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Chapter 7

Summary and conclusions
Secondary metabolites (SMs) are not directly involved in plant growth and development while they are involved in the interaction between plants and their environment (Hartmann 2007). Over the years and with the development of technology, more and more SMs were discovered and an ever increasing number of chemical structures have been identified (Wink 1999). Moreover, within plant species SMs show a high diversity both in terms of composition and concentration (D’Auria and Gershenzon 2005; Fahey et al. 2001; Langenheim 1994).

The interest in the effects of SMs on plant-environment interactions has boosted an increasing number of ecological studies (Schoonhoven et al. 2005). This thesis contributes to this field by using a typical class of SMs in *Jacobaea* plants, pyrrolizidine alkaloids (PAs), as a study system. Based on the chemical structures and the biosynthesis pathway, PAs can be categorized into four groups: senecionine-like PAs, jacobine-like PAs, erucifoline-like PAs and otosenine-like PAs (Cheng et al. 2011a). This clear grouping gave us the opportunity to explore the variation in PA composition and the effects of this variation on plant resistance against insect herbivores.

In chapter 2 I described the seasonal variation of PA concentration and composition in nature. In chapters 3 and 4 I explored the PA diversity by testing two hypotheses: 1) do different structurally related PAs provide resistance against different herbivores? and, 2) do generalist and specialist herbivores exert opposing selection forces on the PA concentration? In chapter 5 I discussed if the PA concentration and composition were induced or affected by the exogenous application of the phytohormone methyl jasmonate. Finally, in chapter 6, I studied the effects of phytohormone application on the composition of SMs, using NMR analyses, and on resistance to different types of herbivores.

**Chapter 2.** How are PAs affected by nature’s seasonal variation? A series of clones of 8 genotypes differing in their alkaloid composition were investigated over a 14 months period in an experimental garden. Besides herbivory, plants in nature also need to deal with other stresses, such as a fluctuating temperature, frost and periods of drought. Plants were harvested every 2 to 3 weeks to study plant growth and PA concentration and composition over the course of the vegetative stage. We chose the parents, 2 F1 and 4 F2 genotypes from a cross between *Jacobaea vulgaris* and *Jacobaea aquatica* that is maintained in tissue culture. We selected the F2 genotypes on the basis of differences in alkaloid concentration and composition. In contrast to other studies we followed the same genotypes over the whole season and therefore could distinguish the seasonal variation from genetic variation.

It was observed that after an steady increase during summer the shoot and root dry mass dropped by about 70% during the winter period (February) before they recovered during the next spring. The variation of the total PA amount was strongly correlated with that of the dry mass and together with the loss of dry mass
in winter a large part of the PAs was lost. Senecionine- and otosenine-like PAs were mainly stored in the roots while the erucifoline- and jacobine-like PAs were mainly stored in shoots. In both roots and shoots the total PA concentration increased gradually until the end of winter and then during spring it decreased, particularly in the shoots. For the 6 genotypes rich in jacobine-like PAs, in the shoots the proportion of jacobine-like PAs showed a decrease during winter, followed by a strong increase in the next spring. In contrast, in the roots a gradual increase of jacobine-like PAs and a decrease of senecionine-like PAs was observed over the whole period. The free base/N-oxide ratio was relatively constant over the seasons until the second spring. Thereafter it sharply increased, most notably in the *J. vulgaris* parent plant. While the genotypes differed in PA composition the direction of the seasonal changes was rather similar for the different genotypes. As a result the variation among genotypes was maintained across seasons.

**Chapter 3.** What is the relative importance of PAs in the oviposition choice of a specialist herbivore? We tested the oviposition preference of the cinnabar moth, *Tyria jacobaeae*, with regard to plant size, nitrogen content, water content and PA concentration and composition. Moths were collected from Meijendel, a regularly defoliated area and from Bertogne, a rarely defoliated area. Ragworts found in coastal areas (Meijendel) are rich in jacobine-like PAs while those that occur inland (Bertogne) are rich in erucifoline-like PAs. Based on the PA composition of the host plants from which the cinnabar moths were collected we selected plant genotypes from the cross described above to represent ranges in both jacobine- and erucifoline-like PA concentrations. We performed choice experiments in experimental cages to study oviposition preference.

Water and nitrogen content did not vary strongly between the plants and were not correlated with oviposition preference. Moths from both origins laid larger egg batches on plants rich in jacobine-like PAs. However, the effects of PA composition were small although significant. In addition we found that moths originating from Meijendel and Bertogne had different oviposition behaviour: moths from Meijendel preferred larger plants and spread their eggs over more egg batches compared to moths from Bertogne. This difference in oviposition behaviour seems adaptive. A preference for larger plants and a bet hedging strategy by producing more and smaller egg batches makes sense for the Meijendel moths that are regularly confronted with complete defoliation of their host plant.

