Summary and Discussion

The diversity of secondary metabolites (SMs) is a consequence of plant dynamic metabolism in interaction with changes of various external stimuli including herbivores. Secondary metabolites show highly divers patterns both among and within species. Within an individual, spatial distribution of SMs can be observed on the levels of organs, tissues and cells. SMs are thought to have evolved as an adaptation to biotic and abiotic stress. This study focused on the role of SMs in plant defence against pests.

Pyrrolizidine alkaloids (PAs) of the genus *Jacobaea* were chosen as a model system to study the diversity of SMs. PAs are present in non-related taxa and reported for the their large variation of concentrations and compositions within a species. Pyrrolizidine alkaloids are known as effective deterrents and toxins to most vertebrates and generalist insects. They are assumed to have evolved as part of the chemical plant defence under selection pressure of herbivores. In chapter 1, the current knowledge about the PA diversity, synthesis and toxicity is discussed. In addition the tools used in this study such as NMR metabolomics and laser microdissection (LMD) are introduced.

Nowadays, the influences of both the biotic and abiotic environments on PA diversity are well recognised. Plant fungal endophytes are part of the biotic environment. However the effect of endophytic fungi in *Jacobaea* plants is still unknown. In chapter 2, the influence of endophytic fungi on PA diversity is studied. Fungal endophytes were eliminated by treating the F2 hybrids of *Jacobaea* plants with three different fungicides: Folicur, Pronto Plus and Switch. Both, fungicide treated and non treated plants were able to perform PA synthesis, indicating that endophytes were not essential for PA production. Thus, *Jacobaea* plants are able to perform *de-novo* PA synthesis. Unexpectedly, one fungal endophyte species was detected in the Folicur treated plants. Based on the b-tubulin gene and the Internal Transcribed Spacer (ITS) region of the rDNA, the detected fungus had a close homology with the *Glomus* genus of mycorrhizal fungi belonging to the Glomeromycota. The Folicur treated plants showed a lower amount of total PAs compared to the control whereas the other fungicide treatments showed no differences. This PA reduction was particularly observed for senecionine- and jacobine- but not for erucifoline- and otosenine-like PAs. Underlining the recent accumulative information on PA synthesis, this result shows that the biotic environment contributes to variation in PA concentration and composition in *Jacobaea* plants. It is unclear how the presence of the *Glomus* fungal endophyte could lower the amount of PAs produced. It may be possible that this fungus may partly inhibit the *de novo* PAs synthesis. Alternatively, the fungus may catabolise PAs. Metabolomic analysis of the fungicide treatments showed that in the Folicur treated plants no unspecific effects of the fungicide treatment occurred.

Therefore, these metabolomic data support that an endophyte of the *Glomus* genus can increase PA variation in *Jacobaea* plants. This may have ecological consequences. The plants may become more susceptible to generalists or become less apparent to specialist herbivores. As shown in chapter 5 of this thesis jacobine-like PAs were present at higher concentrations in plants resistant to the generalist insect herbivore Western Flower thirps, *Frankliniela occidentalis*, compared to the susceptible plants. Furthermore, jacobine-like PAs showed one of the highest toxicities to the generalist Beet Army worm, *Spodoptera exigua*, as shown in chapter 6 of this thesis. On the other hand jacobine chemotypes of *J. vulgaris* were reported more attractive for the specialist Cinnabar moth, *Tyria jacobaea* (Macel and Klinkhamer, 2010). Further experiments to investigate the influence of *Glomus* genus in shaping PA composition may help to explain the results of previous studies that showed that the microbial composition of the soil influences both below and above ground PA concentration and composition (Joosten et al., 2009).

