Pyrrolizidine alkaloid variation in Jacobaea hybrids : influence on resistance against generalist and specialist insect herbivores

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Chapter 1

General introduction

Plants produce a high diversity of secondary metabolites (SMs). The number of SMs which have been identified exceeds 100 000 (Wink, 2009) and the chemical structures of at least 47 000 SMs have been described (De Luca and St Pierre, 2000). With many more SMs yet to be discovered, estimates of the total number of SMs in plants exceed 500,000 (Hadacek, 2002). Within a particular species, or individual plant, a number of major SMs are usually accompanied by several derivatives as minor components (Wink, 2003). For instance, 34 glucosinolates were found in *Arabidopsis thaliana* (Kliebenstein et al, 2001) and more than 20 indole alkaloids were produced in hairy root culture from *Rauvolfia serpintina* (Sheludko et al, 2002). Beside the structural diversity, SMs often show a large variation in concentration. A good example is the variation in the total concentration of the aliphatic glucosinolates in leaves of the ecotypes of *A. thaliana*, which varied nearly 20 fold in total concentration (Kliebenstein et al, 2001). Qualitative and quantitative variation of SMs in plants is determined by genetics (Vrieling et al, 1993; van Dam and Vrieling, 1994; Kliebenstein et al, 2001; Macel et al, 2004), the environment and the interaction between these two (Arany et al, 2009; Lankau and Kliebenstein, 2009; Kirk et al, 2010). As yet, it is poorly understood from an evolutionary point of view how SM diversity emerged and why it is maintained in nature.

In this thesis I will study this evolutionary question from a perspective of the SMs' functions. As a study system I have chosen the pyrrolizidine alkaloids (PAs) of *Jacobaea* (syn. *Senecio*) species. I will investigate whether structurally related PAs in *Jacobaea* species can differentially influence the plants resistance against specialist and generalist insect herbivores. I will study the variation in PA composition and concentration among circa 100 F_2 hybrid genotypes of a cross between *Jacobaea aquatica* (syn. *Senecio aquaticus*) and *Jacobaea vulgaris* (syn. *Senecio jacobaea*). Making use of the independent segregation of different structural types of PAs, I will study the effect of PAs on plant resistance against generalist and specialist insect herbivores.

1. Secondary metabolites

In 1873, Julius Sachs, one of the founders of plant physiology realized that plants contained substances with no obvious function. In 1891, plant physiologist Albrecht Kossel designated the term "secondary" for these low-molecular weight and seemingly non-functional metabolites present in plants. Almost in the same period, others, such as Anton Kerner von Marilaun, Ernst Stahl and Leo Errera found that secondary metabolites protected plants from attack of animals (see reviews by Hadacek, 2002; Hartmann, 2007 and 2008).

Classes of compounds that are regarded as SMs include, amongst others, glucosides, saponins, tannins, alkaloids, essential oils and organic acids. These compounds differ from primary chemicals with respect to function and occurrence. SMs are not directly involved in the growth, development, or reproduction of the plant. Very often they occur in specific taxons (Fraenkel, 1959). But the distinction between primary and secondary metabolism is blurred. Firstly, not all primary metabolites (PMs)

occur in every plant, although primary metabolism involves the essential reactions that occur in all different groups of living organisms. Secondly, some SMs may also function as co-substrates or co-enzymes in primary metabolism (Hadacek, 2002).

Hartmann (1996 and 2007) suggested to base the definition of primary and secondary metabolites on their functions. Thus PMs are universal, conservative, and indispensable chemicals, while SMs are exclusive, diverse, and while dispensable for growth and development, they are indispensable for survival. According to this definition, a metabolite may be a PM as well as a SM. For instance, canavanine from jack bean (*Canavalia ensiformis*) has this dual role as a PM and a SM, because it is both an efficient defense and a nitrogen storage compound (Rosenthal and Rosenthal, 1982).