**Chapter 4.** What is the potential role of herbivores in maintaining PA diversity? Following up a study of Cheng (2012), the generalist herbivore slug *Deroceras invadens* and the specialist flea beetle *Longitarsus jacobaeae* were tested in the same set of F2 hybrids of *Jacobaea* plants differing in PA concentration and composition. No choice and multiple choice feeding tests *in vivo* were conducted respectively for the slug and the flea beetle.
Slug feeding damage was genotype dependent. It was negatively affected by total PA concentration and the concentration of senecionine-like PAs. The common structure of the individual PAs that were significantly negatively correlated with slug feeding is a double bond between the C13 and C19 position. PA concentration and composition did not influence the feeding of flea beetle. Plant size was an important factor for the feeding preference of this specialist.

In this study I combined all the previously tested generalist and specialist herbivores with the same F2 system in order to test the Generalist-Specialist Dilemma. All preferences or performances of the 4 generalist herbivores showed negative correlations with at least one group of PAs while the preference and performance of the specialist *T. jacobaeae* was positively correlated with PAs and *L. jacobaea* was not influenced by PAs. Therefore, this chapter provided evidence for the Generalist-Specialist Dilemma hypothesis.

**Chapter 5.** Are PAs induced by herbivory? Methyl jasmonate (MeJA) application was used to mimic herbivory. A range of concentrations of MeJA was added to the medium of tissue culture plants of *J. vulgaris* and *J. aquatica* under axenic conditions. We chose control plants and plants from one MeJA treatment (1.00 µg µl$^{-1}$) to test if the feeding of the generalist herbivore *Spodoptera exigua* was affected by MeJA application.

The total PA concentration in both species at the whole plant level was marginally or not affected by applying different amounts of MeJA to the roots. This indicated that applying MeJA does not elicit *de novo* synthesis of PAs. In *J. vulgaris*, the total PA concentration in roots decreased and in shoots increased, suggesting that PA reallocation took place from root to shoot. In *J. aquatica*, however, this reallocation was not significant. The latter may be due to the fact that the majority of PAs were already stored in the shoots without applying MeJA. Interestingly, we found that the application of MeJA in both species induced a strong shift in the PA composition especially from senecionine-like PAs to erucifoline-like PAs, indicating that PA conversion took place. For instance, the relative concentration of erucifoline-like PAs increased from an initial 6.2% before MeJA application to 54.7% at the highest concentration (5.00 µg µl$^{-1}$) of MeJA while that of senecionine-like PAs dropped from 81.1% to 28.7% in *J. vulgaris* roots. Senecionine was far less toxic in cell lines of *S. exigua* than erucifoline (Nuringtyas et al. 2014). However, other studies showed that the number of pupae of *Liriomyza trifolii* was positively correlated with erucifoline-like PAs (Cheng 2012) while ragworts of the erucifoline chemotype were the least attacked by *T. jacobaeae* (Macel and Klinkhamer 2010). It remains, however, unclear why no increase in jacobine-like PAs was found because this type of PAs is strongly bioactive as well.

Comparing with the *J. aquatica* control plants, *S. exigua* ate approximately 40% of the leaves treated with MeJA. *S. exigua*, however, barely fed on the leaves
of control plants as well as MeJA treated *J. vulgaris* plants. This result suggested that an increase of erucifoline-like PAs induced by MeJA application played a role in resistance to *S. exigua* in *J. aquatica* while other defence mechanisms might be present in *J. vulgaris*.

**Chapter 6.** What is the relative role of SMs other than PAs in resistance to herbivores? I studied the changes in concentrations of other chemical metabolites by NMR after MeJA and SA application on *J. vulgaris* and *J. aquatica* and explored how the phytohormone treatments affected the feeding of the chewing caterpillar *Mamestra brassicae*, the cell-content feeding leafminer *L. trifolii* and the piercing-sucking thrips *Frankliniella occidentalis*.

Significantly less feeding damage by the herbivores *M. brassicae* and *L. trifolii* was observed on plants treated with MeJA while no significant differences were found between control and SA treated plants in both species. Thrips damage was significantly reduced in *J. aquatica* plants treated with SA while thrips feeding was not affected by SA treatment in *J. vulgaris*. PCA analysis showed a clear distinction between the metabolome of MeJA treated, SA treated and control plants. Among the 16 identified compounds, the peak intensity of jacaranone and isoleucine increased 2-3 times after MeJA treatment. Thus the reduced feeding by the chewing herbivore *M. brassicae* and cell-content feeder *L. trifolii* might be related to the increased concentration of these compounds. Moreover, a previous study showed that the number of emerging pupae of *L. trifolii* was positively correlated with erucifoline-like PAs and the feeding preference of *M. brassicae* was not influenced by senecionine-like PAs (Cheng 2012; Macel et al. 2005). Therefore, it is likely that in MeJA treated plants, PAs were not the cause of the reduced feeding damage by these two herbivores.

