The ecological relevance of chemical diversity in plants: pyrrolizidine alkaloids in Jacobaea species

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Chapter 1

General introduction

Secondary metabolites

Since Friedrich Wilhelm Serturner isolated morphine from opium poppy in 1806, plant secondary metabolites (SMs) have been studied for more than 200 years. The concept of SMs was first introduced by Albrecht Kossel in 1891 (Hartmann 2007) and in the beginning of 20th century Ernst Stahl experimentally showed that SMs serve as defence compounds against snails and other herbivores. SMs have been demonstrated to play a major role in the adaptation of plants to their abiotic and biotic environment (Bourgaud et al. 2001). With the improvement of analytical techniques in the middle of 20th century, more and more SMs were discovered and identified (Bourgaud et al. 2001).

What are secondary metabolites?

Secondary metabolites: a) have no direct implication for the growth and development of plants, b) have a distribution which is sometimes confined to a genus or species, c) are sometimes accumulated in high concentrations (1-3% fresh weight), d) may be very toxic or deterrent to herbivores and pathogens, e) may have a marked biological effect on other organisms, f) frequently have different production and accumulation sites, and g) are often accumulated in the vacuoles in a glycosidic form or in special secretory structures, e.g. trichomes, ducts, canals and laticifers (Figueiredo et al. 2008).

The most characteristic feature of SMs is the high structural diversity among and within plant species. Over 500 000 SMs have been discovered in plants, of which about 100 000 structures have already been described (Hadacek 2002). The diversity within plant species can be well illustrated by the fact that more than 170 SMs belonging to seven major classes have been identified in *Arabidopsis thaliana* (D'Auria and Gershenzon 2005). Around 20 structures in the phenylpropanoids class and 36 different glucosinolates were found (Kliebenstein 2004). However, it is still an open question how such a diversity of SMs evolved and is maintained in plants.

What are the functions of secondary metabolites?

Secondary metabolites, at least the major ones present in plants, apparently function as defence (against herbivores, microbes, viruses or competing plants) and signalling compounds (to attract pollinating or seed dispersing animals) (Wink 2003). They are thus important for the plant's survival and reproduction. The functions of SMs are summarized in Figure 1. They all are involved in the interactions between plants and their environment.

Since herbivores and microbes were already present when the evolution of angiosperms started about 140 million years ago, plants have evolved defence chemicals to ward off, inhibit or kill their enemies (Wink 2003). In the history of evolution, SMs, such as alkaloids, glucosinolates, terpenes and tannins, protected plants against virus, bacteria, fungi, competing plants and most importantly against herbivores, particularly generalist herbivores. SMs do not protect plants against all herbivores. Specialist herbivores have adapted to the SMs in their host plants and can use SMs as cues for finding host plants and oviposition and even sequester SMs for their own benefits (von Nickisch-Rosenegk and Wink 1993).

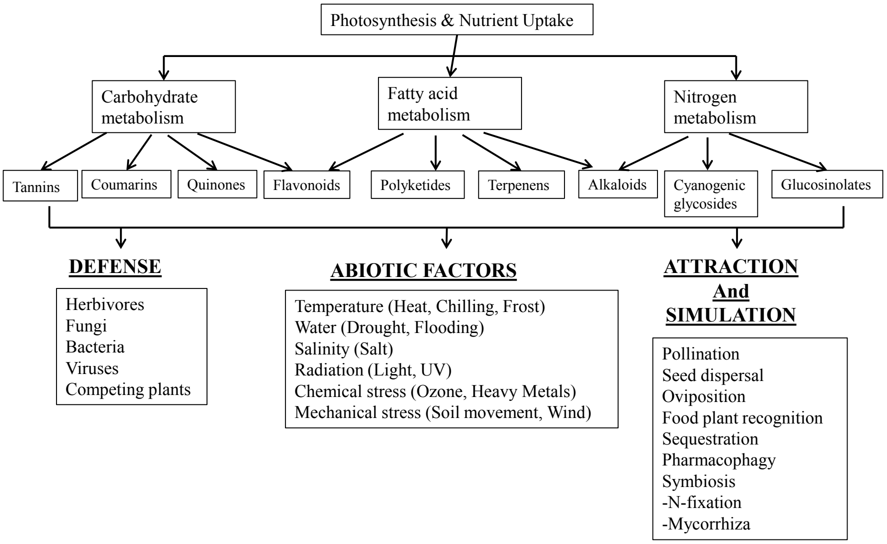

Fig. 1 Secondary metabolites originate from common precursors of primary metabolism. The functions of SMs concern all aspects of plants' chemical interactions with the environment (adapted from Hartmann 1996).