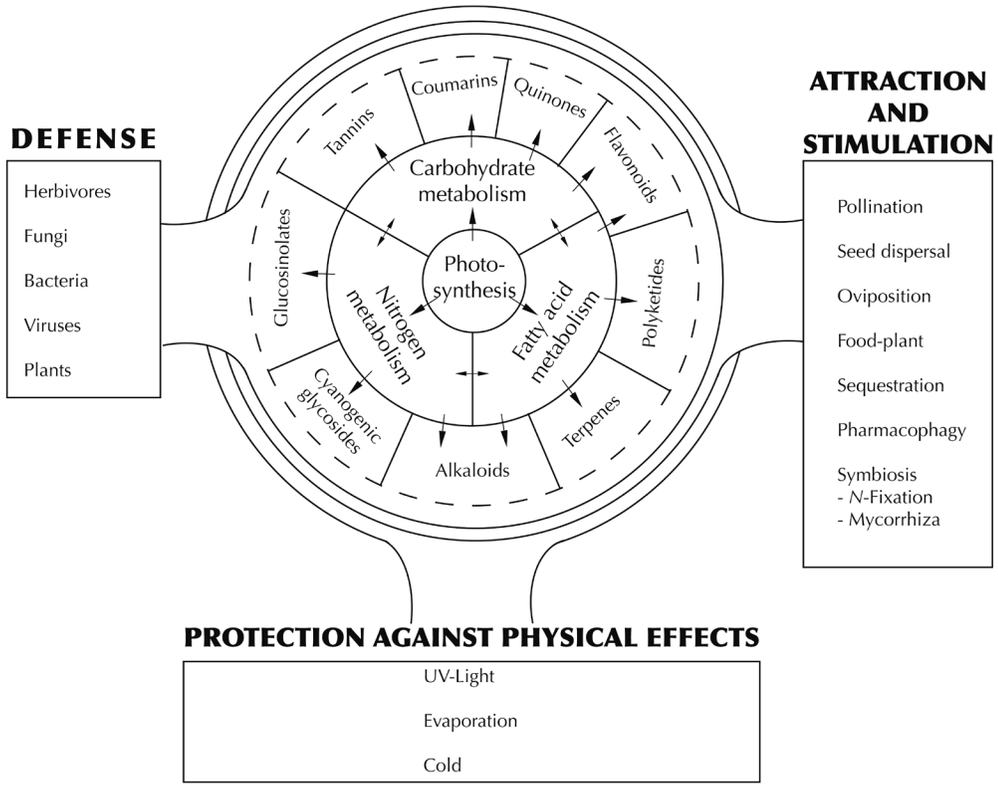

Fig.1 Ecological functions of plant secondary metabolism (from Hartmann, 1996)

1.1. Function of secondary metabolites (SMs) in plants

The idea that SMs have important functions for plants has been widely accepted since the 1970s (Harborne, 1972; Swain, 1977). The functional aspects of plant SMs are illustrated in Fig.1 as proposed by Hartmann (1996). This scheme also illustrates the major groups of SMs and that SMs originate from common precursors of primary metabolism. SMs are involved in all the interactions between plants and their environment (biotic and abiotic). SMs may act as defense compounds against antagonistic organisms such as microbiological attackers, invertebrate and vertebrate herbivores and competing plants. In contrast, specialist herbivores adapted to plant defense can use particular SMs as cue for locating food-plants, oviposition and even utilize SMs for their own benefit. SMs are signaling chemicals in communication with potentially beneficial (and non-beneficial) organisms. They may attract pollinators, seed dispersers (see Hadacek, 2002 and references therein). In addition, SMs may act as protection against abiotic stresses such as high levels of UV radiation, temperature and drought stress (Chen et al, 2009; Vickers et al, 2009). It was also found that plants can release distinct volatile bouquets of SMs when attacked and these SMs can function as indirect defense compounds by attracting carnivores as was shown in controlled experiments in the greenhouse (reviewed by Allison and Hare, 2009) and in the field (Allmann and Baldwin, 2010).

1.2. Hypotheses to explain secondary metabolites (SM) diversity

Several theories and hypotheses have been put forward to explain the SM diversity in plants from the perspective of the function of SMs in plant defense against herbivores. Although these hypotheses are dealing with the diversification of all classes of SMs, they can be used to explain the diversity in structurally related SMs of a specific class. After all, the diversity within a major group of SMs is generally greater than across different kinds of SMs (Langenheim, 1994). For instance, in *A. thaliana* there are more than 170 SMs belonging to 7 different classes, each class containing more than 10 different compounds (D'Auria and Gershenzon, 2005).

