Chemical and morphological factors related to western flowers thrips resistance in the ornamental Gladiolus

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ABSTRACT

In the previous chapter of host plant resistance to western flower thrips in gladiolus we showed that resistant varieties, in comparison to susceptible ones, had smaller mesophyll cells and epidermis cells. The leaves of the resistant plants exhibited a high density of epicuticular papillae. The density of papillae explained 39% of the variation in silver damage. Other factors may be involved in Gladiolus resistance to thrips. In this chapter we address host plant resistance to western flower thrips in *Gladiolus* using an eco-metabolomics approach. We first tested effects of the extracts of six varieties on thrips mortality to confirm that chemical components were involved in thrips resistance. We then compared the ^{1}H NMR profiles and thrips resistance of gladiolus varieties representing a broad range of papillae densities. We observed a number of metabolites that were related to resistance against thrips: two unidentified triterpenoid saponins and the amino acids alanine and threonine. All these compounds were highly correlated amongst each other and to the density of papillae which makes it impossible to distinguish their effects in multivariate analyses. Our findings suggest that papillae are involved in resistance to thrips by producing or storing the compounds that cause resistance.

KEYWORDS: papillae, saponins, alanine, threonine, eco-metabolomics

INTRODUCTION

Plants have to defend themselves against a multitude of herbivores. Plant defense comprises morphological traits, such as spines, trichomes and papillae and chemical traits. Trichomes are epidermal hairs protruding from the surface of leaves and stems (Riddick and Simmons, 2014). Trichomes can impede movement or trap herbivorous insects resulting in their death (Gibson, 1976; Tingey and Sinden, 1982; Maluf *et al.*, 2001). Papillae are protuberances of solid cell wall thickening (Duarte-Silva *et al.*, 2013). They are known to protect cells from pathogen attack due to physical barriers (Maluf *et al.*, 2001 and Underwood, 2012). Prum *et al.* (2013) showed that papillae made it more difficult for beetles to attach to the leaves which suggests that papillae also play a role in defense against insect herbivores. Despite their potential role, papillae have not been studied in great detail yet in relation to plant defense against herbivores.

Besides physical barriers, both trichomes and papillae can produce secondary metabolites that deter or are toxic to herbivores. Among those secondary metabolites are glucosinolates, alkaloids, phenolics, phenylpropanoids, polyketides and terpenoids (Bennett and Wallsgrove, 1994). For instance: Acylsugars (Lucini *et al.*, 2015) and phenols (Mirnezhad *et al.*, 2010) were produced in trichomes of *Solanum pennellii*, phenolics and alkaloids were detected in trichomes of *Withania somnifera* L. (Munien *et al.*, 2015). Papillae stored plant defense compounds such as 2-acetyl-1-pyrroline in rice (Wakte *et al.*, 2007) and cardosin A in *Cynara cardunculus* L. (Ramalho-Santos *et al.*, 1997).

Because trichomes and papillae are part of the plant's defence system they can play an important role in breeding programs aimed at increasing natural host plant resistance. Host plant resistance becomes increasingly important in integrated pest management programs directed at agricultural and horticultural key pests such as western flower thrips (*Frankliniella occidentalis*). This invasive pest is highly polyphagous and attacks fruits, vegetables, and ornamentals (Buitenhuis and Shipp, 2008; Reitz *et al.*, 2019), causing losses of millions of Euros worldwide (Lewis, 1997; Reitz *et al.*, 2019). Thrips are piercing-sucking insects inhibiting plant growth and flower formation (Lewis, 1997). They cause the characteristic silver damage by sucking up a whole cell's content

leaving an empty cell filled with air (Lucini *et al.*, 2015). In addition they are vectors of viral diseases (Kirk, 2002).

Host plant resistance to western flower thrips is mainly chemically based in a number of plants species as has been shown by Leiss *et al.* (2011) applying an eco-metabolomic approach. The ^{1}H NMR (Nuclear Magnetic Resonance) spectroscopy profiles of thrips resistant and susceptible plants were compared to identify metabolites related to constitutive thrips resistance in wild Jacobaea species (Leiss *et al.*, 2009), in the ornamental chrysanthemum (Leiss *et al.*, 2009) and in the vegetables tomato (Mirnezhad *et al.*, 2010; Bac-Molenaar *et al.*, 2019) sweet pepper (Maharijaya *et al.*, 2012; Macel *et al.* , 2019), onion (Diaz-Montano *et al.*, 2010) and carrot (Leiss *et al.*, 2013). Metabolites associated with thrips resistance comprised alkaloids, acylsugars, flavanoids, amino acids and phenylpropanoids.

In my thesis I study resistance in Gladiolus spp. *Gladiolus,* a genus of perennial bulbs belonging to the Iridaceae family. It is a popular decorative plant in summer and thus constitutes an economically important flower crop in the Netherlands. *Gladiolus* comprises 5% of the total Dutch flower production, constituting 21,000 ha of production area and amounting to $ 756 production in value (Benschop *et al.*, 2010). Western flower thrips is a major problem in cultivation of Gladiolus affecting corms, leaves, buds and flowers. Thrips damage results in small corms, which may not germinate, in problems of flower formation and opening and causes an undesirable silvery shiny damage on leaves and flowers (Denmark and Price, 1998).

In the previous chapter of this thesis I observed a negative correlation between the density of epicuticular papillae and feeding damage by thrips in Gladiolus. However, the mechanisms behind this are not known. The density of papillae explained 39% of the variation in silver damage. Thus, other factors may be involved in the Gladiolus resistance to thrips as well. In this chapter we address host plant resistance to western flower thrips in *Gladiolus hybridus* using an eco-metabolomics approach. In the present study we, therefore, first tested the extract of six species differing in resistance to thrips in experiments with artificial diets to confirm that the plant metabolome was involved in thrips resistance. We then compared the ^{1}H NMR profiles and thrips resistance of gladiolus varieties representing a broad range of papillae densities.

MATERIAL AND METHODS

In- vitro Bioassay

We first conducted an *in-vitro* thrips bioassay using leaf extracts to investigate potential effects of plant defense compounds. For this bioassay we used 6 varieties that differed in susceptibility in the whole plant bioassay described in the previous chapter: Charming, Charming Beauty, Nymph, Alba, Elvira and Robinetta. Fifty mg dried leaf material of five replicates of each variety was weighed and pooled for chemical extraction. The samples were extracted with 50% methanol in an ultrasonic bath for 20 min. After filtration, the residue was extracted again. The extraction was repeated three times and the final filtrate was dried in a rotary-evaporator. Nine mg/ml of these extracts were redissolved in a 5% methanol-water solution and the pH was adjusted to seven (Leiss *et al.*, 2013).

For the *in-vitro* thrips bioassay, 96-well plates were filled with 150 µL 2% agarose and 50µL of the extracts to be tested. Methanol-water (5%) and the insecticide abamectin (50µg/ml) were used as a negative and positive control, respectively. Each bioassay consisted of 32 replicates with one column of 8 wells on each of four plates. A single first instar thrips larva was placed into each cap of an 8 caps flat-cap strip. Each cup was sealed with parafilm through which thrips are able to feed. The strips were then placed on top of the 96 -well plates. An adhesive sealing film was placed onto the plate to prevent evaporation and to protect the samples during the assay. All plates were placed up-side down during 48 hours to ensure that thrips got into contact with the extracts. The plates were randomly placed into a growth chamber with standard thrips rearing conditions (L18: D6, 23°C, 65% RH). After 48 hours, the mortality of thrips was recorded. Differences in thrips mortality among varieties were statistically analyzed with a chi-square test. The correlation between thrips mortality in the *in-vitro* bioassay and thrips silver damage in the whole plant non- choice bioassay was analyzed using Pearson correlation test.

Whole Plant Bioassay and ^{1}H NMR- based Metabolomics

To extend our study from the six varieties used above , we used fourteen different gladiolus varieties: Charming, Charming Beauty, Nymph, Alba, Elvira and Robinetta (Gladiolus breeder Gebr P. & M. Hermans, Lisse, The Netherlands), and Ben

Venuto, Red Balance, V-29, Chinon, Live Oak, Deepest Red, Green Star, Essential (VWS B.V.Alkmaar, The Netherlands).

