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CONTENTS

GENERAL INTRODUCTION

Sustainable growth and development require minimizing the natural resources and toxic materials used, and the waste and pollutants generated, throughout the entire production and consumption process. This also applies to the production of food and ornamentals the sustainable production of which requires to minimize the use of pesticides. Breeding for resistance becomes more and more important in this respect. Nowadays for many species we can apply molecular tools, such as gene expression studies and mutants to discover mechanisms of host plant resistance. Such methods and techniques become increasingly cheaper and at some point, will become available for all crops. However, the need to reduce pesticides is extremely urgent as was recently again signaled by the reports on the alarming decline of insect species (Hallmann *et al.*, 2017). For some crops, especially polyploids and crops with large genomes the feasible required molecular tools will most likely not become available soon. So the market still calls for fast and cheap alternatives such as morphological or chemical markers. In order to provide these, we first need to study host plant resistance in plants and how it may change depending on environmental conditions or plant development. Based on these ideas we can than develop markers for herbivore resistance. Gladiolus is such a plant species of which the breeding industry is in search for markers that can assist the breeding for resistance against different insect herbivores with thrips being the most urgent one.

HOST PLANT RESISTANCE

To defend against multiple stresses, every plant species has a palette of different traits to combat them. This includes, among others morphological and chemical traits, as a defence against herbivores. Plant morphological traits includes plant size, trichomes, thickened tough cuticula and leaf surface waxes, all which play a role in resistance against the thrips, *Heliothrips haemorrhoidalis* (Scott Brown and Simmonds, 2006). Especially trichomes in tomato have been well studied as a defence mechanism and combine both morphological and chemical traits that contribute to herbivore resistance (Kang *et al.*, 2014; Balcke

et al., 2017; Chen *et al.*, 2018). In addition to the effect of these traits on thrips preference, thrips damage was positively correlated to plant size in chrysanthemum (de Jager *et al.*, 1996; Kos *et al.*, 2014) and tomato (Mirnezhad *et al.*, 2010).

Resistance strategies can be constitutively expressed or induced after damage (Dicke, 1998). Constitutive defenses are always present in plants, while induced defenses are produced when plants are attacked and damaged. Chemical compounds are produced upon attack that may serve against pathogen infections, may affect herbivores or may attract the natural enemies of herbivores. Constitutive defenses are thought to be costly for plants since they use up resources for their biosynthesis, they may be toxic to the plant itself or ecological costs can be present e.g. because the plant becomes more attractive to specialist herbivores (Gershenzon, 1994; Purrington, 2000). Most qualitative defence compounds occur in concentrations that are very low (<1%) (Zhao *et al.*, 2005) and therefore costs of biosynthesis are assumed to be low at the level of the whole plant. Aerts and co- workers (1991) showed that the carbon atoms needed for alkaloid formation only amount to 0.4% of the carbon atom flux into respiration. However, in locally induced defenses all energy of the induced area goes into the defense related pathways, resulting in local cell death.

Constitutive chemical defenses produced in plants can act as toxic and repellent compounds as well as digestibility reducers (Fürstenberg-Hägg *et al.*, 2013). Different compounds have been reported to be involved in the constitutive defense of different plant species against thrips: pyrrolizidine alkaloids and flavonoids in *Jacobaea vulgaris* (Leiss *et al.*, 2009a), phenylpropanoids (de Jager *et al.*, 1995; Leiss *et al.*, 2009b) and isobutylamides (Tsao *et al.*, 2003) in the ornamental chrysanthemum, acylsugars in tomato plants (Mirnezhad *et al.*, 2010), the flavonoid luteolin, the phenylpropanoid sinapic acid and the amino acid β-alanine in carrots (Leiss *et al.*, 2013). In transgenic potato plants constitutive defense involved cysteine protease inhibitors that affect the oviposition rates (Annadana *et al.*, 2002) and population development (Outchkourov *et al.*, 2004) of *Frankliniella occidentalis*. Moreover, these proteins suppressed the fecundity and reduced the survival rates of *F. occidentalis* in transgenic potato plants (Outchkourov *et al.*, 2004).

Plants induce defenses after wounding. Defense related genes are upregulated which is in general a fast process. However, an increase in the defence compounds resulting from these changes in gene expression is often only seen after a number of days (e.g Boue, 2000). Various signaling compounds like jasmonate, salicylate and ethylene play a major role in inducing these defense responses (Zhao *et al.*, 2005). Though the signaling pathways seem to be very similar in all plant species, they are coupled to totally different secondary metabolite pathways, which includes, among others, sesquiterpenes, triterpenes, anthraquinones, alkaloids, phenylpropanoids, and polyketides. Jasmonic acid was detected as being involved in induced defense against thrips in wheat cultivars (El-Wakeil, Volkmar and Sallam, 2010; El-Wakeil and Volkmar, 2012), tomato (Escobar-Bravo *et al.*, 2017) and chrysanthemum (Chen *et al.*, 2018). In *Nicotiana tabacum* the volatiles α-humulene and caryophyllene oxide were detected after infestation with thrips (Delphia *et al.*, 2007). Wittstock and Gershenzon (2002) pointed to the risk of relying on induced defences only, because the initial attack may be too rapid or too severe for such damage-induced defenses to be deployed effectively. Induced defense should help to reduce the damage after the attack and help other plant parts to be prepared, which means for plant breeding both systems, induced and constitutive defenses, are important as both are needed for a proper resistance of the plant. Recently, plant resistance to herbivores based on chemical traits has been determined using an eco-metabolomic approach. (Leiss *et al.*, 2009b; Leiss *et al.*, 2013). Such an approach is based on the natural variation present among genotypes or varieties of a particular species.

On top of the enormous variation among and within plant species the detection of metabolites involved in resistance is even more complicated by the fact that many plant metabolites interact in their effects. This may explain why, for instance, in some species (e.g. chrysanthemum, Leiss *et al.*, 2009b) resistance against thrips was correlated with chlorogenic acid while in others such as tomato it was not. As a result of the lack of general patterns it remains necessary to study individual species for which resistance is a problem. One such species is the ornamental gladiolus of which many varieties are severely attacked by insect herbivores.

For such plant species we need to investigate defence under various growing conditions because these may alter the attractiveness of plants to herbivores severely.

Such growing conditions include temperature, nutrient, water, oxygen (Pieterse *et al.*, 2009). For instance, two-spotted spider mites preferred plants of *Sorbus aucuparia* L. and *Acer platanoides* L. grown in the shade environment over plants grown in full sunlight. The first contained less phenolics compounds (Giertych *et al.*, 2008). In *Arabidopsis thaliana*, high light intensity led to a significant change in rosette growth and the composition of B-subunits of protein phosphatase 2A, which significantly decreased aphid fecundity in 3 mutant lines but not in the Columbia wild type (Rasool *et al.*, 2014). Nutrient and water availability influenced pyrrolizidine alkaloids concentration which are well-known chemical defenses in e.g. *Senecio jacobaea* and *S. aquaticus* (*Jacobaea aquatica*) (Kirk *et al.*, 2010). Elevated CO_2 reduced resistance in wild-type tomato plants infested with *Helicoverpa armigera* by decreasing jasmonic acid levels (Guo *et al.*, 2012). In broccoli plants, *Brassica oleracea* var. *italica*, water stress differentially influenced induced defences among cultivars (Khan *et al.*, 2011). Moreover, high photosynthetically active radiation (PAR) induced trichome-associated chemical defense which plays a major role in tomato-thrips interaction (Escobar-Bravo *et al.*, 2018).

In addition to abiotic and biotic stresses, plant resistance changes during ontogeny. Ontogenetic shifts in defense traits can be associated with dramatic changes in the levels of herbivory (Barton and Koricheva, 2010). In *Nicotiana attenuata* (Solanaceae) rosette plants, bolting plants, and flowering plants all contained trypsin protease inhibitors in leaves, stems, and flowers, while seed capsules, seeds, and young seedlings did not contain any protease inhibitors (PIs) (van Dam *et al.*, 2001). In *Plantago lanceolata*, Iridoid glycosides concentrations decreased with plant age and even more strongly with leave age (Bowers and Stamp, 1993). Likewise, seedling to early juvenile stages of *Penstemon virgatus* (Plantaginaceae) were better defended against the caterpillar *Junonia coenia* (Nymphalidae) than adult stages (Quintero and Bowers, 2013). In tomato, developing leaves were more resistant against thrips than fully developed leaves (Chen *et al.*, 2018). In general young leaves seem better defended against thripsthan older leaves (Leiss *et al*., 2009b; Mirnezhad *et al.*, 2010). However, Visschers *et al*. (2019) could not observe any differences in thrips damage between old and young leaves. Most likely the balance between the nutritional values of leaf and their

concentrations of toxic compounds determine the pattern of resistance over the leaf canopy.

Given the examples above, exploring resistance means that the plant has to be studied during different life-stages and under different environmental conditions and that both constitutive and inducible defense mechanisms should be included. In this thesis, we studied plant defense of the ornamental plant *Gladiolus* for both morphological and chemical traits related to resistance against thrips.

GLADIOLUS

The Netherlands are a leader in the production of flower bulbs worldwide. They generate $ 756 million in value with 21,000 ha of production area (Benschop *et al.*, 2010). *Gladiolus* is an important flower bulb crop, which comprises 5% of the total bulb production (Benschop *et al.*, 2010). *Gladiolus* is one of the largest genera in Iridaceae family. *Gladiolus* means sword and often Gladiolus is called 'sword lilly' because of the shape of its leaves. The plants are herbaceous, and have lanceolate, unbranched, basal leaves. In addition to bulbs, *Gladiolus* is an important cut flower export product in summer. The *Gladiolus nanus* type, known as mini gladiolus, has been developed for growing in greenhouses in the winter season (Cohen and Barzilay, 1991). In general, *Gladiolus* corms can be planted on beds or ridges of the furrows for flower production. Common *Gladiolus* varieties grow for 110-150 days whilst the nanus type varieties are early flowering *Gladiolus* types that take 70-100 days to bloom.

Besides the use of flowers for decoration purposes, other uses have been explored. Aqueous macerates from *Gladiolus dalenii* corms have been reported to have antidepressant properties in epilepsy-associated depression (Ngoupaye *et al.*, 2013 and Ngoupaye *et al.*, 2014). The extract exhibited both an anticonvulsant and a sedative effect (Ngoupaye *et al.*, 2013). Moreover an antimicrobial and an antifungal activity against *Staphylococcus aureus*, *Bacillus cereus*, *Mycobacterium smegmatis* and *Candida albicans* were reported (Kahriman *et al.*, 2012). These effects were due to the presence of monoterpenes such as limonene and linalool, and sesquiterpene hydrocarbons such as muurolene (Kahriman *et al.*, 2012). *Gladiolus* was reported to contain several triterpenoids and their derivates such as β-amyrin, lupeol, friedelin, betulin, etc (Zhang *et al.*, 2007). The presence of anthraquinone glycosides in *Gladiolus* was reported by

Abdessemed et al. (2011) and Wang (2003). A new oleanan triterpene was identified in some *Gladiolus* species (El-Shanawany *et al.*, 2009).

Gladiolus has many pest problems such as *Thrips simplex* and *F. occidentalis* which are commonly found in *Gladiolus* (Terry and Lewis, 1997). They cause severe damage on corms, leaves and flowers on *Gladiolus*. Control of thrips mainly depends on fumigating, dipping and spraying insecticides on the corms and plants. However, the continuous application of insecticides results in insect resistance, and has negative impacts on human health and the environment.

THRIPS

Thrips are small pest insects (0.5 to 14 mm in length). *F. occidentalis* is a highly polyphagous pest, damaging more than 240 species from 62 different plant families (Kirk and Terry, 2003). Tipping (2008) estimated that they feed on more than 600 different wild and cultivated plants. Thrips may feed on leaves, flowers and fruits. The genus *Frankliniella* contains some of the most important thrips pests such as Western flower thrips [WFT, *F. occidentalis* (Pergande (Thysanoptera:Thripidae)]. It is a significant pest of most crops, including vegetables, ornamentals, fruits and cotton (Lewis, 1997; Kirk and Terry, 2003). Thrips is especially a key pest of ornamental corps (Tommasini and Maini, 1995). In Europe, WFT was first detected in the Netherlands in 1983 and by the end of the 1980's, it has been observed in most European countries (Tommasini and Maini, 1995). It is the most common thrips in Dutch greenhouses as observed by sampling in the period 1994- 2000 (Vierbergen, 2001).

Thrips often go unnoticed because of their behavior. Adults and larvae hide and feed in the protected narrow crevices in flowers and foliage (Jensen, 2000). This makes chemical control difficult. Moreover, thrips developed insecticide resistance, with populations resistant to several different classes of insecticides (Herron and James, 2005). In addition, this pest is an efficient vector of plant viruses, including tomato spotted wilt virus (TSWV), which affects different vegetables crops (Steenbergen *et al.*, 2018).

Oviposition and feeding behavior of thrips cause direct damage to plants (Mouden *et al.*, 2017). Thrips have piercing-sucking mouthparts which allow them to feed on different types of plant cells (Reitz *et al.*, 2019). Thrips feed by penetrating the plant cells and sucking out the cell sap (Capinera, 2004). Feeding causes the plant cells

to become filled with air which cause a characteristic silver leaf scar, so called silver damage (Denmark and Price, 1998). Feeding leads to yield losses due to stunted and deformed plants (Denmark and Price, 1998).

Thrips is commonly found on *Gladiolus* (Terry and Lewis, 1997). They cause damage on the corms resulting in smaller corms, retardation of growth, and poor flowering. Some corms may even fail to germinate. Damage in the buds and flowers may lead to difficulties in flower formation and opening. Moreover, severely damaged flowers may desiccate and fall off. The variation in *Gladiolus* thrips resistance, primarily to *Thrips simplex* and *F. occidentalis*, has been reported by Terry and Lewis (Terry and Lewis, 1997).

METABOLOMIC STUDIES

Metabolomics is an advanced technology aiming at measuring the full suite of metabolites expressed in a cell or tissue both qualitatively and quantitatively. Obviously, that means it is a tool and not a goal. Metabolomics provides comprehensive information related to all metabolites networks (Weckwerth, 2003) and thus on the functioning of the whole system. Metabolomics based on Nuclear Magnetic Resonance spectroscopy (NMR) provides a very fast and detailed analysis of the biomolecular composition of a crude extract (Verpoorte *et al.*, 2008). It has major advantages of high long term reproducibility and a broad range of detected metabolites and simple absolute quantitation of all compounds as signal intensity is only dependent on molar concentration (Kim *et al.*, 2010). However, a relatively low sensitivity and signal overlap in the NMR spectra are the drawback of NMR as a tool in metabolomics studies (Krishnan *et al.*, 2005).

Metabolomics has been applied as a tool to study plant-insect interactions (J William Allwood, Ellis and Goodacre, 2008; Macel *et al.*, 2010). The comparison of the metabolomes of herbivore resistant and susceptible plants allows identification of metabolites related to host plant resistance (Leiss *et al.*, 2011). Identification of such metabolites using NMR has been done successfully in wild *Jacobaea* species (Leiss *et al.*, 2009a), chrysanthemum (Leiss *et al.*, 2009b) and *Barbarea vulgaris* (Kuzina *et al.*, 2009), as well as in crop plants like tomato (Mirnezhad *et al.*, 2010; Bac-Molenaar *et al.*, 2019) and carrot (Leiss *et al.*, 2013).

AIMS AND SCOPE OF THE THESIS

This thesis explores the resistance of the ornamental plant *Gladiolus* to thrips. The aim is to improve our understanding of its defence against WFT by studying both morphological and chemical traits related to resistance against thrips. We focused on how defence traits were influenced by the abiotic and biotic environment and by plant developmental stages.

This general aim was translated to the following research questions:

1. Is leaf morphology related to the defense against thrips in *Gladiolus*?
2. Does chemical defense play a role in *Gladiolus*? And if so which compounds are involved?
3. Does the spectrum of constitutive defense compounds related to thrips resistance alter qualitatively or quantitatively during plant development? Do thrips damage and concentrations of defense compounds differ between plant organs? Do we find significant changes in defense related metabolites depending on the growth conditions?
4. Do we find an increase in resistance related metabolites after thrips infestation?

In the second chapter of this thesis, we explored the natural variation in thrips resistance dwarf *Gladiolus* by performing bioassays and relating differences in resistance to morphological traits as possible mechanical resistance factors (Chapter 2).

In chapter 3 we first explored the natural variation in resistance against thrips of regular, medium and large size *Gladiolus* varieties. Firstly we added extracts of plants to artificial diets to show that resistance was, at least in part, based on the plant's metabolome. We continued by performing a thrips infestation bioassay in which the damage caused by thrips was measured. To determine the chemical factors involved in resistance, all tested plants were subjected to NMR metabolomics analysis to identify possible markers for constitutive resistance. In addition, we studied the correlation between the density of papillae, a morphological structure identified as being related to WFT resistance in chapter 2, and leaf metabolite concentrations.

In chapter 4 we studied the influence of plant development stages on resistance of *Gladiolus* against WFT and the distribution of WFT damage over plant organs in flowering plants. We used 3 development stages (vegetative stage, generative stage with

buds and generative stages with flowers) of susceptible and resistant *Gladiolus* varieties in a thrips infestation bioassay. Furthermore, we grew plants under different conditions to study their effect on the plant's metabolome. Most experiments to study resistance referred to in the literature were performed in climate chambers under controlled growth conditions. But are these results also valid for the performance of plants under field conditions? Therefore, we compared the metabolomes of plants grown in the field and in the climate chamber (Chapter 4).

In chapter 5 we studied metabolic changes in *Gladiolus* after thrips infestation. We subjected six dwarf *Gladiolus* varieties to thrips infestation and measured the metabolomic changes compared to plants that were not infested (Chapter 5).

In chapter 6 we present the summary and conclusions of this thesis and discuss why the density of papillae is a good marker to be used in breeding programs targeted at thrips resistant Gladiolus varieties.

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Morphological traits related to western flower thrips resistance in the ornamental Gladiolus

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ABSTRACT

Host plant resistance can be based on morphological traits or chemical defense compounds. Understanding the mechanisms involved in host plant resistance opens the way for improved resistance breeding programs by using the traits involved as markers. Pest management is a major problem in cultivation of ornamentals. Gladiolus (*Gladiolus hybridus* L.) is an economically important ornamental in the Netherlands. Gladiolus is especially sensitive to attack by Western flower thrips [*Frankliniella occidentalis* (Pergande) (Thysanoptera:Thripidae)]. The objective of this study was, therefore, to investigate morphological markers for resistance breeding to western flower thrips in Gladiolus varieties. We measured thrips damage of fourteen Gladiolus varieties in a whole plant thrips bioassay and related this to morphological traits. Thrips damage varied strongly among the varieties: the most susceptible variety showed 130 times more damage than the most resistant one. Varieties with low thrips damage had smaller mesophyll cells, smaller epidermis cells, and a higher density of epicuticular papillae. In contrast, plant dry mass and leaf length were not correlated with thrips damage. All three traits that were related to thrips damage were highly correlated with each other. In fact, almost without exception all epidermis cells had one papilla. We assume that papillae may inhibit thrips movement or hinder penetration of the epidermis by the cell sucking thrips. In addition, papillae may serve as storage or production sites for plant defense compounds. Our results show that the density of papillae is an important morphological trait related to resistance to thrips. Papillae are easily visualized and may, thus, be used as thrips resistance markers in breeding programs for Gladiolus.

KEYWORDS: Gladiolus, *Frankliniella occidentalis*, host plant resistance, morphological markers, mesophyll, epidermis, papillae

INTRODUCTION

Plants have to defend themselves against a myriad of herbivores and have thus developed different ways of resistance. Host plant resistance can be based on morphological traits or chemical defense compounds. Plant morphological defence traits include smaller size, thorns, trichomes, leaf surface waxes and toughened cuticles (Scott Brown and Simmonds, 2006 and references therein). Chemical defences include the production of toxic and repellent compounds as well as digestibility reducers (Fürstenberg-Hägg *et al.*, 2013). Understanding the mechanisms involved in host plant resistance opens the way for improved resistance breeding programs by using the traits involved as markers. In this respect especially morphological markers can be important because they are mostly easily measured at low costs.

Pest management is a major problem in cultivation of ornamentals, as was again shown by the Russian boycott of Dutch ornamentals because of thrips infestation. Presently, the use of synthetic insecticides is the method of choice for controlling insects in ornamentals. Fumigation or dipping of bulbs and corms during storage and insecticide sprays on foliage and flowers is often applied. The widespread and excessive use of insecticides may cause negative impacts on human health, non-target beneficial organisms and the environment. In addition, it has led to a build-up of insect resistance (Carriere *et al.*, 2012). Therefore, new European Union regulations call for a reduced application of pesticides (Coelho, 2009). Integrated pest management (IPM), using different complementary control tactics is the way forward for bulb flower production (Rossing *et al.*, 1997; Benschop *et al.*, 2010). One of the main strategies to be included is natural host plant resistance.

The Netherlands are a leader in the production of flower bulbs worldwide. They generate $ 756 million in value with 21,000 ha of production area (Benschop *et al.*, 2010). Tulip, lily, narcissus, gladiolus, hyacinths, crocus and iris are the major bulbs produced. Gladiolus, comprises 5% of the total production (Benschop *et al.*, 2010). *Gladiolus hybridus* L. (Iridacaeae), are perennial bulbs belonging to the Iridaceae family. Gladiolus is marketed as cut flowers in summer. The latter are especially sensitive to attack of thrips.

Western flower thrips [*Frankliniella occidentalis* (Pergande (Thysanoptera:Thripidae)] is commonly found on gladiolus (Terry and Lewis, 1997).

Thrips are piercing-sucking insects causing damage to the corms and growing plants. Damage on the corms results in smaller corms, retardation of growth, and poor flowering. Some corms may even fail to germinate. Damage on the leaves, buds and flowers generate the characteristic silver damage (Denmark and Price, 1998). Damage in the buds and flowers may lead to distorted flower formation and opening. Moreover, severely damaged flowers may desiccate and fall off. Variation in gladiolus thrips resistance, primarily to *Thrips simplex* and *F. occidentalis*, has been reported by Terry and Lewis (Terry and Lewis, 1997). Nothing is known yet about the mechanism causing these differences. According to Dutch gladiolus breeders (personal communication), thrips are able to discriminate between different Gladiolus varieties, infesting certain varieties but not others.

The aim of this study is to obtain morphological markers for resistance to western flower thrips that can be used in breeding programs of Gladiolus. We focused on the following traits: plant dry mass, leaf length, size of the epidermis cells, size of the mesophyll cells and the density of papillae at the leaf surface. In particular we wanted to address the following questions: (1) Does thrips damage vary among Gladiolus varieties? (2) Do the varieties differ in potential morphological resistance traits of the leaves? (3) Is thrips damage related to morphological traits?