Firstly, the SM diversity could be the result of Neutral Selectivity. Firn and Jones (2003 and 2009, see also Jones and Firn, 1991) developed the "Screening Hypothesis" to explain the evolution of plant SM diversity. Unlike the theories which emphasize the SMs' function against herbivores, their hypothesis states that most SMs (perhaps more than 90%, as has been estimated from commercial screening programs) have no distinct function for the plants and provide neither cost nor benefit in relation to plant fitness. In other words, most individual SMs are of neutral selection. Nevertheless, SM diversity is favored because it confers the likelihood of producing new active compounds. This can be compared to some extent to commercial screening programs that search for novel bio-active compounds to certain receptor targets. In the screening programs the chance of success is directly related to the number of chemicals that can be screened in a short time, due to a very low frequency of a good match. It is not easy to test this hypothesis in a direct way, because it is difficult to exactly identify the function of each particular SM in plants. However, by applying this hypothesis for a group of SMs, we can test how many compounds from this particular group have an effect on herbivores and how many do not. If a high proportion of SMs (say more than 10%) shows a repellent effect, then the theory of neutral selectivity may not be true for this group of SMs. In contrast to the Screening Hypothesis, many SMs, as for instance, PAs in *Senecio* species, are generally regarded as powerful defense compounds in plants (e.g. Hartmann, 1996).

Secondly, it was hypothesized that the SM diversity was caused by the "Arms Race" between plants and the herbivores. Ehrlich and Raven (1964) proposed that novel SMs increase plant fitness because of the reduction of herbivory. Plant species with the novel SMs have entered a new adaptive zone and the evolutionary radiation might follow. In turn, herbivore species that succeed to counter adapt to these SMs, gain greater fitness and evolutionary radiation happened. The next step is that plants again evolve new SMs. The diversity of plants, herbivorous insects and the SMs that we observe today could be the result of this sequence of evolutionary events. Applying this theory to explain the diversity within a structurally related group of SMs, it follows that individual SMs should differ in their effects on insect herbivores and the SMs that have most recently evolved are more effective than the

older ones. This theory is supported by some experimental evidence (e.g. Berenbaum and Feeny, 1981; Miller and Feeny, 1983, Macel et al, 2005). Cornell and Hawkins (2003) stated that herbivorous insects can adapt to the SMs in host plants, so it may be expected that the more widespread SMs are less toxic than the more narrowly distributed ones. This trend will be stronger with generalist than with specialist herbivores, due to the different adaption strategies of these two groups. Specialist herbivores can quickly adapt to novel, more toxic metabolites in specific host plants, while generalists can tolerate less toxic chemicals by means of feeding on many different hosts and in this way reducing their toxic burden. The Arms Race Theory and other hypotheses of phytochemical coevolution have been criticized on a number of points and some researchers doubt whether insect herbivores could be a selective force on plant SMs (e.g.Thompson, 1988; Jermy, 1993). However, in general, the concept of coevolution has been enthusiastically adopted and it has evoked a lot of new ideas for further development or modification (Futuyma and Agrawal, 2009; Janz, 2011)

Thirdly, the SM diversity could be explained by the acting of Synergistic Effects among SMs. If different compounds can act synergistically on herbivores, then a mixture of structurally related SMs could have a more toxic and deterrent effect on herbivores than the SMs individually. Therefore, plants obtain a benefit if they maintain a high diversity of SMs (Berenbaum et al, 1991; Dyer et al, 2003; Macel et al, 2005).

Finally, the SM diversity may be a result of the Selection from Multiple Herbivores. It can be assumed that each specific SM provides resistance to one or a number of specific herbivores. Several studies have revealed that the relative effects of related compounds may differently affect generalist insect herbivores (Mithen et al, 1995; Juenger and Bergelson, 1998; Juenger and Bergelson, 2000; Macel et al, 2005; Iason et al, 2011). Hence, a mixture of SMs could be selectively advantages by protecting the plants under the pressure from multiple herbivore species including specialists and generalists.