As signal chemicals, SMs can be the attractants of animals for pollination (fragrances, colours) or seed dispersal (Kessler and Baldwin 2007). In addition, SMs may protect plants against abiotic stresses such as UV-light, drought stress and frost (Rivero et al. 2001; Vallat et al. 2005).

Which factors affect secondary metabolite concentration and composition?

A wide range of abiotic environmental factors, e.g. temperature, humidity, light intensity, the supply of water, minerals and ozone, influence the growth of plants and their SM production. Here I list just a few examples. Low temperature is one of the most harmful abiotic stresses affecting temperate plants. Plants have adapted to fluctuations in temperature by adjusting their metabolism during autumn, increasing their content of cryo-protective compounds to maximize their cold tolerance (Janská et al. 2010). Thermal stress induces the accumulation of phenolics in the plant by activating their biosynthesis as well as inhibiting their oxidation in tomato and watermelon (Rivero et al. 2001). Drought stress is usually accompanied by high temperature and little rainfall. Drought caused an increase in the amounts of flavonoids and phenolic acids in willow leaves (Larson 1988). Camphene emissions were negatively correlated with rainfall, further supporting that drought can result in higher production of SMs (Vallat et al. 2005). Light can stimulate gingerol and zingiberene production in *Zingiber officinale* callus culture (Anasori and Asghari 2009). Although UV-B is a minor component of sunlight, it affects the production of various SMs, such as flavonoids, tannins and lignin (Rozema et al. 1997). All the factors mentioned above can change drastically during the seasons. It is therefore to be expected that the concentration and composition of the SMs in plants change significantly during the seasons too. However the seasonal variation in SM composition have not often been studied yet because the majority of studies are conducted under controlled conditions.

In addition to abiotic factors SM concentration and composition are known to be influenced by biotic stresses as well. Many SMs are only produced or their production is strongly upregulated after attack by herbivores or pathogens. The plant's response is regulated by several phytohormones. It is well known that jasmonate (jasmonic acid (JA), methyl jasmonate (MeJA)) and salicylic acid (SA) are signal molecules in response to biotic and abiotic stresses. The JA pathway is hypothesized to be upregulated if the plant is attacked by chewing-biting herbivores, cell-content feeders and necrotrophic pathogens (Glazebrook 2005; Walling 2000). While the SA pathway is hypothesized to be activated especially in response to piercing-sucking insects and biotrophic pathogens (Kawazu et al. 2012; Walling 2000). MeJA and JA are able to elicit the production of several compounds, such as terpenoids and phenolic phytoalexins, in many plant species (van der Fits and Memelink 2000). For instance, jasmonate application increased total shoot glucosinolate levels 1.5-3 times in two *Brassica* species, while glucosinolate levels in the root did not increase (van Dam et al. 2004). Exogenous addition of MeJA to roots of *Nicotiana attenuata* induced *de novo* nicotine synthesis and increased the whole-plant nicotine concentration (Baldwin 1996). Alkaloid concentrations also increased after MeJA treatment in *Catharanthus* and *Cinchona* seedlings (Aerts et al. 1994). In addition, SA application can increase the plant's tolerance to salt and drought stresses (Bastam et al. 2013; Jesus et al. 2015). Salicylic acid increased the release of hyoscyamine and scopolamine (2-12 fold) in root cultures of *Brugamansia candida* (Pitta–Alvarez et al. 2000). However, studies on the effects of phytohormones on SMs focus on the variation of the total concentration of particular groups of SMs such as the total glucosinolate concentration. The variation of SM composition within a group of structurally related SMs after attack by herbivores or phytohormone application has largely been overlooked.

How is the diversity of secondary metabolites maintained?

At least four hypotheses were proposed to explain the diversity in SM composition: a) the neutral selectivity hypothesis states that most SMs have no distinct function for the plants and provide neither costs nor benefits in relation to plant fitness (Firn and Jones 2003; Firn and Jones 2009; Jones et al. 1991), b) the arms race hypothesis proposes that the diversity of SMs is the result of coevolution between plants and herbivores (Ehrlich and Raven 1964), c) the synergistic effects hypothesis proposes that a mixture of SMs has a stronger toxic or deterrent effect on herbivores than individual ones and d) the selection from multiple herbivores hypothesis proposes that each specific SM provides resistance against a specific group of herbivores.

Besides the enormous diversity in composition, SM concentrations also reveal a large variation. Such variation can be explained by shifts in the balance between benefits and costs of SM production (Herms and Mattson 1992; van der Meijden 1996). Plants benefit from the SMs which protect them from external threats. On the other hand, the production of SMs is assumed to be costly and to reduce the investment to growth and reproduction.

