



Universiteit
Leiden
The Netherlands

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

Cheng, D.

Citation

Cheng, D. (2012, April 18). *Pyrrolizidine alkaloid variation in *Jacobaea* hybrids : influence on resistance against generalist and specialist insect herbivores*. Retrieved from <https://hdl.handle.net/1887/18695>

Version: Not Applicable (or Unknown)

License: [Leiden University Non-exclusive license](#)

Downloaded from: <https://hdl.handle.net/1887/18695>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/18695> holds various files of this Leiden University dissertation.

Author: Cheng, Dandan

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

Date: 2012-04-18



The genotype-dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*

Lotte Joosten, Dandan Cheng, Patrick P.J. Mulder, Klaas Vrieling, Johannes A. van Veen, Peter G.L. Klinkhamer

Lotte Joosten and Dandan Cheng contributed equally to this work.

Secondary metabolites such as pyrrolizidine alkaloids (PAs) play a crucial part in plant defense. PAs can occur in plants in two forms: tertiary amine (free base) and *N*-oxide. PA extraction and detection are of great importance for the understanding of the role of PAs as plant defense compounds, as the tertiary PA form is known for its stronger influence on several generalist insects, whereas the *N*-oxide form is claimed to be less deterrent. We measured PA *N*-oxides and their reduced tertiary amines by liquid chromatography–tandem mass spectrometry (LC-MS/MS). We show that the occurrence of tertiary PAs is not an artifact of the extraction and detection method. We found up to 50% of tertiary PAs in shoots of Jacobine – chemotype plants of *Jacobaea vulgaris*. Jacobine and its derivatives (jacoline, jaconine, jacozone and dehydrojaconine) may occur for more than 20% in reduced form in the shoots and more than 10% in the roots. For 22 PAs detected in F₂ hybrids (*J. vulgaris* × *Jacobaea aquatica*), we calculated the tertiary amine percentage (TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100). We found that the TA% for various PAs was genotype-dependent. Furthermore, TA% for the different PAs were correlated and the highest correlations occurred between PAs which share high structural similarity.

Keywords: *Senecio*, *Jacobaea vulgaris*; *Jacobaea aquatica*; *Asteraceae*; Quantitative descriptive analysis; Pyrrolizidine alkaloid; *N*-oxides; Hybrids; Plant defense; Secondary metabolite diversity

This chapter was published as:

Joosten L, Cheng D, Mulder PPJ, Vrieling K, van Veen JA, Klinkhamer PGL. 2011. The genotype dependent presence of pyrrolizidine alkaloids as tertiary amine in *Jacobaea vulgaris*. *Phytochemistry* 72: 214-222

1. Introduction

Pyrrolizidine alkaloids (PAs) are a well known class of defense compounds with a wide variety of structures. From several genera of *Asteraceae*, *Boraginaceae*, *Orchidaceae* and *Fabaceae*, more than 360 structurally different PAs have been isolated (Rizk, 1991; Hartmann and Witte 1995). It is known that PAs are present as mixtures of the tertiary alkaloids and the respective *N*-oxides in plants (Rizk, 1991). It is generally accepted that in *Senecio* and *Jacobaea* plants PAs occur mainly or even exclusively in *N*-oxide form (Hartmann and Toppel, 1987; Hartmann et al, 2004; Cao et al, 2008; Kempf et al, 2010).

In several *Senecio* and *Jacobaea* species, such as *Senecio vulgaris*, PAs are synthesized in the roots primarily as senecionine *N*-oxide (Hartmann and Toppel, 1987; Toppel et al, 1987). Subsequently, senecionine *N*-oxide is transported to the shoot, where by specific enzymes, further diversification into different individual PAs takes place (Hartmann and Dierich, 1998). The water soluble *N*-oxide form is considered to be ideal for phloem transport (Hartmann et al, 1989) and storage in cell vacuoles (von Borstel and Hartmann, 1986; Ehmke et al, 1988).

Generalist insect herbivores reduce *N*-oxides in the gut to tertiary PAs, where these tertiary PAs are passively taken up into the body and when converted into pyrroles they are toxic by acting as highly reactive alkylating agents in mammals and fruit flies (Mattocks, 1986; Frei et al, 1992). Since the PA *N*-oxides are reduced in the herbivore's gut, we could expect that it displays the same degree of toxicity as the respective tertiary amines, however in several studies it was shown that individual PA *N*-oxides showed less deterrent or toxic effects for some generalist insect herbivores compared to the tertiary PAs (Dreyer et al, 1985; van Dam et al, 1995; Macel et al, 2005). van Dam et al (1995) found that three PAs from *Cynoglossum officinale* equally deterred feeding by *Spodoptera exigua* larvae, but the tertiary PA form deterred feeding more efficiently than the corresponding PA *N*-oxides. Macel et al (2005) showed that retrorsine *N*-oxide was significantly less repellent to the locust *Locusta migratoria* compared to the corresponding tertiary PA. After 6 days on a diet of retrorsine *N*-oxide 60% of the thrips *Frankliniella occidentalis* survived against 0% on the tertiary PA. Specialist insects, i.e. some butterflies and moths (Lepidoptera), certain chrysomelid leaf beetles (Coleoptera) and the grasshopper *Zonocerus variegates* are adapted to PAs, sequester the tertiary PAs and specifically convert them into *N*-oxides which they store and utilize for their own chemical defense (Boppre 1986; Lindigkeit et al, 1997; Dobler 2001; Nishida 2002; Narberhaus et al, 2003).

For many years, PAs were typically isolated by acid-base extraction in combination with zinc reduction. Gas chromatography (GC) with flame ionisation detection (FID), nitrogen phosphorus detection (NPD) or mass spectrometric detection (MS) have typically been used as analytical methods. Recently liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been introduced for measuring PAs in plant material. Unlike GC-related methods, LC-MS/MS and NMR can detect both tertiary amines and *N*-oxides without an additional reducing step (Crews et al, 2010; Joosten et al, 2010). However, NMR needs relatively high concentrations of PAs for detection. LC-MS/MS is therefore a suitable and sensitive method to detect both forms of PAs.

We used LC-MS/MS to detect both forms of PAs. We found consistently large amounts of tertiary amines in *Jacobaea vulgaris* plants (Joosten et al, 2009, 2010). However, the general tendency in literature is that tertiary amines are present only in very small amounts and maybe are due to artifacts during extraction or detection (Hartmann and Toppel, 1987; Hartmann, 1999; Hartmann and Ober, 2000). PA *N*-oxides from *Senecio* plants are relatively unstable and are easily converted into their reduced form, the pre-toxic tertiary PAs under various experimental conditions. For example, the

reduction increased upon prolonged heating of the sample (e.g. soxhlet extraction), when the amino acid cysteine was added and in the presence of plant material (Hartmann and Toppel, 1987; Hösch et al, 1996). Therefore we tested our method for possible artifacts by several PA reduction and oxidation experiments with chemical agents and plant material.

Further proof of the presence of tertiary amines in living plant tissue can be obtained by showing that the concentrations of tertiary amines have a genetic basis and result from transformations by specific enzymes. It is already known that variation in composition and concentration of PAs in *J. vulgaris* has a large genetic component (Vrieling et al, 1993; Macel et al, 2004). In order to assess the genetic basis in the variation, the occurrence of the tertiary amine form, we conducted a crossing of *J. vulgaris*, which has high levels of tertiary amines, with the closely related *Jacobaea aquatica* (syn. *Senecio aquaticus*), which has low levels of tertiary amines (Cheng et al, manuscript in preparation).