Beside the variation of SM compositions in plants there is a high variation in concentration of SMs, which could be explained by the balance between benefits and direct costs or ecological costs of SM production. Plants benefit from the SMs because the SMs protect them from herbivores, leading to an increased fitness. On the other hand, production of SMs may reduce the plants' investment to growth and reproduction. This is called The Direct Cost of Resistance. The trade-off between costs and benefits of SM production can be used to explain the variation of SMs maintained in plants (Coley et al, 1985; Herms and Mattson, 1992). Several studies provide support for this idea; while others did not find fitness costs of plant defense (see reviews by Bergelson and Purrington, 1996; Koricheva, 2002; Strauss et al, 2002). In the case of PAs in *Jacobaea* species, costs related to the PA production were regarded absent or small (Vrieling and and van Wijk, 1994a; Vrieling and van Wijk, 1994b; Vrieling et al, 1996). In some cases, the costs of the production of SMs may be not direct, but rather indirect, e.g. may result from increased damage by specialist herbivores. SMs defend plants from generalist herbivores but at the same time they may attract specialist herbivores. The variation in SM concentration in plants can thus be explained by the opposing effect of specialist and generalist herbivores, which is called the Generalist-Specialist Dilemma (van der Meijden, 1996). This hypothesis is supported by the experimental evidence that in plants the concentration of defense chemicals shifted according to the pressure from specialist and generalist herbivores (Lankau, 2007; Arany et al, 2008). Finally, costs could also arise from the negative effects that SMs can exert on beneficial organisms (van der Meijden, 1996). However, this aspect will not be a topic of this thesis.

PAs produced in a hybrid system from a cross between two *Jacobaea* species were chosen as a model to study the SM evolution. PAs from *Jacobaea* species are a well-documented group of SMs and PA biosynthesis has been intensively studied. However, the evolutionary basis of the PA diversity is not clear yet (Langel et al, 2011).

2. Pyrrolizidine alkaloids (PAs)

PAs represent a class of typical SMs, which are constitutively formed in the plants containing them and mediating plant-herbivore interactions (Hartmann, 1999). More than 400 PAs have been identified from approx. 6000 angiosperm species (Chou and Fu, 2006), of which more than 95% belong to four families: Asteraceae, Boraginaceae, Fabaceae and Orchidaceae (Langel et al, 2011).

PAs can occur in plants in two forms: tertiary amine (free base) and *N*-oxide (Rizk, 1991; Wiedenfeld et al, 2008). Hartmann and coworkers showed that PAs are produced as *N*-oxides in the roots and are predominantly present as *N*-oxides in *Senecio* species. The (partial) reduction of *N*-oxides to corresponding tertiary amines can happen spontaneously during alkaloid extraction, resulting in an artificially high amount of tertiary PAs in the sample (Hartmann and Zimmer, 1986; Hartmann and Toppel, 1987, Hösch et al, 1996). However, more recent research indicated that not all PAs are exclusively present as *N*-oxides in the shoots of vegetative *J. vulgaris* plants. Some jacobine-like PAs (jacobine, jacoline, jaconine and jacozine) can regularly occur up to 50% as tertiary amines (Joosten et al, 2009). I will investigate the occurrence of tertiary amines in more detail in Chapter 3.

2.1 PA biosynthesis, translocation and accumulation in Jacobaea/Senecio plants

Pelser et al (2005) reported that 26 PAs (as tertiary amines) were present in 24 species of sect - *Jacobaea*. In *Jacobae*a species, all PAs except senecivernine are derived from senecionine *N*-oxide; senecionine *N*-oxide is synthesized in the roots, via the phloem transported to the shoots, where it is diversified into other PA structures (Hartmann and Toppel, 1987; Hartmann et al, 1989). Aside from structural diversification, PAs do not undergo any significant turnover or degradation (Hartmann and Dierich, 1998). The diversity from senecionine *N*-oxide to other PAs comprises simple one-step or two-step reactions such as hydroxylations, epoxidations, dehydrogenations, and O-acetylations, as well as the more complex conversion of the retronecine into the otonecine base moiety (Hartmann and Dierich, 1998). The first specific compound of PA biosynthesis was identified as homospermidine, which is converted to spermidine and putrescine by the enzyme homospermidine synthase (HSS) (Böttcher et al, 1993). It was shown that the HSS encoding gene originated by gene duplication (Ober and Hartmann, 1999), independently in unrelated angiosperm families (Reimann et al, 2004). The enzymes responsible for the PA diversification are not identified yet. It has been suggested that the genes encoding for the PA pathway-specific enzymes are regulated by a switch-off and switch-on mechanism rather than by gain and loss, since PA distribution appears to be largely incidental in *Senecio* species (Pelser et al, 2005). A schematic diagram representing putative PA biosynthetic pathways is shown in Appendix 1 and the chemical structures of PAs detected in the *Jacobaea* hybrid system that has been used in this study are shown in Appendix 2.

