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Pyrrolizidine alkaloids in and on the leaf surface of *Senecio jacobaea* L.

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Abstract

This is the first study showing that alkaloids are present on the leaf surface of plants. A concentration of 30–230 pmol/cm^2 pyrrolizidine alkaloids (PA's) was detected in 8 different samples taken from *Senecio jacobaea*. PA concentration on the leaves was marginally correlated with PA concentration of the total leaf tissues. The PA spectrum on the leaf differed from the PA spectrum of the total leaf.

Keywords: Pyrrolizidine alkaloids; *Senecio jacobaea*; Leaf surface; Secondary metabolite

1. Introduction

The leaf surface offers the plant the opportunity to display its chemical defense to phytophagous organisms such as insects and mites. Leaf surface chemistry is therefore expected to play a central role in plant recognition and resistance. Many specialist insect herbivores use secondary metabolites as cues to recognize their host-plants and as feeding or egg laying stimulants. However, surprisingly little is known about the chemical information present on the leaf surface. Only a few attempts have been made to analyze secondary metabolites present on the leaf surface (Kennedy et al., 1992; Renwick et al., 1992; Van Loon et al., 1992; Hopkins et al., 1997; Udayagiri and Mason, 1997; Estell et al., 1998; Lin et al., 1998a,b; Griffiths et al., 2001). Especially lepidoptera, diptera and acaridae have to rely to a large extent on the chemical information on the leaf surface for making feeding and oviposition decisions. From an insect point of view the chemical information on the leaf surface should therefore reliably predict the chemical contents, important for the insect, inside the leaf. To our knowledge no reports are known that correlate inner leaf tissue secondary chemistry with leaf surface secondary chemistry.

To shed more light on this important interface of the plants with its surrounding environment we analyzed leaf washings and the corresponding leaf tissues of *Senecio jacobaea* for the presence of pyrrolizidine alkaloids (PAs). Two different washing solutions were used to collect leaf surface metabolites. Despite its poor solubility of PAs water was used to be sure not to disturb (chemically) the leaf surface and cuticle permeability (Derridj, 1996). In addition, an acidic water washing was used from which is known that PAs are readily dissolved.

2. Results and discussion

2.1. Total PAs on the leaf surface

In both the water and the acidic water leaf washings PAs were detected. On average the concentration detected with acidic water spraying was higher than with water spraying (data were log transformed to

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obtain homogeneous variances: $F = 6.634$, Df = 1, 6, $P < 0.05$) (Fig. 1). As expected more PAs were collected from the leaf surface with acidic spraying than with water spraying.

The ratio between PAs on the leaf surface to PAs in the leaf varied from 0.001 to 0.0089, indicating that less