The results of chapter 2 supported the influence of the environment on PA composition and concentration. To better understand the mechanisms behind this we need to know where PAs are synthesised. The biochemistry such as precursors and metabolism of PAs is quite well understood. However, to our knowledge, there has been no study comparing the capacity of different plant organs to synthesise and transform PAs. The PA synthesis has only been compared between detached shoots from flowering plants and *in-vitro* root cultures (Hartmann et al., 1989). In chapter 3 we developed *in-vitro* cultures of roots, shoots and complete plants of five *Jacobaea* genotypes to study PA synthesis in different plant organs. Shoots were able to synthesise *de-novo* PAs while the literature so far suggested that PAs are only produced in the roots (Hartmann and Toppel, 1987). Total PA concentrations in the root cultures were low, in the shoot cultures intermediate (1.5 x the root culture) and in complete plant cultures high (3 x the root culture). This indicates that both roots and shoots are essential for PA synthesis. It may be possible that the lack of shoots may inhibit the transport and distribution of PAs and, subsequently, a feedback mechanism causes the roots not to produce *de-novo* PAs anymore. These results substantiate the future step to apply molecular techniques for detecting the expression of homospermidine synthase (HSS) and deoxyhypusine synthase (DHS). HSS is the enzyme catalysing the formation of the first intermediate of the alkaloid-specific pathway (Hartmann et al., 1988). Generally, HSS is not expressed in the shoots; however DHS from which HSS is derived, is expressed in all above ground organs of *Senecio vernalis* (Moll *et al*., 2002). Possibly, HSS was functionally replaced by DHS in the *Jacobaea* shoot cultures.

The root cultures mainly comprised senecionine- and otosenine-like PAs while jacobine- and erucifoline-like PAs were present in higher proportions in both shoot and complete plant cultures. The high proportion of otosenine-like PAs in the root cultures was observed specifically for onetine. This alkaloid was measured at two times higher concentrations in the roots compared to the shoots and the complete plant cultures. This suggests that the root cultures were able to perform the conversion of the retronecine to the otonecine base structure. The high levels of jacobine- and erucifoline-like PAs in the shoot and complete plant cultures indicate that the epoxidation process to form a cyclic ether ring in the necic acid structure of PAs mainly occurs in the above ground organs. Thus the shoots are essential for jacobine- and erucifoline-like PA diversification. The different PA composition between above and below ground plant parts of *Jacobaea* may be

due to the plants adaptation to its environment, especially in response to pathogens and insects. Indeed, in the cell culture study using *S. exigua*, jacobine and erucifoline were proven to be the most toxic PAs as described in chapter 6. Specifically jacobine was highly accumulated in *Jacobaea* plants resistant to *F. occidentalis* (Chapter 5). Previous reports confirmed the role of jacobine-like PAs in plant defence against leaf feeding insects, especially thrips (*F. occidentalis*) (Leiss et al., 2009; Cheng et al., 2012), and of erucifoline-like PAs against aphids (*M. persicae*) (Dominguez et al., 2008). Compared to the effects on insect herbivores, little is known about the effect of PAs on pathogens. A study using seven strains of the genus *Fusarium* and *Trichoderma* sp showed that PA extracts inhibited the growth rate of the pathogens (Hol and van Veen, 2002). However, all these studies used PA extracts. To our knowledge, no study looked at the effect of individual PAs on pathogens. In this regard, more *in-vitro* studies to confirm the toxicity of individual PAs on other leaf herbivores as well as on pathogens will be of interest to our understanding of the evolution of PA diversity.