Each bulb was planted into a 9 x 9 cm pot filled with a 1 : 1 mixture of potting soil and dune sand. Six replicates of each variety were randomly placed into a growth room (L:D, 18:6, 20^0C) and grown for 10 weeks (Mirnezhad *et al.*, 2010). Three to five replicates were used for a whole plant thrips bioassay while the other replicates were used for counting the density of epicuticular papillae and NMR metabolomics. Five replicates were used for the varieties Charming, Charming Beauty, Nymph, Alba, Elvira and Robinetta and three replicates for the remaining varieties. The results of the thrips bioassay and the morphological data were discussed in the previous chapter. In this chapter we will focus on the metabolomic analyses.

Metabolic Profiling

Extraction of Plant Materials for NMR Metabolomics

Three replicates of each of the fourteen varieties were used for NMR metabolomics. The standard protocol of sample preparation and ^{1}H-NMR profiling described by Kim et al. (Kim, Choi, *et al.*, 2010) was applied. Samples of 30 mg freeze-dried plant material were weighed into a 2 ml microtube and extracted with 1.5 ml of a mixture of phosphate buffer (pH 6.0) in deuterium oxide containing 0.05% trimethylsilylproprionic acid sodium salt-d_4 (TMSP) and methanol-d_4 (1:1). Samples were vortexed at room temperature for 1 min, ultrasonicated for 20 min and centrifuged at 13000 rpm for 10 min. An aliquot of 0.8 ml of the supernatant was transferred to 5 mm NMR tubes for ^{1}H-NMR measurement.

NMR Analysis

^{1}H-NMR spectra were recorded with a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. Deuterated methanol was used as the internal lock. Each ^{1}H-NMR spectrum consisted of 128 scans requiring 10 min and 26 s acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) of 30º (11.3 µs), and relaxation delay (RD) of 1.5s. A pre-saturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. Free induction decay (FIDs) was Fourier transformed with a line broadening (LB) of 0.3 Hz. The resulting

spectra were manually phased and baseline corrected to the internal standard TMSP at 0.00 ppm, using TOPSPIN (version 3.5, Bruker). Two-dimensional J-resolved NMR spectra were acquired using 8 scans per 128 increments for F_1 (chemical shift axis) and 8 k for F_2 (spin-spin coupling constant axis) using spectral widths of 66 Hz and 5000 Hz respectively. Both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex Fourier transformation. J-resolved spectra were tilted by 45^0, symmetrized about F_1, and then calibrated to TMSP, using XWIN NMR (version 3.5, Bruker). ^{1}H-^{1}H correlated COSY spectra were acquired with a 1.0 s relaxation delay and 6361 Hz spectral width in both dimensions. The window function for the COSY spectra was Qsine (SSB = 0).

Data Processing

For the NMR spectra, intensities were scaled to total intensity and reduced to integrated equal widths (0.04 ppm) corresponding to the region of δ 0.32-10.0. The regions of δ 4.7-5.0 and δ 3.30-3.34 were excluded from analysis due to the presence of the residual signals of water and methanol. ^{1}H-NMR spectra were automatically binned by AMIX software (version 3.7, Biospin, Bruker). Data were further analyzed with principal component analysis (PCA), partial least square-discriminant analysis (PLS-DA) and S-plot analysis performed with SIMCA-P software (version 12.0 Umetrics, Umea, Sweden). For PCA, pareto scaling and for PLS-DA and S-plot unit variance scaling was used. The S-plot analysis was validated using a permutation test (N=20), which is a default validation tool in the software package (SIMCA-P).

Differences between gladiolus varieties in the relative concentrations of metabolites related to thrips resistance in the S-plot were analyzed by one-way ANOVA. Pearson correlations were calculated for the relationships between metabolite concentrations, and thrips silver damage, and density of epicuticular papillae. For epicatechin, epigallocatechin and gallic acid Kruskal-Wallis and Spearman rank correlations were used because data were not normally distributed.

RESULTS

***In-vitro* Bioassay**

The extracts of the four varieties with low thrips damage: Alba (χ^2= 7.59, d.f. = 1, P = 0.005) Nymph (χ^2= 10.26, d.f. = 1, P = 0.001), Elvira (χ^2= 13.17, d.f. = 1, P =

0.0003) and Robinetta, (χ^2= 8.89, d.f. = 1, P = 0.002) lead to significantly higher thrips mortality compared to the negative control (Fig.1A). The extracts of the two varieties with high silver damage, Charming Beauty and Charming, showed a thrips mortality comparable to the negative control. Thrips mortality in the *in-vitro* bioassay was negatively correlated to thrips silver damage in the whole plant non-choice bioassay that was described in the previous chapter (r = -0.788, N = 6, P = 0.031) (Fig. 1B). This result implies that chemical compounds played a role in plants resistance to thrips. We, therefore, continued our research with the chemical profiling of all varieties.

Metabolic Profiling

Next to variation in thrips resistance, the gladiolus varieties differed in their metabolomic profiles. PCA which is an unsupervised method did not give a separation of the metabolic profiles based on thrips resistance of the varieties. The supervised analysis-PLS-DA, in contrast to PCA, takes next to the metabolomic matrix also the resistance matrix into account. We, therefore, separated varieties into resistant (0.03 - 0.8% damage), partially resistant (4-20% damage) and susceptible (> 65% damage) varieties. PLS-DA did not result in a clear separation of the metabolomic profiles of the gladiolus varieties either. We, therefore, extended PLS-DA to S-plot analysis, which helps to identify significant signals and to establish their reliability (Wiklund *et al.*, 2008). The S-plot identified candidate signals related to thrips resistant at the upper right quadrant and candidate signals related to susceptibility to thrips at the lower left quadrant in Fig. 2A. Validation of the S-plot analysis by permutation tests resulted in a variance R^2 = 0.93 and a predictive ability Q^2 = 0.88. Q2 values greater than 0.5 are generally accepted as good (Bailey *et al.*, 2004).

Signals Related to Susceptibility

The signals related to thrips susceptibility in the loading plot were observed as phenolic compounds and glucose. The signal epicatechin (δ 6.02), epigallocatechin (δ 6.58) and gallic acid (δ 7.04) were associated with thrips susceptible varieties (Fig.3). In addition, the signals of both α-glucose (δ 5.20) and β-glucose (δ 4.60) were also related to thrips susceptibility.

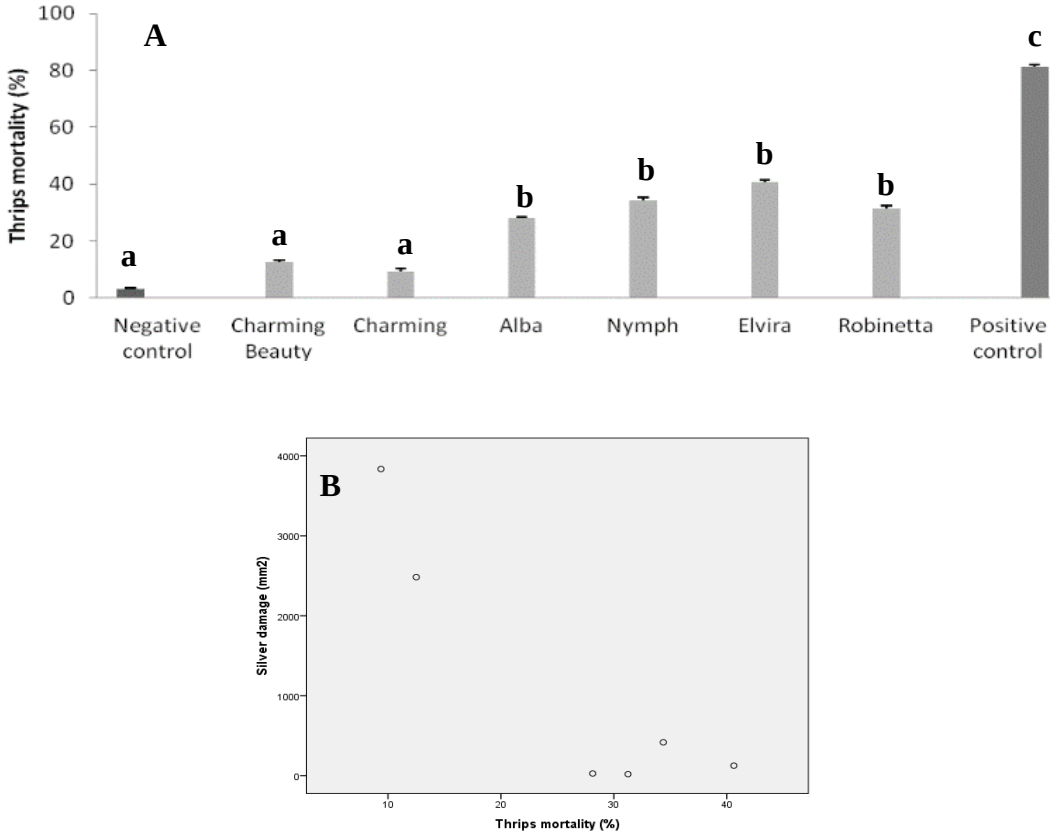


Figure 1. (A) Mortality of thrips feeding on artificial diets (150 µL 2% agarose) with 50µL of leaf extracts of six *Gladiolus* varieties measured in an *in-vitro* bioassay. For each extract 32 thrips were tested. Five percent methanol solution was used as negative and the insecticide abamectin (50 µg/ml) as a positive control. Means and standard errors are presented. Different letters indicate significant differences between varieties at $P \leq 0.05$. (B) The correlation between thrips silver damage, measured in a whole plant non-choice bioassay and thrips mortality measured in the *in-vitro* bioassay of six *Gladiolus* varieties (r = -0.788, N = 6, P = 0.031).

