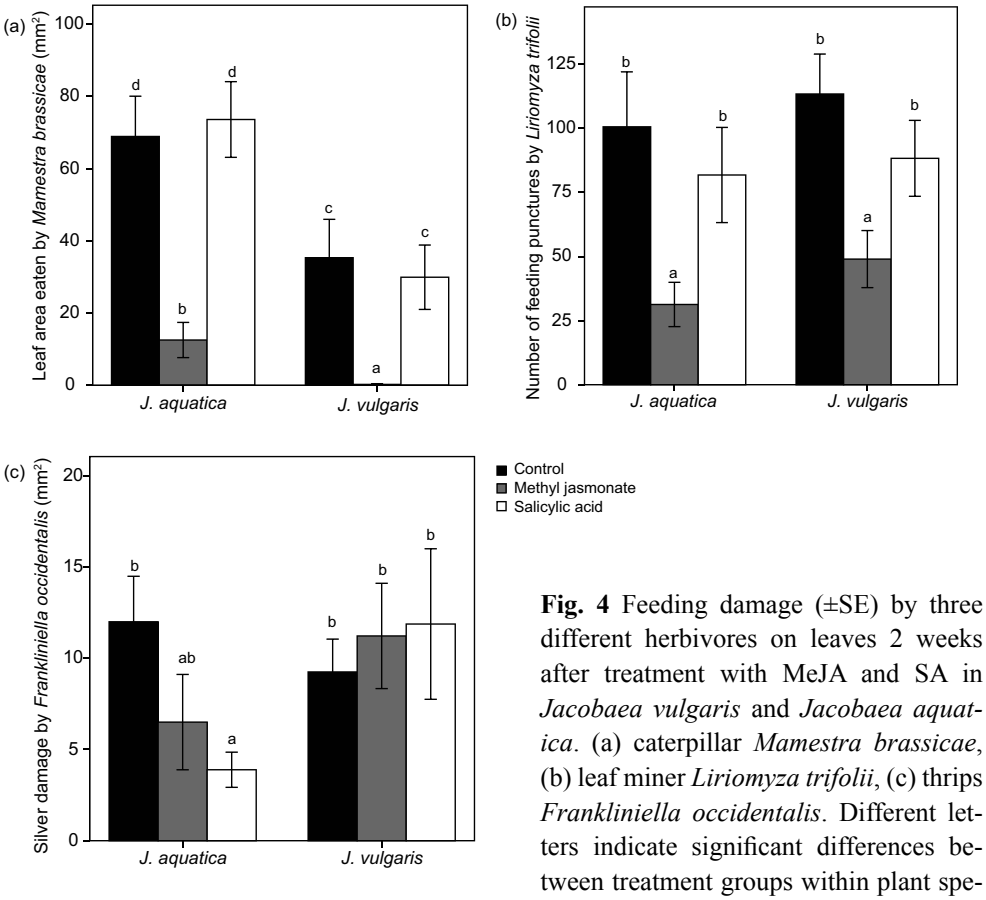

Fig. 4 Feeding damage (±SE) by three different herbivores on leaves 2 weeks after treatment with MeJA and SA in *Jacobaea vulgaris* and *Jacobaea aquatica*. (a) caterpillar *Mamestra brassicae*, (b) leaf miner *Liriomyza trifolii*, (c) thrips *Frankliniella occidentalis*. Different letters indicate significant differences between treatment groups within plant species (post hoc Dunn test, *P*<0.05, n=10).

Damage by the piercing-sucking thrips F. occidentalis

The amount of silver damage by thrips significantly differed between treatment groups in *J. aquatica* (KW test, χ^2=6.938, *df*=2, *P*=0.031) but differences were not significant in *J. vulgaris* (KW test, χ^2=0.089, *df*=2, *P*=0.957). In *J. aquatica* the silver damage was significantly reduced on the plants treated with SA. MeJA treated plants did not differ significantly from the control although damage was reduced 50% compared to the control (Fig. 4c).

