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

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PhD thesis, Leiden University, The Netherlands

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The ecological relevance of chemical diversity in plants:

pyrrolizidine alkaloids in *Jacobaea* species

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Chapter 1

General introduction

Secondary metabolites

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

What are secondary metabolites?

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

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

What are the functions of secondary metabolites?

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

Since herbivores and microbes were already present when the evolution of angiosperms started about 140 million years ago, plants have evolved defence chemicals to ward off, inhibit or kill their enemies (Wink 2003). In the history of evolution, SMs, such as alkaloids, glucosinolates, terpenes and tannins, protected plants against virus, bacteria, fungi, competing plants and most importantly against

herbivores, particularly generalist herbivores. SMs do not protect plants against all herbivores. Specialist herbivores have adapted to the SMs in their host plants and can use SMs as cues for finding host plants and oviposition and even sequester SMs for their own benefits (von Nickisch-Rosenegk and Wink 1993).

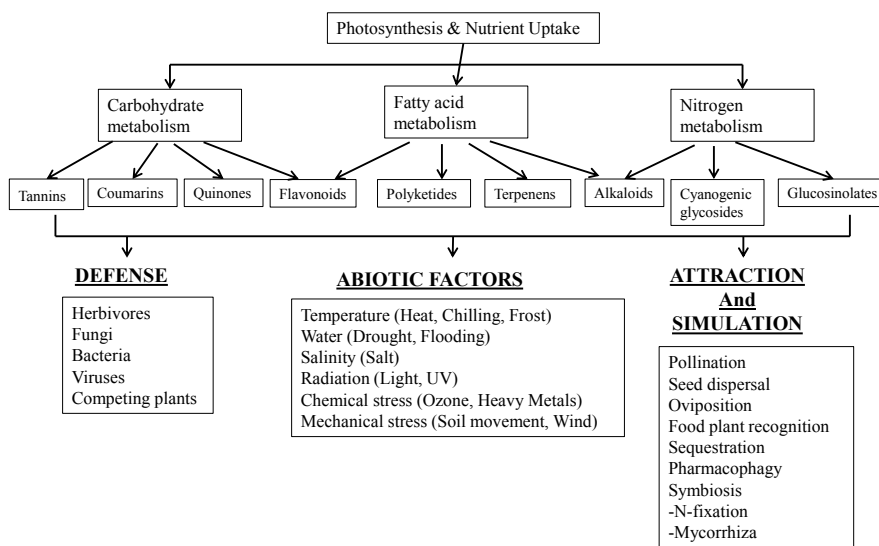


Fig. 1 Secondary metabolites originate from common precursors of primary metabolism. The functions of SMs concern all aspects of plants' chemical interactions with the environment (adapted from Hartmann 1996).

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

Which factors affect secondary metabolite concentration and composition?

A wide range of abiotic environmental factors, e.g. temperature, humidity, light intensity, the supply of water, minerals and ozone, influence the growth of plants and their SM production. Here I list just a few examples. Low temperature is one of the most harmful abiotic stresses affecting temperate plants. Plants have adapted to fluctuations in temperature by adjusting their metabolism during autumn, increasing their content of cryo-protective compounds to maximize their cold tolerance (Janská et al. 2010). Thermal stress induces the accumulation of phenolics in the plant by activating their biosynthesis as well as inhibiting their oxidation in tomato and watermelon (Rivero et al. 2001). Drought stress is usually accompanied

by high temperature and little rainfall. Drought caused an increase in the amounts of flavonoids and phenolic acids in willow leaves (Larson 1988). Camphene emissions were negatively correlated with rainfall, further supporting that drought can result in higher production of SMs (Vallat et al. 2005). Light can stimulate gingerol and zingiberene production in *Zingiber officinale* callus culture (Anasori and Asghari 2009). Although UV-B is a minor component of sunlight, it affects the production of various SMs, such as flavonoids, tannins and lignin (Rozema et al. 1997). All the factors mentioned above can change drastically during the seasons. It is therefore to be expected that the concentration and composition of the SMs in plants change significantly during the seasons too. However the seasonal variation in SM composition have not often been studied yet because the majority of studies are conducted under controlled conditions.

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

How is the diversity of secondary metabolites maintained?

At least four hypotheses were proposed to explain the diversity in SM com-

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

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

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

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

Pyrrolizidine alkaloids

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

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

How are pyrrolizidine alkaloids synthesized and transported?

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

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

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

Effects of PAs on generalist herbivore preference and performance

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

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

Effects of PAs on specialist herbivore preference and performance

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

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

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

What external factors affect the pyrrolizidine alkaloids content?

Effects of abiotic factors on pyrrolizidine alkaloids

Plant organs vary in their response to increased fertilization. Total PA concentration in flowers was constant while the PA concentration significantly decreased with in-

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

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

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

Effects of biotic factors on pyrrolizidine alkaloids

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

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

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

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

Research questions

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

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

Outline of this thesis

In **Chapter 2** I describe the seasonal variation of PAs over 14 months in the field. This is the first detailed report on how the PA concentration and composition of dif-

ferent genotypes are affected by the changing natural conditions.

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

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

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

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

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

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Chapter 2

Seasonal variation in pyrrolizidine alkaloid concentration and composition in clones of *Jacobaea vulgaris*, *Jacobaea aquatica* and their F1 and F2 hybrids

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Abstract

Plants produce a variety of secondary metabolites (SMs). The concentration and diversity of SMs are affected by biotic factors, such as herbivores and pathogens, and abiotic factors, such as temperature and light. Because these factors change during the seasons SMs are also expected to show seasonal variation. We used *Jacobaea vulgaris*, *Jacobaea aquatica*, 2 F1 and 4 F2 hybrids, and their pyrrolizidine alkaloids (PAs) as a system to study such seasonal variation. We used a series of clones of genotypes differing in their alkaloid content to explore how PA concentration and composition varied in their vegetative stage during 14 months in a semi-natural environment. Our results showed that after being planted in April shoot and root dry mass showed a steady increase until October-November, but in the winter period approximately 70% of the biomass was lost, before the plants recovered during the next spring. The total PA concentration in roots and shoots showed a gradual increase until the spring of the next year, when as a result of rapid plant growth the PA concentration dropped, most noticeably in the shoots. The variation of the total PA amount was strongly correlated with that of dry mass and together with the loss of dry mass in winter a large part of the alkaloids was lost. Senecionine- and otosenine-like PAs were mainly stored in the roots while the erucifoline- and jacobine-like PAs were mainly stored in shoots. For the 6 genotypes with higher concentrations of jacobine-like PAs, PA composition showed a decrease of this type of PAs during winter, followed by a strong increase in the next spring. In contrast, the PA composition of the roots over the whole period showed a gradual increase of jacobine-like PAs at the expense of senecionine-like PAs. The ratio of total free base and N-oxide PAs was relatively constant over the seasons until the second spring, when it sharply increased in the shoots of some of the jacobine-rich genotypes, most notably in the parent *J. vulgaris*. While the genotypes differed in PA composition their differences in the variation of the seasonal changes were significant but relatively small. As a result the variation among genotypes was maintained across seasons.

Key words

Secondary metabolites, LC-MS/MS, Genotypes, Senecionine-like PAs, Jacobine-like PAs, PA reallocation, Temperature, Day length

Introduction

Plants produce a staggering diversity of secondary metabolites (SMs) (Hartmann et al. 2005). For a single group of SMs, the concentration and composition may change between developmental stages, tissues and organs, seasons and years (Gols et al. 2007; Liu et al. 1998; Qasem and Hill 1995). SMs are important to plants for coping with the biotic and abiotic stressors of their environments (Iriti and Faoro 2009; Walters 2011). SMs are generally thought to play an important role in the protection of plants against herbivores and pathogens (Stout et al. 2006; van Dam et al. 1995; Wink 1988).

Higher concentrations of SMs can result in more resistant plants. At the same time the production of SMs is thought to be costly. Such costs can reduce plant growth and reproduction (Cipollini et al. 2003; Herms and Mattson 1992; Karban and Baldwin 2007). Hence plants face a dilemma: to grow or to defend (Herms and Mattson 1992). On the one hand, they must grow fast enough to compete with neighbouring plants, on the other hand, they must produce enough defence compounds for survival in the presence of herbivores and pathogens (Siemens et al. 2002).

Secondary metabolite production can be constitutive but is often influenced by many environmental factors, such as temperature, humidity, light intensity and drought (reviewed in Akula and Ravishankar 2011). Temperature strongly influences both plant ontology and metabolic activity. For instance, low and high temperatures caused significant reductions in fresh weight, dry weight, total phenolics, flavonoids and total eleutheroside accumulation in *Eleutherococcus senticosus* (Shohael et al. 2006). Drought conditions were reported to decrease the content of saponins in *Chenopodium quinoa* (Solíz-Guerrero et al. 2002). Low light intensity increased methylxanthine content in leaves of *Ilex paraguariensis* (Coelho et al. 2007), while it decreased resin content in leaves of *Grindelia chiloensis* (Zavala and Ravetta 2001). UV-B light induced the accumulation of glucosinolates in broccoli (Mewis et al. 2012) and flavonoids in *Ribes nigrum* (Schreiner et al. 2012).

In addition to abiotic factors SM concentration and composition are known to be influenced by biotic stresses. For instance, herbivore attack of *Spodoptera littoralis* led to the induction of metabolites in the leaves and roots of maize (Marti et al. 2013). Incubation with bacterial genera *Azospirillum* and *Pseudomonas*, mycorrhizal genus *Glomus* under field conditions led to qualitative and quantitative modifications of SMs in maize roots, particularly of benzoxazinoids and diethylphthalate (Walker et al. 2012). Due to the seasonality of pests, pathogens and climate, we expect the type and level of plant defences to depend on the seasons as well (Brooks and Feeny 2004; Kumar et al. 2012; Shiojiri and Karban 2008).

Seasonal fluctuations of SM concentrations were mostly reported for woody plants. The leaf phenolics of *Quercus robur* L. showed a sharp decline early on in the growing season, followed by relatively stable low amounts until autumn (Salminen

et al. 2004; Somnavilla et al. 2012). In contrast, the flavonoid concentration in the mountain birch *Betula pubescens* subsp. *caerepanovii* increased until early summer, dropped during summer and increased again during autumn (Riipi et al. 2002). Some studies reported seasonal variation in herbaceous plants. For instance, Lubbe et al. (2013) found that the concentration of alkaloids decreased in the leaves of *Narcissus pseudonarcissus* during the growing season while the concentration in roots remained constant. In *Senecio madagascariensis* in Brazil the highest PA concentration occurred in spring (Karam et al. 2011) while in *Senecio riddellii* and *Senecio longilobus* leaves this was in summer (Johnson et al. 1985). Although these studies documented seasonal variation of SMs, there are still very few studies that encompass a time series over all seasons for distinct genotypes.

In this study we used pyrrolizidine alkaloids (PAs) as a study system. PAs are present in several plant families, such as the Apocynaceae, Asteraceae, Boraginaceae, Convolvulaceae, Leguminosae and Orchidaceae (Brehm et al. 2007; Flores et al. 2009; Frölich et al. 2006; Hartmann and Witte 1995; Jenett-Siems et al. 2005; Trigo et al. 2003). In *Jacobaea* (Asteraceae) species, they are synthesized in the roots, and translocated as N-oxides from the roots to the shoots, where further PAs diversification takes place (Hartmann et al. 1989). The shoots are especially essential for PA diversification of jacobine-like PAs (Nuringtyas 2013). PA concentration and composition showed a high heritability (Vrieling et al. 1993). In a climate room PA concentration and composition were strongly genotype-dependent (Cheng et al. 2011a). PAs are well known for their interaction with herbivores. Polyphagous herbivores are generally deterred by the different types of PAs while the specialist herbivores are attracted by PAs (Cheng et al. 2011b; Cheng et al. 2013; Macel et al. 2002; Wei et al. 2015).

To investigate the seasonal variation of SM concentration, we set up a series of clones of the same genotype to specifically explore the effects from seasonal variation. We tracked PA variation over the course of vegetative growth for 14 months. Our aim was to get an understanding of how PA concentration and composition vary when plants are exposed to natural environments and varying climatological conditions and whether genotypic differences are maintained over time. This is, as far as we know, the first study to explore the SM variation with the same genotypes over the whole vegetative growth period. We addressed the following questions: 1) How does the total PA amount vary over the seasons? 2) How does the PA concentration and composition vary over the seasons? 3) Are differences in concentration and composition of PAs between genotypes maintained?

Materials and Methods

Study system

Jacobaea vulgaris subs. *dunensis* was derived from seeds collected at the Meijen-

del Nature Reserve (52°7'54"N, 4°19'46"E, The Netherlands), and *J. aquatica* subs. *aquatica* was derived from seeds collected at the Zwanenwater Reserve (52°48'38"N, 4°41'7"E, The Netherlands). The parents, the F1 and F2 hybrids of these two species are maintained in our lab in tissue culture and can thus easily be multiplied to produce replicates of the same genotype. The cross was documented in detail by Cheng et al. (2011a). In brief, seeds of the parental species were grown until blooming. Both species are self-incompatible, and crosses were performed by rubbing flower heads together. Two rayed F1 offspring were selected and reciprocally crossed with each other to produce two sets of F2 offspring.

Jacobaea vulgaris is a monocarpic biannual / perennial (Chater and Walters 1976; van der Meijden and van der Waals-Kooi 1979). In the first year this species forms a rosette, whereas flowering can occur in the second year or later depending on the size of the plant. After flowering, plants die (Harper and Wood 1957; Wesselingh and Klinkhamer 1996). *Jacobaea aquatica* is a biannual that is phylogenetically closely related to *J. vulgaris* but ecologically and chemically the two species show distinct differences (Cheng et al. 2011a; Pelser et al. 2003). *Jacobaea vulgaris* has a higher concentration of jacobine-like PAs and grows in dry, sandy soils while *J. aquatica* has a higher concentration of senecionine-like PAs and grows in marsh environments (Cheng et al. 2011a). Over 60 herbivores were reported to feed on *J. vulgaris*. This species is regularly completely defoliated by its specialist herbivore *Tyria jacobaeae* (van der Meijden et al. 1990). No severe defoliation events have been reported for *J. aquatica*.

Based on the data of previous field work at Lisse (52°25'12"N, 4°54'10"E, The Netherlands), eight genotypes were chosen for this experiment, including the parental plants *J. vulgaris* and *J. aquatica* (JV and JA), two F1 hybrids (F1A, F1B) and four F2 hybrids (F2A, F2B, F2C, F2D). The four F2 genotypes represented different levels of jacobine-like PAs in the shoots: F2A and F2B (medium), F2C (low) and F2D (high).

Plant growth

Each genotype was cloned into 120 replicates in tissue culture. After two weeks, all the 960 cloned individuals were potted in 0.5 L pots. Potting soil was collected from the experimental field at Lisse (52°25'8"N, 4°54'34"E, The Netherlands). Plants were kept in a greenhouse next to the experimental field for 5 weeks and watered three times per week until planting in the field on April 12, 2012. The experimental field is an open area with rich sandy soil where *J. vulgaris* occurs naturally at its borders. The area was cleared before planting. In the process of growing, plants were also kept free of weeds and their specialist herbivore, the cinnabar moth. Each plant was numbered and they were randomly planted with a distance of 30 cm from each other. Plants were watered only during planting.

Sample collection

We started to harvest plants in May 10, 2012. Two randomly chose plants per genotype were harvested every two weeks from May to September and every three weeks from October to the next June. Because of wintery conditions frozen soil, no data could be obtained from the harvest on March 14, 2013 and due to a broken refrigerator we have no data for the harvest on April 5, 2013. Each harvest included 16 plants (2 individuals $\times$ 8 genotypes). Plants were gently removed from the soil and put in a plastic bag. In summer, when the temperature was high, the plastic bags with the plants were kept in a box cooled with ice. Plants were washed, with scissors separated into roots and shoots and dried with paper tissues. After measuring the fresh weight of the plants, two leaves and two roots were wrapped in aluminium foil and snap frozen in liquid nitrogen. Samples were subsequently freeze dried for 4 days under vacuum with a collector temperature of -80°C (Cryothèque®, Sniders Scientific Company, Tilburg, The Netherlands). The freeze dried samples were ground into fine powder for PA extraction. The dry mass of shoots and roots were additionally measured. Plants were harvested from May 2012 to June 2013. In total on 20 occasions 608 samples were prepared. Due to the fact that most plants of genotype F2C flowered in summer of 2012, there are only 12 harvests for this genotype.

Pyrrolizidine alkaloid extraction and analysis

PA analysis was carried out using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The protocol was described in detail by Cheng et al. (2011a). In brief, 10 mg finely ground powder was extracted by 1.0 ml 2% formic acid solution containing heliotrine ($1\text{ }\mu\text{g ml}^{-1}$) as internal standard. After shaking and centrifuging, 25 μl of the extracted supernatant was diluted with 975 μl of 0.1% ammonia hydroxide solution and 10 μl was injected in a Waters Acquity ultra performance liquid chromatographic system (UPLC) coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA). The instrumental settings used were the same as described by Cheng et al. (2011a). Data were processed in Masslynx 4.1 (Waters Corporation, Milford, MA, USA).

PAs are present in two forms: tertiary amine (free base) and N-oxide. Based on their chemical characteristics and biosynthetic pathway, PAs are classified into four groups: senecionine-like PAs, jacobine-like PAs, erucifoline-like PAs and otosenine-like PAs (Cheng et al. 2011a).

Temperature and day length

A dataset of the daily temperature and day length for the most nearby weather station (Valkenburg Naval Air Base) was obtained from the website of the Royal Netherlands Meteorological Institute (KNMI). Temperature and day length used in our analysis was calculated by averaging the daily temperature and day length data of 14 days previous to the harvest day. The weather station is located at circa 14 km from

the experimental field.

Statistical analysis

The parameters used are calculated by the following equations: shoot/root ratio (SR ratio) = (the mean dry mass of shoots) / (the mean dry mass of roots), PA amount = (total PA concentration) * (dry mass), the relative concentration of each group of PAs (%) = (PA concentration of each group) * 100 / (the total PA concentration in the plant), free base/N-oxide ratio (FN ratio) (%) = (the concentration of free base PAs) * 100 / (the concentration of N-oxide PAs).

Two-way ANOVAs were performed to evaluate the effects of genotype (random factor) and harvest dates (fixed factor) on dry mass, PA concentration, PA amount, FN ratio and SR ratio. General linear models were performed to test the relationship between SR ratio and PA concentration and temperature.

All analyses were performed in SPSS 21.0 for windows and Microsoft Excel 2010.

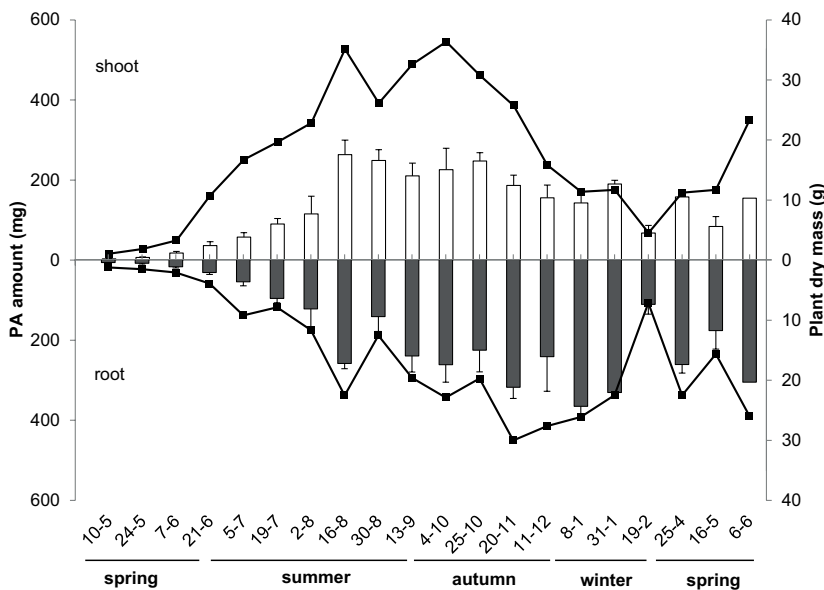


Fig. 1 The mean pyrrolizidine alkaloid (PA) amount ($\pm$ SE, mg) and plant dry mass (g) of 8 genotypes at different harvest dates. The line charts represent the plant dry mass and bar graphs represent the PA amount.

Results

Variation in plant dry mass

Dry mass was significantly different among genotypes and harvest dates (Table 1), while the pattern of seasonal variation was similar for all genotypes except for genotype F2C (Fig. S1). Therefore dry mass is presented graphically as the overall mean

of 8 genotypes in Fig. 1. Shoot dry mass increased over the season and peaked in early October, then decreased until February and increased again during the next spring (Fig. 1). Root dry mass followed a similar pattern but peaked at the end of November and then declined until February after which it increased again until the last harvest in June. In the winter season, between November 2012 and February 2013, almost 70% of the dry mass of shoots and roots was lost.

Variation in shoot/root ratio

The SR ratio was positively correlated with temperature and day length (Fig. S2). The mean SR ratio of the 8 genotypes was the highest in mid-summer and then decreased gradually until mid-winter, after which in spring it started to increase again (Fig. 2).

Although the variation among harvests was the strongest in the F2 hybrids (Fig. S3), all genotypes showed a relatively similar pattern of variation in SR ratio across harvest dates even though differences in this pattern between genotypes were significant (Table 1).

Table 1 Two-way ANOVAs with plant dry mass, total pyrrolizidine alkaloid (PA) amount, total PA concentration, free base/N-oxide (FN) ratio, shoot/root (SR) ratio as dependent variables and with harvest dates and genotypes as independent variable

Dependent variables	Source of variation	DF	Root		Shoot	
			F	P	F	P
Dry mass	Date	19	3.391	<0.001	3.369	<0.001
	Genotype	7	12.648	<0.001	7.488	<0.001
	Date*Genotype	125	1.871	<0.001	2.738	<0.001
Total PA concentration	Date	19	7.138	<0.001	18.581	<0.001
	Genotype	7	37.197	<0.001	21.868	<0.001
	Date*Genotype	125	1.633	0.002	2.119	<0.001
Total PA amount	Date	19	3.112	<0.001	2.511	0.001
	Genotype	7	13.224	<0.001	8.598	<0.001
	Date*Genotype	125	1.677	0.001	2.932	<0.001
Free base/N-oxide ratio	Date	19	2.864	<0.001	1.550	0.08
	Genotype	7	9.605	<0.001	4.934	<0.001
	Date*Genotype	125	2.307	<0.001	2.703	<0.001
SR ratio*	Date	19	4.191	<0.001		
	Genotype	7	6.211	<0.001		
	Date*Genotype	125	1.119	0.253		

*SR ratio is for the whole plant.

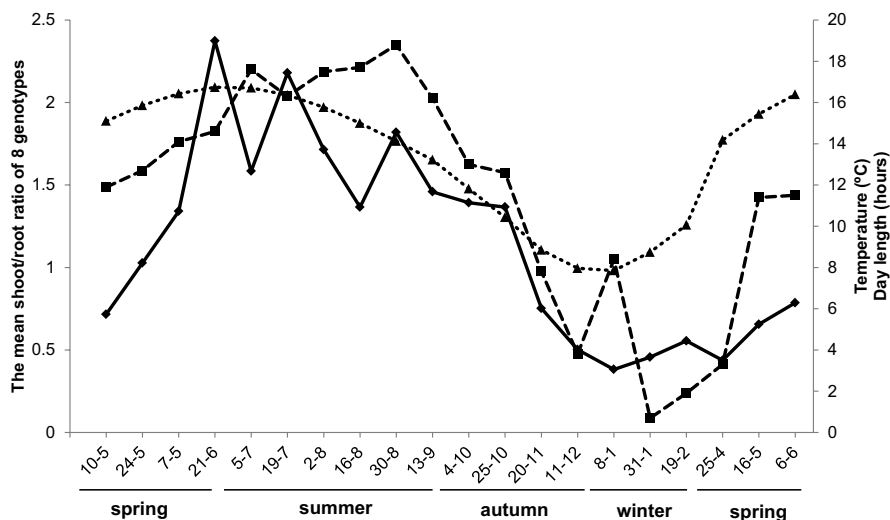


Fig. 2 The mean shoot-root dry mass ratio (solid line) of 8 genotypes, temperature (dashed line) and day length (dot line) at different harvest dates. Temperature and day length are the average of 14 days previous to the harvest day.

Variation in total PA content

Averaged over all 8 genotypes the total amount of PAs stored in the plants increased until late summer (August) (Fig. 1). In the shoots the PA content remained constant until November, and then started to decrease during the winter period. By February 70% of the PAs stored in the shoots had been lost. In the roots the amount of PAs remained rather constant until the end of January, but a sharp decline occurred in February. In total, plants lost approximately 60% of stored PAs during the winter period. The seasonal variation of PA amounts can largely be explained by the seasonal variation in plant dry mass. (Table 2, Fig. 1)

Across harvest dates the variation between genotypes was largely maintained, although the pattern of variation over the seasons differed among genotypes (Table 1, Fig. S4). F2 hybrids contained a smaller total PA amount than the parents and F1 hybrids (Fig. S4). This could largely be explained by smaller amount of dry mass produced by the F2 hybrids compared to the parents and F1 hybrids. Each genotype contained a larger amount of PAs in roots than shoots (Fig. S4).

Roots were relatively rich in senecionine- and otosenine-like PAs while the shoots contained more erucifoline- and jacobine-like PAs (Fig. S5). In the shoots the amount of senecionine-like PAs showed a steady increase until the end of the winter and then decreased dramatically in the next spring (Fig. S5A). The amount of jacobine-like PAs increased until August, remained at similar levels until the end of October and then decreased in winter. The amount of jacobine-like PAs increased again in the next spring (Fig. S5B). The amount of erucifoline-like PAs showed an

increase until August after which it remained at a similar level until the end of winter. In the second spring the amount of erucifoline-like PAs was low (Fig. S5C). The amount of otosenine-like PAs present in the shoots was relatively small, but showed a gradual increase continuing over the whole period until the next spring (Fig. S5D).

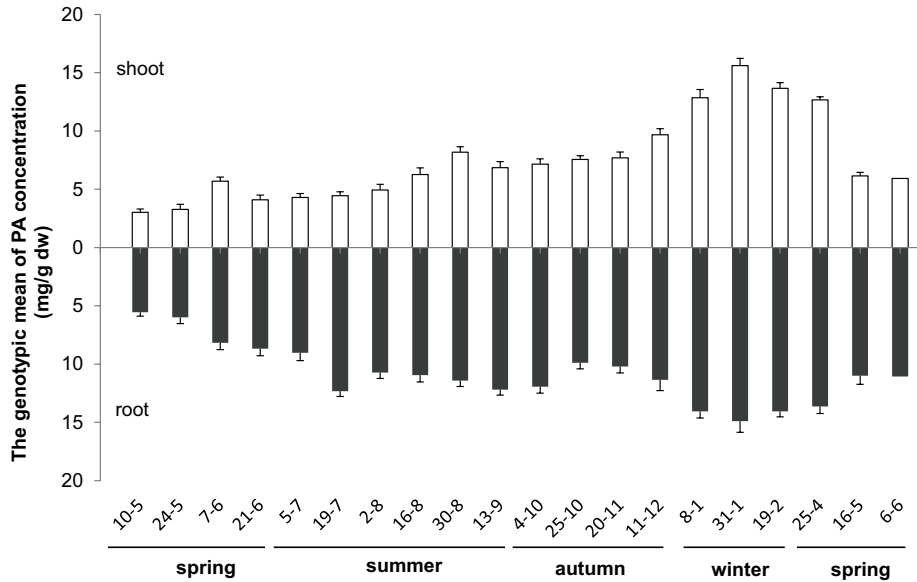


Fig. 3 The mean pyrrolizidine alkaloid (PA) concentration ($\pm$ SE, mg/g dw) of 8 genotypes at different harvest dates.

Variation in PA concentration

The PA concentration averaged over the 8 genotypes increased gradually over the seasons in both shoots and roots (Fig. 3). However, during the spring of the next year PA concentrations dropped significantly in the shoots (Fig. 3). The PA concentration in roots was on average twice in shoots. In the second spring the PA concentration in shoots and roots were 1.5-2 times higher compared to the first spring. The total PA concentration was different among genotypes (Table 1). Differences in the pattern of seasonal variation were relatively small although significant among genotypes (Table 1). Except JA, all genotypes had higher PA concentrations in roots than in shoots (Fig. S6).

The mean PA concentration in roots and shoots averaged over all 8 genotypes was negatively correlated with temperature (Fig. S7A and B) and day length (Fig. S7C and D).

The concentration of senecionine-like PAs in shoots started to increase from October onwards (Fig. S8A) and reached its peak in winter, but decreased to its initial level in the next spring. Roots had about 1.5-7 times higher senecionine-like PA concentrations than shoots (Fig. S8A). The concentration of jacobine-like PAs in

roots increased gradually over the seasons while in shoots it increased until August and then remained at similar levels until the end of the study (Fig. S8B). Concentrations of erucifoline-like PAs were much higher in the shoots than in the roots. The concentration of erucifoline-like PAs peaked in mid-winter and then started to drop in the next spring (Fig. S8C). The concentrations of otosenine-like PAs in general were slightly higher in roots than in shoots. Concentrations in shoots peaked in April and then started to decrease in spring, while remaining constant in the roots (Fig. S8D).

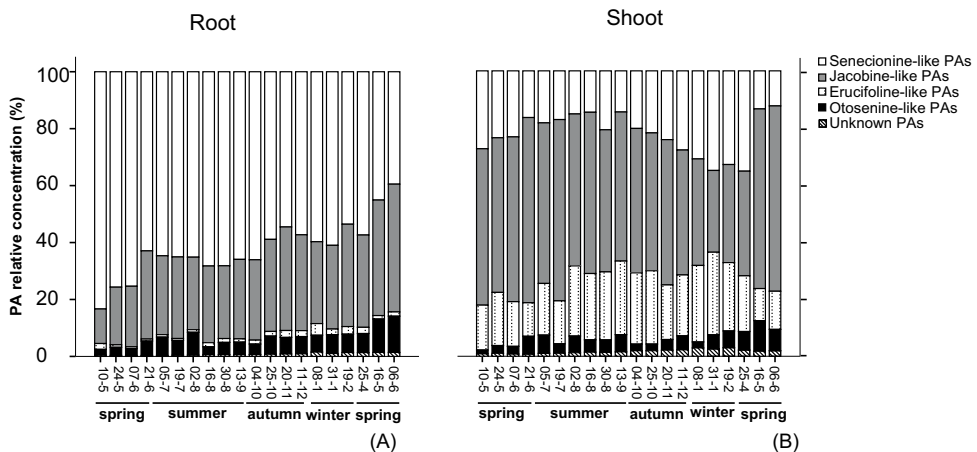


Fig. 4 The relative concentration of different types of pyrrolizidine alkaloids (PAs, %) for 8 genotypes at different harvest dates.

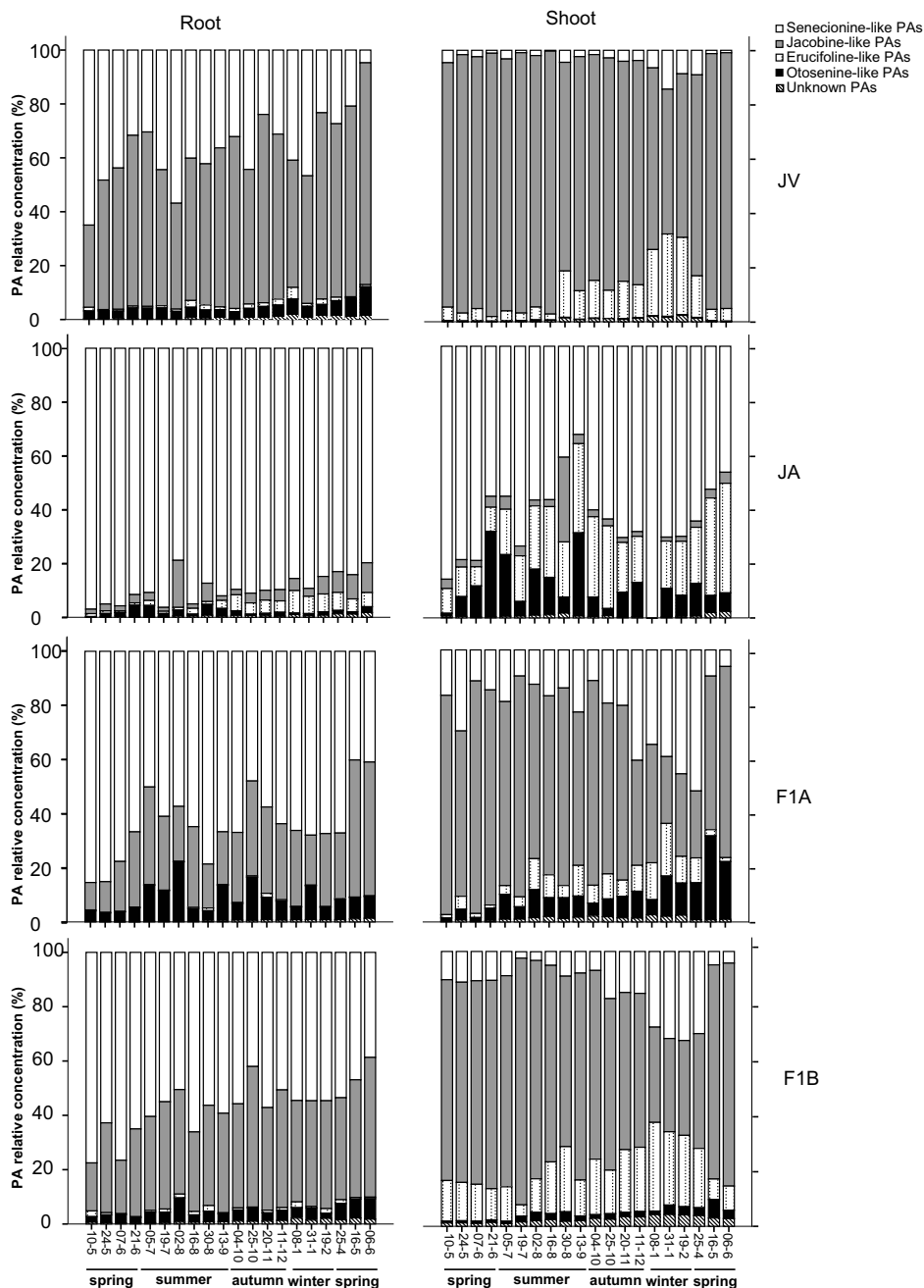
Variation in PA composition

Variation of the relative concentration of four structurally related PA types

For the 8 genotypes combined, in roots senecionine-like PAs were the dominate PA type. However, in the course of time, the relative concentration of senecionine-like PAs gradually decreased at the expense of jacobine- and otosenine-like PAs increased (Fig. 4A). In shoots jacobine-like PAs were the most abundant type of PAs except in winter when the relative concentration of senecionine-like PAs increased (Fig. 4B). The relative concentration of erucifoline-like PAs was close to zero in roots while it was around 20% in shoots except for the second spring when its share decreased to 10% (Fig. 4).

The variation of PA composition differed among genotypes. In the roots, the relative concentration of senecionine-like PAs decreased while that of jacobine-like PAs increased gradually over the seasons except in JA and F2C (Fig. 5), where senecionine-like PAs comprised over 80% of the total content during the whole study. The roots of F2A and F2B, were relatively rich in otosenine-like PAs and their shares increased gradually over time (Fig. 5). In the shoots, jacobine-like PAs were major

type of PAs except in JA and F2C, in which they were practically absent. In the other genotypes the share of jacobine-like PAs showed a similar pattern of variation: the relative contribution was smallest in winter, at the expense of senecionine- or erucifoline-like PAs (Fig. 5).



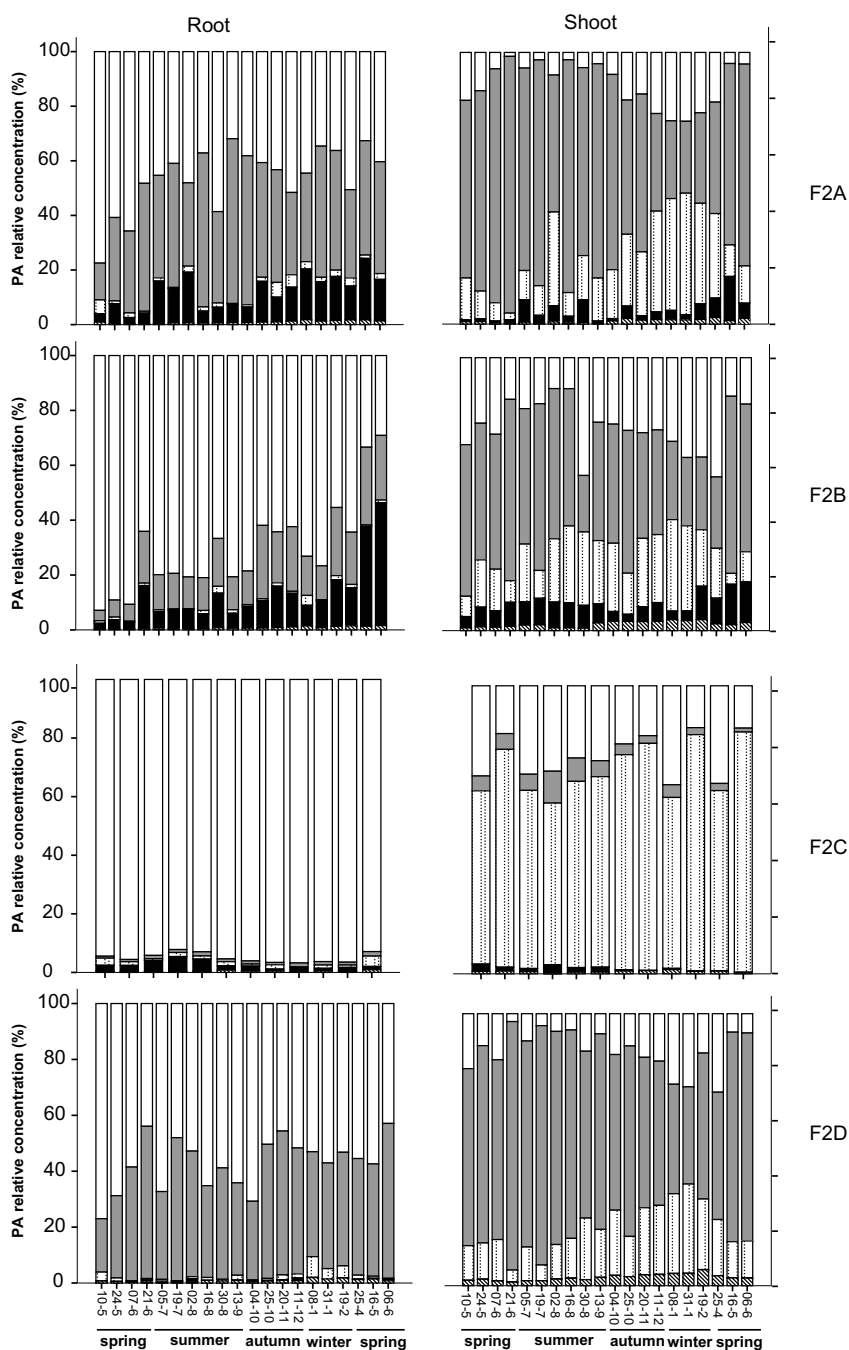


Fig. 5 The relative concentration of pyrrolizidine alkaloids (PAs, %) in roots and shoots in *Jacobaea vulgaris*, *Jacobaea aquatica* and their 2 F1 and 4 F2 hybrids at different harvest dates.

