Chapter 1.

General Introduction

GENERAL INTRODUCTION

Plants synthesise various kinds of compounds, which are classified into primary and secondary metabolites. The primary metabolic pathways refer to the anabolic and catabolic processes required for, among others, respiration, photosynthesis, nutrient assimilation, energy production and growth and development, hence these processes are required for cell maintenance and proliferation (Wink, 1988). Secondary metabolites (SMs), in contrast, are not directly involved in the growth and development of plants. However, they are present in all plants (Wink, 2003) and characterised by their enormous structural diversity (Hartmann, 1996). More than 150,000 SMs have been described so far (Fig 1) (Wink, 2003).

Secondary metabolites are produced by plants via a few basic metabolic pathways, leading to one or a few key-metabolites such as alkaloids, flavanoids, terpenoids, etc (Dixon, 2001; Hartmann, 2007).

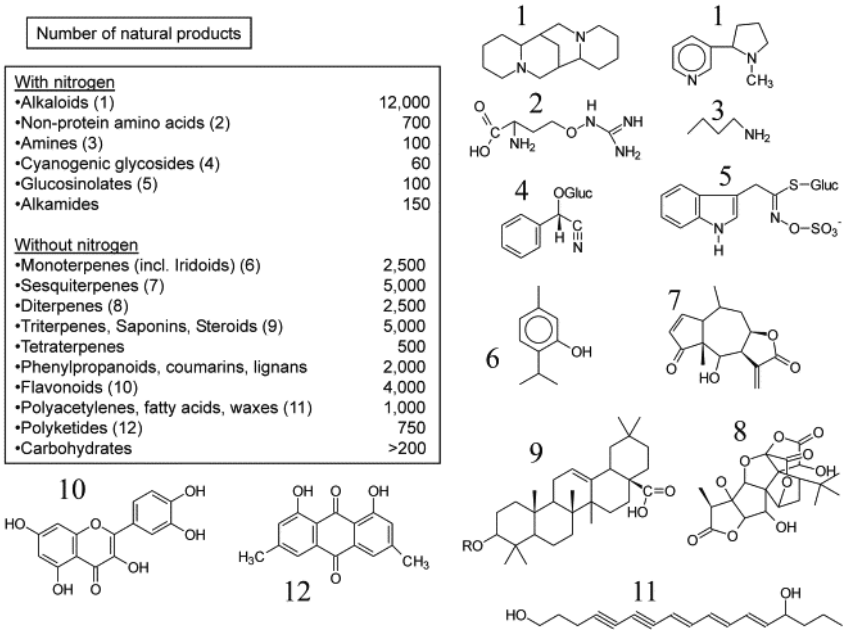

Fig 1. Structural diversity of secondary metabolites (Wink, 2003).

Previously, a number of hypothetical explanations for the function of SMs have been proposed, such as waste and detoxification products, expression of shunt and overflow metabolism, degradation and storage products (reviewed in Hartmann, 1996). However, now it is generally accepted that SMs play a major role in the interaction of a plant with its biotic environment (Després et al., 2007; Dicke, 2000; Wink, 2003). For example, SMs play a major role in plant chemical defence. More than 100 years ago, Ernst Stahl (1888) showed experimentally that SMs serve as defence compounds against snails and other herbivores. Secondary metabolites as a

chemical defence strategy (Hanley et al., 2007; Jansen et al., 2009) involve repellent or toxic compounds as well as digestibility reducers (Loney et al., 2006). Besides functions in plant defence, SMs play a significant role in intra- and inter-specific communication (Hartmann, 2007). As such SMs, like colored anthocyanins or carotenoids in flowers serve to attract pollinators (Harborne and Williams, 2001). Fragrant monoterpenes or sesquiterpenes serve to attract predators of insect herbivores (Birkett et al., 2000). In addition, plants need to defend themselves against microbial infections. Particularly high carbohydrate tissues such as roots, leaves and fruits are rich in nutrients which can promote the growth of microorganisms. Thus, immature fruits are often rich in toxic SMs. In several instances attractant and defensive activities are exhibited by the same compounds: anthocyanins or monoterpenes can be insect attractants in flowers, but are insecticidal and antimicrobial at the same time. In addition, some SMs concomitantly carry out several physiological functions, for example alkaloids and peptides (lectins, protease inhibitors) can serve as mobile and toxic nitrogen transport and storage compounds. Some phenolics, such as flavonoids, may function as plant defence compounds and UV protectants (Harborne, 2001; Wink, 1988).

