Chapter 3.

Shoot and root cultures of *Jacobaea plants* show different capacities to synthesise and transform pyrrolizidine alkaloids (PAs)

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are part of the constitutive chemical defence against herbivores. PA composition in plants is strongly determined by the environment, mostly in an unpredictable way. To understand the mechanisms behind this we need to know the capacity of different plant organs to synthesise and transform PAs.For such an investigation we developed *in-vitro* cultures of roots, shoots and complete plants (roots and shoots) of different *Jacobaea* genotypes*: Jacobaea vulgaris*, *Jacobaea aquatica* and their F1 and F2 hybrids.The cultures were harvested after nine weeks and PA content was measured using LC-MS/MS. We observed that not only roots, as known so far, but also shoots were able to synthesise *de-novo* PAs. Significant differences in total concentration of PAs were observed with the lowest concentration in the roots, followed by shoots and the highest concentration in complete plants.Although the genotypes differed in PA amount and composition they all followed this general pattern. Evaluation of PA composition showed that PLS-DA separated between roots and complete plants, while shoots were intermediate. Senecionine- and otosenine-like PAs were present in both roots and shoots while next to senecionine-like PAs,jacobine- and erucifoline-like PAs occurred in the shoots and complete plants.The *N*-oxides were the major accumulation form of PAs in all organ cultures. Our study indicates that both roots and shoots are essential for PA synthesis, while shoots are essential for PA diversification especially of jacobine-like PAs. This may be an adaption to plant defence since this type of PAs is known to inhibit above ground generalist herbivores.

Keywords: *Jacobaea*, pyrrolizidine alkaloids, organ cultures, synthesis, transformation

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are part of the plant's constitutive chemical defence against herbivores(Ober and Kaltenegger, 2009; Macel et al., 2005). These alkaloids cover more than 400 different structures in approximately 6000 angiosperm species (Chou and Fu, 2006; Hartmann and Ober, 2000) including Asteraceae, Boraginaceae, Fabaceae (mainly the genus *Crotalaria*) and Orchidaceae (Hartmann, 1999). They are known to be powerful deterrents and toxins to most vertebrates (Mattocks, 1986) and generalist insects(Leiss et al., 2009; Cheng et al., 2011a; Macel et al., 2002; Joosten, 2012). However, specialists are not affected (Macel et al., 2002; Macel and Klinkhamer, 2010). Some of the specialists sequester and utilise PAs for their own protection against predators and parasitoids (Hartmann and Ober, 2000) or use them as oviposition stimulants (Macel and Vrieling, 2003; Cheng, 2012). Recent reports showed that hybridization can result in novel patterns of overall PA composition in the roots and shoots of F1 and F2 hybrids of *Jacobaea vulgaris* (syn. *Senecio jacobaea*) and *Jacobaea aquatica* (syn. *Senecio aquaticus*) (Cheng et al., 2011b). Parent and hybrid genotypes are maintained in our laboratory and we have used them as model plants for studies on both ecological and biochemical evolution (Kirk et al., 2004); genotypic-metabolome variation as a result of hybridization (Kirk et al., 2005); compartmentation of metabolites in different leaf tissues (Nuringtyas et al., 2012); as well as plant defence (Leiss et al., 2009; Cheng et al., 2011a; Joosten, 2012). These studies support the hypothesis that PAs evolve as plant defence compounds under the selection pressure of biotic and abiotic environments. Thus, PAs represent an excellent study system for evolutionary ecology and biosynthesis of secondary defence metabolites(Pelser et al., 2005; Hartmann and Ober, 2000)

Pyrrolizidine alkaloids are esters of a necine base with one or more necic acids (Hartmann, 1999). The necine base portion is formed from two molecules of putrescine which is derived from the arginine-agmatine route (Hartmann et al., 1988). The necic acids portion is derived from several common amino acids such as L-threonine, L isoleucine, L-valine and L-leucine(Stirling et al., 1997). Until now, 37 individual PAs have been reported from the 24 species of the genus *Jacobaea* (Pelser et al., 2005; Cheng et al., 2011a). The site of synthesis and accumulation of PAs differs among species. PA synthesis may occur in shoots, like in *Heliotropium indicum* (Frölich et al., 2007) and *Cynoglossum officinale* (van Dam et al., 1995) or in roots like in *Symphytum officinale* (Frölich et al., 2007) and *Senecio vulgaris* (Hartmann et al., 1989). All PAs except senecivernine are derived from senecionine *N*-oxide (Hartmann and Toppel, 1987). From the roots, PAs are translocated to the above ground plant organs via the phloem (Hartmann and Toppel, 1987). In the shoots, senecionine *N*-oxide is biochemically modified by one- or two-step reactions such as hydroxylations, dehydrogenations, epoxydations, *O*-acetylations, to yield the species-specific PAs patterns (Hartmann and Dierich, 1998). Aside from the structural diversification, PAs do not undergo any significant turnover or degradation and they are stored in the vacuoles (Ehmke et al., 1988). According to their biosynthesis, PAs in the *Jacobaea* genus are divided into four groups, including derivatives of: senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Pelser et al., 2005). Based on the dominant PA types present in the *Jacobaea* genus, four chemotypes are known for the intra-species PA variation:jacobine, erucifoline, senecionine and mixed chemotypes(Witte et al., 1992; Macel et al., 2004).