Variation in free base/N-oxide ratio

The free base/N-oxide (FN) ratio remained relatively constant over time until April 2013 (Fig. 6A). It then increased in several genotypes, but the increase was most notable in the shoots of the parent JV (Fig. S9C). The change in overall FN ratio strongly related to the change in FN ratio of the jacobine-like PAs (Fig. 6B). The FN ratio was much higher in shoots than in roots (Fig. S9).

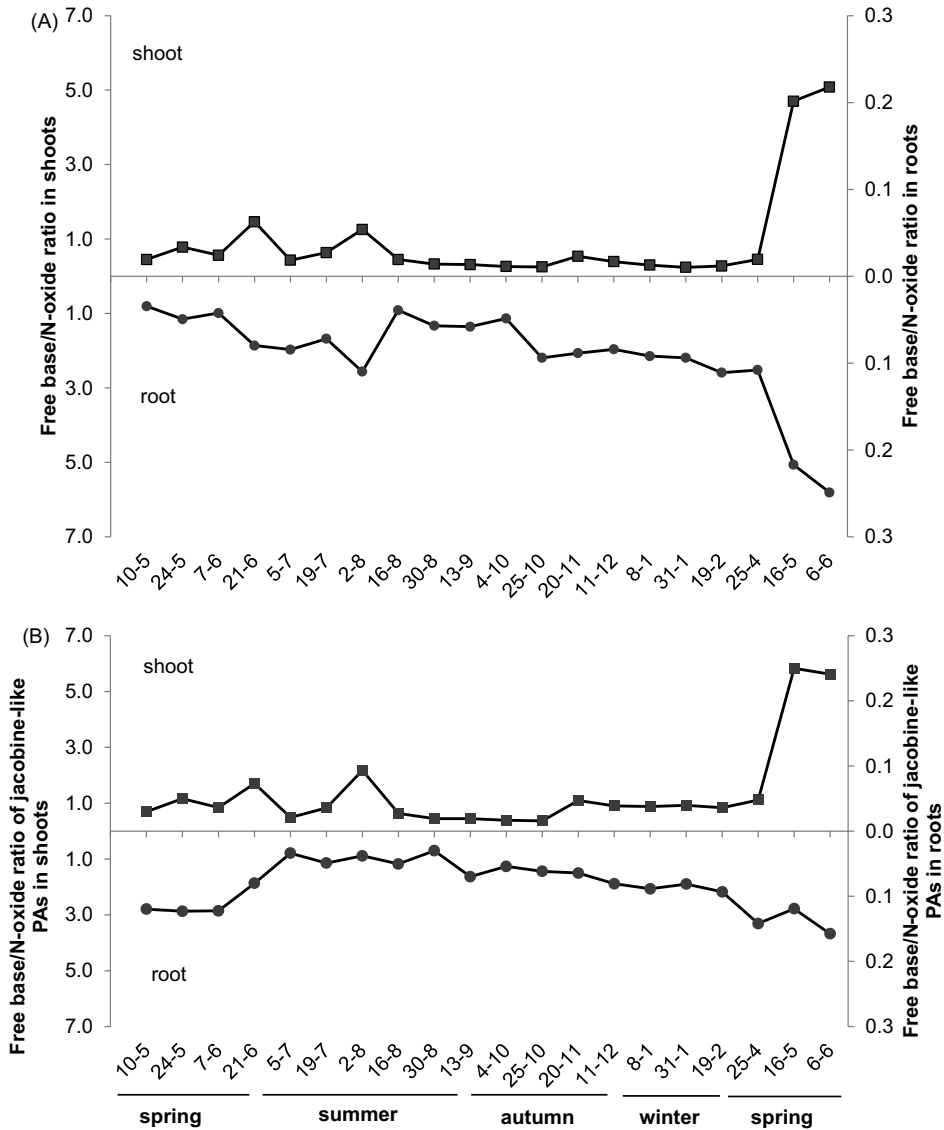


Fig. 6 The total free base/N-oxide ratio averaged over all 8 genotypes (A) and within jacobine-like PAs (B) in roots and shoots at different harvest dates.

Discussion

Seasonal impact on the total PA amount

We found for both roots and shoots a sharp decrease of dry mass and total PA amount in winter. For instance, for shoots over the period from October to February the average dry mass decreased from 35 to 5g and the calculated PA amount decreased from 210 to 70 mg. Interestingly, there was a stronger decrease of the dry mass than of the total PA amount, resulting in an increase of the total PA concentration in winter. Although the total PA concentration peaked in January, the total amount of PAs averaged over the 8 genotypes peaked in August and remained rather stable until November in both shoots and roots. After November, the PA amount in shoots decreased while it remained constant in the roots, indicating that the PAs that were lost in the shoots were not reallocated to the roots. The surprising conclusion is that plants do not seem to invest in the reallocation of PAs from decaying tissues during winter.

PAs are exclusively synthesized in the roots, and there is no substantial degradation of PAs in living tissue (Hartmann and Dierich 1998). Senecionine-like PA N-oxides are the primary products of biosynthetic pathway (Hartmann and Toppel 1987). In this study senecionine-like PAs are mainly stored in the roots. In contrast, the majority of erucifoline-like PAs were present in the shoots and only in very low amounts in the roots. This indicates that the senecionine-like PAs are first transferred from roots to shoots before conversion to erucifoline-like PAs occurs in shoots.

Seasonal impact on the PA concentration

The PA concentration in the roots and shoots gradually increased as the plants grew older. The PA concentration reached its maximum during winter, when the loss of dry mass was stronger than the loss of PAs. The increase in PA concentration was however relatively small given the large decrease in plant dry mass. In spring the increase in dry mass was more significant than the increase in total PA amount resulting in a drop of the PA concentration.

Seasonal impact on PA composition

In the roots the patterns of variation in PA composition were relatively straightforward. When plant grew older the relative concentration of senecionine like PAs declined while, depending on the genotype, that of jacobine-like PAs increased. In the shoots the patterns were more complex. In general the relative concentration of senecionine-like PAs decreased during the growing season but increased again when plants stopped growing in autumn and during winter. In spring when plants started to grow again the relative concentrations sharply decreased. In contrast the relative concentrations of jacobine-like PAs reached their minimum in winter and increased sharply when plants started growing again. The relative concentration of erucifoline-like PAs was highest in the winter season. In *J. vulgaris* the increase of the relative concentration of jacobine-like PAs after winter was at the cost of

both senecionine- and erucifoline-like PAs. In *J. aquatica* which hardly produces jacobine-like PAs the relative concentration of erucifoline-like PAs increased after winter while that of senecionine-like PAs decreased.

One of the most interesting changes was the increase in the F/N ratio in both shoots and roots during the second spring. The increase was most pronounced in JV shoots, but was also seen in the shoots of F1A, F2A and F2B. In roots the largest increase was seen for F2B. This increase was not only caused by the fact that the relative concentration of jacobine-like PAs increased but was also found within the group of jacobine-like PAs. It is hard to judge at this point whether or not these changes are adaptive to the environment. With respect to generalist insect herbivores it has been shown that in general free base PAs are more toxic than their corresponding N-oxides (van Dam et al. 1995). It has also been shown that jacobine- and erucifoline-like PAs are more toxic than senecionine (Nuringtyas et al. 2014). On the other hand, Macel and Klinkhamer (2010) found that at Meijndel the jacobine chemotype of *J. vulgaris* was more severely attacked in May and June by the specialist herbivores *Tyria jacobaeae* and *Longitarsus jacobaeae* and by *Puccinia dioicae* (rust fungus) than erucifoline and mixed chemotype. Cheng et al. (2013) found that *T. jacobaeae* laid more eggs on the plants with higher concentrations of the free base of jacobine-like PAs. It is therefore difficult to understand why in spring *J. vulgaris* plants increase the relative concentration of this type of PAs, because this is the period when *T. jacobaeae* lays its eggs.

Several lines of evidence suggest that after attack by herbivores *Jacobaea* plants increase their erucifoline concentration. Kostenko et al. (2013) found that root herbivory by wireworms (*Agriotes lineatus*) lead to an increase in the concentration of erucifoline-like PAs. Hol et al. (2004) showed that the relative concentration of erucifoline increased while that of jacobine decreased in shoots after *J. vulgaris* shoots were damaged by *Mamestra brassicae*. In addition treatment with methyl jasmonate led to an increase of the erucifoline concentration at the cost of a decrease of the senecionine concentration (see chapter 5 of this thesis). On the one hand such an increase in erucifoline is surprising considering the fact that a number of studies suggested that jacobine-like PAs were more effective in protecting the plant against generalist (Cheng et al. 2011b; Leiss et al. 2009) but on the other hand it is in line with the observation that erucifoline chemotype plants showed less damage when there was a strong pressure of specialist herbivores (Macel and Klinkhamer 2010). Perhaps the increase of erucifoline-like PAs is a compromise between the effects on specialist and generalist herbivores.

Seasonal impact on differences in PA concentration and composition between genotypes

Although PA composition considerably changed over the seasons, generally the changes found in the genotypes were in a similar manner. Compared to the effects of

genotype and harvest date, the interaction between the two variables explained only a small amount of variation. For instance, at each harvest point there was a clear distinction between the 6 genotypes (JV, F1A, F1B, F2A, F2B and F2D) that produced higher levels of jacobine-like PAs and the two genotypes (JA and F2C) that produced much lower levels of these PAs. Therefore, although PA composition changed over seasons within each genotype in a significantly different manner, these differences were not strong enough to overrule the initial genetically derived variation among the genotypes.

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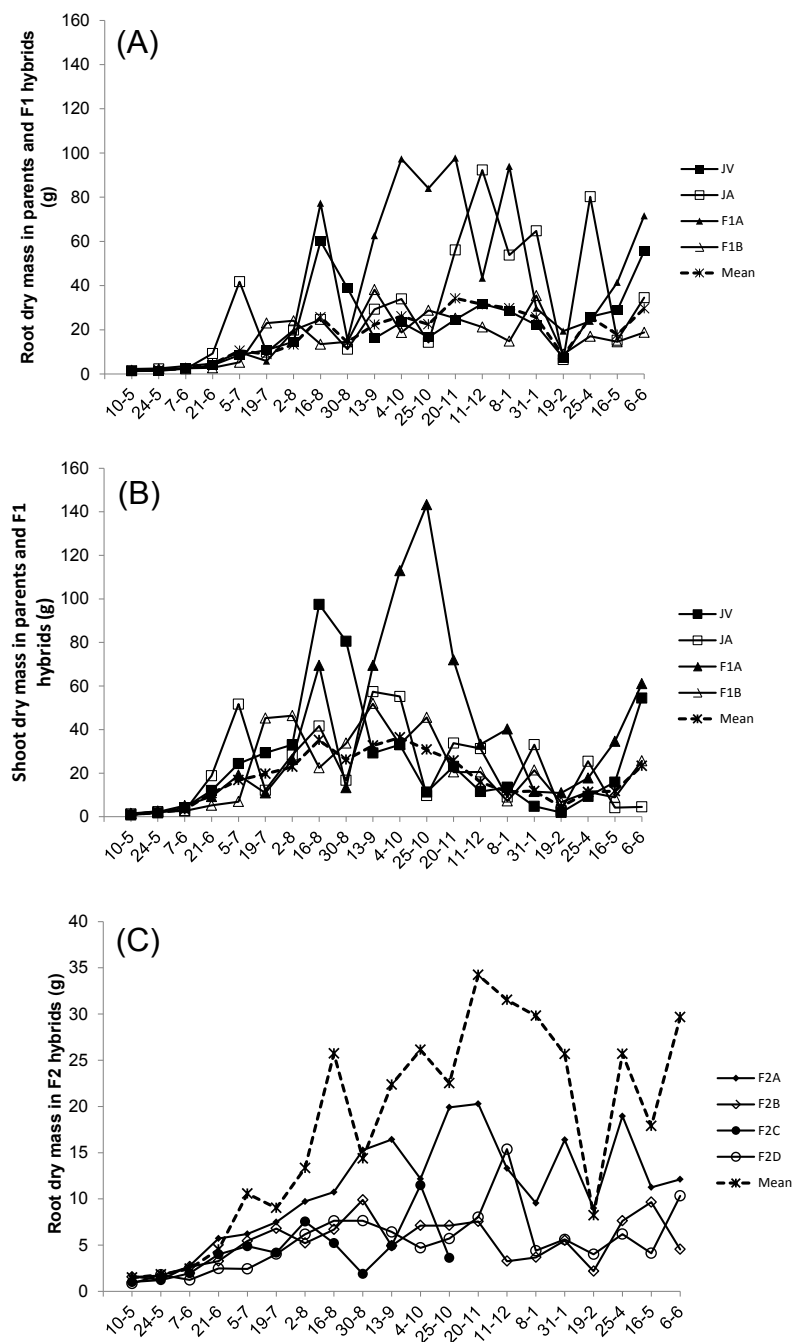
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Supplementary Materials:



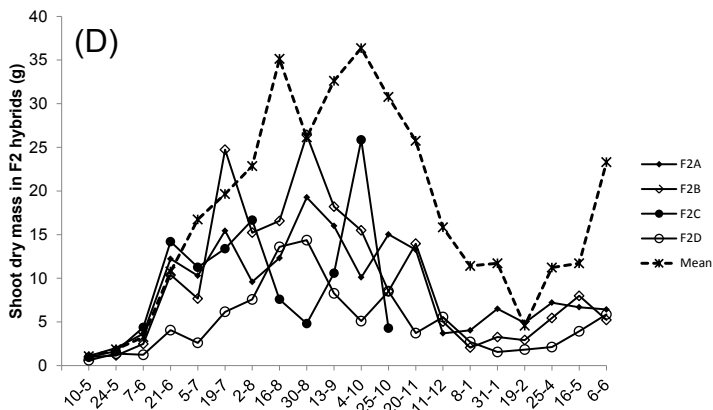


Fig. S1 The dry mass variation in *Jacobaea vulgaris*, *Jacobaea aquatica* and their 2 F1 (F1A and F1B) and 4 F2 (F2A, F2B, F2C and F2D) hybrids at different harvest dates. The dash line represents the mean dry mass over all 8 genotypes in roots or shoots.

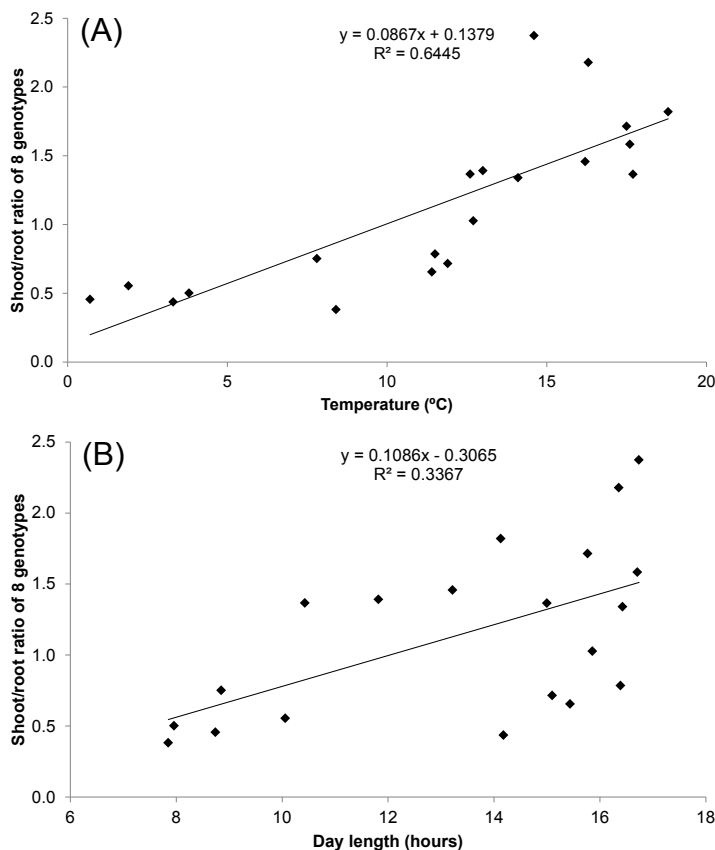


Fig. S2 The relationship between shoot/root dry mass ratio and temperature, day length at different harvest dates. The temperature and day length are the mean of data of 14 days previous to the harvest day.

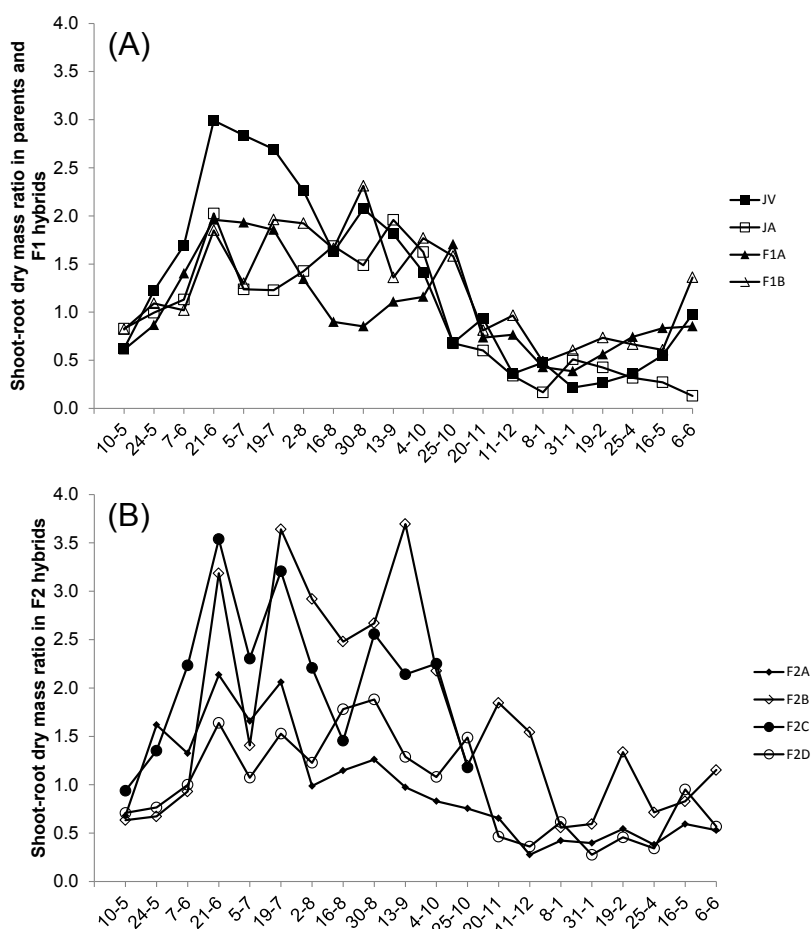
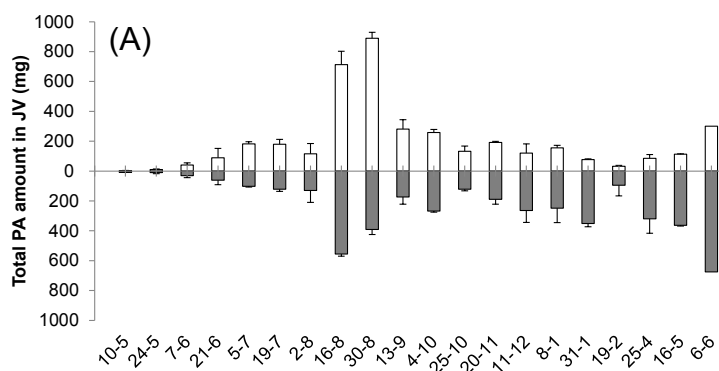
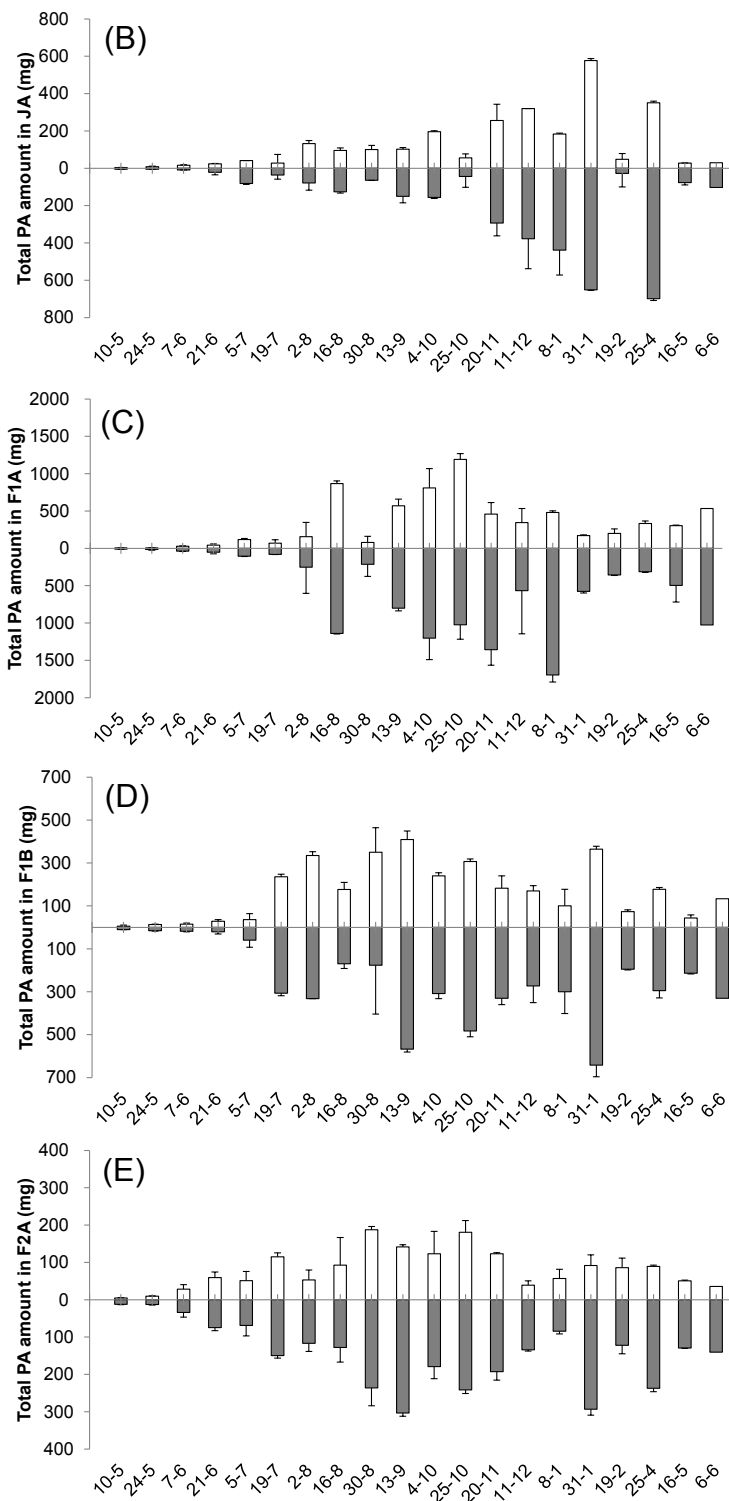


Fig. S3 The shoot/root ratio in *Jacobaea vulgaris*, *Jacobaea aquatica*, 2 F1 (F1A and F1B) and 4 F2 (F2A, F2B, F2C and F2D) hybrids at different harvest dates.





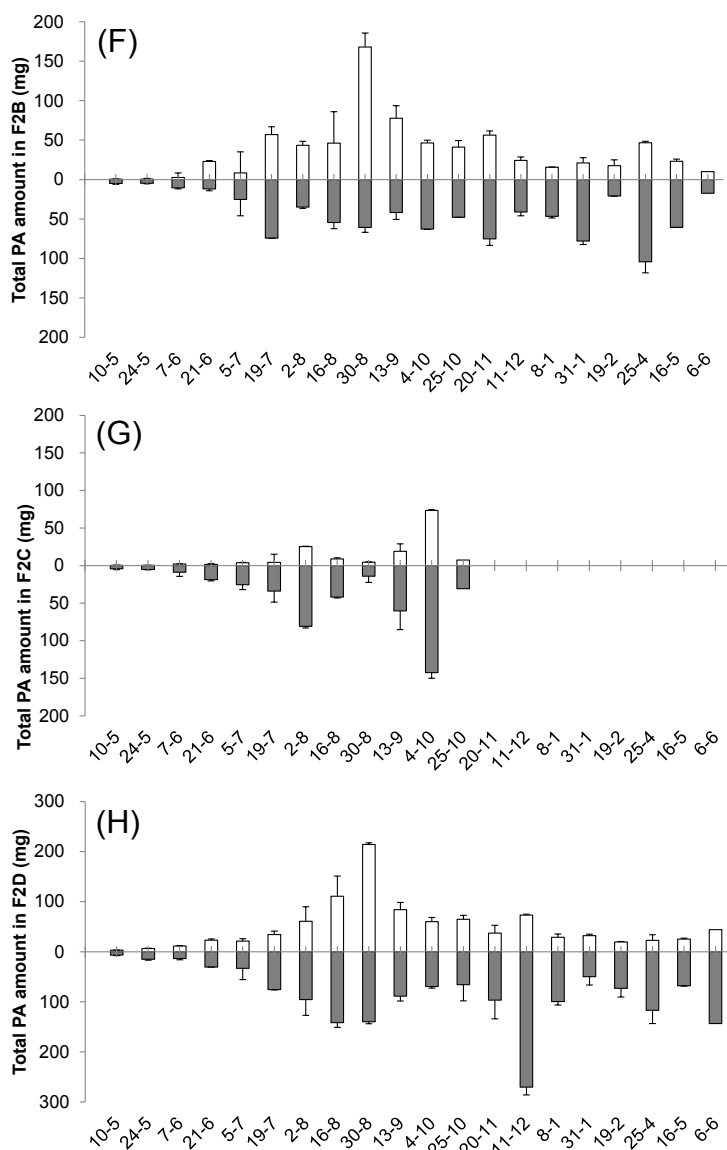
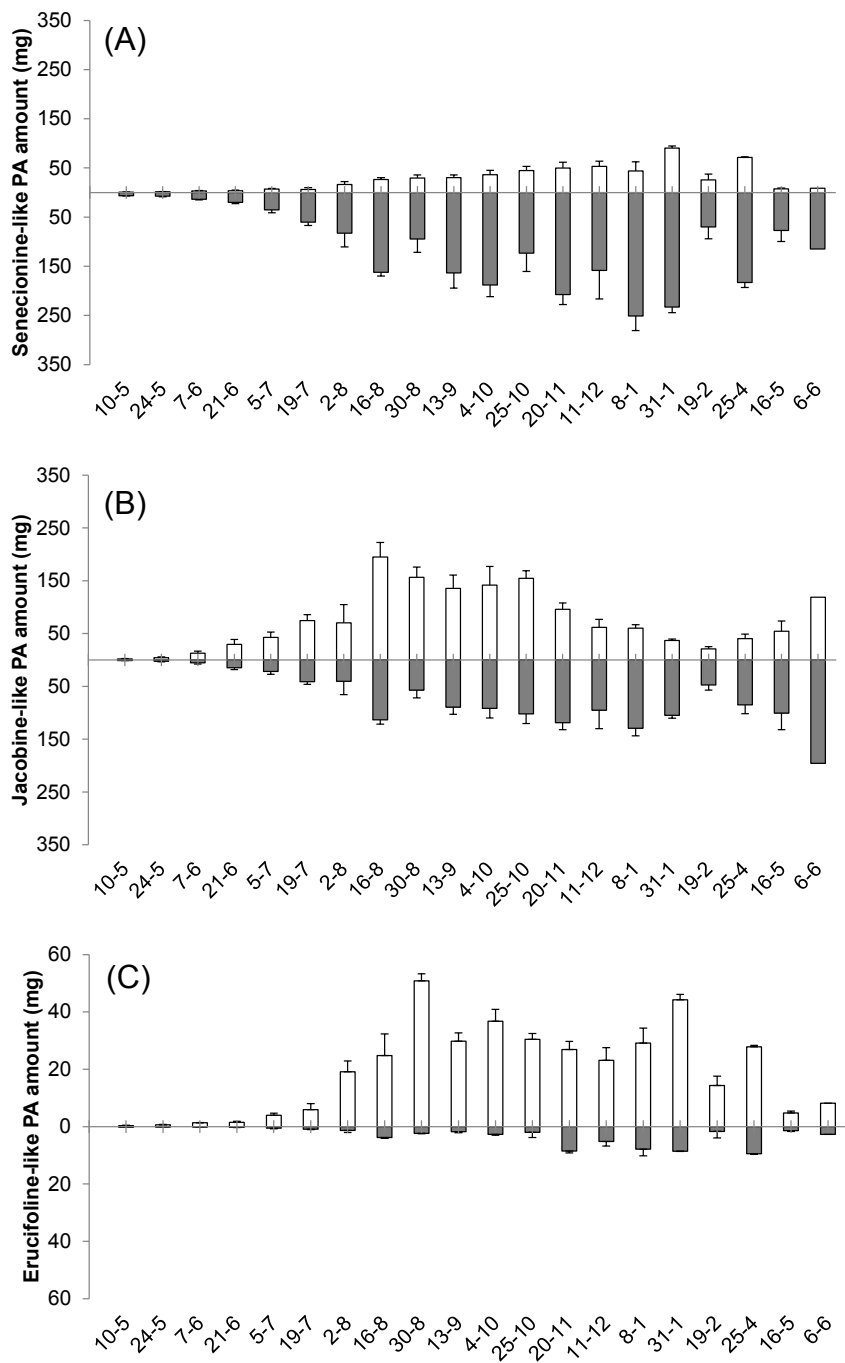


Fig. S4 The pyrrolizidine alkaloid (PA) amount ($\pm$ SE, mg) in *Jacobaea vulgaris*, *Jacobaea aquatica* and their 2 F1 (F1A and F1B) and 4 F2 (F2A, F2B, F2C and F2D) hybrids at different harvest dates. White bars represent shoots and grey bars represent roots.



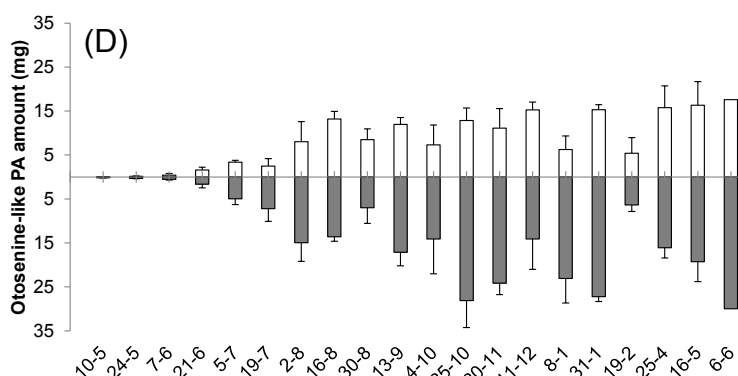
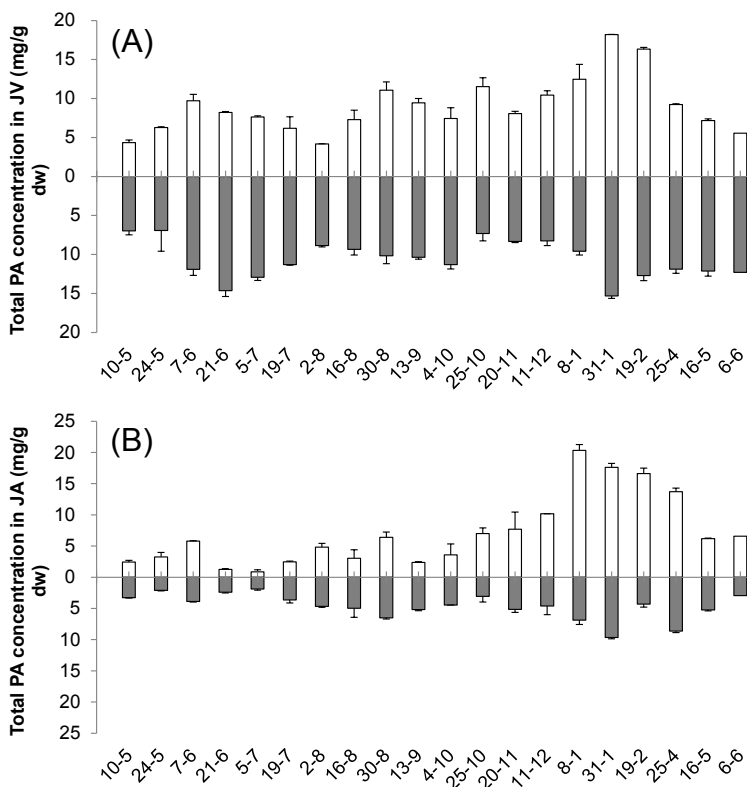
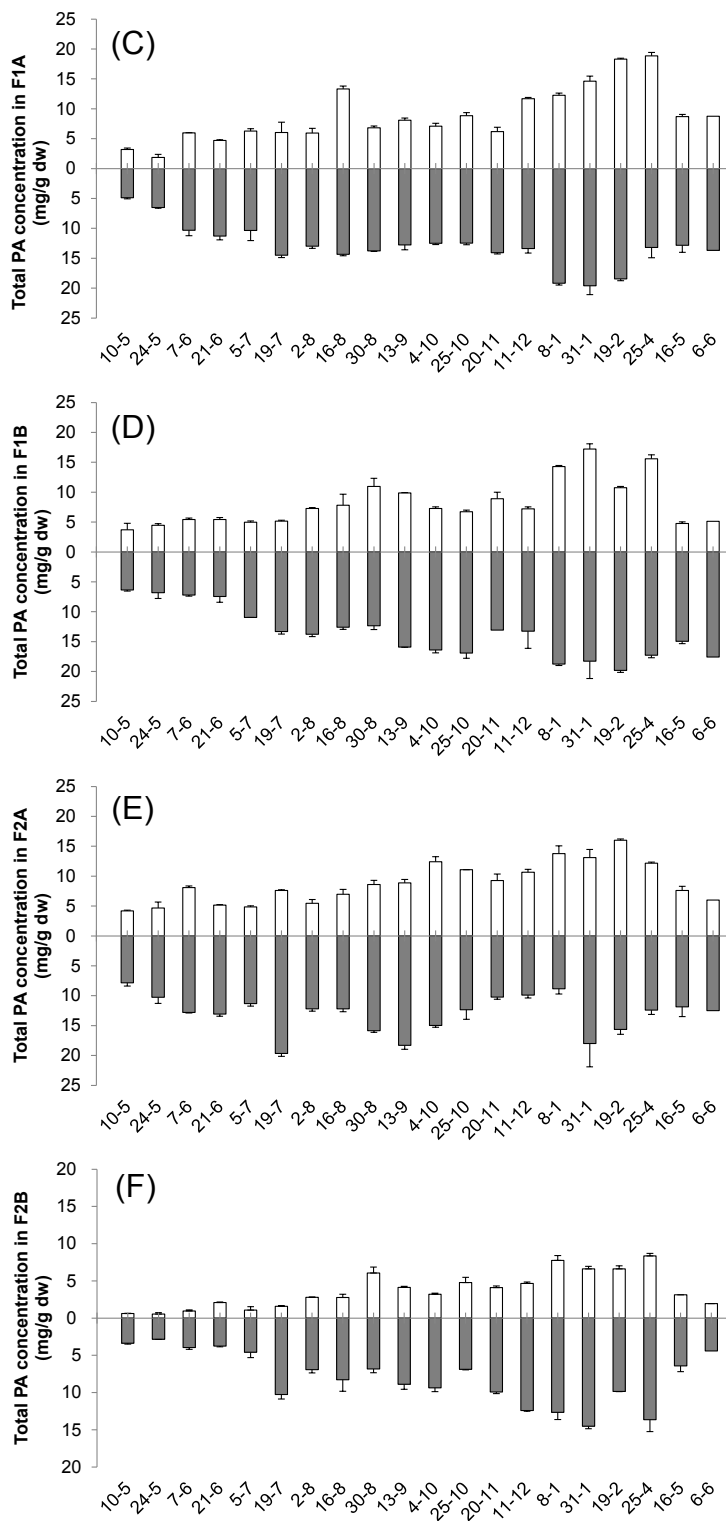


Fig. S5 The mean pyrrolizidine alkaloid (PA) amount ($\pm$ SE, mg) of four structurally related PA groups averaged over all the 8 genotypes at different harvest dates. White bars represent shoots and grey bars represent roots.





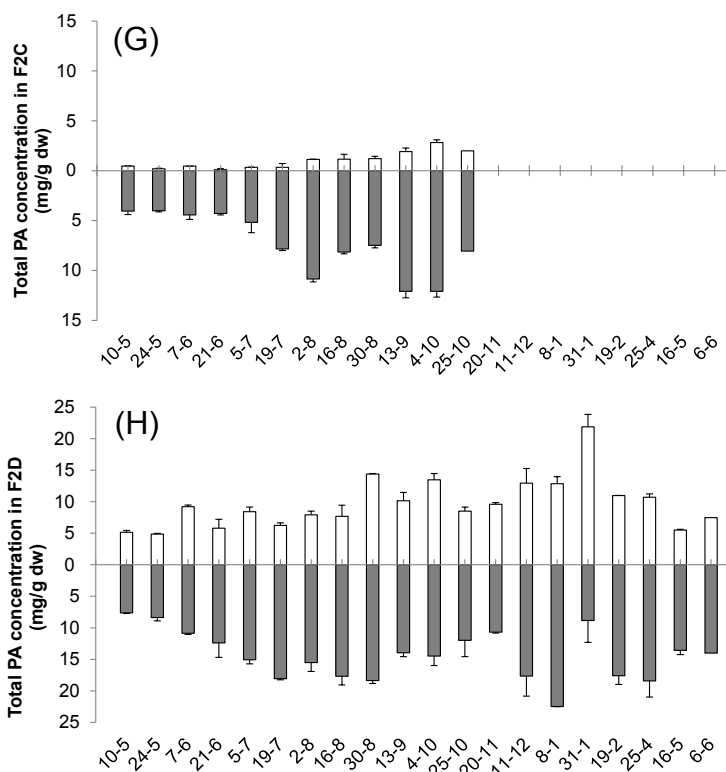
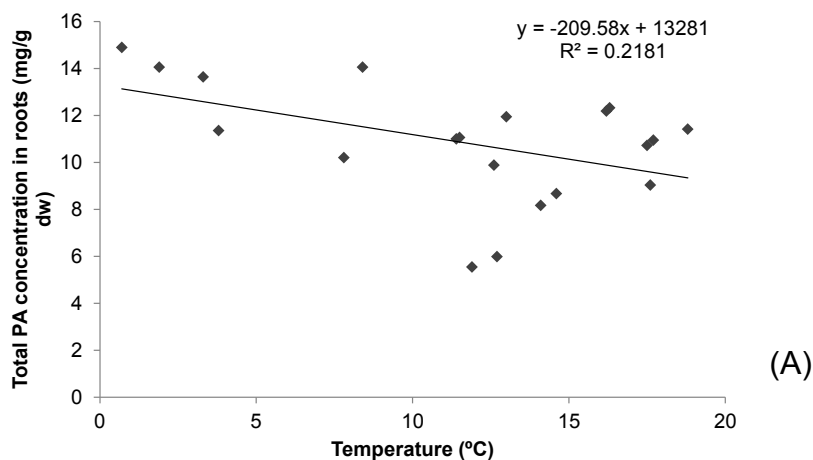


Fig. S6 The mean pyrrolizidine alkaloid (PA) concentration ($\pm$ SE, mg/g dw) in *Jacobaea vulgaris*, *Jacobaea aquatica* and their 2 F1 (F1A and F1B) and 4 F2 (F2A, F2B, F2C and F2D) hybrids at different harvest dates. White bars represent shoots and grey bars represent roots.



