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

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Chapter 6

The role of pyrrolizidine alkaloids in American serpentine leafminer (*Liriomyza trifolii*) resistance in the hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica*

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Jacobaea species (Asteraceae) contain pyrrolizidine alkaloids (PAs), which are deterring and toxic to generalist herbivores. To investigate the function of the diversity of PAs in *Jacobaea* species, we examined the relationship between PA variation and resistance against a generalist leafminer (American serpentine leafminer, *Liriomyza trifolii*) in an artificial *Jacobaea* hybrid family including one *Jacobaea vulgaris*, one *Jacobaea aquatica*, two F_1, and 90 different F_2 hybrid genotypes.

We conducted a leafminer bioassay with replicated individuals (genotypes) from the *Jacobaea* hybrid family. We measured size of the plants and counted the number of pupae from each plant. For the F_2 hybrids we analyzed whether the number of pupae differed among genotypes and we examined the relationship between the number of pupae and the concentration of 37 individual PAs, the sum concentration of the 4 groups of structurally related PAs and the total PA concentration.

We showed that genotypes differed significantly in the number of pupae. On average 47 pupae per plant were collected from the *Jacobaea vulgaris* parent and 15 pupae per plant from the *J. aquatica* parent. The two F_1 hybrids (15 and 16 pupae / plant, respectively) resembled *J. aquatica*. We found that for the F_2 hybrid genotypes plant size had a strong positive effect on the number of the pupae, while the total PA concentration and that of the major PAs (senecionine-like and jacobine-like PAs) were not correlated to the number of pupae. There was however a trend of decreasing the number of pupae with increasing otosenine-like PA concentrations, while there was a slight increase of the number of pupae with increasing erucifoline-like PA concentrations.

This result of *L. trifolii* differs from a previous study with the same plant genotypes on resistance against a generalist thrips (*Frankliniella occidentalis*), in which jacobine-like PAs were found to be positively related to thrips resistance. This difference indicates that the contribution of plant PAs to herbivore resistance is herbivore species-specific.

Key Words: Secondary metabolites, diversity, pupae survival, generalist herbivores, chemical defense

1. Introduction

Pyrrolizidine alkaloids (PAs) are amongst the most well-known plant defence metabolites. They are ester alkaloids composed of a necine base (an amino dihydroxy moiety) and one or two alkyl necic acids (Hartmann, 1999). The PAs occur in two forms *in vivo*: the tertiary amine (free base) form or the *N*-oxide form (Hartmann et al, 1989; Rizk, 1991; Wiedenfeld et al, 2008). PAs are toxic to mammals (Wiedenfeld and Edgar, 2011). PAs have deterring and toxic effects on generalist insects but stimulate the oviposition and feeding of several specialist insects (see reviews by Boppre, 1986, Hartmann, 1999 and Macel, 2011). *In vitro* experiments with isolated PAs showed that structurally different PAs can have different effects on generalist insects; some are more toxic or deterring than others (Macel et al, 2005). It is generally regarded that the tertiary PAs are more toxic than the corresponding *N*-oxides (van Dam et al, 1995; Macel et al, 2005).

Most *Jacobaea* (syn. *Senecio*, Asteraceae) species contain PAs. *Jacobaea vulgaris* (tansy or common ragwort, syn. *Senecio jacobaea*) is native to Europe and west Asia but invasive in North America, Australia and New Zealand. *Jacobaea aquatica* (marsh ragwort, syn. *Senecio aquaticus*) is closely related to, but not a sister species of, *J. vulgaris* (Pelser et al, 2003). Natural hybrids between these species occur in at least one location in The Netherlands (Kirk et al, 2004). Thirty-seven PAs have been detected from the F_1 and F_2 hybrids of these two species and these PAs could be divided into four groups: senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Chapter 2). The cinnabar moth (*Tyria jacobaeae*) preferred, among the F_2 hybrids, those with a high concentration of jacobine-like PAs (Chapter 4). Western flower thrips (*Frankliniella occidentalis*) on the other hand caused more damage on plants with low concentration of jacobine-like PAs (Chapter 5). No specific role of the other structural types of PAs in the *Jacobaea* hybrids has been revealed so far. To understand the role of PA diversity in plant resistance, we need to know whether other generalist insects are deterred by PAs as well and if so, whether they are deterred by the same or by other structural groups of PAs. To address these questions, we performed bioassays with the American serpentine leafminer (*Liriomyza trifolii*).

