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Constitutive and induced defense against thrips in *Gladiolus nanus*

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ABSTRACT

Constitutive defenses are thought to be costly for plants since they require resources for their biosynthesis, or because of their toxicity to the plant itself or because of the ecological consequences of their accumulation. Induced defences against herbivory have the strong disadvantage that the initial attack by the enemies may be too rapid or too severe. In the previous chapter, I showed that differences in resistance and the concentration of the compounds associated with resistance between two varieties (Charming Beauty and Robinetta) remained constant during the ontogeny of the plant and under different growing conditions. Therefore, the aim of the present study was to investigate host plant resistance to thrips in Gladiolus nanus and to study if the compounds that we found to be associated with resistance and susceptibility against thrips in the previous chapters are induced or suppressed upon infestation. We investigated the differences between the metabolomic profiles as reflected by NMR of six Gladiolus nanus varieties. I conducted a whole plant bioassay with two set of plants, a group without thrips and a group with thrips. I then analyzed the metabolomic profiles of all plants tested. Constitutively resistant varieties had higher levels of triterpenoids, flavonoids (kaempferol) as well as some amino acids (alanine, valine and threonine). In our previous study with 16 varieties (chapter 3) we had not identified kaempferol as being associated with resistance. Apparently kaempferol was associated to resistant typically in Gladiolus nanus varieties where the concentration was seven times higher in resistant varieties. I found no significant thrips induced metabolites associated to resistance. Surprisingly, α-glucose and β-glucose which were significantly higher in susceptible varieties were induced by thrips infestation in some varieties.

KEYWORDS: Plant-insect interaction; *Frankliniella occidentalis* (Western flower thrips); Metabolomic; Secondary metabolites; kaempferol

INTRODUCTION

Plants have a wide spectrum of defenses against pests and diseases, ranging from morphological characteristics to chemical and biochemical features, each specific for a given plant species. Chemical and biochemical defenses can be divided into two groups. One concerns constitutive compounds that have an activity against pathogens or insects and that are present already before an attack takes place. Often, however, defence compounds need to be biochemically activated. For example, jasmonate-mediated induced plant resistance yielding toxic and reactive compounds. That means inactive compounds are stored separately from enzymes that can activate the secondary metabolites. The second group concerns compounds and enzymes that are not, or in a low concentration, present in healthy plants, but are induced by external stresses, like wounding or pathogenic infections. These compounds require elicitation of biosynthetic pathways at the level of gene expression. The constitutive compounds are the immediate defense, whereas for the induced compounds and enzymes it may take up to a few days to reach their maximum effect.

Constitutive defenses are thought to be costly for plants since they require resources for their biosynthesis, or because of their toxicity to the plant itself or because of the ecological consequences of their accumulation (Gershenzon, 1994). A meta-analysis on the cost of defence showed that the costs depend strongly on at what level and under which conditions they are measured. Generally costs seemed to be higher when measured under natural conditions implying that to a large extend they are ecological, through their effects on e.g. natural enemies or pollinators, rather than through trade-offs with resource use for other purposes (Koricheva *et al.*, 2004). Induced defences against herbivory have the strong disadvantage that the initial attack by the enemies may be too rapid or too severe (Wittstock and Gershenzon, 2002). The latter may be a lesser problem in case of pathogen infections or when the plant can respond to early signals of potential herbivory such as the presence of eggs on the leaves.

Host plant resistance is an important factor in integrated pest management to control invasive pests such as western flower thrips (WFT, *Frankliniella occidentalis*). WFT is one of the most serious pests for agricultural and horticultural crops (Jensen, 2000). It causes losses of hundreds millions of euros worldwide (Terry and Lewis, 1997).

Thrips cause the characteristic silver damage by sucking up a whole cell's content, leaving an empty cell which is filled with air. Damage in the buds and flowers may lead to malformation of leaves and flowers. Moreover, severely damaged flowers may desiccate and fall off (Denmark and Price, 1998). Besides the physical damage to the plant, thrips are the vectors of viral disease (Kirk and Terry, 2003). Different constitutive compounds are involved in different plant species in thrips resistance, for example: pyrrolizidine alkaloids and a flavonoid in the wild plant *Jacobaea vulgaris* (Leiss *et al.*, 2009), phenylpropanoids in the ornamental chrysanthemum (Leiss *et al.*, 2009), acylsugars in tomato plants (Mirnezhad *et al.*, 2010), the flavonoid luteoline, the phenylpropanoid sinapic acid, amino acid β-alanine in the vegetable carrot (Leiss *et al.*, 2013) and unknown sesquiterpens in pepper (Maharijaya *et al.*, 2012). Thrips infestation can also induce the production of volatile compounds like α-humulene and caryophyllene oxide in tobacco (Delphia *et al.*, 2007) as well as terpenes in tomato (Chen *et al.*, 2018). Such changes in metabolites upon thrips infestation result from elicitation of the the jasmonic acid signaling pathway (Abe *et al.*, 2009 and Escobar-Bravo *et al.*, 2017).