The route of PA diversification still remains unclear. Previously two hypothetical biogenetic scenarios have been proposed. The first scenario suggests that formation of jacobine- and erucifoline-like PAs share the same biosynthetic route with the same enzyme being responsible for the transformation from senecionine *N*-oxide, while the otosenine-like PAs are formed independently as derivatives of senkirkine (chapter 2, Fig S.1A). The second scenario proposes that jacobine- and erucifoline-like PAs are synthesised independently from each other with otosenine-like PAs deriving from the jacobine type (Pelser et al., 2005) (chapter 2, Fig S.1B). In chapter 2, we reported that in the Folicur treated plants only the senecionine- and jacobine-like PAs but not the erucifoline- and otosenine-like PAs were affected by the presence of a mycorhizae closely related to Glomeromycota. These results indicate that the formation of jacobine-like PAs is independent from that of the erucifoline-like PAs as mentioned in the second scenario. However, the otosenine-like PAs are likely to fit the first scenario since this group of PAs was not affected by the treatment. The results of chapter 3 also support this proposition. The high amount of otosenine-like PAs in the root cultures but not in the shoot cultures supports that otosenine-like PAs, especially onetine are not likely to be synthesised from jacoline which has the same necic acid as onetine. We, thus, propose that PA diversification is possibly a combination of both schemes whereby diversification into jacobine-like PAs is independent from erucifoline-like PAs and the otosenine-like PAs are derived from senecionine-like PAs but not from jacobine-like PAs as we show in Fig S.1.C in chapter 2.

In chapter 3 we studied PA composition and distribution in shoots and roots as independent plant organs. Different PA compositions in root and shoots were observed. Jacobine- and erucifoline-like PAs were the most important PAs in the shoot. However, insect herbivores which attack above ground plants have various feeding patterns. Large insects may feed on whole plants or certain plant organs, but smaller ones may only feed on specific leaf tissues or cells. Indeed, plants and their organs possess at least around forty different cell types and twelve of these occur in the leaf. These different cells have their own specific biological functions that play different roles in plant growth, development, reproduction and defence. Therefore, each cell type is directed by its own unique chemical composition. However, less information is available about the variation of SMs within these different tissues or cells. In chapter 4 we used a NMR metabolomic approach, to study

the metabolite profiles of different types of *Jacobaea* leaf tissues: epidermis and mesophyll layers. Orthogonal partial least-squares-discriminant analysis (OPLS-DA) resulted in a clear separation of epidermis and mesophyll extracts. The epidermis contained significantly higher amounts of jacaranone and phenylpropanoids, specifically chlorogenic acid and feruloyl quinic acid if compared to the mesophyll. In contrast, the mesophyll showed significantly higher concentrations of PAs, specifically jacobine and jaconine. Chlorogenic acid (CGA) and PAs are known for their inhibitory effect on herbivores. Chlorogenic acid has been described as an anti-feedant and digestibility reducer against different insects including chewing insects such as caterpillars, leaf beetles and even against sucking insects such as aphids and thrips. Jacobine-like PAs have also been shown to be effective against thrips. Thrips feeding commences with the penetration of the epidermis, followed by ingestion of the contents of sub-epidermal or mesophyll cells. In this case, thrips may encounter CGA in the epidermis as the first line of defence, before encountering the PAs as the ultimate defence in the mesophyll. Therefore, the two layers accumulating two different types of SMs, with different effectiveness, might be one of the plant strategies to ensure that the plants are well protected.

To study the metabolomic profile of different cell types in more detail we conducted a cell specific metabolomic study based on the rapid advancement of single cell technology as described in chapter 5. We applied laser microdissection (LMD), one of the most advanced techniques in single cell isolation, to collect epidermis, palisade- and spongy-mesophyll cells of two *Jacobaea* genotypes: a thrips resistant and a susceptible one. This was followed by cryo probe-NMR to analyse the cell metabolomes. We confirmed that CGA and jacobine *N*-oxide were distributed in different cells of *Jacobaea* leaves. Independent of genotype, CGA accumulated in the epidermis cells while jacobine *N*-oxide was present in the palisade mesophyll cells. In the thrips resistant genotype we detected a higher accumulation of jacobine *N*-oxide but no higher levels of CGA. So far, jacobine-like PAs have been associated with plant defence in *Jacobaea* against generalist herbivores, such as thrips. However, this type of PA is also known to have a positive effect on specialists, which use them as host finding cues. The Cinnabar moth, *Tyria jacobaea*, the specialist thrips *Haplothrips senecionis* and the obligate biotroph Rust fungus *Puccinia lagenophorae* all preferred *Jacobaea* genotypes rich in jacobine-like PAs (Macel and Klinkhamer, 2010). The specific distribution of jacobine *N*-oxide in the palisade-mesophyll cells may thus constitute a strategy to deal with the generalist-specialist dilemma. Thus placing jacobine in the palisade cells, away from the leaf surface may prevent jacobine from being used as a host recognition cue. Further experiments will be needed to prove this hypothesis.