M. brassicae and L. trifolii performance and metabolites

For both plant species, there was significantly lower feeding damage of the caterpillar *M. brassicae* and the leaf miner *L. trifolii* in the MeJA treated plants. From the identified compounds (Fig. 3), jacaranone, isoleucine and asparagine significantly increased after MeJA treatment. Therefore, these three compounds may be involved in the resistance to *M. brassicae* and *L. trifolii*.

Table 3 Summary of Spearman rank correlations between silver damage of thrips and 170 NMR bins in F2 hybrids and number of NMR bins changes in *Jacobaea aquatica* and *Jacobaea vulgaris* after salicylic acid treatment

Plant species	Variables	Positive [a]	Negative
F2 hybrids	Total number of bins [b]	67	96
	Number of significant correlations	17	25
J. aquatica	Bin changes	15↓, 2↑	17↑,8↓
	Significant bin changes [c]	10↓, 2↑	5 ↑
J. vulgaris	Bin changes	8↓,9↑	13↑,12↓
	Significant bin changes [c]		1↑

[a]↑: up-regulated bins; ↓: down-regulated bins.

[b] In the NMR dataset of F2 hybrids, there were 170 bins between δ7.68- δ 0.8, but 7 bins have no signals (δ 4.96, δ 4.92, δ 4.88, δ 4.72, δ 4.68, δ 4.64 and δ 3.32).

[c] The number of NMR bins that are significant after adjusting for multiple comparisons by the Benjamini & Hochberg method.

Comparative studies for thrips F. occidentalis tests

By comparing the results of **t**he thrips bioassays of the F2 hybrids (Cheng et al. 2011b) with the results of the NMR analysis (Kirk et al. 2012), we found 42 NMR bins that were significantly correlated with thrips silver damage, of which 17 were positively correlated and 25 were negatively correlated (Table 3). Of these 42 bins, 17 bins also showed significant changes after SA treatment in *J. aquatica*, including 10 down-regulated NMR bins with positive correlations, 5 up-regulated bins with negative correlations and 2 up-regulated bins with positive correlations. However, only 1 bin with a negative correlation was significantly up-regulated after SA treat-

ment in *J. vulgaris* (Table 3). Because thrips performance was only affected significantly in *J. aquatica*, we will focus only on this plant species hereafter.

NMR bins were suspected to play a role in resistance/susceptibility against thrips if: 1) the bins that were significantly positively correlated with silver damage in the experiment with F2 hybrids were down-regulated after SA treatment, or 2) the bins that were significantly negatively correlated with silver damage in the experiment with F2 hybrids were up-regulated after SA treatment. In total, 10 down-regulated bins with positive correlations with silver damage in F2 hybrids indicating susceptibility and 5 up-regulated bins with negative correlations with silver damage in F2 hybrids indicating resistance were found. Of these 16 bins, 4 down-regulated bins were related to the sugars sucrose and glucose and 1 down-regulated bin was related to 3,5-DCQA. 3 up-regulated bins were related to alanine, threonine and citric acid respectively. 8 bins were too small to identify. Therefore, sucrose, glucose and 3,5-DCQA were correlated with thrips susceptibility while alanine, threonine and citric acid were correlated with thrips resistance in *Jacobaea* plants.

Discussion

The effects of phytohormone application on the metabolite profile

In both species, MeJA application led to significant change in the metabolite profile while this was less clear for the SA application. In general a larger number of NMR signals decreased than increased after SA treatment while the opposite was found after MeJA treatment.

In *Arabidopsis* SA is implicated in the induction of programmed cell death (Brodersen et al. 2005). Six bins in *J. vulgaris* and 56 bins in *J. aquatica* were significantly down-regulated in our study after SA application. These down-regulated bins might be related to cell death. At the same time the difference between the two species indicated that *J. aquatica* is much more sensitive to SA application than *J. vulgaris*.