Here we report on studies to obtain a better understanding of the (bio)chemistry of PAs in above and below ground plant parts of *J. vulgaris* and hence on the mechanisms of their activity as defense compounds against herbivores. Thus, we investigated: (1) the chemical reduction of three different PA *N*-oxides (representatives of the three structural groups) to assess the chemical PA (in)stability towards two different reducing agents; (2) the chemical oxidation of three different tertiary PAs to assess the chemical PA (in)stability towards an oxidation agent; (3) the spontaneous reduction of three different PA *N*-oxides in the presence of possibly reducing agents as well as the spontaneous *N*-oxidation of three different tertiary PA in the presence of possibly oxidation agents naturally occurring in plant material of several different *Asteraceae* species; (4) the spontaneous reduction of PAs during freeze-drying compared to immediate PA extraction from freshly ground material under liquid nitrogen; (5) the PA distribution in five different *J. vulgaris* genotypes by using an LC-MS/MS method for simultaneous measurement of PA *N*-oxides and tertiary PAs and (6) the genotype effect on the tertiary alkaloid relative content (TA%) for different PAs in the hybrids and the correlation between the TA% of different PAs.

2. Material and Methods

2.1. Standard PA extraction for LC-MS/MS

Freeze-dried plant material (approximately 10 mg) was extracted in 1 ml 2% formic acid. Heliotrine was added as internal standard to the extraction solvent at a concentration of 1 µg/ml. The plant extract solution was shaken for 30 min. After centrifugation the residual plant material was removed by filtering the extraction solution through a 0.2 µm nylon membrane (Acrodisk® 13 mm syringe filter). An aliquot of 25 µl filtered solution was diluted with 975 µl water and 10 µl was injected in the LC-MS/MS system.

2.2. Standard PA analysis by LC-MS/MS

A Waters Acquity ultra performance liquid chromatographic (UPLC) system coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, PA, USA) was used for PA determination. Chromatographic separation was achieved on a Waters Acquity BEH C18 150 x 2.1 mm, 1.7 µm, UPLC column, kept at 50 °C and ran with a water/acetonitrile linear gradient containing 6.5 mM ammonia at a flow of 0.4 ml/min. The gradient started at 100% water and during analysis the acetonitrile percentage was raised to 50% in 12 min.

The MS system was operated in positive electrospray mode and data were recorded in multiple monitoring mode using two selected precursor ion to product ion transitions per compound. Cone

and collision energy settings were optimized for the individual compounds. Obtained peak areas were internally calibrated using the internal standard and the individual compounds were quantified against a standard solution of the PAs in an extract of the non-PA containing asterid *Tanacetum vulgare* to mimic the plant matrix. Seventeen individual PA standards were available for this study, representing over 90% of the total amount of PAs present in the plants extracts. Senecionine, seneciophylline, retrorsine and their *N*-oxides as well as senkirkine were available from commercial sources (Phytolab, Vestenbergsgreuth, Germany; Phytoplan, Heidelberg, Germany). Integerrimine was obtained as a kind gift of Dr. Trigo (UNICAMP, Campinas, Brazil). Riddelliine and its *N*-oxide were obtained as a kind gift from Dr. Chou (NCTR, Jefferson, AR, USA). Acetylseneciophylline was obtained by acetylation of seneciophylline with acetic anhydride and pyridine. Jacobine and erucifoline were isolated from *J. vulgaris* plant material (PRISNA, Leiden, The Netherlands). The identity of the standards isolated was confirmed by ¹H-NMR and LC-MS analysis. *N*-oxides of integerrimine, jacobine, erucifoline and acetylseneciophylline were prepared by *N*-oxidation according to the method of Christie et al (1949), adapted by Chou et al (2003). The remaining PAs, being tertiary PAs as well as *N*-oxides, were quantified by using the response of a structurally related standard. Data processing was conducted with Masslynx 4.1 software.

2.3. Chemical reduction of PA *N*-oxides

A mixture of three PA *N*-oxides (senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide, 1 µg/ml) was exposed to the reducing agent sodium metabisulfite (Na₂S₂O₃) in a range of 5 concentrations (0, 0.01, 0.03, 0.1, 0.3, 1 mM) in 2% formic acid solution. After 1, 4 and 24 h of incubation at room temperature the solutions were diluted 10-fold with water and injected in the LC-MS/MS system. The same mixture of standards was also exposed to the amino acid cysteine at three concentrations (1, 10 and 1000 mM), in two different solutions, 2% formic acid and water.

The relative amount of tertiary PA present in a sample was calculated as the measured concentration of tertiary PA divided by the sum of the concentration of tertiary PA and corresponding PA *N*-oxide.

A three-way ANOVA with two replications was used to analyze if PA type (senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide), reducing agent concentration (0, 0.01, 0.03, 0.1, 0.3, 1 mM) and incubation time (1, 4, 24 h) have a significant influence on the relative concentration of tertiary PAs formed by reduction of the added PA *N*-oxides. The analysis was made by General Linear Model (GLM) univariate analyses procedure with the relative concentration of tertiary PAs as the dependent variable and PA type, reducing agent concentration and incubation time as fixed factors. All tests were conducted with SPSS 17.0 for Windows.

2.4. Chemical *N*-oxidation of tertiary PAs

The three individual tertiary PAs (senecionine, jacobine and erucifoline), were added to five concentrations (0.01, 0.03, 0.1, 0.3, 1 mM) of the oxidation agent hydrogen peroxide (HOOH) in 2% formic acid solution. After 1, 4 and 24 h of incubation at room temperature the solutions were diluted 10-fold with water and injected in the LC-MS/MS system.

The relative amount of *N*-oxide present in the sample was calculated as the measured concentration of the PA *N*-oxide divided by the sum of the concentration of PA *N*-oxide and the corresponding tertiary PA.

The same statistical test was used as for the chemical reduction experiment described above, to analyze if PA-structural group (senecionine, jacobine and erucifoline), reducing agent concentration (0, 0.01, 0.03, 0.1, 0.3, 1 M) and incubation time (1, 4, 24 h) did have a significant influence on the relative concentration of PA *N*-oxides formed by *N*-oxidation of the added tertiary PAs.

2.5. PA *N*-oxide reduction and PA *N*-oxidation in the presence of plant material

Three PA *N*-oxides (senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide) and three tertiary PAs (senecionine, jacobine and erucifoline) were added separately to dry plant material of five different flowering *Asteraceae* species collected in the field, *S. gigantea*, *E. cannabinum*, *S. sylvaticus*, *J. erucifolia* (syn. *Senecio erucifolius*) and *J. vulgaris* (erucifoline chemotype). *S. gigantea* contains no PAs, *E. cannabinum* contains the lycopsamine type of PAs, *S. sylvaticus* contains the triangularine type of PAs, and *J. erucifolia* contains the senecionine type of PAs. Dried, ground plant material of the shoot (approximately 100 mg) was wetted with 500 µl distilled water containing senecionine *N*-oxide, jacobine *N*-oxide and erucifoline *N*-oxide (10 µg of each PA) in 3-fold and incubated for 1 h. As a control, from each plant species one sample was wetted with water containing no PA *N*-oxides. After adding 10 ml 2% formic acid and heliotrine as internal standard (1 µg/ml), the plant extract was shaken for 30 min. After centrifugation, the residual plant material was removed by filtering the extraction solution through a 0.2 µm nylon membrane. An aliquot of 25 µl filtered solution was diluted with 975 µl water and injected in the LC-MS/MS system.

2.6. Species description

J. vulgaris (syn. *Senecio jacobaea*) is a suitable system to study PAs. This species is native in Europe and West Asia but invasive in North America, Australia and New Zealand. In previous studies, up to 30 different PAs were detected in *J. vulgaris* (Witte et al, 1992; Macel et al, 2004; Kowalchuk et al, 2006; Joosten et al, 2009). Based on their structural features, major PAs in *J. vulgaris* can be divided into 3 structural groups: senecionine-like, comprising senecionine, integerrimine, retrorsine and (acetyl) seneciophylline; jacobine-like, comprising jacobine, jacoline, jacoline jaconine jaconine, and dehydrojaconine; erucifoline-like, comprising erucifoline and acetylerucifoline (Table 2).

Based on the PA composition, 4 chemotypes of *J. vulgaris* were distinguished: Senecionine-chemotype, largely lacking jacobine- and erucifoline-like PAs; Erucifoline-chemotype, lacking jacobine-like PAs; Jacobine-chemotype, containing high levels of jacobine-like PAs; mixed chemotype, containing both jacobine- and erucifoline-like PAs in similar amounts (Witte et al, 1992; Macel et al, 2004).