Liromyza trifolii is an extremely polyphagous and widespread insect, and it has become an economically important pest in ornamental industry and agriculture (Parrella, 1987; Kang et al, 2009). *Liriomyza trifolii* also has a wide range of weeds and native species as host plants (Stegmaier, 1966; Smith and Hardman, 1986). This leafminer is especially frequent in the Asteraceae. For instance, chrysanthemum was one of the ornamental plants severely damaged by *L. trifolii*. *Senecio glabellus* in Florida (Stegmaier, 1966), *Senecio vulgaris* and *J. vulgaris* in England (Powell, 1981) have been identified as host plants of *L. trifolii*. Furthermore, *L. trifolii* has developed resistance against certain insecticides (Parrella et al, 1984). Therefore, predators and parasites were used as control agents of this insect and resistant lines of crops were selected or developed. Several secondary metabolites have been associated with plant resistance against *L. trifolii*. For example, in specific castor oil plant (*Ricinus communis* L.) lines total phenol concentrations were related to resistance against *L. trifolii* (Anjani et al, 2010). Similarly, trichome-borne acyl sugars from wild tomato *Lycopersicon pennellii* (Hawthorne et al, 1992), cucurbitane glucosides from the cucurbitaceous plant *Momordica charantia* L. (Mekuria et al, 2005; Mekuria et al, 2006), and, phytol, luteolin and various triterpenoids from sweet pepper *Capsicum annuum* (Kashiwagi et al, 2005a; Kashiwagi et al, 2005b) were found to have deterring effects on oviposition and feeding of *L. trifolii*.

We used hybrid plants in this study, because hybrids have several advantages. Interspecific hybrids (specifically segregating generations) often show greater variation in traits compared to parental species. This makes the hybrids useful for studying the relationship between secondary metabolite and herbivores (e.g. Leiss et al, 2009). Interspecific hybrids can have novel patterns of secondary chemical expression or accumulation compared to parental species, and sometimes can be more resistant or susceptible to herbivores than parental species (Rieseberg and Ellstrand, 1993; Fritz, 1999; Orians, 2000; Cheng et al, 2011). Furthermore, segregating hybrids frequently show greater independence between different traits than the parental species (Hochwender et al, 2000; Orians, 2000; Lexer et al, 2003).

We carried out a *L. trifolii* bioassay with an artificial hybrid family including one *J. vulgaris* genotype, one *J. aquatica* genotype, two F_1 and 90 different F_2 hybrids, We determined in an independent set of plants the concentrations of all individual PAs in the shoots of these genotypes (Chapter 2), and investigated the relationship between the PA variation in plants and the plant susceptibility to the leafminer. In this study we address the following questions: 1) Do plant genotypes differ in leafminer resistance? 2) Is leafminer resistance related to the PA variation in the F_2 hybrid genotypes? 3) If so, do different PAs influence resistance to the leafminer differentially? 4) Are there synergistic or antagonistic effects between PAs with respect to leafminer resistance?