Glucose including α and β forms were related to susceptibility in the S-plot (Fig.2A, 2B) and differed significantly (F = 8.352, df = 13, P < 0.000 and F = 8.234, df = 13, P < 0.000, respectively) among varieties (Table 1). These signals were, however, not correlated to thrips silver damage (r = 0.234, N = 14, P = 0.420 and r = 0.265, N = 14, P = 0.360, respectively) (Table 2). Epicatechin, epigallocatechin and gallic acid were also

related to susceptibility in the S-plot. These signals were not detectable in all varieties (Fig S1). The relative concentration of epicatechin, epigallocatechin and gallic acid differed significantly among varieties (H = 52.132, df = 13, P = 0.000; H = 49.133, df = 13, P = 0.000 and H = 48.397, df = 13, P = 0.000, respectively). Epicatechin was marginally significant (ρ = 0.541, N = 14, P = 0.046), it was present however in only three varieties. Epigallocatechin and gallic acid were not related to thrips silver damage when tested as a single factor (ρ = 0.404, N = 14, P = 0.152 and ρ = 0.313, N = 14, P = 0.276, respectively) (Table 2). In conclusion, none of the signals related to susceptibility in the S-plot (epicatechin) was clearly confirmed to be important in subsequent analyses of relative concentrations of the single compounds.

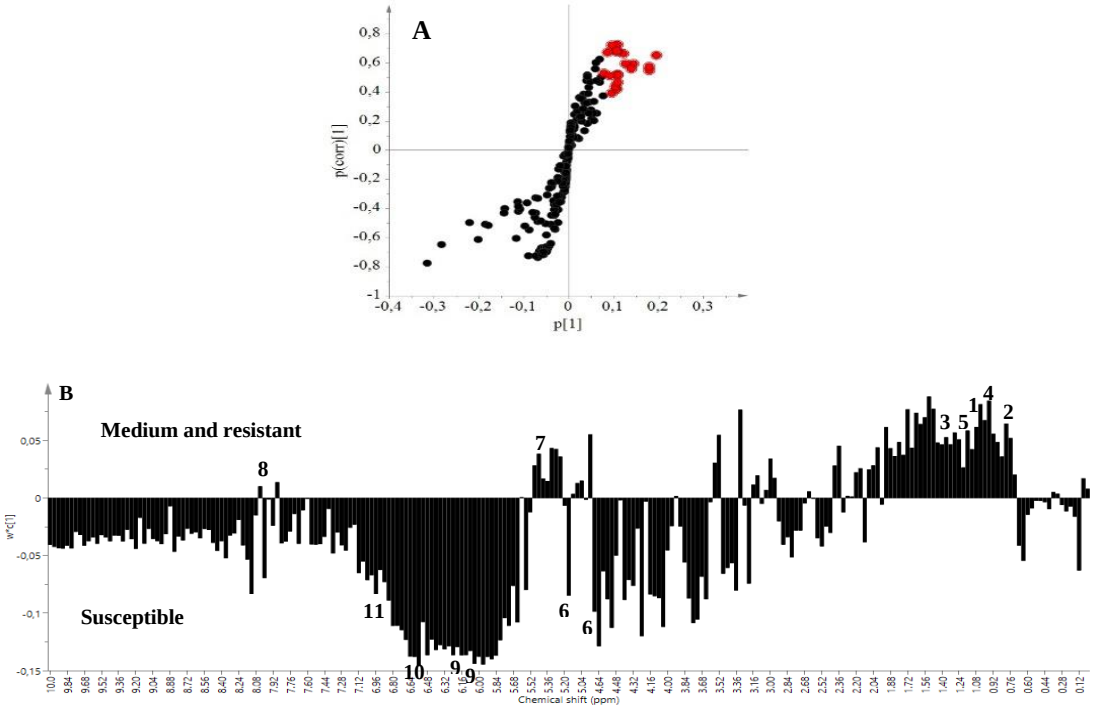


Figure 2. S-plot score (A) and loading plot (B) for gladiolus varieties based on ^{1}H NMR spectra. Metabolites are labeled as (1) signal A, (2) signal B, (3) alanine, (4) valine, (5) threonine, (6) α/β-glucose, (7) sucrose, (8) kaempferol, (9) epicatechin, (10) epigallocatechin and (11) gallic acid.

Signals Related to Resistance

The signals related to thrips resistance in the S-plot were observed in the region of 1.92 to 0.80 ppm (Fig. 2B), some compounds that show signals in this region are terpenoids, saponins and amino acids. The signal A (δ 1.28) and the signal B (δ 0.90) were associated to thrips resistant varieties. These were identified as triterpenoid saponins (Fig.3). In this region we could further identify the signals related to the amino acids valine (δ 1.06), alanine (δ 1.48) and threonine (δ 1.32). In addition, the signal of sucrose (δ 5.40) was related also to thrips resistance.

The relative concentrations of alanine, valine and threonine differed significantly among varieties ($F = 21.754$, $df = 13$, $P = 0.000$; $F = 75.824$, $df = 13$, $P = 0.000$ and $F = 31.460$, $df = 13$, $P = 0.000$). The relative concentrations of alanine valine and threonine were three to four times higher in resistant varieties (Table 1). The relative concentrations of alanine and threonine were negatively correlated to thrips silver damage ($r = -0.612$, $N = 14$, $P = 0.020$ and $r = -0.634$, $N = 14$, $P = 0.015$, respectively) (Table 2) (Fig. 4) while the relative concentration of valine was not significantly correlated to thrips silver damage ($r = -0.100$, $N = 14$, $P = 0.734$) (Table 2).

Table 1. Average relative concentrations, as proportions of the internal standard, detected in the S-plot to be related to thrips resistance for 14 gladiolus varieties divided in three resistance categories. Data are means ± SE. n=14 in all cases.

NO.	Metabolites	Thrips resistance categories		
		Susceptible (N= 2)	**Partial (N= 8)**	**Resistant (N= 4)**
1.	Signal A	0.02±0.004	0.29±0.013	0.54±0.019
2.	Signal B	0.05±0.004	0.17±0.007	0.34±0.013
3.	Alanine	0.12±0.005	0.24±0.008	0.34±0.013
4.	Valine	0.07±0.003	0.47±0.010	0.39±0.026
5.	Threonine	0.18±0.006	0.46±0.017	0.76±0.030
6.	Sucrose	0.88±0.014	1.34±0.039	1.10±0.022
7.	α-Glucose	0.59±0.031	0.62±0.038	0.46±0.022
8.	β-Glucose	0.88±0.044	0.85±0.051	0.65±0.028
9.	Gallic acid	0.10±0.006	0.05±0.004	0.03±0.003
10.	Epigallocatechin	0.15±0.010	0.02±0.003	0.02±0.002
11.	Epicatechin	0.06±0.002	0.005±0.000	0.00±0.000

The relative concentrations of signals A and B were significantly different among the fourteen varieties ($F = 52.216$, $df = 13$, $P = 0.000$ and $F = 44.563$, $df = 13$, $P = 0.000$). Signal A was just not significantly negatively correlated with silver damage ($r = -0.505$, $N = 14$, $P = 0.065$) while, signal B was ($r = -0.557$, $N = 14$, $P = 0.038$) (Table 2) (Fig.4). The relative concentration of sucrose differed among 14 varieties ($F = 14.367$, $df = 13$, $P = 0.000$). Although the concentration of sucrose was about 1.15 times higher in resistant varieties than in susceptible varieties, we did not detect a significant correlation between silver damage and sucrose concentration ($r = 0.083$, $N = 14$, $P = 0.779$) (Table 2). In conclusion, four of the compounds related to resistance in the S-plot were confirmed to be important in subsequent analyses of relative concentrations of the single compounds. These compounds were the amino acids alanine and threonine and the compound related to signal B (δ 0.90). All of these compounds were strongly correlated among each other. In addition, they were also all strongly correlated to the density of papillae (Table 2). Remarkably none of the compounds that were associated with susceptibility in the S-plot were correlated to the density of papillae.