A staggering diversity of SMs is observed within and between plant species. Related plant families generally make use of related chemical structures for defence, such as isoflavonoids in the Leguminosae and sesquiterpenes in the Solanaceae. However, some chemical classes, such as phenylpropanoid derivatives, may be used for defensive functions across taxa (Dixon, 2001). Intra-species diversity is mostly based on variation within a certain group of biosynthetically related compounds (Wink, 2003). In *Catharanthus roseus,* for example, more than 130 terpenoid indole alkaloids have been reported and big differences were observed in the spectra of alkaloids present in different varieties (Jacobs et al., 2004). The same was observed for the glucosinolates in *Brassica napus* (Clossais-Besnard and Larher, 1991). Qualitative and quantitative differences in SMs are also observed between different organs of plants. For example, the highest levels of scopolamine, a tropane alkaloid, are observed in the stem of *Datura stramonium* compared to leaves and roots (Miraldi et al., 2001). Caffeine in *Coffea arabica* is observed mainly in the leaves and seeds but not in the roots (Zheng and Ashihara, 2004).

There are several hypotheses developed to explain this diversity. From the plant's evolutionary point of view, one of the most important explanation is the co-evolution hypothesis (Ehrlich and Raven, 1964). It postulates that the interaction between plants and insects is responsible for the tremendous diversification of plant SMs. It is assumed that new compounds have evolved in a continuous race between plants and insects. A plant that synthesises new compounds is able to escape from herbivory. In turn, insects will adapt to these compounds (Ehrlich and Raven, 1964; Rhoades and Cates, 1976) and the cycle starts again. Alternatively, related compounds may differentially affect different herbivores (Berenbaum and Feeny, 1981).

Plants as complex organisms consist of several organs and tissues. Around forty different cell types occur in plants (Martin et al., 2001) of which twelve in the leaves alone (Nelson et al., 2008). Interestingly, in line with this complexity, there are many types of insect herbivores that attack certain plant organs or tissues. Large herbivores often eat complete plants but some prefer specific plant organs such as leaves, inflorescences, stems or roots. Insects, such as caterpillars,

may consume different plant organs as a whole, while others feed on specific plant tissue only. Examples of the latter comprise leafminers feeding through the mesophyll cells of leaves, thrips sucking up the content of epidermis and mesophyll cells and aphids as phloem feeders. In this regard, plants may adapt their defensive strategy through differential distribution of SMs in specific tissues within an organ. For example, the methoxyphenylphenalenones specifically accumulate in the secretory cavities of *Dilatris pillansii* leaves (Schneider and Hölscher, 2007). Chlorogenic acid in the leaves of *Sorghum bicolor* is specifically accumulated in epidermis cells and much less in mesophyll cells (Kojima and Conn, 1982). Thus, studying the variation and distribution of SMs on the tissue and cell level in the plant will help us to better understand plant defence against insect herbivores.

In this thesis, I focus on the variation of SMs in different organs, tissues, and cells within a plant. As a study system, I used *Jacobaea* (syn. *Senecio*) species. This species is known to constitutively synthesise pyrrolizidine alkaloids (PAs). These alkaloids are present among non related taxa of flowering plants (Hartmann, 2008; Hartmann and Ober, 2000). Pyrrolizidine alkaloids have a high diversity in terms of structure, and distribution among plant organs (Cheng et al., 2011a; Hartmann and Zimmer, 1986). Pyrrolizidine alkaloids are assumed to have evolved as part of the chemical plant defence under the selection pressure of herbivores (Macel, 2003). First, I studied if PA synthesis depends on fungal endophytes as part of the biotic environment. Subsequently, the structural diversity and distribution of PAs within organs, tissues and cells of *Jacobaea* plants were studied. The ecological consequence of the structural diversity of PAs was then evaluated using cell lines and larvae of the Beet Armyworm, *Spodoptera exigua* (family Noctuidae; order Lepidoptera).