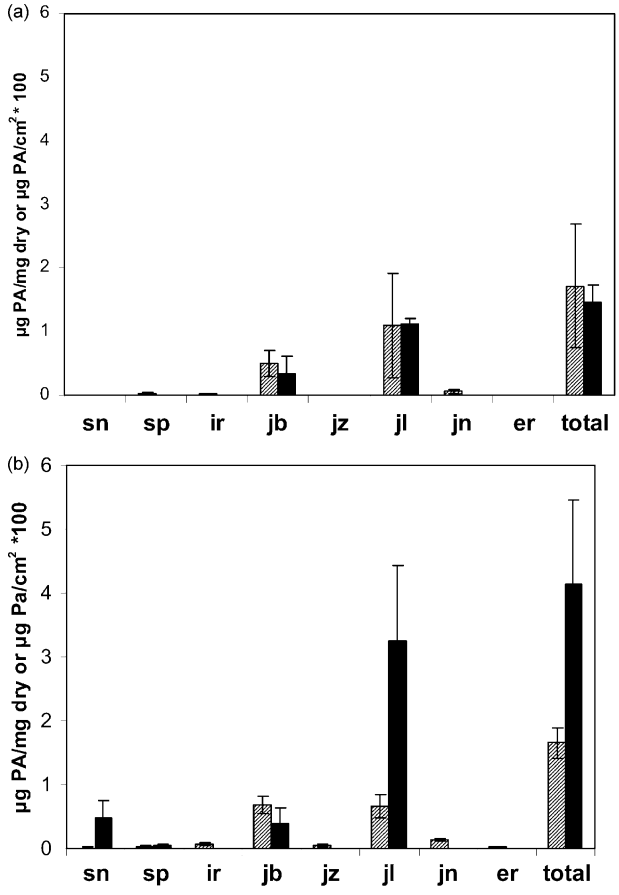

Fig. 1. (a) PAs detected on the leaf surface of *S. jacobaea* in µg PA/cm^2 *100 (hatched bars) and inside the same leaves in µg PA/mg dry weight (filled bars) using water spraying for collection of leaf surface PAs. Data are averaged over four samples. Bars indicate standard errors. sn = senecionine, sp = seneciphylline, ir = integerimine, jb = jacobine, jz = jacozine, jn = jaconine, er = erucifoline. (b) As for (a) but 0.5 mM sulpheric acid spraying was used for collection of PAs from the leaf surface.

than 1% of the PAs of a leaf are found on the surface (Table 1). The concentration of PAs detected on the leaf surface was low, ranging from 10 ng/cm^2 to 76 ng/cm^2 (= 30 – 230 pmol/cm^2). This is in the same range as reports on α-tocopherol in *Populus deltoidus* × *Populus nigra* hybrid (0–17.2 ng/cm^2) (Lin et al. 1998a), *n*-alkanes in *Zea mays* (3–175 ng/cm^2) (Udayagiri and Mason, 1998). Other reports express secondary metabolites in g per g dry (or fresh) weight. Assuming leaves of 1 mm thickness, 10% dry weight and a specific mass of one, the terpenes in *Flourensia cernua* range from 2 to 2220 ng/cm^2 (Estell et al., 1998) and glucosinolates in 18 species of the Cruciferae range from 12 to 308 nM/cm^2 (Griffiths et al., 2001).

Van Loon et al. (1992) found a concentration of 0.75 nmol/cm^2 of the glucosinolate glucobrassicin on the leaves of cabbage. Apparently the concentration of glucosinolates found on the leaf surface of various Cruciferae are circa 1000 times higher as the PA concentration on *S. jacobaea* leaves.

All the aforementioned reports used short dippings in lipid dissolving agents such as hexane (Lin et al., 1998a), pentane (Udayagiri and Mason, 1998), chloroform (Estell et al., 1998) and dichloromethane followed by methanol (Griffiths et al., 2001; Van Loon et al., 1992) to collect leaf surface chemicals. In contrast to watery solutions such solvents will also dissolve part of the epicuticular and sometimes intracuticular waxes and the compounds therein and therefore might not completely reflect compounds accessible to insects (Derridj et al., 1995).

The lepidopteran specialist *Tyria jacobaeae* still made a significant positive choice for oviposition for filter papers coated with a concentration of 50 ng/cm^2 PAs over control filter papers (Macel and Vrieling, 2003). This indicates that the concentrations of PAs detected at the leaf surface of *S. jacobaea* are present in concentrations that are biologically relevant.

Although PAs are produced and maintained in the plants as *N*-oxides (Hartmann and Dierich, 1998) total PAs were measured as free bases. Previous experiments were not able to detect PAs on the leaf surface and

Table 1
Number of genotypes and leaf area used for the collection of PAs for the water and acidic water leaf washings and the ratio of µg PA/cm^2 on the leaf surface and µg of PA/cm^2 in the leaf in *S. jacobaea*

Leaf washing	Sample name	No. of genotypes	Leaf area washed in cm^2	µg PA/cm^2 leafsurface/ µg PA/cm^2 in leaf
Water	D1 wild R1	2	3977	0.003795
Water	D4 wild R2	2	2681	0.007371
Water	D9 wild R3	2	2866	0.001862
Water	D10 1013	3	2131	0.001045
Acidic water	D6 311	1	1109	0.008877
Acidic water	D3 wild R1	2	2494	0.003819
Acidic water	D8 wild R2	3	3240	0.002644
Acidic water	D5/D7 wild R3	3	2537	0.005819

