Pyrrolizidine alkaloid variation in *Jacobaea* plants:
From plant organ to cell level

for Rafif

Tri Rini Nuringtyas

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Pyrrolizidine alkaloid variation in *Jacobaea* plants:
From plant organ to cell level

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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).

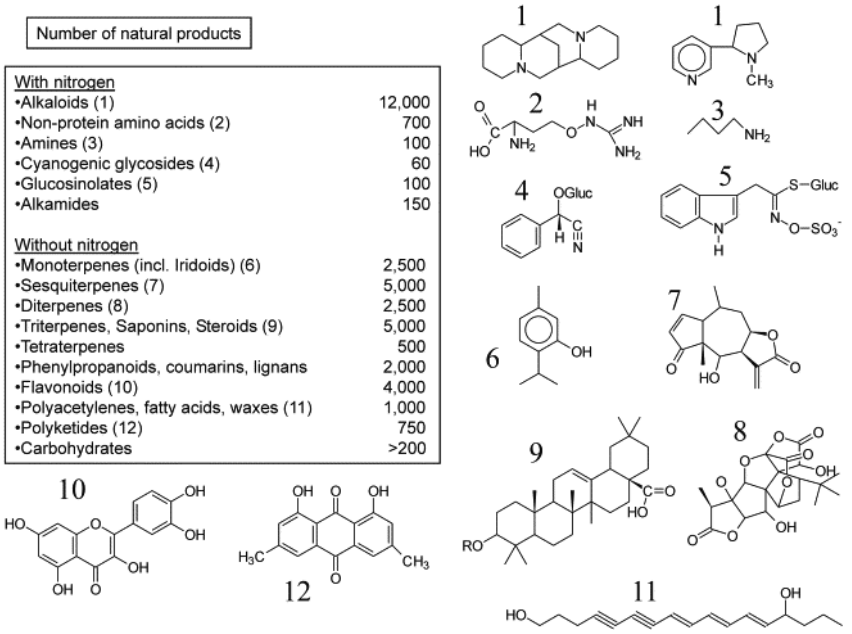

Fig 1. Structural diversity of secondary metabolites (Wink, 2003).

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.

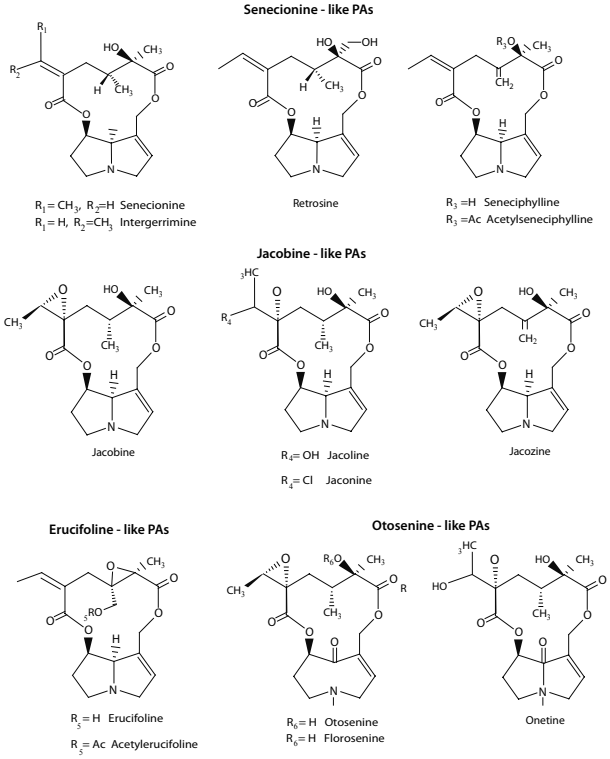

Fig 2. Structure of pyrrolizidine alkaloids in *Jacobaea* plants (adapted with modification from Joosten et al., 2011)

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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Chapter 2.

Role of fungal endophytes on pyrrolizidine alkaloids metabolism in *Jacobaea* plants

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are known as plant defence compounds with highly diverse structures. In *Jacobaea* plants, PAs have been observed to be diverse in both concentration and composition. This diversity may be influenced by biotic and abiotic factors. Plant fungal endophytes are part of the biotic environment. In this study, we eliminated fungal endophytes by treating the plants of F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica* with three fungicides: Folicur, Pronto Plus and Switch. As a result, no detection of fungal endophytes was observed in the Pronto Plus and Switch plants. A fungal endophyte was detected in the Folicur plants. Based on the b-tubulin gene and the Internal Transcribed Spacer (ITS) region of the rDNA, the detected fungus had a close homology with mycorrhizal fungi belonging to the Glomeromycota. The PA content was measured using LC-MS/MS. The Folicur-treated plants showed a lower total PA amount compared to the control whereas the other treatments showed no differences. This suggests that *de-novo* PA synthesis in *Jacobaea* plants is not dependent on endophytes. However, the endophyte present in the Folicur treatment lowered the total amount of PAs produced and changed their composition, leading to relatively low amounts of senecionine- and jacobine-like PAs in contrast to erucifoline- and otosenine-like PAs. In this way endophytes may increase the variation of PAs in *Jacobaea* plants possibly leading to consequences in plant defence against generalist and specialist pathogens and herbivores.

Key Words: *Senecio*, *Jacobaea vulgaris*, *Jacobaea aquatica*, pyrrolizidine alkaloids, fungal endophyte, fungicides.

INTRODUCTION

Plants produce a diversity of secondary metabolites to interact with their dynamic, often hostile environment. These metabolites are distinct from the components of primary metabolism and are not essential for the basic metabolic processes of the plant(Dixon, 2001;Hartmann, 2007). Secondary metabolites may function as a defence against herbivores and pathogens. Pyrrolizidine alkaloids (PAs) belong to one of the secondary metabolite groupsinvolved in the plant's constitutive chemical defence (Ober and Kaltenegger, 2009; Macel et al., 2005).This group of alkaloid is known for its high diversity in occurrence and chemical structure. Pyrrolizidine alkaloids are present in distantly related families such as Asteraceae, Boraginaceae, Fabaceae and Orchidaceae (Hartmann, 1999). Many studies have been conducted to understand the role and meaning of this diversity for plant survival. Accumulated evidences emphasise the importance of both biotic and abiotic factors in PAs diversity.

Pyrrolizidine alkaloids are esters of a necine base with one or more necic acids (Hartmann, 1999). PAs occur in two interchangeable forms: the free base (tertiary amine) and the *N*-oxide form (Hartmann and Dierich, 1998). The PAs in *Jacobaea* plants have been studied intensively and have been reported to be very diverse in both concentration and composition (Pelser et al., 2005; Witte et al., 1992; Macel et al., 2004). At least 37 PAs have been reported from 24 species of the genus *Jacobaea* (Cheng et al., 2011; Pelser et al., 2005). Based on the biosynthesis route, these are dividedinto four main groups : senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Pelser et al., 2005). The PA composition of species such as *Senecio vernalis*, *Senecio vulgaris*, *Senecio inaequidens*, *Jacobaea erucifolia* and *Jacobaea vulgaris* are different (Hartmann and Dierich, 1998). Even within a species, the variation of PA composition like in *J. vulgaris* is high. This intra-specific variation is described by four different chemotypes i.e. the jacobine, erucifoline, senecionine or mixed types (Macel et al., 2004; Witte et al., 1992). PA concentrations differ between organs. For example in *S. vulgaris*, the concentration of PAs in inflorescences is the highest, followed by the leaves, roots and stem (Hartmann and Zimmer, 1986). Composition of PAs differs between organs as well. Generally, the shoots contain higher proportions of jacobine- and erucifoline-like PAs and lower proportions of senecionine- and otosenine-like PAs compared to the roots (Joosten et al., 2009). The PA composition is affected by hybridization (Cheng et al., 2011), plant development stage (Schaffner et al., 2003), and by abiotic factors such as nutrients (Hol et al., 2003), soil types (Joosten et al., 2009), as well as environmental conditions (Hol et al., 2003; Macel and Klinkhamer, 2009, Kirk et al., 2010; Joosten et al., 2009). Furthermore the biotic environment including herbivorous insects (Macel et al., 2005;Leiss et al., 2009), plant fungi(Hol and van Veen, 2002; Hol, 2003) and soil born microorganisms (Joosten et al., 2009; Hol, 2003) can have an effect on PA composition.

Plant endophytes form part of the biotic environment. Plant endophytes refer to bacterial or fungal microorganisms living within plants without causing any visible symptoms or pathogenic effects to the host (Gunatilaka, 2006). Fungi represent the largest group among endophytic microorganisms (Carroll, 1988). Endophytic fungi are ubiquitous and can be extremely diverse in host plants. Almost every organ from every plant species examined harbours at least one species of endophytic fungus (Petrini, 1991). Involvement of endophytic fungi in the biosynthesis of

alkaloids has been observed in several plants. The endophytic fungus *Fusarium solani* produces a quinoline alkaloid, camptothecin, in C*amptotheca acuminata* (Happy Tree plants) (Kusari et al., 2011). *Ipomoea asarifolia* (Ginger Leaf Morning Glory) infected with the endophytic fungus Clavicipitaceous synthesises 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 derivative, the *Neotyphodium* species (Schardl et al., 2004). In general, the symbiotic relationship between fungi and host plants is a mutualistic one since the hosts provide the protection and food for the fungi, whereas in turn the fungi support the host in production of bioactive secondary metabolites (Gunatilaka, 2006). However, more recent evidence suggests that an interaction between host and endophytic fungi does not have to be mutualistic (Jaber and Vidal, 2010; Clay, 1996). It may also be neutral (Saikkonen et al., 1999) or antagonistic (Vicari et al., 2002). Most likely, the type of symbiotic relationship depends on the type of endophytic fungi, plant genotype and environmental conditions (Faeth and Fagan, 2002).
Although plants belonging to the *Jacobaea* genus have been studied intensively, the effect of endophytic fungi on PAs synthesis inthis plant genus is still unknown. Fungal endophytes can be eliminated by treating plants with systemic fungicides. Currently, there are many kinds of synthetic fungicides commercially available with different mode of actions targeting fungal respiration, osmoregulation, methionine or sterol biosynthesis (Leroux et al., 2002).

In this study, we aimed to determine a possible role of endophytic fungi in the biosynthesis of PAs in *Jacobaea* plants. We, therefore, applied different fungicide treatments and studied their effect on PA amount and composition. In addition, we investigated a possible non-specific effect of the fungicide treatments on the plants using a metabolomic approach.

MATERIALS AND METHODS

Set Up of Experiments. F2 hybrid plants of *Jacobaea vulgaris* and *Jacobaea aquatica* were chosen as a study system. Detailed information about the origin of the parent plants and how the hybrids developed had been reported by Cheng et al. (2011). In contrast to the parent plants *J. vulgaris* and *J. aquatica* which accumulate jacobine- and senecionine-like Pas, respectively, F2 hybrids contain all four PA types in comparable proportions. The plants were treated with three different systemic fungicides which have been successfully used to eliminate endophytic fungi in *Ipomoea asarifolia* (Convolvulaceae) (Kucht et al., 2004). The fungicides used were Folicur, Pronto Plus and Switch, exhibiting different ranges and modes of action. Folicur contains 251 g/l tebuconazole as an active ingredient. This active compound claims a broad-spectrum of activity, although it was specifically designed for control of powdery mildew (Ascomycota) and rust fungi (Basidiomycota). Pronto Plus contains 133 g/l tebuconazole and 250 g/l spiroxamine. The addition of spiroxamine suggests that Pronto Plus has a broader spectrum of activity than Folicur. Pronto Plus and Folicur are fungicides that inhibit sterol biosynthesis. Switch contains two active ingredients, 375 g/l cyprodinol and 250 g/l fludioxonil. These anilinopyrimidine fungicides are effective against various Ascomycetes and Adelomycetes. The activity is based on inhibition of methionine biosynthesis and secretion of hydrolytic enzymes (Leroux et al., 2002).

Fungicide Treatment. The F2 hybrid plants were obtained from the tissue culture collection of our department and were transplanted into pots filled with 1:1 sand and soil. Plants were kept in a climateroom (humidity 70%; light 16 h at 20°C; dark 8 h at 20°C). The fungicide concentrations applied were 0.15% for Folicur and Pronto Plus, and 0.1% for Switch. Plants were sprayed with fungicides when six-weeks old. Before spraying five plants were harvested. We refer to these plants as starting materials. For each fungicide treatment, five plants were used. In addition to the treatments, five plants were sprayed with water and these served as control. The plants sprayed with Folicur are referred to as Folicur plants, while plants sprayed with Pronto Plus or Switch are referred to as Pronto Plus and Switch plants, respectively. The fungicides were applied four times with intervals of two weeks in between. One day after the last spraying all plants treated with fungicides as well as the control plants were harvested.

Plant Harvesting. Whole plants including shoots and roots of twelve-weekold plants were removed from the pots, washed, and then frozen in liquid nitrogen. The frozen plants were crushed with mortar and pestle and freeze-dried. The dried samples were weighed and subsequently the samples were kept at -80 ºC until further work-up including endophytes detection, PA analysis and NMR based metabolomics.

*EndophyteDetection***.** To detect the presence of endophytic fungi in the plants, we amplified genes specific for fungi using primers of the β-tubulin gene and the internal transcribed spacer (ITS) region of the rDNA gene cluster. Total DNA was extracted from dry plant material using the Dneasy Plant Mini Kit (QIAGEN, Venlo, The Netherlands). The amplification of the partial β-tubulin gene was carried out using Bt3- LM and Bt10-LM primers (Myllys et al., 2001). Amplification of the ITS region of the rDNA gene cluster was performed with the primers ITS5 and ITS4 (White et al., 1990). Total DNA of *Fusarium oxysporum* (IBL collection) was used as a positive fungal control. Amplification was performed in 20 µl aliquots consisting of 2 µl 10x PCR buffer, 0.5 µl 25 mm $MgCl_2$, 0.5 µl dinucleotide triphosphates (dNTPs) containing 10 µM of each base, 1 µl of each primer 10 µM, 0.2 µl 5 U/µl *Taq* polymerase (QIAGEN, Venlo, The Netherlands) and 1 µl of stock DNA. The PCR conditions were: 3 min at 95 °C; denaturation 30 s at 95 °C, annealing 1 min at 55 ° C (for the ITS region gene) or 1 min at 59 ° C (for the partial β-tubulin gene), extension 1 min at 72 °C (40 cycles), and 15 min at 72°C. The PCR products were separated in a 1.5% agarose gel for evaluation of the product sizes. We then sequenced the β-tubulin and ITS PCR products derived from the control, Folicur plants and the positive fungal control (MACROGEN, Amsterdam, The Netherlands) to identify the genes amplified. We only sequenced the Folicur plants since they were the only treatment containing two ITS PCR products similar in size to that of the control plants and positive fungal control. Alignment assembly and phylogenetic analyses were performed with Geneious 5.5.6 (Biomatters development teams) software. The resulting alignments of the β-tubulin -(701 nucleotides) and the ITS gene (712 nucleotides and 550 nucleotides) were used to retrieve similar sequences from the databases in BLAST searches (Altschul et al., 1990) at the NCBI server http://www.ncbi.nlm.nih.gov/ blast/Blast.cgi. Several sequences, with the closest similarity to our sequences, deriving from different species, were chosen to construct a phylogenetic tree. The fungi *Yarrowia lipolytica* and *Boopis anthemoides* belonging to the *Saccharomycetes* were chosen as additional entries from less closely related fungi to highlight phylogenetic distances. Phylogenetic trees were based on the Jukes-Cantor distance. The bootstraps of the phylogenetic tree were 50% with 100 replicates.

Pyrrolizidine Alkaloid Content. We extracted PAs using aqueous acidic conditions and subsequently measured PAs using liquid chromatography–tandem mass spectrometry (LC-MS/MS). As extraction solvent 2% formic acid was used. Heliotrine at a concentration of 1 µg/ml was added as internal standard. Approximately 10 mg of dried plant powder was extracted by shaking for 1 h with 1 ml of extraction solvent. Solid substances were removed by centrifugation at 9000 rpm for 10 min, followed by filtering the supernatant through a 0.2-µm nylon membrane (Acrodisc 13-mm syringe filter, Pall Life Sciences, Ann Arbor, MI, USA). An aliquot of 25 µl of the filtrate was diluted with 975 ml of water and injected into the LC-MS⁄MS system. The system consisted of a Waters Acquity ultra performance liquid chromatographic (UPLC) unit coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA). Chromatographic separation conditions followed the ones reported by Cheng et al. (2011b). Seventeen individual PA standards (see Joosten et al., 2011, for the source of the standards) were available for this study. For compounds for which no reference standard was available, a semi-quantitative, i.e. indicative value was obtained by comparison with the most closely related isomer. Identification of PAs was based on their retention time, molecular mass and fragmentation pattern and comparison with PA standards and ⁄ or literature data. Data processing was conducted using Masslynx 4.1 software (Waters, Milford, MA, USA). The total PA concentration was defined as the sum of all detected individual PAs. To obtain the amount of PAs per plant, total PA concentration was multiplied with the corresponding plant biomass (dry weight). Except for senecionine *N*-oxide, which is regarded the precursor of all other PAs (Hartmann and Dierich, 1998),we classified the PAs into four groups according to their structural characteristics and biosynthetic pathways (Pelser et al., 2005): senecionine-, jacobine-, erucifoline- and otosenine-like PAs. Each PA group included both the tertiary amine and *N*-oxide forms. The amount of each group of PAs was determined by the changes in the control and treated plants relative to the starting material, thus representing PA production during the period of treatment. The relative concentration of each PA type was calculated as: (absolute concentration of PA type ⁄ total PA concentration) × 100.

NMR Metabolomics. To detect effects on fungicide treatment on the plant metabolome in general, we applied NMR metabolomics. For this 50 mg of dried and powdered material of each plant was extracted using a mixture of KH_2PO_4 buffer (pH 6.0) in D_2O and methanol-d_4 in a ratio of 1:1. The extraction followed the standard NMR based metabolomics procedure for plants (Kim et al., 2010). NMR spectra were recorded at 25°C on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe,Germany) operating at a proton NMR frequency of 600.13 MHz. The NMR conditions followed the ones previously described for *Senecio* plants by Leiss et al. (2009). For the metabolomic analysis, signal intensities of ^{1}H NMR spectra were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to δ 0.4-10.0. The regions of δ 4.75-4.9 and δ 3.28-3.34 were excluded from the analysis because of the residual signal of D_2O and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker, Karlsruhe,Germany). The quantification of the identified metabolites was performed by scaling the intensity of the ^{1}H NMR to the signals of TMSP at 0.00 ppm. Data obtained were subjected to multivariate analysis in order to determine differences in metabolomes between samples.

Data Analysis. The amount of total PA, senecionine *N*-oxide and the different PA-types were analysed using ANOVA with fungicide treatments as a fixed factor and Duncan's multiple

comparisons as a post-hoc test. This statistical analysis was performed using SPSS 19.0. Partial Least Square-Discriminant Analysis (PLS-DA), a supervised multivariate data analysis, was used to cluster the samples based on the relative PA concentrations. This multivariate analysis was performed using SIMCA-P software (version 12.0, Umetrics, Umeå, Sweden). Scaling for PLS-DA was based on the Pareto method. The models were validated by permutation tests through 20 applications and CV-ANOVA, which are the default validation tools in the software package (SIMCA-P). The loading plots were used to identify which ^{1}H NMR signals were important factors for the clustering. The compounds responsible for the clustering were quantified and analysed using ANOVA.

RESULTS

Plant Dry Weight. At harvest, all plants, i.e. the six-week old starting material and the twelve-week old control and fungicide treatments were in the vegetative rosette stage. Dry mass of the starting group, harvested before the treatment, was significantly lower compared to the twelve-week old plants (ANOVA, $F=8.797$, $df= 4$, $P<0.001$). No significant differences in dry weight were observed among the control and fungicide treatments (data not shown).

Endophyte Detection. Endophytic fungi could only be detected in the Folicur plants as indicated by the amplification of the 750 kb β-tubulin gene (Fig.1a). This PCR band was consistently observed in all replicates of the Folicur plants. Amplification of the ITS region of the rDNA gene cluster resulted in a 800 kb DNA fragment in all of the samples. Aside from that, a 600 kb size ITS DNA fragment was observed in the Folicur plants. The DNA fragment had a similar size as the ITS DNA fragment of *F. oxysporum*, which had been used as positive fungal control (Fig. 1b). This indicated that the Folicur plants contained an endophytic fungus in higher concentrations compared to the other treatments. No DNA fragments with similar sizes to these fungal *F.oxysporum* fragment were observed in neither the starting material nor the control, Pronto Plus or Switch plants.

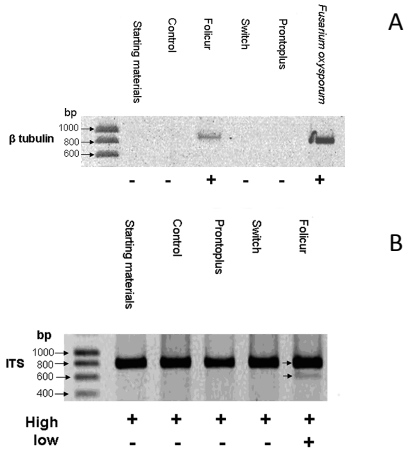

Fig 1. Qualitative analysis of the β tubulin gene (A) and ITS region of the rDNA gene cluster (B) in F2 hybrids of *Jacobaea aquatica* and *Jacobaea vulgaris* after fungicide treatment. + indicates presence and – indicates absence of the corresponding genes. The visible PCR products indicate the concentration of related DNA > 0.28 ng/ml.

Validation of the gene primer showed that the β-tubulin sequence obtained from the positive control *F.oxysporum* showed a high similarity (99% identity) with the β-tubulin sequence of the corresponding accession (AB587041.1) in the NCBI database. The 600 kb partial β-tubulin sequence observed in the Folicur plants was grouped in the same clade as *Glomusclarum,* belonging to the Glomeromycota division, and *Corynespora smithii* belonging to the subdivision of the Pezizomycotina being part of the Ascomycota division (Fig. 2a). The 800 kb sequence of the ITS 4-5 region was observed in the control and treatment samples including Folicur. This sequence was grouped in the same clade as the ITS 4-5 regions of several *Jacobaea* and *Senecio* plants including the parents of the F2 hybrids used in this study: *J. vulgaris* (FJ556936.1) and *J. aquatica* (FJ556950.1) (Fig. 2b). The smaller ITS DNA fragment at 600 kb was grouped in the Glomeromycota clade but also occurred in the Phoma sp. belonging to the Ascomycota. (Fig. 2c). Thus both sequences, the ITS and the β-tubulin sequence indicated that the fungus detected in the Folicur plants, were closely related to the *Glomus* genus within the Glomeromycota division.

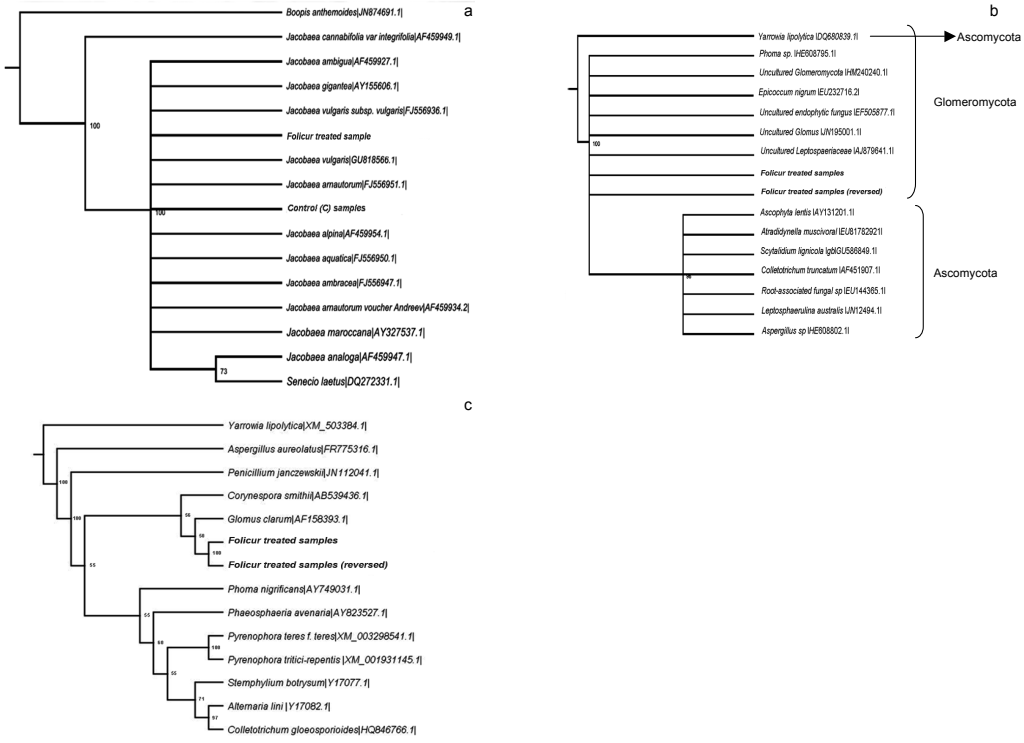

Fig 2. Phylogenies of the ITS sequences at 800 bp (A), and 600 bp (B), as well as of the β tubulin sequences at750 bp (C). Sequences were compared with the highest similarity sequences available in the NCBI database obtained by BLAST. Phylogenetic trees presented are neighbor joining trees based on the Jukes-Cantor distance. The bootstraps were 50% with 100 replicates. *Yarowia lipolytica* and *Boopis anthemoides* were chosen as additional entries from less closely related fungi to highlight phylogenetic distances.

Pyrrolizidine Alkaloid Content. The twelve-week old control group showed a significantly higher total PA concentration compared to the six-week old starting material. The Folicur-treated plants contained lower total PA content compared to the control plants, while the Pronto Plus and Switch plants contained similar amounts of PAs as the control (ANOVA, F=8.741, df= 4, P<0.0001) (Fig. 3a).

The amount of the different PAs, including senecionine *N*-oxide, and the groups of senecionine-, jacobine-, erucifoline- and otosenine-like PAs, showed significant differences between treatments (Fig. 3b). The highest amount of senecionine *N*-oxide was observed in the Switch plants, an intermediate amount in the Pronto Plus and the lowest amount in the Folicur plants and the control (ANOVA, F=9.91, df= 4, P<0.0001). The amount of senecionine-like PAs (without senecionine *N*-oxide) was significantly lower in the Folicur plants compared to all other treatments and the control (ANOVA, F=6.83, df= 4, P=0.001). The same pattern was observed for jacobine-like PAs showing significantly lower amounts in the Folicur plants (ANOVA, F=4.26, df =4, P=0.012). The amount of erucifoline-like PAs in the Folicur plants was significantly lower compared to the other two fungicides treatments but not compared to the control (ANOVA, F=11.72, df= 4, P<0.0001). Otosenine-like PAs occurred only in relatively low amounts in all plant groups, significantly lower in Folicur plants compared to Switch, but did not differ significantly from the Pronto Plus plants or the control respectively (ANOVA, F=4.62, df= 4, P=0.008).

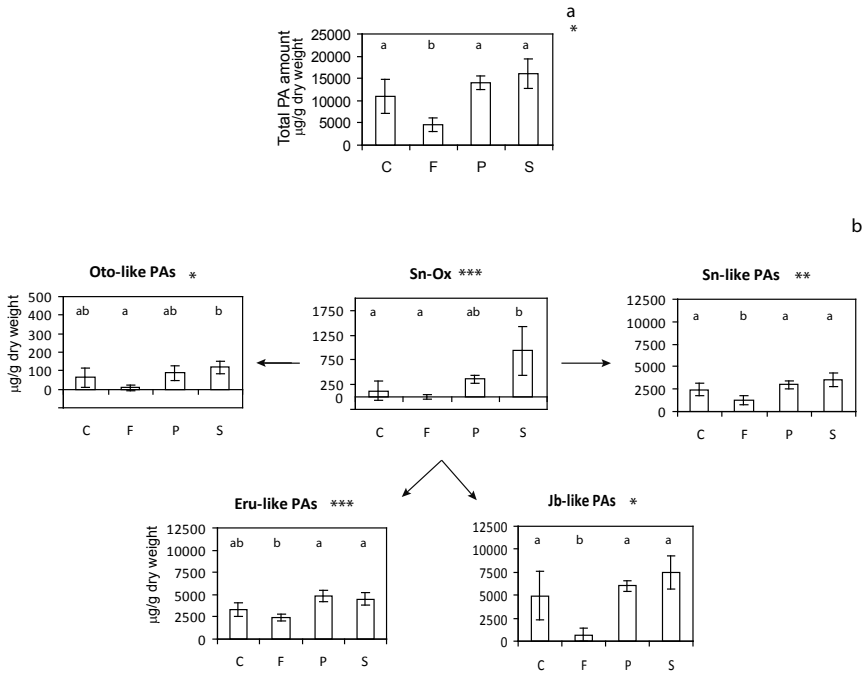

Fig 3. Total PA concentration of F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica* (A). Senecionine *N*-oxide (Sn-ox) and four types of PAs including, senecionine (Sn)-, jacobine (Jb)-, erucifoline (Eru)- and otosenine (Oto)-like PAs composed the total PA amount in each treatment (B). The arrows show that the synthesis of the PAs types is derived from senecionine *N*-oxide. Data present the mean of 5 replicates and the standard error. Treatments were coded as: St=Starting material; C=Control; F= Folicur treatment; P= Pronto Plus treatment; S=Switch treatment. Different letters indicate means that are significantly different at * *P*< 0.05, ** P< 0.01, *** *P* < 0.001.

Partial least square-discriminant analysis (PLS-DA) showed a clear separation between the control and the Folicur plants (Fig. 4). PA-types explained 60% of the total variation between treatments. The control was located in the positive quadrant of PC1 while Folicur plants were located in the negative quadrant. The Pronto Plus and Switch treatments as well as the starting material were located in the middle of the score plot (Fig. 4). PC3 specifically separated the control and Folicur plants from the starting material.The loading plot of the PLS-DA was used to investigate which types of PAs differentiated Folicur from the control. Erucifoline-like PAs were located in the negative quadrant of PC1 coinciding with the Folicur plants. In contrast, the jacobine- and senecionine-like PAs were located in the positive quadrant of PC1 coinciding with the control (Fig. 4). The PLS-DA analysis resulted in a variance R^2 of 0.992 and a predictive ability $Q^2$0.953 indicating a good model.

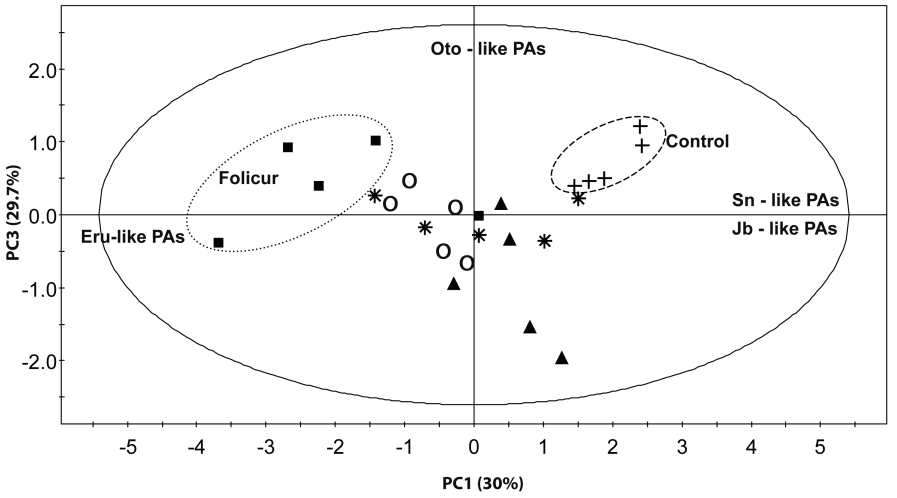

Fig 4. Score and loading plot of PLS-DA based on the relative concentrations of pyrrolizidine alkaloid (PA) types measured by LC/MS/MS in F2 hybrids of *Jacobaea aquatica* and *Jacobaea vulgaris* after fungicide treatment. The ellipse represents the Hotelling T2 with a 95% confidence interval. Symbols depict: starting material (▲); control (+); Folicur treatment (■); Pronto Plus treatment (○); Switch treatment (*).