Thrips is a major problem in Gladiolus cultivation. Gladiolus is a genus of perennial bulbs belonging to the Iridaceae family. *Gladiolus hibridus* L. is commercially important bulbous cut flower plants. Constitutive host plant resistance in gladiolus to WFT was studied in the previous chapters of this thesis. In particular we found that several metabolites including triterpenoid saponins and the amino acids alanine and threonine were associated with resistance. The concentrations of these compounds in leaves were strongly correlated among themselves and with the density of extracuticular papillae (Chapter 3). The latter suggested that papillae play an important role in the production and storage of these defends compounds.

In chapter 4 we showed that differences in resistance and the concentration of the compounds associated with resistance between two varieties (Charming Beauty and Robinetta) remained constant during the ontogeny of the plant and under different growing conditions. The objective of the present study was to investigate host plant resistance to thrips in *Gladiolus nanus* and to study if the compounds that we found to be associated with resistance and susceptibility against thrips in the previous chapters are induced or suppressed upon infestation. We investigated the differences between the metabolomic profiles as reflected by NMR of six gladiolus varieties: with and without thrips infestation.

MATERIAL AND METHODS

Plant Materials

Six *Gladiolus nanus* varieties: Charming Beauty, Charming, Nymph, Alba, Elvira and Robinetta, obtained from Gebr. P. & M. Hermans (Lisse, The Netherlands) were used in this study. We planted single bulbs in a 9 x 9 cm pot filled with a 1: 1 mixture of potting soil and dune sand. Ten replicates of each variety were randomly placed in a growth room (L:D, 18:6, 20^0C) and grown for 10 weeks. The ten replicates of each variety were divided into a group without thrips and a group with thrips infestation Each plant was placed individually in a thrips-proof cage, consisting of a plastic cylinder (80 cm height, 20 cm diameter), closed with a displaceable ring of thrips-proof gauze (Chapter 3). The cages were arranged in a fully randomized design. Plants were infested with two male and 18 female adult western flower thrips. After 10 days silver damage, expressed as the leaf area damaged in mm^2, was visually scored for each plant. All plants from both groups were collected and dried in freeze dryer for two days. For each plants all leaves were ground for NMR metabolomic analyses.

Metabolic Profiling

Extraction of Plant Materials for NMR Metabolomics

The standard protocol of sample preparation and ^{1}H-NMR profiling as described by Kim *et al.* (2010) was used. Briefly, a sample of 30 mg freeze-dried plant material was weighed into a 2 ml microtube and extracted with 1.5 ml of a mixture of phosphate buffer (pH 6.0) in D_2O containing 0.05% trimethylsilylproprionic acid sodium salt-*d4* (TMSP) and methanol-*d4* (1:1). Samples were vortexed at room temperature for 1 min, ultrasonicated for 20 min and centrifuged at 13,000 rpm for 10 min. and an aliquot of 0.8 ml of the supernatant was transferred to a 5 mm NMR tube 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 sample had 128 scans requiring 10 min and 26 s acquisition time with the following parameters: 0.167 Hz/point, pulse width (PW) of 30 (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 decays (FIDs) were Fourier transformed with a line broadening (LB) of 0.3 Hz. Manual phase adjustment and baseline correction were applied as well as calibration with the internal standard TMSP to 0.00 ppm, using TOPSPIN (version 2.0, Bruker). Two dimensional COSY spectra were acquired with a 1.0 sec relaxation delay and 6361 Hz spectral width in both dimensions. The window function for the COSY spectra was Qsine (SSB = 2.0).