Pyrrolizidine alkaloids occur in two interchangeable forms: the free base (tertiary amine) and the *N*-oxide form (Hartmann and Dierich, 1998).The free base from is considered to be non-toxic compared to the *N*-oxide form (Hartmann and Toppel, 1987). The *N*-oxide is the major PA storage form in *S.vulgaris* (Hartmann and Toppel, 1987). Moreover, senecionine *N*-oxide rather than the free base is the specific form for the translocation from root to shoot (Ehmke et al., 1988). Recently, Joosten et al. (2011, 2009) reported that the free base consistently constituted up to 50% of the total alkaloid concentration in the jacobine chemotype of *J. vulgaris*. The relative concentration of free bases for the various PAs was genotype dependent and showed a high correlation with their corresponding *N*-oxide form (Joosten et al., 2011; Cheng et al., 2011b).

Pyrrolizidine alkaloids are present in all organs but are not equally distributed. In *H. indicum*, the highest level of PAs occurs in the inflorescences (Frölich et al., 2007) while in *Phalaenopsis* hybrids maximum amounts of PAs are found in young and developing tissues such as root tips and young leaves (Frölich et al., 2006). A similar pattern is observed in *S. vulgaris*, with the highest levels of PAs in inflorescences, followed by leaves, roots and stems (Hartmann et al., 1989). The composition of PAs in *Jacobaea* and *Senecio* plant genera varies quantitatively and qualitatively among shoot organs, including leaves, stems and inflorescences (Hartmann and Dierich, 1998). Comparison between roots and shoots of vegetative *J. vulgaris* and *J. aquatica* plants and their F2 hybrids showed that the shoots contained higher proportions of jacobine- and erucifoline-like PAs and lower proportions of senecionine- and otosenine-like PAs compared to the roots(Joosten et al., 2009).These examples show that once the PAs are synthesised, they will be distributed over the whole plant. How PAs are accumulated and distributed in a particular organ may depend on several processes such as: (a) the rate of *de-novo* PA synthesis which seems to occur mostly in the roots (b) long-distance translocation of senecionine *N*-oxide through the phloem into the shoots and (c) further structural transformation and organ selective storage.

Thus, the biochemistry and physiology of PAs are quite well understood. In contrast, little is known about the capacity of different plant organs to synthesise and transform PAs. In this regard different study systems with *S. vulgaris* were used. These included detached shoots of flowering plants, *in-vitro* root cultures, and several undifferentiated *in-vitro* cultures including callus, cell suspension, tumor and teratoma shoot cultures. Each of these systems was fed with labelled precursors including ^{14}C putrescine, ^{14}C arginine and ^{14}C spermidine (Hartmann et al., 1989). Only the root cultures produced senecionine *N*-oxide, while all other systems failed to synthesise PAs. However, these results should be interpreted carefully. The undifferentiated *in-vitro* cultures are not comparable with root cultures which consist of differentiated cells. Moreover, the form of a plant organ may affect the capacity of PA synthesis. Van Dam et al. (1995) reported that *C. officinale* was able to synthesise PAs in both the shoot and the root. However, this was only the case when looking at the intact rosette as a shoot while single leaf failed to produce PAs when fed with the precursors. Furthermore, the study of the PA diversification process in different organs may require longer time of observation than the one day used in the previous feeding experiment. Sander and Hartmann (1989) showed that in root cultures of *Jacobaea erucifolia* more senecionine *N*-oxide was transformed into seneciphylline *N*-oxide 10 days after introducing the precursors.

We, therefore, used an alternative approach to understand how organ specificity governs PA

production and variation. We used roots, shoots, and complete plant cultures, with both roots and shoots, deriving from *J. vulgaris*, *J. aquatica* and their hybrids. This enables us to study the potential of different organs for *de-novo* synthesis of PAs.

We specifically wanted to answer the following questions: Do different types of organ cultures have different capacities to produce PAs? Does PAconcentration and composition vary between organs? Do different types of organs have different PA storage forms, i.e. free base or *N*-oxide forms?

MATERIALS AND METHODS

Generation of Organ Cultures. *Jacobaea vulgaris* Gaertn seeds were collected at Meijendel Nature Reserve and *J. aquatica* G. Gaertn, B. Mey and Scherb seeds at the Zwanenwater Reserve. The crosses of these plants were performed by rubbing flower heads together since both species are self–incompatible (Kirk et al., 2004). The parental, F1 and F2 individuals were maintained in tissue cultures. To represent the variation in PAs synthesis in *Jacobaea* plants we included the parental genotypes as well as the hybrids F1A, F1B and one of the F2 in this study. Two-week-old plantlets were used as sources of root, shoot and complete (root and shoot) cultures. The roots and shoots of each plantlet were aseptically excised and separately grown in a modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) optimised for each organ culture, as follows:

a. Root cultures were grown in 250 ml conical flasks containing 80 ml MS medium at pH 6.8, supplemented with 2% sucrose, and plant regulator a-naphtaleneacetic acid (NAA) and indole-3-butyric acid (IBA) in a 3:1 ratio (mg/l). The root cultures were shaken on a gyratory shaker at 108 rpm in the dark and were routinely subcultured at three week intervals.
b. Shoot cultures were grown in closed glass containers on 150 ml of MS medium solid medium at pH 6.0 supplemented with 3% sucrose, 4.5 mM N-benzylaminopurine (BAP) and 0.6% agar for solidification.
c. Complete plant cultures were grown in closed glass containers on the same medium as for the shoot culture but without phytohormones.

We used 5 replicates for each genotype and organ culture giving a total of 75 cultures. All cultures were maintained in a climate room (humidity 70% at 17 ± 2 ºC). The cultures were harvested after nine weeks by a removal from the medium, washing with distilled water and freezing in liquid nitrogen. The shoots and the roots of the complete plant cultures were not separated. The frozen materials were crushed with a mortar and pestle and freeze-dried. Subsequently, the dried samples were kept at 80 ºC until further work-up.