PYRROLIZIDINE ALKALOIDS

Alkaloids form one of the largest classes of secondary metabolites. Type of alkaloids, which has been intensively studied because of its high variability, is the PAs. These alkaloids are present in quite unrelated families such as the Asteraceae, Boraginaceae, Fabaceae and Orchidaceae (Hartmann, 1999).

Pyrrolizidine alkaloids are esters of a necine base with one or more necic acids (Hartmann, 1999). The necine base is formed of two molecules of putrescine deriving from the arginine-agmatine route (Hartmann *et al.*, 1988). The necic acid derives from several common amino acids such as L-threonine, L- isoleucine, L-valine or L-leucine (Stirling *et al.*, 1997). Around 370 different PAs have been identified (Hartmann and Ober, 2000). Based on the complexity and the number of carbon atoms in the necic acid, five major classes of PAs can be distinguished: the senecionine, triangularine, monocrotaline, lycopsamine and phalaenopsine class. The senecionine class comprises more than 100 structures and is the most diverse PAs group. This type of macrocyclic PAs is typically found in the genera *Jacobaea* and *Senecio*. At least 37 PAs have been reported from plants of the genus *Jacobaea* (Cheng et al., 2011a; Pelser et al., 2005).

Insect Plant Defence

For insect herbivores PAs act as feeding deterrents and toxic compounds (Macel et al., 2005;Ober and Kaltenegger, 2009; van Dam et al., 1995). Different structurally related PAs were reported to have different effects on generalist insects. PA toxicity in insects is assumed to be related to the necine base of PAs. In the insect gut PAs are reduced and converted by cytochrome P450 enzymes (CYPs) to the highly reactive pyrrole intermediates (Lindigkeit et al., 1997). These intermediates readily react with the amino groups of proteins as well as with nucleosides in DNA and RNA (Wiedenfeld and Edgar, 2011). In addition, it was shown that the free base form of PAs have a significant binding activity to membranes of muscarinic acetylcholine and serotonine receptors derived from porcine brain (Schmeller et al., 1997). As such PAs may influence neuronal signal transduction as well as central nervous system- and muscular activity.

Generalist insects

The effect of single PAs on generalist insect herbivores depends on PA structure and concentration (Macel et al., 2005; van Dam et al., 1995). Using *in-vivo* plant studies Leiss et al. (2009) observed that jacobine-like PAs i.e. jacobine *N*-oxide and jaconine *N*-oxide were responsible for thrips resistance of F2 hybrids of *J. vulgaris* and *J. aquatica*. In agreement with this result, especially jacobine-like PAs (Cheng et al., 2011a; Joosten, 2012;) and erucifoline-like PAs (Macel, 2003; Macel and Klinkhamer, 2010) were identified to contribute to insect plant defence. However, these results are mainly based on correlative studies. Little is known on the effect of jacobine and erucifoline as individual PAs. Mainly senecionine-like PAs have been individually tested (Lindigkeit et al., 1997; Macel et al., 2005) since these are the only PAs commercially available. Senecionine was reported to be less deterrent than its derivatives e.g. seneciphylline and riddelline for Spruce budworm, *Choristoneura fumeferana* (Bentley et al., 1984). Small structural differences seem to alter the activity of PAs. In two-choice experiments, among closely related senecionine-like PAs, senecionine was less deterrent to the Migratory locust, *Locusta migratoria* compared to seneciphylline, but senecionine and seneciphylline were more toxic to the green peach aphid, *Myzus persicae*, than monocrotaline and senkirkine (Macel et al., 2005). In the same study senkirkine showed high toxicity to Western Flower thrips, *Frankliniella occidentalis*. Senkirkine also showed a strong feeding deterrent activity to *C. fumeferana* (Bentley et al., 1984) . There is only one report on erucifoline, isolated from the Canarian endemic plant *Canariothamnus palmensis*, demonstrating a negative effect on *M. persicae* (Domínguez et al., 2008). Until now, no toxicity studies have been conducted for jacobine-like PAs.