In addition to the investment costs, SMs can also bring about ecological costs. The variation of SM concentration in plants can also be explained by the Generalist-Specialist Dilemma. van der Meijden (1996) proposed that the contrasting effects of plant SMs on specialist and generalist insect herbivores would maintain the variation of plant defence concentration and would prevent plants from having high concentrations of SMs. Generalist herbivores can be deterred by high levels of defence compounds while specialist herbivores may be attracted by the same compounds. Even low concentrations of the defence compounds already may act effectively as oviposition or feeding stimulants. An example of contrasting effects on defensive trait is the opposing selection pressure from specialist aphids and generalist slugs and snails on the defence compound sinigrin in *Brassica nigra* (Lankau and Strauss 2008). By independently manipulating the specialist or generalist through removal experiments in the field, it was found that generalist damage was negatively correlated but specialist loads were positively correlated with sinigrin concentrations. Plants with a high sinigrin concentration had an advantage when specialists were removed, and had a disadvantage when generalists were removed. When both generalists and specialists were present sinigrin concentrations were selectively neutral. This was an elegant study but it still needs further research to test whether this dilemma applies other study systems as well.

To investigate the SM diversity and the role of SM in plant-environment interactions, a typical group of SMs, pyrrolizidine alkaloids (PAs), was studied in this thesis. In the following, I restricted myself to this particular group of SMs.

Pyrrolizidine alkaloids

Pyrrolizidine alkaloids (PAs) are a diverse group of SMs in plants. More than 400

PAs have been identified from ca. 6 000 angiosperm species (Chou and Fu 2006), of which over 95% are mainly present in four families: Asteraceae, Boraginaceae, Fabaceae and Orchidaceae (Langel et al. 2011). PAs are present in two forms: tertiary amines and N-oxides (Rizk 1990).

How are pyrrolizidine alkaloids synthesized and transported?

PA biosynthesis has been extensively studied in *Senecio* (Hartmann and Ober 2000). Senecionine N-oxide was identified as the primary product of biosynthesis in all *Senecio* species studied so far and can be regarded as the backbone structure of most PAs (Hartmann and Toppel 1987). It is synthesized in the roots and translocated to the shoots via the phloem (Hartmann et al. 1989; Sander and Hartmann 1989), where it is transformed into the species-specific PA profiles in *Jacobaea vulgaris* (Hartmann and Dierich 1998). Although PAs are spatially mobile within the plant, they are stored in vacuoles (Ehmke et al. 1988) and typically accumulate in the inflorescences and the peripheral stem tissues, i.e. epidermal and sub-epidermal cell layers (Hartmann et al. 1989; Hartmann and Zimmer 1986). Tracer studies with ^{14}C-labeled senecionine N-oxide applied to a number of *Senecio* species (i.e., *S. erucifolius*, *S. inaequidens*, *S. jacobaea*, *S. vernalis* and *S. vulgaris*) revealed that the structural diversification of senecionine N-oxide requires just one or very few enzymatic modifications (Hartmann and Dierich 1998). Besides structural diversification, PAs do not show any turnover or degradation (Hartmann and Dierich 1998; Sander and Hartmann 1989).

PAs are present in two forms: tertiary amine and N-oxide (Rizk 1990; Wiedenfeld et al. 2008). PA N-oxides are better soluble in water and therefore better suited for phloem transport and vacuolar storage within the plants, so most types of PAs almost exclusively occur as N-oxides (Hartmann et al. 1989). 37 PAs were identified in *Jacobaea* species, and based on chemical structures and the biosynthetic pathway, they are categorized into four groups: senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Cheng et al. 2011a).

What are the roles of pyrrolizidine alkaloids in plant-insect interactions?

Effects of PAs on generalist herbivore preference and performance

Studies using artificial diets or other substrates (e.g. lettuce, filter paper) to which PAs were added showed a negative effect of these alkaloids on different species. Several insects (nymphs, grasshoppers, thrips, caterpillar and aphids) were deterred by PAs or showed lower survival or smaller larval weights (Dreyer et al. 1985; Hagele and Rowell-Rahier 2000; Leiss et al. 2009; Macel et al. 2005; van Dam et al. 1995). In these bioassays, different PA groups or individual compounds were involved and they had different effects on generalist herbivores. In general the tertiary amine PAs were more toxic than the N-oxides as shown in the feeding tests of *Acyrthosiphon pisum* and *Spodoptera exigua* (Dreyer et al. 1985; van Dam et al. 1995).