2. Methods and Material

2.1 Pant origin and growth

The hybrid family was established from two parental individuals of *J. vulgaris* and *J. aquatica*. The *J. vulgaris* parent was grown from a seed collected at Meijendel Nature Reserve (52° 7′ 54″ N, 4° 19′ 46″ E, The Netherlands), and the *J. aquatica* parent was grown from a seed collected at the Zwanenwater Reserve (52° 48′ 38″ N, 4° 41′ 7″ E, The Netherlands). Both species are self-incompatible. Crosses were performed by rubbing flower heads together. Two F_1 offsprings were selected from this initial cross (*J. aquatica* as mother and *J. vulgaris* as father) and were reciprocally crossed with each other to produce F_2 hybrids. The parental, F_1 and F_2 individuals were maintained in tissue culture and were cloned to perform experiments using these replicated individuals (genotypes).

Plants were propagated by tissue culture and potted in 1.3 liter pots filled with 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). Plants were kept in a climate room (humidity 70%, light 16h at 20°C, dark 8h at 20°C) for six weeks before the bioassay.

2.2. Leafminer origin and rearing

Leafminers were originally collected from several greenhouses in The Netherlands and a stock population was established and kept in climate rooms for more than ten years. Leafminers were reared on "Ultra Light", an extreme leafminer-susceptible cultivar of chrysanthemum (*Dendranthema grandiflora*) in a climate room (humidity 60%, light 16h at 25°C, dark 8h at 25°C). Under this rearing condition, about 14 days are required from egg deposition to the emergence of pupae: egg stage requires 2.5 day developing, three active larva instars require 1.5 days each, and the time spent as a pupa is about 7 days. Pupae used for the leafminer bioassay were collected in the morning after they had fallen out of the leaf and were stored in the cold room (5°C). It took one week to collect enough pupae. Pupae were all taken out of the cold room and put in a climate room (25°C) at the same time to synchronize development. Adult leafminers emerged after 7 days and were kept for one day before the bioassay started.

2.3. Leafminer bioassay

We used 12 clonal replicates of each parental and F_1 genotype, and six replicates for each of the 90 F_2 hybrid genotypes. In total, 588 plants were arranged randomly in a climate room (humidity 70%, light 16h at 25°C, dark 8h at 25°C). The number of leaves and the length of the longest leaf of each plant was measured just before the start of the bioassay. In total 1764 adult leafminers (male: female, 1:1) were released at 49 points (one point per 12 plants, 36 leafminers per point, yielding an average of three leafminers per plant) and were allowed to choose host plants freely after releasing. The adult leafminers were allowed to deposit eggs on the plants for 24 hours and were then collected by using insect aspirators. The plants (free of adult leafminers) were moved to another climate room (humidity 70%, light 16h at 25°C, dark 8h at 25°C) and located randomly. After six days, the above ground parts (shoots) were cut just above the shoot crown and harvested. Shoots were stored individually in plastic bags and these were kept in a climate room (humidity 70%, light 16h at 25°C, dark 8h at 25°C) for a week. Development of pupae was checked and the temperature of the climate room was switched from 25°C to 20°C 3 days after plant harvesting to slow the pupae development. Scoring of pupae for each plant began four days later when nearly all larvae had pupated. About 1% of all larvae were then still alive and had not yet pupated. These larvae were assumed to have survived and pupated if the experiment would have lasted longer. Plants were checked carefully for remaining larvae or pupae on the leaf tissue. The work of pupae scoring was completed within a week after the plant harvesting.

2.4. PA data

A similar set of plants was grown under the same conditions as the plants used for the leafminer bioassay for the collection of PA data. This experiment has been described in detail in Chapter 2. The tissue culture derived plants were from the same genotypes and the same number of clones were used as in the leafminer bioassay. The plants were grown in the climate room as the leafminer bioassay and under identical, herbivore-free conditions. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to determine the PA concentration. Extraction of plant material, PA analysis and the determination of the PA profiles for the set of genotypes, all has been described in Chapter 2.