The effects of MeJA on the metabolome have also been studied in other plant species. For instance, MeJA treated *Brassica rapa* and *Fagopyrum esculentum* plants showed a distinct separation from control plants and the phenolic compound content increased significantly after MeJA treatment in these two plant species (Kim et al. 2011; Liang et al. 2006). However, most studies on the effects of exogenous SA application are related to salt tolerance and water deficit (Bastam et al. 2013; Jesus et al. 2015) while few studies are concerned with metabolome and herbivores. Salicylic acid application significantly induced the production of hyoscyamine and scopolamine (2-12 fold) in root cultures of *Brugamansia candida* (Pitta–Alvarez et al. 2000). However, another study in ginger, *Zingiber officinale* Roscoe, showed that the concentration of some of the major flavonoids (quercetin, catechin and kaempferol) decreased significantly after SA treatment (Ghasemzadeh and Jaafar 2012).

In a cell suspension culture of *Catharanthus roseus* SA treated cells showed a high level of sugars up to 48 hours after treatment and one compound, 2,5-dihydroxybenzoic-5-*O*-glucoside, was produced only in SA-treated cells (Mustafa et al. 2009).

The effects of phytohormone treatments on herbivores with different feeding modes

The feeding damage by *M. brassicae* and *L. trifolii* were significantly reduced by MeJA application, which was in line with our hypothesis. JA treatment enhanced the plant resistance to thrips in *Arabidopsis* and *Brassica rapa* (Abe et al. 2008; Abe et al. 2009). JA application also can stimulate tomato resistance to insects by increasing the density of glandular trichomes (Boughton et al. 2005). In our study, however, MeJA application did not have a significant effect on plant resistance against thrips. In contrast we found that silver damage of thrips decreased after SA application only in *J. aquatica*.

Relationship between metabolome changes and herbivory

Isoleucine is the only identified compound that increased after MeJA and SA application in both plant species. It can be conjugated with JA to become JA-Ile (Staswick and Tiryaki 2004). A previous study demonstrated that exogenous MeJA activates defensive systems (such as VOC emissions) in plants by essentially converting itself into JA and JA-Ile and initiating a signal transduction leading to VOC emission and induction of endogenous JA-Ile and JA-Leu, which in turn cause further amplification of VOC emissions (Tamogami et al. 2008). JA-Ile as a defence signal was confirmed in the study that the susceptibility of *Manduca sexta* larvae was associated with reduced levels of JA-Ile in *Nicotiana attenuata* (Kang et al. 2006). In both *J. vulgaris* and *J. aquatica*, isoleucine was significantly increased after MeJA application. Based on these studies, we can speculate that the level of JA-Ile conjugates was increased and consequently led to a higher plant resistance after *Jacobaea* plants were treated by MeJA, which to some extent was supported by the negative correlation between feeding damage and isoleucine in this study (Fig. S1).

Jacaranone significantly increased after MeJA application in both plant species. Jacaranone has been isolated from several *Senecio* species (Kirk et al. 2005; Lajide et al. 1996; Xu et al. 2003). Jacaranone and its analogues have been shown to have insecticidal activity against the housefly *Musca domestica* and can inhibit the growth of generalist herbivore *Spodoptera litura* (Lajide et al. 1996). Leiss et al. (2009) observed a higher concentration of jacaranone in the young leaves of thrips-resistant *Jacobaea* plants. Nuringtyas et al. (2012) found higher jacaranone concentrations in the epidermis leaf tissue of *Jacobaea* plants. Both studies concluded that the higher level of jacaranone was related to thrips resistance. In contrast, our study showed that jacaranone was increased 2-3 times after MeJA application compared to the control while SA treated plants did not differ from the control. Nev-

ertheless in our study thrips damage was not correlated with jacaranone. However, jacaranone levels might be correlated to the performance of chewing herbivores and cell-content feeders (Fig. S1).

Additional evidence from F2 hybrids

Sugars (sucrose and glucose) and 3,5-DCQA showed positive correlations with silver damage in F2 hybrids, indicating that higher concentrations of sugars and 3,5-DCQA were more attractive to thrips. After SA treatment, these compounds were decreased in *J. aquatica*. Also unpublished results from thrips mortality bioassay showed that the mortality of thrips decreased with an increase in the concentration of these compounds (Fig. S2).