Specialist insects

Specialist herbivores (mainly Arctiidae, Danainae, and Ithomiinae butterflies and some Chrysomelidae leaf beetles) are able to overcome PA defence and to even sequester PAs from their host plant for their own defence (Hartmann, 1999). Furthermore, some specialists use PAs for their own benefit as a cue to locate their host plants, an oviposition stimulus (Cheng, 2012; Macel and Vrieling, 2003), for pheromone production (Bernays et al., 2002) and defence against egg (Schulz et al., 2002) and larval predators (Hartmann and Ober, 2000).

Larvae of the cinnabar moth *Tyria jacobaeae* sequester and store PAs from their host plant *J. vulgaris* and retain the alkaloids during all stages of metamorphosis (Aplin and Rothschild, 1972).

The arctiid moth *Utetheisa ornatrix* sequesters PAs, which are then used for egg protection (Dussourd et al., 1989). Besides lepidopterans, the african grasshopper *Zonocerus variegatus* (Bernays et al., 1977), leaf beetles (Chrysomelinae) of the genus *Oreina* (Hartmann et al., 1997) and *Longitarsus* beetles (Haberer and Dobler, 1999) have been found to sequester PAs. Asteraceae of the tribes Eupatorieae and Senecioneae as well as Boraginaceae each have their characteristic types of PAs and the beetles feeding on these plants mirror the pattern of PAs present in the plant reasonably well, including macrocyclic PAs as well as branched mono- and di-esters (Dobler, 2001). Other PA adapted insects such as the African cotton leafworm, *Spodoptera litoralis*, developed the ability to specifically detoxify tertiary alkaloids in the insect body (Hartmann, 1999; Lindigkeit et al., 1997).

One of the most important PA specialists on *J. vulgaris* is *T. jacobaea*. The effect of PA diversity on larval performance of *T. jacobaea* was studied in the laboratory using eleven different *J. vulgaris* populations as well as eight different *Senecio* species with different PA compositions. However, larval performance of this specialist seemed not to be affected by PA composition (Macel et al., 2002). In contrast, a recent study using *Senecio* hybrids showed that cinnabar moth oviposition preference was positively correlated with the concentration of tertiary amines of jacobine- and some otosenine-like PAs (Cheng et al., 2013). Similarly, in a field study, more herbivory by this specialist was found on *J. vulgaris* plants with higher concentrations of both total PA and jacobine (Macel and Klinkhamer, 2010). Macel and Vrieling (2003) reported that an extracted PA mixture, rich in jacobine-like PAs, stimulated oviposition of the cinnabar moth, as did senecionine. In contrast, retrorsine did not stimulate oviposition although it differs only in one OH group at C-12 from senecionine. Thus a small structural difference seemed to alter the stimulatory activity of PAs. However, PAs extracted from the non host plant, *Senecio inaequidens*, that consisted of 81% of the non active retrorsine did stimulate oviposition too (Macel and Vrieling, 2003). A study using the plant *Parsonsia laevigata* showed that females of the large tree nymph butterfly, *Idea leuconoe* deposited eggs in response to a methanolic extract of *P. laevigata* containing macrocyclic PAs including parsonsianine, parsonsianidine, and 17-methylparsonsianidine (Honda et al., 1997).

Pyrrolizidine Alkaloids Biosynthesis and Diversification in Jacobaea and Senecio Species.
In *Senecio vernalis* it was proven that all PAs are derived from senecionine *N*-oxide except for senecivernine. The study was conducted by feeding the plant with radioactive labeled precursors such as arginine, ornithine, putrescine, spermidine, and isoleucine (Hartmann et al., 1989; Sander and Hartmann, 1989). Homospermidine which derives from putrescine and spermidine is the first pathway-specific intermediate of PA biosynthesis. The enzyme catalyzing the formation of homospermidine was identified as a homospermidine synthase (HSS) (Böttcher et al., 1993). The reaction step leading from homospermidine to the necine base moiety has not yet been characterised on the enzymatic level. The biosynthesis of the necic acids moiety of PAs has been less studied. Aside from the knowledge that common amino acids are precursors of necic acids the labeling patterns have been far from complete. Part of the necic acids is derived from 2-aminobutanoic acid (Stirling et al., 1997).