The concentration of each individual PA was averaged across the clonal replicates of each genotype. These genotypic mean concentrations were used in the analyses presented here, because PA expression is genetically controlled under the conditions used in this experiment. According to their structural characteristics, biosynthetic pathways and expression pattern, the 37 PAs identified from the *Jacobaea* hybrids could be classified into four PA types: senecionine-like, jacobine-like, erucifoline-like and otonecine-like. (Pelser et al, 2005; Chapter 2). In this study, the total PA concentration and the sum concentration for each structural group were calculated by summing the concentrations of the individual PAs within that group (Table 1-2).

2.5. Data analysis

One-way ANOVA was conducted with data (the number of pupae per plant) from F_2 plants to check whether the leafminer resistance differed among the F_2 genotypes. In this ANOVA test, numbers of pupae collected from each individual plant (dependent variable) were log-transformed to achieve equal variance among the genotypes, plant genotype was defined as the independent variable and log-transformed plant size (length of the longest leaf × number of leaf) as covariate. Normal distributions were confirmed by testing the residuals of the models using Shapiro tests.

PAs from within structural groups were highly correlated with each other, and it was therefore not possible to investigate the interactions between them. The PAs from different structural groups, however, were generally expressed independently (Chapter 2). We therefore used a multiple-regression model to test for the effects of the four different PA structural classes and the interactions between them on leafminer resistance. In this model, the number of leafminer pupae (represented by log-transformed genotypic mean values) was defined as the dependent variable, and the sum concentrations of each of the PA structural groups and size of the plants (log-transformed and centered genotypic means) were defined as independent variables. To avoid the collinearity between some independent variables and the interactions, the data of independent variables were centered (Quinn and Keough, 2002).

For completeness we tested the correlations between the number of leafminer pupae and individual PAs' concentrations. To exclude the effect of the plant size, in the correlation tests, we represented leafminer resistance by the residuals of a linear regression with the number of pupae as dependent variable and the plant size as independent variable (the data of both variables are log-transformed genotypic means). Correlations tests were conducted using Pearson or Spearman correlations, depending on whether the PA concentration values were normally distributed or not.
All analyses were conducted in R version 2.10.0 (R Development Core Team, 2009).

3 Results

3.1. Variation of leafminer resistance among individual plants

The number of pupae per individual plant ranged from 0 to 92 and on average from every individual plant 15 leafminer pupae emerged. For the genotype means, the number of pupae ranged from 3.2 to 46.8 and half of the genotypes had no more than 15 pupae per plant (Fig.1). The two parental genotypes differed significantly with regard to the number of the pupae: on average, from *J. vulgaris* 47 pupae emerged while from *J. aquatica* on average only 15 pupae emerged. The number of pupae collected from the two F_1 genotypes was very similar to that from *J. aquatica* (Fig.1a). The F_2 genotypes differed significantly in the number of pupae per plant (ANOVA: $df = 89,449$; $F = 1.99$; $P < 0.001$; Fig.1b). More pupae emerged from larger plants ($df = 1,449$; $F = 29.33$; $P < 0.001$).

3.2. Correlation between leafminer resistance and PA concentrations

The plant size had a positive effect on the number of pupae, and alone explained about 23% of the variation in the number of pupae between genotypes (Fig.2a). The multiple regression model which combined the effect of the plant size and PAs showed that the plant size had a positive effect on the number of pupae. The model also showed a negative trend on the number of the pupae when the concentration of otosenine-like PAs increased. Furthermore, there were four kinds of interactions between the factors which significantly affected the number of the pupae as well. Two of them are interactions between PAs and the other two are those between PAs and plants size. However, it is difficult to explain the interactions. The model indicated that the different PA-types can exert synergistic or antagonistic effects on the number of emerging pupae of *L. trifolii*. In addition the effect of PA concentration depended on the plant size as well (Table 1).