In chapter 3 we observed that diversification of PAs into jacobine and erucifoline took place mainly in the shoot culture. This may be the result of an adaptation to plant defence against above ground herbivores. However, the biological effect of jacobine (Leiss et al., 2009a; Cheng et al., 2011; Joosten, 2012) and erucifoline has mainly been based on correlative studies (Macel, 2003; Macel and Klinkhamer, 2010). So far they had not been tested as individual PAs. We, therefore, in chapter 6, isolated jacobine and erucifoline from their respective *Jacobaea* chemotypes and tested these as well as some other commercially available PAs including senecionine, seneciphylline, retrorsine, and senkirkine as free base and *N*-oxide forms against the generalist herbivore *S. exigua*. At the same time CGA was tested individually as well as in combination with the respective PAs. Tests

included *in-vitro* cell culture as well as injection bioassays of larvae. In both bioassays jacobine was the most toxic PA, followed by erucifoline, senkirkine and seneciphylline, while senecionine as well as CGA were not toxic at the tested concentrations. The combination of CGA with PAs reduced PA toxicity in all cases. For all PAs the free base form showed a higher activity compared to the respective *N*-oxide form. These results show that in addition to the necine base, the toxicity of PAs is influenced by the functional groups of the necic acid moiety. Both, jacobine and erucifoline, the most toxic PAs are retronecine macrocyclic diesters with epoxide functional groups. This functional group may facilitate the conversion into the reactive pyrrole intermediates, the basis of PA toxicity. Furthermore, these more complex necic acid moieties could be more resistant to detoxification by esterases and decrease their water solubility for excretion.

In conclusion, we revealed a specific pattern of PA accumulation within *Jacobaea* plants. This specificity opens a new possible explanation for the generalist-specialist dilemma in PAs as defence compounds. PAs which are important defence compounds against generalist insects were accumulated in the middle layer of the leaf possibly reducing their recognition by specialist insects using these PAs as host cues. This strategy is very suitable for plant defence against the larger specialist insects feeding on the whole leaf, such as caterpillars, as well as generalist small insects attacking specifically the mesophyll part of the leaf such as cell feeding insects. In the later case we also observed a multiple defence strategy. Compounds such as CGA are accumulated in the outer layer of the leaf serving as first line defence before cell feeders encounter the ultimate defence compounds, PAs. In addition, CGA reduces PA toxicity and thus separation of these compounds into different tissues ensures PA activity against insects. The finding of cell specific defence may have a major impact on the studies of plant-insect interaction. Indeed each cell type has a specific chemical profile. Thus, the study of SMs at the tissue or cell level should be considered if studying insects that only attack certain tissue or cells. The specific distribution of SMs is also useful when studying the sites where metabolism takes place as well as the sites of metabolite accumulation and storage. In this thesis, the role of jacobine-like PAs in plant defence against generalist herbivores becomes more prominent. Jacobine-like PAs were the ones affected by the presence of mycorrhizae. They were the type of PAs characterising the shoot cultures in contrast to the roots. Moreover, this type of PAs was highly accumulated in the shoots of *F. occidentalis* resistant plants. Using cell culture and injection bioassays the toxicity of jacobine-like PAs against a generalist herbivore was confirmed. These findings point to the role of necic acid in determining PA toxicity. Further studies will be needed to understand the toxicity of jacobine to other generalist insects and to investigate the mechanism by which necic acid supports the formation of pyrolle intermediates, which form the basis of PA toxicity.

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