J. aquatica is a close relative but not a sister species to *J. vulgaris* (Pelser et al, 2003). These two species naturally hybridize in some areas and the hybrids can backcross into the parental populations (Kirk et al, 2004, 2005)

2.7. Effect of freeze-drying on the tertiary PA content

Freeze-drying is a general used method to dry plant material before analyzing PAs in plant material. In this way enzymatic activity can be prevented or at least strongly reduced. We tested if the freeze-drying can lead to spontaneous reduction of PAs. To compare freeze-dried material to the original plant condition we extracted PAs from fresh plant material as control treatment. Liquid nitrogen was used to ground fresh plant material under deep frozen conditions.

2.7.1. Plant material

One genotype of *J. vulgaris* originating from a population near Wageningen was used to study if reduction can take place during freeze-drying. The plants were propagated by tissue culture. In total eight clones per treatment (PA extraction of fresh material versus PA extraction of freeze-dried material) were used. The plants were potted in 1.3 l pots filled with potting soil (Slingerland Potgrond, Zoeterwoude, The Netherlands). The plants were kept in a climate room for 6 weeks (humidity 70%, light 16 h at 20 °C, dark 8 h at 20 °C) and randomly distributed every 8-10 days.

2.7.2. PA extraction from fresh and freeze-dried material

The shoot of each plant was cut lateral in two pieces with scissors so each part had an equal number of leaves of similar size, one shoot part for the control treatment and the other part for the freeze-drying treatment. The control part of the shoot was weighted, immediately ground under liquid N₂ and in frozen condition mixed in 20 ml of 2% formic acid containing 0.2 µg/ml heliotrine as internal standard. From this point on the standard PA extraction for LC-MS/MS was performed as described above. After weighting, the other half of the shoot was immediately stored at -20 °C before being freeze-dried. After freeze-drying, the standard PA extraction for LC-MS/MS was performed.

2.7.3. Data analysis

The 9 major PAs and their corresponding *N*-oxides were included in the analysis. We excluded the minor PAs which had a concentration close to detection limit and for which the ratios were not reliable. The relative concentration of tertiary amine (TA%) were calculated as: TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100. To calculate the percentage of *N*-oxides in fresh material transformed to tertiary amines during freeze-drying, the following formula was used to calculate the relative reduction amount of the *N*-oxides: (tertiary amine concentration in freeze-dried material – tertiary amine concentration in fresh material)/*N*-oxide concentration in fresh material. The difference of total PA, individual PAs and relative concentration of tertiary amines between the two methods were evaluated by paired *t*-test, with the absolute concentration of total PA, individual PA and TA% as the dependent variable, respectively. To test whether different individual PAs had a different amount of reduction from *N*-oxides to tertiary amines, a one-way ANOVA was performed with the relative reduction amount as variable and individual PA as group factor. All tests were conducted with SPSS 17.0 for Windows.

2.8 PA analysis for *J. vulgaris*

2.8.1. Plant material and PA analysis

Five different genotypes of *J. vulgaris* were used representing two chemotypes: three Jacobine-chemotypes and two Erucifoline-chemotypes. Two Jacobine-chemotypes originated from two different populations in Meijndel near The Hague and the third originated from a population near Wageningen. The two Erucifoline-chemotypes originated from a Dutch population near Vilt (Limburg) and a German population near Kassel. The five different genotypes were propagated by tissue culture. In total eight clones per genotype were used. The plants were potted in 1.3 l pots filled with calcareous sandy soil collected from Meijndel, a coastal dune area North of The Hague. The plants were kept in a climate room for 5 weeks (humidity 70%, light 16 h at 20 °C, dark 8 h at 20 °C) and randomly distributed every 8-10 days.

After 5 weeks the plants were harvested in order to determine the PA concentration and composition. The plants were cut with scissors just above the root crown and roots and shoots were immediately stored at -20 °C for 4 days before being freeze-dried for 1 week under vacuum with a

collector temperature of -55 °C (Labconco Free Zone® 12 l Freeze Dry System). PAs were extracted by formic acid, as described above. An aliquot of 25 µl filtered solution was diluted with 975 µl water and injected in the LC-MS/MS system.

2.8.2. Data analysis

A two-way ANOVA was used to analyze if chemotype and plant part (root and shoot) have a significant influence on the TA%. The ANOVA was performed by GLM (General Linear Model) univariate analyses procedure with TA% as the dependent variable, chemotype and plant part as fixed factors. The tests were conducted with SPSS 17.0 for Windows.

2.9. Relative concentration of tertiary amine analysis for *Jacobaea* hybrids

2.9.1. Plant material and PA analysis

F₂ hybrids of two different species were used in this study; *J. vulgaris* subs. *dunensis* and *J. aquatica* subs. *aquatica*. Seeds were collected for *J. vulgaris* at Meijndel, a coastal dune area north of The Hague (The Netherlands) and for *J. aquatica*, a coastal dune area at Zwanenwater Reserve (The Netherlands). Crossings were performed by rubbing flower heads together. This cross resulted in numerous seeds which were germinated. Both species are self incompatible and all F₁ and F₂ seeds are true crosses confirmed by molecular analysis (unpublished data). Two F₁ individuals with rayed flowers were chosen and crossed reciprocally with each other resulting in offspring. The two parental, two F₁ and >100 F₂ individuals were maintained in tissue culture.

The plants used in this study were cloned from the tissue culture material. Beside the two parental genotypes (*J. vulgaris* and *J. aquatica*) and two different F₁ hybrids, 102 different F₂ hybrid genotypes were used. On average 6 cloned replicates per F₂ genotype and 12 cloned replicates per parental and F₁ genotype were grown. In total, 609 plants were used in this study, among which 562 were F₂ individuals.

The plants were potted in 1.3 l pots filled with 95% sandy soil, collected from Meijndel, 5% potting soil (Slingerland Potgrond, Zoeterwoude, The Netherlands) and 1.5 g/l Osmocote (Scotts®, Geldermalsen, The Netherlands, N:P:K = 15:9:11). The plants were randomly distributed and kept in a climate room for 6 weeks (humidity 70%, light 16 h at 20 °C, dark 8 h at 20 °C). After 6 weeks the plants were harvested and prepared for LC-MS/MS analysis as described above.

2.9.2 Data analysis

Of the 37 detected PAs, 9 were otonecine structural group PAs for which no corresponding *N*-oxide exists and 6 were absent or close to the detection limit in some samples. The remaining 22 PAs were used to calculate the relative concentration of tertiary amine as TA% = the tertiary amine concentration/(tertiary amine concentration + the corresponding *N*-oxide concentration) × 100.

The genotype effect on TA% was statistically analyzed by a Kruskal-Wallis test with the TA% as the independent variable and genotype (including parental, F₁ and F₂, 106 genotypes in total) as the grouping variable. Spearman correlation matrix between the 11 kinds of TA% was calculated based on the mean TA% per genotype in root and shoot. *P*-values of the correlations were adjusted by Holm's method (Holm, 1979). To determine if different type of plant material (root/shoot) had a different degree of the correlation between TA%, a paired *t*-test was done with the correlation values as the independent variable.

Depending on the PA-structural group the specific PAs belong to, the correlations were divided into 6 categories: Category 1, correlation between the PAs of the senecionine-like PAs; Category 2, correlation between the PAs of the jacobine-like PAs; Category 3, correlation between the PAs of

the erucifoline-like PAs; Category 4, correlation between the PAs of the senecionine- and jacobine-like PAs; Category 5, correlation between the PAs of the senecionine- and the erucifoline-like PAs; Category 6, correlation between the PAs of the jacobine- and the erucifoline-like PAs. Differences between the correlation values belonging to the different categories were analyzed with a one-way ANOVA with the correlation values as the independent variable and correlation category (Category 1-6) as fixed factor. All tests were conducted with SPSS 17.0 for Windows, except for the correlation matrix and adjustment by Holm's method, which was conducted with R 2.10.0 for Windows.