PA accumulation in a particular tissue depends on a number of interacting processes: (i) synthesis of senecionine *N*-oxide in roots, (ii) continuous long-distance translocation of senecionine *N*-oxide into shoots, (iii) differential senecionine *N*-oxide transformations in different plant organs, (iv) continuous allocation of PAs in the plant, and (v) tissue selective vacuolar storage of PAs (reviewed

by Hartmann and Dierich, 1998). In *Jacobaea erucifolia* (syn. *Senecio erucifolius*), a closely related species of *J. vulgaris*, PA biosynthesis occurs mainly in the root apex and thus coincides with the site of active root growth (Sander and Hartmann, 1989). This is in line with the finding that in young *J. vulgaris* plants the total PA amount in plants was positively correlated to root biomass but negatively correlated to shoot to root ratio, which suggested that PAs are produced by roots at a root-biomass dependent rate and the greater the shoot to root ratio, the greater the overall dilution of alkaloids (Hol et al, 2003;Schaffner et al, 2003).

2.2. The function of PAs in plant defense against herbivores and pathogens

2.2.1. The effect of PAs on vertebrates

PAs are toxic for most vertebrates. Already decades ago, it was understood that, upon ingestion, in particular 1,2-unsaturated PAs were toxic to livestock and humans by causing damage to organs such as liver, lungs, and blood vessels. Most severe damage often occurs to the liver (Bull et al, 1968; McLean, 1970). PAs are not toxic themselves, but require metabolic activation (Mattocks, 1968; Fu et al, 2004; Wiedenfeld, 2011). After ingestion and absorption of PAs, the cytochrome P-450 monoxogenase enzyme complex in the liver can introduce a hydroxyl-group adjacent to the nitrogen-atom in the necine ring system. These hydroxy-PAs are unstable and are rapidly dehydrated to the corresponding 3,4-dehydropyrrolizidine alkaloids (DHPAs). Ring opening at C-7 will produce a stabilized carbonium ion that can react with the nucleophiles such as mercapto, hydroxy and amino functional groups. Such functional groups are present for instance in proteins and PA-protein adducts will be formed *in vivo*. They also react with the amino groups of purine and pyrimidine bases present in DNA and RNA (Fu et al, 2004). The alkylated products can lead to abnormal functions and alkylation of DNA may produce mutations which in the end may result in genotoxic and tumorigenic effects. PA *N*-oxides can be reduced by bacteria and enzymes present in the gut or by the liver microsomes to the corresponding tertiary PAs and they show similar toxicity as the tertiary PAs. Therefore both forms of PAs are considered carcinogenic, mutagenic, genotoxic, fetotoxic and teratogenic (Mattocks, 1968; Mattocks, 1971; Mattocks, 1986; Fu et al, 2004, Wiedenfeld, 2011).

Major detoxification pathways *in vivo* of PAs are: hydrolysis of the ester bonds in PAs leading to necic acids and the necine bases which are not toxic; *N*-oxidation by cytochrome P-450 yielding PA *N*-oxides which are highly water soluble and easily excreted (Mattocks, 1986). Hence, the toxicity level of PAs will depend on: 1)the efficiency of metabolic activation to form DHPAs and the corresponding carbonium ions and: 2) the efficiency of detoxification by ester hydrolysis or *N*-oxidation and excretion via urine (Wiedenfeld and Edgar, 2011). This could explain why the effects of PAs on animals are structure-related and that the sensitivity to PAs is different among different animal species (enzymes involved in PA metabolism differ among species). For instance, typical macrocyclic diesters PAs (e.g. PAs in *J. vulgaris*) are regarded to be more toxic than monoester PAs (e.g. PAs derived from the necine supinidine) (Wiedenfeld and Edgar, 2011). The 1,2-saturated PAs (e.g. PAs derived from the platynecine) are not genotoxic to mammalians (Wiedenfeld et al, 2008).