Pyrrolizidine Alkaloids Analysis. We extracted PAs using a formic acid extraction method and subsequently analysed them by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Samples were extracted using 2% formic acid. Heliotrine, monocrotaline and monocrotaline *N*-oxide, at concentrations of 1 µg/ml, were added to the extraction solvent as internal standards. An amount of 10 mg of dried powder of the culture samples was extracted with

1ml of the extraction solvent. The mixture was shaken for 1 h. Solid substances were removed by centrifugation at 9000 rpm for 10 min, followed by filtering the supernatant through a 0.2-µm nylon membrane (Acrodisc 13-mm filter, Pall Life Scie, Ann Arbor, MI, USA). An aliquot of 25 µl of the filtered solution was diluted with 975 ml water and injected into the LC-MS⁄MS system, consisting of a Waters Acquity Ultra Performance Liquid Chromatographic (UPLC) system coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA). Chromatographic separation conditions followed the reported method on PA extraction (Joosten et al., 2010). Seventeen individual PA standards (see Joosten et al., 2011, for the source of the standards) were available for this study. For compounds without reference standard, a semi-quantitative value was obtained by comparison with the most closely related analogue. Identification of PAs was based on their retention time, molecular mass and fragmentation pattern as well as on comparison with PA standards and⁄or literature data. Data processing was conducted using Masslynx 4.0 software (Waters, Milford, MA, USA). The total PA concentration was calculated as the sum of all detected individual PAs. For further analysis, we classified the PAs into four groups according to their biosynthetic pathways (Pelser et al., 2005): senecionine-, jacobine-,erucifoline- and otosenine-like PAs. The PA composition was evaluated with regard to the relative concentration of each PA- group in comparison with the total PA concentration. The relative concentration of individual PAs was calculated as follows:
((Absolute concentration of PA)⁄(Total PA concentration)) × 100.

Data Analysis: Differences in concentration of total PAs between organ cultures and between genotypes were analysed using General Linear Models (GLM) with organ cultures and genotypes as fixed factors. Statistically significant data were further analysed using a Duncan's test for multiple comparisons in SPSS 19.0. The PA profile was analysed using relative concentrations of individual PAs. The relationship between senecionine *N*-oxide as the first product of the PA biosynthesis and the concentration of total PAs was investigated with a Pearson correlation analysis. Partial Least Square - Discriminant Analysis (PLS-DA), performed with SIMCA-P software (version 12.0, Umetrics, Umeå, Sweden) was used to visualise the variation in PA profiles. Scaling was based on the UV method. The models were validated by permutation test through 20 applications and CV-ANOVA, which is the default validation tool in the software package (SIMCA-P). Using the loading plots of PLS-DA, we determined the individual PAs responsible for the separation. These were further analysed using GLM to determine differences in concentration between organ cultures and genotypes.

RESULTS

Organ Cultures. All organ cultures grew well without callus formation. The starting material for the root cultures had diameter of cultures of 1.5-2 cm on average. At time of harvest, after nine weeks, this had increased to 5-7 cm (Fig.1a and b). The shoot cultures produced twelve - fifteen shoots of 6 - 8 cm height when harvested after nine weeks (Fig.1c). No roots were formed in the shoot culture (Fig.1d), however, a gall-like formation was observed. The complete plant cultures showed development of both shoots and roots (Fig.1e and f).