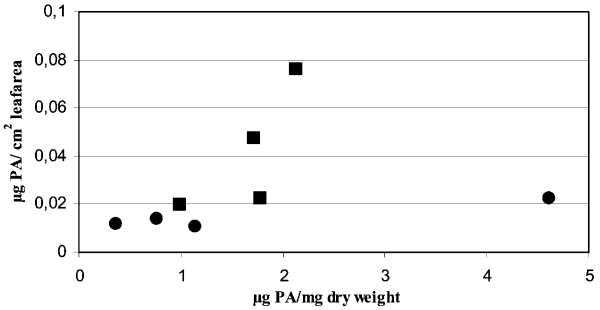

Fig. 2. Relation between PA concentration of *S. jacobaea* on the leaf surface and PA concentration inside the leaf for two methods of collection of PAs from the leaf surface. Circles represent PAs collected with ultra pure water spraying, squares represent PAs collected with 0.5 mM sulpheric acidic spraying. Overall Spearman rank correlation (rho = 0.738, $n = 8$, $P = 0.037$).

therefore it was chosen to maximize the detection of PAs by measuring both PA *N*-oxides and free base together.

Soldaat et al. (1996) did not detect PAs on the leaf surface of *S. jacobaea*. They estimated that the concentration should be below 720 pmol/cm^2. The values detected in the current research are indeed below the detection level of Soldaat et al. (1996).

2.2. Does the outside reflect the inside in total PA concentration?

Total PA concentration in the inner leaf tissues was positively correlated with the concentration of PAs on the leaf collected by water and acidic water sprayings (Fig. 2). The statistical power is not large because only four measurements per treatment are available. The absence of a perfect correlation between leaf surface and the interior of the leaf might be caused by passive differences in diffusion rates of the different PAs (Stammitti et al., 1995) and measurement error. According to Ehmke et al. (1988) PAs are stored in the vacuole in *S. vulgaris* which suggests that the presence of PAs on the leaf surface come from the apoplastic zone and have come about through an active process through the cellular membrane.

2.3. Does the outside PA spectrum reflect the inside PA spectrum?

The PA composition on the leaf surface differs significantly from the composition inside the leaves for the PAs integerimine, jacobine, jacozine, jacoline and jaconine (Table 2). PAs from the tissues are not all represented on the leaf surface in detectable amounts. For both the water and the acidic water extraction it is remarkable that jacobine on the leaf surface is always underrepresented compared to the leaf content while jacoline shows the reversed pattern. The shift from jacobine to jacoline on the leaf surface may be caused by the hydrolysis of the epoxide bond in jacobine (Fig. 3) or an active process from the plant. If jacoline and jacobine are summed there remains a difference between the percentage in and on the leaf (Wilcoxon signed ranks $Z = 2.20$, $n = 8$, $P < 0.05$).

The PA patterns present on leaf surface seem to depend on the method of extraction. With water only two (jacoline, jacobine) PAs are detected instead of four (senecionine, seneciphylline, jacobine, jacoline) with acidic water. Senecionine and seneciphylline are not found on the leaf surface when water was used for collection but were present when acidic water was used.

2.4. Does the extraction method correctly reflect biologically important leaf surface chemicals?

To study the incidence of chemicals which are on the leaf surfaces on insect behavior we need to know if they are really in contact with the insect after alighting. Water soluble substances which leach from the leaves by their removal from the plants by action of aqueous solutions, such as rain, dew, mist and fog (Tukey, 1970) are present on the leaf surface. When other (lipophilic) solvents than water are used one can remove substances which are imbedded in waxes (Derridj et al., 1995) either modify the cuticular permeability to substances coming from the inner tissues (Derridj et al., 1995; Stammitti et al., 1995) and consequently modify their quantities on the leaf surface.