3. Results

3.1. Chemical reduction of PA N-oxides

The chemical reduction of the three PA N-oxides, senecionine N-oxide, jacobine N-oxide and erucifoline N-oxide, with sodium metabisulfite into their tertiary amines showed a significant difference ($F_{2, 87} = 10.8, P < 0.001$) in rate of reduction at any concentration of sodium metabisulfite added. Averaged over all incubation times (1, 4, 24 h) and reducing agent concentrations (0.01, 0.03, 0.1, 0.3, 1 mM) 42.2% (SE \pm 0.63) of the jacobine N-oxide was reduced while 45.8% (SE \pm 0.63) for both senecionine N-oxide and erucifoline N-oxide (Fig.S1). However, the difference is not significant due to the analytical error, which is estimated at 10%.

Exposure of the three PA N-oxides to 1 M cysteine produced no measurable amount of tertiary amines after 24 h under acidic conditions (2% formic acid). However, under neutral conditions (water) with 1 M cysteine a very slow reduction occurred: after 24 h the production of senecionine, jacobine and erucifoline was respectively 1.9%, 4.2% and 2.7% (data not shown). The amounts of tertiary amines formed were too low to draw definitive conclusions about a difference in reactivity of the PA N-oxides towards cysteine and other potential sulfur-containing plant components. It should be pointed out that under the extraction conditions used in this study, the PA N-oxides displayed no measurable reactivity whatsoever towards cysteine. Interestingly, we found that 1 M cysteine catalyzed the isomerisation of senecionine N-oxide into integerrimine N-oxide notably under acidic conditions. After 24 h approximately 30% of senecionine N-oxide has isomerised to integerrimine N-oxide, under neutral condition this was only 14%. In the absence of cysteine the isomerisation in formic acid was less than 1% after 24 h.

3.2. Chemical N-oxidation of tertiary PAs

For the chemical oxidation under acidic conditions of the three macrocyclic tertiary PAs, senecionine, jacobine and erucifoline, with hydrogen peroxide (HOOH) into their N-oxides, relatively high concentrations of peroxide were required to induce oxidation at a measurable rate. Oxidation with HOOH proceeded much faster under neutral conditions (data not shown). Averaged over all incubation times (1, 4, 24 h) and oxidation agent concentrations (0.01, 0.03, 0.1, 0.3, 1 mM), 2.8% (SE \pm 0.14) of the jacobine was oxidized while 1.0 (SE \pm 0.14) and 1.1% (SE \pm 0.14) for senecionine and erucifoline, respectively. The chemical oxidation of senecionine and erucifoline takes place with approximately the same rate, but that the oxidation of jacobine proceeded significantly faster ($F_{2, 105} = 48.6, P < 0.001$). The difference in rate was irrespective to the HOOH concentration. After 24 h with 1 M peroxide approximately 22.2% (SE \pm 1.5) of jacobine had been converted to its N-oxide, while for senecionine the conversion was only 6.4% (SE \pm 1.5) and for erucifoline 7.5% (SE \pm 1.5) (Fig.S2).

3.3. Extraction of tertiary PAs and PA N-oxides in the presence of dried plant material of five different Asteraceae species

The three PA N-oxides, senecionine N-oxide, jacobine N-oxide and erucifoline N-oxide, in presence of dry plant material of 5 flowering Asteraceae species showed no measurable induced formation of tertiary amine PAs by naturally reducing agents if present (data not shown). All PA N-oxides added were recovered with LC-MS/MS after extraction. Only a very small amount (2%) of the added senecionine N-oxide was reduced in the presence of *Solidago gigantea* and *Eupatorium cannabinum* plant material, but the concentrations measured were close to the detection limit. In the presence of *Senecio sylvaticus* no reduction was observed for all three PA N-oxides. In the control samples (no PA N-oxides added) of *Jacobaea erucifolia* and *J. vulgaris* senecionine N-oxide, erucifoline N-oxide and its tertiary PAs were already present in the plant material but jacobine or jacobine N-oxide were not present in detectable amounts. Since senecionine N-oxide and erucifoline N-oxide were naturally present in the plant, we could not draw any conclusions on the reduction of these PAs, as the added N-oxide volumes were negligible. For the jacobine N-oxide added it could be shown that there was no reduction by naturally occurring reducing agents present in *J. erucifolia* and *J. vulgaris*.

The three tertiary PAs, senecionine, jacobine and erucifoline, in presence of dry plant material of several flowering Asteraceae species showed no detectable induced oxidation of PAs by naturally occurring oxidation agents (data not shown). All PAs added were recovered after extraction.

3.4. Effect of freeze-drying on the tertiary PA content

The total PA concentration and the concentration of the individual PAs was not significantly different comparing the freeze-dried with fresh plant material (Table 1). The freeze-dried (lyophilized) materials had a higher TA% for all individual PAs compared to the corresponding fresh materials, which illustrates that the freeze-drying process caused some reduction from N-oxide to tertiary amine. The reduction is not PA specific, because the relative reduction amount was not significantly different between the PAs (Table 1, ANOVA, $F_{8, 63} = 0.69, P = 0.70$).

Table 1 Effect of sample treatment on the observed concentration of total PA, individual PA, relative concentration of tertiary amines (TA%), and relative reduction amount.

PA ^a	Concentration ^b (mg/g dry wt)			TA% ^c			Relative reduction amount ^d (%)
	Freeze-dried	Fresh	Paired t-test	Freeze-dried	Fresh	Paired t-test	
total PA	0.654	0.794	ns	22	13	*	4
sn	0.042	0.047	ns	6	1	*	3
ir	0.014	0.017	ns	5	1	*	2
sp	0.095	0.108	ns	7	2	*	3
acsp	0.012	0.008	ns	4	2	*	5
jb	0.435	0.560	ns	28	17	**	4
jl	0.007	0.008	ns	43	29	*	12
jz	0.011	0.011	ns	20	11	ns	6
er	0.020	0.020	ns	9	3	ns	6
acer	0.014	0.014	ns	5	1	*	3

^a Abbreviations: sn = senecionine; ir = integerrimine; sp = seneciphylline; acsp = acetyl-seneciphylline; jb = jacobine; jl = jacobine; jz = jacobine; er = erucifoline; acer = acetylerucifoline

^b Concentration was the absolute concentration of PAs as tertiary amines and N-oxides

^c TA% = the tertiary amine concentration / (tertiary amine concentration + the corresponding N-oxide concentration) \times 100.

^d Relative reduction amount = (concentration of tertiary amines in freeze-dried material - concentration of tertiary amines in fresh material) / concentration of the corresponding N-oxides in fresh material.

ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

3.5. PA distribution in *Jacobaea vulgaris*

A total of 27 different PAs (*N*-oxides + tertiary amines) were found in roots and shoots of the five genotypes. Dehydrojaconine, spartioidine and senecivernine were found in trace amounts and did only occur in detectable amounts as tertiary PA, while all other individual PAs were found in both forms.

The mean TA% in the roots of Jacobine-chemotypes and both plant parts of Erucifoline-chemotypes were all below 6.2%, while the TA% in the shoots of Jacobine-chemotypes was approx. 6 times higher, resulting in a significant chemotype × plant part interaction (ANOVA, $F_{1,78} = 53.07$, $P < 0.001$). In the roots no significant difference between the chemotypes (Mean TA% roots Jacobine and Erucifoline-chemotype = 5.3% and 5.7%, respectively) was found while in the shoots the difference was highly significant (Mean TA% shoots Jacobine and Erucifoline-chemotype = 37.0% and 6.1%, respectively).