NMR Metabolomics. Metabolites identified in the plant extracts of control and fungicide treatments included sugars, amino acids, organic acids, and phenylpropanoids. The identification of metabolites was based on NMR spectra of known compounds acquired in previous studies on *Jacobaea* plants (Leiss et al., 2009; Nuringtyas et al., 2012) and from comparison with our in house NMR spectra database (Kim et al., 2010).

The metabolite profiles based on the ^{1}H NMR spectra of the different fungicide treatments were compared with the control and starting material using PLS-DA. The PLS-DA score plot explained 66% of the variation and showed a clear separation of the starting material from all treatments (Fig. 5a). Control and fungicide treatments were clustered together in the negative quadrant of PC1. The loading plot showed that amino acids such as alanine and threonine as well as organic acids like malate and citrate were identified as important factors for the clustering of the starting

material. Chlorogenic acid and several primary metabolites like stachyose, mannitol, glutamate, adenosine, and fumarate were important factors for the clustering of the control and fungicide treatments (Fig. 5b).

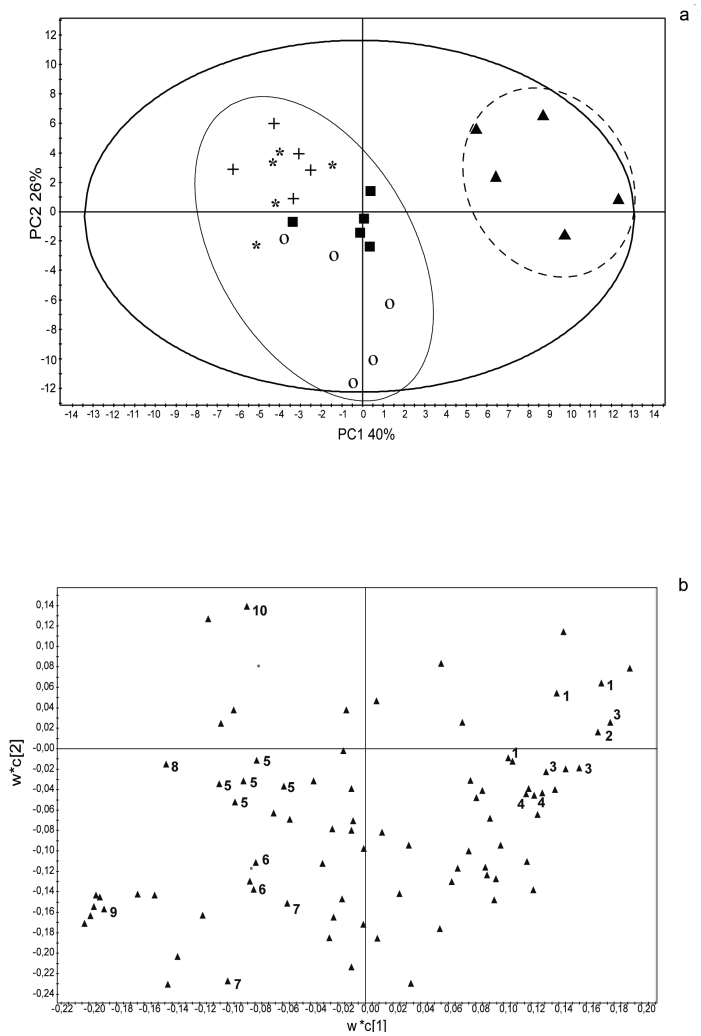

Fig 5. Score (A) and loading plot (B) of PLS-DA of F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica* after fungicide treatment based on 1H NMR spectra. The ellipse represents the Hotelling T2 with a 95% confidence interval. Symbols depict: starting material (▲); control (+); Folicur treatment (■); Pronto Plus treatment (○); Switch treatment (*). Malate(1); Threonine (2); Citrate (3); Alanine (4); Chlorogenic acid (5); Adenosine (6), Glutamate (7); Stachyose (8); Fumarate (9).;Mannitol (10).

Quantitative analysis of the compounds contributing to the separation of the starting material showed significantly higher levels of citrate (*ANOVA*, F=11.09, df= 4, P<0.0001), threonine (*ANOVA*, F=5.58, df= 4, P=0.003) and alanine (*ANOVA*, F=4.30, df= 4, P=0.011) in the starting material compared to the control (Fig. 6). Malate, which was identified in the loading plot of PLS-DA, showed a trend to accumulate in the starting material but failed to be significant. In

addition a higher level of inositol (*ANOVA*, F=3.30, df= 4, P=0.031) was observed in the starting material compared to the treatments. In contrast, significantly lower levels of adenosine (*ANOVA*, F=3.182, df= 4, P=0.036), fumarate (*ANOVA*, F=5.58, df= 4, P=0.003), stachyose (*ANOVA*, F=13.31, df= 4, P<0.0001), glutamate (*ANOVA*, F=4.170, df= 4, P=0.015), and phenylpropanoids such as chlorogenic acid (CGA) (*ANOVA*, F=14.49, df= 4, P<0.0001) and ferulic acid (FQA) (*ANOVA*, F=4.74, df= 4, P=0.07) were measured in the starting material compared to the control. Aside from that, the starting material showed a lower concentration of kaempferol analogues (*ANOVA*, F=3.52, df= 4, P=0.025) compared to the control. The level of mannitol was observed to be lower in the control compared to the starting material and fungicide treatments (*ANOVA*, F=3.52, df= 4, P=0.025).

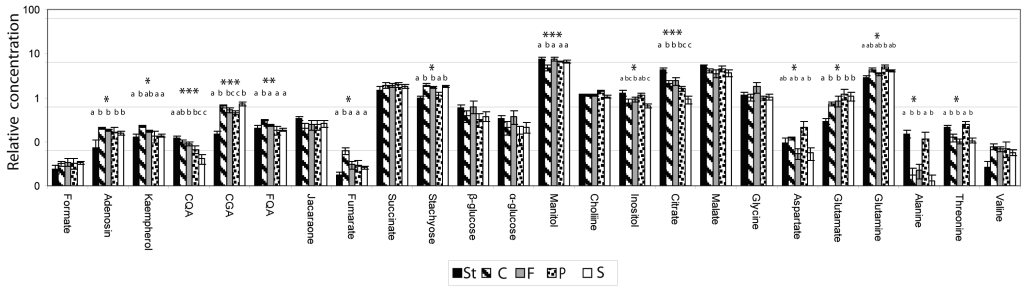

Fig. 6. Quantitfication of metabolites in F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica* after fungicide treatment. Data present the mean of 5 replicates and the standard error. Symbols indicate: St = starting materials, C = control, F = Folicur treatment, P = Pronto Plus treatment, S = Switch. Data were analyzed using ANOVA. Different letters indicate means that are significantly different at * *P* <0.05, ** *P* <0.001, *** *P* <0.0001.

The fungicide-treated plants contained higher concentrations of mannitol but lower concentrations of fumarate and ferulic acid (FQA) than the control plants. Furthermore, the Folicur and Switch plants contained lower levels of the amino acid aspartate (ANOVA, F=4.23, df= 4, P=0.012) compared to the control. In addition to a low level of aspartate, the Switch plants were characterised by low levels of citrate and 5-*O*- caffeoylquinic acid (3-*O*-CQA) (ANOVA, F=8.63, df= 4, P<0.0001). The Pronto Plus plants were characterised by higher levels of the amino acids alanine and threonine while containing a lower level of stachyose.

DISCUSSION

The results of our study showed that *Jacobaea* plants, treated or not treated with fungicides, were able to synthesise PAs. This strongly indicates that endophytic fungi are not involved in PA synthesis. However, endophytic fungi may affect the total amount of PAs produced as well as their composition in the plant. This was observed in the Folicur plants, which contained low levels of total PAs as well as lower levels of senecionine- and jacobine-like PAs compared to the control and other fungicide treatments. A fungal endophyte closely related to Glomeromycota was identified in the Folicur plants at a relatively high concentration.

In all three fungicide treatments, Folicur, Pronto Plus and Switch, PAs were produced. There were no significant differences in the amount and composition of PAs synthesised in the Pronto Plus and Switch treatments and the control. In contrast, the Folicur-treated plants produced less PAs compared to the control and particularly Folicur plants contained less senecionine- and jacobine-like PAs. Only in the Folicur plants an endophyte could be detected. However, we should interpret this result carefully since it is generally accepted that endophytic fungi are ubiquitous and associated with more than 300,000 plant species (Hartley and Gange, 2009;Strobel and Daisy, 2003). Possibly, the concentration of endophytic fungi in the control was too low to be detected. The primers we used in this study are able to amplify the respective gene if the fungal DNA concentration is above 0.28 ng/ml. Fungal endophyte concentration can be as low as 0.1 ng/ml (Musgrave, 1984). However, our results give a strong indication that *de-novo* PA synthesis in *Jacobaea* plants is not depended on endophytes. Our finding is supported by a study reporting PA production of root cultures of *Jacobaea* under sterile conditions (Hartmann et al., 1989; Sander and Hartmann, 1989).

Both the β-tubulin and the ITS genes indicated that the endophyte detected in the Folicur plants showed a high homology with the fungal genus *Glomus*. The Glomeromycota division represents the arbuscularmycorrhizal fungi (AMF). It is known that the roots of *J. vulgaris* have an association with several AMF belonging to the Glomeromycota division (van de Voorde et al., 2010; Harley and Harley, 1987).This type of fungus falls indeed outside the spectrum of activity of tebuconazole, the active compound of Folicur, affecting Ascomycota and Basiodiomycota. Elimination of these fungi may have led to an advantage for the growth of *Glomus*.

Metabolomic analysis of the control and fungicide treatments showed no separation between the fungicide treatments and the control. This indicates that the changes in PA concentration and composition observed in the Folicur plants are a result of the interaction with the *Glomus* mycorrhizal fungus rather than the effect of fungicide treatment. Nothing is known about the role of *Glomus* influencing alkaloid composition. However, a recent study on *Glomus* mycorrhizae showed that the presence of these mycorrhizae reduced alkaloid concentrations in the grass *L. perenne* (Liu et al., 2011). The mechanism by which the *Glomus* mycorrhizae decrease alkaloid concentrations is unclear. The fungus may partly inhibit the *de-novo* PA synthesis or alternatively catabolise PAs. The concentration of senecionine *N*-oxide, the first product of the PA biosynthesis pathway, was significantly lower in the Folicur plants compared to the control and all other treatments. PA diversification is based on the transformation of senecionine *N*-oxide into other

PAs, except senecivernine (Hartmann and Dierich, 1998). The exact sequence of transformation from senecionine *N*-oxide into its derivatives is still under discussion. Two hypothetical biogenetic scenarios have been proposed. The first scenario suggests that formation of jacobine- and erucifoline-like PAs share the same biosynthetic route with the same enzyme being responsible for the transformation from senecionine *N*-oxide, while the otosenine-like PAs are formed independently as derivatives of senkirkine (Pelser et al., 2005) (Fig. S1.a). The second scenario proposes that jacobine- and erucifoline-like PAs are synthesised independently from each other while the otosenine-like PAs are derived from the jacobine ones (Pelser et al., 2005) (Fig. S1. b). The Folicur plants contained less senecionine- and jacobine-like PAs but erucifoline- and otosenine-like PAs were not affected. This indicates that the formation of jacobine-like PAs is independent from that of erucifoline-like PAs, while otosenine-like PAs are not derived from jacobine-ones. We thus propose that in this study PA diversification is a combination of both schemes (Fig. S1.c).

Pyrrolizidine Alkaloidss in the genus *Jacobaea* are known as constitutive defence compounds against pathogens (Joosten and van Veen, 2011) and generalist herbivores (Macel, 2011). Specifically jacobine-like PAs were reported to be involved in host plant resistance to the thrips *Frankliniella occidentalis* (Joosten, 2012; Cheng, et al., 2011; Leiss et al., 2009). In contrast specialist herbivores such as the Cinnabar moth (*Tyriajacobeae*) can use PAs as oviposition cues (Cheng, 2012; Macel and Vrieling, 2003). Jacobine chemotypes of *J. vulgaris* suffered less damage by generalist pathogens and insects in comparison to damage by specialist ones (Macel and Klinkhamer, 2010). An endophyte lowering the levels of total PAs in general and of jacobine-like ones specifically, may, therefore, affect plant defence against generalist and specialists. So far, the mycorrhizal fungus *Glomus mosseae* has been reported to reduce the beneficial effect of *Neotyphodium lolii*, a foliar endophytic fungus present in *L. perenne* on deterrence of the Noctuid moth, *Phlogophora meticulosa* (Vicari et al., 2002).

CONCLUSION

The *de-novo* PA synthesis in *Jacobaea* plants does not seem to be depended on endophytes. However, endophytes, as part of the biotic environment may influence the amount and composition of PAs produced. In this way endophytes may increase the variation of PAs in *Jacobaea* plants possibly leading to consequences in plant defence against generalist and specialist pathogens and herbivores.

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Supplementary data Chapter 2

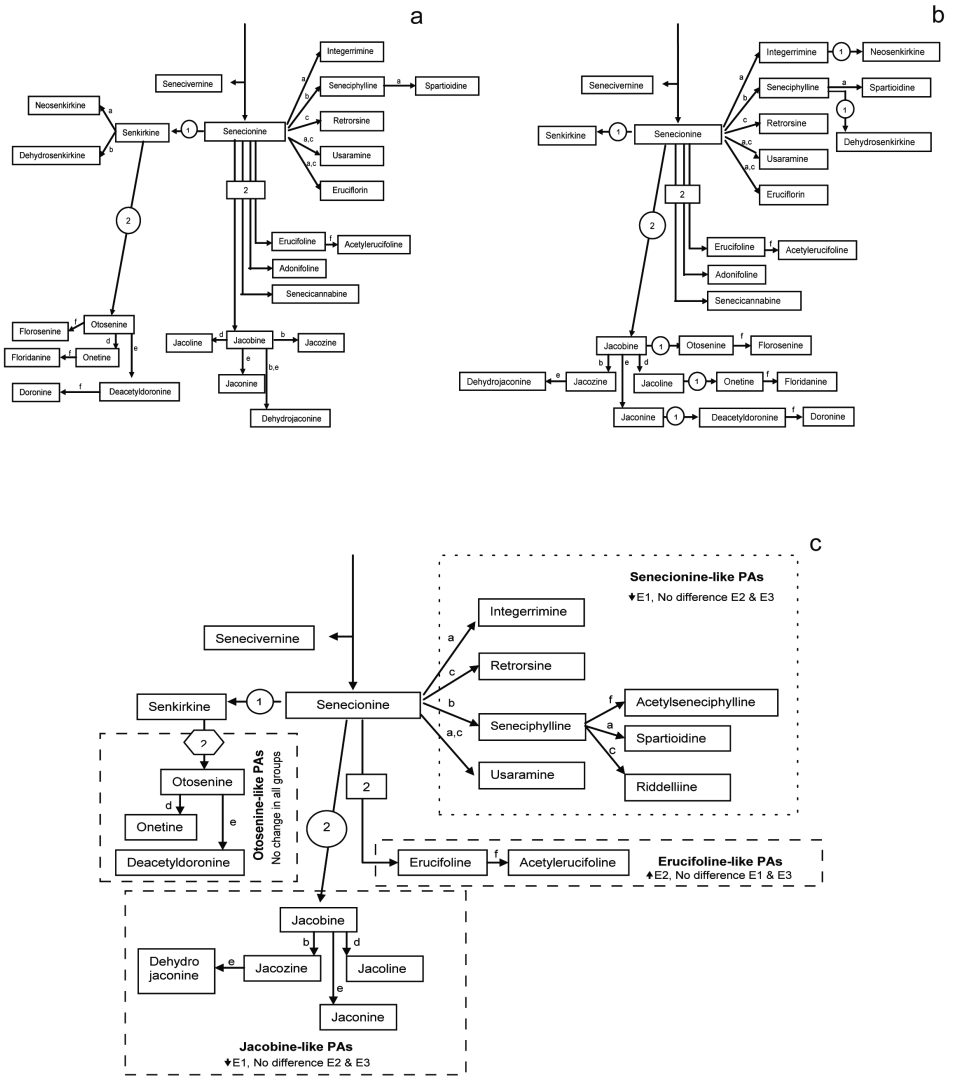

Fig S.1. Putative biosynthetic pathways for the diversification of PAs in the Jacobaea section: With the exception of senecivernine, senecionine is the common precursor of all other PAs. A and B are proposed by Pelser et al. (2005). A. Senkirkine is assumed to be a common precursor of all otonecine derivates; B. Otonecine derivates originate independently from the respective retronecine derivatives. In this case Jacobine- and erucifoline-like PAs are independent from each other. C. Combination of A and B based on the results of our fungicide treatments. The jacobine- and erucifoline-like PAs are independent from each other and the otonecine-like PAs derive from senkirkine. Two main reactions occur: conversion of retronecine to otonecine (reaction 1) and site-specific epoxide formation (reaction 2). Further structural diversification requires six simple one-step reactions marked by letters a-f: a= Z/E isomerization at C20; b= 13, 19-dehydrogenation; c = site-specific hydroxylations; d = hydrolysis of 15,20-epoxide; e = chlorolysis of 15,20-epoxide; f = site-specific O-acetylations.

Table S-1. ^{1}H chemical shifts (d) and coupling constants (Hz) in metabolites of F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica* identified by 1D and 2D NMR spectra in KH_2PO_4 and $MeOD_4$

No	Compounds	Chemical shifts (ppm) and coupling constants (Hz)
1	Adenine	d 8.33 (s), d 8.20 (s)
2	Alanine	d 1.47 (d, J = 7.2 Hz)
3	Aspartate	2.80 (m), d 3.80 (m)
4	3-*O*-caffeoyl quinic acid	d 5.43 (d, J = 5.6 Hz, 3.1 Hz), d 6.35(d, J = 15.9 Hz), d 7.60 (d, J = 15.9 Hz)
5	Chlorogenic acid (5-*O*-caffeoyl quinic acid)	d 5.42 (ddd, J = 10,8 Hz, 9.8 Hz, 5.6 Hz), d 6.28(d, J = 15.9 Hz), d 6.78 (d, J = 8.62 Hz), d 6.95 (dd, J = 8.21 Hz, 1.9 Hz), d 7.05 (d, J = 1.9 Hz), d 7.57 (d, J = 15.9 Hz)
6	Choline	d 3,22 (s)
7	Citrate	d 2.74 (d, *J* = 17.6 Hz), δ 2.56 (d, *J* = 17.6 Hz)
8	Formate	d 8.49 (s)
9	Fumarate	d 6.58 (s)
10	Feruloyl quinic acid	d 5.57 (dt, J = 8.0 Hz, 3.1 Hz), d 6.39 (d, J = 15.9 Hz), d 7.62 (d, J = 15.9 Hz)
11	Glutamine	d 2.15 (m), d 2.47 (m)
12	Glutamate	d 2.07 (m), d 2.36 (m)
13	Glucose	d 4.48 (H-β, d, J = 7,9 Hz), d 5.11 (H-α, d, J = 3,85 Hz)
14	Glycerol	d 3.50 (m), d 3.60 (m)
15	Inositol	d 3.15 (t, J = 9.27 Hz), d 3.43 (dd, J = 2.79 Hz, 9.78 Hz), d 3.96 (t, J = 2.67 Hz)
16	Jacaranone	d 6.16 (d, J = 9.6 Hz), d 7.05 (d, J = 9.6 Hz)
17	Kaempferol analogues	d 7.12 (d, J = 8 Hz), 8.01 (d, J = 8 Hz)
18	Mannitol	d 3.82 (d, J = 3.0 Hz)
19	Malate	d 4.34 (dd, J = 6.6, 4.7Hz), d 2.68 (dd, J = 16.6, 6.6Hz), d 2.78 (dd, J = 16.6, 4.7Hz),
20	Stachyose	d 5.47 (d, J = 3.8 Hz)
21	Succinate	d 2.54 (s)
22	Sucrose	d 5.39 (d, J = 3.8 Hz), d 4.13 (d, J = 8.5 Hz)
23	Threonine	d 1.30 (d, J = 6.6 Hz)
24	Valine	d 1.00 (d, J = 6.8 Hz), d 1.04 (d, J = 6.8 Hz),

Chapter 3.

Shoot and root cultures of *Jacobaea plants* show different capacities to synthesise and transform pyrrolizidine alkaloids (PAs)

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are part of the constitutive chemical defence against herbivores. PA composition in plants is strongly determined by the environment, mostly in an unpredictable way. To understand the mechanisms behind this we need to know the capacity of different plant organs to synthesise and transform PAs.For such an investigation we developed *in-vitro* cultures of roots, shoots and complete plants (roots and shoots) of different *Jacobaea* genotypes*: Jacobaea vulgaris*, *Jacobaea aquatica* and their F1 and F2 hybrids.The cultures were harvested after nine weeks and PA content was measured using LC-MS/MS. We observed that not only roots, as known so far, but also shoots were able to synthesise *de-novo* PAs. Significant differences in total concentration of PAs were observed with the lowest concentration in the roots, followed by shoots and the highest concentration in complete plants.Although the genotypes differed in PA amount and composition they all followed this general pattern. Evaluation of PA composition showed that PLS-DA separated between roots and complete plants, while shoots were intermediate. Senecionine- and otosenine-like PAs were present in both roots and shoots while next to senecionine-like PAs,jacobine- and erucifoline-like PAs occurred in the shoots and complete plants.The *N*-oxides were the major accumulation form of PAs in all organ cultures. Our study indicates that both roots and shoots are essential for PA synthesis, while shoots are essential for PA diversification especially of jacobine-like PAs. This may be an adaption to plant defence since this type of PAs is known to inhibit above ground generalist herbivores.

Keywords: *Jacobaea*, pyrrolizidine alkaloids, organ cultures, synthesis, transformation

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are part of the plant's constitutive chemical defence against herbivores(Ober and Kaltenegger, 2009; Macel et al., 2005). These alkaloids cover more than 400 different structures in approximately 6000 angiosperm species (Chou and Fu, 2006; Hartmann and Ober, 2000) including Asteraceae, Boraginaceae, Fabaceae (mainly the genus *Crotalaria*) and Orchidaceae (Hartmann, 1999). They are known to be powerful deterrents and toxins to most vertebrates (Mattocks, 1986) and generalist insects(Leiss et al., 2009; Cheng et al., 2011a; Macel et al., 2002; Joosten, 2012). However, specialists are not affected (Macel et al., 2002; Macel and Klinkhamer, 2010). Some of the specialists sequester and utilise PAs for their own protection against predators and parasitoids (Hartmann and Ober, 2000) or use them as oviposition stimulants (Macel and Vrieling, 2003; Cheng, 2012). Recent reports showed that hybridization can result in novel patterns of overall PA composition in the roots and shoots of F1 and F2 hybrids of *Jacobaea vulgaris* (syn. *Senecio jacobaea*) and *Jacobaea aquatica* (syn. *Senecio aquaticus*) (Cheng et al., 2011b). Parent and hybrid genotypes are maintained in our laboratory and we have used them as model plants for studies on both ecological and biochemical evolution (Kirk et al., 2004); genotypic-metabolome variation as a result of hybridization (Kirk et al., 2005); compartmentation of metabolites in different leaf tissues (Nuringtyas et al., 2012); as well as plant defence (Leiss et al., 2009; Cheng et al., 2011a; Joosten, 2012). These studies support the hypothesis that PAs evolve as plant defence compounds under the selection pressure of biotic and abiotic environments. Thus, PAs represent an excellent study system for evolutionary ecology and biosynthesis of secondary defence metabolites(Pelser et al., 2005; Hartmann and Ober, 2000)

Pyrrolizidine alkaloids are esters of a necine base with one or more necic acids (Hartmann, 1999). The necine base portion is formed from two molecules of putrescine which is derived from the arginine-agmatine route (Hartmann et al., 1988). The necic acids portion is derived from several common amino acids such as L-threonine, L isoleucine, L-valine and L-leucine(Stirling et al., 1997). Until now, 37 individual PAs have been reported from the 24 species of the genus *Jacobaea* (Pelser et al., 2005; Cheng et al., 2011a). The site of synthesis and accumulation of PAs differs among species. PA synthesis may occur in shoots, like in *Heliotropium indicum* (Frölich et al., 2007) and *Cynoglossum officinale* (van Dam et al., 1995) or in roots like in *Symphytum officinale* (Frölich et al., 2007) and *Senecio vulgaris* (Hartmann et al., 1989). All PAs except senecivernine are derived from senecionine *N*-oxide (Hartmann and Toppel, 1987). From the roots, 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 by one- or two-step reactions such as hydroxylations, dehydrogenations, epoxydations, *O*-acetylations, to yield the species-specific PAs patterns (Hartmann and Dierich, 1998). Aside from the structural diversification, PAs do not undergo any significant turnover or degradation and they are stored in the vacuoles (Ehmke et al., 1988). According to their biosynthesis, PAs in the *Jacobaea* genus are divided into four groups, including derivatives of: senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Pelser et al., 2005). Based on the dominant PA types present in the *Jacobaea* genus, four chemotypes are known for the intra-species PA variation:jacobine, erucifoline, senecionine and mixed chemotypes(Witte et al., 1992; Macel et al., 2004).

Pyrrolizidine alkaloids occur in two interchangeable forms: the free base (tertiary amine) and the *N*-oxide form (Hartmann and Dierich, 1998).The free base from is considered to be non-toxic compared to the *N*-oxide form (Hartmann and Toppel, 1987). The *N*-oxide is the major PA storage form in *S.vulgaris* (Hartmann and Toppel, 1987). Moreover, senecionine *N*-oxide rather than the free base is the specific form for the translocation from root to shoot (Ehmke et al., 1988). Recently, Joosten et al. (2011, 2009) reported that the free base consistently constituted up to 50% of the total alkaloid concentration in the jacobine chemotype of *J. vulgaris*. The relative concentration of free bases for the various PAs was genotype dependent and showed a high correlation with their corresponding *N*-oxide form (Joosten et al., 2011; Cheng et al., 2011b).

Pyrrolizidine alkaloids are present in all organs but are not equally distributed. In *H. indicum*, the highest level of PAs occurs in the inflorescences (Frölich et al., 2007) while in *Phalaenopsis* hybrids maximum amounts of PAs are found in young and developing tissues such as root tips and young leaves (Frölich et al., 2006). A similar pattern is 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 varies quantitatively and qualitatively among shoot organs, including leaves, stems and inflorescences (Hartmann and Dierich, 1998). Comparison between roots and shoots of vegetative *J. vulgaris* and *J. aquatica* plants and their F2 hybrids showed that the shoots contained higher proportions of jacobine- and erucifoline-like PAs and lower proportions of senecionine- and otosenine-like PAs compared to the roots(Joosten et al., 2009).These examples show that once the PAs are synthesised, they will be distributed over the whole plant. How PAs are accumulated and distributed in a particular organ may depend on several processes such as: (a) the rate of *de-novo* PA synthesis which seems to occur mostly in the roots (b) long-distance translocation of senecionine *N*-oxide through the phloem into the shoots and (c) further structural transformation and organ selective storage.

Thus, the biochemistry and physiology of PAs are quite well understood. In contrast, little is known about the capacity of different plant organs to synthesise and transform PAs. In this regard different study systems with *S. vulgaris* were used. These included detached shoots of flowering plants, *in-vitro* root cultures, and several undifferentiated *in-vitro* cultures including callus, cell suspension, tumor and teratoma shoot cultures. Each of these systems was fed with labelled precursors including ^{14}C putrescine, ^{14}C arginine and ^{14}C spermidine (Hartmann et al., 1989). Only the root cultures produced senecionine *N*-oxide, while all other systems failed to synthesise PAs. However, these results should be interpreted carefully. The undifferentiated *in-vitro* cultures are not comparable with root cultures which consist of differentiated cells. Moreover, the form of a plant organ may affect the capacity of PA synthesis. Van Dam et al. (1995) reported that *C. officinale* was able to synthesise PAs in both the shoot and the root. However, this was only the case when looking at the intact rosette as a shoot while single leaf failed to produce PAs when fed with the precursors. Furthermore, the study of the PA diversification process in different organs may require longer time of observation than the one day used in the previous feeding experiment. Sander and Hartmann (1989) showed that in root cultures of *Jacobaea erucifolia* more senecionine *N*-oxide was transformed into seneciphylline *N*-oxide 10 days after introducing the precursors.

We, therefore, used an alternative approach to understand how organ specificity governs PA

production and variation. We used roots, shoots, and complete plant cultures, with both roots and shoots, deriving from *J. vulgaris*, *J. aquatica* and their hybrids. This enables us to study the potential of different organs for *de-novo* synthesis of PAs.

We specifically wanted to answer the following questions: Do different types of organ cultures have different capacities to produce PAs? Does PAconcentration and composition vary between organs? Do different types of organs have different PA storage forms, i.e. free base or *N*-oxide forms?

MATERIALS AND METHODS

Generation of Organ Cultures. *Jacobaea vulgaris* Gaertn seeds were collected at Meijendel Nature Reserve and *J. aquatica* G. Gaertn, B. Mey and Scherb seeds at the Zwanenwater Reserve. The crosses of these plants were performed by rubbing flower heads together since both species are self–incompatible (Kirk et al., 2004). The parental, F1 and F2 individuals were maintained in tissue cultures. To represent the variation in PAs synthesis in *Jacobaea* plants we included the parental genotypes as well as the hybrids F1A, F1B and one of the F2 in this study. Two-week-old plantlets were used as sources of root, shoot and complete (root and shoot) cultures. The roots and shoots of each plantlet were aseptically excised and separately grown in a modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) optimised for each organ culture, as follows:

a. Root cultures were grown in 250 ml conical flasks containing 80 ml MS medium at pH 6.8, supplemented with 2% sucrose, and plant regulator a-naphtaleneacetic acid (NAA) and indole-3-butyric acid (IBA) in a 3:1 ratio (mg/l). The root cultures were shaken on a gyratory shaker at 108 rpm in the dark and were routinely subcultured at three week intervals.
b. Shoot cultures were grown in closed glass containers on 150 ml of MS medium solid medium at pH 6.0 supplemented with 3% sucrose, 4.5 mM N-benzylaminopurine (BAP) and 0.6% agar for solidification.
c. Complete plant cultures were grown in closed glass containers on the same medium as for the shoot culture but without phytohormones.

We used 5 replicates for each genotype and organ culture giving a total of 75 cultures. All cultures were maintained in a climate room (humidity 70% at 17 ± 2 ºC). The cultures were harvested after nine weeks by a removal from the medium, washing with distilled water and freezing in liquid nitrogen. The shoots and the roots of the complete plant cultures were not separated. The frozen materials were crushed with a mortar and pestle and freeze-dried. Subsequently, the dried samples were kept at 80 ºC until further work-up.