SA application did not lead to significant changes in PA concentration and composition (Yan, unpublished data) while there was significantly less thrips silver damage in SA treated plants. This study indicated that the relative role of PAs in resistance to these three herbivores was small. To find candidate compounds in thrips resistance, we combined available data for thrips silver damage (Cheng et al. 2011b) and NMR analysis (Kirk et al. 2012) for a segregation F2 population of a cross between *J. vulgaris* and *J. aquatica* and identified NMR bins that were positively or negatively correlated with silver damage. We then checked whether or not these bins were down-regulated or up-regulated after SA treatment in *J. aquatica*. This analysis lead to three candidate compounds related to susceptibility (sucrose, glucose and 3,5-DCQA) and three compounds related to resistance (citric acid, threonine and alanine). The positive effects of sucrose, glucose and 3,5-DCQA and the negative effects of citric acid on thrips survival were confirmed by results of unpublished in-vitro tests done previously in our research group. Literature data showed that β-alanine was positively correlated to thrips mortality.

**Discussion and conclusions**
The *Jacobaea* hybrid system has been proved to be a good tool to study the interaction between PA variation and herbivore resistance (Cheng, 2012). Based on this hybrid system, we have explored how the biotic and abiotic factors influence the PA diversity. All the herbivore tests were integrated to draw the general conclusion that the selection pressures from different herbivores contribute to the diversity in PA composition and that generalist and specialist herbivores exert a divergent selection pressure on PA concentrations. The PAs showed seasonal variation within each genotype which was the first study to report the seasonal variation of SMs that encompass a time series over all seasons and using distinct genotypes. It showed that the relative differences between genotypes remained constant over time an assumption that is often implicitly made for many studies. If natural selection is to act on PA diversity one of the prerequisites is that PA diversity does not change over time due to environmental differences and my results show that this is the case. These results confirmed the high heritability of PA concentration and diversity estimated from sib families raised at the same time point (Vrieling et al. 1993).

The studies of exogenous application of MeJA can lead to a shift in PA composition and primary metabolites. Shifts in diversity of SMs are underestimated as most studies only report the increase in classes of total metabolites. Importantly the shift in metabolite diversity induced by MeJA application can conversely affect the feeding of chewing herbivores and cell-content feeders. Plants enhance the defense abilities by changing the diversity of SMs. This strongly suggested that SM composition is more important for the plant survival comparing to the total concentration. For instance, senkirkine and seneciphylline showed a lower toxicity than jacobine and erucifoline to the cell lines of *S. exigua* (Nuringtyas et al. 2014). In thrips mortality tests, retrorsine jacobine are much more toxic than senecionine (Liu et al, unpublished). In contrast to MeJA application, SA application did not lead to significant changes in PAs and the metabolite profile changes detected by NMR rendered *J. aquatica* more resistance to piercing-sucking herbivore thrips showing that PAs did not affect thrips resistance. Several metabolites detected by NMR were found to be correlated with resistance (alanine, threonine and citric acid) and susceptibility (sucrose, glucose and 3,5-dicaffeoylquinic acid) to thrips *F. occidentalis*. These compounds are currently tested by *in-vitro* bioassay. Both phytohormone application studies confirmed the hypothesis that chewing herbivore and cell-content feeder activate the JA pathway while the SA pathway can be turn on by piercing-sucking herbivore. The effects of phytohormone application on herbivory might be plant species-dependent. Because all the experiments with phytohormones were conducted under axenic conditions, the differences of herbivore feeding were mainly from the metabolite changes induced by phytohormone application, showing the importance of metabolites in plant resistance.

With respect to the development of different analysis methods of chemical
compounds, metabolites have been extensively applied to the study of plant-environment interactions. In *Jacobaea* study system, the plants benefit from the PA diversity when plants confronted the pressures from the outside, such as herbivory and climatological variation. The PA diversity may be helpful for plants to adapt to the environment. Meanwhile, the environmental pressures exerted different selection forces to keep the PA diversity. In addition, the genetic map of this hybrid system has been constructed and quantitative trait locus analysis is going on. The importance of PAs on plant-insect relationship will be explored further.

The main findings of this study are concluded as following:

- Different generalist herbivores are deterred by different groups of PAs in plants and specialist herbivores can either use PAs as oviposition cues or they are not affected at all by PAs. Hence the selection pressure from different herbivores can drive PA diversity.
- We found only relatively small effects of the total PA concentration on herbivore feeding and oviposition. The latter indicates that the PA composition is more important than the total PA concentration for plant fitness.
- PA concentration and composition change over seasons, but the seasonal variation does not override the initial differences among genotypes.
- There is no reallocation of PAs from shoots to roots during the winter period. During the winter season a large proportion of PAs is lost together with a strong reduction in plant dry mass.
- MeJA application does not increase the total PA concentration, but it leads to a shift in PA composition from senecionine-like PAs to erucifoline-like PAs.
- MeJA treatment rendered plants of both species more resistant to the generalist herbivores *M. brassicae* and *L. trifolii* while resistance to *S. exigua* only increased in *J. vulgaris*. Interestingly, thrips were affected similarly in the two plant species by phytohormone treatment, but the feeding damage was only significantly reduced in SA treated *J. aquatica* plants.
- Jacaranone and isoleucine may be involved in the resistance to the chewing herbivore *M. brassicae* and cell-content feeder *L. trifolii*. Sucrose, glucose and 3,5-DCQA are related to thrips susceptibility while alanine, threonine and citric acid are related to thrips resistance.
Summary and conclusions

References


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