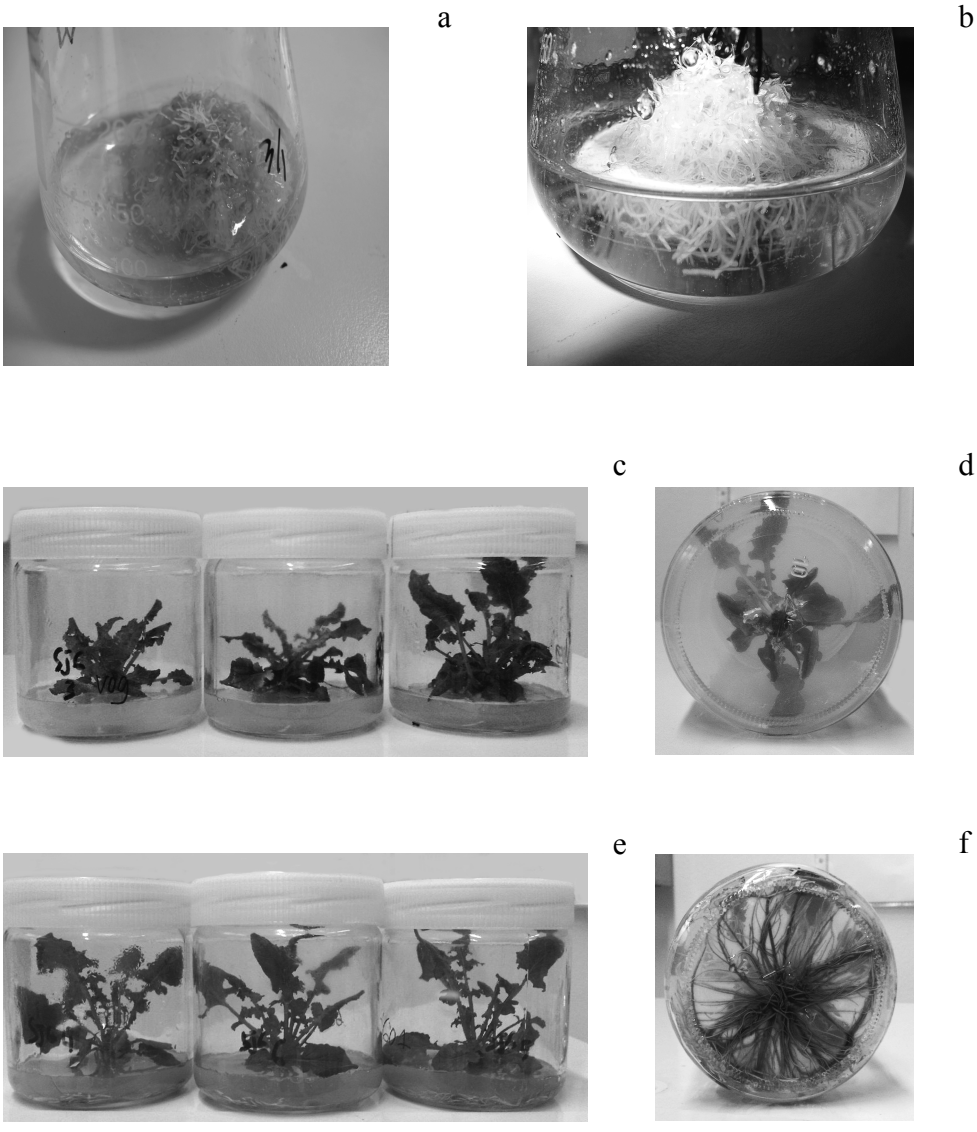

Fig 1. Photographs of organ cultures of *Jacobaea vulgaris and Jacobaea aquatica:* Root cultures of *J. vulgaris* (a) and *J. aquatica* (b).Shoot cultures of *J. vulgaris* showing the above ground part (c) and the base of the shoot cultures without root formation (d). Complete cultures of *J. vulgaris* and *J. aquatica* showing the above ground part (e) and roots formed at the below ground part (f). All photographs were taken at time of harvest, i.e. 9 weeks of growth.

Pyrrolizidine Alkaloid Concentration. The total concentration of PAs differed significantly between organ cultures (ANOVA, F=23,584; df =2; P < 0.001). The complete plant culture showed the highest concentration of total PAs. Compared to the root cultures, complete plant cultures contained three times more and compared to the shoot cultures two times more PAs (Fig.2a). In all of the three cultures, total PA concentration was different among genotypes (Fig. 2b). In the F1A root cultures, PA levels were the highest and in F1B the lowest. The other genotypes

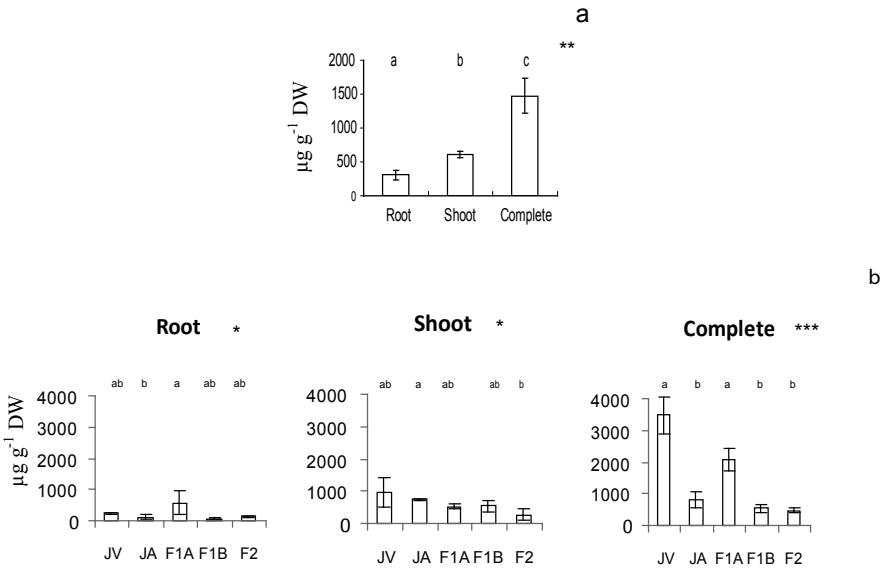

Fig 2. Average of total pyrrolizidine alkaloid (PA) concentration of *Jacobaea vulgaris, Jacobaea aquatica* and their hybrids in root, shoot and complete plant *in-vitro* cultures expressed overall genotypes (a) and expressed per genotype (b): *J. vulgaris* (JV), *J. aquatica* (JA), hybrids of JV and JA: F1A, F1B and F2. Data present the average and standard error of 5 replicates each. Different letters denote significant differences at * *P*< 0.05; ** *P*< 0.001; *** *P*< 0.0001.

contained intermediate PA levels (ANOVA, F=4.292; df =4; P = 0.011). Among the shoot cultures, *J. vulgaris* showed the highest PA level while F2 showed the lowest. The remaining genotypes showed intermediary PA levels (ANOVA, F=3.0; df =4; P = 0.043). In the complete plant cultures *J. vulgaris* and F1A contained significantly higher levels of total PAs compared to the *J. aquatica*, F1B and F2 genotypes (ANOVA, F=16.3; df =4; P < 0.001).