The acidic water spraying may change the ionic relations of the leaves and extracellular space and decrease the buffer capacity of the extracellular compartment and hence change the cuticular permeability (Sherbatskoy and Klein, 1983). In our case where spraying lasts a very short time, this effect is probably not existing. The electrical conductivity of leaf diffusates of *Fagus sylvatica* immersed in 1 mN H_2SO_4 show a slow initial increase throughout 5 h, then a decrease. When there is a possibility of exchange between leaf cations and protons of the efflux medium the time course of exchange is rather longer than our experiment duration of washing. The buffer capacity of isolated cell walls may account for more than half of the total leaf buffer capacity (Leonardi and Flückiger, 1988). The amount of H^+ necessary to decrease the extracellular pH by one unit would be approximately 100 µmol per g of dry weight in *Fagus sylvatica* leaf. Assuming no neutralization of H^+ by Brönsted bases and that 10% of the incident H^+ would infiltrate into the apoplastic space, it would need 70 mm of precipitation with a pH of 3.0 to decrease the extracellular pH by one unit.

Higher quantities of jl are extracted by acidic solution than water in the tissues. Higher ratios of sn and jl are observed in the tissues versus the leaf surface. Probably the permeability of the *Senecio* cuticle is higher for jb

Table 2
Spectra of PAs detected on the leaf surface of *S. jacobaea* and inside the same leaves using water and acidic water spraying for collection of leaf surface PAs[a]

Sample name	Leaf washing	PA	sn	sp	ir[b]	jb[b]	jz[b]	jl[b]	jn[b]	er
D1 wild R1	Water	In leaf	0.7	1.2	3.3	54	0.9	23.8	14.5	1.6
		On leaf	0	0	0	0	0	100	0	0
D4 wild R2	Water	In leaf	1.9	0	7.5	26.3	0	57	7.3	0
		On leaf	0	0	0	23.7	0	76.3	0	0
D9 wild R3	Water	In leaf	0	6.4	3.4	40.6	1.6	38.6	9.4	0
		On leaf	0	0	0	0	0	100	0	0
D10 1013	Water	In leaf	0	0	0	23	0	77	0	0
		On leaf	0	0	0	48.9	0	51.1	0	0
D6 311	Acidic water	In leaf	0	1.8	6.1	29.4	2.2	51.3	6.4	2.7
		On leaf	6.2	0.9	0	3.5	0	89.4	0	0
D3 wild R1	Acidic water	In leaf	0	3.6	1.8	44.2	0.5	40	10	0
		On leaf	2.9	0	0	1.6	0	95	0	0
D8 wild R2	Acidic water	In leaf	0	0	2.9	61.1	5.6	18.9	10.4	1.1
		On leaf	4.21	0	0	2.8	0	93	0	0
D5/D7 wild R3	Acidic water	In leaf	2.5	2.4	5.6	33.8	0	44.1	8.1	0
		On leaf	26.3	2.6	0	24.4	0	46.6	0	0

[a] Individual PAs are calculated as a percentage of the total PAs present. Sn = senecionine, sp = seneciphylline, ir = integerimine, jb = jacobine, jz = jacozine, jn = jaconine, er = erucifoline.
[b] Significant differences ($P < 0.05$) between %PAs found on the leaf surface compared to %PAs found inside the leaf with a Wilcoxon signed ranks test.

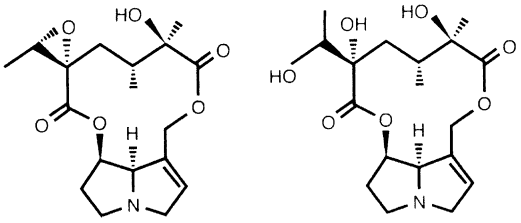

Fig. 3. The PAs jacobine and jacoline.

than for jl and sn. The acidic solution permits to extract PAs better from the leaf surface than water and probably without inducing any leaching.

A way to verify this would be to use mechanical collecting before extracting. Molecules are removed by an acetate film stripping (Silcox and Holloway, 1986) and then PAs are analyzed on the film with water and acidic water extraction.

