Chapter 4.

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

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ABSTRACT

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

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

INTRODUCTION

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

Plant Material

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

Epidermis and Mesophyll Extraction

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

Verification of Epidermal Extracts

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

Chlorophyll extraction and quantification

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

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

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

Microscopic analysis

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

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

Metabolomics

Extraction of plant material

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

One- and two-dimensional NMR analysis

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

Data reduction and quantification of 1H-NMR data

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

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

RESULTS AND DISCUSSION

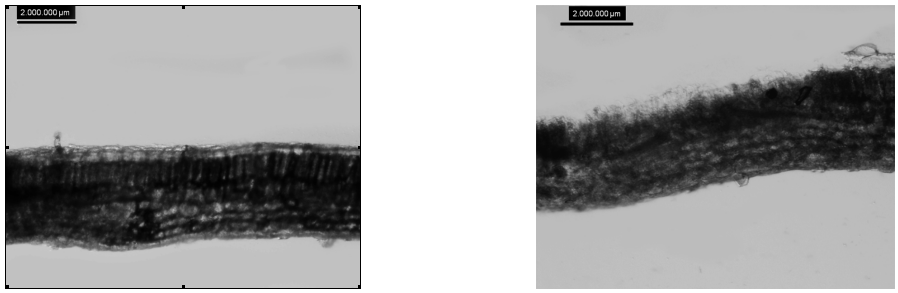
Epidermis and Mesophyll Extraction

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

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

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

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



a b

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

One- and Two-Dimensional NMR Analysis

Identification of metabolites

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

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

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

Comparison of metabolites between epidermis and mesophyll

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

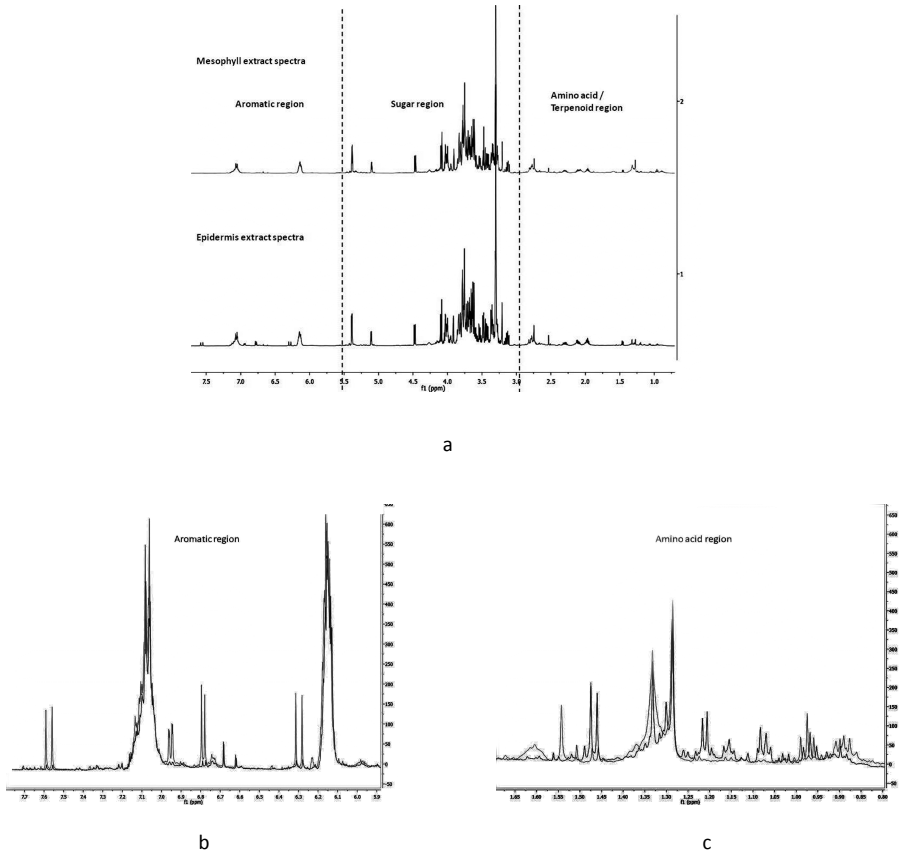


Fig 2. 1H NMR spectra for methanol extracts of *Jacobaea vulgaris* samples. Comparison between epidermal and mesophyll extracts (a); aromatic region (b); methyl region of pyrrolizidine alkaloids (PAs) (c).