Data Processing

^{1}H-NMR spectra were automatically binned by AMIX software (version 3.7, Biospin, Bruker). Spectral intensities were scaled to total intensity and the region of δ 0.32-10.0 was reduced to integrated regions of 0.04 ppm each. The region δ 4.7-5.0 and δ 3.30-3.34 were excluded from the analysis because of the presence of the residual water and methanol signals, respectively. Principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) were performed with SIMCA-P software (version 15.0 Umetrics, Umeå, Sweden). For PCA, Pareto scaling was used whereas for PLS-DA, a unit variance method for scaling was used. The PLS-DA model was validated by application of permutation through 20 applications. twenty permutations were used to validate the PLS-DA model. Variance (R^2) and predictive ability (Q^2) of the models, using three components were calculated. Differences in metabolite concentrations among the six varieties and the thrips infested and non-infested plants for the effects of treatment and variety were analyzed by two-way ANOVA, with treatment and variety as factors. Data were log-transformed to fit a normal distribution. Epicatechin, epigallocatechin and gallic acid were analyzed by Kruskal-Wallis test.

RESULTS

Differences in Resistance to Thrips

Thrips silver damage in the whole plant bioassay differed significantly between varieties (F = 8.680, df = 5, P <0,001). Charming Beauty and Charming as the most susceptible ones showed significantly more damage compared to all other varieties, while Robinetta and Alba showed almost no damage at all (Fig. 1). Charming with a mean of

3159.3 ± 434.8 mm^2, displaying the highest amount of damage showed 130-times more damage than Robinetta with a mean of 23.8 ± 8.9 mm^2.

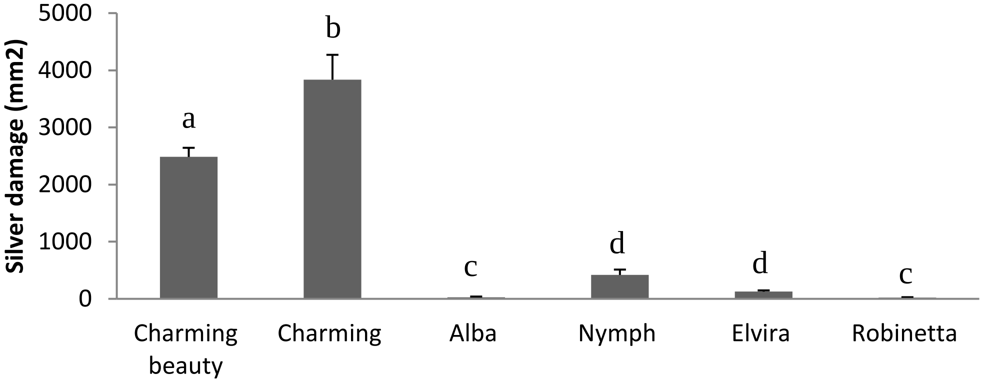

Figure 1. Silver damage, (mm^2) in six *Gladiolus nanus* varieties as measured by a whole plant thrips non-choice bioassay. Data represent mean and standard errors for five replicates. Different letters indicate significant differences between varieties at $P \leq 0.05$.

Metabolic Profiling

The leaf metabolomic profiles of the six *Gladiolus nanus* varieties in two groups were analyzed by multivariate data analysis. Principal component analysis (PCA), an unsupervised method, was applied to reduce the dimensionality of the multivariate data set. The PCA scoring-plot showed that the metabolomics profiles of the groups with and without thrips could not be separated for any of the varieties. The supervised PLS-DA method resulted in a low separation between the two groups for each of the varieties (Fig. 2A). In contrast, the profiles of the leaf metabolomes of the six *Gladiolus* varieties showed a clear separation between the resistant varieties (Robinetta, Alba, Nymph and Elvira) and the susceptible ones (Charming Beauty and Charming) (Fig. 2B). The first and second principle components explained 80.6% and 9.3% of the variance in the dataset, respectively. The model resulted in a variance R^2 of 0.944 and a predictive ability Q^2 of 0.931. The cross validation of the model using CV-ANOVA gave highly significant results (F = 113.708, df = 6, P = 0.000). Since the Q^2 values was greater than 0.5, the model was accepted as good.