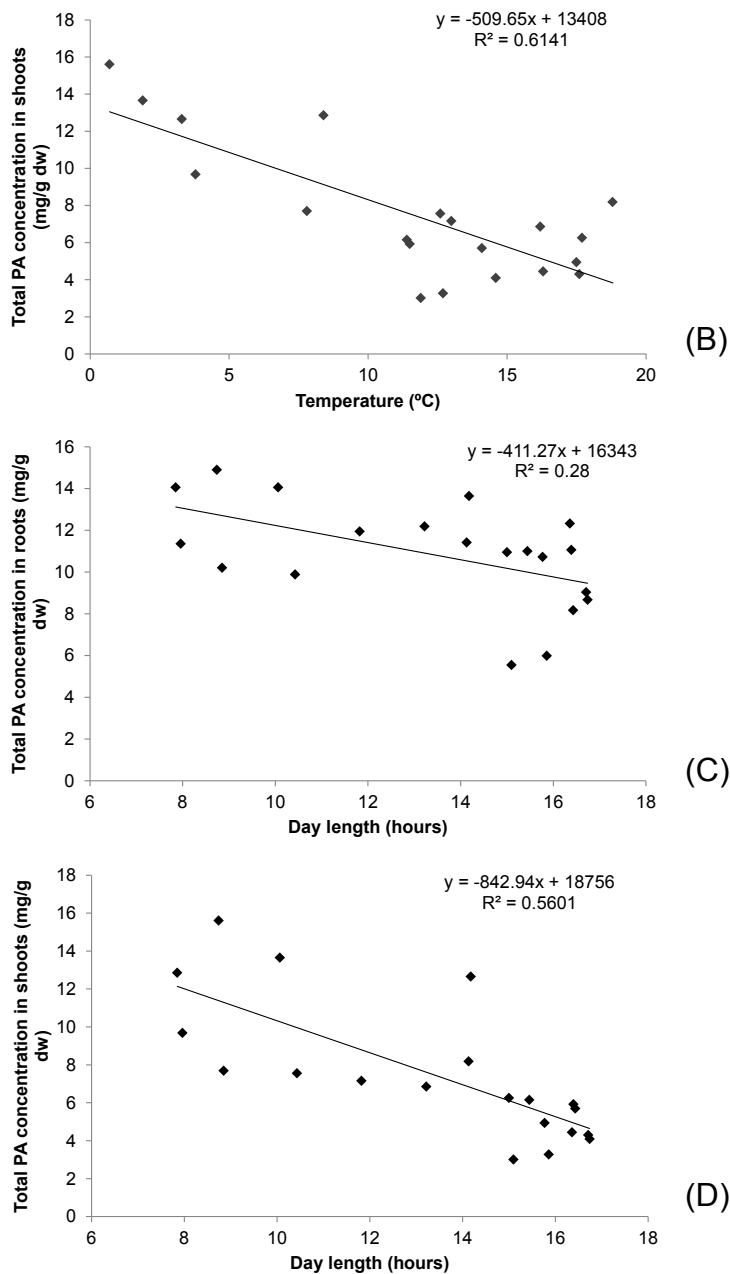
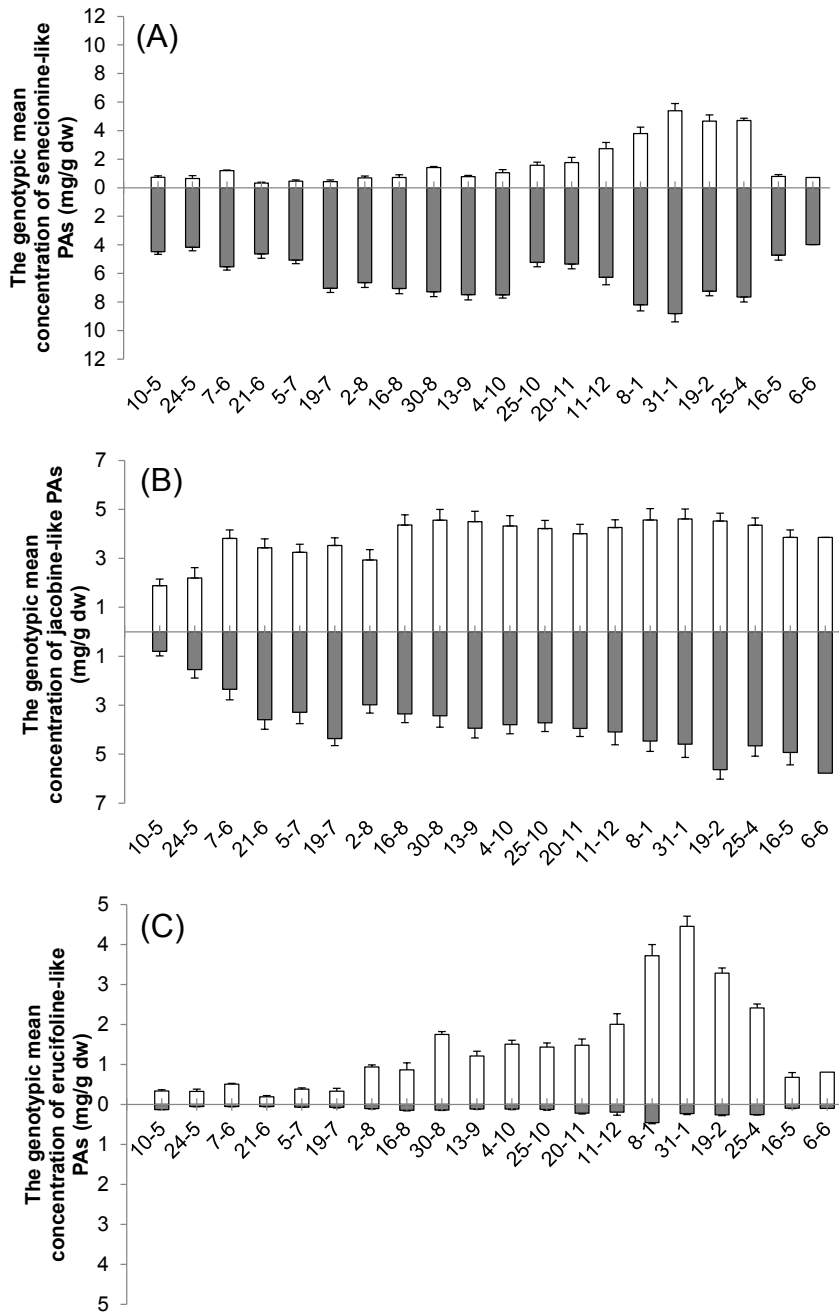


Fig. S7 Relationship between the mean pyrrolizidine alkaloid (PA) concentration (mg/g dw) of 8 genotypes and temperature and day length at different harvest dates in roots and shoots. The temperature and day length are the mean of the data of 14 days previous to the harvest day.



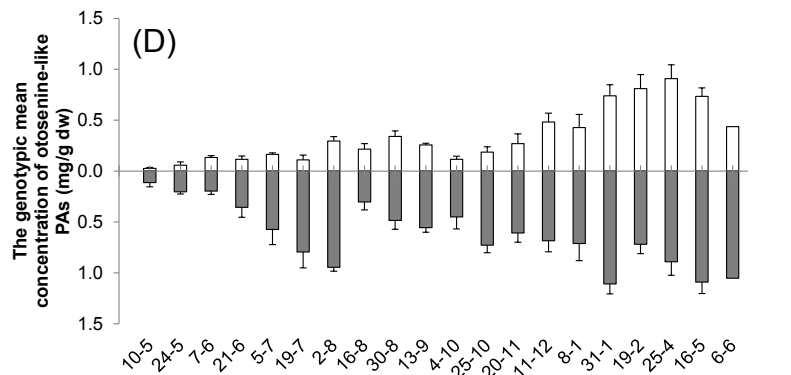
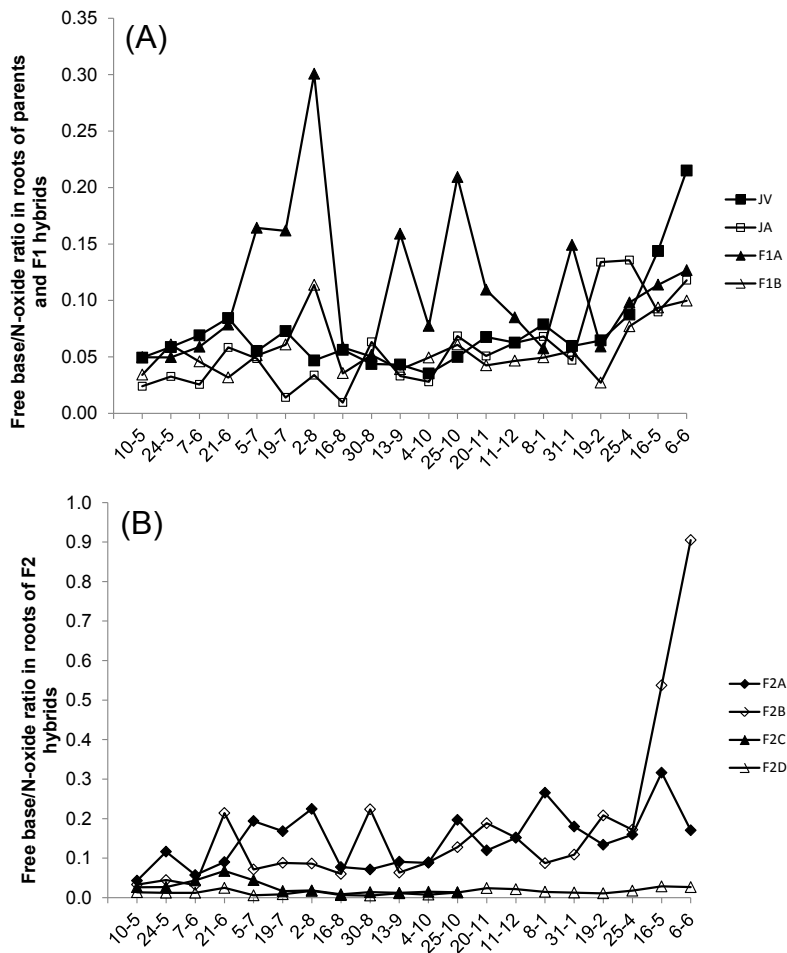


Fig. S8 The mean pyrrolizidine alkaloid (PA) concentration ($\pm$ SE, mg/g dw) of four structurally related PA groups over all 8 genotypes at different harvest dates. White bars represent shoots and grey bars represent roots.



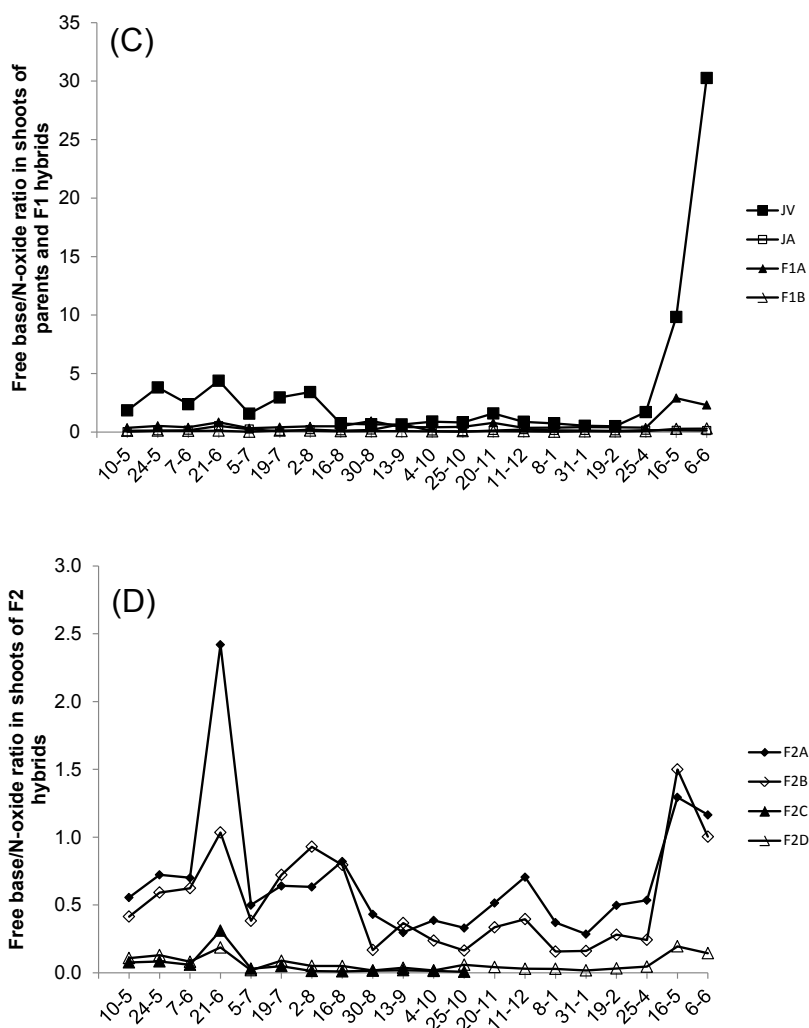


Fig. S9 The free base/N-oxide ratio in roots and shoots in *Jacobaea vulgaris*, *Jacobaea aquatica* and their 2 F1 (F1A and F1B) and 4 F2 (F2A, F2B, F2C and F2D) hybrids at different harvest dates.

Chapter 3

Local adaptation in oviposition choice of a specialist herbivore: the cinnabar moth¹

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¹ This chapter has been submitted to *Entomologia Experimentalis et Applicata* (under review) for publication in an adapted form.

Abstract

Specialist herbivores feed on a restricted number of related plant species and may suffer food shortage if overexploitation leads to periodic defoliation of their food plants. The density, size and quality of food plants are important factors that determine the host plant choice of specialist herbivores. To explore how all these factors influence their oviposition behaviour, we used the cinnabar moth *Tyria jacobaeae* and its host plant, common ragwort *Jacobaea vulgaris*, as a study model. While defoliation by the cinnabar moth is common in the coastal area of The Netherlands, it is relatively rare in inland ragwort population. Ragworts contain pyrrolizidine alkaloids (PAs) and those that are found in coastal areas are rich in jacobine-like PAs while those that occur inland are rich in erucifoline-like PAs. We tested the oviposition preference for plant size, nitrogen and water content and PA composition using cinnabar moth populations from a regularly defoliated area, Meijndel, and Bertogne, a rarely defoliated area. Our results revealed no preference for nitrogen or water content. As for PA composition, they showed that all moths laid larger egg batches on the plants rich in jacobine-like PAs. We also found that Meijndel and Bertogne moths had different oviposition behaviours: the first preferred larger plants and spread their eggs over more egg batches that were, on average, smaller than those of Bertogne moths. These results suggest that both features respond to a strategy of Meijndel moths to cope with potential defoliation.

Key words

Tyria jacobaeae; *Jacobaea* plants; Egg batch; Plant size; Defoliation; Batch size; Jacobine-like PAs; Erucifoline-like PAs

Introduction

Herbivorous insects face a green world as their food source. Generalist insect herbivores feed on a variety of different plant species; specialist herbivores, in contrast, only feed on one or eventually, a restricted number of closely related plant species (Ali and Agrawal 2012). This constitutes a great advantage for generalist herbivores that can benefit from an apparently unlimited availability and broad resource of food rather than the scarce and easily depleted food sources of specialist herbivores (Bernays and Minkenberg 1997). Though unable to use many host plants, specialist herbivores are adapted to tolerate and use plant defences for their own benefit (Berenbaum et al. 1989; Krieger et al. 1971; Whittaker and Feeny 1971).

Yet even then, specialist herbivores, are more prone to suffer food shortage (Dempster and Lakhani 1979). For example, as is usual in predator-prey interactions, the overexploitation of a plant food source can lead to its complete defoliation resulting in a decline in its availability (Chapin III and Slack 1979; Kosola et al. 2001; Larvae 1982; Thalmann et al. 2003). This is followed by a crash of the herbivore population due to starvation (Dempster 1971) which will however, recover in the following years with the regrowth of the food plant (Haukioja et al. 1985; Richards and Caldwell 1985). Specialist herbivores have also evolved strategies to avoid extinction that involve oviposition choices. They can select larger plants (Anthes et al. 2003; Kessler and Baldwin 2002) and adapt egg batch size to the features of the host plant (Pilson and Rausher 1988). For example, if faced with a low plant density, the amount of eggs per plant would be larger as the search for scattered host plants is time-consuming and thus, costly.

Another key factor influencing the fecundity of herbivores is host plant quality (Awmack and Leather 2002; Macel et al. 2002). For herbivores, plant quality is associated to its nutrient and water content as well as to the concentration of secondary metabolites (Slansky Jr and Rodriguez 1987). Plant quality has been reported to affect oviposition choice (Awmack et al. 2002). For example, larger egg batches are laid on high quality hosts since they can potentially support the growth of more offspring (Pilson et al. 1988).

To investigate how the density, size and quality of food plants influence the oviposition behaviour of specialist herbivores, we used the cinnabar moth *Tyria jacobaeae* and its host plant, the common ragwort, *Jacobaea vulgaris*, as a model system. In coastal areas, ragwort density is high and defoliation by the cinnabar moth is common while inland ragwort density is lower and defoliation is rare. The content of pyrrolizidine alkaloids (PAs), a diverse but structurally-related group of secondary metabolites in ragwort, is also different. Coastal ragworts are often rich in jacobine-like PAs while those rich in erucifoline-like PAs are predominant in inland ragworts (Macel et al. 2004; Vrieling and Boer 1999; Witte et al. 1992).

To test whether the different habitats of ragworts could affect the oviposition

choice of cinnabar moths, we used moth populations from Meijendel, a regularly defoliated coastal area and Bertogne, a rarely defoliated inland area, as well as two groups of plants rich in jacobine- or erucifoline-like PAs. We addressed the following questions: do cinnabar moths from Meijendel and Bertogne have equal oviposition choices? Is the oviposition preference of cinnabar moths related to the size of host plants and to the plant quality?

Materials and Methods

Plant sources and growing

The plants used in the oviposition bioassay were propagated from a cross between *Jacobaea vulgaris* and *Jacobaea aquatica* (Cheng et al. 2011). Both species are equally suitable as host plants for the cinnabar moth (Macel et al. 2002). Each hybrid was cloned to obtain replicated individuals. Clonal lines are hereafter referred to as 'genotypes'.

Twenty genotypes were selected based on the data of Cheng et al. (2011). Of these, ten genotypes had a higher jacobine-like PA and low erucifoline-like PA content while the other 10 genotypes were low in jacobine-like PAs and high in erucifoline-like PAs. While maintaining a large variation within the two groups, genotypes were selected in such a way that on average, the total PA concentration and plant mass were as similar as possible.

The 20 genotypes were cloned into 4 replicated individuals respectively. Plants were transferred to 0.8 L pots filled with a mixture of 95% sandy soil (collected from Meijendel), 5% potting soil (Slingerland Potgrond company, Zoeterwoude, The Netherlands) and 1.5 g L⁻¹ Osmocote slow release fertilizer (N:P:K =15:9:11; Scott®, Scotts Miracle-Gro, Marysville, Ohio, USA). They were grown in a climate room (relative humidity: 70%, light 16 h / dark 8 h, 20°C/20°C regime) for 6 weeks and then transferred to a greenhouse with natural light one week before the start of the oviposition bioassay.

Herbivore origin and rearing

The cinnabar moth *Tyria jacobaeae* L., is a specialist univoltine herbivore that feeds mainly on common ragwort (*Jacobaea vulgaris*, formerly known as *Senecio jacobaeae*) and a restricted number of other *Jacobaea* species (Macel et al. 2002; Vrieling 2006). The larvae of the cinnabar moth are completely dependent on ragwort as their food source (Dempster 1971; van der Meijden et al. 1990). Late fifth instar larvae were collected in the coastal dunes of Meijendel (52°48'38"N, 4°41'7"E, The Netherlands) and in Bertogne (50°05'27"N, 5°40'29"E, Belgium) that are approximately 300 km apart. The larvae were kept in glass tubes until pupation; the pupae were then stored in a cold room for 10 months (light/dark, 16h/8h, 4°C) (Zoelen and Kusters 1986). After this, they were removed and sexed before hatching. They were placed in transparent plastic cages (70*70*50 cm) and kept at room temperature under natural

light. Moths started to emerge after 2 weeks, and fed with water and honey. In total, 44 male and 44 female moths were selected from the Meijendel population and 34 male and 34 female moths from the Bertogne population; all were active and healthy.

Oviposition bioassay

The bioassay was conducted in 4 plastic cages (87 cm diameter, 1 m height) covered with gauze in a greenhouse. The setup was the same as that of Cheng et al. (2013). Each cage contained one clonal replicate of the high jacobine-like PA content genotypes and one of the high erucifoline-like PA content genotypes. An equal number of moths of each type was released into two separate cages each. Plants were watered every three days throughout the bioassay. The cages were rotated by 90 degrees every 3 days to avoid position effects on oviposition. After 10 days, the moths were removed and the plants were harvested. Photographs of all the leaves with eggs were used to count the number of egg batches per leaf and the eggs per batch.

The fresh weight, diameter of the shoots and number of leaves were recorded. For PA and nitrogen determination, 2 leaves (from the fourth to eighth leaf) were collected, frozen with liquid nitrogen and lyophilized at -80°C in a freeze dryer (Cryotheque®, Sniders Scientific Company, Tilburg, The Netherlands) for 4 days. The samples were then ground to a fine powder and stored at -20°C freezer until measurement. The remaining leaf material was dried in an oven at 50 °C for 4 days. The dry mass of the shoot was obtained by adding the mass of all the leaves. The water content (%) was calculated as ((fresh weight of shoot)-(dry mass of shoot))*100/(fresh weight of shoot).

Pyrrolizidine alkaloid measurement

Alkaloid content was analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Approximately 10 mg of powdered plant material were extracted with 1 ml 2% formic acid. Heliotrine was added as an internal standard to the extraction solvent at a concentration of 1 µg/ml. This mixture was shaken for 30 minutes, centrifuged for 10 minutes at the speed of 13 000 rpm and filtered through a 0.2 µm nylon membrane (Acrodisc 13-mm syringe filter, Pall Life Sciences, Ann Arbor, MI, USA). An aliquot of 25 µl was diluted with 1 ml 0.05% ammonia in water and 10 µl were injected into a Waters Acquity UPLC system coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters Corporation, Milford, MA, USA). The analytical conditions are described in Cheng et al. (2011). Data was processed with Masslynx 4.1 (Waters Corporation, Milford, MA, USA).

Nitrogen measurement

Nitrogen measurement followed a standard protocol using the Bradford reagent (Kruger 2009). Approximately 5 mg of the powder per samples were diluted with 2 ml TBS (Tris-Cl:NaCl = 1:3, pH = 7.6). The mixture was centrifuged at 13 000 rpm for 20 seconds. The supernatant was transferred quantitatively to a vial and diluted

20 times. A 50 µl aliquot was pipetted into microplate wells (PS microplate 96 wells, Greiner Bio-One, Germany) and 80 µl of Bradford reagent (Bio-Rad Protein Assay, Bio-Rad Laboratories, USA) were added to each well. The samples were incubated at room temperature for 5 minutes and then measured with a Microplate Reader (Model 680XR, Bio-Rad Laboratories, USA) at 575 nm. A calibration curve with known concentrations of bovine serum albumin (BSA) was used to calculate the protein content of the leaf material. Nitrogen content was obtained by dividing the protein content by a factor of 6.25.

Statistical analysis

Three parameters, i.e., number of eggs per plant, number of egg batches per plant, and egg batch size per plant (= (number of eggs per plant)/(number of egg batches per plant)), were used to measure oviposition preference of the cinnabar moths.

Dry mass, fresh weight and diameter of shoot, and the number of leaves were analysed using PCA. One component that explained 71% of the variance was extracted from these four factors used as an estimate of plant size. Linear regressions between plant size and the three oviposition parameters were then calculated.

Water content and nitrogen content in the shoots were used as proxies for plant quality. General linear models were conducted to verify whether water content and nitrogen content were correlated with the oviposition choices.

To confirm if the grouping of ragwort genotypes based on the data of Cheng et al. (2011) was consistent with the PA data in our study, we performed a PCA with all the individual PA concentrations of our study.

General linear models were used to determine whether the moths from Meijendel and Bertogne had different preferences for plants according to their different PA composition. For this, PA composition was defined as a fixed factor, moth origin (Meijendel or Bertogne) as a random factor and the three before mentioned oviposition parameters as dependent variables. As plant size proved to be correlated with the oviposition parameters, the general linear models were rerun after correcting the number of eggs, number of egg batches and egg batch size for the effect of plant size. As this effect depended on the origin of the moths this correction was done separately for the two origins.

All statistical analyses were done with SPSS 21.0 and SIMCA was used for PCA.

Results

Oviposition performance of cinnabar moths

There was no difference among cages in the number of eggs ($F_{3,76}=0.507$, NS), the number of egg batches ($F_{3,76}=1.531$, NS) nor the egg batch size ($F_{3,76}=2.511$, NS).

The Meijendel moths deposited 7704 eggs in 311 egg batches on 39 plants. On average, each plant received 198 eggs in 8.0 egg batches, the mean egg batch

size being 24.8 eggs. Each female moth laid an average 175 eggs in 7.1 egg batches.

Bertogne moths laid 8421 eggs in 254 egg batches on 40 plants. On average, each plant received 211 eggs in 6.4 egg batches, and the mean egg batch size was 33.2 eggs. Each female moth laid 248 eggs on average in 7.5 egg batches.

Effects of plant size on oviposition preference

The number of eggs and egg batches were significantly correlated with plant size for Meijendel moths (Fig. 1A, C and E). In contrast, neither the number of eggs, nor egg batches, egg batch size were correlated significantly with plant size for Bertogne moths (Fig. 1B, D and F).

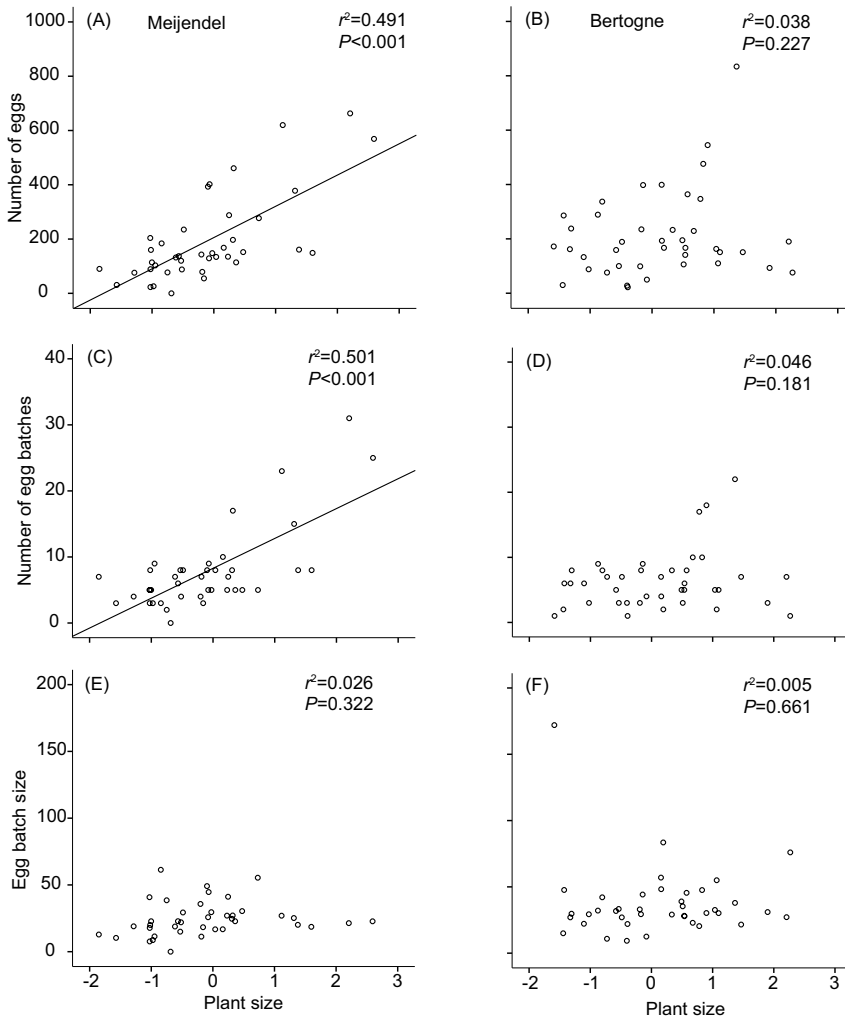


Fig. 1 Scatter plots of number of eggs, number of egg batches, egg batch sizes and plant size for cinnabar moth *Tyria jacobaeae* from Meijendel (A, C, E) and Bertogne (B, D, F) respectively.

Effects of water content and nitrogen content on oviposition preference

The concentration of nitrogen in leaves did not differ significantly amongst genotypes (ANOVA, $F_{19,60}=1.079$, NS) but the water content was significantly different (ANOVA, $F_{19,60}=2.982$, $P=0.001$). However, neither parameter was significantly correlated with the number of eggs, egg batches nor egg batch size for both moth populations (Fig. S1 and S2).

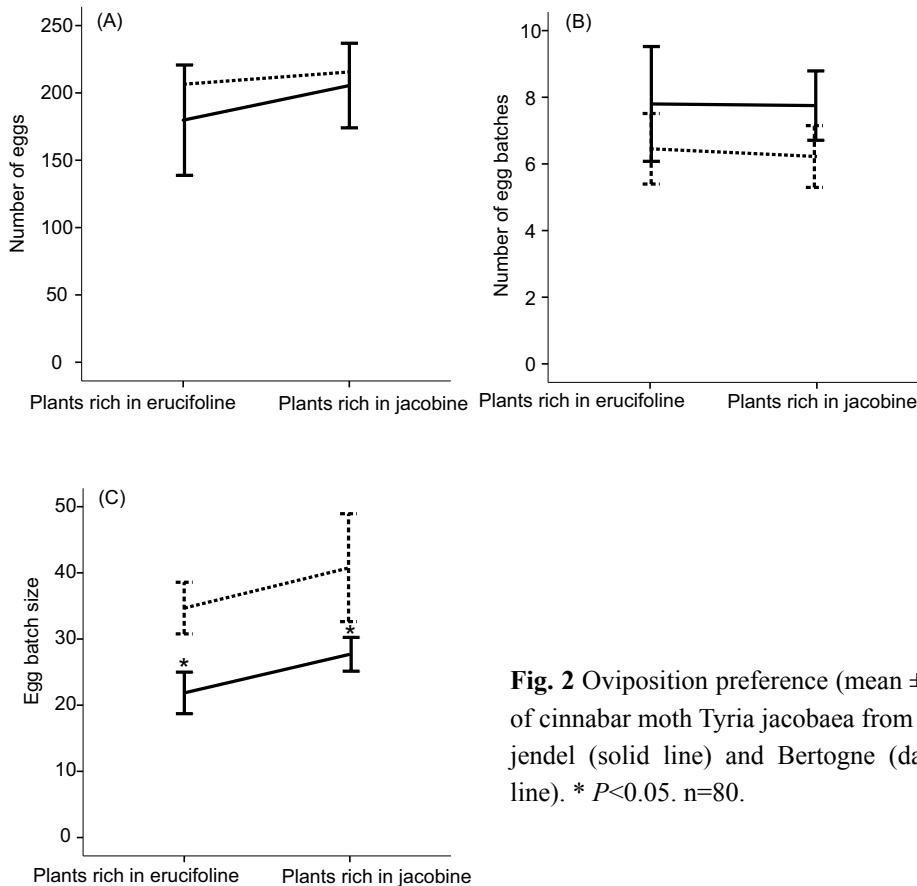


Fig. 2 Oviposition preference (mean $\pm$ SE) of cinnabar moth *Tyria jacobaea* from Mei-jendel (solid line) and Bertogne (dashed line). * $P<0.05$. n=80.

Effects of pyrrolizidine alkaloids on oviposition preference

The PCA plots showed that the genotypes were well separated into a jacobine and erucifoline group (Fig. S3). This grouping was fully consistent with the data obtained by Cheng et al. (2011), proving that the composition of PAs in these plants is a genetically determined trait.

Moths from the two origins laid a similar number of eggs regardless of the PA composition of the plants (Table 1; Fig. 2A). The number of egg batches was marginally, yet not significantly higher for Mei-jendel moths (Fig. 2B). Interestingly, Bertogne moths laid their eggs in significantly larger batches (Fig. 2C). All moths

laid larger egg batches on plants rich in jacobine-like PAs (Table 1; Fig. 2C) and even after correcting for plant size, the egg batch size was still significantly higher for these plants (Table 2).

Table 1 ANOVA with moth origin and pyrrolizidine alkaloid (PA) composition (jacobine or erucifoline-like PA rich plants) as factors and number of eggs, number of egg batches and egg batch size of the cinnabar moth as dependent variables

Dependent variable	Factors	df	F	P
Number of eggs	Moth origin	1, 77	0.766	0.542
	PA composition	1, 77	5.086	0.266
	Moth origin*PA composition	1,76	0.327	0.569
	Error	76		
	Total	80		
Number of egg batches	Moth origin	1, 77	5.198	0.263
	PA composition	1, 77	0.846	0.527
	Moth origin*PA composition	1, 76	0.258	0.613
	Error	76		
	Total	80		
Egg batch size	Moth origin	1, 77	12786.091	0.006
	PA composition	1, 77	2617.928	0.012
	Moth origin*PA composition	1, 76	0.001	0.981
	Error	76		
	Total	80		

Discussion

Our study demonstrated that Bertogne moths laid significantly larger batches of eggs than those from Meijndel. All moths laid larger egg batches on jacobine-like PAs rich plant but only Meijndel moths laid more eggs and egg batches on larger plants. Nitrogen and water content of the host plants did not play a role in the oviposition choice of the cinnabar moths.

Effects of plant characters on oviposition preference

The Meijndel ragwort populations though denser than Bertogne populations are defoliated every 2 or 3 years by the larvae of the cinnabar moth (van der Meijden 1979; van der Meijden et al. 2000). Thus, choosing bigger plants and spreading eggs into more and consequently smaller egg batches might be profitable strategies to increase their chance of survival. In contrast, the ragwort populations or individuals in Bertogne are rarely defoliated and much less dense (van der Meijden personal observation). As the search for host plants for oviposition is costly (Ali et al. 2012),

this low density could explain that cinnabar moths lay larger batches of eggs when they encounter a plant.

Cheng et al. (2013) showed that the fresh weight of plants had a significant positive effect on the number of eggs and egg batches. Our results coincided with this finding since we observed a significant positive correlation between plant size and the number of eggs and egg batches deposited by the Meijendel moths. All these studies suggested that morphological characteristics such as plant size affect the oviposition choices of specialist herbivores.

Table 2 ANOVA with moth origin and PA composition (jacobine- or erucifoline-like PAs rich plants) as factors and the residual ^a of number of eggs, number of egg batches and egg batch size of the cinnabar moth as dependent variables

Dependent variable	Factors	<i>df</i>	<i>F</i>	<i>P</i>
Residual of number of eggs	Moth origin	1, 77	0	1 ^b
	PA composition	1, 77	3.034	0.332
	Moth origin*PA composition	1, 76	0.763	0.385
	Error	76		
	Total	80		
Residual of number of egg batches	Moth origin	1, 77	0	1 ^b
	PA composition	1, 77	0.437	0.628
	Moth origin*PA composition	1, 76	0.717	0.400
	Error	76		
	Total	80		
Residual of egg batch size	Moth origin	1, 77	0	1 ^b
	PA composition	1, 77	465.614	0.029
	Moth origin*PA composition	1, 76	0.003	0.957
	Error	76		
	Total	80		

^a The residuals were from the linear regressions between number of eggs, number of egg batches, egg batch size and plant size, respectively. Plant size was, according to PCA, the component explaining 71% of the variation. Plant size included four factors: dry mass of shoot, fresh weight of shoot, shoot diameter and number of leaves.

^b The plant size depended on the origin of the moths, so the *P* value of moth origin became 1 after being corrected for plant size

Effects of plant quality on oviposition preference

van Der Meijden et al. (1989) found that cinnabar moths selected plants with a high concentration of organic nitrogen and sugars for oviposition. The nitrogen content in the plants used for our study was not significantly different among genotypes perhaps due to the fact that all plants were potted in the same soil mixture and reared under the same conditions. Thus it is difficult to reach a clear conclusion for the role of nitrogen from this experiment. Likewise the water content did not influence the oviposition choice even though it might influence the growth of moth larvae.

All the plants used in this study received eggs but all moths laid larger egg batches on the jacobine-like PA containing plants. This coincided with the results obtained by Macel and Vrieling (2003) which an oviposition preference of moths for filter papers with a PA mixture from *S. jacobaea* rather than control papers without PAs. Similarly, Cheng et al. (2013) found that moths preferred to lay eggs on the plants with a higher free base content of jacobine-like PAs. These studies thus suggest that the type of PAs play a role in the oviposition choice of the cinnabar moth.

Concluding remarks

Cinnabar moths from Meijendel preferred to lay eggs, albeit in smaller batches on larger plants. This was not the case for Bertogne moths. Given the regular defoliation of ragwort populations in Meijendel, laying eggs on bigger plants and spreading them among more egg batches could be a successful strategy to reduce the risk of food shortage and increase the survival of the offspring. While the oviposition choice of cinnabar moths from both populations was not influenced by the nitrogen and water content of the plants, jacobine-like PAs rich plants were clearly preferred. All these observations led us to conclude that the specialist herbivore *T. jacobaeae* has locally adapted its oviposition preference to food quantity but not food quality.