Alanine, threonine and citric acid showed negative correlations with silver damage in F2 hybrids, indicating that these three compounds were deterrent to the thrips. An increase of these compounds should lead to a better defence against thrips. Alanine, threonine and citric acid did increase after SA treatment in *J. aquatica*, but the effects of alanine and threonine on the thrips mortality still need to be tested. The mortality of thrips increased with an increase of the concentration of citric acid (Fig. S2).

In a previous study, concentrations of fumaric acid and adenine were found to be higher in susceptible *J. vulgaris* plants (Leiss et al. 2009). We found that these two compounds decreased significantly after SA treatment in *J. aquatica* but they were not positively correlated with silver damage of thrips in the experiment with the F2 hybrids.

In summary, MeJA treatment significantly reduced the feeding damage of the chewing herbivore *M. brassicae* and the cell-content feeder *L. trifolii* in both plant species while the feeding of piercing-sucking herbivore *F. occidentalis* was negatively affected by SA application in *J. aquatica* only. This is in line with our hypothesis. Based on the differences in metabolite profiles, as measured by ^{1}H-NMR, the plants treated by MeJA can clearly be separated from control and SA treated plants. Both phytohormone applications led to significant changes in a substantial number of bins. In MeJA treated plants 26.9% of the NMR bins were up-regulated while in SA treated plants 3.5% of the NMR bins were down-regulated in *J. vulgaris.* In *J. aquatica* 56.5% of the bins was up-regulated and 32.9% down-regulated. Combining the effects of SA with thrips damage and the NMR data of F2 hybrids, 16 NMR bins were selected and several compounds were identified. Sucrose, glucose and 3,5-DCQA were related to thrips susceptibility while citric acid, alanine and threonine were related to thrips resistance. Isoleucine and jacaranone were related to the resistance of the chewing herbivore *M. brassicae* and the cell-content feeder *L. trifolii*.

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Supplementary Materials:

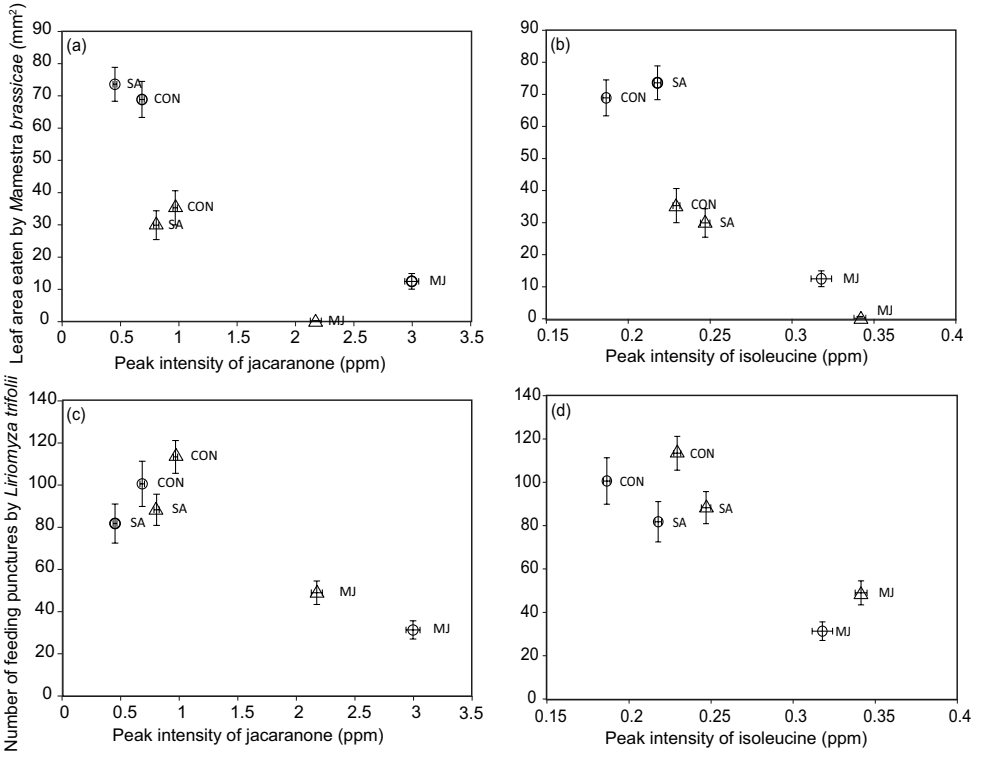

Fig. S1 Feeding damage by caterpillar *Mamestra brassicae* (panels a and b) and leafminer *Liriomyza trifolii* (panels c and d) per treatment against jacaranone and isoleucine in *Jacobaea vulgaris* and *Jacobaea aquatica*. Circles represent *J. aquatica* and triangles represent *J. vulgaris*. Horizontal error bar represents the standard error of peak intensity of metabolites and vertical one represents the standard error of feeding damage of herbivores.

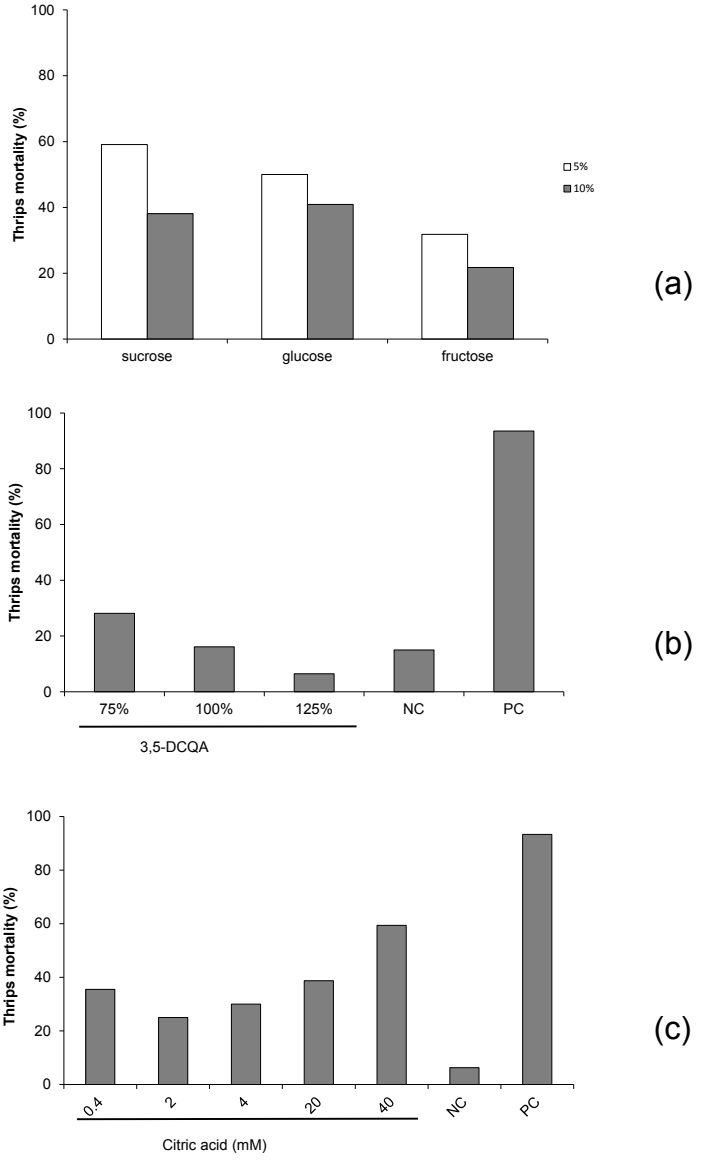

Fig. S2 The mortality of thrips *Frankliniella occidentalis* after feeding with different sugar solutions (a) and 3,5-dicaffeoylquinic acid (3,5-DCQA) (b) and citric acid (c) after 5 days. Negative control (NC): 10% fructose; Positive control (PC): Abamectine (50 ug/ml).