Among the 37 individual PAs, five were negatively related to the number of pupae (two jacobine-like PAs: jacozine and dehydrojaconine; three otosenine-like PAs: otosenine, onetine and desacetyldoronine) and two were positively related to the number of pupae (one senecionine-like PA: riddelliine

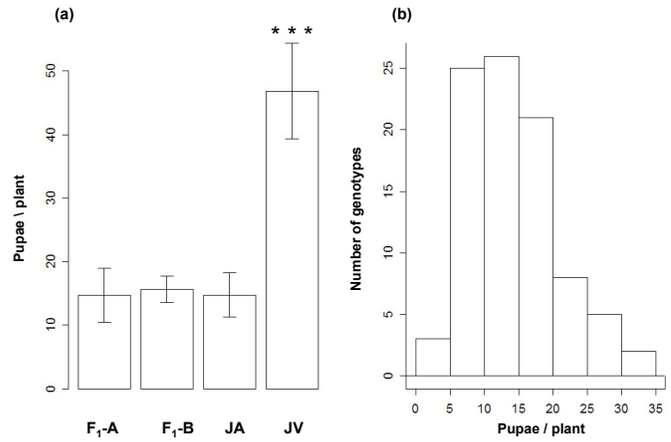

Fig.1 Variation in the number of pupae of the American serpentine leafminer (*Liriomyza trifolii*) collected from J*acobaea aquatica, Jacobaea vulgaris*, and 2 F_1 and 90 F_2 hybrids. (a) Mean number of pupae collected form *J. aquatica* genotype (JA), *J. vulgaris* genotype (JV), and 2 F_1 (F_1-A and F_1-B) genotypes. Each genotype is represented by 12 clonal replicates. Error bars are standard errors. *** $P < 0.001$. (b) Distribution frequency for genotypic mean number of pupae of 90 F_2 hybrids. 3-6 clonal replicates for each genotype. In total, 588 plants were used in the leafminer bioassay.

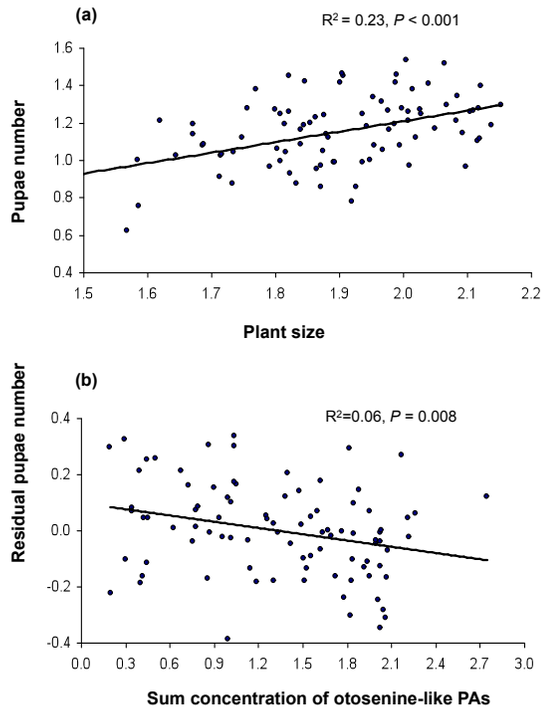

Fig.2 Relationship between American serpentine leafminer (*Liriomyza trifolii*) susceptibility, the plant size and the sum concentration of otosenine-like PAs (µg/g dw) of 90 F_2 hybrid genotypes of *Jacobaea aquatica* and *Jacobaea vulgaris*. Fig.2a: Leafminer susceptibility is represented by the number of pupae per plant. Plant size = length of the longest leaf × number of leaves per plant. Fig.2b: Leafminer susceptibility is represented by the residuals of the number of pupae against the plant size. In all subfigures: data are the log-transformed genotypic mean values.

Table 1 Multiple regression analysis of the number of pupae of American serpentine leafminer (*Liriomyza trifolii*) against the plant size and the sum concentration of four structural groups of pyrrolizidine alkaloids (PAs, µg/g dw) in the 90 F_2 hybrid genotypes from *Jacobaea aquatica* and *Jacobaea vulgaris* (For the regression model: adjusted $R^2 = 0.53$; $df = 31,58$; $F = 4.25$; $P < 0.001$).