Acknowledgements

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Supplementary Materials:

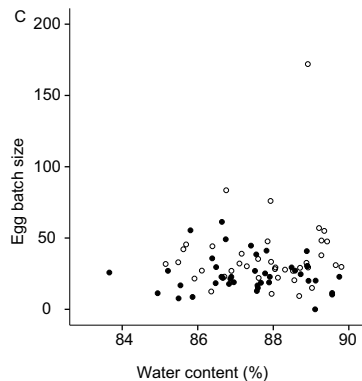
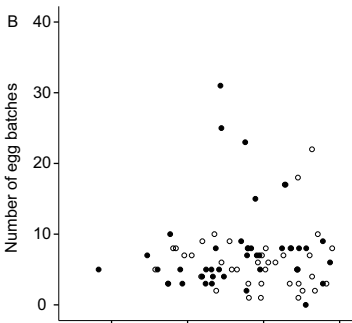
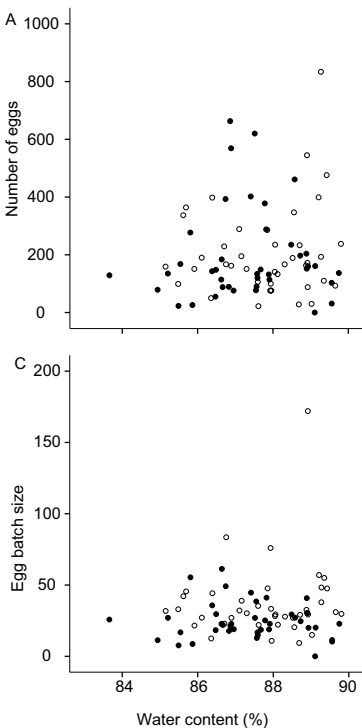


Fig. S1 Scatter plots of water content and number of eggs (A), number of egg batches (B) and egg batch size (C). The open circles represent the moths from Bertogne and the closed circles represent the moths from Meijendel.

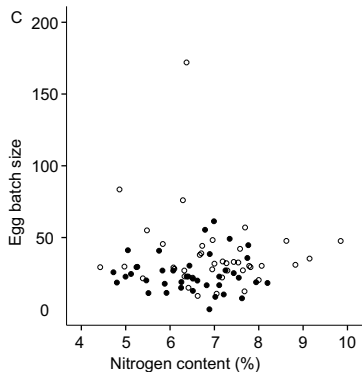
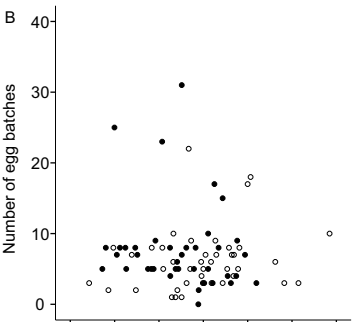
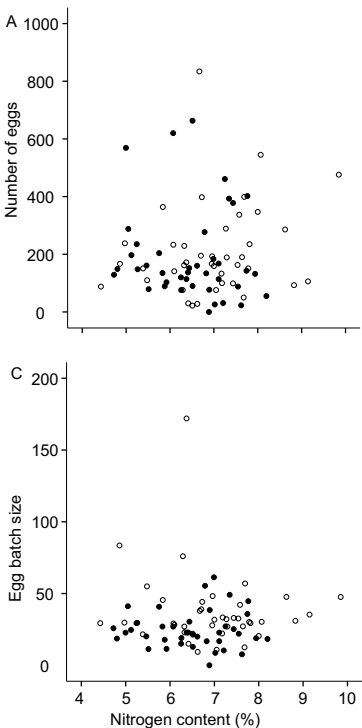


Fig. S2 Scatter plots of nitrogen content and number of eggs (A), number of egg batches (B) and egg batch size (C). The open circles represent the moths from Bertogne and the closed circles represent the moths from Meijendel.

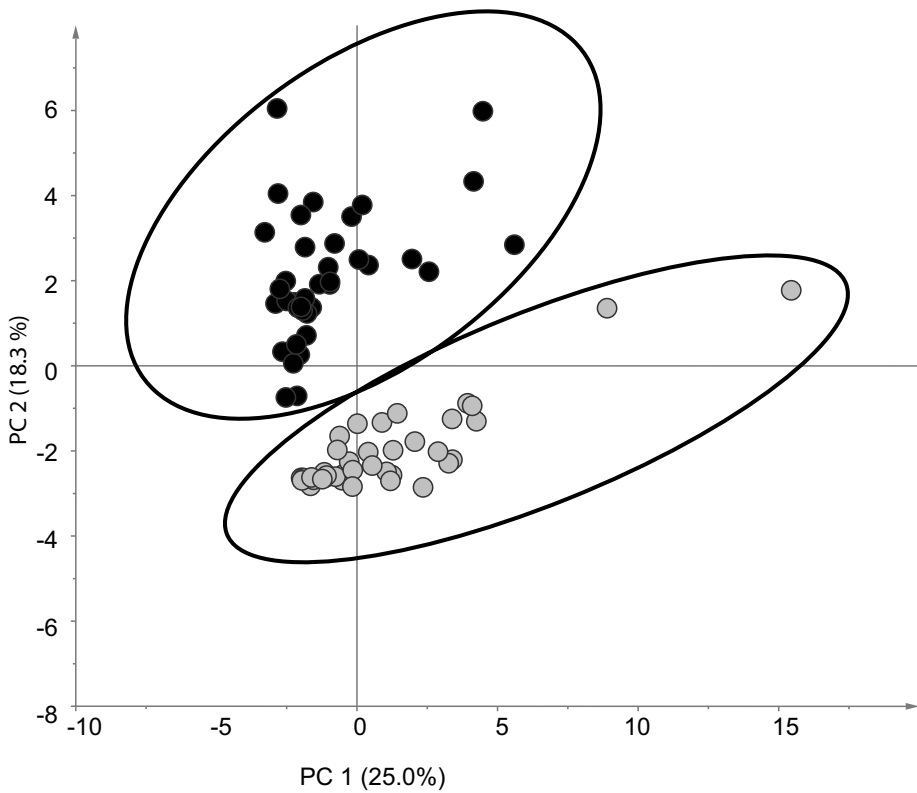


Fig. S3 Principal Component Analysis based on all of the individual pyrrolizidine alkaloid (PA) values obtained in this study. Black circles representing plants rich in jacobine-like PAs and grey circles representing plants rich in erucifoline-like PAs were defined based on PA data of Cheng et al. (2011).

Chapter 4

The role of pyrrolizidine alkaloids in resistance to invertebrate herbivores in *Jacobaea* Species¹

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Abstract

Plants produce a diversity of secondary metabolites (SMs) to protect them from generalist herbivores. On the other hand, specialist herbivores use SMs for host plant recognition, feeding and oviposition cues, and even sequester SMs for their own defense. Therefore plants are assumed to face an evolutionary dilemma stemming from the contrasting effects of generalist and specialist herbivores on SMs. To test this hypothesis, bioassays were performed with F2 hybrids from *Jacobaea* species segregating for their pyrrolizidine alkaloids (PAs), using a specialist flea beetle (*Longitarsus jacobaeae*) and a generalist slug (*Deroceras invadens*). Our study demonstrated that while slug feeding damage was negatively correlated with the concentration of total PAs and that of senecionine-like PAs, flea beetle feeding damage was not affected by PAs. It was positively correlated though, with leaf fresh weight. The generalist slug was deterred by senecionine-like PAs but the specialist flea beetle was adapted to PAs in its host plant. Testing other herbivores in the same plant system it was observed that the egg number of the specialist cinnabar moth was positively correlated with jacobine-like PAs, while the silver damage of generalist thrips was negatively correlated with senecionine- and jacobine-like PAs, and the pupae number of generalist leaf miner was negatively correlated with otosenine-like PAs. Therefore while the specialist herbivores showed no correlation whatsoever with PA concentration, the generalist herbivores all showed a negative correlation with at least one type of PA. We concluded that the generalist herbivores were deterred by different structural groups of PAs while the specialist herbivores were attracted or adapted to PAs in its host plants.

Key Words

Secondary metabolites diversity, F2 hybrids, *Deroceras invadens*, *Longitarsus jacobaeae*, *Jacobaea vulgaris*, feeding damage.

Introduction

Plants have evolved a variety of defense systems to ward off or reduce the attack from other organisms such as herbivores and pathogens. These defenses include secondary metabolites (SMs) as well as a great diversity of external and internal physical barriers, including trichomes, thorns and spines, lignified cell walls and silica crystals (Bennett and Wallsgrove 1994; Lucas et al. 2000; Paré and Tumlinson 1999). As herbivores can cause severe damage resulting in a loss of fitness (Becker 1983; Blundell and Peart 2000), it is expected that plants with high levels of defensive traits are selected in nature (Geber and Griffen 2003). Among these, numerous studies covering a wide range of species have revealed that SMs constitute an important class of defense (Hay and Fenical 1988; Textor and Gershenzon 2009).

Secondary metabolites are regarded as dispensable for basic growth and development yet indispensable for the fitness and survival of an organism (Janz and Nylin 1998). The most characteristic feature of SMs is their great structural multiplicity among and within plant species (Hartmann 1996). More than 500 000 SMs have been discovered in the plant kingdom (Hadacek 2002) and their diversity even within one plant species is enormous. For instance, more than 170 SMs belonging to seven major groups have been identified in *Arabidopsis thaliana* (D'Auria and Gershenzon 2005).

Insect herbivores are considered an important driving force for the diversification of SMs due to their close long-term association with plants (Janz and Nylin 1998). There are several hypotheses on the way SM diversity can increase herbivore deterrence. One of them states that the presence of multiple herbivores selects for increased SM diversity assuming that each specific SM confers resistance against a specific herbivore or a group of specific herbivores (Lason et al. 2011). Hence, a mixture of SMs is selected for by multiple herbivores (Juenger and Bergelson 1998; Juenger and Bergelson 2000; Macel et al. 2005; Mithen et al. 1995).

In addition to structural diversity, SMs frequently exhibit a high genotypic variation in their concentration, probably explained by a balance between the benefits of an increased resistance against herbivores and the costs of production and storage (Coley et al. 1985; Herms and Mattson 1992). The production of SMs, however, can come with an ecological cost, since some herbivores may be attracted to plants with a high SM content (van der Meijden 1996). In fact, while generalist herbivores are usually deterred by high concentrations of SMs specialist herbivores are often adapted to them (van Dam et al. 1995; van der Meijden 1996). Moreover, several specialist herbivores sequester SMs to use them for host plant recognition, feeding and oviposition cues, and even as nuptial gifts (Dussourd et al. 1991; Nishida 2002; Opitz and Müller 2009). Therefore, plants presumably face a dilemma due to potentially contrasting natural selection by generalist and specialist herbivores that is correlated to the production of defense-related chemicals (van der Meijden

1996). However, so far there is only limited experimental evidence for this hypothesis (Lankau 2007; Lankau and Sharon 2008).

To evaluate the role of SMs in the resistance against generalist and specialist herbivores, a series of F2 hybrid plants were fed to a major specialist herbivore flea beetle, *Longitarsus jacobaeae* (Chrysomelidae) and a common generalist slug, *Deroceras invadens* (Agriolimacidae). The hybrid plants were derived from a cross between *Jacobaea vulgaris* (common ragwort, syn. *Senecio jacobaea*) and *Jacobaea aquatica* (marsh ragwort, syn. *Senecio aquaticus*). Both species contain pyrrolizidine alkaloids (PAs), a group of typical SMs with a highly heritable pattern (Vrieling et al. 1993). The F2 hybrid population consists of a set of genotypes that show a significant variation in PA diversity and concentration between plants. Thus, if PAs were to play a role in plant resistance, this should be reflected in differences throughout the set.

We compared the activity of specialist and generalist herbivores on the same genotypes of hybrid plants. For this, we designed two bioassays aimed at seeking answers to the following questions: 1) Is there any difference in the feeding damage to F2 hybrid plants produced by slugs and flea beetles? 2) If so, can its pattern be explained by the type and concentration of PAs? Additionally, considering that another three herbivores had been tested in an identical setup with the same set of genotypes, we analyzed the data from all the five herbivore bioassays and addressed the last question: 3) Does the feeding damage of the different herbivores covary with each other and is it related to the same or different PAs?

Materials and Methods

Study system

Jacobaea vulgaris is native to the Eurasian continent and invaded North America, Australia and New Zealand (Doorduyn et al. 2010). *Jacobaea aquatica*, a European endemic species, though closely related to *J. vulgaris* is not a sister species (Pelsner et al. 2003). Based on morphology and molecular methods, putative hybrids between the two species have been found in Western and Central Europe (Chater and Walter 1976; Kirk et al. 2004). Thus, *Jacobaea* hybrids occur commonly in nature. The hybrids used in this study were developed in our lab (see Cheng et al. 2011a). In brief, two F1 offsprings were used to obtain 87 F2 individuals from a cross between *J. vulgaris* from a dune system, the Meijendel nature reserve (52°7'54"N, 4°19'46"E, The Netherlands) and *J. aquatica* from marsh area, the Zwanenwater nature reserve (52°48'38"N, 4°41'7"E, The Netherlands). The parents, F1 and F2 hybrids are all maintained in tissue culture and can be cloned ad libitum (see Plant Growth). The F2 hybrids were used for this study. *Jacobaea* plants are well known for their content of PAs, which have negative effects on the growth of fungal pathogens (Hol and van Veen 2002) and also play a dual role in plant-insect interactions (Cheng et al. 2011b;

Cheng et al. 2013; Macel 2003).

Plant growth

Plants were propagated by tissue culture and each of the 87 F2 individuals was cloned into six replicate individuals. Plants were potted in 0.8 L pots filled with a mixture of 95% sandy soil (collected from Meijendel), 5% potting soil (Slingerland Potgrond Company, Zoeterwoude, The Netherlands) and 1.5 g/L Osmocote slow release fertilizer (N:P:K=15:9:11; Scott®, Scotts Miracle-Gro, Marysville, Ohio, USA). They were kept in a climate room (humidity 70%, light 16h /dark 8h, 20°C/20°C, light intensity 130 $\mu\text{m}^2/\text{sec}$) for six weeks before starting the generalist herbivore bioassay. A total of 87 and 84 genotypes were used for the slug and flea beetle bioassay respectively (in some cases fewer replicates survived). Some genotypes were represented by four or five replicates.

Generalist herbivore bioassay

Deroceras invadens is a land slug that feeds on many different plant species. It is a significant pest in gardens, greenhouses and pastures and is invasive in The Netherlands and many other parts of the world (Barker 2002). Slugs were collected in a lawn close to the Institute of Biology Leiden (52°09'27"N, 4°28'52"E, The Netherlands). The lawn was sprayed with water in the afternoon and covered with black plastic bags. The next morning *D. invadens* slugs hiding on the inner surface of the bags were collected and kept in a box with wet sand and fed with herbs and grasses from the same lawn. They were starved for 48 hours before the initiation of the bioassay. A subset of eight slugs was examined by Dr. Ton de Winter (malacologist at Naturalis Biodiversity Center, The Netherlands) who identified them all as *D. invadens*.

We used 2 cm diameter leaf discs to conduct a no-choice bioassay. One slug and one leaf disc were placed on a layer of wet sand in a petri dish. Petri dishes were kept in the dark for 48 hours, after which the leaf disc was scanned to measure the percentage of eaten leaf disc. In the cases in which there were no signs of feeding, we replaced the *Jacobaea* leaf disc with a lettuce leaf disc and controlled it throughout 48 hours. It was observed that most slugs started eating the lettuce within the first 30 minutes. If the slug fed on the lettuce leaf disc, it was assumed that the slug refused to eat the *Jacobaea* leaf disc and thus recorded as zero. If the slug did not eat the lettuce leaf disc, it was assumed that other factors besides the food-source could be affecting the non-feeding behavior of the slug and was considered as a missing value in the data analysis. This occurred four times in total. The experiment was conducted 6 times, each time using the same number of slugs as that of genotypes. In total 498 slugs were used in the bioassay. Each slug and each plant were used only once.

Specialist herbivore bioassay

Longitarsus jacobaeae, a flea beetle, is a specialist herbivore on *J. vulgaris*. This beetle is native to Eurasia and was introduced into US, New Zealand and Australia as a ragwort biocontrol agent (Frick 1969; McLaren et al. 1999; Syrett et al. 1991). *Longitarsus jacobaeae* and the cinnabar moth *Tyria jacobaeae* have the same efficient *N*-oxidization enzyme to sequester PAs (Dobler et al. 2000; Narberhaus et al. 2003; Naumann et al. 2002), but they display different oviposition behavior and feed on *Jacobaea* plants in different metamorphic stages. *Longitarsus jacobaeae* lays eggs in the soil surrounding the host plants (Windig 1991) while *T. jacobaeae* lays eggs on the leaves. The adults of *L. jacobaeae* feed on leaves of *J. vulgaris*, leaving a characteristic “shot-hole” pattern (Frick 1969). Flea beetles were collected from *J. vulgaris* plants at the Meijendel nature reserve (52°7'548"N, 4°19'46"E, The Netherlands) with portable vacuum cleaners (DOMO DO211S, 14.4 V, Herentals, Belgium). After collection, they were kept in a climate room (humidity 70%, light 16 h/dark 8 h, 20°C/20°C, light intensity 130 $\mu\text{m}^2/\text{sec}$) at the Institute of Biology Leiden, and fed with *J. vulgaris*. Each beetle was used only once for the bioassay.

We conducted a multiple-choice bioassay with whole leaves in a climate room (humidity 70%, light 16h/ dark 8h, 20°C/20°C, light intensity 130 $\mu\text{m}^2/\text{sec}$). Flea beetles were starved for 48 hours before initiating the experiment. Firstly, a plastic box filled with floral foam was placed in an insect cage (Bug Dorm 32.5×32.5×77.0 cm). Water was added until the floral foam reached full water capacity. Then the middle leaf of each genotype plant was cut with a scalpel and inserted into the floral foam in a random position in a grid, 5 cm apart from each other. All the leaves kept fresh and none wilted during the experiment. We released the same number of beetles as genotypes into the insect cage in each trial. The beetles were allowed to feed for 48 hours. The experiment was carried out six times and 484 beetles were released in this bioassay. Herbivory was measured as the number of holes and leaf surface area consumed (mm^2). Just before the experiment the fresh weight of the leaves was recorded as an indicator of plant size.

Pyrrolizidine alkaloid data

Three to six replicates per genotype in F2 hybrids were analyzed by LC-MS/MS as described by Cheng et al. (2011a) and the mean PA values obtained were used for our analysis. In the F2 hybrid cross, 37 individual PAs were detected (Cheng et al. 2011a). Based on their presumed biosynthesis, PAs have been classified into four main groups: senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Cheng et al. 2011a; Pelser et al. 2003). These alkaloids exist in two forms: tertiary amine and *N*-oxide (Hartmann 1999). A large variation in the concentration of *N*-oxides (267.4 to 3927.9 $\mu\text{g/g}$) has been reported in F2 hybrid shoots (Cheng et al. 2011a).

The PA concentration and composition have proved to possess a high narrow sense heritability (Vrieling et al. 1993). To further confirm that PA concentration is strongly genotype-dependent, we chose 20 genotypes from the F2 hybrids that had

also been used by Cheng et al. (2011a) and grew them under the same conditions (Cheng et al. 2011a). The PA content was determined again by LC-MS/MS. The results showed that the PAs of the plant measured by Cheng et al. (2011a) were highly correlated with the values obtained for this independent set of genotypes. There was a high correlation between the total PA concentration (Spearman correlation: $r=0.7$, $P=0.001$) and that of four groups of PAs (Spearman correlation: $r>0.7$, $P<0.001$) of the two sets of plants with the same genotypes.

Statistical analysis for slug bioassay

We used a general linear model to check whether the trials influenced the feeding damage of the slug *D. invadens*. The percentage of feeding damage was used as the dependent variable, and trials were considered as a random factor. The percentage of feeding damage was arcsine square root transformed to achieve normality. Normality was confirmed by Shapiro-Wilk tests. As there were significant differences among trials, we decided to use the unstandardized residuals (slug feeding residual, SFR) for further tests. General linear models were then performed on SFR with plant genotype as a random factor to determine whether slug resistance differed among the F2 hybrids.

We calculated genotypic mean SFR and used these in linear multiple regression to test whether the four structural groups of PAs were correlated with slug feeding. The genotypic mean concentrations of the four structural PA groups were log transformed and considered as independent variables.

Only the concentration of spartioidine and spartioidine N-oxide were normal distributed, so all the other individual PAs were log transformed to achieve normality. However, even after log transformation five PAs were not normally distributed and in these cases we used a Spearman rank correlation test. In all other cases a two-tailed Pearson correlation test was applied. To correct for multiple testing, the P -values of the individual tests were adjusted with the sequential Bonferroni method.

Statistical analysis for flea beetle bioassay

Two variables were used to measure flea beetle feeding damage: the number of holes and the total area consumed (mm^2). As both variables were highly correlated with each other ($R^2=0.884$, $n=484$, $P<0.001$), only the total area consumed was used for further analysis.

We applied a general linear model to correct the data for differences among trials and used the unstandardized residuals (beetle feeding residual, BFR). In this model, feeding damage by the flea beetle was defined as the dependent variable and the trial was defined as the random factor. We also used general linear models to determine whether feeding damage by the flea beetle differed in relation to the plant genotype. The BFR was considered as the dependent variable, and plant genotype as the random factor with the fresh weight of the leaf as a covariate.

A multiple regression analysis was conducted to determine whether the four structural groups of PAs affected the feeding choice of flea beetles. The BFR was considered as the dependent variable. The genotypic mean concentrations of the four structural PAs groups and the leaf fresh weight were used as independent variables. The PA concentrations were log transformed. Two-tailed Pearson or Spearman rank correlation tests were conducted between BFR and the concentrations of individual PAs.

Comparison of proxies for herbivore feeding

Three other herbivores have been tested using the same set of F2 genotypes: the specialist lepidopteran cinnabar moth *Tyria jacobaeae* (oviposition bioassay), the generalist thrips *Frankliniella occidentalis* (silver damage bioassay), and the generalist leaf miner *Liriomyza trifolii* (pupae number bioassay) in previous studies (Cheng et al. 2011b; Cheng 2012; Cheng et al. 2013). Two-tailed Pearson correlation tests were performed to check for correlations among the different bioassays. The BFR corrected for leaf fresh weight and SFR were used for the feeding damage of flea beetles and slugs respectively. In the case of the cinnabar moth oviposition bioassay, egg batch numbers were corrected for cage number. The silver damage was log transformed for normality and the number of leaf miner pupae was corrected for plant size. The Pearson correlations between herbivore feeding estimates and PAs were summarized. The concentration of total tertiary amines, total *N*-oxides and four groups of PAs were also log transformed.

All analyses were conducted in SPSS 19.0.

Results

Slug herbivore feeding damage and response to pyrrolizidine alkaloids

Slug feeding damage was below 60% in all F2 genotypes and below 30% in 67 of them (Fig. S1a). As feeding of the slugs differed among trials ($F_{5, 492}=5.68$; $P<0.001$) we used slug-feeding residuals (SFR) corrected for trials for the next tests. The SFR were genotype-dependent ($F_{86, 411}=2.69$; $P<0.001$).

A significant negative correlation was observed between the total PA, *N*-oxides and senecionine-like PAs concentrations and SFR (Fig. 1a, b) while the other groups of PAs had no significant correlation with SFR (Fig. 1c, d, e). The multiple regression analysis also showed that senecionine-like PAs had a significant negative effect on SFR. In contrast to the simple correlation between the four structural PA groups and SFR, the otosenine-like PAs had a positive effect on SFR while the other two groups exerted no effect (Table 1).

After correction for the number of tests by the sequential Bonferroni test, two-tailed Pearson or Spearman rank correlation tests showed that SFR was negatively correlated with the concentration of seneciphylline, seneciphylline *N*-oxide, spartioidine, spartioidine *N*-oxide, senecivernine and jacozone *N*-oxide (Table S1,

Fig. S1).

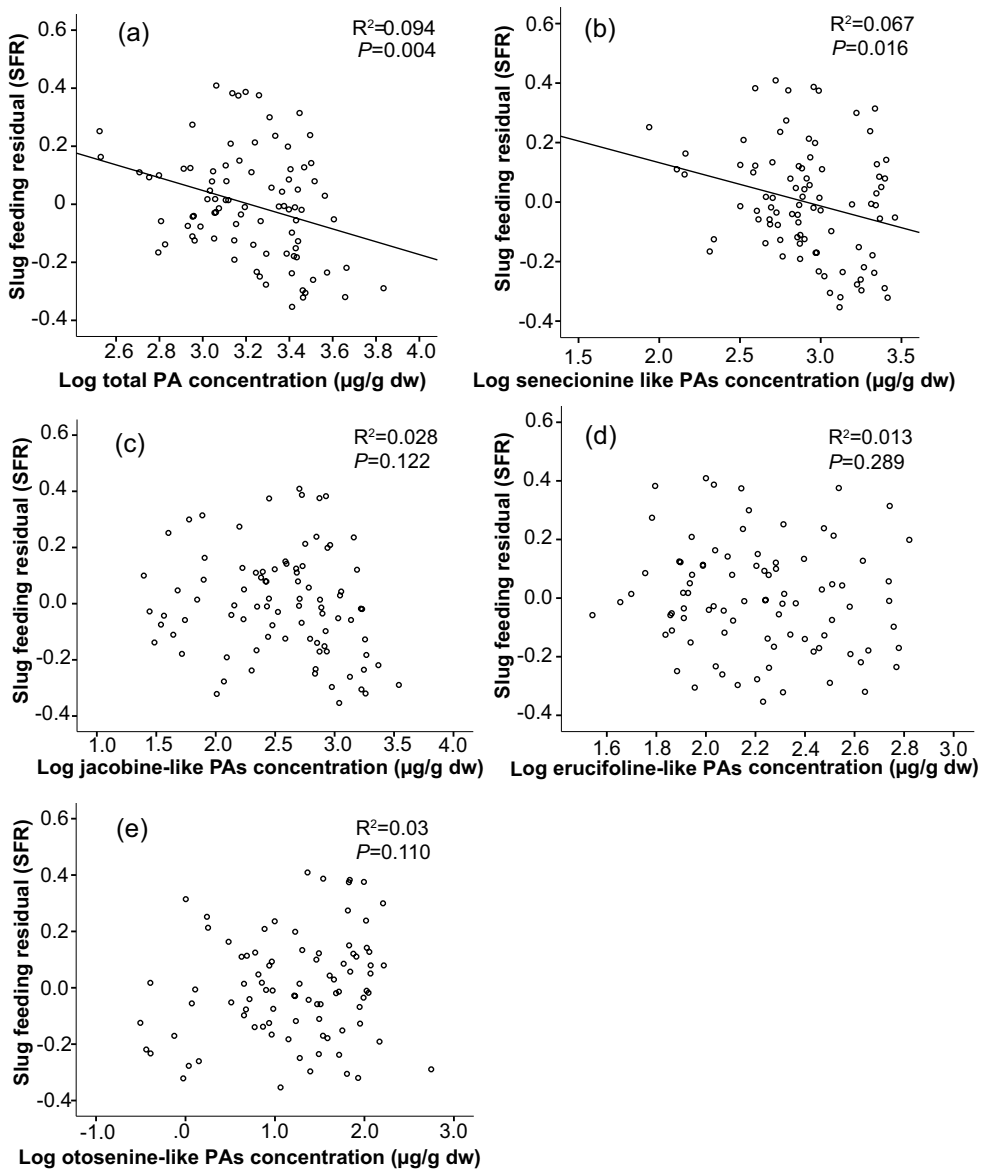


Fig. 1 Scatter plots of the slug arcsine square root-transformed percentage of feeding damage corrected for trial (SFR) and the concentration of total pyrrolizidine alkaloids (PAs) (a), senecionine-like PAs (b), jacobine-like PAs (c), erucifoline-like PAs (d), otosenine-like PAs (e) of 87 F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. All PAs were log transformed to obtain normal distribution. Each dot represents the genotypic mean value of one of the 87 F2 genotypes.

Table 1 Multiple regression of the slug feeding residual (SFR) against the sum concentration of the four main structural groups of pyrrolizidine alkaloids (PAs, $\mu\text{g/g}$ dw) in the host plants of 87 F2 hybrid genotypes from a cross between *Jacobaea vulgaris* and *Jacobaea aquatica*. For the regression model: adjusted $R^2=0.0973$; $F_{4,82}=3.318$; $P=0.014$

Predictors ^a	Estimate	<i>t</i> value
(Intercept)	-0.001	-0.062
Sn	-0.049	-2.479*
Jb	-0.030	-1.556
Er	-0.007	-0.354
Ot	0.042	2.195*

^a Sn, Jb, Er and Ot are the sum concentration of senecionine-, jacobine-, erucifoline- and otosenine- like PAs

* $P<0.05$

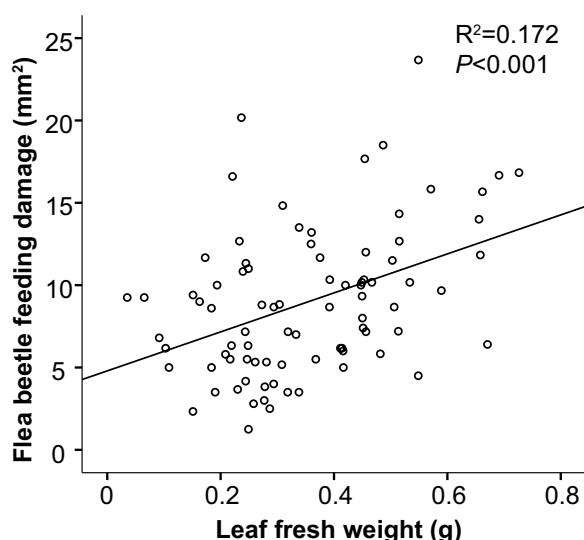


Fig. 2 Scatter plot of the genotypic mean of leaf fresh weight and genotypic mean of flea beetle feeding damage. Each dot is the mean of 4-6 replicates of 84 F2 genotypes.

Flea beetle preference and response to pyrrolizidine alkaloids

The genotypic mean leaf area removed by flea beetle feeding ranged from 0 to 25 mm² (Fig. S1b). As the feeding damage of the flea beetle differed among trials ($F_{5,478}=4.147$; $P=0.001$) we used flea beetle feeding residual (BFR) corrected for trials for the next tests.

While BFR did not differ among plant genotypes ($F_{83,397}=1.098$; $P=0.277$) leaf fresh weight had a positive effect on the BFR ($F_{1,397}=9.084$; $P=0.003$).

Multiple regression analysis showed a significant positive correlation between BFR and leaf fresh weight (Table 2, Fig. 2), but there were no significant correlations between the four structural groups of PAs and BFR (Table 2).

No correlation was observed between the individual PAs and BFR (data not

shown). There was, however, a significant positive correlation with the fresh weight of the leaf ($R^2=0.172$, $P<0.001$) (Fig. 2).

Table 2 Multiple regression of flea beetle feeding residual (BFR) corrected for trials against the sum concentration of four structural groups of pyrrolizidine alkaloids (PAs, $\mu\text{g/g dw}$) in the 84 F2 hybrid genotypes from a cross between *Jacobaea vulgaris* and *Jacobaea aquatica*. For the regression model: adjusted $R^2=0.2425$; $F_{15,68}=2.771$; $P=0.002$

Predictors ^a	Estimate	<i>t</i> value
(Intercept)	-5.773	-1.112
Fw	11.680	3.706**
Sn	0.234	0.162
Jb	-0.117	-0.126
Er	0.276	0.180
Ot	0.492	0.771

^a Sn, Jb, Er, Ot are log transformed concentration of senecionine-, jacobine-, erucifoline- and otosenine- like PAs. Fw is the genotypic mean of leaf fresh weight

** $P<0.01$

Table 3 Pearson correlation tests among five different herbivore feeding^a bioassays of *Jacobaea* F2 hybrids from a cross between *Jacobaea vulgaris* and *Jacobaea aquatica*

	Specialist herbivores		Generalist herbivores		
	Flea beetle (N=87)	Cinnabar moth- (N=35)	Slug (N=84)	Thrips (N=98)	Leafminer (N=90)
Flea beetle					
Cinnabar moth	0.057				
Slug	-0.191	-0.136			
Thrips	-0.101	-0.047	0.334**		
Leafminer	0.094	-0.189	-0.091	-0.095	

^a Proxies for herbivore feeding used: flea beetle feeding residual, corrected for leaf fresh weight, for the flea beetle (*Longitarsus jacobaeae*) bioassay; the number of egg batches, corrected for cage number, for the cinnabar moth (*Tyria jacobaeae*) oviposition bioassay; the slug feeding residual (SFR) for the slug (*Deroceras invadens*) bioassay; the log transformed silver damage for the Western flower thrips (*Frankliniella occidentalis*) bioassay and the number of pupae, corrected for plant size, for the American serpentine leafminer (*Liriomyza trifolii*) bioassay.

** $P<0.01$

Table 4 Summary of Pearson correlations between pyrrolizidine alkaloid (PA) concentrations and the proxies of herbivore feeding^a in the five bioassays conducted with *Jacobaea* F2 hybrids from a cross between *Jacobaea vulgaris* and *Jacobaea aquatica*

PA	Specialist herbivores		Generalist herbivores		
	Flea beetle	Cinnabar moth	Slug	Thrips	Leafminer
	(N=87)	(N=35)	(N=84)	(N=94)	(N=90)
Total tertiary amines	0.079	0.391*	-0.206	-0.266**	-0.235*
Total N-oxide	0.042	0.043	-0.283**	-0.282**	-0.060
Senecionine-like PAs	0.030	-0.042	-0.259*	-0.204*	-0.091
Jacobine-like PAs	0.017	0.350*	-0.167	-0.297*	-0.188
Erucifoline-like PAs	-0.004	-0.078	-0.115	-0.159	0.226*
Otosenine-like PAs	0.088	0.303	0.173	0.030	-0.267*

^a The feeding damage data used is the same as that in Table 3

* $P < 0.05$, ** $P < 0.01$

Discussion

The results of this study revealed that PAs, one of the major groups of SMs of *Jacobaea* hybrid plants, deterred the generalist herbivore *D. invadens*. We also observed that the feeding damage inflicted by this generalist slug was genotype-dependent, a fact that could be partly explained by the genotype-dependent PA concentrations. Cheng et al. (2011a) had shown that PA concentrations varied widely with the genotype. A negative correlation was observed for senecionine-like PAs and considering half of total *N*-oxide concentration is derived from senecionine-like PAs, a correlation was established between slug feeding damage and total *N*-oxide concentration.

Among the 17 senecionine-like PAs, seneciphylline, seneciphylline *N*-oxide, spartioidine, spartioidine *N*-oxide and senecivernine were negatively correlated with slug feeding (Table S1). It is important to note that there is a significant difference among the concentration of these five PAs. The concentration of seneciphylline *N*-oxide is more than ten times that of the other four PAs combined. Interestingly, all these PAs have a double bond between C13 and C19 except for senecivernine (Fig.S3). In fact, eight of the senecionine-like PAs have a double bond at C13, four of which were found to be negatively correlated with slug feeding. These PAs are highly correlated with each other and also correlated with the other senecionine-like PAs that do not have the C13 double bond. Apart from this type of PAs, three jacobine-like PAs also have a double bond at C13 and are negatively correlated with slug feeding. These three PAs were significantly correlated with those senecionine-like

PAs with a double bond at C13 while they were not correlated to senecionine-like PAs without this double bond. These observations strongly suggest that the presence of this double bond is related to the slug deterrence.

The multiple regression analysis of *D. invadens* showed that otosenine-like PAs had positive effects on slug feeding. Even though we did not find significant simple correlations between slug feeding and the total otosenine-like ($r=0.173$) or individual otosenine-like PAs (Table S1), the correlation coefficient was significantly positive. Furthermore, the concentration of otosenine-like PAs is relatively low, often below 2% of the total PA content.

In this study, the specialist *L. jacobaeae* preferred feeding on larger leaves. Adult herbivory by flea beetles has been reported to be proportional to plant size in the field (Windig 1993). Specialist herbivory is often restricted to one type of food source and they may easily face food shortage, so it is a useful strategy to feed on big plants. Plant size was also shown to be a significant factor in the oviposition choice for the specialist moth *T. jacobaeae* (Cheng et al. 2013).

A previous study had revealed that there was no significant positive correlation between the cinnabar moth oviposition preference of *J. vulgaris* plants and their PA content (Macel et al. 2002). However, that study included only a limited number of PAs. With the help of more sensitive technology and extended analyses, including measurements of the tertiary amine and *N*-oxide forms, Cheng et al. (2013) was able to show that *T. jacobaeae* preferred laying eggs on plants with higher amounts of tertiary amines of jacobine-like PAs. *Longitarsus jacobaeae* has not been as extensively studied as *T. jacobaeae*. Kirk et al. (2012) studied the feeding preference of several *Longitarsus* species, including *L. jacobaeae*, on a range of different *Jacobaea* and *Senecio* plants. Their conclusion that the PA content in plants did not affect flea beetle feeding preference is consistent with our observations on the absence of correlations with the genotype and with the four structural PA groups. However, even though *L. jacobaeae* is apparently not attracted by PAs, it does sequester them (Dobler et al. 2000).

As mentioned above, Cheng et al. (2013) conducted an oviposition bioassay using the specialist *T. jacobaeae* and found that they preferred plants with higher amounts of tertiary amines of jacobine-like PAs. In the bioassay with the generalist *Frankliniella occidentalis*, feeding damage has been reported to be negatively correlated with the total PA content and with that of jacobine-like PAs (Cheng et al. 2011b). The *Liriomyza trifolii* pupae bioassay has revealed a weak negative correlation between the number of pupae and the concentration of otosenine-like PAs (Cheng 2012). Our study showed that senecionine-like PAs deterred the feeding of the slug *D. invadens*. Therefore, it appears that different groups of PAs are attractive or deterring to different herbivores. Collectively, these findings suggest that PA diversity is maintained by the selection of different generalist and specialist herbivores.

Comparing the different bioassays, we only found a significant positive correlation between slug feeding damage and thrips silver damage (Table 3). There were no other significant correlations. It is noteworthy that five over six correlations between the feeding damage of generalist and specialist herbivores were negative (Table 3). The results showed with the exception of the flea beetle, the amount of herbivore damage was genotype-dependent (Table 4), suggesting that there are multiple resistance mechanisms against herbivores in *Jacobaea* plants. In general only about 10 percent of the variance in resistance can be explained by PAs implying that other secondary or primary metabolites, or physical barriers may also play an important role (Nuringtyas et al. 2012).

Due to practical issues, we designed no-choice and multiple-choice bioassays. Many studies have revealed that both approaches often yield the same results. For instance, *L. jacobaeae* feeding preferences in a multiple-choice experiment were highly correlated with the results from a no-choice experiment (Spearman rank correlation: $r=0.779$, $n=17$, $P<0.001$) (Kirk et al. 2012). In addition, the feeding damage of thrips (*Frankliniella occidentalis*) on F2 hybrids (on a subset of the F2 hybrids plants of our study) from a choice bioassay was consistent with the results obtained in a non-choice bioassay (Leiss et al. 2009). It is thus most likely that the approach did not affect our conclusions.