MATERIAL AND METHODS

Plant Materials

Fourteen different gladiolus varieties differing in size were used. Six small varieties (Charming, Charming Beauty, Nymph, Alba, Elvira and Robinetta obtained from Gebr P. & M. Hermans, Lisse, The Netherlands), eight medium to large size varieties (Ben Venuto, Red Balance, V-29, Chinon, Live Oak, Deepest Red, Green Star, and Essential obtained from 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 to ten replicates of each variety were randomly placed into a growth room (L:D, 18:6, 20 ^{0}C) and grown for 10 weeks. Three to five replicates of each variety were used for a whole plant thrips bioassay while the remaining replicates were used for measuring the morphological parameters.

Plant Resistance to Thrips

A non-choice whole plant bioassay was conducted as described in Leiss *et al.* (2009). Plants were 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. The cages were arranged in a fully randomized design in a climate chamber (L18: D6, 20 °C). Two male and 18 female adult western flower thrips were added and left for two weeks. Thereafter, silver damage, expressed as the leaf area damaged in mm^2, was visually scored for each plant.

Morphological Measurements

Morphological resistance traits were measured on the longest leaf of each replicate. In addition, plant dry mass was obtained after drying plants for 3 days in an oven at 50 °C. We measured the length of the leaves and of epidermis- and mesophyll cells as well as the density of the epicuticular papillae which form a convex outgrowth of epidermal cells (Koch *et al.*, 2008). The density of papillae was measured as number of papillae per 2100 μm^2. To measure these traits cross sections of fresh leaves were examined under a confocal laser scanning and a visual light microscope (Zeiss LSM Exciter) with a 20x magnification. Measurements were conducted using image J software. To visualize the leaf surface of Gladiolus varieties, we choose Charming Beauty and Robinetta as representatives of a variety with high and low thrips damage respectively, for scanning-electron microscopy (SEM). We used a JSM6400 scanning electron microscope (JEOL, Tokyo, Japan). Leaf discs were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7) followed by dehydration with a graded series of acetone solutions (70%, 80%, 90% 96% and 100% acetone) for10 min each. Before imaging, specimens were oriented, mounted on metal stubs and sputter-coated with gold (Polaron 5000 Sputtering System).

Statistical Analysis

Differences between varieties in plant dry mass and morphological traits were analyzed with one-way ANOVA and subsequent post-hoc analysis with Bonferroni correction. Silver damage did not fit a normal distribution and was, therefore, Ln-transformed. Correlations between thrips silver damage and morphological traits were analyzed using Pearson correlations.

RESULTS

Differences in Resistance to Thrips

Thrips silver damage in the whole plant bioassay differed significantly among varieties (F = 11.445, df = 13, P = 0.000). 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, displaying the highest amount of damage, (mean 3159.3 ± 434.8 mm^2), showed 130-times more damage than Robinetta (mean 23.8 ± 8.9 mm^2).

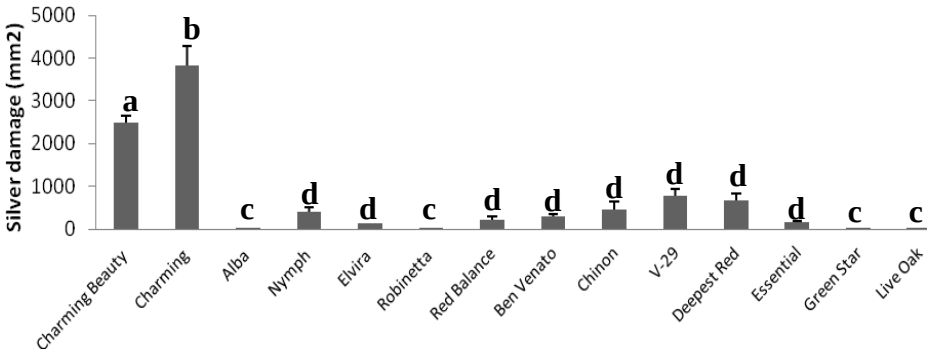


Figure 1. Silver damage, (mm^2) in fourteen Gladiolus varieties as measured by a whole plant thrips non-choice bioassay. Data represent mean and standard errors for three to five replicates. Different letters indicate significant differences between varieties at $p \leq 0.05$.

Morphological Differences

Leaf length (F = 15.522, df = 13, P = 0.000) differed significantly among varieties. Nymph, Elvira and Robinetta were significantly shorter than the other varieties (Fig. 2A). The dry mass of varieties differed significantly (F = 70.531, df = 13, P = 0.000) with large size varieties yielding more than double the dry mass compared to the small ones (Fig. 2B). The length of epidermis- (F = 125.459, df = 13, P = 0.000) (Fig. 3A) and mesophyll cells (F = 90.136, df = 13, P = 0.000) (Fig. 3B) also differed significantly between varieties. For both cell types the cells in the most susceptible varieties, Charming and Charming Beauty, were two times longer than those of the resistant varieties. The density of epicuticular papillae differed between varieties in a similar way as both cell lengths (F = 29.363, df = 13, P = 0.000) (Fig. 3C). Charming and Charming Beauty had two times less papillae compared to the resistant varieties, Alba, Robinetta, Green Star

and Live Oak. The lengths of the epidermis and mesophyll cells were strongly correlated to the density of papillae (Table 1). This strong correlation is explained by the fact that as a rule each epidermis cell produces one papilla (Figs. 4E-F). The different leaf cell forms and the density of papillae of the thrips susceptible variety Charming Beauty compared to the thrips resistant variety Robinetta are depicted as microscopy images in Fig. 4A-F.

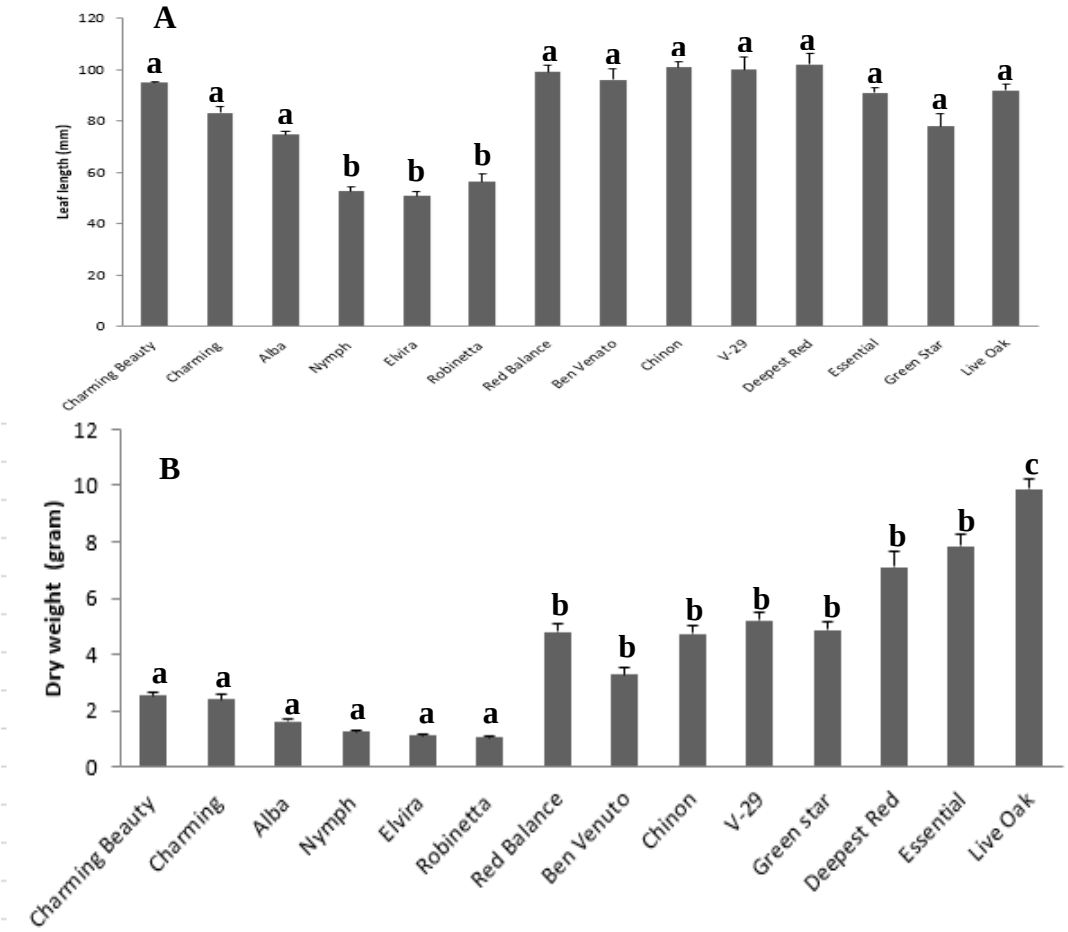


Figure2. Leaf length (A) and dry mass (B) of fourteen Gladiolus varieties. Data represent means and standard errors for three to five replicates. Different letters indicate significant differences between varieties at $p \leq 0.05$.

Table 1. Pearson correlations (*N* =14) between Ln-thrips silver damage (mm^2) and epidermis length (µm), mesophyll length (µm), density of papillae (per 2100 μm^2), leaf length (cm) and dry mass (gram) in Gladiolus varieties (*N* = 14). Data represent means of three to five replicates. Data in bold shows significant level at $p \leq 0.05$.

	Epidermis length (µm)	Mesophyll length (µm)	Density of papillae (per 2100 μm^2)	Leaf length (cm)	Dry mass (gram)
Ln Silver damage	**r = 0.596** **P = 0.024**	**r = 0.603** **P = 0.022**	**r = -0.628** **P = 0.016**	r = 0.320 P = 0.264	r = -0.222 P = 0.445
Epidermis length		**r = 0.931** **P = 0.000**	**r = -0.873** **P = 0.000**	**r = 0.704** **P = 0.005**	r = 0.310 P = 0.281
Mesophyll length			**r = -0.909** **P = 0.000**	**r = 0.777** **P = 0.001**	r = 0.315 P = 0.273
Denstiy of papillae				**r = -0.669** **P = 0.009**	r = -0.389 P = 0.170
Leaf length					r = 0.441 P = 0.114

The Relationship between Thrips Damage and Morphological Characteristics

Silver damage was significantly positive correlated with the lengths of the epidermis (r = 0.596, N = 14, P = 0.024) (Fig. 5A) and mesophyll cells (r = 0.603, N = 14, P = 0.022) (Fig. 5B) while it was significantly negatively correlated with the density of papillae (r = -0.628, N = 14, P = 0.016) (Fig. 5C). Silver damage did not correlate with leaf length (r= 0.320, N = 14, P = 0.264) and not with plant dry mass (r = -0.222, N = 14, P = 0.445).

DISCUSSION

Gladiolus varieties showed a broad range of variation in thrips resistance as demonstrated by the more than 130-fold difference in silver damage between the most resistant and the most susceptible varieties. Such a large variation is not uncommon for ornamentals. Chrysanthemum varieties also exhibited around 100-fold variation in thrips damage (de Jager *et al.*, 1995 and Kos *et al.*, 2014). Gaum *et al.* (1994) found that variation of thrips resistance was 6 times lower in resistant varieties compared to susceptible ones in a study with 25 rose varieties.

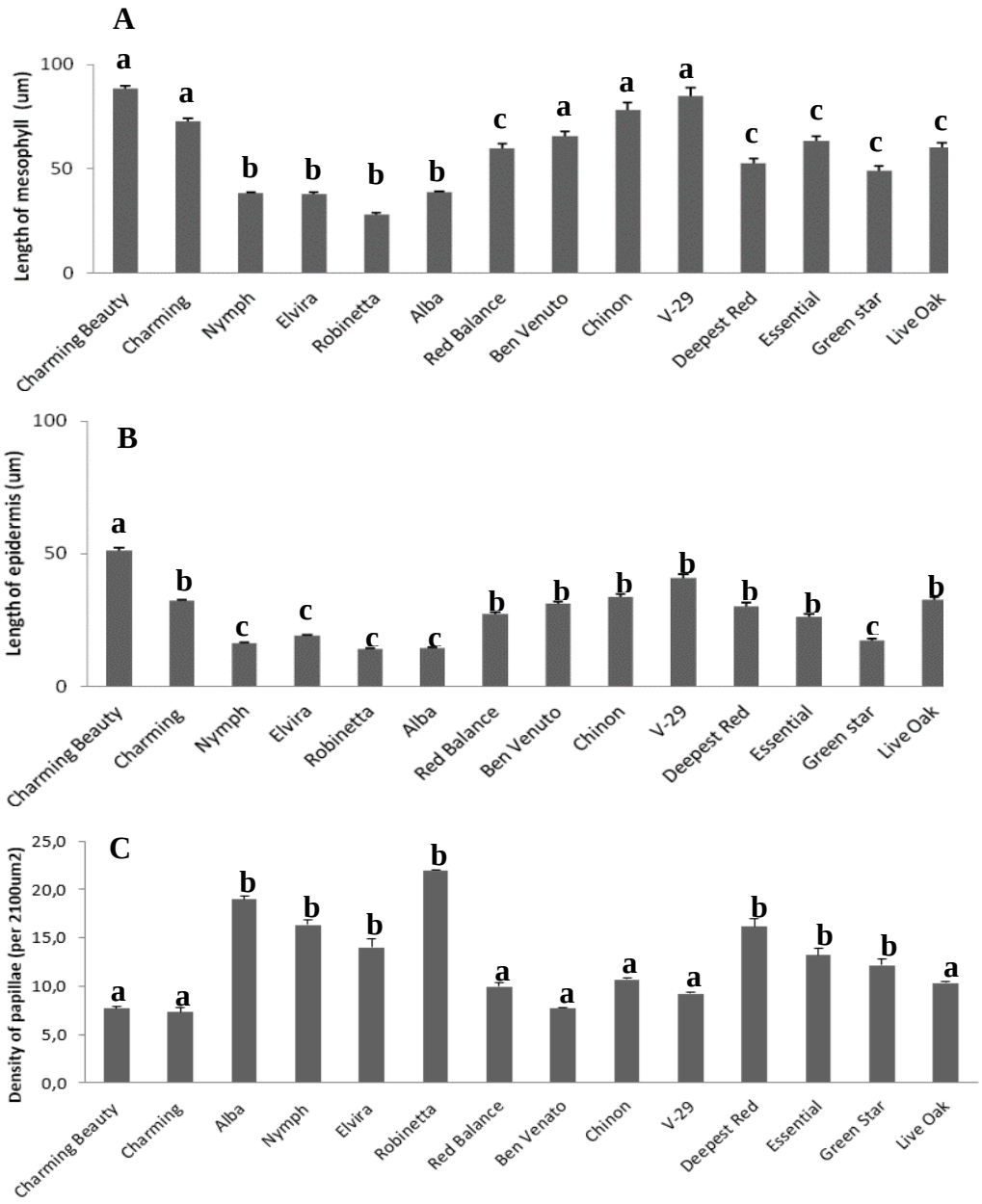
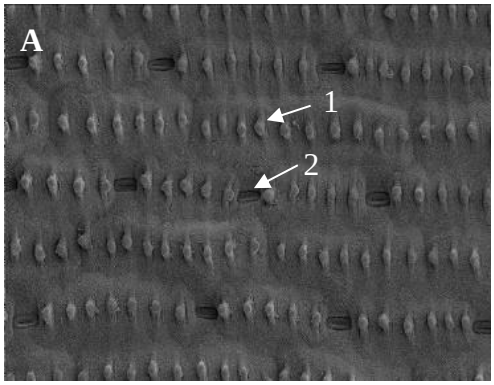


Figure 3. The length of mesophyll (A), epidermis (B) cells and the density of papillae (C) in fourteen Gladiolus varieties. Data represent means and standard errors for three to five replicates. Different letters indicate significant differences between varieties at $p \leq 0.05$.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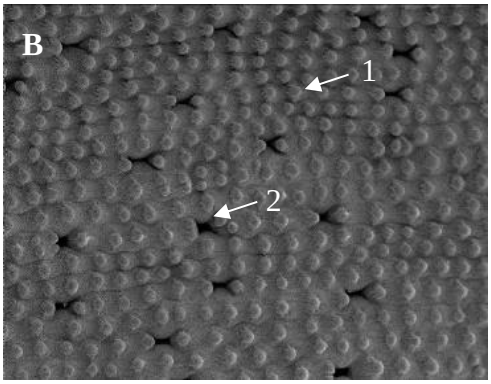
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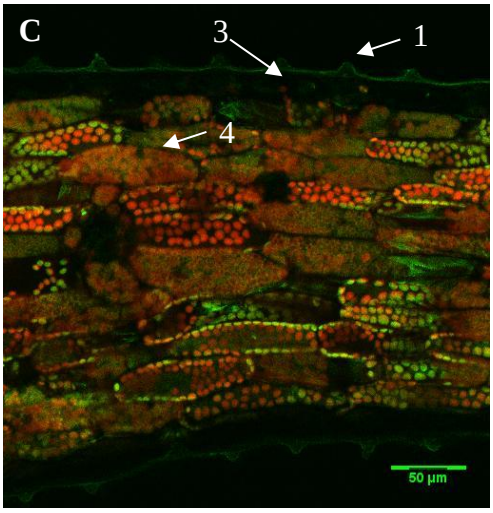
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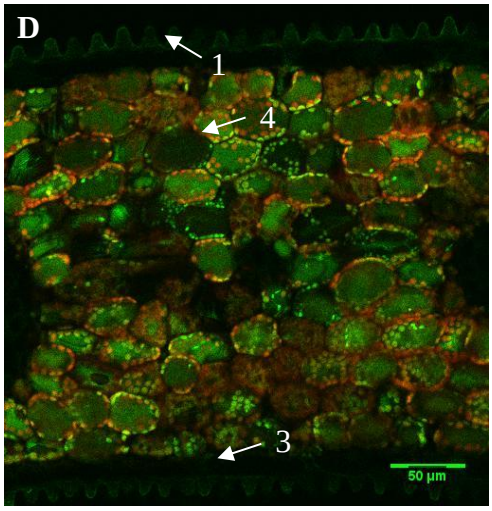
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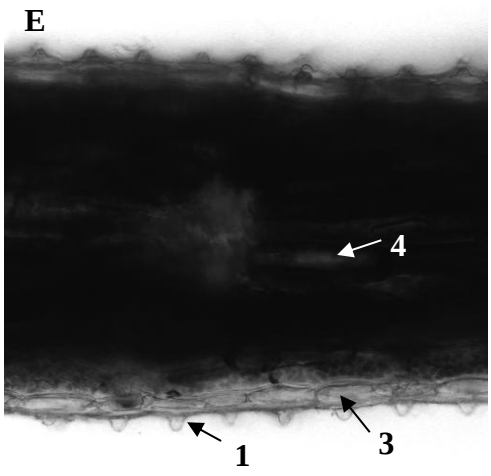
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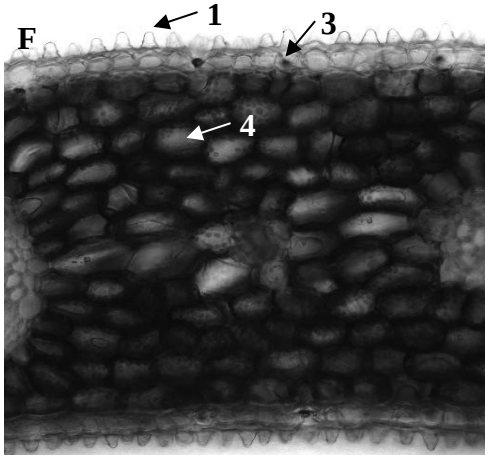
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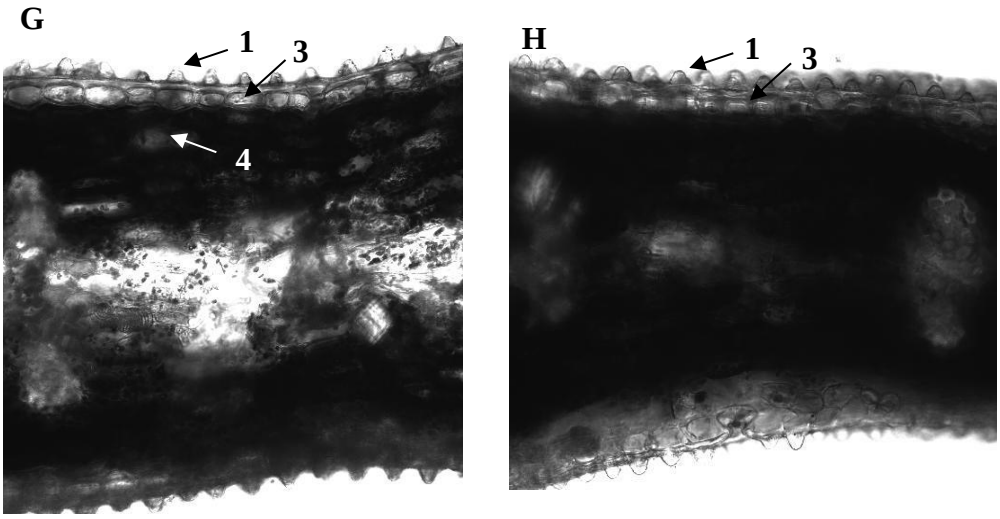

Figure 4. Leaf surface scanning electron photomicrographs of the thrips susceptible Gladiolus variety Charming Beauty (A) and the thrips resistant variety Robinetta (B). Cross leaf sections of Charming Beauty (C) and Robinetta (D) with confocal laser scanning, respectively. Cross leaf sections of Charming Beauty (E), Robinetta (F), Live Oak (G) and Green Star (H) with visual light microscopy, respectively. Arrows indicate papillae (1), stomata (2), epidermis (3) and mesophyll (4).

The density of papillae was negatively correlated to thrips damage while the lengths of the mesophyll and epidermis cells were positively correlated with thrips damage. As a rule, an epidermis cell produced one papilla. Thus, varieties with smaller leaf cells had a higher density of epicuticular papillae. Statistically it is not possible to make a distinction between the effects of cell length and the density of papillae on silver damage. Papillae may inhibit the movement of thrips or hinder penetration of the epidermis by the cell sucking thrips. However, Prüm *et al.* (2013) reported that papillae may slightly enhance adhesity to leaves in Colorado beetle. In line with our study, Scott Brown and Simmonds (2006) who studied effects of leaf morphology on *Heliothrips haemorrhoidalis* reported that this thrips had a preference for leaves with smooth surfaces, while trichomes and leaf surface wax structures inhibited thrips. Trichomes were also implicated to be related to thrips resistance in tomato (Boughton *et al.*, 2005) and chili peppers (Yadwad *et al.*, 2008).