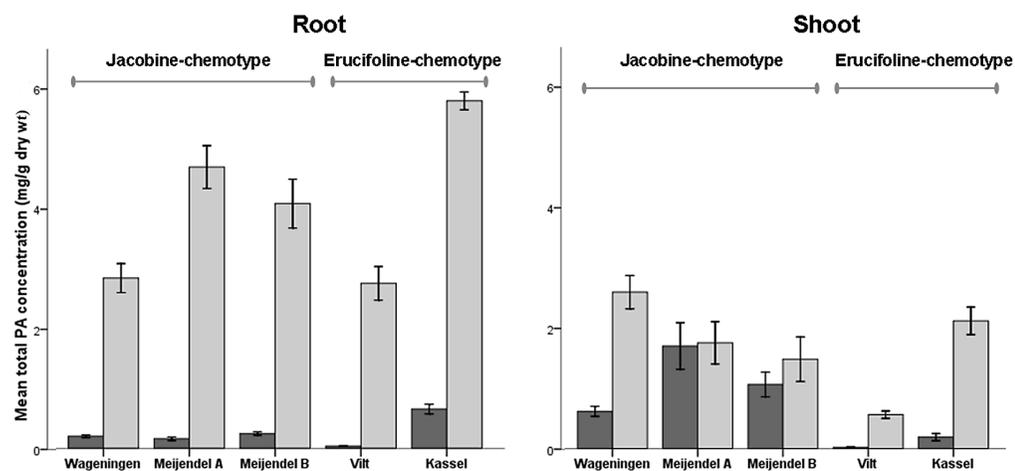


Fig.1 The absolute mean total PA concentration in dry root and shoot material per genotype (n = 8). Light bar = PA *N*-oxides and dark bar = tertiary PAs. Error bars: ±1SE. Above the bars, the genotype the chemotype is indicated.

In the roots of all genotypes on average 94.7% of all PAs were in *N*-oxide form (Fig.1). Senecionine *N*-oxide, seneciophylline *N*-oxide and acetylseneciophylline *N*-oxide were the most abundant PAs in the roots with on average 71.0% of the total PA root concentration (Fig.4). The Jacobine-chemotypes from Meijendel (Meijendel A and B) contained jacobine *N*-oxide as one of the dominant root PAs, while the Erucifoline-chemotypes (Vilt and Kassel) contained erucifoline *N*-oxide as a dominant PA (Fig.2), with respectively 14.3% (for jacobine) and 14.9% (for erucifoline) of the total PA root concentration.

The four most dominant PAs in the shoots of the Erucifoline-chemotypes were senecionine, seneciophylline, erucifoline and acetylerucifoline. In the shoots of this chemotype, a lower concentration of PAs were in the tertiary PA form as compared to the Jacobine-chemotypes with only 3.6% and 8.2% of the total shoot PA concentration for Vilt and Kassel, respectively (Fig.1).

The TA% in the shoots was higher in the Jacobine-chemotypes. In particular, the chemotypes from Meijendel contained a high percentage of tertiary PAs (Fig.1). In the shoots of this chemotype, on average 45.5% of the total shoot PA concentration occurred as tertiary PA. In the Jacobine-chemotype from Wageningen, tertiary forms comprised nearly 20% of the total shoot PA concentration (Fig.1).

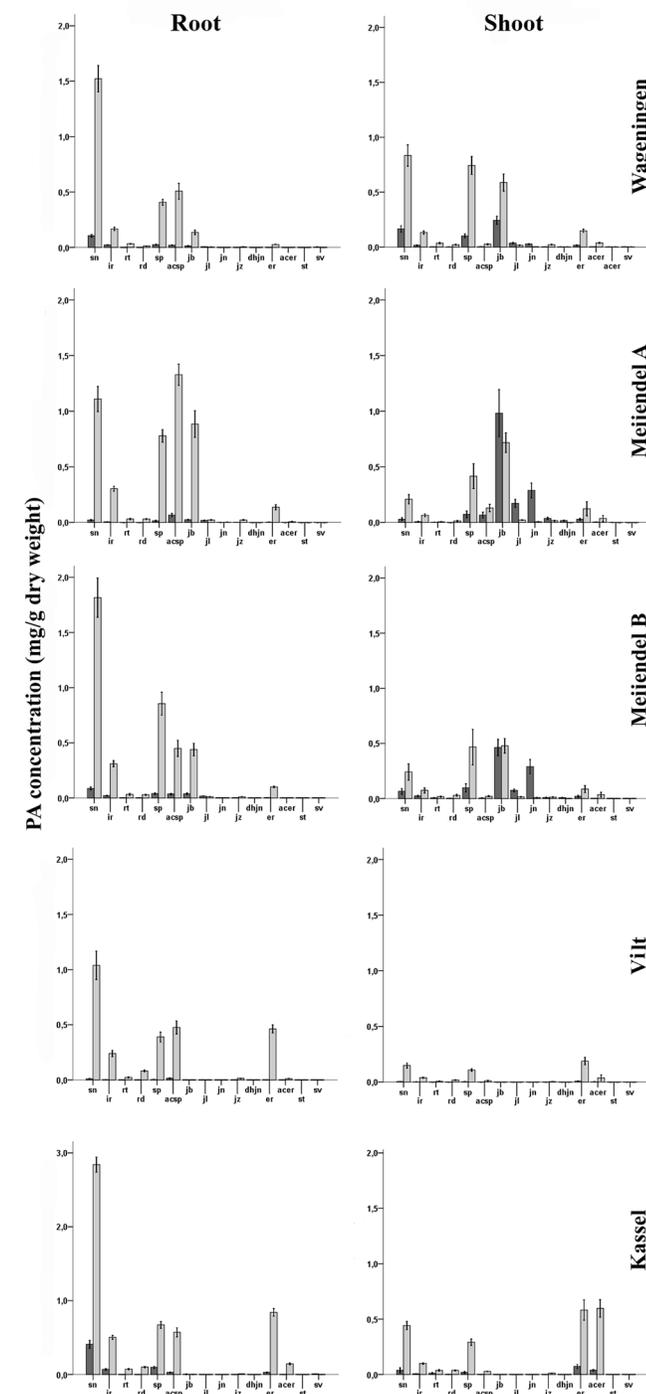


Fig.2 The PA composition of *J. vulgaris* in absolute concentration per individual PA of dry root and shoot material (n = 8) Light bar = PA *N*-oxides and dark bar = tertiary PAs. For abbreviations see legend Table 2. Error bars: ±1SE.

The TA% is in fact only determined by the presence of the jacobine-like PAs. Jacobine and its derivatives jaconine, jacoline, jacozine and dehydrojaconine showed the highest percentage in reduced form (Fig.2). In the two Jacobine-chemotypes from Meijndel on average only 17.0% of the total senecionine and seneciphylline concentration was present as tertiary PAs while for jacobine this was 54.1%.

3.6. Relative tertiary amine concentration in *Jacobaea* hybrids

Of the 37 detected PAs in the *Jacobaea* hybrids, 9 were otonecine-group PAs with no corresponding *N*-oxides and 6 were absent or close to the detection limit in some samples. The remaining 22 PAs were used to calculate the relative concentration of tertiary amine as TA%.

The TA% of the senecionine-like and erucifoline-like PAs in the roots were lower than 10%, which demonstrates that more than 90% of these PAs were present in *N*-oxide in the roots. But the jacobine-like PAs had TA% ranging from 10% till 56%. Except for senecionine, integerrimine and acetylerucifoline, the TA% of all the other PAs was genotype dependent in the roots. In the shoots, the TA% were higher than those in the roots (for all 11 PAs, paired *t*-test, *df* = 608, *P* < 0.001). Particularly for jaconine, the TA% was up to 80% in the shoots. The TA% of all the individual PAs were genotype dependent in the shoots (Table 2).

Generally there was a significant positive correlation between the TA% both in the roots and in the shoots. The correlation coefficients were not significantly different between the shoots and roots (paired *t*-test, *df* = 54, *t* = -0.393, *P* = 0.696), but correlation coefficients differed between structural groups (ANOVA, $F_{5, 104} = 10.69$, *P* < 0.001). Correlation coefficients of TA% within structural groups are always higher than TA% correlation between different structural groups (Fig.S3).

Table 2 The concentration of tertiary and *N*-oxide PA, TA% and the genotype effect on the TA% in two parental, two F1 and 102 F2 genotypes from a cross between *J. vulgaris* and *J. aquatica*.