DISCUSSION

We showed that variation in the plant´s metabolome causes variation in thrips mortality in *in-vitro* bio-assays. This variation was highly correlated to thrips damage in whole plant bioassays. We then identified two amino acids and two triterpenoid saponins that were associated with thrips resistance by correlating the relative concentrations in the NMR analyses of the leaf metabolome with thrips resistance of varieties differing in papillae density. No compound was clearly related to thrips susceptibility. Remarkably, all the compounds that were correlated with resistance were highly correlated among each other and with papillae density. These finding suggests that papillae are involved in resistance to thrips by producing or storing the compounds that cause resistance. If this indeed would be the case it also suggests that the physical effect of papillae on thrips resistance is relatively small because e.g. threonine explains slightly more of the variation in thrips resistance than the density of papillae. Threonine explained 40% of the variation in silver damage. However, the strong correlation among factors identified as being associated with thrips resistance makes it hard to separate their effects from each other. Likewise, due the correlations between compounds and between metabolites and density

Table 2. Pearson correlations between the concentrations of metabolites detected in the S-plot to be related to thrips resistance and silver damage and the density of papillae in fourteen gladiolus varieties. Silver damage was ln transformed to obtain normally distributed data. * = p < 0.05, ** = p< 0.01, N=14 in all cases. Spearman correlations were used for the relationships between silver damage, epicatechin (EC), epigallocatechin (EGC), gallic acid and density of papillae. Data in bold are significant at p ≤ 0.05.

		Ln damage	Papillae	Signal A	Signal B	Alanine	Valine	Threonine	Sucrose	α-glucose	β-glucose	EC	EGC	Gallic acid
Ln damage	Correlation	1	**-.628***	-.505	**-.557***	**-.612***	-.100	**-.634***	.083	.234	.265	**.541***	.404	.313
	Sig. (2-tailed)		**.016**	.065	**.038**	**.020**	.734	**.015**	.779	.420	.360	**.046**	.152	.276
Papillae	Correlation	**-.628***	1	**.869****	**.881****	**.761****	-.034	**.904****	-.117	-.424	-.442	-.506	-.275	-.019
	Sig. (2-tailed)	**.016**		**.000**	**.000**	**.002**	.907	**.000**	.692	.131	.114	.065	.342	.950
Signal A	Correlation	-.505	**.869****	1	**.987****	**.801****	-.119	**.947****	-.119	-.384	-.390	-.444	-.208	.233
	Sig. (2-tailed)	.065	**.000**		**.000**	**.001**	.686	**.000**	.685	.175	.168	.112	.477	.423
Signal B	Correlation	**-.557***	**.881****	**.987****	1	**.814****	-.160	**.942****	-.207	-.427	-.430	-.433	-.166	.235
	Sig. (2-tailed)	**.038**	**.000**	**.000**		**.000**	.585	**.000**	.478	.128	.125	.122	.571	.418
Alanine	Correlation	**-.612***	**.761****	**.801****	**.814****	1	.357	**.873****	-.204	-.180	-.194	**-.602***	-.428	.094
	Sig. (2-tailed)	**.020**	**.002**	**.001**	**.000**		.210	**.000**	.483	.539	.507	**.023**	.127	.748
Valine	Correlation	-.100	-.034	-.119	-.160	.357	1	.021	.345	.128	.096	-.419	-.594*	-.403
	Sig. (2-tailed)	.734	.907	.686	.585	.210		.944	.227	.663	.743	.135	.025	.153
Threonine	Correlation	**-.634***	**.904****	**.947****	**.942****	**.873****	.021	1	-.194	-.258	-.266	**-.552***	-.316	.048
	Sig. (2-tailed)	**.015**	**.000**	**.000**	**.000**	**.000**	.944		.507	.374	.358	**.041**	.270	.870
Sucrose	Correlation	.083	-.117	-.119	-.207	-.204	.345	-.194	1	-.076	-.123	-.353	**-.576***	-.058
	Sig. (2-tailed)	.779	.692	.685	.478	.483	.227	.507		.795	.676	.215	**.031**	.845
α-glucose	Correlation	.234	-.424	-.384	-.427	-.180	.128	-.258	-.076	1	**.996****	.054	-.111	.126
	Sig. (2-tailed)	.420	.131	.175	.128	.539	.663	.374	.795		**.000**	.854	.705	.667
β-glucose	Correlation	.265	-.442	-.390	-.430	-.194	.096	-.266	-.123	**.996****	1	.108	-.047	.139
	Sig. (2-tailed)	.360	.114	.168	.125	.507	.743	.358	.676	**.000**		.714	.874	.636
EC	Correlation	.541*	-.506	-.444	-.433	**-.602***	-.419	**-.552***	-.353	.054	.108	1	**.932****	-.013
	Sig. (2-tailed)	.046	.065	.112	.122	**.023**	.135	**.041**	.215	.854	.714		**.000**	.964
EGC	Correlation	.404	-.275	-.208	-.166	-.428	-.594*	-.316	**-.576***	-.111	-.047	**.932****	1	.074
	Sig. (2-tailed)	.152	.342	.477	.571	.127	.025	.270	**.031**	.705	.874	**.000**		.801
Gallic acid	Correlation	.313	-.019	.233	.235	.094	-.403	.048	-.058	.126	.139	-.013	.074	1
	Sig. (2-tailed)	.276	.950	.423	.418	.748	.153	.870	.845	.667	.636	.964	.801	

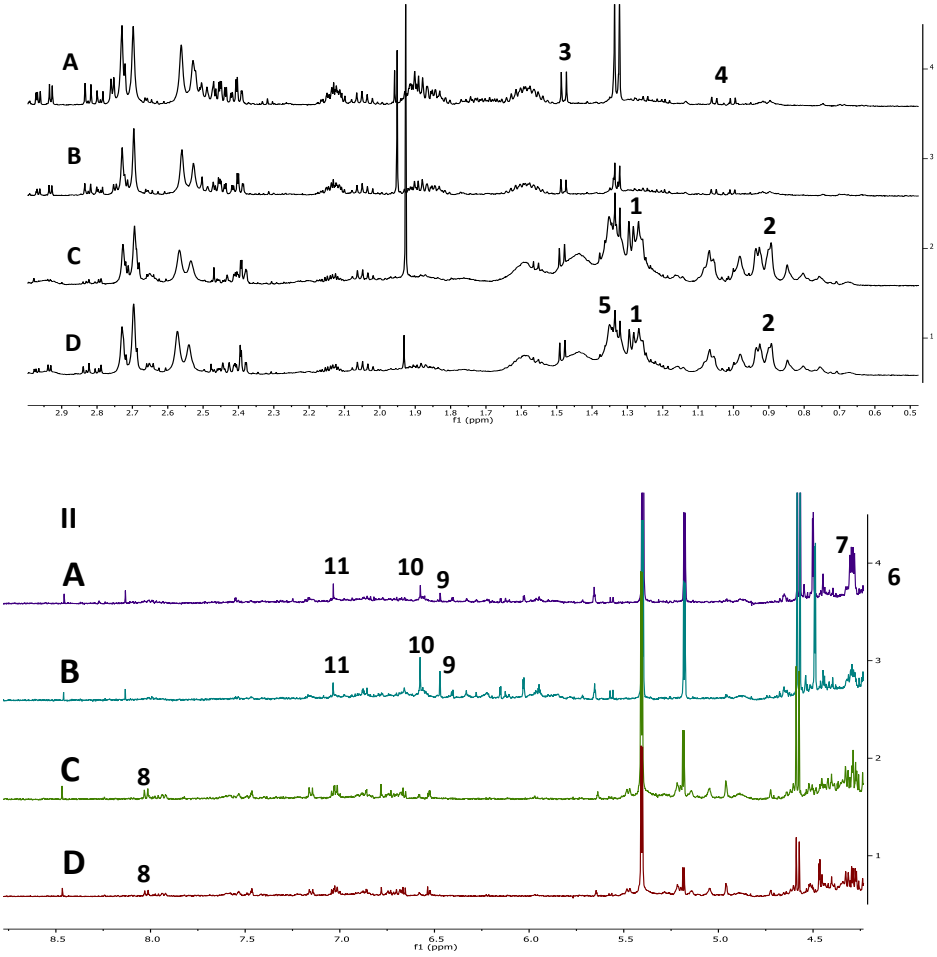


Figure 3. ^{1}H-NMR spectra (CH3OH-d_4-KH_2PO_4 in D_2O extracts of (A) and (B) the susceptible variety Charming Beauty and (C) and (D) the resistant variety Robinetta, in the range of d 0.5 - 3.0 (I) and d 4.5 - 8.5 (II). Assignments: (1) signal A, (2) signal B, (3) alanine, (4) valine, (5) threonine, (6) α/β-glucose, (7) sucrose, (8) kaempferol, (9) epicatechin, (10) epigallocatechin and (11) gallic acid.

of papillae, no particular single compound can be pinpointed to be related to thrips resistance.