The *Jacobaea* F2 hybrids study system has already been used in a number of herbivore bioassays (Cheng et al. 2011b; Cheng 2012; Cheng et al. 2013; Leiss et al. 2009). Hybridization has been proved to lead to a greater quantitative and qualitative variation of SMs (Cheng et al. 2011a; Fritz 1999; Orians 2000). The other advantage of using an F2 progeny is that specific traits can be studied against an average equal genetic background (Hochwender et al. 2000; Lexer et al. 2003). The considerable and novel PA diversity present in *Jacobaea* F2 hybrids can lead to variations in the performance of insect herbivores (Cheng et al. 2011a; Macel et al. 2003; van Dam et al. 1995). Hybrids are thus very useful to investigate the functions of plant secondary metabolites in resistance to herbivores (Cheng et al. 2011c).

In summary, our results showed that the generalist herbivore slug was deterred by senecionine-like PAs but the PA content in *Jacobaea* plants did not affect the specialist herbivore flea beetle. Leaf fresh weight was positively correlated with flea beetle feeding, suggesting that plant size could be more important than PA concentration for specialist herbivores. Altogether, the results of the current experiment combined with previous herbivore tests on the same experimental system (Cheng et al. 2011b; Cheng et al. 2012; Cheng et al. 2013) produced only partial evidence to support the Generalist-Specialist dilemma. The concentration of at least one PA type was negatively correlated with generalists while the specialist caterpillar showed positive correlations with some PA types but no significant correlations with the specialist flea beetle. Additionally, we found no negative correlations between specialist

and generalist feeding. In part, this may be caused by unknown factors that influence specialists and generalists in the same way. Thus, though the Generalist-Specialist dilemma was only partly proved here, our results clearly revealed that the divergent selection pressure from the different insect herbivores are important for the evolution and function of PA diversity.

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Supplementary Materials:

Table S1 Two tailed Pearson/Spearman correlation tests between slug feeding residual (SFR) and the concentrations of individual pyrrolizidine alkaloids (PAs, $\mu\text{g/g dw}$) in the 87 F2 genotypes from a cross between *Jacobaea vulgaris* and *Jacobaea aquatica*

Group	PA	r/r_s	P
Senecionine-like PAs	senecionine	-0.201	0.062
	senecionine <i>N</i> -oxide	-0.184	0.087
	integerrimine	-0.160	0.139
	integerrimine <i>N</i> -oxide	-0.131	0.227
	retrorsine	-0.125	0.249
	retrorsine <i>N</i> -oxide	-0.005	0.962
	usaramine	-0.112	0.300
	usaramine <i>N</i> -oxide ^a	-0.041	0.706
	riddelliine	0.205	0.057
	riddelliine <i>N</i> -oxide	-0.124	0.251
	seneciphylline	-0.374	<0.001*
	seneciphylline <i>N</i> -oxide	-0.342	0.001*
	spartioidine	-0.380	<0.001**
	spartioidine <i>N</i> -oxide	-0.345	0.001*
	acetyl­seneciphylline	0.068	0.531
	acetyl­seneciphylline <i>N</i> -oxide	0.638	0.265
	senecivernine	-0.341	0.001*
Jacobine-like PAs	jacobine	-0.113	0.297
	jacobine <i>N</i> -oxide ^a	-0.171	0.114
	jacoline	-0.100	0.356
	jacoline <i>N</i> -oxide ^a	-0.156	0.149
	jaconine	-0.093	0.394
	jaconine <i>N</i> -oxide ^a	-0.159	0.142
	jacozine	-0.238	0.027
	jacozine <i>N</i> -oxide	-0.336	0.001*
	dehydrojaconine	-0.259	0.015
	erucifoline	-0.095	0.384

Erucifoline-like PAs	erucifoline <i>N</i> -oxide	-0.137	0.204
	acetylerucifoline	0.045	0.682
	acetylerucifoline <i>N</i> -oxide	0.011	0.920
Otosenine-like PAs	senkirkine ^a	-0.002	0.985
	otosenine	0.163	0.131
	onetine	0.123	0.256
	desacetyldoronine	0.137	0.207
	florosene	0.141	0.195
	floridanine	0.088	0.426
	doronine	0.161	0.142

^a Spearman correlation tests were carried out for PAs with not normally distributed concentrations

* $P < 0.05$, ** $P < 0.01$ indicates significance after the sequential Bonferroni test

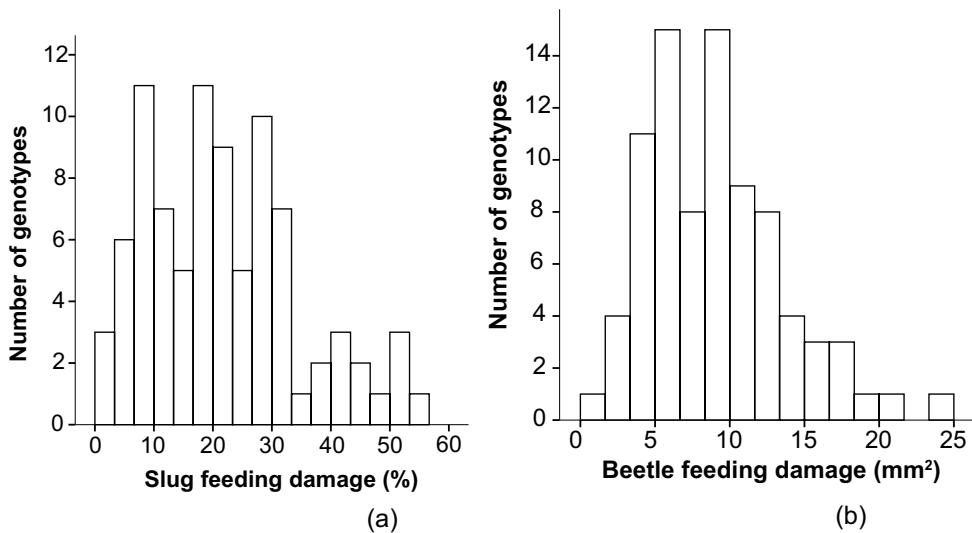


Fig. S1 (a) Frequency distribution of genotypic mean percentage of slug feeding damage of 87 F2 hybrids. Each genotype is the mean of 4-6 replicates. In total, 498 plants were used in the slug bioassay. (b) Frequency distribution of the genotypic mean square millimeter of flea beetle feeding damage of 84 F2 hybrids. Each genotype is the mean of 4-6 replicates. In total, 484 plants were used in the flea beetle bioassay.

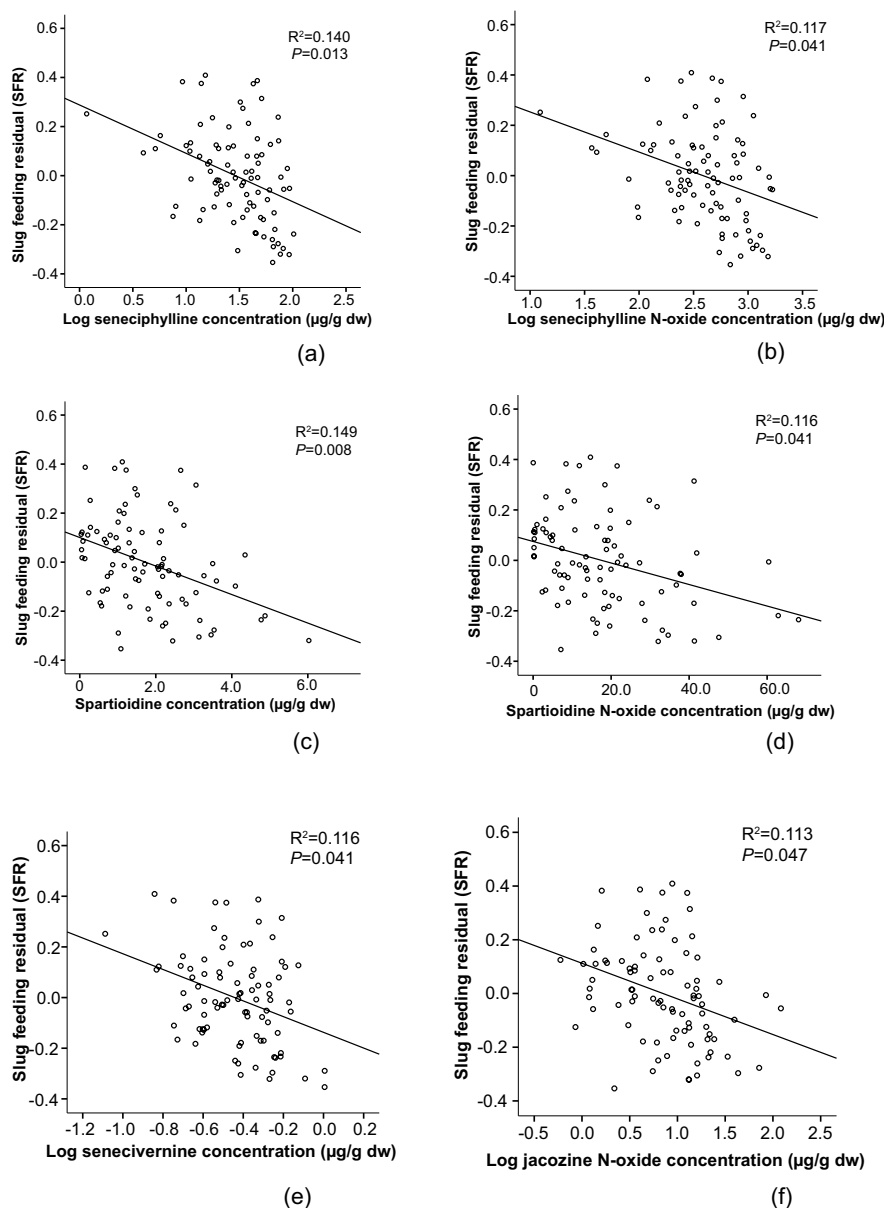


Fig. S2 Scatter plots between slug arcsine square root transformed percentage of feeding damage corrected for trial (SFR) and the concentration of individual pyrrolizidine alkaloids (PAs): seneciphylline (a), seneciphylline N-oxide (b), spartioidine (c), spartioidine N-oxide (d), senecivernine (e), jacozone N-oxide (f) of 87 F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*. The genotypic mean of PA concentrations (except for spartioidine and spartioidine N-oxide) were log transformed. Each P value was corrected by sequential Bonferroni test. Each dot represents the genotypic mean value of one of 87 F2 genotypes.

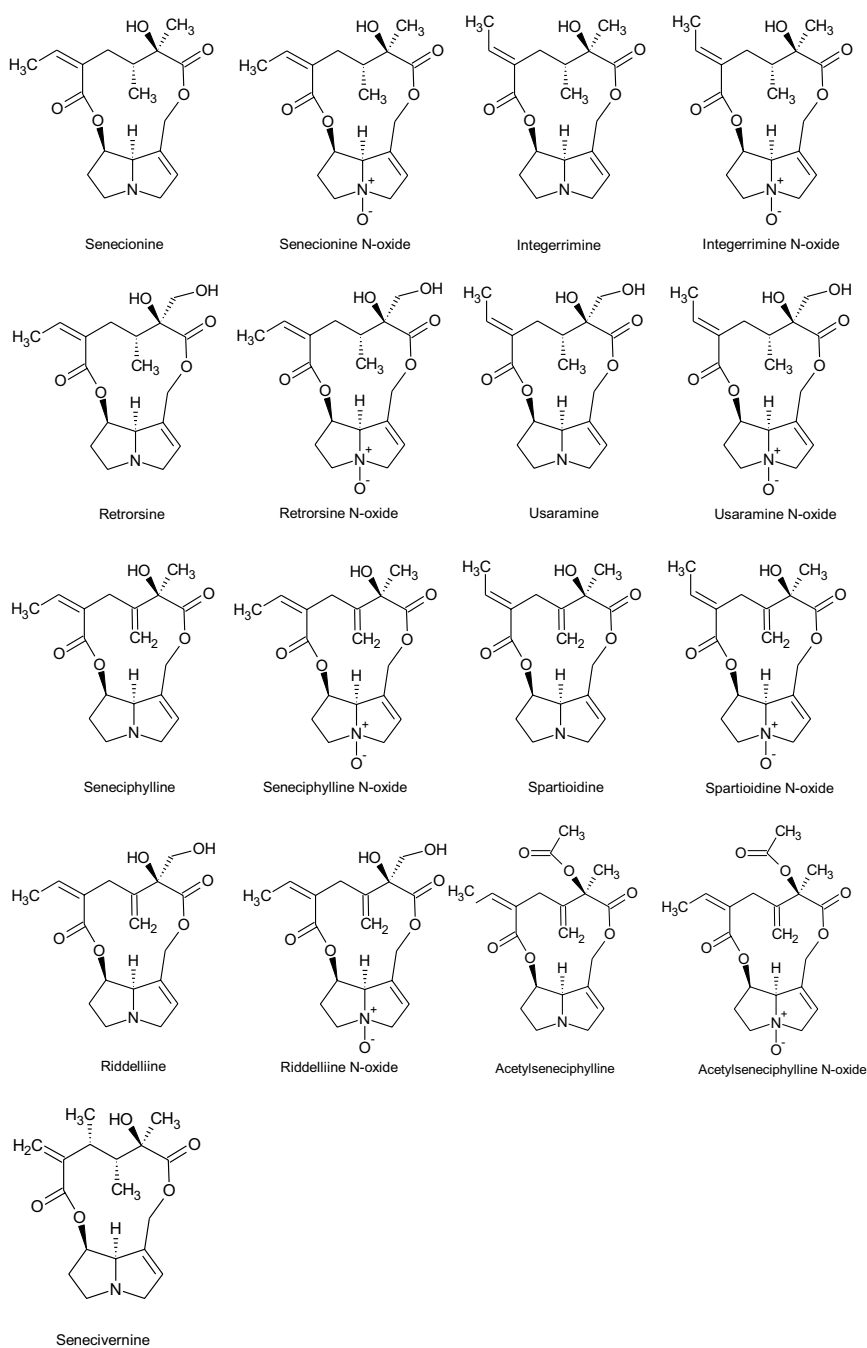


Fig. S3 Chemical structures of senecionine-like PAs.

Chapter 5

The effects of exogenous application of methyl jasmonate on the concentration and composition of pyrrolizidine alkaloids and resistance towards *Spodoptera exigua* in two *Jacobaea* species¹

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¹ This chapter has been submitted to *Plant Science* for publication in an adapted form.

Abstract

Treating plants with methyl jasmonate (MeJA) can mimic the effects of herbivory by activating the octadecanoid pathway in plants. Many studies have shown that the concentration and composition of pyrrolizidine alkaloids (PAs), the secondary metabolites of *Jacobaea* species, play a role in resistance against herbivores. The aim of this study was to determine whether the application of MeJA changed the PA concentration and composition and if affected herbivory by the generalist herbivore *Spodoptera exigua*. A range of concentrations of MeJA was added to the medium of *Jacobaea vulgaris* and *Jacobaea aquatica* tissue culture plants under axenic conditions. After two weeks, plants were harvested and PA concentration and composition were measured by LC-MS/MS. While the application of MeJA to the roots only marginally affected the total PA concentration in whole plants, the total PA concentration decreased in roots but increased in shoots of *J. vulgaris*. This indicates that applying MeJA does not elicit *de novo* synthesis and that PAs are reallocated from root to shoot. In *J. aquatica* the total PA concentration was maintained and this reallocation was not significant due to the fact that the majority of PAs were already stored in the shoots before the application of MeJA. Interestingly, we found that the application of MeJA in both species induced a strong shift in the PA composition especially from senecionine-like PAs to erucifoline-like PAs indicating that PA conversion took place. Our studies further showed that root induction could lead to PA variation in the shoots of *Jacobaea* plants. In the bioassay, *S. exigua* preferred feeding on control leaves of *J. aquatica* than those treated with MeJA while for *J. vulgaris* the leaves of control and MeJA treated plants were hardly eaten. This result suggests that an increase of erucifoline-like PAs induced by the application of MeJA application play a role in resistance to *S. exigua* in *J. aquatica* while other defence mechanisms might be present in *J. vulgaris*.

Keywords

Jacobaea vulgaris, *Jacobaea aquatica*, *Spodoptera exigua*, Feeding damage, Tissue culture, Reallocation, Conversion, Biosynthetic pathway, Senecionine, Erucifoline.

Introduction

Secondary metabolites (SMs) are regarded as indispensable for the fitness and survival of an organism (Wink 2003). The synthesis of many SMs in plants is in part controlled by the jasmonic acid (JA) pathway (Gundlach et al. 1992; Karban and Baldwin 2007), which is activated especially in response to chewing/biting herbivores, cell-content feeders and necrotrophic pathogens (Glazebrook 2005; Kawazu et al. 2012; Walling 2000). Herbivore attack may cause an increase in endogenous jasmonate levels that leads to the production of defensive proteins and SMs or morphological changes, such as an increase in trichome density (Farmer et al. 2003; Traw and Dawson 2002; Traw and Bergelson 2003; Zhao et al. 2005). The exogenous application of jasmonate, including JA and methyl jasmonate (MeJA), is widely used as a biochemical tool to elicit the production of SMs and to study plant defence responses (Chen et al. 2006; Largia et al. 2015; Liang et al. 2006). In a literature review we found that with the exception of three studies, only changes in levels of total groups of SMs or one major compound was studied after MeJA application (Table S1). However, one same group of SMs within a plant species generally includes many structurally diverse compounds. For example, 35 different glucosinolates have been identified in *Arabidopsis thaliana* (D'Auria and Gershenzon 2005). Interestingly not a single report shows changes in the qualitative composition of SMs after MeJA treatments although it is well known that this type of change can affect herbivores or pathogens profoundly (e.g. Berenbaum et al. 1991; Bruinsma et al. 2007; Cheng et al. 2013; Hung et al. 1995; Kowalchuk et al. 2006).

To explore the influence of jasmonate on the concentration and qualitative composition of SMs, we used *Jacobaea vulgaris* and *Jacobaea aquatica* as a study system. All *Jacobaea* PAs are classified into four groups according to their basic structure: jacobine-, erucifoline-, ostenoline- and senecionine-like PAs (Cheng et al. 2011a; Hartmann 1999). The diversity of PA structures in these plants provides an opportunity to zoom in on the variation in composition of SMs within plant species. These alkaloids have been extensively studied as they play a role in defence against insect herbivores as well as soil-born microbes (Hol and van Veen 2002; Macel 2011). An experiment with cell lines of *Spodoptera exigua* showed jacobine and erucifoline to be the most toxic (Nuringtyas et al. 2014). We therefore expected that if MeJA application induced an increase of PAs, these would consequently lead to a reduction of the herbivory by a chewing generalist insect herbivores such as *S. exigua*.

The variation of PA concentration and composition has a genetic basis (Macel et al. 2004; Vrieling et al. 1993), but it is also influenced by environmental conditions. Macel and Klinkhamer (2010) observed that the PA composition of *J. vulgaris* plants changed after they were transferred from the laboratory to experimental sites in natural populations. Soil-type and soil-borne microorganisms also proved to affect

PA composition in *J. vulgaris* (Joosten et al. 2009). To avoid the effect of potential interactions with microorganisms on the jasmonate application we therefore performed the experiments under axenic conditions.

Thus, to investigate the effects of exogenously applied MeJA on PA concentration and composition and on herbivory, we used *J. vulgaris* and *J. aquatica* tissue culture plants and the generalist herbivore *S. exigua*. Specifically, we addressed the following questions: 1) Does a MeJA treatment lead to dose-related changes in the total PA concentration? 2) Does a MeJA treatment lead to dose-related changes in PA composition? 3) Does MeJA application lead to PA reallocation and conversion? 4) Does MeJA application affect the herbivory by *S. exigua*?

Materials and Methods

Plant species

The *J. vulgaris* and *J. aquatica* individuals used in this study are the parental plants of a well-studied hybrid cross maintained in tissue culture (Cheng et al. 2011a). *Jacobaea vulgaris* was derived from seeds collected at the Meijndel Nature Reserve (52°7'54"N, 4°19'46"E, The Netherlands), and *J. aquatica* from the seeds collected at the Zwanenwater Reserve (52°48'38"N, 4°41'7"E, The Netherlands). The two genotypes representing the two species were multiplied into 70 replicates each in Murashige and Skoog medium (MS-0 medium) with 0.1 mg benzylaminopurine. After application each plant was first kept in 5 ml of MS-0 medium without MeJA for two weeks to induce a fully developed root system (Fig. S3). Plants were checked weekly for fungal or bacterial infections in the medium. Infected plants were not included in the experiment. Plants were grown in a climate room (humidity 50%, light 16h /dark 8h, 20°C/20°C).

Methyl jasmonate treatment

Methyl jasmonate was dissolved in a 1% solution of ethanol in water in a concentration range of 0.00 to 5.00 $\mu\text{g } \mu\text{l}^{-1}$. The plants were assigned to one of seven treatments: control, 0.00 (ethanol control), 0.05, 0.10, 0.50, 1.00 and 5.00 $\mu\text{g } \mu\text{l}^{-1}$ MeJA. Due to the fact that the PAs are produced in the roots, for each treatment, except the control, 100 μl of the solution was added to the agar-based medium. A test with a solution containing a dye showed that within a few hours this amount of solution diffused into the medium. The control and ethanol control (100 μl 1% ethanol aqueous solution=0.00 $\mu\text{g } \mu\text{l}^{-1}$ MeJA) were added to evaluate for the effect of the ethanolic solvent. Each treatment was replicated 5 times. The addition of the solution was done in a flow cabinet in order to keep the plants and the medium axenic. In addition, thirteen more replicates of each species with an ethanol control and 1.00 $\mu\text{g } \mu\text{l}^{-1}$ of MeJA were cultured for a herbivory test with *S. exigua*.

Plant harvest

Two weeks after application of MeJA, the plants were harvested. Shoots and roots were separated, wrapped in aluminium foil and immediately submerged in liquid nitrogen. All samples were freeze dried at -80°C (Cryotheque®, Sniders Scientific Company, Tilburg, The Netherlands) for 4 days. One root sample of *J. aquatica* was lost, leaving 139 samples in total for biomass and PA analysis. The dry mass of roots and shoots were recorded.

Pyrrolizidine alkaloid analysis

Pyrrolizidine alkaloids are present in two forms: tertiary amines and N-oxides. Based on their chemical structure and biosynthetic pathway, PAs are classified into four groups: senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Cheng et al. 2011a). Pyrrolizidine alkaloid analysis was carried out using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The protocol was described in detail by Cheng et al. (2011a). In brief, 10 mg freeze dried material was ground well and extracted with 1.0 ml of 2% formic acid solution containing heliotrine ($1 \mu\text{g ml}^{-1}$) as an internal standard. After shaking and centrifugation, 25 μl of the supernatant was diluted with 975 μl of 0.1% ammonia hydroxide solution and 10 μl were injected into a Waters Acquity Ultra Performance Liquid Chromatographic system (UPLC) coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters Corporation, Milford, MA, USA). The instrumental settings used were the same as described by Cheng et al. (2011a). Data were processed in Masslynx 4.1 (Waters Corporation, Milford, MA, USA).

Spodoptera exigua feeding bioassay

The eggs of *S. exigua* were obtained from ENTOCARE (Wageningen, The Netherlands) and cultured in a climate room (humidity 50%, light 16h /dark 8h, 20°C/20°C). After the larvae hatched, they were reared on an artificial diet (Singh 1983). Third instar caterpillars were used in a feeding bioassay.

Similar sized leaves from ethanol control plants and plants treated with MeJA ($1.00 \mu\text{g } \mu\text{l}^{-1}$) were placed on agar in a petri dish to maintain the humidity and freshness of the leaves. The caterpillars were allowed to feed for 4 hours after they had been starved for 12 hours. Thirteen replicate trials were done for each plant species. The area of leaves before and after the test was measured by scanning them, and the area of eaten leaf surface was calculated by subtracting these two areas.

Statistical analysis

One-way ANOVA was used to test if plant dry mass, total PA concentration and PA relative concentration differed among MeJA treatments. Dry mass, total PA concentration and PA relative concentrations were considered as independent variables and MeJA treatment was considered as a fixed factor. The PA relative concentration was calculated as follows: (absolute concentration of an individual PA or a group of PAs) *100/(total PA concentration). Significant ANOVAs were followed by Tukey HSD

post hoc tests with $\alpha=0.05$. The MeJA treatments in ANOVA tests were conducted for the treatments (0.00, 0.05, 0.10, 0.50, 1.00 and 5.00 $\mu\text{g } \mu\text{l}^{-1}$ application of MeJA) and the two controls (control and ethanol control). The second ANOVA was done to test whether the addition of the 1% ethanol solution (0 $\mu\text{g } \mu\text{l}^{-1}$ application of MeJA) to the agar could have influenced plant dry mass, PA concentration or PA composition.

Linear regressions were conducted to verify the correlation between the MeJA concentration and total PA concentration and relative concentration of PAs. The control was not included in the regression analysis. Total PA concentrations (in whole plant, roots and shoots) and relative concentration of PAs (in whole plant, roots and shoots) were defined as dependent variables and MeJA treatments were defined as independent variables.

Pearson correlations were performed to determine if there was a relationship between the concentration of senecionine- or erucifoline-like PAs and the MeJA concentration in the whole plant. They were also performed to determine whether the PA concentration in the shoots was correlated with that in the roots. For all correlations both the PA and the MeJA concentrations were log transformed. In addition, Pearson correlations were performed between senecionine- and erucifoline-like PAs in roots and shoots to make inferences about the biosynthetic pathway.

Wilcoxon matched pairs tests were performed to check whether the leaf area eaten by *S. exigua* was different between ethanol control and MeJA treated leaves of *J. vulgaris* and *J. aquatica*.

All the analyses were conducted in SPSS 21.0.

Results and Discussion

We used 1% ethanol as a solvent for the MeJA. We therefore firstly compared control plants and plants grown on agar to which only ethanol was added (the ethanol control) to test if it had an effect on PA variation. We then compared the MeJA treatments with the ethanol control to investigate the effects of MeJA on PA variation.

Variation of pyrrolizidine alkaloid concentration

PA concentration in whole plant

Ethanol had no effect on the total PA concentration of whole plants (Fig 1A and B). The MeJA treatments had a small but significant effect on the total PA concentration in the whole *J. vulgaris* plants (Fig 1A, $F_{5,24}=4.151$; $P=0.007$) but it did not affect the total PA concentration in *J. aquatica* (Fig 1B, $F_{5,24}=2.531$; $P=0.058$). However, post-hoc tests yielded no significant differences in the total PA concentration between MeJA treatments in *J. vulgaris* either (Fig. 1A). Regression analysis confirmed that the total PA concentration was not significantly related to the MeJA concentration (*J. vulgaris*: $r=-0.316$; $N=30$; $P=0.089$; *J. aquatica*: $r=-0.280$ $N=29$;

$P=0.141$) for neither species. These results indicate that MeJA application did not elicit *de novo* synthesis of PAs. In one of those studies, the total glucosinolate content in leaf surface extracts was similar for control and JA treated *Brassica oleracea* plants (Bruinsma et al. 2007). In another case, the foliar concentration of terpenes and formylated phloroglucinol compounds of *Eucalyptus grandis* was unaffected by MeJA treatments (Henery et al. 2008) and lastly, the oil yield of *Satureja hortensis* was not significantly affected by the foliar application of JA (Pirbalouti et al. 2014).

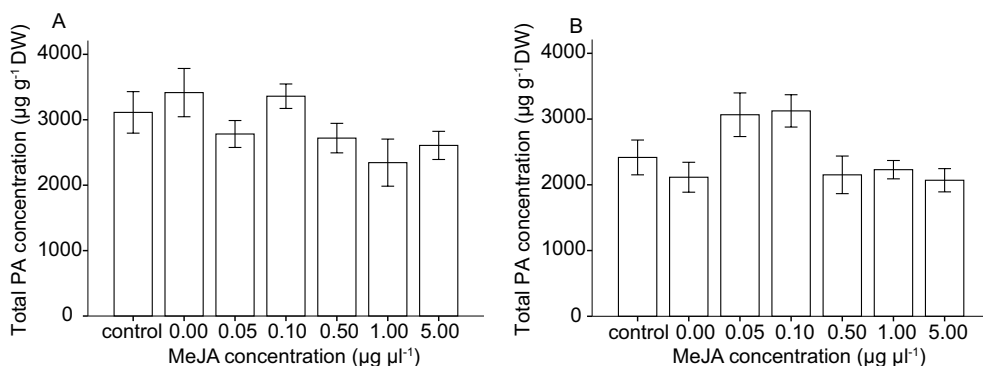


Fig. 1 Effect of methyl jasmonate (MeJA) treatments on the total pyrrolizidine alkaloid (PA) concentration ($\pm$ SE) in whole plants of *Jacobaea vulgaris* (A) and *Jacobaea aquatica* (B). Error bars indicate standard error.

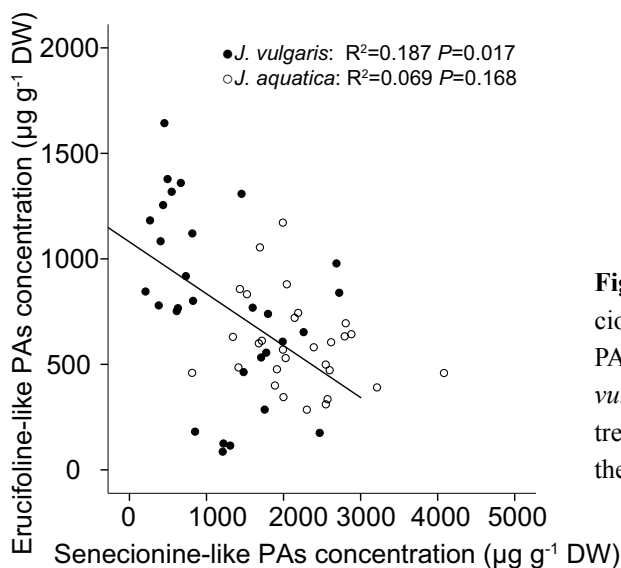


Fig. 2 Correlations between senecionine-like PAs and erucifoline-like PAs in whole plants of *Jacobaea vulgaris* and *Jacobaea aquatica*. The trend line was drawn based only on the samples of *J. vulgaris*.

At the whole plant level, the concentration of senecionine-like PAs decreased while the concentration of erucifoline-like PAs increased with increasing MeJA concentrations in *J. vulgaris* (Fig. S1A and B) and *J. aquatica* (Fig. S1C and D). More-

over, the concentration of senecionine-like PAs was negatively correlated with that of erucifoline-like PAs in *J. vulgaris* (Fig. 2), indicating that PA conversion took place from senecionine-like PAs to erucifoline-like PAs. This negative correlation was not observed in *J. aquatica* (Fig. 2). Kostenko et al. (2013) also found an increase of acetylerucifoline and acetylerucifoline N-oxide concentration in the shoots of *J. vulgaris* after root herbivory by the wireworm *Agriotes linearis* while most senecionine-like PAs decreased. Thus, whenever changes in the PA composition have been detected they have followed a similar pattern, consisting in an increase in erucifoline-like PAs and a decrease of senecionine-like PAs. Previous studies showed that senecionine or senecionine N-oxide had no significant influence on herbivory and mortality of *S. exigua* (Macel et al. 2005; Nuringtyas et al. 2014). In contrast, erucifoline and jacobine were proven to be the most toxic PAs in *S. exigua* cell lines (Nuringtyas et al. 2014).

Pyrrolizidine alkaloid concentration in roots and shoots

The control and the ethanol control did not differ significantly with respect to the total PA concentration in the roots of *J. vulgaris* (Fig. 3A, $F_{1,8}=0.014$; $P=0.909$) and in the shoots of *J. aquatica* (Fig. 3D, $F_{1,8}=1.442$; $P=0.264$). In contrast plants from the ethanol control had a lower total PA concentration in the shoots of *J. vulgaris* (Fig. 3B, $F_{1,8}=5.750$; $P=0.043$) and in the roots of *J. aquatica* (Fig. 3C, $F_{1,8}=8.606$; $P=0.016$) compared to the control plants.

The different MeJA treatments had significant effects on total PA concentration in the roots and shoots of *J. vulgaris* (Fig. 3A and B, root: $F_{5,24}=8.437$; $P<0.001$; shoot: $F_{5,24}=60248$; $P=0.001$). Linear regressions showed that the total PA concentration in *J. vulgaris* roots decreased while that in the shoots increased with increasing MeJA concentration (Fig. S2A and B). The significant negative correlation of PA concentration between roots and shoots suggested that PA reallocation took place from roots to shoots in *J. vulgaris* (Fig. 4). In the control treatment of *J. vulgaris* the total PA concentration in the roots was almost four times than that of the shoots (Fig. 3A and B). Even though the shoot dry mass was slightly higher than root dry mass, it showed that almost 80% of the total amount of PAs were stored in the roots.

In the roots of *J. aquatica*, the total PA concentration decreased significantly with increasing MeJA concentrations (Fig. 3C and S2C, $F_{5,23}=6.573$; $P=0.001$) while MeJA treatments did not induce a change in total PA concentration in the shoots of *J. aquatica* (Fig. 3D and S2D, $F_{5,24}=1.225$; $P=0.328$). In contrast to *J. vulgaris*, the total PA concentration of *J. aquatica* was almost three times higher in the shoots than in the roots of plants from the control treatment implying that approximately 75% of the total PA amount was stored in the shoots in this species (Fig. 3C and D). Therefore, even if PAs of *J. aquatica* were also reallocated from roots to shoots, this would not lead to significant changes in the shoots because the majority of the PAs were already there before the treatment (Fig. 4).

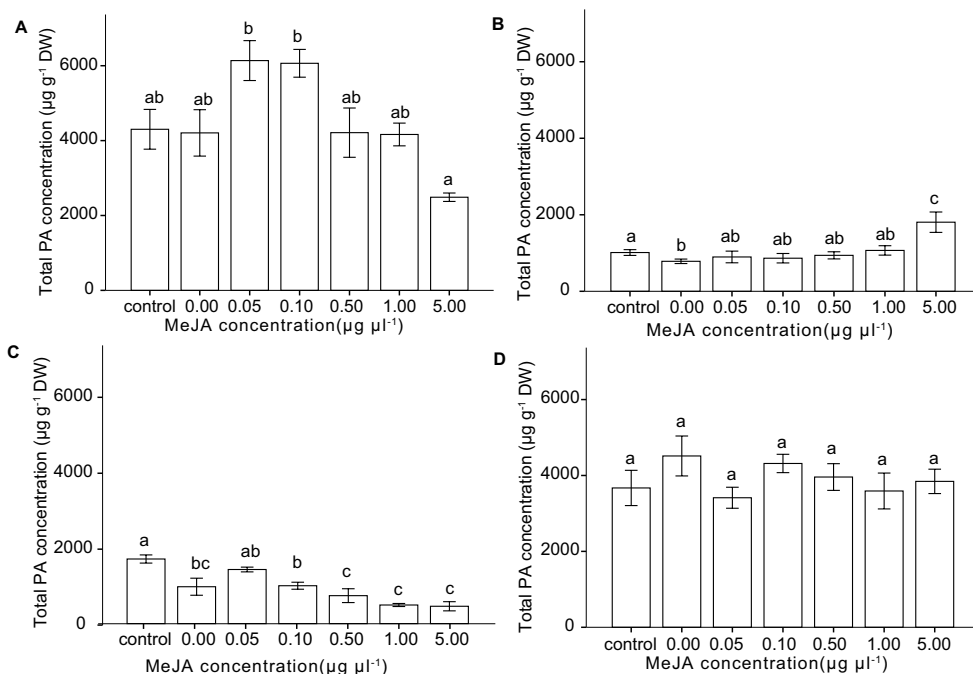


Fig. 3 Effect of methyl jasmonate (MeJA) treatments on total pyrrolizidine alkaloid (PA) concentration ($\pm$ SE, $\mu\text{g g}^{-1}$ dw) in roots and shoots of *Jacobaea vulgaris* and *Jacobaea aquatica*. (A) *J. vulgaris* root, (B) *J. vulgaris* shoot, (C) *J. aquatica* root, (D) *J. aquatica* shoot. Letters show statistically significant differences (one-way ANOVA followed by Tukey HSD post hoc test with $\alpha=0.05$). Error bars indicate standard error.

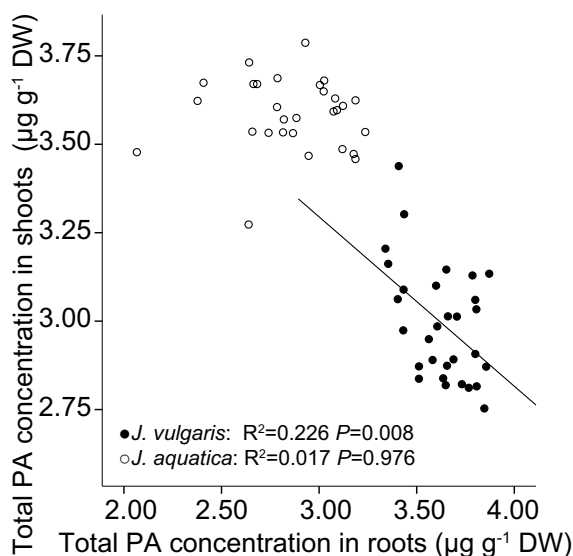


Fig. 4 Correlations between total pyrrolizidine alkaloid (PA) concentration in roots and shoots in *Jacobaea vulgaris* and *Jacobaea aquatica* observed for all methyl jasmonate treatments.

Table 1 Summary of ANOVAs: Effects of the solvent and methyl jasmonate (MeJA) treatments on the relative concentration of four structurally related pyrrolizidine alkaloid (PA) groups in whole plant, roots and shoots of *Jacobaea vulgaris* and *Jacobaea aquatica*

Species	Part	Dependent variable	Controls ^a			MeJA Treatments ^b		
			<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>
<i>J. vulgaris</i>	Whole ^c	Senecionine-like PAs	1, 8	0.016	0.903	5, 24	40.618	<0.001
		Jacobine-like PAs		0.202	0.665		3.104	0.027
		Erucifoline-like PAs		1.141	0.317		81.361	<0.001
		Otosenine-like PAs		0.730	0.418		2.336	0.073
	Root	Senecionine-like PAs	1, 8	0.325	0.584	5, 24	60.153	<0.001
		Jacobine-like PAs		0.150	0.709		4.139	0.007
		Erucifoline-like PAs		0.649	0.444		89.862	<0.001
		Otosenine-like PAs		0.096	0.765		3.431	0.018
	Shoot	Senecionine-like PAs	1, 8	2.041	0.191	5, 24	1.302	0.296
		Jacobine-like PAs		5.220	0.052		13.210	<0.001
		Erucifoline-like PAs		3.342	0.105		22.701	<0.001
		Otosenine-like PAs		2.368	0.162		2.144	0.095
<i>J. aquatica</i>	Whole	Senecionine-like PAs	1, 8	6.534	0.34	5, 24	26.880	<0.001
		Jacobine-like PAs		11.985	0.009		6.951	<0.001
		Erucifoline-like PAs		18.093	0.003		31.993	<0.001
		Otosenine-like PAs		6.778	0.031		0.178	0.968
	Root	Senecionine-like PAs	1, 8	3.363	0.104	5, 23	36.549	<0.001
		Jacobine-like PAs		1.011	0.344		1.361	0.275
		Erucifoline-like PAs		6.221	0.037		36.675	<0.001
		Otosenine-like PAs		2.686	0.140		1.180	0.349
	Shoot	Senecionine-like PAs	1, 8	9.097	0.017	5, 24	23.23	<0.001
		Jacobine-like PAs		13.276	0.007		9.879	<0.001
		Erucifoline-like PAs		2.246	0.172		24.260	<0.001
		Otosenine-like PAs		8.094	0.022		0.088	0.993

^a Controls include control (no solvent) and ethanol control (100 μ l of 1% ethanol solvent).