Plant Part	Structural group	Pyrrolizidine alkaloid	Code	Concentration (mg/g dry wt)		TA% ^a	x ^{2b}	P ^c
				Tertiary amine	-oxide			
Roots	Senecionine-like	senecionine	sn	0.053	1.435	4	111.7	ns
		integerrimine	ir	0.007	0.232	3	97.9	ns
		retrorsine	rt	0.002	0.037	5	131.8	*
		seneciphylline	sp	0.025	0.601	4	144.6	**
		acetyl-seneciphylline	acsp	0.047	0.996	5	133.9	*
	Jacobine-like	jacobine	jb	0.029	0.250	13	245.2	***
		jacoline	jl	0.009	0.013	45	252.1	***
		jaconine	jn	0.033	0.025	56	166.7	***
		jacozine	jz	0.001	0.009	10	268.2	***
	Erucifoline-like	erucifoline	er	0.003	0.039	9	144.5	**
acetylerucifoline		acer	0.000	0.009	6	98.4	ns	
Shoots	Senecionine-like	senecionine	sn	0.011	0.177	8	144.2	**
		integerrimine	ir	0.003	0.063	7	132.6	**
		retrorsine	rt	0.001	0.009	17	163.6	***
		seneciphylline	sp	0.038	0.513	9	134.2	**
		acetyl-seneciphylline	acsp	0.009	0.148	10	147.5	**
	Jacobine-like	jacobine	jb	0.077	0.234	24	311.9	***
		jacoline	jl	0.023	0.016	55	354.8	**
		jaconine	jn	0.252	0.047	80	376.4	***
		jacozine	jz	0.003	0.012	31	343.3	***
	Erucifoline-like	erucifoline	er	0.015	0.138	15	203.5	***
		acetylerucifoline	acer	0.004	0.052	11	134.9	*

^a TA% = the tertiary amine concentration / (tertiary amine concentration + the corresponding *N*-oxide concentration) × 100.

^b Kruskal-Wallis test with the concentration data from two parental, two F1 and 102 F2 genotypes. Ca. 12 replicates per parental and F1 and ca. 6 replicates per F2 hybrid, in total n = 609 plants.

^c ns: not significant, * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001

4. Discussion

We observed that the tertiary amine proportion was different among PAs and genotypes. Two possible and nonexclusive hypotheses may explain this pattern. Firstly, the chemical transformation and perhaps allocation of PA *N*-oxides, is accompanied by a continuous slow reduction of the original *N*-oxides. Thus, the most peripheral “on a time scale oldest” PAs like jacoline and jaconine, which are far down the pathway (Fig.S4), show the highest TA% and the “youngest” PAs, i.e. senecionine or integerrimine, have the lowest TA%. The observation that the TA% values in shoots are always higher than the values for the respective PAs in roots goes in the same direction (Hartmann, 2010, personal communication). Secondly, specific (re-)oxidation of the tertiary PAs might explain the pattern. The reduction of PA *N*-oxides in the plant is an unspecific, chemical process induced by the presence of endogenous reducing compounds and (traces of) transition metal salts. Meanwhile, there is a, biochemically based, process operating to re-oxidize the reduced tertiary amines for PA transport. Enzyme(s) that may be involved seem to work well for senecionine-like and erucifoline-like PAs but work less well for jacobine-like PAs. Possibly, the substrate specific enzyme is affected when alterations at positions 15 and 20 (addition of O, H₂O, HCl, Fig.S5) are made. This perhaps makes the epoxidized PAs less accessible for the enzyme, which results in a lower conversion rate. So, the second hypothesis could explain the TA% difference among the PAs and the genotypes. Furthermore, it may get more support from a biochemical point of view, since the plant has to use an enzyme to produce the backbone senecionine *N*-oxide at the beginning of the PA pathway. Senecionine *N*-oxygenase (SNO) was isolated (from the larvae of specialist insect *Tyria jacobaeae*, less relevant for plants) and *Crotalaria scassellatii* seedlings. The enzymes were tested with different PAs as substrates and showed that they specifically oxidized tertiary PAs (Lindigkeit et al, 1997; Chang and Hartmann, 1998). These enzymes might be highly preserved and similar in the various PA containing plants. A very interesting follow-up of this study could be the identification, isolation and characterization of this putative *N*-oxidation enzyme(s) and exploration of genetic variation concerning these enzymes. It would also be interesting to see if the TA% can be influenced by external factors, like a high metal content in the soil, or by application of reducing compounds to the leaves.

Our results showed that jacobine-like PAs had a higher TA% than the other PAs. This coincides with the role of jacobine-like PAs as important defense compounds. Several studies showed that jacobine and jaconine were especially feeding deterrent for generalist insect herbivores (Macel et al, 2005; Leiss et al, 2009), while some specialists, preferred plants containing high concentrations of jacobine (Macel and Klinkhamer, 2010). From an evolutionary and ecological point view, it represents a next step in the arm-race between plants and herbivores as a number of studies show that tertiary amines are more toxic than their respective *N*-oxides (Dreyer et al, 1985; van Dam et al, 1995; Macel et al, 2005). Further research on the chemistry and biology of PA *N*-oxides and tertiary PAs and their influence on generalist and specialist insects are required to better understand the ecological significance of these highly interesting compounds.

We showed that the occurrence of tertiary PAs is not an artifact of the freeze drying, extraction or detection method. The three main PA *N*-oxides of *J. vulgaris* showed no significant differences during the reduction experiments. Jacobine was significantly more reactive compared to senecionine and erucifoline towards chemical *N*-oxidation with oxidation agent HOOH.

These results strongly indicate that the high levels of free bases found for jacobine and other

jacobine-like PAs are not caused by an intrinsic structural instability of the PA molecule or by chemical attack. Also it was not observed that naturally occurring agents in plant material caused reduction or oxidation of the added PAs during our extraction method. Plant material of different species did not induce any transformation of PAs from one form into the other. From our results we can conclude that the high percentages in tertiary form for jacobine-like PAs are not due to instability or higher sensitivity for reducing agents in the extraction and analytical process, but likely are the result of a change induced by (bio) chemical processes in the plant itself. We cannot exclude that a minor amount of reduction occurs during harvesting and the freeze-drying, but it seemed to affect all PA *N*-oxides to the same extent. We did find that in the Jacobine-chemotype plants a much higher level of tertiary PA present compared to the Erucifoline-chemotypes. By crossing *J. vulgaris* Jacobine-chemotype with the closely related *J. aquatica*, which lacks jacobine, and measuring PA *N*-oxide and tertiary amine concentrations, we showed that the TA% was genotype-dependent. This means that the variation found for relative tertiary amine content has a genetic base, since the environmental conditions of the plants during growth and analysis were kept equal for all plants.

Acknowledgements

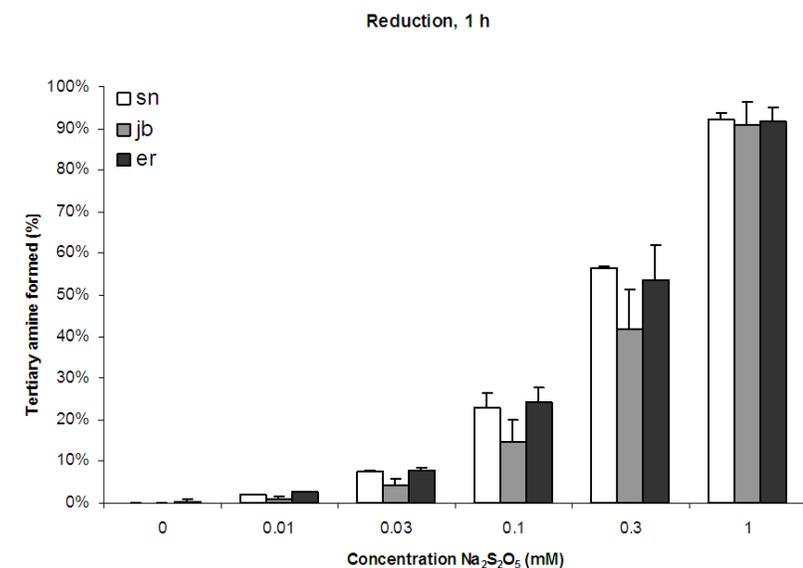
We are grateful to Kelly Stolk, Anniek de Vreede, Cilke Hermans, Karin van der Veen-van Wijk, Ciska Raaijmakers and Henk Nell for their technical assistance. We thank Prof. Dr. Thomas Hartmann for his helpful comments on the manuscript and the hypothesis on slow unspecific reduction of PA *N*-oxides. We thank the China Scholarship Council (CSC) of the Ministry of Education for financial support.