Both saponins and the amino acids alanine and threonine have been mentioned in the literature in relation to resistance against insect herbivores. The alanine

concentration was higher in a variety of peach that was resistant against the Mediterranean fly (or medfly) than in a susceptible variety, while for threonine such a difference was not detected (Capitani *et al.*, 2012). Besides, Leiss *et al.* (2013) reported that alanine occurred in higher concentration in the leaves of thrips resistant carrots. In contrast, Dillon and Kumar (2017) reported that the threonine concentration was significantly higher in *Sorghum bicolor* seedlings resistant to the stem borer *Chilo partellus* than in the seedlings of a susceptible variety, while alanine concentrations did not significantly differ. In an experiment with artificial diets, similar as the one used in this chapter (Leiss *et al.*, 2013), both alanine and threonine lead to increased mortality compared to the negative control while valine did not (Fig. S2). These results confirmed the notion that these amino acids may be involved in thrips resistance. However, the correlation we found for two amino acids alanine and threonine does not necessarily mean that these compounds indeed themselves confer resistance to thrips. It is likely that that these amino acids are associated with a pool of metabolites that support the synthesis of compounds such as triterpenoid saponins, kaempferol flavonoids which may act as resistance metabolites. More detailed metabolomic studies including fluxomics can shed more light on this.

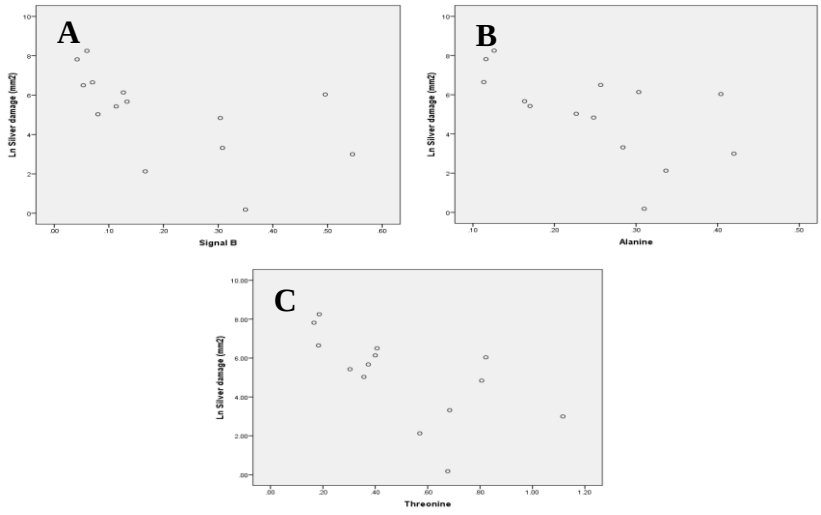


Figure 4. Relationships between the relative concentrations of (A) signal B, (B) alanine and (C) threonine and silver damage in fourteen gladiolus varieties.

Saponins are well-known to infer resistance against pathogens. They are reported to be involved in resistance to insect herbivores as well. Saponins were shown to be important as a defensive chemical in *Aesculus pavia* against the leafminer *Cameraria ohridella* (Ferracini *et al.*, 2010). This leafminer caused heavy damage to the white-flowering horse chestnut in Europe. Among the Aesculus genus, *A. pavia* L. a HBT genotype, characterized by red flowers, showed an atypical resistance towards this pest. This resistance appeared to be based on exogenous saponins that were translocated from roots/stem to the leaf tissues. Saponins were reported to mediate the resistance in *Barbarea vulgaris* and counter adaptations in the flea beetle *Phyllotreta nemorum* (Kuzina *et al.*, 2009; Nielsen *et al*., 2010). Saponins from resistant varieties of garden pea inhibited development of the Azuki bean beetle *Callosobruchus chinensis* whereas saponin extracts from non-resistant legumes did not (Applebaum *et al.*, 1969). The mechanism through which saponins contribute to resistance are largely unknown. Ishaaya suggested (1986) that they slow down the passage of food through the gut whereas Shaney *et al.* (1970) suggested that saponins block the uptake of sterols which is essential to insects. De Geijter *et al.* (2007) reviewed the effects of saponins on insect herbivores and concludes: "these interesting plant compounds offer new strategies to protect crops in modern agriculture and horticulture with integrated pest management (IPM) programs against pest insects, either by spraying or by selecting high-saponin varieties of commercial crops.

Papillae as storage sites of plant defense secondary compounds have been reported in rice (Wakte *et al.*, 2007) and cardoon (Ramalho-Santos *et al.*, 1997). Our study indicates that chemical compounds stored in papillae may confer resistance in gladiolus species. This can be further examined by histochemical studies or by analyzing the expression of genes that encode the committed steps in the synthesis of triterpenoid saponins (Haralampidis et al. 2002). This offers a promise for further research on the mechanisms involved in resistance. At this point we should be careful however because we conducted a correlative study and correlation does not mean causation. Thus, other associated characteristics may be involved in the mechanism of resistance. Meanwhile papillae density may provide an easy marker in Gladiolus breeding programs targeted at increased resistance against thrips.

ACKNOWLEDGEMENTS

We thank the Dutch Gladiolus breeder Gebr. Hermans and VWS B.V. (Alkmaar, The Netherlands) for providing the different Gladiolus varieties. Suzanne Kos, Rita Rakhmawati and Mariá José Rodriguez-Lopez from Plant Sciences and Natural Products, Institute of Biology (IBL), Leiden University are thanked for their technical assistance. Dinar Sari Cahyaningrum Wahyuni holds a grant of the Directorate General of Higher Education (DHGE) of the Republic of Indonesia.

REFERENCES

Applebaum, S. W., Marco, S. and Birk, Y. (1969) 'Saponins as possible factors of resistance of legume seeds to the attack of insects', *Journal of Agricultural and Food Chemistry*, 17(3), pp. 618–622.

Bac-Molenaar, J. A., Mol, S., Verlaan, M. G., van Elven, J., Kim, H. K., Klinkhamer, P. G. L., Leiss, K. A. and Vrieling, K. (2019) 'Trichome Independent Resistance against Western Flower Thrips in Tomato', *Plant and Cell Physiology*, 60(5), pp. 1011–1024.

Bailey, N. J. C., Wang, Y., Sampson, J., Davis, W., Whitcombe, I., Hylands, P. J., Croft, S. L. and Holmes, E. (2004) 'Prediction of anti-plasmodial activity of Artemisia annua extracts: application of 1H NMR spectroscopy and chemometrics', *Journal of Pharmaceutical and Biomedical Analysis*, 35(1), pp. 117–126.

Bennett, R. N. and Wallsgrove, R. M. (1994) 'Secondary metabolites in plant defence mechanisms', *New Phytologist*, 127(4), pp. 617–633.

Benschop, M., Kamenetsky, R., Le Nard, M., Okubo, H. and De Hertogh, A. (2010) 'The Global Flower Bulb Industry: Production, Utilization, Research', *Horticultural Reviews*, 36, p. 1.

Buitenhuis, R. and Shipp, J. L. (2008) 'Influence of plant species and plant growth stage on Frankliniella occidentalis pupation behaviour in greenhouse ornamentals', *Journal of Applied Entomology*, 132(1), pp. 86–88.