3. Experimental

3.1. Origin of plant material and growth conditions

Seeds were obtained from a *S. jacobaea* stock from the plant ecology lab in Leiden. These plants originated from the dune area Meijendel close to The Hague. The seeds were germinated and plants were grown in mould in a greenhouse from May to September under a natural photoperiod at 23±2 °C. Plants received nutrient solution during two months after planting and ample water. Plants were used for assaying three months after sowing.

To obtain enough leaf area for washing in almost all cases more than 1 genotype was used (Table 1). It was chosen to pool genotypes, as Soldaat et al. (1996) could not detect PAs on the leaf surface using small leaf areas.

3.2. Sampling the leaf surface for PAs

Plants were analyzed at the rosette stage. Young, not completely developed and very old leaves were excluded from the washings. Care was taken to sample intact leaves without any damage. Ten leaves were sampled per plant and 2–3 plants were gathered and constituted one replicate. Leaf washing was carried out between 2 and 5 pm solar hour. PAs were washed out from the leaves following the method described by Fiala et al. (1990) and Derridj (1996). The cut ends of the leaves were dipped in liquid paraffin at a low melting point 42 °C to seal injuries due to the cutting. Leaves were fixed on a plastic plate at 60° inclination on which they were washed with a sprayer (Maugiere, France) with ultra-pure water (Millipore, conductivity = 10 MΩ) or ultra pure water containing 0.50 mM per liter of sulpheric acid. The spraying was 20 cm from the leaf surface and lasted 30 s for 25 ml. 25 ml were used to collect substances from a 200 cm^2 leaf area. This ratio between water volume sprayed and leaf area washed was kept among replicates. Leaf areas according to the sample varied from 1108 cm^2 to 3240 cm^2. The spraying pressure (1.7 MPa) was maintained constant by neutral and inert nitrogen gas (quality U GA 1240) resulting in a drop size of 0.1–0.3 mm diameter. Drops covered the leaf surface uniformly. When all the liquid was sprayed,

the spraying was prolonged with a nitrogen flow to push out drops in a sterile dish at the bottom of the plate in which washing was collected. Both leaf sides were sprayed and washings were immediately filtrated (0.2 μm) after each collection to remove epiphytic micro-organisms. The water washings were immediately evaporated at 40 °C after collection and stored in a freezer (−80 °C) prior to analysis. The acidic washings were reduced 10 times in volume (40 °C) avoiding dryness and then, filtrated (0.20 μm) and kept for chemical analyses at 4 °C.

3.3. PA determination of the leaf and leaf washings

Leaf material was dried for 3 days at 50 °C after collection of PAs from the leaf surface and stored at −20 °C until analysis. Leaves were grounded and about 10 mg was used for PA extraction following the modified method of Hartmann and Zimmer (1996) (de Boer, 1999). The acidic water leaf washings were stored at 4 °C and the evaporated water leaf washings were stored at −20 °C until analysis. The evaporated water leaf washings were dissolved in 2.5–6 ml 0.25 M H_2SO_4. To all the samples, water and the acidic water leaf washings, an excess amount of zinc powder was added and all were gently shaken for 24 h to reduce the PA *N*-oxides to tertiary bases.

All samples were brought to pH = 10 with concentrated NH_4OH. The water and the acidic water leaf washings were four times shaken with an equal volume of CH_2Cl_2. The CH_2Cl_2 fractions of each sample were collected, combined and evaporated to dryness. Subsequently samples were dissolved in 400 μl MeOH. Total PA content of extracts of the leaf washings and the leaves were determined spectrophotometrically, modified after Mattocks (1967).

Separate PAs were determined by GC-FID, modified after Vrieling et al. (1993), on a chrompack model 437A equipped with a WCOT fused silica column, 50 m × 0.25 mm ID, 0.4 μm (Chrompack CP-Sil 8); split ratio 1:60, oven 265 °C, injection and detection 300 °C, FID. Carrier gas H_2, injection volume circa 0.4 μl.

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