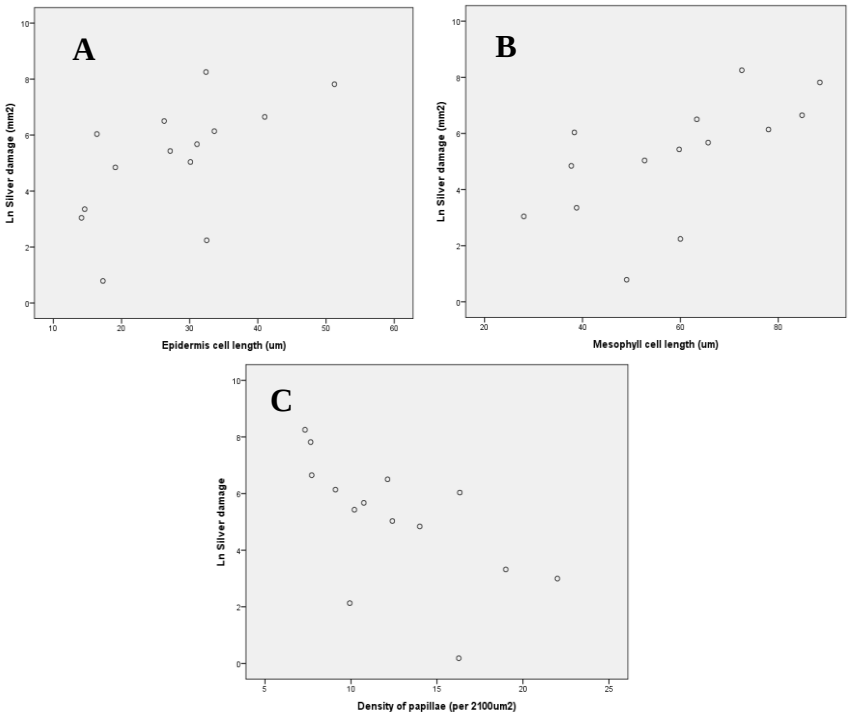

Figure 5. Relationships between Ln-thrips silver damage, measured in a whole plant non-choice bioassay, and cell length of the epidermis (A) (r = 0.596, N = 14, P = 0.024), cell length of the mesophyll (B) (r = 0.603, N = 14, P = 0.022) and density of epidermal papillae (C) (r = -0.628, N = 14, P = 0.016) in fourteen Gladiolus varieties.

Besides forming a physical barrier, papillae may store plant secondary compounds. The epidermal papillae of *Pandanus amaryllifolius* Roxb. are the storage site of the basmati rice aroma compound, 2-acetyl-1-pyrroline (Wakte *et al.*, 2007). Cardinosin A, an aspartic proteinase, suggested to be involved in plant defence against pathogens, is stored in the stigmatic papillae of *Cynara cardunculus* L. (Ramalho-Santos *et al.*, 1997). Similarly, Gladiolus varieties with higher densities of papillae may contain higher amounts of defence compounds. The density of papillae explained 39% of the variation in silver damage. From Fig.1 it becomes clear that the density of papillae sets an upper limit to silver damage. However, other factors may be involved as well as can be seen from the two varieties with low silver damage in relation to their density of the papillae. This will lead to false negatives when this morphological marker would be the

only marker used in breeding programs for thrips resistance in Gladiolus. Next to papillae, chemical traits are likely candidates to be involved in thrips resistance. This needs to be further studied to develop additional markers.

In conclusion: our study revealed that morphological traits play an important role in thrips resistance in the ornamental Gladiolus. Varieties with low thrips damage exhibited high densities of epicuticular papillae. Papillae, in contrast to the correlated cell length, are easily visualized and counted. They, therefore, constitute promising morphological thrips resistance markers facilitating host plant resistance breeding in Gladiolus.

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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.

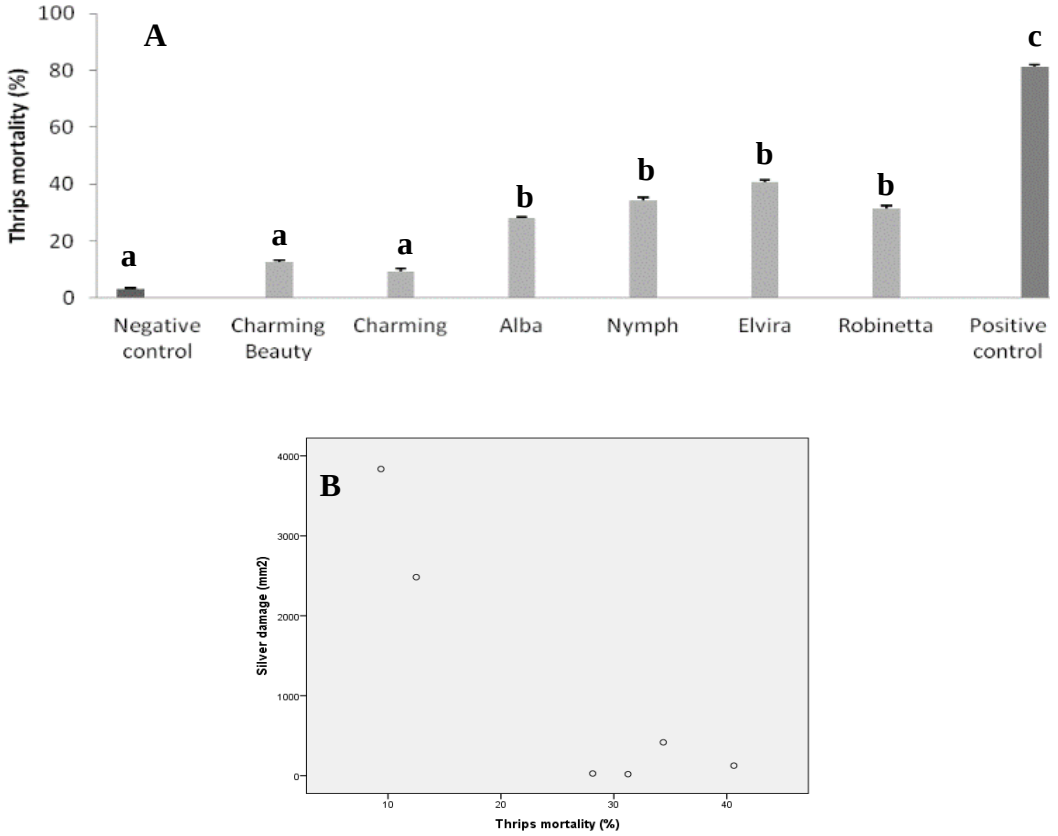

Figure 1. (A) Mortality of thrips feeding on artificial diets (150 µL 2% agarose) with 50µL of leaf extracts of six *Gladiolus* varieties measured in an *in-vitro* bioassay. For each extract 32 thrips were tested. Five percent methanol solution was used as negative and the insecticide abamectin (50 µg/ml) 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.

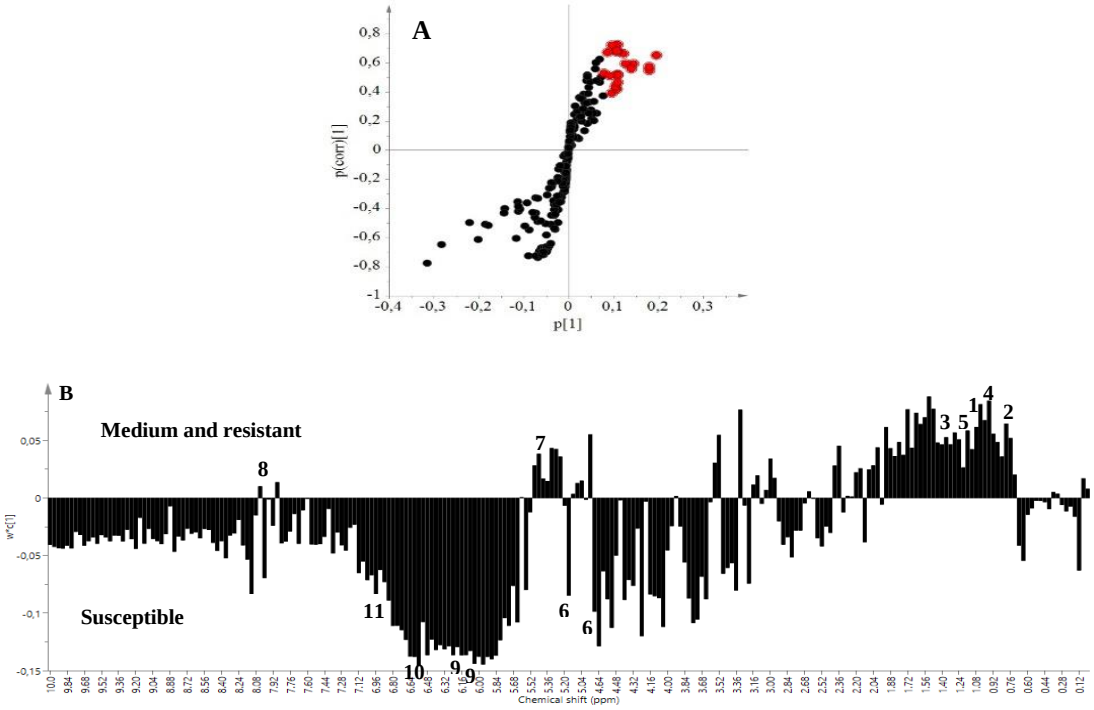

Figure 2. S-plot score (A) and loading plot (B) for gladiolus varieties based on ^{1}H NMR spectra. Metabolites are labeled as (1) signal A, (2) signal B, (3) alanine, (4) valine, (5) threonine, (6) α/β-glucose, (7) sucrose, (8) kaempferol, (9) epicatechin, (10) epigallocatechin and (11) gallic acid.

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	

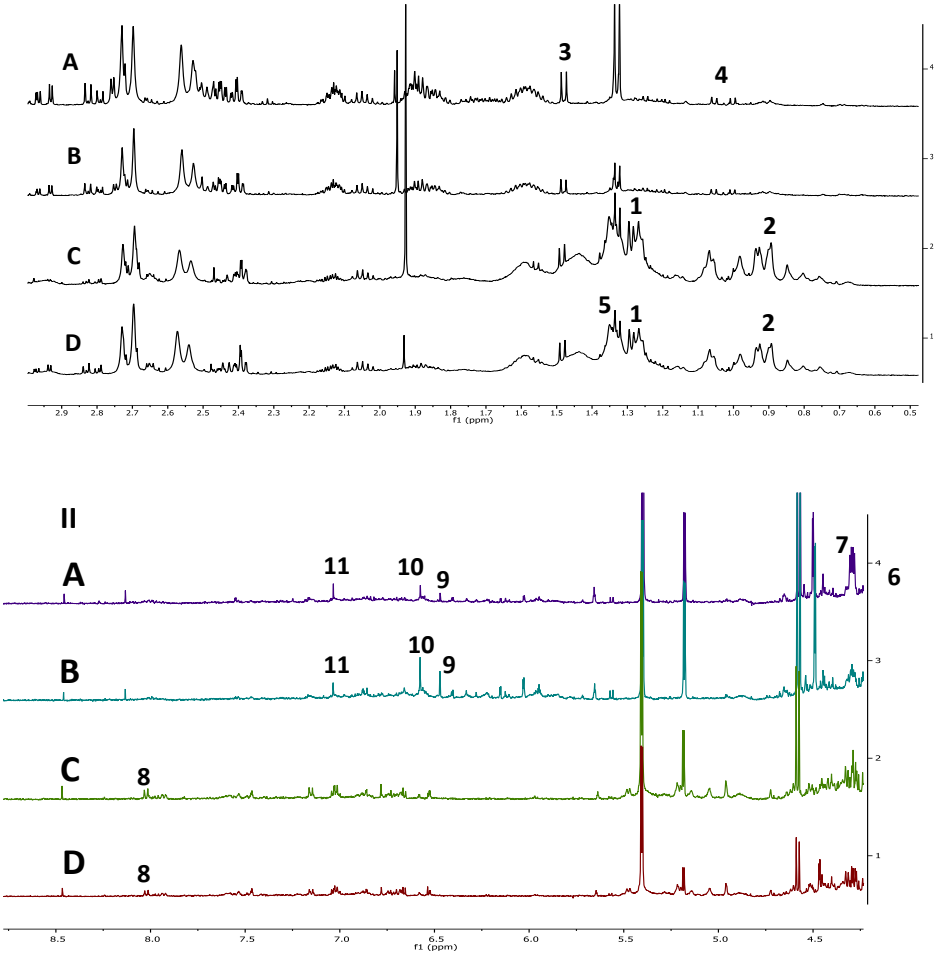

Figure 3. ^{1}H-NMR spectra (CH3OH-d_4-KH_2PO_4 in D_2O extracts of (A) and (B) the susceptible variety Charming Beauty and (C) and (D) the resistant variety Robinetta, in the range of d 0.5 - 3.0 (I) and d 4.5 - 8.5 (II). Assignments: (1) signal A, (2) signal B, (3) alanine, (4) valine, (5) threonine, (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.

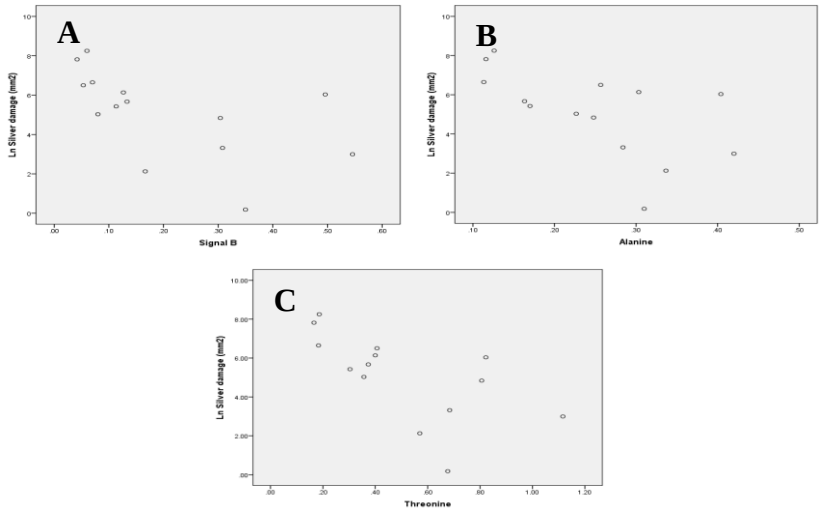

Figure 4. Relationships between the relative concentrations of (A) signal B, (B) alanine and (C) threonine and silver damage in fourteen gladiolus varieties.

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.

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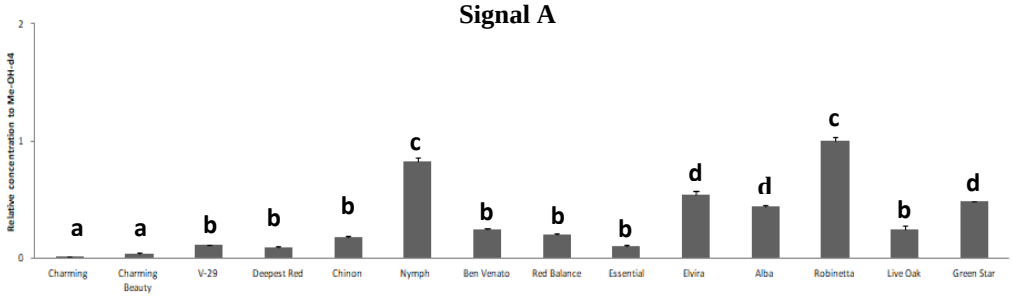
Signal A



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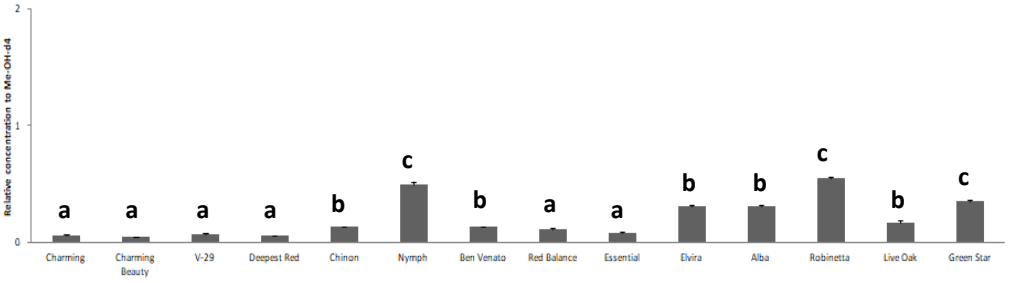

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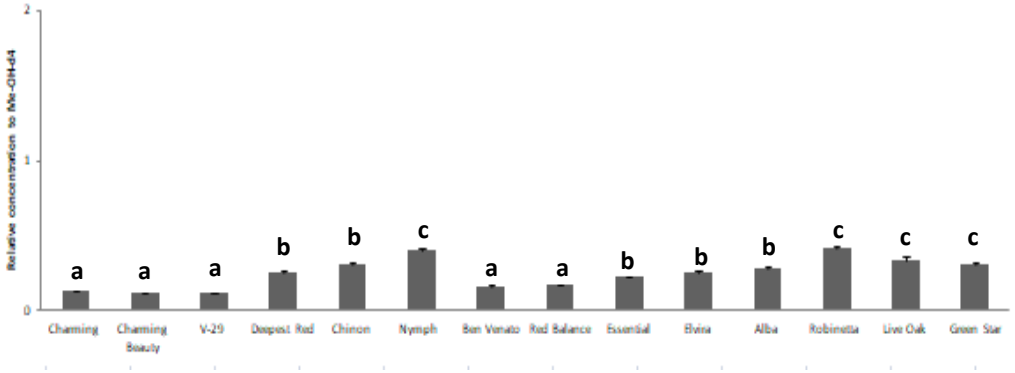

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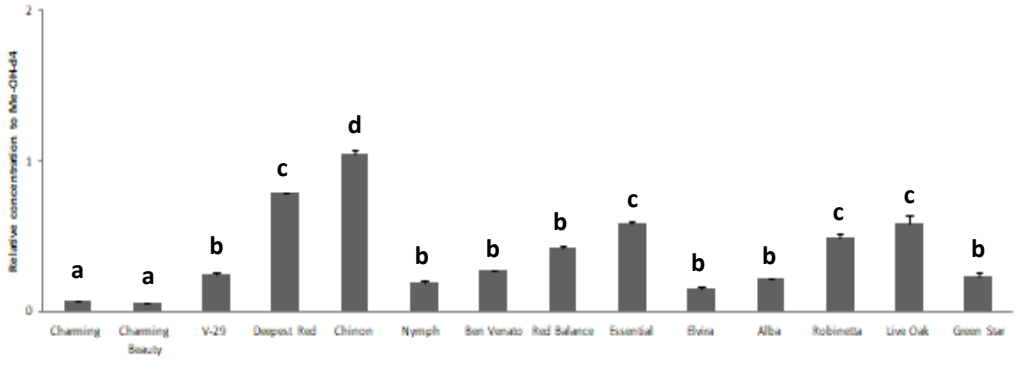