Capitani, D., Sobolev, A. P., Tomassini, A., Sciubba, F., De Salvador, F. R., Mannina, L. and Delfini, M. (2012) 'Peach fruit: metabolic comparative analysis of two varieties with different resistances to insect attacks by NMR spectroscopy', *Journal of Agricultural and Food Chemistry*. ACS Publications, 61(8), pp. 1718–1726.

Denmark, H. A. and Price, J. F. (1998) 'Gladiolus Thrips, Thrips simplex (Morison)(Insecta: Thysanoptera: Thripidae)', *DPI Entomology Circular*, 322.

Dhillon, M. K. and Kumar, S. (2017) 'Amino acid profiling of Sorghum bicolor vis-à-vis Chilo partellus (Swinhoe) for biochemical interactions and plant resistance', *Arthropod-Plant Interactions*. Springer, 11(4), pp. 537–550.

Diaz-Montano, J., Fuchs, M., Nault, B. A. and Shelton, A. M. (2010) 'Evaluation of onion cultivars for resistance to onion thrips (Thysanoptera: Thripidae) and Iris yellow spot virus', *Journal of Economic Entomology*, 103(3), pp. 925–937.

Duarte-Silva, A. G., Carvalho-Silva, M. and Camara, P. E. A. S. (2013) 'Morphology and development of leaf papillae in the Pilotrichaceae', *Acta Botanica Brasilica*, 27(4), pp. 737–742.

Ferracini, C., Curir, P., Dolci, M., Lanzotti, V. and Alma, A. (2010) 'Aesculus pavia foliar saponins: defensive role against the leafminer Cameraria ohridella', *Pest Management*

Science, 66(7), pp. 767–772.

de Geyter, E., Lambert, E., Geelen, D. and Smagghe, G. (2007) 'Novel advances with plant saponins as natural insecticides to control pest insects', *Pest Technol*, 1(2), pp. 96–105.

Gibson, R. W. (1976) 'Trapping of the spider mite Tetranychus urticae by glandular hairs on the wild potato Solanum berthaultii', *Potato Research*, 19(2), pp. 179–182.

Haralampidis, K., Trojanowska, M. and Osbourn, A. E. (2002) 'Biosynthesis of triterpenoid saponins in plants', in *History and Trends in Bioprocessing and Biotransformation*. Springer, pp. 31–49.

Ishaaya, I. (1986) 'Nutritional and allelochemic insect-plant interactions relating to digestion and food intake: some examples', in. Springer, pp. 191–223.

Kim, H. K., Choi, Y. H. and Verpoorte, R. (2010) 'NMR-based metabolomic analysis of plants', *Nature protocols*. Nature Publishing Group, 5(3), p. 536.

Kirk, W. D. J. (2002) 'The pest and vector from the West: Frankliniella occidentalis BT - Thrips and Tospoviruses: Proceedings of the 7th International symposium on Thysanoptera', in. Australian National Insect Collection Canberra, Australia, pp. 33–42.

Kuzina, V., Ekstrom, C. T., Andersen, S. B., Nielsen, J. K., Olsen, C. E. and Bak, S. (2009) 'Identification of Defense Compounds in Barbarea vulgaris against the Herbivore Phyllotreta nemorum by an Ecometabolomic Approach', *Plant Physiology*, 151(4), pp. 1977–1990.

Lea, P. J. and Azevedo, R. A. (2017) 'Amino Acids', in Thomas, B., Murray, B. G., and Murphy, D. J. B. T.-E. of A. P. S. *Encyclopedia of Applied Plant Sciences.* Oxford: Academic Press, pp. 56–66.

Leiss, K. A., Choi, Y. H., Abdel-Farid, I. B., Verpoorte, R. and Klinkhamer, P. G. L. (2009) 'NMR metabolomics of thrips (Frankliniella occidentalis) resistance in Senecio hybrids', *Journal of Chemical Ecology*, 35(2), pp. 219–229.

Leiss, K. A., Choi, Y. H., Verpoorte, R. and Klinkhamer, P. G. L. (2011) 'An overview of NMR-based metabolomics to identify secondary plant compounds involved in host plant resistance', *Phytochemistry Reviews*, 10(2), pp. 205–216.

Leiss, K. A., Cristofori, G., van Steenis, R., Verpoorte, R. and Klinkhamer, P. G. L. (2013) 'An eco-metabolomic study of host plant resistance to Western flower thrips in cultivated, biofortified and wild carrots', *Phytochemistry*, 93, pp. 63–70.

Leiss, K. A., Maltese, F., Choi, Y. H., Verpoorte, R. and Klinkhamer, P. G. L. (2009) 'Identification of chlorogenic acid as a resistance factor for thrips in chrysanthemum.', *Plant physiology*, 150(3), pp. 1567–1575.

Lewis, T. (1997) *Thrips as crop pests*. Cab International.

Lucini, T., Faria, M. V, Rohde, C., Resende, J. and de Oliveira, J. R. F. (2015) 'Acylsugar and the role of trichomes in tomato genotypes resistance to Tetranychus urticae', *Arthropod-Plant Interactions*, 9(1), pp. 45–53.

Maharijaya, A., Vosman, B., Verstappen, F., Steenhuis-Broers, G., Mumm, R., Purwito, A., Visser, R. G. F. and Voorrips, R. E. (2012) 'Resistance factors in pepper inhibit larval development of thrips (F rankliniella occidentalis)', *Entomologia experimentalis et applicata*, 145(1), pp. 62–71.

Macel, M., Visschers, I. G. S., Peters, J. L., Kappers, I. F., de Vos, R. C. H. and van Dam, N. M. (2019) 'Metabolomics of Thrips Resistance in Pepper (Capsicum spp.) Reveals Monomer and Dimer Acyclic Diterpene Glycosides as Potential Chemical Defenses', *Journal of Chemical Ecology*. Springer, 45(5–6), pp. 490–501.

Maluf, W. R., Campos, G. A. and das Grasas Cardoso, M. (2001) 'Relationships between trichome types and spider mite (Tetranychus evansi) repellence in tomatoes with respect to foliar zingiberene contents', *Euphytica*, 121(1), pp. 73–80.

Mirnezhad, M., Romero-González, R. R., Leiss, K. A., Choi, Y. H., Verpoorte, R. and Klinkhamer, P. G. L. (2010) 'Metabolomic analysis of host plant resistance to thrips in wild and cultivated tomatoes', *Phytochemical Analysis*, 21(1), pp. 110–117.

Munien, P., Naidoo, Y. and Naidoo, G. (2015) 'Micromorphology, histochemistry and ultrastructure of the foliar trichomes of Withania somnifera (L.) Dunal (Solanaceae)', *Planta*, 242(5), pp. 1107–1122.

Nielsen, J. K., Nagao, T., Okabe, H. and Shinoda, T. (2010) 'Resistance in the Plant, Barbarea vulgaris, and Counter-Adaptations in Flea Beetles Mediated by Saponins', *Journal of Chemical Ecology*, 36(3), pp. 277–285.

Prüm, B., Bohn, H. F., Seidel, R., Rubach, S. and Speck, T. (2013) 'Plant surfaces with cuticular folds and their replicas: Influence of microstructuring and surface chemistry on the attachment of a leaf beetle', *Acta Biomaterialia*, 9(5), pp. 6360–6368.

Ramalho-Santos, M., Pissarra, J., Veríssimo, P., Pereira, S., Salema, R., Pires, E. and Faro, C. J. (1997) 'Cardosin A, an abundant aspartic proteinase, accumulates in protein storage vacuoles in the stigmatic papillae of Cynara cardunculus L.', *Planta*, 203(2), pp. 204–212.

Reitz, S. R., Gao, Y., Kirk, W. D. J., Hoddle, M. S., Leiss, K. A. and Funderburk, J. E. (2020) 'Invasion biology, ecology, and management of western flower thrips', *Annual Review of Entomology*, 65, 17-37.

Riddick, E. W. and Simmons, A. M. (2014) 'Do plant trichomes cause more harm than good to predatory insects?', *Pest Management Science*, 70(11), pp. 1655–1665.

Sawai, S. and Saito, K. (2011) 'Triterpenoid biosynthesis and engineering in plants', *Frontiers in plant science*. Frontiers Research Foundation, 2, p. 1-8.

Shany, S., Birk, Y., Gestetner, B. and Bondi, A. (1970) 'Preparation, characterisation and some properties of saponins from lucerne tops and roots', *Journal of the Science of Food and Agriculture*, 21(3), pp. 131–135.