Pyrrolizidine Alkaloids Analysis. We extracted PAs using a formic acid extraction method and subsequently analysed them by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Samples were extracted using 2% formic acid. Heliotrine, monocrotaline and monocrotaline *N*-oxide, at concentrations of 1 µg/ml, were added to the extraction solvent as internal standards. An amount of 10 mg of dried powder of the culture samples was extracted with

1ml of the extraction solvent. The mixture was shaken for 1 h. Solid substances were removed by centrifugation at 9000 rpm for 10 min, followed by filtering the supernatant through a 0.2-µm nylon membrane (Acrodisc 13-mm filter, Pall Life Scie, Ann Arbor, MI, USA). An aliquot of 25 µl of the filtered solution was diluted with 975 ml water and injected into the LC-MS⁄MS system, consisting of a Waters Acquity Ultra Performance Liquid Chromatographic (UPLC) system coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA). Chromatographic separation conditions followed the reported method on PA extraction (Joosten et al., 2010). Seventeen individual PA standards (see Joosten et al., 2011, for the source of the standards) were available for this study. For compounds without reference standard, a semi-quantitative value was obtained by comparison with the most closely related analogue. Identification of PAs was based on their retention time, molecular mass and fragmentation pattern as well as on comparison with PA standards and⁄or literature data. Data processing was conducted using Masslynx 4.0 software (Waters, Milford, MA, USA). The total PA concentration was calculated as the sum of all detected individual PAs. For further analysis, we classified the PAs into four groups according to their biosynthetic pathways (Pelser et al., 2005): senecionine-, jacobine-,erucifoline- and otosenine-like PAs. The PA composition was evaluated with regard to the relative concentration of each PA- group in comparison with the total PA concentration. The relative concentration of individual PAs was calculated as follows:
((Absolute concentration of PA)⁄(Total PA concentration)) × 100.

Data Analysis: Differences in concentration of total PAs between organ cultures and between genotypes were analysed using General Linear Models (GLM) with organ cultures and genotypes as fixed factors. Statistically significant data were further analysed using a Duncan's test for multiple comparisons in SPSS 19.0. The PA profile was analysed using relative concentrations of individual PAs. The relationship between senecionine *N*-oxide as the first product of the PA biosynthesis and the concentration of total PAs was investigated with a Pearson correlation analysis. Partial Least Square - Discriminant Analysis (PLS-DA), performed with SIMCA-P software (version 12.0, Umetrics, Umeå, Sweden) was used to visualise the variation in PA profiles. Scaling was based on the UV method. The models were validated by permutation test through 20 applications and CV-ANOVA, which is the default validation tool in the software package (SIMCA-P). Using the loading plots of PLS-DA, we determined the individual PAs responsible for the separation. These were further analysed using GLM to determine differences in concentration between organ cultures and genotypes.

RESULTS

Organ Cultures. All organ cultures grew well without callus formation. The starting material for the root cultures had diameter of cultures of 1.5-2 cm on average. At time of harvest, after nine weeks, this had increased to 5-7 cm (Fig.1a and b). The shoot cultures produced twelve - fifteen shoots of 6 - 8 cm height when harvested after nine weeks (Fig.1c). No roots were formed in the shoot culture (Fig.1d), however, a gall-like formation was observed. The complete plant cultures showed development of both shoots and roots (Fig.1e and f).

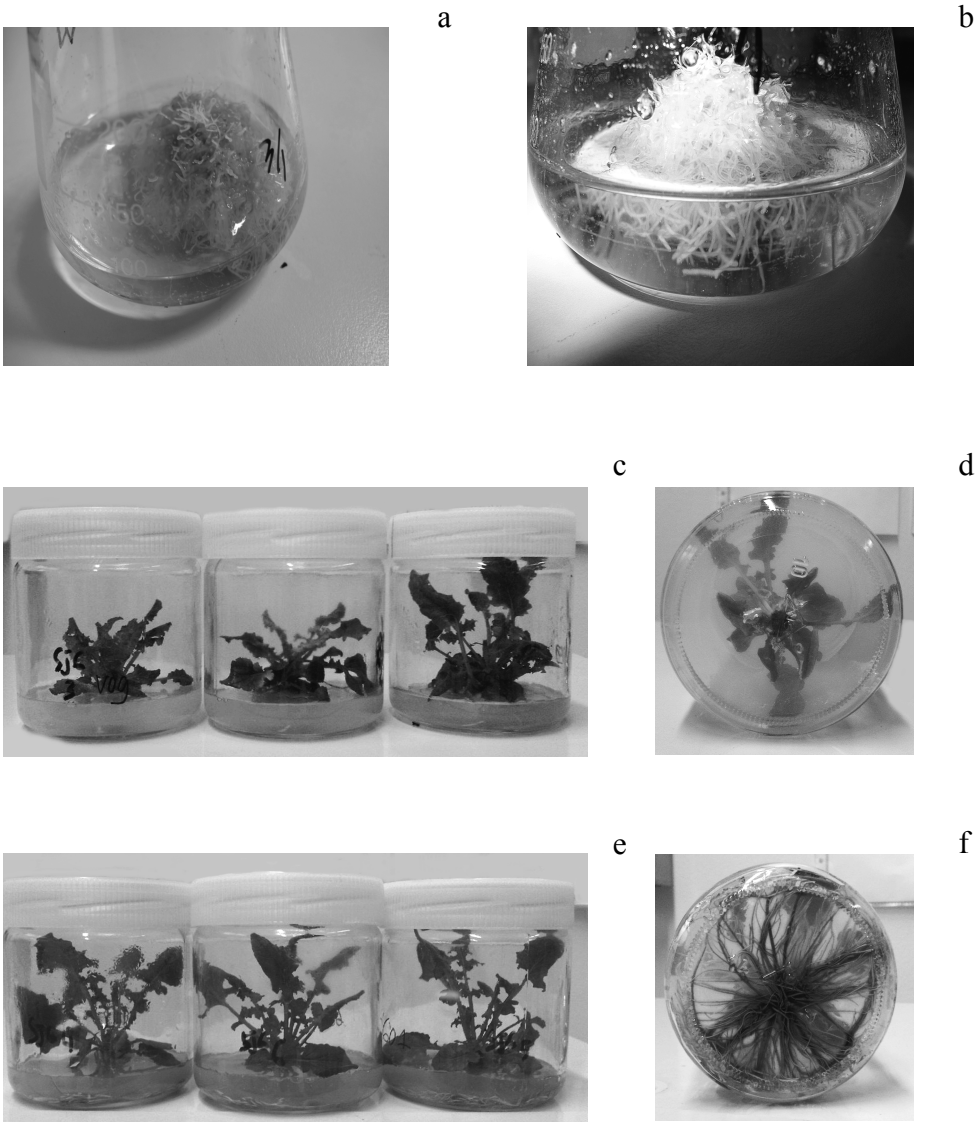

Fig 1. Photographs of organ cultures of *Jacobaea vulgaris and Jacobaea aquatica:* Root cultures of *J. vulgaris* (a) and *J. aquatica* (b).Shoot cultures of *J. vulgaris* showing the above ground part (c) and the base of the shoot cultures without root formation (d). Complete cultures of *J. vulgaris* and *J. aquatica* showing the above ground part (e) and roots formed at the below ground part (f). All photographs were taken at time of harvest, i.e. 9 weeks of growth.

Pyrrolizidine Alkaloid Concentration. The total concentration of PAs differed significantly between organ cultures (ANOVA, F=23,584; df =2; P < 0.001). The complete plant culture showed the highest concentration of total PAs. Compared to the root cultures, complete plant cultures contained three times more and compared to the shoot cultures two times more PAs (Fig.2a). In all of the three cultures, total PA concentration was different among genotypes (Fig. 2b). In the F1A root cultures, PA levels were the highest and in F1B the lowest. The other genotypes

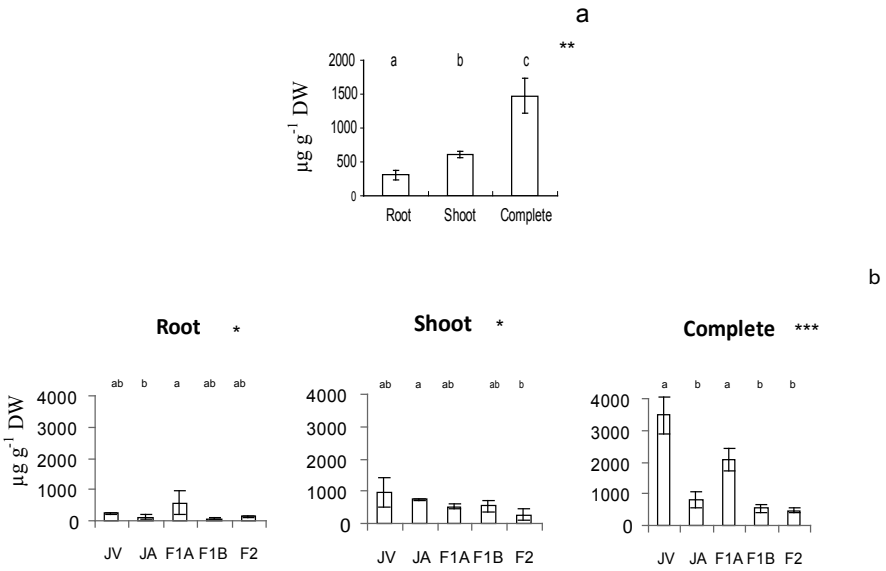

Fig 2. Average of total pyrrolizidine alkaloid (PA) concentration of *Jacobaea vulgaris, Jacobaea aquatica* and their hybrids in root, shoot and complete plant *in-vitro* cultures expressed overall genotypes (a) and expressed per genotype (b): *J. vulgaris* (JV), *J. aquatica* (JA), hybrids of JV and JA: F1A, F1B and F2. Data present the average and standard error of 5 replicates each. Different letters denote significant differences at * *P*< 0.05; ** *P*< 0.001; *** *P*< 0.0001.

contained intermediate PA levels (ANOVA, F=4.292; df =4; P = 0.011). Among the shoot cultures, *J. vulgaris* showed the highest PA level while F2 showed the lowest. The remaining genotypes showed intermediary PA levels (ANOVA, F=3.0; df =4; P = 0.043). In the complete plant cultures *J. vulgaris* and F1A contained significantly higher levels of total PAs compared to the *J. aquatica*, F1B and F2 genotypes (ANOVA, F=16.3; df =4; P < 0.001).

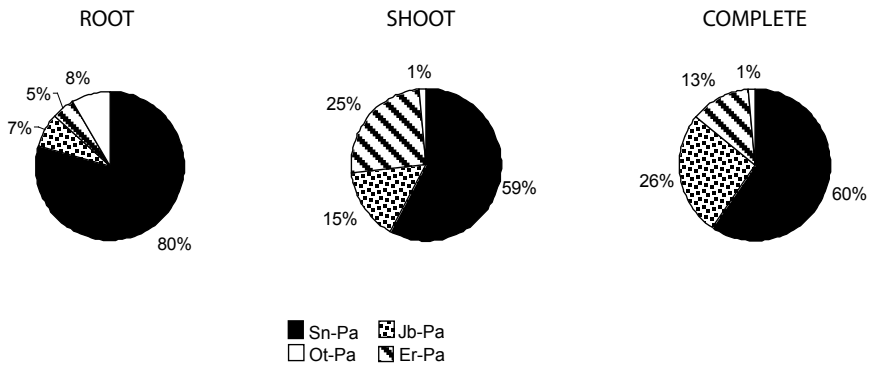

Fig 3. Overall pyrrolizidine alkaloid (PA) composition of *Jacobaea vulgaris, Jacobaea aquatica* and their hybrids based on four structural PAs in root, shoot cultures and complete plant cultures. Data present the average of relative concentration and standard error of 25 replicates of each culture. Sn-PA = senecionine-like PAs, Jb-PA = jacobine-like PAs, Ot-PA = otosenine-like PAs, Er-PA = erucifoline-like PAs

Pyrrolizidine Alkaloid Composition. A total of 36 different PAs were detected in the five genotypes used in this study. Several PAs including usaramine, riddeline, spartiodine, integerrimine, and acetylerucifoline were detected in trace amounts. In general, PA composition in the shoot and complete plant cultures was more variable compared to the root cultures (Fig. 3). In the latter all PAs consisted of senecionine-like PAs in contrast to shoots and complete plant cultures. Jacobine-like PAs were at a maximum in complete plant cultures, followed by the shoot and the root cultures, respectively. In contrast, erucifoline-like PAs showed the highest percentage in shoot cultures followed by the complete plant and in the root cultures. Otosenine-like PAs contributed 8% of the total PAs in root cultures, which equalled the jacobine contribution, but contributed only marginally, with below 1%, to the PAs in shoots and complete plant cultures.

The PA composition differed among genotype (data not shown). In all culture types *J. vulgaris* contained significantly more jacobine-like PAs compared to *J. aquatica*. Hybrids contained intermediate amounts of PAs in comparison to their parents with the exception of F1B which contained significantly more erucifoline-like PAs in the shoot culture.

The PA composition was further evaluated based on individual PAs using PLS-DA. The score plots of PLS-DA showed a clear separation of root cultures from complete plant cultures. Most of the complete plant cultures were located in the positive quadrant while the root cultures were in the negative quadrant of PC1.The shoot cultures were situatedin the middleof the plot. The PC1 determined the separation between the root cultures from complete plant cultures and explained 31% of the PA variation (Fig. 4a). The loading plots of the PLS-DA showed that the root cultures were strongly affected by senecionine-like PAs especially acetylseneciphylline *N*-oxide, retrorsine *N*-oxide, retrorsine and onetine, an otosenine-like PA. In contrast, in the positive quadrant of PC1 we observed a strong effect of jacobine-like PAs including jacobine, jacobine *N*-oxide, jaconine and jaconine *N*-oxide located in the lower part and erucifoline-like PAs including erucifoline and acetylerucifoline *N*-oxide located in the upper part of the quadrant. In the positive quadrant we can also observe senecionine *N*-oxide (Fig.4b). This PLS-DA obtained two principal components with a predictive ability Q^2 of 0.57. The validation of the model used CV-ANOVA and resulted in a *P* value of 0.00011 indicating a good model.

Based on the loading plot of the PLS-DA (Fig.4b), we further analysed the individuals PAs responsible for the separation of the root cultures from complete plant cultures. These PAs comprised senecionine *N*-oxide, seneciphylline *N*-oxide, acetylseneciphylline *N*-oxide, retrorsine *N*-oxide, jacobine *N*-oxide, jacobine, jaconine *N*-oxide, jaconine, erucifoline *N*-oxide, acetylerucifoline *N*-oxide and onetine (Table 1). In general, the root cultures always showed lower concentrations of individual PAs compared to the shoots and complete plant cultures except for onetine (ANOVA, F=8.47; df =2; P =0.001). The concentration of senecionine *N*-oxide, the first alkaloid in the PA synthesis pathway, differed significantly between organ cultures (ANOVA, F=23.6; df =2; P <0.0001). This PA showed a much lower concentration in the root cultures compared to the shoot and complete plant cultures, which contained 3 and 10 times more of senecionine *N*-oxide, respectively. Senecionine *N*-oxide was positively correlated to the concentration of total PAs in root (N= 5, R= 0.913, P=0.030) and complete cultures (N= 5, R= 0.973, P=0.005)

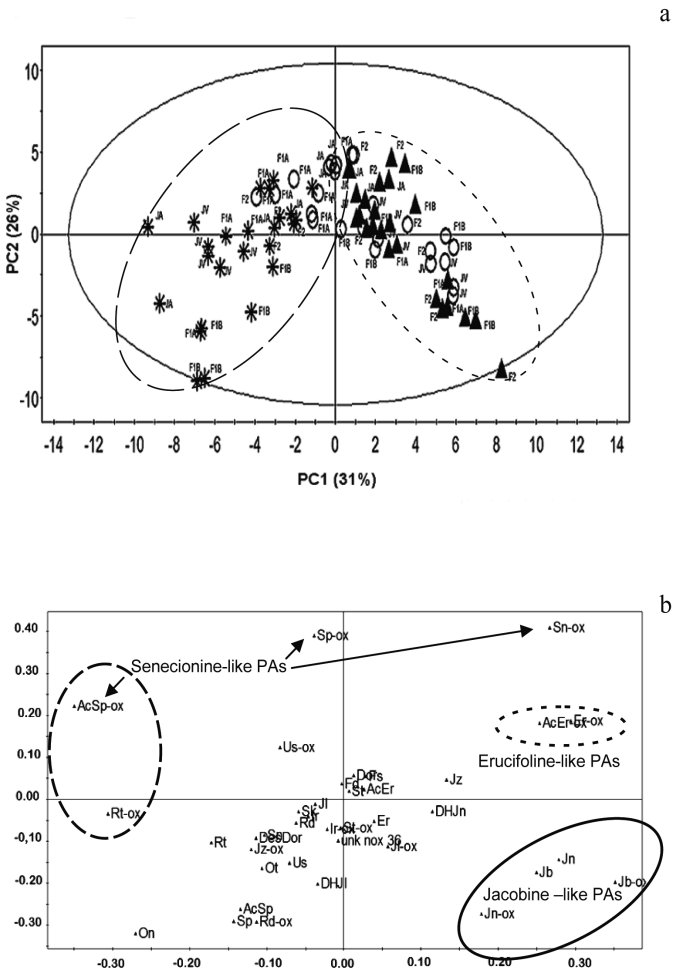

Fig 4. Score (a) and loading plots (b) of PLS-DA for pyrrolizidine alkaloid (PA) composition of *Jacobaea vulgaris* (JV), *J. aquatica* (JA) and their hybrids (F1A, F1B and F2) in three types of *in-vitro* cultures: root (*), shoot (o), and complete plants (▲). The ellipse represents the 95% confidence limit.

but not in the shoot culture (N= 5, R= 0.746, P=0.147). The other senecionine-like PAs such as seneciphylline *N*-oxide, acetylseneciphylline *N*-oxide and retrorsine *N*-oxide contained comparable levels between root and shoot cultures but 1.5 to 3 times significantly lower levels compared to complete plant cultures (ANOVA, F =7.43; df =2; P =0.001). In the root cultures, jacobine-like PAs including jacobine *N*-oxide, jacobine, jaconine and jaconine *N*-oxide were only present in trace amounts. Complete cultures contained the maximum amount of jacobine-like PAs with a four times higher level compared to shoot cultures. Erucifoline-like PAs, including erucifoline *N*-oxide and acetylerucifoline *N*-oxide were at the lowest concentration in the root cultures (ANOVA, F =55.65; df =2; P <0.0001). No significant difference was observed between the concentration of erucifoline-like PAs of shoot and complete plant cultures.

Table 1. Pyrrolizidine alkaloid (PA) concentration of 11 individual PAs in shoot, root and complete plant cultures of *Jacobaea vulgaris*, *Jacobaea aquatica* and their hybrids measured using LC-MS-MS.

PA	Code	Retention time (min)	Precursor mass (m/z)	Concentration (mg/g DW)			*P*
				Root	Shoot	Complete	
Senecionine *N*-oxide	Sn-ox	6.97	352.2	24.4[a]	77.8[b]	230.5[c]	***
Seneciphylline *N*-oxide	Sp-ox	6.36	350.2	131.5[a]	168.3[a]	380.6[b]	***
Acetylseneciphylline *N*-oxide	Acsp-ox	8.86	392.2	64.5[a]	95.3[a]	200.1[b]	*
Retrorsine *N*-oxide	Rt-ox	6.01	368.2	9.7[a]	5.5[a]	14.4[b]	***
Jacobine *N*-oxide	Jb-ox	5.49	368.2	1.6[a]	28.2[b]	114.1[c]	***
Jacobine	Jb	7.89	352.2	0.2[a]	8.5[b]	64.7[c]	***
Jaconine *N*-oxide	Jn-ox	5.77	404.2	1.2[a]	5.4[b]	28.6[c]	***
Jaconine	Jn	8.75	388.2	0.3[a]	14.3[b]	70.6[c]	***
Erucifoline *N*-oxide	Er-ox	4.80	366.2	7.0[a]	82.8[b]	90.6[b]	***
Acetylerucifoline *N*-oxide	Acer-ox	7.17	408.2	0.3[a]	22.0[b]	47.3[b]	***
Onetine	On	4.35	400.2	5.0[a]	1.5[b]	2.5[b]	*

Data present the average of 25 replicates of each culture.
The letters indicate significant differences between different types of cultures: root, shoot and complete plants at * *P*< 0.05, and *** *P*< 0.0001

Pyrrolizidine Alkoloids Forms. All PAs were present in both the *N*-oxide and the free base form except the otosenine-like PAs, dehydrojaconine and dehydrojacoline, which were detected in the free base form only. In general, the *N*-oxide form constituted more than 70% of the total PAs in all cultures except for F1B in the root culture which amounted to 53.4% (data not shown). The total *N*-oxide concentration of the different organ cultures showed significant differences (ANOVA, F=3.2; df =8; P = 0.004) (Fig. 5). The root cultures contained the lowest levels of total *N*-oxide followed by the shoot cultures with a two fold increase, while the complete plant cultures, with a four fold increase contained the highest levels. Interestingly, no significant differences in the concentration of the free base form could be observed between the root and shoot cultures while the complete plant culture showed a three fold higher concentration of the free base in comparison with the root and shoot cultures (ANOVA, F=3.0; df =8; P = 0.008) (Fig.5).

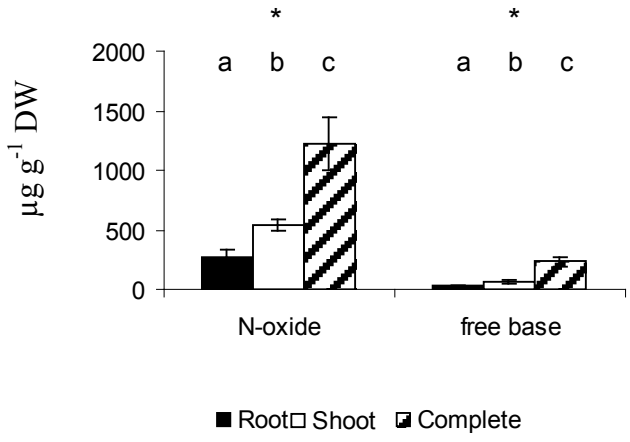

Fig 5. Average of total *N*-oxide and free base forms of pyrrolizidine alkaloid (PA) concentration of *Jacobaea vulgaris, Jacobaea aquatica* and their hybrids in root, shoot and complete plant *in-vitro* cultures expressed overall genotypes. Closed bar = root, open bar = shoot, stripped bar = complete plant. Data present the average and standard error of 25 replicates each. Different letters denote significant differences at * *P*< 0.05.

DISCUSSION

In this study, we report for the first time that both shoot and root cultures of *Jacobaea* plants were able to synthesise PA. The root cultures showed the lowest total PA level followed by shoot and complete plant cultures. The PA composition of root cultures differed from the shoot and the complete plant cultures. The *N*-oxide form was the main accumulation form of PAs in all culture types.

The low level of PAs in the root cultures compared to the shoot and the complete plant cultures may indicate that the root cultures have a limiting capacity to accumulate PAs. Following synthesis, PAs are distributed through the whole plant (Hartmann et al., 1989) to function as plant defence compounds. The lack of shoots may inhibit the distribution of PAs and subsequently the roots as a feedback mechanism will not produce any *de-novo* PAs anymore. Shoots alone were also able to synthesise PAs, as we show in this study, however the levels of total PAs were lower compared to the complete plant. It seems that both the presence of roots and shoots are essential for PA synthesis. Indeed the PA levels produced by the complete plant are for most individual PAs much higher than the sum of PAs produced in roots and shoots cultures.

Pyrrolizidine Alkaloid synthesis in the shoots of the genus *Jacobaea* has so far not been reported. Up to now it was thought that PA biosynthesis in these plants exclusively occurs in the roots (Hartmann et al., 1989). However, PA synthesis in the shoots has been described in other plant species such as *H. indicum* (Frölich et al., 2007) and *C. officinale* (van Dam et al., 1995). The latter study emphasised the importance of the rosette form for PA production. In *C. officinale* only intact rosettes as shoots produced PAs while single leaves failed to do so. The shoot cultures, which we used were indeed rosette forms whereas Hartmann et al. (1989), reporting no PA synthesis in the shoots, used *S. vulgaris* shoots from flowering plants instead. PA production in the shoot is probably related to active metabolism in the central meristem of the rosette (McKey, 1974). In this regard, further studies to investigate the expression of homospermidine synthase (HSS), an enzyme catalyzing the formation of the first intermediate of the alkaloid-specific pathway in the central meristem of the rosette of *Jacobaea* plants will be needed. HSS is derived from deoxyhypusine synthase (DHS), an enzyme which catalyses the first step in the activation of the translation initiation factor 5A (eIF5A) (Ober and Hartmann, 1999). Generally, HSS is not expressed in the shoot. In contrast to HSS, DHS was reported to be expressed in all above ground organs of *Senecio vernalis*, including the shoots (Moll et al., 2002). Thus, it may be a possibility that the enzyme HSS was functionally replaced with the enzyme DHS. The heterologous expression of both enzymes deriving from *S. vernalis* revealed that DHS also accepts putrescine as a substrate to form homospermidine in plants. Thus, both enzymes show the same kinetic properties to catalyse the synthesis of homospermidine from putrescine and spermidine (Ober et al., 2003). Further studies using molecular techniques to detect the role of HSS and DHS expression in PAs synthesis of shoots will be needed.Another explanation for the PA production in the shoots is the observation of a small gall-like formation at the basal part of the shoot cultures. Possibly these galls replaced the function of roots to absorb nutrients from the medium and as such were able to express HSS. Clearly, more studies on the role of HSS and DHS in PA synthesis of *Jacobaea* shoots are needed.

The PA composition between the different organ cultures differed considerably. While the root cultures mainly contained senecionine- and otosenine-like PAs, the shoot and complete plant cultures, next to the senecionine-like PAs, contained more jacobine- and erucifoline-like PAs. Likewise, senecionine-like PAs constituted the main type of PAs detected in roots of *Senecio* (Hartmann and Toppel, 1987) and *Jacobaea* plants (Hartmann and Toppel, 1987; Sander and Hartmann, 1989). A similar pattern was reported by Joosten et al. (2009) where shoots of complete *J. vulgaris* plants, grown in soil contained higher proportions of jacobine- and erucifoline-like PAs and lower proportions of senecionine- and otosenine-like PAs compared to the roots (Joosten et al., 2009). The senecionine-like PAs in our study included senecionine *N*-oxide, seneciphylline *N*-oxide, acetylseneciphylline *N*-oxide and retrorsine *N*-oxide. Senecionine *N*-oxide in the roots showed significantly lower concentrations compared to the shoot while no differences in the concentrations of the other senecionine-like PAs were observed. The level of senecionine *N*-oxide as the first product of the PA biosynthesis from which all other PAs are derived, except senecivernine, was positively correlated with the total concentration of PAs produced in the roots and complete plants. This correlation explains why senecionine *N*-oxide is associated with the classification of complete plants in the PLS-DA loadings plot. Diversification of PAs takes mainly place in the shoots with different diversification rates depending on the type of PA (Hartmann and Dierich, 1998). Therefore, we did not observe a correlation between the amount of senecionine *N*-oxide and total PAs in the shoot cultures.

Next to the higher amount of senecionine-like PAs we observed a high proportion of otosenine-like PAs in the root cultures. Specifically onetine was measured at two times higher concentrations compared to the shoot and complete plant cultures. This may suggest that the root cultures are able to perform conversion of retronecine base structure to otonecinebase structure. The exact diversification mechanism from senecionine *N*-oxide into otosenine-like PAs is still unclear. Pelser et al. (2005) proposed two different putative diversification routes either from senkirkine or jacobine-like PAs. The amount of otosenine-like PAs in the roots was higher than that of the jacobine-like PAs. It is, therefore, unlikely that in our study the otosenine-like PAs have been synthesised from jacobine.

The PA composition of the shoot cultures was very similar to those of the complete plant cultures. It seems that the absence of roots does not influence the PA composition in the shoots. The higher proportion of jacobine- and erucifoline-like PAs in the shoot and complete cultures suggests that specifically the epoxidation process which transforms senecionine *N*-oxide into jacobine and erucifoline-like PAs occurs mainly in the shoot. Nothing is known about this transformation though, since, so far only the transformation of senecionine *N*-oxide into seneciphylline *N*-oxide and retrorsine *N*-oxide has been investigated (Hartmann et al., 1989; Hartmann and Dierich, 1998).

The different patterns between above and below ground plant parts of *Jacobaea* plants in PA diversification may be the result of the plants adaptation to its environment especially to pathogens and insects. The high proportion of senecionine-like PAs in the root cultures observed in our study may be associated with plant defence against soil pathogens. Retrorsine and retrorsine *N*-oxide, both senecionine-like PAs occurring in the roots, inhibited mycelium growth of several root-associated fungi (Hol and van Veen, 2002). Moreover, integerrimine and a mixture of

integerrimine and retrorsine inhibited the growth of the soil inhabiting *Fusarium oxysporum* (Hol, 2003). Among the senecionine-like PAs, so far senkirkine is the only one reported to actively deter Lepidoptera (Bentley et al., 1984;Macel et al., 2005). This deterrent activity was associated with the otonecine base structure of senkirkine which might be more toxic compared to the retronecine base structure of the other senecionine-like PAs. While the association of a high proportion of senecionine-like PAs in the roots with plant defence against soil pathogensis still under discussion evidence accumulates for the role of jacobine-like PAs in plant defence against leaf feeding insects. Jacobine-like PAs including, jaconine (Joosten, 2012), jacobine *N*-oxide and jaconine *N*-oxide (Leiss et al., 2009; Cheng et al., 2011a; Joosten, 2012) as well as jacoline *N*-oxide (Cheng et al., 2011a) have been reported to be active in the above ground defence of *Jacobaea* plants, particularly against the generalist herbivore Western Flower thrips (*Frankliniella occidentalis*). Unfortunately, so far no *in-vitro* bioassays on the negative effect of jacobine-like PAs on generalist insects have been performed since these compounds are not commercially available and thus need to be isolated from the respective *Jacobaea* plants. However, such a study is currently underway in our laboratory. It has been indicated that *J. vulgaris* plants with a high level of jacobine-like PAs also suppressed the growth of microbes leading to a lower diversity of fungi in the rhizosphere (Kowalchuk et al., 2006).Very little is known about the effect of erucifoline- and otosenine-like PAs on plant defence. To our knowledge, only one study tested the toxicity of erucifoline to several generalist insects observing an anti-feeding activity of erucifoline on the Green Peach aphid (*Myzus persicae*) (Domínguez et al., 2008). A decreasing trend of the number of pupae of the American Serpentine leafminer (*Liriomyza trifolii*) was observed with an increase in the concentration of otosenine-like PAs, and a slight increase of number of pupae with an increase in the concentration of erucifoline-like PAs (Cheng, 2012). In a recent study Joosten (2012) reported a significant negative correlation between erucifoline-like PAs and fungal diversity in the roots of the plant.

In our study we observed that the *N*-oxide formwas the major PA form for accumulation in all organ cultures. Up to now it was thought that the non-toxic *N*-oxide form is the major storage form of PAs in *Jacobaea* plants (Hartmann and Toppel, 1987). However, Joosten et al. (2009; 2011) demonstrated that also the toxic free base form can contribute substantially to PA storage: up to 50% in the shoots of a jacobine- chemotype of *J. vulgaris*.In our study, the *N*-oxide form prevails with 82% in the roots and 89% in the shoots compared to the free base form with 18% and 11% respectively. Possibly, the aseptic growing conditions of the organ cultures may have influenced the accumulation of the *N*-oxide form. Due to the absence of biotic interactions in such a sterile environment plants may choose for the non-toxic storage form.