PAs are stored in the vacuoles (Ehmke et al., 1988) but the sites of PA synthesis differs among species. PA synthesis is reported to occur in the roots like in *Symphytum officinale* (Frölich et al.,

2007) and *Jacobaea* species (syn. *Senecio*, Asteraceae) (Hartmann et al., 1989) or in the shoots like in *Heliotropium indicum* and *Cynoglossum officinale* (Frölich et al., 2007). From the root of *Jacobaea* plants, PAs are translocated to the above ground plant organs via the phloem (Hartmann and Toppel, 1987). In the shoots, senecionine *N*-oxide is biochemically modified in one or two steps through reactions like hydroxylation, dehydrogenation, epoxydation, *O*-acetylation to yield the species-specific PA patterns (Hartmann and Dierich, 1998). Based on this diversification process, the macrocyclic diester PAs i.e. the senecionine class of the *Jacobaea* plants can be divided into four major types: senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Figure 2).

Thus, the biochemistry and physiology of PAs are quite well understood. However, much information is still lacking. The enzymes responsible for PA diversification are not known and thus the exact mechanism of diversification is not clear yet. Pelser et al. (2005) suggested that the mechanism of the diversification may be based on a specific-genetic control by means of a transient switch-off and switch-on of single enzymes or their encoding genes.

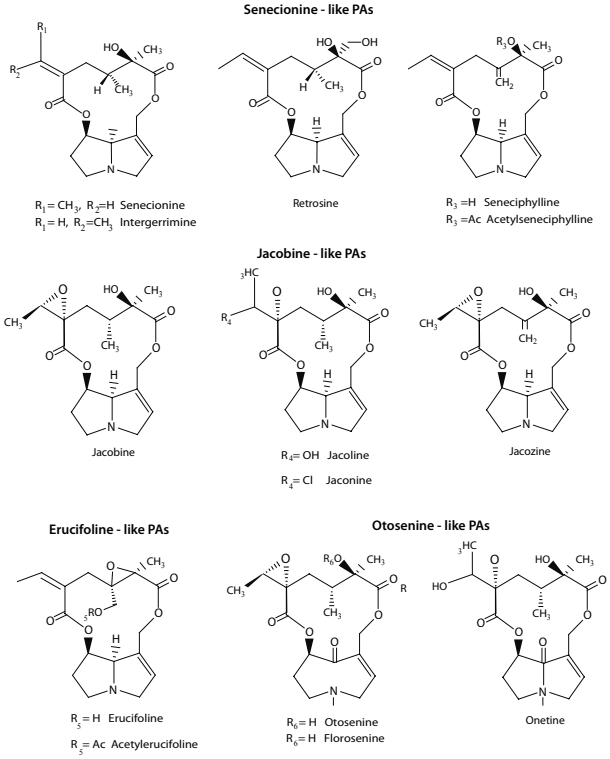

Fig 2. Structure of pyrrolizidine alkaloids in *Jacobaea* plants (adapted with modification from Joosten et al., 2011)

Pyrrolizidine alkaloids occur in two interchangeable forms: the free base (tertiary amine) and the *N*-oxide form (Hartmann and Dierich, 1998). It has been reported that the *N*-oxide is the major PA storage form in *Senecio* and *Jacobaea* plants (Hartmann and Toppel, 1987). Moreover, the *N*-oxide of senecionine rather than the free base has been reported as the form for translocation from roots to shoots (Hartmann et al., 1989). Recently, Joosten et al. (2009, 2011) reported that

the free base form consistently presented up to 50% of the total alkaloid content in the jacobine chemotype of *J. vulgaris*. The ratio of free base and its corresponding *N*-oxide varied depending on the genotype (Joosten et al., 2011). The possibility of an interchange between the PA forms as a result of biotic or abiotic stress is of interest for further studies. More recently, it has been reported that in *J. vulgaris* the concentration of the *N*-oxide form in the leaves decreased upon root herbivore attack (Kostenko et al., 2013).