References

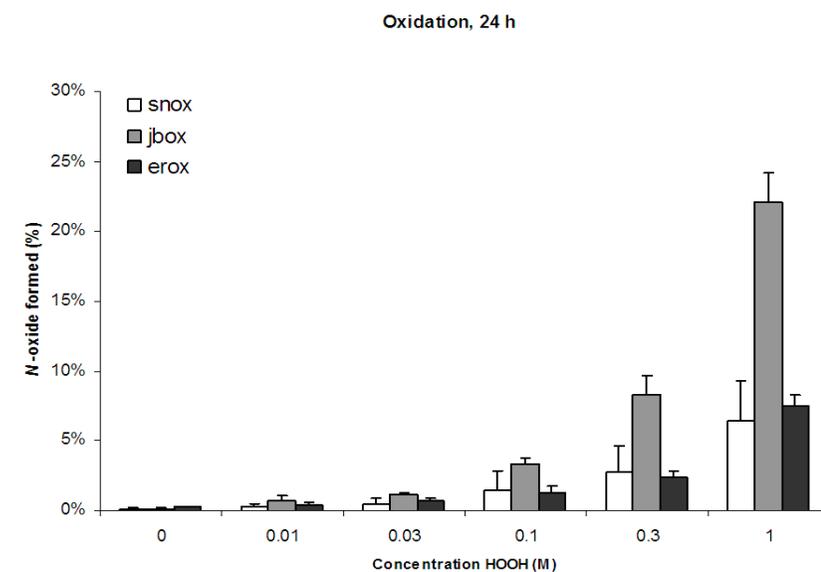
- Boppre M. 1986. Insects pharmacophagously utilizing defensive plant-chemicals (pyrrolizidine alkaloids). *Naturwissenschaften* 73: 17-26.
- Cao Y, Colegate SM, Edgar JA. 2008. Safety assessment of food and herbal products containing hepatotoxic pyrrolizidine alkaloids: Interlaboratory consistency and the importance of *N*-oxide determination. *Phytochemical Analysis* 19: 526-533.
- Chang A, Hartmann T. 1998. Solubilization and characterization of a senecionine *N*-oxygenase from *crotalaria scassellatii* seedlings. *Phytochemistry* 49: 1859-1866.
- Chou MW, Wang YP, Yan J, Yang YC, Beger RD, Williams LD, Doerge DR, Fu PP. 2003. Riddelliine *N*-oxide is a phytochemical and mammalian metabolite with genotoxic activity that is comparable to the parent pyrrolizidine alkaloid riddelliine. *Toxicology Letters* 145: 239-247.
- Christie SMH, Kropman M, Leisegang EC, Warren FL. 1949. The *Senecio* alkaloids. Part iii. The structure of retrorsine and isatidine, and the isomerism of retronecic acid and isatinecic acid. *J. Chem. Soc.*: 1700-1702.
- Crews C, Berthiller F, Krska R. 2010. Update on analytical methods for toxic pyrrolizidine alkaloids. *Analytical and Bioanalytical Chemistry* 396: 327-338.
- Dobler S. 2001. Evolutionary aspects of defense by recycled plant compounds in herbivorous insects. *Basic and Applied Ecology* 2: 15-26.
- Dreyer DL, Jones KC, Molyneux RJ. 1985. Feeding deterrence of some pyrrolizidine, indolizidine, and quinolizidine alkaloids towards pea aphid (*Acyrtosiphon pisum*) and evidence for phloem transport of indolizidine alkaloid swainsonine. *Journal of Chemical Ecology* 11: 1045-1051.
- Ehmke A, Borstel K, Hartmann T. 1988. Alkaloid *N*-oxides as transport and vacuolar storage compounds of pyrrolizidine alkaloids in *Senecio vulgaris* L. *Planta* 176: 83-90.
- Frei H, Luthy J, Brauchli J, Zweifel U, Wurgler FE, Schlatter C. 1992. Structure/activity relationships of the genotoxic potencies of 16 pyrrolizidine alkaloids assayed for the induction of somatic mutation and recombination in wing cells of *Drosophila melanogaster*. *Chemico-Biological Interactions* 83: 1-22.
- Hartmann T. 1999. Chemical ecology of pyrrolizidine alkaloids. *Planta* 207: 483-495.
- Hartmann T, Dierich B. 1998. Chemical diversity and variation of pyrrolizidine alkaloids of the senecionine type: Biological need or coincidence? *Planta* 206: 443-451.
- Hartmann T, Ehmke A, Eilert U, von Borstel K, Theuring C. 1989. Sites of synthesis, translocation and accumulation of pyrrolizidine alkaloid *N*-oxides in *Senecio vulgaris* L. *Planta* 177: 98-107.
- Hartmann T, Ober D. 2000. Biosynthesis and metabolism of pyrrolizidine alkaloids in plants and specialized insect herbivores. In: Leeper FJ, Vederas JC, eds. *Biosynthesis: Aromatic polyketides, isoprenoids, alkaloids*. Heidelberg, Germany: Springer, 207-243.
- Hartmann T, Theuring C, Beuerle T, Ernst L, Singer MS, Bernays EA. 2004. Acquired and partially *de novo* synthesized pyrrolizidine alkaloids in two polyphagous arctiids and the alkaloid profiles of their larval food-plants. *Journal of Chemical Ecology* 30: 229-254.
- Hartmann T, Toppel G. 1987. Senecionine *N*-oxide, the primary product of pyrrolizidine alkaloid biosynthesis in root cultures of *Senecio vulgaris*. *Phytochemistry* 26: 1639-1643.
- Hartmann T, Witte L. 1995. Pyrrolizidine alkaloids: Chemistry, biology and chemoeology aspects. In: Pelletier SW, ed. *Alkaloids: Chemical and biological perspectives*. Elmsford, NY: Pergamon, 155-233.
- Holm S. 1979. A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* 6: 65-70.
- Hösch G, Wiedenfeld H, Dingermann T, Röder E. 1996. A new high performance liquid chromatography method for the simultaneous quantitative analysis of pyrrolizidine alkaloids and their *N*-oxides in plant materials. *Phytochemical Analysis* 7: 284-288.

