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**Author:** Wahyuni, D.S.C.

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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. Leaf 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. Cardiosin 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 then 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 result 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 than 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 Jankapaa *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 not 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 Jankapaa *et al.* (2012) as light-intensity dependent compounds in *Arabidopsis thaliana*. Valine was strikingly higher in plants grown under low light (30  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) conditions, alanine had higher concentrations in high light (600  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and normal light (300  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). 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 deterred *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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