Contamination with PAs of livestock forage, honey and pollen (Deinzer et al, 1977; Kempf et al, 2010; Dübecke et al, 2011), herbal medicine and tea (Wiedenfeld and Edgar, 2011), and even milk (Dickinson et al, 1976; Deinzer et al, 1982; Hoogenboom et al, 2011) has been reported. PA-containing plants may contaminate food or they may be consumed as vegetables by mistake (Wiedenfeld and Edgar, 2011). Therefore, much attention has been paid to PAs because of their potential threat to human and animal health (Boppré, 2011),

2.2.2. The effect of PAs on invertebrates

The toxicity mechanism of PAs to insect herbivores is not as clear as that to mammals. Frey et al (1992) suggested that the same mechanisms may be involved for both kinds of animals. Bioassays have demonstrated that structurally different PAs differentially affect insect herbivores. A particular PA that was effective against one insect did not necessarily deter other insect species (Bentley et al, 1984; Dreyer et al, 1985; van Dam et al, 1995; Macel et al, 2005; Dominguez et al, 2008). PA mixtures often have a stronger effect on insects compared to individual PAs, indicating the presence of synergistic effects (Macel et al, 2005). Generally, the PA *N*-oxides are less deterrent than the corresponding tertiary PAs (Dreyer et al, 1985; van Dam et al, 1995; Macel et al, 2005).

PA-adapted specialist insects detoxify PAs through *N*-oxidation (Lindigkeit et al, 1997; Naumann et al, 2002). PAs may stimulate the feeding and oviposition of the larva and adult of specialist insects (Boppré, 1986; Honda et al, 1997; Kelley et al, 2002; Macel and Vrieling, 2003; Bernays et al, 2004). The oviposition-stimulating effect of PAs on *T. jacobaeae* was observed to be different among structurally different PAs but concentration-dependent effects were not found (Macel and Vrieling, 2003). Larval feeding-preference of specialist insects (the sawfly *Aglaostigma discolour* and the beetle *Oreina cacaliae*) on a PA-containing plant species (*Adenostyles alliariae*) was not affected by the isolated PAs individually added to artificial diets (Hagele and Rowell-Rahier, 2000). Some specialist insects take up PAs from the host plants and utilize them for their own defense or as sexual pheromones (see review by Trigo, 2011). *Tyria jacobaeae* even produces its own specific PAs by metabolizing PAs that were taken up from host plants (Rothschild et al, 1979).

There are some *in vivo* studies which indicated the negative influence of PAs in plants on generalist herbivorous insects. Negative correlations between concentration of total PAs and of individual PAs such as jacobine and the resistance to the generalist insect herbivore *Franklinella occidentalis* were observed in *J. vulgaris* and the hybrids of *J. vulgaris* and *J. aquatica* (Macel, 2003; Leiss et al, 2009). In field experiments, the total amount of PAs in plants of *J. vulgaris* was negatively correlated to the performance of the generalist aphid *Brachycaudus cardii* and the specialist aphid *Aphis jacobaeae* (Vrieling et al, 1991). It has also been observed that the young leaves of *J. vulgaris* plants were less damaged by generalist herbivorous insects than the old leaves, and the young leaves always contained higher amounts of PAs than the older leaves (de Boer, 1999; Leiss et al, 2009).

No relationship was found between the PAs in *J. vulgaris* plants and the specialist *T. jacobaeae* with regard to both preference and performance in bioassays under controlled conditions (Vrieling and de Boer, 1999). Similar results were found in a bioassay with *T. jacobaeae* and eight *Senecio* species (Macel et al, 2002). However, in a field study Macel and Klinkhamer (2010) found that the damage that was mainly caused by specialist herbivorous insects such as *T. jacobaeae, Longitarsus jacobaeae* and *Haplothrips senecionis*, on *J. vulgaris* plants was positively correlated to the concentration of total PAs and individual PAs (jacobine and jacobine *N*-oxide). This finding suggests that plants with higher concentrations of PAs are more attractive to the specialists. The studies published thus far seem to contradict one another. It is not clear yet whether PA variation in plants has an effect on specialist herbivores preference and performance.

2.2.3. The effect of PAs on pathogens

It was demonstrated by means of *in-vitro* bioassays that PAs isolated from plants can significantly inhibit the growth of many different fungal species (Jain and Sharma, 1987; Marquina et al, 1989; Reina et al, 1995; Reina et al, 1997; Reina et al, 1998; Hol and van Veen, 2002; Singh et al, 2002). It was shown that rhizosphere fungal communities were influenced by the PA content and composition of *J. vulgaris*. High PA concentrations decreased the diversity in the rhizosphere (Kowalchuk et al, 2006). At the other hand, there are indications that soil-born microorganisms can influence the concentration of individual PAs in *J. vulgaris* (Joosten et al, 2009).