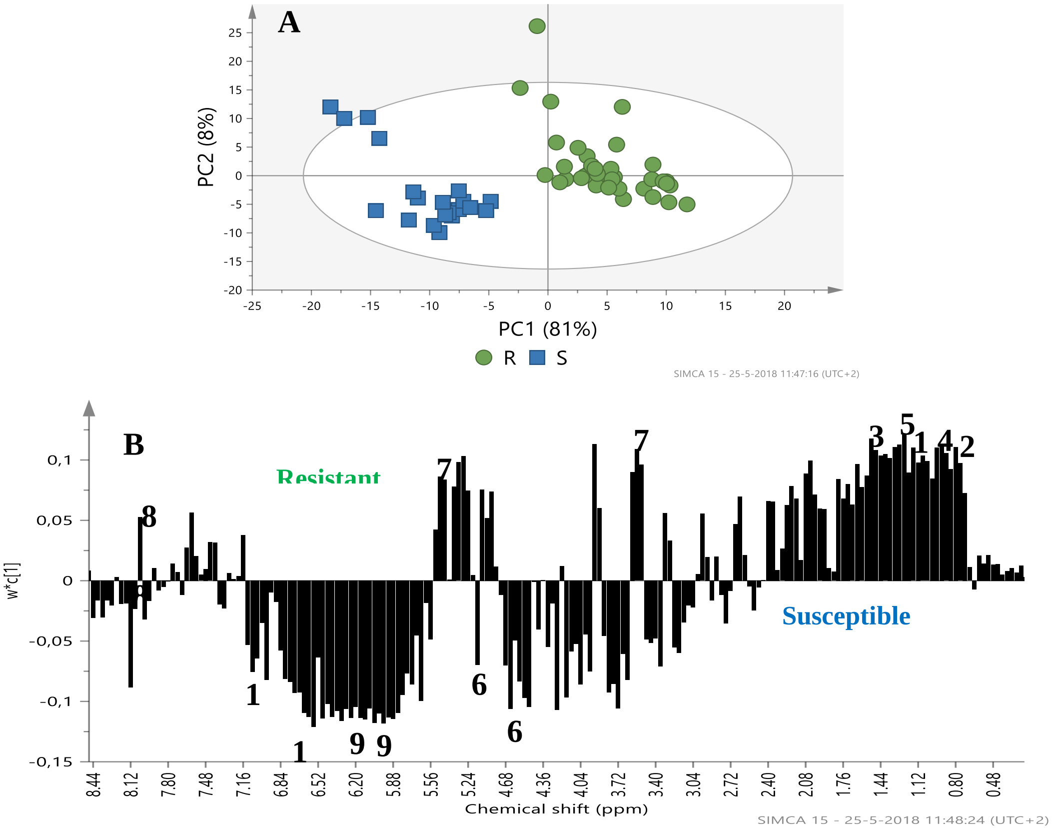

Figure 2. (A) PLS-DA score plot of six dwarf *Gladiolus nanus* varieties based on ^{1}H-NMR spectra related to their resistance, (■) susceptible (Charming Beauty and Charming varieties) and (●) resistant (Alba, Nymph, Elvira and Robinetta varieties). Loading plot of PLS-DA (B) based on thrips resistance, (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.

Relative Quantification of Metabolites

The signals that were related to the thrips resistant varieties are shown in the loading plot (Fig. 2C). They were identified as belonging to triterpenoid saponins (signal A and signal B), alanine, valine, threonine, kaempferol and sucrose. The score plot showed that α-glucose, β-glucose, epicatechin, epigallocatechin and gallic acid were related to the susceptible varieties. Signal intensity, as proportion of the internal standard, was used to quantify the metabolites.