In-vivo tests also presented good evidence of the detrimental role of PAs on generalist herbivores (Cheng 2012; Cheng et al. 2011b; Kostenko et al. 2013; Kostenko et al. 2012). Silver damage of thrips and larvae number of leafminer were negatively correlated with jacobine-like PAs and otosenine-like PAs. Correlative studies, however, do not show causation and the results of these correlative studies still needs to be backed-up by confirmation in *in-vitro* test. Unfortunately *in-vitro* tests are often limited by the availability of the relevant compounds.

Effects of PAs on specialist herbivore preference and performance

Specialist herbivores do not suffer from negative effects of PAs present in their host plants. The most widely studied specialist herbivore is the cinnabar moth *Tyria jacobaeae*. The pupal weight and larval development did not differ significantly among 11 populations of *J. vulgaris* with varying PA composition constituting different chemotypes (Macel et al. 2002). In natural populations, the total PA content was not an important factor in host plant selection of the cinnabar moth (van der Meijden et al. 1989). However, in a cage experiment the moths preferred laying eggs on *Jacobaea* plants with a high concentration of tertiary amines of jacobine-like PAs (Cheng et al. 2013). In a field experiment, it was found that chemotypes containing jacobine were more severely attacked by specialist herbivores than chemotypes without jacobine in the period of the year prior to *T. jacobaeae* damage (Macel and Klinkhamer 2010).

Another specialist herbivore of ragwort is the flea beetle *Longitarsus jacobaeae*. The relation between feeding damage of *L. jacobaeae* and PA concentration in plants ranged from positive to negative (Stastny et al. 2005; Vrieling and van Wijk 1994). With 17 plant species containing PAs, Kirk et al. (2012) found no evidence that intra-generic differences in PA profiles affect feeding by this specialist herbivore.

In PA diet feeding test, Hagele and Rowell-Rahier (2000) applied seneciphylline N-oxide on the leaves of *Petasites fragrans* (Asteraceae) and observed that the specialist herbivores (*Oreina cacaliae*, *Oreina speciosissima*, *Aglaostigma discolour*) did not show any preference for leaves with or without seneciphylline N-oxide. Interestingly, the larvae of the arctiid moth *Callimorpha dominula* did not discriminate seneciphylline N-oxide when naive, but preferred to feed on this PA when experienced. This moth is probably a PA-specialist (Hagele and Rowell-Rahier 2000). Altogether, these studies suggest that specialist herbivores exert none or a negative selection on PAs in plants.

What external factors affect the pyrrolizidine alkaloids content?

Effects of abiotic factors on pyrrolizidine alkaloids

Plant organs vary in their response to increased fertilization. Total PA concentration in flowers was constant while the PA concentration significantly decreased with increasing nutrients in both roots and shoots (Hol 2011; Hol et al. 2003). The total PA concentration in roots and shoots was influenced by both soil moisture and nutrient availability (Salmore and Hunter 2001). Kirk et al. (2010) found that the PA concentration was higher in plants grown on sandy soils without nutrients compared to plants grown on sandy soils with nutrients. This discovery was consistent with resource availability hypothesis, which postulates that plants should make higher investments in defense in the absence of abundant resources (Coley et al. 1985). Therefore PA concentration are expected to be higher when nutrient availability is low. A proximate explanation for this is given by the fact that when plants are grown in soils with low nutrient availability they produce more roots and less shoots. Because PAs are produced in the roots a higher root/shoot ratio gives a higher production capacity for PAs relative to plant size.

The influence of soil moisture and nutrient availability on PA diversity in the shoots was small. In the roots, PA richness increased with increasing soil moisture. This suggests that differences in PA richness may play a large role in below-ground plant defense because pathogenic pressure will rise increasing soil moisture (Brockett et al. 2012; Kirk et al. 2010; Schafer and Kotanen 2003; van de Voorde et al. 2012).

The concentration of PAs in *J. vulgaris* was influenced by the interaction of season and vegetation successional stage (Carvalho et al. 2014). Karam et al. (2011) found that the combined aerial plant parts (stems, flowers, and leaves) had the highest concentration of total PAs in the spring (October), and in contrast, the lowest PA concentration was measured during the summer collection (January) in *Senecio madagascariensis* in Brazil. In *Senecio riddellii* and *Senecio longilobus* leaves the highest PA concentrations were observed in summer (between July and September) in the United States while in *S. jacobaea* no evident relationship between the PA content and the time of year was found (Johnson et al. 1985). Although these studies indicated seasonal variation of SMs, there are still no studies that encompass a time series over all seasons and that used distinct genotypes.