	Predictors [a]	Estimate	t value
Factors	Sn-sum	-0.04	-0.58
	Jb-sum	-0.01	-0.24
	Er-sum	0.13	1.92
	Oto-sum	-0.07	-2.24 *
	Plant size	0.56	4.34 ***
Interactions between factors [b]	Sn-sum : Jb-sum	0.49	3.45 **
	Jb–sum : Er-sum	-0.55	-2.41 *
	Er–sum : Plant size	1.06	2.07 *
	Sn-sum : Oto-sum : Plant size	1.65	2.11 *

[a] sn - sum, jb - sum, er - sum, oto - sum: the sum concentration of senecionine-, jacobine- erucifoline- and otosenine-like PAs. Pant size = length of the longest leaf × number of leaves per plant,
[b] The interactions which do not significantly affect the number of pupae are not shown,
* P <0.05, ** P < 0.01, *** P < 0.001

Table 2 Pearson / Spearman correlation tests between the concentrations of pyrrolizidine alkaloids (PAs) and the susceptibility to the American serpentine leafminer (*Liriomyza trifolii*) in 90 F_2 hybrid genotypes of *Jacobaea aquatica* and *Jacobaea vulgaris*. Leafminer susceptibility is represented by the residuals of the number of pupae against the plant size. The data of the number of pupae and the plant size are the genotypic mean values. Size = length of the longest leaf × number of leaves per plant.

Group	PA	r / r_s	P
Senecionine-like PAs	senecionine	-0.084	0.430
	senecionine *N*-oxide	-0.078	0.463
	integerrimine	-0.060	0.576
	integerrimine *N*-oxide	-0.087	0.416
	retrorsine	-0.006	0.953
	retrorsine *N*-oxide	0.165	0.121
	usaramine	-0.069	0.516
	usaramine *N*-oxide [a]	-0.018	0.869
	riddelliine	0.073	0.495
	riddelliine *N*-oxide	0.208	0.049 *
	seneciphylline	-0.120	0.261
	seneciphylline *N*-oxide	-0.073	0.496
	spartioidine	0.054	0.610
	spartioidine *N*-oxide	0.138	0.194
	acetylseneciphylline	-0.147	0.167
	acetylseneciphylline *N*-oxide	-0.183	0.084
	senecivernine	-0.029	0.788
Jacobine-like PAs	jacobine	-0.159	0.135
	jacobine *N*-oxide	-0.144	0.176
	jacoline	-0.155	0.145
	jacoline *N*-oxide	-0.130	0.220
	jaconine	-0.185	0.082
	jaconine *N*-oxide	-0.127	0.232
	jacozine	-0.243	0.021 *
	jacozine *N*-oxide	0.095	0.376
	dehydrojaconine	-0.227	0.031 *
Erucifoline--like PAs	erucifoline	0.122	0.252
	erucifoline *N*-oxide	0.211	0.046 *
	acetylerucifoline	0.175	0.098
	acetylerucifoline *N*-oxide	0.193	0.069
Otosenine--like PAs	senkirkine [a]	-0.148	0.164
	otosenine	-0.226	0.032 *
	onetine	-0.257	0.015 *
	desacetyldoronine	-0.246	0.020 *
	florosenine [a]	-0.067	0.532
	floridanine [a]	-0.090	0.399
	doronine [a]	-0.147	0.167

[a] For PAs with concentrations that were not normally distributed Spearman rank correlation tests were carried out.
Significance codes: ns not significant, *$P < 0.05$, ** $P < 0.01$. Corrected according to the Bonferroni method ($\alpha = 0.05/37 = 0.001$), no correlations are significant.