We did not detect senecivernine in any of the organ cultures. This PA was reported to be present in *J. vulgaris* (Pelser et al., 2005) and in the F1 and F2 crosses of *J. vulgaris* and *J. aquatica* (Cheng et al., 2011b). The absence of senecivernine was consistently observed in all types of cultures and genotypes used. Similarly, Toppel et al. (1987) did not observe any senecivernine in root cultures of *Senecio squalidus*, while the root from complete plants, grown in soil, contained senecivernine in amounts comparable to senecionine *N*-oxide (Toppel et al., 1987). The lack of senecivernine, therefore, seems to be a general pattern for the organ cultures. If so, the sterile-growing conditions may have affected the synthesis of senecivernine. Senecivernine is the only PA which does not derive from senecionine *N*-oxide (Pelser et al., 2005). Thus, possibly the pre-

cursor for senecivernine was not present in the organ cultures. However, nothing is known about the synthesis of this PA.

Comparing the potential of different plant organs for synthesis and diversification of PAs we detected that not only roots, as known so far, but also shoots of *Jacobaea* plants were able to synthesise *de-novo* PAs. Senecionine- and otosenine-like PAs were present in both roots and shoots while next to the senecionine-like PAs more jacobine- and erucifoline-like PAs occurred in the shoots. The latter maybe an adaption of plant defence against above ground plant herbivores.

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Chapter 4.

Differential tissue distribution of metabolites in *Jacobaea vulgaris*, *Jacobaea aquatica* and their crosses

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ABSTRACT

Plants are attacked by many different herbivores. Some will consume whole leaves or roots, while others will attack specific types of tissue. Thus, insight into the metabolite profiles of different types of leaf tissues is necessary to understand plant resistance against herbivores. *Jacobaea vulgaris*, *Jacobaea aquatica* and three genotypes of their crossings were used to study the variation in metabolomic profiles between epidermis and mesophyll tissues. Extracts of epidermis and mesophyll tissues were obtained using carborundum abrasion (CA). Subsequently, ^{1}H nuclear magnetic resonance (NMR) spectroscopy and multivariate data analyses were applied to compare the metabolome profiles. Orthogonal partial least-squares-discriminant analysis (OPLS-DA) resulted in a clear separation of epidermis and mesophyll extracts. The epidermis contained significantly higher amounts of jacaranone and phenylpropanoids, specifically chlorogenic (5-*O*-CQA) and feruloyl quinic (FQA) acids compared to the mesophyll. In contrast, the mesophyll showed significantly higher concentrations of pyrrolizidine alkaloids (PAs), specifically jacobine and jaconine. The tissue specific distribution of these compounds was constant over all genotypes tested. Phenylpropanoids, 5-*O*-CQA and FQA, as well as PAs are known for their inhibitory effect on herbivores, especially against thrips. Thrips feeding commences with the penetration of the epidermis, followed by ingestion of sub-epidermal or mesophyll. Thrips thus may have to encounter phenylpropanoids in the epidermis as the first line of defence, before encountering the PAs as the ultimate defence in the mesophyll. The finding of tissue specific defence may have a major impact on studies of plant resistance. We cannot judge resistance using analyses of a whole roots, leafs or flowers. In such a whole-organism approach, the levels of potential defence compounds are far below the real ones encountered in tissues involved in the first line of defence. Instead, it is of great importance to study the defence compounds in the specific tissue to which the herbivore is confined.

Keywords: *Jacobaea*, NMR metabolomics, Epidermis, Compartmentation, Pyrrolizidine alkaloids, Phenylpropanoids.

INTRODUCTION

Plants synthesise various kinds of compounds, which are classified into primary and secondary metabolites. Primary metabolites are substances involved in the basic functions of the living cell, such as respiration and biosynthesis of essential cell compounds. Secondary metabolites, in contrast, are not directly involved in the growth and development of plants. However, they play an indispensable role in the interaction of a plant with its biotic environment, such as the defence against herbivores and pathogens (Choudhary et al., 2008; Macias et al., 2007; Verpoorte et al., 2007).

The most striking feature of secondary metabolites is their enormous structural diversity. Most plant species are characterised by their individual bouquet of secondary compounds (Berenbaum and Zangerl, 2008; Hartmann, 1996). But even within species, populations and individual plants, large quantitative and qualitative differences are observed. From an evolutionary point of view this variation is hypothesised to result from a co-evolutionary arms race. Plants evolve new defence compounds and subsequently, herbivores adapt to these, where after the plants evolve new compounds again (Ehrlich and Raven, 1964; Berenbaum and Feeny, 1981). Alternatively, it has been suggested that different compounds act against different herbivores or that synergistic effects select for diverse patterns of defence compounds (Berenbaum et al., 1991; Adams and Bernays, 1978).

Plants can be attacked by a myriad of herbivore species. All of them have characteristic feeding patterns. Large herbivores may eat complete plants but some prefer different plant organs such as leaves, inflorescences, stems or roots. Insects, such as caterpillars, may chew on different plant organs as a whole, while other insects feed on specific plant tissues only. Examples of the latter comprise leafminers feeding through the mesophyll cells of leaves, thrips sucking up the content of epidermis cells and aphids as phloem feeders. These different organs, tissues and cells have their own specific biological functions that play different roles in plant growth, development and reproduction and as a consequence have a different selective value (Murata et al., 2008). Therefore, the chemical composition of these different organs and different cell types are destined to be different in order to optimise plant defence (Martin et al., 2001).

Yet, relatively little information is known about differences in secondary metabolites within a plant. Most studies on this topic deal with differences between organs comparing, for instance, roots and shoots or shoots and inflorescences (Moco et al., 2009; Hartmann and Zimmer, 1986). Such studies can reveal that different organs can be involved in the synthesis, accumulation or further transformation of secondary metabolites into their derivates. For example, in *Senecio* species, PAs are synthesised in the roots (Hartmann et al., 1989) while in *Heliotropium indicum,* PAs are synthesised in the shoots (Frölich et al., 2007). Less is known about the variation of secondary metabolites at the tissue level within a plant organ. Previous studies focused on elucidating the synthesis of secondary compounds at the tissue level using molecular biology or enzyme activity approaches. In this way the biochemical specialization of epidermis cells in the early steps of vindoline biosynthesis was revealed, while the last part of vindoline synthesis occurs in idioblast cells of the mesophyll (Murata et al., 2008). Vindoline and catharantine are components of the

commercially important anticancer dimers, vinblastine and vincristine (Murata and Luca, 2005). Another study showed that chlorogenic acid and the key enzyme involved in its synthesis were highly increased in the epidermis cells of *Sorghum bicolor* compared to the mesophyll (Kojima and Conn, 1982). Variation of the whole set of metabolites among cell types is virtually unexplored. However, one might ask: what is the relevance of determining secondary metabolites at the level of a shoot or a leaf when a particular insect is only feeding on a certain tissue? In this paper, we seek answers to this question and focus on the differences in secondary metabolite profiles among different cell tissues of the leaf.

Plants in the genus *Jacobaea* (Syn. *Senecio*, Asteraceae) represent an excellent study system with respect to the evolutionary ecology and biosynthesis of secondary defence metabolites (Pelser et al., 2005; Hartmann and Ober, 2000). The genus is characterised by PAs which are frequently observed in Asteraceae, Boraginaceae, the genus Crotalaria (Fabaceae), several orchid genera (e.g., Phalaenopsis), and few genera of the Apocynaceae (Hartmann and Dierich, 1998). Pelser et al. (2005) divided the PAs synthesised in *Jacobaea* into seven groups, including derivatives of: retronecine and otonecine, senecionine, senkirkine, jacobine, erucifoline and otosenine. In the present study, we used *Jacobaea vulgaris* Gaertn and *J. aquatica* G. Gaertn, B. Mey and Scherb as well as their crosses, as representative plants of the genus *Jacobaea. Jacobaea vulgaris* and *J. aquatica* are non-sister species living in contrasting natural habitats. *Jacobaea vulgaris* grows mostly on dry sandy soils while *J. aquatica* prefers wet environments. Both species differ in herbivore susceptibility and in the type of herbivores that feed on them in their natural populations (Kirk et al., 2010). Crosses of the two species do naturally occur in the Zwanenwater Reserve (The Netherlands). Currently, F1 and F2 crosses are available in our laboratory and have been used as a model plant for studies on both ecological and biochemical evolution (Kirk et al., 2004) as well as genotypic-metabolome variation (Kirk et al., 2005) and plant defence (Leiss et al., 2009a; Cheng et al., 2011).

The broad range of detection and high reproducibility of Nuclear Magnetic Resonance Spectroscopy (NMR) make it a good choice to study the variability of metabolite content in plants (Kim et al., 2010). The range of compounds detected is not limited by their volatility, or presence of chromophores. Furthermore, the intensity of NMR signals is directly proportional to their molar concentrations and thus qualitative and quantitative differences can be compared between samples. However, the spectral complexity and lower sensitivity can be a drawback (Kim et al., 2006).

The purpose of this study was to compare the metabolomes of epidermis and mesophyll tissues from the leaves of *J. vulgaris* and *J. aquatica,* and their F1 and F2 crosses. In order to address the following questions: (1) Are the metabolomic patterns between the two tissue types different? (2) Do the relative concentrations of both primary and secondary metabolites differ between the two tissue types? (3) Are the relative concentrations of secondary metabolites in the epidermis and mesophyll genotype specific or can we detect general patterns?

MATERIALS AND METHODS

Plant Material

Plants of *Jacobaea vulgaris* Gaertn and *Jacobaea aquatica* G. Gaertn, B. Mey and Scherb and their crosses F1 (F1A and F1B) and F2 were used as different genotypes in this study. Plants were derived from a tissue culture collection of our department. The parental, F1 and F2 individuals were cloned in order to obtain sufficient amounts used for the study. We used five replicates for each genotype giving a total of twenty five plants. The tissue culture plants were transplanted to pots (11 cm diameter) filled with a 1:1 mixture of dune sand and potting soil. The plants were maintained in a growth chamber (16:8 L: D, 20:15°C) for twelve weeks. Leaves of 4-6 cm length were chosen for epidermis and mesophyll extraction.

Epidermis and Mesophyll Extraction

The epidermis was extracted using the carborundum abrasion (CA) technique of Murata et al. (2008) with some minor modifications. Several leaves of one plant were collected until a weight of 2 g was reached. The leaves were abraded with carborundum F (Fisher Scientific) using a cotton swab. Even pressure was applied to damage the leaf surface. The epidermis was rubbed 3-4 times per leaf and then dipped in 10 ml 50% MeOH at room temperature. Each abraded leaf was gently agitated for 1 min to produce a crude epidermal cell extract. The extraction solvent was maintained at a volume of 10 ml until all leaves were agitated. The abraded leaves, considered as the mesophyll, were immediately ground with liquid nitrogen and extracted in 10 ml of 50% MeOH. Epidermis and mesophyll extracts were dried using a rotary-evaporator. A 50% methanol solvent was chosen to reduce the extraction of non polar compounds, such as waxes, that are usually present on the surface of the leaf. Moreover, based on previous experience in our laboratory, extraction with the 1:1 mixture of methanol-buffer resulted in a broader range of extracted metabolites (Kim et al., 2006).

Verification of Epidermal Extracts

Extraction of the epidermis was verified by chlorophyll measurements as well as microscopic observations. In contrast to the mesophyll, the epidermis does not contain chlorophyll. Thus the presence of chlorophyll in the epidermal extract indicates contamination with mesophyll. Microscopy gave insight into possible damage of the tissues used before and after the CA treatment.

Chlorophyll extraction and quantification

Two hundred microlitter of either epidermal or mesophyll extract was mixed with 1.8 ml acetone and incubated for 30 min at -20 °C. The solution was then centrifuged at 13000 rpm for 20 min. The supernatant was separated and absorbance at 662 and 645 nm was measured. The chlorophyll content was calculated using the following formula (Lichtenthaler, 1987):

$\text{Chlorophyll}_a = 11.75\ A_{662} - 2.350\ A_{645}$

$\text{Cholorophyll}_b = 18.61\ A_{645} - 3.960\ A_{662}$

Microscopic analysis

Microscopic slides of fresh and abraded leaves were prepared using a hand microtome and

examined under a light stereo-microscope (Leica MZ16FA, Wetzlar, DE) using a 40x magnification. The leaf surface and the leaf longitudinal sections before and after CA treatment were examined for cell damage.

Metabolomics

Extraction of plant material

The 50 dried epidermal and mesophyll extracts were used for NMR metabolomics. One ml of methanol-d_4 was added to each sample for NMR extraction. The mixture was vortexed at room temperature for 2 min, and ultrasonicated for 15 min. Subsequently, the mixtures were centrifugated for 15 min at 13000 rpm. An aliquot of 800 µl of the supernatant was transferred to a 5 mm NMR tube.

One- and two-dimensional NMR analysis

Proton NMR spectra were recorded at 25°C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. Deuterated methanol was used as the internal lock. Each ^{1}H NMR spectrum consisted of 128 scans requiring 10 min and 26 s acquisition time with the following parameters: 0.16 Hz/point, pulse width (PW) = 30 (11.3 µs) and relaxation delay (RD) = 1.5 s. A pre-saturation sequence was used to suppress the residual water signal with low power selective irradiation at the water frequency during the recycle delay. Free induction decays (FIDs) were Fourier transformed with a line broadening (LB) = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated to methanol at 3.30 ppm, using XWIN NMR (version 3.5, Bruker). 2D *J*-resolved NMR spectra were acquired using 8 scans per 128 increments at 66Hz for the spin-spin coupling constant axis (f1) and 8 k for the chemical shift axis (f2) at 5000 Hz. A relaxation delay of 1.5 s was employed, giving a total acquisition time of 56 min. Data sets were zero-filled to 512 points in f1, and both dimensions were multiplied by sine-bell functions (SSB=0) prior to double complex Fourier Transformation. *J*-Resolved spectra, tilted by 45°, were symmetrised about f1, and then calibrated, using XWIN NMR (version 3.5, Bruker). ^{1}H-^{1}H correlated spectroscopy (COSY) and heteronuclear multiple bonds coherence (HMBC) spectra were also recorded. The COSY spectra were acquired with 1.0 s relaxation delay and a spectral width of 6361 Hz in both dimensions. The window function for COSY spectra was sine-bell (SSB=0). The HMBC spectra were obtained with 1.0 s relaxation delay, a spectral width of 30,183 Hz in f2 and 27,164 Hz in f1. Qsine (SSB=2.0) was used for the window function of the HMBC. The optimised coupling constant for HMBC was 8 Hz.

Data reduction and quantification of 1H-NMR data

Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) from δ 0.4 to 10.0. The regions of δ 4.7– 4.9 and δ 3.28 – 3.34 were excluded from the analysis due to the residual signals of water and methanol. Bucketing was performed using AMIX (Bruker) with scaling on total intensity. In order to cluster the samples, Orthogonal Partial Least Square - Discriminant Analysis (OPLS-DA) were performed with SIMCA-P software (version 12.0, Umetrics, Umeå, Sweden). Scaling was based on Pareto. The OPLS-DA models were validated by CV-ANOVA methods which are the default validation tool in the software package (SIMCA-P). The relative quantification of the identified metabolites was performed by measuring the ^{1}H NMR

signal area of the corresponding signal compared to the methanol signal. To analyse differences in metabolites between genotypes one-way ANOVA for the corresponding 1H NMR signals were performed using SPSS statistic 17.0. Also the ratio of metabolites in epidermis and mesophyll of the different genotypes was analysed by one-way ANOVA. Differences in metabolite concentration between epidermis and mesophyll were analysed by t-tests for normally distributed population except for CGA signal and chlorophyll data which analysed using Man–Whitney U tests.

RESULTS AND DISCUSSION

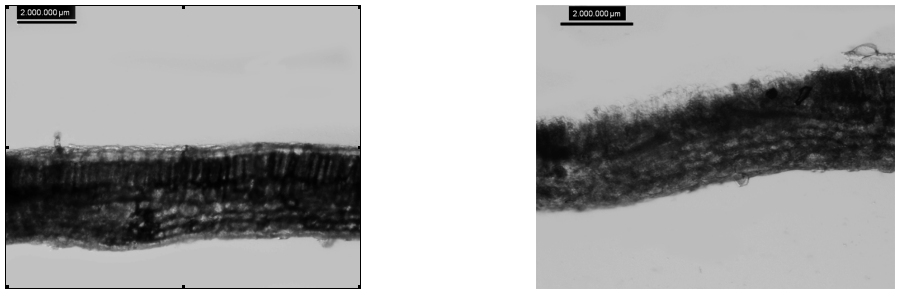
Epidermis and Mesophyll Extraction

The carborondum abrasion (CA) technique for isolation of epidermis extracts (Murata and de Luca, 2005) was coupled with our laboratory standard 1H NMR preparation for metabolomic studies (Kim et al., 2010) to obtain metabolomic profiles of epidermis and mesophyll leaf tissue. The purity of the epidermis extract obtained was determined by measuring the chlorophyll concentration of both epidermis and mesophyll extracts. The leaf tissue structure before and after the CA treatment was also analysed using a microscope. A good quality epidermis extract should have at least a twenty times lower chlorophyll content compared to the mesophyll extract (Murata and de Luca, 2005). The epidermis extracts obtained in this study all met this criterion (Table 1). Leaf cross-sections showed that the epidermis layer was removed by the CA treatment (Figures 1.a and b).

Table 1. Relative levels of chlorophyll in epidermis and mesophyll leaf extracts

Samples	Epidermis	Mesophyll
J. vulgaris	0.75±0.22	97.92±11.72
J. aquatica	1.21±0.13	109.22±30.15
F1A cross	0.36±0.11	53.76±7.43
F1B cross	1.66±0.11	82.45±7.43
F2 cross	1.53±0.17	61.31±8.89

The values expressed in the table are in $\mu g\ g^{-1}$ fr.wt .



a b

Fig 1. Microscopic comparison of *Jacobaea* leaves before and after carborundum abrasion. Cross-section of leaf before treatment showing the epidermis (a); and cross-section of leaf after treatment showing the removed epidermis (b).

One- and Two-Dimensional NMR Analysis

Identification of metabolites

^{1}H NMR measurements of both epidermal and mesophyll extracts allowed the identification of a number of different metabolites including amino acids, organic acids, carbohydrates, phenylpropanoids and PAs (Table 2). The identification of metabolites was based on NMR spectra of known compounds acquired in previous studies on *Jacobaea* plants (Pieters et al., 1989; Witte et al., 1992; Segall and Dallas, 1983; Leiss et al., 2009a). To improve the sensitivity and to resolve overlapping signals in the ^{1}H NMR, two-dimensional NMRs (^{1}H-^{1}H *J*-resolved, COSY, HMBC) were also recorded.

The ^{1}H NMR spectra could be divided into three regions representing the aromatic, carbohydrate and amino acid regions at d 7.8 - 5.5, δ 5.5 – 3.0 and d 3.0 - 1.0, respectively (Figure 2.a). Visual inspection of the NMR spectra illustrated considerable differences between the epidermal and mesophyll extracts in the aromatic (Figure 2.b) and the methyl signal region of the PAs (Figure 2.c). In the aromatic region, the signals of two isomeric phenylpropanoids, 3-*O*-caffeoyl quinic (3-*O*-CQA) – and 5-*O*-caffeoyl quinic acid (chlorogenic acid/CGA), as well as of 5-*O*-feruloyl quinic acid (FQA) were identified. Jacaranone signals were present in both tissue extracts at d 6.16 (H-2, H-6, d, J = 9.6 Hz), and d 7.05 (H-3, H-5, d, J = 9.6 Hz). Several PAs were identified. The 2D *J*-resolved spectra of the plant extracts enabled us to distinguish a characteristic doublet at d 6.22 (d, J = 1.8 Hz) which was assigned to the H-2 of senecionine *N*-oxide. jaconine *N*-oxide, jacobine *N*-oxide and jacobine free base were identified by the characteristic singlets of H-2 at d 6.27, 6.30, and 6.18, respectively. However, due to the low concentration of PAs in both epidermis and mesophyll extracts, the H-2 of the PAs could not clearly be quantified. Therefore, the methyl groups were used for PA determination. This, however, only allows a distinction between the type of PAs but not between free base and *N*-oxide. In the further discussion we will, therefore, use the sum of both *N*-oxide and free base for each PA type. Moreover, formic acid at d 8.49 (s) and fumaric acid at d 6.58 (s) were identified.

In the carbohydrate region, the anomeric protons of glucose, fructose, trehalose, raffinose, stachyose and sucrose were identified (Table 2). ^{1}H-^{1}H-*J*-resolved spectra were applied to the identification of raffinose, stachyose and sucrose and the observed patterns were similar to those previously reported (Leiss et al., 2009a). In the amino acid region, proline, arginine, glutamine, alanine, valine and threonine were identified (Table 2). The methyl signals of the PAs present at d 1.9 – 0.8 were not in a crowded area and could be clearly distinguished as senecionine and jacobine type of PAs (Table 2).

Comparison of metabolites between epidermis and mesophyll

The OPLS-DA was introduced as an improvement of the PLS-DAto discriminate two or more classes using multivariate data (Bylesjö et al., 2006). The advantage of OPLS-DA compared to PLS-DA is that a single component is used as a predictor for the class, while the other components describe the variation orthogonal to the first predictive component (Westerhuis et al., 2010). To distinguish between metabolites from epidermis and mesophyll tissues in this study, PLS-DA analysis was extended to OPLS-DA.

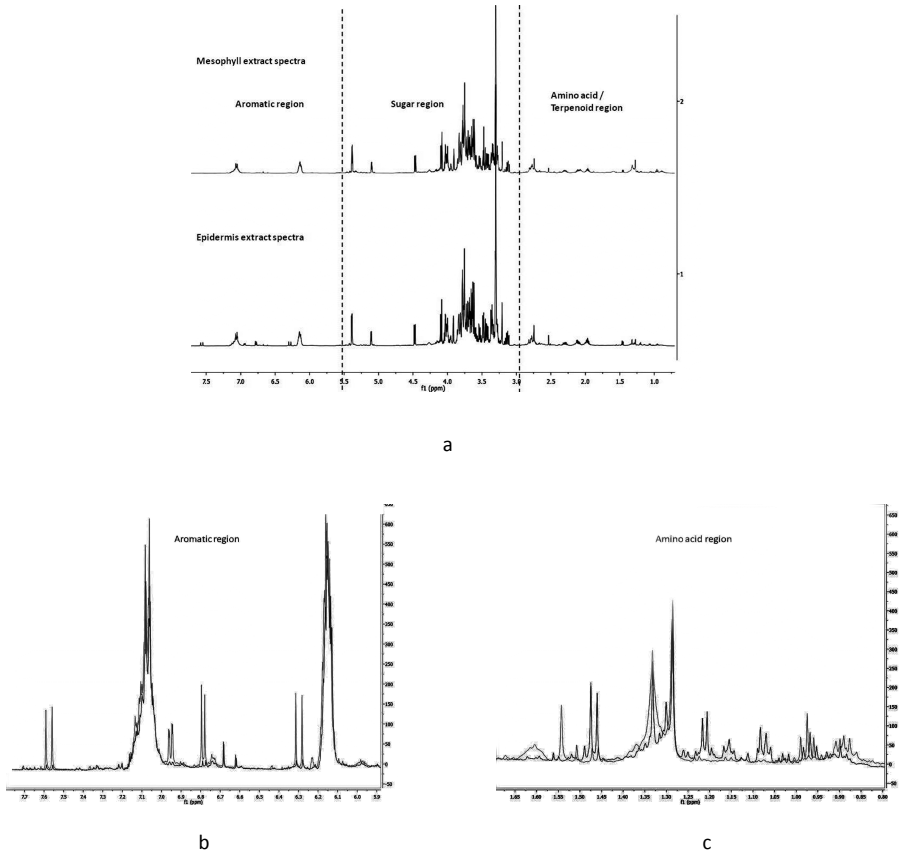


Fig 2. 1H NMR spectra for methanol extracts of *Jacobaea vulgaris* samples. Comparison between epidermal and mesophyll extracts (a); aromatic region (b); methyl region of pyrrolizidine alkaloids (PAs) (c).

The OPLS-DA analysis showed a clear separation between epidermal and mesophyll extracts (Figure 3.a). The model resulted in a variance R^2 of 0.574 and a predictive ability Q^2 of 0.515. The cross validation of the model using CV-ANOVA gave highly significant results (F=4.39, df= 48, P=0.002). The loading plot of the OPLS-DA showed that signals of phenylpropanoids were present in the negative quadrant of PC1, which represents the epidermis, while signals of PAs, sucrose and amino acids were present in the positive quadrant representing the mesophyll (Figure 3.b)

The epidermis showed a much higher concentration of phenylpropanoids compared to the mesophyll. The epidermis had a ten times higher concentration of 5-*O*-CQA (t=9.26, df=48, p<0.0001) and showed almost twice the amount of 3-*O*-CQA (t=7.24, df=48, p<0.0001) and 5-*O*-FQA (t=2.88, df=48, p<0.05) with respect to the mesophyll (Figure 4.a). In contrast, the typical *Jacobaea* alkaloids, specifically, jacobine (t=-8.118, df= 48, p<0.0001 and jaconine (t=-7.12, df=48,

Table 2. 1H chemical shifts (d) and coupling constants (Hz) in metabolites of leaves of *Jacobaea vulgaris, Jacobaea aquatica* and their crosses identified by 1D and 2D NMR spectra in MeOH-d_4

No	Compounds	Chemical shifts (ppm) and coupling constants (Hz)
1	Adenosine	d 8.33 (H-8, s), d 8.20 (H-2, s)
2	Alanine	d 1.47 (H-3, d, J = 7.2 Hz)
3	Arginine	d 3.64 (H-3, t, J = 6.1 Hz), d 3.15 (H-5, t, J = 6.8 Hz)
4	3-*O*-caffeoyl quinic acid	d 5.43 (H-3, d, J = 5.6 Hz, 3.1 Hz), d 6.35(H-8', d, J = 15.9 Hz), d 7.60 (H-7', d, J = 15.9 Hz).
5	Chlorogenic acid (5-*O*-caffeoyl quinic acid)	d 5.42 (H-5, ddd, J = 10,8 Hz, 9.8 Hz, 5.6 Hz), d 6.28(H-8', d, J = 15.9 Hz), d 6.78 (H-5', d, J = 8.62 Hz), d 6.95 (H-6, dd, J = 8.21 Hz, 1.9 Hz), d 7.05 (H-2', d, J = 1.9 Hz), d 7.57 (H7', d, J = 15.9 Hz),
6	Choline	d 3,22 (s)
7	Formic acid	d 8.49 (s)
8	Fumaric acid	d 6.58 (s)
9	Fructose	d 4.03 (H-1, d, J = 3.5 Hz);
10	Feruloyl quinic acid	d 5.57 (H-3, dt, J = 8.0 Hz, 3.1 Hz), d 6.39 (H-8', d, J = 15.9 Hz), d 7.62 (H-7', d, J = 15.9 Hz)
11	Glutamine	d 2.36 (H-3, m), d 2.09 (H-4, m)
12	Glucose	d 4.48 (H-β, d, J = 7,9 Hz), d 5.11 (H-1α, d, J = 3,85 Hz)
13	Inositol	d 3.15 (H-5, t, J = 9.27 Hz), d 3.43 (H-1, H-3, dd, J = 2.79 Hz, 9.78 Hz), d 3.96 (H-2, t, J = 2.67 Hz)
14	Jacaranone	d 6,16 (H-2, H-6, d, J = 9.6 Hz), d 7.05 (H-3, H-5, d, J = 9.6 Hz)
15	Jacobine *N*-Oxide	d 6,27 (H-2, brs), d 4.74 (H-3a, dd, J = 6.74Hz, 14 Hz), d 5.55 (H-9a, d, J = 11.9 Hz), d 4.01 (H-9b, d, 11.9 Hz), d 5.20 (H-7, t, J = 5.0 Hz), d 3.99 (H-6a, dd, J= 14.2 Hz, 5.8 Hz) d 1.26 (H-18, s) , d 1.15 (H-19a, d, J = 6.23 Hz), δ 3.01(H-20, d, J = 5.4 Hz) , d 1.20 (H-21a, d, J = 5.39Hz).
16	Jaconine *N*-Oxide	d 6,22 (H-2, brs), d 1.29 (H-18, s) , d 1.20 (H-21a, d, J = 5.39Hz), d 1.15 (H-19, d, J = 6.23 Hz)
17	Mannitol	d 3.82 (H-1, d, J = 3.0 Hz)
18	Proline	d 4.06 (H-2, dd, J = 8.6 Hz, 6.4 Hz), d 2.31 (H-3, m)
19	Raffinose	d 5.42 (H-1', d, J = 3.93 Hz)
20	Stachyose	d 5.47 (d, J = 3.8 Hz)
21	Succinic acid	d 2.54 (s)
22	Sucrose	d 5.39 (H-1, d, J = 3.8 Hz), d 4.13 (H-1', d, J = 8.5 Hz)
23	Senecionine *N*-Oxide	d 6.22 (H-2, d, J = 1.9 Hz), d 4.57 (H-3a, d, J = 6.2 Hz), d 4.49 (H-3b, d, J = 6.2 Hz), 1.89 (H-21, dd, J = 5.77 Hz, 8.85 Hz), d 5.98 (H-20, m), d 1.51 (H-18, s), d 0.88 (H-19, d, J 6.7 Hz), d 4.58 (H-3a, m)
24	Threonine	d 1.30 (H-5, d, J = 6.6 Hz)
25	Trehalose	d 5.08 (H-1, d, J = 3.8 Hz)
26	Valine	d 1.00 (H-3, d, J = 6.8 Hz), d 1.04 (H-4, d, J = 6.8 Hz),

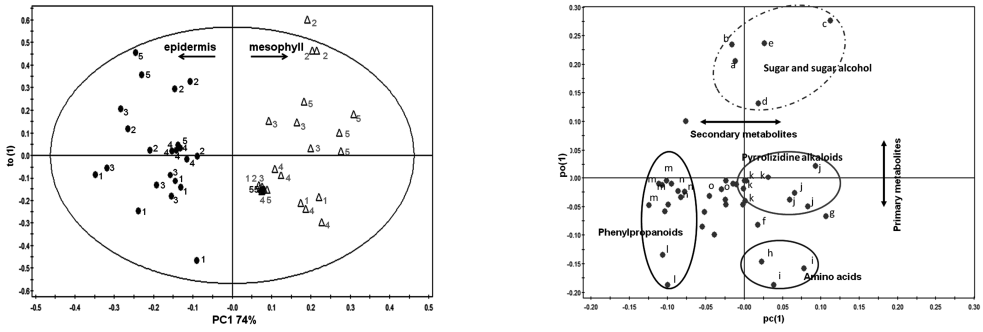


Fig 3. Score (A) and loading plots (B) for epidermis (•) and mesophyll (Δ) of OPLS-DA based on 1H NMR signals of extracts from *Jacobaea vulgaris*, *J. aquatica* and their crosses. *J. vulgaris* (1); *J. aquatica* (2); F1A cross (3); F1B cross (4); F2 cross (5). sucrose (a); glucose (b); fructose (c); stachyose (d); mannitol (e); choline (f); inositol (g); proline (h); arginine (i); jacobine (j); senecionine (k); ferulic acid (l); chlorogenic acid (m); 3-*O*-caffeoyl quinic acid (n); jacaranone (o).