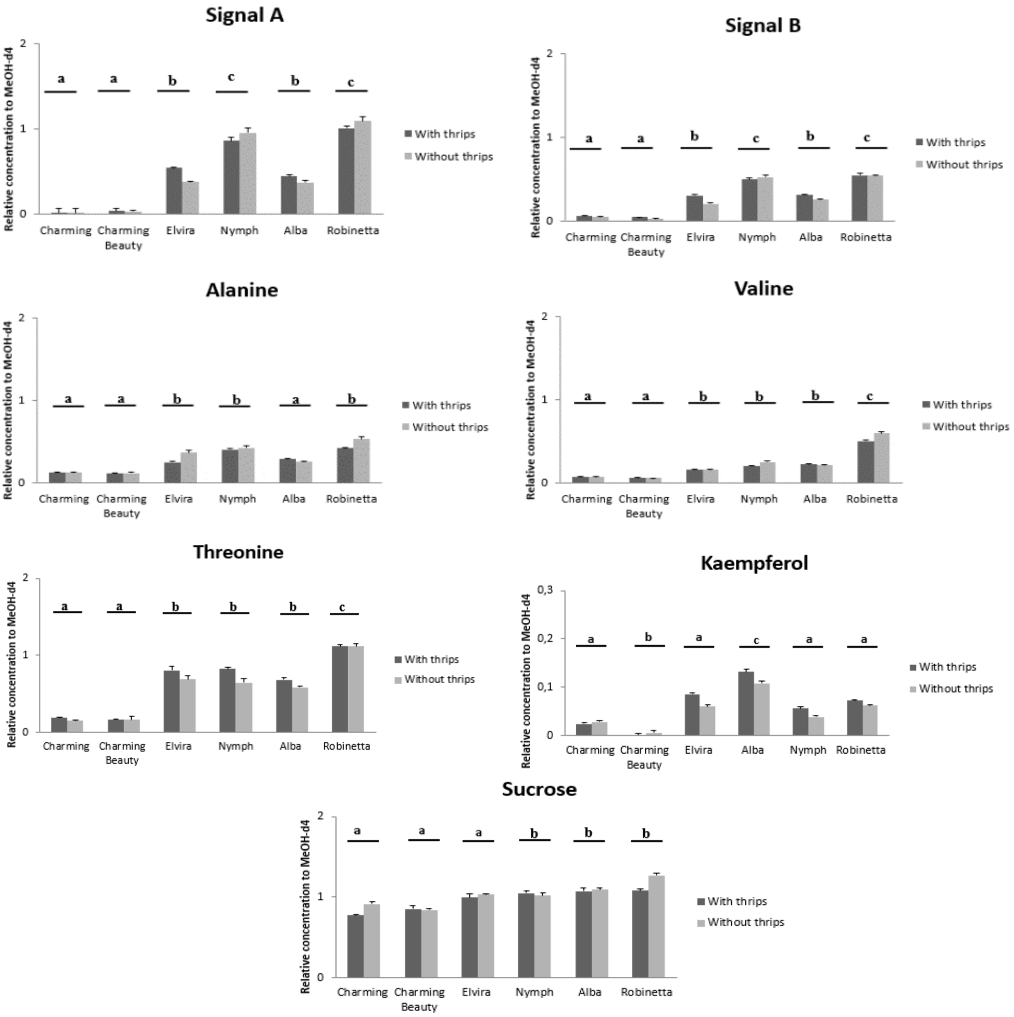

Figure 3. Relative concentration of metabolites related to resistant varieties, as proportions of the internal standard, in ^{1}H-NMR spectra of two triterpenoid saponins (signal A, signal B), alanine, valine, threonine, kaempferol and sucrose in the leaves of *Gladiolus nanus* varieties. Data present the mean of five replicates ± SE of the mean. Data were analyzed by a two-way ANOVA with cultivar and treatment as factors. Letters refer to significant differences among varieties (P<0.05). Differences within varieties between plants with and without thrips were not significant.