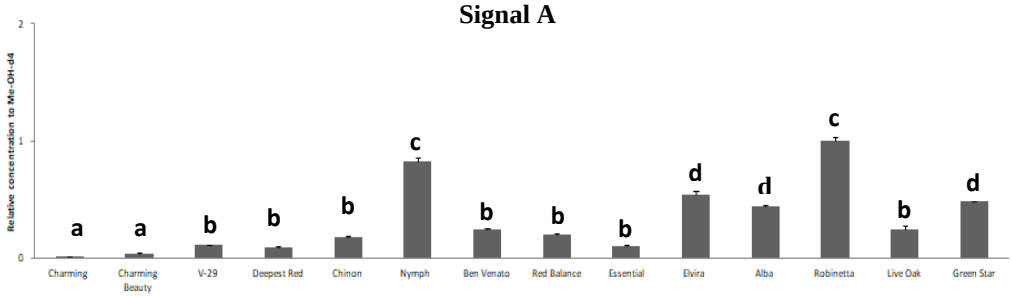
Tingey, W. M. and Sinden, S. L. (1982) 'Glandular pubescence, glycoalkaloid composition, and resistance to the green peach aphid, potato leafhopper, and potato fleabeetle in Solanum berthaultii', *American Potato Journal*, 59(3), pp. 95–106.

Underwood, W. (2012) 'The plant cell wall: a dynamic barrier against pathogen invasion', *Frontiers in Plant Science*, 3, p. 6.

Wakte, K. V, Nadaf, A. B., Krishnan, S. and Thengane, R. J. (2007) 'Studies on lower epidermal papillae, the site of storage of basmati rice aroma compounds in Pandanus amaryllifolius Roxb', *Current Science*, 93(2), pp. 238–242.

Wiklund, S., Johansson, E., Sjostrom, L., Mellerowicz, E. J., Edlund, U., Shockcor, J. P., Gottfries, J., Moritz, T. and Trygg, J. (2008) 'Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models', *Analytical Chemistry*, 80(1), pp. 115–122.

Signal A



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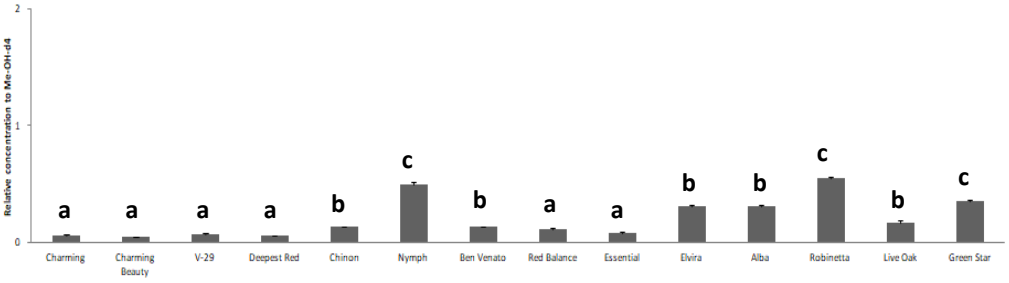

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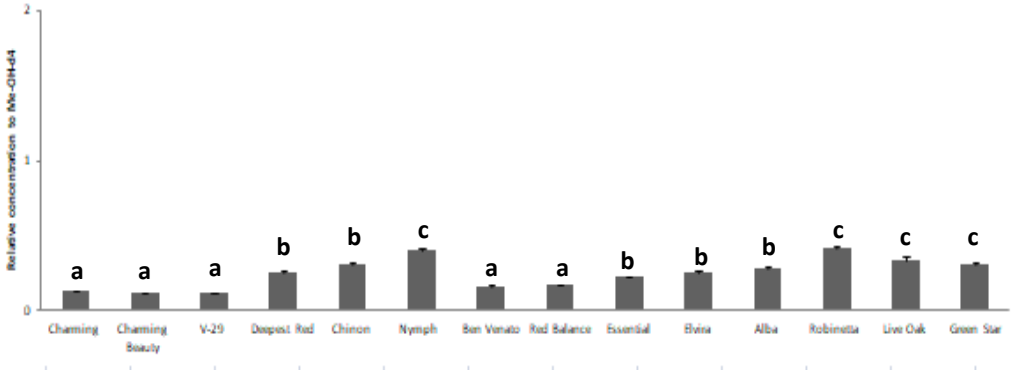

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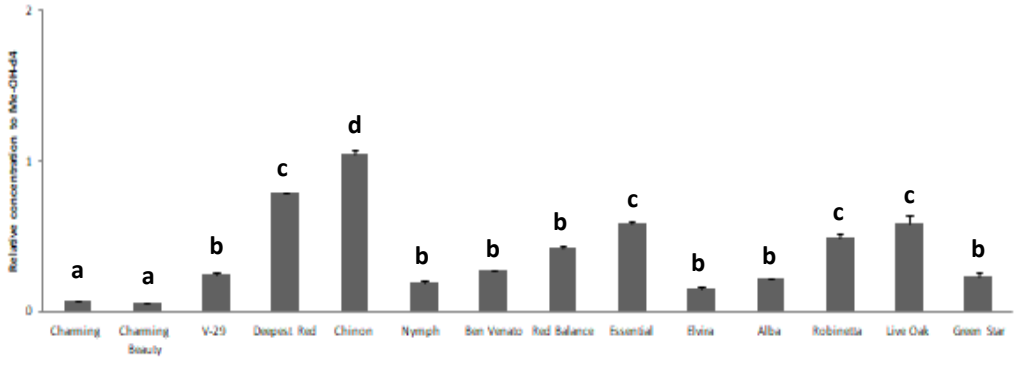

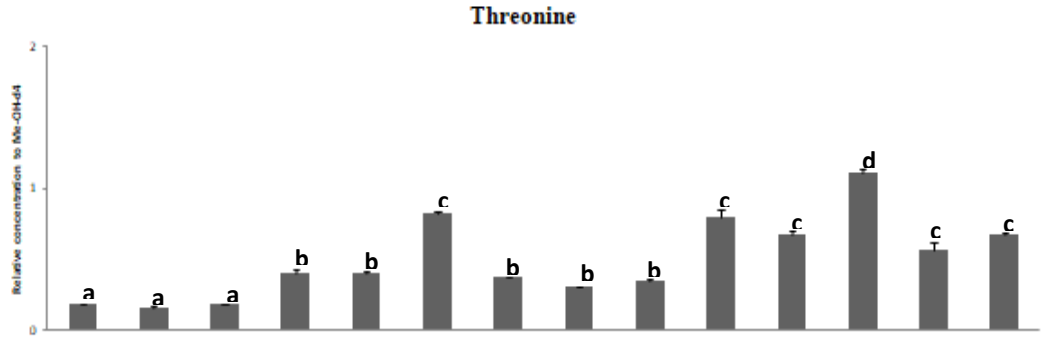
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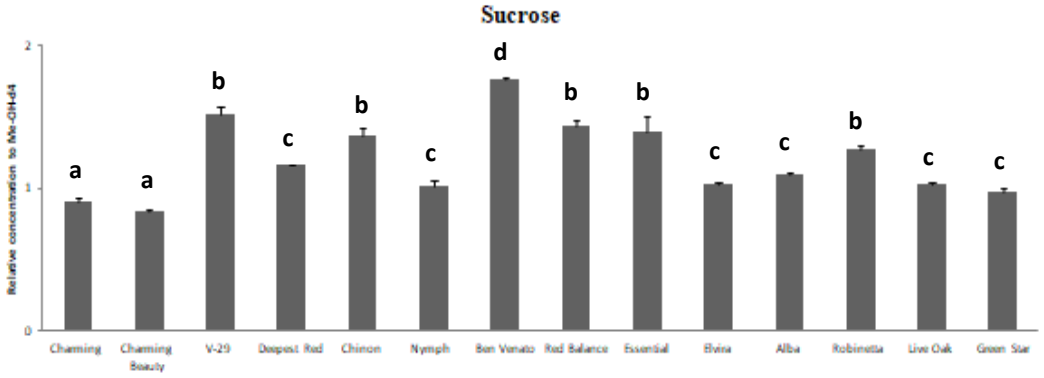
Sucrose
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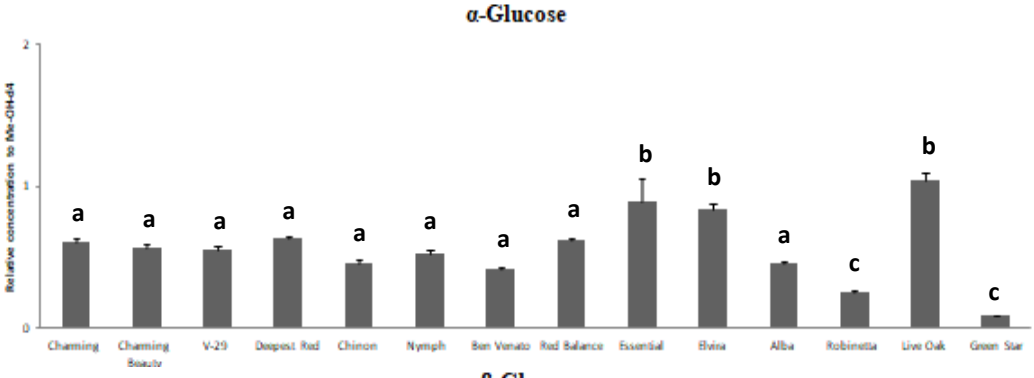
Charming
Charming Beauty
V-29
Deepest Red
Chinon
Nymph
Ben Venato
Red Balance
Essential
Elvira
Alba
Robinetta
Live Oak
Green Star