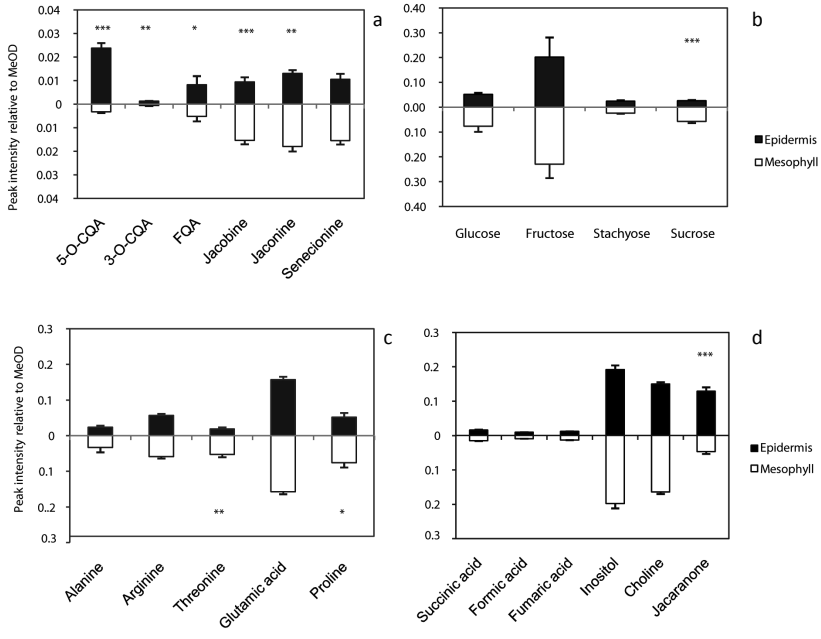


Fig 4. Quantitative expression of phenylpropanoids and PAs (a) sugars (b) amino acids (c) acids, sugar alcohols and jacaranone (d) of epidermis and mesophyll leaf tissues of *Jacobaea vulgaris, J. aquatica* and their crosses. Data present the mean of 5 replicates ± SE of the mean. Data were analyzed by a t-test and Man–Whitney U test for chlorogenic acid. Significant differences between epidermis and mesophyll are indicated as *** $P <$ 0.0001, ** $P <$ 0.001, * $P <$ 0.05.

p<0.0001) showed two times higher concentration levels in the mesophyll extracts (Figure 4.a). However, for senecionine no significant differences could be observed between the two tissue extracts. Of all sugars identified, only sucrose (t=-4.28, df=48, p<0.0001) showed significantly higher amounts in the mesophyll (Figure 4.b). The amounts of amino acids were similar in both epidermis and mesophyll (Figure 4.c) except for threonine which was four times higher (t=-13.93, df=48, p<0.0001) and proline which was two times higher in the mesophyll extract (t=-3.251, df=48, p<0.05). Organic acids, such as succinic and formic acid, showed similar concentrations in both tissues (Figure 4.D). The amount of jacaranone was threefold increased in the epidermis compared to the mesophyll (t=6.039, df=48, p<0.001) (Figure 4.d).

The ratio of each metabolite between epidermis and mesophyll gives a picture of the vertical distribution of the metabolites between genotypes. Most genotypes tested showed a consistent pattern in the distribution of metabolites over epidermis and mesophyll (Figure 5). Among the twenty six metabolites identified in this study only five showed a different pattern across genotypes: 3-*O*-FQA (F=4.141, df=4, P< 0.05), threonine (F=5.710, df=4, P< 0.01), sucrose (F=4.688, df=4, P< 0.01), fumaric acid (F=6.372, df=4, P< 0.01), and mannitol (F=11.275, df=4, P< 0.001).

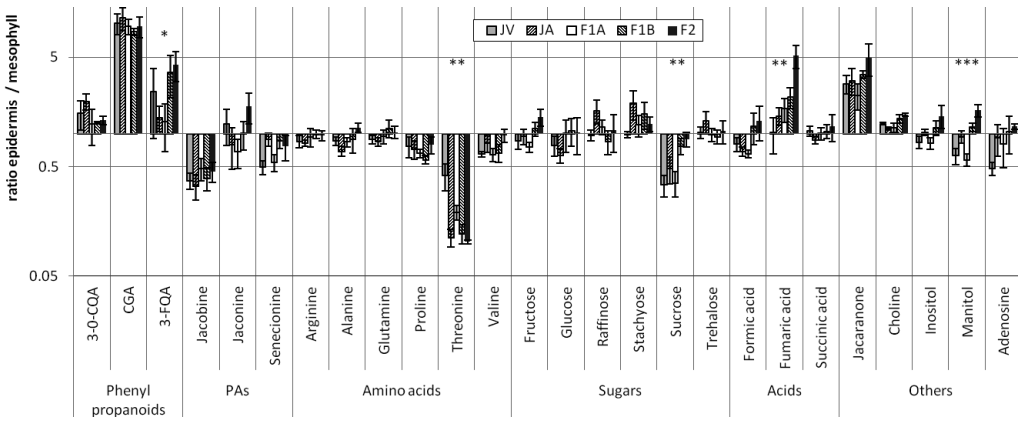


Fig 5. Ratio of metabolites in epidermis and mesophyll extracts. Data were analyzed by ANOVA. Significant differences between *Jacobaea vulgaris* (JV), *J. aquatica* (JA), their crosses: F1A, F1B, and F2 are indicated as *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$.

DISCUSSION

In this study, we showed that different plant defence compounds are concentrated in different leaf tissues. Our result presents an example of the vertical distribution of different metabolites within the leaf. Primary metabolites were present in similar concentrations in epidermis and mesophyll while secondary compounds, important for plant protection, showed a different pattern of distribution in these tissues. Phenylpropanoids and jacaranone were concentrated in the epidermis while PAs were concentrated in the mesophyll. Similarly, a study on UVB protection mechanisms showed an increased concentration of phenylpropanoids in the epidermis of *Arabidopsis* mutants (Bharti and Khurana, 1997). Kojima and Conn (1982) studying tissue distribution of CGA in leaves of *Sorghum bicolor* observed that 60% of this compound was contained in the epidermis. Concurrently, they described a 6-18 fold higher activity of phenylalanine ammonia-lyase, the key enzyme of chlorogenic acid synthesis in the epidermis in contrast to chlorogenic acid oxidase, which was predominantly present in the mesophyll (Kojima and Conn, 1982).

The high concentration of phenylpropanoids in the epidermis has been proposed to play a role as protectants against ultra violet radiation (Bharti and Khurana, 1997). We suggest that in addition they may provide protection against herbivores. Chlorogenic acid has been described as an anti-feedant and digestibility reducer against different insects based on both *in-vivo* and *in-vitro* studies. This includes chewing insects such as caterpillars (Mallikarjuna et al., 2004; Ahmad et al., 2003; Johnson and Felton, 2001; Simmonds and Stevenson, 2001; Beninger et al., 2004) and leaf beetles (Ikonen et al., 2002; Ahmad et al., 2003) as well as sucking insects such as thrips (Leiss et al., 2009b) and aphids (Miles and Oertli, 1993). Chlorogenic acid also showed negative effects on the growth of fungi such as *Phytophtora capsicii* (Lizzi et al., 1995), bacteria like *Pseudomonas syringae* (Niggeweg et al., 2004), and baculovirus (Hoover et al., 1998). When ingested by insects, oxidases catalyse the oxidation of chlorogenic acid to chlorogenoquinone which binds to free amino acids and proteins. This leads to a reduced bioavailability of amino acids and decreased digestibility of dietary proteins (Felton et al., 1992). Besides chlorogenic acid, increased amounts of the phenylpropanoid feruloyl quinic acid were observed in the epidermis. Feruloyl quinic acid has been reported to be involved in thrips resistance in *Chrysanthemum* (Leiss et al., 2009b). It is known as a precursor of lignin, which is involved in the rigidity of cell walls (Bennett and Wallsgrove, 1994) and has been linked to resistance against cereal stemborers (Santiago et al., 2006; Wang et al., 2006), cereal aphids (Havlickova et al., 1996) and cereal midges (Abdel-Aal et al., 2001). (Bennett and Wallsgrove, 1994)As such, feruloyl quinic acid is also involved in resistance to pathogens such as the fungi *Fusarium gramineum* in maize (Bily et al., 2003) and *Sclerotium rolfsii* in chick pea (Singh et al., 2003) as well as the bacteria *Clavibacter michiganense* (Beimen et al., 1992).

Jacaranone was previously isolated from *Jacaranda* sp (Ogura et al., 1976). Analogues of this compound were observed in different *Senecio* species (Xu et al., 2003; Kirk et al., 2005; Wang et al., 2010, Lajide et al., 1996). Jacaranone has antioxidant properties (Jo et al., 2005) and has been reported as having anti-insecticidal activity against houseflies (Xu et al., 2003) and growth inhibition of the insect generalist *Spodoptera littoralis* (Lajide et al., 1996). Leiss et al. (2009b) observed higher concentrations of jacaranone in the young leaves of thrips-resistant plants of

the genus *Jacobaea*. The higher concentrations of jacaranone in the epidermis may thus support the potential of this compound in plant defence against herbivores.

PAs have been reported to reduce the larval survival of generalist herbivores such as *Frankliniella occidentalis* and *Myzus persicae,* and to deter the feeding by *Locusta migratoria* (Macel et al., 2005). Jacobine *N*-oxide and jaconine *N*-oxide were reported to be involved in host plant resistance to the thrips *F. occidentalis* in *Jacobaea* plant (Cheng et al., 2011; Leiss et al., 2009a). The exact mechanism of toxicity of PAs to insects is still unknown. Perhaps more than for their role in resistance against insect herbivores, these alkaloids are notorious for their toxicity to mammalian grazers. Studies on mammals showed that PAs are not toxic when ingested but that they are converted by P-450s mixed function oxidases into hepatoxic pyrroles in the liver (McLean, 1970; Winter et al., 1988).

Both, phenylpropanoids (Leiss et al., 2009b) and PAs (Cheng et al., 2011; Leiss et al., 2009a; Macel et al., 2005) have been shown to be involved in resistance to thrips, which are piercing-sucking insects. Feeding commences with the penetration of the epidermis, followed by ingestion of sub-epidermal or mesophyll cells (Harrewijn et al., 1996, Kindt et al., 2003). Ingestion of the whole cell contents causes air to enter leading to the characteristic silver damage (de Jager et al., 1995). Leiss et al. (2009a) applying NMR detected higher amounts of the PAs jacobine *N*-oxide and jaconine *N*-oxide, in thrips resistant *Jacobaea* leaves but no elevated levels of phenylpropanoids. However, whole leaves were used in this experiment possibly diluting the higher concentration of phenylpropanoids in the epidermis. A negative correlation between thrips silver damage and the concentration of jacobine-like PAs was described by Cheng et al. (2011). Macel et al. (2005) also reported that thrips silver damage was reduced with increasing jacobine concentrations in *in-vitro* experiments. The relatively high concentrations of PAs in the mesophyll and the relatively high concentrations of phenylpropanoids in the epidermis might suggest that the PAs are more toxic than phenylpropanoids for thrips. Sucking insects may have to encounter phenylpropanoids in the epidermis as the first line of defence, where the anti-oxidative properties of these compounds also protect the plant against reactive oxygen species (ROS) formed by UV radiation, before encountering the more toxic PAs as the ultimate defence in the mesophyll. Possibly these two compounds may have additive or synergistic effects on herbivores.

The differences in metabolite patterns and their concentrations between epidermis and mesophyll were consistent across genotypes. Higher concentrations of phenylpropanoids in the epidermis and higher amounts of PAs in the mesophyll were observed over all genotypes. Our findings suggest that the distribution patterns of these metabolites are common in at least plants of the genus *Jacobaea*.

CONCLUDING REMARKS

Our findings may have important consequences for the analyses of ecological experiments on plant-herbivore interactions. If we want to analyse the effects of secondary metabolites on insects or pathogens we should to take into account which tissue is actually attacked. If insects are confined to feed on particular plant tissue we may expect, from an evolutionary point of view, that defence compounds against the insect will accumulate there. However, if the attacker has a choice in the type of tissue it can feed on, it may avoid the tissue that has the highest concentration of defence compounds. Given the complexity of the distribution patterns of secondary metabolites in different plant tissues, further studies are clearly needed. Confirmation of our results for other *Jacobaea* species will show whether the tissue-specific distribution of plant defence compounds is a general phenomenon or species specific. We cannot judge resistance only on the analysis of a whole root, leaf or flower. In such a whole organism approach the levels of potential defence compounds are far below the real ones encountered in tissues involved in the first line of defence. Instead it is of great importance to study the defence compounds in the specific tissue the herbivore is confined to. Further studies on different plant tissues taking into account the compartmentation of defence compounds in different cell types are thus of great importance.

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Chapter 5.

Cells specific metabolomics on F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*

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ABSTRACT

Plants are complex organisms that consist of different cell types and each cell type contains its unique chemical composition to support its specific function. We therefore applied laser microdissection coupled with NMR metabolomics to study the distribution of primary and secondary metabolites in epidermis, palisade and spongy mesophyll leaf cells of *Jacobaea* plants. Two genotypes, of F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica,* genotype A resistant and genotype B susceptible to western flower thrips, with 3 replicates each, were used. For each cell type approximately 3000 cells were collected and analysed. The partial least square discriminant analysis (PLS-DA) showed a clear separation of the three different cell types for both genotypes. Regarding primary metabolites the epidermis cells showed lower levels of glucose, threonine and the organic acids malate and succinate but higher levels of cholin as well as of amino acid alanine in comparison with the two mesophyll cell types. With regard to secondary metabolites the epidermis cells contained lower concentrations of the PA jacobine *N*-oxide, which was concentrated in the palisade cells, but higher concentrations of the phenylpropaonid chlorogenic acid and the flavanoid kaempferol glucoside. Although both genotypes showed almost the same metabolite profiles over the three cell types, the resistant genotype contained significantly more fructose, threonine and jacobine *N*-oxide compared with the susceptible genotype. The epidermis as outer cell layer confers protection to the cells. Therefore, it was rich in metabolites ameliorating the negative effects of abiotic factors such as UV-light, drought, frost and salt and rich in plant defence compounds against general pathogens and herbivores. However, jacobine *N*-oxide compounds may also act as host finding cues for specialist herbivores. In order to solve this generalist-specialist dilemma the distribution of the defence compounds may adapt as in the case of the PA jacobine *N*-oxide, which did not accumulate in the epidermis but in the palisade mesophyll beneath. In contrast the mesophyll cells, containing chlorophyll, were generally rich in metabolites related to respiration and energy generation. As such our study emphasises the importance of studying the cell-specific distribution and function of metabolites in distinguished cell types.

Keywords: cell type specific metabolomics, chlorogenic acid, laser microdissection, *Jacobaea* sp., jacobine, NMR, pyrrolizidine alkaloids

INTRODUCTION

Plants, as complex organisms distribute specific functions to different types of organs and tissues. These distinct functions result from the integrated activity of individual cells (Schad et al., 2005). Around forty different cell types occur in plants (Martin et al., 2001) of which twelve are in the leaves alone (Nelson et al., 2008). Epidermal cells, as the outer leaf tissue, represent the outer barrier of the leaves with its environment. As such they are involved in gas exchange, water homeostasis, and plant protection (Gutiérrez-Alcalá et al., 2000). Hence, the internal leaf tissue is needed for photosynthesis. Despite this common function, a number of cell types such as the palisade and the spongy mesophyll as well as the bundle sheath cells can be distinguished (Langdale, 1998). These different cell types have their own specific biological functions that play different roles in plant growth and development (Murata et al., 2008). Therefore, each cell type contains its own unique chemical composition to support its specific function (Day et al., 2005; Martin et al., 2001). Studying the chemical composition of each cell type will help us to identify how different compounds are compartmentalised and to understand how and where these compounds are biosynthesised and what their functions in plant survival are.

Efforts to conduct cell specific studies have been started since a few decades ago. In the early phase, most of these studies mainly used *in-situ* methods such as enzyme histochemistry and RNA hybridization that rely on localization of an indicator in a cellular region. In such approach the cells remain in the context of the tissue (Outlaw and Zhang, 2001). Recently, at least two approaches are available for collecting specific cell types. The first is to sample single cells from living plant tissues using microcapillaries (Karrer et al., 1995). The second is the removal of the sample interest from the tissue context. Several protocols have been developed to remove specific cells, including protocols for the isolation of leaf surface cells like glandular trichomes that protrude on the leaf surface (Lange et al., 2000; Gang et al., 2002; Wagner et al., 2004) and isolation of epidermal cells by carborondum abrasion (CA) (Murata and de Luca, 2005). Among the different cell types of the leaf, epidermis cells have been studied more frequently. It was revealed that in most plant species, the epidermis cell plays specialised roles in biosynthesis and accumulation of a wide range of secondary metabolites (SMs), including flavanoids (Kutchan, 2005), terpenes (Dudareva et al., 2005), and alkaloids, (Murata and de Luca, 2005). The latter study was conducted using RNA isolation and gene expression analysis on *Catharanthus roseus* describing the spatial distribution of the intermediate compounds, vindoline and catharanthine, as members of the monoterpenoid indole alkaloids pathway (Murata and de Luca, 2005). These alkaloids are the components of the commercially important anticancer dimers, vinblastine and vincristine.

The most advanced technique in the single cell isolation is the use of laser microddissection (LMD). In this technique, the tissue is stabilised before the cells are dissected. Laser microdissection has been proven to be an effective technique to cut and collect single cell types. Firstly, LMD was applied to isolate cancer cells (Emmert-Buck et al., 1996) but has in the mean while been adapted to plant sciences for isolation of different cell types in proteomic studies (Banks et al., 1999) and gene expression studies (Nakazono et al., 2003). As such it has been used to determine the SMs content of specific plant cells. The stone cells of Norway Spruce, *Picea abies*, bark contained, next to lignin also stilbene astringin and dihydroflavonol dihydroxyquercetin 3'-*O*-β-D- glucopyranoside

(Li et al., 2007). Thus, the stone cells function as more than just repositories for lignin but may also be involved in chemical as well as physical defence against bark beetles and their associated microorganisms. Until now only a few studies used LMD for cell metabolomics. Application of LMD on cell specific metabolomics will allow us to detect the potential key metabolites in each cell type (Day et al., 2005). This approach is important especially if we do not have a prior knowledge about the compounds in the plant. For conventional metabolomics usually whole plant organs such as leaves, flowers, and roots are used mostly ignoring the different cell types contained in such organs. This leads to averaging and diluting the information of the different micro-metabolomes (Schad et al., 2005). An example of cell specific metabolomics comprises the comparison of metabolites in the vascular bundles with non-vascular cells in *Arabidopsis thaliana* (Schad et al., 2005). This study revealed a high accumulation of simple sugars such as galactose, fructose and glucose in the vascular bundles cells while disaccharide sucrose and TCA (threecarboxilic acid) cycle intermediates such as malic acid, isocitric acid and citric acid were higher in the leaves section without the vascular bundle cells. This study emphasises the effectiveness of this technique to identify the distribution of major small metabolites such as simple sugars, amino acids, etc. in the different type of cells.

Plants in the genus *Jacobaea* (Syn. *Senecio*, Asteraceae) represent an excellent study system with respect to the evolutionary ecology and biosynthesis of secondary defence metabolites (Pelser et al., 2005; Hartmann and Ober, 2000). These plants are known to constitutively synthesise pyrrolizidine alkaloids (PAs) (Hartmann and Zimmer, 1986). Aside from that, several SMs known as defence compounds such as chlorogenic acid (CGA), kaempherol glucoside and jacaranone have been reported to be present in these plants. Plants are attacked by many types of herbivorous insects. However, these differ in the specific plant tissue they will attack. For example, caterpillars chew on different plant organs as a whole, leafminers chew through the leaf mesophyll, thrips and mites suck up the content of epidermis cells and aphids feed on the phloem. Indeed, in a previous study, we reported a tissue specific distribution of the defence compounds chlorogenic acid (CGA) and PAs in *Jacobaea* plants (Nuringtyas et al., 2012). While CGA was highly accumulated in the epidermis, the PAs were concentrated s in the mesophyll. In regards with specialist herbivores, this high accumulation of PAs in the mesophyll tissues may serve as a defensive strategy to reduce the apparent of *Jacobaea* plants to specialist. The high accumulation of CGA in the epidermis may also serve as first line defence against certain types of insects which only attack epidermis such as thrips and mites. Thus, for plant host resistance study, this result emphasises that using leaf-level analysis one may fail to identify SMs with a specific compartmentation since the metabolites may be diluted by the other metabolites present in higher concentration in the leaves in other cells types. Considering the importance of specific compartmentation of defence compounds, in this current study, we chose two different genotypes: a thrips resistant and a susceptible one which may represent the variation within *Jacobaea* plants.

In this study, we applied the LMD technique to isolate the different cell types of the leaves: epidermis, palisade- and spongy-mesophyll cell followed by NMR metabolomics. We specifically addressed the following questions: Is the distribution of metabolites cell-specific, i.e. is the distribution of metabolites different between epidermis, palisade and spongy mesopyll cells? If so, which metabolites contribute to this difference? Are the differences observed common to both genotypes?

MATERIALS AND METHODS

Plant Materials. F2 hybrids of *Jacobaea vulgaris* Gaertn and *Jacobaea aquatica* G. Gaertn, B. Mey and Schreb were used. We chose two genotypes, A and B, as representatives of intra-species variation in *Jacobaea* plants. These genotypes have been used in a previous study on host plant resistance to western flower thrips (Cheng et al., 2011), in which genotype A was resistant and genotype B susceptible. The genotypes are maintained in the department tissue culture collection. We used three replicates for each genotype giving a total of six plants. The tissue culture plants were transplanted to pots (11 cm diameter) filled with a 1:1 mixture of dune sand and potting soil. The plants were maintained in a growth chamber (16:8 L:D, 20:15±2 °C) for four weeks. Each day two leaves of 2-3 cm length were harvested for cell collection over a period of five days.

Tissue Preparation. The harvested leaves were microwaved for 10 s at 400 W to deactivate enzymes. A 5x7 mm^2 leaf slice was dissected using a scalpel. In order to obtain sufficient rigidity when freezing the tissue using Jung tissue freezing medium (Leica Microsystem, Nussloch, Germany) the slice was cut across the leaf midrib. After embedding the slice in the freezing medium, it was frozen in liquid nitrogen and immediately cut using a cryostat microtome (Leica CM1850, Bensheim, Germany) adjusted to -20°C. Serial sections of 30µm thickness were cut and mounted directly on a microscopic glass slide 76x26 mm ((ROTH, Germany). Each slide consisted of 20 sections.

Laser Microdisection (LMD). Three types of cells were collected from each plant: epidermis, palisade- and spongy-mesophyll cells, amounting to eighteen samples in total. Collection of a sufficient amount of cells for NMR analysis took about 5 days per plant. Cells were collected separately per day and pooled before NMR analysis. Collections of cells of the two respective genotypes were alternated weekly. The serial sections were used in a Leica LMD6000 system for cell collection. Cutting was performed in the visible light mode at 40x magnification. While cutting, the number of cells collected was counted. Each cell type was collected separately in the cap of a 0.5 ml microcentrifuge tube. The caps were filled with pure methanol to ensure that the cells were immediately extracted in the solvent. A preliminary study showed that CGA, due to laser heating, started to degrade after 1.5 h and was almost totally degraded after 3 h during LMD. Therefore, the cell collection per slide was limited to 1.5 h. At the end of dissection, the tube with samples was carefully closed and the tubes were centrifugated at 12,000 rpm, 4°C for 20 min. For ^{1}H NMR analysis about 0.4 mg material, equivalent to about 3000 cells was needed. Therefore, the tubes were weighed after centrifugation. The supernatant was collected and transferred to fresh microtubes to be dried under a stream of nitrogen and kept at 4°C until ^{1}H NMR analysis. The daily cell collections were pooled per cell type and genotype by adding 50 µl methanol. The samples were centrifugated at 12,000 rpm, 4°C for 20 min. The methanolic extract was transferred to a fresh microtube and samples were dried under a stream of nitrogen.

Metabolomics.

Sample preparation for ^{1}HNMR analysis. The eighteen dried extracts of epidermis, palisade- and spongy-mesophyll cells were subjected to NMR metabolomics. For NMR analysis 100 µl of freshly opened methanol-d_4 (99.96% Deutero GmbH, Germany) were added. The methanol-*d4* contained 0.17 mmol of 2,4,6 Trichloronitrobenzene (TCNB) as internal standard. The mixture was vortexed

at room temperature for 30 s and ultrasonicated for 3 min. Subsequently, the mixtures were centrifugated for 5 min at 12,000 rpm, 4°C for 20 min. An aliquot of 75 µl of the supernatant was transferred to a capillary NMR tube.

NMR analysis.^{1}H NMR spectra were recorded with a Bruker Avance 500 NMR spectrometer equipped with a cryogenic TXI probehead (Bruker, Karlsruhe, Germany) at 30ºC. Each ^{1}H NMR spectrum consisted of 1054 scans requiring 3 h acquisition time.

Quantification. For the quantification of identified metabolites using NMR spectroscopy, the peak area of selected proton signals belonging to the target compounds, and the peak area of IS, were integrated manually for all the samples.

The following equation was applied for the calculations (van Beek et al., 2007):

$$C = \left(\frac{\text{Integral (Target)}}{\text{Integral (IS)}} \times \frac{\text{* MW (Target)}}{\text{**MW (IS)}} \times \text{Weight (IS)} \right) / \text{number of cells}$$

Target = signal of the target compound,
IS = signal of internal standard,
* MW (Target) = molecular mass/ number of protons involved in the target signal,
**MW (IS) = molecular mass / number of protons involved in the internal standard (IS) signal,
C = concentration in one cell, Weight is in micrograms. The concentration of TCNB in each NMR tube was fixed as 0.17 mmol.

Metabolite concentration was expressed in mol per cell. The total concentration of each metabolite per leaf was calculated by adding the concentrations of the respective three cell types.

Data reduction and quantification of ^{1}H NMR data. Spectral intensities were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) from d 0.4–10.0. The regions of d 4.75–4.93 and d 3.28–3.34 were excluded from the analysis due to the residual signals of water and methanol. Bucketing was performed using AMIX (Bruker) with scaling on total intensity. Partial least square-discriminant analysis (PLS-DA) was performed with the SIMCA-P software (v. 12.0. Umetrics, Umeå. Sweden). The scaling method for both analyses was uv. The PLS-DA model was validated using the permutation method through 20 applications and CV-ANOVA, which are default validation tools in the software. Differences in concentration of the identified compounds between different cells types were analysed using ANOVA with cell type nested within genotypes. Statistic analysis was performed using SPSS statistic 19 (Chicago, IL, USA).

RESULTS

Identification of Metabolites. ^{1}H NMR analysis of epidermis, palisade, and spongy cell extracts allowed the identification of different metabolites including amino acids, organic acids, carbohydrates, phenylpropanoids and PAs (Table 1). Identification of metabolites was based on NMR spectra of known compounds acquired in previous studies on *Jacobaea* plants (Leiss et al., 2009a; Nuringtyas et al., 2012) and from comparison with our in house NMR spectra database (Kim et al., 2010).

Table 1. 1H chemical shifts (d) and coupling constants (Hz) in epidermis, palisade and spongy cell extracts of F2 hybrids of *Jacobaea* leaves identified by 1D NMR spectra in MeOH-d_4

No	Compounds	Chemical shifts (ppm) and coupling constants (Hz)
1	Alanine	d 1.46 (H-3, d, J = 7.2 Hz)
2	Chlorogenic acid (5-*O*-caffeoyl quinic acid)	d 5.42 (H-5, ddd, J = 10,8 Hz, 9.8 Hz, 5.6 Hz), d 6.28(H-8', d, J = 15.9 Hz), d 6.78 (H-5', d, J = 8.62 Hz), d 6.95 (H-6, dd, J = 8.21 Hz, 1.9 Hz), d 7.05 (H-2', d, J = 1.9 Hz), d 7.57 (H7', d, J = 15.9 Hz),
3	Choline	d 3,22 (s)
4	Fructose	d 4.03 (H-1, d, J = 3.5 Hz);
5	Feruloyl quinic acid	d 5.57 (H-3, dt, J = 8.0 Hz, 3.1 Hz), d 6.39 (H-8', d, J = 15.9 Hz), d 7.62 (H-7', d, J = 15.9 Hz)
6	Glutamine	d 2.32 (H-3, m), d 2.05 (H-4, m)
7	Glucose	d 4.48 (H-β, d, J = 7,9 Hz), d 5.11 (H-1α, d, J = 3,85 Hz)
8	Inositol	d 3.15 (H-5, t, J = 9.27 Hz), d 3.43 (H-1, H-3, dd, J = 2.79 Hz, 9.78 Hz), d 3.96 (H-2, t, J = 2.67 Hz)
9	Jacobine *N*-oxide	d 6,25 (H-2, brs), d 4.74 (H-3a, dd, J = 6.74Hz, 14 Hz), d 5.55 (H-9a, d, J = 11.9 Hz), d 4.01 (H-9b, d, 11.9 Hz), d 5.20 (H-7, t, J = 5.0 Hz), d 3.99 (H-6a, dd, J= 14.2 Hz, 5.8 Hz) d 1.34 (H-18, s) , d 1.15 (H-19a, d, J = 6.23 Hz), d 3.01(H-20, d, J = 5.4 Hz) , d 1.20 (H-21a, d, J = 5.39Hz).
10	Kaempferol glucoside	d 7.18 (H-3 and H-5, d, J = 8 Hz), d 7.38 (H-2 and H-6, d, J = 8 Hz)
11	Malate	d 4.28(H-2, dd, J = 9.8 Hz, 3.3 Hz), d 2.38(H-3a, dd, J = 16.3 Hz, 6.8 Hz), d 2.68(H-3b, dd, J = 15.32 Hz, 3.2 Hz),
12	Succinate	d 2.52 (s)
13	Sucrose	d 5.39 (H-1, d, J = 3.8 Hz), d 4.13 (H-1', d, J = 8.5 Hz)
14	Threonine	d 1.32 (H-5, d, J = 6.6 Hz)
15	Fatty acid	0.98 (H-v, t, J = 7.5), 1.31 (CH3, brs), 1.6 (m), 2.02 (m), 2.35 (m)

The ^{1}H NMR spectra obtained were divided into three regions representing the aromatic, carbohydrate and amino acid regions at d 7.8 –5.5, d 5.5 – 3.0 and d 3.0 –1.0, respectively (Fig. 1). In the aromatic region phenylpropanoids signals including CGA and 5-*O*-feruloyl quinic acid (ferulic acid, FQA) were observed. Aside from that, we could identify kaempferol glucoside based on its characteristic doublet signals at d 7.38 (*J* = 8.0 Hz) and d 7.18 (*J* = 8.0 Hz). The identification of this metabolite was confirmed by comparison with our in house NMR library as well by reported data (Leiss et al., 2009b). In the carbohydrate region, the protons of sucrose, glucose, raffinose, stachyose and fructose were identified. In addition, signals at d 3.15 (t, *J* = 9.3 Hz), d 3.43 (dd, *J* = 9.8 Hz, 2.8 Hz), and d 3.96 (t, *J* = 2.7 Hz) were assigned to inositol. Another sugar alcohol identified was mannitol with its characteristic doublet signal at d 3.80 (*J* = 3.0 Hz). In the amino acid region, glutamine, alanine and threonine were identified. Signals of fatty acids were detected at d0.98 (t, *J* = 7.5 Hz) and d 1.31 (brs). Jacobine *N*-oxide, belonging to the PAs, was identified based on the broad singlet of H-2 at d 6.25, characteristic of PAs, and the presence of doublet signals at d 1.20 (*J* = 5.4Hz) and d 1.15 (*J* = 6.2 Hz) in combination with a singlet at d 1.34 (Fig. 2). This identification was confirmed by comparing the sample spectra with a jacobine *N*-oxide standard. The methyl signals were not in the crowded area thus they were used for subsequent PA quantification.