Diversity of Pyrrolizidine Alkaloids in Jacobaea Species

Inter-species variation.
Species specific PA compositions were observed in the *Jacobaea* and *Senecio* genera (Hartmann and Dierich, 1998). For example *S. vulgaris* contained a high proportion of senecionine-like PAs while *J. erucifolia* contained a high proportion of erucifoline-like PAs. PA composition varied in response to both biotic and abiotic stress (Hartmann, 2007) .

Intra-species variation
A high intra-species variation in PA composition is well known from various *Jacobaea* and *Senecio* species such as *J. vulgaris* (Macel et al., 2004)*, S. vulgaris* and *S. vernalis* (Borstel et al., 1989). Different chemotypes based on the major PAs present were observed, e.g. jacobine, erucifoline, senecionine or mixed types (Macel et al., 2004; Witte et al., 1992). A study on PA variation using F2 hybrids of *J. vulgaris* and *J. aquatica* showed that several F2 hybrids over-expressed otosenine-like PAs, whereas others contained relatively high proportions of erucifoline-like PAs compared to their parents (Cheng et al., 2011b) .

Intra-plant variation
PAs are present in all plant organs but are not equally distributed. In *H. indicum,* the highest level of PAs occured in the inflorescences (Frölich et al., 2007) while in *Phalaenopsis* hybrids maximum amounts of PAs were found in young and developing tissues such as root tips and young leaves (Frölich et al., 2006). A similar pattern was observed in *S. vulgaris*, with the highest levels of PAs in inflorescences, followed by leaves, roots and stems (Hartmann et al., 1989).

The composition of PAs in *Jacobaea* and *Senecio* plant genera may vary quantitatively and qualitatively among shoot organs, i.e. leaves, stems and inflorescences (Hartmann and Dierich, 1998). PA composition in the roots and shoots of *J. vulgaris* and *J. aquatica* plants and their F2 hybrids was different (Cheng et al., 2011b; Joosten et al., 2009). Generally the shoots contained higher levels of jacobine- and erucifoline-like PAs and lower levels of senecionine- and otosenine-like PAs compared to the roots. How PAs are accumulated and distributed in particular organs may depend on several processes such as: (a) the rate of *de-novo* PA synthesis which seems to occur mostly in the roots of *Jacobaea* plants; (b) long-distance translocation of senecionine *N*-oxide into the shoots through the phloem which is specific only for the *N*–oxide form (c) further structural transformation and organ selective storage which depends on many external factors such as herbivory and microbial infections (Hartmann and Ober, 2000). Since PAs are spatially mobile, the pattern of PAs in different organs within a plant may change in response to biotic and abiotic factors.

Environmental Effects on Pyrrolizidine Alkaloids

Abiotic environment.

Variation in environmental factors such as climate, light, humidity and nutrients cause large variations in concentration, allocation, and diversity of SMs including PAs (Close et al., 2005; Loney et al., 2006). Higher light intensity increased PA production (Vrieling and Wijk, 1994). Increasing nutrient supplies lead to an increased shoot:root ratio (Hol et al., 2003). Since PAs in *Jacobaea* and *Senecio* plants are produced in the root, higher nutrient supplies are expected to lead to lower PA concentrations. Under nitrogen- and phosphorous-limited conditions no trade-off between PA production and growth (Vrieling and van Wijk, 1994). Increasing nutrients led to a significant reduction in total PA concentration of both roots and shoots, and all individual PAs except jacobine decreased in concentration. However, the total amount of PAs was not influenced by nutrient supply (Hol et al., 2003). A more recent study showed that total PA concentration of *J. vulgaris* grown in Meijendel soil was higher compared to the same plants grown in Heteren soil (Macel and Klinkhamer, 2010). However, it was not clear whether nutrient contents, structure of the soil or the biotic environment such as microorganisms or root herbivores influence the PA production.