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

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

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

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

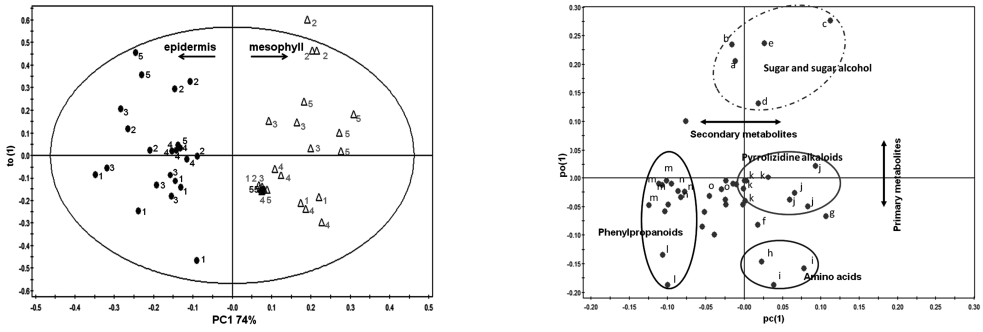


Fig 3. Score (A) and loading plots (B) for epidermis (•) and mesophyll (Δ) of OPLS-DA based on 1H NMR signals of extracts from *Jacobaea vulgaris*, *J. aquatica* and their crosses. *J. vulgaris* (1); *J. aquatica* (2); F1A cross (3); F1B cross (4); F2 cross (5). sucrose (a); glucose (b); fructose (c);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

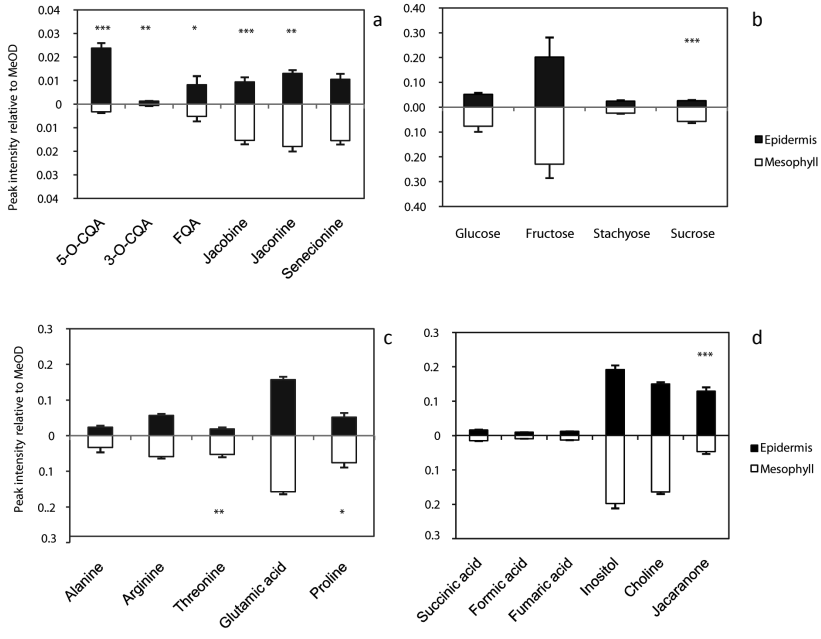


Fig 4. Quantitative expression of phenylpropanoids and PAs (a) sugars (b) amino acids (c) acids, sugar alcohols and jacaranone (d) of epidermis and mesophyll leaf tissues of *Jacobaea vulgaris, J. aquatica* and their crosses. Data present the mean of 5 replicates ± SE of the mean. Data were analyzed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

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

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

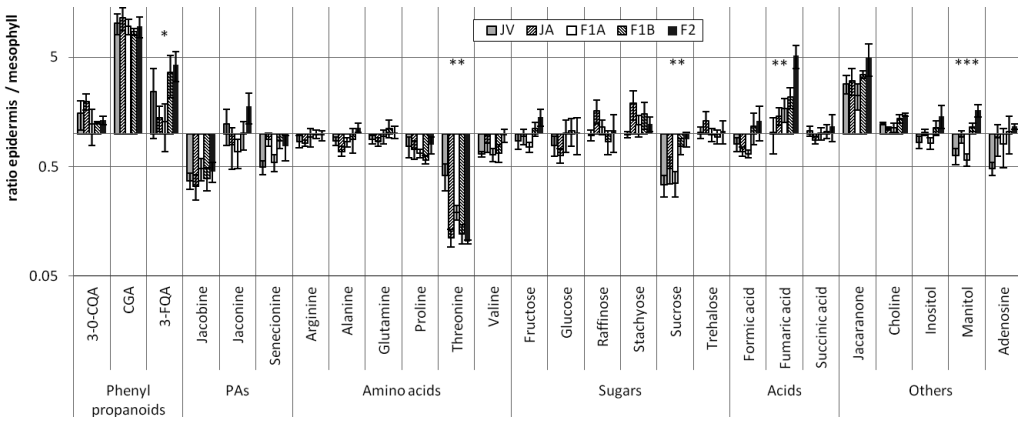


Fig 5. Ratio of metabolites in epidermis and mesophyll extracts. Data were analyzed by ANOVA. Significant differences between *Jacobaea vulgaris* (JV), *J. aquatica* (JA), their crosses: F1A, F1B, and F2 are indicated as *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$.

DISCUSSION

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

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

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

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

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

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

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

CONCLUDING REMARKS

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

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