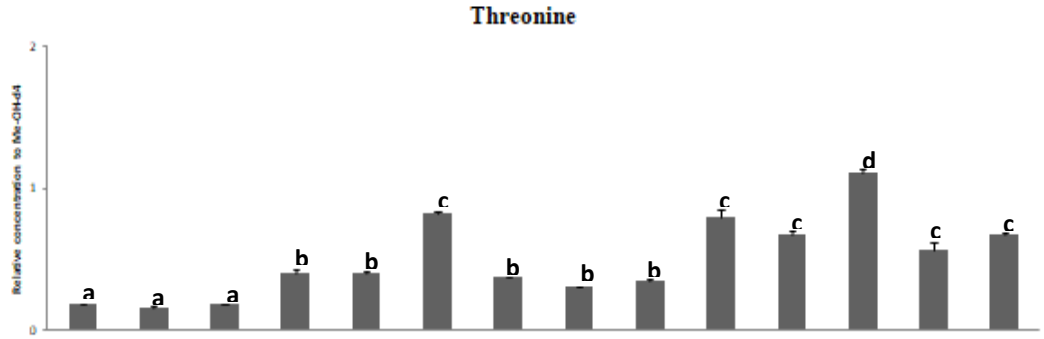
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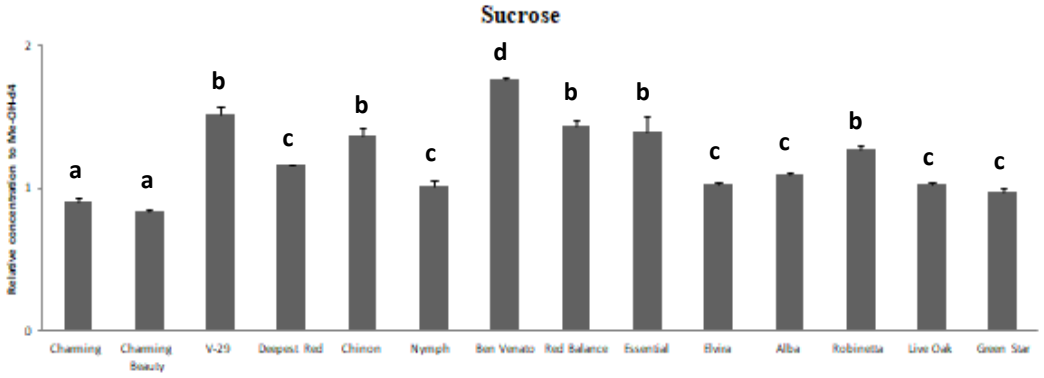
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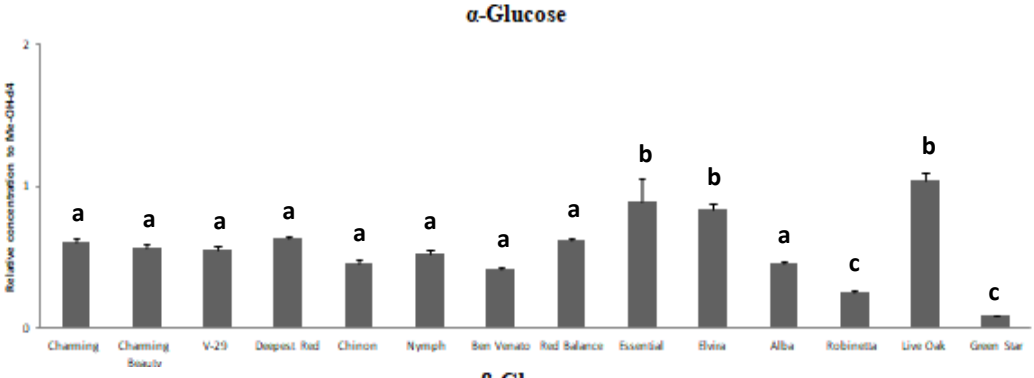
Charming
Charming Beauty
V-29
Deepest Red
Chinon
Nymph
Ben Venato
Red Balance
Essential
Elvira
Alba
Robinetta
Live Oak
Green Star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Alba
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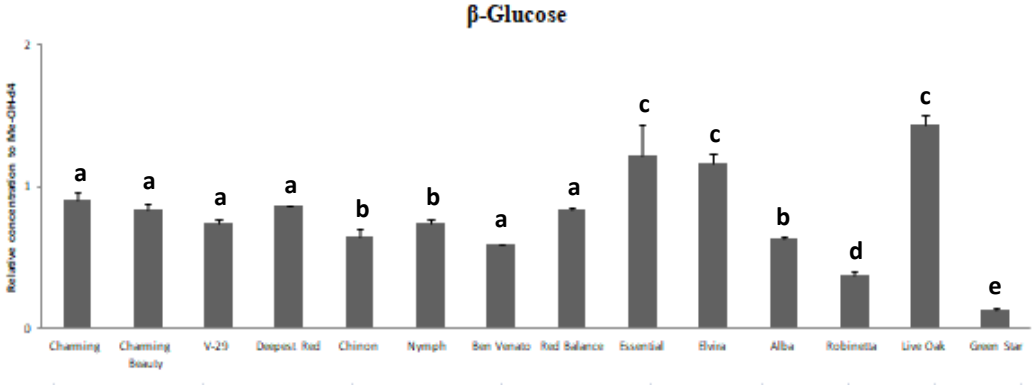
Live Oak
Green Star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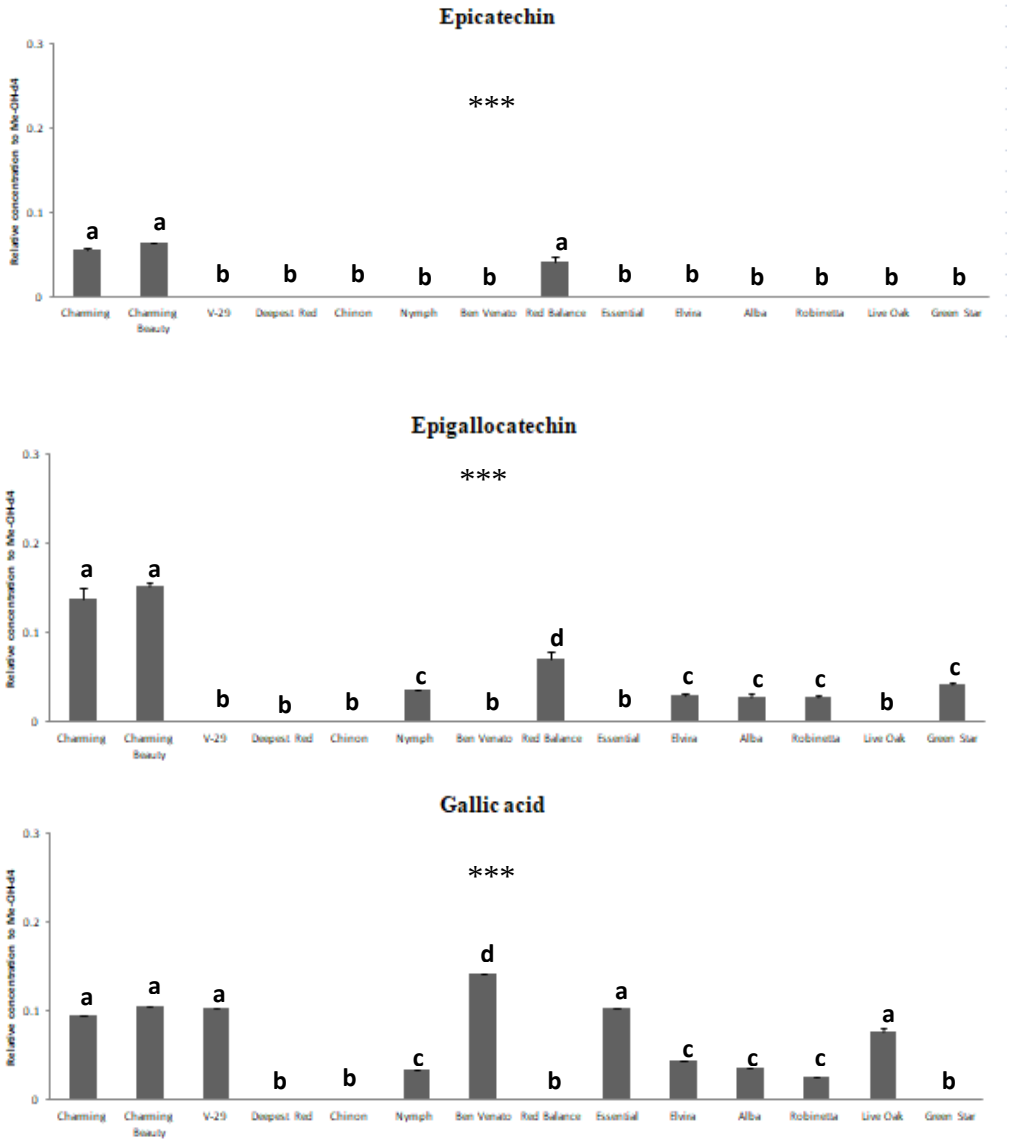

Figure S1. Relative concentration, as proportions of the internal standard, in ^{1}H NMR spectra of alanine, valine, threonine, sucrose, α-glucose and β-glucose in fourteen gladiolus varieties. Data present the mean of three to five replicates of leaves ± SE of the mean. Signal A, signal B, alanine, 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 ($P < 0.001$).

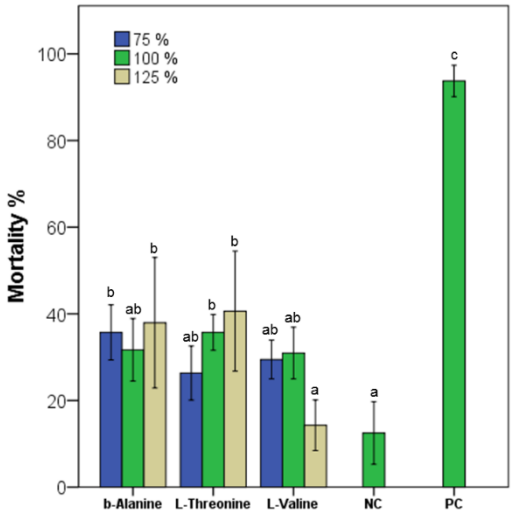

Figure S2. Thrips mortality in artificial diets to which three amino acids were added at three concentrations corresponding to 75%, 100% and 125% of the average plant concentration of 75%, 100% and 125%. Different letters above the bars indicate significant differences between treatment (Leiss *et al.*,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

Resistance against *Frankliniella occidentalis* during different plant life-stages and under different environmental conditions in the ornamental *Gladiolus*

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ABSTRACT

Defence systems of plants change during their phenology. In general plants are best protected during the life-stages that contribute most to fitness. In breeding programmes it is important to be able to phenotype individuals as early as possible. This is especially true if no molecular markers are available as in the case of many ornamental species. In the previous chapters of this thesis I studied chemical defence against western flower thrips (WFT) in Gladiolus. Metabolites that were associated with resistance included triterpenoid saponins and the amino acids alanine and threonine. These compounds occurred at higher concentration in resistant varieties than susceptible ones. In the previous chapters I studied plants in the vegetative stage, in this chapter I want to investigate whether differences in defence against WFT are consistent across developmental stages for plants grown under different conditions. I first conducted a whole plant bioassay with plants in three developmental stages of the varieties Charming beauty and Robinetta as examples of a susceptible and resistant variety, respectively. I analyzed the metabolomic profiles of the leaves, buds and flowers previous to infection and measured silver damage caused by thrips. I then compared the metabolite profiles and the silver damage between the two varieties and among three plant stages and plant organs within each variety. Damage in Charming Beauty was more than 500- fold higher compared to damage in Robinetta at all plant development stages. Relative concentrations of triterpenoid saponins and amino acids that were associated to resistance in the previous chapter were higher in Robinetta at all plant stages. In Charming Beauty leaves showed more damage than buds and flowers. The relative concentrations ofalanine, valine and threonine were higher in buds and flowers than in leaves. Metabolomic profiles of the leaves did not change significantly during plant development. In addition, I grew plants in the climate room, in a bulb field and I transferred plants from the field to the

climate room. Metabolomic differences between the two varieties remained constant across growing conditions. Results showed that the chemical thrips resistance markers that I identified in an earlier chapter based on analysis of vegetative plants grown in climate rooms are reliable over the plant's lifetime and for plants grown under field conditions.

KEYWORDS: Ontogeny; climate and field-grown plants; *Frankliniella occidentalis*; Gladiolus; eco-metabolomics

INTRODUCTION

Plant defences are not fixed throughout a plant's life. Major changes occur depending on growing conditions, plant development and the level of biotic and abiotic stressFor breeders such changes may present a problem when they want to detect robust chemical markers for resistance in their breeding programs.

Plant resistance to herbivores has mostly been studied under controlled conditions in growth cabinets or climate chambers to minimize the effects of external variables on the plant metabolome. Under laboratory conditions photoperiod, light intensity, temperature, and humidity are controlled, whereas in the field those conditions are highly variable. These external variables may thus cause variation in the levels of defense compounds and consequently affect plant resistance to herbivores. For instance the concentration of triterpenoid saponins in plants is affected by habitat, season, age of plants, light, temperature, and water (Szakiel *et al.*, 2011). Amino acid levels were reported to depend on light conditions (Jänkänpää *et al.*, 2012). Also drought affects amino acid contents and through this herbivore feeding performance (Rani and Prasannalaxmi, 2014).

During a plant's lifetime major changes in its defence system occur. This can be the result of aging tissues (Boege and Marquis, 2005; Leiss *et al.*, 2009) or these changes can be associated with developmental switches such as from seedling to vegetative or from vegetative to flowering stages (Barton and Koricheva, 2010). Generally it is assumed that plant parts that most strongly contribute to fitness are defended best (De Jong and Van Der Meijden, 2000). For instance young leaves are in general better protected from generalist herbivores than older leaves (Sun *et al.*, 2014) and buds and flowers are better protected than leaves (Van Dam *et al.*, 2001). The ultimate choice of herbivores will be determined by both the nutritional value of the tissue and the level of defence. While for herbivores, such as thrips, young flowers with pollen can represent a high nutritional value (Damle *et al.*, 2005) they may at the same time be better protected and have accumulated higher defence levels than other plant tissues such as leaves (Damle *et al.*, 2005). The effect of developmental stage or plant age on resistance has been well studied for a number of insect herbivores among which Western flower thrips. The preference pattern for WFT was not fully consistent across species. In a greenhouse study

with *Impatiens wallerana* the rank order of WFT preference was 1. plants with flower buds, 2. plants with fully opened flowers with pollen, 3. plants with fully opened flowers without pollen and 4. plants with foliage without flowers (Ugine *et al.*, 2006). In *Calystegia sepium* WFT numbers increased during bud development and opening and reached a peak just before flowers started to wilt (Kirk, 1985). In both *Impatiens wallerana* and *Calystegia sepium* WFT preferred flowers over leaves (Kirk, 1985; Ugine *et al.*, 2006). In tomato (Mirnezhad *et al.*, 2010) and in Senecio (Leiss *et al.*, 2009) WFT damage was higher in older leaves In tomato this difference became stronger after external application of JA (Chen *et al.*, 2018). Although from an evolutionary point of view it makes sense that tissues that contribute less to fitness are not optimally defended, it presents a problem to growers (de Jager *et al.*, 1993). While high infestation levels on older leaves may not reduce flower or seed production they may lead to unmarketable products or higher levels of virus infections as e.g. in the case of thrips (Kirk and Terry, 2003).

For plant breeders potential changes in the plant's defence system during plant development presents a problem because selection in breeding programs is based on the analyses of early life stages. The question is whether or not predictions of resistance in young plants are good indicators of resistance later in life. Especially, for herbivores that show a clear preference for particular plant organs such as buds, flowers or seeds this question is highly relevant. In this paper we will study defence of Gladiolus against WFT at three developmental stages and under different growing conditions. WFT is one of the most serious pests in agricultural and horticultural crops worldwide (Jensen, 2000) causing losses of millions of euros. WFT is highly polyphagous, invading fruit, vegetables and ornamentals (Buitenhuis and Shipp, 2008). Thrips have piercing-sucking mouthparts which allow them to feed on different types of plant cells (Ullman *et al.*, 1997). After sucking up the cell's content, these fill with air leading to the characteristic silver damage. Moreover, they are the vectors of viral diseases (Kirk and Terry, 2003).

In gladiolus too, thrips infestation presents a severe problem. Differences in thrips resistance for different varieties of Gladiolus have been reported by Terry and Lewis (1997) and are reported in chapters 2 and 3 of this thesis. Plant breeders are in need for morphological or chemical markers to assist breeding programs and to make full use

of the natural variation that is present in gladiolus with respect to thrips resistance. For gladiolus varieties differing in resistance against thrips, under climate room conditions, we detected, in a multivariate analysis of NMR data, signals related to thrips resistance. These were a signal at δ 0.90 ppm linked to triterpenoid saponins and the amino acids alanine and threonine. Subsequent correlation analyses only gave significant relationships with signal of 0.90 ppm, linked to triterpenoid saponins, alanine and threonine. All these signals were highly correlated among each other and with density of papillae (chapter 3). Most likely these defence compounds are produced and/or stored in the extracuticular papillae.

The experiments described in chapters two and three were based on vegetative plants under controlled conditions. The objective of this study was to investigate the effect of environmental conditions and plant developmental stages on plant resistance. We investigated resistance against WFT for plants grown under natural field conditions of a plant breeder, for plants transferred from the field to a climate room and for plants grown during the whole experiment in a climate room. The vegetative life stage comprises about 80% of the total life-cycle of gladiolus. However, success in later developmental stages of the plants is crucial for bulb and flower production. We, therefore, compared metabolomic profiles and WFT infestation for three developmental stages: vegetative stage, generative stage with buds and generative stage with flowers.

For our experiments we used the gladiolus varieties Robinetta and Charming Beauty which in previous chapters were shown to be highly resistant and susceptible in the vegetative stage, respectively (chapters 2 and 3). We specifically addressed the following questions:

- Do Robinetta and Charming Beauty show consistent differences in WFT resistance over all development stages?
- Does WFT damage differ between plant organs?
- Does WFT damage to leaves differ among plant development stages?
- Are differences between the metabolomic profiles of Robinetta and Charming Beauty consistent across developmental stages?
- Do the concentrations of defence compounds related to WFT resistance differ among plant organs?

- Do the concentrations of compounds that were related to WFT resistance alter with the development stages of the plant?
- Are the metabolic profiles of the plants dependent on the growing conditions?
- And if so: Is there a change in the concentration of compounds related to thrips resistance?

MATERIAL AND METHODS

Plant Varieties

Two *Gladiolus nanus* varieties, (Charming Beauty and Robinetta), were obtained from the *Gladiolus* breeder Gebr. P. & M. Hermans (Lisse, The Netherlands).

Plant Developmental Stages

We grew plants outdoors in a field at Lisse to mimic the natural growing conditions. Plants at three development stages, i.e. vegetative, generative with buds and generative with flowers were collected from the field by carefully digging out the plants with their root system. Consequently, they were then potted and placed in a climate room (L:D, 18:6, 20^0C) for 7 days of further growth before they were infested by thrips. Robinetta was planted in the field 25 days earlier as Charming beauty on May 2013. Because we harvested all the plants in a particular stage at the same day Robinetta plants had been in the field for a longer time period. Vegetative plants of Charming Beauty and Robinetta were thus collected after 65- and 90-days growth in the field, respectively. Plants with buds that just started to develop were collected after 75 days and 100 days in the field, respectively and plants with fully developed buds that started to open flowers were collected after 85 and 110 days, respectively. After collecting, plants were transferred to a climate chamber.

Different Growing Conditions

Vegetative plants were grown under three different conditions: field, field transition and climate chamber. Plants grown in the climate room during the whole experiment were the same as the ones from chapter 3. These plants were planted as bulbs to 9 x 9 cm pots filled with a 1 : 1 mixture of potting soil and dune sand. They were randomly placed in a climate chamber (L:D, 18:6, 20^0C, 70% relative humidity and 90-120 µmol photons m^{-2} s^{-1}) and grown for 70 days. Plants grown in the field were the same as the ones from the development study of this chapter. Field-grown plants were planted

and grown for 65 days (Charming Beauty) and 90 days (Robinetta). Part of these were carefully dug out from the field and transferred immediately into a climate room for 7 days. Plants from all conditions were harvested at the vegetative stage. Four to six replicates of all three conditions were used for the NMR metabolomics study.

Whole Plant Bioassay

For each of the two varieties, three to four plants per developmental stage were tested in a non-choice whole plant bioassay. Each plant was placed individually in a WFT proof cage, consisting of a plastic cylinder (80 cm height, 20 cm diameter), closed with a displaceable ring of WFT proof gauze (Chapter 3). The cages were arranged in a fully randomized design. Two adult males and 18 adult females of western flower WFT were released in each cage and left for 10 days. Thereafter, silver damage, expressed as the leaf area damaged in mm^2, was visually scored for each plant. Silver damage in the buds and flowers in flowering plants were counted in mm^2.

We calculated total damage per plant as the sum of the silver damage in all plant organs present in a certain stage. Because WFT damage in Robinetta was zero in many samples we could not use a two-way ANOVA to test for the effects of variety and developmental stage on silver damage. Instead we tested for the effects of developmental stage for each variety separately. We used the Kruskal-Wallis test to do so for Robinetta and we used one-way ANOVA for Charming Beauty. Differences in total damage between the two varieties were analyzed by using a Mann-Whitney U test.

Metabolic Profiling

Extraction of Plant Materials for NMR Metabolomics The dried plant material was used to test for differences among leaves of the three developmental stages and for differences among buds and flowers in flowering plants for the two varieties using the standard protocol of sample preparation and ^{1}H-NMR profiling described by Kim et al. (2010).

Samples of 30 mg freeze-dried plant material were weighed into a 2 ml microtube and extracted with 1.5ml 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 were 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 following parameters: 0.16 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 (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 and 8 k for F_2 using spectral widths of 5000 Hz in F_2 (chemical shift axis) and 66 Hz in F_1 (spin-spin coupling constant axis). Both dimensions were multiplied by sine-bell functions (SSB = 0) prior to double complex Fourier transformation. J-resolved spectra were tilted by 45^{o}, 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 sec relaxation delay and 6361 Hz spectral width in both dimensions. The window function for the COSY spectra was Qsine (SSB = 0).

Data Processing

Spectral intensities were scaled to total intensity and reduced to integrated equal width (0.04 ppm) for 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). Plant development stages data were further analyzed with principal component analysis (PCA) performed with SIMCA-P software (version 15.0 Umetrics, Umea, Sweden). Pareto scaling was used for PCA analysis. With the PCA we tested for differences in metabolomics profiles between the two varieties. Besides, different

environmental conditions data were further analyzed with partial least square-discriminant analysis (PLS-DA) which used unit variance scaling.

The peak area of triterpenoid saponins at δ 1.28 and 0.92 ppm (signals that were related to resistance in chapter 3) were close to zero in all plant development stages in Charming Beauty we, therefore, analyzed differences in these signals with the Kruskal-Wallis test. The relative concentrations of threonine, valine, alanine, sucrose, α-glucose and β-glucose were ln-transformed to fit a normal distribution. For leaves, differences between the two varieties in the peak areas of triterpenoid saponins were analyzed with a Kruskal-Wallis test, while differences between the two varieties in other metabolites were analyzed with one-way ANOVA. Differences in relative concentrations of triterpenoid saponins between plant organs were analyzed with a Kruskal-Wallis test while differences in other metabolites were analyzed with one-way ANOVA within variety. Differences in the relative concentrations of compounds between leaves at different developmental stages were analyzed with one-way ANOVA within variety. Data were subsequently analyzed with the Scheffe post-hoc test. Differences in metabolite concentrations between plants grown under different conditions, were analyzed separately using one-way ANOVA. Data was log-transformed to fit a normal distribution. Triterpenoid saponins, threonine and kaempferol were analyzed by Kruskal-Wallis tests.

RESULTS

Differences in total WFT damage between the two varieties. Total WFT damage differed significantly between Charming Beauty and Robinetta ($U = 55.000$, $df = 1$, $P = 0.000$) (Fig. 1). Hardly any damage occurred in Robinetta at all developmental stages. The average total damage across all three developmental stages was: 565.22 ± 77.4 mm^2 in Charming Beauty and 3.3 ± 1.9 mm^2 in Robinetta.

WFT Damage in Different Plant Organs. In Charming Beauty damage to buds accounted for 35% of the total damage in the bud stage. Damage to flowers accounted for 16% from the total damage in this stage, while no damage to buds occurred in this stage. In Robinetta damage to all plant organs was low and in buds and flowers it was even zero (Fig. 1).

WFT Damage on Leaves at Different Plant-Stages. WFT damage on leaves differed significantly among the three plant development stages in Charming Beauty (F

= 16.593, df =2, *P* = 0.023) (Fig. 1). Damage in the vegetative stage was two times higher than in the generative stage with buds or flowers. In Robinetta WFT damage at all three developmental stages was close to zero and did not differ significantly developmental stages (H = 2.333, df = 2, P = 0.311) (Fig. 1).