Effects of biotic factors on pyrrolizidine alkaloids

In rosettes of *S. jacobaea*, neither biomass removal nor incision were able to induce PAs (Vrieling and Bruin 1987). By removing half of the leaf area with a pair of scissors, van Dam et al. (1993) found that the PA concentration in damaged leaves slightly decreased within 6-12 hours but the induction ratio was back to the initial value after 24 hours. Root damage increased PA concentration in the roots while there was only a weak response in the shoots of *J. vulgaris* (Gera Hol et al. 2004).

Kostenko et al. (2013) found that root herbivory by the wireworms *Agriotes linearus* had a strong negative effect on the total PA concentration in the shoots of *J. vulgaris* while there was no significant effect on the PA concentration and composition in the roots. They did find an increase of acetylerucifoline and acetylerucifoline

N-oxide concentration in the shoots after root herbivory while most senecionine-like PAs decreased. Shoot herbivory by *Mamestra brassicae* decreased the total PA concentration in the roots (Gera Hol et al. 2004). The erucifoline concentration in the shoots increased while the concentration of senecionine in the roots decreased after shoot herbivory (Gera Hol et al. 2004). Altogether these results indicate that the effects of herbivory depend on the herbivore species. However, so far we only have a fragmented picture. Phytohormones are widely used to mimic herbivory. As mentioned above, alkaloid concentrations increased after MeJA treatment in *Catharanthus* and *Cinchona* seedlings (Aerts et al. 1994). It is unclear whether this also happens in *Jacobaea* species because there are no studies on the effects of exogenous application of phytohormones on PA content in these species yet.

In this thesis I used the PA containing species, *Jacobaea vulgaris*, *Jacobaea aquatica* and their hybrids. The parental plants, the 2 F1 hybrids and 102 F2 hybrids are kept in tissue culture. This hybrid system gives a good opportunity to study the interaction between plants and their environments. Firstly, the segregating hybrids have a large variation in PA content and herbivore resistance (Cheng et al. 2011a). Moreover the separate traits can be studied against the same genetic background. Because all the individuals are maintained in tissue culture, they can be cloned into any number of replicates for the experiments. The same genotypes can be used within a single experiment and in different experiments.

Research questions

In this thesis, I investigate how the PAs play a role in plant-herbivore interaction and how the external factors influence the PA concentration and composition. The following questions are addressed:

1) How does seasonal variation affect PA concentration and composition of different genotypes of *Jacobaea* plants? (chapter 2)
2) Do structurally related PAs differ in their effects on specialist and generalist herbivores in *Jacobaea* hybrid plants? (chapters 3 and 4)
3) Does exogenous application of methyl jasmonate and salicylic acid change PA concentration and composition and other metabolites in *J. vulgaris* and *J. aquatica*? (chapters 5 and 6)
4) Does exogenous application of methyl jasmonate and salicylic acid change the feeding behaviour of herbivores with different modes in *J. vulgaris* and *J. aquatica*? (chapters 5 and 6)

Outline of this thesis

In **Chapter 2** I describe the seasonal variation of PAs over 14 months in the field. This is the first detailed report on how the PA concentration and composition of different genotypes are affected by the changing natural conditions.

In **Chapter 3** I describe the oviposition preference of the specialist *T. jacobaeae* using different hybrids between *J. vulgaris* and *J. aquatica*. The cinnabar moths were collected from a regularly defoliated population (Meijendel) and a rarely defoliated population (Bertogne). Except PAs, other factors, such as plant size and plant quality were tested. I aimed to explore the role of PAs, relative to these other factors, in the preference of a specialist herbivore.

In **Chapter 4** I explore the role of specialist and generalist herbivores in maintaining PA diversity. A series of hybrids from a cross between *J. vulgaris* and *J. aquatica* were used in bioassays with a generalist herbivore, the slug *Deroceras invadens*, and a specialist herbivore the flea beetle *Longitarsus jacobaeae*. I investigated whether the feeding damage was genotype-dependent and whether the feeding preference was related to PAs. In the end of this chapter, I summarize all the herbivore bioassays conducted with the same hybrid system to test the Generalist-Specialist Dilemma.

In **Chapter 5** I test how the PA concentration and composition are induced by MeJA application. Axenic tissue culture plants were used to study how MeJA application induced PA variation in concentration and composition and if this variation conversely affected herbivory.

In **Chapter 6** I applied MeJA and SA to investigate if chemical compounds, other than PAs, were induced and if the herbivores of three different feeding types were influenced after phytohormone application as well. This chapter is the first step to study the relative role of PAs in resistance to herbivores.

In **Chapter 7** I summarize the findings presented in this thesis.

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