Partial Least Square-Discriminant Analysis of ^{1}H-NMR Data. *Partial Least Square-Discriminant Analysis* score plot showed a clear separation of the three different cell types for both genotypes (Fig 3a and b). In both cases, the epidermis cells were located in the positive quadrant of PC1 while the palisade mesophyll cells were in the negative quadrant. The spongy mesophyll cells were located in the middle between epidermis and palisade mesophyll cells. The PLS-DA score plot of genotype A explained a total variation of 51.7% while the score plot of genotype B explained 66.2%. The permutation test of both genotype A and genotype B PLS-DA showed that the R^2 and Q^2 of the permuted Y vectors were lower than the original ones (Fig. 3c & d). The CV-ANOVA of both PLS-DA gave significant results (A: F = 7.01, df = 4, P = 0.049. B: F = 14.03, df = 4, P = 0.0012). Although the distribution pattern of the metabolites over the three leaf cell-types was the same in both genotypes, significant differences in the amount of specific metabolites between genotype A and B were observed. The PLS-DA score plot showed a clear separation of genotype A and B cell extracts (Fig. 4a). All cell extracts of genotype A were located in the negative quadrant of PC 1 while those of the B were in the positive quadrant. PC1 explained 33.6% of the variation while PC2 explained 19.0%, amounting to a total of 52.6% of explained variation. The model resulted in a variance R^2 of 0.652 and a predictive ability Q^2 of 0.526. The permutation test showed that the Q^2 of the permuted Y vectors was lower than the original one (Fig. 4b). Additional cross validation using CV-ANOVA gave a significant result (F = 8.83, df = 17, P = 0.005). The loading plot of the PLS-DA showed that the separation of the genotype A was highly affected by the primary metabolites fructose, succinate and threonine as well by the secondary metabolites jacobine *N*-oxide, CGA, FQA, and kaempferol glucoside, (Fig. 4c).

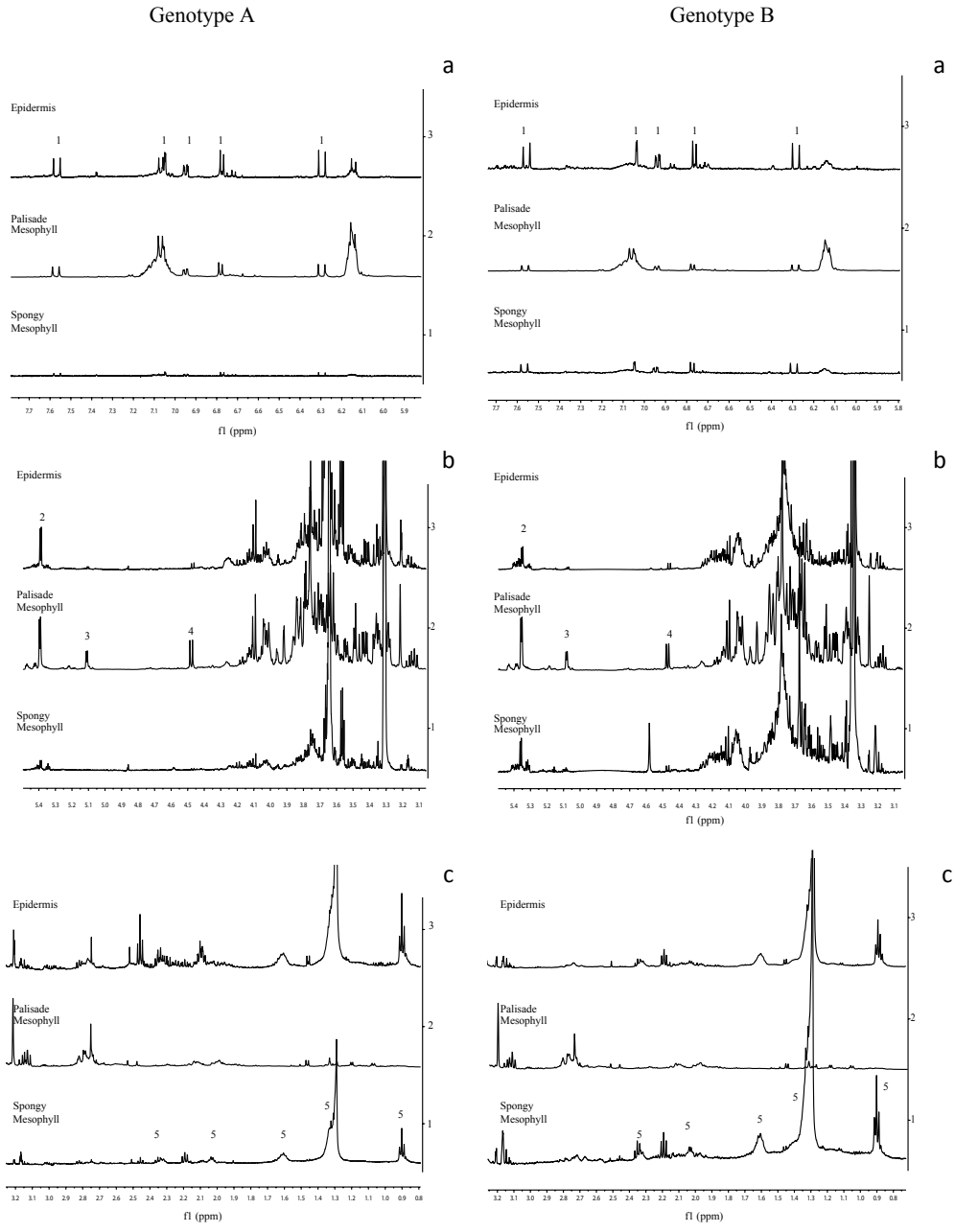

Fig 1. ^{1}H NMR spectra of the aromatic (A), sugar (B) and methyl (C) region for methanol extracts of epidermis, palisade- and spongy mesophyll leaf cells of genotypes A and B of F2 *Jacobaea* hybrids. Metabolites are labeled as chlorogenic acid (1), sucrose (2), α glucose (3), β glucose (4) and fatty acids (5).

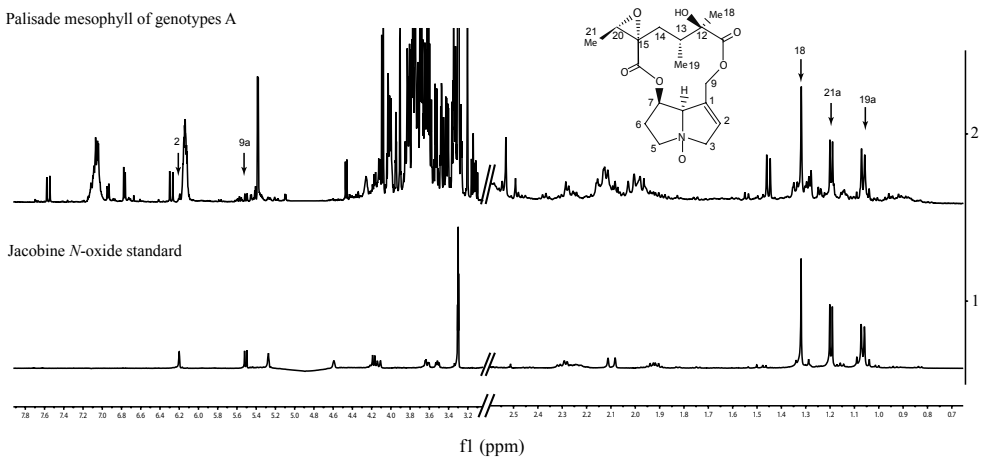

Fig 2. [1]H NMR spectra for methanol extracts of palisade mesophyll leaf cells of genotypes A of the F2 *Jacobaea* hybrids compared to the jacobine *N*-oxide standard.

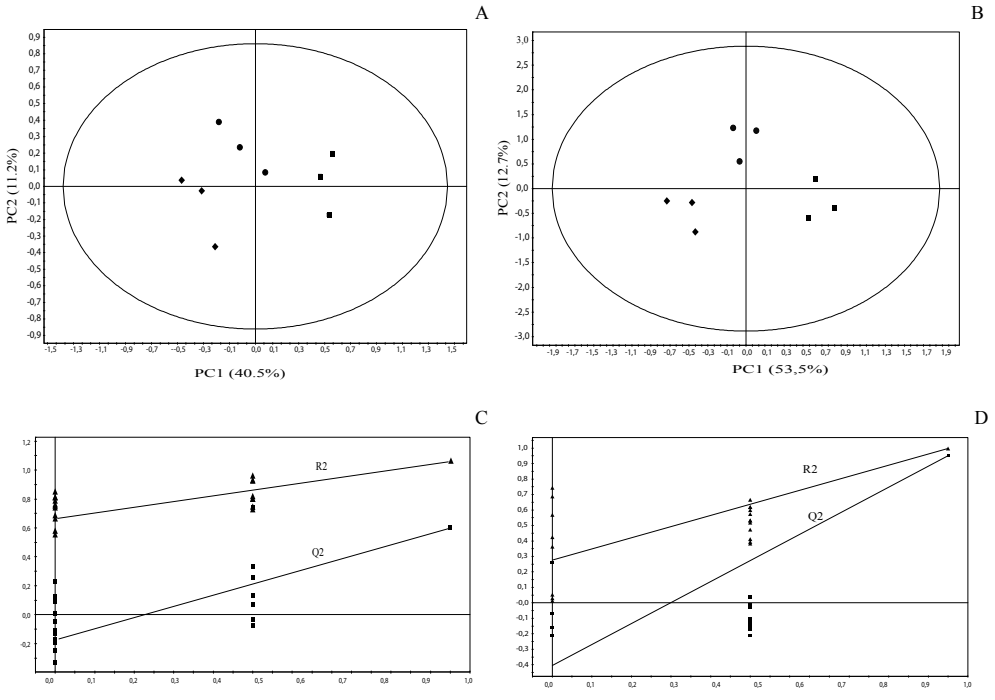

Fig 3. PLS-DA score plots for different cell types: epidermis (■), palisade mesophyll (♦) and spongy mesophyll (●) based on [1]H NMR signals of genotypes A (A) and genotypes B (B) of F2 *Jacobaea* hybrids and permutation validation (20 permutations with three components) of the PLS-DA plot of A (C) and B (D) genotypes.

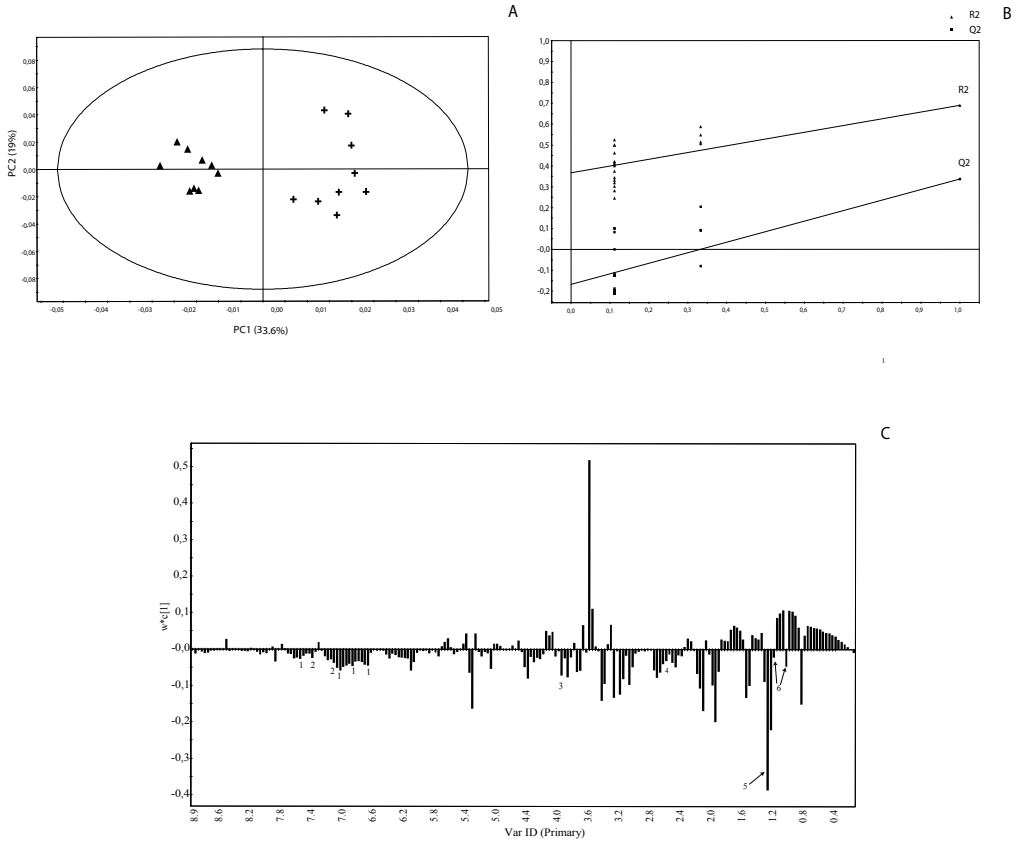

Fig 4. Score (A) loading plot (B) and permutation validation (20 permutations with three components) plot (C) of PLS-DA based on ^{1}H NMR signals of genotypes A (▲) and genotypes B (+) of F2 *Jacobaea* hybrids. Metabolites are labeled as chlorogenic acid (1), kaempferol glucoside (2), fructose (3), succinate (4), threonine (5) and jacobine *N*-oxide (6).

Quantification of Metabolites. Plant cell types differed significantly in primary and secondary metabolites as depicted in Figs. 5 and 6. In regard to the primary metabolites the epidermis cells were low in both alpha glucose ($F = 4.06$, $df = 2$, $P < 0.04$) and beta glucose ($F = 14.08$, $df = 2$, $P < 0.0001$) levels and threonine ($F = 4.52$, $df = 2$, $P = 0.04$) compared to the spongy mesophyll cells. With regard to the organic acids, epidermis contained lower levels of malate ($F = 11.17$, $df = 2$, $P = 0.006$) compared to palisade- and spongy- mesophyll cells and succinate ($F = 11.17$, $df = 2$, $P = 0.001$) compared to the palisade mesophyll cells. In contrast they were rich in cholin, a quaternary ammonium salt, ($F = 38.32$, $df = 2$, $P < 0.0001$) and amino acid alanine ($F = 4.59$, $df = 2$, $P = 0.029$) compared to both palisade- and spongy- mesophyll cells. Looking at the SMs the epidermis cells contained lower concentrations of the PA jacobine *N*-oxide ($F = 14.66$, $df = 2$, $P < 0.0001$), which was concentrated in the palisade cells, but higher concentrations of the phenylpropanoid CGA ($F = 19.65$, $df = 2$, $P < 0.0001$) compared to the palisade- and spongy-mesophyll. Similar distribution patterns with CGA was also observed the flavanoid kaempferol glucoside ($F = 13.01$, $df = 2$, $P = 0.001$) that is higher level in epidermis cell compared to the spongy mesophyll cells.

Significant quantitative differences in metabolites between gentoypes occured in the primary metabolites fructose and threonine as well as in the SMs jacobine *N*-oxide. Genotype A contained one-fourth more of fructose (F = 438.84, df = 1, P = 0.002), double as much threonine (F = 232.04, df = 1, P = 0.030) and two folds of the PA, jacobine *N*-oxide (F = 32.628, df = 1, P = 0.031) compared to genotype B. In the PLS-DA loadings plots, succinate was identified as important factor for the separation between the two genotypes. However in the quantitative analysis, it did not show any difference. This may due to the presence of other signals which may influence the PLS-DA analysis.

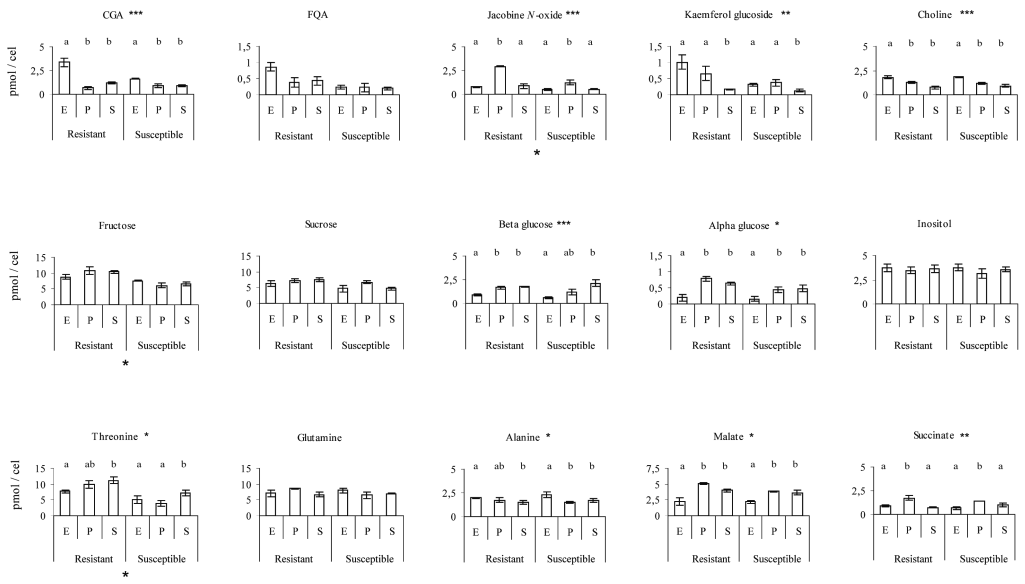

Fig 5. Concentration of metabolites in epidermis (E), palisade- (P) and spongy mesophyll (S) leaf cells of genotypes A and B of F2 *Jacobaea* hybrids. Means of three replicates and the standard error are presented. Data were analyzed by nested ANOVA with cell type nested within genotypes. Significant differences between A and B genotypes are indicated at the base of the graphs, whereas significant differences between cell types are indicated at the top of the graph whereby * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

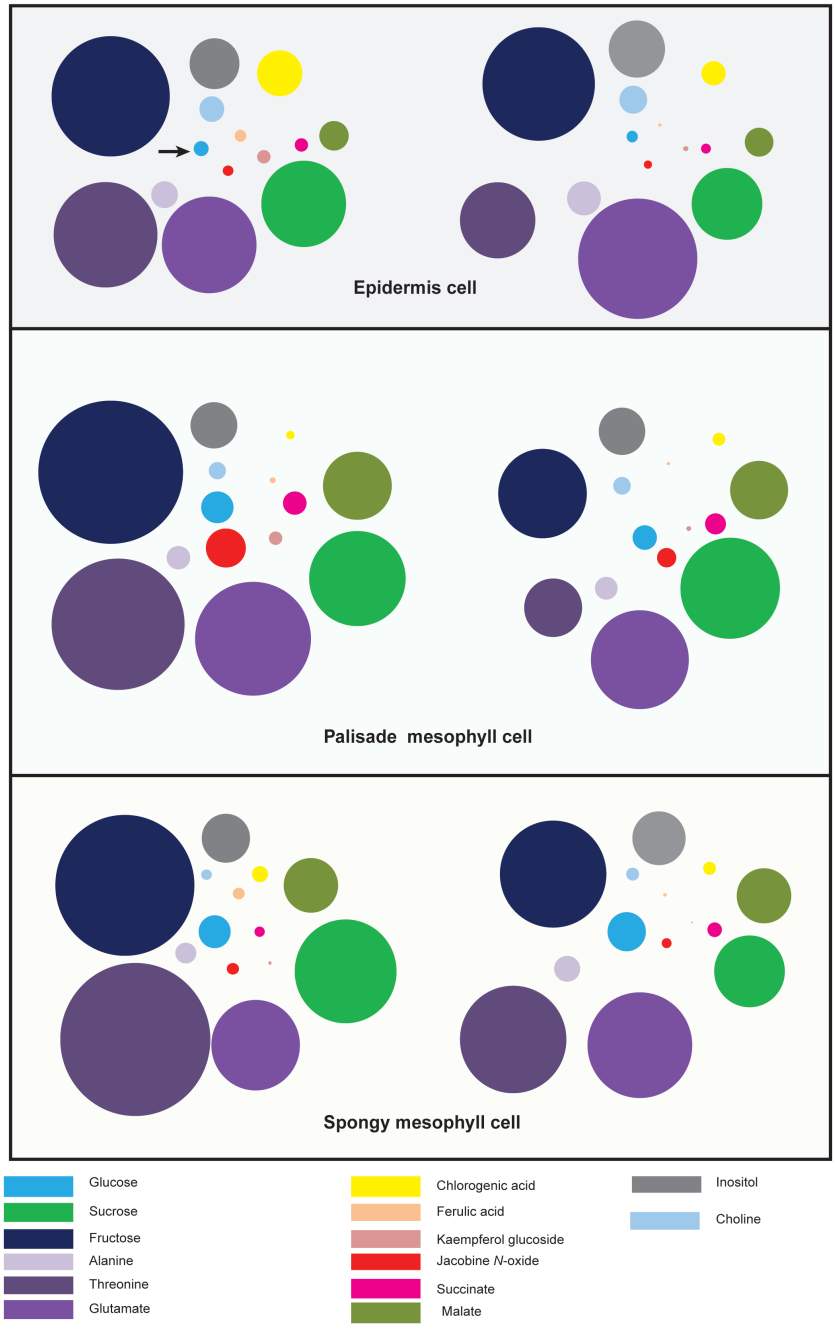

Fig 6. The distribution of metabolites in epidermis (E), palisade- (P) and spongy mesophyll (S) leaf cells of F2 *Jacobaea* hybrids. The area of the circles represent the ratio of respective metabolites levels compared to glucose levels in epidermis of the genotype A. → indicated the glucose which was used to determine the ratio.

DISCUSSION

Cell-specific metabolomics of epidermis, palisade- and spongy- mesophyll cells showed that metabolites contained in these three cell types were cell specific. A similar pattern of cell-specific distribution of primary and secondary metabolites was observed for both genotypes tested, indicating that such a specific distribution may be of general occurrence.

Regarding primary metabolites the epidermis cells showed lower levels of glucose, threonine and the organic acids malate and succinate but higher levels of cholin as well as of the amino acids alanine in comparison with the two mesophyll cell types. The epidermis cell in contrast to the mesophyll cell is free of chlorophyll. Chlorophyll enables photosynthesis of which glucose is the primary product. In our earlier study (Nuringtyas et al., 2012), we also reported low levels of glucose in the epidermal leaf tissue. Similarly, malate, as a key metabolite, is involved in respiration and energy generation through photosynthesis (Martinoia and Rentsch, 1994). Cholin, as an intracellular salt, plays a role in protecting plants against unfavourable conditions such as salt, drought or cold stress (Sakamoto and Murata, 2002). Higher accumulation of alanine may serve as a source of beta-alanine and beta-alanine betain which was known as one of the most effective osmoprotectant (Rathinasabapathi et al., 2000). The amino acid threonine has been indicated as a precursor of the necid acid of PAs (Stirling et al., 1997). As for threonine also the levels of PAs, as SMs were increased in the mesophyll cells in contrast to the epidermis cell. The same finding but then on the tissue level (Nuringtyas et al., 2012) confirms our result. Thus, SMs showed a cell-specific distribution pattern. The epidermis cells contained lower concentrations of the PA Jacobine *N*-oxide, which was concentrated in the palisade cells, but higher concentrations of the phenylpropaonid CGA and the flavanoid kaempferol glucoside.

In the genus *Jacobaea* PAs are associated with plant defence against generalist herbivores as described in the review by Macel (2011). Particularly the jacobine type PAs, including jacobine *N*-oxide, are involved in host plant resistance to Western Flower thrips (Leiss et al., 2009a, Cheng et al., 2011). However, Macel and Klinkhamer (2010) indicated that jacobine has a positive effect on specialist feeding. In a field study they observed a positive correlation between jacobine concentration and damage of the cinnabar moth (*Tyria jacobaeae*), the thrips *Haplothrips senecionis* and the Rust fungus *Puccinia dioicae,* respectively. We showed that jacobine *N*-oxide was highly accumulated in the cells of the palisade mesophyll, which may constitute a strategy to deal with the generalist-specialist dilemma. Jacobine serves as a plant defence compound against generalists but at the same time attracts specialists. The cinnabar moth can use PAs as an oviposition cue (Macel and Vrieling, 2003; Cheng et al., 2013). Placing jacobine in the palisade cells may ensure plant defence against the chewing larvae but prevent the use of jacobine as a host recognition cue for oviposition on the leave surface. Similarly, *P. diociae*, as an obligate biotroph pathoghen, may be inhibited to use jacobine on the leave surface for host recognition. Thrips, which are piercing-sucking insects, commence feeding with probing of the epidermis followed by ingestion of the sub-epidermal cells (Harrewijn et al., 1996; Kindt et al., 2003). High concentrations of jacobine in the sub-epidermal palisade cells may thus prevent the specialist thrips *H. senecionis* from recognising its host and at the same time prevent from feeding by the generalist thrips *F. occidentalis*. The accumulation of jacobine in the palisade cells confirms our

earlier finding reporting a high concentration of PAs, including jacobine, jaconine and senecionine, as both, the free base and *N*-oxide forms, in the mesophyll tissue of *Jacobaea* plants. However, in that study the two different mesophyll cell-types could not be distinguished. Due to the micro-metabolomic approach, looking at single cells, the amount of material analysed in the current study allowed to detect only the most abundant PA: jacobine *N*-oxide.

In line with our previous study (Nuringtyas et al., 2012), CGA was highly accumulated in the epidermis cells. A high concentration of phenylpropanoids, including CGA, in the epidermis of *Arabidopsis* mutants was associated with its function as a UV-B protector in plants (Bharti and Khurana, 1997). Treatment of wild tobacco (*Nicotiana attenuata*) plants with UV-B radiation increased the level of CGA and subsequently decreased the amount of leaf damage caused by the thrips *F. occidentalis* (Demkura et al., 2010). Accumulation of CGA has been reported to be related to thrips resistance in chrysanthemum (Leiss et al., 2009b). From the biosynthesis point of view, the higher amounts of CGA are based on the activity of phenylalanine ammonia-lyase, the key enzyme of CGA biosynthesis which is situated in the epidermis (Kojima and Conn, 1982).

Another secondary metabolite associated with plant defence, kaempferol glucoside, was accumulated in the epidermis and palisade cells. Kaempferol glucoside has been identified in *Jacobaea* hybrids earlier (Leiss et al., 2009; Kirk et al., 2005). Kaempferol is known to confer a deterrent effect on onion thrips, *Thrips palmi*, (Wu et al., 2007), generalist caterpillars (Onyilagha et al., 2004) and aphids (Lattanzio et al., 2000). In a study on tissue localization of phenolics, high concentrations of kaempferol glucosides were detected in the epidermis of Broad Bean, *Vicia fab*a, (Hutlzer et al., 1998). Schnitzler et al. (1996) reported the accumulation of kaempferol glucosides, as UV-B pigments, in the epidermis of Scots Pine, *Pinus sylvestris*.

While the above patterns of cell-specific metabolites were the same for both genotypes tested, the thrips resistant genotype A contained higher amounts of fructose, threonine and jacobine *N*-oxide compared to the susceptible genotype B. Carbohydrates, especially sucrose and fructose are strong feeding stimulants for herbivorous insects (Bernays and Simpson, 1982). Naturally, they are also important nutrients for the insects to synthesise body tissue and to serve as an energy source (Schoonhoven et al., 2008). Threonine, as described above functions as a pre-cursor for the necic acid of PAs (Stirling et al., 1997) and may thus serve to supply the synthesis of jacobine. Higher amounts of jacobine *N*-oxide in *Jacobaea* plants resistant to *F. occidentalis*, in contrast to susceptible ones have been reported by Leiss et al. (2009a) and Cheng et al. (2011). The plant defence compounds CGA and kaempferol glucoside were both somewhat increased in the resistant genotype but this was not significantly different.

CONCLUSION

Cell-specific metabolomics of epidermis, palisade- and spongy- mesophyll cells showed that metabolites contained in these three cell types were cell-specific for both genotypes indicating that this may be a general pattern. The epidermis as outer cell layer confers protection to the cells. Therefore, it was rich in metabolites ameliorating the negative effects of abiotic factors such as UV-light, drought, frost and salt and rich in plant defence compounds against general pathogens and herbivores. However, the later compounds may also act as host finding cues for specialist herbivores. In order to solve this generalist-specialist dilemma the distribution of the defence compounds may adapt as in the case of the PA jacobine *N*-oxide, which did not accumulate in the epidermis but in the palisade mesophyll beneath. In contrast the mesophyll cells, containing chlorophyll, were generally rich in metabolites related to respiration and energy generation. As such our study emphasises the importance to study the cell-specific distribution and function of metabolites in distinguished cell types.

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Chapter 6.

Toxicity of pyrrolizidine alkaloids to *Spodoptera exigua* using insect cell lines and injection bioassays

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are known as feeding deterrents and toxic compounds to generalist herbivores. Among the PAs of *Jacobaea vulgaris,* jacobine and erucifoline are the most effective against insect herbivores as indicated by correlative studies. So far, little is known on the effect of jacobine and erucifoline as individual PAs. We, therefore, isolated these PAs from their respective *Jacobaea* chemotypes. These and other commercially available senecionine-like PAs including, senecionine, seneciphylline, retrorsine, and senkirkine were tested as free base and *N*-oxide forms. A range of concentrations from 0–70 ppm was added to *Spodoptera exigua* cell lines and injected into the haemolymph of 3rd instar larvae. Both bioassays led to similar results in the order of PA toxicity, indicating that the cell lines are a valuable tool for a first toxicity screen. Testing individual PAs, jacobine and erucifoline appeared to be the most toxic PAs proving their major role in plant defence against generalist herbivores. Senkirkine and seneciphylline showed a lower toxicity than jacobine and erucifoline but higher than retrorsine. Senecionine was not toxic at the tested concentrations. In all toxic PAs the free base form was more toxic than the *N*-oxide form. Combination of toxic PAs with chlorogenic acid, another reported defence compound in *Jacobaea*, resulted in a reduction of PA toxicity. Our results stress that structural variation of PAs influences their effectiveness in plant defence.

Key Words- *Spodoptera exigua*, cell line bioassay, insect injection bioassay, pyrrolizidine alkaloids, toxicity.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are a class of secondary plant metabolites well known for their negative effects on insect herbivores and vertebrates. In insect herbivores PAs can act as feeding deterrents and toxic compounds (Macel et al., 2005; Ober and Kaltenegger, 2009; van Dam et al., 1995). In vertebrates they can have hepatoxic, pulmotoxic (Mattocks, 1986; Cheeke, 1988) and carcinogenic effects (Frei et al., 1992).

Pyrrolizidine alkaloids are esters of a necine base with one or more necic acids (Hartmann, 1999). In members of the *Jacobaea* genus (Asteraceae), PAs exist as macrocyclic diesters with two types of necine bases: retronecine and otonecine (Fig. 1). So far over thirty five PAs have been reported in *Jacobaea* plants (Cheng et al., 2011; Pelser et al., 2005). Based on the biosynthetic route, these alkaloids are divided into four main groups: senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Pelser et al., 2005). Most of the PAs occur in two interchangeable forms: free base (tertiary amine) and *N*-oxide (Hartmann and Dierich, 1998). So far the *N*-oxide is accepted as the major storage form in plants (Hartmann and Toppel, 1987; van Dam et al., 1995) and generally the concentration of this form is higher than that of the related free base (Joosten et al. 2011). The free base form is considered to be more toxic than the *N*-oxide one (van Dam et al., 1995; Hartmann, 2007).

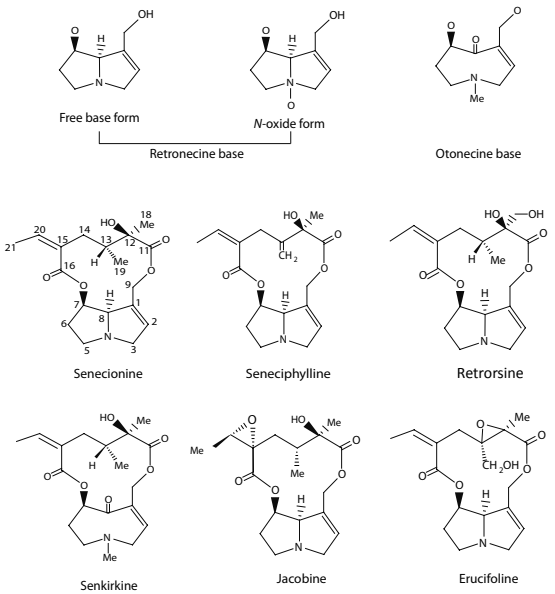

Fig 1. Chemical structures of pyrrolizidine alkaloids tested in insect cell line and injection bioassays.