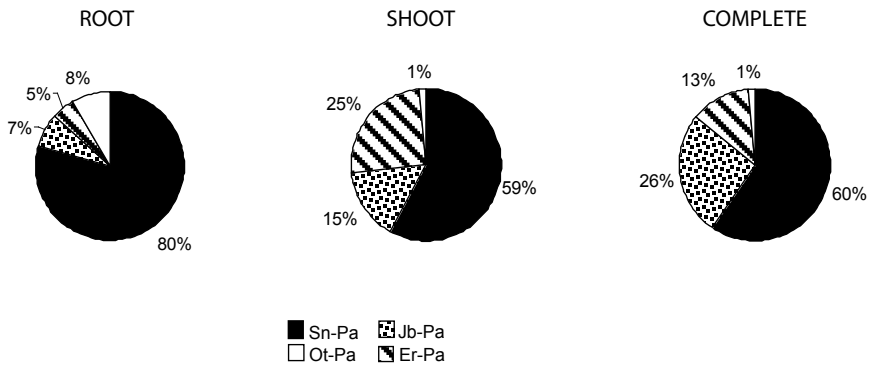

Fig 3. Overall pyrrolizidine alkaloid (PA) composition of *Jacobaea vulgaris, Jacobaea aquatica* and their hybrids based on four structural PAs in root, shoot cultures and complete plant cultures. Data present the average of relative concentration and standard error of 25 replicates of each culture. Sn-PA = senecionine-like PAs, Jb-PA = jacobine-like PAs, Ot-PA = otosenine-like PAs, Er-PA = erucifoline-like PAs

Pyrrolizidine Alkaloid Composition. A total of 36 different PAs were detected in the five genotypes used in this study. Several PAs including usaramine, riddeline, spartiodine, integerrimine, and acetylerucifoline were detected in trace amounts. In general, PA composition in the shoot and complete plant cultures was more variable compared to the root cultures (Fig. 3). In the latter all PAs consisted of senecionine-like PAs in contrast to shoots and complete plant cultures. Jacobine-like PAs were at a maximum in complete plant cultures, followed by the shoot and the root cultures, respectively. In contrast, erucifoline-like PAs showed the highest percentage in shoot cultures followed by the complete plant and in the root cultures. Otosenine-like PAs contributed 8% of the total PAs in root cultures, which equalled the jacobine contribution, but contributed only marginally, with below 1%, to the PAs in shoots and complete plant cultures.

The PA composition differed among genotype (data not shown). In all culture types *J. vulgaris* contained significantly more jacobine-like PAs compared to *J. aquatica*. Hybrids contained intermediate amounts of PAs in comparison to their parents with the exception of F1B which contained significantly more erucifoline-like PAs in the shoot culture.

The PA composition was further evaluated based on individual PAs using PLS-DA. The score plots of PLS-DA showed a clear separation of root cultures from complete plant cultures. Most of the complete plant cultures were located in the positive quadrant while the root cultures were in the negative quadrant of PC1.The shoot cultures were situatedin the middleof the plot. The PC1 determined the separation between the root cultures from complete plant cultures and explained 31% of the PA variation (Fig. 4a). The loading plots of the PLS-DA showed that the root cultures were strongly affected by senecionine-like PAs especially acetylseneciphylline *N*-oxide, retrorsine *N*-oxide, retrorsine and onetine, an otosenine-like PA. In contrast, in the positive quadrant of PC1 we observed a strong effect of jacobine-like PAs including jacobine, jacobine *N*-oxide, jaconine and jaconine *N*-oxide located in the lower part and erucifoline-like PAs including erucifoline and acetylerucifoline *N*-oxide located in the upper part of the quadrant. In the positive quadrant we can also observe senecionine *N*-oxide (Fig.4b). This PLS-DA obtained two principal components with a predictive ability Q^2 of 0.57. The validation of the model used CV-ANOVA and resulted in a *P* value of 0.00011 indicating a good model.

Based on the loading plot of the PLS-DA (Fig.4b), we further analysed the individuals PAs responsible for the separation of the root cultures from complete plant cultures. These PAs comprised senecionine *N*-oxide, seneciphylline *N*-oxide, acetylseneciphylline *N*-oxide, retrorsine *N*-oxide, jacobine *N*-oxide, jacobine, jaconine *N*-oxide, jaconine, erucifoline *N*-oxide, acetylerucifoline *N*-oxide and onetine (Table 1). In general, the root cultures always showed lower concentrations of individual PAs compared to the shoots and complete plant cultures except for onetine (ANOVA, F=8.47; df =2; P =0.001). The concentration of senecionine *N*-oxide, the first alkaloid in the PA synthesis pathway, differed significantly between organ cultures (ANOVA, F=23.6; df =2; P <0.0001). This PA showed a much lower concentration in the root cultures compared to the shoot and complete plant cultures, which contained 3 and 10 times more of senecionine *N*-oxide, respectively. Senecionine *N*-oxide was positively correlated to the concentration of total PAs in root (N= 5, R= 0.913, P=0.030) and complete cultures (N= 5, R= 0.973, P=0.005)