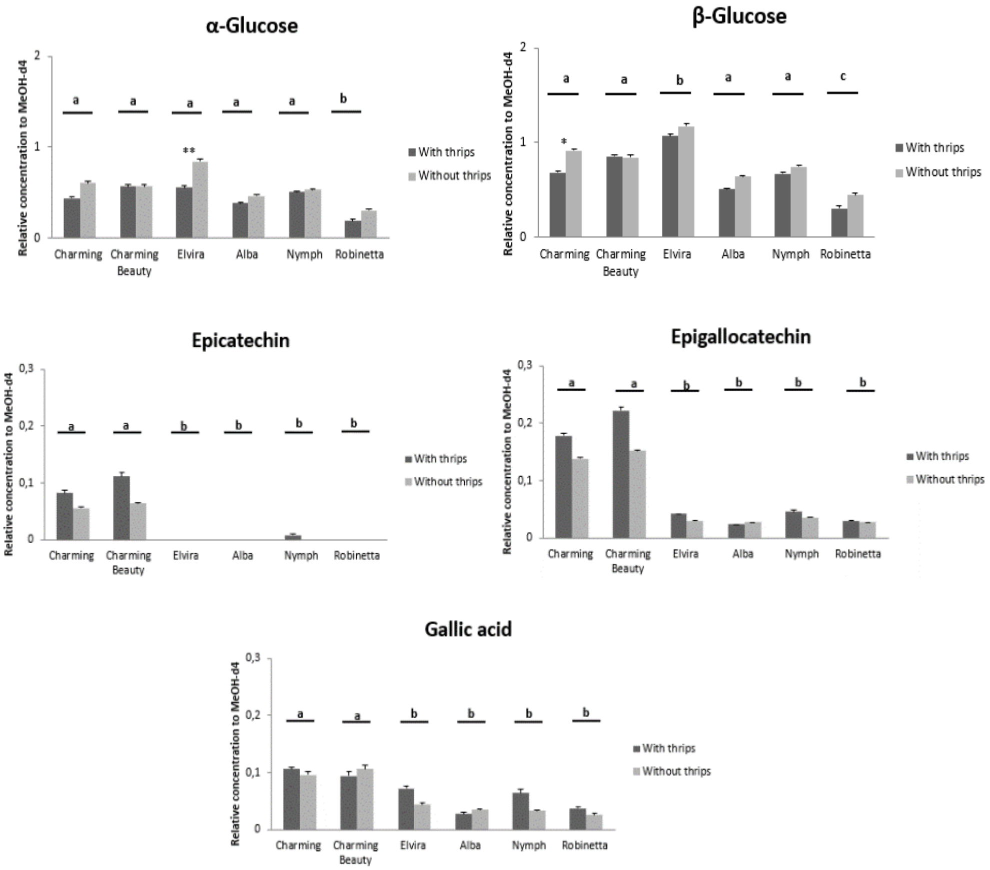

Figure 4. Relative peak intensities of metabolites related to susceptibility, as proportions of the internal standard, in ^{1}H-NMR spectra of α-glucose, β-glucose, epicatechin, epigallocatechin and gallic acid in the leaves of *Gladiolus nanus* varieties. Data present the mean of five replicates ± SE of the mean. α-glucose, β-glucose were analyzed by ANOVA. Letters and asterisk refer to significant differences among varieties at the 0.05 level and significant differences in particular variety, respectively. Epicatechin, epigallocatechin and gallic acid were analyzed by Kruskal-Wallis test.

All compounds that we identified as being associated with resistance in the previous chapters of this thesis gave similar results. None of the metabolites related to resistant varieties was induced by thrips infestation and all of them were significantly different among varieties (Fig. 3). Kaempferol which was associated to resistance typically in *Gladiolus nanus* was seven times higher in resistant varieties. We found no differences in the effect of thrips infestation among varieties as can be seen from the nonsignificant interaction between the effects of thrips infestation and varieties in the ANOVA (Table 1).

Metabolites associated to the susceptible varieties were significantly different among varieties (Fig. 4). The concentration of epicatechin, epigallocatechin and gallic acid were close to zero for the resistant varieties. However, in the two susceptible varieties the concentrations of epicatechin, epigallocatechin and gallic acid were not significantly affected by thrips infestation (H = 0.695, df =1, *P* = 0.404; H = 0.695, df =1, *P* = 0.404 and H = 1.434, df =1, *P* = 0.231). α-glucose and β-glucose were induced by thrips infestation in some varieties (F = 7.820, df =1, *P* = 0.007 and F = 4.350, df =1, *P* = 0.042, respectively) although their concentrations were higher in susceptible varieties.

Table 1. Result of two-way ANOVA with treatment and variety as factors of metabolites associated to both resistant and susceptible in *Gladiolus nanus*.

No.	Metabolites	Treatment (df=1)	Varieties (df=5)	Interaction (df=5)
1.	Signal A	F = 0.062, *P* = 0.804	F = 137.125, *P* = 0.000	F = 1.891, *P* = 0.113
2.	Signal B	F = 2.841, *P* = 0.398	F = 111.453, *P* = 0.000	F = 1.883, *P* = 0.331
3.	Alanine	F = 3.051, *P* = 0.087	F = 35.298, *P* = 0.000	F = 1.549, *P* = 0.193
4.	Valine	F = 2.471, *P* = 0.122	F = 108.450, *P* = 0.000	F = 1.492, *P* = 0.210
5.	Threonine	F = 3.009, *P* = 0.089	F = 58.400, *P* = 0.000	F = 0.642, *P* = 0.682
6.	Kaempferol	F = 3.752, *P* = 0.059	F = 32.427, *P* = 0.000	F = 0.933, *P* = 0.468
7.	Sucrose	F = 2.059, *P* = 0.158	F = 10.702, *P* = 0.000	F = 0.871, *P* = 0.507
8.	α-glucose	F = 7.820, *P* = 0.007	F = 12.114, *P* = 0.000	F = 1.274, *P* = 0.291
9.	β-glucose	F = 4.350, *P* = 0.042	F = 17.594, *P* = 0.000	F = 0.388, *P* = 0.854