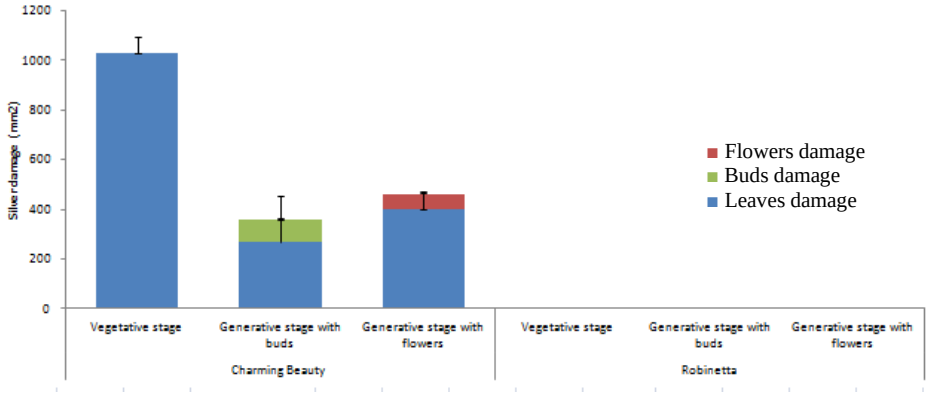

Figure 1. Plant silver damage (mm^2) in Charming Beauty and Robinetta at three plant development stages: vegetative, generative with buds and generative with flowers as measured by a whole plant western flower thrips non-choice bioassay. Bars represent total plant damage, colours within bars represent different plant organs. Differences in total plant damage within the three developmental stages were tested with one-way ANOVA (Charming Beauty) and Kruskal-Wallis (Robinetta). Data represent mean and standard errors for three to four replicates. Different letters above the bars refer to significant differences within development stages at the 0.05 level. *** Indicate significant differences between the varieties (P < 0.000).

Differences in Metabolite Profiles in Leaves Between the Two Varieties

PCA is an unsupervised method which enables to identify the differences or similarities among samples. Charming Beauty and Robinetta differed in their leaf metabolomic profiles at all plant stages although the differences in flowers were relatively small (Fig. 2A). The separation was mainly due to PC1 which explained 41% of the variation in leaf metabolites. The loading plot showed that the signals in the region between δ 1.92-0.80 ppm had a low score and thus were associated with Robinetta the WFT resistant variety (Fig. 2B). The signals at δ 1.28 (signal A) and 0.90 ppm (signal B) were related to triterpenoid saponins (see also chapter 3). In this region we could further

identify signals related to the amino acids valine (δ 1.06) alanine (δ 1.48), and threonine (δ 1.32). The relative concentrations of the triterpenoid saponins that were related to signal A and signal B were significantly higher in Robinetta (H = 16.323, df = 1, P = 0.000 and H = 14.449, df = 1, P = 0.000, respectively) than in Charming Beauty (Fig. 3). The relative concentrations of alanine, valine and threonine were about three to four times higher in Robinetta than in Charming Beauty (F = 73.702, df =1, *P* = 0.000; F = 334.108, df =1, *P* = 0.000; F = 584.607, df =1, *P* = 0.000, respectively) (Fig. 3).

Signals with a high score on the loading plot, that thus were associated with Charming Beauty, were in the sugar region δ 5.0-3.0 ppm. However, the relative concentrations of the sugars we could identify, sucrose (δ 5.40), α-glucose (δ 5.20) and β-glucose (δ 4.60) did not differ significantly between Charming Beauty and Robinetta (F = 1.284, df =1, P = 0.272; F = 0.351, df =1, P = 0.561 and F = 0.219, df =1, P = 0.645, respectively) (Fig. 4).

Differences Between Metabolomics Profiles of Plant Organs

The PCA analysis of the metabolomic profiles of the three plant organs showed clear differences for Charming Beauty (Fig.5A). PC1, which explained 42% of the variation, separated the flowers from the leaves and buds. The loading plot for PC1 showed that the region between δ 5.40-3.00 ppm which represents sugar compounds was responsible for this separation (Fig. 5B). In Robinetta too plant organs were separated by their metabolomics profiles in the PCA (Fig. 6A). The separation was mainly due to PC1 which explained 57% of the variation in plant metabolites. Signals with low value on the loading plot, and thus associated with buds and leaves belonged to the region δ 2.5-0.80 ppm. These signals were related to amino acids and saponins. Other signals with a negative value on the loading plot in the region δ 4.20-3.20 ppm, which we identified as being from sucrose, were associated with buds and leaves. Signals with positive values on the loading plot and thus associated with flowers, in the range from δ 4.00-3.28 ppm (Fig. 6B) were identified as glucose.

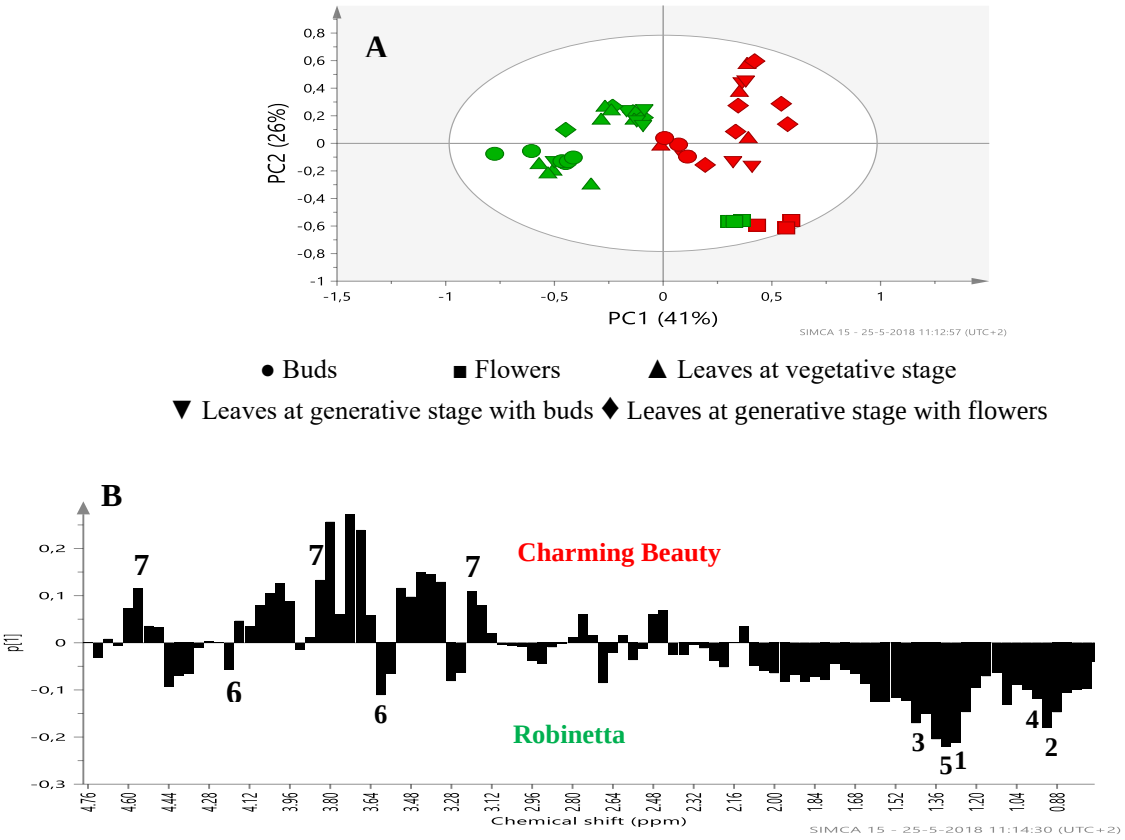

Figure 2. PCA score plot (A) and loading plot (B) for two varieties, Robinetta (green) and Charming Beauty (red) based on ^{1}H NMR spectra. (●) buds, (■) flowers (▲) leaves at vegetative stage, (▼) leaves at generative stage with buds (♦) leaves at generative stage with flowers. Metabolites are labeled as triterpenoids saponins (1 and 2), alanine (3), valine (4), threonine (5), sucrose (6) and glucose (7).

The relative concentration of signal A did not show significant differences among plant organs in Charming Beauty (H = 2.333, df = 2, P = 0.311). The relative concentration of signal B was slightly higher in buds compared to flowers and leaves (H = 6.706, df = 2, P = 0.035). Threonine, alanine and valine were two times higher in buds and flowers in Charming Beauty than in leaves (F = 5.335, df =2, P = 0.039; F = 29.535, df =2, P = 0.000; F = 16.347, df =2, P = 0.002, respectively) (Fig. 7). The concentrations of α- and β-glucose were about two times higher in flowers than in leaves and buds (F = 31.846, df =2, P = 0.000 and F = 27.131, df =2, P = 0.001, respectively) (Fig. 7). However,

the relative concentration of sucrose (δ 5.40 ppm) was lower in flowers than in leaves and buds (F = 5.502, df =2, P = 0.020) (Fig. 8).

In Robinetta signals A and signal B were about 50% higher in leaves and buds than in flowers (F = 63.507, df =2, P = 0.000 and F = 14.969, df =2, P = 0.005, respectively). Threonine was higher in leaves and buds than flowers (F = 61.767, df =2, P = 0.000). Alanine was similar in concentration in all plant organs (F = 3.056, df =2, P = 0.122). Valine concentration was about 50% higher in leaves and buds (F = 7.368, df =2, P = 0.004) than in flowers (Fig. 7). Relative concentrations of α- and β-glucose were about two times higher in flowers than in leaves and buds (F = 5.543, df =2, P = 0.043 and F = 404.909, df =2, P = 0.000, respectively) (Fig. 7). In contrast, sucrose was lower in flowers than in leaves and buds (F = 10.648, df =2, P = 0.011) (Fig. 8).

Differences Between Metabolomics Profiles of Leaves at Different Developmental Stages

The PCA analysis of metabolomic profiles did not separate the leaves of the three developmental stages in both Charming Beauty and Robinetta (Figs. S1A, 1B). In addition, the relative concentrations of the two triterpenoid saponins (signal A and signal B), the amino acids and the sugars did not differ among the leaves from different plant developmental stages (Fig. 3, 4).

Metabolic Profiling of Plants Grown Under Different Conditions

Visual inspection of the NMR-metabolomic profiles of plants grown under different conditions (field, climate room and transferred to climate room from field) clearly showed differences between varieties and among growing conditions (Fig. 9). To further analyze these results multivariate data analysis was applied. First principal component analysis (PCA), was used. However, there was no clear clustering of the different samples within each variety. Apparently, the variability of the samples was too high to give a clear separation. Using the three growing conditions we then applied PLS-DA, for each variety. The climate room grown samples clearly separated from the other two groups of field grown plants and plants transferred from the field to the climate room. The latter two overlapped in the PLS-DA scoring plots of both Charming Beauty (Fig. 10A) and in Robinetta (Fig. 10B). The first component explained 80% and 81% of the

variance in the dataset in Charming Beauty and in Robinetta, respectively. The climate chamber-grown plants were clustered at the negative side of PC1.

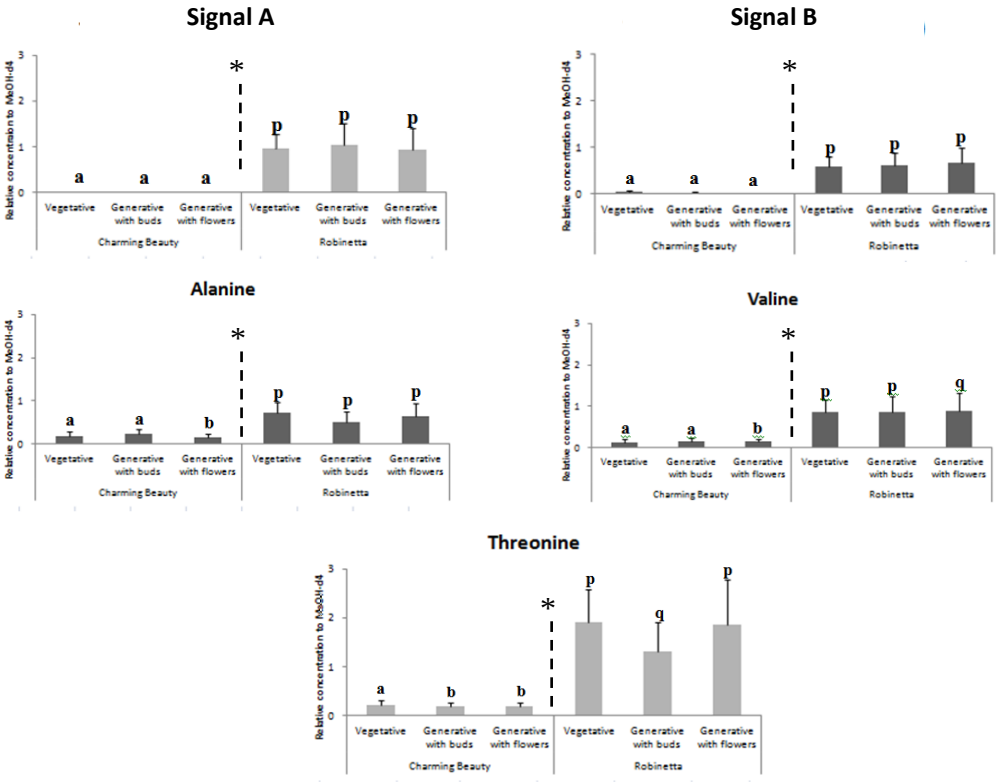

Figure 3. Relative concentration, as proportion of the internal standard, in ^{1}H NMR spectra of triterpenoid saponins (signal A and signal B), threonine, valine and alanine in leaves of three plant development stages of Charming Beauty and Robinetta. Data present the mean± SE of four to six for replicates of leaves at the vegetative, generative with buds and generative with flower stages. Differences in relative concentrations of triterpenoid saponins and amino acids within variety and between the two varieties were analyzed by a Kruskal-Wallis test and a one-way ANOVA, respectively. Different letters refer to significant differences among development stages within varieties at the 0.05 level. *** indicate significant differences between varieties ($P < 0.000$).

The important question one may ask is if there is a consistent difference between the two varieties independent of the growing conditions. All compounds that were associated with resistance in the previous chapter were higher in Robinetta, the resistant variety, for all three growing conditions (Fig. 11). Between growing conditions there were

some metabolomic differences, with a trend for triterpenoids to be lower under climate room conditions. A similar trend seemed to be present for the amino acids alanine, valine and threonine and sucrose (Fig. 11). In contrast the concentrations of kaempferol were significantly higher when plants were grown in the climate room.

All compounds that were associated with susceptibility, although they were not confirmed in single correlation analyses in the previous chapter, were higher in Charming Beauty, the susceptible variety, for all three growing conditions (Fig. 12). These compounds were I affected by the growing conditions in a different manner. Concentrations of α-glucose and β-glucose were lower in the climate room whereas concentrations of gallic acid and epigallocatechin were higher and the concentration of epicatechin was not affected by growing conditions.

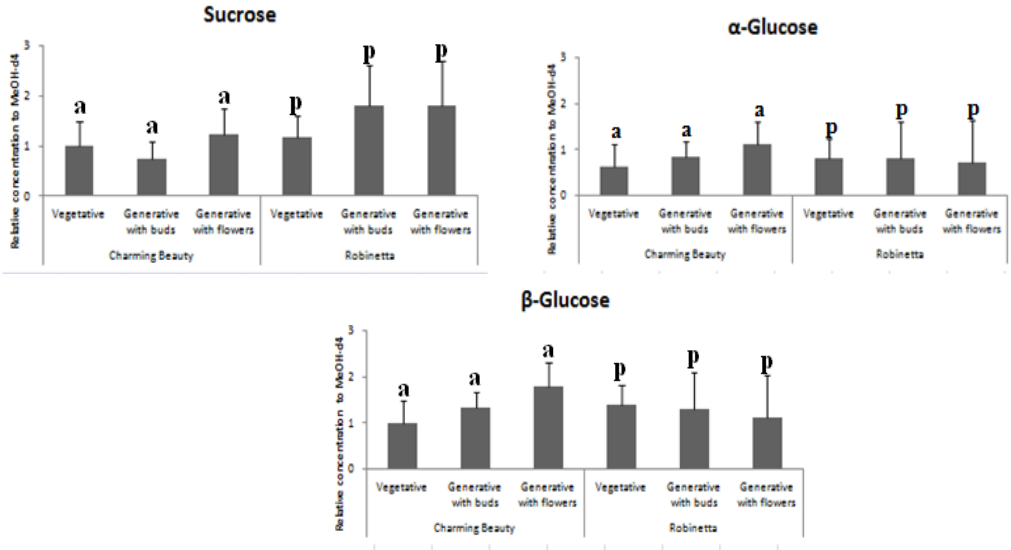

Figure 4. Relative concentration, as proportions of the internal standard, in ^{1}H NMR spectra of sucrose, α-glucose and β-glucose in leaves of the plant development stages of Charming Beauty and Robinetta, respectively. Data present the mean± SE of four to six for replicates of leaves, buds and flowers. Differences in the relative concentrations of sucrose, α-glucose and β-glucose between the two varieties and within variety were analyzed by one-way ANOVA. Letters refer to significant differences among development stages within variety at the 0.05 level. Differences between varieties were not significant at all plant stages and between varieties.

Other metabolites that changed due to different growth location were luteolin and apigenin as well as the organic acids formic acid, malic acid (Fig. 13). Luteolin and apigenin were significantly higher in field grown plants (Fig. 13) while formic acid and malic acid (Fig. 13) were higher in the climate chamber.

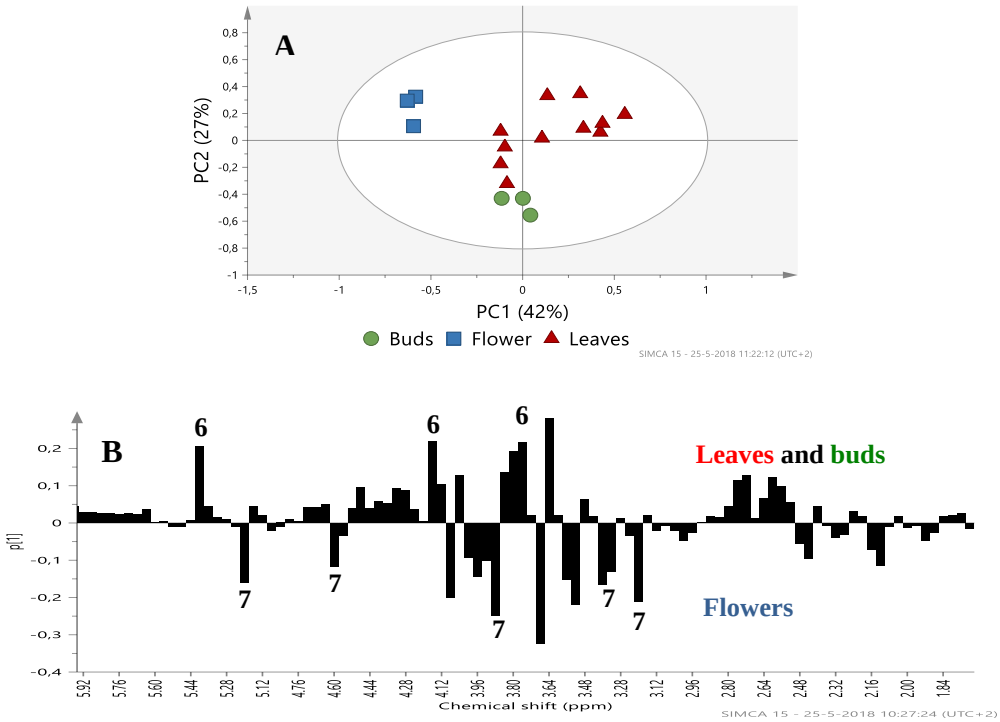

Figure 5. PCA score plot (A) and loading plot PC1 (B) for Charming Beauty based on ^{1}H NMR spectra. (▲) Leaves, (●) buds (■) flowers from plants at the two generative stages. Metabolites are labeled as sucrose (6) and glucose (7).

DISCUSSION

Robinetta and Charming Beauty showed consistent differences in WFT resistance over all development stages. Robinetta as the resistant variety exhibited more than 500- fold less silver damage at all plant development stages compared to Charming Beauty. Metabolomic profiles differed between the two varieties throughout all three plant stages. They revealed triterpenoid saponins and amino acids as metabolites associated with the resistant variety, as in the previous chapter. Those compounds were

consistently higher in Robinetta overall plant stages. Threonine was 10 times higher and triterpenoid saponins, valine and alanine were about five times higher in Robinetta. With the exception of valine all these compounds were also found to be negatively correlated with thrips resistance in chapter 3 where we studied thrips resistance in a series of 14 cultivars.

In Charming Beauty leaves were more damaged than buds and flowers: 50% of all damage occurred to the leaves. Metabolomic profiles differed among plant organs. Triterpenoid saponins were slightly higher in buds and amino acids were two to three times higher in buds and flowers compared to leaves. Patterns in metabolites related to resistance were, therefore, in line with patterns in silver damage. However, leaves represent a relatively larger area compared to buds and flowers so that differences in

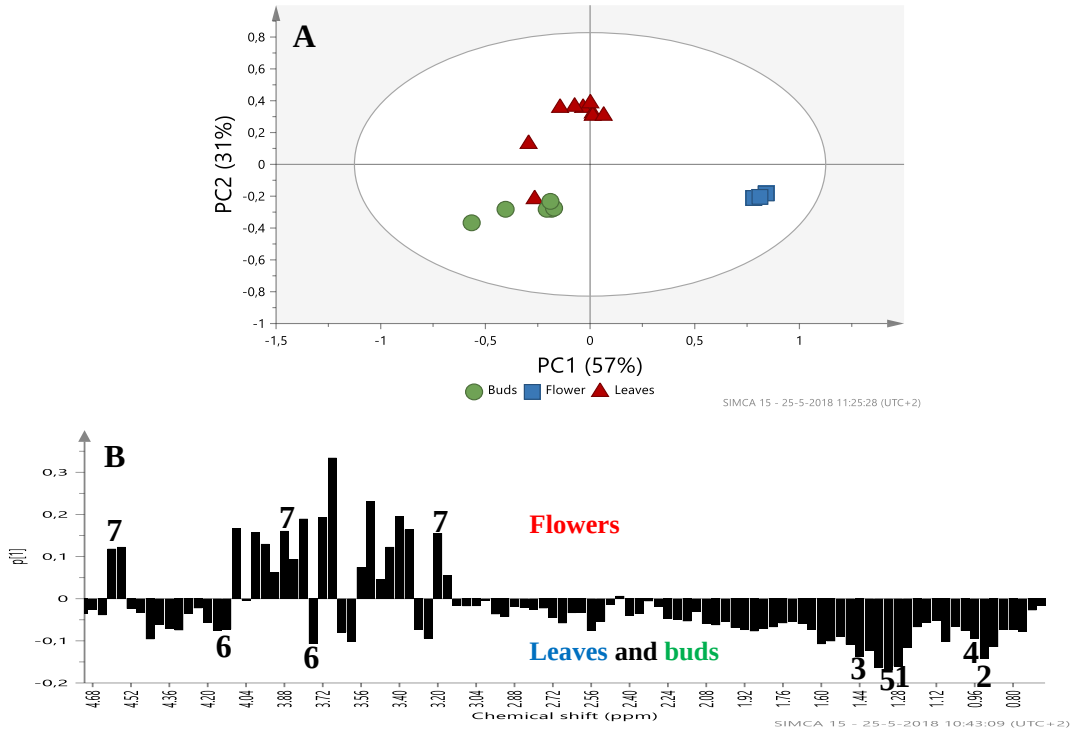

Figure 6. PCA score plot (A) and loading plot (B) for Robinetta based on ^{1}H NMR spectra. (▲) Leaves, (●) buds and (■) flowers from plants at the two generative stages. Metabolites are labeled as signal A (1), signal B (2), alanine (3), valine (4), threonine (5), sucrose (6) and glucose (7).