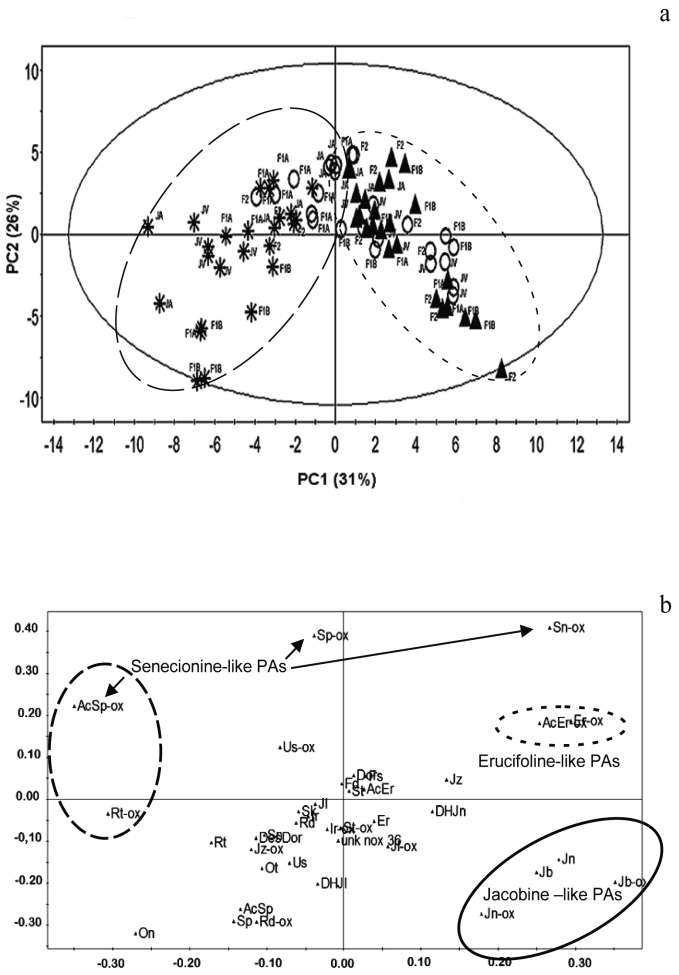

Fig 4. Score (a) and loading plots (b) of PLS-DA for pyrrolizidine alkaloid (PA) composition of *Jacobaea vulgaris* (JV), *J. aquatica* (JA) and their hybrids (F1A, F1B and F2) in three types of *in-vitro* cultures: root (*), shoot (o), and complete plants (▲). The ellipse represents the 95% confidence limit.

but not in the shoot culture (N= 5, R= 0.746, P=0.147). The other senecionine-like PAs such as seneciphylline *N*-oxide, acetylseneciphylline *N*-oxide and retrorsine *N*-oxide contained comparable levels between root and shoot cultures but 1.5 to 3 times significantly lower levels compared to complete plant cultures (ANOVA, F =7.43; df =2; P =0.001). In the root cultures, jacobine-like PAs including jacobine *N*-oxide, jacobine, jaconine and jaconine *N*-oxide were only present in trace amounts. Complete cultures contained the maximum amount of jacobine-like PAs with a four times higher level compared to shoot cultures. Erucifoline-like PAs, including erucifoline *N*-oxide and acetylerucifoline *N*-oxide were at the lowest concentration in the root cultures (ANOVA, F =55.65; df =2; P <0.0001). No significant difference was observed between the concentration of erucifoline-like PAs of shoot and complete plant cultures.

Table 1. Pyrrolizidine alkaloid (PA) concentration of 11 individual PAs in shoot, root and complete plant cultures of *Jacobaea vulgaris*, *Jacobaea aquatica* and their hybrids measured using LC-MS-MS.

PA	Code	Retention time (min)	Precursor mass (m/z)	Concentration (mg/g DW)			*P*
				Root	Shoot	Complete	
Senecionine *N*-oxide	Sn-ox	6.97	352.2	24.4[a]	77.8[b]	230.5[c]	***
Seneciphylline *N*-oxide	Sp-ox	6.36	350.2	131.5[a]	168.3[a]	380.6[b]	***
Acetylseneciphylline *N*-oxide	Acsp-ox	8.86	392.2	64.5[a]	95.3[a]	200.1[b]	*
Retrorsine *N*-oxide	Rt-ox	6.01	368.2	9.7[a]	5.5[a]	14.4[b]	***
Jacobine *N*-oxide	Jb-ox	5.49	368.2	1.6[a]	28.2[b]	114.1[c]	***
Jacobine	Jb	7.89	352.2	0.2[a]	8.5[b]	64.7[c]	***
Jaconine *N*-oxide	Jn-ox	5.77	404.2	1.2[a]	5.4[b]	28.6[c]	***
Jaconine	Jn	8.75	388.2	0.3[a]	14.3[b]	70.6[c]	***
Erucifoline *N*-oxide	Er-ox	4.80	366.2	7.0[a]	82.8[b]	90.6[b]	***
Acetylerucifoline *N*-oxide	Acer-ox	7.17	408.2	0.3[a]	22.0[b]	47.3[b]	***
Onetine	On	4.35	400.2	5.0[a]	1.5[b]	2.5[b]	*

Data present the average of 25 replicates of each culture.
The letters indicate significant differences between different types of cultures: root, shoot and complete plants at * *P*< 0.05, and *** *P*< 0.0001

Pyrrolizidine Alkoloids Forms. All PAs were present in both the *N*-oxide and the free base form except the otosenine-like PAs, dehydrojaconine and dehydrojacoline, which were detected in the free base form only. In general, the *N*-oxide form constituted more than 70% of the total PAs in all cultures except for F1B in the root culture which amounted to 53.4% (data not shown). The total *N*-oxide concentration of the different organ cultures showed significant differences (ANOVA, F=3.2; df =8; P = 0.004) (Fig. 5). The root cultures contained the lowest levels of total *N*-oxide followed by the shoot cultures with a two fold increase, while the complete plant cultures, with a four fold increase contained the highest levels. Interestingly, no significant differences in the concentration of the free base form could be observed between the root and shoot cultures while the complete plant culture showed a three fold higher concentration of the free base in comparison with the root and shoot cultures (ANOVA, F=3.0; df =8; P = 0.008) (Fig.5).

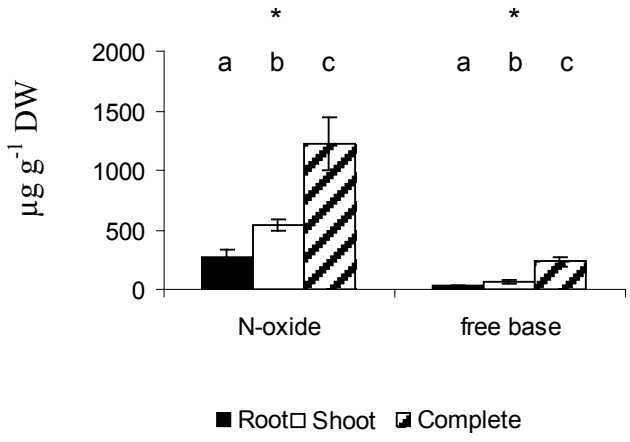

Fig 5. Average of total *N*-oxide and free base forms of pyrrolizidine alkaloid (PA) concentration of *Jacobaea vulgaris, Jacobaea aquatica* and their hybrids in root, shoot and complete plant *in-vitro* cultures expressed overall genotypes. Closed bar = root, open bar = shoot, stripped bar = complete plant. Data present the average and standard error of 25 replicates each. Different letters denote significant differences at * *P*< 0.05.