^b MeJA Treatments include 100 μ l of 0.00, 0.05, 0.10, 0.50, 1.00 and 5.00 μ g μ l⁻¹ MeJA in 1% ethanol.

^c Whole indicates whole plant.

Variation of pyrrolizidine alkaloid composition

The relative concentration of each PA group referred below represents (absolute con-

centration of that group PAs) / (total PA concentration)*100 in whole plant in roots and shoots. For instance, the relative concentration of erucifoline-like PAs in whole plant=(the total erucifoline-like PAs in whole plant)/(the total PA concentration in whole plant)*100.

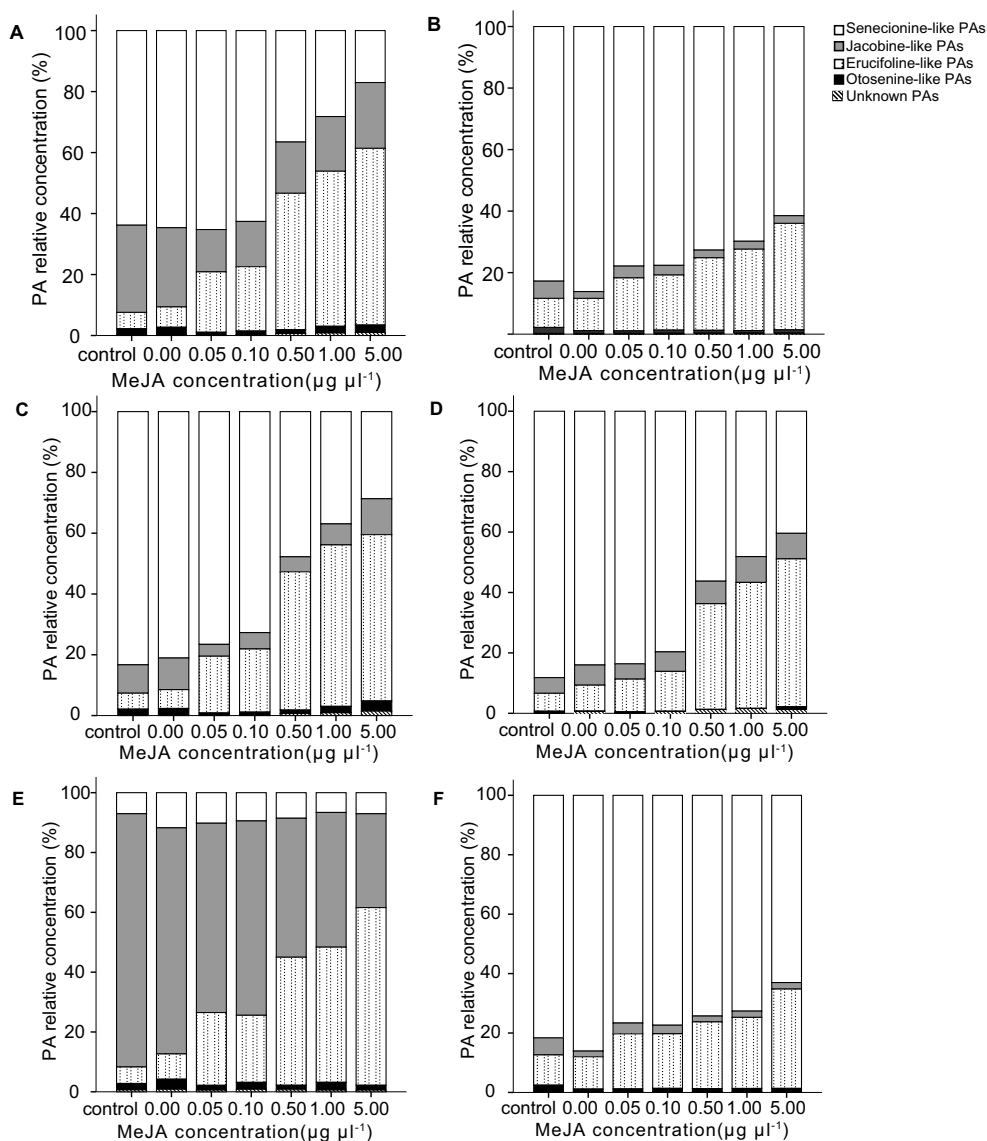


Fig. 5 Relative concentration of the four groups of pyrrolizidine alkaloids (PAs) in whole plants, roots and shoots of *Jacobaea vulgaris* and *Jacobaea aquatica* after methyl jasmonate (MeJA) treatments. (A) *J. vulgaris* whole plant, (B) *J. aquatica* whole plant, (C) *J. vulgaris* root, (D) *J. aquatica* root, (E) *J. vulgaris* shoot, (F) *J. aquatica* shoot.

Relative concentration of pyrrolizidine alkaloid groups in whole plant

No significant effects of ethanol were found for the relative concentration of the four groups of PAs in *J. vulgaris* (Table 1, Fig. 5A). However, it did have a significant effect in three groups of PAs in *J. aquatica*, in which an increase in the relative concentration of erucifoline-like PAs, a decrease of jacobine-like PAs and otosenine-like PAs was observed (Table 1, Fig. 5B).

At the whole plant level, MeJA application induced a decrease of the relative concentration of senecionine- and jacobine-like PAs and an increase of the relative concentration of erucifoline-like PAs in both species. It did not affect the relative concentration of otosenine-like PAs in neither of the species (Table 1, Fig. 5).

Relative concentration of different pyrrolizidine alkaloid structural groups in root and shoot

In the roots of *J. vulgaris*, the relative concentration of senecionine-like PAs decreased significantly with MeJA concentration while that of the other three groups increased, most notably erucifoline-like PAs (Table 1, Fig. 5C). For instance, the relative concentration of erucifoline-like PAs increased from an initial 6.2% before MeJA application to 54.7% at the highest concentration $5.00 \mu\text{g } \mu\text{l}^{-1}$ of MeJA while that of senecionine-like PAs dropped from 81.1% to 28.7% (Fig. 5C). In the case of shoots, the relative concentration of jacobine-like PAs decreased while erucifoline-like PAs increased as MeJA concentration increased (Fig. 5E). For instance, the relative concentration of erucifoline-like PAs significantly increased from an initial 8.5% before applying MeJA to 59.4% with the highest MeJA concentration ($5.00 \mu\text{g } \mu\text{l}^{-1}$) while the relative concentration of jacobine-like PAs dropped from 75.6% to 31.4% (Fig. 5E).

In both roots and shoots of *J. aquatica*, the relative concentration of erucifoline-like PAs increased at the expense of senecionine-like PAs with increasing MeJA concentrations (Fig. 5D and F). For instance, in the roots of *J. aquatica* the relative concentration of erucifoline-like PAs increased from 8.6% to 48.9% after applying $5.00 \mu\text{g } \mu\text{l}^{-1}$ MeJA while senecionine-like PAs significantly dropped from 84.0% to 40.4% (Fig. 5D).

The range of the concentrations of applied MeJA could mimic different levels of herbivore attacks to the roots. Low levels of MeJA (0.05 and $0.10 \mu\text{g } \mu\text{l}^{-1}$) already resulted in large changes of the PA composition in both species (Fig. 5). With increasing amounts of MeJA the relative concentration of erucifoline-like PAs also continued to increase in both roots and shoots. Perhaps because the roots were in direct contact with MeJA, the increase in the relative concentration of erucifoline-like PAs in the shoots was less notable than in the roots. In relation to increasing MeJA concentration, the relative concentration of senecionine-like PAs decreased, particularly in the roots while jacobine-like PAs decreased in the shoots of *J. vulgaris* (Fig. 5E). Interestingly, root herbivory (Kostenko et al. 2013) or MeJA application (this

paper) was not found to induce an increase of jacobine-like PAs. These PAs were shown to deter generalist herbivores (Cheng et al. 2011b; Leiss et al. 2009). The plants respond to MeJA application by converting senecionine-like PAs to erucifoline-like PAs. While *J. vulgaris* and *J. aquatica* grow in different habitats and have a different alkaloid composition, the shift of PA composition appeared to respond similarly to MeJA application. This suggests that the MeJA effect on PAs may be a general phenomenon in *Jacobaea* species. The overall impact on *J. aquatica* was lower due to the fact that the majority of PAs were stored in the shoots and therefore transport from roots to shoots could easily pass unnoticed. In contrast, in *J. vulgaris* the majority of PAs were stored in the roots.

The significant changes of PA composition were observed in the shoots even though MeJA was applied to the roots. These results are in line with a previous study that showed that root herbivory can produce an alteration in the level and distribution of SMs of the shoots (Wäckers and Bezemer 2003). Therefore root induction can lead to an indirect effect of defence in the shoots as well.

The application of jasmonate has been widely used in many plant species, but almost all the studies only reported increases of total SM concentration but provided no details of the qualitative changes in their composition. For instance, a paper on jasmonate application to shoots of two *Brassica* species reported a 1.5-3 times increase in glucosinolate levels in shoots (van Dam et al. 2004). The exogenous addition of MeJA to roots of *Nicotiana attenuata* was observed to induce *de novo* nicotine synthesis and increase whole-plant nicotine concentration (Baldwin 1996) and alkaloid concentrations also increased after MeJA treatment of *Catharanthus* and *Cinchona* seedlings (Aerts et al. 1994). In the references review of Table S1, there is however, only one study that showed how different levels of JA impacted significantly on the chemical composition of an essential oil but not on oil yield (Pirbalouti et al. 2014). In this study, we did find large changes in PA composition after treating plants with MeJA. Surprisingly this is one of the first reports that explicitly demonstrates that MeJA application can change the qualitative profile of a group of SMs in plants.

Herbivory by Spodoptera exigua

In *J. aquatica*, the leaf area eaten by *S. exigua* caterpillars was significantly different between the ethanol control and MeJA treatment (Fig. 6A, Wilcoxon test, $Z=-2.275$, $P=0.023$). The caterpillars hardly ate neither the *J. vulgaris* ethanol control plants nor the MeJA-treated plants and consequently we found no significant differences for this species (Fig. 6A, Wilcoxon test, $Z=0$, $P=1$).

Jacobine and erucifoline have been proved to be the most toxic PAs to cell lines of *S. exigua* (Nuringtyas et al. 2014). In *J. aquatica*, jacobine-like PA concentrations were not influenced by MeJA treatment (Fig. 6B) while erucifoline-like PA concentrations were significantly increased after applying MeJA (Fig. 6C). This

increase was accompanied by a decrease of herbivory by *S. exigua* (Fig. 6A). In *J. vulgaris*, a significant increase of the concentration of erucifoline-like PAs was also observed after MeJA treatment (Fig. 6C). As herbivory by *S. exigua* of *J. vulgaris* was also very low for the control plants, other defence mechanism may play a role in the resistance of *J. vulgaris* to *S. exigua*.

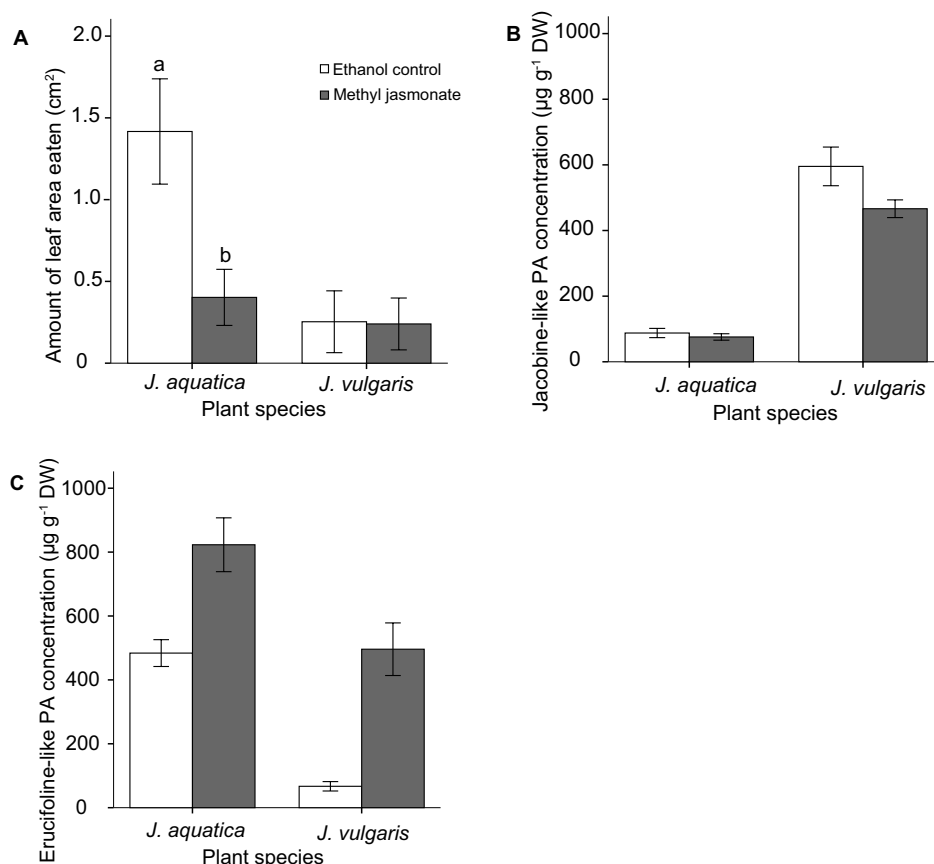


Fig. 6 Leaf area eaten by *Spodoptera exigua* (A), jacobine-like PA concentration (B) and erucifoline-like PA concentration (C) of *Jacobaea vulgaris* and *Jacobaea aquatica* in a feeding bioassay between ethanol control and methyl jasmonate treated leaves. Letters indicate significant differences with Wilcoxon matched pairs test. Error bars indicate standard error.

Concluding remarks

To avoid the side effects of potential inductions from different soil-types or soil-borne microorganisms (Joosten et al. 2009; Macel and Klinkhamer 2010), we conducted this experiment with tissue culture plants in an axenic environment. With this we ensured that the application of MeJA was the only factor that could lead to PA variation. This study is one of the first experiments to show the effects of MeJA on

SM composition. While the effects of MeJA on PA concentration of the whole plant were absent or marginal, we found large changes in the PA composition and distribution. Of these, the most pronounced was an increase of the relative concentration of erucifoline-like PAs and a decrease in senecionine-like PAs. In the feeding bioassay with *S. exigua*, MeJA application reduced the feeding damage of leaves of *J. aquatica* while it had no effect on *J. vulgaris* feeding damage with the same herbivore. Since previous studies have proved that senecionine did not influence the mortality in cell lines of *S. exigua* while erucifoline strongly increased their mortality, our results suggest that the change in PA composition after MeJA application may be one of the reasons for the reduced herbivory in *J. aquatica*.

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Supplementary Materials:

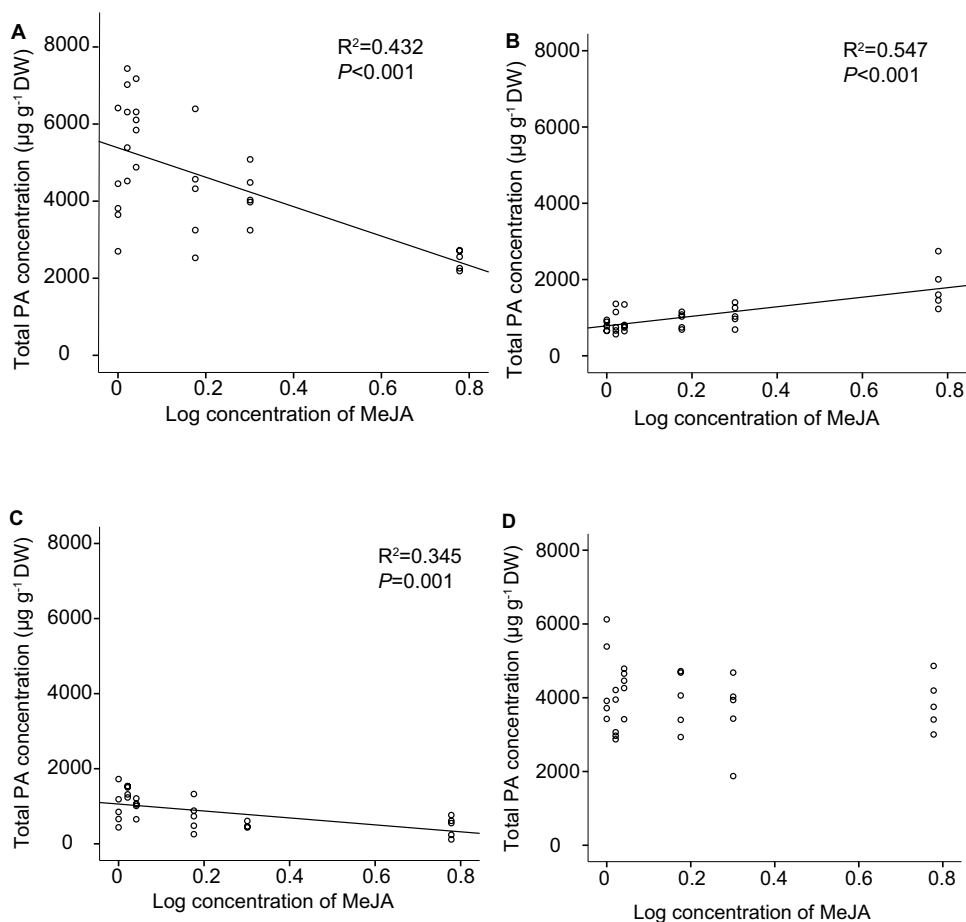


Fig. S1 Correlations between pyrrolizidine alkaloid (PA) concentration and methyl jasmonate (MeJA) treatments (0.00, 0.05, 0.10, 0.50, 1.00 and 5.00 µg µl⁻¹) in the whole plant of *Jacobaea vulgaris* and *Jacobaea aquatica*. (A) senecionine-like PAs in *J. vulgaris*; (B) erucifoline-like PAs in *J. vulgaris*; (C) senecionine-like PAs in *J. aquatica*; (D) erucifoline-like PAs in *J. aquatica*. MeJA concentration was log transformed.

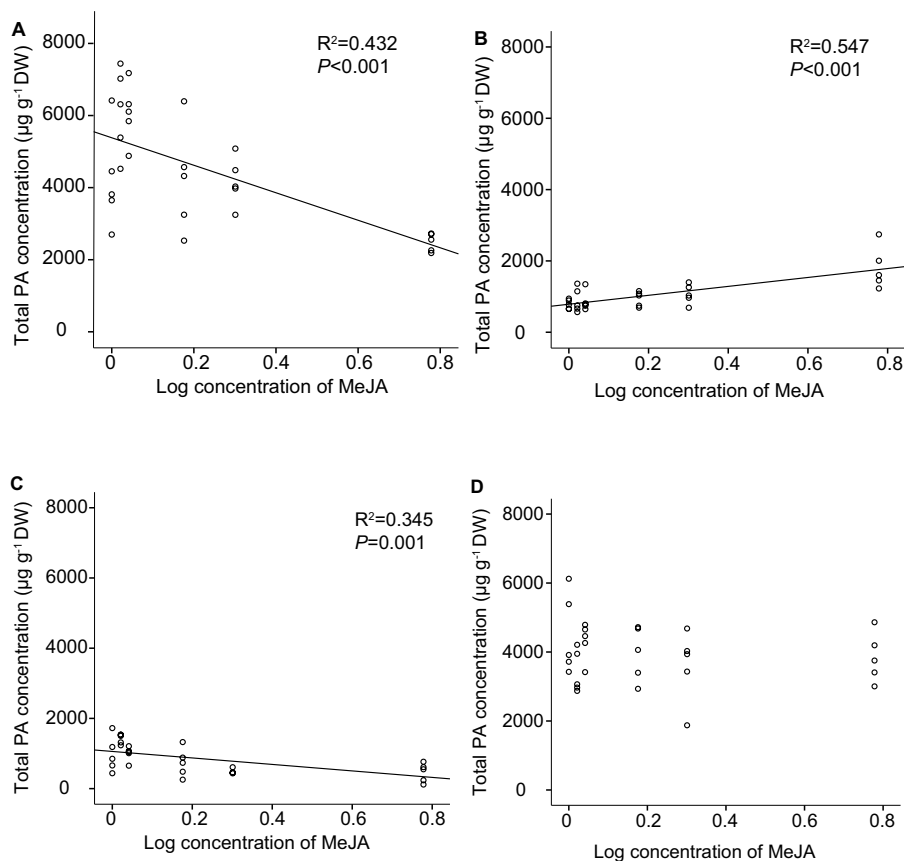


Fig. S2 Regression of total pyrrolizidine alkaloid (PA) concentration on methyl jasmonate (MeJA) concentration (0.00, 0.05, 0.10, 0.50, 1.00 and 5.00 µg µl⁻¹) in roots and shoots of *Jacobaea vulgaris* and *Jacobaea aquatica*. (A) *J. vulgaris* root, (B) *J. vulgaris* shoot, (C) *J. aquatica* root, (D) *J. aquatica* shoot. MeJA concentration was log transformed.



Fig. S3 Photographs of tissue culture plants of *Jacobaea vulgaris* (A) and the root system of the plants (B).

Table S1:

Family	Species	Compound	Type of plants studied	The media of plant growth	Site of application	
Solanaceae	<i>Brugmansia suaveolens</i>	Tropane alkaloids scopolamine	Whole plants	Pots	Root	
Solanaceae	<i>Nicotiana attenuata</i>	Nicotine	Hydroponically grown plants	Pots/Field	Root & Shoot	
Apocynaceae	<i>Catharanthus roseus</i>	Terpenoids Indole alkaloids	Seedlings	MS solid medium	Root	
Solanaceae	<i>Datura innoxia</i>	Cytokinin Alkaloids	Seedlings	Pots	Leaves	
Brassicaceae	<i>Brassica kaber</i>	Glucosinolates	Seedlings	Pots/Field	Leaves	
Brassicaceae	<i>Brassica oleracea</i> L. var. <i>italica</i>	Glucosinolates	Seedlings	Coconut fiber with the solution tested	Cotyledons	
Brassicaceae	<i>Brassica rapa</i>	Glucosinolates	Seedlings	Nutrient solution	Root	
Brassicaceae	<i>Brassica rapa</i> ssp. <i>rapa</i>	Glucosinolates	Seedlings	Nutrient solution	Root	
Brassicaceae	cultivars	Glucosinolates	Seedlings	Field	Foliar spray	
Brassicaceae	<i>Arabidopsis thaliana</i>	Glucosinolates	Seedlings	Pots	Rosette Leaves	
Brassicaceae	<i>Arabidopsis thaliana mutant</i>	Glucosinolates	Seedlings	Promix-BX	Leaves	
Tropaeolaceae	<i>Tropaeolum majus</i>	Glucosinolates	Seedlings	Pots	Leaves	
Brassicaceae	<i>Brassica napus</i> cultivar <i>Brassica oleracea</i> cultivar	Glucosinolates	Seedlings	Pots	All aerial plant tissues	
Brassicaceae	F1 broccoli cultivars	Glucosinolates	Seedlings	Pots	Broccoli florets	
Brassicaceae	Broccoli and radish cultivars	Glucosinolates	Seedlings	Growth pad	Cotyledons	
Brassicaceae	Broccoli cultivars	Glucosinolates	Plants	Field	Aerial plant tissue	
Brassicaceae	<i>Brassica rapa</i> ssp. <i>chinensis</i>	Glucosinolates	Seedlings	Pots	Sprout and mature leaves	
Brassicaceae	Cauliflower	Glucosinolates	Plants	Field	Leaves	

	Time period	Solvent	Concentration-Increase/ decrease	Technology	Reference
	10 days	Distilled water	Increase	GC-MS	(Arab et al., 2012)
	5 & 7 days	Lanolin	Increase	HPLC	(Baldwin, 1996)
	One month	60% Ethanol	Increase	HPLC	(El-Sayed and Verpoorte, 2004)
	35 days	Distilled water	Decrease (cyto- kinin) Increase (alkaloids)	GC	(Gendy and Rabie, 2000)
	6, 12, 25 July; After 5days	Water	Increase	Affinity chroma- tography	(Cipollini and Sipe, 2001)
	3, 5, 7 days	0.04% Ethanol	Increase	LC-MS, HPLC- DAD	(Perez-Balibrea et al., 2011)
	10, 15, 20, 25, 30 days	Water	Increase	HPLC	(Smetanska et al., 2007)
	10, 15, 20, 25, 30 days	Water	Increase	HPLC	(Schreiner et al., 2011)
	About 2 months	Water	Increase	HPLC	(Fritz et al., 2010)
	1 week	1.5% Ethanol and 0.125% Triton	Increase	HPLC	(Mewis et al., 2005)
	4 days	4% Ethanol	Increase	HPLC	(Traw et al., 2003)
	1 week	0.2% Triton X	Increase	HPLC	(Bloem et al., 2014)
	4 days	0.1% Triton X-100	Increase	HPLC LC-MS	(Ku et al., 2014)
	4 days	0.1% Triton X-100	Increase	HPLC LC-MS/MS	(Ku et al., 2014)
	5 days	0.2% Ethanol	Increase	HPLC-DAD- ESI-MS	(Baenas et al., 2014)
	4 days	0.1% Triton X-100	Increase	HPLC	(Ku et al., 2013)
	48 hours	0.01% Tween 20	Increase	HPLC-DAD- UV	(Wiesner et al., 2013)
	4 days	0.1% Triton X-100	Increase	HPLC	(Ku et al., 2013)

Brassicaceae	<i>Brassica napus</i> L.	Glucosinolates	Seedlings	Pots	Rape leaves Spray or MJ vapour	
Brassicaceae	<i>Brassica napus</i> and <i>Brassica rapa</i>	Glucosinolates	Seedlings	Field	Shoot spray	
Brassicaceae	<i>Brassica oleracea</i>	Glucosinolates	Seedlings	Pots	Root & shoot	
Brassicaceae	<i>Brassica nigra</i> <i>Brassica oleracea</i>	Glucosinolates	Seedlings	Pots	Shoots	
Brassicaceae	<i>Brassica oleracea</i> L. var. italica	Glucosinolates	Seedlings	Coconut fiber with the solution tested	Cotyledons	
Brassicaceae	<i>Brassica oleracea</i>	Glucosinolates	Plants	Field	Plant surfaces were fully saturated with MeJA	
Brassicaceae	<i>Brassica rapa</i>	Glucosinolates	Seedlings	Lab and field	Leaves surface	
Brassicaceae^b	<i>Brassica oleracea</i>	Glucosinolates	Brussels sprouts plants	Pots	Sprouts	
Brassicaceae	<i>Brassica rapa</i> <i>Sinapis alba</i>	Glucosinolates	Root culture	MS medium	Root	
Lamiaceae	<i>Ocimum basilicum</i> L.	Phenolic compound	Seedlings	Pots	Seeds (24 hours) & Roots (10days)	
Araliaceae	<i>Panax ginseng</i> Meyer	Ginsenoside	Plants	Perlite and peat moss	Root	
Lamiaceae^{b,c}	<i>Satureja hortensis</i> L.	Essential oil	Seedlings	Pots	Foliar	
Brassicaceae	<i>Brassica oleracea</i> var. italica	Phenolic compound	Seedlings	Paper soaked in elicitor solution	Seeds (soak for 4hrs)	
Gentianaceae	<i>Exacum affine</i>	Phenolic acid	Shoot culture	MS medium	Shoot	
Scrophulariaceae	<i>Bacopa monnieri</i>	Bacoside A	Shoot culture	Liquid MS medium	Shoot	
Plantaginaceae	<i>Digitalis lanata</i>	Lanatoside C	Shoot culture	MS medium	Shoot	
Polygonaceae	<i>Fagopyrum esculentum</i> Moench	Anthocyanins Proanthocyanidins	Seedlings	Water	Root zone	
Celastraceae	<i>Trigonella foenum-graecum</i> L.	Diosgenin	Seedlings	MS salt solution	Seedling	

	72 hours	0.2% Triton 100	Increase	HPLC	(Doughty et al., 1995)
	5 days	Water	Increase	chromatography	(Cipollini and Sipe, 2001)
	7 days	0.5%Ethanol (root); 0.1%Triton & 0.5% Ethanol (shoot)	Increase	HPLC	(van Dam and Oomen, 2008)
	7 days	0.1% Triton X-100	Increase	HPLC	(van Dam et al., 2004)
	3, 5, 7 days	0.04% Ethanol	Increase	LC-MS	(Perez-Balibrea et al., 2011)
	4 days	0.1% Triton X-100	Increase	HPLC	(Liu et al., 2014)
	2 weeks&2 days	2% Ethanol	Increase	HPLC	(Loivamaki et al., 2004)
	24 hours	0.1 % Tween	No influence	HPLC	(Bruinsma et al., 2007)
	7, 14 ,28 days	5% Ethanol	Increase	HPLC	(Kastell et al., 2013)
	10 days (but the seeds soaked in MeJA for 24 hours before germination)	1% Ethanol	Increase	HPLC/UV	(Koca and Karaman, 2015)
	2 days	Water	Increase (total)	HPLC	(Oh et al., 2014)
	15 days	Ethanol	No influence	GC/MS	(Pirbalouti et al., 2014)
	11days	Water	Decrease	HPLC	(Carvacho et al., 2014)
	1, 3, 7 days	0.1 % Ethanol	Increase	HPLC	(Skrzypczak-Pietraszek et al., 2014)
	Weekly for 5 weeks	Ethanol	Increase	HPTLC	(Sharma et al., 2013)
	28 days	95% Ethanol	Increase	HPLC	(Perez-Alonso et al., 2012)
	3 days	Ethyl alcohol	Decrease Increase	HPLC	(Horbowicz et al., 2011)
	5 days	100% Ethanol	Increase	HPLC	(Debjani and Bratati, 2011)

Polygonaceae	<i>Fagopyrum esculentum</i> M.	Phenolic compounds	Seedlings	Cheese cloth tray	Sprout	
Asteraceae	<i>Artemisia annua</i> L.	Artemisinin	Plants	Peat soil	Shoot	
Myrtaceae^b	<i>Eucalyptus grandis</i>	Terpene	Plants	Pots	Ramet	
Asteraceae	<i>Lactuca sativa</i>	Phenolic compounds Carotenoids	Seedlings	Pots	Seedlings	
Hypericaceae	<i>Hypericum perforatum</i> <i>H. sampsonii</i>	Hypericins Hyperforin	Plantlet	MS medium	Plantlet	
Brassicaceae	<i>Raphanus sativus</i> L.	Phenolics isothiocyanate	Seedlings	Pots	Sprout	
Solanaceae	<i>Solanum lycopersicum</i>	Caffeoylputrescine	Seedlings	Pots	Leaves	
Lamiaceae	<i>Ocimum basilicum</i> L.	Phenolic content	Seedling	Pots	Leaves	
Rubiaceae	<i>Psychotria brachyceras</i>	Brachycerine (alkaloids)	Seedlings (hydroponically grown cuttings)	MS liquid medium	Shoot	
Solanaceae	<i>Nicotiana attenuata</i>	Phenolics Diterpene glycosides	Rosette plants	Field	Leaves	
Asteraceae	<i>Echinacea pallida</i>	Alkamides Ketoalkene	Plants	Soil	Leaves	

^a In Web of Science, we searched “jasmonate AND secondary metabolites” in the field of topic. In total 437 articles (before January 1, 2015) were obtained. Firstly, we ruled out about 55% of the articles which focused on transcript and protein level. Then we excluded most of the articles which studied cell culture or root culture, which are not real plants. The articles which investigated in metabolite levels and used treatment with jasmonate in real plants were selected in this table.

^b The study showed that the secondary metabolites content was not influenced by MeJA application.

^c The composition of secondary metabolites was changed after MeJA application.

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	0, 1, 3, 5, 7 days	0.25% Ethanol	Increase	UPLC-Q-TOF	(Kim et al., 2011)
	0, 2, 4, 6, 8, 10 days	0.8% Ethanol	Increase	HPLC GC-MS	(Wang et al., 2010)
	7 days	Ethanol	No influence	HPLC	(Henery et al., 2008)
	4 hours	0.25% Ethanol	Increase	HPLC	(Kim et al., 2007)
	25 days	Ethanol	Increase	HPLC	(Liu et al., 2007)
	0, 6, 12, 24 , 48 hours	0.25% Ethanol	Increase Decrease	HPLC	(Kim et al., 2006)
	3 weeks	Ethanol	Increase	LC/MS/MS	(Chen et al., 2006)
	0, 1, 2, 4 days	Water	Increase	HPLC	(Kim et al., 2006)
	2,4,6 days	50% Ethanol	Increase	HPLC	(Gregianini et al., 2004)
	4 days	Water	Increase	HPLC	(Keinanen et al., 2001)
	24 hours	Water	Increase	HPLC	(Binns et al., 2001)

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Chapter 6

**The effects of phytohormone application
on metabolite profile and the feeding of
chewing, piercing-sucking and cell-content
herbivores: a study in *Jacobaea vulgaris* and
*Jacobaea aquatica***

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Abstract

Inducible defences are common strategies in plants. In a previous study methyl jasmonate (MeJA) application on the roots did not increase the total pyrrolizidine alkaloid (PA) concentration while the PA composition was shifted from less toxic senecionine-like PAs to more toxic erucifoline-like PAs. Here, we analyse whether levels of primary and secondary metabolites are influenced by MeJA and salicylic acid (SA) application. Furthermore, we tested whether herbivores with different feeding modes were affected by application of these phytohormones. We used *Jacobaea vulgaris* and *Jacobaea aquatica* as plant species and the chewing caterpillar *Mamestra brassicae*, the cell-content feeder leafminer *Liriomyza trifolii* and the piercing-sucking thrips *Frankliniella occidentalis* as herbivores. PCA analysis showed a clear distinction between the metabolome of MeJA treated, SA treated and control plants. Both phytohormone applications led to significant changes in bins (compounds) of the ¹H-NMR spectrum. More bins were up-regulated than down-regulated after MeJA treatment while the reverse was true for the SA treatment. We found significant less feeding damage of *M. brassicae* and *L. trifolii* in plants treated by MeJA while no significant difference was found between control and SA treated plants in both plant species. SA treatment rendered *J. aquatica* more resistant to thrips. Because the concentration of many bins changed, it is difficult to identify the bins that are related to resistance. For thrips resistance we could combine available data for thrips silver damage and NMR analysis for a segregation F2 population of a cross between *J. vulgaris* and *J. aquatica* and identified NMR bins that were positively or negatively correlated with thrips resistance. We then checked whether or not these bins were down-regulated or up-regulated after SA treatment in *J. aquatica*. This analysis led to three candidate compounds related to susceptibility (sucrose, glucose and 3,5-dicaffeoylquinic acid (3,5-DCQA)) and three compounds related to resistance (citric acid, threonine and alanine). The positive effects of sucrose, glucose and 3,5-DCQA and the negative effects of citric acid on thrips survival were confirmed by results of unpublished mortality bioassay done previously in our research group. Literature data showed that β-alanine was positively correlated to thrips mortality.

Key words

Metabolome, ¹H-NMR, Methyl jasmonate, Salicylic acid, Jacaranone, Isoleucine, Alanine, Threonine

Introduction

Plants have to cope with various enemies, such as herbivores and pathogens in the natural environment. They therefore have evolved different strategies including chemical defences and mechanical defences, such as trichomes and thorns. Chemical defences are mainly based on secondary metabolites (SMs) of different chemical origins, often characteristic for certain plant taxa and effective at least against generalist herbivores (Schoonhoven et al. 2005). Besides constitutive defences, many metabolites can be induced when the plants are attacked by pathogens or herbivores (Karban and Baldwin 2007).

Induction of chemical defence after attack is mediated by phytohormones like jasmonate (JA), ethylene (ET) and salicylic acid (SA). As a general rule, the JA pathway that frequently acts synergistically with ET is hypothesized to be up-regulated if the plant is attacked by chewing-biting herbivores, cell-content feeders and necrotrophic pathogens (Glazebrook 2005; Walling 2000). While the SA pathway is hypothesized to be activated especially in response to piercing-sucking insects and biotrophic pathogens (Kawazu et al. 2012; Walling 2000). Many studies revealed that the JA and SA pathways are not distinct but interact by a complex network of regulatory interactions (Beckers and Spoel 2006; Pieterse et al. 2001). These interactions include priming (De Vos et al. 2006), complementary additive or synergistic effects (Devadas et al. 2002; Schenk et al. 2000) and mutual antagonism (Diezel et al. 2009; Zhang et al. 2013).

Direct exogenous application of jasmonate has been commonly used to simulate attack by herbivores and to analyse the subsequent plant responses (Howe and Jander 2008). For instance, glucosinolate and nicotine concentrations rise after JA treatment in *Brassicae* and *Nicotiana attenuata* (Baldwin 1996; van Dam et al. 2004). The growth of both *Mamestra brassicae* and *Pieris rapae* was retarded when feeding on the leaves of JA -induced plants of *Brassicae oleracea* (van Dam and Oomen 2008). The role of SA in plant responses to pathogens has been well documented, but its effects on plant responses to insects are not so well understood (Penninckx et al. 1996; Tateda et al. 2014).

These studies showed the effects of JA application on the production of SMs or the performance of herbivores. However, there are not many studies that couple the induction of SMs to the changes in herbivore resistance, to identify the candidate compounds involved (but see Schweiger et al. 2014; Sutter and Müller 2011).

A plant's resistance to herbivore attack is thought to be principally determined by its SMs, which can be remarkably plastic and responsive to different grades and types of herbivory (Schwachtje and Baldwin 2008). The role of primary metabolites (PMs) is often neglected in plant defence. For instance, isoleucine (Ile) produced at the attack site is rapidly conjugated to jasmonate (JA), forming JA-Ile, a key activator of defence signalling (Chini et al. 2007; Thines et al. 2007). The

hundreds of genes down- or up-regulated during the plant-herbivore or -pathogen interaction have been analysed with microarray studies, and almost all aspects of metabolism are represented, with a substantial fraction coming from primary metabolism (Both et al. 2005; Hui et al. 2003). Therefore, the role of primary metabolism in plant defence may be underestimated.