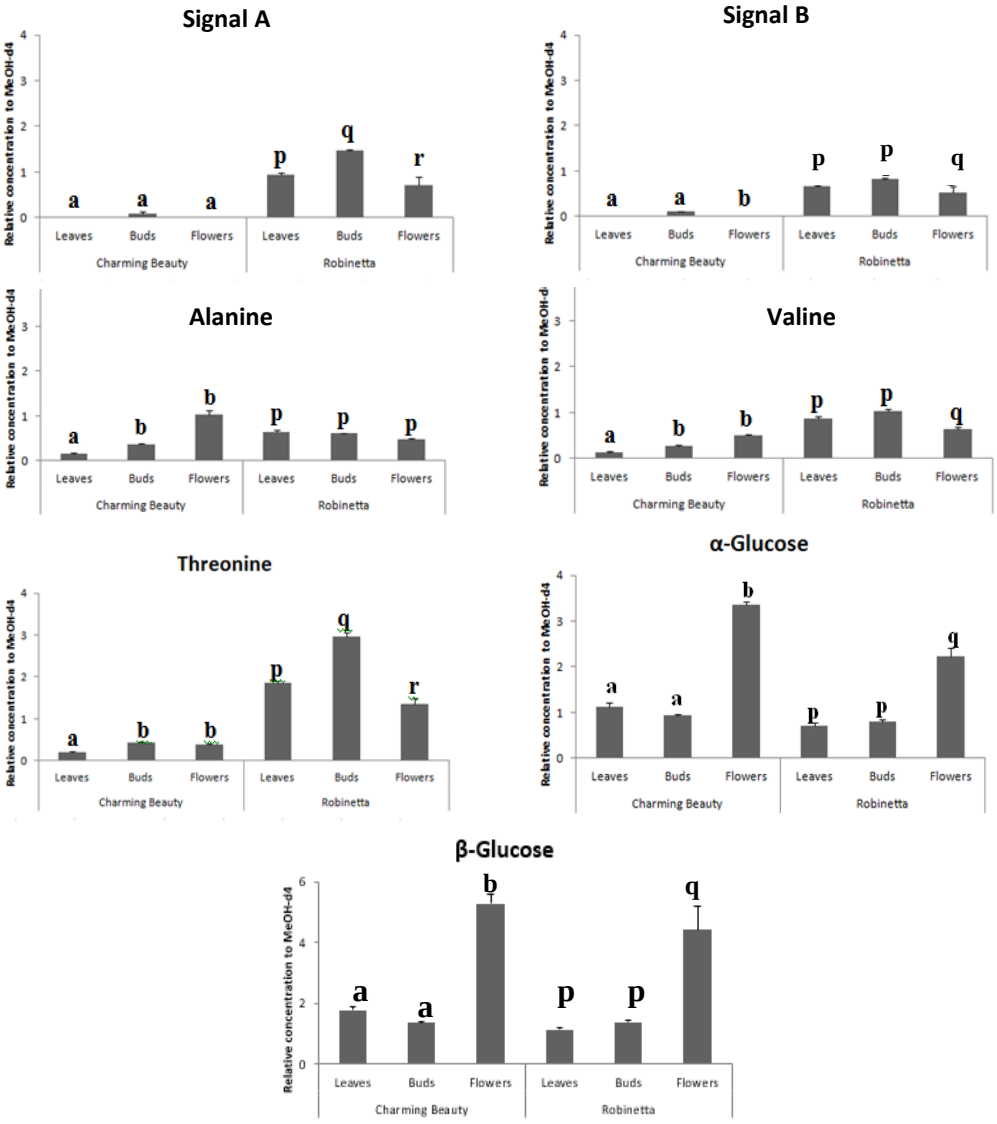

Figure 7. Relative concentrations, as proportions of the internal standard, in ^{1}H NMR spectra of triterpenoids, threonine, valine, alanine, α-glucose and β-glucose in different plant organs of Charming Beauty and Robinetta. Data present the mean of four to six for replicates of leaves, buds and flowers for Charming Beauty and Robinetta ± SE of the mean, respectively. Relative concentration of metabolites in leaves is the average of the two generative stages. Differences in relative concentration of triterpenoid saponins and amino acids within variety and between the two varieties were analyzed by Kruskal-Wallis test and one-way ANOVA, respectively. Different letters refer to significant

differences among plant organs within varieties at the 0.05 level. Differences between varieties were not significant at the 0.05 level.

damage between organs may not solely be attributed to variation in metabolites. Although the silver damage on leaves was higher in the vegetative stage than in the two generative stages, we did not observe significant differences in leaf metabolites related to resistance (or to susceptibility) between leaves of different developmental stages. While in Robinetta damage was always much lower than in Charming Beauty, the concentrations of all compounds we identified in the previous chapter as being related to thrips resistance, were much higher. In Robinetta, the relative concentrations of the triterpenoid saponins (signals A and B) and threonine, and valine were much higher in leaves and buds than in flowers.

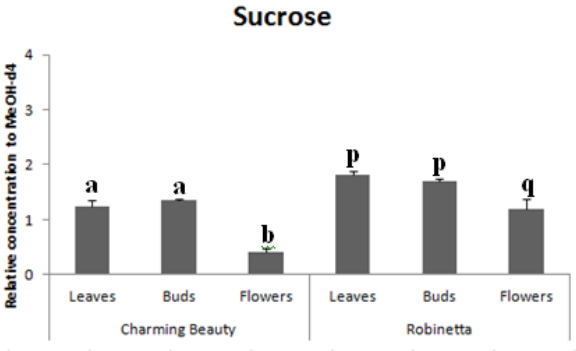

Figure 8. Relative concentration, as proportion of the internal standard, in ^{1}H NMR spectra of sucrose, in the plant organs of Charming Beauty and Robinetta, respectively. Data present the mean ± SE of four to six for replicates of leaves at vegetative, generative with buds and generative with flowers stages. Relative concentration of metabolites in leaves is the average of the two generative stages. Differences in relative concentration of sucrose within variety and between the two varieties was analyzed by one-way ANOVA within variety. Different letters refer to significant differences among plant organs within varieties at the 0.05 level. Differences between varieties were not significant at the 0.05 level.

Whereas in many plants species old leaves are more attractive to WFT than young leaves we observed an opposite pattern in Gladiolus. Damage to leaves was highest in the vegetative life-stage when leaves were on average young. However, vegetative and generative plant stages have similar leaf numbers while leaf area expands with age.

Moreover, the concentration of defence compounds in leaves did not drop during successive life-stages. Having a higher concentration of defence compounds in buds and flowers is a way to protect the most valuable organs with respect to plant fitness from WFT. Similarly, Damle *et al.* (2005) reported an accumulation of proteinase inhibitors in flowers as a protection against *Helicoverpa armigera* on tomato (*Lycopersicon esculentum* Mill). The pattern of damage across plant organs in Charming Beauty contrasted with the ornamental chrysanthemum, on which WFT preferred flowers over leaves (de Jager *et al.*, 1993). In the latter species WFT is attracted to pollen and it may find shelter in the flowers. In contrast to what we observed for Gladiolus, WFT caused more damage on plants with flower buds, than on plants with fully opened flowers or on plants with only leaves in *Impatiens wallerana* (Ugine *et al.*, 2006).

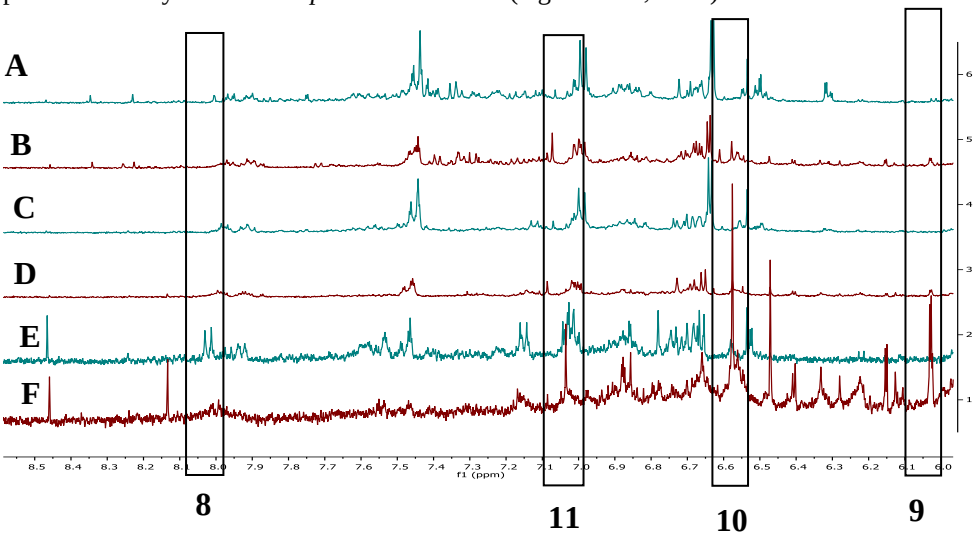

Figure 9. Comparison of ^{1}H-NMR spectra of phenolics regions of (A and B) Robinetta and Charming Beauty of field, (C and D) field transition and (E and F) climate chamber, respectively. Metabolites associated with resistance kaempferol (8), epicatechin (9), epigallocatechin (10) and gallic acid (11).

Differences in resistance between the susceptible variety Charming Beauty the resistant variety Robinetta remained constant across developmental stages. Furthermore, in leaf metabolites that were identified as associated with resistance in the previous chapter remained similar between the two varieties during developmental stages. These

results strongly suggest that markers for resistance in early developmental stages remain valid throughout the plant's life.

The effect of the environment on the metabolomic profile is clear between plants grown in the field and in the climate room, but the transition from the field into the climate chamber does not seem to cause much changes in the metabolome. Metabolites that were affected by the growing conditions included the flavonoids kaempferol, apigenin, and luteolin, as well as some organic acids: formic acid, gallic acid and malic acid. Climate chambers generally have a lower photosynthetic active radiation (PAR) level and UV-B dose compared to field conditions (Deckmyn and Impens, 1997). In the present study, light in the climate chamber was lower than in field conditions which might have caused the chemical variation. Kaempferol was at higher levels in the climate room grown plants. This is in accordance with the results reported by Muller *et al.* (2015) for the perennial semi-aquatic plant *Hydrocotyle leucocephala* showing higher kaempferol concentrations for plants grown in climate chambers compared to plants grown in natural light conditions in the field. In contrast, luteolin and apigenin, were higher in field and field transition-grown plants. Markham *et al.* (1998), reported that in the thallus of the common liverwort, *Marchantia polymorpha* the flavonoids, luteoline and apigenin, had a strong positive correlation to UV-B levels. Formic acid, gallic acid and malic acid were higher in climate room-grown plants whereas Jankanpaa *et al.* (2012) reported that malic acid was more abundant in high-light plants than in low-light plants of *Arabidopsis*.

Concentrations of metabolites previously found to be related to thrips resistance were similar in each of the three environments while differences between the two varieties remained Consequently, the environment seemed no to have affected the compounds related to constitutive thrips resistance in *Gladiolus*. In other words, resistance in *Gladiolus* seems mainly genetically determined.

Unlike secondary metabolites, amino acids belong to the primary metabolites and are part of the plants primary metabolism which is responsible for plant growth and development. Amino acids were reported by Jankanpaa *et al.* (2012) as light-intensity dependent compounds in *Arabidopsis thaliana*. Valine was strikingly higher in plants grown under low light (30 µmol photons m^{-2} s^{-1}) conditions, alanine had higher concentrations in high light (600 µmol photons m^{-2} s^{-1}) and normal light (300 µmol

photons m^{-2} s^{-1}). Threonine had accumulated in Arabidopsis one hour after transfer from a growth chamber into the field. In the present study, alanine, valine and threonine were slightly lower in the climate chamber with lower light intensity (Fig. 11).

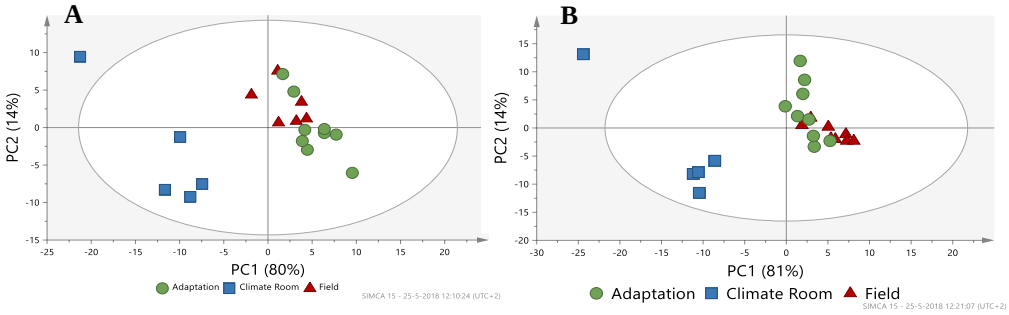

Figure 10. Score plot of PLS-DA based on (■) climate chamber-grown plants, (▲) field-grown plants, field adaptation-grown plants (●), and of Charming Beauty (A) and Robinetta (B), respectively.

All together our results show that differences in plant defence compounds related to thrips resistance between a resistant and a susceptible variety persist during plant development and under different growing conditions. Therefore, they seem useful for breeding programs targeted at resistance. However, when breeding for resistance it is important not to impair bulb or flower production. These metabolites associated with resistance are among the most expensive defence metabolites (triterpenoid saponins) for plants to synthesize (Gershenzon, 1994). Thus, the higher expenditure in resistance may be one of the factors leading to a smaller dry mass of Robinetta compared to Charming Beauty (Chapter 2). More research on the costs of resistance would be needed for a successful breeding program.

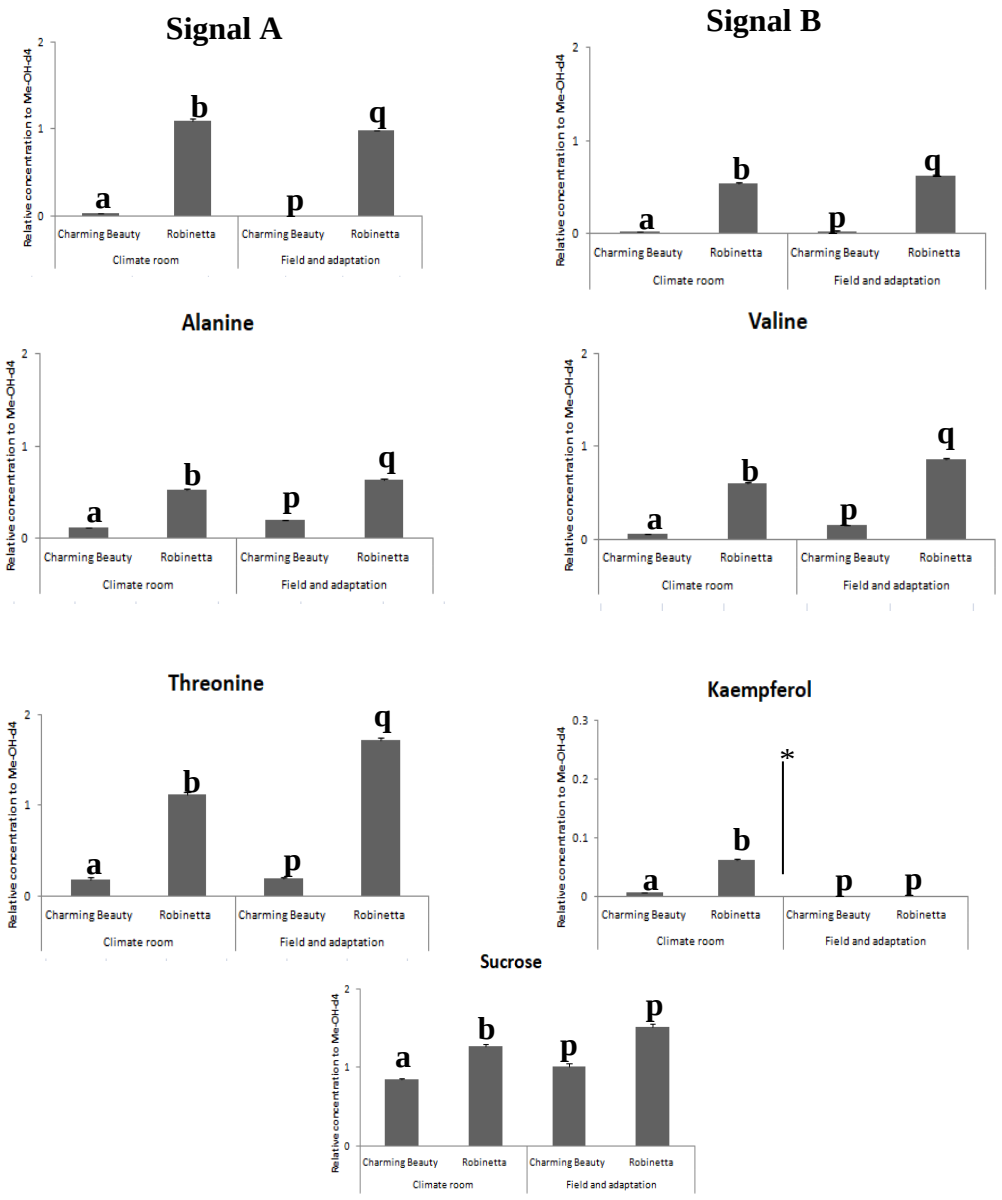

Figure 11. Relative concentration, as proportion of the internal standard, in ^{1}H-NMR spectra of signal A, signal B, alanine, valine, threonine, sucrose and kaempferol as the metabolites associated with the resistant variety Robineta. Data present the mean of four to six replicates ± SE of the mean. Signal A, signal B, threonine and kaempferol were analyzed by Kruskal-Wallis test. Alanine, valine and sucrose were analyzed by one-way

ANOVA. Different letters refer to significant differences between varieties in each growing condition at the 0.05 level., while * indicate significant differences between growing condition at the 0.05 level.

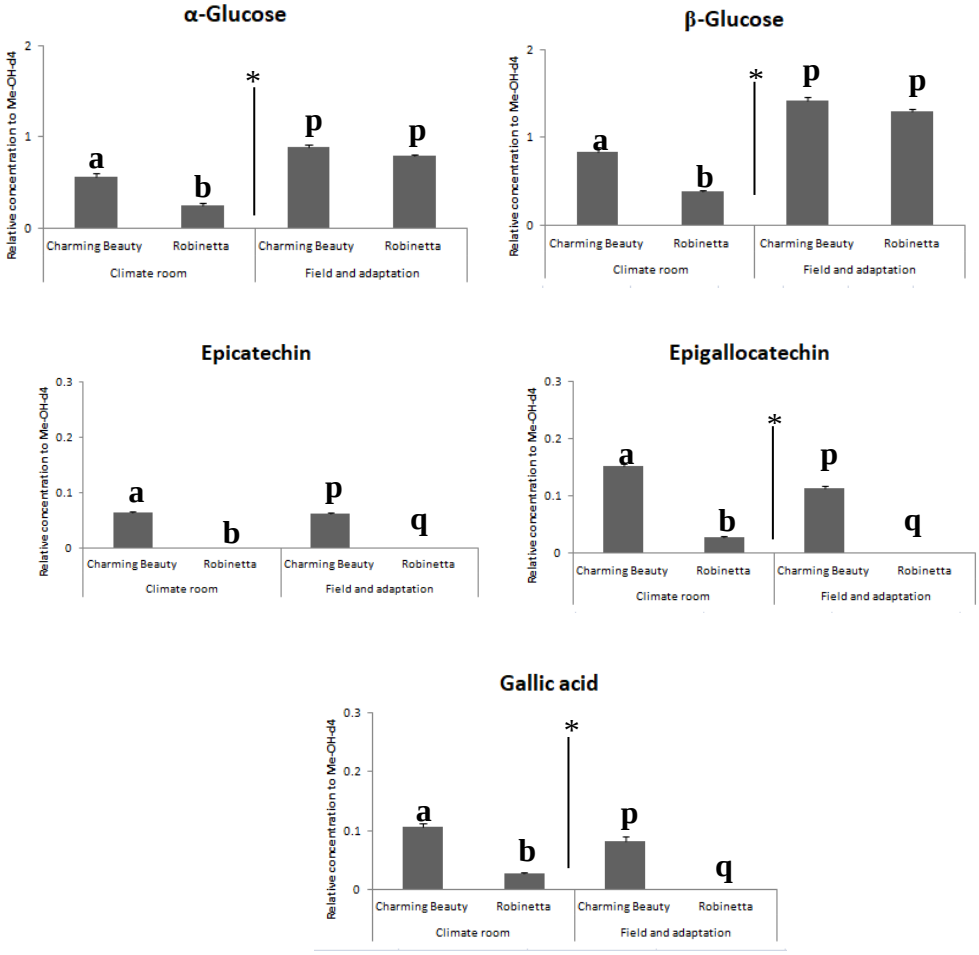

Figure 12. Relative concentration, as proportion of the internal standard, in ^{1}H-NMR spectra of α-glucose, β-glucose, epicatechin, epigallocatechin and gallic acid as the metabolites associated with susceptible variety Charming Beauty Data present the mean of four to six replicates ± SE of the mean. α-glucose and β-glucose were analyzed by one-way ANOVA while epicatechin, epigallocatechin and gallic acid kaempferol were analyzed by Kruskal-Wallis test. Different letters refer to significant differences between varieties in each growing condition at the 0.05 level, while * indicate significant differences between growing condition at the 0.05 level.