DISCUSSION

In this study, we report for the first time that both shoot and root cultures of *Jacobaea* plants were able to synthesise PA. The root cultures showed the lowest total PA level followed by shoot and complete plant cultures. The PA composition of root cultures differed from the shoot and the complete plant cultures. The *N*-oxide form was the main accumulation form of PAs in all culture types.

The low level of PAs in the root cultures compared to the shoot and the complete plant cultures may indicate that the root cultures have a limiting capacity to accumulate PAs. Following synthesis, PAs are distributed through the whole plant (Hartmann et al., 1989) to function as plant defence compounds. The lack of shoots may inhibit the distribution of PAs and subsequently the roots as a feedback mechanism will not produce any *de-novo* PAs anymore. Shoots alone were also able to synthesise PAs, as we show in this study, however the levels of total PAs were lower compared to the complete plant. It seems that both the presence of roots and shoots are essential for PA synthesis. Indeed the PA levels produced by the complete plant are for most individual PAs much higher than the sum of PAs produced in roots and shoots cultures.

Pyrrolizidine Alkaloid synthesis in the shoots of the genus *Jacobaea* has so far not been reported. Up to now it was thought that PA biosynthesis in these plants exclusively occurs in the roots (Hartmann et al., 1989). However, PA synthesis in the shoots has been described in other plant species such as *H. indicum* (Frölich et al., 2007) and *C. officinale* (van Dam et al., 1995). The latter study emphasised the importance of the rosette form for PA production. In *C. officinale* only intact rosettes as shoots produced PAs while single leaves failed to do so. The shoot cultures, which we used were indeed rosette forms whereas Hartmann et al. (1989), reporting no PA synthesis in the shoots, used *S. vulgaris* shoots from flowering plants instead. PA production in the shoot is probably related to active metabolism in the central meristem of the rosette (McKey, 1974). In this regard, further studies to investigate the expression of homospermidine synthase (HSS), an enzyme catalyzing the formation of the first intermediate of the alkaloid-specific pathway in the central meristem of the rosette of *Jacobaea* plants will be needed. HSS is derived from deoxyhypusine synthase (DHS), an enzyme which catalyses the first step in the activation of the translation initiation factor 5A (eIF5A) (Ober and Hartmann, 1999). Generally, HSS is not expressed in the shoot. In contrast to HSS, DHS was reported to be expressed in all above ground organs of *Senecio vernalis*, including the shoots (Moll et al., 2002). Thus, it may be a possibility that the enzyme HSS was functionally replaced with the enzyme DHS. The heterologous expression of both enzymes deriving from *S. vernalis* revealed that DHS also accepts putrescine as a substrate to form homospermidine in plants. Thus, both enzymes show the same kinetic properties to catalyse the synthesis of homospermidine from putrescine and spermidine (Ober et al., 2003). Further studies using molecular techniques to detect the role of HSS and DHS expression in PAs synthesis of shoots will be needed.Another explanation for the PA production in the shoots is the observation of a small gall-like formation at the basal part of the shoot cultures. Possibly these galls replaced the function of roots to absorb nutrients from the medium and as such were able to express HSS. Clearly, more studies on the role of HSS and DHS in PA synthesis of *Jacobaea* shoots are needed.

The PA composition between the different organ cultures differed considerably. While the root cultures mainly contained senecionine- and otosenine-like PAs, the shoot and complete plant cultures, next to the senecionine-like PAs, contained more jacobine- and erucifoline-like PAs. Likewise, senecionine-like PAs constituted the main type of PAs detected in roots of *Senecio* (Hartmann and Toppel, 1987) and *Jacobaea* plants (Hartmann and Toppel, 1987; Sander and Hartmann, 1989). A similar pattern was reported by Joosten et al. (2009) where shoots of complete *J. vulgaris* plants, grown in soil contained higher proportions of jacobine- and erucifoline-like PAs and lower proportions of senecionine- and otosenine-like PAs compared to the roots (Joosten et al., 2009). The senecionine-like PAs in our study included senecionine *N*-oxide, seneciphylline *N*-oxide, acetylseneciphylline *N*-oxide and retrorsine *N*-oxide. Senecionine *N*-oxide in the roots showed significantly lower concentrations compared to the shoot while no differences in the concentrations of the other senecionine-like PAs were observed. The level of senecionine *N*-oxide as the first product of the PA biosynthesis from which all other PAs are derived, except senecivernine, was positively correlated with the total concentration of PAs produced in the roots and complete plants. This correlation explains why senecionine *N*-oxide is associated with the classification of complete plants in the PLS-DA loadings plot. Diversification of PAs takes mainly place in the shoots with different diversification rates depending on the type of PA (Hartmann and Dierich, 1998). Therefore, we did not observe a correlation between the amount of senecionine *N*-oxide and total PAs in the shoot cultures.