In this study, we investigated how the plant metabolite profile, and the amount of damage inflicted by herbivores with different feeding modes, were affected by MeJA and SA application. *Jacobaea vulgaris* and *Jacobaea aquatica* both notorious for their pyrrolizidine alkaloids (PAs) were used to address these questions. A previous study (chapter 5 of the thesis) showed that the total PA concentration was not affected by root application of MeJA while a significant shift in PA composition occurred from senecionine-like PAs to erucifoline-like PAs. However, it is unclear in as much other plant chemical components are affected and therefore we used nuclear magnetic resonance (NMR) spectroscopy to profile the major organic molecules present in leaf tissue. A chewing herbivore *Mamestra brassicae*, a cell-content feeder *Liriomyza trifolii* and a piercing-sucking herbivore *Frankliniella occidentalis* were used in this study. We compared our results with a previous experiment (Cheng et al. 2011b) on thrips resistance off a population of 102 segregating F2 genotypes from a cross between the two species from the present study and combined these results with the results from an ¹H-NMR analysis of the same genotypes (Kirk et al. 2012). The combination of the two experiments and the present experiment reduces the chance on false positives. Our experiments were designed to answer the following questions: (1) Do plant metabolite profiles change after MeJA and SA application? (2) Are the chewing herbivore and the cell-content feeder negatively affected by MeJA application while the piercing-sucking herbivore is negatively affected by SA application? (3) Are the changes in the metabolome related to the changes in herbivore feeding? (4) Do the effective compounds also confer resistance to thrips attack in an independent study using F2 hybrids between *J. vulgaris* and *J. aquatica* in this study? (5) Are the identified metabolites involved in resistance to thrips also effective if tested separately?

Materials and Methods

Plant species

The *Jacobaea vulgaris* and *Jacobaea aquatica* individuals used in this study are the parental plants of a well-studied hybrid cross (Cheng et al. 2011a). *J. vulgaris* was derived from the seeds collected at the Meijendel Nature Reserve (52°7'54"N, 4°19'46"E, The Netherlands), and *J. aquatica* was derived from the seeds collected at the Zwanenwater Reserve (52°48'38"N, 4°41'7"E, The Netherlands). *J. vulgaris* is attacked by over 60 species of herbivores and grows in dry and sandy soils while *J. aquatica* is fed on by relatively few herbivores and grows in marshy environment.

Genotypes of both species are maintained in tissue culture and can be easily be cloned to produce replicates. Each plant was grown in 5 ml of MS medium to induce a fully developed root system in a climate room (humidity 50%, light 16h /dark 8h, 20°C/20°C).

Plant treatments

After two weeks of growth in Murashige and Skoog (MS-0) medium, the shoots were randomly assigned to one of the following treatments: (1) Control (CON) in which the shoot was dipped in 0.1 % Triton water solution for 30 seconds; (2) Methyl jasmonate (MeJA) in which the shoot was dipped in 5 mM methyl jasmonate (dissolved in 0.1% Triton solution) for 30 seconds; (3) Salicylic acid (SA) in which the shoot was dipped in 5 mM salicylic acid (dissolved in 0.1% Triton solution) for 30 seconds. Each treatment was replicated 10 times for both species. After dipping, the plants were put on a paper tissue to absorb solution left on the leaves. The treated plants were propagated in a new tube with MS medium for another two weeks. After that, three leaf discs (1.2 cm diameter) per plant were punched for herbivore tests. The remaining leaf material were wrapped in aluminium foil, put in liquid nitrogen and then kept in a -80 °C freezer until freeze drying.

Herbivore source and rearing

Eggs of *Mamestra brassicae* were obtained from Entomology Lab, WUR (Wageningen, The Netherlands) and caterpillars were reared on cabbage (*Brassica rapa*) in a climate room (humidity 50%, light 16h /dark 8h, 20°C/20°C).

Leafminer *Liriomyza trifolii* with a cell-content feeding mode and thrips *Frankliniella occidentalis* with a piercing-sucking feeding mode were both from cultures maintained in our lab. Both species were reared on chrysanthemum (*Dendranthema grandiflora*) plants in a climate room (humidity 50%, light 16h /dark 8h, 20°C/20°C).

Herbivore performance tests

The non-choice feeding bioassays were conducted in petri dishes (60mm * 15mm) lined with a layer of agar to maintain a consistent humidity. Leaf-discs were gently pressed on the agar with the abaxial side up. Each trial had 10 replicates per treatment per species totalling 10 replicates * 3 treatments * 2 plant species = 60 assays.

In tests with *M. brassicae*, each trial included one leaf-disc and one 2nd instar caterpillar. The caterpillars fed for four hours and the damaged leaf-discs were scanned. The leaf area eaten was estimated to the nearest mm² by subtracting the damaged leaf area from the whole leaf-disc.

For the bioassay with *L. trifolii* we used two female leafminers and one leaf-disc in each trial. After 24 hours, the number of feeding punctures were counted under microscope.

For *F. occidentalis* we used four female thrips and one leaf-disc for each trial.

After 7 days, for each leaf the amount of silver damage was visually scored in mm² using a microscope.

Nuclear magnetic resonance measurements

Freeze-dried plant material was extracted and analysed by NMR spectroscopy according to Kim et al. (2010). Briefly, 20 mg of lyophilized sample was taken into a 2.0 ml microtube and 0.5 ml methanol-*d*₄ and 0.5 ml D₂O (KH₂PO₄ buffer, pH= 6.0) containing 0.005% TMSP-*d*₄ (trimethyl silyl propionic acid sodium salt-*d*₄, w/v, Sigma-Aldrich, Zwijndrecht, the Netherlands) were added. The mixture was extracted at room temperature for 20 min with ultrasonication (Branson 5510E-MT, Branson Ultrasonics, Danbury, CT, USA) and subjected to centrifugation at 17,000 ×g at room temperature for 5 min. 300 µl of the supernatant was transferred to a 3 mm NMR tube and analysed.

The ¹H-NMR spectra were recorded using a Bruker DMX 600 spectrometer (Bruker, Karlsruhe, Germany). For each sample, 64 scans were recorded with the following parameters: 0.167 Hz/point, pulse width (PW) = 4.0 µs, acquisition time (AQ) = 3.17 s, dummy scans (DS) = 4 and relaxation delay (RD) = 1.5 s. Free induction decays (FIDs) were Fourier transformed with line broadening (LB) = 0.3 Hz. Phase adjustment and baseline correction were applied manually.

The AMIX software (Bruker) was used to reduce the ¹H-NMR spectra to an ASCII file. The spectra, which were measured in chemical shift (ppm), were binned (bucketed) into bins of equal width (0.04 ppm). Spectral intensity was scaled to internal standard (TMSP) and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 10.00–0.40. The region of δ 4.88–4.72 was excluded from the analysis because of the residual signal of H₂O and CH₃OH-*d*₄, respectively. The regions of δ 10.00–7.68 and δ 0.8–0.4 were excluded from the analysis because these contained no significant peaks.

Correlation with datasets of previous studies

We used the NMR measurements (Kirk et al. 2012) and silver damage data of thrips (Cheng et al. 2011b), obtained for a segregating population of 102 F2 hybrids from a cross between the two species, and used these in the present study to correlate the NMR bins from the NMR spectra with silver damage. This analysis for the F2 genotypes allows for an independent assessment of the metabolites involved in the resistance against thrips. The NMR bins that were positively or negatively correlated with thrips resistance were then checked whether or not these bins were down-regulated or up-regulated after SA treatment in *J. aquatica*. From the significant bins in both tests, the candidate compounds involved in thrips resistance were identified. These compounds were among the compounds tested previously in thrips mortality bioassay in our research group.

Statistical analysis

Principal component analysis (PCA) was performed with the SIMCA-P (ver. 13.0, Umetrics, Umeå, Sweden). The effects of the phytohormone treatments were evaluated by analysis of 170 NMR data bins. For each bin it was tested if the metabolite profile changed after phytohormone treatment. The number of up- and down-regulated bins were tested by χ^2 tests.

The NMR bins were compared between each phytohormone treatment and the control group with Mann-Whitney U-tests. *P*-values of the bins in a specific pairwise comparison were adjusted according to Benjamini & Hochberg method (Benjamini and Hochberg 1995) to correct for multiple testing. Volcano plots including all the bins of a given treatment-control comparison with fold changes (peak intensity in treatment bin divided by peak intensity in control bin) were plotted in R 3.0.2.

Different herbivore performances between control, MeJA treatment and SA treatment were evaluated using Kruskal-Wallis one-way analysis of variance, followed by post hoc Dunn's tests in R 3.0.2.

In the available datasets of the F2 hybrids for thrips damage and NMR, spearman rank correlations between peak intensity of the NMR bins and thrips silver damage were performed. Significant bins were then compared with the NMR bins of the SA application in *J. aquatica*. We selected the NMR bins that were increased after SA application and negatively correlated with silver damage in the F2 hybrids and the NMR bins that were reduced after SA application and positively correlated with silver damage in the F2 hybrids. For the selected bins we identified the metabolites represented by these bins.

Results

Metabolome profiles of leaf tissue

For the dataset obtained from the ^1H -NMR analysis, a 13-component model explained 97.5% of the variance, with the first two components explaining 63.0 % (the third one explaining 9.2%) in *J. vulgaris* (Fig. 1a). In *J. aquatica*, a 13-component model explained 98.4% of the variance, with the first two components explaining 64.8% (the third one explaining 10.0%) (Fig. 1b). PCA plots of the first two components showed that the plants treated by MeJA were clearly separated from the control and SA treated plants for both species. The separation between the control plants and the SA treated plants was less clear.

Compared to the control group, significant up- or down-regulated NMR bins were found for both phytohormone treatments. MeJA application caused significant changes in 51 bins and 122 bins in *J. vulgaris* and *J. aquatica*, respectively (Table 1, Fig. 2). SA application led to significant changes in 7 bins and 78 bins in *J. vulgaris* and *J. aquatica*, respectively (Table 1, Fig. 2). Among the significant NMR bins, MeJA treatment resulted in more up-regulated (44) than down-regulated (7) bins in *J. vulgaris* ($\chi^2=565.14$, $df=1$, $P<0.001$) and in *J. aquatica* (96 and 26 bins, respective-

ly) ($\chi^2=4557.5$, $df=1$, $P<0.001$) (Table 1, Fig. 2). For the SA treatments 1 NMR bin was up-regulated and 6 bins were down-regulated in *J. vulgaris* ($\chi^2=20.513$, $df=1$, $P<0.001$) while in *J. aquatica* 22 were up-regulated and 56 bins were down-regulated ($\chi^2=110.831$, $df=1$, $P<0.001$) (Table 1, Fig. 2).

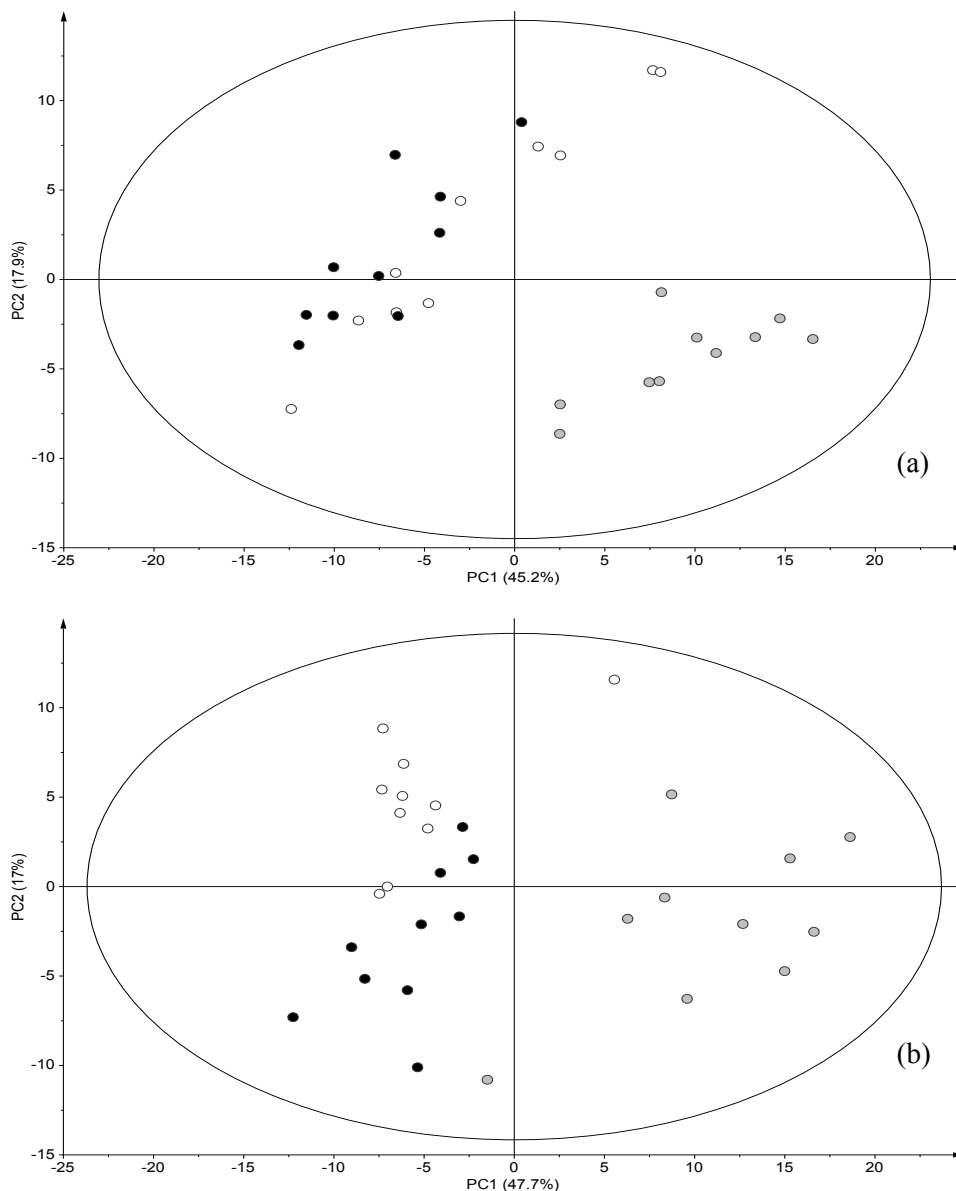


Fig. 1 Plot of principal components 1 and 2 based on PCA of the signal intensity of 170 ^1H -NMR bins of *Jacobaea vulgaris* (a) and *Jacobaea aquatica* (b). White dots represent control plants, grey dots represent plants treated with methyl jasmonate (MeJA) and black dots represent plants treated with salicylic acid (SA), $n=10$ for each treatment.

Table 1 Summary of up- or down-regulated NMR bins after methyl jasmonate (MeJA) and salicylic acid (SA) treatments in *Jacobaea vulgaris* and *Jacobaea aquatica*

Plant species	<i>J. vulgaris</i>		<i>J. aquatica</i>	
Treatments	MeJA	SA	MeJA	SA
Total bins	170	170	170	170
Down-regulated bins	65	117	42	116
Up-regulated bins	105	53	128	54
Significant bins ^a	51	7	122	78
Significant down-regulated bins	7	6	26	56
Significant up-regulated bins	44	1	96	22

^a The number of NMR bins that are significant after adjusting for multiple comparisons by the Benjamini & Hochberg method

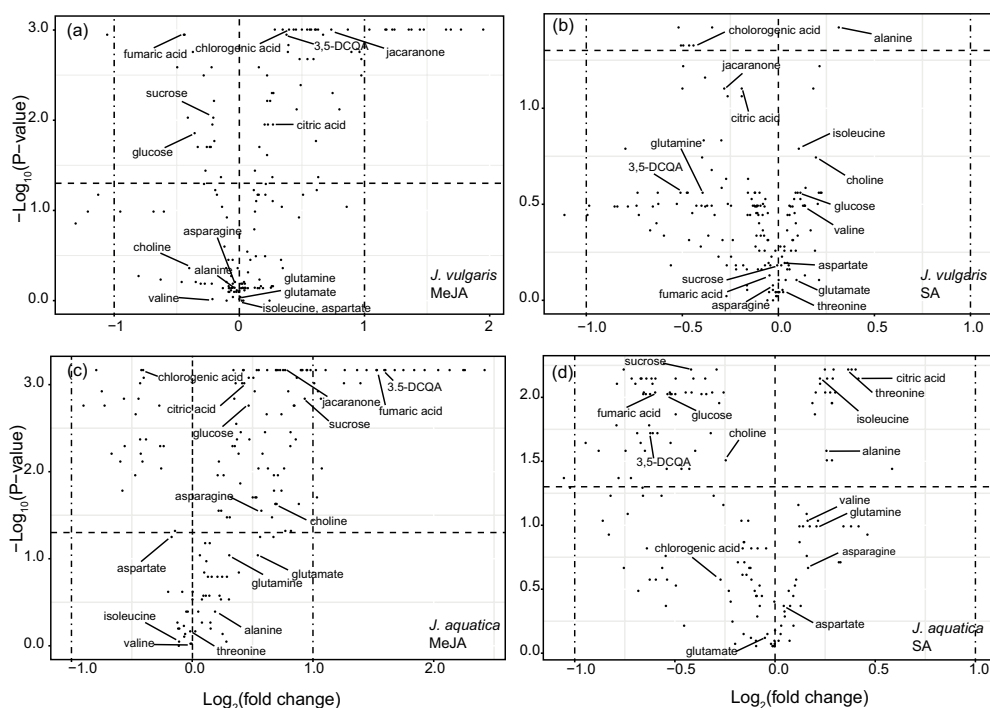


Fig. 2 Volcano plots of 246 bins of NMR analysis of shoots of *Jacobaea vulgaris* and *Jacobaea aquatica*, 2 weeks after phytohormone treatment with methyl jasmonate (MeJA) or salicylic acid (SA). For each bin, the negative log10 of the unadjusted P value (Mann-Whitney U-test, comparison with the control) is plotted against the log₂ of the mean fold change. The horizontal dashed line is $P = 0.05$ and the significant bins ($P < 0.05$) are above this line. The identified metabolites with significant P value of Mann-Whitney U-test were labeled.

Table 2 ^1H chemical shift (d) of metabolites present in leaves of *Jacobaea vulgaris* and *Jacobaea aquatica* identified by 1D NMR spectra in MeOH-d_4

No	Compounds	Chemical shift (ppm)
1	Alanine	δ 1.48 (d)
2	Asparagine	δ 2.94 (d), 2.96 (d)
3	Aspartate	δ 2.71 (d), 2.68 (d)
4	Chlorogenic acid	δ 7.61 (d)
5	Choline	δ 3.22 (s)
6	Citric acid	δ 2.55 (s)
7	3,5-dicaffeoylquinic acid	δ 6.90 (d), 6.88 (d), 6.49 (d)
8	Fumaric acid	δ 6.53 (s)
9	Glucose	δ 5.18 (d)- α , δ 4.59 (d)- β
10	Glutamate	δ 2.40 (m)
11	Glutamine	δ 2.46 (m), 2.13 (m)
12	Isoleucine	δ 0.96 (t)
13	Jacaranone	δ 6.24 (d), 7.14 (d)
14	Sucrose	δ 5.41 (d)
15	Threonine	δ 1.34 (d)
16	Valine	δ 1.06 (d), 1.00 (d)

In total, 16 up- or down-regulated compounds were identified in the leaf tissue (Table 2), including 3 secondary metabolites (jacaranone, chlorogenic acid and 3, 5-dicaffeoylquinic acid) and 13 primary metabolites. Of these primary metabolites, there are 2 sugars (sucrose and glucose), 8 amino acids (asparagine, aspartate, glutamine, glutamate, alanine, threonine, valine and isoleucine), fumaric acid, citric acid and choline. Jacaranone significantly increased compared to the control after MeJA application in both species, while chlorogenic acid decreased significantly by both phytohormone applications in *J. vulgaris* but not in *J. aquatica* (Fig. 3). In *J. aquatica* 3,5-dicaffeoylquinic acid (3,5-DCQA) increased significantly after MeJA application, while it decreased after SA treatment, but in *J. vulgaris* there was no significant differences among the treatments (Fig. 3b). Both MeJA and SA treatment induced a significant decrease of sucrose and glucose concentrations in *J. aquatica*. In contrast, only glucose decreased after MeJA application in *J. vulgaris*. Fumaric acid decreased after MeJA treatment in *J. vulgaris* while in *J. aquatica* it caused a decrease (Fig. 3). Citric acid increased significantly after MeJA and SA treatment in *J. aquatica*. However, in *J. vulgaris* it increased after MeJA treatment while it decreased after SA treatment. Choline increased in *J. vulgaris* while it decreased in *J. aquatica* after SA application. MeJA application induced a decrease of choline in

both species (Fig. 3). All the amino acid showed similar responses after plant phytohormone treatments in both species, except glutamine. Glutamine decreased in *J. vulgaris* after SA application while it increased in *J. aquatica* (Fig. 3).

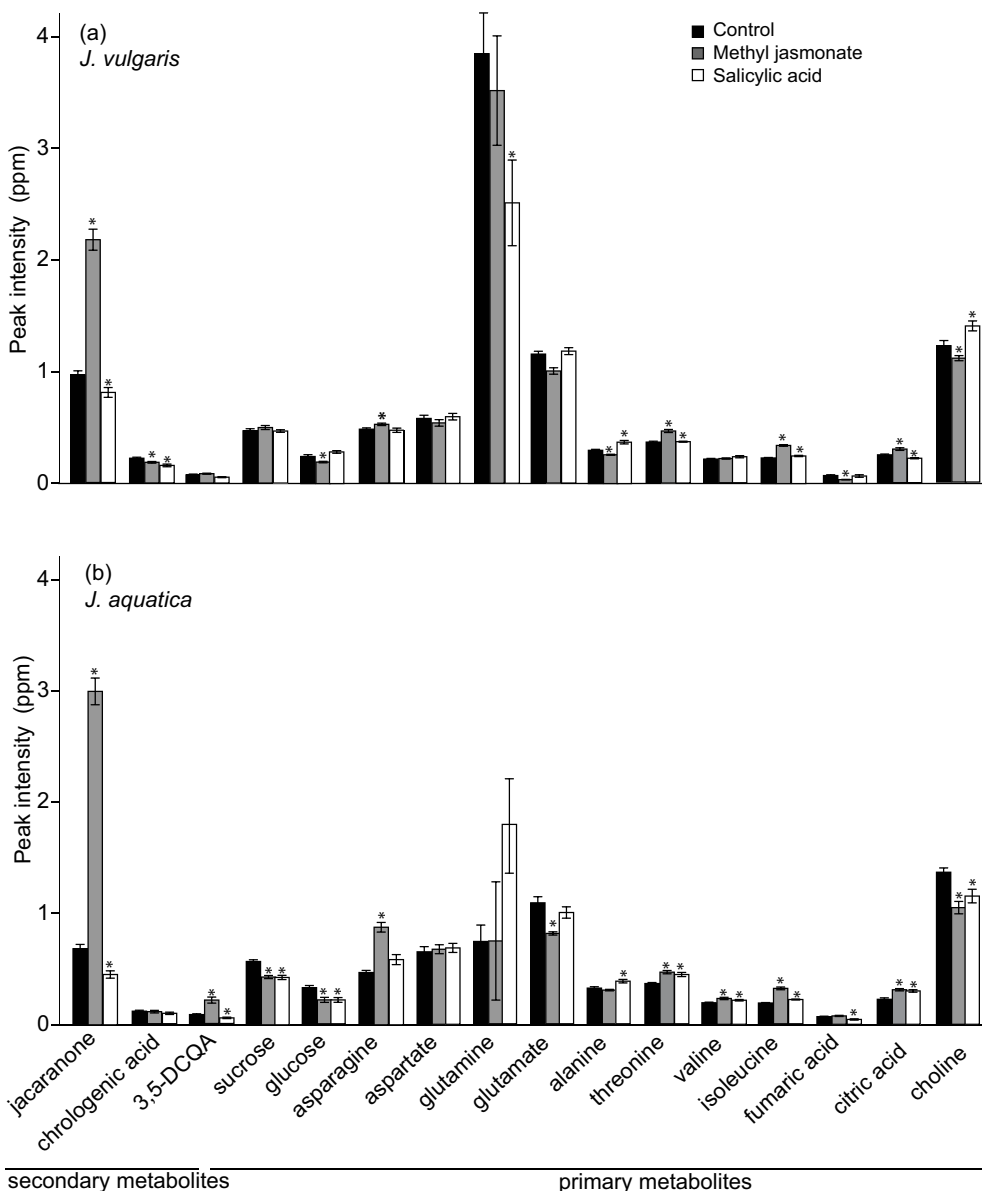


Fig. 3 Semi-quantitative expression of secondary and primary metabolites identified in *Jacobaea vulgaris* and *Jacobaea aquatica* leaves after phytohormone application. The Y-axis represents peak intensity relative to the internal standard TMSP. Asterisks indicate metabolites that differed significantly from the control after correction for multiple testing with the Benjamini Hochberg method.

Herbivore performance

Damage by the chewing caterpillar *M. brassicae*

The leaf area eaten by caterpillars significantly differed between phytohormone treatments for both plant species (KW test, *J. vulgaris*: $\chi^2=14.551$, $df=2$, $P<0.001$; *J. aquatica*: $\chi^2=14.837$, $df=2$, $P<0.001$). The leaf area eaten was significantly lower on plants treated with MeJA compared to plants treated with SA and control plants (Fig. 4a).

Damage by the cell-content feeding leafminer *L. trifolii*

Similar to *M. brassicae*, the number of leaf punctures by leafminers significantly differed between the phytohormone treatments for both plant species (KW test, *J. vulgaris*: $\chi^2=8.240$, $df=2$, $P=0.016$; *J. aquatica*: $\chi^2=7.747$, $df=2$, $P=0.021$). The number of leaf punctures was significantly reduced in the MeJA treatment group compared to the SA treatment group and the control group (Fig. 4b).

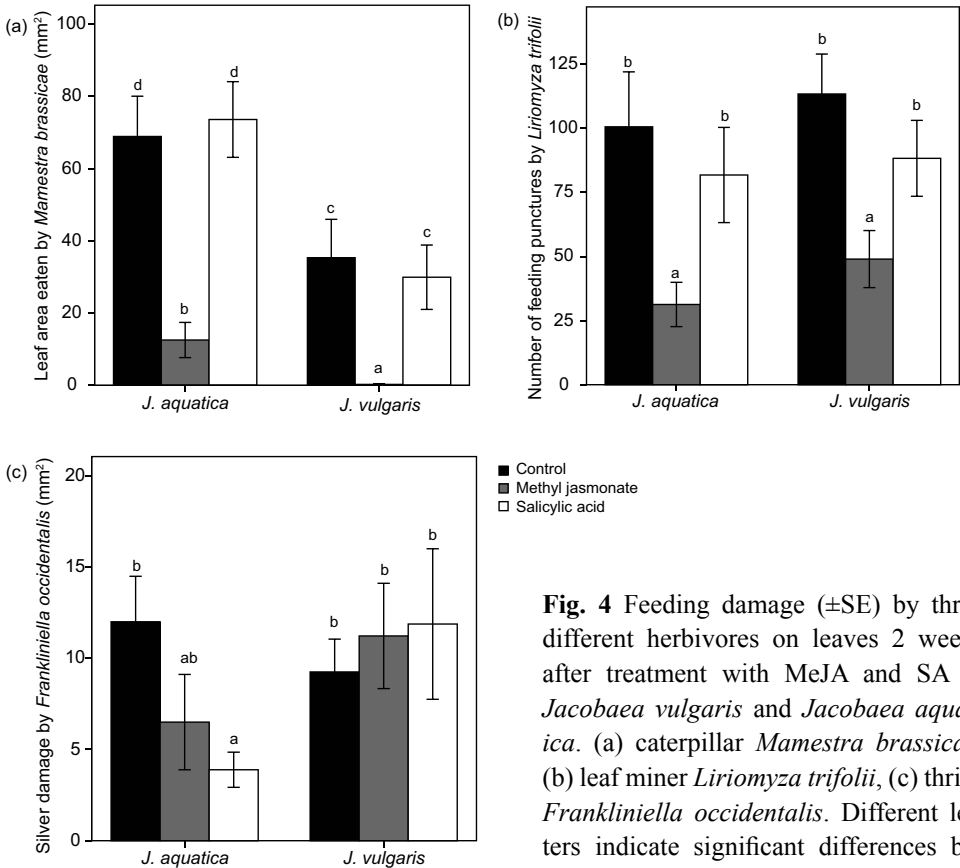


Fig. 4 Feeding damage ($\pm$ SE) by three different herbivores on leaves 2 weeks after treatment with MeJA and SA in *Jacobaea vulgaris* and *Jacobaea aquatica*. (a) caterpillar *Mamestra brassicae*, (b) leaf miner *Liriomyza trifolii*, (c) thrips *Frankliniella occidentalis*. Different letters indicate significant differences between treatment groups within plant species (post hoc Dunn test, $P<0.05$, $n=10$).

Damage by the piercing-sucking thrips *F. occidentalis*

The amount of silver damage by thrips significantly differed between treatment groups in *J. aquatica* (KW test, $\chi^2=6.938$, $df=2$, $P=0.031$) but differences were not significant in *J. vulgaris* (KW test, $\chi^2=0.089$, $df=2$, $P=0.957$). In *J. aquatica* the silver damage was significantly reduced on the plants treated with SA. MeJA treated plants did not differ significantly from the control although damage was reduced 50% compared to the control (Fig. 4c).

M. brassicae and *L. trifolii* performance and metabolites

For both plant species, there was significantly lower feeding damage of the caterpillar *M. brassicae* and the leaf miner *L. trifolii* in the MeJA treated plants. From the identified compounds (Fig. 3), jacaranone, isoleucine and asparagine significantly increased after MeJA treatment. Therefore, these three compounds may be involved in the resistance to *M. brassicae* and *L. trifolii*.

Table 3 Summary of Spearman rank correlations between silver damage of thrips and 170 NMR bins in F2 hybrids and number of NMR bins changes in *Jacobaea aquatica* and *Jacobaea vulgaris* after salicylic acid treatment

Plant species	Variables	Positive ^a	Negative
F2 hybrids	Total number of bins ^b	67	96
	Number of significant correlations	17	25
<i>J. aquatica</i>	Bin changes	15↓, 2↑	17↑, 8↓
	Significant bin changes ^c	10↓, 2↑	5 ↑
<i>J. vulgaris</i>	Bin changes	8↓, 9↑	13↑, 12↓
	Significant bin changes ^c		1↑

^a↑: up-regulated bins; ↓: down-regulated bins.

^bIn the NMR dataset of F2 hybrids, there were 170 bins between $\delta 7.68$ - $\delta 0.8$, but 7 bins have no signals ($\delta 4.96$, $\delta 4.92$, $\delta 4.88$, $\delta 4.72$, $\delta 4.68$, $\delta 4.64$ and $\delta 3.32$).

^cThe number of NMR bins that are significant after adjusting for multiple comparisons by the Benjamini & Hochberg method.

Comparative studies for thrips *F. occidentalis* tests

By comparing the results of the thrips bioassays of the F2 hybrids (Cheng et al. 2011b) with the results of the NMR analysis (Kirk et al. 2012), we found 42 NMR bins that were significantly correlated with thrips silver damage, of which 17 were positively correlated and 25 were negatively correlated (Table 3). Of these 42 bins, 17 bins also showed significant changes after SA treatment in *J. aquatica*, including 10 down-regulated NMR bins with positive correlations, 5 up-regulated bins with negative correlations and 2 up-regulated bins with positive correlations. However, only 1 bin with a negative correlation was significantly up-regulated after SA treat-

ment in *J. vulgaris* (Table 3). Because thrips performance was only affected significantly in *J. aquatica*, we will focus only on this plant species hereafter.

NMR bins were suspected to play a role in resistance/susceptibility against thrips if: 1) the bins that were significantly positively correlated with silver damage in the experiment with F2 hybrids were down-regulated after SA treatment, or 2) the bins that were significantly negatively correlated with silver damage in the experiment with F2 hybrids were up-regulated after SA treatment. In total, 10 down-regulated bins with positive correlations with silver damage in F2 hybrids indicating susceptibility and 5 up-regulated bins with negative correlations with silver damage in F2 hybrids indicating resistance were found. Of these 16 bins, 4 down-regulated bins were related to the sugars sucrose and glucose and 1 down-regulated bin was related to 3,5-DCQA. 3 up-regulated bins were related to alanine, threonine and citric acid respectively. 8 bins were too small to identify. Therefore, sucrose, glucose and 3,5-DCQA were correlated with thrips susceptibility while alanine, threonine and citric acid were correlated with thrips resistance in *Jacobaea* plants.

Discussion

The effects of phytohormone application on the metabolite profile

In both species, MeJA application led to significant change in the metabolite profile while this was less clear for the SA application. In general a larger number of NMR signals decreased than increased after SA treatment while the opposite was found after MeJA treatment.

In *Arabidopsis* SA is implicated in the induction of programmed cell death (Brodersen et al. 2005). Six bins in *J. vulgaris* and 56 bins in *J. aquatica* were significantly down-regulated in our study after SA application. These down-regulated bins might be related to cell death. At the same time the difference between the two species indicated that *J. aquatica* is much more sensitive to SA application than *J. vulgaris*.

The effects of MeJA on the metabolome have also been studied in other plant species. For instance, MeJA treated *Brassica rapa* and *Fagopyrum esculentum* plants showed a distinct separation from control plants and the phenolic compound content increased significantly after MeJA treatment in these two plant species (Kim et al. 2011; Liang et al. 2006). However, most studies on the effects of exogenous SA application are related to salt tolerance and water deficit (Bastam et al. 2013; Jesus et al. 2015) while few studies are concerned with metabolome and herbivores. Salicylic acid application significantly induced the production of hyoscyamine and scopolamine (2-12 fold) in root cultures of *Brugamansia candida* (Pitta-Alvarez et al. 2000). However, another study in ginger, *Zingiber officinale* Roscoe, showed that the concentration of some of the major flavonoids (quercetin, catechin and kaempferol) decreased significantly after SA treatment (Ghasemzadeh and Jaafar 2012).

In a cell suspension culture of *Catharanthus roseus* SA treated cells showed a high level of sugars up to 48 hours after treatment and one compound, 2,5-dihydroxybenzoic-5-*O*-glucoside, was produced only in SA-treated cells (Mustafa et al. 2009).

The effects of phytohormone treatments on herbivores with different feeding modes

The feeding damage by *M. brassicae* and *L. trifolii* were significantly reduced by MeJA application, which was in line with our hypothesis. JA treatment enhanced the plant resistance to thrips in *Arabidopsis* and *Brassica rapa* (Abe et al. 2008; Abe et al. 2009). JA application also can stimulate tomato resistance to insects by increasing the density of glandular trichomes (Boughton et al. 2005). In our study, however, MeJA application did not have a significant effect on plant resistance against thrips. In contrast we found that silver damage of thrips decreased after SA application only in *J. aquatica*.

Relationship between metabolome changes and herbivory

Isoleucine is the only identified compound that increased after MeJA and SA application in both plant species. It can be conjugated with JA to become JA-Ile (Staswick and Tiryaki 2004). A previous study demonstrated that exogenous MeJA activates defensive systems (such as VOC emissions) in plants by essentially converting itself into JA and JA-Ile and initiating a signal transduction leading to VOC emission and induction of endogenous JA-Ile and JA-Leu, which in turn cause further amplification of VOC emissions (Tamogami et al. 2008). JA-Ile as a defence signal was confirmed in the study that the susceptibility of *Manduca sexta* larvae was associated with reduced levels of JA-Ile in *Nicotiana attenuata* (Kang et al. 2006). In both *J. vulgaris* and *J. aquatica*, isoleucine was significantly increased after MeJA application. Based on these studies, we can speculate that the level of JA-Ile conjugates was increased and consequently led to a higher plant resistance after *Jacobaea* plants were treated by MeJA, which to some extent was supported by the negative correlation between feeding damage and isoleucine in this study (Fig. S1).

Jacaranone significantly increased after MeJA application in both plant species. Jacaranone has been isolated from several *Senecio* species (Kirk et al. 2005; Lajide et al. 1996; Xu et al. 2003). Jacaranone and its analogues have been shown to have insecticidal activity against the housefly *Musca domestica* and can inhibit the growth of generalist herbivore *Spodoptera litura* (Lajide et al. 1996). Leiss et al. (2009) observed a higher concentration of jacaranone in the young leaves of thrips-resistant *Jacobaea* plants. Nuringtyas et al. (2012) found higher jacaranone concentrations in the epidermis leaf tissue of *Jacobaea* plants. Both studies concluded that the higher level of jacaranone was related to thrips resistance. In contrast, our study showed that jacaranone was increased 2-3 times after MeJA application compared to the control while SA treated plants did not differ from the control. Nev-

ertheless in our study thrips damage was not correlated with jacaranone. However, jacaranone levels might be correlated to the performance of chewing herbivores and cell-content feeders (Fig. S1).

Additional evidence from F2 hybrids

Sugars (sucrose and glucose) and 3,5-DCQA showed positive correlations with silver damage in F2 hybrids, indicating that higher concentrations of sugars and 3,5-DCQA were more attractive to thrips. After SA treatment, these compounds were decreased in *J. aquatica*. Also unpublished results from thrips mortality bioassay showed that the mortality of thrips decreased with an increase in the concentration of these compounds (Fig. S2).

Alanine, threonine and citric acid showed negative correlations with silver damage in F2 hybrids, indicating that these three compounds were deterrent to the thrips. An increase of these compounds should lead to a better defence against thrips. Alanine, threonine and citric acid did increase after SA treatment in *J. aquatica*, but the effects of alanine and threonine on the thrips mortality still need to be tested. The mortality of thrips increased with an increase of the concentration of citric acid (Fig. S2).

In a previous study, concentrations of fumaric acid and adenine were found to be higher in susceptible *J. vulgaris* plants (Leiss et al. 2009). We found that these two compounds decreased significantly after SA treatment in *J. aquatica* but they were not positively correlated with silver damage of thrips in the experiment with the F2 hybrids.

In summary, MeJA treatment significantly reduced the feeding damage of the chewing herbivore *M. brassicae* and the cell-content feeder *L. trifolii* in both plant species while the feeding of piercing-sucking herbivore *F. occidentalis* was negatively affected by SA application in *J. aquatica* only. This is in line with our hypothesis. Based on the differences in metabolite profiles, as measured by ¹H-NMR, the plants treated by MeJA can clearly be separated from control and SA treated plants. Both phytohormone applications led to significant changes in a substantial number of bins. In MeJA treated plants 26.9% of the NMR bins were up-regulated while in SA treated plants 3.5% of the NMR bins were down-regulated in *J. vulgaris*. In *J. aquatica* 56.5% of the bins was up-regulated and 32.9% down-regulated. Combining the effects of SA with thrips damage and the NMR data of F2 hybrids, 16 NMR bins were selected and several compounds were identified. Sucrose, glucose and 3,5-DCQA were related to thrips susceptibility while citric acid, alanine and threonine were related to thrips resistance. Isoleucine and jacaranone were related to the resistance of the chewing herbivore *M. brassicae* and the cell-content feeder *L. trifolii*.