- Joosten L, Mulder PPJ, Klinkhamer PGL, van Veen JA. 2009. Soil-borne microorganisms and soil-type affect pyrrolizidine alkaloids in *Jacobaea vulgaris*. *Plant and Soil* 325: 133-143.
- Joosten L, Mulder PPJ, Vrieling K, van Veen JA, Klinkhamer PGL. 2010. The analysis of pyrrolizidine alkaloids in *Jacobaea vulgaris*; a comparison of extraction and detection methods. *Phytochemical Analysis* 21: 197-204.
- Kempf M, Heil S, Hasslauer I, Schmidt L, von der Ohe K, Theuring C, Reinhard A, Schreier P, Beuerle T. 2010. Pyrrolizidine alkaloids in pollen and pollen products. *Molecular Nutrition & Food Research* 54: 292-300.
- Kirk H, Choi YH, Kim HK, Verpoorte R, van der Meijden E. 2005. Comparing metabolomes: The chemical consequences of hybridization in plants. *New Phytologist* 167: 613-622.
- Kirk H, Macel M, Klinkhamer PGL, Vrieling K. 2004. Natural hybridization between *Senecio jacobaea* and *Senecio aquaticus*: Molecular and chemical evidence. *Molecular Ecology* 13: 2267-2274.
- Kowalchuk GA, Hol WHG, Van Veen JA. 2006. Rhizosphere fungal communities are influenced by *Senecio jacobaea* pyrrolizidine alkaloid content and composition. *Soil Biology & Biochemistry* 38: 2852-2859.
- Leiss KA, Choi YH, Abdel-Farid IB, Verpoorte R, Klinkhamer PGL. 2009. NMR metabolomics of thrips (*Frankliniella occidentalis*) resistance in *Senecio* hybrids. *Journal of Chemical Ecology* 35: 219-229.
- Lindigkeit R, Biller A, Buch M, Schiebel HM, Bopp M, Hartmann T. 1997. The two faces of pyrrolizidine alkaloids: The role of the tertiary amine and its *N*-oxide in chemical defense of insects with acquired plant alkaloids. *European Journal of Biochemistry* 245: 626-636.
- Macel M, Bruinsma M, Dijkstra SM, Ooijendijk T, Niemeyer HM, Klinkhamer PGL. 2005. Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. *Journal of Chemical Ecology* 31: 1493-1508.
- Macel M, van Dam NM, Keurentjes JJB. 2010. Metabolomics: The chemistry between ecology and genetics. *Molecular Ecology Resources* 10: 583-593.
- Macel M, Vrieling K, Klinkhamer PGL. 2004. Variation in pyrrolizidine alkaloid patterns of *Senecio jacobaea*. *Phytochemistry* 65: 865-873.
- Mattocks A. 1986. *Chemistry and toxicology of pyrrolizidine alkaloids*: Academic Press London.
- Narberhaus I, Theuring C, Hartmann T, Dobler S. 2003. Uptake and metabolism of pyrrolizidine alkaloids in longitarsus flea beetles (Coleoptera:Chrysomelidae) adapted and non-adapted to alkaloid-containing host plants. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology* 173: 483-491.
- Nishida R. 2002. Sequestration of defensive substances from plants by Lepidoptera. *Annual Review of Entomology* 47: 57-92.
- Pelser PB, Gravendeel B, van der Meijden R. 2003. Phylogeny reconstruction in the gap between too little and too much divergence: The closest relatives of *Senecio jacobaea* (Asteraceae) according to DNA sequences and AFLPs. *Molecular Phylogenetics and Evolution* 29: 613-628.
- Rizk AM. 1991. *Naturally occurring pyrrolizidine alkaloids*. Boca Raton, Florida, USA: CRC Press.
- Toppel G, Witte L, Riebesehl B, von Borstel K, Hartmann T. 1987. Alkaloid patterns and biosynthetic capacity of root cultures from some pyrrolizidine alkaloid producing *Senecio* species. *Plant Cell Reports* 6: 466-469.
- van Dam NM, Vuister LWM, Bergshoeff C, de Vos H, van der Meijden ED. 1995. The 'raison d'etre' of pyrrolizidine alkaloids in *Cynoglossum officinale* deterrent effects against generalist herbivores. *Journal of Chemical Ecology* 21: 507-523.
- von Borstel K, Hartmann T. 1986. Selective uptake of pyrrolizidine *N*-oxides by cell suspension cultures from pyrrolizidine alkaloid producing plants. *Plant Cell Reports* 5: 39-42.
- Vrieling K, de vos H, van Wijk CAM. 1993. Genetic analysis of the concentrations of pyrrolizidine alkaloids in *Senecio jacobaea*. *Phytochemistry* 32: 1141-1144.
- Witte L, Ernst L, Adam H, Hartmann T. 1992. Chemotypes of 2 pyrrolizidine alkaloid-containing *Senecio* species. *Phytochemistry* 31: 559-565.

Supplementary Material



- **Fig.S1** Reduction of PA *N*-oxides after incubation (1h, n = 2) in 2% formic acid solution with sodium metabisulfite (Na₂S₂O₅) in five different concentrations (0.01, 0.03, 0.1, 0.3, 1 mM). sn = senecionine; jb = jacobine; er = erucifoline. Error bars: ±1SD. The tertiary amine formed is the relative amount of tertiary PA present in a sample, which was calculated as the measured concentration of tertiary PA divided by the sum of the concentration of tertiary PA and corresponding PA *N*-oxide.



- **Fig.S2** Oxidation of PA tertiary amines after incubation (24h, n = 2) in 2% formic acid solution with hydrogen peroxide (HOOH) in 5 different concentrations (0.01, 0.03, 0.1, 0.3, 1 M). snox = senecionine *N*-oxide, jbox = jacobine *N*-oxide, erox = erucifoline *N*-oxide. Error bars: ±1SD. The *N*-oxide formed is the relative amount of *N*-oxide present in the sample which was calculated as the measured concentration of the PA *N*-oxide divided by the sum of the concentration of PA *N*-oxide and the corresponding tertiary PA.

sn	0.85 ^{***}	0.38 ^{**}	0.79 ^{***}	0.68 ^{***}	0.25 ^{ns}	0.17 ^{ns}	0.17 ^{ns}	0.15 ^{ns}	0.45 ^{***}	0.46 ^{***}
0.89 ^{***}	ir	0.35 ^{**}	0.77 ^{***}	0.60 ^{***}	0.32 [*]	0.21 ^{ns}	0.21 ^{ns}	0.06 ^{ns}	0.46 ^{***}	0.44 ^{***}
0.55 ^{***}	0.55 ^{***}	rt	0.42 ^{***}	0.38 ^{***}	0.37 ^{**}	0.35 ^{**}	0.40 ^{***}	0.48 ^{***}	0.55 ^{***}	0.49 ^{***}
0.83 ^{***}	0.80 ^{***}	0.58 ^{***}	sp	0.66 ^{***}	0.35 ^{**}	0.23 ^{ns}	0.24 ^{ns}	0.29 [*]	0.68 ^{***}	0.63 ^{***}
0.81 ^{***}	0.84 ^{***}	0.56 ^{***}	0.80 ^{***}	acsp	0.27 [*]	0.28 [*]	0.29 [*]	0.34 ^{**}	0.5 ^{***}	0.59 ^{***}
0.43 ^{***}	0.38 ^{**}	0.32 [*]	0.51 ^{***}	0.50 ^{***}	jb	0.82 ^{***}	0.86 ^{***}	0.46 ^{***}	0.6 ^{***}	0.44 ^{***}
0.43 ^{***}	0.37 ^{**}	0.26 ^{ns}	0.52 ^{***}	0.49 ^{***}	0.80 ^{***}	jl	0.87 ^{***}	0.49 ^{***}	0.56 ^{***}	0.48 ^{***}
0.51 ^{***}	0.43 ^{***}	0.34 ^{**}	0.54 ^{***}	0.55 ^{***}	0.79 ^{***}	0.82 ^{***}	jn	0.53 ^{***}	0.59 ^{***}	0.49 ^{***}
0.15 ^{ns}	0.10 ^{ns}	0.31 [*]	0.20 ^{ns}	0.18 ^{ns}	0.19 ^{ns}	0.07 ^{ns}	0.17 ^{ns}	jz	0.58 ^{***}	0.43 ^{***}
0.50 ^{***}	0.41 ^{***}	0.51 ^{***}	0.52 ^{***}	0.44 ^{***}	0.43 ^{***}	0.45 ^{***}	0.52 ^{***}	0.31 [*]	er	0.75 ^{***}
0.21 ^{ns}	0.18 ^{ns}	0.14 ^{ns}	0.15 ^{ns}	0.15 ^{ns}	0.02 ^{ns}	0.03 ^{ns}	0.17 ^{ns}	0.28 ^{ns}	0.21 ^{ns}	acer

- Fig.S3** The correlations between genotype mean TA% of the PAs. Two parental, two F1 and 102 F2 genotypes were used. Numbers in the cells are the correlation coefficients. The background color of the cells is related to the number: black (>0.75); dark grey (0.50~0.75); light grey (0.25 ~ 0.50); white (< 0.20 and (or) p-value is not significant). ns: not significant, * p<0.05, **p<0.01, ***p<0.001. In the cells along the diagonal line are the codes for PAs. Sn = senecionine; ir = integerrimine; rt = retrorsine; sp = seneciphylline; acsp = acetylseneciphylline; jb = jacobine; jl = jacoline; jz = jacozine; er = erucifoline; acer = acetylerucifoline. Correlation coefficients above the diagonal line are for shoots, below the diagonal for roots.
- Fig.S4,5** are Appendix 1-2 at the end of this thesis