DISCUSSION

In this chapter we confirmed that constitutively resistant varieties had higher levels of triterpenoids, flavonoids (kaempferol) as well as some amino acids (alanine, valine and threonine). In our previous study with 14 varieties (chapter 3) we had not identified kaempferol and valine as being associated with resistance. Apparently kaempferol was only associated to resistance in *Gladiolus nanus* varieties grown in the climate chamber. This might be due to variation in light based on the different growing conditions (Chapter 4). In Robinetta and Charming Beauty grown in climate chambers the relative concentration of kaempferol was 0.062±0.0025 and 0.006±0.0001, respectively; while it was very close to zero for both varieties when plants grew in the field. For the perennial semi-aquatic plant *Hydrocotyle leucocephala* similar results were found (Muller *et al.*, 2015). It was assumed that the lower levels of photosynthetic active radiation (PAR) and UV-B caused this increase in kaempferol concentrations in climate rooms compared to natural conditions (Deckmyn and Impens, 1997). Kaempferol deterred the generalist caterpillar, *Mamestra configurata* in a choice experiment (Onyilagha *et al.*, 2004). Moreover, kaempferol content was two times higher in cowpea lines (*Vigna unguiculate* L. Walp.) resistant to *Aphis fabae* (Lattanzio *et al.*, 2000). The concentration of kaempferol glycoside, was higher in *Jacobaea vulgaris* hybrids resistant to *F. occidentalis* (Kirk *et al.*, 2005; Leiss *et al.*, 2009) and it deterred *Thrips palmi* in *Solidago altissima* L. (Wu *et al.*, 2007).

Plants have to defense itself against a variety of threats and the response of the plant upon attack may be specific for a given enemy. Nevertheless, it is often unclear if particularly changes in the plant's metabolome indeed contribute to increased resistence against a specific pest species. Surprisingly we did not find major metabolomics changes upon infestation with thrips for any of the metabolites associated with the resistant varieties. Upon attack plant metabolite concentrations can either increase or decrease. For instance, kaempferol glycosides concentrations decreased while *p*-coumaroyl-tryptamine increased after infestation of maize by *Spodoptera littoralis* (Marti *et al.*, 2013). We found that none of the metabolites that were associated with resistance decreased upon infestation. Meanwhile, the concentration of glucose, which was associated with susceptibility, decreased.

Both, before and after thrips infestation, the metabolomic profile of different varieties of Gladiolus were clearly separated from each other in multivariate analyses. In contrast, the metabolomics profiles were not strongly affected after thrips infestation of the total leaves of the plant after 10 days infestation. However, the local changes after the limited damage, might be lost in the overall metabolome. Specific analysis of the tissues around the damage, might be of interest. For the most susceptible variety (Charming beauty) that showed damage all over the plant this most likely rules out the possibility that locally induction occurred. For the most resistant variety (Robinetta) with very local spots of damage we cannot rule out this possibility and a more analysis would be necessary to investigate whether selecting for increased local induced defences could lead to significant reduction of damage levels. A further limitation of this study is that induction was measured at only one point in time. Perhaps earlier or later stages after infection would show some level of induction of defence compounds. The analysis of early defense responses involving the signal transduction pathway such as a Reactive Oxygen Species (ROS) burst, hypersensitive response or Nitric Oxides (NO) accumulation are worth to further study.

Triterpenoids as defense compounds in *Gladiolus* leaves can be stored in the papillae leaf surfaces (Chapter 3) as the concentration of the compounds that we found to be associated to defence were all strongly correlated with the density of epicuticular papillae. In this chapter we clearly found that the differences among varieties in concentrations of the compounds related to defence were not affected by thrips infection. Because the concentration of these compounds did not change during plant developmental stages and were not strongly affected by environmental conditions as well (Chapter 4) they seem to be promising markers in breeding programmes aimed at increasing resistance.

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