Biotic environment.

Plants are members of complex communities and interact with their biotic environment including antagonistic and beneficial organisms (Pieterse and Dicke, 2007). This biotic environment may consist of other plants, insects and microbes. Several reports have shown the influence of these biotic agents on the concentration and composition of PAs. It was hypothesised that above ground herbivory will cause an increase in PA production while below ground herbivory will decrease it (van Dam, 2009). The composition of PAs in the shoot was indeed affected by below ground herbivory (Kostenko et al., 2013; Martijn Bezemer et al., 2013). The levels of *N*-oxides in shoots decreased by 52 % in the plants exposed to root herbivory. Furthermore, Kostenko et al. (2013) reported that root herbivory in *J. vulgaris* had a strong negative effect on the total concentration of PAs in shoot tissues. Hol et al. (2004) also showed that shoot herbivory decreased PA concentrations in *J. vulgaris* roots. The interaction of plant-producing PAs with microorganisms may work in two ways. The diversity of microorganisms may affect PA concentration and composition and in turn PAs may affect soil bacteria and fungi when released via root exudates or leakages from damaged roots (Wu et al., 2010). Several studies showed that PAs can inhibit fungal growth and play a significant role in shaping the soil fungal community of the rhizosphere (Hol and van Veen, 2002; Kowalchuk et al., 2006).

Plant endophytes are part of the biotic environment. Plant endophytes refer to bacterial or fungal microorganisms living within plants for at least a part of their life cycle without causing any visible symptoms or pathogenic effects to the host (Gunatilaka, 2006; Kusari et al., 2011). The interaction between the plant and endophytes can be characterised as extreme mutualism, antagonism or neutral (Clay, 1996; Jaber and Vidal, 2010; Kusari et al., 2011; Vicari et al., 2002). Examples of mutualistic interactions are the involvement of endophytic fungi in the synthesis of SMs. In the biosynthesis of alkaloids, involvement of endophytic fungi has been observed in several plants. Happy Tree plants (C*amptotheca acuminata)* together with the endophytic fungus *Fusarium solani* produce the indole quinoline alkaloid camptothecine (Kusari et al., 2011). *Ipomoea asarifolia*

plants (Convolvulaceae) infected with the endophytic fungus Clavicipitaceous synthesise ergoline alkaloids (Ahimsa-Müller *et al.*, 2007). Another example is Loline, a fungal alkaloid, produced in Cool Season grasses (Poaceae; subfamily Pooideae) which are infected by an endophytic fungus of the genus *Epichloë* (Clavicipitaceae) and its asexual relative, a *Neotyphodium* species (Schardl et al., 2004). However, such a mutualistic interaction may turn into a negative one when the surrounding environment changes. For example the presence of the foliar endophyte, *Neotyphodium lolii,* in the perennial ryegrass *Lolium perenne* increases the survival of the herbivorous fifth-instar caterpillars of the angel shade *Phlogophora meticulosa* when phosphorous is limiting (Vicari et al., 2002). Most likely, the type of symbiotic relationship depends on the type of fungi, plant genotype and environmental conditions (Faeth and Fagan, 2002). The possible role of endophytic fungi in the PA synthesis of *Jacobaea* plants has not been studied yet.

METABOLOMIC STUDIES

Recent advanced technologies aiming at studying the full suite of metabolites in a plant are called metabolomics. Metabolomics deal with all observable metabolites in a plant both qualitatively and quantitatively. Analyzing the metabolome provides a comprehensive insight into the metabolic status of a plant under different conditions (Weckwerth, 2003;Bundy et al., 2009). It provides insight in the diversity of SMs on all levels, including the highly compartmentalised metabolic networks. Plant metabolism has four dimensions, three of space and one of time. The different pathways have different cellular compartmentations (organ, tissues and cells) and differ through time (diurnal, seasonal, and developmental).