Next to the higher amount of senecionine-like PAs we observed a high proportion of otosenine-like PAs in the root cultures. Specifically onetine was measured at two times higher concentrations compared to the shoot and complete plant cultures. This may suggest that the root cultures are able to perform conversion of retronecine base structure to otonecinebase structure. The exact diversification mechanism from senecionine *N*-oxide into otosenine-like PAs is still unclear. Pelser et al. (2005) proposed two different putative diversification routes either from senkirkine or jacobine-like PAs. The amount of otosenine-like PAs in the roots was higher than that of the jacobine-like PAs. It is, therefore, unlikely that in our study the otosenine-like PAs have been synthesised from jacobine.

The PA composition of the shoot cultures was very similar to those of the complete plant cultures. It seems that the absence of roots does not influence the PA composition in the shoots. The higher proportion of jacobine- and erucifoline-like PAs in the shoot and complete cultures suggests that specifically the epoxidation process which transforms senecionine *N*-oxide into jacobine and erucifoline-like PAs occurs mainly in the shoot. Nothing is known about this transformation though, since, so far only the transformation of senecionine *N*-oxide into seneciphylline *N*-oxide and retrorsine *N*-oxide has been investigated (Hartmann et al., 1989; Hartmann and Dierich, 1998).

The different patterns between above and below ground plant parts of *Jacobaea* plants in PA diversification may be the result of the plants adaptation to its environment especially to pathogens and insects. The high proportion of senecionine-like PAs in the root cultures observed in our study may be associated with plant defence against soil pathogens. Retrorsine and retrorsine *N*-oxide, both senecionine-like PAs occurring in the roots, inhibited mycelium growth of several root-associated fungi (Hol and van Veen, 2002). Moreover, integerrimine and a mixture of

integerrimine and retrorsine inhibited the growth of the soil inhabiting *Fusarium oxysporum* (Hol, 2003). Among the senecionine-like PAs, so far senkirkine is the only one reported to actively deter Lepidoptera (Bentley et al., 1984;Macel et al., 2005). This deterrent activity was associated with the otonecine base structure of senkirkine which might be more toxic compared to the retronecine base structure of the other senecionine-like PAs. While the association of a high proportion of senecionine-like PAs in the roots with plant defence against soil pathogensis still under discussion evidence accumulates for the role of jacobine-like PAs in plant defence against leaf feeding insects. Jacobine-like PAs including, jaconine (Joosten, 2012), jacobine *N*-oxide and jaconine *N*-oxide (Leiss et al., 2009; Cheng et al., 2011a; Joosten, 2012) as well as jacoline *N*-oxide (Cheng et al., 2011a) have been reported to be active in the above ground defence of *Jacobaea* plants, particularly against the generalist herbivore Western Flower thrips (*Frankliniella occidentalis*). Unfortunately, so far no *in-vitro* bioassays on the negative effect of jacobine-like PAs on generalist insects have been performed since these compounds are not commercially available and thus need to be isolated from the respective *Jacobaea* plants. However, such a study is currently underway in our laboratory. It has been indicated that *J. vulgaris* plants with a high level of jacobine-like PAs also suppressed the growth of microbes leading to a lower diversity of fungi in the rhizosphere (Kowalchuk et al., 2006).Very little is known about the effect of erucifoline- and otosenine-like PAs on plant defence. To our knowledge, only one study tested the toxicity of erucifoline to several generalist insects observing an anti-feeding activity of erucifoline on the Green Peach aphid (*Myzus persicae*) (Domínguez et al., 2008). A decreasing trend of the number of pupae of the American Serpentine leafminer (*Liriomyza trifolii*) was observed with an increase in the concentration of otosenine-like PAs, and a slight increase of number of pupae with an increase in the concentration of erucifoline-like PAs (Cheng, 2012). In a recent study Joosten (2012) reported a significant negative correlation between erucifoline-like PAs and fungal diversity in the roots of the plant.

In our study we observed that the *N*-oxide formwas the major PA form for accumulation in all organ cultures. Up to now it was thought that the non-toxic *N*-oxide form is the major storage form of PAs in *Jacobaea* plants (Hartmann and Toppel, 1987). However, Joosten et al. (2009; 2011) demonstrated that also the toxic free base form can contribute substantially to PA storage: up to 50% in the shoots of a jacobine- chemotype of *J. vulgaris*.In our study, the *N*-oxide form prevails with 82% in the roots and 89% in the shoots compared to the free base form with 18% and 11% respectively. Possibly, the aseptic growing conditions of the organ cultures may have influenced the accumulation of the *N*-oxide form. Due to the absence of biotic interactions in such a sterile environment plants may choose for the non-toxic storage form.

We did not detect senecivernine in any of the organ cultures. This PA was reported to be present in *J. vulgaris* (Pelser et al., 2005) and in the F1 and F2 crosses of *J. vulgaris* and *J. aquatica* (Cheng et al., 2011b). The absence of senecivernine was consistently observed in all types of cultures and genotypes used. Similarly, Toppel et al. (1987) did not observe any senecivernine in root cultures of *Senecio squalidus*, while the root from complete plants, grown in soil, contained senecivernine in amounts comparable to senecionine *N*-oxide (Toppel et al., 1987). The lack of senecivernine, therefore, seems to be a general pattern for the organ cultures. If so, the sterile-growing conditions may have affected the synthesis of senecivernine. Senecivernine is the only PA which does not derive from senecionine *N*-oxide (Pelser et al., 2005). Thus, possibly the pre-

cursor for senecivernine was not present in the organ cultures. However, nothing is known about the synthesis of this PA.

Comparing the potential of different plant organs for synthesis and diversification of PAs we detected that not only roots, as known so far, but also shoots of *Jacobaea* plants were able to synthesise *de-novo* PAs. Senecionine- and otosenine-like PAs were present in both roots and shoots while next to the senecionine-like PAs more jacobine- and erucifoline-like PAs occurred in the shoots. The latter maybe an adaption of plant defence against above ground plant herbivores.

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