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Supplementary Materials:

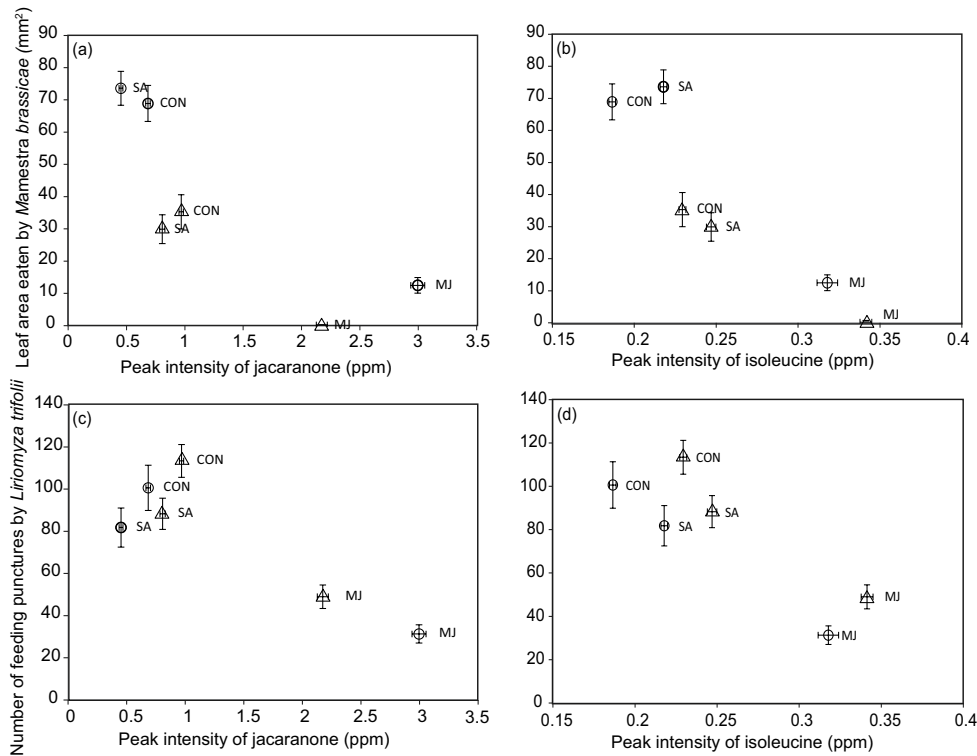


Fig. S1 Feeding damage by caterpillar *Mamestra brassicae* (panels a and b) and leafminer *Liriomyza trifolii* (panels c and d) per treatment against jacaranone and isoleucine in *Jacobaea vulgaris* and *Jacobaea aquatica*. Circles represent *J. aquatica* and triangles represent *J. vulgaris*. Horizontal error bar represents the standard error of peak intensity of metabolites and vertical one represents the standard error of feeding damage of herbivores.

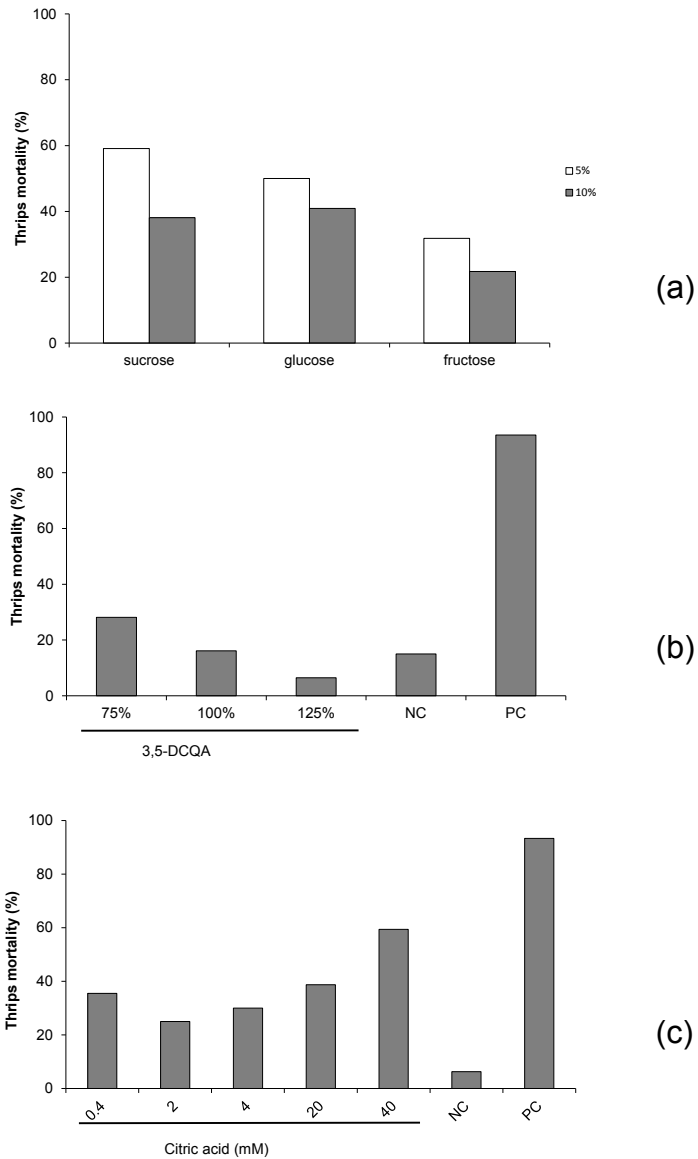


Fig. S2 The mortality of thrips *Frankliniella occidentalis* after feeding with different sugar solutions (a) and 3,5-dicaffeoylquinic acid (3,5-DCQA) (b) and citric acid (c) after 5 days. Negative control (NC): 10% fructose; Positive control (PC): Abamectine (50 ug/ml).

Chapter 7

Summary and conclusions

Secondary metabolites (SMs) are not directly involved in plant growth and development while they are involved in the interaction between plants and their environment (Hartmann 2007). Over the years and with the development of technology, more and more SMs were discovered and an ever increasing number of chemical structures have been identified (Wink 1999). Moreover, within plant species SMs show a high diversity both in terms of composition and concentration (D'Auria and Gershenzon 2005; Fahey et al. 2001; Langenheim 1994).

The interest in the effects of SMs on plant-environment interactions has boosted an increasing number of ecological studies (Schoonhoven et al. 2005). This thesis contributes to this field by using a typical class of SMs in *Jacobaea* plants, pyrrolizidine alkaloids (PAs), as a study system. Based on the chemical structures and the biosynthesis pathway, PAs can be categorized into four groups: senecionine-like PAs, jacobine-like PAs, erucifoline-like PAs and otosenine-like PAs (Cheng et al. 2011a). This clear grouping gave us the opportunity to explore the variation in PA composition and the effects of this variation on plant resistance against insect herbivores.

In chapter 2 I described the seasonal variation of PA concentration and composition in nature. In chapters 3 and 4 I explored the PA diversity by testing two hypotheses: 1) do different structurally related PAs provide resistance against different herbivores? and, 2) do generalist and specialist herbivores exert opposing selection forces on the PA concentration? In chapter 5 I discussed if the PA concentration and composition were induced or affected by the exogenous application of the phytohormone methyl jasmonate. Finally, in chapter 6, I studied the effects of phytohormone application on the composition of SMs, using NMR analyses, and on resistance to different types of herbivores.

Chapter 2. How are PAs affected by nature's seasonal variation? A series of clones of 8 genotypes differing in their alkaloid composition were investigated over a 14 months period in an experimental garden. Besides herbivory, plants in nature also need to deal with other stresses, such as a fluctuating temperature, frost and periods of drought. Plants were harvested every 2 to 3 weeks to study plant growth and PA concentration and composition over the course of the vegetative stage. We chose the parents, 2 F1 and 4 F2 genotypes from a cross between *Jacobaea vulgaris* and *Jacobaea aquatica* that is maintained in tissue culture. We selected the F2 genotypes on the basis of differences in alkaloid concentration and composition. In contrast to other studies we followed the same genotypes over the whole season and therefore could distinguish the seasonal variation from genetic variation.

It was observed that after an steady increase during summer the shoot and root dry mass dropped by about 70% during the winter period (February) before they recovered during the next spring. The variation of the total PA amount was strongly correlated with that of the dry mass and together with the loss of dry mass

in winter a large part of the PAs was lost. Senecionine- and otosenine-like PAs were mainly stored in the roots while the erucifoline- and jacobine-like PAs were mainly stored in shoots. In both roots and shoots the total PA concentration increased gradually until the end of winter and then during spring it decreased, particularly in the shoots. For the 6 genotypes rich in jacobine-like PAs, in the shoots the proportion of jacobine-like PAs showed a decrease during winter, followed by a strong increase in the next spring. In contrast, in the roots a gradual increase of jacobine-like PAs and a decrease of senecionine-like PAs was observed over the whole period. The free base/N-oxide ratio was relatively constant over the seasons until the second spring. Thereafter it sharply increased, most notably in the *J. vulgaris* parent plant. While the genotypes differed in PA composition the direction of the seasonal changes was rather similar for the different genotypes. As a result the variation among genotypes was maintained across seasons.

Chapter 3. What is the relative importance of PAs in the oviposition choice of a specialist herbivore? We tested the oviposition preference of the cinnabar moth, *Tyria jacobaeae*, with regard to plant size, nitrogen content, water content and PA concentration and composition. Moths were collected from Meijendel, a regularly defoliated area and from Bertogne, a rarely defoliated area. Ragworts found in coastal areas (Meijendel) are rich in jacobine-like PAs while those that occur inland (Bertogne) are rich in erucifoline-like PAs. Based on the PA composition of the host plants from which the cinnabar moths were collected we selected plant genotypes from the cross described above to represent ranges in both jacobine- and erucifoline-like PA concentrations. We performed choice experiments in experimental cages to study oviposition preference.

Water and nitrogen content did not vary strongly between the plants and were not correlated with oviposition preference. Moths from both origins laid larger egg batches on plants rich in jacobine-like PAs. However, the effects of PA composition were small although significant. In addition we found that moths originating from Meijendel and Bertogne had different oviposition behaviour: moths from Meijendel preferred larger plants and spread their eggs over more egg batches compared to moths from Bertogne. This difference in oviposition behaviour seems adaptive. A preference for larger plants and a bet hedging strategy by producing more and smaller egg batches makes sense for the Meijendel moths that are regularly confronted with complete defoliation of their host plant.

Chapter 4. What is the potential role of herbivores in maintaining PA diversity? Following up a study of Cheng (2012), the generalist herbivore slug *Deroceras invadens* and the specialist flea beetle *Longitarsus jacobaeae* were tested in the same set of F2 hybrids of *Jacobaea* plants differing in PA concentration and composition. No choice and multiple choice feeding tests *in vivo* were conducted respectively for the slug and the flea beetle.

Slug feeding damage was genotype dependent. It was negatively affected by total PA concentration and the concentration of senecionine-like PAs. The common structure of the individual PAs that were significantly negatively correlated with slug feeding is a double bond between the C13 and C19 position. PA concentration and composition did not influence the feeding of flea beetle. Plant size was an important factor for the feeding preference of this specialist.

In this study I combined all the previously tested generalist and specialist herbivores with the same F2 system in order to test the Generalist-Specialist Dilemma. All preferences or performances of the 4 generalist herbivores showed negative correlations with at least one group of PAs while the preference and performance of the specialist *T. jacobaeae* was positively correlated with PAs and *L. jacobaea* was not influenced by PAs. Therefore, this chapter provided evidence for the Generalist-Specialist Dilemma hypothesis.

Chapter 5. Are PAs induced by herbivory? Methyl jasmonate (MeJA) application was used to mimic herbivory. A range of concentrations of MeJA was added to the medium of tissue culture plants of *J. vulgaris* and *J. aquatica* under axenic conditions. We chose control plants and plants from one MeJA treatment ($1.00 \mu\text{g } \mu\text{l}^{-1}$) to test if the feeding of the generalist herbivore *Spodoptera exigua* was affected by MeJA application.

The total PA concentration in both species at the whole plant level was marginally or not affected by applying different amounts of MeJA to the roots. This indicated that applying MeJA does not elicit *de novo* synthesis of PAs. In *J. vulgaris*, the total PA concentration in roots decreased and in shoots increased, suggesting that PA reallocation took place from root to shoot. In *J. aquatica*, however, this reallocation was not significant. The latter may be due to the fact that the majority of PAs were already stored in the shoots without applying MeJA. Interestingly, we found that the application of MeJA in both species induced a strong shift in the PA composition especially from senecionine-like PAs to erucifoline-like PAs, indicating that PA conversion took place. For instance, the relative concentration of erucifoline-like PAs increased from an initial 6.2% before MeJA application to 54.7% at the highest concentration ($5.00 \mu\text{g } \mu\text{l}^{-1}$) of MeJA while that of senecionine-like PAs dropped from 81.1% to 28.7% in *J. vulgaris* roots. Senecionine was far less toxic in cell lines of *S. exigua* than erucifoline (Nuringtyas et al. 2014). However, other studies showed that the number of pupae of *Liriomyza trifolii* was positively correlated with erucifoline-like PAs (Cheng 2012) while ragworts of the erucifoline chemotype were the least attacked by *T. jacobaeae* (Macel and Klinkhamer 2010). It remains, however, unclear why no increase in jacobine-like PAs was found because this type of PAs is strongly bioactive as well.

Comparing with the *J. aquatica* control plants, *S. exigua* ate approximately 40% of the leaves treated with MeJA. *S. exigua*, however, barely fed on the leaves

of control plants as well as MeJA treated *J. vulgaris* plants. This result suggested that an increase of erucifoline-like PAs induced by MeJA application played a role in resistance to *S. exigua* in *J. aquatica* while other defence mechanisms might be present in *J. vulgaris*.

Chapter 6. What is the relative role of SMs other than PAs in resistance to herbivores? I studied the changes in concentrations of other chemical metabolites by NMR after MeJA and SA application on *J. vulgaris* and *J. aquatica* and explored how the phytohormone treatments affected the feeding of the chewing caterpillar *Mamestra brassicae*, the cell-content feeding leafminer *L. trifolii* and the piercing-sucking thrips *Frankliniella occidentalis*.

Significantly less feeding damage by the herbivores *M. brassicae* and *L. trifolii* was observed on plants treated with MeJA while no significant differences were found between control and SA treated plants in both species. Thrips damage was significantly reduced in *J. aquatica* plants treated with SA while thrips feeding was not affected by SA treatment in *J. vulgaris*. PCA analysis showed a clear distinction between the metabolome of MeJA treated, SA treated and control plants. Among the 16 identified compounds, the peak intensity of jacaranone and isoleucine increased 2-3 times after MeJA treatment. Thus the reduced feeding by the chewing herbivore *M. brassicae* and cell-content feeder *L. trifolii* might be related to the increased concentration of these compounds. Moreover, a previous study showed that the number of emerging pupae of *L. trifolii* was positively correlated with erucifoline-like PAs and the feeding preference of *M. brassicae* was not influenced by senecionine-like PAs (Cheng 2012; Macel et al. 2005). Therefore, it is likely that in MeJA treated plants, PAs were not the cause of the reduced feeding damage by these two herbivores.

SA application did not lead to significant changes in PA concentration and composition (Yan, unpublished data) while there was significantly less thrips silver damage in SA treated plants. This study indicated that the relative role of PAs in resistance to these three herbivores was small. To find candidate compounds in thrips resistance, we combined available data for thrips silver damage (Cheng et al. 2011b) and NMR analysis (Kirk et al. 2012) for a segregation F2 population of a cross between *J. vulgaris* and *J. aquatica* and identified NMR bins that were positively or negatively correlated with silver damage. We then checked whether or not these bins were down-regulated or up-regulated after SA treatment in *J. aquatica*. This analysis lead to three candidate compounds related to susceptibility (sucrose, glucose and 3,5-DCQA) and three compounds related to resistance (citric acid, threonine and alanine). The positive effects of sucrose, glucose and 3,5-DCQA and the negative effects of citric acid on thrips survival were confirmed by results of unpublished *in-vitro* tests done previously in our research group. Literature data showed that β -alanine was positively correlated to thrips mortality.

Discussion and conclusions

The *Jacobaea* hybrid system has been proved to be a good tool to study the interaction between PA variation and herbivore resistance (Cheng, 2012). Based on this hybrid system, we have explored how the biotic and abiotic factors influence the PA diversity. All the herbivore tests were integrated to draw the general conclusion that the selection pressures from different herbivores contribute to the diversity in PA composition and that generalist and specialist herbivores exert a divergent selection pressure on PA concentrations. The PAs showed seasonal variation within each genotype which was the first study to report the seasonal variation of SMs that encompass a time series over all seasons and using distinct genotypes. It showed that the relative differences between genotypes remained constant over time an assumption that is often implicitly made for many studies. If natural selection is to act on PA diversity one of the prerequisites is that PA diversity does not change over time due to environmental differences and my results show that this is the case. These results confirmed the high heritability of PA concentration and diversity estimated from sib families raised at the same time point (Vrieling et al. 1993).

The studies of exogenous application of MeJA can lead to a shift in PA composition and primary metabolites. Shifts in diversity of SMs are underestimated as most studies only report the increase in classes of total metabolites. Importantly the shift in metabolite diversity induced by MeJA application can conversely affect the feeding of chewing herbivores and cell-content feeders. Plants enhance the defense abilities by changing the diversity of SMs. This strongly suggested that SM composition is more important for the plant survival comparing to the total concentration. For instance, senkirkine and seneciophylline showed a lower toxicity than jacobine and erucifoline to the cell lines of *S. exigua* (Nuringtyas et al. 2014). In thrips mortality tests, retrorsine jacobine are much more toxic than senecionine (Liu et al, unpublished). In contrast to MeJA application, SA application did not lead to significant changes in PAs and the metabolite profile changes detected by NMR rendered *J. aquatica* more resistance to piercing-sucking herbivore thrips showing that PAs did not affect thrips resistance. Several metabolites detected by NMR were found to be correlated with resistance (alanine, threonine and citric acid) and susceptibility (sucrose, glucose and 3,5-dicaffeoylquinic acid) to thrips *F. occidentalis*. These compounds are currently tested by *in-vitro* bioassay. Both phytohormone application studies confirmed the hypothesis that chewing herbivore and cell-content feeder activate the JA pathway while the SA pathway can be turn on by piercing-sucking herbivore. The effects of phytohormone application on herbivory might be plant species-dependent. Because all the experiments with phytohormones were conducted under axenic conditions, the differences of herbivore feeding were mainly from the metabolite changes induced by phytohormone application, showing the importance of metabolites in plant resistance.

With respect to the development of different analysis methods of chemical

compounds, metabolites have been extensively applied to the study of plant-environment interactions. In *Jacobaea* study system, the plants benefit from the PA diversity when plants confronted the pressures from the outside, such as herbivory and climatological variation. The PA diversity may be helpful for plants to adapt to the environment. Meanwhile, the environmental pressures exerted different selection forces to keep the PA diversity. In addition, the genetic map of this hybrid system has been constructed and quantitative trait locus analysis is going on. The importance of PAs on plant-insect relationship will be explored further.

The main findings of this study are concluded as following:

- Different generalist herbivores are deterred by different groups of PAs in plants and specialist herbivores can either use PAs as oviposition cues or they are not affected at all by PAs. Hence the selection pressure from different herbivores can drive PA diversity.
- We found only relatively small effects of the total PA concentration on herbivore feeding and oviposition. The latter indicates that the PA composition is more important than THE total PA concentration for plant fitness.
- PA concentration and composition change over seasons, but the seasonal variation does not override the initial differences among genotypes.
- There is no reallocation of PAs from shoots to roots during the winter period. During the winter season a large proportion of PAs is lost together with a strong reduction in plant dry mass.
- MeJA application does not increase the total PA concentration, but it leads to a shift in PA composition from senecionine-like PAs to erucifoline-like PAs.
- MeJA treatment rendered plants of both species more resistant to the generalist herbivores *M. brassicae* and *L. trifolii* while resistance to *S. exigua* only increased in *J. vulgaris*. Interestingly, thrips were affected similarly in the two plant species by phytohormone treatment, but the feeding damage was only significantly reduced in SA treated *J. aquatica* plants.
- Jacaranone and isoleucine may be involved in the resistance to the chewing herbivore *M. brassicae* and cell-content feeder *L. trifolii*. Sucrose, glucose and 3,5-DCQA are related to thrips susceptibility while alanine, threonine and citric acid are related to thrips resistance.

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Samenvatting en conclusies

Secundaire metabolieten (SM's) zijn niet direct betrokken bij de groei en ontwikkeling van planten, maar spelen een rol in de interactie tussen planten en hun omgeving (Hartmann 2007). Mede dankzij technologische ontwikkelingen zijn in de loop der jaren steeds meer SM's ontdekt en een alsmaar toenemend aantal chemische structuren geïdentificeerd (Wink 1999). Er blijkt een grote diversiteit in SM's te zijn binnen plantensoorten, zowel qua samenstelling als concentratie (D'Auria en Gershenzon 2005; Fahey et al. 2001; Langenheim 1994).

De belangstelling voor de effecten van SM's op plant-omgeving interacties heeft tot een sterke toename van het aantal ecologische studies geleid (Schoonhoven et al. 2005). Dit proefschrift is er daar één van, met het gebruik van pyrrolizidine alkaloiden (PA's), een specifieke klasse van SM's in *Jacobaea* planten, als modelsysteem. Op basis van de chemische structuren en de biosynthese, kunnen PA's worden onderverdeeld in vier groepen: senecionine-achtige PA's, jacobine-achtige PA's, erucifoline-achtige PA's en otosenine-achtige PA's (Cheng et al. 2011a). Deze duidelijke groepering gaf ons de mogelijkheid om de variatie in PA samenstelling en de gevolgen van deze variatie voor plantenresistentie tegen insecten herbivoren te bestuderen.

In hoofdstuk 2 beschreef ik de seizoensgebonden variatie van PA concentratie en samenstelling in een natuurlijke situatie. In hoofdstuk 3 en 4 onderzocht ik de PA diversiteit aan de hand van twee hypothesen: 1) Verschaffen verschillende, structureel verwante PA's resistentie tegen verschillende herbivoren? En, 2) Oefenen generalistische en specialistische herbivoren tegengestelde selectiekrachten uit op de samenstelling en concentratie van PA's? In hoofdstuk 5 besprak ik of de PA concentratie en samenstelling werd geïnduceerd of anderszins beïnvloed door de externe toediening van het fytohormoon methyljasmonaat. In hoofdstuk 6, ten slotte, bestudeerde ik de effecten van fytohormoon toediening op de samenstelling van PA's, met behulp van NMR analyses, en op de resistentie tegen verschillende typen herbivoren.

Hoofdstuk 2. Hoe worden PA's beïnvloed door natuurlijke seizoensgebonden variatie? Een serie klonen van 8 genotypen, die verschilden in hun alkaloiden samenstelling, werd gedurende 14 maanden bestudeerd in een proeftuin. Naast schade door herbivoren hebben planten in hun natuurlijke omgeving ook te maken met andere soorten stress, zoals een fluctuerende temperatuur, vorst en perioden van droogte. Gedurende de gehele periode van het vegetatieve stadium werden elke 2 tot 3 weken planten geoogst om hun groei en PA concentratie en samenstelling te bestuderen. We kozen de ouders, 2 F1 en 4 F2 genotypen van een kruising tussen *J. vulgaris* en *J. aquatica* die in stand wordt gehouden in weefselkweek. De F2 genotypen werden gekozen op basis van hun verschillen in alkaloiden concentratie en samenstelling. In tegenstelling tot andere studies volgden wij dezelfde genotypen

gedurende het hele seizoen en konden daardoor onderscheid maken tussen seizoensgebonden en genetische variatie.

Het drooggewicht van zowel spruit als wortel vertoonde een gestage toename in de zomer, maar nam tijdens de winter periode (februari) met ongeveer 70% sterk af, waarna een herstel volgde tijdens de volgende lente. De variatie in de totale hoeveelheid PA's was sterk gecorreleerd met die van het drooggewicht. En met het verlies van drooggewicht in de winter ging ook een groot deel van de PA's verloren. Senecionine- en otosenine-achtige PA's werden voornamelijk opgeslagen in de wortels, terwijl de erucifoline- en jacobine-achtige PA's hoofdzakelijk werden opgeslagen in de spruit. De totale PA concentratie nam in zowel wortels als spruit geleidelijk toe tot het einde van de winter, om vervolgens in het voorjaar weer te dalen, met name in de spruit. Voor de 6 genotypen rijk aan jacobine-achtige PA's gold dat in de spruit het aandeel van jacobine-achtige PA's in de winter daalde en vervolgens in het volgende voorjaar weer sterk steeg. In de wortels daarentegen werd een geleidelijke toename van jacobine-achtige PA's en een afname van senecionine-achtige PA's waargenomen gedurende de gehele onderzochte periode. Tot aan de tweede lente was de vrije base / N-oxide-verhouding relatief constant. Daarna nam deze sterk toe, met name in de *J. vulgaris* moederplant. Terwijl de genotypen verschilden in PA samenstelling, waren de veranderingen die gedurende de verschillende seizoenen plaatsvonden onderling goed vergelijkbaar. Zodoende bleef de variatie tussen de genotypen behouden tijdens de seizoenen.

Hoofdstuk 3. Wat is het relatieve belang van PA's in de ovipositie voorkeur van een specialistische herbivoor? We testten de ovipositie voorkeur van de sint-jacobsvlinder, *Tyria jacobaeae*, in relatie tot de plantgrootte, het stikstofgehalte, watergehalte, de PA concentratie en PA samenstelling. Sint-jacobsvlinders werden verzameld in Meijndel, een gebied waar zebra-rupsen regelmatig alle jacobskruiskruidplanten volledig kaal vreten en in Bertogne, waar complete kaalvraat door zebra-rupsen zelden voorkomt. Jakobskruiskruid planten in kustgebieden (Meijndel) zijn rijk aan jacobine-achtige PA's, terwijl die in het binnenland (Bertogne) rijk zijn aan erucifoline-achtige PA's. Op basis van de PA samenstelling van de gastheerplanten waarvan de sint-jacobsvlinders werden verzameld kozen we uit de hierboven beschreven kruising genotypen die varieerden in zowel jacobine- als erucifoline-achtige PA concentraties. We bestudeerden de ovipositie voorkeur van sint-jacobsvlinders door keuze-experimenten uit te voeren in kooien.

Tussen de planten was weinig variatie in het water- en stikstofgehalte en er was geen correlatie met ovipositie voorkeur. De effecten van de PA samenstelling waren klein maar significant. Sint-jacobsvlinders uit beide gebieden legden grotere eipakketten op planten rijk aan jacobine-achtige PA's. Daarnaast vonden we dat sint-jacobsvlinders afkomstig uit Meijndel en Bertogne verschillend eileggedrag vertoonden: in vergelijking met sint-jacobsvlinders uit Bertogne hadden sint-jac-

cobsvlinders uit Meijndel voorkeur voor grotere planten en verspreidden hun eieren over meer eipakketten. Dit verschil in eileggedrag lijkt adaptief. Een voorkeur voor grotere planten en het spreiden van kansen door het produceren van meer en kleinere eipakketten is zinvol voor de sint-jacobsvlinders uit Meijndel die regelmatig worden geconfronteerd met de volledige kaalvraat van hun waardplant.

Hoofdstuk 4. Wat is de mogelijke rol van herbivoren in het behoud van PA diversiteit? Als vervolg op een studie van Cheng (2012), werden experimenten uitgevoerd met de generalistische naaktslak *Deroceras invadens* en de specialistische aardvlo *Longitarsus jacobaeae*, waarbij dezelfde F2 hybriden van *Jacobaea* planten variërend in PA concentratie en samenstelling werden gebruikt.

Niet-keuze en meerkeuze toetsen werden uitgevoerd met respectievelijk de naaktslakken en de aardvlooien. Vraatschade van de naaktslakken was genotype afhankelijk. Het werd negatief beïnvloed door de totale PA concentratie en de concentratie van senecionine-achtige PA's. De individuele PA's die significant negatief gecorreleerd bleken met naaktslakvraat hebben als overeenkomst dat ze in hun structuur allemaal een dubbele binding hebben tussen C13 en C19. PA concentratie en samenstelling hadden geen invloed op de vraatschade van de aardvlooien. Plantgrootte was een belangrijke factor voor de voedingskeuzes van deze specialist.

Teneinde de 'generalist-specialist dilemma' hypothese te testen combineerde ik in deze studie de resultaten van alle eerder met hetzelfde F2 systeem geteste generalistische en specialistische herbivoren. Alle voorkeuren of prestaties van de 4 generalistische herbivoren toonden negatieve correlaties met ten minste één groep van PA's. Daarentegen waren de voorkeur en de prestaties van de gespecialiseerde *Tyria jacobaeae* positief gecorreleerd was met PA's terwijl *L. jacobaea* niet beïnvloed werd door de PA's. Hiermee levert dit hoofdstuk bewijs voor de 'generalist-specialist dilemma' hypothese.

Hoofdstuk 5. Worden PA's geïnduceerd door vraat van herbivoren? De toediening van methyljasmonaat (MeJA) werd gebruikt om insectenvraat te simuleren. Aan het medium van *Jacobaea vulgaris* en *Jacobaea aquatica* weefselkweekplanten werd onder steriele omstandigheden MeJA toegevoegd in een reeks van concentraties. Om te testen of de vraat van de generalistische herbivoor *Spodoptera exigua* beïnvloed werd door de MeJA toediening werden controle planten en planten van één MeJA behandeling ($1.00 \mu\text{g } \mu\text{l}^{-1}$) gekozen.

In beide plantensoorten werd de PA concentratie in de gehele plant niet of maar marginaal beïnvloed door de toevoeging van verschillende hoeveelheden MeJA aan de wortels. Dit wijst erop dat MeJA geen *de novo* synthese van PA's veroorzaakt. In *J. vulgaris* nam de totale PA concentratie in de wortels af, terwijl deze in de spruit toenam, wat suggereert dat er herverdeling van de wortel naar de spruit plaatsvindt. In *J. aquatica* daarentegen was deze herverdeling niet significant. Dit laatste zou kunnen komen door het feit dat ook zonder de toediening van MeJA de meerderheid

van de PA's al in de spruit is opgeslagen. Interessant is dat de toediening van MeJA in beide soorten een sterke verschuiving in PA samenstelling veroorzaakte, met name van de senecionine-achtige PA's naar de erucifoline-achtige PA's, wat erop wijst dat er PA conversie plaats vindt. In *J. vulgaris* wortels, bijvoorbeeld, nam de relatieve concentratie van erucifoline-achtige PA's toe van 6.2% voor MeJA toediening, naar 54.7% bij de toediening van de hoogste concentratie ($5.00 \mu\text{g } \mu\text{l}^{-1}$) MeJA, terwijl de relatieve concentratie van senecionine-achtige PA's daalde van 81.1% naar 28.7%. Senecionine was in vergelijking met erucifoline veel minder toxisch in cellijnen van *S. exigua* (Nuringtyas et al. 2014). Andere studies daarentegen toonden aan dat de hoeveelheid poppen van *Liriomyza trifolii* positief gecorreleerd waren met erucifoline-achtige PA's (Cheng 2012) terwijl jacobskruiskruidplanten van het erucifoline chemotype minder aangevallen werden door *T. jacobaeae* (Macel and Klinkhamer 2010). Het blijft echter onduidelijk waarom er geen toename werd gevonden in jacobine-achtige PA's terwijl dit type PA's ook zeer bioactief is.

Vergeleken met de *J. aquatica* controle planten at *S. exigua* ongeveer 40% van de bladeren die behandeld waren met MeJA. *S. exigua* at daarentegen nauwelijks van de bladeren van zowel de controle als met MeJA behandelde *J. vulgaris* planten. Dit resultaat suggereert dat in *J. aquatica* de door MeJA toediening geïnduceerde toename van erucifoline-achtige PA's een rol speelt in de resistentie tegen *S. exigua* terwijl in *J. vulgaris* een ander afweermecanisme aanwezig moet zijn.

Hoofdstuk 6. Wat is met betrekking tot herbivorenresistentie in *J. vulgaris* en *J. aquatica* de relatieve rol van andere SM's, naast die van PA's? Met behulp van NMR bestudeerde ik in *J. vulgaris* en *J. aquatica* na MeJA en salicylzuur (SA) toediening de veranderingen in concentraties van andere metabolieten en onderzocht hoe de fytohormoon behandelingen de vraat beïnvloeden van de kauwende rups *Mamestra brassicae*, de mineervlieg *Liriomyza trifolii* en de trips *Frankliniella occidentalis*, die cellen aanprikt en leegzuigt.

Voor beide plantensoorten werd er door de herbivoren *M. brassicae* en *L. trifolii* significant minder vraatschade veroorzaakt aan planten die behandeld waren met MeJA, terwijl er geen significante verschillen werden gevonden tussen controle planten en planten behandeld met SA. Tripsschade was significant minder in *J. aquatica* planten behandeld met SA, terwijl in *J. vulgaris* de tripsschade niet beïnvloed werd door SA behandeling. PCA analyses lieten een duidelijk onderscheid zien tussen de metabool profielen van MeJA behandelde, SA behandelde en controle planten. Van de 16 geïdentificeerde stoffen lieten jacaranone en isoleucine na MeJA behandeling een twee- tot drievoudige toename zien van de piekintensiteit. De verminderde vraatschade van de kauwende herbivoor *M. brassicae* en de zich met cel-inhoud voedende *F. occidentalis* zou dus het gevolg kunnen zijn van de toegenomen concentratie van deze stoffen. Een eerdere studie toonde reeds aan dat de hoeveelheid poppen van *L. trifolii* positief gecorreleerd was met erucifoline-achtige

PA's en dat de voorkeur van *M. brassicae* niet beïnvloed werd door senecionine-achtige PA's (Cheng 2012; Macel et al. 2005). Dit maakt het waarschijnlijk dat PA's niet de veroorzakers zijn van de verminderde vraatschade in de met MeJA behandelde planten van deze twee herbivoren.

SA toediening leidde niet tot significante veranderingen in PA concentratie en samenstelling (Yan, niet gepubliceerde data) terwijl er significant minder trips zilverschade was in de met SA behandelde planten. Deze studie lijkt aan te geven dat de rol van PA's op de resistentie tegen deze drie herbivoren klein is. Om stoffen te identificeren die mogelijk een rol spelen bij trips resistentie combineerden we beschikbare data voor tripsschade (Cheng et al. 2011b) en NMR analyses (Kirk et al. 2012) van een F2 populatie van een kruising tussen *J. vulgaris* en *J. aquatica*. We identificeerden de NMR bins die positief of negatief gecorreleerd waren met trips zilverschade en controleerden of deze waren afgenomen of toegenomen na SA toediening in *J. aquatica*. Deze analyse leidde tot drie kandidaat stoffen die gerelateerd konden worden aan trips gevoeligheid (sucrose, glucose en 3,5-DCQA) en drie kandidaat stoffen die gerelateerd konden worden aan trips resistentie (citroenzuur, threonine en β -alanine). De positieve effecten van sucrose, glucose en 3,5-DCQA en de negatieve effecten van citroenzuur op trips overleving waren in overeenstemming met de (niet gepubliceerde) resultaten van *in-vitro* testen eerder uitgevoerd in onze onderzoeksgroep. Verder lieten literatuur data zien dat β -alanine positief gecorreleerd was met trips mortaliteit.

Conclusies

- Verschillende generalistische herbivoren worden afgeweerd door verschillende groepen van PA's aanwezig in de planten terwijl specialistische herbivoren PA's of als ovipositie stimulant kunnen gebruiken of er helemaal niet door beïnvloed worden. De selectiedruk van verschillende herbivoren kan dus leidend zijn in het in stand houden van PA diversiteit.
- We vonden alleen relatief kleine effecten van de PA concentratie op herbivoren-vraat en ovipositie. Dit lijkt erop te wijzen dat voor plantfitness de PA samenstelling belangrijker is dan de PA concentratie.
- PA concentratie en samenstelling veranderen tijdens de seizoenen, maar de seizoensgebonden variatie heft de initiële verschillen tussen de genotypen niet op.
- Er vindt geen herverdeling van PA's van de spruit naar de wortel plaats tijdens de winterperiode. Tijdens de winter verdwijnt een groot deel van de PA's te samen met een sterke teruggang in plant biomassa.
- MeJA toediening veroorzaakt geen toename in totale PA concentratie, maar leidt wat PA samenstelling betreft tot een verschuiving van senecionine-achtige PA's naar erucifoline-achtige PA's.

- MeJA behandeling van *J. vulgaris* and *J. aquatica* resulteerde voor beide plantensoorten in een hogere resistentie tegen de generalistische herbivoren *M. brassicae* en *L. trifolii*, terwijl resistentie tegen *S. exigua* alleen toenam in *J. vulgaris*. Interessant was dat in de twee plantensoorten trips op dezelfde manier werd beïnvloed door fytohormoon toediening, maar dat de tripsschade alleen significant kleiner was in de met SA behandelde *J. aquatica* planten.
- Jacaranone en isoleucine zijn mogelijk betrokken bij de resistentie tegen de kauwende herbivoor *M. brassicae* en van *L. trifolii* die zich met de inhoud van cellen voedt. Sucrose, glucose en 3,5-DCQA zijn gerelateerd aan gevoeligheid voor trips terwijl alanine, threonine en citroenzuur gerelateerd zijn aan trips resistentie.

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Xianqin Wei
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Curriculum Vitae

Xianqin Wei was born on 26th November, 1986 (the 25th day of the 10th month that year according to the traditional Chinese calendar) in Weifang, Shandong, China. After taking the National College Entrance Examination in the summer of 2004, she entered Qingdao Agricultural University. She obtained the bachelor's degree in Biological Science in 2008. She then continued with a master study in Botany at the Sichuan University. In 2011, she obtained the Master of Science degree with her thesis: "Cytotaxonomic study and morphology of *Allium* L. from Southwestern China" under the guidance of Prof. Dr. Xingjin He. In the same year, she was awarded a CSC grant to pursue a PhD at the Institute of Biology, Leiden University, The Netherlands. She started her scientific journey in the area of Plant Ecology and Phytochemistry under the supervision of Prof. Dr. Peter G. L. Klinkhamer, Dr. Klaas Vrieling and Dr. Patrick P. J. Mulder. Her PhD research mainly involved exploring the diversity of pyrrolizidine alkaloids in *Jacobaea* species. The work is presented in this thesis. She has attended the PhD courses of the Faculty of Science, Leiden University and presented her work at national and international conferences.

Publications

Scientific publication

Xianqin Wei, Klaas Vrieling, Patrick P. J. Mulder, Peter G. L. Klinkhamer (2015) Testing the Generalist-Specialist Dilemma: the role of pyrrolizidine alkaloids in resistance to invertebrate herbivores in *Jacobaea* species. *Journal of Chemical Ecology* 41: 159-167

Manuscripts submitted for publication

Xianqin Wei, Klaas Vrieling, Patrick P. J. Mulder, Peter G. L. Klinkhamer (submitted) The effects of exogenous application of methyl jasmonate on the concentration of composition of pyrrolizidine alkaloids and resistance towards *Spodoptera exigua* in two *Jacobaea* species.

Xianqin Wei, Klaas Vrieling, Patrick P. J. Mulder, Peter G. L. Klinkhamer (in review) Local adaptation in oviposition choice of the cinnabar moth.

Manuscripts in preparations

Xianqin Wei, Klaas Vrieling, Hye Kyong Kim, Peter G. L. Klinkhamer. The effects of phytohormone application on metabolite profiles and the feeding of the chewing, piercing-sucking and cell-content herbivores: a study in *Jacobaea vulgaris* and *J. aquatica*

Xianqin Wei, Klaas Vrieling, Patrick P. J. Mulder, Peter G. L. Klinkhamer. Seasonal variation in pyrrolizidine alkaloid concentration and composition in clones of *Jacobaea vulgaris*, *Jacobaea aquatica* and their F1 and F2 hybrids