N-oxide; one erucifoline-like PA: erucifoline *N*-oxide). In fact, all correlation coefficients were quite small and none of the correlations remained significant after Bonferroni correction (Table 2). For the four groups of PAs: the sum concentration of otosenine-like PAs was negatively related to the number of pupae (Fig.2b); erucifoline-like PAs were positively related to the number of pupae (Pearson correlation test: $df = 88$, $r = 0.23$, $P = 0.029$); while no correlation was found for the jacobine-like, senecionine-like PAs or total PA (three Pearson correlation tests: $df = 88$, $P > 0.05$).

4. Discussion

Our study shows that the number of leafminer pupae per plant varied among the plant genotypes. Significantly more pupae developed in larger plants than in smaller ones (Fig.2a). In contrast to our expectation, the effects of major PAs on leafminer resistance were rather weak. Only otosenine-like PAs, a group of PAs that occur often in relatively low concentrations, were slightly negatively correlated to the number of the pupae, which shown by multiple regression and correlation analysis (Fig.2b, Table 2). Surprisingly we even found a weak positive correlation between the number of pupae and the group of erucifoline-like PAs. However, we think it is less likely that erucifoline-like PAs really have positive effects on leafminers. After all, PAs are well known for their negative effects on generalist herbivores (e.g. Macel, 2011). Moreover, erucifoline-like PAs' effects on leafminers were not significant as shown by multiple regression analyses. Multiple regression analyses also showed that there were synergistic and antagonistic effects of PAs and/or the plant size on leafminer pupae (Table 1). However, it is difficult to explain the biological meaning of these interactions.

The sum concentration of the major PA groups (senecionine- and jacobine-like PAs) and total PAs were not related to the number of the pupae. This is a strong indication that the major PAs in *Jacobaea* are not deterring or toxic to the leafminer, at least not in the concentrations present in the plants. The high number of pupae collected from *J. vulgaris* suggests that *J. vulgaris* is in fact the more suitable host plant for *L. trifolii*. Actually, *J. vulgaris* and *S. vulgaris* have been found susceptible to infestation with *L. trifolii* in the field (Powell, 1981). *Senecio glabellus* was identified as one of the host plants of *L. trifolli* in Florida (Stegmaier, 1966). This indicates that *L. trifolii is well* adapted to the PAs present in these plants. For *J. vulgaris* four chemotypes based on their PA profiles were distinguished: 'jacobine chemotype' dominated by jacobine and its derivatives as major PAs; 'erucifoline chemotypes' dominated by erucifoline-like PAs; 'senecionine chemotype' with senecionine-like PAs as dominating PAs; and 'mixed chemotype' with both jacobine- and erucifoline-like PAs as dominating PAs (Witte et al, 1992; Macel et al, 2004). *Senecio vulgaris* has only senecionine-like PAs (Hartmann and Zimmer, 1986; Borstel et al, 1989) and the same is true for *Senecio glabellus* (Ray et al, 1987). None of these species are rich in otosenine-like PAs. This coincides with our finding that the otosenine-like PAs are negatively related to the number of pupae. To confirm that otosenine-like PAs have negative effect on leafminer resistance, it is necessary to perform *in vitro* experiments with pure isolated compounds as some previous work (Hawthorne et al, 1992; Kashiwagi et al, 2005a).

Different to the results for western flower thrips (*Frankniella occidentalis*) obtained with the same genotypes (Chapter 5), PAs do not play an important role in plant resistance against leafminers. This conclusion is in line with a previous study using isolated PAs in artificial diets of generalist herbivores, which showed that six individual PAs (senecionine, retrorsine, seneciphylline, monocrotaline, heliotrine and senkirkine) differed in their toxic or deterrent effect on *Frankliniella occidentalis* (the western flower thrips), *Myzus persicae* (the green peach aphids) and *Locusta migratoria* (grasshopper) while none of the individual PAs deterred feeding by *Spodoptera exigua* (small mottled willow moth) or *Mamestra brassicae* (cabbage moth) (Macel et al, 2005).

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