The plant defence mechanism of *Jacobaea* has been extensively studied. However, most of these studies have been limited to the PAs. Application of metabolomics in this study area may serve as an alternative approach to study plant insect interactions. The comparison of herbivore resistant and susceptible plant metabolomes allows identification of different metabolites related to host plant resistance. This approach, the so called eco-metabolomics, has been applied by Leiss et al. (2009) in *Jacobaea* plants. In this study, the metabolomes of plants resistant and susceptible to *F. occidentalis* were compared resulting in the identification of a kaempferol glucoside and jacaranone as secondary metabolites involved in host plant resistance against thrips next to the PAs jacobine and jaconine.

The NMR metabolomics approach is known for its high long term reproducibility, speed and broad range of metabolites detected (Verpoorte et al., 2007). The broad range of metabolites detected by NMR makes this approach a good candidate for macroscopic metabolomics giving a total representative view of all metabolites present both qualitatively and quantitatively (Kim et al., 2006). However the application of NMR is limited by a relatively low sensitivity and a considerable signal overlap in the NMR spectra (Kim et al., 2010). Research efforts to overcome these issues are in progress. As such, low temperature probes, CryoProbe (Bruker Biospin GmbH, Rheinstetten, Germany) or Cold Probe (Varian, Palo Alto, CA, USA) have been developed and claimed to give a 16-fold increase of sensitivity. Moreover, the overlapping signal issue can be solved to a great extent by using two-dimensional NMR, leading to a much better resolution. These developments

make it possible to use NMR to study the metabolome at even relatively low concentrations such as at tissue or cell type level. Micro- or cell specific-metabolomics becomes important considering that plants contain at least 40 different cell types (Martin et al., 2001). Only a few studies have used micro metabolomics for plant studies so far. From those studies, two used laser microdisection, one of the most advanced techniques in single cell isolation. Laser microdissection has been proven to be an effective technique to cut and collect single cell types.

The metabolites in the vascular bundles of *Arabidopsis thaliana* were compared with non-vascular cells using this technique (Schad et al., 2005). Another study on the metabolites of the stone cells in the bark of Norway spruce, *Picea abies*, revealed that these cells are more than just repositories for lignin (Li et al., 2007). They also contained the stilbene astringin and adihydroflavonol, which may be involved in chemical and physical defence against bark beetles and their associated microorganisms. In this thesis we used metabolomics to study the distribution of plant defence related SMs, with particular focus on PAs, in *Jacobaea* plants at the organ, tissue and cell level.

AIMS

The aim of this thesis is to understand the diversity of PAs in *Jacobaea* plants with respect to their spatial distribution and its consequences for generalist insects. This question is broken down into several questions that will be answered in the respective chapters of this thesis. The questions addressed are:

1. Do endophytic fungi play a role in the biosynthesis of PAs in *Jacobaea* plants?
2. Do different organs of *Jacobaea* plants differ in their capacity to produce PAs? Do they differ in PA distribution?
3. Do different leaf tissues of *Jacobaea* plants differ in PA distribution?
4. Do different leaf cell types o*f Jacobaea* differ in PA distribution?
5. Do different types and forms of PAs have different toxic effects on *S. exigua*?

OUTLINE OF THESIS

In this thesis chapter 2 reports on the role of endophytes in the production of PAs in *Jacobaea.* Plants were treated with different systemic fungicides to eliminate endophytic fungi and the effect on PA concentration and composition was determined. Chapter 3, 4 and 5 deal with the variation of PA distribution at organ, tissue and cell levels. Chapter 3 describes the capacity of different plant organs to produce PAs, using different types of *in-vitro* organ cultures including roots, shoots and complete plants. Chapter 4 reports on the metabolomics of different leaf tissues, focusing on differences of PA distribution between epidermis and mesophyll. Chapter 5 deals with a metabolomic study on the different cell types of *Jacobaea* leaves resistant to thrips. Laser micro dissection coupled with NMR was used to study epidermis and mesophyll cells. Chapter 6 describes structure activity relationships for the effect of different PAs on *S. exigua* larvae and cell cultures. The results on the variation of PA distribution in *Jacobaea* plants and their consequence to generalist insects are summarised and discussed in Chapter 7.

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