When ingested by vertebrates the *N*-oxide form is not toxic but when entering the gut the *N*-oxide is reduced to its free base, which is then converted by cytochrome P450 enzymes (CYPs) into unstable pyrrole intermediates in the liver. These intermediates readily react with the amino groups of proteins as well as with nucleosides in DNA and RNA (Wiedenfeld and Edgar, 2011).

Similarly, in insects, PAs are reduced in the gut and converted by CYPs to the highly reactive pyrrole intermediates (Lindigkeit et al., 1997). Besides these, the potential of PAs as neurotoxins has been demonstrated. The free base form of PAs showed a significant binding activity to membranes of muscarinic acetylcholine and serotonine receptors derived from porcine brain, which may influence neuronal signal transduction as well as central nervous system- and muscular- activity (Schmeller et al., 1997).

As such structural variation of PAs may influence their effectiveness in plant defence. Several *in-vivo* plant studies have addressed this question. Especially jacobine- (Leiss et al., 2009a; Cheng et al., 2011; Joosten, 2012) and erucifoline like PAs (Macel, 2003; Macel and Klinkhamer, 2010) were identified to contribute to plant defence. However, these results are mainly based on correlative studies. Little is known on the effect of jacobine and erucifoline as individual PAs. There is only one report on erucifoline, isolated from the Canarian endemic plant *Canariothamnus palmensis*, demonstrating a negative effect on green peach aphid *Myzus persicae* (Dominguez et al., 2008). Mainly senecionine-like PAs have been individually tested (Lindigkeit et al., 1997; Macel et al., 2005) since these are the only PAs commercially available. However, in contrast to senecionine-, jacobine and erucifoline like PA are mainly involved in plant defence against generalist insects. In the current study we, therefore, isolated jacobine and erucifoline from their respective *Jacobaea* chemotypes for application in lepidopteran insect bioassays.

Most studies on insect toxicity use feeding bioassays in which insects are presented with artificial diets or leaf surfaces to which the compounds to be tested have been added. However, this approach is often tedious, time consuming and the amounts of PAs eaten by the insects may vary. Moreover, such assays need relatively large amounts of the test compounds (Decombel et al., 2004). The latter is especially problematic for PAs, of which only a few are commercially available and which, therefore, need to be isolated first in order to test them. For insect toxicity bioassays the amount of compound needed can be greatly reduced by using injection bioassays in which the compound is directly injected into the haemolymph. However, for routine screening purposes this method remains too laborious. Using insect cell lines as a bioassay system appears to form an interesting alternative to test PA toxicity (Dinan et al., 1990). Cell based bioassays are commonly used in life sciences to study biological activities, toxicity and cellular processes. Cell lines of the beet armyworm, *Spodoptera exigua* (family Noctuidae; order Lepidoptera), have been successfully used for screening insecticide activities (Decombel et al., 2004). In the present study we, therefore, compared the toxicity of individual PAs in, *S. exigua* cell lines with that of an insect injection bioassays.

In this study, we specifically addressed the following questions:

1. Do different types of PAs have different toxic effects on *S. exigua*?
2. Do the free base and *N*-oxide forms of PAs have different toxic effects on *S. exigua*?
3. Next to PAs chlorogenic acid (CGA) is a common defence compound occurring in *J. vulgaris* (Nuringtyas et al, 2012, Leiss et al., 2009). Does the combination of PAs and CGA lead to additive or synergistic effects of toxicity to *S. exigua*?
4. Is the toxicity of PAs to *S. exigua* measured in cell lines comparable to that measured in an injection bioassay?

METHODS AND MATERIALS

Pyrrolizidine Alkaloids. We tested crude PA extracts containing a mixture of PAs present in the *Jacobaea* plants as well as individual PAs including senecionine-like PAs: senecionine, seneciphylline, senkirkine (Carl Roth, Karlsruhe, Germany), retrorsine and retrorsine *N*-oxide (Sigma, St. Louis, USA) as well as jacobine and erucifoline (Fig. 1). Jacobine and erucifoline were isolated from the respective chemotypes of *J. vulgaris*. For PA extraction, a modified procedure of Hartmann and Toppel (1987) was used, in which 0.05 M H_2SO_4 was replaced with 3% formic acid. Since the extraction of the erucifoline chemotype resulted in a low amount of extract, this extract was used only for isolation of erucifoline. The larger amount of extract obtained from the jacobine chemotype was used for isolation of jacobine as well as for preliminary toxicity bioassays to define the range of PAs concentrations to be tested. Isolation of jacobine and erucifoline was carried out using Centrifugal Partition Chromatography (CPC) and HPLC (Hartmann and Zimmer 1986; Hösch et al. 1996). Except for senkirkine all the PAs tested occur in plants as both *N*-oxide or free base form. To obtain the *N*-oxide form the PA extract as well as the corresponding free base, individual PAs were oxidised with *m*-chloroperbenzoic acid and purified using column chromatography with an alkaline alumina gel (Craig and Purushothaman 1970) except for retrorsine *N*-oxide which was available commercially. The result of the *N*-oxidation process was confirmed by Thin Layer Chromatography (TLC) and ^{1}H Nuclear Magnetic Resonance Spectroscopy (NMR).

Spodoptera exigua Cell Culture Bioassay.
Pyrrolizidine alkaloid extracts. PA extracts were dissolved directly in DMSO. For a first preliminary experiment, we used a concentration range of 0 – 220 ppm. The range of 0 –70 ppm appeared to be most effective and was thus used in the later experiments.

Individual PAs. Each individual PA was dissolved directly in DMSO. Seven different concentrations in the range of 0 – 70 ppm were used.

Individual PAs combined with CGA. Individual PAs and CGA, both dissolved in DMSO, were combined. Individual PAs, which proved to be toxic and senecionine as a representative of a non-toxic PA were included in this bioassay. Seven concentrations of PAs ranging from 0–70 ppm were used to which 45 ppm CGA, which is representative for the amount of CGA in plants (Leiss et al., 2009b) was added. In addition, seven concentrations of CGA in the range of 0–70 ppm were tested.

Cell Lines. A sample of *S. exigua* cell line SE301 (Hara et al., 1995) was obtained from a cell culture routinely grown at the Laboratory of Virology, Wageningen University, The Netherlands. These cells were originally derived from neonate larvae. Cell lines were propagated at 27 °C in a Hyclone CCM3 medium (Thermo Scientific, Utah, USA) enriched with 5% Fetal Bovine Serum (FBS; Gibco, Auckland, NZ). To prevent contamination during the experiments, antibiotic gentamycine (Sigma, St. Louis, MO, USA) was added to the medium at 50 µg/ml. The cell line was sub-cultured every four days in 25 cm^2 tissue culture flasks (Corning Inc, New York City, USA).

Cell Culture Bioassay. The PA extract and individual PAs in both *N*-oxide and free base form were tested in a dose-response experiment. Two kinds of controls were applied, a negative control

consisting of 2 µl DMSO and a positive control consisting of 2 µl of 5 ppm Abamectine (Sigma, Chemical, St. Louis, USA), which is equivalent to the concentration inhibiting cell growth by 50% (IC_{50}). Abamectine is the active compound of the commercial insecticide Agri-Mek (Syngenta). Cells were collected two days after sub-culturing when they were in the exponential growth phase. Cells were diluted with fresh medium without serum to a density of 10^5 cells/ml. After loading each well of a 96-well culture plate (Costar, Corning Inc, New York, USA) with 200 µl of the cell solution (2.10^4 cells), 2 µl of compound solution to be tested was added with a micropipette (Gilson, USA). For each concentration tested nine replicates were used. Each compound was applied in threefold using three different plates. The compounds to be tested were randomly applied to each plate. After incubation for two days at 28ºC and 90% humidity, the cell numbers per cultured well were counted with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell-counting technique, described below. The IC_{50} concentrations were calculated using probit analysis.

MTT Assay. MTT (Sigma) solution was prepared as a 5 mg/ml stock in fetal bovine serum (FBS; Gibco, Auckland, NZ, USA). This solution was sterilised by filtration through a 0.2 mm filter (Acrodisc, Pall Co., Ann Arbor, MI, USA) and stored at 2–8°C. Twenty µl MTT solution was added to each well and incubated at 37°C for 3 h. At the end of the incubation period, the medium was removed carefully without disturbing the cells. The converted dye was solubilised with 200 µl acidic isopropanol (0.04 M HCl in absolute isopropanol). Subsequently, the optical densities (OD) were recorded at 575 nm in an automatic 96-well microtiter plate reader (Sigma Aldrich). In initial tests, we established a good linear relation between OD and cell numbers of the *S. exigua* cell cultures (Fig. S.1).

Spodoptera exigua Injection Bioassay.
Pyrrolizidine alkaloid preparation. An initial test with 2% DMSO resulted in 50 % larval mortality. Therefore, individual PAs were dissolved in 50% ethanol. We selected a number of PAs to represent PAs with the highest, intermediate and the lowest IC_{50} values based on the cell line bioassay: jacobine, retrorsine, seneciphylline and senecionine. Since the highest toxicity observed corresponded to an IC_{50} of 35 ppm we used three concentrations in the range of 0 – 50 ppm for injection which equals 0 – 50 µg/larva.

Insects. Third instars caterpillars of *S. exigua* were obtained from a laboratory culture reared on an artificial diet (Singh 1983) in a growth chamber at 30ºC, 16h/8h L/D photoperiod, 70% RH. The larvae used were 100–120 µg in weight.

Injection bioassay. One microlitre of individual PAs was injected into the haemolymph of a third instar larvae of *S. exigua.* For each concentration twenty four larvae were used. Injections were performed under a binocular microscope using a microsyringe (SGE GC, Australia) which was inserted into the 5^{th} larval segment. During injection, the larvae were held immobile with a thin metal scalpel. After injection, the larvae were kept together on an artificial diet in a plastic container (diameter 8.5 cm, height 8 cm) covered with tissue paper for proper aeration. Containers were randomly placed in a growth chamber at 27ºC, 16h/8h L/D photoperiod, 70% RH. After 24h, larval mortality was counted. A larva was considered dead when no response to the touch

of a glass rod was observed. Two kinds of controls were used. First, an empty injection, and second an injection with the solvent only. Each bioassay was conducted twice. The number of surviving larvae of the treatment was compared with that of the solvent control using a Chi-square test. Furthermore, the LC_{50}, the concentration causing 50% larval mortality, was calculated using probit analysis. The correlation between the two injection bioassays was analysed with a Pearson correlation analysis. All statistic analyses were performed using IBM SPSS statistics 19 (Chicago, IL, USA).

RESULTS

Cell Culture Bioassay.

PA extracts. In the initial experiments with concentrations ranging from 0–220 ppm, 90 % of the larvae died at concentrations of 90 ppm and higher (Fig. 2a). Therefore, we narrowed the concentration range of the PA extract to 0-70 ppm. The free base form with an IC_{50} of 30.03 ppm showed a higher toxicity compared to the *N*-oxide form with an IC_{50} of 50.24 ppm (Fig. 2b).

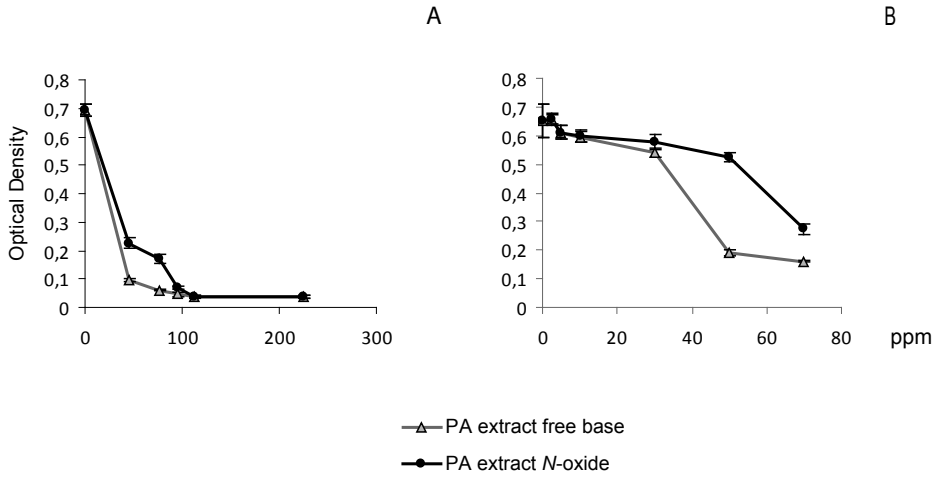

Fig 2. The bioactivity of PA extracts as free base and *N-oxide* form in *Spodoptera exigua* cell lines at concentrations of 0-220 ppm (A) and 0-70 ppm (B), measured as optical density at 575 nm. Data present the average of nine replicates and the standard errors are indicated

Individual PAs. All *N*-oxide forms tested had an IC_{50} value > 70 and we considered them, therefore, as non- toxic at the tested doses (Fig. 3). Except for senecionine and retrorsine all free-base forms, were toxic, jacobine showed the highest toxicity with an IC_{50} of 34.95 ppm followed by erucifoline at 36.92 ppm (Fig. 3). The senecionine-like PAs senkirkine and seneciphylline showed an IC_{50} value of 43.15 and 47.75 ppm, respectively.

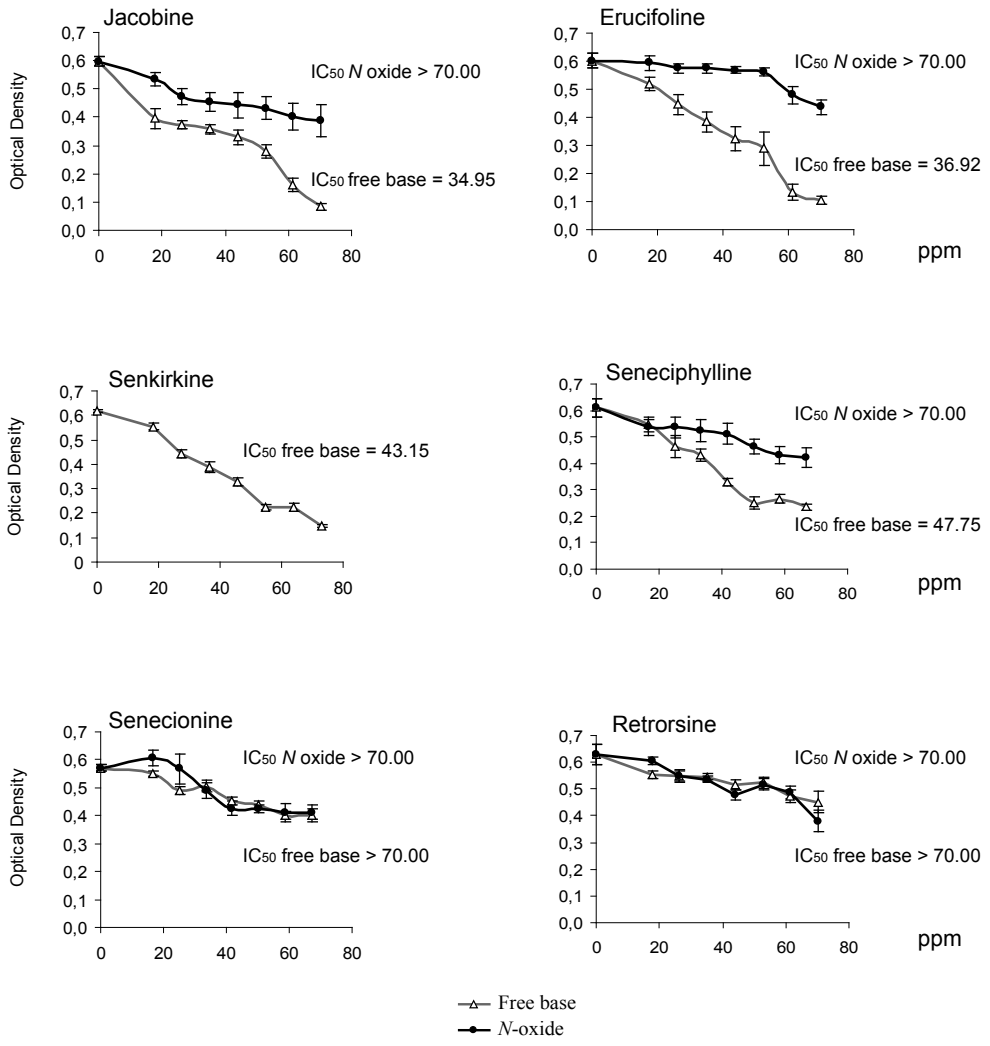

Fig 3. The bioactivity of individual PAs as free base and *N-oxide* form in *Spodoptera exigua* cell line at concentrations of 0-70 ppm measured as optical density at 575 nm. Data present the average of nine replicates and the standard errors are indicated. The IC_{50} is equivalent to the concentration inhibiting cell growth by 50%

Individual PAs combined with CGA. Chlorogenic acid showed an IC_{50} of 67.70 ppm (Fig. 4). In general, the combination of CGA at 45 ppm that is IC_{24} and PAs resulted in a reduction of PA toxicity as expressed by the lower IC_{50} values of the mixture compared to the respective individual PAs (Fig. 4). The strongest reduction of toxicity was observed in a mixture with erucifoline amounting to an IC_{50} >70 ppm, indicating that erucifoline toxicity was highly reduced. Similarly, the IC_{50} of jacobine and seneciphylline increased when CGA was added. Senecionine did not show any toxic effect either as individual PA or in combination with CGA.

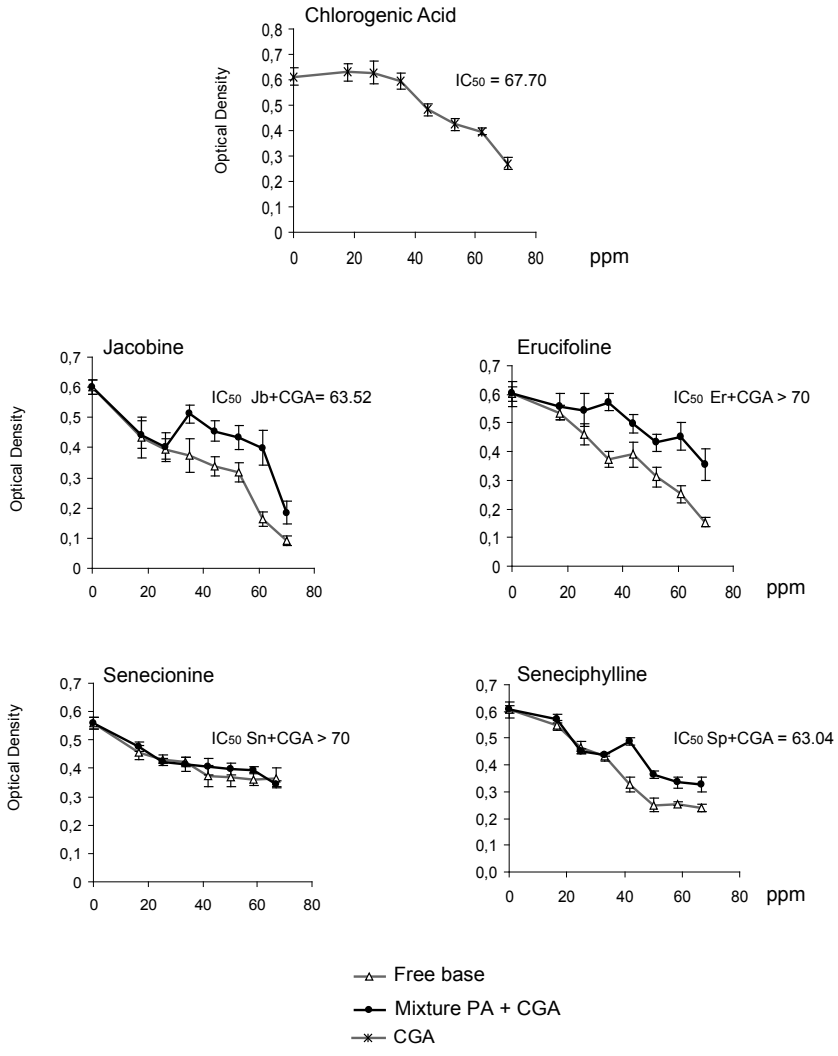

Fig 4. The effect of 45 ppm chlorogenic acid on the toxicity of individual PAs in *Spodoptera exigua* cell lines at concentrations of 0-70 ppm measured as optical density at 575 nm. Data present the average of nine replicates and the standard errors are indicated. The IC_{50} is equivalent to the concentration inhibiting cell growth by 50%

Injection bioassay.

Except for seneciphylline all *N*-oxide alkaloids showed lower toxicity than the respective free base form (Fig. 5). The free base form of jacobine with an LC_{50} of 22.9 ppm was most toxic followed by seneciphylline and retrorsine an LC_{50} of 32.35 and 43.16 ppm, respectively. At the concentrations tested, senecionine as free base form was also not toxic. The bioassay was conducted twice and the results of both bioassays were significantly correlated for each PA tested (data not presented). In contrast to the free base form, the *N*-oxide form of the PAs showed no biological relevant toxicity in all PAs except for a low toxicity of the *N*-oxide. The result of the two experiments showed a

similar response as presented in Fig. 5. The free base form of jacobine was most toxic followed by seneciphylline and retrorsine, respectively. Senecionine as free base was also not very toxic at the doses tested.

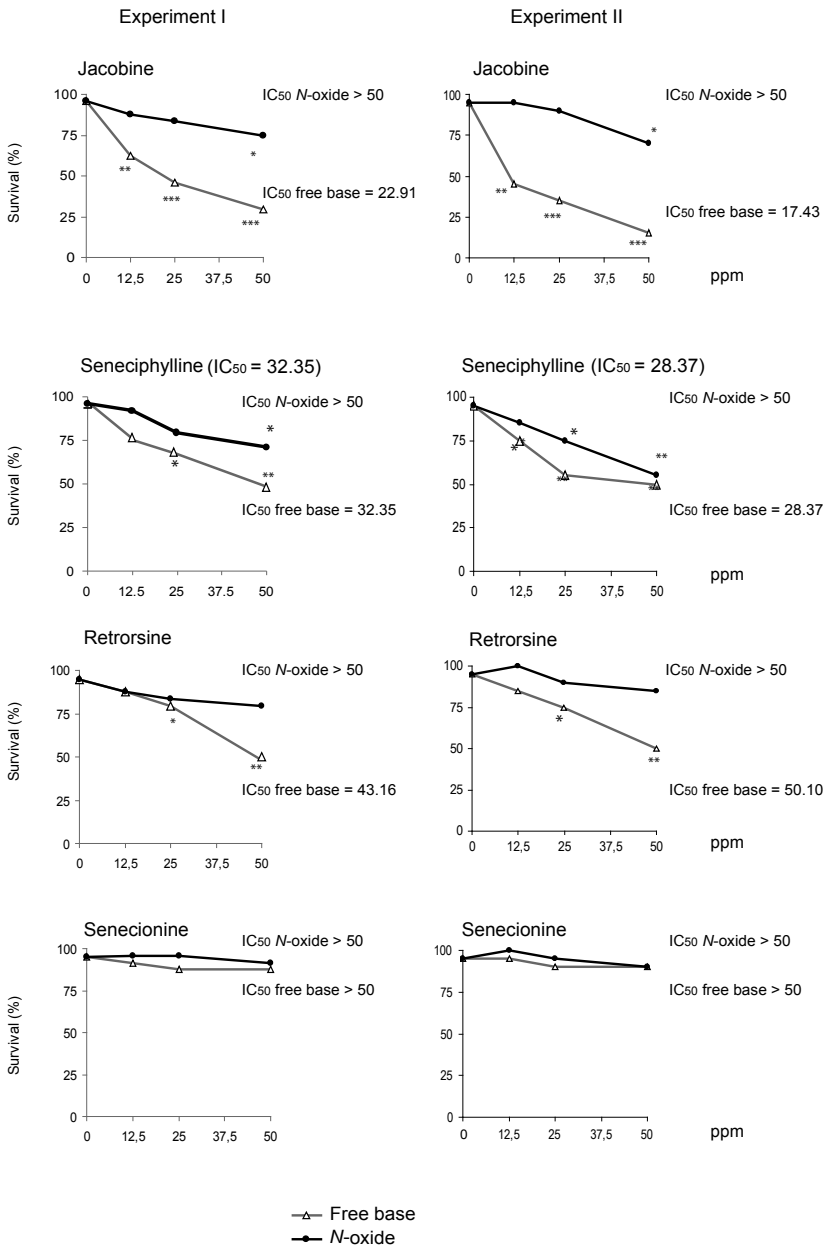

Fig 5. Survival of third instar larvae of *Spodoptera exigua* upon haemolymph injection with individual PAs at concentrations of 0-50 ppm. Data present the percentage survival from 24 larvae for each compound and concentration tested and the standard error. Significant differences between treatments and control are indicated as *** = P< 0.0001, ** = P < 0.001, * = P<0.05. The LC_{50} is equivalent to the concentration which caused death by 50% of the population.

DISCUSSION

Comparing the toxicity of PAs to the generalist herbivore *S. exigua* using cell line and injection bioassays, we observed in both assays similar results for the order of PAs toxicity. Jacobine was the most toxic, followed by erucifoline, senkirkine and seneciphylline, while senecionine was not toxic at the tested concentrations. Decombel et al. (2004) showed that the cytochrome P450 (CYPs) responsible for the formation of the highly reactive pyrrole intermediates were present in both *S. exigua* cell lines as well as the *in-vivo* insects. Therefore, most likely similar toxicity mechanisms are present in the two different systems, indicating that *S. exigua* cell lines are a valuable tool for a first screen of PA toxicity.

Our results confirm the prominent role of jacobine and erucifoline-like PAs in plant defence against leaf feeding insects. In *in-vivo* plant studies mainly the jacobine-like PAs were observed to be correlated to the above ground defence of *Jacobaea* plants against the generalist herbivore western flower thrips, *Frankliniella occidentalis*, including, jaconine (Joosten, 2012), jacobine *N*-oxide and jaconine *N*-oxide (Cheng et al., 2011; Joosten, 2012; Leiss et al., 2009a) as well as jacoline *N*-oxide (Cheng et al., 2011). Erucifoline, isolated from an extract of *C. palmensis*, demonstrated a negative effect on the green peach aphid (Dominguez et al.,, 2008). Comparing jacobine and erucifoline chemotypes in the laboratory Macel (2003) observed a negative effect of the jacobine chemotypes on *S. exigua*, *F. occidentalis*, and *M. persicae*, while the erucifoline chemotype affected the cabbage moth, *Mamestra brassicae*. Comparing the same chemotypes in the field, the erucifoline chemotypes showed less damage compared to the jacobine ones (Macel and Klinkhamer, 2010). This result was explained by the presence of the specialist cinnabar moth, *Tyria jacobaea*, which uses jacobine as an oviposition cue for host finding (Cheng et al., 2013).

Both, jacobine and erucifoline, the most toxic PAs in our study are retronecine macrocyclic diesters with epoxide functional groups. These functional groups may provide suitable sites for chemical modification by CYPs resulting in the formation of toxic pyrroles (Wiedenfeld and Edgar 2011). Furthermore, the presence of epoxide functional groups may inhibit detoxification by esterases (Culvenor et al. 1976). The ester bonds of PAs can be hydrolysed by esterases to form non-toxic necine and necid acid (Wiedenfeld and Edgar 2011). The rate of hydrolysis depends on the level of steric hindrance of the ester linkages. The more complex the PAs the higher the level of steric hindrance. Jacobine and erucifoline are both relatively complex branched structures, which may inhibit hydrolysis.

Senkirkine and seneciphylline in our study showed lower toxicity than jacobine and erucifoline. Senkirkine has been reported to significantly reduce the survival of western flower thrips at a concentration present in plants (Macel et al., 2005) and to deter sixth instar larvae of the spruce budworm, *Choristoneura fumiferana* (Bentley et al., 1984). Senkirkine, compared to senecionine was more genotoxic to the fruit fly, *Drosophila melanogaster* (Frei et al., 1992). Seneciphylline has been observed to be a feeding deterrent to the pea aphid, *Acrythosiphon pisum* (Dreyer et al., 1985) and the crane fly, *Cyllindrotoma distinctissima* (Hagele and Rowell-Rahier, 2000). The migratory locust, *Locusta migratoria* was more strongly deterred by seneciphylline compared to other senecionine-like PAs and was toxic to *M. persicae* (Macel et al., 2005). The necic acid

moiety of senkirkine is identical to that of senecionine, but contains an otonecine base instead of retronecine. In contrast to senecionine, it is not easily oxidised to an *N*-oxide. That means that fast excretion in the form of a more water soluble *N*-oxide is hindered and possibly explains the higher toxicity of senkirkine compared to senecionine. Seneciphylline contains a retronecine base, as does senecionine which is not toxic, but contains a methylene group at C-13 of the necic acid. Addition of a methylene group at the lactone ring of eremophilanes increased antifeedant and insecticidal activity against *S. littoralis* (Tan et al., 1998).

Senecionine was not toxic at the tested concentrations for both cell lines and *S. exigua* larvae. Macel et al. (2005) also did not observe deterrent feeding activity of senecionine in *S. exigua* larvae even at three times the plant concentration of 1.5 mg/g fresh weight. Dominguez et al. (2008) studying the effect of senecionine on *S. littoralis* did not report any effect either. However, senecionine has been reported to significantly reduce larval survival of *M. persicae* and to deter feeding of *L. migratoria* (Macel et al., 2005). Senecionine is the backbone of all PAs, thus it represents the simplest structure of the macrocyclic PAs, which might explain its non-toxic nature. It may be easily detoxified by hydrolysis and rapidly removed from the body. Indeed, larvae of *Spodoptera littoralis*, injected with 3.5 µg senecionine *N*-oxide were able to completely eliminate this PA within 24 hours (Lindigkeit et al., 1997). The rapid excretion of senecionine avoids its conversion into the toxic pyrrole. Excretion of senecionine by *S. exigua* may explain why this PA was not toxic in our study in contrast to its toxic effect on aphids and locusts. Our results showed that both retronecine- and otosenine based PAs were toxic to *S. exigua*. However, within the retronecine based PAs toxicity depended on the structure of the necic acid.Supporting the important of necic acid, Dreyer et al. (1985) after removing the necic acid of riddelliine, a senecionine-like PA, demonstrated that the remaining retronecine base was 100 times less toxic to *A. pisum* compared to the complete macrocyclic riddelliine.

The cell line and injection bioassay led to comparable results except for retrorsine, which showed toxicity in the insect but not in the cell line bioassay. Retrorsine is a monohydroxylated derivative of senecionine. The hydroxylation may increase the water solubility of the PA possibly facilitating excretion. Injection of retrorsine into the haemolymph may lead to high concentration; thus retrorsine may immediately reach essential organs before effective metabolism and excretion occur.

Both bioassays, the cell line and the injection bioassay, indicated that the free base form of the PAs was more toxic than the *N*-oxide form. Similarly, for the deterrent activity, *S. exigua* larva clearly preferred the *N*-oxide form of a *Cynoglossum officinale* PA extract to the free base form (van Dam et al., 1995). Also for other generalist insects such as *A. pisum* (Dreyer et al., 1985), *F. occidentalis*, *L. migratoria* (Macel et al., 2005) and *M. persicae* (Reina et al., 2001) the free base form was the deterrent one. The free base form was reported to show a significant binding activity to muscarinic acetylcholine and serotonine receptors (Schmeller et al., 1997). This might cause short-term physiological disturbance contributing to mortality. Further studies on the mechanisms of toxicity are required to be able to better understand the role of PAs in plant defence.