2.2.4. PAs and plant inducible defense against aboveground herbivores

It has been suggested that whether or not PAs are involved in inducible defense depends on the life history and ecological environment of the plants (van Dam et al, 1993). In general, the difference in constitutive PA levels in *J. vulgaris* was greater than that caused by induction. It was observed that the total PA concentration in *J. vulgaris* decreased within 6-12 h, but returned to the initial value 24 h after the mechanically induced damage (van Dam et al, 1993). Vrieling and Bruin (1987) did not found a significant change of total PA concentrations in the shoots of *J. vulgaris* after artificial damage. Aboveground herbivory did not change the total concentration of PAs in the shoots, and the effect on PA composition was genotype-dependent (Hol et al, 2004).

2. 3. PA variation in *Jacobaea/Senecio* plants

2.3.1. Inter-species variation

A large variety of PA profiles can be found among *Senecio* and *Jacobaea* species (Langel et al, 2011). PA profiles are species-specific (Hartmann and Dierich, 1998). For instance, some species are very rich in jacobine-like PAs such as *J. vulgaris*, while erucifoline-like PAs dominate in *J. erucifolia*. However, PA profiles do not represent the phylogenetic relationships between the *Senecio/Jacobaea* species. This indicates that the flexible PA profiles in these species are probably helpful to protect plants from multiple herbivores, because flexible PA mixtures are more difficultly adapted by herbivores (Pelser et al, 2005; Langel et al, 2011).

2.3.2. Intra-species variation

PA profiles also vary within species. The existence of different chemotypes of *J. vulgaris* is a well known example for the intra-species PA variation. Based on the evaluation of the PA profiles of more than 100 *J. vulgaris* populations in Europe, it was concluded that there existed two different chemotypes: the 'jacobine chemotype', which is dominated by jacobine and its derivatives as major PAs; the 'erucifoline chemotype', dominated by erucifoline-like PAs (Witte et al, 1992). Besides these two chemotypes, later on also a 'senecionine chemotype' (with senecionine-like PAs as dominating PAs) and a 'mixed chemotype' (with both jacobine- and erucifoline-like PAs as dominating PAs) were described (Macel et al, 2004). The distribution of the chemotypes showed a geographic pattern: The jacobine chemotype mostly occurs in the coastal areas and the erucifoline chemotype is mainly found inland of Europe (Witte et al, 1992; Vrieling and de Boer, 1999; Macel et al, 2004). Plants from the same population often belong to the same chemotype but still they shown significant variation in relation to PA composition. For instance, although plants collected at Meijendel (the Netherlands) mainly contained jacobine, the percentage of jacobine ranged from 41 to 100% of the total PA content and the percentage of erucifoline ranged from 0 to 19% (Macel et al, 2004).

2.3.3. Intra-plant variation

PAs are not equally distributed over the organs of individual plants. PAs are stored in vacuoles and typically accumulate in the inflorescences and the peripheral stem tissues, i.e. epidermal and sub-epidermal cell layers in the plants, as has been shown for *Senecio vulgaris* (Hartmann et al, 1989). The total concentration of PAs in vegetative *J. vulgaris* plants was found to decrease with leaf age (de Boer, 1999), and inflorescences often have a higher concentration of PAs than the leaves in reproductive *J. vulgaris* plants (Witte et al, 1992).

PA composition differs in the root and shoot of the vegetative plants of *J. vulgaris*, *J. aquatica* and the F_2 hybrids: Generally, shoots have more variation in the composition and more jacobine-like PAs compared to the roots (Joosten et al, 2009). In reproductive *J. vulgaris* plants, leaves have less senecionine-like PAs but more jacobine- or erucifoline-like PAs. In *J. vulgaris* erucifoline chemotype the proportion of acetylerucifoline was however much higher in the leaves than in inflorescences (Witte et al, 1992).

The PA concentration on the leaf surface of *J. vulgaris* is much lower (less than 1%) compared to the interior of the leaves. The concentration at the surface of the leaves was only marginally correlated with that of the interior, and the PA composition on the leaf surface also differed from the PA spectrum inside (Vrieling and Derridj, 2003).