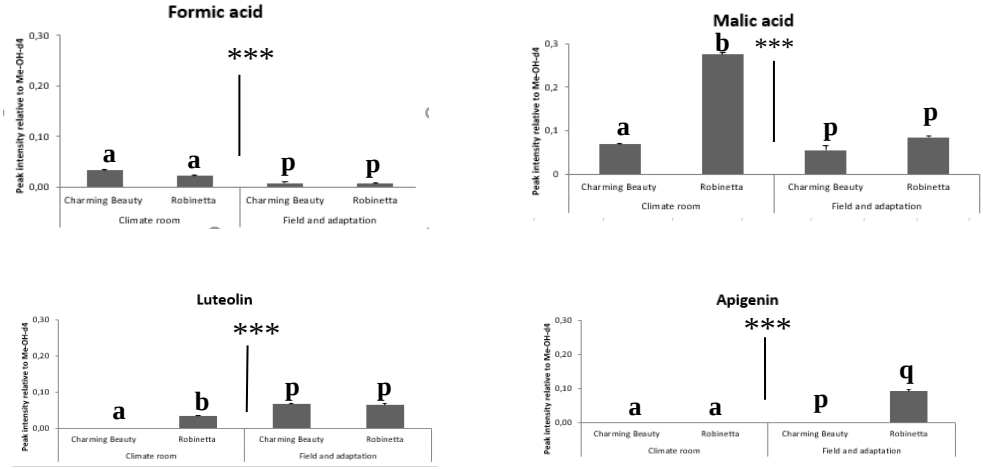

Figure 13. Relative concentration, as proportions of the internal standard, in ^{1}H-NMR spectra of formic acid, malic acid, luteolin and apigenin. Metabolites related to different growing places. Data present the mean of four to six replicates ± SE of the mean. Formic acid and malic acid were analyzed by one-way ANOVA while luteolin and apigenin were analyzed by Kruskal-Wallis tests. Different letters refer to significant differences between varieties in each growing condition at the 0.05 level., while *** indicate significant differences between growing condition at the 0.05 level.

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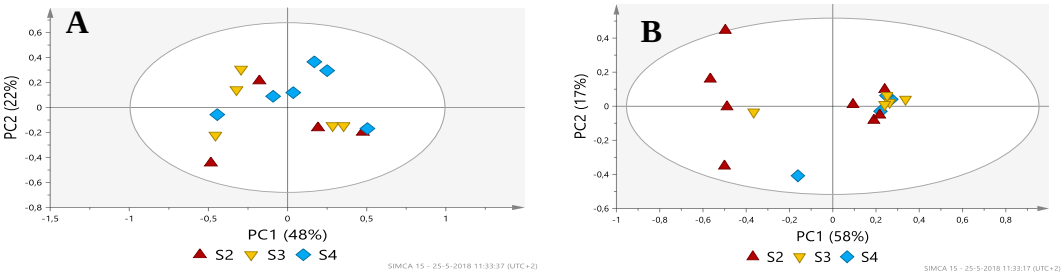

(▲) Vegetative stage (▼) Generative with buds stage

(♦) Generative with flowers stage

Figure S1. PCA score plot for Charming Beauty (A) and Robinetta (B) based on ^{1}H NMR spectra. Leaves at different developmental stages were included in each variety: (▲) vegetative, (▼) generative with buds and (♦) generative with flowers.

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.

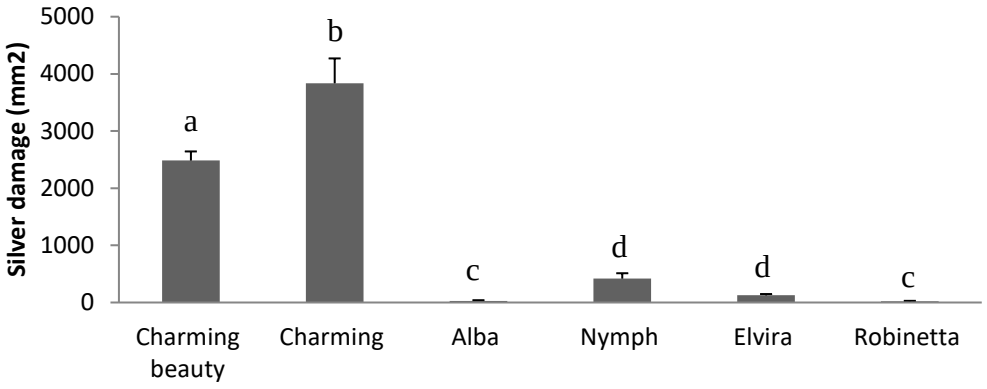

Figure 1. Silver damage, (mm^2) in six *Gladiolus nanus* varieties as measured by a whole plant thrips non-choice bioassay. Data represent mean and standard errors for five replicates. Different letters indicate significant differences between varieties at $P \leq 0.05$.

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.

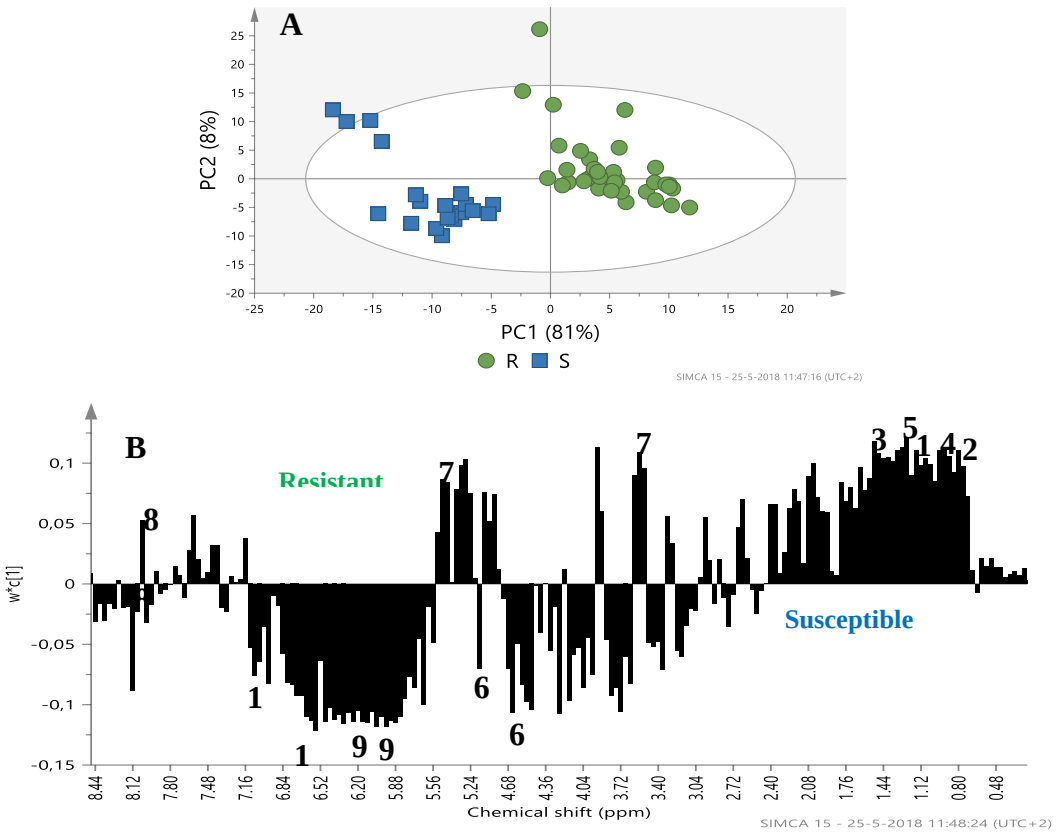

Figure 2. (A) PLS-DA score plot of six dwarf *Gladiolus nanus* varieties based on ^{1}H-NMR spectra related to their resistance, (■) susceptible (Charming Beauty and Charming varieties) and (●) resistant (Alba, Nymph, Elvira and Robinetta varieties). Loading plot of PLS-DA (B) based on thrips resistance,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

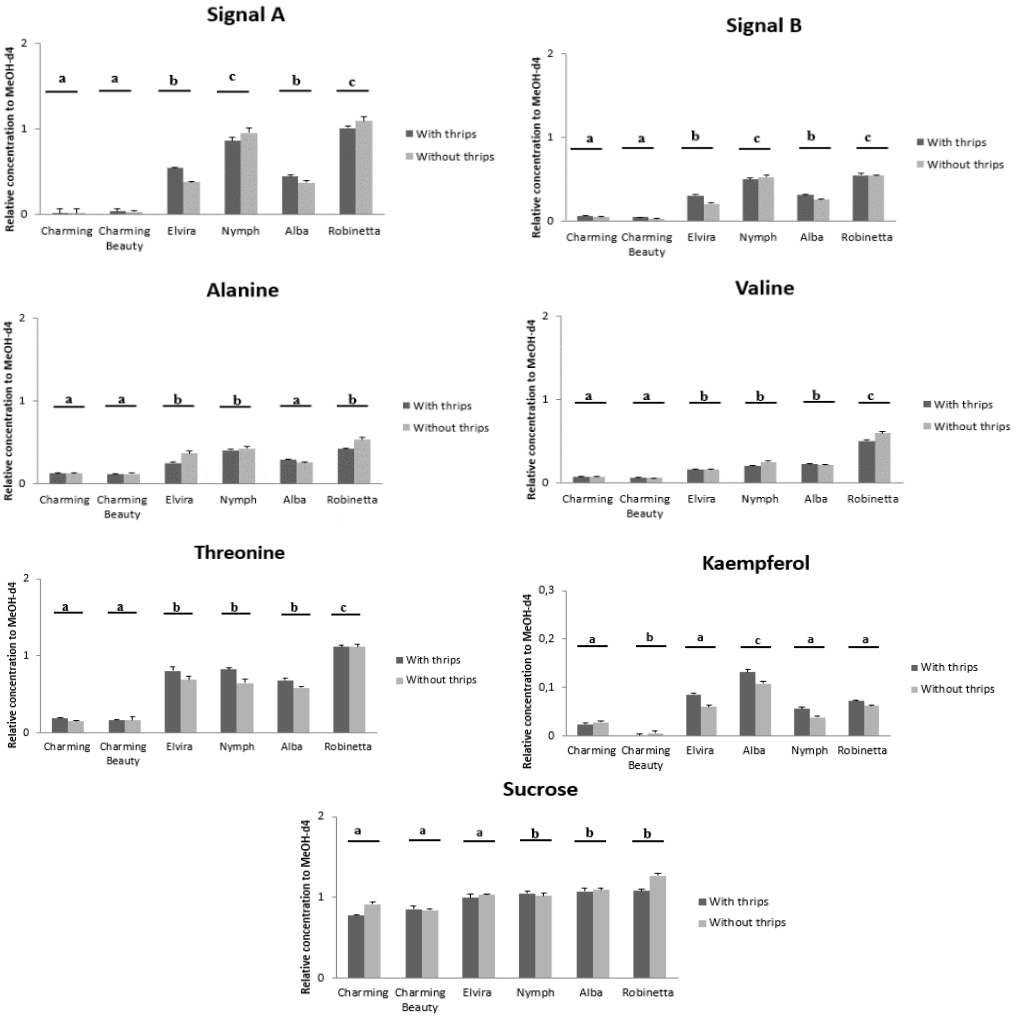

Figure 3. Relative concentration of metabolites related to resistant varieties, as proportions of the internal standard, in ^{1}H-NMR spectra of two triterpenoid saponins (signal A, signal B), alanine, valine, threonine, kaempferol and sucrose in the leaves of *Gladiolus nanus* varieties. Data present 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.

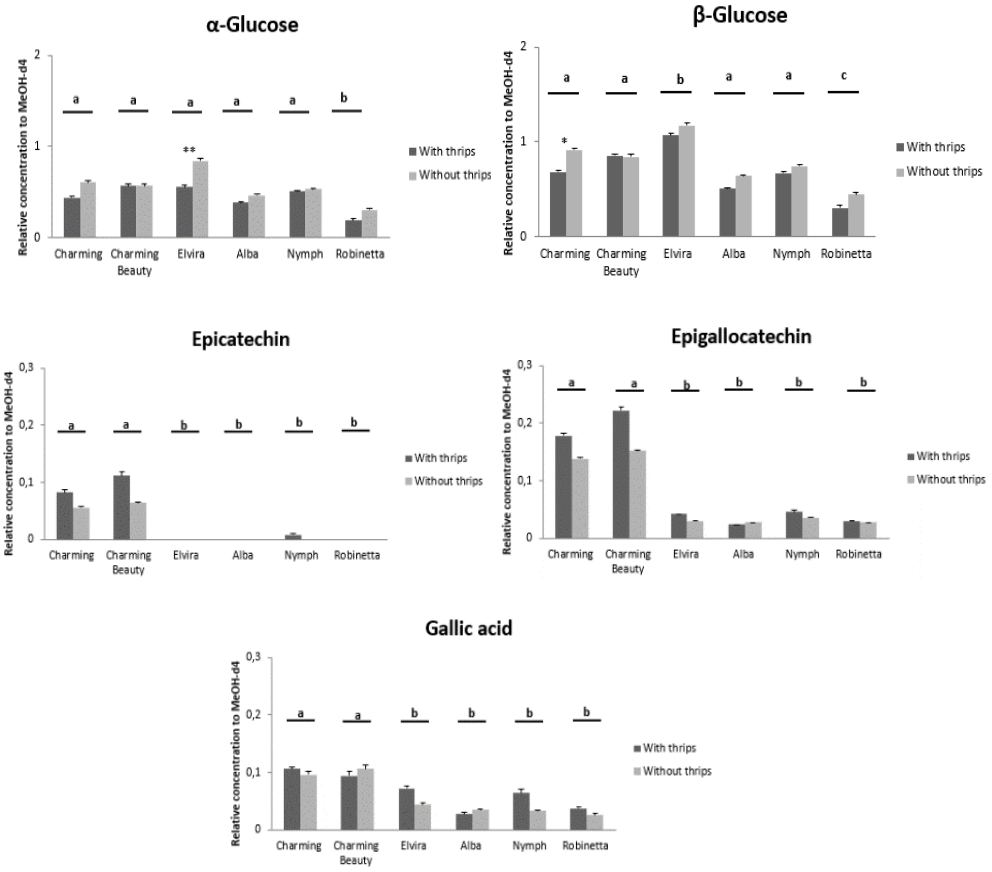

Figure 4. Relative peak intensities of metabolites related to susceptibility, as proportions of the internal standard, in ^{1}H-NMR spectra of α-glucose, β-glucose, epicatechin, epigallocatechin and gallic acid in the leaves of *Gladiolus nanus* varieties. Data present the mean of five replicates ± SE 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 non-

significant 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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GENERAL DISCUSSION AND CONCLUSION

Breeding for resistance becomes more and more important because we want to reduce the use of pesticides. Nowadays for many species we can use a molecular toolbox. However, for some crops, especially polyploids and crops with large genomes this is not yet feasible. A fast and cheap alternative can be to make use of morphological or chemical markers. In order to do so we first need the study resistance mechanism in plants and how they may change depending on environmental conditions or plant development. Based on these ideas we developed markers for thrips resistance in gladiolus.

Gladiolus ornamental flowers exhibited a broad range of variation in thrips resistance in the field. Therefore, thrips damage to fourteen varieties including dwarf, medium and large size gladiolus were compared (Chapter 2). We found a 130-fold difference in silver damage between the most resistant and the most susceptible varieties. Leave morphology was strongly involved in thrips resistance. The density of papillae was negatively correlated to thrips silver damage while the lengths of the mesophyll- and epidermis cells were positively correlated with thrips damage. As a rule, an epidermis cell produced a single papilla. Thus, varieties with smaller leaf cells had a higher density of epicuticular papillae and these were the most resistant ones. The density of papillae explained 39% of the variation in silver damage. Papillae can inhibit the movement of thrips or hinder penetration of the epidermis by the cell sucking thrips. Scott, Brown and Simmonds (2006) who studied effects of leaf morphology on *Heliothrips haemorrhoidalis* reported that this thrips had a preference for leaves with smooth surfaces, while trichomes and leaf surface wax structures inhibited thrips movement. Besides forming a physical barrier, papillae may store plant secondary compounds. Cardinosin A, an aspartic proteinase, suggested to be involved in plant defence against pathogens, is stored in the stigmatic papillae of *Cynara cardunculus* L.(Ramalho-Santos *et al.*, 1997). Therefore, we expected that Gladiolus varieties with higher densities of papillae contained higher amounts of defence compounds.

We, therefore, (chapter 3) extended our study with experiments that included a broader range of varieties and that focused on the plant metabolome. First, we checked if the plant metabolome was involved in thrips resistance by testing the effects of the leave extracts of the varieties of the previous experiments on thrips mortality in an *in-vitro* experiment. Thrips mortality in this experiment was strongly negatively correlated with silver damage in the whole plant bio-assay. We than used fourteen varieties of dwarf, medium to large sized Gladiolus to investigate metabolites associated with thrips resistance and susceptibility. NMR-based metabolomics indicated that two unidentified triterpenoid saponins (Signal A and signal B), two amino acids (alanine and threonine) were related to thrips resistance. All these compounds were highly correlated amongst each other and to the density of papillae. In contrast, no compounds were significantly correlated to susceptibility although they were identified as VIPs in multivariate statistics. The correlations between the concentrations of the compounds related to resistance and the density of papillae strongly suggests that the papillae serve as storage organ or produce the compounds associated to resistance. The strong correlation between them makes it, however, unclear what their relative contributions to resistance are. Saponins were shown to be important as a defensive chemical in *Aesculus pavia* against the leafminer *Cameraria ohridella* (Ferracini *et al.*, 2010), in *Barbarea vulgaris* against the flea beetle *Phyllotreta nemorum* (Kuzina *et al.*, 2009) (Nielsen *et al.*, 2010). The alanine concentration was higher in a variety of peach that was resistant against the Mediterranean fruitfly (*Ceratitis capitata*), while for threonine such a difference was not detected (Capitani *et al.*, 2012). In contrast, Dillon and Kumar (Dhillon 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. Such a difference in the bioactivity of a particular compound across different plant species is not unusual and may result from the fact that mostly multivariate correlations are used and the concentrations of plant metabolites are highly correlated among themselves. Alternatively, these differences in bioactivity of compounds across plant species may results from synergistic or antagonistic effects within different metabolomics backgrounds. 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 suggests that chemical compounds stored in papillae may confer resistance in gladiolus species. This offers an existing 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 and/ or the compounds identified may provide easy morphological and/ or chemical markers in Gladiolus breeding programs targeted at increased resistance against thrips.

Markers for resistance in breeding programs should be relevant throughout the plant's ontogeny and under different environmental conditions. However, chemical defense might be changed as plants grow, starting with the seedling stage, passing through the vegetative juvenile stage and becoming mature at the reproduction stage. Ontogenetic shifts in defense traits might be associated with dramatic changes in levels of herbivory experienced (Boege and Marquis, 2005). Besides, plant resistance to herbivores which mostly has been studied under controlled conditions in growth cabinets could be different when studied under natural growing conditions in the field where conditions such as photoperiod, light intensity, temperature and humidity are highly variable. Therefore, in chapter 4, we first compared resistance among 3 development plant stages: vegetative, generative stage with buds and generative stage with flowers. Second, we compared resistance between plants grown under natural field conditions at the site of a breeder that were transferred to a climate room for the resistance test and plants grown during the whole experiment in a climate room. For this study, the Gladiolus varieties Robinetta and Charming Beauty which previously were highly resistant and highly susceptible in the vegetative stage, respectively were used. Robinetta showed more than 500-fold less damage than Charming Beauty with consistent differences in WFT resistance over all development stages. Metabolomic profiles differed between the two varieties throughout all three plant stages. It revealed triterpenoids saponins and amino acids as metabolites associated with the resistant variety, as in the previous chapter. Threonine was 10 times higher and triterpenoid saponins, valine and alanine were about five times higher in Robinetta. Most likely, however, valine is not a reliable marker for resistance because we found no correlation with resistance in chapter 3 where we studied thrips resistance in a

series of cultivars. In Charming beauty, the susceptible cultivar, leaves were more damaged than buds and flowers. However, leaves represent a relatively larger area compared to buds and flowers so that differences in damage between organs may not solely be attributed to variation in metabolites. Although the silver damage on leaves was higher in the vegetative stage than in the two generative stages we did not observe significant differences in leaf metabolites related to resistance (or to susceptibility) between leaves of different developmental stages. While in Robinetta damage was always much lower that in Charming Beauty all the concentrations of the compounds we identified in the chapter 3 as being related to resistance were much higher. In Robinetta, the relative concentrations of the triterpenoids saponins (signals A and B) were higher in leaves and buds than in flowers while the concentrations of threonine, alanin and valine were much higher in leaves and buds than in flowers. As expected, the concentration of compounds that were related to defence in chapter 3 of this thesis are higher in buds and flowers than in leaves in the susceptible variety Charming Beauty. Having a higher concentration of defence compounds such as alanine and threonine in buds and flowers is a way to protect the most valuable organs with respect to plant fitness from WFT. Similarly, Damle *et al.* (2005) reported an accumulation of proteinase inhibitors in flowers as a protection against *Helicoverpa armigera* on tomato (*Lycopersicon esculentum* Mill). The pattern of damage across plant organs in Charming Beauty contrasted with the ornamental chrysanthemum, on which WFT preferred flowers over leaves (De Jager *et al.*, 1993). Differences in resistance between the susceptible variety Charming Beauty and the resistant variety Robinetta remained constant across developmental stages. Furthermore, we found no differences in resistance of leaves among developmental stages for both varieties which was accompanied by the absence of differences among developmental stages of metabolites in leaves that were identified as associated with resistance in the previous chapter. Together, these results strongly suggest that markers for resistance in early developmental stages remain valid throughout the plant's life.

In the second part of this chapter we investigated the effect of environmental conditions on thrips resistance. The environment clearly affected the metabolomic profiles. Phenolic compounds such as flavonoids kaempferol, apigenin, and luteolin, as

well as some organic acids: formic acid, gallic acid and malic acid were metabolites affected by the growing conditions. Clearly light intensity in the climate chamber was lower than under field conditions which may have caused the chemical variation. Luteolin and apigenin, were higher under field conditions and in plants transitioned from the field to the climate room than in plants grown in the climate room. Markham et al (Markham *et al.*, 1998), reported that in the thallus of the common liverwort, *Marchantia polymorpha* the flavonoids, luteoline and apigenin, increased with higher UV-B levels. In contrast, Kaempferol was at higher levels in the climate room grown plants. The latter is in line with the results of Muller *et al.* (2015) for the perennial semi-aquatic plant *Hydrocotyle leucocephala* showing higher kaempferol concentration for plants grown in climate chambers. Formic acid, gallic acid and malic acid were higher in climate room-grown plants whereas Jankanpaa *et al.* (Jänkänpää *et al.*, 2012) reported that malic acid was more abundant in high-light plants than in low-light plants of *Arabidopsis*. Concentrations of metabolites previously found to be related to thrips resistance (triterpenoid saponins, alanine and threonine) were similar in each of the three environments while differences between the two varieties remained constant. Consequently, the environment seemed no to have affected the compounds related to thrips resistance in *Gladiolus*. In other words, resistance in *Gladiolus* seems mainly genetically determined without a strong genotype-environment interaction. Amino acids belong to the primary metabolites and are part of the plants primary metabolism which is responsible for plant growth and development. Amino acids were reported by Jankanpaa *et al.* (2012) as light-intensity dependent compounds in *Arabidopsis thaliana*. Valine was strikingly higher in plants grown under low light (30 µmol photons m^{-2} s^{-1}) conditions, alanine had higher concentrations in high light (600 µmol photons m^{-2} s^{-1}) and normal light (300 µmol photons m^{-2} s^{-1}). Threonine had accumulated in Arabidopsis one hour after transfer from a growth chamber into the field. In the present study, alanine, valine and threonine were slightly lower in the climate room. All together these results show that differences in plant defence compounds related to thrips resistance between a resistant and a susceptible variety persist during plant development and under different growing conditions.