α-Glucose
Relative concentration to Me-OH-d4
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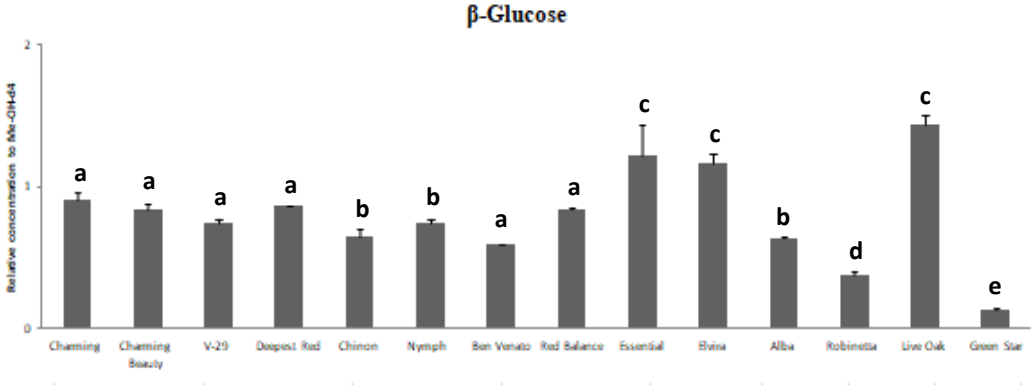
a a a a a a a a b b a c b c
Charming
Charming Beauty
V-29
Deepest Red
Chinon
Nymph
Ben Venato
Red Balance
Essential
Elvira
Alba
Robinetta
Live Oak
Green Star


β-Glucose
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a a a a b b a a c c b d c e
Charming
Charming Beauty
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Elvira
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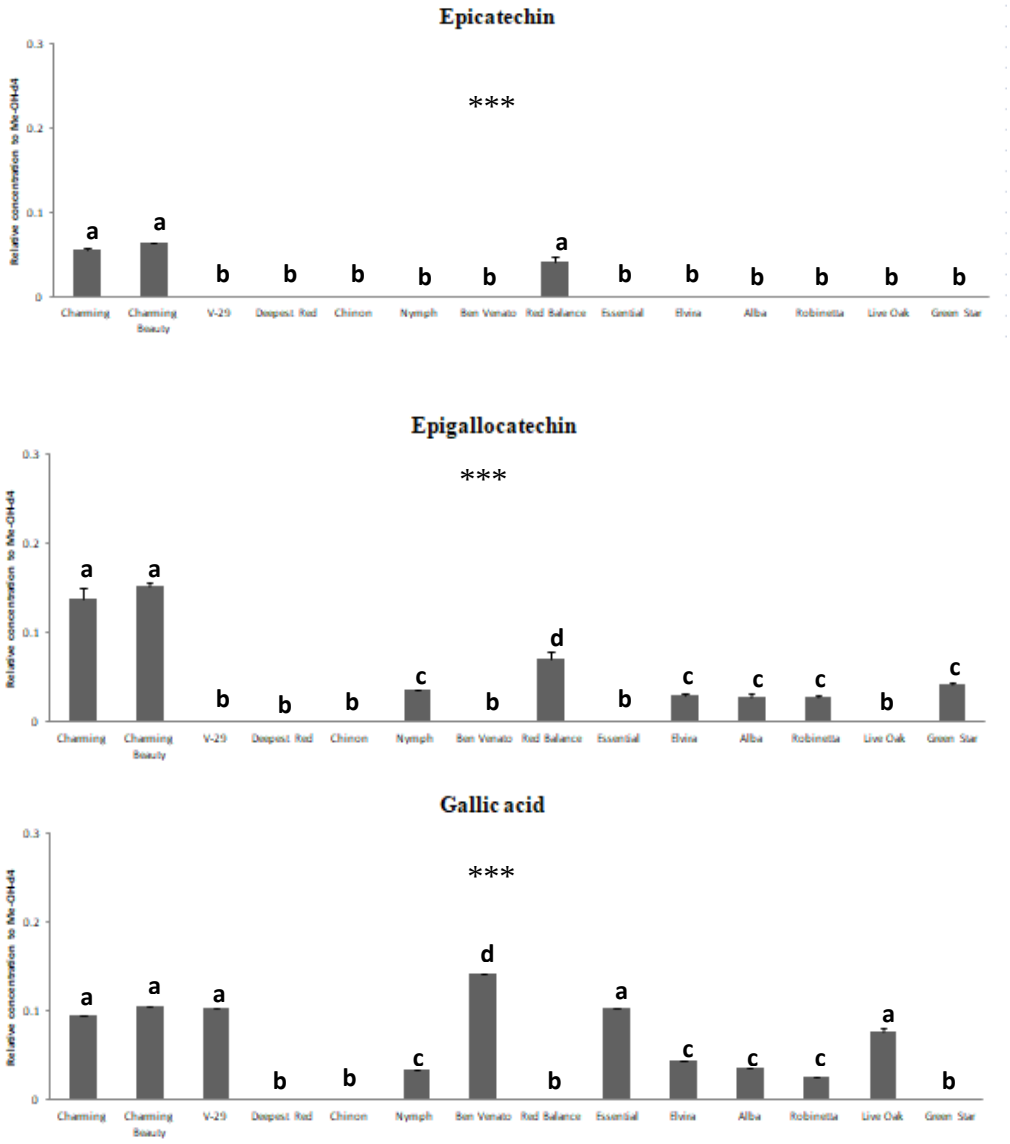

Figure S1. Relative concentration, as proportions of the internal standard, in ^{1}H NMR spectra of alanine, valine, threonine, sucrose, α-glucose and β-glucose in fourteen gladiolus varieties. Data present the mean of three to five replicates of leaves ± SE of the mean. Signal A, signal B, alanine, valine, threonine, sucrose, α-glucose and β-glucose were analyzed by one-way ANOVA. Different letters above the bars indicate significant differences between varieties. Epicatechin, epigallocatechin and gallic acid were

analyzed by Kruskall-Wallis test. *** indicate significant differences between varieties ($P < 0.001$).

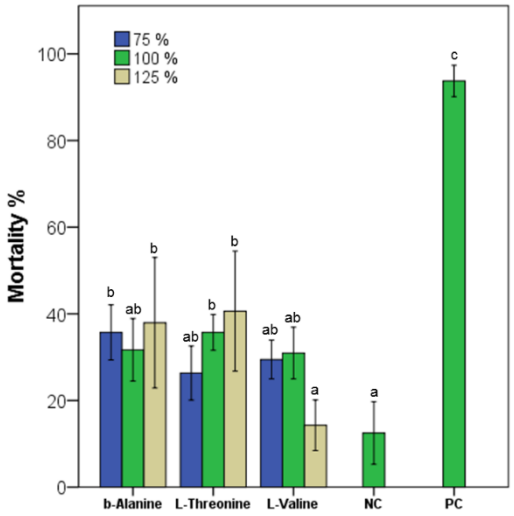

Figure S2. Thrips mortality in artificial diets to which three amino acids were added at three concentrations corresponding to 75%, 100% and 125% of the average plant concentration of 75%, 100% and 125%. Different letters above the bars indicate significant differences between treatment (Leiss *et al.*, unpublished results).