2.3.4. Genetic control and environmental influence on PA variation

It has been estimated that under climate room conditions 50-100% of the variation in total PA concentration is due to genetic variation (Vrieling et al, 1993). PA measurements on replicated genotypes illustrated that the PA concentration and composition were genotype-dependent (Macel et al, 2004; Joosten et al, 2009). PA accumulation in plants is also affected by abiotic environmental factors such as nutrients and water. It was found that *J. vulgaris* plants grown under drought or nutrient stress conditions tend to have higher PA concentrations, than those grown under normal conditions (Vrieling and van Wijk, 1994). Increased nutrient availability leads to a significant reduction in total PA concentration in shoots of *J. vulgaris* plants (Hol et al, 2003). It was postulated that in this particular situation of rich nutrient supply, the decreased PA level in shoots may have resulted from a dilution effect: The increase of the nutrient supply will favor an increase of shoot over root biomass ratio and as PA production is correlated with root growth, plants under nutrient rich conditions produce relatively less PAs. Some genotypes of *J. vulgaris*, *J. aquatica* and their hybrids produce different PA concentrations and compositions under different nutrient and water treatment conditions, so it seems that PA expression is affected by genotype and environment interactions (Kirk et al, 2010).

3. Research questions

In this thesis, I will investigate whether the structurally related PAs differentially influenced generalist and specialist insect herbivores in *Jacobae*a hybrids. I will address the following questions:

1. Do the F_2 hybrids from a cross between *J. vulgaris* and *J. aquatica* display a greater PA variation compared to their parents? Is PA variation, especially the production of tertiary PAs, dependent

on the plant genotype?
2. Is herbivore resistance against generalist and specialist insect herbivores dependent on the plant hybrid genotype?
3. Does herbivores resistance against generalist and specialist insect herbivores relate to PA composition and concentration?
4. Do the effects of PAs on herbivore resistance differ among different PAs and does it make a difference whether they are present as tertiary amines or *N*-oxides?
5. Do different PAs act synergistically in their effects on herbivores?

Up to now, most conclusions about the effects that PAs have on insects and pathogens are based on *in vitro* experiments, while these effects of PAs are not always apparent in *in vivo* experiments (see reviews by Joosten and van Veen, 2011; Macel, 2011; Trigo, 2011). Also, most of the previous studies were hampered by the fact that less sensitive methods were used to detect PAs and often no distinction was made between tertiary amines and *N*-oxides. Research on individual PAs is difficult as the majority of the PAs cannot be obtained commercially unless at a very high cost. *In vivo* experiments have an advantage over *in vitro* bioassays, in the sense that these can overcome the need for PAs as isolated compounds. The disadvantage of *in vivo* experiments is that the species or genotypes that are used may differ in other characteristics as well that are relevant for herbivory. Therefore it can be difficult to sort out the effect of PAs. Many of these disadvantages can be overcome by using segregating hybrids instead of randomly chosen genotypes: Firstly, a greater variation of SMs and herbivore resistance can occur among these hybrids compared to genotypes within a single species (Fritz, 1999; Orians, 2000; Cheng et al, 2011). Secondly, traits will segregate independently so that trait variation can be studied against an on average equal genetic background (Hochwender et al, 2000; Lexer et al, 2003). Therefore, I will use *Jacobaea* hybrids as a study system.

4. Outline of the thesis

In Chapter 2, the PA variation in the shoots and roots of F_2 hybrids, obtained from a cross between *J. vulgaris* and *J. aquatica*, will be studied. I will investigate whether there are any novel PAs, or novel PA compositions, present in the hybrids, and whether transgressive segregation of PA concentrations occurs. I will investigate whether the PA expression is different among the plant genotypes. The PA variation patterns and the implications for PA biosynthesis and PA genetic control will be discussed. For a long time it has been assumed that in *Senecio* species PAs are present mainly as *N*-oxides and that tertiary amines were mostly artifacts formed by (spontaneous) reduction of *N*-oxides during PA extraction. The presence of significant amounts of specific tertiary PAs in the plants of *J. vulgaris*, *J. aquatica* and their hybrids will be described and discussed in Chapter 3.

The oviposition preference of *T. jacobaeae* among the hybrids of *J. vulgaris* and *J. aquatica* is studied in Chapter 4. The resistance of *Jacobaea* hybrids against two generalist insect herbivores, *F. occidentalis* (western flower thrips) and *Liriomyza trifolii* (American serpentine leafminer), will be studied in Chapter 5 and Chapter 6, respectively. Through the use of bioassays, I will explore the relationship between herbivore resistance and PA variation and the possible synergism among PAs with respect to plant resistance against these insects.

Finally, the relation between PA variation in the *Jacobaea* hybrids and performance and preference of insects among the plants will be discussed and the conclusions will be summarized in the last chapter (Chapter 7).

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