Constitutive defenses, as studied in the previous chapters, are thought to be costly for plants since they require resources for their biosynthesis (Boege and Marquis, 2005). Therefore, defence systems are often only switched on when an infestation actually takes place. Such induced defences are known from many plant species. We, therefore, used six varieties of Gladiolus nanus with and without thrips infestation to investigate host plant resistance to thrips and to study if the compounds that we found to be associated with resistance and susceptibility against thrips in the previous chapters were affected by thrips infestation. We confirmed that constitutively resistant varieties had higher levels of triterpenoids, well as some amino acids (alanine, valine and threonine). In addition, we found that kaempferol was associated to resistance in *Gladiolus nanus*. Leiss *et al.* (2009) and Kirk *et al.* (2005) found that kaempferol glycoside, was higher in genotypes of *Jacobaea vulgaris* that were resistant against *F. occidentalis*. Kaempferol concentration was two times higher in resistant cowpea lines (*Vigna unguiculate* L. Walp.) to Aphids (*Aphis fabae*) (Lattanzio *et al.*, 2000). Wu *et al.* (2007) reported that kaempferol glycosides detered *Thrips palmi* in golden-rod (*Solidago altissima* L.).

Plants have to defend themselves against all threats outside with a pathway which may differ for each pest and disease. As a result, besides the triterpenoid saponins and amino acids mentioned above, thrips are resistant against phenolic compound kaempferol as one of resistant metabolite typically in *Gladiolus nanus*. Phenolic compounds such as kaempferol glycosides revealed the opposite pattern in response to *Spodoptera littoralis* infestation in maize (Marti *et al.*, 2013). Kaempferol 3-*O*-rutinoside was reduced in herbivores-attacks. Forkner *et al.* (2004) reported that tannins as phenolic compounds functioning as anti-herbivores against the leaf-chewing insects *Acronicta increta* (Noctuidae) and *Attelabus* sp. (Curculionidae) on *Quercus velutina* and *Q. alba*. Both metabolomic profiles before and after thrips infestation of different varieties of Gladiolus were clearly separated from each other in multivariate analyses. In contrast metabolomics profiles were not strongly affected by thrips infestation. Damage after infestation may occur in a small part of a leaf only and may cause some local metabolic changes because of induced defense. However, the local changes after the limited damage, might be lost in the overall metabolome. We measured metabolomic profiles at the level of the total leaves of the plant. For the most susceptible cultivar (Charming

Beauty) with severe that showed damage all over the plant this most likely rules out the possibility that locally induction occurred. For the most resistant cultivar Robinetta with very local spots of damage we cannot rule out this possibility and more analysis would be necessary to investigate whether selecting for increased local induced defences could lead to significant reduction of damage levels. A 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.

In conclusion, we showed that the density of papillae and chemical traits (triterpenoids and amino acids) are useful markers for breeding programs targeted at increased resistance in Gladiolus. Epicuticular papillae are easily detected since they located at the leaves surface. They may inhibit the movement of thrips or hinder penetration of the epidermis by the cell sucking thrips. The density of papillae explained 39% of the variation in silver damage. The density of papillae is highly correlated with a number of metabolites that were associated with resistance. Concentrations of triterpenoid saponins, alanine and threonine were higher in resistant varieties. Moreover, these compounds were highly correlated amongst each other and to the density of papillae. Most likely these compounds are produced and/or stored in the papillae. Because of the high correlation of the concentration of these compounds with papillae density it is not possible to determine what actually causes resistance. Meanwhile, papillae density and chemical compounds may provide an easy marker in Gladiolus breeding programs targeted at increased resistance against thrips. Concentrations of metabolites associated to thrips resistant remained constant during the ontogeny of the plant and under different growing conditions. These findings provide useful information for breeders because it implies that these markers can be determined in young plants and under different conditions. In dwarf gladiolus, *Gladiolus nanus*, also kaempferol was associated to resistance. However, it was only found in in plants grown in the climate chamber. Further studies will be needed to understand the mechanism of thrips resistance in Gladiolus in more detail. Such experiments should include the identification and isolation of the triterpenoid saponins and experiments with identified compounds spiked to artificial diets of thrips.

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Algemene discussie en conclusie

Resistentieveredeling wordt steeds belangrijker voor het verminderen van het gebruik van pesticiden. Daarvoor kunnen we tegenwoordig een moleculaire toolbox gebruiken. Voor sommige gewassen is deze toolbox nog niet toereikend vanwege hoge ploidie-graad of vanwege de grootte van het genoom. Een snel en goedkoop alternatief kan zijn om gebruik te maken van morfologische of chemische markers. Om dit te doen moeten resistentiemechanismen in de planten worden onderzocht en hoe deze kunnen veranderen onder invloed van omgevingsfactoren of plantontwikkeling. Op basis van deze ideeën hebben we markers ontwikkeld voor tripsresistentie in gladiolen.

Gladiolen zijn sierbloemen met een breed scala aan variaties in tripsweerbaarheid. Tripsschade werd bij veertien variëteiten, waaronder dwerg-, middelgrote en grote gladiolen, vergeleken (Hoofdstuk 2). We vonden een 130-voudig verschil in zilverschade tussen de meest resistente en de meest vatbare variëteiten. De morfologie van het blad droeg sterk bij aan de tripsresistentie. De dichtheid van papillen was negatief gecorreleerd met tripsschade, terwijl de lengtes van de mesofyl- en epidermiscellen positief gecorreleerd waren met tripsschade. In de regel produceerde een epidermiscel een enkele papil. Dus rassen met kleinere bladcellen hadden een hogere dichtheid aan epicuticulaire papillen en waren daarom het meest resistent. De dichtheid van papillen verklaarde 39% van de variatie in zilverschade. Papillen kunnen de beweging van trips remmen en het binnendringen van de epidermis door trips belemmeren. Scott, Brown en Simmonds (2006) die de effecten van bladmorfologie op *Heliothrips haemorrhoidalis* bestudeerden, meldden dat deze trips een voorkeur had voor bladeren met gladde oppervlakken, terwijl trichomen en wasstructuren van het blad de tripsbeweging remden. Naast het vormen van een fysieke barrière, kunnen papillen secundaire metabolieten bevatten. Cardinosine A, een asparaginezuurproteïnase, waarvan wordt aangenomen dat het betrokken is bij de afweer van planten tegen pathogenen, wordt opgeslagen in de stigmatische papillen van *Cynara cardunculus* L. (Ramalho-Santos et al., 1997). We verwachtten daarom dat Gladiolus-variëteiten met hogere dichtheden van papillen grotere hoeveelheden afweerstoffen bevatten.

Om die reden hebben we onze studie uitgebreid met experimenten die een breder scala aan variëteiten omvatten en die gericht waren op het metaboloom van de plant (hoofdstuk 3). Eerst hebben we gekeken of het plantmetaboloom betrokken was bij tripsresistentie door de effecten van de bladextracten van de variëteiten van de vorige experimenten op tripssterfte te testen in een in vitro experiment. Tripssterfte in dit

experiment was sterk negatief gecorreleerd met zilverbeschadiging in de bio-toets van de hele plant. Vervolgens gebruikten we veertien soorten dwerggladiolen van gemiddelde tot grote grootte om metabolieten te onderzoeken die geassocieerd zijn met tripsresistentie en -gevoeligheid. NMR-gebaseerde metabolomics gaven aan dat twee niet-geïdentificeerde triterpenoïde saponinen (signaal A en signaal B), twee aminozuren (alanine en threonine) gerelateerd waren aan tripsresistentie. Al deze inhoudsstoffen waren sterk gecorreleerd met elkaar en met de dichtheid van papillen. Daarentegen waren er geen inhoudsstoffen significant gecorreleerd met gevoeligheid, hoewel ze werden geïdentificeerd als VIP's in multi-variate toetsen. De correlaties tussen de concentraties van de inhoudsstoffen gerelateerd aan resistentie en de dichtheid van papillen suggereert sterk dat de papillen dienen als opslagorgaan van inhoudsstoffen die bijdragen aan resistentie of dat deze stoffen door de papillen worden geproduceerd. De sterke correlatie tussen hen maakt het echter onduidelijk wat hun relatieve bijdragen aan resistentie zijn. Saponinen bleken belangrijk te zijn als chemische afweerstof in *Aesculus pavia* tegen de mineervlieg *Cameraria ohridella* (Ferracini et al., 2010), in *Barbarea vulgaris* tegen de vlokever *Phyllotreta nemorum* (Kuzina et al., 2009) (Nielsen et al., 2010). De alanineconcentratie was hoger in een perzikenras dat resistent was tegen de mediterrane fruitvlieg (*Ceratitis capitata*), terwijl voor threonine een dergelijk verschil niet werd waargenomen (Capitani et al., 2012). Dillon en Kumar (Dhillon en Kumar, 2017) rapporteerden daarentegen dat de threonine-concentratie significant hoger was in zaailingen van tweekleurige Sorghum die resistent zijn tegen de stengelboorder *Chilo partellus* dan in de zaailingen van een vatbaar ra, terwijl de alanineconcentraties niet significant verschilden. Een dergelijk verschil in de bioactiviteit van een bepaalde inhoudsstof tussen verschillende plantensoorten is niet ongebruikelijk en kan het gevolg zijn van het feit dat meestal multivariate correlaties worden gebruikt en de concentraties van plantmetabolieten onderling sterk gecorreleerd zijn. Als alternatief kunnen deze verschillen in bioactiviteit van inhoudsstoffen tussen plantensoorten het gevolg zijn van synergetische of antagonistische effecten met de achtergrondmetabolieten. Papillen als opslagplaatsen van secundaire plantafweerinhoudsstoffen zijn gerapporteerd in rijst (Wakte et al., 2007) en kardoen (Ramalho-Santos et al., 1997). Onze studie suggereert dat chemische inhoudsstoffen die in papillen zijn opgeslagen, resistentie kunnen verlenen aan gladiolen. Dit biedt een kansen voor verder onderzoek naar de mechanismen die betrokken zijn bij resistentie tegen thrips. Op dit punt moeten we echter voorzichtig zijn, omdat we een correlatieve studie hebben uitgevoerd en correlatie betekent niet altijd een causaal verband. Zo kunnen andere bijbehorende kenmerken betrokken zijn bij het resistentiemechanisme. Ondertussen kunnen de dichtheid van papillen en/of de geïdentificeerde inhoudsstoffen gemakkelijke morfologische en/of chemische markers opleveren in veredelingsprogramma’s voor gladiolen die gericht zijn op verhoogde resistentie tegen trips.

Markers voor resistentie in kweekprogramma's moeten relevant zijn gedurende in meerdere groeifases van de plant en onder verschillende omgevingsomstandigheden. De chemische afweer kan echter veranderen naarmate planten groeien, beginnend in de zaailingfase, via de vegetatieve juveniele fase naar de reproductiefase. Ontogenetische verschuivingen in verdedigingskenmerken kunnen worden geassocieerd met dramatische veranderingen in niveaus van ervaren herbivorie (Boege en Marquis, 2005). Bovendien kan de resistentie van planten tegen herbivoren, die meestal onder gecontroleerde omstandigheden in klimaatcellen is bestudeerd, verschillen wanneer deze wordt bestudeerd onder natuurlijke groeiomstandigheden in het veld, waar omstandigheden zoals fotoperiode, lichtintensiteit, temperatuur en vochtigheid dagelijks variëren. Daarom hebben we in hoofdstuk 4 tripsresistentie vergeleken tussen 3 ontwikkelingsstadia van de plant: vegetatief, generatief stadium met knoppen en generatief stadium met bloemen. Ten tweede hebben we de resistentie vergeleken tussen planten die onder natuurlijke omstandigheden op de locatie van een veredelaar waren gekweekt en die voor de resistentietest naar een klimaatkamer werden overgebracht, en planten die gedurende het hele experiment in een klimaatkamer waren gekweekt. Voor deze studie werden de Gladiolus-variëteiten Robinetta en Charming Beauty gebruikt, die respectievelijk zeer resistent en zeer vatbaar waren in de vegetatieve fase. Robinetta vertoonde meer dan 500 keer minder schade dan Charming Beauty met consistente verschillen in tripsresistentie over alle ontwikkelingsstadia. Metabole profielen verschilden tussen de twee variëteiten in alle drie de plantstadia. Triterpenoïden, saponinen en aminozuren werden aangewezen als metabolieten die geassocieerd zijn met het resistente ras, zoals in het vorige hoofdstuk. Threonine was 10 keer hoger en triterpenoïde saponinen, valine en alanine waren ongeveer vijf keer hoger in Robinetta. In Charming beauty, de vatbare cultivar, waren de bladeren meer beschadigd dan de knoppen en bloemen. Bladeren vertegenwoordigen echter een relatief groot oppervlak in vergelijking met knoppen en bloemen, zodat verschillen in tripsschade tussen organen mogelijk niet alleen kon worden toegeschreven aan variatie in metabolieten. Hoewel de zilverschade op bladeren hoger was in de vegetatieve fase dan in de twee generatieve stadia, zagen we geen significante verschillen in bladmetabolieten gerelateerd aan resistentie (of gevoeligheid) tussen bladeren van verschillende ontwikkelingsstadia. Terwijl in Robinetta de schade altijd veel lager was, waren alle concentraties van de inhoudsstoffen die we in hoofdstuk 3 identificeerden als gerelateerd aan resistentie in het vatbare ras Charming Beauty veel hoger. In Robinetta waren de relatieve concentraties van de triterpenoïden saponinen (signalen A en B) hoger in bladeren en knoppen dan in bloemen, terwijl de concentraties van threonine, alanine en valine veel hoger waren in bladeren en knoppen dan in bloemen. Zoals verwacht, is de concentratie van inhoudsstoffen die gerelateerd waren aan afweer in hoofdstuk 3 van dit proefschrift hoger in knoppen en bloemen dan in bladeren in de vatbare variëteit Charming Beauty. Het hebben van een hogere concentratie aan afweerstoffen zoals alanine en threonine in knoppen en bloemen is een manier om de meest waardevolle

organen met betrekking tot plantfitness te beschermen tegen trips. Ook Damle et al. (2005) rapporteerden een accumulatie van proteïnase-remmers in bloemen als bescherming tegen *Helicoverpa armigera* op tomaat (*Lycopersicon esculentum* Mill). Het patroon van schade aan plantorganen in Charming Beauty contrasteerde met chrysant, waarop trips de voorkeur gaf aan bloemen boven bladeren (De Jager et al., 1993). Verschillen in resistentie tussen het vatbare ras Charming Beauty en het resistente ras Robinetta bleven constant in de ontwikkelingsstadia. Bovendien vonden we geen verschillen in resistentie van bladeren tussen ontwikkelingsstadia voor beide variëteiten, wat gepaard ging met het ontbreken van verschillen tussen ontwikkelingsstadia van metabolieten in bladeren die in het vorige hoofdstuk als geassocieerd met resistentie werden geïdentificeerd. Samen suggereren deze resultaten sterk dat markers voor resistentie in vroege ontwikkelingsstadia geldig blijven gedurende het hele leven van de plant.

In het tweede deel van dit hoofdstuk onderzochten we het effect van omgevingscondities op tripsresistentie. De omgeving had duidelijk invloed op de metabole profielen. Fenolen zoals flavonoïden kaempferol, apigenine en luteoline, evenals enkele organische zuren zoals mierenzuur, galluszuur en appelzuur waren inhoudsstoffen die werden beïnvloed door de groeiomstandigheden. Het was duidelijk dat de lichtintensiteit in de klimaatkamer lager was dan onder veldomstandigheden die mogelijk de chemische variatie veroorzaakten. Luteoline en apigenine waren hoger onder veldomstandigheden en in planten die van het veld naar de klimaatkamer werden overgebracht dan in planten die in de klimaatkamer werden gekweekt. Markham et al (Markham et al., 1998) rapporteerden dat in de thallus van het gewone levermos, *Marchantia polymorpha*, de flavonoïden, luteoline en apigenine, toenamen met hogere UV-B-niveaus. Kaempferol was meer aanwezig in planten die in de klimaatcel waren opgekweekt. Dit laatste is in lijn met de resultaten van Muller et al. (2015) voor de meerjarige semi-aquatische plant *Hydrocotyle leucocephala* met een hogere kaempferolconcentratie voor planten die in klimaatkamers worden gekweekt. Mierenzuur, galluszuur en appelzuur waren hoger in planten die in de klimaatkamer werden gekweekt, terwijl Jankanpaa et al. (Jänkänpää et al., 2012) meldden dat appelzuur overvloediger voorkwam in Arabidopsis met veel licht dan in planten met weinig licht. Concentraties van metabolieten waarvan eerder werd vastgesteld dat ze verband hielden met tripsresistentie (triterpenoïde saponinen, alanine en threonine) waren vergelijkbaar in elk van de drie omgevingen, terwijl de verschillen tussen de twee variëteiten constant bleven. Bijgevolg leek het milieu geen invloed te hebben gehad op de inhoudsstoffen die verband houden met tripsresistentie in Gladiolus. Met andere woorden, resistentie bij Gladiolus lijkt voornamelijk genetisch bepaald zonder een sterke genotype-omgeving interactie. Aminozuren behoren tot de primaire metabolieten en maken deel uit van het primaire metabolisme van planten, dat verantwoordelijk is voor de groei en ontwikkeling.

Aminozuren werden gerapporteerd door Jankanpaa et al. (2012) als lichtintensiteitsafhankelijke stoffen in *Arabidopsis thaliana*. Valine was opvallend hoger in planten die onder omstandigheden met weinig licht (30 µmol fotonen m-2 s-1) werden gekweekt, alanine had hogere concentraties bij veel licht (600 µmol fotonen $m^{-2}s^{-1}$) en normaal licht (300 µmol fotonen $m^{-2}s^{-1}$). Threonine had zich in Arabidopsis één uur na het overbrengen van een groeikamer naar het veld opgehoopt. In de huidige studie waren alanine, valine en threonine iets lager in de klimaatkamer. Al met al laten deze resultaten zien dat verschillen in plantafweerstoffen die verband houden met tripsresistentie tussen een resistente en een vatbare variëteit blijven bestaan tijdens de ontwikkeling van de plant en onder verschillende groeiomstandigheden.

Constitutieve afweermechanismen, zoals bestudeerd in de vorige hoofdstukken, worden geacht duur te zijn voor planten, aangezien hun biosynthese energie kost (Boege en Marquis, 2005). Om die reden worden verdedigingssystemen in planten vaak pas ingeschakeld bij een daadwerkelijke aanval door een plaag of ziekte. Dergelijke geïnduceerde afweermechanismen zijn bekend voor vele plantensoorten. Daarom hebben we zes variëteiten *Gladiolus nanus* met en zonder tripsplaag gebruikt om de resistentie van waardplanten tegen trips te onderzoeken en om te onderzoeken of de inhoudsstoffen die we in de vorige hoofdstukken met resistentie en vatbaarheid voor trips in verband brachten, ook door trips werden geinduceerd. We bevestigden dat constitutief resistente variëteiten hogere niveaus van triterpenoïden hadden, evenals enkele aminozuren (alanine, valine en threonine). Bovendien ontdekten we dat kaempferol geassocieerd was met resistentie in *Gladiolus nanus*. Leiss et al. (2009) en Kirk et al. (2005) ontdekten dat kaempferol glycoside hoger was in genotypen van Jacobaea vulgaris die resistent waren tegen *F. occidentalis*. Ook was de kaempferolconcentratie twee keer hoger in ogenbonen-lijnen (*Vigna unguiculate* L. Walp.) die resistent waren tegen luizen (Aphis fabae) (Lattanzio et al., 2000). Wu et al. (2007) meldden dat kaempferolglycosiden *Thrips palmi* in guldenroede (*Solidago altissima* L.) afschrikken.

Planten moeten zich tegen aanvallen verdedigen middels verdedigingsroutes die per plaag of ziekte kunnen verschillen. Als gevolg hiervan zijn trips, naast de hierboven genoemde triterpenoïde saponinen en aminozuren, resistent tegen de fenolverbinding kaempferol als een van de resistente metabolieten die typisch in Gladiolus nanus voorkomen. Fenolen zoals kaempferol-glycosiden vertonen een tegenovergesteld patroon als reactie op infectie met *Spodoptera littoralis* in maïs (Marti et al., 2013). Kaempferol 3-O-rutinoside was verminderd bij aanvallen van herbivoren. Forkner et al. (2004) rapporteerden dat tannines net als fenolen functioneren als anti-stoffen tegen de bladetende insecten *Acronicta increta* (Noctuidae) en *Attelabus sp*. (Curculionidae) op *Quercus velutina* en *Q. alba*. Metaboliet profielen van verschillende soorten Gladiolus werden duidelijk van elkaar gescheiden in multivariate analyses, zowel voor als na het inbrengen van trips. Daarentegen werden metaboliet-profielen niet duidelijk beïnvloed

door de aanwezigheid van trips. Het is mogelijk dat trips slecht een klein deel van het blad beschadigen, waardoor de afweer enkel lokaal wordt aangeschakeld en dus de metaboliet profielen alleen lokaal veranderen. De lokale veranderingen na de beperkte schade kunnen echter verloren gaan in het algehele metaboliet karakterisering. Metaboliet profielen werden bepaald op basis van een monster van een geheel blad. Voor de meest gevoelige cultivar (Charming Beauty) die tripsschade vertoonde over de hele plant, sluit dit hoogstwaarschijnlijk de mogelijkheid uit dat lokale afweerinductie heeft plaatsgevonden. Voor de meest resistente cultivar Robinetta met zeer lokale tripsschade kunnen we deze mogelijkheid niet uitsluiten en zou meer analyse nodig zijn om te onderzoeken of het selecteren op verhoogde lokaal geïnduceerde afweer zou kunnen leiden tot een significante vermindering van de schade. Een beperking van deze studie is dat aanschakeling van de afweer slechts op één moment in de tijd werd gemeten. Misschien zou op een eerder of later moment na infectie wel een zekere mate van verandering in metaboliet-profielen kunnen worden waargenomen.

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