Chlorogenic acid was moderately toxic to the *S. exigua* cell lines. Feeding deterrence of CGA to *S.*

exigua larvae has been observed by Felton et al. (1989, 1991). A negative effect on various other caterpillars was observed *in-vitro* (Bernays et al. 2002) and *in-vivo* (Elliger et al., 1981; Huang and Renwick, 1995; Mallikarjuna et al., 2004). A combination of PAs and CGA decreased the toxicity of PAs in our experiments and in the case of erucifoline toxicity was lost completely. The negative effect of CGA on caterpillars is based on its prooxidant effect as has been demonstrated for *S. exigua* (Felton et al., 1989, 1991). CGA is readily oxidised to chlorogenoquinone which binds to proteins, free amino and nucleic acids. As such CGA possibly competes with the PAs for enzymatic oxidation. Furthermore, the unstable pyrroles formed through PA reduction may compete with chlorogenoquinone for binding to amino and nucleic acids. CGA in the leaves of *Jacobaea* plants was highly accumulated in the epidermis in contrast to the PAs, which were concentrated in the mesophyll (Nuringtyas et al. 2012). Based on our results, such tissue specific distribution would prevent the antagonistic activity of CGA on PA toxicity.

CONCLUSION

The comparison of PA toxicity to the generalist herbivore *S. exigua* using cell line and injection bioassays, led to similar results in the order of PA toxicity, indicating that the insect cell lines are a valuable tool for a first toxicity screening. Testing individual PAs, jacobine and erucifoline appeared to be the most toxic PAs proving their major role in plant defence against generalist herbivores, which so far has only been shown in correlation studies. As such our study shows that structural variation of PAs influences their effectiveness in plant defence. For a better understanding of toxicity, metabolism studies in cell cultures will be the next step in unravelling the biological activity of PAs.

ACKNOWLEDGEMENTS

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Supplementary data Chapter 6

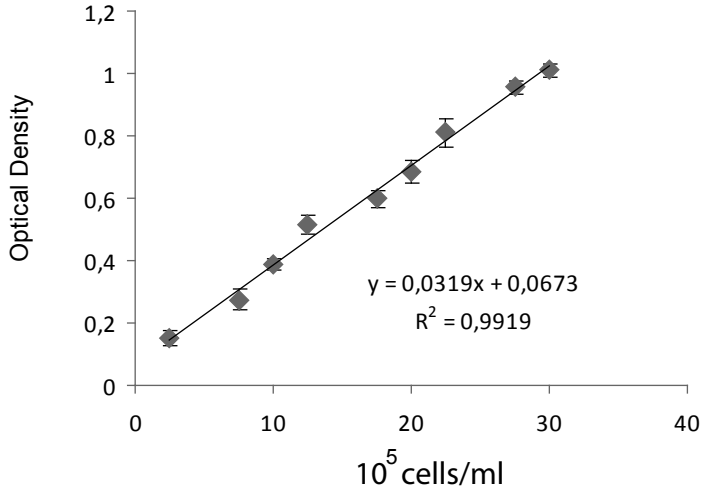

Fig S1. Standard curve for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in a *Spodoptera exigua* cell line measured as optical density at 575 nm. Data present the average of nine replicates with the corresponding standard errors are indicated.

Summary and Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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Samenvatting en Discussie

Planten bevatten veel verschillende secundaire metabolieten (SM's). Diversiteit van SM's vinden we op alle nivo's: tussen soorten, binnen soorten, tussen planten van een soort en tussen weefsels en cellen van een individu. Het onderzoek in de proefschrift richt zich op de rol van de diversiteit van SMs in de verdediging van planten tegen herbivoren.

Pyrrolizidine alkaloïden (PA's) in soorten van het geslacht *Jacobaea* werden gekozen als systeem om deze diversiteit te bestuderen. PA's zijn bekende gifstoffen voor de meeste gewervelde herbivoren en generalistische fytofage insecten. Er zijn grote verschillen in concentraties en samenstelling van PA's binnen een plantensoort.

In hoofdstuk 1 wordt de huidige kennis over de diversiteit in PAs, synthese en toxiciteit besproken. Bovendien worden de methoden die in deze studie gebruikt zijn, zoals Nuclear Magnetic Resonance (NMR) metabolomics en laser microdissectie (LMD), geïntroduceerd.

Hoofdstuk 2 beschrijft de invloed van endofytische schimmels op PA diversiteit. *Jacobaea* planten werden behandeld met drie verschillende fungiciden (Folicur, Pronto Plus en Switch). Na behandeling met Pronto plus en Switch waren de planten vrij van endofytische schimmels. In de met Folicur behandelde planten vonden we onverwacht een endofytische schimmelsoort die een sterke homologie vertoonde met het geslacht *Glomus*, een mycorrhiza behorende tot de Glomeromycota. Alle planten, zowel met als zonder fungicide behandelding, synthetiseerden PA's. Dit geeft aan dat endofyten niet onmisbaar zijn voor PA productie. De met Folicur behandelde planten hadden een lager PA gehalte dan de onbehandelde controlegroep terwijl de planten met de andere twee fungicide behandelingen niet verschilden van de controle groep. Vooral lagere concentraties van senecionine- en jacobine-achtige PA's veroorzaakten een daling van het PA gehalte.. Dit resultaat bevestigde dat de biotische omgeving bijdraagt aan variatie in PA concentratie en compositie in *Jacobaea* planten. Het is vooralsnog onduidelijk hoe de aanwezigheid van deze endofytische schimmel de PA concentratie verlaagt. De verlaagde jacobine gehaltes kunnen belangrijke ecologische concequenties hebben omdat ze enerzijds de plant resistenter kunnen maken tegen generalistische herbivoren (hoofdstuk 5) en anderzijds de planten aantrekkelijker kunnen maken voor specialistische herbivoren.

Er is nog weinig onderzoek gedaan naar de mate waarin verschillende plantenorganen PA's kunnen synthetiseren en transformeren. De PA synthese is door Hartmann et al. (1989) vergeleken tussen bloeiende planten waarvan de wortels waren verwijderd en in-vitro wortelkweken. In hoofdstuk 3 is beschreven hoe we weefsel kweken van wortels, spruiten en complete planten gebruikten om de PA synthese te bestuderen. Spruiten konden PA's de novo synthetiseren. Dit is in tegenspraak met eerdere studies die veronderstelden dat PA's alleen geproduceerd worden in de wortels

(Hartmann en Toppel, 1987). De PA concentraties waren laag in de wortelkweken, gemiddeld in de spruitkweken (1,5 x de wortelkweken) en hoog in kweken van complete planten (3 x de wortelkweken). Dit geeft aan dat zowel wortels als spruiten belangrijk zijn voor PA synthese. De wortelkweken bevatten voornamelijk senecionine- en otosenine-achtige PA's terwijl jacobine- en erucifoline-achtige PA's in hogere concentraties aanwezig waren in zowel spruitkweken als kweken van complete planten. Onetine concentraties was twee maal zo hoog in de wortelkweken dan in de spruitkweken kweken van complete planten. Dit suggereert dat de wortelkweken retronecine kunnen transformeren tot de otonecine basisstructuur. De hoge niveaus van jacobine- en erucifoline- achtige PA's in de spruitkweken en kweken van complete planten geven aan dat de epoxidatie die leidt tot een cyclische ether ring in de necic acid (necicine zuur) structuur van PA's vooral voorkomt in de bovengrondse organen. Spruiten zijn dus essentieel voor de diversificatie van jacobine- en erucifoline-achtige PA's.

In studies met celkweken van de generalistische insect herbivoor *Spodopter exigua*, bleken jacobine en erucifoline de meest toxische PA's (hoofdstuk 6). De jacobine concentratie was hoog in planten die resistent waren tegen de trips *Franklieniella. occidentalis* (hoofdstuk 5). Eerdere studies bevestigden de rol van jacobine-achtige PA's in de afweer van planten tegen herbivore insecten zoals trips, Leiss et al., 2009; Cheng et al., 2012) en van erucifoline-achtige PA's tegen bladluizen (*M. persicae*, Dominguez et al., 2008). In vergelijking met de effecten op insect herbivoren, is er weinig bekend over het effect van PA's op pathogenen. Een studie met zeven stammen van de genera *Fusarium* en *Trichoderma* toonde aan dat PA extracten de groei remden van pathogenen (Hol en Van Veen, 2002). Meer in-vitro studies naar de toxiciteit van de afzonderlijke PA's voor andere blad-herbivoren en pathogenen zijn van belang voor een beter begrip van de evolutie van PA diversiteit.

De route van de PA diversificatie is nog onduidelijk. In de literatuur zijn twee hypothetische scenario's voor de biosynthese voorgesteld. In het eerste scenario delen jacobine- en erucifoline-achtige PA's dezelfde biosyntheseroute met hetzelfde enzym dat verantwoordelijk is voor de transformatie van senecionine N-oxide, terwijl otosenine-achtige PA's onafhankelijk als derivaten van senkirkine worden gevormd (hoofdstuk 2, Fig S.1A). In het tweede scenario worden jacobine- en erucifoline-achtige PA's onafhankelijk gesynthetiseerd terwijl otosenine-achtige PA's worden gevormd uit jacobine-achtige PA's (Pelser et al., 2005; hoofdstuk 2, Afb S.1B). In hoofdstuk 2 hebben we laten zien dat door de Folicur behandeling alleen de senecionine- en jacobine-achtige PA's, maar niet de erucifoline- en otosenine-achtige PA's werden beïnvloed Deze resultaten geven aan dat de vorming van jacobine-achtige PA's onafhankelijk is van die van de erucifoline-achtige PA's zoals volgens het tweede scenario. Echter, de otosenine-achtige PA's passen waarschijnlijk beter in het eerste scenario omdat deze groep van PA's niet wordt beïnvloed door de behandeling. De resultaten van hoofdstuk 3 ondersteunen dit. De hoge hoeveelheid otosenine-achtige PA's in de wortelkweken in vergelijking met de spruitkweken maakt waarschijnlijk dat otosenine-achtige PA's, en speciaal onetine, niet worden gesynthetiseerd uit jacoline dat dezelfde necic acid groep heeft als onetine. We veronderstellen dat PA diversificatie een combinatie is van beide voorgestelde syntheseroutes waarbij de diversificatie in jacobine-achtige PA's onafhankelijk is van die van erucifoline-achtige PA's en de otosenine-achtige PA's zijn afgeleid van senecionine-achtige PA's en niet van jacobine-achtige PA's (Figuur S.1.C in hoofdstuk 2).

Grote insecten kunnen zich voeden met hele spruiten of bepaalde plantenorganen, maar kleinere insecten zullen zich voeden met vooral specifieke bladweefsels. Planten bezitten ongeveer veertig verschillende celtypen en twaalf ervan komen voor in het blad. De verschillende celtypen hebben hun eigen specifieke biologische functies in de groei, de ontwikkeling, de voortplanting en de verdediging van planten. Er is echter weinig informatie beschikbaar over de variatie van SM's tussen deze verschillende weefsels of celtypen. In hoofdstuk 4 gebruikten we NMR om de metabolietprofielen in epidermis en mesofyl te bestuderen. De epidermis bevatte een aanzienlijk grotere hoeveelheid jacaranone en fenylpropanoïden (vooral chlorogeenzuur en feruloyl kininezuur) terwijl het mesofyl hogere concentraties aan PA's bevatte. Chlorogeenzuur (CGA) en PA's zijn bekend om hun remmend effect op herbivoren zoals trips. Vóórdat trips geconfontreerd wordt met de PA's als de ultieme verdediging in de mesofyl komen tripsen eerst CGA tegen in de epidermis. Dus de twee cel lagen met hoge concentraties van twee verschillende soorten SM's met verschillende doeltreffendheid zou een strategie van de plant kunnen zijn om zich te verdedigen.

Om de metabolietprofielen van verschillende celtypes meer in detail te bestuderen voerden we een celspecifieke metaboloom studie uit met behulp van laser microdissection. We isoleerden epidermis- en palissade- en spons mesofylcellen van een trips resistent en een vatbaar genotype. De CGA concentratie was hoog in de epidermiscellen terwijl jacobine N-oxide vooral aanwezig was in de palissade mesofylcellen. Dit is in overeenstemming met de resulaten van hoofdstuk 4. In het genotype dat resistent was tegen trips vonden we een hogere concentratie van jacobine N-oxide maar geen hogere concentratie van CGA. Jacobine-achtige PA's zijn geassocieerd met verdediging van planten tegen generalistische herbivoren. Jacobine-achtige PA's kunnen echter ook gebruikt worden door specialistische insecten om hun waardplant te herkennen. Rupsen van de jacobsvlinder, *Tyria jacobaea*, de specialistische trips, *Haplothrips senecionis,* en de obligate biotrophe roest, *Puccinia lagenophorae,* werden allemaal vaker gevonden op genotypen met een hoog gehalte van jacobine-achtige PA's (Macel en Klinkhamer, 2010). De specifieke verdeling van jacobine N-oxide in de palissade mesofylcellen kan een strategie zijn om om te gaan met het generalist-specialist dilemma. Het concentreren van jacobine in de palissade cellen onder het bladoppervlak kan verhinderen dat jacobine wordt gebruikt voor gastheer herkenning door specialisten.

Het effect van jacobine (Leiss et al., 2009a; Cheng et al., 2011; Joosten, 2012) en van erucifoline op herbivoren is voornamelijk bestudeerd in correlatieve studies (Macel, 2003; Macel en Klinkhamer, 2010). De effecten van individuele PA's zijn niet getest in bioassays. We hebben daarom, in hoofdstuk 6, jacobine en erucifoline geisoleerd uit *Jacobaea* chemotypen. We hebben de effecten van deze PA's evenals enkele andere commercieel verkrijgbare PA's waaronder senecionine, seneciphylline, retrorsine en senkirkine, in vrije base en N-oxide vorm getest op de generalistische herbivoor *S. exigua* Tegelijkertijd werd CGA individueel getest en in combinatie met de verschillende PA's. Twee soorten testen werden uitgevoerd: een met in vitro celkweek van S. exigua en een met injectie van PA's in larven. In beide bioassays was jacobine de meest giftige PA terwijl senecionine en CGA niet toxisch waren. De combinatie van CGA met PA's verminderde de toxiciteit van PA's in alle gevallen. Voor alle PA's vertoonde de vrije base vorm een hogere activiteit dan de N-oxide vorm. Deze resultaten tonen aan dat naast de necine basis, de toxiciteit van PA's wordt beïnvloed door de functionele necic acid groep. Zowel, jacobine als erucifoline, de twee

meest toxische PA's, zijn retronecine macrocyclische di-esters met functionele epoxide groepen. Deze meer complexe necic acid groepen zijn resistenter tegen detoxificatie door esterases en ze kunnen een verlaagde water oplosbaarheid hebben waardoor ze moeilijker worden uitgescheiden.

Concluderend: we onthulden een specifiek patroon van PA accumulatie binnen *Jacobaea* planten. Deze specificiteit geeft een nieuwe mogelijke verklaring voor een oplossing van het generalist-specialist dilemma. PA's die belangrijke afweerstoffen tegen generalistische insecten zijn komen vooral voor in de middelste laag van het blad waardoor ze minder goed gebruikt kunnen worden voor gastheer herkenning en vraat stimulering door gespecialiseerde insecten. Planten lijken een meervoudige verdedigings strategie te hebben ontwikkeld. Verbindingen zoals CGA worden geaccumuleerd in de buitenste laag van het blad en dienen als eerstelijns verdediging. PA's in het mesofyl vormen de tweede verdedigingslijn. Bovendien vermindert CGA de toxiciteit van PA's en dus zorgt een scheiding van deze verbindingen in verschillende weefsels voor verhoogde PA activiteit tegen insecten. Elk celtype heeft een specifiek chemisch profiel. Daarom moet de studie van SMs zich richten op die weefsels die voor een specifieke herbivoor relevant zijn. In dit proefschrift wordt de rol van jacobine-achtige PA's in de afweer van planten tegen de generalistische herbivoren beter belicht. Jacobine-achtige PA's werden beïnvloed door de aanwezigheid van een mycorrhiza. Jacobine-achtige PA's komen vooral in hogere concentraties voor in de spruiten. Bovendien komen deze PAs in hogere concentraties voor in planten die resistent zijn tegen *F. occidentalis*. We bevestigden de toxiciteit van jacobine-achtige PA's voor een generalistische herbivoor. Deze resultaten wijzen dat een necic acid groep de toxiciteit van PA's verhoogt.

Ringkasan

Tumbuhan mensintesis berbagai macam senyawa yang dapat diklasifikasikan menjadi metabolit primer dan sekunder. Salah satu karakter dari MS adalah keanekaragamannya yang sangat tinggi (Hartmann, 1986). Karakter ini dapat ditemukan baik antar spesies maupun dalam satu spesies. Distribusi spatial MS bahkan dapat ditemukan dalam satu individu baik pada tingkat organ, jaringan maupun sel. Keanekaragaman MS merupakan konsekuensi dari metabolisme tumbuhan yang dinamis untuk merespon perubahan yang terjadi di lingkungan biotik dan abiotik. Serangga herbivora termasuk dalam lingkungan biotik. Berbagai hipotesis telah diajukan untuk menerangkan keanekaragaman ini. Salah satu penjelasan dari sudut pandang evolusi adalah hipotesis ko-evolusi (Ehrlich dan Raven, 1964). Hipotesis ini menjelaskan bahwa interaksi antara tumbuhan dengan serangga memberi kontribusi yang penting terhadap keanekaragaman MS. Tumbuhan menghasilkan senyawa baru untuk mengatasi serangga, selanjutnya serangga beradaptasi untuk menetralisir senyawa baru tersebut. Siklus ini selalu berulang kembali (Ehrlich and Raven, 1964; Rhoades and Cates, 1976). Hipotesis lain menyatakan bahwa MS yang berbeda mempunyai efek yang berlainan terhadap berbagai jenis serangga (Berenbaum and Feeny, 1981).

Dalam disertasi ini dipelajari variasi MS pada tingkatan organ, jaringan dan sel dalam satu tumbuhan. Alkaloid pirolisidin (AP) dari genus *Jacobaea* dipilih sebagai model sistem karena AP memiliki keanekaragaman yang tinggi baik dari segi konsentrasi maupun komposisi. Alkaloid ini memiliki distribusi yang luas dan dapat ditemukan pada tumbuhan yang tidak berkerabat dekat. Alkaloid pirolisidin di alam dapat ditemukan dalam dua bentuk yaitu basa bebas dan *N*-oksida. Pada genus *Jacobaea,* AP dibagi menjadi empat grup yaitu AP tipe senesionin, jakobin, erusifolin dan otosenin.Tujuan dari disertasi ini adalah untuk memahami lebih mendalam keanekaragaman AP pada genus *Jacobaea* mencakup distribusi secara spatial di dalam satu individu dan konsekuensi dari keanekaragaman dan distribusi tersebut terhadap serangga polifagus.

Pada bab 1 dijabarkan keanekaragaman struktur, sintesis dan toksisitas AP. metabolomik NMR dan mikrodiseksi laser (LMD) sebagai salah satu pendekatan terkini dalam fikokimia juga diperkenalkan.

Pengaruh lingkungan biotik dan abiotik terhadap keanekaragaman AP telah banyak diketahui. Jamur endofit merupakan bagian dari lingkungan biotik. Sampai saat ini efek jamur endofit terhadap AP dari *genus Jacobaea* belum diketahui. Pada bab 2 diteliti pengaruh jamur endofit terhadap keanekaragaman AP. Pada percobaan ini, jamur endofit dieliminasi dengan memperlakukan tumbuhan dengan tiga macam fungisida sistemik, yaitu Folicur, Prontoplus dan Switch. Hasil penelitian menunjukan bahwa baik tumbuhan perlakuan maupun kontrol mampu memproduksi AP. Hasil ini menunjukkan bahwa sintesis AP *de novo* dapat dilakukan secara mandiri oleh genus *Jacobaea*. Hasil tidak terduga ditemukan pada tumbuhan yang diperlakuan dengan Folicur. Pada tumbuhan ini ditemukan jamur endofit yang memiliki kesamaan yang tinggi dengan jamur genus *Glomus* yaitu sejenis jamur mikoriza yang tergolong dalam Glomeromycota. Kelompok perlakuan ini mempunyai kandungan AP total yang lebih rendah dibandingkan dengan kontrol. Rendahnya kandungan AP terjadi pada AP tipe senesionin dan jakobin dan tidak pada tipe otosenin dan erusifolin. Hasil analisis metabolomik pada semua sampel menunjukan bahwa tumbuhan yang diperlakukan dengan Folikur tidak menunjukan perbedaan profil metabolom dengan kontrol sehingga rendahnya kandungan AP terutama

tipe jakobin bukan disebabkan oleh perlakuan fungisida akan tetapi karena keberadaan jamur *Glomus*.

Untuk lebih memahami mekanisme pengaruh lingkungan terhadap keanekaragaman AP, maka dilakukan evaluasi kemampuan setiap organ dari tumbuhan *Jacobaea* dalam mensintesis AP. Studi yang mempelajari kapasitas organ tumbuhan dalam memproduksi AP dilakukan dengan membandingkan tunas daun yang berasal dari tumbuhan berbunga dengan kultur akar *in vitro* (Hartmann et al., 1989). Pada bab 3 ini dikembangkan kultur *in vitro* akar, tunas dan tumbuhan utuh dari genus *Jacobaea*. Hasil penelitian menunjukan bahwa kultur tunas ternyata mampu menghasilkan AP walaupun literatur yang ada menyatakan bahwa produksi AP hanya terjadi di akar (Hartmann & Toppel, 1987). Kultur akar mempunyai konsentrasi AP paling rendah, disusul oleh kultur tunas sebanyak 1,5 kali dari kultur akar dan kultur utuh dengan kandungan AP paling tinggi yaitu 3 kali dari kultur akar. Tingginya konsentrasi AP pada kultur utuh menunjukkan bahwa untuk keberlangsungan sintesis AP diperlukan organ akar dan tunas. Absennya tunas pada kultur akar mungkin menyebabkan hilangnya organ untuk mengakumulasi AP. Akumulasi AP pada kultur akar dapat menginisiasi penghambatan umpan-balik sehingga produksi AP *de novo* terhenti. Kompositi utama AP pada kultur akar terdiri dari AP tipe senesionin dan otosenin. Pada kultur tunas dan kultur utuh diketahui AP tipe jakobin dan erusifolin mempunyai proporsi yang lebih tinggi dibanding dua tipe yang lainnya. Diversifikasi AP dari senesionin *N* oksida menjadi berbagai jenis AP terjadi di dalam tunas (Hartmann and Dierich, 1998). Dalam penelitian ini, proporsi AP tipe otosenin terutama onetin dalam kultur akar diketahui dua kali lebih tinggi dibandingkan dalam kultur tunas dan kultur utuh. Hasil ini menunjukan bahwa kultur akar mampu melakukan diversifikasi struktur basa retronesin menjadi otonesin. Kultur tunas dan utuh mempunyai kandungan AP tipe jakobin dan erusifolin yang tinggi. Hal ini menunjukkan bahwa proses epoksidasi pembentukan cincin eter secara spesifik hanya terjadi di organ bagian atas tanah.

Tumbuhan adalah organisme komplek yang memiliki kurang lebih empat puluh jenis sel dan dua belas diantaranya adalah sel penyusun daun. Berbagai jenis sel ini memiliki fungsi dan peran yang berbeda dalam pertumbuhan, perkembangan, reproduksi dan pertahanan. Oleh karena itu, setiap tipe sel diharapkan memiliki komposisi kimia yang unik. Pada bab 4 dipelajari profil metabolomik jaringan penyusun daun yaitu epidermis dan mesofil dengan metabolomic NMR. Hasil analisa deskriptif dengan *Orthogonal Partial Least-Squares-Discriminant Analysis* (OPLS-DA) menunjukan pemisahan yang jelas antara ekstrak epidermis dan mesofil. Lapisan epidermis mengandung lebih tinggi senyawa jakaranon dan fenilpropanoid, terutama asam klorogenat (AKG) dan asam ferulat dibanding lapisan mesofil. Lapisan mesofil mangandung lebih tinggi AP terutama jakobin dan jakonin. Asam klorogenat dan AP telah diketahui aktifitasnya sebagai pengusir serangga. Asam klorogenat berfungsi sebagai *anti feedant* yang mengurangi daya cerna tumbuhan oleh serangga pengunyah seperti ulat, kumbang daun dan bahkan serangga pengisap seperti apid dan trip. Akumulasi MS pada dua lapisan jaringan daun yang berbeda ini merupakan contoh menarik bagaimana tumbuhan memperkuat dirinya secara berlapis untuk memperkuat sistem pertahanannya.

Dengan menggunakan teknologi terkini untuk isolasi sel tunggal, pada bab 5 dipelajari profil metabolomik tiga tipe sel yang berbeda pada daun yaitu sel epidermis, mesofil palisade dan mesofil spon dengan menggunakan LMD dan metabolomik NMR. Dua macam genotip *Jacobaea* yang resisten dan rentan terhadap serangga trip *Frankliniella occidentals* digunakan sebagai sampel. Hasil penelitian ini mengkonfirmasi akumulasi AKG yang tinggi dalam sel

epidermis dan jakobin *N*-oksida dalam sel mesofil palisade. Pada genotipe yang resisten terhadap trip dideteksi akumulasi jakobin *N*-oksida lebih tinggi dibanding pada tumbuhan yang rentan. Sejauh ini, AP tipe jakobin telah dilaporkan sebagai AP yang berperan penting dalam pertahanan genus *Jacobaea* terutama untuk menghadapi herbivora polifagus seperti trip *F. occidentalis*.

Pada bab 3 telah ditunjukkan bahwa diversifikasi senesionin *N*-oksida menjadi AP tipe jakobin dan erusifolin ditemukan terutama dalam daun. Hal ini mungkin merupakan hasil adaptasi tumbuhan untuk mempertahankan diri dari tekanan serangga. Bukti yang mendukung pendapat tersebut sampai saat ini diperoleh dari hasil analisa statistik korelasi. Pada bab 6 dilakukan pengujian secara *in vitro* dua macam AP yaitu jakobin dan erusifolin pada serangga polifagus. Kedua macam AP tersebut tidak tersedia secara komersial sehingga terlebih dahulu dilakukan isolasi. Selanjutnya, kedua AP tersebut dan beberapa AP yang tersedia secara komersial diujikan pada *Spodoptera exigua.* Semua AP yang diujikan dalam dua bentuk yaitu basa bebas dan *N*-oksida. Selain itu, AKG juga diujikan baik secara individual maupun berkombinasi dengan AP. Pengujian mencakup uji *in vitro* dengan kultur sel dan uji injeksi pada larva. Pada kedua uji ini, diketahui jakobin merupakan AP yang paling toksik diikuti dengan erusifolin dan senkirkin. Baik senesionin maupun AKG tidak menunjukan efek toksik pada konsentrasi uji. Untuk semua pengujian, AP dalam bentuk basa bebas mempunyai aktivitas yang lebih tinggi dibandingkan bentuk *N*-oksidanya. Kombinasi antara AKG dengan AP ternyata menurunkan toksisitas AP. Hasil ini menunjukkan bahwa selain karena basa nesinnya, toksisitas AP dipengaruhi oleh gugus fungsional pada struktur asam nesiknya. Jakobin dan erusifolin, keduanya adalah AP yang paling toksik dan keduanya mempunyai gugus epoksi sebagai gugus fungsionalnya. Gugus fungsional ini mungkin memudahkan pembentukan senyawa antara pirol yang reaktif, yang bersifat toksik. Pada akhirnya, disertasi ini telah berhasil mengungkapkan adanya pola distribusi AP spefisik dalam satu individu tumbuhan genus *Jacobaea*. Spesifitas distribusi ini mendukung munculnya penjelasan baru tentang dilema pertahanan tanaman dalam menghadapi serangga polifagus dan monofagus. Alkaloid pirolisidin yang berperan penting untuk pertahanan menghadapi serangga polifagus diakumulasi dalam lapisan mesofil sehingga mengurangi kemampuan serangga monofagus untuk mengenali senyawa tersebut. Strategi ini cocok digunakan untuk pertahanan tumbuhan dalam menghadapi serangga berukuran besar seperti ulat maupun serangga polifagus berukuran lebih kecil yang hanya menyerang lapisan mesofil daun. Akumulasi AKG di lapisan epidermis berperan sebagai pertahanan awal sebelum serangga bertemu dengan senyawa yang lebih toksik yaitu AP. Lebih lanjut, AKG diketahui mengurangi toksisitas AP. Untuk itu pemisahan kedua jenis MS ini penting untuk menjaga aktivitas AP. Hasil ini mungkin akan berpengaruh besar terhadap penelitan interaksi serangga dan tumbuhan. Telah diketahui bahwa setiap tipe sel akan mempunyai profil kimiawi yang khas, sehingga penelitian mengenai metabolit sekunder pada tingkatan jaringan dan sel harus dipertimbangkan apabila mempelajari serangga yang hanya menyerang suatu jaringan atau sel tertentu saja. Dalam disertasi ini, peran AP tipe jakobin pada pertahanan tumbuhan menghadapi serangga polifagus menjadi semakin jelas. Senyawa AP tipe jakobin adalah AP yang dipengaruhi oleh keberadaan jamur mikorizae. Alkaloid tipe ini pula yang menjadi ciri dari kultur tunas dan bukan kultur akar. Lebih lanjut, AP tipe ini terakumulasi lebih tinggi pada daun tumbuhan yang resisten terhadap trip. Dengan menggunakan uji *in vitro* kultur sel dan injeksi larva, diketahui bahwa AP tipe jakobin yang paling toksik untuk serangga polifagus. Penelitian lebih lanjut perlu dilakukan untk memahami toksisitas jakobin terhadap serangga polifagus lainnya.

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Curriculum Vitae

Tri Rini Nuringtyas was born on 27th March, 1973, in Yogyakarta, Indonesia. In 1991, she was admitted to Gadjah Mada University (UGM) for the Bachelor of Science Programme in Environmental Biology at the Faculty of Biology. She completed her degree in 1997 with the undergraduate research project, "Specific proteins of bacteria resistance to copper". Her career at the university started in 1996, when she became a student assistant of the Biochemistry Laboratory at the Faculty of Biology, and later joined the permanent academic staff in 1999. Since then, she has continued to work in the laboratory as the coordinator of the practical work of basic and technical biochemistry courses. In 2001, she was granted a scholarship from the South East Asian Ministry of Education Organization (SEAMEO-SEARCA) to continue her study in Molecular Biology and Biotechnology (MBB) at the University of the Philippines Los Banos (UPLB). She obtained her master's degree in 2004 based on the thesis project, "Isolation and molecular characterization of the ethylene receptor ETR1 from 'Carabao' mango (*Mangifera indica* L) fruit". For her academic achievement, she received an honor distinction from Gama Sigma Delta (The Honor Society of Agriculture). Upon the completion of her study at UPLB, she returned to the Faculty of Biology at UGM as a lecturer, teaching Basic Biochemistry, Technical Biochemistry, Enzymology, and Biotechnology. Her present research interests focus on plant secondary metabolites, including research on the attractant properties of plant terpene and alkaloid crude extracts on wild silk moth (*Attacus atlas* L) and the anti-cancer properties of Red Fruit (*Pandanus conoideus* Lamk). In 2008, she was granted a scholarship from the Directorate General of Higher Education (DGHE) of the Republic of Indonesia to pursue her PhD. She started her PhD study in January, 2009, at the Institute of Biology (IBL) at Leiden University with the Plant Ecology and Phytochemistry Section and the Natural Product Laboratory. Currently she returned to her position as a lecturer at the Faculty of Biology (UGM), where she initiated the formation of a host plant resistance research group in collaboration with the Entomology Laboratory at UGM.

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